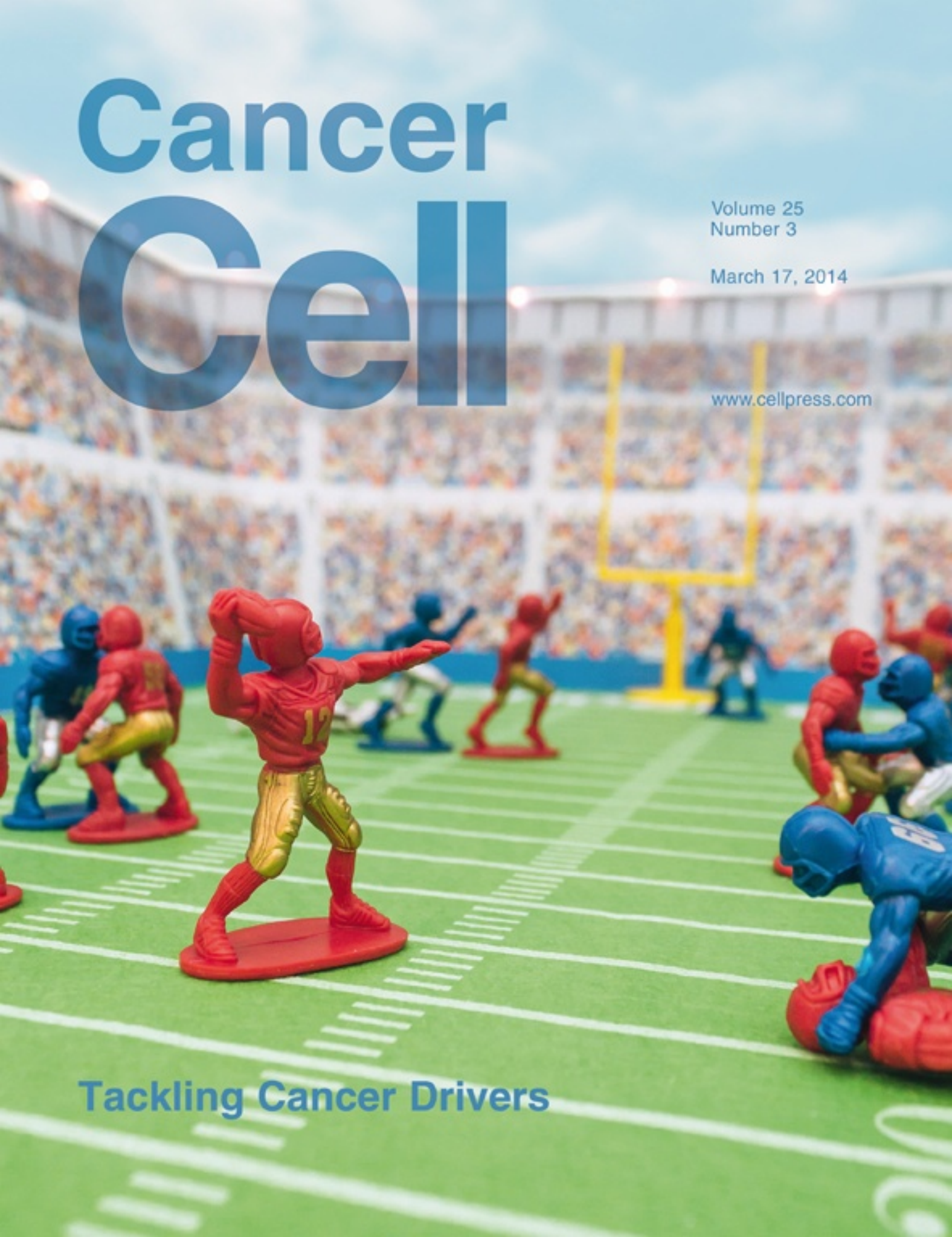

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Tackling Cancer Drivers

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Previews

RNA Identity Crisis: Hepatitis B Walks the *LINE*

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Hepatitis B virus (HBV) integration into the host genome has been implicated in the causation of hepatocellular carcinoma (HCC). In this issue of *Cancer Cell*, Lau and colleagues report an HBV integration site recurrent in HCC that generates a chimeric transcript with oncogenic function as an RNA (but not protein) through stimulation of Wnt/β-catenin signaling.

Hepatocellular carcinoma (HCC) is one of the few cancers directly caused by a virus (both hepatitis B virus [HBV] and hepatitis C virus) and is thus preventable by vaccination. Worldwide, HCC is the fifth most common cancer and the second most common cause of cancer mortality, especially impacting developing countries (Jemal et al., 2011). HBV is endemic to sub-Saharan Africa and East Asia; thus, the disparity of HCC epidemiology in developed versus developing nations reflects the intimate relationship that HBV plays in causing HCC. Globally, the leading cause of HCC is HBV. Other major causative factors include hepatitis C virus and alcohol consumption (El-Serag and Rudolph, 2007).

HBV has been implicated in HCC development via three mechanisms: direct action from the viral protein X (HBx) stimulating cell proliferation, chronic hepatic inflammation caused by immune response to the virus, and disruption of chromosomes and genes by viral insertion into the host genome. The ability of HBV to integrate into the human genome has been implicated in HCC oncogenesis for many decades (Brechot et al., 1980), but the mechanism through which HBV integration gives rise to HCC is not clearly understood. High-throughput sequencing-based approaches now enable large-scale detection of viral integration and are a promising avenue for understanding how HBV integration leads to HCC. A recent transcriptome sequencing (i.e., RNA-seq) study of HBV-positive HCC samples discovered multiple recurrent insertion sites of the virus into various protein-coding genes (Sung et al., 2012), and, in this issue of *Cancer Cell,* Lau et al. (2014) discover a highly recurrent insertion event in HCC wherein HBV integrates into a transposable *LINE1* element.

Transposable elements are DNA particles that can change their genomic location. They are widespread in the human genome, where they are known to still be mobile. By nature of being able to "jump around" the genome, potentially landing within genes, transposable elements are known mutagens. *LINE1* elements are known to be particularly mobile, and their insertion into and disruption of genes has been implicated in many diseases, including cancer (Lee et al., 2012), with a recent study describing a handful of *LINE1* insertions that contribute to HCC (Shukla et al., 2013). These studies implicate LINEs in carcinogenesis through a mechanism of LINE element insertion into genes, disrupting normal cellular function. However, the study by Lau et al. (2014) is intriguing for its discovery not of a LINE element insertion into a host gene, but of an HBV gene insertion into a LINE element, which in turn produces an oncogenic HBV-*LINE1* chimeric transcript.

In their study, Lau et al. (2014) analyzed RNA-seq data from six HCC cell lines, searching for viral-human transcript fusions. They found multiple instances of chimeric transcripts resulting from HBV integration, but, remarkably, one particular insertion event of the gene encoding hepatitis B X protein (*HBx*) into a *LINE1*-element on chromosome 8p11.21 (*HBx-LINE1*) was recurrent among the HCC tissue samples used for validation of the fusion candidates. This chimeric transcript was found in 21 of the 90 HCC samples analyzed, while the next most prevalent fusion was only found in 2 samples. In addition to its recurrence, Lau et al. (2014) found that *HBx-LINE1* is also a significant prognosticator of survival in these HCC patients. Through a series of RNAi experiments in a cell line harboring the *HBx-LINE1* fusion, the authors show that *HBx-LINE1* confers migration, invasion, and colony formation capabilities. Additionally, *HBx-LINE1* was found to facilitate an epithelial-to-mesenchymal transition, supporting cell motility. The authors further investigated this function of *HBx-LINE1*, showing that the chimeric transcript is capable of activating Wnt signaling as manifested by its ability to induce nuclear localization of β-catenin.

Addition of *HBx-LINE1* to an immortalized human hepatocyte cell line was able to recapitulate many of the oncogenic behaviors lost by RNAi knockdown. Interestingly, only the full length chimeric *HBx-LINE1* transcript was able to reconstitute oncogenesis; vectors containing just the *HBx* portion or just the *LINE1* portion were unable to do so. The *HBx-LINE1* transcript produces an 87 amino acid protein with 5 of the amino acids coded for by *LINE1*, but the authors present compelling evidence that this protein is not actually participating in the oncogenic activities observed. They generated a mutant *HBx-LINE1* vector with an early stop codon and validated that this transcript did not produce the 87 amino acid protein. However, this mutant *HBx-LINE1* was able to fully recapitulate all observable oncogenic functions, suggesting that *HBx-LINE1* may serve a role

similar to a long noncoding RNA (lncRNA) as a functional mRNA molecule. Finally, Lau et al. (2014) generated a transgenic mouse model expressing the chimeric transcript and showed that, when exposed to a carcinogenic agent, these mice acquired more hepatic tumors with stronger nuclear localization of β-catenin than control mice treated with carcinogen.

lncRNAs are long, spliced, polyadenylated RNA molecules that do not have protein coding potential. They have been widely studied in the past decade and have been implicated in multiple pathologies, including cancer (Prensner and Chinnaiyan, 2011). The *HBx-LINE1* transcript identified by Lau et al. (2014) seems to function similar to an lncRNA, despite coding for a large protein. This phenomenon is compelling, because it highlights the possibility that many of the thousands of coding genes may have mRNAs that possess functions independent of the protein they generate. One other prominent example of mRNAs serving a role independent of the proteins they encode is competing endogenous RNAs (ceRNAs). mRNAs serve as ceRNAs by acting as miRNA "sponges," which provide miRNA binding sites to modulate free miRNA levels in the cell (Salmena et al., 2011). The field of mRNA functionality (outside of protein-coding capacity) is still in its infancy.

Lau et al.'s study of the *HBx-LINE1* transcript falls at an exciting intersection of cancer biology with the fields of retrotransposons, viral DNA mutagenesis, and functional mRNA. Of the HCC samples analyzed by Lau et al. (2014), 23.3% bear the precise integration identified of *HBx* into *LINE1*, strongly suggesting that formation of *HBx-LINE1* is selected for in HCC oncogenesis. Analogous to recurrent protein-coding fusions such as *BCR-ABL* in leukemia, *EML4-ALK* in lung cancer, and *TMPRSS2-ERG* in prostate cancer (Prensner and Chinnaiyan, 2009) (Figure 1A), *HBx-LINE1* likely represents the oncogenic driver mutation in a subset of HCC. However, unlike those examples, *HBx-LINE1* is, to date, unique, in that it does not mediate oncogenic activity through the protein it produces (Figure 1B). Further investigation into the mechanism by which the *HBx-LINE1* chimeric transcript participates in the causation of HCC will be beneficial to understanding HCC biology, developing therapies targeting HCC, and deciphering oncogenic mechanisms of long RNAs (whether they are coding or noncoding).

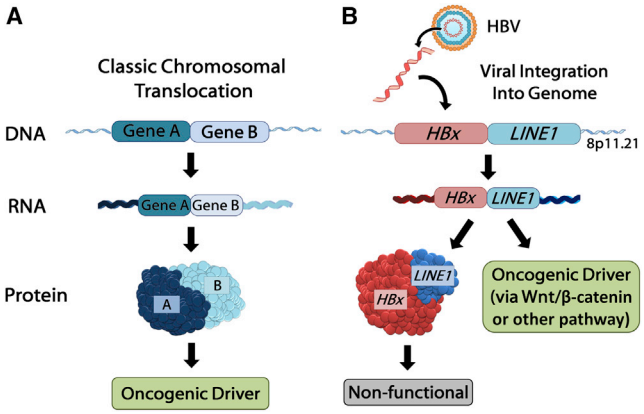

Figure 1. Coding and Noncoding Oncogenic Gene Fusions in Cancer

(A) Schematic of classic fusion events giving rise to cancer via expression of a chimeric protein. Well-known oncogenic fusions include *BCR-ABL* in chronic myelogenous leukemia, *EML4-ALK* in lung cancer, and *TMPRSS2-ERG* in prostate cancer.

(B) Lau et al. (2014) discovered that approximately 23% of HBV-positive hepatocellular carcinomas harbor an integration of the hepatitis B X protein gene (*HBx*) into a *LINE1* element in chromosome 8. This integration leads to expression of the *HBx-LINE1* fusion transcript, which mediates its oncogenic effects independent of the protein it encodes.

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A Jagged Road to Lymphoma Aggressiveness

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In this issue of *Cancer Cell*, Cao and colleagues identify an FGF4/Jagged1-driven crosstalk between tumor cells and their vascular niche that activates Notch signaling, sustaining the aggressiveness of certain mouse and human B cell lymphomas. These findings identify new therapeutic opportunities to target pathogenic angiocrine functions in cancer.

The Notch signaling pathway plays important roles in development, tissue homeostasis, and cancer. In mammals, Notch signaling is mediated by four Notch receptors (Notch1–4) interacting with ligands of the Delta-like (Dll1, Dll3, and Dll4) or Jagged (Jagged1 and Jagged2) families. During physiological signaling, ligand binding leads to intramembrane receptor proteolysis and release of intracellular Notch, which translocates to the nucleus and initiates target gene transcription. In cancer, activating *NOTCH1* mutations were initially identified in a majority of T cell acute lymphoblastic leukemias (Figure 1A) (Weng et al., 2004). A first class of point mutations in the extracellular heterodimerization domain disrupts stability of a negative regulatory region, leading to constitutive Notch activation even in the absence of ligand. A second type of mutations truncates the C-terminal PEST domain, leading to decreased proteasomal degradation and increased half-life of activated Notch. Although first associated with transformation of the T cell lineage, activating *NOTCH* mutations were subsequently identified in several subtypes of B cell malignancies, including chronic lymphocytic leukemia, mantle cell lymphoma, splenic marginal zone lymphoma, and diffuse large B cell lymphoma (Figure 1A) (Kiel et al., 2012; Kridel et al., 2012; Lee et al., 2009; Martínez-Trillos et al., 2013; Rossi et al., 2013). In almost all of these cases, point mutations were identified only within the region encoding the PEST degron domain of either *NOTCH1* or *NOTCH2*, suggesting sensitization to ligand-mediated receptor activation rather than true constitutive signaling. Moreover, systematic immunohistochemical scoring demonstrates NOTCH1 activation at a frequency that markedly exceeds the reported prevalence of *NOTCH1* mutations in specific lymphoid malignancies, suggesting that other mechanisms must exist to activate Notch signaling in these diseases (Kluk et al., 2013).

Results presented by Cao et al. (2014) in this issue of *Cancer Cell* identify the capacity of endothelial cells within the tumor microenvironment to induce ligand-mediated Notch activation in adjacent lymphoma cells, leading to enhanced tumor growth and aggressive in vivo behavior (Figure 1B). Using complementary in vitro and in vivo models of mouse and human Myc-driven lymphoma interacting with a vascular niche, the authors describe reciprocal interactions involving FGF4-dependent induction of the expression of the Notch ligand Jagged1 in endothelial cells, leading in turn to Notch2-mediated signaling in tumor cells with lymphoma-initiating characteristics. As a first step, Cao et al. (2014) built on past work from their laboratory using E4ORF1-transduced endothelial cells, which can be maintained in culture without serum or recombinant angiogenic factors and allow for detailed analysis of their angiocrine functions (Butler et al., 2010). Co-culture of these endothelial cells with Eμ-Myc-driven mouse B lymphoma cells selected for cells with increased in vitro growth, chemoresistance, in vivo repopulation potential, and invasiveness. Using a combination of genetic and pharmacological methods, the authors demonstrated that this phenomenon required *Jagged1* expression by the endothelial cells and *Notch2,* but not *Notch1*, expression in lymphoma cells. FGF4 release by the lymphoma cells induced *Jagged1* expression, suggesting that specific lymphomas capable of FGF4 production might be uniquely sensitive to this mechanism (although alternative pathways might exist to induce *Jagged1*). The effects of Notch2 appeared entirely dependent on the Notch target gene *Hey1*, while mechanisms operating downstream of this transcriptional repressor remain to be investigated. To gain insight about the potential human relevance of these findings, Cao et al. (2014) analyzed a panel of primary human Burkitt's lymphomas, demonstrating the existence of HEY1-positive cancer cells in proximity of JAGGED1-expressing endothelial cells in these tumors. Moreover, knockdown of *NOTCH2* in these primary human tumor cells followed by transfer into immunodeficient mouse recipients recapitulated observations made with Eμ-Myc mouse tumors, suggesting the existence of shared pathogenic mechanisms at least in these Myc-driven lymphoid malignancies.

It remains to be determined how broadly applicable the specific observations presented in this paper will be. Of note, tumor-vasculature interactions involving Notch signaling were reported recently in other contexts, such as glioblastoma multiforme and colorectal cancer. An interesting implication of these findings is that Notch activation might be at play in malignant tissues even in the absence of activating *NOTCH* mutations, provided the tumor microenvironment constitutes a good source of Notch ligands. In lymphoid malignancies, PEST

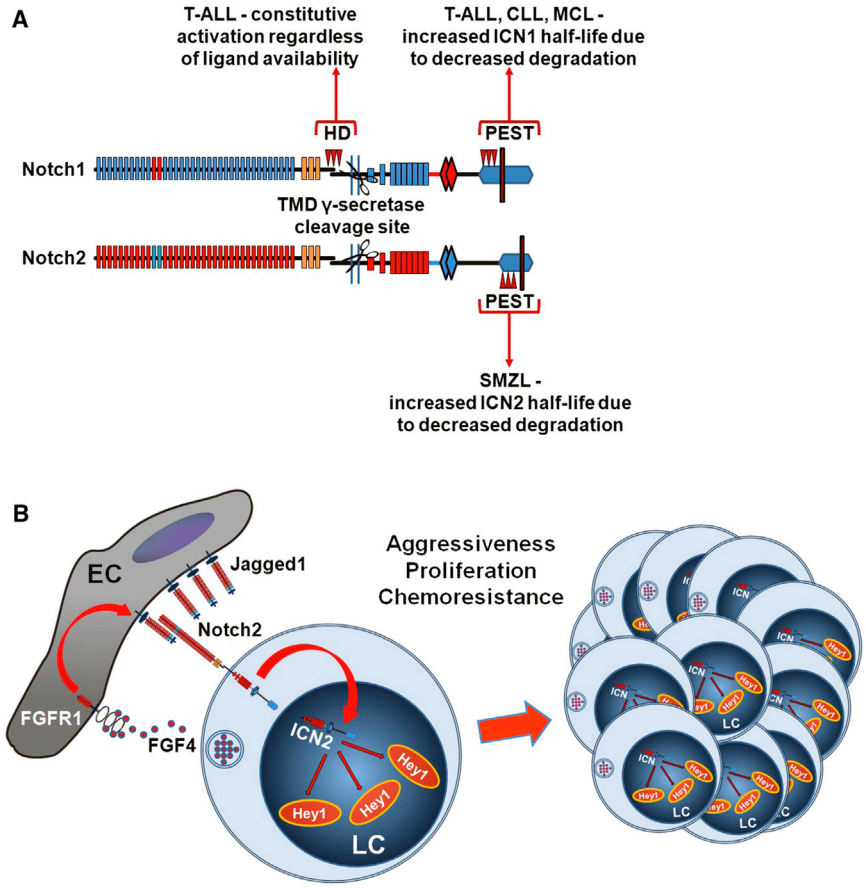

Figure 1. Emerging Roles of Notch in Lymphoma Pathogenesis

(A) Structure of Notch1 and Notch2 receptors with sites of activating mutations previously reported in lymphoid malignancies. T-ALL, T cell acute lymphoblastic leukemia/lymphoma; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma; ICN, intracellular Notch; HD, heterodimerization domain; TMD, transmembrane domain.

(B) Proposed model for crosstalk between lymphoma cells (LC) and endothelial cells (EC), with Jagged1/Notch2-driven effects on lymphoma aggressiveness (Cao et al., 2014). FGFR1, fibroblast growth factor receptor 1; FGF4, fibroblast growth factor 4; ICN, intracellular Notch.

domain mutations are predicted to matter only upon exposure of the tumor cells to Notch ligands, because these genetic events lead to stabilization of cleaved active Notch only after ligand-receptor interaction. Thus, PEST domain mutations may function as a sensitizer for the exposure of malignant cells to Notch ligands in their immediate environment. In other words, the type of ligand-dependent mechanisms identified by Cao et al. (2014) could synergize with PEST domain Notch mutants to potently activate the pathway. In terms of the overall importance of Notch as an oncogenic pathway in B cell neoplasms, another word of caution is that Notch signaling can have versatile functions. Tumor suppressive effects of the pathway have been reported, for example, in myeloid neoplasms, squamous cell carcinomas, and certain B cell malignancies (Zweidler-McKay et al., 2005). It is possible that Notch activation could have different effects in lymphomas originating from cells arrested at specific stages of differentiation, such as before, during, or after the germinal center reaction. To fully investigate the spectrum of Notch effects in lymphoma, future work should ideally focus on in vivo models and the combined use of loss-of-function and gain-of-function experimental approaches.

The vascular niche represents an attractive potential source of Notch ligands in the tumor microenvironment. In lymphoma, past observations revealed increased Notch activity in a high proportion of aggressive and highly vascularized angioimmunoblastic T cell lymphomas (Kluk et al., 2013). However, other cellular sources of Notch ligands must be considered as well. Using standardized immunohistochemistry to specifically detect the cleaved and active form of NOTCH1 in tumor tissues, Aster and colleagues reported evidence of high Notch activity within secondary lymphoid organs, but with rapid loss of the signal in tumor areas that extend beyond the lymph node capsule (Kluk et al., 2013). Although anecdotal, these findings suggest that cellular elements specific to the lymph node microenvironment might represent an important source of Notch ligands in vivo. Moving forward, additional work may reveal specific mechanisms for Notch ligand induction in this context and cooperativity with genetic Notch activation, perhaps leading to the maintenance of lymphoma-initiating cells in vivo. As a practical implication, future investigations are predicted to underestimate or to altogether miss the importance of Notch signaling in lymphoid malignancies if tumor cells are not adequately exposed to Notch ligands in culture (e.g., in the absence of cocultured cells expressing relevant Notch ligands) or in vivo (e.g., in conventional subcutaneous xenografts).

The translational impact of the findings reported by Cao et al. (2014) is significant, because they imply a larger array of potential tumor targets for the use of therapeutic Notch inhibition than predicted only by *NOTCH* mutational analysis. Therefore, emerging data implicating a central role for Notch ligands derived from the tumor vasculature or other sources suggest that our current understanding of Notch in lymphomagenesis has only exposed the tip of the iceberg. It is time to dive deeper.

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Leukemia Propagating Cells Akt Up

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Individual cancer cells can exhibit striking differences in tumorigenic potential following experimental transplantation, but the molecular pathways that regulate this activity remain poorly understood. In this issue of *Cancer Cell*, Blackburn and colleagues report that Akt signaling regulates both leukemia-propagating potential and proliferation rate via distinct pathways in T-ALL.

Cancers arise as clonal outgrowths from individual transformed cells, but the resultant population of tumor cells often harbors extensive phenotypic variability. Of particular interest is the observation that different cells of an individual cancer can exhibit marked differences in disease-propagating activity following transplantation into unaffected recipients, suggesting fundamental differences in their tumorigenic properties. One model to explain these differences is the cancer stem cell model, which posits that a subpopulation of cancer cells harbors a tumor's disease-propagating potential, and this cancer stem cell population gives rise to a "bulk" cancer cell population that lacks tumor-propagating potential. This model is well supported by experimental evidence in several tumor types, including acute myeloid leukemia (Lapidot et al., 1994), but equally convincing evidence argues against this model in other tumors such as melanoma (Quintana et al., 2008). Independent of the cancer stem cell model, genetic mutations, epigenetic alterations, and/or environmental influences, can give rise to distinct tumor subclones with extensive functional variation within the disease-propagating population.

Limiting-dilution transplantation experiments to assess tumor-propagating potential have emerged as a promising experimental tool to interrogate functional variability among tumor cell populations. In this issue of *Cancer Cell*, Blackburn and colleagues began their investigation of phenotypic variegation in T cell acute lymphoblastic leukemia (T-ALL) leukemia-propagating cells (LPC) using the limiting-dilution transplantation assay in a zebrafish model of Myc-induced T-ALL (Blackburn et al., 2014). In this transplantation tour-de-force, 16 fluorescently labeled primary T-ALLs were subjected to serial limiting-dilution transplantation into syngeneic recipients (n = 6,024 transplantations) to assess the evolution of LPC frequency and time to T-ALL onset (latency) in distinct subclones derived from a common ancestral progenitor.

The authors show that, although the LPC frequency of individual clones is relatively stable following serial transplantation, most primary T-ALLs (81%) harbor clones with functional heterogeneity, as evidenced by variations in latency and LPC frequency. Importantly, they demonstrate that LPC frequency can be uncoupled from leukemic growth rate and disease latency, arguing that the pathways that control tumor latency versus LPC potential can evolve independently. The authors then investigated potential molecular mechanisms involved and found that evolution of increasing LPC frequency was associated with Akt pathway activation. Expression of constitutively active Akt cooperated with transgenic Myc or intracellular (constitutively active) notch1a to accelerate leukemia onset and increase LPC frequency, implicating Akt signaling as a key regulator of these phenotypes.

The authors then investigated the contribution of two key pathways downstream of Akt to these phenotypes: Gsk3β-dependent inhibition of Myc degradation and Tsc-dependent activation of Rheb, which is best known as a positive regulator of mammalian target of rapamycin complex 1 (mTorc1) activity. Surprisingly, expression of constitutively active Rheb increased LPC frequency without accelerating tumor onset. By contrast, expression of a Myc T58A mutant that is resistant to proteasomal

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The authors then investigated the contribution of two key pathways downstream of Akt to these phenotypes: Gsk3β-dependent inhibition of Myc degradation and Tsc-dependent activation of Rheb, which is best known as a positive regulator of mammalian target of rapamycin complex 1 (mTorc1) activity. Surprisingly, expression of constitutively active Rheb increased LPC frequency without accelerating tumor onset. By contrast, expression of a Myc T58A mutant that is resistant to proteasomal

degradation induced proliferation and accelerated T-ALL onset, but had no effect on LPC frequency. These genetic studies suggest that Akt stimulates leukemic proliferation and accelerates the onset of leukemia by stabilizing Myc, whereas increases in LPC frequency reflect Akt-mediated effects on the Rheb-mTorc1 pathway (Figure 1).

The finding that tumor-propagating activity can be regulated independent from proliferation rate or latency raises an intriguing question: what are the cellular mechanisms that specifically enhance tumor-propagating activity? One intriguing possibility is an alteration in the kinetics of asymmetric cell division within T-ALL cancer stem cells, leading to an increase in cancer stem cell frequency within the T-ALL population. This possibility obviously requires that T-ALL fit the cancer stem cell model, and, although not yet demonstrated irrefutably, the available data are consistent with this possibility. Indeed, identifiable subsets of human and murine T-ALL cells harbor increased LPC activity, although discrepancies in the immunophenotypic subpopulation harboring this activity in human T-ALL have been reported (see, for example, Chiu et al., 2010). These discrepancies may reflect biologic differences among distinct molecular T-ALL subtypes or potentially result from differences in experimental methodology, which can have a substantial impact on the observed frequency of tumor-propagating cells in specific subpopulations (Quintana et al., 2008; Taussig et al., 2008). In addition, LPC potential was recently shown to be enriched within a MYC-high subpopulation of murine T-ALL, and transplantation of these MYC-high LPCs resulted in generation of leukemias that recapitulate the variability of MYC expression observed in primary mouse T-ALLs (King et al., 2013). Furthermore, depletion or inhibition of MYC eliminates the LPC population and prevents disease initiation, demonstrating a critical role for MYC in LPC function (King et al., 2013; Roderick et al., 2014). However, in the zebrafish, overexpression of stabilized Myc had no additional effect on LPC frequency above that induced by wild-type Myc overexpression. Potential explanations for this might be that the mouse studies involved *Myc* under the control of its endogenous gene-regulatory elements, leading to differentiation stage-specific regulation of *Myc* expression. Alternatively, the effects of MYC activity on LPC function may require MYC levels above certain thresholds, but far exceeding such a threshold may have little additional effect on LPC potential.

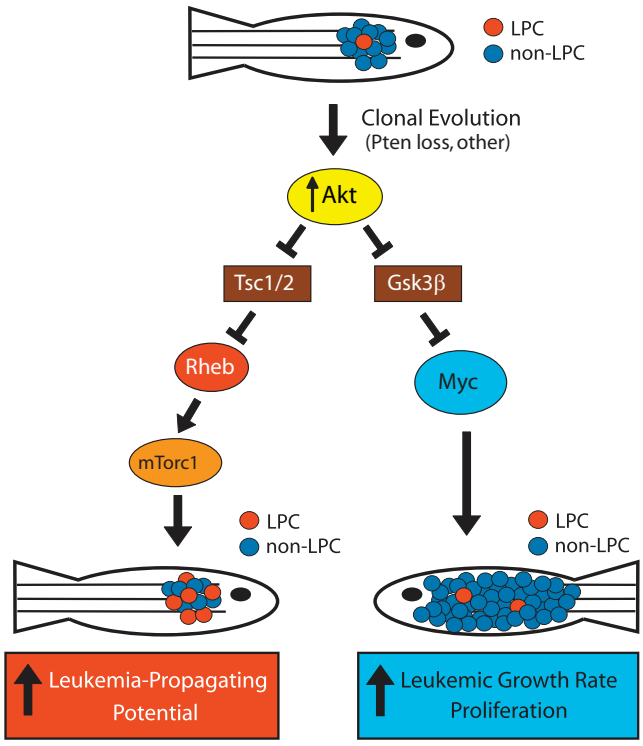

Figure 1. Akt Regulates Leukemia-Propagation Potential and Proliferation via Distinct Downstream pathways

Clonal evolution can lead to the emergence of subclones with increased leukemia-propagating potential, often due to activation of the Akt pathway. Akt directly phosphorylates and inactivates the Tsc complex, resulting in relief of repression of Rheb, a GTPase best known as a positive regulator of mTorc1 activity. Rheb activation results in a specific increase in T-ALL cells with leukemia-propagating potential without increasing proliferation or shortening time to T-ALL development, an effect that is likely mediated by mTorc1. Akt also phosphorylates Gsk3β, resulting in inhibition of Gsk3β-dependent phosphorylation of Myc at T58, leading to stabilization of Myc protein levels. Overexpression of a Myc T58A mutant that mimics the effect of the Akt-Gsk3β pathway on Myc stabilization accelerates T-ALL proliferation, but had no measurable impact on leukemia-propagating potential. As previewed in this article, Blackburn et al. (2014) show that these Akt-positive LPCs can be targeted by the combination of Akt inhibitor and dexamethasone.

Nonetheless, the available data can also be explained by several possibilities independent of the cancer stem cell model. For example, although the cancer stem cell model posits unidirectional differentiation of stem cells into "bulk" tumor cells, evidence suggests that this phenotypic change may be bidirectional, as some bulk tumor cells can give rise to stem cell-like progenitors (Chaffer et al., 2011). Thus, Akt-Rheb pathway activation may promote the clonal evolution of non-LPC into LPC, with the Akt-Gsk3β-Myc pathway independently controlling proliferation of the tumor cell population.

Alternatively, Akt-Rheb-mTor pathway activation may promote leukemic cell fitness through the transplantation procedure. Blackburn et al. (2014) used transplantation of fluorescently sorted cells in syngeneic zebrafish, which avoids a number of potentially problematic experimental steps, such as the barrier to cross-species transplantation resulting from the failure of some growth factor ligand-receptor interactions across species or the use of antibodies against cell surface antigens that can trigger tumor cell destruction (Taussig et al., 2008). However, none of the available methods to assess tumor-propagating activity avoid all potential problems, and the methods used by the authors require T-ALL cells to survive outside of their native microenvironment during cell sorting and heterotopic transplantation, and the use of immunocompetent recipients allows the possibility of an immune response against tumor antigens. Nevertheless, despite the seemingly artificial nature of such experimental barriers, a tumor cell's resistance to proapoptotic stimuli and to an antitumor immune response may

be highly relevant to clinical resistance to conventional cytotoxic chemotherapy (Ni Chonghaile et al., 2011). Moreover, transplantation of human T-ALL into mice is associated with clonal evolution that closely mimics the evolution observed in human patients between diagnosis and relapse (Clappier et al., 2011), highlighting the relevance of this approach to treatment resistance in humans.

The findings of Blackburn et al. (2014) have clear potential translational implications, because they suggest that AKT activation in T-ALL cells with LPC potential may be poised to mediate treatment resistance and relapse. The authors show that these cells can be effectively targeted by the combination of dexamethasone and AKT inhibitors, potentially due to reversal of AKT-mediated dexamethasone resistance (Piovan et al., 2013), thus providing an additional rationale for clinical trials testing such a strategy in high-risk T-ALL.

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The AML Salad Bowl

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Tumors arise from single cells but become genetically heterogeneous through continuous acquisition of somatic mutations as they progress. In this issue of *Cancer Cell*, Klco and colleagues used whole genome sequence analysis to demonstrate the correlation of genetic clonal architecture with functional heterogeneity in acute myeloid leukemia.

A major tenet of cancer biology is that tumors are clonal reflecting their origins from single cells. This is best illustrated by early seminal cytogenetic studies demonstrating that all cells of a patient's leukemia, for example, may harbor a specific chromosomal aberration. However, it has also been recognized for decades that leukemias and solid tumors are heterogeneous in their genetic composition such that, despite sharing a specific chromosomal aberration, not all cells of a given cancer demonstrate a completely identical cytogenetic profile. This intratumoral genetic heterogeneity extends to the level of individual genes and DNA mutations as shown by next-generation sequencing technologies and is fully expected based on the fact that tumor (and normal) cells acquire new mutations with each cell division. At a practical level, somatically acquired mutations that accumulate at defined frequencies can distinguish individual cells or tumor subclones and serve as a clock to mark and track their divergence from a common ancestor cell. The complexity of clonal architecture has been shown in hematological malignancies, including acute lymphoblastic leukemia (ALL) (Anderson et al., 2011) and acute myeloid leukemia (AML) (Ding et al., 2012) as well as other cancer types such as breast carcinoma (Shah et al., 2009), and is likely a universal feature of all cancers. It is also known that subpopulations of cells in an individual tumor can be morphologically or functionally distinct, e.g., display sensitivity or resistance to therapeutic agents. However, the relationship between intratumoral genetic heterogeneity and cancer cell function has not been well defined. Nevertheless, clonal evolution has major implications for understanding the cellular hierarchies and

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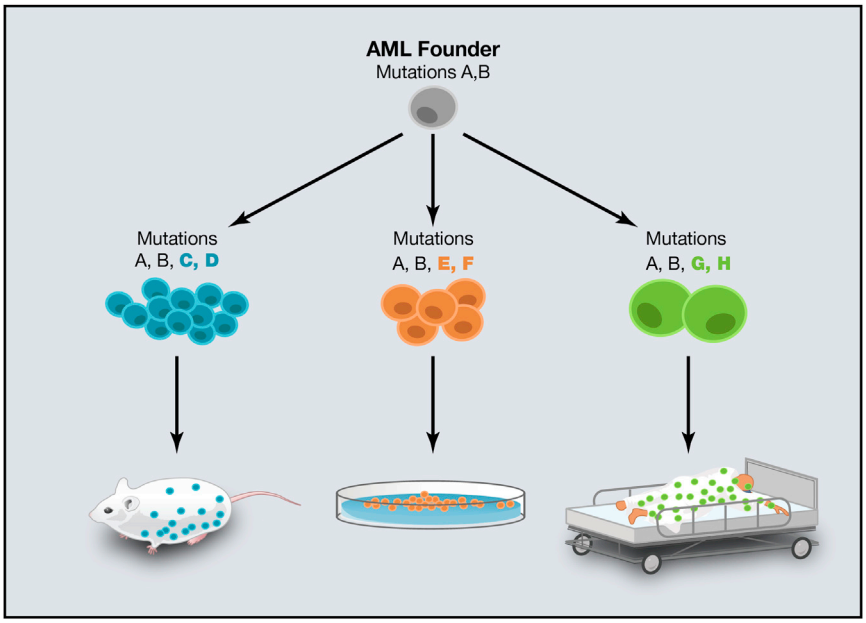

Figure 1. Functional and Genetic Heterogeneity of Primary AML
A founding AML clone arises with accumulation of a set of acquired "signature" mutations. During tumor progression, the founding clone evolves into genetically distinct subclones through the acquisition of new mutations. The complete spectrum of mutations defines morphological and phenotypic properties of the subclones that correlate with distinct functional characteristics, such as in vitro growth, engraftment in immune-deficient mice, or relapse in patients.

interrelationships in tumors, as well as the development and application of targeted therapies in the rapidly unfolding era of personalized medicine. In this issue of *Cancer Cell*, Klco et al. (2014) explored the correlation of clonal architecture with functional heterogeneity in AML. Rather than a melting pot blend of operational and genomic diversity, the data support that AML comprises a salad bowl of distinct subclones whose functional differences may be genetically determined (Figure 1).

Whole genome (and capture-based targeted) sequences were analyzed to determine the somatic mutations present in unfractionated bone marrow cells of patients at presentation with de novo AML encompassing a range of morphological and genetic subtypes. The spectrum of mutations and their fractional representation was used to define the founding clone, from which all leukemic cells were descended, and also identify leukemic cell subpopulations possessing the "signature" variants of the founding clone as well as additional subclonal sequence variants that arose during tumor evolution. Sequence analysis of single cells purified by cell sorting in several AMLs verified the identity of subclonal genotypes and the allele fractions deduced from unfractionated bone marrow samples.

The genetically defined subclones were evaluated under various biological and experimental conditions. The clonal architecture present in the bone marrow was consistently detected in the peripheral blood, indicating no major differences in trafficking properties among different AML subclones, unlike the regional intratumoral and metastatic variation reported in solid tumors (Navin et al., 2011). Mutations found in AML blast cells were often present in morphologically more mature myelomonocytic cells, demonstrating maintenance of at least minimal differentiation potential despite the presence of AML driver genes that otherwise antagonize maturation. Somatic mutations in rare peripheral blood B and T lymphocytes suggested the acquisition of some mutations in leukemic multi-potential hematopoietic stem-progenitor cells or even in pre-leukemic hematopoietic stem cells consistent with recent observations (Shlush et al., 2014). In some cases, morphologic or phenotypic features, as well as in vitro growth properties, correlated with distinct subclones, suggesting functional variation in differentiation potential that may be genetically determined.

The in vivo functional heterogeneity of cells comprising leukemia samples at disease presentation was interrogated by transplantation into immune-compromized mice. Unexpectedly, none of the resulting xenografts displayed a clonal architecture that was identical to that of the transplanted AML. Rather, subclones showed variable engraftment potential, and single subclones generally predominated in the engrafted mice despite the presence of multiple subclones in the injected sample. Relapsing AML subclones were not predicted by engraftment outcome or by the presence of recurring AML mutations. Thus, in many cases, there was no apparent relationship between the engrafting cells and the evolutionary hierarchy of the leukemia subclones in the patient. However, these results should be interpreted with caution. Although the functional heterogeneity among AML clones was clearly demonstrated, the engrafted subclone in some cases was dictated by the recipient mouse strain used. This underscores that xeno-engraftment can be affected by a variety of technical factors that were not optimized, such as mouse strain and preconditioning, route of injection, number of injected cells, and time for engraftment/disease assessment. The application of next-generation sequencing techniques in future studies should illuminate the relative influence of these various factors on the clonal compositions and clinical significance of engrafting leukemia cells.

AML cells capable of engrafting in xenograft assays, and thus establishing disease in mice, are operationally defined as leukemia-initiating cells or leukemia stem cells (LSCs) (Lapidot et al., 1994). Previous studies have shown that LSCs defined by this experimental approach may be phenotypically heterogeneous (Goardon et al., 2011). The studies of Klco et al. (2014) extend this to suggest that LSCs may also be genetically heterogeneous. Importantly, the founding AML clone defined genetically may not necessarily be the same as the LSC clone defined functionally by xenotransplantation. This likely reflects that xeno-transplant models exert selective growth pressures through the mouse

microenvironment or lack of immune system that are not equivalent to those encountered by leukemia cells in the patient. The authors' results highlight the clonal and functional diversity of LSCs and suggest that future studies should include an integration of genetic and functional data as part of their characterization.

The authors correlated the presence of specific subclones marked by their respective mutational spectra with functional readouts, but the mutations that mechanistically account for the observed functional differences are unknown. It will be important to define the genetic (or epigenetic) determinants that underlie the observed biology, particularly the genes or pathways that may promote engraftment in various immune-compromised mouse models and relapse in patients.

Xenograft models also serve an instrumental role in the preclinical stages of cancer therapeutics development. Indeed, almost every FDA-approved anticancer drug in the modern era has been tested in such models. However, they are known to have limitations for predicting clinical responses in solid tumors (Sharpless and Depinho, 2006), and the results of Klco et al. (2014) underscore potential constraints of the approach to evaluate drug efficacy in AML, although previous studies have demonstrated their value in predicting early relapse in ALL (Meyer et al., 2011). Clonal analysis through whole genome sequencing provides a powerful approach for assessing the fidelity of xenografts in support of ongoing efforts to devise novel targeted therapeutics directed at the pathways and mutant factors that sustain critical functions of founding clones and/or cancer stem cells.

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Therapeutic Opportunities for Medulloblastoma Come of Age

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In this issue of *Cancer Cell*, Kool and colleagues reveal clear genetically defined subclasses of the sonic hedgehog (SHH) subclass of medulloblastoma. This molecular dissection of the SHH subclass is not simply a cutting-edge advance; the data have profound impact on clinical trial design and decision-making.

Twenty years ago, the world health organization guidelines and revered neuropathologists encouraged us to "lump" any small round blue cell tumor in the brain into a single category for the purpose of clinical trials and therapeutics. The only way to improve survival in the "age of the lumpers" was to intensify therapy, so we collectively brought the kids to the very edge of life and death. This resulted in improved survival rates at the expenses of both short- and long-term toxicity (Jakacki et al., 2012; Packer et al., 2006).

It is now the age of the "splitters". In the wake of a half dozen outstanding medulloblastoma genomics papers in 2012, Kool et al. (2014, in this issue of *Cancer Cell*) now further show us that there are at least three distinct molecular subclasses within the sonic hedgehog (SHH)-driven subclass of medulloblastoma. These data create a double-edged sword, establishing clear responder hypotheses while revealing that, as we

microenvironment or lack of immune system that are not equivalent to those encountered by leukemia cells in the patient. The authors' results highlight the clonal and functional diversity of LSCs and suggest that future studies should include an integration of genetic and functional data as part of their characterization.

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In this issue of *Cancer Cell*, Kool and colleagues reveal clear genetically defined subclasses of the sonic hedgehog (SHH) subclass of medulloblastoma. This molecular dissection of the SHH subclass is not simply a cutting-edge advance; the data have profound impact on clinical trial design and decision-making.

Twenty years ago, the world health organization guidelines and revered neuropathologists encouraged us to "lump" any small round blue cell tumor in the brain into a single category for the purpose of clinical trials and therapeutics. The only way to improve survival in the "age of the lumpers" was to intensify therapy, so we collectively brought the kids to the very edge of life and death. This resulted in improved survival rates at the expenses of both short- and long-term toxicity (Jakacki et al., 2012; Packer et al., 2006).

It is now the age of the "splitters". In the wake of a half dozen outstanding medulloblastoma genomics papers in 2012, Kool et al. (2014, in this issue of *Cancer Cell*) now further show us that there are at least three distinct molecular subclasses within the sonic hedgehog (SHH)-driven subclass of medulloblastoma. These data create a double-edged sword, establishing clear responder hypotheses while revealing that, as we

further define the molecular drivers for each patient, we will at some point lose our gold-standard phase III trials as a definitive tool because of small patient numbers.

Among the four molecular subclasses of medulloblastoma, SHH-driven tumors represent about 25%–30% of all patients. SHH pathway drives the majority of medulloblastomas in infants and adults, whereas tumors with the SHH molecular signature are uncommon in children between the ages of 4 and 17.

Therapeutic agents targeting the SHH pathway were developed following the observation that cyclopamine derivatives caused medulloblastoma cell death and regression in preclinical models (Berman et al., 2002). Newer-generation drugs targeting the smoothened protein are now in clinical trials. They are not expected to affect disease caused by *SUFU* mutations or *GLI2*- or *MYCN*-amplifications. Whereas certain SMO mutations were previously shown to reduce affinity for cyclopamine-like SMO inhibitors, it is not yet clear whether clinically relevant SMO mutations interfere with the activity of modern antagonists. This is why precise genomic analyses for each child are warranted.

In the study published in this issue of *Cancer Cell*, nonoverlapping mutations in known SHH pathway genes occurred in 87% of the 133 cases analyzed. This includes mutations in *SHH*, *SMO*, *PTCH1*, or *SUFO* as well as *TP53* mutations with concordant *GLI2* or *MYCN* amplification and occurred in about half of the remaining cases (Figure 3 of Kool et al., 2014). The results of the current study (stratified by age) and my perspective on therapeutic opportunities are highlighted below.

Infants (0–4 years): *PTCH1* and *SUFU* mutations each drive approximately one-third of infant medulloblastomas. A small fraction of the remainder are driven by *SMO* or *SHH* mutations or *MYCN* amplification. *TP53* mutations are absent in this age group. Infants respond exceptionally well to surgery and traditional chemotherapy, although toxicity is a major problem (Geyer et al., 2005). The likelihood of testing SMO inhibitors in infants with recurrent SHH-driven medulloblastoma is vanishingly small, because these tumors are rare and few children relapse. Were it not for concerns about SMO inhibitors interfering with cerebellar or bone growth plate development, it would be appealing to consider whether newly-diagnosed infants with complete surgical resection and *PTCH1* mutations could postpone or avoid chemotherapy if treated with a SMO inhibitor. As SHH-driven cerebellar granule cell proliferation is largely complete by six months of age, the older children in this group could be candidates for SMO inhibitors if current clinical trials reveal the absence of bone growth toxicity.

Children (4–17 years): nearly half of the patients in this age range have a mutation in *PTCH1* as an apparent driver. Whether SMO antagonists could replace selected more toxic chemotherapy agents in newly-diagnosed patients would be a very interesting question to address in a randomized clinical trial. Of the remaining patients, most have a *TP53* mutation and amplification of *GLI2* and *MYCN*. These events occur in conjunction with or as a consequence of chromothripsis. These patients experience the poorest outcome among SHH-driven tumors (Li et al., 1988; Zhukova et al., 2013), and most cases are associated with large cell anaplasia (LCA) pathology. Because of the poor outcome of LCA patients, the Children's Oncology Group now treats children with LCA on high-risk protocols (Gajjar et al., 2013). This involves doubling/nearly doubling the amount of craniospinal irradiation. It is possible, but far from certain, that increased radiation will help these patients survive. But if so, the cost may be second malignancies as patients with germline *TP53* mutation (Li Fraumeni syndrome) are at increased risk of second malignancy (Li et al., 1988).

Adults (over 17 years): over half appear to be driven by *PTCH1* mutations, whereas most remaining cases seem to be driven by *SMO* mutations. Because of this, Kool et al. (2014) suggest that 82% of adult cases are potentially responsive to SMO antagonists. This may be an overestimate, because certain SMO mutations render certain SMO antagonists relatively ineffective. The authors make a compelling case for adding a PI3K/AKT inhibitor to SMO antagonists in cases where immunohistochemistry reveals elevated p-AKT or p-S6. In the absence of p-AKT or p-S6 activation, nearly 100% of adult medulloblastoma patients (n = 41) were alive following standard therapy, whereas survival was quite poor among those with elevated p-AKT or p-S6. The number of younger patients with elevated p-AKT or p-S6 is too small to draw conclusions.

Nearly half of the children with malignant brain cancer will die from their disease. Of those who survive, only one-fourth will be able to participate in a long-term relationship or job as adults because of the neurotoxicity of craniospinal irradiation and chemotherapy. There is room to be bold in our research and clinical trial design. Might we find ways to eliminate radiotherapy and alkylating chemotherapy altogether for the Li Fraumeni syndrome patients with germline *TP53* mutations that are at very high risk for second malignancy? Might we treat adult patients with both a SMO inhibitor and a PI3K/AKT inhibitor if they have SHH-driven medulloblastomas with elevated p-AKT or p-S6? Why not do this at the time of initial presentation rather than waiting for recurrence?

Because clinical trials will necessarily be much smaller than those in the past, it will be necessary to find agents with profound effects on the disease in order to have sufficient statistical power. Paying close attention to basic pharmacological properties (e.g., crossing the blood brain barrier) is critical. Reconsidering trials in which targeted therapies are used for cytoreduction following biopsy and prior to surgery might be indicated for some groups of patients to reduce the incidence of surgery-induced cerebellar autism (a.k.a. posterior fossa syndrome), a brutal reminder of our current somewhat heavy-handed approaches. Such "therapeutic window" trials would also offer a sense of drug activity in newly-diagnosed patients using quantitative response criteria.

Patients represented in this study are the poster children for asking the FDA to relax barriers to use genomic data to guide therapeutic decisions in those with rare diseases, particularly for clinical trials conducted by cooperative groups. Pediatric neuro-oncologists work together as a global community to help our patients (Gottardo et al., 2014). The very fact that Kool et al. (2014) collected 133 cases of this rare cancer speaks to our collaborative nature, and many other rare-disease groups are similarly collaborative and committed to making thoughtful decisions. Caring for children with brain cancer is difficult enough without having

to fight to use the knowledge that we have in hand, such as the elegant work by Kool et al. (2014).

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PI3Kδ Inhibition Hits a Sensitive Spot in B Cell Malignancies

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A PI3Kδ-selective inhibitor shows impressive clinical activity in chronic lymphocytic leukemia and indolent B cell non-Hodgkin's lymphomas. In these malignancies, the PI3K pathway is not mutationally activated as in many other cancers, but it is important for mediating supportive cues from the cancer microenvironment and the B cell antigen receptor.

Major cancer drug development efforts currently focus on targeting PI3K pathway components based on the overwhelming genetic evidence in support of a role for this pathway in malignancy (Fruman and Rommel, 2014). The PI3K pathway has multiple nodes and is mediated by eight distinct PI3K catalytic subunits, among which the p110 subunits (p110α, β, γ, and δ) transduce signals downstream of tyrosine kinases, G protein-coupled receptors, Ras, and other small GTPases. Whereas p110α and p110β show broad tissue distribution, p110γ and p110δ are highly expressed in leukocytes and control immune responses. *PIK3CA*, which encodes p110α, is frequently mutated and activated in solid tumors, but not in hematological malignancies. Constitutively activating p110δ mutations have recently been found in a human primary immunodeficiency disorder without apparently predisposing to cancer (Fruman and Rommel, 2014). Other PI3K catalytic subunits have not been found to be mutated in disease.

Two recent studies report impressive clinical activity of idelalisib, an oral ATP-competitive and reversible p110δ inhibitor, in relapsed chronic lymphocytic leukemia (CLL) and indolent non-Hodgkin's lymphomas (iNHLs). All patients were heavily pretreated and/or not sufficiently fit to undergo standard cytotoxic chemotherapy. Idelalisib was tested in combination with rituximab in CLL (Furman et al., 2014) and as a single agent in iNHL (Gopal et al., 2014). Rituximab is currently commonly used, although not approved, as a single agent for treating relapsed CLL.

NHLs are a heterogeneous group of lymphomas that arise primarily in the lymph nodes, and CLL is usually an indolent tumor with accumulation of malignant B cells in lymphatic tissues, bone marrow, and blood. The course of these diseases is highly variable, with some patients never needing treatment. For those who do, the standard approach is chemotherapy, which can lead to significant toxicities and immunosuppression, and most patients will relapse. Recent years have witnessed the development of more targeted therapies for hematological malignancies, in particular, small molecule inhibitors of kinases that mediate intracellular signal transduction in lymphocytes, such as Syk and Btk. Such agents would be expected to be more tumor selective and better tolerated. The rationale for targeting p110δ in B cell malignancy is its key role in signal transduction downstream of various B cell receptors, including the B cell antigen receptor (BCR), cytokine/chemokine receptors, and adhesion molecules (Figure 1), contributing to the regulation of proliferation, differentiation, migration, and survival (Fruman and Rommel, 2011).

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PI3Kδ Inhibition Hits a Sensitive Spot in B Cell Malignancies

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A PI3Kδ-selective inhibitor shows impressive clinical activity in chronic lymphocytic leukemia and indolent B cell non-Hodgkin's lymphomas. In these malignancies, the PI3K pathway is not mutationally activated as in many other cancers, but it is important for mediating supportive cues from the cancer microenvironment and the B cell antigen receptor.

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Two recent studies report impressive clinical activity of idelalisib, an oral ATP-competitive and reversible p110δ inhibitor, in relapsed chronic lymphocytic leukemia (CLL) and indolent non-Hodgkin's lymphomas (iNHLs). All patients were heavily pretreated and/or not sufficiently fit to undergo standard cytotoxic chemotherapy. Idelalisib was tested in combination with rituximab in CLL (Furman et al., 2014) and as a single agent in iNHL (Gopal et al., 2014). Rituximab is currently commonly used, although not approved, as a single agent for treating relapsed CLL.

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The CLL study was a randomized, double-blind phase III trial with 220 patients, who were all given rituximab in combination with either idelalisib or placebo (Furman et al., 2014). The study was stopped early by the safety monitoring board based on overwhelming efficacy, with the idelalisib group showing a significantly higher overall response rate (81% versus 13%) and overall survival (92% versus 80% at 12 months). Median duration of progression-free survival was 5.5 months in the non-idelalisib cohort and was not reached in the idelalisib group. The iNHL study was a single arm, phase II trial in which 125 patients were given idelalisib continuously until disease progression or toxicity (Gopal et al., 2014). There was a 57% overall response rate that was similar across all iNHL subtypes. Median progression-free survival was 11 months, and median overall survival was 20.3 months, with overall survival at 1 year estimated to be 80%. Overall, idelalisib was well tolerated, with rapid, durable responses independent of previous therapies or the genetic landscape of the tumors. Of the patients treated with idelalisib, ≥90% showed a reduction in lymph node size.

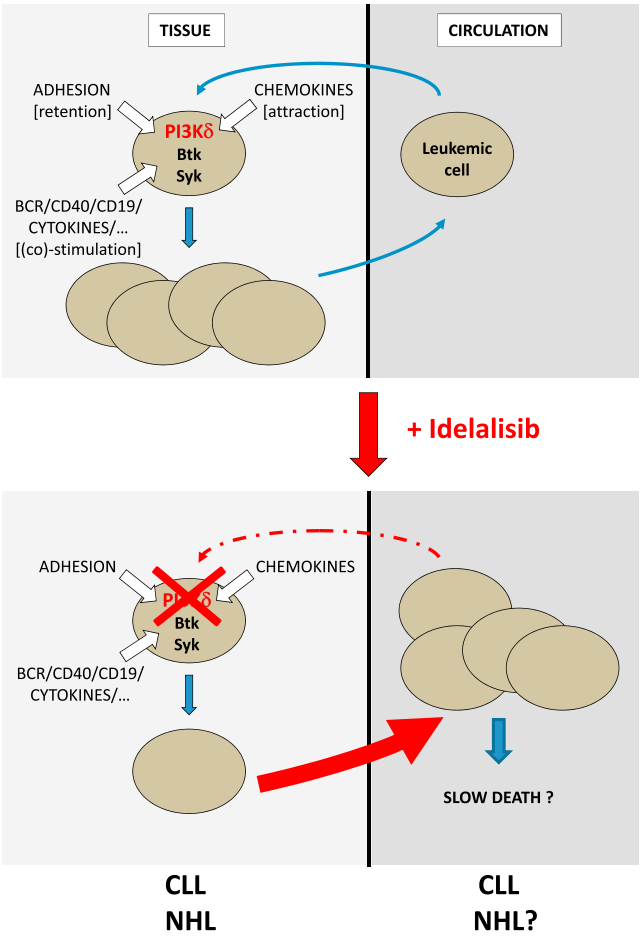

Figure 1. Role of PI3Kδ in CLL Cell "Life Cycle"
CLL cells circulate in the peripheral blood and are attracted by chemokine gradients to lymph nodes and bone marrow, where they encounter signals from adhesion molecules (such as integrins and selectins), cytokines and chemokines, costimulatory molecules (such as CD40), and possibly also BCR signaling. PI3Kδ, Btk, and Syk are important intracellular transducers of these stimuli, and interference with their activity results in both release of the CLL cells into the circulation (lymphocytosis) and blockade of their re-entry. Such cycles have not been demonstrated for NHL, but these cells are also expected to require these signaling molecules for trophic signals from their microenvironment.

What is the basis for this impressive clinical impact of p110δ blockade? An important clue comes from the observation that idelalisib rapidly induces lymphocytosis, an increase in the number of circulating lymphocytes, in CLL patients, correlating with lymph node shrinkage, a phenomenon also observed with inhibitors of Syk and Btk (Burger and Montserrat, 2013). In B cells, Btk, Syk, and p110δ are part of a signaling network involved in the interaction and communication of B cells with their environment, which is of critical importance in the life cycle of CLL cells (Figure 1). This cycle involves migration of leukemic cells from the peripheral blood to the lymph nodes, spleen, and bone marrow, where they become activated by microenvironmental stimuli, including costimulatory molecules, chemo/cytokines, cell adhesion, and possibly also antigen-stimulation of the BCR, leading to their survival and proliferation. CLL cells then egress back into the circulation, where they are mostly noncycling and are thought to die unless they home back to the tissue compartment (Burger and Montserrat, 2013).

Inhibition of p110δ, like that of Btk and Syk, may interfere with the CLL cycle at various levels, leading to mobilization of tissue-resident CLL cells into the blood and blocking re-entry to the stroma. Sustained interference with this recirculation cycle may make CLL cells "run out of fuel," and the lack of trophic signals sensitize them to combination treatments. It is likely that the dynamic life cycle of CLL could explain why this disease, among the different B cell malignancies, is the most responsive to B cell signaling blockers. Indeed, the in vivo mechanism of action of p110δ inhibition in NHL is less clear, although intracellular signaling in these B cell malignancies is also known to be partially controlled by PI3Kδ. While interference with BCR signaling is often posited as the key mechanism of therapeutic activity of inhibitors targeting p110δ, Btk, or Syk, its relative importance compared to other B cell stimuli is unclear. It is also not clear if p110δ inhibitors have direct antiproliferative or cytotoxic effects in vivo, given that they only have a modest impact on in vitro cell proliferation and survival of primary B cell malignancies.

Residual PI3K activity present in immune cells upon p110δ inhibition by idelalisib might explain its lack of overt immune suppression. Inhibitors that block multiple p110 isoforms are in clinical trials for treating solid tumors (Fruman and Rommel, 2014). As these compounds more effectively block leukocyte signaling than p110δ-selective inhibitors (Iyengar et al., 2013), should they also be tested in hematological malignancies? While broad(er) spectrum PI3K inhibitors would indeed hit the malignant cells harder, they might be less well tolerated and be more immune suppressive upon long-term administration. Normal B cell development is only modestly affected upon p110δ inactivation, but is blocked upon co-inactivation

of p110α (Ramadani et al., 2010). Combined inactivation of the leukocyte selective p110δ and p110γ during mouse development results in severe immune defects (Ji et al., 2007), but temporary co-targeting of these kinases might be of therapeutic benefit, as shown in a mouse model of T cell leukemia (Subramaniam et al., 2012).

Many new targeted therapies for B cell malignancy are being developed, offering a bewildering number of possible drug combinations and dose regimens. One consideration will be drug tolerability, and the available evidence suggests that p110δ inhibitors score well in this respect. It will be important to assess how long p110δ has to be inhibited in order to obtain durable effects. Not all patients respond to p110δ or other targeted therapies, and it will be critical to identify biomarkers that predict and/or monitor drug responsiveness (Woyach et al., 2014).

The research and development that underpins the progress represented by idelalisib is a prime example of the identification and exploitation of a key vulnerability in cancer cells. The hope is to uncover more such druggable critical nodes in the future and to do it quicker.

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Cancer Cell
Perspective

Dragging Ras Back in the Ring

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Ras proteins play a major role in human cancers but have not yielded to therapeutic attack. Ras-driven cancers are among the most difficult to treat and often excluded from therapies. The Ras proteins have been termed "undruggable," based on failures from an era in which understanding of signaling transduction, feedback loops, redundancy, tumor heterogeneity, and Ras' oncogenic role was poor. Structures of Ras oncoproteins bound to their effectors or regulators are unsolved, and it is unknown precisely how Ras proteins activate their downstream targets. These knowledge gaps have impaired development of therapeutic strategies. A better understanding of Ras biology and biochemistry, coupled with new ways of targeting undruggable proteins, is likely to lead to new ways of defeating Ras-driven cancers.

Fifty years have passed since the transforming power of Ras genes was first recognized. Harvey sarcoma virus, Kirsten sarcoma virus, and Rasheed sarcoma virus contain Ras genes (so named for their role in forming *rat* sarcomas; reviewed in Barbacid, 1987; Karnoub and Weinberg, 2008). These retroviruses initiated tumors efficiently and, using temperature-sensitive mutants, were shown to be necessary for tumor maintenance (Shih et al., 1979). They formed part of a fascinating collection of retroviruses that was assembled in the 1970s, each able to transform cells in culture and in avian and rodent models. These experiments were, essentially, unbiased screens for genes that cause cancer; the nature of the proteins that the genes encoded was completely unknown. Remarkably, the majority of these viruses encoded proteins that were later identified as components of the tyrosine kinase-Ras signaling pathway (Vogt, 2012), even though the biochemical nature of these proteins was unknown, and tyrosine kinase activity had not been discovered (Eckhart et al., 1979). Of the hundreds of mutant proteins now known to contribute to cancer that could have been identified in these assays, including those involved in DNA repair, cellular metabolism, RNA splicing, and the other hallmarks of cancer (Hanahan and Weinberg, 2011), those in the tyrosine kinase-Ras pathway stand out as the major drivers and have been the richest source of targets of successful cancer therapies (Abl, epidermal growth factor receptor [EGFR], Her2/neu, B-Raf, Kit, ALK, etc.). These successes can therefore be attributed to the central, dominant role of this pathway in cancer, as well as the fortuitous abundance of druggable targets.

However, specific therapies have not been developed for mutant Ras proteins themselves or for the cancers that they drive. Worse yet, tumors driven by Ras genes are excluded from treatment with other targeted therapies. Early efforts to block Ras cancers by preventing Ras farnesylation, once thought to be an essential posttranslational modification for Ras activity, were thwarted by the unexpected presence of a backup system (geranylgeranyltransferase) that restored activity of K-Ras and N-Ras after farnesyltransferase treatment. Likewise, efforts to kill Ras cancers by blocking one of Ras' major downstream effectors, Raf kinase (Figure 1), ran into the unexpected discovery that, in Ras-transformed cells, Raf inhibitors activate the pathway rather than inhibit it (see below and discussion in Holderfield et al., 2013 and Lito et al., 2013). MAP kinase kinase (MEK) inhibitors and phosphatidylinositol 3-kinase (PI3K) inhibitors have not yet shown significant clinical activity in Ras cancers, for reasons relating to feedback loops and poor therapeutic windows, among other issues discussed below.

A convergence of urgent unmet clinical needs and advances in drug discovery has energized new efforts to target Ras cancers within academic centers and in the biopharmaceutical industry. To catalyze these renewed efforts, the National Cancer Institute recently launched a national Ras program at Frederick National Laboratory for Cancer (see http://RasCentral.org), whose goal is to fill critical knowledge gaps that are essential to target Ras cancers effectively and to engage the research community toward solving the Ras problem. Here, we will discuss some of these knowledge gaps, as well as recent advances and the challenges that lie ahead.

Ras Mutations in Cancer

Ras genes were the first oncogenes identified in human cancer cells. In a series of classic experiments, the groups of Weinberg, Cooper, Barbacid, and Wigler independently identified the transforming genes from T24/EJ bladder carcinoma cells as H-Ras (Der et al., 1982; Parada et al., 1982; Santos et al., 1982; Taparowsky et al., 1982). More than 30 years later, Ras genes are well established as the most frequently mutated oncogenes in human cancer (Table 1), though H-Ras itself is rarely one of them. Although these numbers are, by now, painfully familiar, they underscore major gaps in our knowledge of Ras biology. Most obviously, we do not understand why K-Ras mutation is much more frequent in human cancer than N-Ras or H-Ras, even though each of these is a powerful transforming gene in model systems, and all forms are expressed widely in adult tissues and in tumors.

A simple explanation for the high frequency of K-Ras mutations, relative to H-Ras and N-Ras, is that the K-Ras protein has unique properties that favor oncogenesis. At first sight, this seems unlikely because the Ras proteins are highly conserved, especially in their effector-binding regions where they are actually identical. However, K-Ras, but not N-Ras or H-Ras, confers

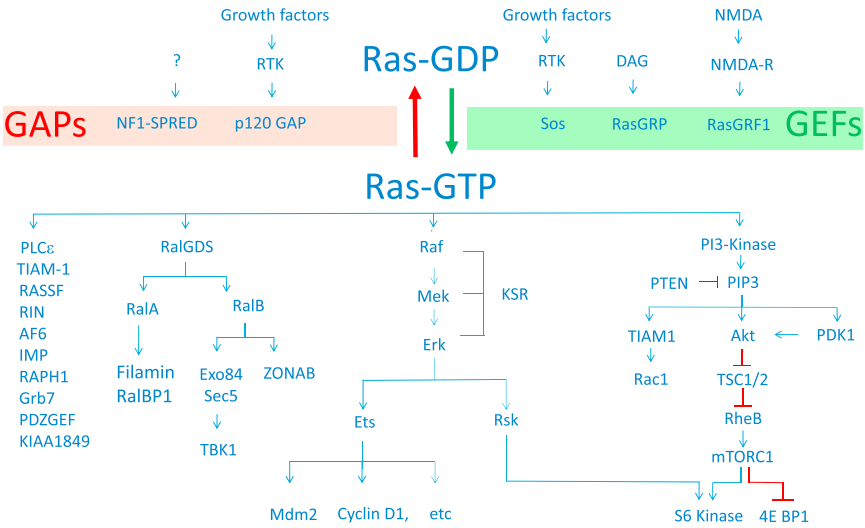

Figure 1. Simplified View of the Ras Pathway

Ras proteins are converted from their GDP state to their GTP state by GEFs, in response to upstream signals (Bos et al., 2007). GAPs convert Ras-GTP back to Ras-GDP. p120 GAP does this when recruited to activated RTKs. The signal that directs NF1 (neurofibromin)/SPRED to inactivate Ras is not known. Several other GAPs are capable of downregulating Ras (Bos et al., 2007). Ras-GTP binds and activates multiple downstream effectors. The group of proteins shown on the left includes potential effectors whose significance is less well understood relative to RalGDS, Raf kinases, and PI3Ks (Gysin et al., 2011). Protein families are represented as single proteins to simplify the schematic; in addition, feedback loops are not included.

stem-like properties on certain cell types (Quinlan and Settleman, 2009). K-Ras-4B, the most highly expressed splice variant of K-Ras, binds calmodulin; H-Ras and N- Ras do not (Villalonga et al., 2001). We believe that this unique property of K-Ras-4B confers stem-like properties to cells expressing oncogenic K-Ras-4B proteins (M. Wang and F.M., unpublished data).

Analysis of human syndromes caused by germline mutations in H-Ras or K-Ras supports the idea that K-Ras is a stronger oncogene. Unexpectedly, humans can tolerate germline-activating mutations in H-Ras—the same activating mutations that drive somatic mutations. Costello syndrome, which is characterized by germline H-Ras mutations, is associated with a broad spectrum of developmental abnormalities and a high risk for rhabdomyosarcomas and neuroblastomas (reviewed in Rauen, 2013). It is puzzling that these individuals do not succumb to malignancies associated with sporadic H-Ras mutations (Table 1). Although fully activating alleles of H-Ras can be tolerated, fully activated alleles of K-Ras may not. Variant alleles of K-Ras that account for a small fraction of Noonan's syndrome and cardiofaciocutaneous syndrome are weakly activated relative to their sporadic oncogenic counterparts (Schubbert et al., 2007).

Further support for the idea that K-Ras has functions distinct from H-Ras and N-Ras comes from analyses of the roles of Ras genes in development. Mice that lack K-Ras die during embryogenesis, whereas mice lacking H-Ras and/or N-Ras are viable (Johnson et al., 1997). However, replacing K-Ras with H-Ras at the K-Ras genomic locus allows mice to develop, suggesting that differential regulation of K-Ras and H-Ras gene expression determines their relative importance in development rather than the properties of the proteins themselves (Potenza et al., 2005). Furthermore, Balmain and colleagues discovered that these H-Ras knock-in mice develop tumors in response to carcinogens at normal frequencies, except that they are now driven by H-Ras instead of K-Ras (To et al., 2008). These data argue strongly that the locus is critical and that the specific Ras paralog encoded at that locus does not affect the frequency at which tumors arise. Equally important, they find that K-Ras-4A, not K-Ras-4B, is necessary for lung tumor initiation, although K-Ras-4B is much more highly expressed during progression. This supports the idea that K-Ras-4B is the more important target in established tumors but raises the concern that K-Ras-4A may have an important role in minor stem-like populations of established tumors. These findings point toward an urgent need to validate K-Ras-4A and K-Ras-4B as drug targets, a major issue that has not yet been addressed.

Different frequencies of K-Ras, N-Ras, and H-Ras mutations in human tumors may also reflect differences in gene expression resulting from differential codon usage; rare codons limit K-Ras expression and thus allow more efficient oncogenesis by preventing oncogene-induced senescence (Lampson et al., 2013). In addition, different rates of DNA repair have been reported for the K-Ras gene relative to N-Ras and H-Ras (Feng et al., 2002).

The underlying reasons for different frequencies of specific activating mutations are not well understood either. Some of these differences reflect different mutagenic insults to the genome; the G12C mutation, for example, is a hallmark of exposure to tobacco smoke and, accordingly, is the most common mutation in K-Ras in lung cancer (reviewed in Prior et al., 2012; Table 2). Other differences in frequency may reflect different biological properties of mutant proteins. For example, G12C and G12V K-Ras mutations in lung adenocarcinoma preferentially activate the RalGDS pathway, whereas G12D prefers the Raf/mitogen-activated protein kinase (MAPK) and PI3K pathways (Ihle et al., 2012). In addition, mutations at codon 61 have a more profound effect on intrinsic GTPase when these Ras proteins are bound to Raf kinase. This may drive a stronger signal through this effector pathway and account for higher frequency of N-Ras position 61 mutations in melanoma, a disease frequently driven by hyperactivation of Raf kinase through B-Raf mutations (Buhrman et al., 2010).

From a clinical viewpoint, lung adenocarcinomas driven by K-Ras mutations at G12C and G12V have a worse outcome than G12D, possibly because these mutations engage different downstream effectors as described above (Figure 1; Ihle et al., 2012). As MEK and PI3K inhibitors are tested in the clinic, it will be important to ask whether Ras alleles respond differently to these treatments. Patients suffering from cancers driven by any of these Ras mutations are excluded from treatment with

Table 1. Frequency of Ras Isoform Mutations in Selected Human Cancers

Primary Tissue	*KRAS* (%)	*HRAS* (%)	*NRAS* (%)	Total (%)
Pancreas	71	0	<1	71
Colon	35	1	6	42
Small intestine	35	0	<1	35
Biliary tract	26	0	2	28
Endometrium	17	<1	5	22
Lung	19	<1	1	20
Skin (melanoma)	1	1	18	20
Cervix	8	9	2	19
Urinary tract	5	10	1	16

Data were compiled from the Catalogue of Somatic Mutations in Cancer (COSMIC) version 67. All human cancers that had total Ras mutation frequencies above 15% are listed.

Table 2. Incidence of *KRAS* Mutations in Three Human Cancers

	All *KRAS*	G12C	G12D	G12V	G13D
Colorectal	60,000	5,700	25,000	15,700	13,600
Lung	45,600	23,000	9,200	11,900	1,500
Pancreas	32,200	1,000	19,500	11,500	200
Total new cases/year	137,800	29,700	53,700	39,100	15,300

Shown are the numbers of new cancer cases per year in the United States that contain the most frequent *KRAS* mutant alleles. Data are based on estimated new case incidence values from the National Cancer Institute and primary tumor mutation frequency data from COSMIC v.67.

cetuximab (colorectal cancer) or erlotinib (lung adenocarcinoma) because these treatments are ineffective for cancers with these Ras mutations and may even increase rates of progression. Likewise, malignant melanomas with mutant N-Ras are excluded from treatment with vemurafenib. However, surprisingly, K-Ras-G13D-bearing colorectal cancers may show clinical benefit when treated with cetuximab. This result challenges our understanding of how these Ras mutations actually function in clinical situations (De Roock et al., 2010).

Even the prototypic oncogenes of Harvey and Kirsten sarcoma viruses are not fully understood; each has a codon 12 mutation, but each also carries a mutation of alanine 59 to threonine, which becomes phosphorylated by guanosine triphosphate (GTP). This must have helped Scolnick and colleagues (Shih et al., 1979) identify Ras' crucial guanosine diphosphate (GDP)/GTP properties; without covalent phosphorylation, association with these nucleotides would have been very hard to detect. However, how phosphorylation at threonine 59 contributes to Ras' potent oncogenicity is unclear. This A59T mutation inhibits Ras-Raf interaction (Shirouzu et al., 1994) and is extremely rare in human cancer. These anecdotes simply remind us that after 50 years, we still have a lot to learn about the biological and biochemical functions of Ras proteins.

Although K-Ras has emerged as by far the major Ras gene mutated in human cancer, it is surprising that other activating mutations in other members of the Ras superfamily, such as R-Ras or Rap proteins, occur very rarely. This is surprising because these proteins share identical or near-identical effector-binding regions. However, only H-Ras, N-Ras, and K-Ras are capable of binding and activating Raf kinases, and this unique property may well account for their predominance as human oncogenes. In contrast, the closely related R-Ras proteins bind and activate PI3Ks but are rarely mutated in human cancer (Rodriguez-Viciana et al., 2004).

Activating mutations in Ras genes, coupled with a long history of Ras biology, implicate these mutant Ras proteins as major drivers in many cancers. Loss of the Ras GTPase-activating protein (GAP) neurofibromin inculpates hyperactive wild-type Ras proteins as drivers in many more cancers. Somatic loss of neurofibromin expression by mutation, deletion, or by other means occurs in about 14% glioblastoma, 13%–14% melanoma, 8%–10% lung adenocarcinoma, and at single-digit frequency in most other cancers (E.A. Collisson, personal communication). Neurofibromin must now be considered as a major tumor suppressor, along with p53 and phosphatase and tensin homolog, in human cancers.

Loss of neurofibromin is usually mutually exclusive with Ras mutation and receptor tyrosine kinase (RTK) activation, suggesting that these genetic events represent different ways of activating similar pathways. However, the precise consequences of losing neurofibromin are not entirely clear. Levels of Ras-GTP are high in cells lacking neurofibromin, but which forms of hyperactive wild-type Ras proteins are most important to the malignant phenotype is a more difficult question. Perhaps elevated H-Ras, N-Ras, K-Ras-4A, and K-Ras-4B all contribute to some extent. However, neurofibromin is also a GAP for R-Ras proteins, and hyperactivation of these proteins can also contribute to the malignant phenotype because R-Ras proteins activate p110α, p110γ, and p110δ isoforms of PI3Ks (Marte et al., 1997; Huang et al., 2004).

Recently, Legius and colleagues (Brems et al., 2007) discovered mutations in the Sprouty-related protein, SPRED1, in a form of neurofibromatosis type I (NF1) in which the neurofibromin gene is wild-type. This disease is now called Legius syndrome (Brems et al., 2007). SPRED1 has a well-established pedigree as a negative regulator of the Raf/MAPK pathway, though the mechanism has been unclear. However, the fact that loss of neurofibromin is, to a significant extent, phenocopied by loss of SPRED1, supports the idea that NF1 is a disease of hyperactive Ras and that the major function of neurofibromin is to turn Ras off. The neurofibromin protein itself is over 2,800 amino acids in length, and the GAP domain only accounts for about 300 amino acids, raising the possibility that neurofibromin has other functions that are not directly related to negative regulation of Ras. Most attempts to identify additional functions have failed, however, and it seems most likely that neurofibromin senses an unidentified cellular metabolite and downregulates Ras accordingly, just as p120 Ras-GAP senses phosphotyrosine residues, and downregulates Ras when it binds to these residues on activated receptors in the plasma membrane (reviewed in Bos et al., 2007). Whatever neurofibromin senses (if this model is correct) is likely to be conserved between *S. cerevisiae* and humans because the *S. cerevisiae* IRA1 and IRA2 proteins look very much like neurofibromin. Unfortunately, the complete lack of any recognizable domains or motifs outside the GAP domain and a SEC14 domain has not helped in identifying what these proteins recognize.

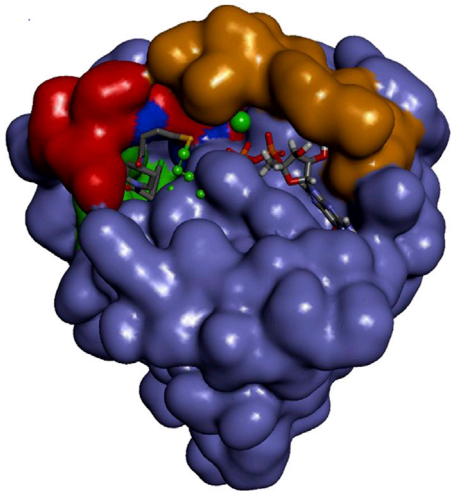

Figure 2. Structure Showing Small Molecule-Directed Electrophilic Attack of K-Ras-G12C
K-Ras-G12C (Protein Data Bank 4LUC_A) is displayed in surface representation. The cocrystallized ligands, GDP and N-(1-[(2,4-dichlorophenoxy)acetyl] piperidin- 4-yl)-4-sulfanylbutanamide, are shown in stick mode. The location of calcium ion is shown as a green ball. Switch 1 (28–38) and switch 2 (57–63) are highlighted by orange and red colors, respectively. Key ligand-interacting residue (C12, V9, V7, F78, I100, M72, Q99, and R68) positions are colored green. Position of C12 residue is shown in ball and stick (green). Note that residues 58 and 60 are part of both switch 2 and the key ligand-interacting group (shown in blue).

By comparing proteins that bind to wild-type SPRED1 versus mutants from Legius syndrome, we found that neurofibromin binds directly to SPRED proteins, via their EVH1 domains, and that SPRED proteins bring neurofibromin to the plasma membrane (Stowe et al., 2012). SPRED proteins also bind to c-Kit, and perhaps to other RTKs, suggesting that neurofibromin regulates Ras locally in response to specific receptor signaling, rather than simply suppressing Ras throughout the plasma membrane. In this case, loss of neurofibromin may lead to local activation of Ras that is coupled to specific receptors, suggesting that inhibitors of these receptors might reverse the effects of neurofibromin loss. The recent Cancer Genome Atlas analysis of mutations in lung cancer revealed an intriguing overlap between neurofibromin loss and Met amplification, suggesting a functional connection that merits further investigation (E.A. Collisson, personal communication).

Validation of Ras as a Target

Ras oncogenes can certainly initiate cancer in model organisms and probably do so in humans. However, their role in maintaining tumors is less clear. There is significant evidence that supports K-Ras as a continued candidate for direct therapeutic targeting, dating back to the classic studies of temperature-sensitive mutants of Ras, by Scolnick, Lowy, and colleagues and including microinjection studies with antibodies that block Ras activity (Kung et al., 1986) or block specific mutant alleles of Ras (Feramisco et al., 1985). Ablation of K-Ras in mouse models of lung adenocarcinoma (Fisher et al., 2001) or pancreas cancer (Ying et al., 2012) led to dramatic tumor regression, just as ablation of H-Ras leads to tumor regression in mouse models of melanoma (Chin et al., 1999). On the other hand, K-Ras knockdown in human cell lines resulted in a spectrum of responses, revealing a range of K-Ras dependencies (Singh et al., 2009). Assessment of Ras dependency in 3D culture systems suggests that this assay system is a more stringent measure of Ras dependency. These studies raise the question of what is the most relevant system to measure this essential parameter and, in general, responses to candidate therapeutics targeting K-Ras. Furthermore, the degree to which Ras genes are knocked down may be critical. Genetic ablation is obviously different than small interfering RNA- or small hairpin RNA (shRNA)-mediated knockdown. It is also clear that knocking down activated Ras can lead to hyperactivation of upstream pathways, such as EGFR signaling (Young et al., 2013). Presumably, these pathways are suppressed in cells with activated Ras and rebound when the suppressor is removed. Although this rebound effect may not be sufficient to sustain a malignant phenotype, it may offset pro-apoptotic effects associated with oncogene inactivation.

Do K-Ras Therapies Have to Be Allele Specific?

The most specific way to block oncogenic Ras would be to target the activating substitution itself. The first example was recently published by Shokat and colleagues, who identified electrophilic compounds that react covalently with cysteine-12 in G12C mutant K-Ras (Ostrem et al., 2013). These compounds interact selectively with the GDP form of K-Ras-G12C protein (Figure 2) and bind at a pocket near switch 2 that had not been apparent from analysis of crystal structures. A similar approach led to the identification of a GDP analog that covalently and specifically binds G12C and renders this oncogenic protein inactive (Lim et al., 2014). Perhaps other compounds could be identified that interact specifically with the G12D and G13D mutant forms using similar strategies. These brilliant experiments remind us that these proteins are in dynamic and flexible states that might present more opportunities for small molecule attack than was previously realized. Indeed, it is well established that Ras-GTP exists in two states, only one of which is active and each with distinct binding properties for effectors, GAPs, and nucleotide (Geyer et al., 1996; Liao et al., 2008).

The idea of targeting the GDP-bound form of an oncogenic mutant seems counterintuitive because we often think of oncogenic mutants as being locked in their GTP-bound states, signaling persistently downstream. However, codon 12 mutants retain measurable intrinsic GTPase activity, even though they are all refractory to GAP-mediated GTPase stimulation. Although GTP hydrolysis rates are slow, the GDP off rates are also slow, and indeed, oncogenic mutants often exist with similar levels of GTP and GDP: if intrinsic GTPase and GDP off rates were identical, Ras proteins would be 50% GTP bound and 50% GDP bound. This presents an opportunity for targeting the GDP-bound state and trapping it in the off state and so preventing recharging with GTP.

As an alternative to targeting specific Ras mutants, such as G12C, compounds could be developed that target individual Ras isoforms but do not discriminate between wild-type and mutant Ras proteins. This could be achieved by targeting specific hypervariable regions at the C terminus where the Ras proteins differ most widely (Figure 3). The C-terminal hypervariable region of K-Ras-4B is very different from the hypervariable regions of other Ras proteins and is involved in the specific

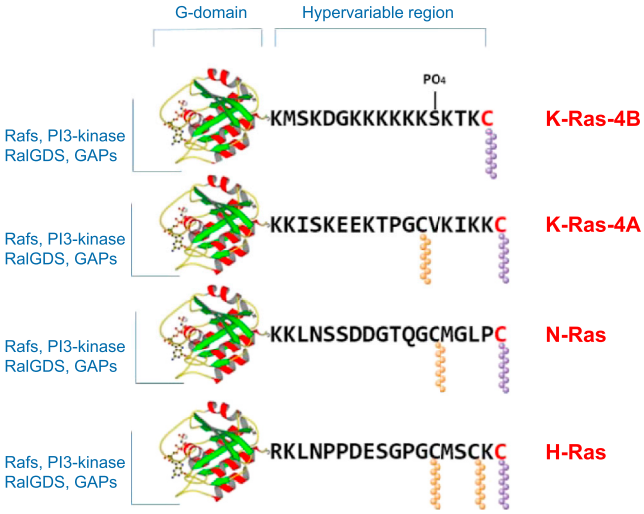

Figure 3. Schematic Representation of the Ras Isoforms
The structures of the G domain of H-Ras, N-Ras, and K-Ras have been solved and are virtually identical, but the structure of processed hypervariable regions has not been solved and is therefore depicted as a linear sequence. Lipid modifications with farnesyl (purple) and palmitoyl (orange) chains are shown.

interaction of K-Ras-4B with calmodulin (Lopez-Alcalá et al., 2008). Because K-Ras-4B seems to be the major form of K-Ras in established tumors, these specific biochemical properties may afford unique opportunities for therapeutic attack. Mouse models suggest that such compounds would be well tolerated because animals lacking any single isoform of Ras are viable (A. Balmain, personal communication).

Targeting GDP/GTP Binding and Exchange

Ras proteins bind GDP and GTP with picomolar affinity. It is generally accepted that oncogenic Ras proteins cannot be attacked with nucleotide analogs because high GTP concentrations make competition impossible. The high affinity for GTP is also considered a barrier, though it is easy to imagine that analogs could be developed with equally high affinity. This approach to targeting Ras has therefore been abandoned. However, Ras proteins in their GTP state exist in complexes with effectors (Raf kinases, RalGDS, PI3K, other Ras-binding proteins), as well as regulators (GAPs and guanine nucleotide exchange factors [GEFs]). The effects of most of these proteins on nucleotide binding have not been measured. GEFs, of course, greatly reduce the affinity for nucleotides, allowing GDP to be released rapidly and replaced by GTP. Although oncogenic mutants do not need GEFs to put them in the active state, they are still sensitive to GEF-mediated exchange and cycle through a complex state in which nucleotide-free Ras protein is bound to the GEF; this may provide a potential opportunity for a mutant-specific nucleotide analog to bind. In support of this, we noted many years ago that antibodies directed against specific codon 12 mutants were effective at reversing transformation in cells, as cited above, yet these antibodies do not bind to nucleotide-loaded Ras (Clark et al., 1985). We therefore speculate that oncogenic Ras exists in a nucleotide-free state frequently enough to make it vulnerable to attack.

Whether oncogenic Ras proteins are regulated at all by Sos and other GEFs has been surprisingly difficult to determine definitively, partly because there are many types of GEFs in mammalian cells. Furthermore, GEFs such as Sos have allosteric sites for Ras binding as well as sites for GDP/GTP exchange, and it is hard to measure GTP loading on individual Ras isoforms in cells. However, it is clear that mutant Ras proteins are not 100% GTP bound, and GEFs could increase the fraction of Ras-GTP to some extent. However, targeting Sos or other GEFs for treating mutant Ras cancers does not appear an attractive proposition. Oncogenic mutants may or may not depend on GEFs, to some degree, but wild-type Ras proteins most certainly do. For these reasons, recent efforts to target mutant Ras that led to compounds that bind at the Sos-binding site may seem disappointing (Maurer et al., 2012; Sun et al., 2012). However, the compounds that these groups discovered could be excellent starting points toward the discovery of compounds that have selectivity for mutant forms of K-Ras or block effector interactions.

Restoring GTP Hydrolysis

Mutations at codons 12, 13, and 61 inhibit GAP-mediated GTP hydrolysis. As a result, mutant Ras proteins accumulate with elevated GTP-bound proportion. Trahey and McCormick discovered GAP while seeking to explain how relatively small changes in intrinsic GTPase between wild-type and mutant Ras proteins accounted for profound differences in transforming activity (Trahey and McCormick, 1987). Intrinsic rates of GTP hydrolysis are five orders of magnitude slower than rates catalyzed by GAPs and therefore do not contribute significantly to steady-state levels of Ras-GTP. However, once Ras proteins bind effectors, GAPs can no longer interact, and intrinsic GTPase may become important in determining how long Ras and its effectors remain engaged. Indeed, effector binding may well affect intrinsic GTPase activity of Ras as it does for heterotrimeric G proteins. If indeed intrinsic GTPase limits signal output, perhaps assays for compounds that stimulate intrinsic GTPase of Ras effector complexes may merit consideration. Mattos and colleagues recently showed that the Ras-binding domain of Raf (the RBD) has a profound effect on suppressing intrinsic hydrolysis rates of Ras Q61 mutants, but not wild-type Ras or G12V mutants (Buhrman et al., 2010). They propose that suppression of intrinsic GTPase stabilizes Ras-Raf complexes and increases signal output to the MAPK pathway selectively; this accounts for the preference of Q61 mutants over G12 mutants in melanoma, a disease that is clearly Raf-MAPK driven (Buhrman et al., 2010).

In the 1980s, several groups, including those at Cetus and Hoffmann La Roche, screened for compounds that restore GTP hydrolysis to mutant Ras, in the presence or absence of GAP. These screens failed to find compounds that increased GTPase rates. Furthermore, as structures of Ras proteins emerged, mostly from Wittinghofer's group, it became clear that codon 12 substitutions presented a steric block to GAP-mediated GTP hydrolysis that could not be overcome by a small molecule. These studies were mostly based on G12V mutations because these were the most widely used at that time. Whether the same conclusion can be applied to other mutations such as G12D or G13D remains to be seen because structures of these proteins bound to GAP have not been solved.

The approach of restoring GTP hydrolysis to mutant proteins received a brief infusion of hope when Scheffzek and colleagues showed that G12V H-Ras could indeed hydrolyze a GTP analog diaminobenzophenone-phosphoroamidate-GTP in which the aromatic amino group mimics the catalytic effects of GAP's arginine finger (Ahmadian et al., 1999). A small molecule that provided this local charge might therefore trick mutant Ras into GTP hydrolysis. At first sight, the GTD-/GTP-binding site of Ras does not offer any room for such a molecule to bind. However, these issues deserve rethinking—perhaps G12D offers more possibilities for this kind of attack than G12V, for example.

Targeting Ras Posttranslational Modification Pathways

Ras proteins are processed in several steps (reviewed in Gysin et al., 2011), including farnesylation, proteolytic cleavage at the C terminus by RCE1, and carboxymethylation by isoprenylcysteine carboxyl methyltransferase (ICMT). K-Ras-4A, H-Ras, and N-Ras are further processed by palmitoylation (Figure 3). These reactions are not only essential for plasma membrane localization but also for Raf kinase activation. The failure of farnesyltransferase inhibitors has been well documented. By sheer bad luck, the forms of Ras that play the major roles in human cancer, K-Ras and N-Ras, can be geranylgeranylated when farnesyltransferase is inhibited, allowing newly synthesized Ras proteins to be inserted correctly in the membrane and to function normally. H-Ras, on the other hand, is not geranylgeranylated, suggesting that tumors driven by mutant H-Ras, such as bladder cancer or thyroid cancer, might be susceptible to farnesyltransferase inhibition. Targeting RCE1 or ICMT has also been evaluated, though the consequences of blocking these enzymes are difficult to predict or understand. For example, inhibition of either enzyme can actually lead to increased Ras-mediated tumorigenesis (Court et al., 2013; Wahlstrom et al., 2007).

Palmitoylation and depalmitoylation of H-Ras, K-Ras-4A, and N-Ras proteins provide dynamic aspects to membrane localization and may present therapeutic opportunities for these proteins. Recent work demonstrated that acyl protein thioesterase 1 (APT1), which is responsible for Ras depalmitoylation, could be targeted by palmostatin B to selectively inhibit the growth of N-Ras mutant leukemia cells (Xu et al., 2012). In contrast, K-Ras-4B localization seemed relatively static and stable: specific localization of K-Ras-4B to plasma membranes is based on electrostatic interactions between lysine residues in the hypervariable region and phospholipids in the membrane. However, another therapeutic opportunity has been presented by the discovery that PDE6δ acts as a solubilizing factor that modulates Ras proteins by sustaining their dynamic distribution in cellular membranes. A small molecule was identified that prevents association of K-Ras-4B, and other proteins, with PDEδ and so delocalizes these proteins and inhibits downstream signaling (Zimmermann et al., 2013). Little is still known about the trafficking of Ras to and from the membrane, and there are likely to be additional factors or chaperones involved in the movement of the proteins that could serve as targets for small molecules. In a related study, K-Ras-4B was shown to undergo retrograde trafficking from the plasma membrane to endomembranes. This may serve as an additional pathway to specifically disrupt for therapeutic benefit (Bivona et al., 2006).

In addition to these processing events, several recent papers have highlighted other posttranslational modifications of K-Ras that could serve as therapeutic targets. Mono-ubiquitination at Lys147 has been shown to enhance GTP loading and effector-binding affinity of K-Ras (Sasaki et al., 2011), suggesting that targeting of ubiquitin pathway enzymes might have an effect on K-Ras activity. Acetylation of Lys104 was shown to decrease GEF-induced nucleotide exchange, leading to reduced transformation efficiency in cells, and the deacetylases SIRT2 and HDAC6 were shown to regulate the level of acetylation of K-Ras (Yang et al., 2013), suggesting that inhibitors of these enzymes might have an effect on the oncogenic potential of mutant K-Ras tumors. Finally, nitrosylation of Cys118 in H-Ras has been shown to activate Ras by enhancing nucleotide dissociation, leading to higher levels of GTP-bound protein. The eNos protein was identified as a strong enhancer of nitrosylation and therefore could also be a therapeutic target to attack mutant Ras (Lim et al., 2008).

Downstream Pathways and Drug Targets

When it became clear that targeting mutant Ras proteins directly was technically impossible with the tools available at that time, the search for drugs that block Ras activity moved downstream. In the early 1990s, the MAPK pathway and the PI3K pathway were known to be downstream of Ras (Figure 1). In 1993, four groups showed that Ras binds directly to Raf kinase (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang et al., 1993), and later, activation of Raf kinase by Ras was achieved in vitro (Stokoe and McCormick, 1997). This was unexpectedly difficult; for one thing, Raf activation by Ras required fully processed Ras in a lipid environment, and direct binding of unprocessed Ras failed to activate the kinase. Furthermore, autophosphorylation rapidly shut down Raf kinase in vitro; we had to preincubate processed Ras with Raf in the absence of ATP before measuring Raf kinase activity. This autophosphorylation accounts, at least in part, for paradoxical activation of Raf kinase by Raf inhibitors, a phenomenon that was discovered 16 years later (reviewed in Holderfield et al., 2013). These issues complicate the development of in vitro assays for compounds that prevent Ras-dependent activation of Raf kinase, an obvious system for therapeutic intervention. Direct blocking of Ras-Raf binding with small molecules does not appear to be a promising approach because the binding surface (two antiparallel β strands) offers no foothold in which a compound could bind. However, for example, preventing binding using peptides or by indirect allosteric approaches has been considered (see Wu et al., 2013).

The drug discovery group at Onyx Pharmaceuticals began screening for Raf kinase inhibitors in 1992 after it was able produce active c-Raf kinase in baculovirus (by coinfection with v-Src) and to reconstitute the MAPK pathway in vitro (Macdonald et al., 1993). It was then assumed that in cancer cells with mutant Ras, the Raf/MAPK pathway would be hyperactive and that drugs that inhibit Raf would be effective ways of treating Ras mutant cancers. It was also assumed that MEK and extracellular signal-regulated kinase (ERK) inhibitors would have the same effect. In hindsight, most of the assumptions were incorrect: the Raf/MAPK pathway is not often hyperactive in human cancer cells with mutant Ras, as measured by steady-state levels of

phospho-MEK or phospho-ERK. Raf inhibitors lead to paradoxical activation of Raf kinase following exposure to Raf inhibitors, especially in Ras mutant cancers (reviewed in Lito et al., 2013). MEK and ERK inhibitors do not show paradoxical activation but are generally ineffective on their own because they relieve feedback inhibition on upstream kinases, leading to activation of PI3K, among other effects (Mirzoeva et al., 2009; Corcoran et al., 2012; Turke et al., 2012; Montero-Conde et al., 2013), and because they lack a clear therapeutic window. MEK1/MEK2 isoforms have a high degree of amino acid identity, suggesting redundant roles in signaling. The same is true for ERK1/ERK2. However, knocking out the gene encoding MEK1, *Map2k1* (Giroux et al., 1999), or the gene encoding ERK2, *Mapk1* (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003), is embryonic lethal, indicating a requirement for signaling from a particular isoform, at least in the context of embryogenesis. Although *Map2k2*$^{-/-}$ (MEK2 null) and *Mapk3*$^{-/-}$ (ERK1 null) mice are viable, in vivo ablation of MEK1 in a *Map2k2*$^{-/-}$ background (Scholl et al., 2007; Blasco et al., 2011) or ERK2 in a *Mapk3*$^{-/-}$ background (Chan et al., 2013) results in apoptosis and lethality in adult mice. This may suggest a limited therapeutic window for any pan inhibitor of these kinases, and the clinical toxicity of potential drugs in this target class bears this out.

The Onyx/Bayer screen for c-Raf inhibitors led to the discovery and development of sorafenib. However, it was disappointing when sorafenib failed to show clinical benefit in early clinical trials against Ras mutant cancers, and this lack of response was difficult to understand because sorafenib does indeed inhibit Raf kinase. Despite this, sorafenib and fluorosorafenib (regorafenib) have since been approved for the treatment of renal cell carcinoma, hepatocellular carcinoma, thyroid cancer, colorectal cancer, and gastrointestinal stromal cancer. In hepatocellular carcinoma, biomarker analysis in Phase II clinical trials showed a clear correlation between levels of phospho-ERK and clinical response, suggesting that inhibition of Raf kinase is responsible for part of the clinical benefit (Abou-Alfa et al., 2006), but in the other indications, it appears likely that inhibition of vascular endothelial growth factor receptor 2 or other kinases is responsible. Hopefully, a clearer picture will emerge through analysis of exceptional responders or through deciphering mechanisms of drug resistance.

Inhibitors of PI3K pathway have not yet fared much better in the clinic, also because of feedback mechanisms that activate upstream signaling, as well as poor therapeutic index. However, the relative failure of these downstream approaches does not mean that they are not critical to Ras oncogenesis. Indeed, ablation of c-Raf (but not B-Raf) in mice inhibits development and delays progression of Ras-driven tumors in a lung adenocarcinoma model (Blasco et al., 2011). However, in an in vivo pancreatic cancer mouse model, B-Raf was shown to be required for tumor progression (Sobczak et al., 2008). This suggests tissue-specific signaling cascades and will require more investigation. Genetic disruption of Ras binding to PI3K-α has a similar effect. We can therefore assume that small molecules that can block downstream signaling without triggering feedback and with the correct specificity and biochemical properties may still be effective, but more work needs to be done to develop such compounds effectively.

The third direct effector arm of Ras signaling that plays a major role in human cancer is the RalGDS (Ral guanine nucleotide dissociation stimulator) pathway (Figure 1). Perhaps the best evidence of the importance of this effector pathway comes from demonstration that mice null for RalGDS have reduced skin carcinogen-induced tumor incidence, size, and progression to malignancy compared to wild-type mice (González-García et al., 2005). These data, and many others (Martin et al., 2011; Kashatus, 2013), support a role for RalGDS both in vitro and in vivo as an important effector pathway utilized by oncogenic Ras to drive tumorigenesis that could potentially be exploited for therapeutic intervention, although the absence of somatic mutations in this effector pathway makes its precise role less clear than the Raf/MAPK and PI3K pathways. On the other hand, Ral signaling is upstream of NF-κB and TBK1, both of which have been implicated as essential genes downstream of K-Ras (Neel et al., 2011; Kashatus, 2013).

Other potential Ras effectors that could be important in cancer and therefore a source of potential therapeutic targets include phospholipase CE and Tiam1, a GEF that stimulates the activation of Rac (Figure 1). Rac1 is necessary for K-Ras tumor initiation, further implicating the importance of this pathway in K-Ras tumorigenesis, though not yet providing obvious therapeutic targets (Gysin et al., 2011). Likewise, cyclin D1, NF-κB, and Myc are necessary for Ras tumorigenesis; further analysis of the role of these pathways may lead to new therapeutic insights. For example, Puyol et al. (2010) recently demonstrated that germline or conditional deletion of *Cdk4* led to senescence in lung cells expressing activated K-Ras. Furthermore, treatment with a Cdk4 inhibitor reduced the growth of K-Ras-driven tumors. Finally, unbiased shRNA screens have revealed potential targets for K-Ras cancers. These include STK33, TBK1, and GATA-2. So far, STK33 inhibition does not appear to be a useful approach to K-Ras cancers (Weiwer et al., 2012). TBK1 inhibitors are still being investigated: this target is of particular interest because it is part of the well-validated RalGDS pathway. GATA2 is also of considerable interest; genetic ablation leads to tumor regression in mouse models of adenocarcinoma of the lung, and whereas this transcription factor may appear to be the least druggable of targets, its role in regulating the proteasome suggested therapeutic approaches that appear very promising (Kumar et al., 2012).

Future Prospects

In this Review, we have summarized some of the challenges of targeting Ras cancers. Despite the tremendous progress that has been made, we still have to learn a great deal about these cancers before we can be confident that we can treat them effectively. Recent experience in targeting Raf and MEK has underscored how a pathway that appeared simple and linear is extremely complex and poorly understood at the level of detail required to shut it down effectively. Nobody expected that Raf inhibitors would activate Raf kinase in Ras-transformed cells, for example, or that inhibition of downstream kinases like MEK would lead to activation of upstream signaling. We need a much deeper analysis of the molecular mechanisms underlying Ras regulation and effector engagement before we can expect to interfere with these mechanisms effectively. It seems to us more likely that these deeper insights will lead to productive

approaches for intervention than to the conclusion that Ras is indeed undruggable. New technologies and insights and fresh eyes are likely to solve this problem. We are also optimistic that completely different approaches to treating cancer will contribute to eliminating Ras cancers, including new ways of knocking down/out genes using RNAi and CRISPR technologies and delivering these payloads to tumors (Davis et al., 2010), as well as new ways of deploying the immune system. In this respect, it is noteworthy that anti-CTLA-4 therapy appears to be equally effective in treating melanoma driven by N-Ras or B-Raf; therefore, Ras cancers may not be excluded from these approaches as they have been from others. All of these considerations lead us to be optimistic about future prospects of finally delivering the knockout punch.

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Cancer Cell
Perspective

ERBB Receptors: From Oncogene Discovery to Basic Science to Mechanism-Based Cancer Therapeutics

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ERBB receptors were linked to human cancer pathogenesis approximately three decades ago. Biomedical investigators have since developed substantial understanding of the biology underlying the dependence of cancers on aberrant ERBB receptor signaling. An array of cancer-associated genetic alterations in ERBB receptors has also been identified. These findings have led to the discovery and development of mechanism-based therapies targeting ERBB receptors that have improved outcome for many cancer patients. In this Perspective, we discuss current paradigms of targeting ERBB receptors with cancer therapeutics and our understanding of mechanisms of action and resistance to these drugs. As current strategies still have limitations, we also discuss challenges and opportunities that lie ahead as basic scientists and clinical investigators work toward more breakthroughs.

ERBB Family: EGFR, HER2, HER3, and HER4

The ERBB family of transmembrane receptor tyrosine kinases (RTKs) consists of the epidermal growth factor receptor EGFR (ERBB1), HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4). Binding of ligands to the extracellular domain of EGFR, HER3, and HER4 induces the formation of kinase active hetero-oligomers (Yarden and Sliwkowski, 2001). HER2 does not bind any of the ERBB ligands directly, but it is in a conformation that resembles a ligand-activated state and favors dimerization (Cho et al., 2003; Garrett et al., 2003). Activation of HER2 and EGFR induces transphosphorylation of the ERBB dimer partner and stimulates intracellular pathways such as RAS/RAF/MEK/ERK, PI3K/AKT/TOR, Src kinases, and STAT transcription factors (reviewed in Yarden and Pines, 2012). Although HER3 can bind ATP and catalyze autophosphorylation, it has a weak kinase activity compared to that of its ERBB coreceptors (Shi et al., 2010). However, upon transphosphorylation by another ERBB family member, HER3 serves as an efficient phosphotyrosine scaffold, leading to potent activation of downstream signaling. The specificity and potency of intracellular signaling cascades are determined by the expression of positive and negative regulators, the specific composition of activating ligand(s), receptor dimer constituents, and the array of proteins that associate with the tyrosine phosphorylated C-terminal domain of the ERBB receptors (Avraham and Yarden, 2011).

Over the past several years, it has become evident the ERBB family members have a prominent role in the initiation and maintenance of several solid tumors. This has led to the development and widespread implementation of specific ERBB inhibitors as cancer therapies. In this Perspective, we will focus on the therapeutic approaches for targeting ERBB family members in cancer, with a particular emphasis on *HER2*-amplified breast cancer and *EGFR* mutant lung cancer.

Links to Cancer

HER2

The first evidence for a role of ERBB2 or HER2 (for *h*uman *EGFR2*) in cancer was inferred from the connection to its rat ortholog, Neu, a mutant cDNA isolated from carcinogen-induced neuroblastomas (Schechter et al., 1984). (Please note that in this Perspective, ERBB2 and HER2 will be used when discussing mouse and human ERBB2, respectively.) Although rodent *Neu* is mutated, human *HER2* is typically amplified in human cancers such as breast, gastric, and esophageal cancer (Table 1). Overexpression of either rat or human wild-type ERBB2 was shown to transform diploid cells. Consistent with its oncogenic activity, overexpression of wild-type Neu or HER2 under the control of a mammary-specific promoter leads to metastatic mammary tumors in transgenic mice (Andrechek et al., 2000; Finkle et al., 2004). In a seminal study, Slamon et al. found that *HER2* is amplified in about 20% of breast cancers (Slamon et al., 1987). This was the first report of an oncogenic alteration associated with poor outcome in cancer patients, suggesting a causal relationship to cancer virulence. Further evidence linking HER2 with cancer progression is the improvement in survival of patients with *HER2*-amplified early-stage breast cancer treated with the HER2 antibody trastuzumab. More-recent studies using next-generation sequencing have identified less-frequent activating mutations in *HER2* in several cancer types without *HER2* gene amplification (discussed below).

A recent study of >500 breast tumors by The Cancer Genome Atlas (TCGA) Network has shed light into the biological heterogeneity of clinical HER2-overexpressing cancers ($HER2^+$ as defined by gene amplification) by further parsing into HER2-enriched (HER2E) and luminal subtypes as defined by gene expression (Cancer Genome Atlas Network, 2012). HER2E-$HER2^+$ tumors had higher frequencies of aneuploidy, somatic mutation, and *TP53* mutation, as well as amplification of FGFRs, EGFR, CDK4, and cyclin D1. Luminal-$HER2^+$ breast cancers

Table 1. Alterations of ERBB Receptors and Ligands in Human Cancer

Molecule	Alteration	Cancer Types	Notes	References
EGFR	mutation (L858R, etc.)	NSCLC (adenocarcinoma)	substitutions, deletions and insertions	Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004
EGFR	vIII	glioma	deletion of exons 2–7 in the ectodomain	Sugawa et al., 1990
EGFR	amplification	NSCLC (squamous), head and neck, glioma, esophageal, colorectal, anal (?)		Yarden and Pines, 2012
HER2	amplification	breast, gastric, esophageal		Cancer Genome Atlas Network, 2012
HER2	mutation	breast (lobular), lung, gastric, bladder, endometrial	unclear whether all those reported are activating or gain of function	Cancer Genome Atlas Network, 2012
HER3	mutation	breast, gastric		Jaiswal et al., 2013
HER4	mutation	melanoma, NSCLC, medulloblastoma		Gilbertson et al., 2001; Prickett et al., 2009
TGF-α	overexpression	prostate, lung, pancreas, ovary, colon, head and neck	androgen-independent prostate cancer; poor prognosis when associated with high EGFR	Rubin Grandis et al., 1998; Yarden and Sliwkowski, 2001
Neuregulin-1	overexpression	colorectal, head and neck	linked to sensitivity to ERBB3 inhibitors and resistance to EGFR inhibitors	Wilson et al., 2011; Yonesaka et al., 2011

showed higher expression of a luminal gene cluster including GATA3, BCL2, and ESR1 and harbored a higher rate of GATA3 mutations. It is anticipated that because of these molecular differences, the clinical management of HER2E and luminal subtypes of $HER2^+$ breast cancers will also be different. Finally, not all tumors of the HER2E gene expression subtype are *HER2* amplified. One implication of these data is that some breast cancers with a single copy of *HER2* harbor an expression signature of HER2 dependence and, as such, may benefit from anti-HER2 therapy. Consistent with this speculation are the results of the NSABP B-31 adjuvant trastuzumab trial, in which 9.7% of patients that did not meet criteria for HER2 overexpression by fluorescence in situ hybridization (FISH) or immunohistochemistry (IHC) also benefitted from adjuvant trastuzumab (Paik et al., 2008).

Somatic mutations in HER2 have been reported in several human cancers (Table 1). Most are missense mutations in the tyrosine kinase and extracellular domains or duplications/insertions in a small stretch within exon 20. *HER2* mutations are almost exclusively observed in cancers without *HER2* gene amplification. Several of these mutants have increased signaling activity, and are most commonly associated with lung adenocarcinoma and lobular breast, bladder, gastric, and endometrial cancers (Cancer Genome Atlas Network, 2012).

EGFR

The EGF receptor was originally identified as an oncogene because of its homology to v-ERBB, a retroviral protein that enables the avian erythroblastosis virus to transform chicken cells (Downward et al., 1984). Subsequently, EGFR overexpression was shown to be transforming in laboratory models, and *EGFR* gene amplification was reported in a wide range of carcinomas. Early studies by Mendelsohn and colleagues demonstrated that antibodies directed against EGFR block growth of A431 cells, demonstrating that EGFR signaling could drive cancer cell growth and setting the stage for clinical use of EGFR inhibitors (Kawamoto et al., 1983).

An oncogenic mutation that deletes exons 2–7 in the receptor ectodomain, denoted *EGFRvIII*, is found in about 40% of high-grade gliomas with wild-type *EGFR* amplification (Sugawa et al., 1990). EGFRvIII exhibits constitutive dimerization, impaired downregulation, and aberrant tyrosine kinase activity, all resulting in enhanced tumorigenicity (Nishikawa et al., 1994). In addition to glioblastoma multiforme (GBM), EGFRvIII has been found in a fraction of breast, lung, head and neck, ovarian, and prostate cancers (Moscatello et al., 1995). Because its expression is restricted to tumor tissues, EGFRvIII has been therapeutically targeted with specific antibodies and vaccines. There is clinical evidence suggesting that the presence of EGFRvIII can predict clinical responses of GBMs to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib (Haas-Kogan et al., 2005; Mellinghoff et al., 2005). The second most common EGFR variant in GBM is EGFRc958, observed in about 20% of tumors with wild-type *EGFR* amplification. EGFRc958 lacks amino acids 521–603 and displays increased, ligand-dependent kinase activity (Frederick et al., 2000).

The causal role of EGFR in tumorigenesis was further solidified in 2004 when somatic, activating mutations in EGFR were discovered in a subset of non-small-cell lung cancers (NSCLCs) (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004) (Table 1). The discovery was spurred by efforts to understand why occasional NSCLCs were highly sensitive to small-molecule EGFR TKIs. It is now well established that lung cancers harboring these *EGFR* mutations are highly responsive to single-agent EGFR inhibitors with RECIST response rates of ~55%–75% (Mok et al., 2009; Rosell et al., 2012; Sequist et al., 2013b). *EGFR* mutations are primarily localized within two hot spots of the kinase domains, a series of overlapping deletions in exon 19 and a leucine-to-arginine substitution at amino acid position 858 (L858R) (reviewed in Pao and Chmielecki, 2010). In addition,

mutations are also rarely observed elsewhere in the kinase domain, including insertions in exon 20 (Yasuda et al., 2013). The prevalence of the mutations differs among distinct human populations. They are found in ~8%–10% of Caucasians, but in a higher proportion of East Asians. Lung cancers with *EGFR* mutations are most highly associated with adenocarcinoma histology and in patients with a minimal smoking history. Of note, cancers with *EGFR* mutations often have amplification of the mutant *EGFR* allele as well (Cappuzzo et al., 2005). Cell culture and transgenic mouse model studies have shown that mutant EGFR has transforming activity (Greulich et al., 2005; Ji et al., 2006; Politi et al., 2006).

EGFR is important for the growth of some colorectal cancers (CRCs) and head and neck cancers. In these cancers, genetic alterations in *EGFR* have not been consistently identified. However, the efficacy of the EGFR antibody cetuximab demonstrates the importance of EGFR signaling in these tumors. Although some reports suggest that *EGFR* amplification correlates with response to cetuximab (Moroni et al., 2005), this alteration is not currently used as a predictive biomarker. Importantly, cetuximab provides clinical benefit primarily in colorectal cancers that do not harbor *KRAS* mutations (Cunningham et al., 2004) and in those with high expression of the EGFR ligands amphiregulin and epiregulin (Khambata-Ford et al., 2007). Presumably, cetuximab is effective in sensitive cancers because it blocks ligand-dependent activation of EGFR and downregulates the receptor from the cell surface (Fan et al., 1994). Thus, in these colorectal cancers, we suspect that ligand-dependent activation of EGFR drives progression of these cancers. Currently, cetuximab is most often administered with chemotherapy in *KRAS* wild-type colorectal cancers. Similarly, in head and neck cancers, cetuximab is primarily used in conjunction with chemotherapy (Vermorken et al., 2008) and radiotherapy (Bonner et al., 2006). Despite conflicting reports on the utility of EGFR expression by IHC for patient selection in head and neck cancers (and CRCs), there currently are no validated predictive biomarkers of response to EGFR inhibitors in head and neck cancers (Burtness et al., 2005; Cunningham et al., 2004; Licitra et al., 2011, 2013; Vermorken et al., 2008). It is notable that cetuximab appears to be more effective than EGFR TKIs in cancers with ligand-dependent activation of EGFR, whereas TKIs are more effective in cancers with *EGFR* mutations. We speculate that this is so because mutant EGFR activation is not ligand dependent and because TKIs have higher affinity for mutant EGFR than for wild-type EGFR, thus leading to a significant therapeutic window. In contrast, antibodies such as cetuximab are more effective in EGFR wild-type cancers because they are highly effective at blocking ligand-dependent activation of EGFR and are pharmacologically stable.

ERBB3 and ERBB4

ERBB3 has been linked to cancer, primarily due to its mechanistic role in promoting signaling from oncogenic HER2 and EGFR (discussed below). However, somatic mutations scattered throughout the *ERBB3* gene were recently identified in subsets of breast and gastric cancers (Table 1). Many of the mutations were located in the extracellular domain, and they appear to have oncogenic potential, function in a ligand-independent manner, and require heterodimerization with HER2 for transforming activity (Jaiswal et al., 2013). Future studies are needed to determine whether cancers with ERBB3 mutations are particularly sensitive to ERBB3- and/or HER2-targeted drugs. Similarly, mutations in ERBB4 were identified in cancer, particularly melanoma (Prickett et al., 2009), lung adenocarcinoma (Ding et al., 2008), and medulloblastoma (Gilbertson et al., 2001). Although laboratory studies demonstrated that melanoma cell lines harboring ERBB4 mutations were sensitive to lapatinib, it remains unknown whether targeting of ERBB4, or any other ERBB family member, will have therapeutic value in these cancers.

ERBB Ligands

Overproduction of ligands is one mechanism by which cancers aberrantly activate ERBB receptors. The source of these can be tumor cells or the tumor stroma. There are three groups of ligands. One group specifically binds EGFR and includes EGF, transforming growth factor α (TGF-α), amphiregulin (AR), and epigen (EPG). A second group binds both EGFR and HER4 and includes betacellulin (BTC), HB-EGF, and epiregulin (EPR). The third group includes all of the neuregulins (NRG1–NRG4), of which NRG1 and NRG2 bind HER3 and HER4, whereas NRG3 and NRG4 only bind HER4 (Hynes and MacDonald, 2009).

In transgenic mouse studies, mice that coexpress TGF-α and Neu in mammary epithelium developed multifocal mammary cancers that arise after a significantly shorter latency than those expressing either gene alone (Muller et al., 1996). TGF-α is also co-overexpressed with EGFR in lung, colorectal, ovary, and head and neck squamous cancers, where it is associated with poor patient prognosis (Rubin Grandis et al., 1998; Yarden and Sliwkowski, 2001) (Table 1). Recent reports suggest that in addition to overexpression, mistrafficking, and/or ''extracrine'' (*ex*osomal *t*argeting *r*eceptor *a*ctivation) signaling by ERBB ligands may also contribute to epithelial cell transformation (Singh and Coffey, 2014). For example, altered trafficking of EREG to the apical cell surface leads to prolonged EGFR phosphorylation and more proliferative and more invasive tumors (Singh et al., 2013). Further, significantly enhanced levels of invasiveness are observed when breast cancer cells are incubated with exosomes containing high levels of AREG compared to incubation of cells with exosomes containing low levels of AREG or recombinant EGFR ligands (Higginbotham et al., 2011), suggesting a gain-of-function mode of EGFR signaling that might act in more distant environments. Other roles of ligand-dependent activation of EGFR were discussed above.

An autocrine loop has been described in ovarian cancer cells and tumors that overexpress NRG1 and HER3, where suppression of HER3 with RNAi or with a neutralizing HER3 antibody suppressed ovarian cancer growth in laboratory models (Sheng et al., 2010) (Table 1). A NRG1-mediated autocrine loop inducing HER3 activation was also discovered in head and neck cancer cells without *HER2* amplification. These cells were particularly sensitive to the EGFR/HER2 TKI lapatinib (Wilson et al., 2011), suggesting that NRG1-driven tumors depend on HER3 activated by HER2 and/or EGFR. Finally, Hegde et al. found high levels of NRG1 and its receptor, HER4, in NSCLC residual tumor cells that remained after cytotoxic chemotherapy. Inhibition of HER3/HER4 signaling with a NRG1-blocking antibody increased the magnitude and duration of response to chemotherapy in these in vivo models (Hegde et al., 2013). This causal association of ERBB ligand overexpression and drug resistance is not limited to NRG1 or to chemotherapy. For example, HGF has been found

to confer resistance to the BRAF inhibitor vemurafenib in BRAF mutant melanoma cells (Wilson et al., 2012).

Downstream Signaling

Oncogenic addiction to EGFR and HER2 are intimately linked to regulation of downstream signaling. In cancers highly sensitive to inhibition of EGFR or HER2 inhibitors, EGFR or HER2 is the main driver of downstream signaling, particularly via the PI3K/AKT and MEK/ERK pathways. Thus, in cancers addicted to EGFR or HER2, inhibition of the respective RTK leads to concomitant loss of flux through these pathways. Loss of these signaling events leads to growth arrest and converges on the BCL-2 family of proteins to promote apoptosis (reviewed in Niederst and Engelman, 2013).

In EGFR and HER2 driven cancers, HER3 is an important heterodimer partner because it potently activates the phosphatidylinositide-3 kinase (PI3K)/AKT survival pathway via its six docking sites for the p85 regulatory subunit of PI3K. Although HER2 potently activates ERK signaling, it does not bind p85 or directly activate PI3K/AKT. Thus, HER2-mediated activation of HER3 is essential for stimulation of the PI3K/AKT pathway. In transgenic mice, genetic ablation of ERBB3 in the mammary gland via Cre-mediated recombination abrogates ERBB2-driven mammary hyperplasias, DCIS, invasive cancers, and metastases (Vaught et al., 2012). Similarly, small hairpin RNA (shRNA)-mediated knockdown of HER3 but not EGFR inhibits viability of HER2-overexpressing breast cancer cells. Further, HER3 but not EGFR, is always phosphorylated in human *HER2*-amplified breast cancers (Lee-Hoeflich et al., 2008), suggesting that it is an obligatory cobiomarker of aberrant HER2 activity and dependence. More recently, an inducible HER3 shRNA (Lee-Hoeflich et al., 2008) and a HER3-neutralizing antibody (Garrett et al., 2013b) were shown to inhibit growth of established *HER2*-amplified xenografts, further suggesting that HER3 is essential for the survival of HER2-dependent tumors. Analogous to HER2-induced signal transduction, mutant EGFR often activates PI3K via HER3 (Engelman et al., 2005), and maintenance of HER3 signaling can promote resistance to EGFR inhibitors (Engelman et al., 2007; Schoeberl et al., 2010). However, unlike HER2, EGFR is also able to signal to PI3K via GAB1 in a HER3-independent manner (Mattoon et al., 2004; Turke et al., 2010), suggesting that *EGFR* mutant cancers may be better equipped than *HER2*-amplified cancers to adapt to the loss of HER3 function.

HER2-amplified tumors have a strong dependence on PI3K/AKT signaling, as sustained blockade of this pathway appears to be required for the antitumor effect of HER2 antagonists (Chakrabarty et al., 2013; Yakes et al., 2002). Comprehensive cancer cell line panels screened for sensitivity to pan-PI3K, p110α-specific, and AKT inhibitors have consistently shown preferential activity of these drugs against *HER2*-amplified breast cancer lines (Heiser et al., 2012; O'Brien et al., 2010). Further, genetic ablation of p110α has been shown to abrogate ERBB2-induced mammary tumor formation in transgenic mice (Utermark et al., 2012). Preclinical studies have shown that, compared to HER2-amplified cancers, *EGFR* mutant cancers are less sensitive to single-agent PI3K/AKT inhibitors. Rather, inhibition of the PI3K and MEK pathways is necessary in order to induce apoptosis and cause tumor regressions (Faber et al., 2010). Importantly, mechanisms of de novo and acquired resistance to HER2- and EGFR-directed therapies involve persistence or reactivation of PI3K/AKT signaling via alternate amplified RTKs and/or mutations in the PI3K pathway (Rexer and Arteaga, 2013).

Other downstream signaling pathways, such as Src kinases, JAK/STAT, and WNT, are also activated by ERBB receptors (Yarden and Sliwkowski, 2001). Examples below suggest that they are involved in and/or mediate resistance to ERBB-receptor-targeted therapies. However, evidence that ERBB receptors depend on Src, JAK/STAT, or WNT for their effects on transformation and cancer progression is less clear and will not be discussed further.

Feedback Activation of ERBB Signaling Promoting Resistance to Inhibition of Alternative Kinases

More recently, EGFR and HER3 activation have been observed as important cellular adaptations to inhibitors of downstream signaling. For example, in *BRAF* mutant CRC, BRAF inhibitors fail to inhibit ERK signaling in sustained fashion due to activation of EGFR which, in turn, reactivates ERK in the presence of the BRAF inhibitor (Corcoran et al., 2012; Prahallad et al., 2012). However, combined inhibition of EGFR and BRAF blocks reactivation of ERK and leads to regressions of BRAF mutant CRC in vivo. This combination is now being actively developed in clinic for this subset of CRCs. Similarly, inhibition of the MEK pathway in many cancers, including KRAS mutant cancers, activates ERBB signaling by releasing a negative feedback on ERBB dimerization (Turke et al., 2012). This further suggests the ERBB activation could mitigate the responsiveness of other cancers to MEK inhibition.

Analogous to the effects of inhibition of the MEK pathway, inhibition of the PI3K pathway leads to potent activation of HER3-dependent signaling in *HER2*-amplified breast cancers (Chakrabarty et al., 2012; Chandarlapaty et al., 2011). In these cancers, coinhibition of HER3 and PI3K provided substantially greater antitumor efficacy. In other examples, EGFR activation has been observed as a resistance mechanism to small molecules targeting other tyrosine kinases. For example, EGFR activation is a resistance mechanism to ALK and MET inhibitors in *ALK*-positive lung and *MET*-amplified gastric cancers, respectively. Inhibition of EGFR resensitizes the resistant cancers to their respective TKI (Katayama et al., 2012; McDermott et al., 2010; Qi et al., 2011; Sasaki et al., 2011). Thus, activation of ERBB family members has emerged as a common mechanism of adaptation upon inhibition of downstream signaling, and inhibition of ERBB family members may be used to augment the efficacy of other pathway inhibitors.

Mechanisms of Action of EGFR and HER2 Inhibitors

HER2

Trastuzumab is a humanized immunoglobulin G_1 (IgG_1) antibody that binds to an epitope in juxtamembrane region IV of the HER2 receptor. It inhibits cleavage of the HER2 ectodomain, uncouples ligand-independent HER2-containing dimers leading to partial inhibition of downstream signaling, and triggers antibody-dependent, cell-mediated cytotoxicity (ADCC) (Clynes et al., 2000; Ghosh et al., 2011; Junttila et al., 2009; Molina et al., 2001; Yakes et al., 2002) (Table 2). This last mechanism cooperates with the recruitment of a T cell population mediating an adaptive immune (memory) response that enhances tumor

Table 2. ERBB Receptor Inhibitors: Mechanisms of Action and Key Clinical Trials

Drug	Type of Molecule	Mechanism of Action	FDA Approval	Key Clinical Trial(s)
Trastuzumab	humanized IgG_1, binds juxtamembrane domain IV	inhibits ectodomain cleavage and ligand-independent HER2-containing dimers; ADCC and adaptive immunity to HER2	1998 (metastatic breast); 2006 (adjuvant early breast); 2010 (advanced gastric)	Slamon et al., 2001; Piccart-Gebhart et al., 2005; Robert et al., 2006; Romond et al., 2005; Bang et al., 2010
Pertuzumab	humanized IgG_1, binds heterodimerization domain II	inhibits ligand-induced HER2-containing dimers	2012 (metastatic breast); 2013 (neoadjuvant breast)	Baselga et al., 2012b; Gianni et al., 2012; Schneeweiss et al., 2013
Lapatinib	small molecule	reversible, ATP-competitive TKI	2006 (advanced breast)	Geyer et al., 2006
Trastuzumab emtansine (T-DM1)	antibody-drug conjugate	same as trastuzumab plus inhibition of microtubules and cell lysis (DM-1)	2013 (advanced breast)	Verma et al., 2012
Erlotinib	small molecule	reversible, ATP-competitive TKI of EGFR	2004 (third-line advanced NSCLC); 2005 (pancreas cancer); 2013 (first-line EGFR mutant NSCLC)	Mok et al., 2009; Moore et al., 2007; Shepherd et al., 2005
Afatinib	small molecule	irreversible, ATP-competitive TKI of EGFR and HER2	2013 (metastatic EGFR mutant NSCLC)	Sequist et al., 2013b
Neratinib	small molecule	irreversible, ATP-competitive TKI of HER2	N/A	trials in patients with HER2 mutant tumors in progress
Cetuximab	human-murine chimeric IgG_2, binds ligand-binding domain	inhibits ligand-dependent activation of EGFR	2004 (originally for late line $EGFR^+$ CRC, but now only used in earlier-line wild-type KRAS CRC); 2006 (head and neck with radiotherapy or chemotherapy)	Van Cutsem et al., 2009; Vermorken et al., 2008; Bonner et al., 2006
Panitumumab	human IgG_1, binds ligand-binding domain	inhibits ligand-dependent activation of EGFR	2006 (originally for late-line $EGFR^+$ CRC, but now only used in earlier-line wild-type KRAS CRC)	Van Cutsem et al., 2007
AZD9291	small molecule	irreversible, ATP-competitive TKI of mutant EGFR (third generation)	NA	trials in EGFR mutant lung cancer in progress
CLO-1686	small molecule	irreversible, ATP-competitive TKI of mutant EGFR (third generation)	NA	trials in EGFR mutant lung cancer in progress

NA, not applicable.

eradication (Park et al., 2010; Stagg et al., 2011). The importance of the immune response is underscored by the finding that the therapeutic effect of trastuzumab was markedly diminished in mice that were engineered to be deficient in natural killer (NK) cells and macrophages capable of binding the Fc region of trastuzumab (Clynes et al., 2000). Pertuzumab is a monoclonal antibody that recognizes an epitope in heterodimerization domain II of HER2, thus blocking ligand-induced HER2-HER3 dimerization, resulting in partial inhibition of PI3K/AKT signaling (Agus et al., 2002). Because pertuzumab and trastuzumab bind to different epitopes in the HER2 ectodomain (Franklin et al., 2004), hence their complementary abilities to disrupt HER2-containing dimers, the combination of pertuzumab and trastuzumab has shown synergy in preclinical studies (Scheuer et al., 2009) and clinical trials (Baselga et al., 2012b; Gianni et al., 2012) and is now approved for treatment of patients with $HER2^+$ breast cancer. *T*rastuzumab-*d*erivative of *m*aytansine 1 (T-DM1 or trastuzumab emtansine) is an antibody-drug conjugate in which one molecule of trastuzumab is covalently bonded via a noncleavable linker to 3.5 molecules of a maytansinoid that inhibits microtubule polymerization (DM1). After binding to the receptor, the T-DM1/HER2 complex is internalized followed by degradation in lysosomes, release of DM1, and subsequent cell lysis (Lewis Phillips et al., 2008). T-DM1 binds to HER2 with similar affinity as trastuzumab, thus retaining the ability of the naked antibody to inhibit ligand-independent HER2-containing dimers and signal transduction as well as to mediate ADCC (Junttila et al., 2011).

Lapatinib is an ATP-competitive, reversible small-molecule inhibitor of the HER2 and EGFR tyrosine kinases (Konecny et al., 2006). In $HER2^+$ breast cancers, lapatinib quickly disables HER2 signaling, resulting in inhibition of the PI3K/AKT and MAPK pathways, and it has shown clinical activity in $HER2^+$ breast cancers that have progressed on trastuzumab (Geyer et al., 2006). Lapatinib also binds the inactive conformation of EGFR (Wood et al., 2004), but it has not been active against cancers for which EGFR antibodies or TKIs are approved. Afatinib (Minkovsky and Berezov, 2008) and neratinib (Burstein et al., 2010) are irreversible, covalent HER2/EGFR TKIs with activity against HER2, HER4,

EGFR, and some HER2 insertion mutants (Bose et al., 2013). Of note, the clinical efficacy of all therapeutic inhibitors of HER2 has been predominantly limited to breast cancers that overexpress HER2 as measured by intense membrane staining in the majority of tumor cells with HER2 antibodies (3+ by IHC) or excess copies of the *HER2* gene determined by FISH.

EGFR

Gefitinib and erlotinib are ATP-competitive EGFR TKIs (Table 2). Biochemical and crystallography analyses demonstrate that the mutants possess a higher affinity for the first-generation EGFR inhibitors gefitinib and erlotinib compared to the wild-type receptor (Carey et al., 2006; Yun et al., 2007). Thus, the mutant enzymes are inhibited at lower concentrations of drug, which leads to a favorable therapeutic index. As will be discussed in greater detail below, EGFR mutant lung cancers often develop a second mutation in the gatekeeper residue, T790M, as they become resistant to gefitinib or erlotinib. Thus, there have been intense efforts to develop a drug that can inhibit T790M EGFR to overcome resistance. One such effort was the development of second-generation EGFR inhibitors, such as afatinib and dacomitinib. These drugs are irreversible ATP competitors that form covalent links with the Cys773 residue of EGFR. Although these second-generation drugs have the capacity to inhibit the EGFR T790M, they do so at concentrations that also inhibit wild-type EGFR. Thus, there is not a favorable therapeutic index, and dose-limiting toxicities due to inhibition of wild-type EGFR (such as rash and diarrhea) prevent increasing doses high enough to fully suppress T790M. Thus, they have been largely ineffective at overcoming T790M-mediated resistance in the clinic. Pao et al. found that mouse lung transgenic tumors expressing T790M EGFR are sensitive to the combination of afatinib and cetuximab (Regales et al., 2009). This combination has progressed to the clinic, where it has demonstrated significant clinical activity against T790M EGFR lung cancers, although it is also associated with significant toxicity (Janjigian et al., 2011).

More recently, third-generation EGFR inhibitors have been developed. The first of such compounds, WZ-4002, was designed to be much more potent against the resistant T790M mutation than the wild-type receptor, thus restoring a favorable therapeutic index in which the drugs can be dosed high enough to inhibit T790M without inducing toxicity from inhibiting wild-type EGFR (Walter et al., 2013; Zhou et al., 2009). Of note, this drug is not a quinazoline derivative like the first- and second-generation EGFR inhibitors. WZ-4002 has not been developed clinically, whereas two drugs with similar properties, AZD9291 and CLO-1686, have been (Walter et al., 2013). Clinical data are emerging for these compounds, and the high rate of clinical responses, with minimal toxicity, is increasing enthusiasm for the class of drugs (Ranson et al., 2013; Sequist et al., 2013a; Soria et al., 2013).

In contrast to the EGFR TKIs, the EGFR-neutralizing antibody cetuximab blocks ligand binding to the EGFR. Thus, it is most effective in cancers that harbor ligand-activated, wild-type *EGFR*. In colorectal cancers with wild-type *KRAS*, inhibition of EGFR leads mainly to loss of downstream ERK signaling. However, since mutant KRAS directly activates ERK, cetuximab fails to suppress ERK in these cancers, most likely explaining the lack of clinical activity (Ebi et al., 2011). As a result, cetuximab is now used primarily in cancers with wild-type *KRAS*. Panitumumab is another EGFR-targeted antibody that has activity in wild-type *KRAS* CRC. Unlike cetuximab, it is an IgG_2, and is predicted not to engage immune effector cells to mediate ADCC. Despite this difference, phase III studies have demonstrated clinical efficacy similar to that of cetuximab (Douillard et al., 2014; Jonker et al., 2007; Van Cutsem et al., 2007, 2009). Thus, it seems plausible that the primary mechanism of action of cetuximab and panitumumab is due to its inhibition of EGFR signaling and not engagement of ADCC.

HER3 Inhibitors

Several HER3-neutralizing antibodies are in clinical development. MM-121 and U3-1287 (formerly AMG-888) bind the extracellular domain of HER3, block heregulin-induced phosphorylation, and reduce expression of HER3 at the cell surface (Garrett et al., 2011; Schoeberl et al., 2010). MM-121 (IgG_2) is most effective against tumors with ligand-dependent activation of HER3 (Sheng et al., 2010). U3-1287 synergizes with trastuzumab and lapatinib to suppress the growth of *HER2*-amplified xenografts (Garrett et al., 2013a) and has single-agent activity against transgenic mouse mammary cancers induced by Polyomavirus middle T antigen (Cook et al., 2011). RG7116 is an IgG_1 that selectively binds domain 1 of human HER3. It blocks ligand binding and downregulates HER3 from the cell surface. Through glycoengineering of its Fc moiety, RG7116 mediates enhanced ADCC that correlates with HER3 receptor density (Mirschberger et al., 2013). At this time, these antibodies have completed phase I safety and dose-finding trials, but their clinical efficacy remains to be shown.

LJM716 is a novel anti-HER3 antibody that binds an epitope within domains 2 and 4 in the receptor's extracellular domain, thus trapping HER3 in an inactive conformation. In contrast to the other anti-HER3 antibodies, it blocks both ligand-induced and ligand-independent HER3 dimerization and activation (Garner et al., 2013). This property may be particularly advantageous in *HER2*-amplified breast cancers, in which HER2 appears to activate HER3 in a ligand-independent manner. Accordingly, in laboratory studies, LJM716 reduced growth of established *HER2*-amplified xenografts when given as a single agent and synergized with PI3K inhibitors to suppress growth of *HER2*-amplified/*PIK3CA* mutant tumors (Garrett et al., 2013b).

More recently, bispecific antibodies targeting HER3 have been introduced. MM-111 is an antibody that docks onto HER2 and subsequently binds HER3, thus blocking ligand-dependent activation of HER2/HER3 dimers (McDonagh et al., 2012). Finally, MEHD7945A is a two-in-one IgG_1 generated by phage display engineering that specifically binds HER3 and EGFR with high affinity, thus blocking TGF-α- and HRG-induced activation of both receptors and downstream PI3K/AKT and ERK signaling. MEHD7945A mediates ADCC in vivo and demonstrates superior antitumor activity against multiple tumor models compared to monospecific antibodies (Schaefer et al., 2011). Currently, HER3 inhibitors are being developed in combination with trastuzumab, EGFR antibodies and TKIs, PI3K inhibitors, and cytotoxic chemotherapy. In addition to *HER2* amplification and *EGFR* mutation, high heregulin expression and *HER3* mutations are being explored as predictive biomarkers of response in clinical trials.

Mechanisms of Resistance to ERBB Inhibitors

Although ERBB-targeted therapies have provided substantial benefit to patients with advanced cancer, cancers ultimately

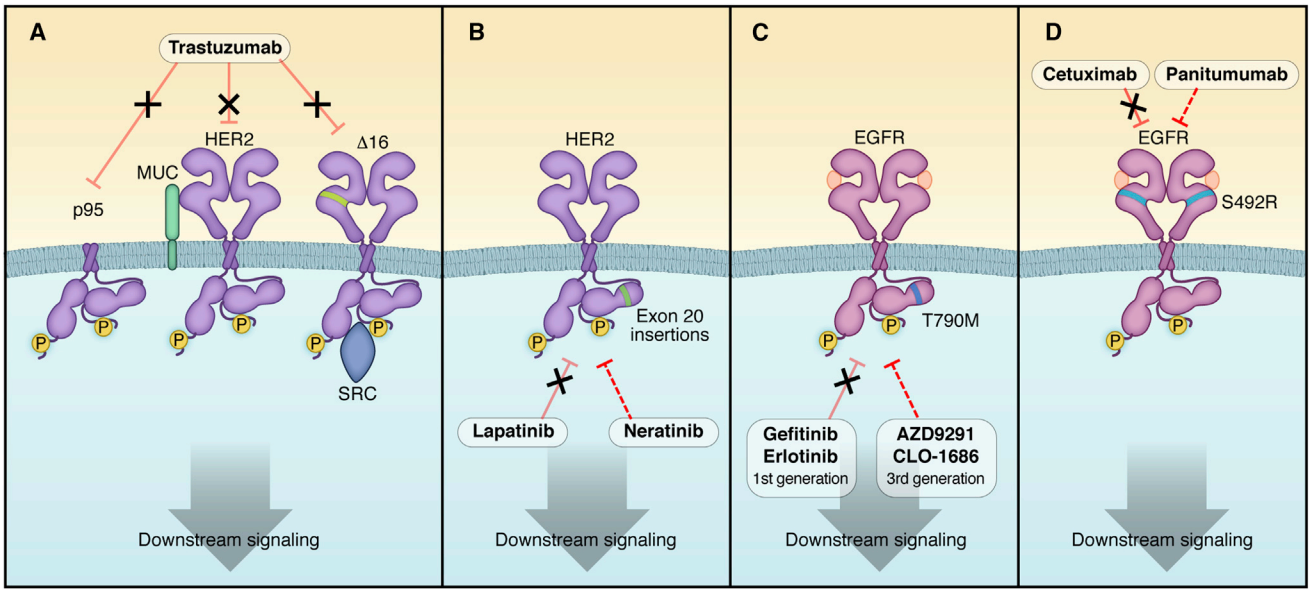

Figure 1. Schema Depicting Intragenic Alterations Leading to Resistance to HER2 and EGFR Inhibitors

(A) HER2 truncations (p95) and splice variants (Δ16) are not inhibited by trastuzumab. In addition, expression of specific mucin isoforms can prevent trastuzumab from binding HER2 (Price-Schiavi et al., 2002). Not shown in the figure, pertuzumab and T-DM1 cannot recognize p95 either.

(B) HER2s harboring exon 20 insertions are not inhibited by lapatinib, but may be sensitive to irreversible HER2 inhibitors afatinib and neratinib. They are also resistant to trastuzumab.

(C) The EGFR T790M gatekeeper mutation leads to acquired resistance to first generation EGFR inhibitors, but is effectively inhibited by third-generation EGFR inhibitors.

(D) An EGFR mutation in the extracellular domain is associated with acquired resistance to cetuximab, but may still be sensitive to another anti-EGFR antibody, panitumumab. Dashed lines indicate inhibition via alternative antibodies and inhibitors.

have developed resistance to the current approaches. In this Perspective, we will discuss both de novo and acquired resistance. The distinction is primarily a clinical one: de novo or intrinsic resistance refers to cancers that do not exhibit an initial response, whereas acquired resistance develops after an initial, often marked and durable, clinical response. It is important to appreciate that the same molecular mechanism may cause both types of resistance, underscoring the robustness of the biological principles underlying how cancers evade these therapies.

Mechanisms of resistance have been discovered by several approaches, including the maintenance of cell lines and xenografts in the presence of drug until resistance emerges or infection of sensitive cell lines with open reading frame (ORF) or shRNA libraries to identify genes whose expression or loss leads to resistance. These efforts have also been coupled to biopsy programs, in which cancers are systematically biopsied upon the development of resistance to interrogate acquired molecular changes upon treatment pressures (Sequist et al., 2011; Yano et al., 2011; Yu et al., 2013). However, there are significant limitations with many of the laboratory studies. Although EGFR TKIs are primarily used as single agents for *EGFR* mutant lung cancers, HER2-directed therapies and EGFR antibodies are generally used in combination with chemotherapy in the clinic. However, most laboratory studies have modeled resistance to these agents as single therapies, thus not recapitulating the selective pressure of combination therapies applied in the clinic. Other data about potential resistance mechanisms have been derived from correlative clinical trials in which patients have been treated with anti-HER2 drug(s) in combination with chemotherapy, a variable not always considered in the interpretation of the studies of drug resistance. Finally, even though combinations of HER2 antagonists are increasingly used in the clinic, resistance to these combinations has yet to be modeled widely in the laboratory.

Intrinsic HER2 Alterations

Some resistance mechanisms affect the capacity for HER2 inhibitors to directly engage HER2. Anido et al. described p95-HER2, a truncated form of HER2 lacking the trastuzumab binding region, which may arise from alternate transcription initiation sites in *HER2* (Anido et al., 2006) (Figure 1A). Patients with metastatic breast cancer harboring cytosolic expression of p95-HER2 exhibit a very low response rate to trastuzumab compared to those patients without p95-HER2 in their tumors (Scaltriti et al., 2007). This form of HER2 retains kinase activity, and tumors with p95-HER2 may still be susceptible to kinase inhibition with a TKI, as suggested by the observation that p95-HER2 tumors exhibit a similar response rate to the combination of capecitabine and lapatinib compared to breast cancers expressing full-length HER2 (Scaltriti et al., 2010).

A splice variant that eliminates exon 16 in the extracellular domain of the HER2 receptor has also been identified in $HER2^{+}$ primary breast cancers and cell lines (Kwong and Hung, 1998) (Figure 1A). This variant does not eliminate the trastuzumab epitope on HER2, but stabilizes HER2 homodimers and prevents their disruption upon binding by the antibody, resulting in trastuzumab resistance in cell lines. The Δ16 isoform was found to interact directly with Src, and treatment with the Src inhibitor dasatinib overcame the resistance to the antibody

conferred by the alternative splicing variant (Mitra et al., 2009). However, clinical evidence of an association between HER2-Δ16 and resistance to trastuzumab has not been shown.

HER2 mutations have been found in a small proportion of lung, gastric, colorectal, breast, and head and neck cancers (Lee et al., 2006; Ross et al., 2013; Stephens et al., 2004; Willmore-Payne et al., 2006). These mutants of HER2 are resistant to lapatinib and trastuzumab (Figure 1B), but are sensitive to the covalent HER2 TKI neratinib (Bose et al., 2013; Wang et al., 2006). To the best of our knowledge, *HER2* mutations in *HER2* gene-amplified breast tumors are very rare. As such, they have not been identified as a resistance mechanism to trastuzumab. One possible reason is that these mutations may comprise only a portion of the amplified HER2 alleles and, therefore, exist below the limits of sensitivity of traditional DNA sequencing methods (Zito et al., 2008). It is possible that cancer cells harboring these mutations will be selected, or acquired, after the selective pressure of anti-HER2 treatment. If so, they may only be detected in tumors that are progressing after primary HER2-targeted therapy. However, comprehensive studies profiling $HER2^+$ tumors that have progressed on primary anti-HER2 therapies have not been reported.

Intrinsic EGFR Alterations

In EGFR mutant lung cancer, the most common mechanism of acquired resistance to EGFR inhibitors is the development of a mutation in the gatekeeper residue of EGFR, T790M (Kobayashi et al., 2005; Pao et al., 2005). T790M abrogates the inhibitor effects of gefitinib and erlotinib by increasing the affinity of the receptor for ATP (Yun et al., 2008), thereby lessening the potency of first-generation EGRF inhibitors (Figure 1C). At least 50% of biopsies from patients with acquired resistance harbor the T790M mutation. Recent studies suggest that highly sensitive methods can detect the T790M mutation in ~35% of pretreatment biopsies. This suggests, but does not prove, that it preexists in a small fraction of cells and that those cells are selected for during the course of treatment (Maheswaran et al., 2008; Rosell et al., 2011). Currently, the third-generation EGFR inhibitors (discussed above) are in early clinical trials to overcome this resistance.

An analogous finding has been observed in wild-type KRAS CRCs that develop resistance to cetuximab. A small study reported the development of a S492R mutation in the extracellular domain of EGFR that interferes with cetuximab binding, but does not interfere with ligand-dependent activation or abrogate receptor engagement by panitumumab (Montagut et al., 2012) (Figure 1D).

Bypass Track Resistance

Other than the immune effects of ERBB antibodies, it is believed that most of activity of these drugs is due to suppression of downstream signaling, particularly PI3K/AKT and MEK/ERK. Thus, many cancers are resistant to single-agent ERBB inhibitors because at least one of these critical downstream pathways is maintained despite inhibition of the targeted receptor. This type of resistance, also termed "bypass track" resistance, is often used to describe resistance resulting from maintenance of these key downstream signaling pathways despite adequate inhibition of the respective RTK (reviewed in Niederst and Engelman, 2013; Figure 2).

Ligand- and RTK-Mediated Resistance. One of the earliest validated observations that RTK bypass signaling induces resistance to ERBB inhibitors was in *EGFR*-mutant NSCLCs. Amplification of the *MET* gene was found in *EGFR* mutant cancers with acquired resistance to EGFR TKIs but not in pretreatment biopsies (Bean et al., 2007; Engelman et al., 2007). In these resistant cancers, MET reactivates both PI3K/AKT and MEK/ERK signaling despite the inhibition of EGFR. The combination of MET and EGFR inhibitors was sufficient to block downstream signaling and induce marked tumor regressions (Engelman et al., 2007; Turke et al., 2010). Activation of MET by its ligand hepatic growth factor (HGF) was also sufficient to promote resistance through activation of downstream signaling (Yano et al., 2008). MET has also been implicated in trastuzumab resistance. HGF-induced signaling through MET was shown to abrogate the action of trastuzumab (Shattuck et al., 2008; Turke et al., 2010). Further, gene amplification of MET and HGF was reported in a cohort of $HER2^+$ patients who did not respond to trastuzumab and chemotherapy (Minuti et al., 2012). Thus, MET activation by either gene amplification or ligand stimulation can cause bypass resistance to EGFR and HER2 inhibitors.

Reactivation of EGFR and HER3 can also serve as a mechanism of resistance to ERBB inhibitors. In laboratory models of *HER2*-amplified breast cancer treated with trastuzumab, increased levels of EGFR and ERBB ligands led to an increase in active EGFR/HER3 and EGFR/HER2 dimers to promote resistance (Ritter et al., 2007). This is consistent with data showing that trastuzumab is unable to block ligand-induced HER2-containing heterodimers (Agus et al., 2002). Similarly, activation of TGFβ receptors can increase ERBB ligand production and cleavage, particularly TGF-α, amphiregulin, and heregulin, via activation of the TACE/ADAM17 sheddase; this results in activation of HER3 and PI3K and promotes drug resistance (Wang et al., 2008). Further, a gene signature of TGFβ activity was developed and shown to correlate with resistance to trastuzumab and poor clinical outcome in patients (Wang et al., 2008).

Similarly, in *EGFR* mutant cancers, *MET* amplification leads to resistance to EGFR TKIs through reactivation of HER3 (Engelman et al., 2007). In a subset of *EGFR* mutant lung cancers, amplification of HER2, presumably involving HER3 reactivation, was also identified as a resistance mechanism to EGFR TKIs (Takezawa et al., 2012). Consistent with these data, blockade of HER3 with the neutralizing antibody MM-121 increases the efficacy of cetuximab in a mouse model of EGFR mutant lung cancer (Schoeberl et al., 2010). Along those lines, a selective ADAM inhibitor, INCB3619, which prevents the processing and activation of multiple ERBB ligands including heregulin, inhibits HER3 signaling and enhances gefitinib-mediated inhibition of EGFR in NSCLC (Zhou et al., 2006). Further supporting a role of amplified HER2-HER3 signaling in resistance to EGFR-targeted therapies, colorectal cancer patients with de novo or acquired resistance to cetuximab-based therapy exhibit HER2 amplification in their tumor or high levels of circulating heregulin (Yonesaka et al., 2011). Finally, using patient-derived colon cancer xenografts, Bertotti et al. identified *HER2* gene amplification as a predictor of resistance to cetuximab among KRAS wild-type tumors (Bertotti et al., 2011).

IGF-I receptors have also been implicated in driving resistance to both EGFR and HER2 inhibitors. Overexpression of IGF-1R or an increase in levels of IGF-1R/HER2 heterodimers can potently activate PI3K/AKT signaling and confer resistance to

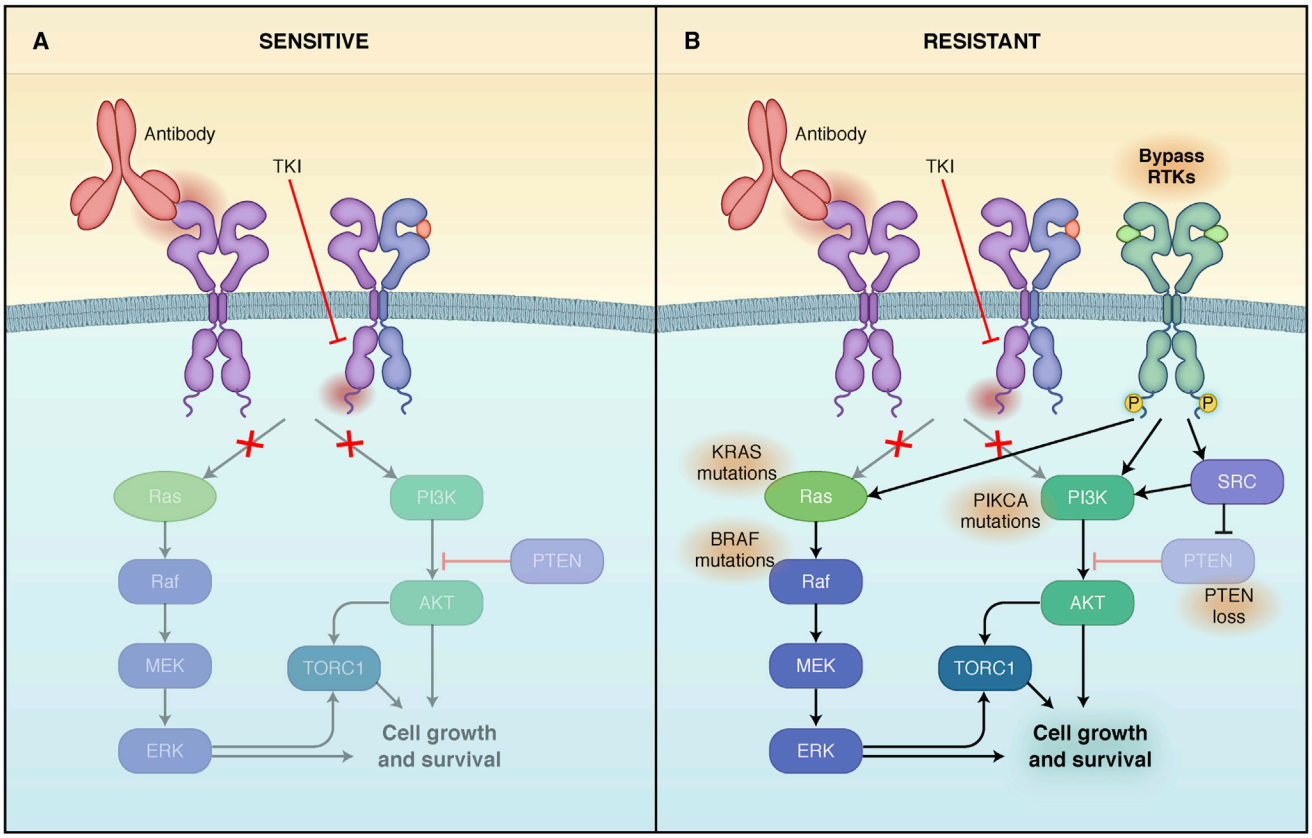

Figure 2. Schematic Depicting Resistance to EGFR and HER2 Inhibitors due to Activation of Bypass Track Signaling
(A) Model of a sensitive EGFR or HER2-addicted cancer treated with an ERBB small-molecule inhibitor or antibody resulting in suppression of downstream signaling. EGFR or HER2 homodimers and heterodimers are shown.
(B) Model of a EGFR mutant or *HER2*-amplified cancer with resistance due to maintenance of downstream signaling in the presence of the EGFR or HER2 inhibitors. Activation of signaling can be caused by activation of other RTKs or mutational activation of downstream signaling.

trastuzumab in laboratory studies (Huang et al., 2010). Inhibition of IGF-1R with a neutralizing antibody or a small-molecule TKI, or targeting of the HER2 kinase with lapatinib was found to overcome IGF-1R-mediated resistance to trastuzumab (Nahta et al., 2007). In a neoadjuvant trial of chemotherapy plus trastuzumab, a high level of IGF-1R expression measured by IHC correlated with a poor clinical response (Harris et al., 2007). Similarly, activation of IGFIR, via loss of expression of *IGFBP3* and *IGFBP4*, which encode insulin-like growth factor binding proteins 3 and 4, respectively, maintains PI3K/AKT signaling despite blockade of EGFR and promotes resistance to EGFR inhibitors in multiple cell lines (Guix et al., 2008). In these cases, inhibition of IGF1R resensitized to EGFR inhibition. In addition, inhibition of IGF-IR also suppressed the development of "persistor cells," the small population of PC9 EGFR mutant cells that survives the initial inhibition of EGFR, described by Settleman and colleagues (Sharma et al., 2010).

In addition to the bypass pathways mentioned above, numerous other RTK-mediated resistance mechanisms have been observed. The EphA2 receptor has been shown to confer resistance to trastuzumab in cell lines, and EphA2 expression was shown to predict poor outcome patients with $HER2^+$ breast cancer (Zhuang et al., 2010). Most recently, the erythropoietin (Epo) receptor was found to be coexpressed in cell lines and primary tumors that overexpress HER2. In these cell lines, concurrent treatment with recombinant erythropoietin conferred trastuzumab resistance. In patients with $HER2^+$ breast cancer, the concurrent administration of erythropoietin and trastuzumab correlated with a shorter progression-free and overall survival compared to patients not receiving erythropoietin (Liang et al., 2010). Finally, in erlotinib-resistant *EGFR* mutant lung cancer cells and lapatinib-resistant *HER2*-amplified breast cancer cells, levels of the AXL RTK were markedly increased (Liu et al., 2009; Zhang et al., 2012). Targeting of AXL was able to resensitize some of these resistant cancers to the original TKI.

We should note that most of these RTK-mediated mechanisms do not necessarily involve genetic activation of the RTK, as mainly protein assays (i.e., IHC for IGF-IR, AXL, EphA2, etc.) have been employed to measure their levels in tumor tissues. Such correlations do not *prove* that the putative bypass RTK is causal to drug resistance in the clinic or in a particular patient. Ultimately, clinical efficacy using specific drugs that target the bypass RTK will be needed for true validation.

Intracellular Kinases. Molecules in the pathways downstream of RTKs can be aberrantly activated as a result of genetic alterations, also resulting in drug resistance (Figure 2). Somatic alterations in the PI3K/AKT pathway are the most frequent in breast cancer, occurring in approximately 30% of $HER2^+$ tumors. These

include mutation and/or amplification of the genes encoding the PI3K catalytic subunits p110α (*PIK3CA*) and p110β (*PIK3CB*), the PI3K regulatory subunit p85α (*PIK3R1*), the PI3K effectors AKT1, AKT2, and PDK1, and loss of the lipid phosphatases PTEN and INPP4B (reviewed in Engelman, 2009). It is generally accepted that the antitumor activity of HER2 inhibitors depends on inhibition of PI3K-AKT downstream of HER2. Thus, one would expect that activating mutations in the PI3K pathway would confer resistance to HER2 inhibitors.

Constitutive activation of PI3K, via overexpression of *PIK3CA* mutants, conferred resistance to the antibody in laboratory studies (Chakrabarty et al., 2010; Eichhorn et al., 2008; Serra et al., 2008). Patients with "hot-spot" *PIK3CA* mutations and undetectable or low PTEN measured by IHC exhibited a poorer outcome after treatment with chemotherapy and trastuzumab compared to patients without those alterations (Berns et al., 2007; Dave et al., 2011; Esteva et al., 2010). In the EMILIA and Neo-ALTTO randomized trials in HER2$^+$ breast cancer, patients with *PIK3CA* mutant tumors did not benefit from lapatinib and capecitabine (Baselga et al., 2013b) and from lapatinib and trastuzumab (Baselga et al., 2013a), respectively. It remains to be determined whether T-DM1, because of its ability to deliver high levels of cytotoxic chemotherapy to HER2-overexpressing cells, trumps this mechanism of resistance.

One of the first discoveries linking constitutive activation of PI3K signaling and resistance to HER2 inhibitors was accomplished by Berns et al. Using a large-scale small interfering RNA genetic screen, they identified PTEN as the only gene whose knockdown conferred trastuzumab resistance (Berns et al., 2007). However, the association of PTEN loss with drug resistance in the clinic is less clear. In one early study in patients with metastatic breast cancer, loss or low levels of PTEN correlated with a lower response to trastuzumab (Nagata et al., 2004). This correlation was not found in patients with early breast cancer treated with adjuvant trastuzumab (Perez et al., 2013). We speculate that this was because of the concomitant administration of chemotherapy in an adjuvant setting (Rexer et al., 2013).

Similar findings have been observed in cancers with acquired resistance to EGFR inhibitors. Introduction of *PIK3CA* mutations into EGFR mutant lung cancer cell lines is sufficient to maintain PI3K signaling and promote resistance (Engelman et al., 2006). Accordingly, PIK3CA mutations have been identified in biopsies of *EGFR* mutant cancers with acquired resistance to EGFR inhibitors (Sequist et al., 2011). Similarly, a report found that *PTEN* loss may be associated with resistance to EGFR inhibitors (Sos et al., 2009). In addition to reactivation of PI3K, reactivation of ERK signaling can promote resistance to EGFR inhibitors, as evidenced by the finding of a *BRAF* mutation in an *EGFR* mutant lung cancer with acquired resistance (Ohashi et al., 2012). In a second example, an *EGFR* mutant cell line made resistant to third-generation EGFR inhibitors developed amplification of ERK and was resensitized upon inhibition of MEK (Ercan et al., 2012).

A compelling recent discovery underlying this type of resistance mechanism was the study of KRAS wild-type colorectal cancers that had developed resistance to cetuximab. By performing repeat biopsies and evaluating circulating tumor DNA, investigators observed the emergence of *KRAS* mutations as a resistance mechanism (Diaz et al., 2012; Misale et al., 2012). From a signaling perspective, one would expect that the *KRAS* mutant clones fail to downregulate the ERK pathway in response to cetuximab, underlying the resistance. As the presence of KRAS mutations predicts for lack of initial response to cetuximab, these findings underscore the convergence of intrinsic and acquired resistance mechanisms.

Src family kinase (SFK) signaling has been implicated by several studies in promoting resistance to HER2 inhibitors. In HER2$^+$ breast cancer cells with acquired resistance to lapatinib, upregulation of SFK activity, particularly Yes, was observed in several resistant cell lines. Resistance was associated with recovery of PI3K/AKT signaling despite inhibition of HER2. Addition of a Src TKI partially blocked PI3K/AKT and restored sensitivity to lapatinib (Rexer et al., 2011). In another study, the authors suggest that PTEN was no longer capable of dephosphorylating and suppressing Src in trastuzumab-resistant HER2$^+$ cells, and the addition of a Src kinase inhibitor overcame trastuzumab resistance (Zhang et al., 2011). Src activity is also involved in the resistance conferred by the Δ16 HER2 isoform and the EpoR (Mitra et al., 2009). Src is thought to mediate resistance in part via phosphorylation and inhibition of PTEN, leading to constitutive PI3K signaling (Liang et al., 2010).

Defects in Apoptosis and Cell-Cycle Control

Inhibition of a driver oncogene such as EGFR and HER2 results in proliferation arrest and apoptosis. Therefore, alterations in the normal apoptotic machinery can also induce resistance to EGFR- and HER2-targeted therapies. Indeed, we observed that levels of the proapoptotic BH3-only Bcl2 family member, BIM, are predictive of response to targeted therapy in *EGFR* mutant lung cancers, *HER2*-amplified breast cancers, and *PIK3CA* mutant cancers (Faber et al., 2011). BIM protein normally is induced after inhibition of EGFR and HER2 in these cancers. In this study, although erlotinib or lapatinib inhibited EGFR and HER2 and downstream signaling in *EGFR* mutant and *HER2*-amplified cancers, respectively, only those cell lines with high levels of BIM underwent marked apoptosis. This suggests that BIM levels are a biomarker predictive of response to a TKI in an oncogene-addicted cancer. Other groups have reported similar results in EGFR mutant lung cancers (Ng et al., 2012) and *HER2* gene-amplified breast cell lines with and without activating mutations (Tanizaki et al., 2011). In cancers with concurrent *PIK3CA* mutant cells, however, both the growth inhibitory effect and induction of BIM after treatment with lapatinib were blunted (Tanizaki et al., 2011).

Survivin, a member of the inhibitor of apoptosis (IAP) protein family that inhibits the activity of caspases, has been shown to be a point of convergence of several pathways that can lead to resistance to HER2 inhibitors. In HER2$^+$ breast cancer cells, inhibition of HER2-PI3K reduces survivin expression resulting in apoptosis. *HER2*-amplified breast cancer cells with acquired resistance to lapatinib upregulate ERα, which, in turn, induces FoxO3a-mediated transcription of survivin (Xia et al., 2006). In turn, high survivin levels allow for escape from lapatinib. Accordingly, elevated levels of survivin and MCL-1 have been found in trastuzumab-resistant cells (Chakrabarty et al., 2013).

Altered control of progression through the cell cycle in response to HER2 inhibition also plays a role in resistance. Cell lines made resistant to trastuzumab by chronic exposure

exhibited focal amplification of cyclin E. CDK2 inhibitors reduced growth of these trastuzumab-resistant xenografts (Scaltriti et al., 2011). Further, in a cohort of patients with $HER2^+$ breast cancers treated with trastuzumab, amplification of cyclin E was associated with a diminished clinical response. Downregulation of the Cdk inhibitor $p27^{KIP1}$ and a resulting increase in Cdk activity has also been associated with trastuzumab resistance (Nahta et al., 2004; Yakes et al., 2002). Indeed, modulation of levels of $p27^{KIP1}$ appears to be a common endpoint for several of the resistance pathways noted above, including signaling from IGF-1R and MET (Nahta et al., 2005; Shattuck et al., 2008).

Tumor Host Factors

Host factors that affect the immunomodulatory function of trastuzumab can also contribute to trastuzumab resistance. In mice lacking FcγRIII and, thus, deficient in NK cells and macrophages capable of binding the Fc region of trastuzumab, the therapeutic effect of trastuzumab was markedly diminished (Clynes et al., 2000). Polymorphisms in the gene encoding FcγRIII in humans were associated with resistance to trastuzumab in patients with metastatic $HER2^+$ breast cancer (Musolino et al., 2008). In the same study, PBMCs from patients with *FCGR3* polymorphisms associated with an improved outcome after trastuzumab induce a stronger trastuzumab-mediated ADCC in vitro. A follow-up study found that the quantity and lytic efficiency of $CD16^+$ lymphocytes are the major factors affecting the level of ADCC induced by trastuzumab. This, in turn, correlates with tumor response (Gennari et al., 2004). We should note, however, that a large trial of trastuzumab-based adjuvant chemotherapy in patients with early $HER2^+$ breast cancer did not show an association between *FCGR3A* and *FCGR2A* polymorphisms with patient outcome (Hurvitz et al., 2012).

Strategies to Overcome Resistance: Combination Therapies

Clinical experience has validated EGFR and HER2 as effective drug targets. However, in the metastatic setting, these inhibitors do not lead to cures, and cancers ultimately develop resistance. Thus, there is a great need to identify therapeutic strategies that will improve upon the current approaches. We believe that one strategy will include maximal blockade of the oncogene target itself as well as inhibition of the key bypass tracks that promote resistance. The growing number of escape routes will likely necessitate combinations of multiple agents, whose delivery will require innovative dosing and scheduling.

HER2

All currently available HER2 inhibitors target or exploit mechanisms of HER2 function. As single drugs, however, they do not potently suppress HER2 signaling. This may explain the generally short-lived responses of metastatic $HER2^+$ breast cancers to single-agent HER2 inhibitors. Trastuzumab and pertuzumab, in particular, are each weak signaling inhibitors, possibly because they incompletely block HER2-containing dimers (Junttila et al., 2009). Treatment with lapatinib (and most likely other HER2 TKIs) leads to an increase in HER2 and HER2-containing dimers at the plasma membrane and fails to completely and persistently inhibit the HER2 kinase (Garrett et al., 2011; Scaltriti et al., 2009). This may be explained by both the narrow therapeutic index of current HER2 TKIs and the challenge of complete and sustained inhibition of an amplified drug target, i.e., HER2, with small molecules. Moreover, inhibition of the HER2 kinase leads to an initial reduction of PI3K/AKT signaling, which releases negative feedbacks resulting in upregulation of HER3 and other RTKs, as well as survival factors such as BCL2 and ERα, thereby mitigating the efficacy of HER2 inhibitors (Chakrabarty et al., 2012; Chandarlapaty et al., 2011; Garrett et al., 2011; Muranen et al., 2012; Xia et al., 2006). We also recognize that *HER2*-amplified breast cancers vary with respect to their addiction to HER2 signaling, although they are grouped together using clinical criteria (FISH, IHC) for HER2 overexpression. Thus, we speculate many *HER2*-gene-amplified cancers are not truly "addicted" to HER2 signaling and, as such, are not sensitive to HER2 TKIs. Finally, resistance to T-DM1 may be due to several reasons, including the sparing of $HER2^-$ tumor cells within heterogeneous cancers containing $HER2^+$ and HER^- tumor cells, a scenario not uncommon in clinical practice. Finally, T-DM1, trastuzumab, and pertuzumab cannot bind p95-HER2 and, thus, would be inactive against tumor cells with an abundance of cytosolic fragments of HER2.

One strategy to address the limitations of anti-HER2 drugs as single agents has been to combine multiple HER2 antagonists that have different but complementary mechanisms of action. Clinical experience had suggested that trastuzumab-refractory tumors remained dependent on HER2 as continuing trastuzumab in new treatment regimens beyond progression to trastuzumab demonstrated clinical benefit (von Minckwitz et al., 2009). Currently, dual blockade of HER2 is well entrenched in the clinic. For example, the combination of trastuzumab and lapatinib is superior to each agent alone in both the metastatic (Blackwell et al., 2010) and neoadjuvant (Baselga et al., 2012a) settings. Similarly, the combination of trastuzumab and pertuzumab was shown to be superior to each antibody alone in both neoadjuvant trials in patients with early disease (Gianni et al., 2012) and in patients with advanced disease (Baselga et al., 2012b), as assessed by progression-free survival. The combination of T-DM1 and pertuzumab is also in progress. This novel approach would incorporate dual receptor blockade with two HER2 antibodies (trastuzumab and pertuzumab) plus the delivery of a potent cytotoxic (DM1) to *HER2*-amplified cells while mostly sparing host tissues (Phillips et al., 2014).

At the time of writing this Perspective, several novel anti-HER2 combinations are being tested in clinical trials. Some of these include a third drug targeted to the HER2 network (Table 3). It is anticipated that for a cohort of $HER2^+$ breast cancers that escape anti-HER2 dual therapy, a third drug targeted against a signaling hub in the receptor network might be necessary. Supporting this possibility, a recent study showed that transgenic mammary tumors expressing HER2 and $PIK3CA^{H1047R}$ were completely resistant to the combinations of trastuzumab plus pertuzumab and trastuzumab plus lapatinib. Addition of the pan-PI3K inhibitor BKM120 to each combination resulted in inhibition of tumor growth, but only partially and temporarily (Hanker et al., 2013). Currently, a main clinical focus is the addition of PI3K inhibitors and/or HER3-neutralizing antibodies (Garrett et al., 2013b) to the established combinations of anti-HER2 therapies (Table 3). More-recent data suggest that blockade of mTOR downstream HER2 with the TORC1 inhibitor everolimus, while maintaining trastuzumab therapy, can induce clinical responses in $HER2^+$ cancers that have progressed on trastuzumab (Hurvitz et al., 2013; Morrow et al., 2011; O'Regan et al., 2013).

Table 3. Anti-ERBB Combinations

Combination	Mechanism(s) of Action	Relevant Clinical Trials
Trastuzumab + lapatinib (or neratinib, afatinib)	ADCC, partial disruption of HER2-HER3 dimers, inhibition of HER2 and EGFR tyrosine kinases	Baselga et al., 2012a; Blackwell et al., 2010
Trastuzumab + pertuzumab (only approved combination)	More-complete inhibition of ligand-induced and ligand-independent HER2-containing heterodimers, ADCC, downregulation of HER2 from cell surface	Baselga et al., 2012b; Gianni et al., 2012; Schneeweiss et al., 2013
T-DM1 + pertuzumab	Same as above plus inhibition of polymerization of microtubules with DM1	MARIANNE (NCT01120184)
Trastuzumab + everolimus	ADCC, disruption of ligand-independent HER2-HER3 dimers, inhibition of TORC1	BOLERO-3 (NCT01007942)
Trastuzumab + pertuzumab + PI3K inhibitor	Inhibition of ligand-induced and ligand-independent HER2-containing heterodimers, ADCC, ATP-competitive inhibition of catalytic activity of p110	
Trastuzumab + HER3-neutralizing antibody	ADCC, partial disruption of HER2-HER3 dimers, inhibition of heregulin binding, downregulation of HER3 and/or HER3 dimerization	
Trastuzumab + HER3 antibody + PI3K inhibitor	Same as above plus direct inhibition of p110	
T-DM1 + PI3K inhibitor	ADCC, partial disruption of HER2-HER3 dimers, inhibition of polymerization of microtubules, direct inhibition of p110	
Afatinib + cetuximab	Combined targeting of EGFR T790M to compensate for complete inhibition of target by either approach alone. Afatinib may also target resistance due to HER2 activation	NCT01090011
EGFR + PI3K inhibitor	Block resistance due to reactivation of PI3K signaling	
Erlotinib + MET inhibitor	Block MET-dependent resistance to EGFR inhibitors	MetLung Trial and others
EGFR inhibitor + IGF-IR antibody	Block IGF-IR-dependent resistance to EGFR inhibitors	
Erlotinib + hydroxychloroquine	Effort to block the survival of "drug-tolerant" cells after treatment with EGFR TKIs (Sharma et al., 2010)	Goldberg et al., 2012
Irreversible EGFR inhibitor + MET inhibitor	Overcome both T790M- and MET-mediated resistance	

Along the same lines, neratinib in combination with the TORC1 inhibitor temsirolimus recently demonstrated clinical activity in HER2 mutant lung cancers (Gandhi et al., 2014). Finally, one proposed novel strategy is the combination of trastuzumab with anti-PD1 and anti-CD37 monoclonal antibodies. In this case, anti-PD1 would inhibit IFNγ-activated T cells, and anti-CD37 would block $CD8^+$ T cells; both of these T cell subtypes are required for the adaptive immune response triggered by trastuzumab (Stagg et al., 2011).

EGFR

One major mechanism of resistance to EGFR inhibitors in EGFR mutant cancers is the development of the T790M gatekeeper residue. The newer third-generation EGFR TKIs that suppress T790M are showing remarkable progress in this subset of cancers (Ranson et al., 2013; Sequist et al., 2013a; Soria et al., 2013). Ultimately, clinical trials will be needed to determine whether third-generation EGFR inhibitors become first-line therapy for *EGFR* mutant lung cancers. We anticipate that metastatic *EGFR* mutant lung cancers will likely become resistant to drugs that target T790M since there are several additional potential resistance mechanisms. Thus, it will most likely be necessary to combine inhibitors of bypass tracks with T790M-specific inhibitors to provide greater durations of remission and prolongation of patient survival.

In general, combinations that overcome resistance to EGFR inhibitors have generally required continued inhibition of EGFR combined with a drug that blocks the bypass track (reviewed in Niederst and Engelman, 2013). For example, in *EGFR* mutant lung cancers that are resistant via *MET* amplification, combined EGFR and MET inhibition is required to suppress downstream PI3K/AKT and MEK/ERK and induce tumor regressions in vivo (Turke et al., 2010). In similar examples of resistance mediated by IGF-IR and AXL, inhibition of the bypass RTK in combination with the EGFR is needed to overcome resistance (Cortot et al., 2013; Guix et al., 2008; Zhang et al., 2012). Thus, one central strategy involving combinations centers on maintaining potent inhibition mutant EGFR while adding different inhibitors to these accessory pathways. This has been employed in early clinical trials that have combined EGFR inhibitors with MET inhibitors, PI3K inhibitors, and IGF-IR inhibitors (Table 3). However, none of these trials utilized third-generation EGFR inhibitors, which are the only drugs that appear to be capable of overcoming T790M. The advent of these third-generation inhibitors may now unleash the potential of targeting bypass tracks once T790M is effectively inhibited.

In *KRAS* wild-type colorectal cancers, the recent finding that resistant cancers develop EGFR mutations that abrogate cetuximab binding or *KRAS* mutations suggests approaches analogous to those discussed above. Again, the development of point mutations in EGFR suggests that alternative approaches to suppress EGFR may be warranted. Preclinical studies suggest panitumumab may overcome this type of resistance (Montagut et al., 2012). In colorectal cancers that develop *KRAS* mutations upon development of resistance, current trials are examining the

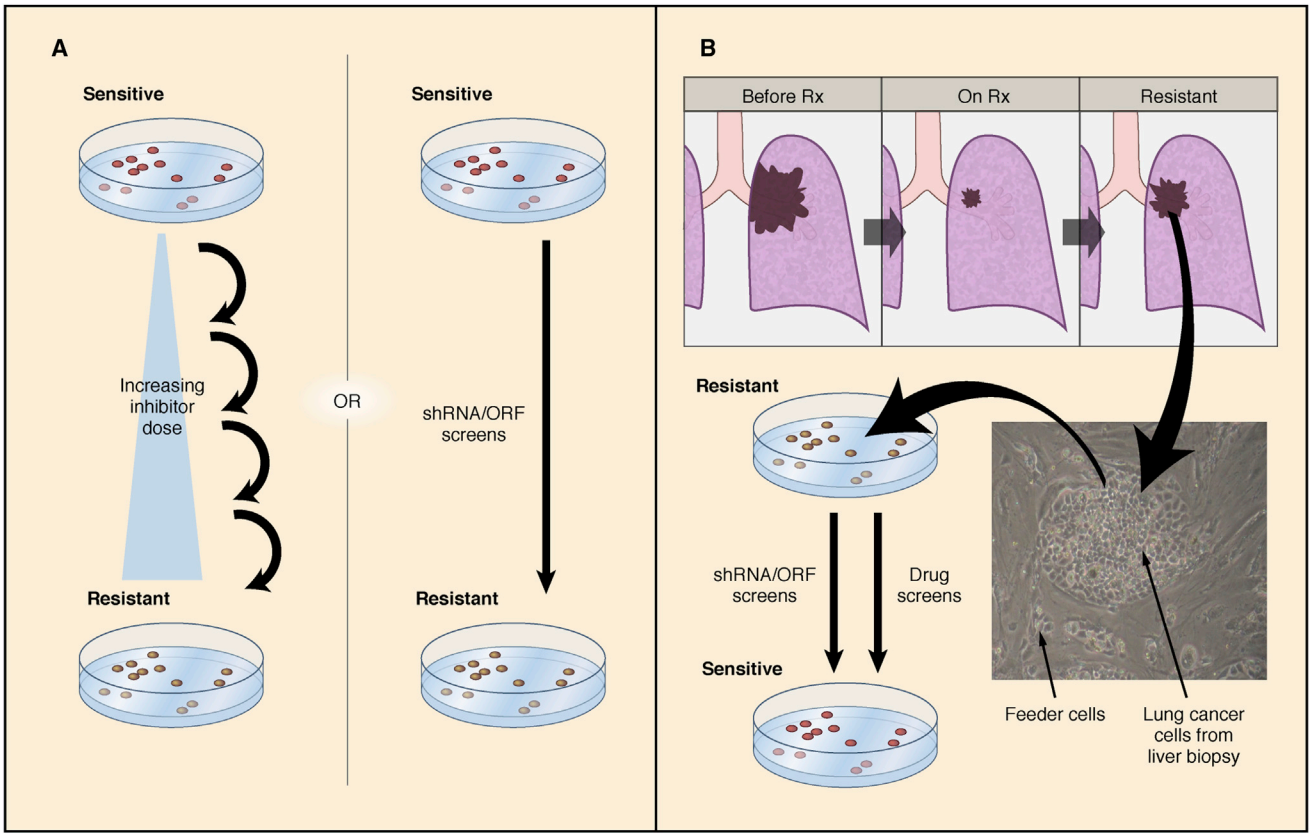

Figure 3. Developing Laboratory Models to Discover Mechanisms of Resistance
(A) Resistance mechanisms can be discovered by culture of sensitive cell lines in the presence of a specific HER2 or EGFR inhibitor until resistance develops or by introduction of shRNA or ORF libraries to determine genes whose overexpression or suppression will lead to resistance.
(B) Alternatively, when resistance develops in the clinic, a cell line can be generated from a biopsy of the resistant lesion, and the resulting resistant line can be screened with drugs and/or shRNA libraries to determine strategies to resensitize them.

efficacy of EGFR inhibitors in combination with inhibitors to overcome resistance (Misale et al., 2014).

Projections to the Future: Novel Approaches

Monitoring Tumor Evolution

One of the major challenges will be to determine the optimal combinations for individual patients. Some examples have clear biomarkers pointing to specific combinations, such as the use of combined HER2 and PI3K inhibitors for *HER2*-amplified breast cancers harboring *PIK3CA* mutations, as well as EGFR and MET inhibitors for *EGFR* mutant lung cancers harboring *MET* amplifications. However, several of the other combinations do not have straightforward biomarkers for patient selection. In the cases of IGF-IR and AXL, it is quite unlikely that expression of the RTK alone will accurately identify those cancers in which those proteins are driving resistance. Thus, more-precise assessments of RTK and signaling activation via novel proteomic methods would be potentially valuable to identify the most appropriate combinations.

As the cancers progress through therapies, there will be a need to continually interrogate the cancer to understand how it has adapted to treatment pressures and become drug resistant. Many centers have utilized repeat biopsy programs to perform biopsies after the development of resistance to targeted therapies to determine how the cancer has evolved. Indeed, with the increasing use of next-generation sequencing approaches, it is likely that the genetic landscape for resistance mechanisms will increase dramatically over the coming years. Even though this approach has great promise to discover resistance mechanism, it may also have potential limitations when the results are used to determine the next course of therapy for an individual patient as the (acquired) alterations identified in a single biopsy may not reflect all of the resistant clones in multiple metastatic sites in an individual patient (Bean et al., 2007; Engelman et al., 2007; Turke et al., 2010). Thus, noninvasive measures such as molecular interrogation of circulating tumor cells or plasma DNA may help capture the heterogeneity of resistance in patients, as was done to identify the development of *KRAS* mutations in colorectal cancers that acquire resistance to cetuximab (Diaz et al., 2012; Misale et al., 2012). As efforts to identify new therapeutic strategies to overcome resistance are intensified, the development of cell lines and patient-derived xenografts from resistant biopsies may facilitate the identification of new therapeutic strategies. Recent advances in technology may help bring these "live" biopsies directly into the laboratory for interrogation (Liu et al., 2012). Such models could be interrogated by high-throughput shRNA and drug screens to identify novel therapeutic approaches to overcome resistance (Figure 3).

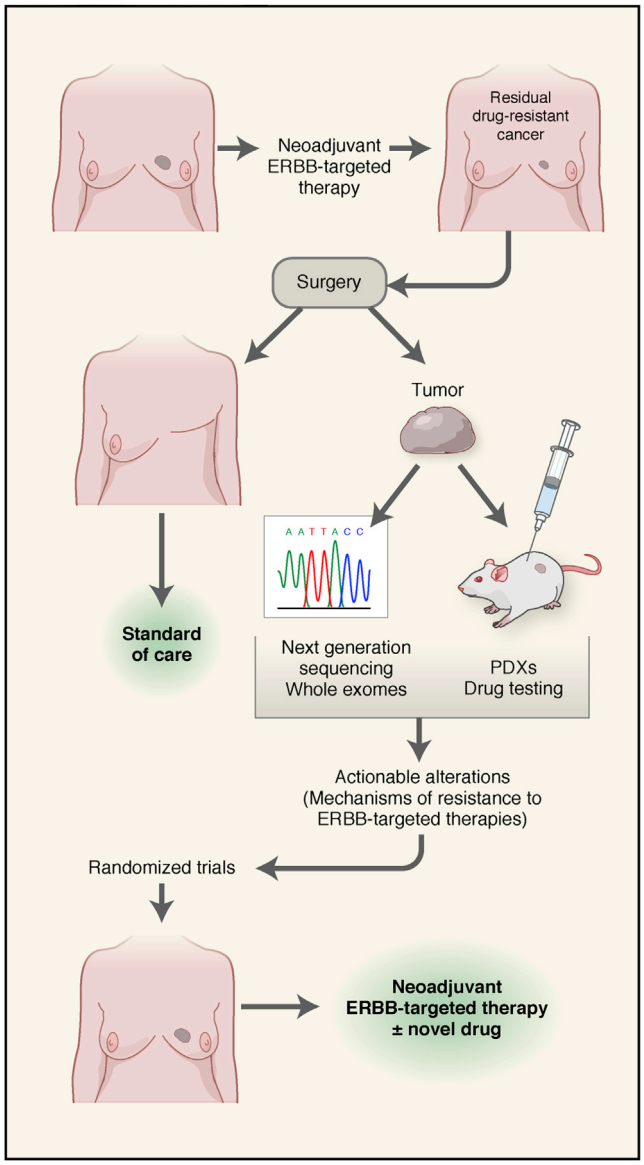

Figure 4. Targetable Alterations in Residual Breast Cancers after Neoadjuvant Anti-HER2 Therapy May Identify Actionable Mechanisms of Drug Resistance

Systemic neoadjuvant anti-HER2 therapy reduces or eliminates the primary HER2$^+$ tumor as well as micrometastases (top row). We propose that "drug-resistant" residual cancers in the breast after neoadjuvant therapy harbor targetable genomic alterations causally associated with resistance to HER2 inhibitors. Molecular profiling of these residual tumors should identify these genomic alterations. Further, patient-derived xenografts (PDXs) generated with these residual cancers can be used to test novel combinations with activity against these drug-resistant cancers that can be later applied to patients on an individual basis. Drugs that target novel mechanisms of resistance identified in the residual tumors can be examined in subsequent neoadjuvant trials (bottom row).

In addition to profiling of metastatic recurrences, the increasing use of neoadjuvant anti-HER2 therapy in patients with newly diagnosed HER2$^+$ breast cancers provides a novel platform for discovery of mechanisms of resistance and tumor heterogeneity. At least two studies have shown that some patients with HER2$^+$ tumors convert to HER2$^-$ after neoadjuvant trastuzumab and chemotherapy, and these patients exhibit a shorter relapse-free survival compared to those with residual tumors that remain HER2 amplified (Hurley et al., 2006; Mittendorf et al., 2009). These results suggest, first, that with heterogeneity in HER2 overexpression in the primary tumor, the antioncogene therapy eliminates the HER2-dependent compartment and enriches for HER2-negative clones. Second, patients with HER2$^+$ tumors that change to HER2$^-$ upon primary anti-HER2 therapy are at a high risk of early recurrence. This neoadjuvant approach facilitates interrogation of the drug-resistant cancer and the identification of targetable mechanisms potentiallly driving subsequent metastatic recurrences (discussed below).

Innovative Trial Designs

For both HER2- and EGFR-driven cancers, it is becoming apparent that new treatment paradigms will be necessary to lead to durable remissions or even cures. Ultimately, we posit that combination therapies will be needed and that it is more rational to consider a proactive regimen that employs alternating regimens of combinations that eliminate cancer cells before they adapt and become resistant rather than treating cancers after the development of clinically overt resistance. However, the large number of potential resistance mechanisms will most likely necessitate the use of more drugs than will be tolerable if they are all delivered simultaneously and each drug is dosed to achieve continual target suppression. In the development of combinations, the use of mutant specific inhibitors will be highly attractive components because of their greater therapeutic windows. However, even with such an approach, given the large number of potential resistance mechanisms, it may become necessary to use even more-creative approaches to proactively kill the various resistant clones as they emerge. In the future, we envision developing regimens that rotate and intercalate tolerable combinations to prevent or substantially delay the development of resistance. In particular, regimens that include immunotherapy and other disparate approaches may be needed.

In breast cancer, the increasing use of neoadjuvant therapy lends itself to some innovative possibilities to develop novel therapeutic regimens, accelerate drug approvals, and discover mechanisms of drug resistance. Achievement of a pathological complete response (path CR) in the breast and axillary lymph nodes after neoadjuvant trastuzumab or chemotherapy has been associated with improved long-term outcome (Gianni et al., 2010; Liedtke et al., 2008). Because of this association, the FDA recently proposed that randomized neoadjuvant trials can be considered for accelerated drug approval using path CR as a surrogate that is "reasonably likely to predict longer term benefit," at least for some subtypes of breast cancer, particularly the HER2$^+$ subtype (Prowell and Pazdur, 2012). Recently, the FDA approved the HER2 antibody pertuzumab as neoadjuvant treatment in patients with HER2$^+$ early breast cancer (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm370393.htm). This approval was based on the results of two neoadjuvant studies, NeoSphere and TRYPHAENA, where the combination of pertuzumab and trastuzumab was superior to trastuzumab alone. The potential impact of this recommendation is quite transformative, as it can accelerate the approval of novel and effective combinations. Further, the early delivery of these anti-HER2 combinations to patients with treatment-naive HER2$^+$ tumors should at least

partially trump acquired drug resistance. The use of the preoperative therapy setting as a clinical research platform in which novel combinations and regimens can be compared and triaged using path CR as a clinical endpoint predictive of long-term outcome has been discussed recently (Bardia and Baselga, 2013).

Another benefit of a preoperative approach is that, except for patients who experience a complete response, tumor tissue is always available at the time of surgery. These ''drug-resistant'' residual cancers should harbor mechanisms and/or biomarkers of resistance to the primary therapy and, potentially, a similar molecular profile to that of drug-resistant micrometastases that can be interrogated with massive parallel sequencing of DNA extracted from the mastectomy specimen (Balko et al., 2012, 2014). Thus, we propose that ''drug-resistant'' $HER2^+$ residual cancers in the breast harbor targetable genomic alterations causally associated with resistance to neoadjuvant anti-HER2 therapy (Figure 4). Molecular profiling of these residual tumors should identify these alterations. In addition, patient-derived xenografts generated with these residual cancers can be used to test novel combinations with activity against these drug-resistant cancers that can be later applied to patients on an individual basis. Drugs that target novel mechanisms of resistance identified in the residual tumors can be examined in subsequent randomized neoadjuvant trials. In the future, we anticipate that tumor types other than HER2-overexpressing breast cancer could also effectively utilize neoadjuvant trials to accelerate drug development and discover mechanisms of resistance.

Conclusions

Ultimately, to cure ERBB-dependent cancers, we will most likely have to incorporate therapeutics that are toxic to cancer cells via mechanisms that are not solely based on suppressing ERBB signaling, the associated bypass tracks, and antibodies targeting ERBB receptors to induce ADCC. The timing of treatment may also make a difference. For example, deploying ERBB-targeted combinations early in the natural history of these cancers, i.e., in the adjuvant setting to treat micrometastatic subclinical disease, may yield better outcomes than treating patients with metastatic disease, where the effect will not be curative. We feel that optimizing the timing and intensity of this approach will provide substantial clinical benefit to patients and will serve as the foundation for incorporating complementary, independent therapeutic strategies that may ultimately lead to highly durable responses and further cures.

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Cancer Cell
Perspective

Mutant p53 in Cancer: New Functions and Therapeutic Opportunities

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Many different types of cancer show a high incidence of *TP53* mutations, leading to the expression of mutant p53 proteins. There is growing evidence that these mutant p53s have both lost wild-type p53 tumor suppressor activity and gained functions that help to contribute to malignant progression. Understanding the functions of mutant p53 will help in the development of new therapeutic approaches that may be useful in a broad range of cancer types.

p53 is one of the most intensively studied tumor suppressor proteins, with mutations that lead to loss of wild-type p53 activity frequently detected in many different tumor types. Perturbations in p53 signaling pathways are believed to be required for the development of most cancers, and there is evidence to suggest that restoration or reactivation of p53 function will have significant therapeutic benefit. For the first 10 years of investigation, p53 was considered to be the product of an oncogene, with many studies describing proliferative and transforming activities for p53. This mistake in the initial classification of p53 was the result of a simple error; the *TP53* gene that had been cloned and used in the initial experiments encoded a mutant version of the wild-type gene. The tumor suppressor credentials of wild-type p53 are no longer in doubt, but the early studies provided a tantalizing hint of what has become an extremely active area of study—the suggestion that mutations in p53 can result in both loss of wild-type activity and gain of a novel transforming function. Moving in a circle in the past 30 years, we have come back around to considering that p53, albeit mutant versions of p53, can function as oncoproteins. In this review, we highlight recent progress in our understanding of how mutant p53 functions, discuss the avenues that are being explored to target mutant p53 tumors, and explore future directions for mutant p53 research.

TP53 is the most commonly mutated gene in human cancer (Kandoth et al., 2013). Alterations have been found in virtually every region of the protein (Leroy et al., 2013), but only a handful of the most frequently occurring mutations have been studied in depth for their contribution to cancer progression. In some cases, frameshift or nonsense mutations result in the loss of p53 protein expression, as seen with other tumor suppressors. However, more frequently, the tumor-associated alterations in p53 result in missense mutations, leading to the substitution of a single amino acid in the p53 protein that can be stably expressed in the tumor cell. These substitutions occur throughout the p53 protein, but most commonly cluster within the DNA binding region of p53, with six "hotspot" amino acids that are most frequently substituted. These mutations generally lead to a loss or diminution of the wild-type activity of p53, and because p53 normally acts as a tetramer, these mutant proteins may also function as dominant negative inhibitors over any remaining wild-type p53. Indeed, in a mouse model, the expression of mutant p53 has been shown to dampen (but not prevent) the therapeutic response to restoration of wild-type p53 (Wang et al., 2011). However, it is becoming clear that at least some of these mutant p53 proteins give rise to a more aggressive tumor profile, indicating that they have acquired novel functions in promoting tumorigenesis.

Gain of Function of Mutant p53

The concept that mutant p53 may show a neomorphic gain of function (GOF) was first suggested 20 years ago (Dittmer et al., 1993), when the introduction of mutant p53 into p53 null cells was shown to give rise to a new phenotype. Since then, a large number of publications have demonstrated many GOFs in numerous cell lines with a variety of p53 mutations, summarized in Table 1. The GOF acquired by mutant p53 is further supported by the finding that patients carrying a *TP53* missense mutation (leading to expression of a mutant p53 protein) in the germline have a significantly earlier cancer onset than patients with mutations in *TP53* that result in loss of p53 protein expression (Bougeard et al., 2008; Zerdoumi et al., 2013). Consistently, in vivo experiments showed that mice expressing mutant p53 display a tumor profile that is more aggressive and metastatic than p53 null or p53 wild-type mice (Doyle et al., 2010; Lang et al., 2004; Morton et al., 2010; Olive et al., 2004), although some tissue specificity of this effect has been suggested by further studies showing that introduction of similar p53 mutations in the lung did not reveal any detectable GOF activity over p53 loss (Jackson et al., 2005). Nevertheless, numerous in vitro and xenograft models have confirmed the ability of mutant p53s to drive enhanced invasion and motility, with evidence that mutant p53 can enhance signaling through receptors such as transforming growth factor β (TGF-β) receptor, epidermal growth factor receptor, and MET (Adorno et al., 2009; Grugan et al., 2013; Muller et al., 2009, 2012; Sauer et al., 2010; Wang et al., 2013a). In part,

Table 1. The Different GOF Roles of Mutant p53 in Cells

Mutation	Cell Line	Mutant p53 Expression	Reference
Invasion			
R172H (human R175H), 175H	PDAC	endogenous (also stable/transient)	Muller et al., 2012
R175H	KLE	endogenous (also stable/transient)	Dong et al., 2009
R175H, R273H, R248Q, R280K,	H1299	stable/transient	Adorno et al., 2009; Coffill et al., 2012; Muller et al., 2009; Noll et al., 2012; Yoshikawa et al., 2010
G266E	MDA MB435	endogenous	Yeudall et al., 2012
R273H	A431	endogenous	Muller et al., 2009
R280K	MDA MB231	endogenous	Coffill et al., 2012; Girardini et al., 2011; Muller et al., 2009
Increased (Altered) Migration[a]			
R172H	MEF	endogenous	Adorno et al., 2009
R175H, H179L, R248Q, R273H, D281G	H1299	stable/transient	Adorno et al., 2009; Muller et al., 2009, 2012; Noll et al., 2012; Yeudall et al., 2012
R175H, R248Q	HEC-50	stable/transient	Dong et al., 2012
R248Q	HEC-1	endogenous	Dong et al., 2012
R248W	HCT116$^{-/-}$	endogenous	Muller et al., 2012
R249S	KNS-62	endogenous	Vaughan et al., 2012b
R267P	H1437	endogenous	Vaughan et al., 2012b
R273H	HT29, A431, U373, SNB19	endogenous	Huang et al., 2013; Muller et al., 2012
R280K	MDA MB231	endogenous	Adorno et al., 2009; Girardini et al., 2011; Li et al., 2011a
Proliferation, Propagation of Cell Cycle			
P278S	ABC1	endogenous	Vaughan et al., 2012a
R172H (human R175H)	MEF	endogenous	Lang et al., 2004
R175H	SK-BR3, VMRC	endogenous	Bossi et al., 2006; Vaughan et al., 2012a
R175H, R248H	BE-13	stable/transient	Hsiao et al., 1994
R175H, R273H, D281G	H1299	stable/transient	Liu et al., 2011; Scian et al., 2004b)
C176F, P223L, R273H, R282Q	PC-3	stable/transient	Shi et al., 2002
M246I	H23	endogenous	Vaughan et al., 2012b
R248W, D281G	10(3)	stable/transient	Loging and Reisman, 1999; Scian et al., 2004a
R249S	KNS-62	endogenous	Vaughan et al., 2012a
R267P	H1437	endogenous	Vaughan et al., 2012a; Vaughan et al., 2012b
R273C	H1048	endogenous	Vaughan et al., 2012b
R273H	HT-29, MDA MB468, H2405	endogenous	Bossi et al., 2006; Gurtner et al., 2010; Vaughan et al., 2012a; Wang et al., 2013a
R273H/ P309S	SW480	endogenous	Bossi et al., 2006; Yan et al., 2008
R273H/ R248W	Mia-Paca-2	endogenous	Yan et al., 2008
R280T	SWO-38	endogenous	Lin et al., 2012
Drug Resistance/Avoidance of Cell Death			
A135V, R248W, R273H	M1/2 cells, LN-308	stable/transient	Li et al., 1998; Matas et al., 2001; Pohl et al., 1999; Trepel et al., 1998
R175H	MEC, 10(3), HEC-50	stable/transient	Dong et al., 2012; Murphy et al., 2000; Pugacheva et al., 2002
R175H	SK-BR3	endogenous	Bossi et al., 2006; Di Agostino et al., 2006; Vaughan et al., 2012b
R175H, P223L + V274F	Pc-3	stable/transient	Gurova et al., 2003; Zalcenstein et al., 2003

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Table 1. *Continued*

Mutation	Cell Line	Mutant p53 Expression	Reference
R175H, R245S, R273H, D281G	Saos-2	stable/transient	Atema and Chène, 2002; El-Hizawi et al., 2002; Kawamata et al., 2007; Tsang et al., 2005; Wong et al., 2007
R175H, R248W, R273H	SKOV-3	stable/transient	Buganim et al., 2006; Liu et al., 2011; Pugacheva et al., 2002
R175H, R248W, R273H	H1299	stable/ transient	Blandino et al., 1999; Di Como et al., 1999; Pugacheva et al., 2002; Zalcenstein et al., 2006
Y220S	fibroblasts	stable/transient	Capponcelli et al., 2005
M237?	T98G	endogenous	Wang et al., 2013b
R248Q	HEC-1	endogenous	Dong et al., 2012
G266E	MDA MB435	endogenous	Vaughan et al., 2012b
R273?	U138	endogenous	Wang et al., 2013b
R273C	C33A, H1048	endogenous	Liu et al., 2011; Vaughan et al., 2012b
R273H	C33A	endogenous	Liu et al., 2011
R273H	HT-29, MDA MB468	endogenous	Bossi et al., 2006; Vaughan et al., 2012b
R273H/ P309S	SW480	endogenous	Bossi et al., 2006; Di Agostino et al., 2006
R273H/ R248W	Mia-Paca-2	endogenous	Do et al., 2012
V143A, R175H, R248W, R273H	Hep3B	stable/transient	Schilling et al., 2010
Anchorage-Independent Growth/Anoikis			
Y126C, R175H, H214R, G245S, R273C, R273H, V273F, R280T, R282Q	SAOS-2	stable/transient	Dittmer et al., 1993; Shi et al., 2002; Sun et al., 1993
P151S	TU-138	endogenous	Xie et al., 2013
Increased Colony Formation			
V143A	BEAS-2B	stable/transient	Gerwin et al., 1992
V143A, R175H, R248W, R273H	H1299	stable/transient	Kalo et al., 2012; Liu et al., 2011; Weisz et al., 2004
V143A, Y163C, R175H, L194R, R273H, D281G, R282W	10(3)	stable/transient	Scian et al., 2004a
G144P, R158H, Y163N, H168Y, V173L, Y234C, R248W	REF[b]	stable/transient	Smith et al., 1999
C174Y	Saos-2	stable/transient	Preuss et al., 2000
R172H (human R175H)	MEF	endogenous	Lang et al., 2004
R175H	SK-BR3	endogenous	Bossi et al., 2006
C194T	T47D	endogenous	Nguyen et al., 2013; Vikhanskaya et al., 2007
A220G	Huh-7	endogenous	Vikhanskaya et al., 2007
R270C	IP3	stable/transient	Halevy et al., 1990
R273H	HT-29, MDA MB 468, U373, SNB19	endogenous	Bossi et al., 2006, 2008; Huang et al., 2013; Wang et al., 2013a
R273H	MCF10A[b]	stable/transient	Nguyen et al., 2013
R273H/ P309S	SW480	endogenous	Bossi et al., 2006; Yan and Chen, 2009, 2010; Yan et al., 2008
R273H/ R248W	Mia-Paca-2	endogenous	Yan and Chen, 2009; Yan et al., 2008
Genomic Instability			
R172H (human R175H)	primary mouse oral tumor	endogenous	Acin et al., 2011
R175H	MEC	stable/transient	Murphy et al., 2000
R175H, R248W, R273H	MEF	stable/transient	Agapova et al., 1996
N236S (human N239S)	MEF	endogenous	Jia et al., 2012

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Table 1. *Continued*

Mutation	Cell Line	Mutant p53 Expression	Reference
R248W	primary mouse cells	endogenous	Song et al., 2007
R248W, R273H	K562 KMV	stable/transient	Restle et al., 2008
Spheroid Disorganization/Mammary Architecture Disruption			
R273H, R280K	MDA MB 468, MDA MB231	endogenous	Freed-Pastor et al., 2012
R175H, G245S, R248W, R273H	MCF10A[b]	stable/transient	Zhang et al., 2011
Stem Cell Dedifferentiation/Propagation			
V143A, R175H, R273H	10(3)	stable/transient	Yi et al., 2012
R172H (human R175H)	MEF	endogenous	Sarig et al., 2010
Xenograft Growth (Cell Line Injected Subcutaneously or in the Mammary Fat Pad)			
V143A, R175H, R248W, R273H, R281D, D281G	(10) 3	stable/transient	Dittmer et al., 1993; Lányi et al., 1998
R172H (human R175H)	primary mouse oral tumor	endogenous	Acin et al., 2011
R175H, R273H,	H1299	stable/transient	Liu et al., 2011
N236S (human N239S)	MEF	endogenous	Jia et al., 2012
R267P	H1437	endogenous	Vaughan et al., 2012a
R273C	H1048	endogenous	Vaughan et al., 2012b
R273H	HT29, MDA MB 468	endogenous	Bossi et al., 2008; Wang et al., 2013a
P278S	ABC1	endogenous	Vaughan et al., 2012a
R280K	MDA MB 231	endogenous	Adorno et al., 2009
R280T	SAOS-2	stable/transient	Sun et al., 1993
Intravenous Injection (Formation of Lung Metastasis)			
R175H, R248G, R213G	BE-13[c]	stable/transient	Hsiao et al., 1994
C236F	D3S2	endogenous	Adorno et al., 2009
R280K	MDA MB231	endogenous	Adorno et al., 2009
Elongated Cell Morphology/EMT			
C135Y, R175H, R273H	HEC-50	stable/transient	Dong et al., 2012
V143A	HCT116$^{-/-}$	stable/transient	Roger et al., 2010
R175H	H1299	stable/transient	Adorno et al., 2009
R175H, R273H	10(3)	stable/transient	Gloushankova et al., 1997
R248Q	HEC-1	endogenous	Dong et al., 2012
R273H	SW620	endogenous	Roger et al., 2010
R175H, G245S, R248W, R273H	MCF10A[b]	stable/transient	Zhang et al., 2011
Polyploidy			
V143A	NHF3 cells[b]	stable/transient	Gualberto et al., 1998
R248W, R249S, R175H	H1299	stable/transient	Noll et al., 2012
Angiogenesis			
Δ126	T24	endogenous	Zhu et al., 2013
R175H[d]	H1299	stable/transient	Fontemaggi et al., 2009
Y220S	fibroblasts	stable/transient	Capponcelli et al., 2005
Cell Survival			
V157F	Hs578T	endogenous	Braicu et al., 2013
C194T	T47D	endogenous	Lim et al., 2009
P223L/V274F	DU-145	endogenous	Zhu et al., 2011
R273H	MDA MB468, U373, SNB19	endogenous	Huang et al., 2013; Lim et al., 2009
R273H	H1299	stable/transient	Kalo et al., 2012

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Table 1. *Continued*

Mutation	Cell Line	Mutant p53 Expression	Reference
R280K	MDA MB231	endogenous	Ali et al., 2013; Hui et al., 2006
R280T	5637	endogenous	Zhu et al., 2013
Mammosphere Formation			
R175H	MESC, HEC-50	endogenous	Lu et al., 2013; Dong et al., 2012
R248Q	HEC-1	endogenous	Dong et al., 2012

The different cellular processes in which mutant p53 has been shown to play a role are indicated. Literature was selected based on the following search criteria in Pubmed: "Mutant p53" and "Gain of Function" or "Mutant p53" and "acquired functions." Only studies in which a clear gain of function effect was shown are included (i.e., mutant p53 compared to a p53 null in the same cell line). These comprise studies in which mutant p53 was overexpressed in a p53 null cell line and compared to a vector control, or studies in which endogenous mutant p53 was knocked down or knocked out compared to control cells. Studies describing the activity of mutant p53 in cells that express wild-type p53 are not included to avoid complications from possible dominant negative effects. Indicated are the different mutations, cell lines, endogenous expression, or stable/transient transfection, and the references. The studies in this table were manually selected from >400 publications and we apologize to those authors whose papers we have inadvertently missed.

[a]Increased (altered) migration comprises wound scratch assays, scattering, migration in three-dimensional culture conditions, and Boyden chamber migration (frequently referred to as transwell invasion without addition of a matrix such as Matrigel).

[b]Cells were depleted for endogenous wild-type p53 expression.

[c]These are T cell acute lymphoblastic leukemia cells and therefore increased hematological disease rather than promoted lung metastases.

[d]H1299 cells expressing p53 R175H promoted the angiogenesis of HUVEC cells.

these responses reflect an ability of mutant p53 to promote integrin/RCP driven recycling (Muller et al., 2009, 2012) or increase the expression of growth factor receptors (Sauer et al., 2010; Wang et al., 2013a). Although mutant p53s have generally lost the ability to bind consensus p53 DNA binding regions in target gene promoters, their activity appears to reflect an ability to regulate gene expression directly (Weisz et al., 2007), although cytoplasmic and mitochondrial activities of mutant p53 in regulating apoptosis and autophagy have also been described (Chee et al., 2013; Frank et al., 2011; Morselli et al., 2008). Whereas various different mutant p53s can bind directly to DNA with some degree of selectivity (Brázdová et al., 2013; Göhler et al., 2005; Quante et al., 2012) and may thereby directly control the transcription of some genes (Weisz et al., 2007), there is increasing evidence that an indirect effect on gene expression through binding to other transcription factors underlies the novel activities of mutant p53s. For example, several studies have revealed a role for TAp63, a p53 family protein and transcription factor, which interacts with mutant but not wild-type p53 (Gaiddon et al., 2001; Strano et al., 2002). By inhibiting TAp63, mutant p53 can regulate a pro-invasive transcription program that includes regulation of the expression of Dicer, DEPDC1, Cyclin G2, and Sharp1 (Adorno et al., 2009; Girardini et al., 2011). The Dicer regulation by mutant p53 may be of particular importance, because several miRNAs that can in turn regulate genes involved in invasion have been described to be regulated by mutant p53, although this may not always involve TAp63 or Dicer inhibition (Dong et al., 2012; Neilsen et al., 2012; Tucci et al., 2012; Wang et al., 2013a).

Mutant p53 inhibition of TAp63 can be modeled by deletion of TAp63, which results in an aggressive tumor profile and metastases similar to that seen in mice expressing mutant p53 (Su et al., 2010). However, a direct comparison of mutant p53 expression with loss of TAp63 in a mouse model of pancreatic ductal adenocarcinoma (PDAC) showed that loss of TAp63 is less potent in inducing metastases, suggesting that mutant p53 does more than inhibiting TAp63 (Tan et al., 2013). This is not surprising, because mutant p53 interacts with a wide variety of other proteins, resulting in interference in a multitude of cellular pathways, some of which are likely to contribute to metastasis (Freed-Pastor and Prives, 2012; Muller and Vousden, 2013; Walerych et al., 2012). Besides inhibiting p63, mutant p53 inhibits and interacts with other proteins including the MRE11-Rad51-NSB complex, p73, and SP-1 to induce genomic instability, chemoresistance, or proliferation (Chicas et al., 2000; Gaiddon et al., 2001; Song et al., 2007). Furthermore, mutant p53 can also promote the function of proteins including SREBP, NF-Y, VDR, ETS2, or NRF2, resulting in increased proliferation, cholesterol synthesis, accumulation of reactive oxygen species, and enhanced cell survival (Do et al., 2012; Freed-Pastor et al., 2012; Kalo et al., 2012; Liu et al., 2011; Stambolsky et al., 2010). All of these proteins and pathways affected by mutant p53 are thoroughly described in three recent reviews (Freed-Pastor and Prives, 2012; Muller and Vousden, 2013; Walerych et al., 2012).

More recent studies are identifying further GOF activities of mutant p53, such as a role in cell reprogramming and expansion or in the maintenance and interaction with tumor stroma. Wild-type p53 was characterized as a suppressor of somatic stem cell reprogramming, the process in which differentiated somatic cells can be reprogrammed into a pluripotent stem cell to allow for unlimited expansion (Kawamura et al., 2009; Marión et al., 2009). Loss of p53 promoted the dedifferentiation of somatic cells and some, but not all, mutant p53s could potentiate the reprogramming (Sarig et al., 2010; Yi et al., 2012). An expansion of hematopoietic and mesenchymal stem cell progenitors is also seen in mutant p53 R248Q transgenic mice (Hanel et al., 2013). Consistently, in breast tissue with a *Wnt* transgene, loss of wild-type p53 generally promoted the formation of one distinct tumor, whereas mutant p53 R175H expression promoted the initiation of multiple different tumors that could be expanded in mammosphere assays (Lu et al., 2013). Together, these data suggest that mutant p53 can initiate tumor formation by promoting the generation and expansion of pluripotent stem cells.

The role of stroma tissue, including extracellular matrix, proteases, cytokines, immune cells, epithelial cells, and cancer-associated fibroblasts (CAFs), in tumorigenesis has become very evident (Pietras and Ostman, 2010). CAFs, the most abundant cell type in the stroma, secrete cytokines, hormones, and growth factors including hepatocyte growth factor and TGF-β (Bhowmick et al., 2004; Ostman and Augsten, 2009), both of which have been shown to mediate mutant p53-dependent invasion and metastasis (Adorno et al., 2009; Muller et al., 2012). In addition, a recent report highlights an important function for mutant p53 in promoting the inflammatory environment of colorectal tumors by prolonging NF-κB activation and cell survival (Cooks et al., 2013). It seems clear, therefore, that the presence of a mutant p53 in tumor cells will have an influence on how the tumor and stromal cells interact. In co-culture experiments, H1299 cells (regardless of p53 status) upregulated interferon-β (IFN-β) secretion in CAFs. This would normally cause inhibition of cell migration, but mutant p53-expressing tumor cells counteracted this response by enhancing STAT phosphorylation to promote invasion (Madar et al., 2013). Although interesting, these experiments are difficult to interpret, because the IFN-β secreted by the fibroblasts also reduced mutant p53 expression (Madar et al., 2013). Alternatively, it is possible that *TP53* mutations occur in the stroma surrounding tumors to promote tumor growth (Narendran et al., 2003; Patocs et al., 2007). Mutant p53-expressing fibroblasts were shown to promote tumor growth better than p53 null fibroblasts, suggesting that mutant p53 has a pro-oncogenic GOF role not only in tumor cells, but also in stromal cells (Addadi et al., 2010). However, whether stromal cells that have sustained mutations in p53 are prevalent, and how they are affected by (or affect) tumor cells remains unclear.

Are All Mutant p53s the Same?

Although most experimental studies have focused on the activity of a few most commonly detected p53 mutations that are clustered at codons 175, 245, 248, 249, 273, and 282, almost every codon within the DNA binding domain of p53 has been found to be mutated in cancer. Mutations have also been found in other domains, but their contribution to carcinogenesis is largely unknown (Leroy et al., 2013). Different tumor types show different spectra of *TP53* mutations—in some cases, reflecting the mutagenic event was thought to contribute to that type of cancer (e.g., aflatoxin and liver, UV light, and skin) or geographic variation in other cases. The frequency of missense mutations also differs in different subclasses of tumors of the same organ. For example, luminal breast cancers almost all carry point mutations in *TP53*, while alterations resulting in p53 truncations were more frequently detected in basal breast tumors (Dumay et al., 2013).

Whereas p53 mutants are often considered to be equivalent, evidence is accumulating to indicate that different mutants show a distinct profile with respect to loss of wild-type p53 activity, the ability to inhibit wild-type p53, and the acquisition of gain of function (Table 1; Halevy et al., 1990; Petitjean et al., 2007). The large number of p53 mutations complicates such analyses, as does the realization that different mutants may function differently in different tissues, potentially reflecting differences in the expression of targets of mutant p53 such as TAp63. To date, mutant p53s have been considered in two different categories: the first affecting amino acids that contact DNA and so preventing wild-type transcriptional activity without dramatically affecting the conformation of the p53 protein (known as contact mutants), and the second comprising mutations that clearly disrupt the three-dimensional structure of the protein (termed conformational mutants). Data from cell lines suggest that conformational and contact mutants can cooperate via different mechanisms with the H-Ras signaling pathway, leading to similar gene expression profiles and tumorigenesis (Solomon et al., 2012). However, this classification of mutants is clearly an oversimplification, because different mutations can lead to subtly different alterations in the structure and conformational stability of the p53 protein (Joerger and Fersht, 2007). Various mouse models have shown that both conformational and contact mutants can promote metastasis compared to p53 null mice. These differences appear to be dependent on the nature of the substitution, but caution should be taken when interpreting data from mouse models using different strain backgrounds that are being studied in different laboratories, and in some cases mutate the mouse gene and in others examine humanized TP53 sequences in the mouse. Models of R172H or R270H (prototype examples of a conformation and a contact hotspot mutation, equivalent to R175H and R273H in humans) both showed GOF activity (Lang et al., 2004; Olive et al., 2004), whereas no GOF was seen in R246S (the mouse equivalent of human R249S) and the humanized G245S mutant p53 mouse models, although the R246S could dominant-negatively inhibit wild-type p53 to promote cell survival after radiation exposure (Hanel et al., 2013; Lee et al., 2012). R248Q (humanized) p53 knock-in mice showed an earlier onset of tumor formation with a significantly reduced lifespan compared to p53 null mice (Hanel et al., 2013), although this reduction in overall survival was not evident in any of the other mutant p53 models. Consistently, Li-Fraumeni patients carrying an R248Q mutation display an earlier onset of cancer compared to inherited null mutations or the G245S mutation (Hanel et al., 2013). These findings suggest that the R248Q p53 functions in a different manner than other p53 mutants that have been studied so far. Remarkably, not only the position of the mutation, but also the nature of the substitution may influence the activity of the resulting mutant protein. For example, both R248Q and R248W are structural mutants, but the humanized R248W p53 knock-in mouse does not display reduced lifespan or earlier disease onset (Song et al., 2007). Understanding the consequences of each p53 mutation in relationship to disease progression and response to therapy therefore promises to be an extremely complex undertaking.

Consequences of Mutant p53 Expression to Tumor Therapy

The realization that loss of p53 and expression of mutant p53 may not be analogous has also raised the question of whether the presence of a mutant p53 protein may affect the response to therapy. Whereas there is evidence that the presence of mutant p53 may dampen the response to restoration of wild-type p53 (Wang et al., 2011), reflecting a dominant negative activity of mutant p53, more recent studies have indicated that the retention of wild-type p53 can be detrimental to the therapeutic response in breast cancer. This effect is seen in tumors that express both mutant and wild-type p53 alleles (Jackson et al., 2012). Such studies highlight the possibility that in some

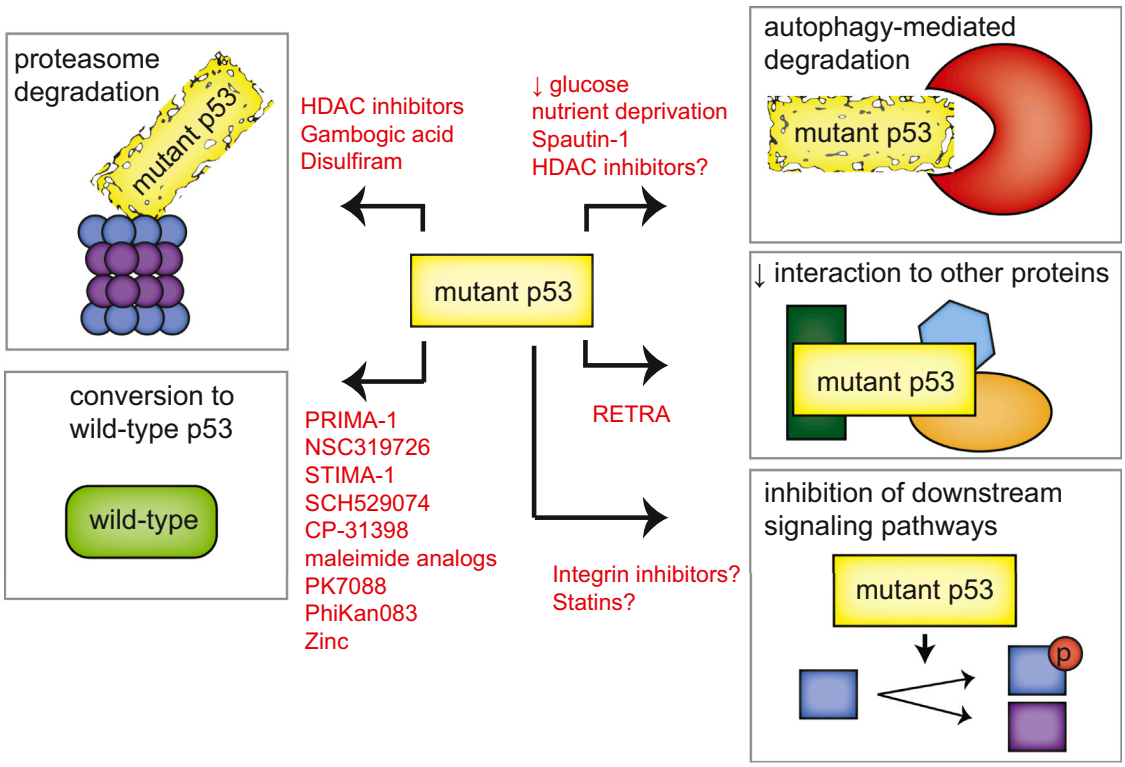

Figure 1. Strategies that Are Currently Being Explored to Target Mutant p53
Depicted in red are schematics of the strategies that are currently being explored to target p53 mutant-expressing cancers. These strategies include promotion of mutant p53 degradation through the proteasome and autophagy pathways, restoration of wild-type p53 activity, interference with the interaction between mutant p53 and other proteins, and interference in signaling pathways downstream of mutant p53.

tumor types wild-type p53 can be dominant over mutant, and that studies of patient response based on p53 status must take into account heterozygosity at the *TP53* locus, as well as the presence of mutant or wild-type p53 (Jackson and Lozano, 2013).

Therapeutic Strategies to Restore Wild-Type Activity to Mutant p53

With so many different mutations and phenotypes it is not surprising that a variety of strategies are being explored to target tumors expressing mutant p53s (summarized in Figure 1). Wild-type p53 is a potent inducer of apoptosis and senescence when expressed in tumor cells, making the reactivation of some level of wild-type function in mutant p53 (which is generally expressed at high levels in cancer cells) an attractive therapeutic avenue. Interestingly, loss of wild-type function introduced by some destabilizing tumor-derived mutations can be rescued by additional point mutations that serve to stabilize the conformation of p53 protein, showing that the loss of structure is intrinsically reversible (Joerger and Fersht, 2008). In addition, a variety of compounds that might restore wild-type p53 function have been characterized and are reviewed in several recent publications (Lehmann and Pietenpol, 2012; Maslon and Hupp, 2010; Wiman, 2010). Small molecules that bind to a site in p53 formed in the Y220C mutant (PhiKan083 and PK7088) function by stabilizing the structure of this mutant p53, and so increasing the level of p53 with a wild-type conformation and activity (Boeckler et al., 2008; Liu et al., 2013). Other compounds bind to multiple mutant p53 proteins (e.g., PRIMA-1, or the soluble derivative PRIMA-met/APR-246, CP-31398, and SCH29074; Bykov et al., 2002; Demma et al., 2010; Foster et al., 1999), interacting with the DNA binding domain, thereby promoting proper folding of the mutant protein and restoration of p53 function. However, the precise mechanistic function of these compounds and others, such as maleimide analogs and STIMA-1, remain to be elucidated (Bykov et al., 2005; Zache et al., 2008).

Whereas wild-type p53 requires binding to the metal ion Zn(2+) to fold correctly (Loh, 2010; Verhaegh et al., 1998), the R175H p53 mutant was found to be impaired in zinc binding (Butler and Loh, 2003). Loss of metallothioneins that chelate and store intracellular zinc promotes a wild-type conformation of misfolded p53 (Puca et al., 2009) and addition of zinc to the conformational mutants G245C and G245D p53 partially restored the wild-type conformation (Pintus et al., 2013). The potential use of zinc to recover wild-type folding has therefore been explored and this approach has been shown to restore chemosensitivity to anticancer drugs in cells expressing endogenous mutant p53 (Puca et al., 2011). In addition, the thiosemicarbazone metal ion chelator NSC31926 was found to restore wild-type function in a variety of different mutant p53-expressing cell lines, possibly through increasing the bioavailability of zinc to (mutant) p53 (Yu et al., 2012).

Of all the compounds that restore wild-type activity, the most progress has been made with PRIMA-1 analogs, with the

demonstration of safety in a phase I clinical study (Lehmann et al., 2012). PRIMA-1 is rapidly converted to other compounds, including MQ, which can bind to both mutant p53 and wild-type p53 (Lambert et al., 2009), although the precise mechanisms underlying the p53 reactivation are currently unknown. Under some circumstances, p53 can adopt an unfolded conformation and behave like a mutant p53 protein to promote invasion (Trinidad et al., 2013). Unfolded wild-type p53 seen in tumor cells grown under hypoxia (Gogna et al., 2012) could be restored by PRIMA-1 treatment (Rieber and Strasberg-Rieber, 2012). It will therefore be interesting to explore whether both wild-type and mutant p53 tumors might benefit from PRIMA-1 treatment.

Therapeutic Strategies to Promote Mutant p53 Degradation

An alternative approach to targeting mutant p53 is to remove the proteins by enhancing turnover (Figure 1). Both wild-type and mutant p53 can be targeted for proteasomal degradation in otherwise normal cells by the ubiquitin ligase MDM2. Inhibition of MDM2 in response to stress underlies the activation of wild-type p53, but is also thought to lead to the overexpression of mutant p53 seen in cancer cells. Indeed, stress induced stabilization of mutant p53 seems to be a prerequisite for its GOF (Suh et al., 2011). In addition to MDM2, another chaperone-associated E3 ubiquitin ligase, CHIP, was shown to be important for mutant p53 degradation (Esser et al., 2005; Lukashchuk and Vousden, 2007). To be stabilized, mutant p53 interacts with the Hsp70 and Hsp90 chaperone complex that requires an interaction with HDAC6 for proper functioning (Li et al., 2011b). Abrogation of HDAC6 binding results in the dissociation of the heat shock proteins from mutant p53 and allows for mutant p53 degradation by MDM2 and CHIP (Li et al., 2011b). HDAC inhibitors such as SAHA show promise in destabilizing mutant p53 by preventing HDAC6 from interacting with Hsp90 (Li et al., 2011a). However, SAHA and the pan-HDAC inhibitor NaB were recently shown to not only regulate mutant p53 stability, but also its transcription via the p53 activator HoxA5 (Yan et al., 2013). This activity was not confined to mutant p53 and also extended to decreasing wild-type p53 expression (Yan et al., 2013), indicating that care should be taken to determine the p53 status of tumors when HDAC inhibitors are used as therapeutic agents. Small molecule activators of SIRT1 have also been shown to lead to the deacetylation of p53 and reduction of overall mutant p53 levels (Yi et al., 2013). In other studies, Stathmin—a transcriptional target of wild-type p53 and mutant p53 (through the regulation of miR-223)—promoted mutant p53 activity by regulating phosphorylation and stability in ovarian cancers (Sonego et al., 2013).

Autophagy also plays a role in mutant p53 degradation. Macro-autophagy is the process by which intracellular contents such as proteins or organelles are engulfed and degraded through lysosomes. This can provide a means to recycling intracellular content, providing an alternative energy source to allow cells to survive transient starvation, and also functioning to remove damaged or excess organelles (Mizushima et al., 2008). The role of autophagy in cancer is complex and can both promote and inhibit tumor development, depending on the targets of the autophagic process and the timing during tumor evolution (Liu and Ryan, 2012). Macro-autophagy induced by glucose restriction selectively promoted mutant p53 degradation, whereas wild-type p53 was stabilized under similar conditions (Rodriguez et al., 2012). The degradation of mutant p53 was promoted by proteasomal inhibition and depended on functional autophagy machinery (Choudhury et al., 2013; Rodriguez et al., 2012). Glucose starvation combined with confluent growth conditions could promote mutant p53 degradation by a specialized form of autophagy known as chaperone-mediated autophagy (Vakifahmetoglu-Norberg et al., 2013). In contrast to the findings of Rodriguez et al. (2012), degradation of mutant p53 via this specialized autophagy pathway was enhanced by inhibition of macro-autophagy (Vakifahmetoglu-Norberg et al., 2013), suggesting conditional aspects to glucose deprived mutant p53 degradation. Furthermore, both mutant and wild-type p53 can inhibit autophagy when localized in the cytoplasm (Morselli et al., 2008; Tasdemir et al., 2008), indicating that the relationship between autophagy and mutant p53 is complex.

Therefore, while targeting mutant p53 for degradation seems feasible, there remains a concern as to how effective simple removal of mutant p53 (without replacement by degradation-resistant wild-type p53) might be in driving a therapeutic response. Some comfort has been provided by many studies showing reduction of mutant p53 levels (either by siRNA or spautin treatment) results in increased apoptosis, indicating that these cells may have become dependent on mutant p53 for their survival (Table 1; Ali et al., 2013; Braicu et al., 2013; Huang et al., 2013; Hui et al., 2006; Lim et al., 2009; Vakifahmetoglu-Norberg et al., 2013; Xie et al., 2013; Zhu et al., 2011, 2013). However, whether decreasing mutant p53 levels is sufficient as a means of therapy in vivo and in the long term requires confirmation.

Targeting Mutant p53 Regulated Pathways

Instead of targeting mutant p53 directly, another approach is to identify commonalities in the mechanisms through which mutant p53 proteins function and to target and exploit these downstream pathways (Figure 1). Despite the clear differences between mutant p53s, a large number of them interact and inhibit p63 and p73. A small molecule named RETRA, identified by serendipity in a screen to identify drugs to stabilize wild-type p53, has been suggested to destabilize the p73 mutant p53 interaction (Kravchenko et al., 2008). RETRA-induced release of p73 resulted in the activation of p73 target genes and a concomitant decreased tumor cell survival and suppression of xenograft tumor growth (Kravchenko et al., 2008). Whether RETRA impairs the interaction of mutant p53s with other target proteins has not been reported, but this could be a more general approach to block the oncogenic effect of mutant p53s that share binding partners.

Downstream pathways activated by mutant p53 may also be targets for therapeutic intervention. An attractive possibility here is the cholesterol synthesis pathway through which mutant p53 disrupts the morphology of mammary tumors (Freed-Pastor et al., 2012). Inhibition of cholesterol synthesis restored the morphology and decreased survival of mutant p53 cells (Freed-Pastor et al., 2012). This is of particular interest because statins (cholesterol inhibitors) are among the most commonly prescribed drugs worldwide to prevent cardiovascular diseases and have shown promise as preventive anticancer agents (Singh

and Singh, 2013). It will therefore be interesting and relatively straightforward to determine the utility of statins as a therapeutic strategy for mutant p53 tumors.

Finally, several studies have described a role for mutant p53 in enhancing receptor tyrosine kinase (RTK) signaling (Adorno et al., 2009; Muller et al., 2009; Sauer et al., 2010; Wang et al., 2013a). A multitude of inhibitors of the kinase activity of RTKs or their downstream mediators have been described, including EGFR inhibitors, MET inhibitors and MAPK inhibitors. Selective efficacy of these compounds in the treatment of mutant p53 expressing cancers remains to be explored. The specific role of RTK and integrin recycling may also provide an additional attractive target, since various integrin antibodies and drugs that inhibit integrin recycling are currently on the market and have shown some promise as anticancer agents (Desgrosellier and Cheresh, 2010).

Future Directions

A number of hurdles still need to be overcome before the studies of mutant p53 can be translated into clinical practice. While there is clear evidence that mutant p53 promotes various oncogenic responses, the relative importance of survival, motility, invasion, and metabolic changes, or the critical pathways through which these responses are mediated remain unclear. How different mutations affect p53 function also remains underexplored, as does the comparative importance of loss of wild-type, dominant-negative, and GOF phenotypes. The fact that most mutant p53s are expressed at very high levels in cancer cells (leading to the immunohistochemical detection of p53 being used as a proxy for the presence of mutant p53) makes these proteins tremendously attractive therapeutic targets, and the efficacy of inhibiting the activity of these mutant p53s or even re-establishing some wild-type function, as described above, holds great promise. Such approaches depend, however, on designing efficient mechanisms through which to target mutant p53, an understanding of the activities and function of the many different mutants, and the capacity to identify which mutation a tumor carries (the latter likely to be the most easily attainable goal).

Maybe a more effective approach will be to explore the possibility of synthetic lethality as a therapeutic strategy. Recently, a computational approach using gene expression from the NCI-60 panel, the GBM (glioblastoma multiforme) project and the TCGA (the cancer genome) project revealed a number of genes and pathways that may result in synthetic lethality when targeted in mutant p53-expressing tumors (Wang and Simon, 2013). The majority of these genes were involved in the cell cycle, perhaps reflecting the loss of wild-type p53 function, and an interesting candidate identified in several of the data sets is polo-like kinase 1 (PLK1), which is involved in the regulation of mitosis. PLK1 was found to be upregulated in breast cancers with mutant p53 expression; the presence of both coincided with a worse prognosis than cancers with either PLK1 upregulation or mutant p53 expression alone (King et al., 2012). Because PLK1 can be inhibited by a variety of compounds (Strebhardt, 2010), it will be interesting to follow up this lead.

Conclusions

Recent data reveal that mutant p53 is not just one protein, but a multitude of proteins that can contribute to a wide range of oncogenic processes. Designing drug strategies to target mutant p53 tumors is therefore highly challenging and will require a deeper understanding of the degradation pathways, interaction partners, and downstream signaling pathways in mutant p53 cells. However, we are optimistic that our ever-expanding knowledge of mutant p53 function will translate into some useful therapeutic strategies in the future.

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Cancer Cell
Perspective

From Fly Wings to Targeted Cancer Therapies: A Centennial for Notch Signaling

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Since Notch phenotypes in *Drosophila melanogaster* were first identified 100 years ago, Notch signaling has been extensively characterized as a regulator of cell-fate decisions in a variety of organisms and tissues. However, in the past 20 years, accumulating evidence has linked alterations in the Notch pathway to tumorigenesis. In this review, we discuss the protumorigenic and tumor-suppressive functions of Notch signaling, and dissect the molecular mechanisms that underlie these functions in hematopoietic cancers and solid tumors. Finally, we link these mechanisms and observations to possible therapeutic strategies targeting the Notch pathway in human cancers.

This year will be the centennial of the discovery of a signaling pathway that has fascinated developmental, molecular, and cancer biologists around the world. Mutant Notch phenotypes in the fly wing were characterized by John S. Dexter 100 years ago (Dexter, 1914), and, rapidly thereafter, Thomas Hunt Morgan identified the mutant alleles (Morgan, 1917). Almost seven decades later, after the molecular biology revolution, Spyros Artavanis-Tsakonas and Michael Young cloned the Notch receptor and attributed the wing-notching phenotype to gene haploinsufficiency (Kidd et al., 1986; Wharton et al., 1985). These studies brought about a revolution in a large number of fields, including developmental and stem cell biology, neuroscience, and (related to this review) cancer biology (Fortini et al., 1993). Indeed, in the early 1990s, gain-of-function mutations of the pathway were identified in cancer (Ellisen et al., 1991; Gallahan and Callahan, 1997; Gallahan et al., 1987; Jhappan et al., 1992). A deluge of reports followed, cementing the role of Notch signaling as oncogenic but also tumor suppressive, depending on the context. In this review, we attempt to provide a detailed characterization of Notch functions in both solid and hematopoietic cancers. In addition, we discuss the molecular mechanisms that underlie such functions, as well as approaches to target Notch signaling in human cancers.

A Brief Description of the Notch Signaling Pathway

There are four Notch receptors (named Notch1–Notch4) in mammals. Notch1 and Notch2 each have 36 epidermal growth factor (EGF)-like repeats, while Notch3 and Notch4 have 34 and 29 repeats, respectively, which affects their affinity for corresponding ligands (Haines and Irvine, 2003; Okajima and Irvine, 2002; Rebay et al., 1991; Figure 1). Notch receptors are single-pass type I transmembrane molecules coded by a single precursor that becomes a noncovalently linked heterodimer. This heterodimer consists of an N-terminal extracellular (NEC) fragment and a C-terminal transmembrane-intracellular subunit (NTM) as a result of cleavage by a furin-like protease in the *trans*-Golgi network (Blaumueller et al., 1997; Figures 1 and 2). The Notch pathway is normally activated upon interactions with ligands such as Delta-like and Jagged, which are also transmembrane proteins containing EGF-like repeats. In mammals, there are three Delta-like ligands (Dll1, Dll3, and Dll4) and two Jagged ligands (Jag1 and Jag2). The Notch pathway is activated in a strictly controlled fashion: ADAM10/17 metalloproteases cause an S2 cleavage in the receptor, followed by a third cleavage (S3 cleavage) mediated by the presenilin-γ-secretase complex, which is composed of presenilin 1 (PSEN1), PSEN2, nicastrin (NCSTN), presenilin enhancer 2 (PEN2), and anterior pharynx-defective 1 (APH1) (Shah et al., 2005). This series of events releases the intracellular portion of the Notch receptor (termed ICN), which then translocates into the nucleus to mediate target gene activation (De Strooper et al., 1999; Schroeter et al., 1998). Notch-ICN is a transcriptional activator (Bray, 2006) consisting of ankyrin repeats, a RAM (RBP-Jκ associated molecule) domain, a transactivation domain (TAD), a nuclear localization signal (NLS), and a PEST domain that regulates protein stability (Figures 1 and 2). Notch ligands are also cleaved by γ-secretase and ADAM metalloprotease complexes, thus providing an additional level of regulation of the pathway (LaVoie and Selkoe, 2003; Six et al., 2003). Despite the overall similarities between the receptors, the differences in the ligand-binding extracellular domains and the transactivation intracellular domains lead to distinct ligand affinities and capacities to activate downstream transcription.

In the nucleus, Notch binds to initially inactive CBF1-Su(H)-LAG1 (CSL) (aka RBP-Jκ)-containing complexes and mediates their conversion to a transcriptional activator followed by the recruitment of the coactivator protein mastermind-like 1 (MAML1) (Figure 2; Nam et al., 2006; Wilson and Kovall, 2006; Wu et al., 2000). The ankyrin repeats seem to play an important role in MAML1 recruitment. The list of target genes regulated by Notch is very much dependent on cell type and can include genes whose products are involved in fundamental aspects of cell biology, such as cell-cycle regulation (Joshi et al., 2009; Lewis et al., 2007), cellular differentiation, and metabolism (Palomero et al., 2006). Common targets of the pathway include the HES and HEY families of transcription repressors (Iso et al.,

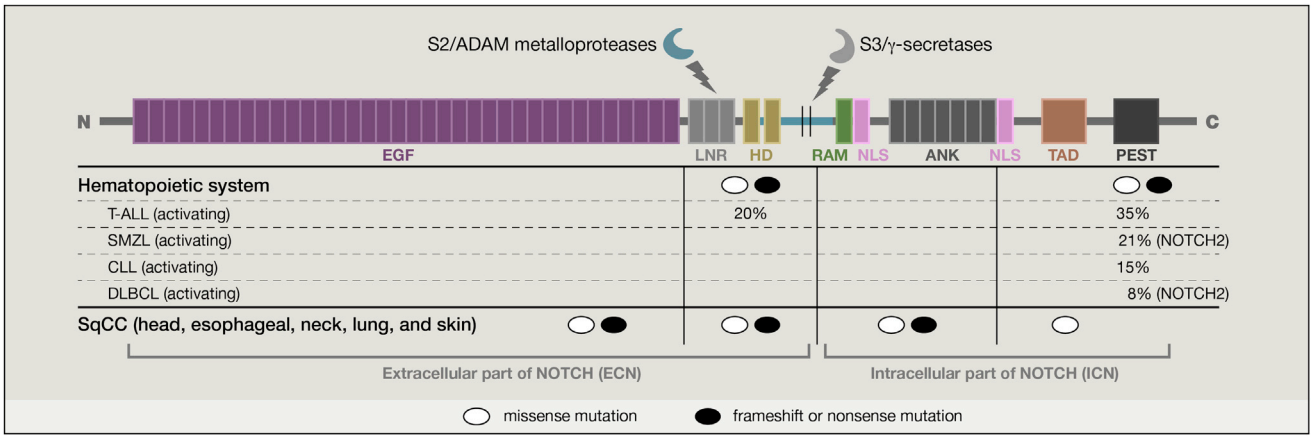

Figure 1. Protein Structure and Mutations of a Typical Notch Receptor
The structure of the NOTCH1 receptor and genetic alterations of the protein in representative types of cancer are depicted. ADAM metalloproteases and the γ-secretase complex cleave the receptor and free the ICN domain. Major mutations are clustered according to their effects on protein activity. Both gain- and loss-of-function mutations are shown. The majority of the T-ALL mutations are clustered in the heterodimerization (HD) and PEST domains that control processing of the receptors by proteases and the stability of the protein, respectively. Different characteristic cases of hematopoietic disorders (affecting NOTCH2 as well) are shown. In CLL tumors, there is an apparent mutational hotspot at the PEST domain of NOTCH1. In the case of SqCC mutations, mutations are mainly clustered in the EGF repeat region, potentially affecting interaction with the ligands. T-ALL, T cell acute lymphoblastic leukemia; SMZL, splenic marginal zone lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; SqCC, squamous cell carcinoma. Percentages are approximations based on current literature.

2001a, 2001b; Jarriault et al., 1995), as well as the MYC transcription factor (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). The binding of Notch on DNA appears to be a rapid and dynamic process controlled by the kinase CDK8 and the ubiquitin ligase Fbxw7, followed by phosphorylation, ubiquitination, and proteasomal degradation of Notch (Fryer et al., 2004; Mukherjee et al., 2005; O'Neil et al., 2007; Thompson et al., 2007), which shuts off the pathway (Figure 2).

Various tools have been developed to study the transcriptional activity of the pathway, such as chromatin immunoprecipitation sequencing (ChIP-seq) and ChIP-chip to map Notch1 binding on the genome (Castel et al., 2013; Ntziachristos et al., 2012; Palomero et al., 2006; Wang et al., 2011a), and mouse models that allow efficient tracing of receptor cleavage/activity in many different tissues (Hansson et al., 2006; Liu et al., 2011; Mizutani et al., 2007; Souilhol et al., 2006). Recently, the group of Artavanis-Tsakonas (Fre et al., 2011; Šale et al., 2013) and members of our laboratory (Oh et al., 2013) traced Notch pathway activity in vivo by using reporter systems for Notch receptor expression and Hes1 activity by coupling them to fluorescent proteins (Figure 3). Since there are several unanswered questions regarding Notch ligand expression, even under physiological conditions, an exciting next step could involve the development of fluorescent tools to probe ligand expression together with pathway activation in real time within a living organism.

NOTCH Signaling Pathway in Cancer

The Notch pathway is genetically altered in a large number of hematopoietic and solid tumors (Figure 1). Intriguingly, these alterations can lead to either activation or repression of the pathway depending on the context and the activation status of other potentially oncogenic pathways (Table 1; Figure 4). Interestingly, it appears that there are multiple and distinct modes of aberrant regulation of the pathway and its targets in cancer. They include activating and inactivating mutations, receptor/ligand overexpression, epigenetic regulation, and effects of posttranslational modifications, most notably receptor and ligand fucosylation (especially O-fucosylation) (Haines and Irvine, 2003; Lei et al., 2003; Okajima et al., 2003) and ubiquitination (Fryer et al., 2004; Thompson et al., 2007). We initially discuss T cell acute lymphoblastic leukemia (T-ALL), a disease in which Notch has a well-characterized oncogenic role. Subsequently, we present several other cases of hematopoietic and solid tumors in which Notch has tumor-suppressive or oncogenic roles, along with its potential mechanisms of action and partners.

T-ALL

NOTCH1 is a master transcription factor that controls innate and adaptive immunity, and plays an important role in directing hematopoietic development toward T cells (Aifantis et al., 2008; Li and von Boehmer, 2011; Radtke et al., 2013). The very first finding of Notch pathway alterations in cancer was made by Ellisen et al. (1991), who revealed a rearrangement between the intracellular part of NOTCH1 (ICN1) and the T cell receptor beta (TRB) locus that leads to high-level expression of truncated, constitutively active NOTCH1 in leukemia. Subsequent in vitro studies (Capobianco et al., 1997) and animal modeling (Girard et al., 1996; Pear et al., 1996) by other groups further confirmed this finding and proved that ICN1 is a strong oncogenic allele. Most importantly, 10 years ago, the Aster and Look laboratories reported the first activating NOTCH1 mutations in human T-ALL, occurring in approximately 50% of all cases (Weng et al., 2004).

The majority of these mutations encompass single amino acid substitutions, insertions, and deletions located in exons 26 and 27 of the genetic locus, which encode the N-terminal and C-terminal components, respectively, of the heterodimerization domain. These mutations lead to lower protection of S2 cleavage of Notch, resulting in either ligand-independent activation or

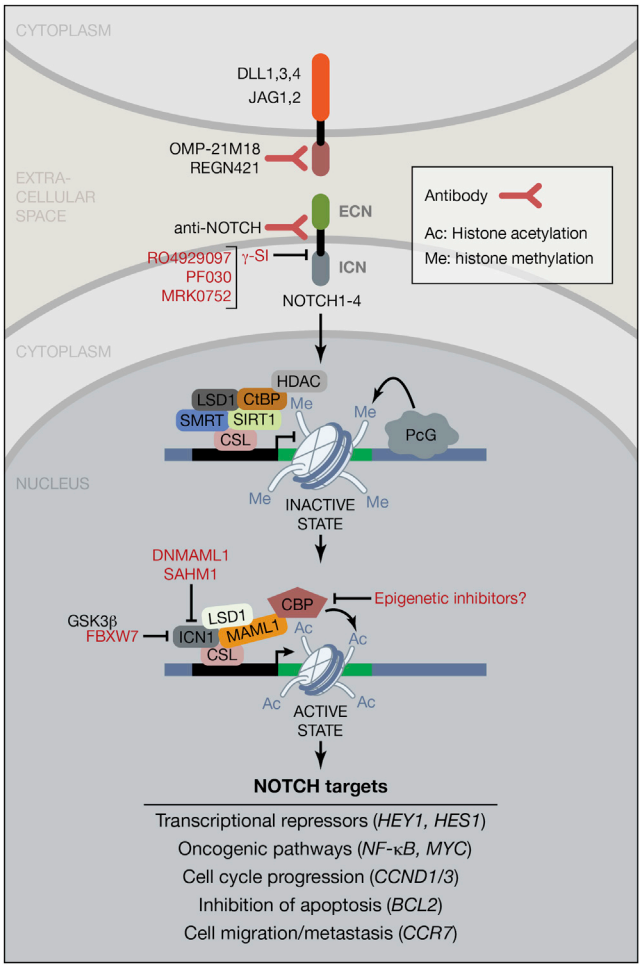

Figure 2. Overview of the Notch Signaling Pathway
A visual description of the signaling cascade is shown for the signal-receiving cell (i.e., the cell expressing the Notch receptor). The pathway inhibitors used include antibodies against NOTCH receptors and DLL ligands, GSIs, and small peptides that inhibit formation of the transcriptional complex. Antibody-based treatments are shown in purple, GSI compounds are in pink, and peptide-based drugs are in red. Potential epigenetic inhibitors (in green) can include BRD inhibitors such as JQ1. HDAC, histone deacetylase; ICN1, intracellular part of NOTCH1; LSD1, lysine-specific demethylase 1; SMRT, Silencing-Mediator for Retinoid/Thyroid hormone receptors; GSK3β, glycogen synthase kinase 3 beta; DNMAML1, dominant-negative MAML1.

hypersensitivity of the pathway to ligands. Another rare group of mutations, known as juxtamembrane expansion (JME) mutants, also augments NOTCH1 activation at the cell membrane (Sulis et al., 2008; Figure 1). Finally, PEST domain mutants encompass another category of NOTCH1 mutations in 20%–25% of T-ALLs. PEST domain alterations lead to truncation or loss of the domain due to frameshift or nonsense nucleotide substitutions, which impair proteasomal degradation mediated by the ubiquitin ligase FBXW7 and lead to higher ICN1 cellular concentrations (Weng et al., 2004). The importance of ICN1 degradation in physiology is further evidenced by the fact that 15% of T-ALL cases harbor mutations or deletions in *FBXW7* (Asnafi et al., 2009; O'Neil et al., 2007; Thompson et al., 2007). These changes are localized in three arginine residues that are critical for its interaction with ICN1. Mutations in the PEST domain and FBXW7 do not occur concurrently, which implies that they play the same role to increase the stability of ICN1 (Asnafi et al., 2009; O'Neil et al., 2007).

The fact that FBXW7 mutations directly affect cells with leukemia-initiating properties though the stabilization and overexpression of MYC, another well-characterized substrate of this ubiquitin ligase, further demonstrates that NOTCH and MYC actions are intertwined in cancer cells (King et al., 2013). Interestingly, NOTCH1 mutations in T-ALL were shown to have a favorable prognosis and better outcome after treatment in a number of studies, including the ALL-Berlin-Frankfurt-Munster 2000 study (Breit et al., 2006), the Japan Association of Childhood Leukemia Study that examined NOTCH1 and FBXW7 mutational status in T-ALL and T cell lymphoblastic lymphoma patients (Park et al., 2009), and the Lymphoblastic Acute Leukemia in Adults (LALA)-94 and GRAALL-2003 trials (Asnafi et al., 2009). Finally, another report on 134 pediatric patients from the EORTC-CLG 58881 and 58951 protocols concluded that NOTCH1 and FBXW7 mutations are associated with improved early chemotherapeutic response and lower minimal residual disease levels (Clappier et al., 2010). It remains to be seen whether Notch pathway inhibition can be used successfully to target T-ALL, especially in relapsed disease that is refractory to conventional chemotherapy-based treatments.

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. Recently, it was demonstrated using next-generation sequencing-based approaches that 10%–12% of CLL cases exhibit activating mutations of NOTCH1, underlining the significance of such mutations as a prognostic marker. The vast majority of these mutations are in the PEST domain, leading to truncated protein variants with a longer half-life (Figure 1). Interestingly, there seems to be a mutational hotspot in this disease, with P2515Rfs being the most prevalent mutation (Fabbri et al., 2011; Puente et al., 2011; Rossi et al., 2012a). Mutations of *NOTCH1* are mutually exclusive with *TP53* abnormalities, and survival outcomes are poor in both cases (Rossi et al., 2012a; Wickremasinghe et al., 2011). *NOTCH1* and *SF3B1* (a splicing factor) mutations were associated with decreased overall survival, and both retained independent prognostic significance for survival outcomes (Oscier et al., 2013). In a study by Fabbri et al. (2011), mutational activation of NOTCH1 was found to occur with significantly higher frequency during disease progression toward the high-risk Richter transformation (30%) and chemorefractory CLL (20%). That study and a very recent large-scale clinical analysis of CLL patients (Weissmann et al., 2013) confirmed that NOTCH1 mutations are an adverse prognostic parameter in this disease.

Lymphoma

Non-Hodgkin lymphoma (NHL) is a heterotypic mix of diseases, the most prevalent being Burkitt lymphoma, follicular lymphoma (FL, the most indolent of NHL cases) (Pasqualucci et al., 2014; Roulland et al., 2011), and diffuse large B cell lymphoma (DLBCL). In one study, Burkitt lymphomas, which are mainly characterized by the upregulation of MYC due to its translocation to the immunoglobulin locus, displayed recurrent gain-of-function NOTCH1 mutations in 8%–9% of patients (Love et al., 2012). FL and DLBCL are malignancies of B cell origin and together account for 60% of new NHL diagnoses in

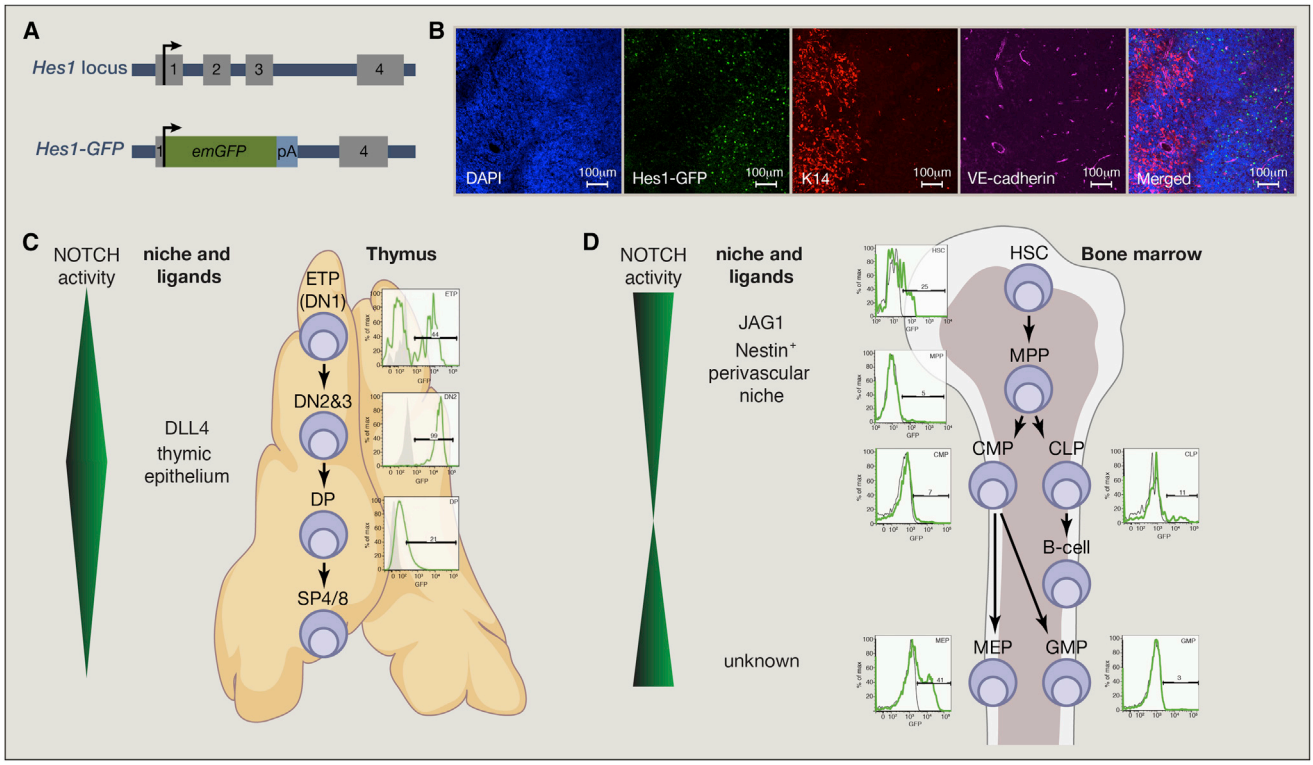

Figure 3. In Vivo Mapping of Notch Pathway Activity Using a Hes1^{GFP} Reporter

(A) Targeting strategy for the generation of transgenic animals expressing Emerald GFP (emGFP) from the endogenous Hes1 locus.

(B) Immunofluorescence staining for the thymus of Hes1^{GFP} mice. DAPI stains DNA (nucleus), VE-cadherin is a vascular endothelial marker, and K14 is a marker of thymic medullary cells.

(C) Increased levels of Notch pathway help differentiation of thymic T cell progenitors through the DN2/3 CD4$^-$8$^-$ differentiation stage, and the pathway activity is decreased immediately at the DP stage.

(D) Activity of the Notch pathway in the mouse bone marrow is detected at the HSC level and is decreased as cells differentiate. Subsequently, it is reactivated at the level of a megakaryocytic-erythrocytic progenitor (MEP). HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor; DN, double negative (CD4$^-$CD8$^-$); DP, double positive (CD4$^+$CD8$^+$); SP, single positive.

North America. FL and the germinal center B cell (GCB) DLBCL subtype are derived from GCB cells, whereas the more aggressive activated B cell (ABC) DLBCL subtype is most likely derived from cells that have exited the germinal center. NOTCH2 is mutated in ~8% of DLBCL cases (Lee et al., 2009). These are mainly gain-of-function mutations that affect the PEST domain (and thus the stability of the protein), as well as copy number alterations (Morin et al., 2011). Interestingly, NOTCH2 is required for B cell development in the splenic marginal zone (SMZ) environment and has been implicated in SMZ lymphoma (SMZL) (Kiel et al., 2012; Rossi et al., 2012b), as 20% of SMZL cases exhibit gain-of-function NOTCH2 mutations accompanied by mutations of NOTCH1, SPEN, and DTX1 (Rossi et al., 2012b). It was suggested that these genetic changes are associated with adverse prognosis (Kiel et al., 2012). Finally, Jundt and colleagues have characterized an activating role for NOTCH1 in classic Hodgkin lymphoma (Schwarzer et al., 2012; Schwarzer and Jundt, 2011). These authors suggested that NOTCH1 is activated through the upregulation of its ligands within the tumor niche and suppresses genes that are important for B cell identity, such as E12/E47 and early B cell factor (EBF) (Jundt et al., 2008). Additional studies are required to better define Notch receptor and ligand expression, and targeted signaling pathways in the distinct subtypes of lymphoma.

Acute Myeloid Leukemia and Myelomonocytic Neoplasms

Several years ago, emerging evidence indicated that the Notch pathway could have tumor-suppressive roles in various types of tumors, in stark contrast to its oncogenic role in the aforementioned hematopoietic malignancies (Nicolas et al., 2003; Rangarajan et al., 2001). In contrast to the tumorigenic role of NOTCH1 in T-ALL, our laboratory and others recently characterized a tumor-suppressive role of the Notch pathway in myeloid malignancies. We showed that deletion of nicastrin (*Ncstn*), an essential component of the γ-secretase complex, leads to the induction of chronic myelomonocytic leukemia (CMML) (Klinakis et al., 2011), a disease characterized by increased extramedullary hematopoiesis, monocytosis, myeloproliferation, and frequent progression to acute myeloid leukemia (AML). This is a Notch-mediated effect, as compound deletion of *Notch1/2* in vivo led to similar effects. In support of this, an analysis of the conditional model for the deletion of FX (the homolog of human GDP-L-fucose synthase) or O-fucosyltransferase 1 (*Pofut1*) showed myeloid hyperplasia (Yao et al., 2011), underlining the importance of Notch receptor fucosylation for ligand

Table 1. Oncogenic and Tumor-Suppressive Roles of Notch Signaling in Human Cancers

Tumor Type	Oncogene or Tumor Suppressor	Mutations (%) and Noteworthy Observations	References
T cell acute lymphoblastic leukemia (T-ALL)	oncogene	50%–60% *NOTCH1*, 30% *FBXW7* role in cancer initiation and maintenance	Malyukova et al., 2007; Weng et al., 2004
Chronic lymphocytic leukemia (CLL)	oncogene	5%–12% *NOTCH1* role in cancer initiation and survival	Fabbri et al., 2011; Puente et al., 2011
Melanoma	oncogenic	~50% *NOTCH1* overexpression in human samples possible role in metastasis	Balint et al., 2005; Bedogni et al., 2008
Cholangiocarcinoma (CCC)	oncogenic	35% *FBXW7* Notch1 promotes tumor initiation and maintenance	Akhoondi et al., 2007; Zender et al., 2013
Colorectal cancer	oncogenic	8%–9% FBXW7 crosstalk with Wnt and Hippo signaling	Miyaki et al., 2009 ; Akhoondi et al., 2007
Lung adenocarcinoma	oncogenic	10% *NOTCH1* role in initiation and maintenance (Notch1), and metastasis (Jagged2) specific role for Notch3 in tumor propagation	Licciulli et al., 2013; Westhoff et al., 2009; Zheng et al., 2013
Glioblastoma	oncogenic	role in tumor propagation and radioresistance	Chu et al., 2013; Wang et al., 2010
Renal cell carcinoma	oncogenic	role in progression and maintenance	Sjölund et al., 2008
Ovarian cancer	oncogenic	role in maintenance and therapy response	Cancer Genome Atlas Research Network, 2011; McAuliffe et al., 2012
Prostate	oncogenic	activation of the pathway associated with tumor progression, metastasis, and recurrence	Marignol et al., 2013; Santagata et al., 2004
Breast cancer	mostly oncogenic	*NOTCH1* and *NOTCH4* fusions potential *NOTCH2* dominant-negative truncated mutant other alterations activate Notch signaling, but hyperactive Notch signaling may inhibit cancer growth	Fu et al., 2010; Imatani and Callahan, 2000; Jhappan et al., 1992
Pancreatic ductal adenocarcinoma (PDAC)	mostly oncogenic	Notch2 loss inhibits progression and maintenance overexpression of ligands (Jagged2 [90%], Dll4 [50%], but Notch1 loss may promote tumor initiation	Hanlon et al., 2010; Mazur et al., 2010; Mullendore et al., 2009
Cervical cancer	mostly oncogenic	pathway activation in human tumors, but dose-dependent effects possible role in tumor-propagating cells	Bajaj et al., 2011; Maliekal et al., 2008; Zagouras et al., 1995
Head and neck squamous cell carcinomas (HNSCC)	mostly oncogenic	possible bimodal pattern of Notch pathway alterations with a small subset of tumors with inactivating *NOTCH1* mutations, but a larger group with pathway activation	Sun et al., 2014
Hepatocellular carcinoma (HCC)	oncogenic and tumor suppressive	context-dependent effects that may be related to various molecular subtypes	Qi et al., 2003; Villanueva et al., 2012
Medulloblastoma	oncogenic and tumor suppressive	opposite roles for Notch1 and Notch2	Fan et al., 2004
B cell acute lymphoblastic leukemia (B-ALL)	tumor suppressive	no mutations role in maintenance (activation induces growth arrest and death)	Zweidler-McKay et al., 2005
Acute myeloid leukemia (AML)	tumor suppressive	Notch1 and Notch2 are expressed, but the pathway is not active role in cancer initiation and maintenance	Kannan et al., 2013; Lobry et al., 2013
Small cell lung carcinoma (SCLC)	tumor suppressive	no mutations inhibits tumor maintenance (possible similar role in other neuroendocrine tumor types)	Sriuranpong et al., 2001

(*Continued on next page*)

Table 1. *Continued*

Tumor Type	Oncogene or Tumor Suppressor	Mutations (%) and Noteworthy Observations	References
Lung squamous cell carcinoma (SqCC)	tumor suppressor	5%–12.5% *NOTCH1* and *NOTCH2*	Cancer Genome Atlas Research Network, 2012; Wang et al., 2011b
Cutaneous squamous cell carcinoma (SqCC)	tumor suppressor	60%–75% *NOTCH1* and *NOTCH2*	Wang et al., 2011b
Chronic myelomonocytic leukemia (CMML)	tumor suppressor	12% various pathway genes (*NCSTN*, *APH1*, *MAML1*, and *NOTCH2*)	Klinakis et al., 2011
		role in cancer initiation	

The cancers indicated in this table have been selected for historical reasons (first examples of mutations in the Notch pathway), because they affect large populations of cancer patients, or because of the particular insight of some studies into the role of Notch signaling in cancer. Selected observations and references are shown for the selected tumor types; see text for additional references and details. In particular, data from large cancer genome efforts indicate that many alterations in the extended Notch pathway exist in human tumors; most of these alterations are awaiting additional analyses and functional validation.

binding and pathway activation. Ablation of MAML1 can lead to similar phenotypes (Chen et al., 2008). Mechanistically, the tumor-suppressor role of NOTCH in this disease is mediated by direct repression of the *PU.1* and *CEBP*α promoters by HES1. Subsequent screening of primary CMML samples for Notch pathway mutations showed that *NCSTN*, *MAML1*, *APH1A*, and *NOTCH2* are mutated and genetically inactivated in about 12% of CMML patients. These mutations are unique to CMML and are not found in other myeloproliferative disorders, such as polycythemia vera and myelofibrosis. Notch-inactivating mutations co-occurred with other described myeloid mutations in genes such as *TET2*, *FLT3*, and *ASXL1* (Klinakis et al., 2011). Based on these findings, we were able to show that the combination of Notch pathway and TET2 inactivation leads to AML. AML cells specifically express NOTCH2 on their surface, but show no signs of pathway activity. Interestingly, reactivation of the Notch pathway in established AML leads to complete disease remission (Kannan et al., 2013; Lobry et al., 2013). This observation provides a rationale for the use of specific NOTCH2-activating antibodies or specific agonists as a viable therapeutic strategy in this type of leukemia. Mechanistically, there might be several ways to suppress Notch pathway activity in AML. Initially, AML cells might reside in microenvironments that lack Notch ligands. Another putative mechanism is epigenetic silencing achieved by DNA and histone methylation of target gene promoters/transcriptional start sites. In agreement with this possibility, we found that Notch target genes are characterized by H3K27me3 marks (Lobry et al., 2013), and mice carrying the R132H mutation of isocitrate dehydrogenase 1 (IDH1) (Figueroa et al., 2010; Gross et al., 2010; Xu et al., 2011) develop a myeloproliferative disease characterized by marked DNA hypermethylation of Notch pathway genes such as Lfng, Maml3, and Hes5 (Sasaki et al., 2012).

B Cell ALL

Interestingly, Notch signaling also appears to act as a tumor suppressor in B cell ALL (B-ALL). In agreement with the AML findings, a study by Zweidler-McKay et al. (2005) showed that Notch pathway reactivation leads to growth inhibition and induces apoptosis in human B-ALL cells. In a recent follow-up publication, it was shown that several Notch pathway targets in B-ALL are suppressed by DNA cytosine hypermethylation on their promoters, followed by histone H3K27 and H3K9 trimethylation (Kuang et al., 2013). The parallel between AML and B-ALL is intriguing and potentially can be explained by a recent Notch activity mapping effort (Oh et al., 2013) that demonstrated activity of the pathway in T cell progenitors and pre-erythrocytes, and a lack of pathway activation in the B cell and myelomonocytic lineages. These findings provide support for the notion that NOTCH plays a key role as a developmental regulator in determining the fate of progenitors in the hematopoietic system. In this model, NOTCH action needs the addition of other oncogenic stimuli to transform cells. In agreement with this idea, we found that Notch pathway inactivation can lead to an increased frequency of granulocyte-monocyte progenitors (GMPs), which can initiate diseases such as CMML and AML upon further alterations (Klinakis et al., 2011).

Notch Signaling in Solid Tumors

A number of recent reviews have thoroughly summarized the current knowledge about Notch signaling in solid tumors (Nowell and Radtke, 2013; Ranganathan et al., 2011; South et al., 2012; see also Table 1). Recent studies, including resources from The Cancer Genome Atlas (TCGA), have provided genomic data that underscore the prevalence and complexity of Notch pathway alterations in human cancers; however, they have not provided detailed functional interpretations of these alterations. Our goal in the section below is not to provide an exhaustive list of the solid tumor types in which Notch signaling is altered and the possible consequences of these alterations. Rather, we aim to highlight some key observations regarding a few prominent tumor types and draw some points of discussion from relevant studies, including the large number of partners used by Notch (Figure 4) and the distinct roles of the four Notch receptors.

Breast Cancer

Breast cancer is a very prevalent form of cancer in which the Notch pathway may act as a tumor suppressor or an oncogene depending on the subtype. One of the first indications that Notch signaling may play a role in solid tumors came from experiments with mouse mammary tumor viruses (MMTVs). Integration of the MMTV genome next to the "*Int-3*" locus resulted in an activating mutation of *Notch4*, leading to the constitutive activation of the receptor and breast cancer development (Gallahan and Callahan, 1997; Jhappan et al., 1992; Robbins et al., 1992). Since this seminal discovery, a number of studies have confirmed that activation of Notch signaling plays an oncogenic role in

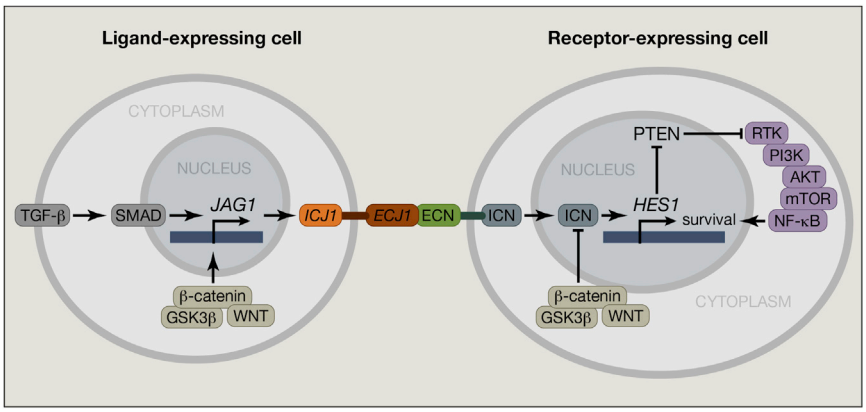

Figure 4. Simplified Scheme of Notch Interactions with Other Signaling Pathways in Cancer

The TGFβ, PI3K, NFκB, and WNT pathways are some of the most important pathways that interact with NOTCH. Notably, Jagged 1 is activated by the TGFβ pathway and in turn activates NOTCH receptors in neighboring cells. Phosphorylation of NOTCH from the WNT-induced GSK3β leads to ubiquitination through FBXW7 and final degradation. Also, a classical NOTCH target, HES1, represses PTEN, a competitor of another pathway with oncogenic roles, PI3K, which in turn activates NFκB, a pathway that is important for leukemia progression. Important parameters of the interactions, such as regulation of NFκB pathway by NOTCH through HES1 action, or interaction of NOTCH with the WNT member DVL (Dishevelled) protein, which inhibits both WNT and NOTCH pathways, are not shown in this figure. ICJ1, intracellular part of JAG1; ECJ1, extracellular part of JAG1.

breast cancer (Colaluca et al., 2008; Pece et al., 2004; Robinson et al., 2011; Xu et al., 2012). In breast cancer cells, Notch signaling can be activated by functional interactions with other signaling pathways, including the Ras and Wnt pathways (Ayyanan et al., 2006; Fitzgerald et al., 2000; Izrailit et al., 2013; Meurette et al., 2009; Weijzen et al., 2002). Recent observations indicate that Notch4 may play a more specific role than other Notch receptors in breast cancer stem cells (Harrison et al., 2010). In contrast, a recent study indicated that hyperactivation of NOTCH3 may actually be detrimental to breast cancer cells by inducing senescence (Cui et al., 2013). Interestingly, mammary epithelial cells respond differently to different levels of activation of the Notch pathway (Mazzone et al., 2010). Thus, although accumulating evidence indicates that Notch is protumorigenic in breast cancer, in certain contexts, specific (high) levels of activation may be tumor suppressive. Alternatively, different Notch receptors may have unique signaling outputs in mammary epithelial cells or in different subtypes of breast cancer. Once more, the notion of a ''differentiation switch'' could explain the many faces of Notch signaling in this type of tumor.

Lung Cancer

Lung adenocarcinoma (LAC) is a major subtype of lung cancer. Initial observations suggested that Notch signaling promotes the expansion of LAC cells in culture (Dang et al., 2003; Eliasz et al., 2010; Haruki et al., 2005). More recent in vivo studies demonstrated that Notch signaling is a key promoter of LAC development and maintenance (Allen et al., 2011; Licciulli et al., 2013; Maraver et al., 2012), and that NOTCH3 plays a unique role in the self-renewal of LAC tumor-propagating cells (Zheng et al., 2013). Expression of JAG2 at the surface of lung adenocarcinoma cells leads to homotypic interactions with Notch receptors and promotes the metastatic potential of these LAC stem cells (Yang et al., 2011). Thus, although mutations and other alterations may not be frequent in LAC (Westhoff et al., 2009), Notch pathway activity correlates significantly with worse survival in lung cancer patients (Hassan et al., 2013; Zheng et al., 2013), and activation of Notch may be important for the sustained growth of LAC. Targeting NOTCH3 and/or JAG2 may benefit a very large number of lung cancer patients worldwide.

Squamous cell lung carcinoma (SqCC) is the second major type of non-small cell lung cancer. In stark contrast to LAC, Notch signaling is thought to be a tumor suppressor of SqCC development, as evidenced by the identification of loss-of-function mutations in human tumors (Wang et al., 2011b). These mutations mainly cluster in the EGF-like repeat region of NOTCH1 and thus have the potential to disrupt ligand binding or to produce truncated receptors (Figure 1). Although functional validation of these observations is still missing owing to the current lack of appropriate mouse models, numerous observations indicate that inactivation of Notch signaling promotes the development of squamous cell carcinoma in other tissues, including cutaneous and head-and-neck tumors (Agrawal et al., 2012; Pickering et al., 2013; Proweller et al., 2006; Rothenberg and Ellisen, 2012; Wang et al., 2011b). These observations suggest that loss of Notch pathway activity may be critical for the growth of tumor cells with squamous differentiation characteristics.

Small cell lung carcinoma (SCLC) is a neuroendocrine subtype of lung cancer. Although it accounts for a smaller fraction of lung cancer cases (~12%–15%), it has the highest mortality rate. Genomic studies have failed to identify recurrent mutations in the Notch pathway in SCLC (Peifer et al., 2012; Rudin et al., 2012). However, early observations indicated that hyperactivation of Notch signaling blocks the cell cycle of SCLC cells (Sriuranpong et al., 2001, 2002). A tumor-suppressive role for Notch in SCLC is supported by evidence that Notch may play a similar role in other neuroendocrine tumors, such as medullary thyroid carcinoma (Cook et al., 2010). Thus far, however, no functional evidence has been obtained in vivo that activation of Notch may block SCLC development or maintenance, and it is still possible that subpopulations of cells in SCLC tumors may display some Notch activity and contribute to SCLC growth (Kluk et al., 2013; Salcido et al., 2010).

Thus, three different subtypes of lung cancer display strikingly different roles for Notch signaling in cancer development: (1) actively oncogenic with rare genetic alterations in LAC, (2) tumor suppressive with inactivating mutations in SqCC, and (3) possibly tumor suppressive with no sign of mutations in SCLC. It is possible that these differences are related to the role of Notch in cell-fate decisions during lung embryonic development.

Liver Cancer

Genome sequencing analyses did not reveal recurrent mutations in Notch pathway genes in hepatocellular carcinoma (HCC), a leading cause of cancer-related deaths worldwide (Fujimoto et al., 2012; Guichard et al., 2012). Nevertheless, Notch signaling has been of interest to liver cancer biologists because of the prominent role of Notch signaling in liver development, including mutations in *NOTCH2* or *JAG1* in patients with Alagille syndrome (syndromic bile duct paucity) (McDaniell et al., 2006; Oda et al., 1997). Haploinsufficient mutations in a specific ligand and a specific receptor in the Notch pathway would suggest that Notch signaling could play very context- and level-dependent roles in liver tumors. Initial observations suggested that low levels of Notch correlate with high activity of the Wnt pathway, a major oncogenic pathway in HCC (Wang et al., 2009). Also, high levels of active Notch1 may inhibit the expansion of HCC cells (Qi et al., 2003), and deletion of *Notch1* in the liver of mice results in hyperproliferative hepatocytes, suggesting a tumor-suppressive role for Notch in HCC (Croquelois et al., 2005). Similarly, Notch signaling has a tumor-suppressive effect in HCC initiated by inactivation of the RB pathway (Viatour et al., 2011). However, more recent reports have provided evidence that Notch signaling is active and oncogenic in HCC (Dill et al., 2013; Tschaharganeh et al., 2013; Villanueva et al., 2012), and may be important for the development of tumors following hepatitis B virus infection (Jeliazkova et al., 2013). These observations suggest that the role of Notch signaling in HCC may differ among the distinct molecular subgroups of this cancer type, and underscore the need to further explore the molecular contexts associated with the tumor-suppressive or oncogenic roles of Notch in the liver.

In contrast to the complex roles of Notch signaling in HCC, accumulating evidence supports a protumorigenic role for Notch signaling in cholangiocarcinoma (CCC). Mutations of the Notch repressor *FBXW7* are found in a subset of human tumors (Akhoondi et al., 2007). Similar to the disruption of bile ducts in Alagille patients, activation of Notch2 in liver progenitors and adult hepatocytes promotes biliary tubulogenesis (Jeliazkova et al., 2013). Finally, constitutive activation of NOTCH1 is sufficient to initiate CCC development in mice (Zender et al., 2013).

It is likely that the sometimes contradictory consequences of Notch activation in liver cells are due to a combination of the strength of the downstream signal, the timing of the activation, the cell type in which this activation occurs, and the receptor involved (Ortica et al., 2014). There seems to be a consensus that higher Notch levels in liver progenitors favor bile duct differentiation over hepatocytic differentiation. Possibly, activation of Notch (e.g., NOTCH2) in these progenitors promotes CCC while suppressing HCC (Guest et al., 2014). It is also possible that Notch switches from a suppressive role in the early stages of HCC development to a more oncogenic role. Although it was proposed that Notch signaling plays a role in liver cancer invasion and metastasis (Lim et al., 2011; Zhou et al., 2013), more work is required to further support this notion.

Colorectal Cancer

The intestinal epithelium exhibits an unprecedented self-renewal rate that appears to be linked to a high susceptibility to malignant transformation. Notch signaling has been known for many years now to be involved in both the control of homeostatic self-renewal in stem cell populations and the development of colorectal cancer (CRC) (Fre et al., 2005; Radtke and Clevers, 2005; van Es et al., 2005). Although mutations in *NOTCH* genes are rare, Notch signaling is overexpressed or constitutively activated in CRC in part because of mutations in regulators of Notch signaling, including *FBXW7* (although FBXW7 clearly controls other cellular pathways beyond Notch) (Akhoondi et al., 2007; Babaei-Jadidi et al., 2011; Camps et al., 2013; Miyaki et al., 2009; Sancho et al., 2010; Zhu et al., 2013). In addition, Notch activation has been linked to activation of Wnt signaling and Hippo/YAP signaling in CRC cells, although the various levels of crosstalk between these pathways are still not fully understood (Camargo et al., 2007; Fre et al., 2009; Kim et al., 2012; Kwon et al., 2011; Peignon et al., 2011; Rodilla et al., 2009; Tschaharganeh et al., 2013). In particular, Jagged1, expressed on tumor cells themselves or produced from endothelial cells, is thought to be a key ligand for Notch activation in CRC cells (Lu et al., 2013; Rodilla et al., 2009; Tschaharganeh et al., 2013). Another Notch ligand, DLL4, plays a non-cell-autonomous role in CRC development, in large part by controlling the development of blood vessels necessary for tumor growth (Fischer et al., 2011; Ridgway et al., 2006). Expression of miR-34a in CRC stem cells may help control Notch output and generate a bimodal Notch response (Bu et al., 2013). Finally, Notch signaling may play a crucial role not only in the early stages of CRC development, by controlling the fate of stem cells and cancer stem cells, but also in the later stages of tumor invasion and metastasis (Sonoshita et al., 2011).

Pancreatic Cancer

The major and most lethal type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC). An early study detected evidence of Notch pathway activation in PDAC and showed that Notch lies downstream of TGFß during ductal metaplasia, an early stage of PDAC development (Miyamoto et al., 2003). Mouse genetics studies have demonstrated that activation of Notch signaling cooperates with oncogenic K-Ras to promote both initiation and dysplastic progression from acinar cells by inducing their rapid reprogramming to a duct-like phenotype (De La O et al., 2008). Indeed, pharmacological inhibition of Notch signaling slows the progression of the disease in mutant mice and prevents the expansion of some human PDAC cell lines (Cook et al., 2012; Mizuma et al., 2012; Plentz et al., 2009), possibly in part because of an inhibition of PDAC stem cells (Bailey et al., 2014). Genetic inactivation of *Notch2*, but not Notch1 (Avila et al., 2012; Mazur et al., 2010), inhibits PDAC development initiated by oncogenic K-Ras. In fact, loss of *Notch1* function may even promote PDAC development, although the basis of this observation remains unknown (Hanlon et al., 2010).

Melanoma

The Notch pathway has been found to be active in melanoma (Asnaghi et al., 2012). NOTCH1 appears to promote disease progression (Rangarajan et al., 2001; Zhang et al., 2012) and growth of melanocytes under hypoxic conditions (Bedogni et al., 2008). There are no documented gain-of-function mutations that affect the pathway in this disease (Hodis et al., 2012), suggesting that the pathway might be affected through transcriptional and epigenetic control, possibly via the

contrasting actions of BRN2, a possible activator, and MITF, which acts as a repressor of the Notch pathway (Thurber et al., 2011). Whatever the mechanism of activation, recent preclinical studies have reported that γ-secretase inhibitors (GSIs) can reduce the tumor-initiating potential, and suggested that combining GSIs with chemotherapy could be a useful new therapeutic approach for melanoma (Huynh et al., 2011).

In conclusion, Notch signaling plays distinct roles in different types of tumors, both solid and liquid (hematopoietic). This review is by no means exhaustive, but provides a detailed overview of Notch signaling in cancer. Most of the studies cited here are recent, emphasizing the increasing interest in the role of Notch signaling in cancer during the last decade. Mechanistically, more work is required to pinpoint specific molecular pathways and gene targets in each tumor type, but emerging technologies and, most notably, DNA and RNA next-generation sequencing-based approaches will continue to help us further dissect the role of this pathway in tumor initiation and progression.

A Perspective on Two Decades of Notch-Centered Cancer Research: Remaining Intriguing Questions

This brief overview of some of the most common and/or lethal human cancers, both hematopoietic and solid, highlight several key aspects of the role of Notch signaling in cancer development that hold true for other tumors in which Notch signaling is also altered, including myeloma, prostate, ovarian, skin, and brain cancers. Obviously, there are several outstanding questions that have to be addressed to not only help us better understand pathway function in cancer but also enable more efficient therapeutic targeting (see below). An initial question is whether Notch pathway mutations are tumor initiating or tumor propagating. Most likely, both types of mutations can be described depending on the tumor type. We discussed an intriguing example in myeloid neoplasms where Notch signaling loss of activity seems to expand the frequency of leukemia-initiating cells, but requires secondary mutational events to lead to full-blown disease (Klinakis et al., 2011; Lobry et al., 2013). A similar scenario might play out in B-ALL, as it was shown that Notch activity directs lymphocyte progenitors exclusively to the T cell lineage, at the expense of B cell differentiation (Pui et al., 1999; Radtke et al., 1999). On the other hand, it is intriguing to ask whether NOTCH1-activating mutations in T-ALL occur to simply define lineage, by locking cells in a specific differentiation status (T cell in this case), or to truly transform the cells. Further studies that allow genetic sequencing of both leukemia and normal stem cell/progenitor populations, preferably at the single-cell level, might be able to address such questions.

One particularly interesting aspect of Notch signaling in cancer progression that has been emerging in the last few years is its potential impact on metastasis, which may be linked to the role of Notch in cancer stem cells (see Giancotti, 2013, for a recent discussion). Early studies identified JAG1 expression as a marker of metastatic prostate cancer (Santagata et al., 2004) and found a role for Notch in controlling the epithelial-mesenchymal transition (EMT) (Timmerman et al., 2004). Indeed, JAG1 expression on tumor cells may help promote the spread of breast cancer cells to the bone microenvironment by activating Notch signaling in bone cells (Sethi et al., 2011). Activation of Notch during EMT and metastasis may be under the control of the miR-200 microRNA (Brabletz et al., 2011; Yang et al., 2011). This prometastatic function of Notch signaling may be promoted by its crosstalk with the machinery that responds to hypoxic environments (Sahlgren et al., 2008; Yeung et al., 2011). Furthermore, an increasing number of studies are connecting Notch signaling to molecules and pathways involved in tumor invasion and metastatic growth, including Tenascin C (Oskarsson et al., 2011) and regulators of polarity (McCaffrey et al., 2012) in breast cancer.

As discussed above, multiple studies have shown Notch signaling in tumor cells to be involved in various aspects of angiogenesis, especially via the DLL4 and JAG1 ligands (Benedito et al., 2009; Li and Harris, 2005; Phng and Gerhardt, 2009; Zeng et al., 2005). In particular, the Notch ligand DLL4 is upregulated in the angiogenic vasculature in response to vascular endothelial growth factor (VEGF), and blockade of DLL4 was shown to lead to markedly increased nonproductive tumor vascularity, which inhibits tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006). Although these observations seemed promising clinically (Hoey et al., 2009), long-term blockade of DLL4 was shown to lead to the development of vascular neoplasms (Yan et al., 2010), potentially limiting the therapeutic potential of DLL4-blocking strategies. Thus, activation of Notch signaling may contribute to tumor spread via multiple mechanisms, such as maintaining the self-renewal of cancer stem cells, contributing directly to the cellular processes involved in tumor invasion (e.g., EMT and response to hypoxia) (Wang et al., 2011c), controlling neovascularization, and playing a key role in the metastatic niche. Such issues could be more important in solid tumors than in leukemia; however, it is intriguing to define Notch-ligand-expressing niches in different types of hematopoietic tumors, and to test whether ligand expression is important for leukemia cell homing to different tissues and response to drug treatments. For example, targeting the expression or function of a specific ligand could affect NOTCH1-expressing T-ALL homing and metastasis. As most cancer patients die from metastatic disease, it will be important in the near future to continue to investigate the molecular and cellular bases of tumor spread in connection with Notch signaling.

Therapeutic Targeting of the Notch Pathway in Tumors

Because proteolytic cleavage of NOTCH receptors by the presenilin/γ-secretase complex is a prerequisite for the activation of signaling (in the absence of downstream activating mutations), small-molecule GSIs efficiently block NOTCH1 activity in T-ALL cells. Thus, the use of GSIs to inhibit NOTCH signaling has been proposed as a molecular targeted therapy for treatment of this disease (Aster and Blacklow, 2012; Palomero and Ferrando, 2009). However, animal studies have shown that systemic inhibition of NOTCH signaling results in "on-target" gastrointestinal toxicity because of the accumulation of secretory goblet cells in the intestine due to alterations in the differentiation of intestinal stem cells following Notch inactivation. Phase 1 clinical trials further confirmed these treatment side effects. As a result, inhibition of the pathway using GSIs alone may not be the most viable therapeutic choice in the future. An alternative to single GSI treatment would be a combinatorial use of

glucocorticoids and GSIs, because glucocorticoids ameliorate the GSI-induced gut toxicity by inducing the expression of Cyclin D2, thereby protecting the animals from developing intestinal goblet cell metaplasia (Real et al., 2009).

However, targeting of Notch signaling is not restricted to the use of GSIs. α-Secretase inhibitors (ASIs) against the ADAM10/17 metalloproteases that mediate receptor S2 cleavage are available (Zhou et al., 2006) and are currently being tested (Purow, 2012). Furthermore, using phage display technology, pharmaceutical companies have generated highly specialized antibodies against NOTCH1 and NOTCH2 that act mainly by stabilizing the negative regulatory region of the receptors and protecting against proteolytic cleavage, thus inhibiting the production of ICN1/2 (Wu et al., 2010). These antibodies lead to lower levels of gastrointestinal toxicity and other side effects resulting from the pan-Notch pathway inhibition achieved by GSIs. Selective blocking of NOTCH1 inhibits tumor growth in preclinical models through at least two mechanisms: inhibition of cancer cell growth and deregulation of angiogenesis. Soluble extracellular fractions of Notch receptors and ligands can also act as decoys and inhibit the pathway in a dominant-negative manner. In one study, a Notch1 decoy decreased tumor cell viability in xenograft models (Funahashi et al., 2008). However, under different conditions, a DLL1 decoy was found to play either an activating or an inhibitory role (Hicks et al., 2002). Thus, a better understanding of the dynamics by which decoys work is needed before they can be considered as a viable therapeutic strategy.

Other types of experimental inhibitors entail synthetic peptides that mimic MAML1 but lack its active domains. Although these peptides are widely used for basic research purposes, their use for therapeutic purposes is still limited. Moellering et al. (2009) generated a synthetic, cell-permeable, α-helical peptide (SAHM1) that blocks MAML1 recruitment and NOTCH-mediated transcription as it binds with high affinity to the interface on the NOTCH-CSL transactivation complex. Treatment of human T-ALL cell lines and a mouse model of NOTCH1-driven T-ALL with SAHM1 resulted in strong NOTCH-specific inhibition of cell proliferation and leukemia progression (Moellering et al., 2009).

Another intriguing idea for the treatment of tumors that are induced by NOTCH and depend on pathway activity is to not target the Notch pathway itself, and instead focus on its signaling targets. Several such efforts are currently under way. Briefly, investigators have demonstrated in vivo T-ALL remission by targeting (1) the NOTCH1-induced IKK kinase complex, which plays a pivotal role in controlling the NF-kB pathway, which in turn is strongly related to NOTCH in leukemia (Figure 4; Dan et al., 2008; Espinosa et al., 2010; Vilimas et al., 2007); (2) the CyclinD:CDK4/6 kinase complex, which is hyperactivated in this type of acute leukemia (Sawai et al., 2012); and (3) the bromodomain-containing protein BRD4 (King et al., 2013). BRD proteins can be transcriptional coactivators and share common binding patterns with T-ALL oncogenes NOTCH1 and MYC in promoters and enhancers of key genes for the induction and progression of the disease. Filippakopoulos et al. (2010) recently modified a thienodiazepine molecule so that it inhibits binding of BRD to the acetylated residues of histone H4. We were able to show that such drugs can target both NOTCH1- and MYC-regulated transcription in T-ALL, leading to complete disease remission in vivo (King et al., 2013). Such "epigenetically"-targeted therapies might be particularly attractive considering the ability of Notch to alter locus accessibility and initiate transcription. We recently connected NOTCH1 binding to loss of H3K27me3 on target promoters, and demonstrated an antagonism between NOTCH1 binding and Polycomb complex 2 (PRC2) recruitment and activity (Ntziachristos et al., 2012). Based on these findings, H3K27me3 demethylation inhibitors might be an attractive therapy option in NOTCH1-induced T-ALL (or CLL). Finally, recent evidence suggests that it may be possible to inhibit Notch signaling by interfering with its trafficking in cancer cell secretory pathways (Ilagan and Kopan, 2013; Krämer et al., 2013).

Although a number of "anti-Notch" strategies are emerging, it may be as important to specifically activate Notch in tumors where activation of the Notch pathway is tumor suppressive. As discussed above for AML, in cases where tumor cells express a Notch receptor (NOTCH2) but do not show signs of pathway activation, providing a ligand for these receptors or treating them with activating antibodies may be sufficient to activate the pathway in certain contexts and inhibit tumor growth. Furthermore, in cases where Notch receptors are not expressed (e.g., they are transcriptionally silenced), or some of their key target genes are silenced, approaches to derepress the expression of these genes may be useful to slow cancer growth (Stockhausen et al., 2005).

Future Directions in the Understanding and Treatment of Notch-Induced Tumors

The NOTCH pathway has been the intense focus of cancer researchers for the last two decades. Unfortunately, there are still no FDA-approved, Notch-targeted therapies. Retrospectively, this is not surprising, as we now know that the pathway plays key roles in several tissues, including adult differentiating and regenerating tissues, which explains the potential side effects of general inhibitors of the Notch pathway, such as GSIs. The critical question is whether one can successfully target the Notch pathway to significantly inhibit cancer growth. Another major conundrum comes from the notion that Notch may play distinct roles during several stages of the tumorigenic process, an idea that has not been thoroughly examined. It is likely that inhibition of Notch signaling in tumors initiated by Notch-activating mutations will have a therapeutic effect, as tumors are often addicted to early initiating events. However, tumors in which alterations in Notch pathway members occur late during tumor evolution may rapidly invent ways to get around the targeting of Notch.

We suggest that specificity should be the key for future attempts to target NOTCH activity in cancer cells. One must have a complete map of both Notch receptor and ligand expression in different cancers and their microenvironments to be able to use antibodies or other small molecules that specifically inhibit only the relevant molecules. Targeted (*NOTCH*-focused) sequencing of tumors is also important to obtain a clear idea of the type of mutation and its potential impact on pathway activity. Importantly, a large number of tumors that contain Notch-activating mutations, such as the ICN1 translocation, cannot be treated with GSIs. In contrast, receptor-specific antibody

agonists could be of significant clinical value for tumors in which Notch signaling has a tumor-suppressive function. Myeloid neoplasms are a cancer subtype that could benefit from targeted pathway activation, as we have shown that in such tumors the pathway is inactive but the NOTCH2 receptor is expressed on the surface of the cells and can be activated by ligand binding, leading to cell death. Strategies to activate NOTCH in some cancers are worth testing, first in preclinical models and hopefully in the near future in patients. Moreover, both Notch agonists and antagonists could also be used in combination with current treatments, including chemotherapy and more recent targeted-therapies. In an intriguing example of such treatments, anti-DLL4 antibodies were combined with either chemotherapy or Avastin or VEGF traps to target tumor angiogenesis (Lobov et al., 2011; Noguera-Troise et al., 2006). Another example is the combination of NOTCH receptor inhibition (using GSIs or antibodies) and glucocorticoids for the treatment of T-ALL (Real et al., 2009).

As with other signaling pathways involved in cancer, identification and targeting of NOTCH-interacting partners and targets could be pivotal for the development of antitumor therapy protocols. Some attempts have been made to identify such genes/proteins using whole-proteome (mass spectrometry) (Yatim et al., 2012) and genome/transcriptome (RNA-seq, gene array, and ChIP-seq for NOTCH1 and HES1) (Ntziachristos et al., 2012; Wang et al., 2011a) approaches. These studies suggest that, apart from a small fraction of "universal" targets, including members of the HES and HEY families, Notch pathway activity controls the expression of a large number of tissue- and cell-type-specific gene targets. Indeed, we have shown that NOTCH2-HES1 signaling can regulate the expression of CEBPA and PU1, two key regulators of myeloid differentiation, but these genes are not affected by NOTCH pathway regulation in T-ALL. Thus, the ability to target the function of tissue-specific Notch pathway targets could offer more targeted therapies with fewer side effects. In a similar fashion, it will be intriguing to define the biochemical composition of the nuclear Notch complex in different tissues to see whether there is a specificity that can guide small-molecule-inhibition efforts.

Overall, it is fair to say that it took the scientific community almost a century to reach the point where the basic molecular tenets of NOTCH signaling are well understood. Similarly, although we have known for the last two decades that Notch signaling is involved in cancer, only recently have we developed the means (e.g., small molecules and antibodies) to effectively target pathway activation in this disease. There is still a significant need for further research efforts to better define the pathway and to propose drugs or drug combinations that can affect Notch signaling specifically in cancer, avoiding harmful side effects and improving both survival and quality of life for patients with Notch-induced tumors.

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Angiocrine Factors Deployed by Tumor Vascular Niche Induce B Cell Lymphoma Invasiveness and Chemoresistance

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SUMMARY

Tumor endothelial cells (ECs) promote cancer progression in ways beyond their role as conduits supporting metabolism. However, it is unknown how vascular niche-derived paracrine factors, defined as angiocrine factors, provoke tumor aggressiveness. Here, we show that FGF4 produced by B cell lymphoma cells (LCs) through activating FGFR1 upregulates the Notch ligand Jagged1 (Jag1) on neighboring ECs. In turn, upregulation of Jag1 on ECs reciprocally induces Notch2-Hey1 in LCs. This crosstalk enforces aggressive CD44$^+$ IGF1R$^+$CSF1R$^+$ LC phenotypes, including extranodal invasion and chemoresistance. Inducible EC-selective deletion of *Fgfr1* or *Jag1* in the *Eμ-Myc* lymphoma model or impairing Notch2 signaling in mouse and human LCs diminished lymphoma aggressiveness and prolonged mouse survival. Thus, targeting the angiocrine FGF4-FGFR1/Jag1-Notch2 loop inhibits LC aggressiveness and enhances chemosensitivity.

INTRODUCTION

Vascular endothelial cells (ECs) are a specialized component of the tumor microenvironment that can orchestrate tumor growth and invasion (Beck et al., 2011; Bergers and Hanahan, 2008; Butler et al., 2010a; Calabrese et al., 2007; Carmeliet and Jain, 2011; Charles et al., 2010; Ghajar et al., 2013; Lu et al., 2013; Weis and Cheresh, 2011). During regeneration, tissue-specific ECs provide instructive paracrine cues, known as angiocrine growth factors, that trigger proliferation of repopulating progenitor cells (Brantley-Sieders et al., 2011; Butler et al., 2010a, 2010b, 2012; Ding et al., 2014, 2010, 2011, 2012; Potente et al., 2011; Red-Horse et al., 2007). However, the mechanism by which EC-derived angiocrine factors influence tumor behaviors is unknown (Gilbert and Hemann, 2010; Leite de Oliveira et al., 2012; Nakasone et al., 2012; Schmitt et al., 2000).

Notch signaling is a pivotal modulator of lymphomagenesis (Aster et al., 2008; Espinosa et al., 2010; Liu et al., 2010; Lobry et al., 2013), enhancing Myc activity and upregulating receptors such as insulin growth factor-1 receptor (IGF1R) (Medyouf et al., 2011; Weng et al., 2006). The Jagged (Jag) and Delta-like (Dll) families of Notch ligands induce Notch signaling (Gridley, 2010; Siekmann and Lawson, 2007). Both Jag1 and Dll4 are preferentially expressed by ECs during tumor progression but have

Significance

Blood vessels within lymphomas are not just passive conduits delivering nutrients but contain specialized ECs that constitute a maladapted niche actively instigating aggressiveness during tumor progression. Here, we show that tumor ECs supply angiocrine factors to promote chemotherapy resistance and extranodal invasiveness of CD44$^+$IGF1R$^+$CSF1R$^+$ LCs. LCs produce FGF4 to induce expression of Notch ligand Jag1 in ECs. In turn, EC-derived angiocrine Jag1 activates Notch2 in LCs to promote tumor invasiveness and chemoresistance. Interfering with the FGF4/Jag1 cross-talk between LCs and ECs decreases tumor progression and promotes sensitivity to chemotherapy, thereby increasing survival of tumor-bearing mice. Targeting protumorigenic angiocrine factors supplied by lymphoma blood vessels promises efficacious approaches to block tumorigenesis and restore chemotherapy sensitivity without compromising tumor blood perfusion.

distinct roles in neoplastic tissue (Rehman and Wang, 2006; Sethi et al., 2011; Vilimas et al., 2007). Dll4 is expressed by sprouting ECs and appears to regulate EC expansion (proliferative angiogenesis), whereas juxtacrine activation of Notch receptors on tumor cells appears to be mediated by EC-derived Jag1 (inductive angiogenesis) (Lu et al., 2013; Sonoshita et al., 2011; Noguera-Troise et al., 2006; Ridgway et al., 2006). However, mechanisms controlling expression of these Notch ligands in tumor ECs are undefined (Benedito et al., 2009; Corada et al., 2010; High et al., 2008; Hoey et al., 2009; Hofmann et al., 2010; Tung et al., 2012). Moreover, the paucity of EC-specific mouse genetic models has handicapped elucidation of the EC-derived angiocrine signals regulating the fate and behavior of tumors.

Malignant lymphoma cells (LCs) are composed of heterogeneous cell subpopulations, with a subset of LCs possessing more aggressive features (Dierks et al., 2007; Hoey et al., 2009; Kelly et al., 2007). Although chemotherapy eliminates the majority of proliferating LCs, a subpopulation of aggressive LCs manifests resistance, ultimately leading to lymphoma relapse. Because the surrounding microenvironment can support tumor cells (Hanahan and Coussens, 2012; Lane et al., 2009; Memarzadeh et al., 2007; Rakhra et al., 2010; Reimann et al., 2010; Scadden, 2012; Trimboli et al., 2009; Zhang et al., 2012), we reasoned that elucidating the microenvironmental signals (i.e., tumor vascular niche) influencing aggressive LCs, such as lymphoma initiating cells (LICs), could provide effective lymphoma treatment strategies.

RESULTS

ECs Support Expansion of LCs with Aggressive Features

To identify the crosstalk between ECs and LCs without the confounding influence of supplementation with exogenous serum and angiogenic growth factors, we devised a serum- and growth factor-free platform to propagate LCs in coculture with ECs. To this end, we transduced ECs, such as human umbilical vein ECs, with the adenoviral E4ORF1 gene. E4ORF1 transduced ECs (VeraVec ECs)—for simplicity referred to here as ECs—are nontransformed but have low-level Akt signaling that permits their serum-free survival while retaining their tissue-specific vascular attributes as well as the capacity to form functional contact-inhibited monolayers in vitro and perfused, patent blood vessels in vivo (Butler et al., 2010b, 2012; Nolan et al., 2013; Seandel et al., 2008). Indeed, because maintenance of VeraVec ECs does not require recombinant angiogenic factors (e.g., vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF2)), serum, or other xenobiotic factors, these ECs can be used in coculture models to screen and to identify the instructive vascular niche-like functions and angiocrine factors supporting the expansion of organ-specific stem and progenitor cells (Butler et al., 2010b; Ding et al., 2010, 2011, 2014) and possibly tumor cells. To reveal the angiocrine influence of ECs on LCs, we compared expansion of $B220^+CD19^+$ LCs isolated from *Eμ-Myc* mice in three conditions: in serum-containing medium (LC^{Serum}), in serum- and growth factor-free medium (LC), or in serum- and growth factor-free medium with cocultured ECs (LC^{EC}). We found that serum-free coculture of LCs with ECs supported greater LC proliferation than serum alone (Figures 1A and 1B; Figures S1A and S1B available online). Subcutaneous coinjection of LCs with ECs into immunodeficient NOD-SCID-IL2R$\gamma^{-/-}$ (NSG) mice significantly enhanced tumor growth, compared with LCs injected alone (Figure S1C). The growth rate of LC^{EC} in wild-type (WT) C57/B6 mice was significantly higher than LC^{Serum} early after subcutaneous injection (Figures S1D and S1E). We then assessed the serial methylcellulose colony formation (MCF) capacity of expanded LCs. LC^{EC} had 5-fold greater MCF potential than did LC^{Serum} (Figures 1C and 1D). Limiting dilution transplantation into NSG mice showed that LC^{EC} cells contained more lethal LCs than did LC^{Serum} cells (Figure 1E). Thus, ECs establish an inductive vascular niche that enforces outgrowth of aggressive LCs.

We then evaluated whether ECs could confer chemotherapy resistance to LCs by treating LC^{EC} and LC^{Serum} with doxorubicin. Indeed, LC^{EC} were less sensitive to doxorubicin than LC^{Serum} in vitro (Figures 1F and 1G). Doxorubicin prolonged survival of NSG mice transplanted with LC^{Serum} but not mice transplanted with LC^{EC} (Figure 1H). Thus, coculture with ECs promoted a chemoresistant phenotype in the $B220^+CD19^+Myc^+$ B cell LCs.

To investigate the mechanism underlying the aggressiveness of LC^{EC}, we profiled the transcriptome of LC^{EC}. Coculture with ECs induced transcripts characteristic of LICs, including CD44, IGF1R, and CSF1R (Hanahan and Weinberg, 2011; Medyouf et al., 2011) (Figure 1I; Table S1). Flow cytometry analysis and quantitative PCR (qPCR) confirmed that EC coculture-mediated upregulation of corresponding proteins was due to generation of a $CD44^+IGF1R^+CSF1R^+$ LC subset (Figures 1J, S1F, and S1G). To assess the functional activity of the $CD44^+IGF1R^+CSF1R^+$ subpopulation, we obtained clonally derived LCs by serial dilution in coculture with ECs (Figure 1K) and compared their activity to $CD44^-IGF1R^-CSF1R^-$ LCs. The $CD44^+IGF1R^+CSF1R^+$ LCs yielded more serial methylcellulose colonies, caused higher lethality in limiting dilution transplantation, and were less sensitive to doxorubicin than $CD44^-IGF1R^-CSF1R^-$ LCs (Figures 1L–1O and S1H–S1J). Thus, EC coculture enabled outgrowth of more aggressive and chemoresistant $CD44^+IGF1R^+CSF1R^+$ LCs.

ECs Support LCs via Jag1-Dependent Juxtacrine Activation of Notch2 Pathway

We next investigated the mechanism by which ECs stimulate aggressiveness in LCs. Transcription profiling showed upregulation of the Notch downstream transcriptional effector Hey1 in LC^{EC} (Figure 1I). qPCR confirmed specific upregulation of Hey1 in LC^{EC} (Figure 2A) and in the aggressive $CD44^+IGF1R^+CSF1R^+$ LC subpopulation (Figures S2A– S2C). To test whether Hey1 upregulation confers aggressive LC features, we studied how loss and gain of function of Notch1, Notch2, and Hey1 in LCs altered their expansion (Figures 2B and S2D–S2F). Genetic silencing using small hairpin RNA (shRNA) to Notch2 (shNotch2) or Hey1 (shHey1), but not Notch1 (shNotch1), abrogated EC-driven expansion of LCs. In contrast, Hey1 overexpression phenocopied the effect of EC coculture (Figures 2C, 2D, S2G, and S2H). Notch pathway inhibition using the γ-secretase inhibitor compound E similarly abrogated LC growth after coculture with ECs. Immunoblot and immunostaining for Notch intracellular domains and chromatin immunoprecipitation (ChIP) of

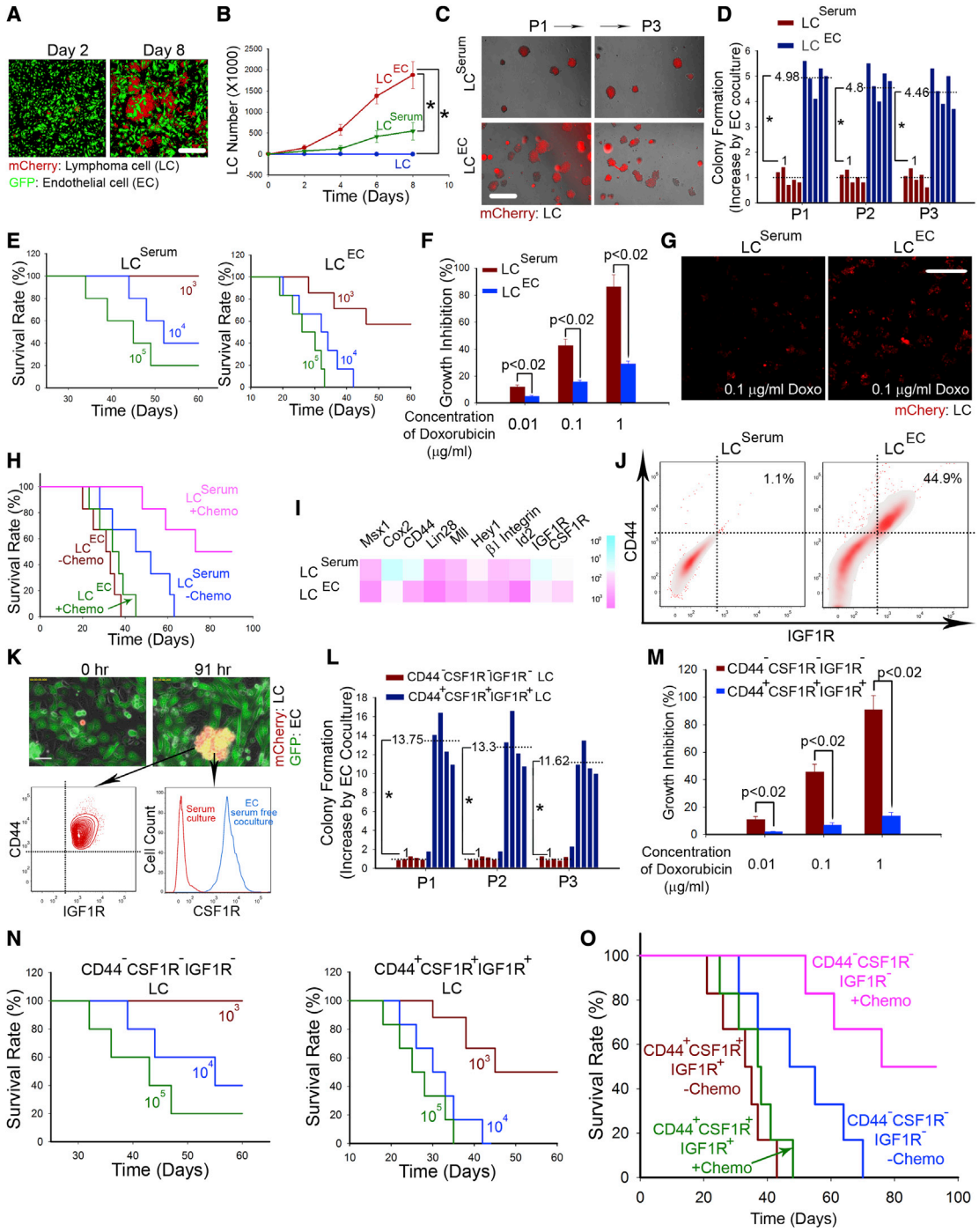

Figure 1. Expansion of *Myc*$^+$ LCs with Aggressive LIC Features after Coculture with ECs in Serum and Cytokine-free Conditions

(A) Representative images of *Eμ-Myc* mouse LCs cultured with EC. Scale bar, 200 μm.

(B) Quantification of LC number of *Eμ-Myc* mouse LCs cultured in the absence of ECs (LC), with EC (LC^{EC}), or with serum supplementation (LC^{Serum}). *p < 0.02; n = 5. All data are presented as means ± SEM throughout.

(C and D) Representative image (C) and the colony number (D) of colony-forming capacity of LCs after serial passage. Five clones were passed every step. *p < 0.02. Scale bar, 500 μm.

(E) Survival curve of NSG mice i.p. transplanted with the indicated numbers of LCs; n = 6–8.

(F and G) Quantification (F) and representative image (G) of proliferation of LCs after treatment of indicated concentrations of doxorubicin; n = 5. Scale bar, 1,000 μm.

(H) Survival of NSG mice inoculated with 1 × 10^5 indicated LCs and treated or not treated with doxorubicin (chemo) as indicated; n = 6–8.

(I) Heat map presenting the expression level of indicated transcripts in LC^{EC} and LC^{Serum}.

(legend continued on next page)

RBPJ showed selective activation of Notch2, but not Notch1, in LC^{EC} (Figures 2E, 2G, S2I, and S2J). Therefore, coculture with ECs selectively activates Notch2 in LCs, resulting in Hey1-dependent expansion of LCs.

Next, we tested how disruption of Notch signaling affected in vivo hepatic tumor seeding following intrasplenic injection (Ding et al., 2010) (Figure 2H). Knockdown of Notch2 or Hey1 or administration of compound E significantly reduced hepatic tumor load, but Notch1 knockdown had little effect (Figures 2I, 2-K, S2K, and S2L). Thus, juxtacrine activation of Notch2-Hey1 by ECs promotes extranodal invasion, a feature of aggressive lymphomas.

Although ECs express several Notch ligands, Jag1 was primarily upregulated by ECs after coculture with LCs (Figure 2L). This finding implicates EC Jag1 as the ligand activating Notch2 in LCs because LCs express negligible Jag1 (Figure S2M). To test this, we knocked down Jag1 in ECs (EC^{shJag1}) and cocultured these feeder cells with *Myc*$^+$ LCs expressing a Notch reporter (RBPJ-driven GFP). EC^{shJag1} were less effective than control ECs transduced with scrambled shRNA (EC^{Srb}) in supporting serum- and growth factor-free expansion of LCs and in inducing Hey1 upregulation and Notch activation (Figures 2M–2P). Notably, inhibition of the Notch2-Hey1 pathway in CD44$^+$ IGF1R$^+$ LCs after coculture with ECs had little effect on the aggressive feature (Figures S2N and S2O). Thus, propagation of aggressive LICs in vitro is driven by EC Jag1 expression.

EC-Specific Upregulation of Jag1 in Human Lymphoma Tissue Accompanies Propagation of Perivascularly Localized Aggressive LCs

To investigate whether human LCs upregulate Jag1 in ECs, we stained primary human Burkitt's lymphoma sections for Jag1 and Hey1. Jag1 was specifically expressed by tumor ECs but not by LCs in all tested lymphomas (Figures 3A and 3B; Figure S3A and Table S2). Notably, Hey1 was preferentially expressed in LCs adjacent to Jag1$^+$ tumor ECs (Figures 3A and 3C). We next investigated whether ECs foster propagation of human Burkitt's LCs that harbor a translocated MYC gene under the control of immunoglobulin heavy chain gene regulatory elements, as modeled by the *Eμ-Myc*$^+$ mouse. Coculture of CD19$^+$ human Burkitt's LCs with ECs triggered Notch2-mediated signaling in LCs and outgrowth of the CD44$^+$IGF1R$^+$ subpopulation (Figures 3D–3F and S3B–S3F). Expansion of human lymphoma colony-forming cells and hepatic lymphoma load was also promoted by coculture with ECs, compared with serum culture (Figures 3G–3J). Disruption of the Notch2-Hey1 pathway during coculture by knockdown of Jag1 in ECs (EC^{shJag1}) or Notch2 in LCs ($LC^{shNotch2}$) or use of compound E abrogated EC-dependent expansion, colony formation, and extranodal invasiveness of human LCs (Figures 3G–3N and S3G–S3J). Notably, after intrasplenic injection into NSG mice, human LC^{EC} induced Jag1 expression in the ECs surrounding the lymphoma nodules but not tumor-free hepatic regions (Figure 3O). Thus, expansion of invasive human Burkitt's LCs is also driven by instructive angiocrine factors supplied by the vascular niche.

LCs Induce FGFR1 Signaling in ECs to Prime a Jag1$^+$ Vascular Niche that Reciprocally Reinforces Lymphoma Propagation and Chemoresistance

We have found that during organ regeneration, activation of VEGF-A/VEGFR2 and FGF/FGF receptor 1 (FGFR1) signaling in ECs induces expression of angiocrine factors (Ding et al., 2010, 2011, 2014). Hence, we tested whether LCs might coopt these mechanisms to upregulate Jag1 in tumor ECs and form a malignant vascular niche. Both microarray expression and qPCR analysis showed that coculture with ECs upregulated FGF4 in mouse and human LCs (Figure 4A). Whereas normal human lymph nodes have negligible FGF4, we found significant expression of FGF4 protein in human Burkitt's lymphoma tissue and preferential activation of FGFR1 in the lymphoma-associated ECs (Figure S4A). To examine whether FGFR1-mediated signaling in ECs was necessary for Jag1 induction, we exposed control ECs and FGFR1-deficient ECs to serum-free or conditioned media (CM) derived from LCs. CM derived from mouse LC^{EC} activated Jag1 expression in ECs in a FGFR1-dependent manner (Figures 4B and 4C). Notably, during EC-LC coculture, shRNA knockdown of *FGFR1* in ECs or of *FGF4* in LCs blocked Jag1 induction and FGFR1 signaling in ECs (Figures 4D and S4B–S4D). Importantly, after intrasplenic injection, *FGF4*-deficient human LCs failed to upregulate Jag1 in the ECs of liver lymphoma nodules of recipient mice (Figure 4E). Therefore, LCs supply FGF4 to activate FGFR1 on ECs to reinforce Jag1--mediated vascular niche function, driving Notch2-dependent expansion of aggressive LCs.

To determine whether this "feed-forward" loop drives lymphoma tumorigenesis in vivo, we conditionally deleted Fgfr1 specifically in adult ECs by crossing VE-cadherin-Cre^{ERT2} mice with *Fgfr1*$^{loxP/loxP}$ mice (Figure 4F) and using tamoxifen to delete *Fgfr1* specifically in ECs (*Fgfr1*$^{i\Delta EC/i\Delta EC}$) (Figures S4E and S4F). To control for Cre-mediated toxicity, we used EC-specific haplodeficient *Fgfr1*$^{i\Delta EC/+}$ mice as control mice. We used a murine B6RV2 lymphoma transplantation model to examine the lymphoma growth. *Fgfr1*$^{i\Delta EC/i\Delta EC}$ mice, but not *Fgfr1*$^{i\Delta EC/+}$ mice, were inhospitable to subcutaneously and intrasplenically injected B6RV2 LCs, resulting in reduced tumor growth and hepatic colonization (Figures 4G–4K). Therefore, activation of FGFR1 in ECs is required for LCs to establish a protumorigenic vascular niche.

We next crossed *Eμ-Myc* mice with *Fgfr1*$^{i\Delta EC/i\Delta EC}$ and control mice (Figure 5A) to assess the role of FGFR1 in establishing a

(J) Representative flow cytometry graph of CD44 and IGF1R expression in LCs.
(K) Representative time-lapse microscopy image of single colony expansion of LC^{EC} (top panel) and flow cytometry analysis of the clonally derived CD44$^+$ IGF1R$^+$CSF1R$^+$ LCs (bottom panel). Scale bar, 25 μm.
(L and M) Serial colony forming ability (L) and growth inhibition after doxorubicin treatment (M) comparing CD44$^+$IGF1R$^+$CSF1R$^+$ and CD44$^-$IGF1R$^-$CSF1R$^-$ LCs. *$p < 0.025$; $n = 4$.
(N) Tumorigenicity of indicated LCs was compared by limiting dilution transplantation into NSG mice; $n = 6$ and 10 in CD44$^+$IGF1R$^+$CSF1R$^+$ and CD44$^-$IGF1R$^-$CSF1R$^-$ LC-injected groups, respectively.
(O) Survival of NSG mice implanted with 10^5 indicated LCs and then treated with or without 50 mg/kg doxorubicin; $n = 6$–10.
See also Figure S1 and Table S1.

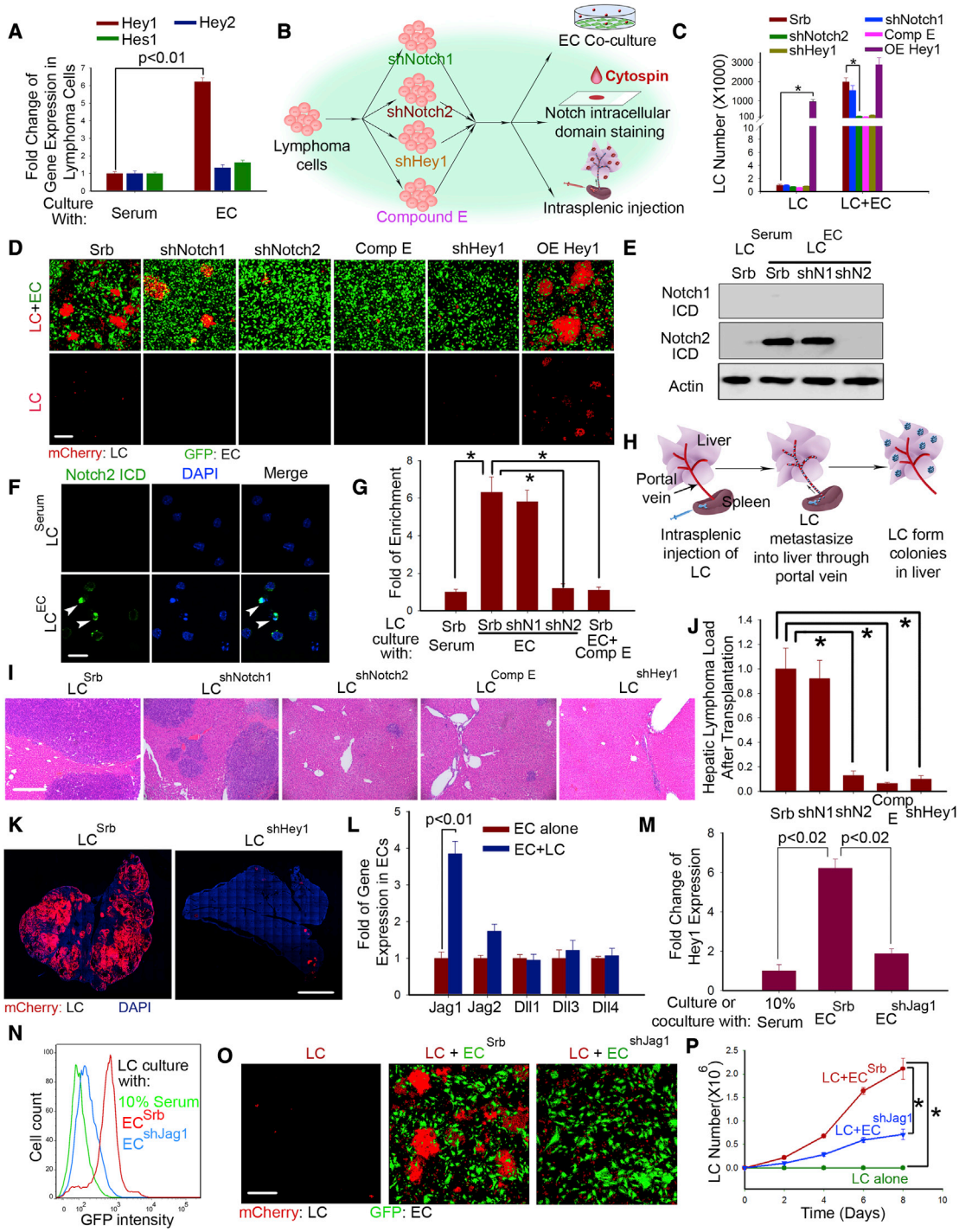

Figure 2. Angiocrine Effects of Endothelial Jag1 on Notch2 Activation and Propagation of *Myc*$^+$ Mouse LCs

(A) Expression level of Notch pathway effecter Hey1, Hes1, and Hey2 in LCs.

(B) Approaches to define Notch pathway activation in mouse LCs. Notch1, Notch2, and Hey1 in LCs were silenced by shRNA (shNotch1, shNotch2, and shHey1), and Notch pathway was blocked by compound E. Cell expansion, Notch activation, colony formation, and hepatic invasiveness were then compared.

(C and D) Quantification (C) and representative image (D) of expansion of LCs cocultured with ECs (LC+EC) or cultured in serum-free medium (LC). Srb, scrambled shRNA; OE Hey1, overexpression of Hey1; Comp E, compound E; n = 4. Scale bar, 25 μm.

(E and F) Notch1 and Notch2 intracellular domains (ICDs) were detected in LCs by immunoblot (E), and Notch2 ICD in LCs was examined by immunostaining (F). White arrowheads indicate nuclear Notch2 ICD. Scale bar, 10 μm.

(G) ChIP analysis of RBPJ activity in LCs after Notch inhibition. shN1 and shN2 denote shRNA against Notch1 and Notch2, respectively; n = 4.

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malignant vascular niche. Jag1 was preferentially expressed by VE-cadherin$^+$ ECs within the lymphoma of control (*Myc*$^+$*Fgfr1*$^{i\Delta EC/+}$) mice, but not by ECs of *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ lymphomas (Figure 5B). Complementary expression of Hey1 was seen in the perivascular LCs of control but not *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ mice. As a result, *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ mice survived longer than *Myc*$^+$*Fgfr1*$^{i\Delta EC/+}$ controls, owing to reduced tumor cell proliferation (Figures 5C–5I).

Because ECs promoted the expansion of chemoresistant LCs, we investigated whether EC-specific deletion of *Fgfr1* sensitized *Eμ-Myc* lymphomas to doxorubicin treatment. Survival of *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ mice, but not *Fgfr1*$^{i\Delta EC/+}$ mice, was prolonged by chemotherapy (Figure 5I). LCs treated with doxorubicin upregulated FGF4 expression, enabling a vascular niche that confers chemoresistance to LCs (Figure 5J). Hence, FGFR1-mediated signaling deploys Jag1 in tumor ECs, establishing a protumorigenic vascular niche that shelters resident LCs from chemotherapy-induced cytotoxicity.

Endothelial Jag1 in Tumor Capillaries Subverts Indolent LCs to Manifest Aggressive Features

To investigate whether enhanced chemoresistance and invasiveness of LCs upon being cocultured with Jag1$^+$ ECs were due to selective enrichment of an aggressive LC subclone or bestowing aggressive attributes to indolent LCs by tumorigenic vascular niche, we cocultured clonally derived CD44$^-$IGF1R$^-$CSF1R$^-$ LCs (triple-negative LCs) with ECs (Figure 6A). After coculture with ECs, the triple-negative LC progeny acquired ~14-fold greater MCF capacity (Figures 6B and 6C). Immunophenotypic analysis showed that 9.5% of LCs from the single triple-negative clone are CD44$^+$IGF1R$^+$ after being cocultured with EC (Figure 6D). Notably, after EC coculture, triple-negative LCs were more lethal to NSG mice following intraperitoneal (i.p.) injection (Figure 6E). All of these effects were attenuated by coculture with Jag1-deficient ECs (Figures 6B–6E). Therefore, the aggressive lymphoma tumor phenotype is conferred upon LCs by angiocrine signals emanating from a tumorigenic vascular microenvironment.

We then assessed tumorigenicity and extranodal invasiveness of LCs in *Jag1*$^{i\Delta EC/+}$ control and *Jag1*$^{i\Delta EC/i\Delta EC}$ mice (Figure 6F). LCs transplanted into *Jag1*$^{i\Delta EC/i\Delta EC}$ mice yielded smaller tumors than did the same LCs inoculated into *Jag1*$^{i\Delta EC/+}$ mice (Figures 6G and 6H; Figure S5A). To test whether host EC Jag1 altered CD44$^-$IGF1R$^-$CSF1R$^-$ LCs in vivo, individual triple-negative LC clones were injected subcutaneously into *Jag1*$^{i\Delta EC/i\Delta EC}$, *Jag1*$^{i\Delta EC/+}$ (Figure 6I), or WT mice (Figure S5B). Following expansion in the recipient mice, transplanted triple-negative LCs acquired over 6-fold greater triple-positive immunophenotype and ~5-fold higher MCF in *Jag1*$^{i\Delta EC/+}$ mice than in *Jag1*$^{i\Delta EC/i\Delta EC}$ mice (Figure 6J). Therefore, host EC-derived Jag1 subverts indolent LCs to manifest aggressive phenotypes.

Deletion of *Jag1* in ECs of *Eμ-Myc* Mice (*Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$) Abolishes de Novo Generation of Aggressive LCs

To assess the lymphoma-promoting role of EC Jag1, we selectively deleted *Jag1* in ECs of *Eμ-Myc* mice (*Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$), using *Myc*$^+$*Jag1*$^{i\Delta EC/+}$ as controls. We then isolated nodal B cell LCs and assayed for their aggressive attributes (Figure 7A). During serial methylcellulose culture, LCs from *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice had fewer CD44$^+$IGF1R$^+$ LCs and yielded substantially fewer colonies than LCs from control mice (Figures 7B–7D). LCs derived from *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice were less capable in killing the recipient mice and were more sensitive to doxorubicin compared with LCs isolated from *Myc*$^+$*Jag1*$^{i\Delta EC/+}$ mice (Figures 7E and 7F). LCs from *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice also gave rise to fewer and smaller hepatic tumors after intrasplenic seeding (Figures 7G and 7H). Therefore, the protumorigenic Jag1$^+$ vascular niche endows aggressive features to LCs, driving lymphomagenesis, extranodal invasiveness, and chemoresistance.

Vascular Niche-Derived Jag1 Confers Notch-Dependent Chemoresistance to Myc$^+$ LCs

To unravel the role of EC-derived Jag1 in lymphoma pathogenesis and development of chemoresistance, we administered doxorubicin to *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ and control mice (Figures 8A and 8B). Notch activity was tracked in LCs by crossing *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice with transgenic Notch reporter (TNR) mice expressing GFP upon Notch activation (Butler et al., 2010b). Genetic ablation of Jag1 in ECs reduced tumor load and improved survival of *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice, compared with control *Myc*$^+$*Jag1*$^{i\Delta EC/+}$ mice (Figure 8B; Figure S6A). Moreover, LCs from *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice were more sensitive to chemotherapy, manifesting as enhanced survival (64% after 8 months) compared with a median survival of ~150 days in controls. Notch was activated in LCs positioned adjacent to VE-cadherin$^+$ ECs in control mice, but not in *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice (Figures 8C and 8D), indicating that Jag1 supplied by ECs activate Notch signaling in LCs. The enhanced survival of *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice was associated with greater LC death (Figures 8E and 8F). Notably, when EC Jag1 is intact, a subset of perivascularly localized LCs with activated Notch signaling (reported by TNR-GFP cells) was protected from doxorubicin-induced cell death. Therefore, Jag1-expressing ECs establish a chemoresistant microenvironment for LCs via juxtacrine Notch activation (Figure 8G).

To assess whether aberrant EC Jag1 expression could cause vascular abnormalities that might compromise tumor blood supply, we examined vascular perfusion in *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice by intravenously injecting B4-isolectin to label patent ECs. The majority of VE-cadherin$^+$ ECs within the lymphoma were recognized by B4-isolectin. Furthermore, tissue staining using the hypoxia marker pimonidazole showed little hypoxia

(H–K) Aggressive features of LCs were tested after disruption of Notch2-Hey1 pathway. Extranodal invasiveness of LCs was examined by intrasplenic injection into NSG mice (H). Hepatic tumor load was examined by H&E staining (I) and fluorescent microscopy (K). Quantification of hepatic tumor load is shown in (J); n = 4. Scale bars, 100 μm in (I) and 1 mm in (K).

(L) Expression of Notch ligands in feeder ECs after coculture with LCs (EC+LC); n = 4.

(M and N) LCs were cocultured with ECs transduced with Scrambled shRNA (EC^{Srb}) and *Jag1* shRNA (EC^{shJag1}). Notch activation in LCs was tested by Hey1 upregulation (M) and fluorescent intensity of RBPJ-driven GFP reporter in LCs (N); n = 5.

(O and P) Representative image (O) and expansion (P) of LCs cultured alone or cocultured with EC^{ShJag1} or EC^{Srb}. *p < 0.025; n = 5. Scale bar, 50 μm.

See also Figure S2.

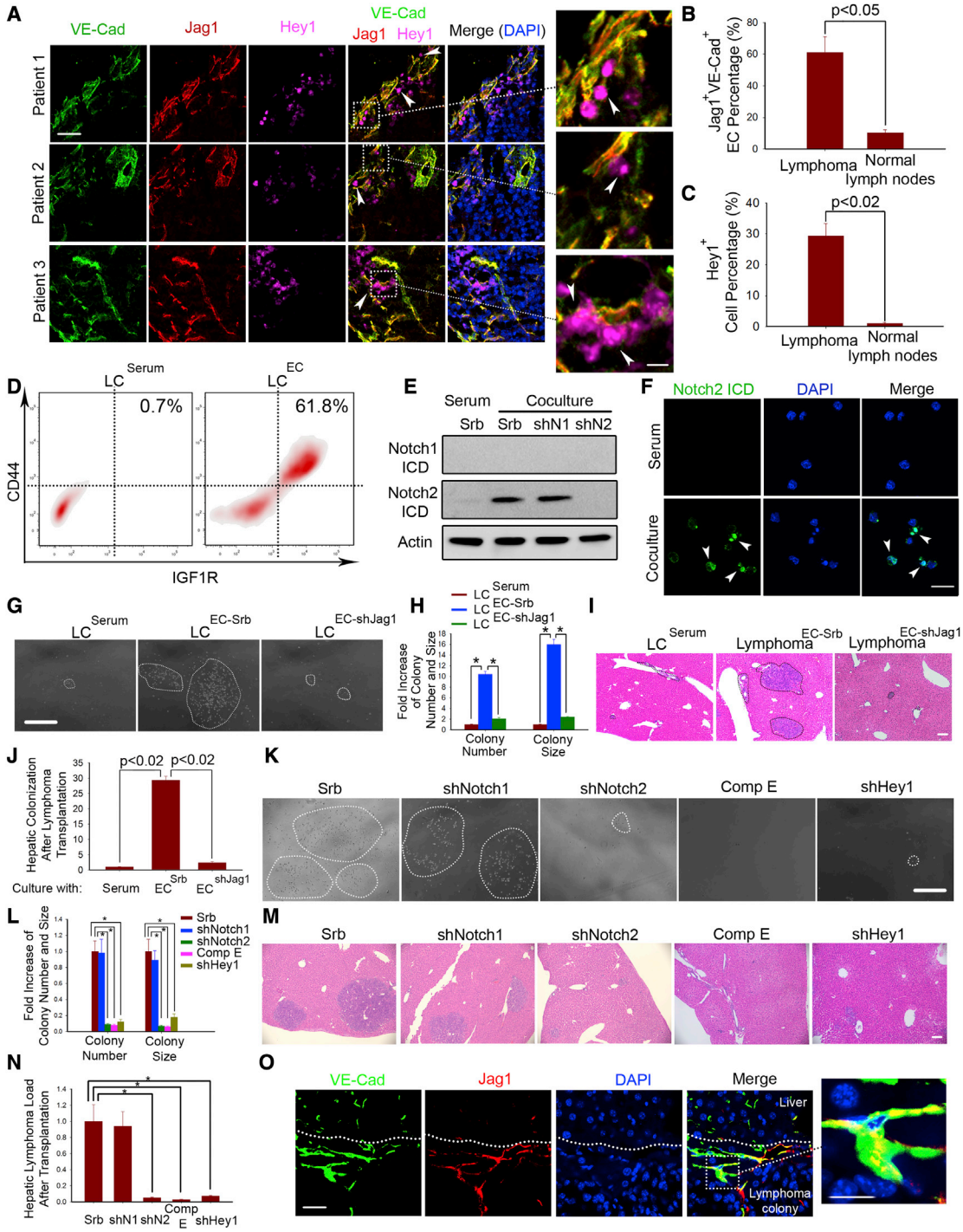

Figure 3. Influence of Angiocrine Jag1 on Expansion and Aggressive Features of Human B Cell LCs

(A–C) Expression of Hey1 and Jag1 in patient Burkitt's lymphoma with *MYC* translocation were examined. VE-cadherin (VE-Cad) was stained to identify ECs (A). Quantification of Jag1 expression in lymphoma ECs (B) and Hey1 in perivascular LCs (C) is shown. Scale bars, 100 μm and 20 μm in inset.

(D) CD44 and IGF1R expression on human LCs cultured with ECs (LC^{EC}) or with serum (LC^{Serum}).

(E and F) Immunoblot analysis (E) and immunostaining (F) analysis of Notch ICD in LC cultured with serum or with indicated ECs. Srb, Scrambled. Scale bar, 25 μm.

(G and H) Representative image (G) and quantification (H) of colony formation capacity of human LC^{Serum} and LCs cocultured with ECs transduced with Scrambled ($LC^{EC\text{-}srb}$) or *Jag1* shRNA ($LC^{EC\text{-}shJag1}$); n = 4. Scale bar, 100 μm.

(I and J) Representative image of hepatic lymphoma (I) and quantification of tumor colony number burden (J) of human $LC^{EC\text{-}srb}$ or $LC^{EC\text{-}shJag1}$ intrasplenically injected into NSG mice. LC^{Serum} were also injected for comparison. Scale bar, 50 μm.

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in the lymphoma mass of *Myc*$^{+}$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice (Figures S6B–S6D). Therefore, vascular blood supply and oxygen delivery were not compromised in *Myc*$^{+}$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice. Taken together, we employed a variety of mouse/human lymphoma models to demonstrate that angiocrine expression of Jag1 activates lymphoma Notch to promote tumorigenicity without affecting the passive perfusion function of tumor vasculature.

DISCUSSION

Tumor-initiating cells are believed to acquire aggressive phenotypes via cell-autonomous mechanisms. Here, we challenge this paradigm and demonstrate that in certain lymphomas, the protumorigenic vascular niche dictates tumor aggressiveness. We show that tumor ECs convert indolent CD44^{-}IGF1R^{-}CSF1R^{-} LCs to more aggressive CD44^{+}IGF1R^{+}CSF1R^{+} LIC-like cells manifesting greater tumorigenicity, extranodal invasion, and chemoresistance. The majority of these aggressive tumor features were dependent upon FGF4-driven Jag1 expression by tumor ECs. Disruption of this juxtacrine/angiocrine loop at any level—FGF4, FGFR1, Jag1, or Notch2—severely diminished the aggressiveness of both human and murine lymphomas. Thus, *Eμ-Myc*-driven oncogenesis is not sufficient to provoke aggressive lymphoma behaviors. The LC phenotype is plastic and is determined by cues emanating from the malignant vascular niche rather than by cell-autonomous signaling pathways alone.

The properties of the malignant vascular niche are co-opted via activation of FGF4/FGFR1 signaling in tumor ECs. This signaling induces host ECs to express Jag1 in proximity to neighboring LCs. Importantly, the FGFR1-Jag1 feed-forward signaling loop promotes LC chemoresistance and is further reinforced by chemotherapy administration. In this way, angiocrine Jag1 functionalizes a chemoresistance niche that activates Notch signaling in perivascular LCs and spares them from chemotherapeutics such as doxorubicin. These results suggest a paradigm of tumor propagation whereby a dynamic neoplasm-primed tumor microenvironment shelters tumor cells from chemotherapy and instructively directs them to grow locally and invade distal organs. Thus, targeting the malignant vascular niche should sensitize LCs to chemotherapy and improve outcomes.

Indeed, EC-specific deletion of *Fgfr1* or *Jag1* in the *Fgfr1*$^{i\Delta EC/i\Delta EC}$ and *Jag1*$^{i\Delta EC/i\Delta EC}$ mice enhanced chemosensitivity and improved mouse survival by abolishing Notch activation in *Myc*$^{+}$ LCs after doxorubicin treatment. Similarly, in human lymphoma specimens, we found Jag1 upregulated in tumor ECs and the Notch pathway activated in perivascular human LCs, as originally observed in the murine lymphoma models. These findings highlight the functional interplay between ECs and tumor cells that depends on the FGF-Notch paracrine/juxtacrine loop. Upregulation of endothelial Jag1 is central to this loop because it endows both mouse and human LCs with aggressive LC features. Similarly, as-yet unrecognized and distinct angiocrine pathways are likely activated within the microenvironment of other tumor types.

Blockade of Jag1 expressed by tumor ECs reduced lymphoma progression without compromising perfusion of the tumor vasculature. Jag1 appeared dispensable for most homeostatic vascular functions because deletion of endothelial Jag1 in adult mice caused no excess mortality or morbidity. Thus, inhibiting the instructive angiocrine signals from a tumor-primed vascular niche can effectively target aggressive LC features. Anti-angiocrine therapeutics need not interfere with tumor perfusion and therefore should not be compromised by tumor hypoxia and rebound angiogenesis that can lead to paradoxical tumor growth (Ebos et al., 2009; Pàez-Ribes et al., 2009).

Comparative analyses of human and mouse lymphoma tissues suggest that our findings may have clinical relevance. Jag1 is upregulated in ECs in human lymphoma specimens. Jag1 upregulation in ECs endows both mouse and human LCs with aggressive LC features. Whether induced expression of Jag1 and activation of Notch pathway in human tumor ECs may also portend poor prognosis is unknown and can only be determined in double-blind multicenter clinical studies. Based on our data, we speculate that patients harboring tumors with the capacity of inducing functional Notch ligands in tumor ECs may be at higher risk for tumor relapse and chemoresistance and be treated with more aggressive therapeutic protocols.

Taken together, our findings demonstrate that tumor cells prime a maladapted vascular niche that reciprocally confers tumors with aggressive, lethal properties: augmenting tumor growth, fostering chemoresistance, and promoting extranodal invasion. Indeed, function of lymphoma-propagating or -initiating cells depends on the protumorigenic state of the vascular niche and cannot be entirely attributed to cell-autonomous malignant properties of tumor cells. For example, an authentic tumor-initiating cell may fail to engraft host tissues with an inhospitable vascular niche, and assays used to identify tumor-initiating cells need to be modified to account for the activation state of vascular niche. Similarly, differences in host EC functions may underlie the tumor tropisms that select common metastatic sites. This study introduces promising therapeutic approaches to improve clinical outcomes for patients with aggressive lymphomas by ejecting LCs from the protumorigenic vascular niche to limit local tumor growth and extranodal invasion while sensitizing LCs to chemotherapy.

EXPERIMENTAL PROCEDURES

Transgenic Reporter and Gene-Targeted Animals

Jag1$^{loxP/loxP}$ mice were provided by Dr. Thomas Gridley (Jackson Laboratories), and *Fgfr1*$^{loxp/loxp}$ mice were obtained from Dr. Michael Simons (Yale University School of Medicine). Generation of inducible EC-specific *Fgfr1* knockout mice was carried out as described (Ding et al., 2010, 2011; Wang et al., 2010). In brief, *Fgfr1*$^{loxp/loxp}$ or *Jag1*$^{loxp/loxp}$ mice were bred with *VE-cadherin-Cre*^{ERT2} (*cdh5-PAC-Cre*^{ERT2}) transgenic mice and treated

(K–N) Inhibition of Notch1, Notch2, and Hey1 was performed in human LCs before EC coculture, and EC-dependent expansion (K and L) and hepatic tumor load of (M and N) of LCs were determined; n = 4. Scale bars, 1000 μm in (K) and 50 μm in (M).
(O) Jag1 expression in host ECs within the hepatic lymphoma nodule was assessed 14 days after intrasplenic injection of human LCs into NSG mice. Lymphoma mass in the liver is denoted by a dotted line. Scale bar, 50 μm (20 μm in inset).
See also Figure S3 and Table S2.

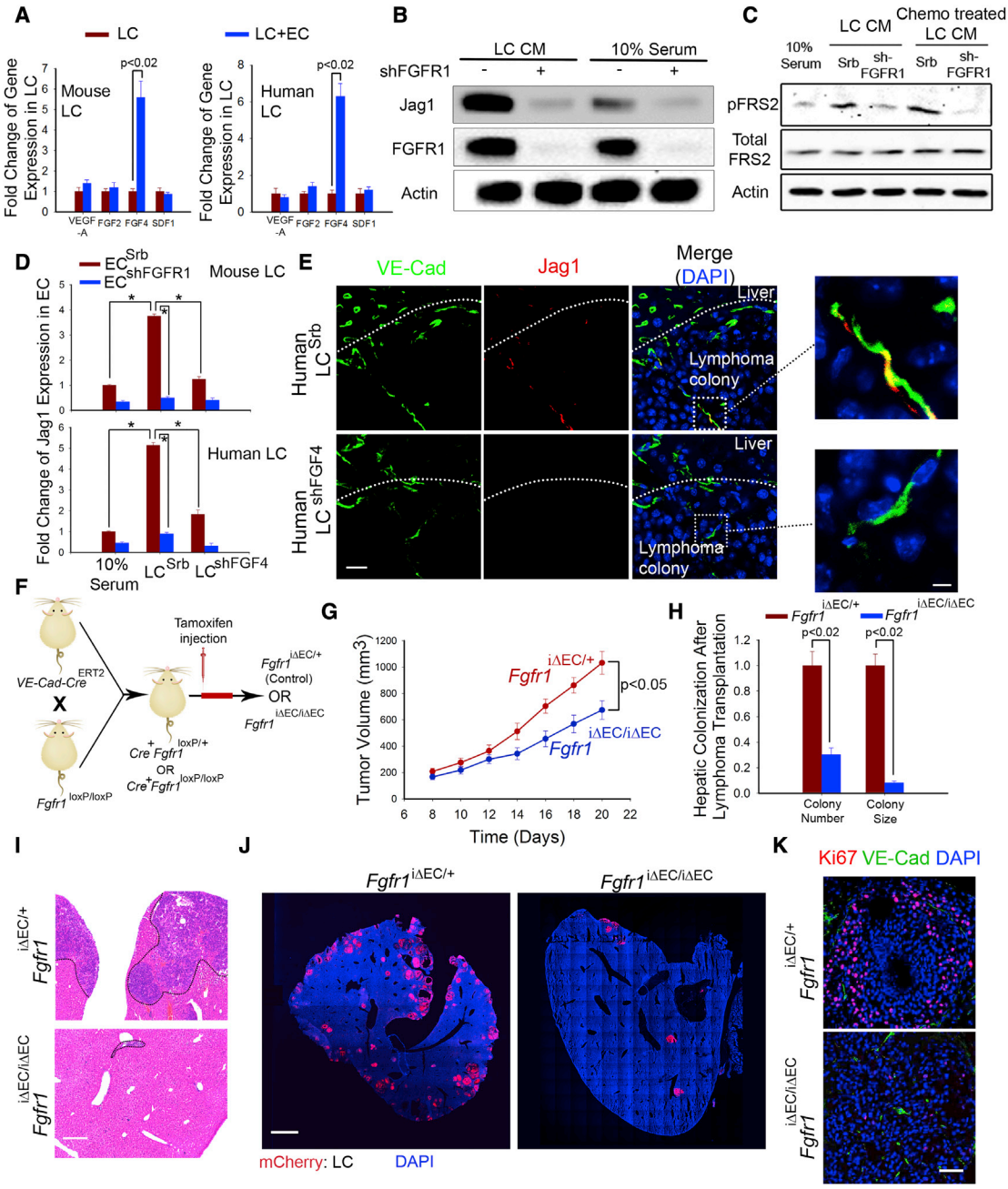

Figure 4. Reciprocal Instigatory Interactions between LCs and Cocultured ECs In Vitro and Host ECs In Vivo

(A) Expression of angiogenic factors VEGF-A, SDF1, FGF2, and FGF4 in mouse and human LCs cocultured with ECs (LC + EC) or in serum-containing medium (LC); n = 4.

(B–D) ECs transduced with *FGFR1* shRNA were stimulated with LC CM. LCs were transduced with Scrambled (LC^{Srb}) and *Fgf4* shRNA (LC^{shFGF4}). Jag1 and FGFR1 protein levels (B) and FGFR1 activation (as determined by phosphorylation of FRS-2) (C) in ECs were examined and quantified (D); n = 4.

(E) After human LCs were transplanted into NSG mice via intrasplenic injection, Jag1 induction in ECs associated with hepatic lymphoma nodule was determined. Lymphoma mass is delineated from normal tissue by a dotted line. Jag1 expression in lymphoma ECs is indicated in the inset. Scale bar, 100 μm and 20 μm in inset.

(F and G) Schematic representation of generating $Fgfr1^{i\Delta EC/i\Delta EC}$ and control $Fgfr1^{i\Delta EC/+}$ mice (F) and quantification of growth of subcutaneously injected B6RV2 mouse LCs in these mice (G).

(H–K) B6RV2 lymphoma in the liver was examined in $Fgfr1^{i\Delta EC/i\Delta EC}$ and control mice (H). Histological studies of hepatic tumor load were done with H&E staining (I) and fluorescent microscopy scan (J) of liver lobe 14 days after intrasplenic injection of LCs. Proliferation of LCs was determined by Ki67 staining (K). Scale bar, 50 μm in (I) and (K) and 1 mm in (J).

See also Figure S4.

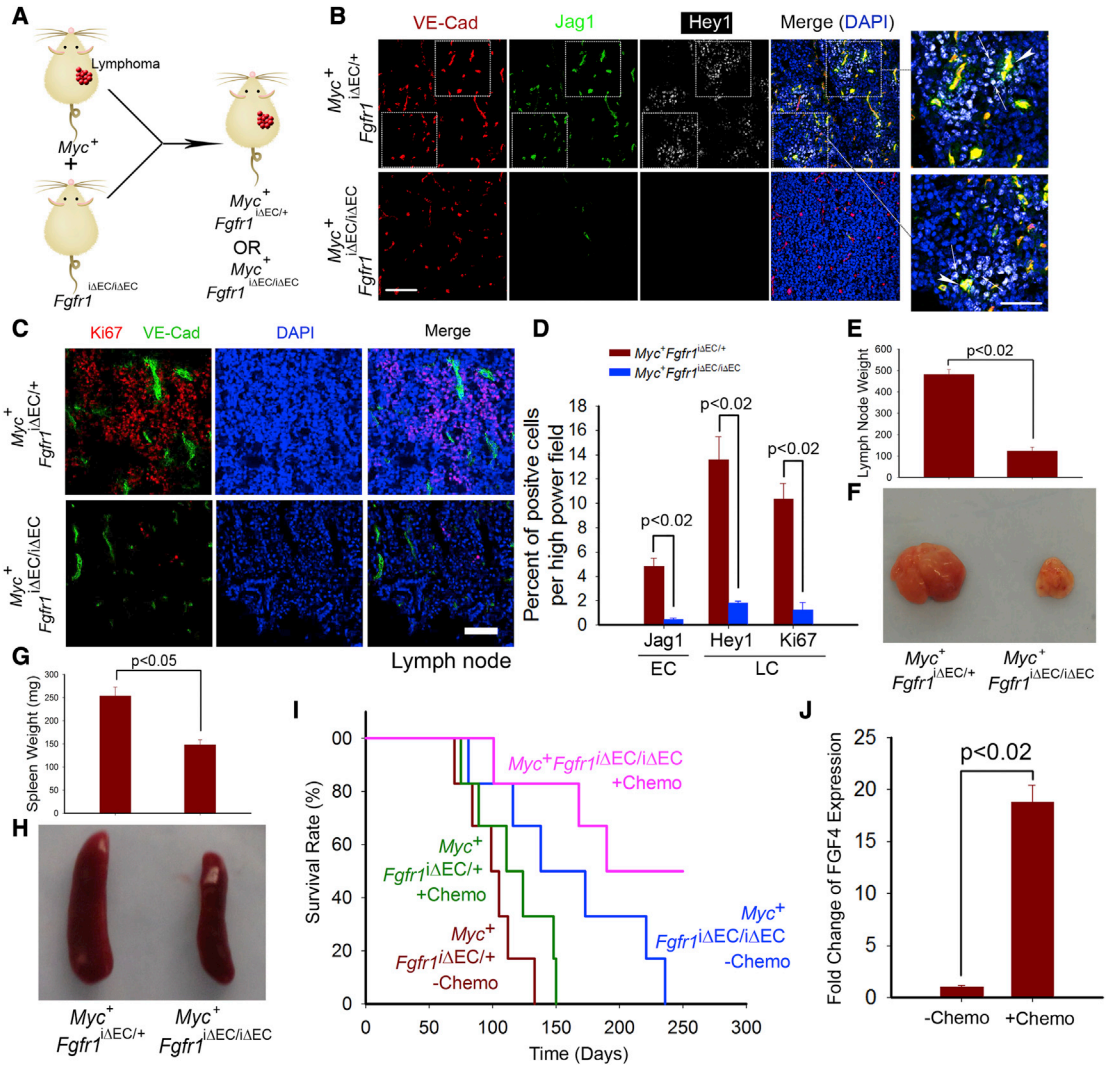

Figure 5. Lymphoma Propagation and Chemoresistance in *Eμ-Myc* Mice with EC-Specific Deletion of *Fgfr1*: *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$

(A) Generation of *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ and control *Myc*$^+$*Fgfr1*$^{i\Delta EC/+}$ mice.

(B–D) Expression of Jag1 and Hey1 in lymphoma tissue was determined in *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ and control mice (B). White arrow indicates expression of Jag1 on VE-cadherin$^+$ ECs. LC proliferation was tested by Ki67 staining (C). Quantification of Jag1 upregulation in ECs, Hey1, and proliferation marker Ki67 in LCs is presented in (D); n = 4. Scale bar, 50 μm in B (20 μm in inset).

(E–H) Weight (E and G) and representative images (F and H) of lymph node (E and F) and spleen (G and H) in *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ and control mice; n = 4.

(I) Survival of *Myc*$^+$*Fgfr1*$^{i\Delta EC/i\Delta EC}$ and control mice with or without treatment of 100 mg/kg of doxorubicin every week; n = 5–6.

(J) FGF4 mRNA expression in LCs after treatment of doxorubicin; n = 4.

with tamoxifen to induce EC-specific gene deletion (*Fgfr1*$^{i\Delta EC/i\Delta EC}$ or *Jag1*$^{i\Delta EC/i\Delta EC}$), as described in Supplemental Experimental Procedures. Lymphomagenesis was induced by transgenic Eμ-driven Myc. *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice were crossed with TNR mice in which the activation of the Notch pathway results in GFP expression. All animal experiments were carried out under the guidelines of the National Institutes of Health and approved by institutional animal care and use committee at Weill Cornell Medical College, using age/weight/genetic background-matched animals.

Human Burkitt's Lymphoma Tissues/Samples

Burkitt's lymphoma patient specimens were obtained from Weill Cornell Medical College. The procedure was approved by the institutional review board at Weill Cornell Medical College. Patient-related information is identified in Table S2. Human Burkitt's LCs without Epstein-Barr-virus infection were purchased from American Type Culture Collection (ATCC).

In Vitro Modeling of Vascular Niche for LC Coculture

To maintain EC survival in serum/growth factor-free conditions without confounding effects of supplementation with serum, bovine brain extracts and recombinant angiogenic factors (i.e., VEGF-A, FGF2, epidermal growth factors, PDGFs, and angiopoietins); primary freshly purified ECs, such as human umbilical vein ECs; or adult tissue-specific mouse ECs were transduced with E4ORF1 gene (VeraVec ECs; Angiocrine Bioscience). VeraVec ECs maintain their native vascular and microvascular attributes and produce physiological levels of tissue-specific angiocrine factors (Butler et al., 2010b, 2012; Nolan et al., 2013; Seandel et al., 2008). As such, VeraVec ECs establish a responsive unbiased vascular niche model to unequivocally interrogate the role of angiocrine factors in fostering the homeostasis of tumor cells and LICs. Both *Eμ-Myc* mouse LCs and human Burkitt's LCs (ATCC) harboring the c-MYC translocation were utilized for coculture with VeraVec ECs as described in

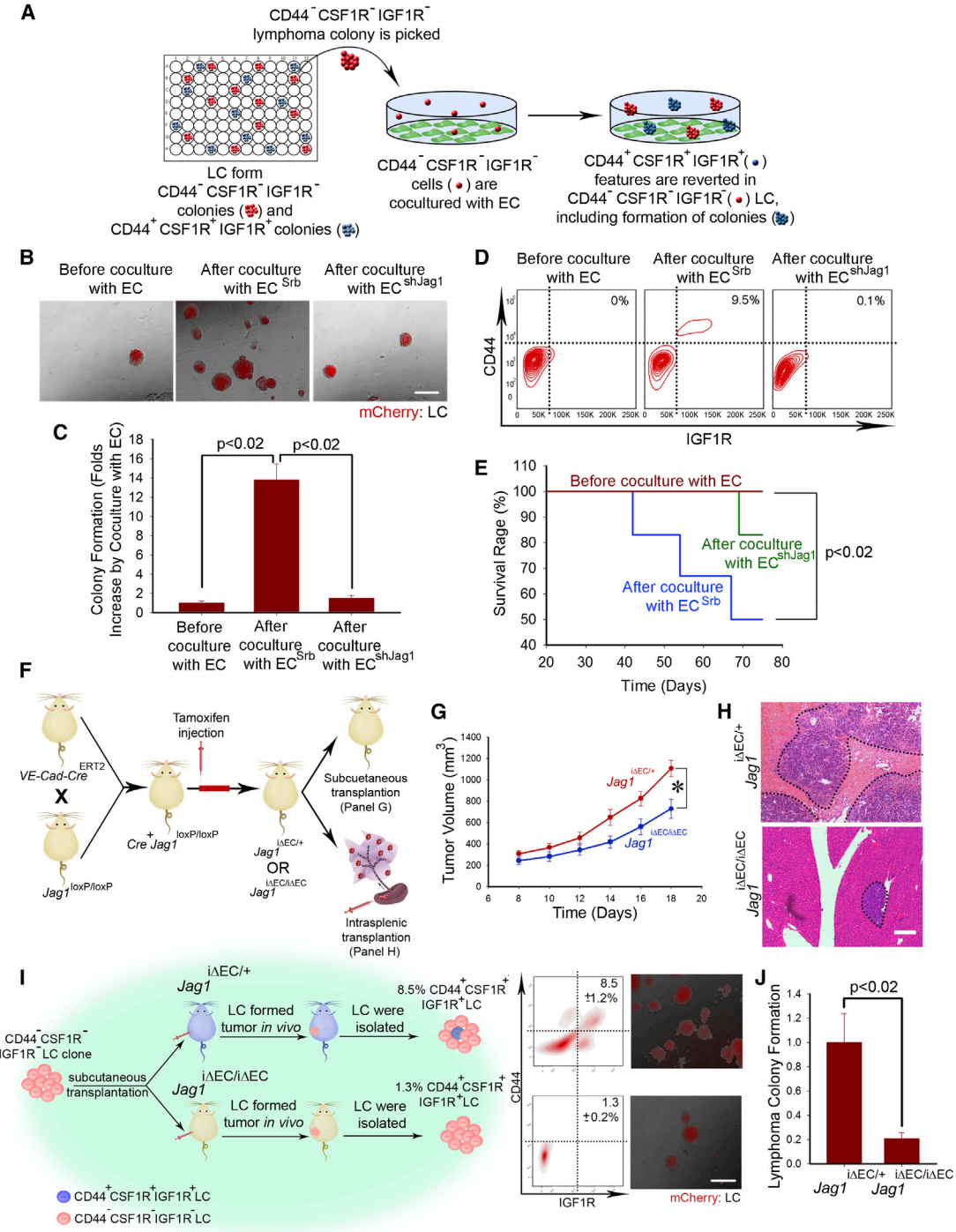

Figure 6. Acquisition of Aggressive LIC-like Features in LCs by Jag1-Expressing Vascular Niche

(A–E) Aggressive traits of CD44$^-$IGF1R$^-$CSF1R$^-$ indolent LC colonies after coculture with ECs was investigated (A). CD44$^-$IGF1R$^-$CSF1R$^-$ LC colonies were cocultured with EC^{Srb} and EC^{ShJag1} and tested for colony-forming capacity (B and C), CD44 and IGF1R expression (D), and lethality in NSG mice after injection of 5×10^3 indicated LCs (E).

(F) Different LC colonies were transplanted into mice with EC-specific deletion of *Jag1* (*Jag1*$^{iΔEC/iΔEC}$). *Jag1*$^{iΔEC/+}$ mice were used as control.

(G and H) Propagation of CD44$^+$IGF1R$^+$CSF1R$^+$ LCs in *Jag1*$^{iΔEC/iΔEC}$ and control mice was determined after subcutaneous (G) and intrasplenic transplantation. Representative H&E staining of liver is shown in (H); n = 4. Scale bar, 50 μm.

(I and J) Acquisition of aggressive LIC features in CD44$^-$IGF1R$^-$CSF1R$^-$ LCs after transplantation to control and *Jag1*$^{iΔEC/iΔEC}$ mice. LCs were isolated at day 28 after subcutaneous injection from enlarging tumor mass and analyzed for CD44, IGF1R, and CSF1R (I) and serial colony formation capacity (J). Each derived clone was injected into three mice.

See also Figure S5.

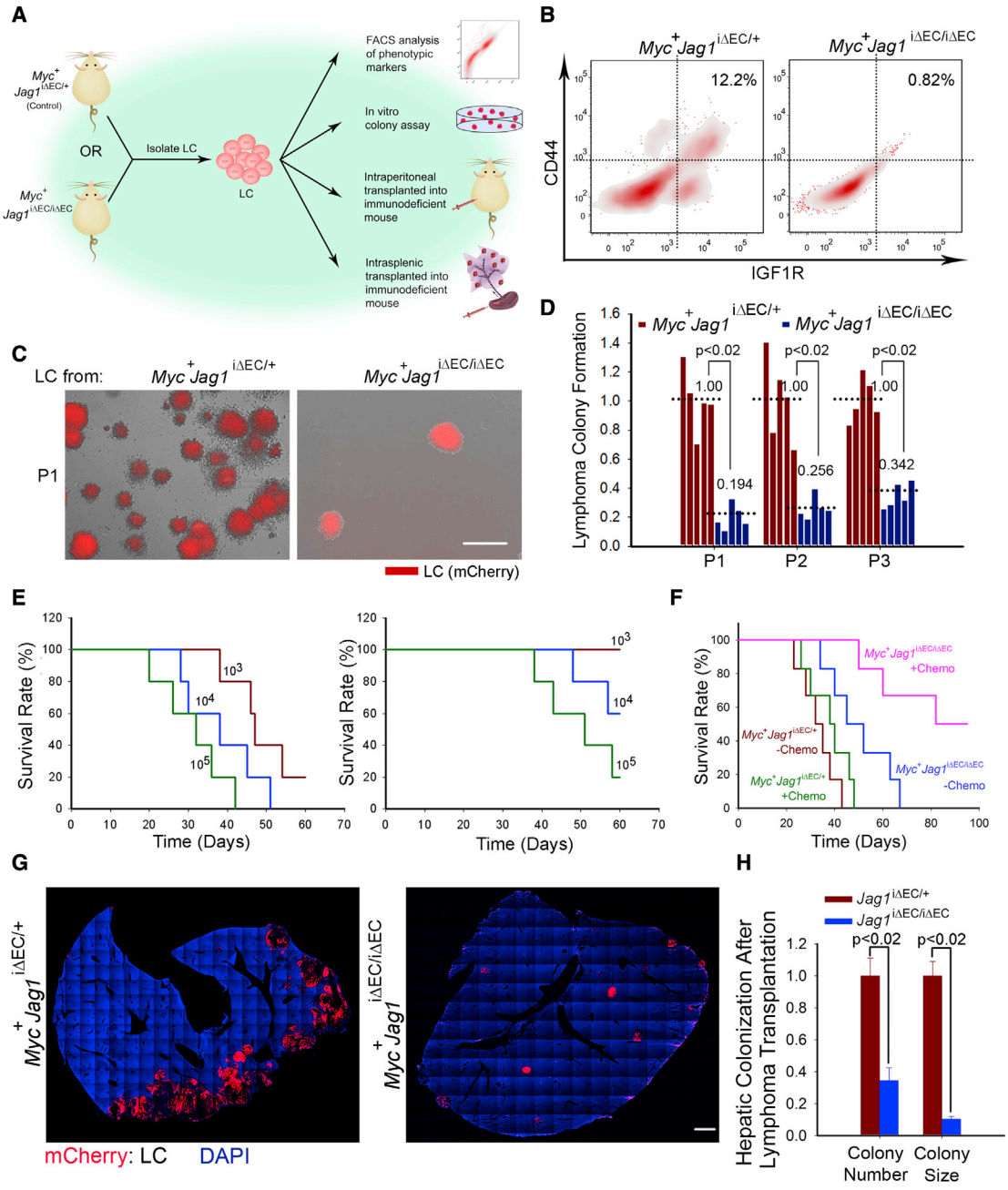

Figure 7. Generation of Invasive and Chemoresistant Triple-Positive LC Subpopulation in *Myc*$^+$ Mice with Conditional Deletion of *Jag1* in ECs: *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$

(A) Analysis of aggressive attributes of LCs from *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ and control *Myc*$^+$*Jag1*$^{i\Delta EC/+}$ mice. FACS, fluorescence-activated cell sorting.

(B) Percentage of CD44$^+$IGF1R$^+$ LC subset in *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ and control mice.

(C and D) Colony-forming capacity of LCs. Five clones isolated from *Myc*$^+$ mice were picked for each passage. Representative images (C) and quantification of colony (D) are shown. Scale bar, 500 μm.

(E) Lethality of LCs from control (left) and *Myc*$^+$*Jag1*$^{i\Delta EC/i\Delta EC}$ (right) mice after limiting dilution transplantation into NSG mice.

(F) Survival of NSG mice injected with 10^5 indicated LCs with or without treatment of 50 mg/kg doxorubicin; n = 5–8.

(G and H) Representative image (G) and quantification of tumor colony number (H) of hepatic tumor in NSG mice after injection of LCs. Scale bar, 1mm.

Supplemental Experimental Procedures. For simplicity, we refer to VeraVec ECs as ECs.

To investigate the angiocrine contribution of vascular niche to aggressiveness of LCs/LICs, *Jag1* shRNA or scrambled shRNA (Open Biosystems) was used to knock down Jag1 in ECs. Experimental procedures of shRNA knockdown of Notch pathway (Notch1, Notch2, and Hey1) in LCs and Notch ligand Jag1 in ECs are described in Supplemental Experimental Procedures.

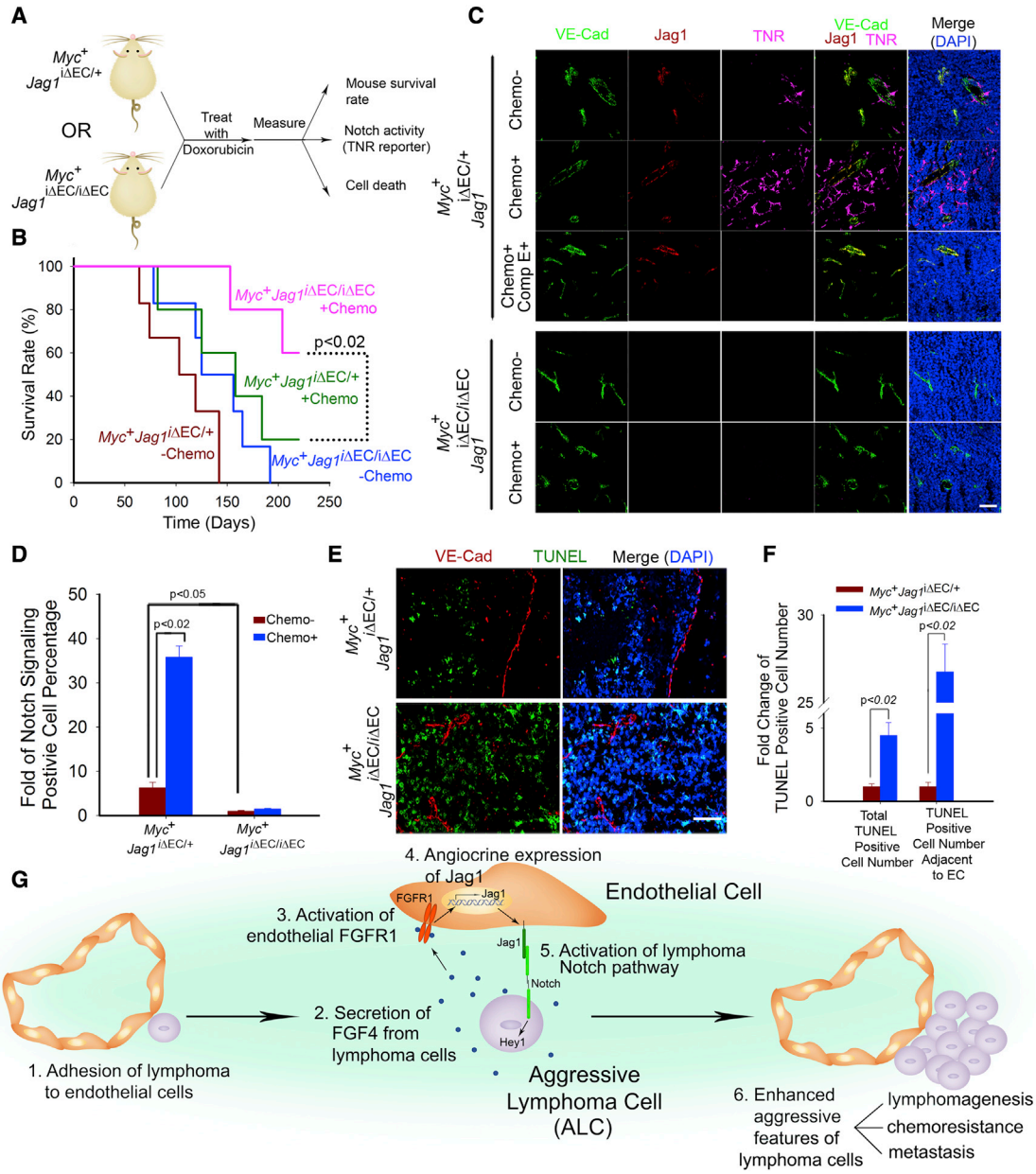

Figure 8. Chemoresistance of Lymphoma in *Myc*$^{+}$*Jag1*$^{i\Delta EC/i\Delta EC}$ and Control *Myc*$^{+}$*Jag1*$^{i\Delta EC/+}$ Mice

(A) To test the role of angiocrine Jag1 in stimulating chemoresistance, control and *Myc*$^{+}$*Jag1*$^{i\Delta EC/i\Delta EC}$ mice were treated with doxorubicin at 100 mg/kg once a week for 4 consecutive weeks.

(B) Survival rate of *Myc*$^{+}$*Jag1*$^{i\Delta EC/i\Delta EC}$ and control mice. Chemo indicates mouse group treated with 100 mg/kg doxorubicin.

(C and D) Jag1 expression in VE-cadherin^{+} ECs and Notch activation (GFP expression) in LCs were measured with or without chemotherapy (C). Quantification of GFP intensity is shown (D). Compound E was injected into control mice to compare the degree of Notch inhibition; n = 4. Scale bar, 50 μm.

(E and F) Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining image (E) and quantification of TUNEL^{+} cells (F) in the lymphoma of Myc^{+} mice after chemotherapy; n = 4. Scale bar, 50 μm.

(G) Angiocrine Jag1 activates Hey1 to stimulate the emergence of LCs exhibiting aggressive LIC features. Expanding LCs reciprocally activate FGFR1 on ECs and induce Jag1 upregulation, further reinforcing Jag1-mediated angiocrine support of aggressive LCs with LIC attributes.

See also Figure S6.

Subcutaneous Inoculation and Hepatic Tumor Seeding Model

To monitor tumor propagation in vivo, 5 × 10^{5} human LCs or 1 × 10^{5} murine *Eμ-Myc* LCs were injected i.p., or 2 × 10^{6} LCs were injected subcutaneously into immunodeficient NSG mice and mice with the indicated genetic backgrounds. A liver-seeding model via intrasplenic transplantation of LCs was performed as described (Ding et al., 2010). Briefly, the mice were anesthetized, and 5 × 10^{5} mCherry-labeled human LCs or 1 × 10^{5} mouse LCs were injected into the parenchyma of the spleen. Splenectomy was carried out after the injection. The mice were sacrificed 14 days after intrasplenic transplantation, and hepatic tumor load was analyzed by

hematoxylin and eosin (H&E) staining and immunofluorescence whole scan of liver lobe.

γ-secretase inhibitor compound E was utilized to abolish Notch pathway activation in LCs. LCs were incubated with 1 μM compound E. For in vivo Notch inhibition, compound E was i.p. injected to mice at 2 mg/kg. Notch2 activation was also determined as described in Supplemental Experimental Procedures.

Flow Cytometric Analysis of LCs

For flow cytometry analysis, LCs were filtered through a 30 μm strainer, pre-blocked with Fc block (CD16/CD32; BD Biosciences), and then incubated with the primary antibodies CD44, CD19, and B220 (eBiosciences); IGF1R (Abcam); and Notch1 and Notch2 (Biolegend). Primary antibodies were conjugated to Alexa Fluor dyes using antibody labeling kits (Invitrogen) following the instructions of the manufacturer. GFP-expressing cells were detected by their own fluorescence, as described in Supplemental Experimental Procedures.

Immunofluorescent Staining

For immunofluorescence study, cryopreserved sections were incubated with antibodies to mouse VE-Cadherin (R&D Systems), Jag1 (Abcam), and Ki67 (Dako) supplemented with 10% normal donkey serum/1% BSA/0.1% Tween 20, followed by incubation with fluorophore-conjugated second antibodies (Jackson ImmunoResearch). Images were captured on AxioVert LSM710 microscope (Zeiss).

Methylcellulose Colony Assay

Mouse and human LCs formed colonies upon serum culture (LC^{Serum}) or EC coculture (LC^{EC}). Single colonies of LC^{Serum} and LC^{EC} were dispersed in methylcellulose. Colonies formed were randomly picked and serially passaged. Colony number was quantified upon each serial passage.

Statistical Analysis of Data

All data are presented as means ± SEM. Comparisons between different groups were made using Student's t test and ANOVA. Statistical significance was considered as $p < 0.05$.

ACCESSION NUMBERS

The microarray data are deposited at Gene Expression Omnibus under accession number GSE46368.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.02.005.

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Clonal Evolution Enhances Leukemia-Propagating Cell Frequency in T Cell Acute Lymphoblastic Leukemia through Akt/mTORC1 Pathway Activation

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SUMMARY

Clonal evolution and intratumoral heterogeneity drive cancer progression through unknown molecular mechanisms. To address this issue, functional differences between single T cell acute lymphoblastic leukemia (T-ALL) clones were assessed using a zebrafish transgenic model. Functional variation was observed within individual clones, with a minority of clones enhancing growth rate and leukemia-propagating potential with time. Akt pathway activation was acquired in a subset of these evolved clones, which increased the number of leukemia-propagating cells through activating mTORC1, elevated growth rate likely by stabilizing the Myc protein, and rendered cells resistant to dexamethasone, which was reversed by combined treatment with an Akt inhibitor. Thus, T-ALL clones spontaneously and continuously evolve to drive leukemia progression even in the absence of therapy-induced selection.

INTRODUCTION

Cancer is an evolutionary process whereby transformed cells continuously acquire genetic and/or epigenetic lesions to generate functionally distinct tumor cells. Natural selection then favors the clones with the best fitness for driving cancer progression, therapy resistance, and relapse (Aparicio and Caldas, 2013). Genetic heterogeneity is increasingly recognized as an important biomarker of cancer progression and outcome. For example, increased tumor cell heterogeneity was recently correlated with chemotherapy resistance in renal cell carcinoma (Gerlinger et al., 2012) and metastasis in pancreatic adenocarcinoma (Yachida et al., 2010). Similar associations have been reported in acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and chronic lymphocytic leukemia (CLL), where genetic diversity within the primary leukemia was correlated with an increased likelihood of drug resistance, disease progression, and relapse (Anderson et al., 2011; Ding et al., 2012; Landau et al., 2013; Mullighan et al., 2008; Notta et al., 2011). While these studies have provided valuable insight into intratumoral heterogeneity and patient outcome, analyses of bulk patient samples often identifies large numbers of mutations within a single tumor, making it difficult to determine how genetic diversity and acquired mutations promote cancer progression.

Significance

Uncovering the consequences of acquired mutations resulting from clonal evolution will be critical for understanding tumor progression and relapse. Our findings demonstrate that a subset of single T-ALL cells spontaneously acquired Akt pathway activation, which decreased latency, increased the frequency of relapse-driving leukemia-propagating cells (LPCs) and mediated resistance to chemotherapy in the absence of prior drug exposure. These data suggest that diagnosis clones can stochastically acquire mutations necessary to survive treatment and drive relapse even before a patient receives therapy, with acquired mutations being independently selected based on important cancer phenotypes. Our work also indicates that combination therapy of dexamethasone and an Akt inhibitor can successfully kill LPCs in a subset of refractory and relapsed T-ALL.

Understanding the consequences of genetic heterogeneity necessarily require detailed functional analysis of multiple single cells contained within the same primary tumor.

Recent advances in genomic technologies have provided unique insights into the clonal relationships between cancer cells and in some cases have documented the order by which genetic changes accumulate following progression and relapse. For example, the clonal relationship between primary and relapsed ALL was identified using copy number aberration analysis in matched patient samples. Continued clonal evolution and acquisition of de novo mutations occurred in a majority of relapse samples (Clappier et al., 2011; Mullighan et al., 2008), with most relapse disease arising from the evolution of an underrepresented clone contained within the primary leukemia. Whole genome sequencing studies have revealed that AML also undergoes clonal evolution from diagnosis to relapse, with five of eight patients developing relapse from a genetically distinct, minor clone that survived chemotherapy (Ding et al., 2012). Finally, 60% of CLL exhibited continued clonal evolution, where high clonal heterogeneity in the primary leukemia was associated with disease progression and prognosis (Landau et al., 2013), suggesting that clonal evolution is common and likely an important driver of cancer progression. While these studies have detailed lineage relationships between leukemic clones and often identified genetic lesions correlated with progression and relapse, the functional effects of these mutations have not been fully assessed.

Cancer progression and relapse are driven by distinct and often-rare cancer cells referred to as tumor-propagating cells, or in blood cancers as leukemia-propagating cells (LPCs). If LPCs are retained following treatment, they will ultimately initiate relapse disease (Clarke et al., 2006). Despite the substantial number of genetic lesions that have been identified in relapse samples and the contention that these mutations likely modulate response to therapy, acquired mutations that increase the overall frequency of tumor-propagating cells following continued clonal evolution at the single cell level have not been reported. Such mutations would increase the pool of cells capable of driving continued tumor growth and progression, thereby increasing the likelihood of relapse. Although we have previously found that LPC frequency can increase in a given leukemia over time (Smith et al., 2010), it is unclear whether this was the result of continued clonal evolution or if a clone with inherently high LPC frequency simply out competed other cells within the leukemia.

T-ALL is an aggressive malignancy of transformed thymocytes with an overall good prognosis. Yet despite major therapeutic improvements for the treatment of primary T-ALL, a large fraction of patients relapse from retention of LPCs following therapy, often developing leukemia that is refractory to chemotherapies including glucocorticoids (Einsiedel et al., 2005; Pui et al., 2008). Importantly, T-ALL exhibits clonal evolution at relapse, suggesting that this process is an important driver of therapy resistance, enhanced growth and leukemia progression (Clappier et al., 2011; Mullighan et al., 2008). Primary T-ALL is characterized by changes in several molecular pathways, including mutational activation of *NOTCH* and inactivation of *CDKN2A*, *FBXW7*, and *PTEN* (Van Vlierberghe and Ferrando, 2012). The Myc pathway is also a dominant oncogenic driver in vast majority of human T-ALL, resulting in part from NOTCH1 pathway activation (Palomero et al., 2006). Myc has also been recently shown to be a critical regulator of T-ALL progression (King et al., 2013), suggesting that identifying collaborating genetic events that synergize with Myc to enhance LPC frequency, leukemic cell growth, and resistance to therapy will likely be important to understanding human disease.

RESULTS

Continued Clonal Evolution Generates Subclonal Variation to Enhance LPC Frequency and Shorten Disease Latency

To gain insight into the functional role that tumor heterogeneity has on leukemia-propagating potential and latency, a cell transplantation-based screen was completed in which syngeneic zebrafish were engrafted with single, fluorescently labeled clones isolated from primary *Myc*-induced T-ALL (Figure 1A). This approach mimics the process by which a single cell can reinitiate leukemia at relapse. Importantly, zebrafish *Myc*-induced T-ALL are molecularly similar to the subset of human T-ALL that expresses *SCL* and *LMO2*, mimicking an aggressive and common form of human disease (Blackburn et al., 2012; Langenau et al., 2005). Moreover, zebrafish *Myc*-induced T-ALL is heterogeneous and is often comprised of numerous clones that harbor unique T cell receptor beta (*tcr*β) rearrangements, making it the ideal system with which to define the functional effects of intratumoral heterogeneity and clonal evolution. *Myc*-induced leukemias were generated to express a variety of fluorescent proteins including AmCyan, GFP, zsYellow, dsREDexpress, and mCherry. The monoclonality of each transplanted T-ALL was confirmed by genomic DNA analysis of *tcr*β rearrangements (Table S1 available online) and comparative genomic hybridization arrays (aCGH). Each monoclonal, primary transplanted T-ALL was then assessed for differences in latency, determined as the time required for >50% of the animal to be overtaken by fluorescently labeled T-ALL and in the overall frequency of relapse-driving LPCs, as determined by limiting dilution transplantation into syngenic recipients (Figure 1A). Sort purity following FACS ranged from 87%–98% and viability was >95% for all analyses. In total, 47 primary transplanted, fluorescently labeled monoclonal T-ALL were derived from 16 different primary leukemias.

Array CGH analysis revealed that clones from the same leukemia often shared common focal amplifications and deletions irrespective of *tcr*β rearrangement status. For example, all four clones from T-ALL #1 shared regional losses within chr1 and identical gains in chr2, chr7, chr12, chr14, and chr25, despite harboring different *tcr*β rearrangements (Figure S1A). Clones had additional genetic changes that were specific to each subclone, suggesting that zebrafish T-ALL clones were derived from a common ancestor and had undergone a branched evolution similar to that found in human patients (Mullighan et al., 2008) (Figures S1A and S1B). Clones isolated from the same primary T-ALL also commonly had different functional phenotypes. From primary T-ALL #1, clone 1.1 harbored a V15C1 *tcr*β rearrangement and generated leukemias that contained 1 LPC for every 12 cells. By contrast, clone 1.4 was genetically distinct with a V17C2 rearrangement and had a significantly lower LPC

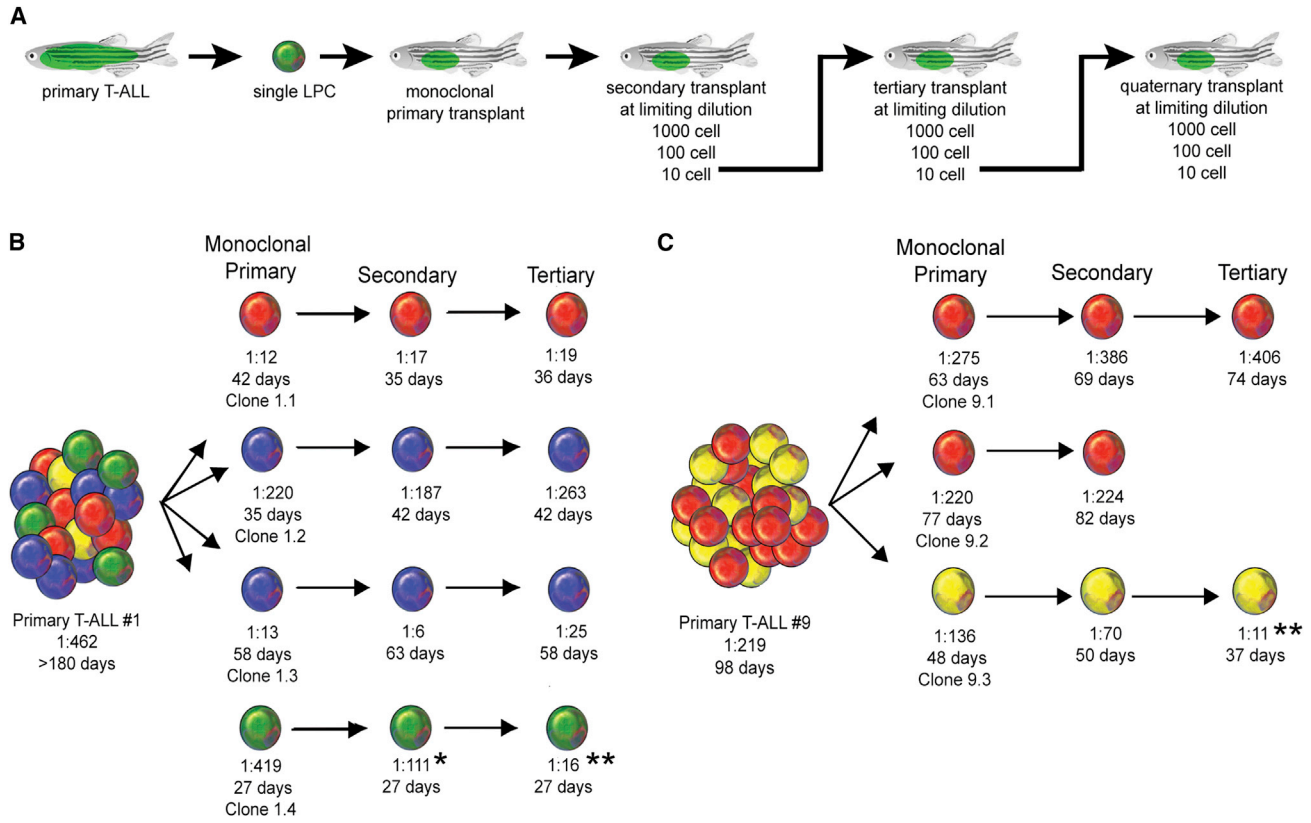


Figure 1. Clonal Evolution Drives Intratumoral Heterogeneity and Can Lead to Increased Leukemia Propagating Cell Frequency

(A) A schematic of the cell transplantation screen designed to identify phenotypic differences between single leukemic clones.

(B and C) Schematic of results from primary T-ALL #1 (B) and T-ALL #9 (C). *Denotes a significant increase in LPC frequency from monoclonal primary to secondary transplant ($p = 0.02$). **Denotes a significant increase in LPC frequency from monoclonal primary transplant T-ALL compared with tertiary transplanted leukemia ($p < 0.0001$). Clones are color-coded based on *tcr*β-rearrangements.

See also Figure S1 and Table S1.

frequency of 1 in 419 cells ($p < 0.0001$, Figure 1B; Table 1; Table S1). Functional variation was also observed within related clones. For example, clones 1.2 and 1.3 had the same V36C1 rearrangement, but had an ~17-fold difference in the overall numbers of LPCs ($p < 0.0001$), suggesting that continued clonal evolution resulted in increased LPC function and frequency. Finally, individual clones isolated from T-ALL #1 also had significant differences in latency, with the median time to T-ALL onset ranging from 27 to 58 days in transplant animals (Figure 1B; Table S1, $p < 0.01$). In total, functional variation between single clones was documented in 13 of 16 the primary T-ALL examined (Figures 1B, 1C, and S1B; Table S1), indicating that functional heterogeneity between leukemic clones is common.

To directly assess if continued clonal evolution can impart functional consequences to LPC frequency and latency, T-ALL generated from single cells were serially passaged at limiting dilution and recipient animals assessed for changes in time to leukemia regrowth and overall LPC frequency (n = 3,767 transplant animals assessed). From this analysis, 6 of 47 clones underwent continued clonal evolution that resulted in an average 20-fold increase in LPC frequency when compared with the initial monoclonal, primary transplanted T-ALL (range: 7- to 30-fold, Figures 1B and 1C; Table 1). For example, clone 1.4 had an LPC frequency of 1:419, which increased following serial passaging to 1:16 (Figure 1B; Table 1). Further, two clones simultaneously evolved both elevated LPC frequency and reduced latency following serial passaging (clones 10.1 and 14.1, Table 1). Outgrowth of a contaminating clone was excluded in all cases by analysis of *tcr*β rearrangements. The remaining 41 clones had no significant change in latency or LPC frequency following serial passaging (Table S1). Together, these data indicate that continued clonal evolution occurs in a small subset of T-ALL clones and can lead to spontaneous changes within single cells to alter both LPC frequency and latency.

Independent Pathways Can Regulate LPC Frequency and Growth Rate

In analyzing the functional differences between clones and the subclonal variation acquired following serial passaging, we found that LPC frequency and latency often evolve independently, suggesting that these processes can be regulated by different molecular mechanisms (Table S1). To confirm this observation, equal numbers of LPCs from selected clones were transplanted into recipient fish, and time to leukemia regrowth was assessed. From this analysis, we confirmed that

Table 1. Summary of Clones that Evolved Increased LPC Frequency

	Limiting Dilution Transplant[a]				
T-ALL	1,000 Cell	100 Cell	10 Cell	LPC Frequency[b]	Latency (days)[c]
Primary 1	7/8	3/20	2/38	1:462 (871, 245)	>180
Clone 1.4					
Primary transplant	4/5	2/8	1/17	1:419 (1,050, 167)	27.1 ± 2.1
Secondary transplant	5/5	4/8	2/11	1:111 (259, 49)	26.8 ± 2.2
Tertiary transplant	5/5	8/8	5/11	1:16 (36, 7)**	27.1 ± 2.1
Primary 6	5/5	9/18	6/45	1:114 (193, 67)	45
Clone 6.1					
Primary transplant	5/5	4/10	1/15	1:182 (417, 79)	43.0 ± 2.4
Secondary transplant	3/3	6/6	3/10	1:25 (58, 10)	53.4 ± 2.9
Tertiary transplant	3/3	6/6	6/20	1:26 (57, 13)***	45.0 ± 3.0
Primary 8	4/5	5/34	5/82	1:447 (793, 251)	115
Clone 8.4					
Primary transplant	3/3	2/6	2/10	1:267 (786, 91)	35.0 ± 3.1
Secondary transplant	2/2	5/6	3/10	1:43 (88, 19)	38.5 ± 2.4
Tertiary transplant	2/2	6/6	7/11	1:9 (21, 5)**	33.3 ± 1.8
Primary 9	5/5	8/26	4/52	1:219 (378, 126)	98
Clone 9.3					
Primary transplant	4/4	3/6	1/12	1:136 (365, 50)	47.8 ± 2.2
Secondary transplant	2/2	4/6	2/8	1:70 (171, 29)	50.2 ± 2.8
Tertiary transplant	2/2	5/5	6/10	1:11 (24, 4)**	37.6 ± 2.9
Primary 10	5/5	6/20	3/42	1:225 (418, 121)	49
Clone 10.1					
Primary transplant	3/3	2/6	0/10	1:263 (780, 88)	78.4 ± 4.1
Secondary transplant	3/3	6/6	4/9	1:16 (42, 7)**	32.7 ± 2.0
Tertiary transplant	3/3	6/6	3/9	1:22 (58, 9)**	32.2 ± 1.5#
Primary 14	6/7	5/36	3/72	1:509 (892, 290)	82
Clone 14.1					
Primary transplant	3/3	1/5	0/10	1:356 (1,101, 115)	63.0 ± 2.9
Secondary transplant	3/3	4/6	2/10	1:74 (177, 31)	51.3 ± 4.3
Tertiary transplant	2/2	5/5	5/9	1:12 (25, 5)**	37.1 ± 2.7##

[a]The number of engrafted animals over the number of total animals transplanted at each dose.
[b]The 95% confidence interval for LPC frequency is shown in parenthesis. Clones that increased the overall fraction of LPCs following serial passage are indicated. **$p < 0.0001$; ***$p = 0.0003$.
[c]Data are presented with ±SE where applicable. Clones that exhibited diminished latency from the primary to tertiary transplantation are noted. #$p < 0.0001$; ##$p = 0.016$.

clones with similar LPC frequency exhibited wide differences in time to leukemia onset (Figure 2A). Analysis of all serially passaged clones revealed no significant correlation between LPC frequency and latency (n = 120, Figure 2B). 5-Ethynyl-2'-deoxyuridine (EDU) incorporation (Figure 2C) and phospo-H3 staining (data not shown) showed that proliferation rate correlated with time to leukemia regrowth in vivo, but did not correlate with overall LPC frequency. Apoptosis rates did not differ between clones (Figures S2A and S2B). Together, these data suggest that the pathways that regulate T-ALL latency/proliferation and LPC frequency need not be controlled by the same molecular mechanism; rather they can be evolved independently in a subset of T-ALL clones.

To directly assess which of these processes dominate in driving T-ALL progression, in vivo competition experiments were performed between clones that exhibited inherent differences in latency and LPC frequency and differed in fluorescent protein expression. In each experiment, recipient fish were transplanted with 25 LPCs from two fluorescent clones and leukemia growth was assessed at 15 and 45 days posttransplantation. T-ALL clones with short latencies but low LPC frequency consistently out competed clones with longer latencies but high LPC frequency (Figures 2D and 2E). These data suggest that although leukemia propagating potential is required to reinitiate disease, clones with high proliferative capacity likely out compete other cells during disease progression and relapse. Moreover, these data support our conclusion that T-ALL latency and leukemia-propagating potential need not be regulated by the same molecular mechanisms.

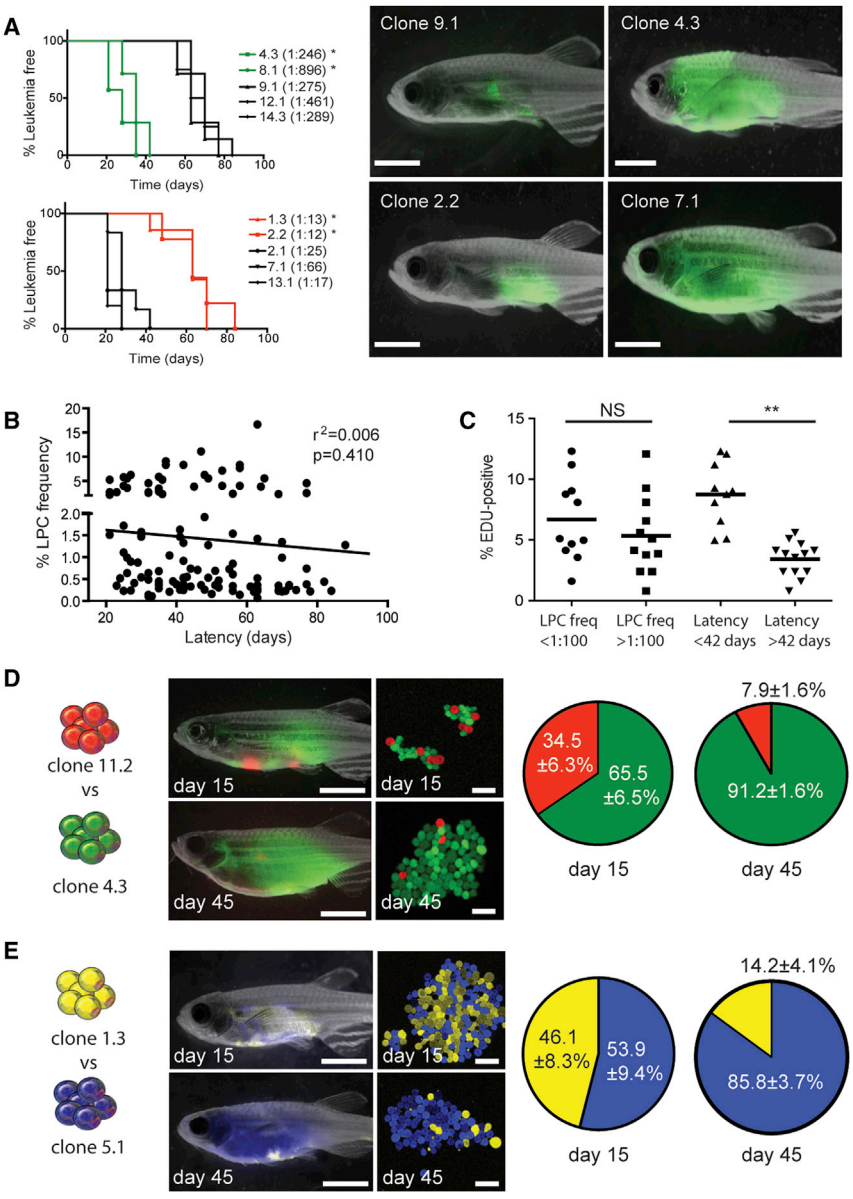


Figure 2. Mechanisms that Drive Leukemia Propagating Cell Frequency and Latency Can Evolve Independently

(A) Fish were transplanted with 25 LPCs from various clones and assessed for time to leukemia onset (n = 8–10 animals transplanted per individual clone). *Denotes significant differences in latency between clones that have low (upper left panel) or high (lower left panel) LPC frequencies (<0.001). Representative fluorescent images of animals following 28 days of engraftment are shown.

(B) Correlation between LPC frequency and T-ALL latency across all clones.

(C) EDU analysis of selected clones and correlation with LPC frequency and latency. Each datum point represents a single clone. NS, not significant. **Denotes a significant difference in the percent of cells that are EDU-positive (p = 0.0004).

(D) Animals were transplanted with 25 LPCs from clone 11.2 (dsRED-positive, 1:78 LPC frequency, 88 days latency) and 25 LPCs from clone 4.3 (GFP-positive, 1:246 LPC frequency, 28 days latency). Representative images of whole fish and confocal images of T-ALL cells harvested at 15 days and 45 days posttransplantation. The percentages of dsRED-positive and GFP-positive cells at 15 days and 45 days were analyzed by FACS. Data are represented as ± SE (n = 4–7 transplant recipients per time point).

(E) Twenty-five LPCs from clone 1.3 (zsYellow-positive, 1:13 LPC frequency, 58 days latency) were competed with 25 LPCs from clone 5.1 (amCyan-positive, 1:184 LPC frequency, 30 days latency), and analyzed as in (D). Scale bars represent 5 mm in images of whole fish and 40 μm in confocal images. See also Figure S2.

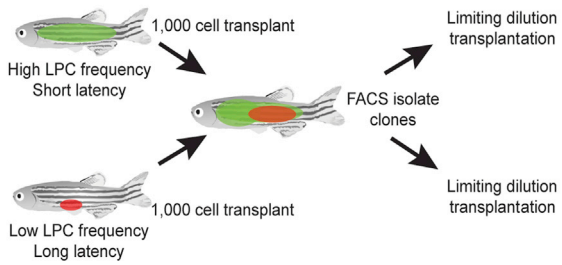
Cell Intrinsic Processes Control LPC Frequency and Growth

We next performed a series of competition experiments to determine whether clones within a heterogeneous leukemia could functionally impact the LPC frequency and/or latency of other, genetically distinct clones. Clones that had different functional properties and labeled with different fluorescent proteins were transplanted into recipient animals at limiting dilution and assessed for the ability to alter the cellular fate of cotransplanted T-ALL cells. In the first set of experiments (Figure 3A), fluorescently labeled clones with high LPC frequency and short latencies were cotransplanted with clones that had low LPC frequency and long latencies. After T-ALL formation, fluorescent T-ALL cells were isolated from recipient fish by FACS (>94% purity and >98% viability) and transplanted at limiting dilution. Mixing had no long-term effect on altering LPC frequency (Figure 3B) or latency (Figure 3C) in any clone tested. Next, we determined if continuous juxtacrine or paracrine signaling from clones with high LPC frequency was necessary to alter the frequency of LPCs in clones with low leukemia propagating potential. Specifically, T-ALL clones with high LPC frequency were transplanted into recipient fish and allowed to engraft for 7 days. Engrafted animals were then transplanted at limiting dilution with a clone with low LPC frequency (Figure S3A). Animals were assessed for engraftment of the second clone at 45 days, and again, cell extrinsic signaling from clones with high LPC frequency did not impart elevated leukemia propagating potential to cells with low LPC frequency (Figure S3B). These data show that cell autonomous processes regulate growth kinetics and overall frequency of LPCs in most T-ALL clones.

Clonal Evolution Can Activate Akt Signaling to Increase Both LPC Frequency and Growth in T-ALL

Although our data suggest that increased LPC frequency and growth rate can be acquired in clones through independent mechanisms, mutations in genes and pathways that simultaneously enhance both of these processes would likely be

A



B

T-ALL	Fluorescence	Limiting Dilution Transplant 1,000 cell	100 cell	10 cell	LPC Frequency
2.1§	GFP	3/3	5/5	6/10	1:11 (25,5)
11.2§	dsRED	2/2	3/6	1/10	1:131 (356,48)
Post-mix 2.1	GFP	3/3	4/4	3/8	1:21 (56,8)
Post-mix 11.2	dsRED	3/3	4/6	2/12	1:78 (1854,34)
13.1§	mCherry	3/3	6/6	6/9	1:9 (22,4)
9.2	GFP	3/3	2/6	0/10	1:263 (781,89)
Post-mix 13.1	mCherry	2/2	4/4	4/8	1:14 (38,6)
Post-mix 9.2	GFP	2/2	2/8	1/12	1:256 (671,94)

C

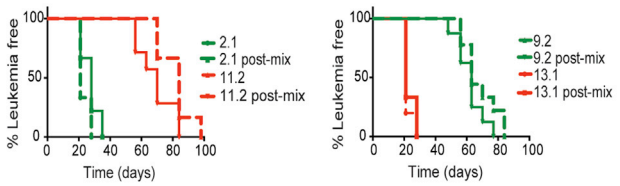


Figure 3. LPC Frequency and Latency Are Regulated Cell-Autonomously in T-ALL

(A) Schematic of the experimental design.

(B) Summary of results with 95% confidence intervals shown in parenthesis. §Indicates an independent limiting dilution cell transplantation experiment confirming similar results as those shown in Table S1.

(C) Kaplan-Meier analyses of leukemia regrowth in animals transplanted with individual clones alone or following mixing. Tumor-negative animals were excluded from analysis.

See also Figure S3.

selected for within a heterogeneous leukemia. Thus, we completed a systematic genetic analysis to identify the pathways associated with elevated LPC frequency and reduced latency. We did not identify shared, recurrent genetic amplifications or deletions among the clones that had evolved high LPC frequency following serial passaging (n = 5 clones analyzed by array CGH at both primary and tertiary transplant). We next analyzed 45 of 47 monoclonal primary transplanted T-ALL for recurrent point mutations common to human T-ALL pathogenesis, including six paired samples that consisted of a primary monoclonal transplanted T-ALL with low LPC frequency and the matched tertiary transplant that had evolved high LPC frequency. In total, *notch1b*, *il7r*, *akt2*, *akt3*, *n-ras*, *h-ras*, *k-ras*, *fbxw7*, *ptpn11a*, *pik3ca*, *pikr1*, *pten-a*, and *pten-b* were assessed for known hotspot mutations in human T-ALL. Although we observed *notch1* mutations in several clones (11 of 44), they were not exclusively associated with clones that had high LPC frequency, consistent with our previous report that activated Notch signaling does not elevate LPC frequency in *Myc*-driven T-ALL (Blackburn et al., 2012). Moreover, most *notch1* mutations did not occur within the amino acid residues known to alter protein stability or lead to constitutively active Notch signaling (Table S2). Genetic alterations in other genes were not detected (Table S2).

Because our data suggested that both genetic and epigenetic modifications likely play a role in clonal evolution (Figures S4A and S4B), we next assessed clones with high LPC frequency for changes in the expression of a wide range of genes implicated in human T-ALL (Figure S4C; Table S3). Endogenous *myc-a*, *myc-b*, or transgenic *Myc* expression were not altered following serial transplant, obviating the possibility that *Myc* transcript levels accounted for phenotypic changes in LPC frequency and/or latency in the *Myc*-induced zebrafish model (Figure S4C). Additionally, expression of genes that have been previously linked to T-ALL proliferation and self-renewal, including the Notch target gene *hes1*, *lmo2*, and *tal1* (McCormack et al., 2010; Van Vlierberghe and Ferrando, 2012), were not elevated in clones with high LPC frequency. In contrast, genes known to regulate the Akt pathway were frequently misexpressed in clones with high LPC frequency (Table S3). For example, serially passaged clone 8.4 had acquired elevated expression of *n-ras* and *h-ras*, clone 10.1 increased expression of *ptpn11*, and clone 14.1 lost expression of both *pten-a* and *pten-b* (Figure S4C; Table S3).

Based on these findings, we next assessed whether activation of the Akt pathway was directly associated with high LPC frequency. Immunohistochemistry (IHC) showed that 11 of 19 monoclonal T-ALL with high LPC frequency expressed high levels of phosphorylated Akt (pAkt), while only 1 of 26 clones with low LPC frequency exhibited pAkt staining (Figures 4A and S4D). Western blot analysis confirmed IHC results for the subset of clones analyzed (Figure S4E). Additionally, three of six T-ALL clones showed a marked increase in pAkt staining following continued clonal evolution, concurrent with an increase in LPC frequency (Figure 4B). These same clones had exhibited robust gene expression changes associated with activation of the Akt pathway, noted above (clones 8.4, 10.1, and 14.1, Figure S4C). Acquired Akt activation in these evolved clones was confirmed by western blot analysis for phosphorylation of Akt at serine 473 and phosphorylation of the downstream target S6-kinase (S6K, Figure 4C). Interestingly, treatment of evolved clone 10.1 with the epigenetic modifying drugs 5-azacytadine and sodium butyrate reduced LPC frequency and eliminated Akt signaling (Figures S4B and S4F), suggesting that the Akt signaling pathway can be regulated by epigenetic mechanisms and likely plays a functionally important role in regulating the overall numbers of LPCs in T-ALL.

To assess if clones had become dependent on Akt signaling for growth and LPC function in vivo, zebrafish were transplanted at limiting dilution with monoclonal primary transplant clone 10.1, which had low LPC frequency and was pAkt-negative, or tertiary transplant clone 10.1, which concurrently evolved high LPC frequency and pAkt-positivity. Transplanted animals were immediately placed in water containing DMSO or MK2206, an allosteric inhibitor of Akt, at a dose that effectively reduced phosphorylation of both Akt and S6K in vivo (Figure S4G). Zebrafish received drug treatment for 5 days, and transplanted animals were subsequently followed for altered LPC frequency (Figure 4D) and T-ALL latency (Figures 4E–4G). MK2206 treatment had no effect on modulating LPC frequency or latency in clone 2.2, which

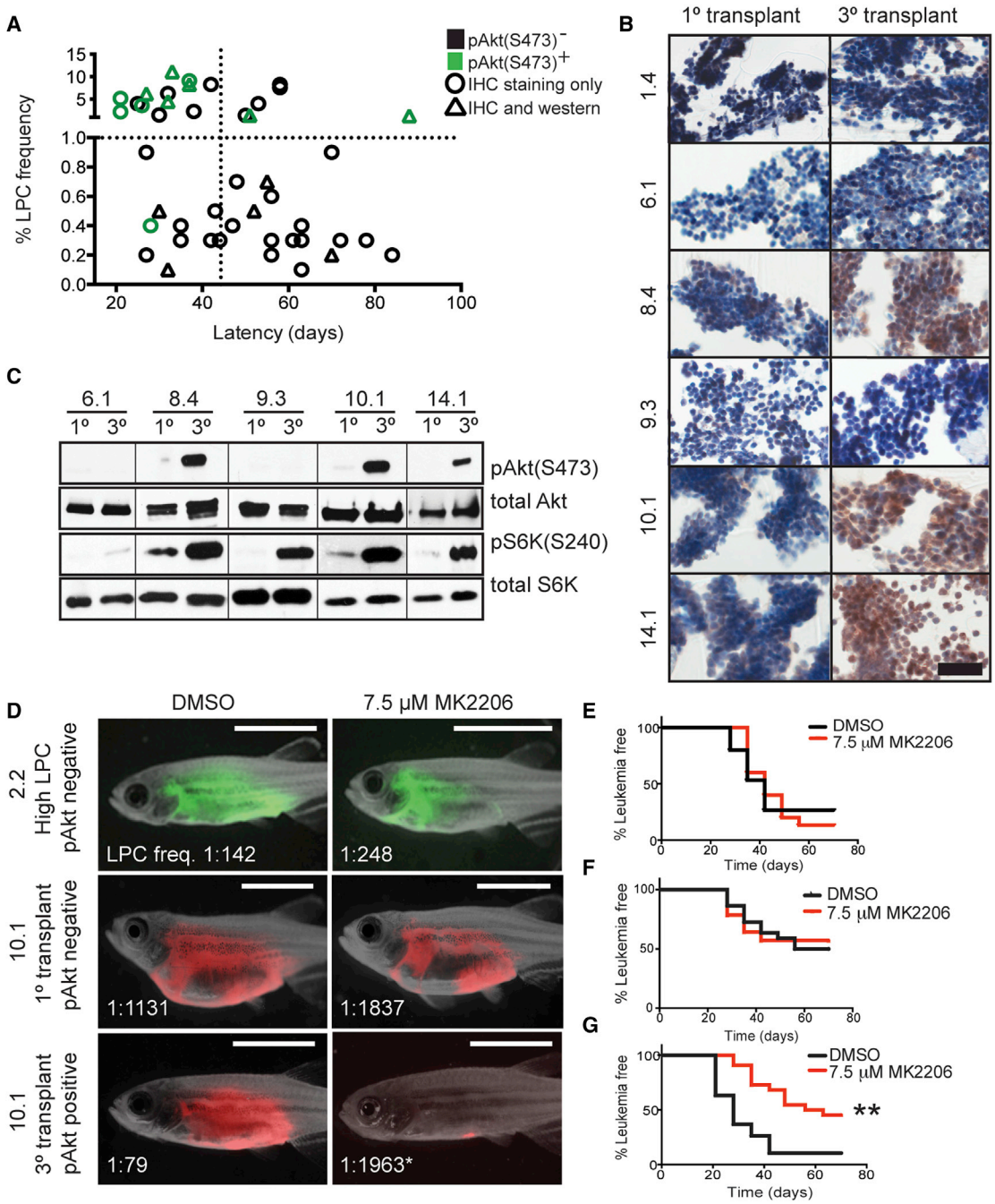


Figure 4. Akt Pathway Activation Is Acquired by a Subset of Cells following Clonal Evolution and Drives Elevated LPC Frequency and Growth

(A) Graphical summary of pAkt(S473) IHC from 46 monoclonal T-ALL. Green denotes samples that are pAkt-positive, and black have low or absent pAkt staining. Triangles represent clones that were confirmed for pAkt status by western blot analysis. The vertical dotted line demarcates clones with short (<45 days) or long latencies, and the horizontal dotted line identifies clones with low (<1.0%) or high LPC frequency. pAkt-positivity is significantly associated with high LPC frequency ($p < 0.0001$) and short latency ($p = 0.017$) by Fisher's exact test.

(B) IHC analysis of pAkt staining in T-ALL clones. Scale bar represents 50 μm.

(C) Western blot analysis of selected clones from (B).

(D) Animals were transplanted with the clones indicated and treated with MK2206 or DMSO for 5 days. Representative images at 28 days posttransplantation with LPC frequencies noted. *Denotes a significant change in LPC frequency following MK2206 treatment ($p < 0.001$). Scale bar represents 5 mm.

(E–G) Kaplan-Meier analyses for T-ALL regrowth following DMSO or MK2206 treatment for clone 2.2 (E), primary monoclonal transplant clone 10.1 (F), and tertiary transplant clone 10.1 (G). **Denotes a significant change in T-ALL latency following MK2206 treatment ($p < 0.0001$).

See also Figure S4, Table S2, and Table S3.

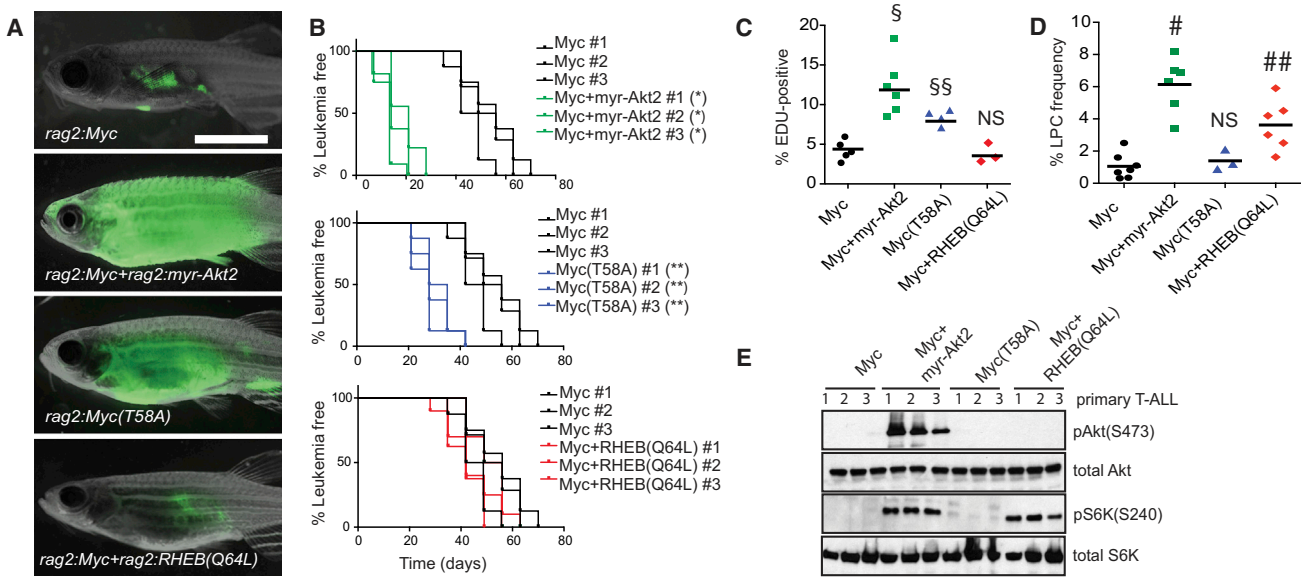


Figure 5. The Akt Pathway Increases LPC Frequency through Downstream Activation of mTORC1 and Shortens Latency by Augmenting Myc Stability

(A) Representative images of zebrafish that were transplanted with 25 LPCs from T-ALL expressing GFP and the indicated constructs (three T-ALL per genotype, n = 35 animals transplanted per primary leukemia) at 28 days posttransplantation.

(B) Kaplan-Meier analyses of time to T-ALL regrowth for each genotype and compared to Myc alone expressing T-ALL. *Denotes a significant difference in latency of $p < 0.0001$. **Indicates a significant difference in latency of $p = 0.003$.

(C) EDU analysis of transgenic T-ALL. Each datum point represents the percent EDU-positive cells for one T-ALL. §Represents a significant difference of $p < 0.0001$. §§Denotes a significant difference of $p = 0.004$, when compared to Myc alone expressing T-ALL. NS, no significant difference.

(D) Graph showing LPC frequency within each transgenic group. Each point represents data for one primary T-ALL. #Denotes a significant difference in LPC frequency of $p < 0.0001$. ##Indicates a significant difference in LPC frequency of $p = 0.0025$ when compared to Myc alone expressing T-ALL. NS, no significant difference.

(E) Western blot analysis.

See also Figure S5 and Table S4.

exhibited high LPC frequency but lacked Akt pathway activation, or in the pAkt-negative primary monoclonal transplanted clone 10.1. However, MK2206 treatment reduced the overall frequency of LPCs by 25-fold in evolved, pAkt-positive clone 10.1, reverting it to a similar LPC frequency and latency as its pAkt-negative ancestral clone (Figures 4D, 4F, 4G and S4H). Importantly, 15 of 17 zebrafish transplanted with pAkt-positive serially passaged clone 10.1 and treated with DMSO developed T-ALL, while only 11 of 21 zebrafish treated with the MK2206 developed T-ALL ($p = 0.034$, Fisher's exact test, Figure S4H). These data suggest that the Akt inhibitor not only reduced LPC frequency, but also efficiently killed LPCs, and provides further evidence that Akt plays an important role in LPC function.

Akt Pathway Activation Enhances the Frequency of LPCs through mTORC1

To provide conclusive genetic evidence that Akt signaling enhances LPC frequency, transgenic zebrafish were generated that expressed (1) *rag2:Myc* + *rag2:GFP*, (2) *rag2:Myc* + *rag2:myristoylated-Akt2 (myr-Akt2)* + *rag2:GFP*, or (3) *rag2:myr-Akt2* + *rag2:GFP*. The myristoylated-Akt2 transgene leads to constitutive activation of Akt signaling in the *Myc*-induced zebrafish T-ALL model (Gutierrez et al., 2009) and significantly enhanced time to primary T-ALL onset when compared to *Myc*-alone expressing T-ALL (25 ± 4 days compared with 62 ± 17 days, Figures S5A and S5B). In contrast, zebrafish that expressed only *rag2:myr-Akt2* failed to develop T-ALL over the 180 days of observation (n = 7) and are in agreement with earlier work indicating that Akt signaling induces T-ALL in zebrafish with very low penetrance (Gutierrez et al., 2011). Upon transplantation of 25 LPCs, T-ALL that expressed both *Myc* and *myr-Akt2* reformed leukemia 35 days faster than those that expressed *Myc* alone (Figures 5A and 5B), likely due to a substantial increase in proliferation in *Myc* + *myr-Akt2* cells (Figure 5C). Constitutive Akt signaling also increased the overall LPC frequency by an average of 5.8-fold when compared to those that expressed only *Myc* (Figure 5D; Table S4). Activation of the Akt pathway by the *myr-Akt2* transgene was verified by western blot analysis for phosphorylated Akt and S6K (Figure 5E). Importantly, constitutive activation of Akt also shortened latencies in both primary and transplant T-ALL in the *notch1a*^{ICD}-induced zebrafish model (Figures S5C and S5D) and significantly increased the LPC frequency by 12-fold when compared to *notch1a*^{ICD}-alone expressing T-ALL (Figure S5E; Table S4), demonstrating that the Akt pathway can synergize with multiple oncogenes to enhance LPC frequency.

To rapidly identify candidate molecular pathways acting downstream of Akt that are required for continued T-ALL maintenance and growth, monoclonal T-ALL were treated ex vivo with inhibitors to various Akt target pathways. All pAkt-positive

clones could be partially killed with MK2206 and the mTORC1 inhibitors Torisel and rapamycin, but not with β-catenin or NF-κβ pathway inhibitors (Figure S5F). These compounds had no effect on the pAkt-negative clones. Efficacy of Torisel in blocking S6K phosphorylation was verified by western blot analysis (Figure S5G). Based on this data, transgenic zebrafish were generated that overexpressed constitutively active RHEB(Q64L), which specifically activates the mTORC1 pathway (Jiang and Vogt, 2008). Akt is also known to stabilize the Myc protein through downregulation of GSK-3β, preventing phosphorylation of Myc at threonine 58 and blocking its degradation through the ubiquitin/proteasome pathway (Bonnet et al., 2011). Additionally, we had observed that a subset of pAkt-positive clones overexpressed the Myc target genes *ocd1*, *cd25a*, and *cad*, without concurrent increase in transgenic or endogenous *Myc* transcript levels (Figure S4C; Table S3), suggesting that Myc protein may be stabilized in these cells. Therefore, zebrafish T-ALL were also generated that overexpressed Myc(T58A), a mutant that is inefficiently degraded by the proteasome and mimics the effects of pAkt stabilization. Zebrafish expressing either *rag2:Myc* + *rag2:RHEB(Q64L)* + *rag2:GFP* and *rag2:Myc(T58A)* + *rag2:GFP* developed early onset primary T-ALL when compared with animals that expressed only *rag2:Myc* + *rag2:GFP* (Figures S5A and S5B). Importantly, *rag2:RHEB(Q64L)* + *rag2:GFP* animals did not develop T-ALL over 180 days of observation, indicating that mTORC1 activation alone may not be sufficient for T-ALL formation (n = 9).

To assess the effects of pAkt regulated pathways on leukemia regrowth and LPC frequency, T-ALL cells from each genotype were transplanted at limiting dilution and assessed for time to leukemia onset or overall LPC frequency. T-ALL expressing *Myc(T58A)* reinitiated leukemia 19 ± 4.3 days faster than *Myc*-expressing T-ALL, while *RHEB(Q64L)* overexpression had no effect on latency (Figures 5A and 5B). Similarly, *Myc(T58A)* but not *RHEB(Q64L)* T-ALL cells were significantly more proliferative than *Myc*-induced T-ALL (Figure 5C). Apoptosis rates did not differ between any transgenic T-ALL (Figure S5H), suggesting that proliferation rather than apoptosis rates are largely responsible for differences in latency. Limiting dilution cell transplantation experiments revealed that stabilized Myc(T58A) had no effect on LPC frequency. By contrast, *RHEB(Q64L)* expression significantly enhanced LPC frequency by an average of 4-fold when compared to *Myc*-alone expressing leukemias (Figure 5D; Table S4). Phosphorylation of S6K and expression of Myc target genes were verified in all transgenic T-ALL analyzed (Figures 5E and S5I). No overt differences in morphology and T-ALL molecular subtype were observed between any transgenic T-ALL (Figures S5J and S5K). These data show that the Akt signaling may act through two independent downstream pathways to increase T-ALL growth, likely by stabilizing Myc protein to enhance proliferation rates, and to increase the overall frequency of LPCs through activation of mTORC1.

Akt Activation following Clonal Evolution Also Renders Cells Resistant to Glucocorticoid Therapy

Treatment of T-ALL with the glucocorticoid dexamethasone often leads to acquired drug resistance in patients, which can be partly attributed to *PTEN* loss and activation of the Akt pathway (Beesley et al., 2009; Piovan et al., 2013; Schult et al., 2012; Silva et al., 2008). Based on these reports, we questioned if T-ALL clones spontaneously developed dexamethasone resistance following clonal evolution and activation of the Akt signaling pathway. Indeed, ex vivo drug treatment showed that the three T-ALL clones that were pAkt-negative as primary monoclonal T-ALL were killed by dexamethasone, while these same clones had become refractory to glucocorticoid-induced cell killing following clonal evolution and Akt pathway activation (Figure 6A). Combined treatment with either the Akt inhibitor MK2206 or the PIK3C inhibitor PI103 resensitized pAkt-positive clones to dexamethasone-induced killing (Figures 6A and S6A), suggesting that inhibition of Akt is responsible for the reduced viability of these clones. Similar results were also obtained using additional pAkt-positive and pAkt-negative primary monoclonal transplant clones (Figure S6B) and human T-ALL cells (Figure 6B), where pAkt status predicted a reduced response to dexamethasone-induced killing, which could be restored with the addition of MK2206 (Chan et al., 2007; Palomero et al., 2007). Finally, drug combinations were also tested in vivo. Similar to the ex vivo results, dexamethasone treatment killed T-ALL in animals transplanted with pAkt-negative, primary monoclonal transplant clone 14.1, but the evolved, pAkt-positive cells were largely refractory to treatment (Figure 6C). The pAkt-positive T-ALL was effectively killed by the combination of dexamethasone and MK2206, resulting in almost complete T-ALL regression. These experiments provide evidence that an Akt inhibitor can resensitize refractory T-ALL cells to dexamethasone-induced killing in vivo and demonstrate that T-ALL clones can spontaneously develop resistance to chemotherapy as a result of clonal evolution and that this can occur without selection induced by prior drug exposure.

DISCUSSION

The transformation from a normal to a tumorigenic cell is generally thought to follow a Darwinian evolutionary model, ultimately culminating in clonal evolution producing tumor cells that are well-adapted to proliferate and to overcome the selective pressures of metabolic stress, hypoxia, radiotherapy, and chemotherapy (Gerlinger and Swanton, 2010). In response to therapy, established cancers can continue to amass genetic and epigenetic lesions to enhance their propensity to form relapse. In support of this theory, genetic heterogeneity has been described in most cancer types and continued clonal evolution at relapse has been documented in several cancers. Moreover, heterogeneity has been correlated with important clinical features including progression, therapy resistance, and relapse (Almendro et al., 2013; Gerlinger and Swanton, 2010). Although the acquisition of genetic mutations at relapse has been well described, few studies have documented continued clonal evolution in the absence of therapy and the spontaneous acquisition of drug-resistant clones. For example, untreated chronic lymphocytic leukemias exhibit continued clonal evolution, ultimately resulting in spontaneous acquisition of genetic lesions that impart drug-resistance (Landau et al., 2013). However, these studies failed to correlate clonal evolution with specific features of progression, including shortened latency and enhanced LPC frequency, nor did they identify mechanistic drivers responsible for therapy resistance following continued clonal evolution. Our work follows

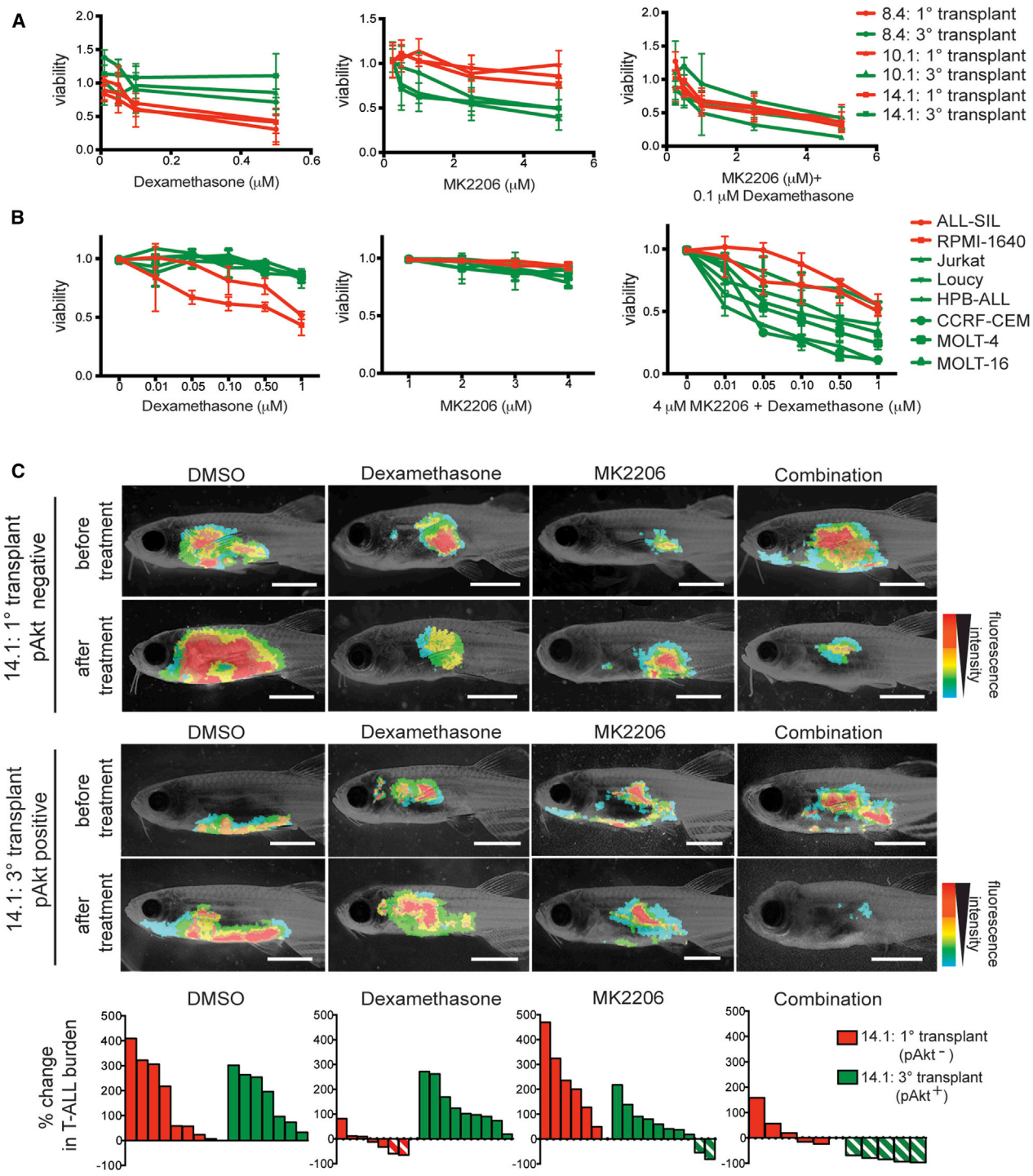


Figure 6. Dexamethasone Resistance Is Acquired following Akt Pathway Activation in Clonally Evolved Cells and Can Be Overcome by Combined Treatment with MK2206

(A) Primary monoclonal T-ALL that were pAkt-negative (red) and tertiary transplanted T-ALL that were pAkt-positive (green) were treated ex vivo as indicated and assessed for viability (n = 6 replicates per clone). Error bars are ± SE.

(B) Human cell lines with (green) or without (red) active Akt signaling were treated in vitro as indicated. Each point is the average viability after 24 hr of drug treatment (n = 3 replicates per cell line). Error bars ± SE.

(C) Representative images of leukemic fish prior to or 4 days after drug treatment with DMSO, 350 mg/l dexamethasone, 3.5 μM MK2206, or 350 mg/l dexamethasone + 3.5 μM MK2206. Clone name and pAkt status are shown to the left. Waterfall plots at the bottom summarize the in vivo T-ALL responses. Each bar denotes the percent change in T-ALL burden within a single animal, those with diagonal lines indicate >50% reduction in T-ALL burden.

See also Figure S6.

clonal evolution within single leukemic cells and directly associates functional consequences of clonal evolution with specific cellular features including therapy resistance. Moreover, we have discovered key oncogenic pathways responsible for changing the cellular phenotypes of leukemic cells following clonal evolution.

Utilizing the zebrafish transgenic T-ALL model and large-scale cell transplantation experiments, leukemias were generated from single LPCs isolated from heterogeneous primary T-ALL samples. This process of single cell cloning effectively created a homogenous background in which additional mutational events could be readily identified and directly associated with cellular phenotype. This large-scale screen allowed us to answer fundamental questions regarding the role that tumor cell heterogeneity has in cancer progression at the single cell level. First, we found that continued clonal evolution reduced latency, increased the frequency of LPCs, and modulated chemotherapy resistance, all of which can contribute to disease progression and relapse. These processes were independently evolved in a subset of T-ALL clones, yet in others, they were coevolved simultaneously through activation of the Akt signaling pathway. Our data suggest that multiple, parallel pathways can modulate each of these processes within a single leukemic cell. Additionally, while it is widely recognized that LPCs are required for malignancy and relapse (Clarke et al., 2006), a role for continued clonal evolution in enhancing the overall frequency of LPCs has gone largely unreported. In vivo competition experiments further suggest that mechanisms regulating LPC frequency and latency are regulated cell autonomously, without influence from secreted factors or continued juxtacrine signaling from neighboring clones. Our data also show that highly proliferative clones have the greatest fitness to regrow in a transplantation setting, which may explain, in part, why relapsed cancers are often more aggressive than primary disease.

Although we found that three of the six clones that evolved increased LPC frequency acquired Akt pathway activation, we were unable to pinpoint the precise mechanism of Akt pathway activation in zebrafish, besides a correlation with loss of *pten* expression and elevated expression of *ras* family members and *ptpn11a*. In humans, the primary activator of Akt signaling is likely *PTEN* inactivation, which occurs in >18% of T-ALL and is associated with poor prognosis (Gutierrez et al., 2009). *PTEN* deletion was also acquired upon relapse in 12% of matched primary and relapse patient xenograft samples, and PTEN knockdown in a single primary human T-ALL provided a competitive growth advantage and increased engraftment potential upon xenograft transplant (Clappier et al., 2011), suggesting that *PTEN* loss and Akt activation plays an important role in T-ALL progression and relapse. Additionally, RAS is known to activate the PI3K/Akt signaling cascade and is activated in a subset of human T-ALL (Kawamura et al., 1999; Trinquand et al., 2013; Zhang et al., 2012). Activation of the RAS pathway potently induced T-ALL in mouse models, largely though activation of the PI3K/Akt pathway (Kong et al., 2013; Shieh et al., 2013). Together, these data suggest that the RAS and PI3K/Akt pathways may be more commonly activated in T-ALL than previously thought. As suggested by our data, Akt pathway activation likely plays a critical role in human T-ALL progression by shortening T-ALL latency and enhancing the overall frequency of LPCs contained within the leukemia, both of which would provide a selective advantage to clones during disease progression and relapse. Importantly, we observed that Akt signaling synergized with both the *Myc* and intracellular *notch1a* oncogenes to drive T-ALL progression in the zebrafish models, suggesting that the Akt pathway likely collaborates with a variety of oncogenic drivers to enhance LPC frequency in T-ALL. Finally, transgenic epistasis experiments showed that Myc stabilization reduced T-ALL latency while mTORC1 acts downstream of pAkt to regulate LPC frequency. Although it is well known that mTORC1 regulates normal hematopoietic stem cell self-renewal and has prominent roles in leukemia initiation (Kalaitzidis et al., 2012), a role for mTORC1 in regulating the frequency of LPCs in T-ALL is largely unknown.

Acquired Akt pathway activation also rendered clonally evolved T-ALL cells insensitive to dexamethasone, a glucocorticoid used as a front-line therapy for T-ALL. Remarkably, inhibition of Akt or PI3K could restore sensitivity to dexamethasone-induced killing and specifically targets the LPC fraction. While Akt pathway activation is known to render T-ALL cells resistant to dexamethasone (Beesley et al., 2009; Chiarini et al., 2010; Piovan et al., 2013), our data demonstrate that T-ALL clones can also spontaneously develop chemotherapy resistance without any prior exposure to drug. Thus, Akt pathway activation is likely stochastically activated in a subset of clones following continued clonal evolution and selected within untreated, primary leukemias based on its role in elevating LPC frequency and overall growth. Sadly, patients with clones that have Akt pathway activation would likely already contain cells that are insensitive to dexamethasone therapy. As an important corollary, T-ALL clones found in patients undergoing dexamethasone therapy likely encounter extreme selection pressure to activate the PI3K/Akt pathway in an effort to suppress responses to dexamethasone. Once the PI3K/Akt pathway is activated, T-ALL cells become simultaneously resistant to therapy, have shortened latency, and have increased numbers of LPCs capable of driving relapse. While MK2206 has met with limited success in clinical trials for leukemia and solid tumors due to toxicity issues (Yap et al., 2011), our data provide a strong rationale for utilizing PI3K/Akt inhibitors in preclinical testing against T-ALL in combination with glucocorticoids, providing powerful combinatory therapies that will eradicate T-ALL cells, reduce the overall numbers of LPCs, and decrease T-ALL growth.

EXPERIMENTAL PROCEDURES

Animal Use, Creation of Transgenic Zebrafish, and T-ALL Transplantation

Zebrafish studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, under protocol #2011N000127. CG1-strain zebrafish were used exclusively for these studies. Creation of mosaic transgenic animals and DNA constructs have been described previously (Blackburn et al., 2012) and are fully described in the Supplemental Experimental Procedures. Because primary *Myc*-induced zebrafish T-ALL contains, on average, <1 LPC per 100 FACS isolated cells (Smith et al., 2010), monoclonal T-ALL could be generated from animals engrafted with ten T-ALL cells and in some cases 100 cells. The statistical likelihood of generating T-ALL from a single LPC by 10- or 100-cell transplants is noted for each clone in Table S1. Monoclonality was verified by detection of a single *tcr*β rearrangement (Blackburn et al., 2012) (Table S1) and in some cases by array CGH. Serial passaging of monoclonal T-ALL was accomplished using similar

protocols as outlined previously (Blackburn et al., 2011). Kaplan-Meier analyses were performed using a Log-rank (Mantel-Cox) test in GraphPad Prism. Analyses of limiting dilution cell transplantation data and calculation of 95% confidence intervals were completed using Extreme Limiting Dilution Analysis software (Hu and Smyth, 2009). All other statistical calculations were completed using Student's t test, except where noted.

Chemical Treatment of T-ALL Cells and T-ALL-Bearing Zebrafish

Human T-ALL cell lines were cultured as described (Blackburn et al., 2012) and drug-treated as outlined in the Supplemental Experimental Procedures. Ex vivo drug treatment of zebrafish T-ALL cells was completed using zebrafish kidney stromal (ZKS) conditioned media as described the Supplemental Experimental Procedures. For in vivo drug treatment, drugs were mixed in zebrafish system water and animals submersed in drug containing water at the doses and times described in the main text. Additional experimental conditions are described in the Supplemental Experimental Procedures.

Immunohistochemistry, Western Blot Analysis, EDU Staining, Real-Time Reverse Transcriptase PCR and Genomic DNA Sequencing

Paraffin embedding, sectioning and immunohistochemical analysis of zebrafish tissue were performed essentially as described (Langenau et al., 2003). Antibodies and dilutions used for western blot analysis are noted in the Supplemental Experimental Procedures. EDU staining was performed using the Click-iT Alexa Fluor 647 imaging kit (Invitrogen) according to manufacturer's protocol. Real-time reverse transcriptase PCR (RT-PCR) and sequencing of genomic DNA was performed as previously described (Blackburn et al., 2012), utilizing gene-specific primers (Supplemental Experimental Procedures). Gene expression was normalized to *ef1-α* and β-*actin* controls to obtain relative transcript levels using the $2^{-\Delta\Delta CT}$ method. For all analyses, cells were isolated from fish with >50% T-ALL burden.

ACCESSION NUMBERS

The NCBI Gene Expression Omnibus accession number for the aCGH data reported in this paper is GSE54482.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.01.032.

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Functional Heterogeneity of Genetically Defined Subclones in Acute Myeloid Leukemia

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SUMMARY

The relationships between clonal architecture and functional heterogeneity in acute myeloid leukemia (AML) samples are not yet clear. We used targeted sequencing to track AML subclones identified by whole-genome sequencing using a variety of experimental approaches. We found that virtually all AML subclones trafficked from the marrow to the peripheral blood, but some were enriched in specific cell populations. Subclones showed variable engraftment potential in immunodeficient mice. Xenografts were predominantly comprised of a single genetically defined subclone, but there was no predictable relationship between the engrafting subclone and the evolutionary hierarchy of the leukemia. These data demonstrate the importance of integrating genetic and functional data in studies of primary cancer samples, both in xenograft models and in patients.

INTRODUCTION

Cancer arises through an evolutionary process of somatic mutation and selection. Although it may be depicted as a linear sequence of mutational events that produces a homogeneous cell population, tumor evolution is associated with significant intratumoral heterogeneity (reviewed in Swanton, 2012). All cells in a tumor contain shared somatic mutations that reflect its clonal origin (the "founding clone"), but additional mutations are present in subpopulations of cells that define tumor subclones. This heterogeneity, and the presence of subclonal alterations, was recognized even in early models of tumor evolution, demonstrating that subclonal cytogenetic aberrations can define "sublines" within a tumor (Nowell, 1976)—which would now be defined as subclones.

New sequencing technologies have greatly improved the characterization of genetic heterogeneity in cancer. Previous work on acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) demonstrated that these myeloid disorders exhibit clonal heterogeneity that evolves upon disease progression and/or relapse (Ding et al., 2012; Ley et al., 2010; Mardis et al., 2009; Walter et al., 2012; Welch et al., 2012). Similar observations have been made in other malignancies. Work from Gerlinger et al. (2012) found that renal cell carcinomas can show striking clonal variation within different geographic regions of a single tumor, and recent analysis of clonal architecture in breast cancer demonstrated a hierarchy that elucidated the phylogeny of mutational events within individual tumors (Ding et al., 2012; Navin et al., 2010; Nik-Zainal et al., 2012; Shah et al., 2009).

Significance

Although clonal heterogeneity in many tumor types has been clearly demonstrated, the functionality of tumor subclones and their relationships to "tumor initiating/stem cells" are not yet clear. Using acute myeloid leukemia (AML) as a model, we found that subclones can correspond to different cellular populations within a single AML sample and can have different functional properties in vitro and in immunodeficient mice. Specifically, xenotransplantation results in a dramatic decrease in the subclonal complexity of AML samples; engrafting subclones are not defined by recurrent mutations (e.g., *FLT3*) and do not reliably predict relapse. These studies suggest that engrafting potential is not uniform among AML subclones and that functional differences among subclones need to be considered in studies of primary cancer samples.

Table 1. Features of 19 De Novo AML Samples

UPN	AML No.	FAB	Somatic Fusions and Mutations[a]
933124	1	M1	*DNMT3A* L723fs, *FLT3*-ITD, *NPMc* *SMC3* G662C
426980	28	M2	*IDH2* R140L, *IDH2* R140W
452198	31	M5	*DNMT3A* R882H, *FLT3* D835H, *FLT3*-ITD, *IDH1* R132H, *NPMc*,*IDH2* R140Q
869586	43	M4	*RUNX1* G164fs, *WT1* A382fs, *PHF6* G288V
161510	54	M2	*IDH1* R132H, *NRAS* G13D, *WT1* E143fs
255108	60	M0	*FLT3*-ITD, *NPMc*, *WT1* e7+1, *STAG2* e21+2
296361	62	M5	*DNMT3A* R882H, *FLT3*-ITD, *NPMc*, *IDH1* R132H
303818	63	M0	*SETBP1* D868N, *ASXL1* Q588*, *KRAS* I36M
348685	70	M4	*FLT3*-ITD, *WT1* R370fs
375182	71	M5	*DNMT3A* R882H, *FLT3*-ITD, *NPMc*
433325	75	M2	*CEPBA* R343fs, *DNMT3A* Q515*, *NPMc*, *NRAS* G13D, *PTPN11* I545L, *WT1* A381fs, *SMC3* T1174I
492687	78	M2	*FLT3*-ITD, *FLT3*-D835Y, *RUNX1* G305*
498463	79	M1	*DNMT3A* P718L[b], *IDH1* R132C, *NRAS* G13D, *EZH2* R690H
605322	83	M1	*DNMT3A* D781G, *DNMT3A* R320*, *FLT3* D839G, *IDH1* R132C, *TET2* P1115fs, *TET2* E1352V, *PTPN11* G503E
708512	87	M4	*DNMT3A* R882H, *FLT3* D835E, *IDH1* R132H, *NPMc*, *KIT* D816V
721214	88	M1	*DNMT3A* R882H, *FLT3*-ITD, *NPMc*
737451	91	M5	*DNMT3A* R882H, *NPMc*
809653	93	M1	*NRAS* G13D, *TP53* E286G
852559	94	M1	*PICALM-MLLT10 PTPN11* P491L, *PHF6* F214fs

UPN, unique patient number.
[a]Mutations found in significantly mutated genes (Cancer Genome Atlas Research Network, 2013) are listed, in addition to *ASXL1* and *SETBP1* variants in AML63.
[b]Homozygous variant.

In addition to genetic heterogeneity, functional heterogeneity also exists within a primary tumor and has largely been studied in the context of identifying cells capable of initiating tumors when transferred into immunodeficient mice. However, the relationship of these initiating cells (also referred to as cancer stem cells) to the clonal organization of a tumor is not yet clear. Previous studies of acute lymphoblastic leukemia (ALL) and colorectal cancer have begun to address this relationship: tumor subclones can be dynamic with serial passaging, and some display enhanced engraftment potential (Anderson et al., 2011; Clappier et al., 2011; Kreso et al., 2013; Notta et al., 2011; Schmitz et al., 2011). However, studies of leukemia samples have thus far followed copy number alterations, and/or used ALL samples with single, well-defined initiating events (*BCR-ABL1* or *ETV6-RUNX1* gene fusions), or have used distinct clinical subsets that do not reflect the full spectrum of this disease. In addition, the regional heterogeneity of solid tumors (Ding et al., 2012; Gerlinger et al., 2012; Nik-Zainal et al., 2012; Shah et al., 2009; Sottoriva et al., 2013) may introduce sampling bias when assessing clonal heterogeneity (especially in xenotransplantation models), making it difficult to generalize the results to other cancers. From the studies published to date, it also is not yet clear whether functional differences among tumor subclones can be observed beyond these experimental systems or whether they can be identified directly in patient samples.

In this study, we sought to explore the relationship between functional and genetic heterogeneity by following genetically defined subclones under different experimental and biological conditions in de novo AML samples with a wide range of phenotypic and genetic characteristics.

RESULTS

Sequencing and Somatic Mutation Identification of De Novo AMLs

We used whole-genome sequencing (WGS) to discover somatic mutations in the unfractionated bone marrow cells of 19 patients with de novo AML using previously described approaches (Ding et al., 2012; Ley et al., 2010; Welch et al., 2012). Most of the assessed AMLs had a normal karyotype (11/18; 61%), and they encompassed a range of French-American-British (FAB) subtypes and mutational spectra (Table 1 and Table S1 available online). All but one of the samples (AML54/UPN161510) have been analyzed previously by either exome sequencing (14 samples; Cancer Genome Atlas Research Network, 2013) or WGS (four samples; Ding et al., 2012; Ley et al., 2008, 2010; Welch et al., 2012), although samples with existing WGS were reanalyzed to identify additional somatic variants. AML-associated single-nucleotide variants (SNV) and coding insertion-deletion (indel) mutations discovered by WGS were confirmed using targeted, deep digital sequencing with custom capture arrays (Tables S1, S2, and S3), which demonstrated high reproducibility with repeated targeted sequencing of the same bone marrow samples (Figure S1A). The majority of the identified variants in each AML sample formed a variant cluster with a variant allele fraction (VAF) of 45%–50%, which corresponds to heterozygous somatic mutations present in nearly all cells in the sample; this variant cluster marks the founding clone, from which all leukemic cells descend. A smaller number of variants were present in clusters at lower VAFs and represent leukemic subclones that possess all of the founding clone variants as well as additional subclonal somatic variants that arose during the evolution of the tumor (Figure S1B). Although these subclones are derived from the founding clone and the subclonal variants have lower VAFs, it is important to note that, in many cases, they represent the most abundant leukemic cell population in the bone marrow.

Hierarchy of AML Mutations within Peripheral Blood Cells

AML subclones were identified using bone marrow samples from AML patients, but leukemic cells also circulate in the peripheral blood. Peripheral blood involvement can vary substantially

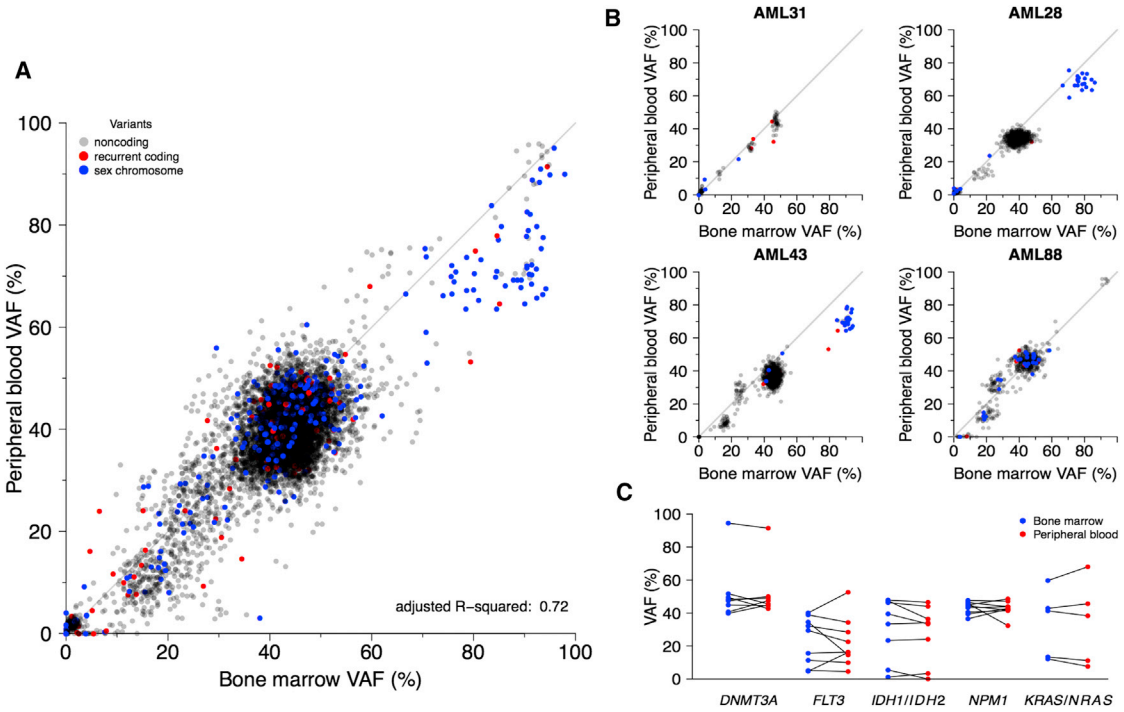


Figure 1. Mutational and Subclonal Comparison of Peripheral Blood and Bone Marrow Leukemia Samples

Unfractionated bone marrow and peripheral blood leukemia samples were characterized by targeted capture followed by deep sequencing.

(A) Similarity of the variant allele fractions (VAFs) of somatic variants for 19 AML cases (all variants with coverage >50× from all samples are shown). Variants present in coding regions of the genome are shown red, noncoding in gray, and sex chromosome variants in blue.

(B) Four representative cases of the peripheral blood versus the bone marrow samples, demonstrating cases with strong concordance (AML31 and AML28) and more variable subclonal distributions (AML43 and AML88).

(C) Comparison of the VAFs of recurrent AML mutations in paired peripheral blood and bone marrow samples, including coding mutations in *DNMT3A*; *FLT3* (both ITD and D835); *NPMc*; and canonical *IDH1*, *IDH2*, and *KRAS*/*NRAS* mutations.

See also Figure S1 and Tables S1, S2, and S3.

across patients, and some studies have reported that the allelic burdens of single AML mutations can be different in the peripheral blood and bone marrow (Jilani et al., 2003; Krönke et al., 2011; Ma et al., 2009). This raises the possibility that AML subclones may differ in their ability to leave the bone marrow and circulate in the peripheral blood. To determine whether AML subclones peripheralize in different ways, we used targeted sequencing to compare the clonal architecture of peripheral blood samples to concurrently obtained bone marrow samples. We found a similar clonal architecture in the peripheral blood in 13 of the 19 cases (68%; Figures 1A, 1B, and S1C); the remaining samples showed small but significant relative differences (Figure S1C). However, all variants detected in the bone marrow sample were present in the peripheral blood, including common recurrent coding mutations associated with AML (Figure 1C).

The founding clone variants were commonly present at VAFs of ~50% in the peripheral blood leukemia samples, suggesting that nearly all cells contained these leukemic variants, despite variable percentages of leukemic blasts (Table S1). To determine whether hematopoietic cells other than blasts are derived from the leukemic clone, we used multicolor flow cytometry to isolate myeloid blasts ($CD45^{dim}$, SS^{low}, and $CD33^{+}$) and lymphocytes ($CD45^{bright}$, SS^{low}, and $CD33^{-}$) from all 19 cases (Figure 2A); monocytes ($CD45^{int/bright}$, SS^{int}, and $CD33^{bright}$) and mature myelomonocytic cells ($CD45^{int}$, SS^{high}, and $CD33^{dim/neg}$) were collected from a subset of cases. These different peripheral blood populations were then analyzed by targeted deep sequencing of all known somatic variants (Table S4). As expected, sorted blasts contained all founding clone variants and also the vast majority of the subclonal variants identified in unfractionated marrow (Figure 2B). Surprisingly, somatic variants were also detected in other purified myeloid cells without blastic morphology (Figures 2C and S2A), regardless of the morphologic features of the leukemia (represented by the FAB subtypes). Whereas some of the variants showed either enrichment or depletion in these cell populations (i.e., those that appear above or below the line Y = X in Figure 2C; see also Table S5), the founding clone variants had VAFs of ~50%, indicating they were present in nearly all of the cells analyzed (and thus are unlikely to represent technical contamination from flow sorting). Although AML is functionally defined by a differentiation block leading to an accumulation of blasts, some cells clonally related to the leukemia are still capable of myeloid differentiation. These mature cells are either derived from the leukemic clone or a related "preleukemic" hematopoietic stem-progenitor cell (HSPC) (Jan et al., 2012; Krönke et al., 2013; Shlush et al., 2012).

Other studies have shown that lymphocyte populations can harbor mutations or fusion events identified in concurrent

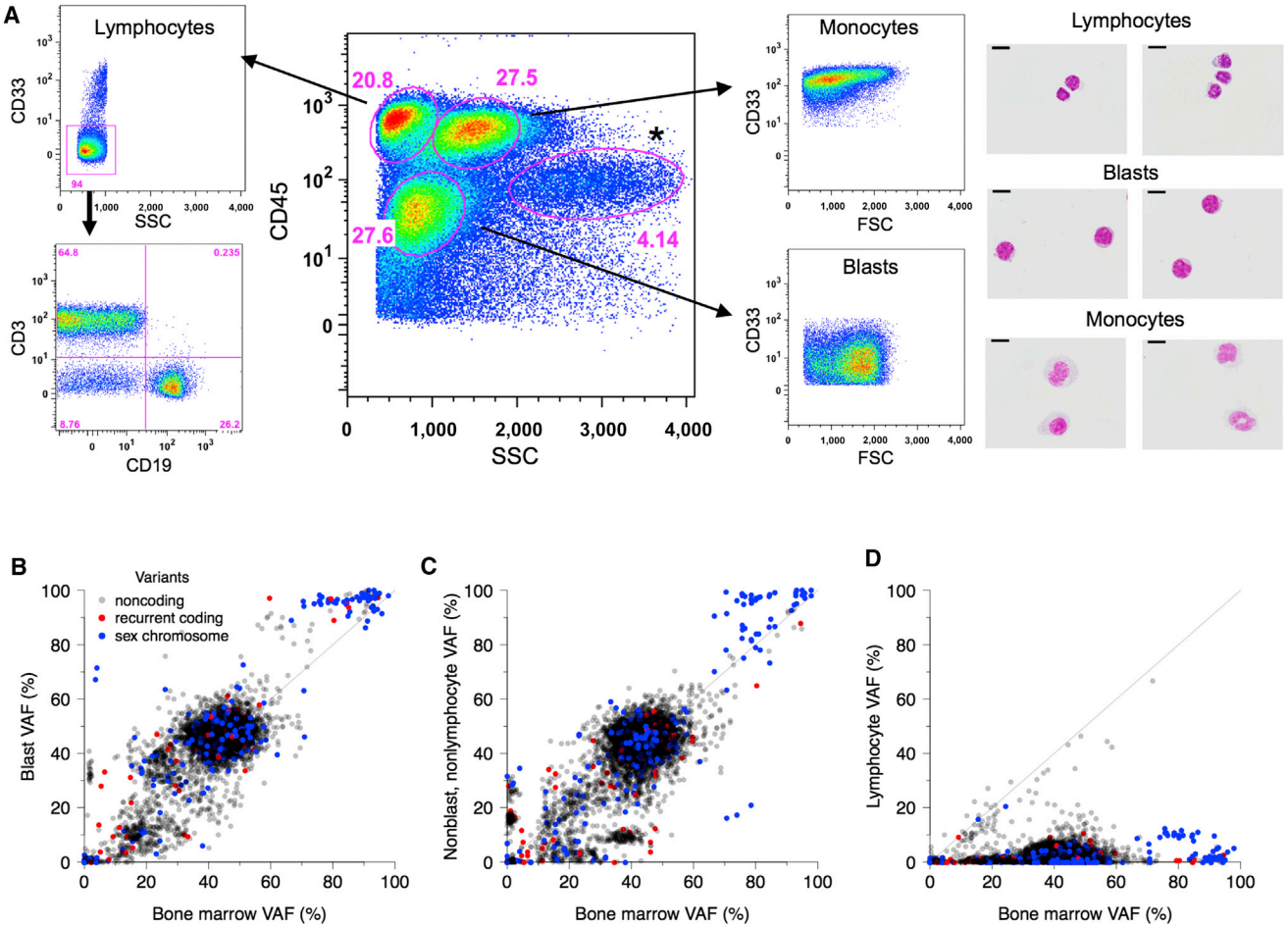


Figure 2. Distribution of Leukemia Variants and Subclones in Peripheral Blood Cells

(A) Representative sample demonstrating the isolation of different cell populations by flow cytometry using a combination of side-scatter, CD45, and CD33, as well as CD19 and CD3 when sufficient cells were available. *, maturing myelomonocytic cells. Scale bar represents 10 μm.

(B–D) Correlation of the bone marrow VAF with flow-sorted and enriched blasts (B), nonblast, nonlymphocytes, including maturing myelomonocytic cells and monocytes (C), and lymphocytes (D). Variants present in coding regions of the genome are shown red, noncoding in gray, and sex chromosome variants in blue. See also Figure S2 and Tables S4, S5, and S6.

myeloid malignancies (Miyamoto et al., 2000; Smith et al., 2010). However, we found that the majority of the somatic mutations we identified in the unfractionated de novo leukemia samples were either not detected in peripheral blood lymphocytes or were present at very low levels (mean VAF = 1.79%; 95%ile VAF = 6.06%; Figure 2D). This was also true for three cases with separately collected B and T lymphocytes (Figure S2B). In several cases, rare variants (99 of 6,805 total variants from all cases; 1.45%) demonstrated VAFs of >10%, implying they were present in a substantial fraction of the cells in the lymphocyte pool. The majority of these occurred in noncoding regions and are therefore not likely to be relevant for AML pathogenesis (Table S6). In one case (AML71), a recurrent AML-associated mutation in *DNMT3A* at codon R882 had a VAF of 10.5% (8 out of 68 reads), but no other cases had previously identified recurrent AML mutations (Cancer Genome Atlas Research Network, 2013) with VAFs >10% in the lymphocyte pools. We suspect that the mutations with higher VAFs in peripheral blood lymphocytes are present in multipotential HSPCs; these may have been acquired during embryogenesis or early hematopoietic development (i.e., not in the leukemic clone) or they arose in a preleukemic HSPC population that can contribute to lymphopoiesis. The variants with very low VAFs were probably caused by leukemic contamination of the flow-purified lymphocytes; morphologic assessment of these samples revealed that 2%–5% of the cells in the lymphocyte pools had myeloid morphology (Figure S2C). In addition, low levels of sequence contamination (0.5%–1%; Figure S2D) occurred among indexed samples that were pooled together for targeted sequencing.

In sum, these data show that most AML subclones have an equal propensity to circulate into the peripheral blood. In addition, the majority of myeloid cells in the peripheral blood are derived from a common myeloid-skewed, transformed HSPC, either because this transformed cell defaults toward myeloid differentiation or because the AML-causative mutations block the potential for lymphoid differentiation. A consequence of this is that the VAFs of founding clone variants in the peripheral blood samples are influenced not only by the blast percentage, but

also by the percentage of "contaminating" normal lymphocytes in the sample (Figure S2E).

AML Subclones Can Have Distinct Cellular Phenotypes

Although all AML subclones were represented among the different myeloid populations in the peripheral blood samples (Table S5), some cases showed dramatic differences in the subclonal architecture in purified cell populations. A particularly striking example was AML31, an acute monocytic leukemia characterized by leukemic cells with predominantly monocytic features (Figure 3A) and only rare blasts (~3%) in the peripheral blood. To enhance tracking of subclones in this AML across samples, we assigned leukemic variants to distinct clusters using a computational subclone identification method (C.A.M., Brian S. White, Nathan D. Dees, M.G., Obi L. Griffith, John S. Welch, Ravi Vij, Michael H. Tomasson, T.A.G., M.J.W., Matthew J. Ellis, William Schierding, J.F.D., T.J.L., E.R.M., R.K.W., and Li Ding, unpublished data) that clusters variants with similar VAFs across multiple samples. This approach identified three subclones (subclones 1–3): Subclones 1 and 2 were present in the de novo bone marrow but disappeared at relapse, and subclone 3 was rare at presentation but dominant at relapse (Figure 3B; Ding et al., 2012). Targeted sequencing of purified myeloid blasts from de novo AML31 (identified by dim CD45 expression, low side scatter [SSC], and the presence of Auer rods: Figure 3A, inset) revealed an enrichment of variants associated with subclone 3 (Figure 3C), the main leukemic population present in the relapse sample. Consistent with this observation, AML31 exhibited predominantly blastic features at relapse, rather than the monocytic morphology of the de novo leukemia. In addition, cells from AML28 with flow characteristics of maturing myelomonocytic cells (Figure 3D) showed enrichment of subclonal variants (subclone 4 variants) previously found at disease relapse (Ding et al., 2012). Characterization of the myeloid blasts and monocytes in AML87, an acute myelomonocytic leukemia (FAB M4), also revealed that different subclones had distinct morphologic properties (Figure 3E). Whereas such dramatic enrichment was not observed in all cases (see Figures 2 and S3; Table S5), these data demonstrate that some subclones correspond to distinct populations of cells with characteristic morphologic and/or immunophenotypic properties. This implies that some subclones are functionally distinct in their ability to differentiate into more mature cells.

Single-Cell Genotyping Verifies Imputed AML Subclones

To confirm the identity of subclones at the single-cell level and to establish their hierarchy within the tumor, we used an amplicon-based sequencing approach to genotype the founding clone and subclonal SNVs in single cells purified by cell sorting. Leveraging the findings for AML28 (where purified blasts and maturing myelomonocytic cells were enriched for different subclonal variants; see Figure 3D), we isolated individual cells from total myeloid cells (excluding lymphocytes) and from maturing myelomonocytic cells identified by flow cytometric and immunophenotypic features (Figures 4A) and verified these populations by morphologic examination (Figure S4A). Whole-genome amplification was then used to prepare DNA from each cell for subsequent PCR amplification and sequencing of ten known somatic mutations and nine known heterozygous SNPs (used to assess the frequency of allele-specific amplification and to establish accurate genotyping criteria; Figures S4B–S4F). The fraction of single cells harboring each of the ten leukemic variants was in close agreement with predictions based on the variant allele fractions found in the unfractionated sample (Figures 4B and 4C). Founding clone variants and variants in subclones 1 and 2 were present in the majority of the purified myeloid cells, but subclone 4 variants were not detected in the total myeloid cell pool (Figure 4B). In contrast, subclone 4 variants were significantly enriched in the purified maturing myelomonocytic cells (Figure 4C). We next assembled the genotypes for each individual cell to establish the relationships of the subclonal variants within the tumor. This demonstrated that the predominant genotype among all myeloid cells included variants in subclones 1 and 2, in addition to founding clone variants; cells with subclone 1, 2, and 3 variants were the next most common (Figure 4D). It also established that subclone 3 arose from subclone 2 and implied the existence of an ancestral cell that contained only subclone 1 and founding clone variants. The subclone 4 genotype was most common in cells with the maturing myelomonocytic phenotype and arose directly from the founding clone, i.e., independently from subclones 1, 2, and 3 (Figure 4E). We also performed genotyping of single cells isolated from the unfractionated peripheral blood of AML31, which also confirmed the allele fractions and subclone genotypes imputed from the unfractionated bone marrow sample (Figures 4F and 4G).

Some AML Subclones Have Unique Functional Properties In Vitro

The findings noted above suggest that some subclones have distinct functional properties, including different capacities for hematopoietic differentiation in vivo. In addition, the presence of multiple subclones in some samples implies that these subclonal populations may have cell-intrinsic advantages that allow them to expand faster than the founding clone. To determine whether subclones have different growth properties in an experimental system, we used an established in vitro stromal coculture method (Klco et al., 2013) to expand three different de novo AML samples with well-defined subclonal populations at presentation and relapse (AMLs 1, 31, and 43; Ding et al., 2012). These de novo AML samples were cultured on stromal cells in the presence of human hematopoietic cytokines for 7 days (Figures 5A and 5B) and then analyzed by targeted sequencing of all known variants in these genomes. Although the subclonal composition of AML1 (Figure 5C), AML43 (Figure 5D), and AML88 (Figure S5A) were stable after 1 week of expansion, the subclonal architecture of AML31 changed dramatically—subclone 3 variants showed substantial enrichment in culture, increasing from a mean VAF of ~2% to almost 20% within 7 days (Figures 5E and S5B). As described above, this subclone was highly enriched in myeloid blasts and was also the dominant subclone at relapse.

Preferential Subclone Engraftment in Immunodeficient Mice

Xenotransplantation studies are commonly used to study functional heterogeneity of primary AML samples, including the identification of phenotypes associated with leukemia-initiating cell populations. To examine the patterns of subclonal engraftment

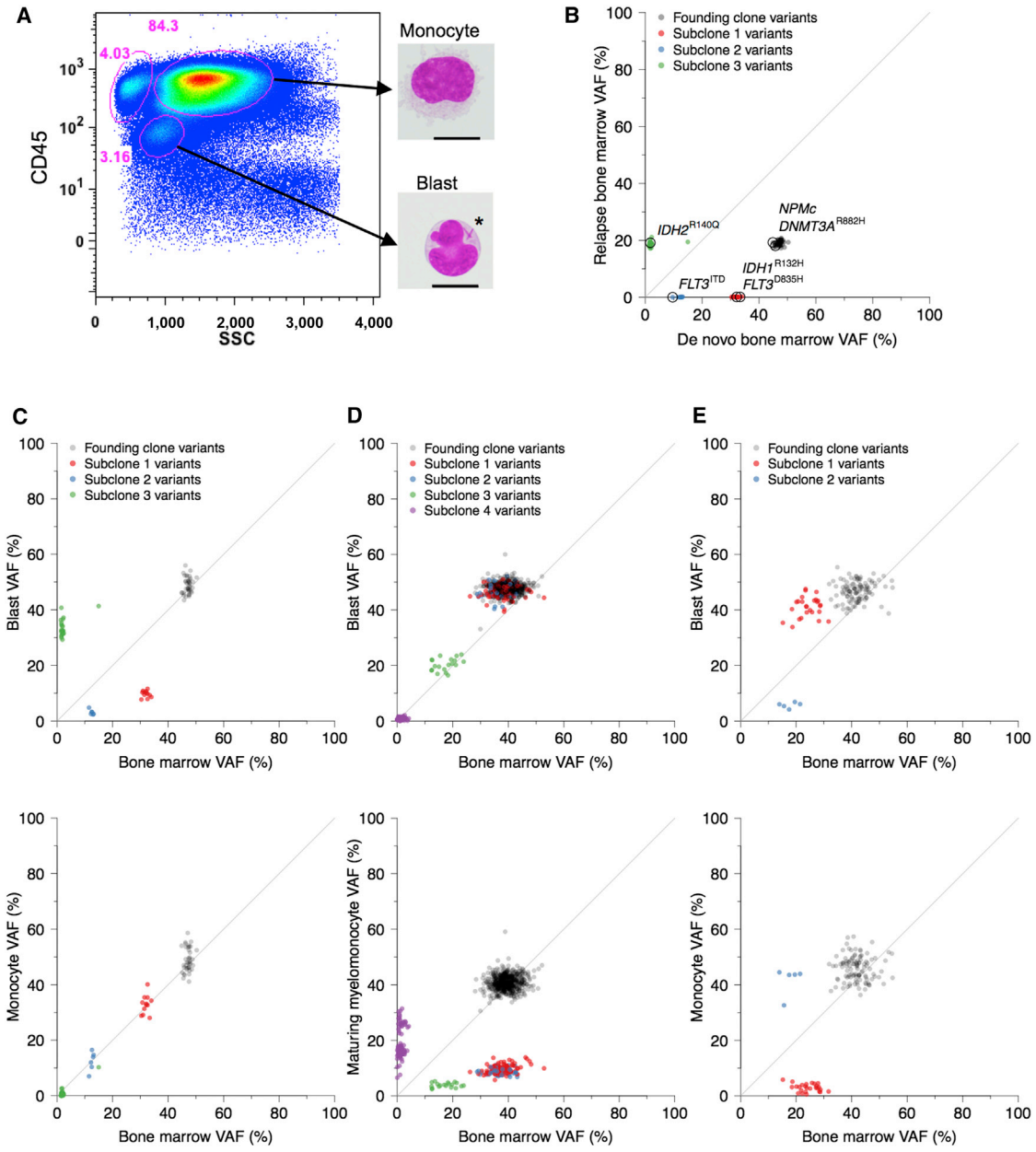


Figure 3. Subclonal Enrichment in Distinct Myeloid Subpopulations

(A) Cells from AML31 were flow-sorted, and distinct morphologic populations were confirmed by morphologic examination. Note Auer Rod present in the myeloblast (*). Scale bar represents 10 μm.

(B) Clonality plot demonstrating the relationship of the de novo AML31 tumor and the relapse leukemia (Ding et al., 2012). Important coding mutations are highlighted.

(C–E) Clonality plots demonstrating the relationship of the de novo leukemias to different cell populations; (C) AML31, blasts (top) and monocytes (bottom); (D) AML28, blasts (top) and maturing myelomonocytic cells (bottom); and (E) AML87, blasts (top) and monocytes (bottom). For all clonality plots, only variants in computationally identified clusters (SciClone; C.A.M., Brian S. White, Nathan D. Dees, M.G., Obi L. Griffith, John S. Welch, Ravi Vij, Michael H. Tomasson, T.A.G., M.J.W., Matthew J. Ellis, William Schierding, J.F.D., T.J.L., E.R.M., R.K.W., and Li Ding, unpublished data) that are diploid (copy number = 2) and with coverage depth >50× are shown.

See also Figure S3.

following xenotransplantation, we transferred cells from nine different AML samples into unconditioned immunodeficient mice (one million viable cells/mouse via lateral tail vein injections), including six samples that were concurrently injected into mice from the NOD-*scid*-IL2Rγ^{null} (NSG) strain (Ito et al., 2002; Sanchez et al., 2009; Sarry et al., 2011) and also the NSG-SGM3 strain (Wunderlich et al., 2010), which expresses the human hematopoietic cytokines stem cell factor, granulocyte

macrophage-colony stimulating factor, and interleukin 3; cells from AML1, AML62, and AML88 were injected only into NSG-SGM3 mice. A total of 73 mice (31 NSG and 42 NSG-SGM3) were injected; mice were sacrificed at 12–16 weeks or at the first sign of illness. Eight of the nine AML samples engrafted, with the NSG-SGM3 strain achieving equivalent or higher engraftment efficiencies in all cases, as expected (Figure S6A; Table S6).

To assess the subclonal composition of the xenografts, human leukemia cells were purified from 52 bone marrow xenografts by flow cytometry using antibodies to human CD45, CD33, and/or CD34 for subsequent sequencing. Flow cytometric characterization showed substantial immunophenotypic variability among the xenografts that was in part dependent on the mouse strain used. For example, AML31 xenografts (n = 11) showed higher CD34 expression in NSG-SGM3 mice compared to NSG mice (Figure 6A). Sequence analysis of AML31 xenografts revealed that subclone 3 was the dominant cell type in all five NSG-SGM3 mice (mean VAF 37%–46%), whereas the majority of the NSG animals (5/6) preferentially engrafted with subclone 1 (Figure 6B), which was the most abundant cell type in the de novo sample. This pattern supports the single-cell analysis of AML31, which showed that subclones 1 and 3 arose from the founding clone independently; accordingly, variants assigned to subclones 1 and 3 were mutually exclusive in the xenografts and were never present at similar allele fractions. These results also suggest that subclone 3 possesses a cell-intrinsic functional advantage, which appears to be enhanced by the cytokine milieu of the NSG-SGM3 mice; it was able to engraft and outcompete other subclones, despite being present in only a small fraction of the total cells in the injected sample (mean VAF: 2.5%). Notably, all the xenografts had significant subclonal restriction, and no xenografts had a subclonal architecture that was identical to that of the input leukemia.

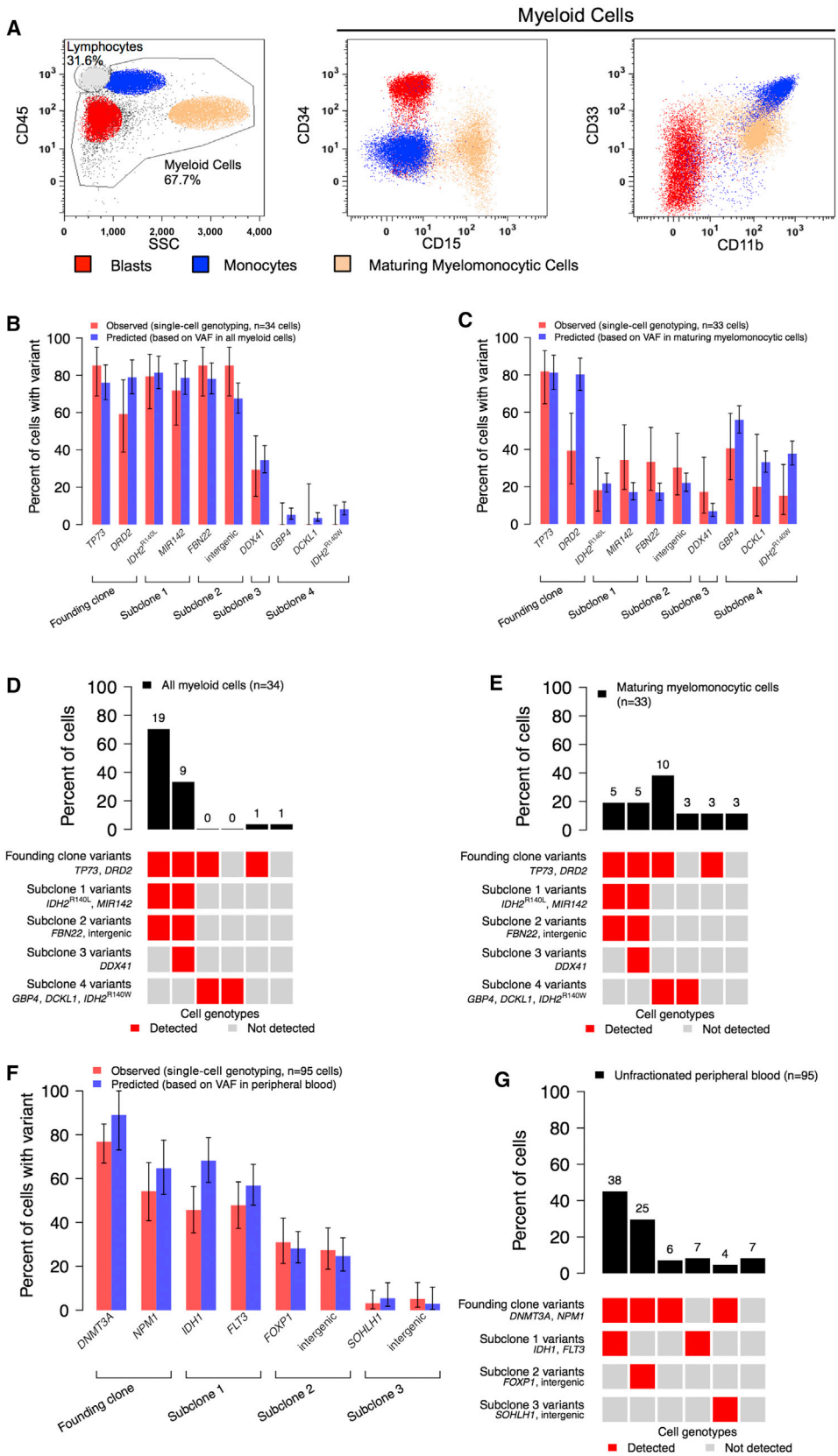
Immunophenotypic and sequence analysis of the xenografts from the other seven AML samples also demonstrated subclonal restriction (Figures S6B and S6C). In addition, immunophenotypic differences between the xenografts obtained from identical human AML samples injected into NSG and NSG-SGM3 mice were observed in other cases. For example, AML63 xenografts from NSG mice consistently displayed a $CD34^{+}CD33^{-}$ immunophenotype, whereas xenografts obtained from NSG-SGM3 mice were $CD34^{-}CD33^{+}$ (Figure S6B). In contrast to AML31, all of the AML63 xenografts were composed of the same subclone, suggesting that, in this sample, the same initiating subclone can display disparate surface antigen markers based on the cytokine milieu in which it develops.

The studies noted above tracked variants previously discovered by WGS (mean coverage ~30×), which has a limit of detection for somatic variants of ~10%. Thus, some rare variants that became dominant in the xenografts were probably below the limit of detection in the primary sample examined by whole-genome sequencing. In addition, previous studies have suggested that mutations can arise during passaging of human cells in immunodeficient mice (Li et al., 2013). To discover additional somatic variants within the engrafted subclones, we performed targeted capture sequencing of the 264 genes that are recurrently mutated in AML and other myeloid neoplasms (Cancer Genome Atlas Research Network, 2013). In most cases, there were no mutations in the xenografts that had not been identified in the initial tumor. However, we discovered a canonical *IDH1* R132H mutation in all six of the AML62 xenografts (mean VAF of xenografts: 45.86%; range: 36.84%–58.44%). In retrospect, this mutation was then detected in the de novo sample (5.44%; 8 of 147 reads). Similar analysis demonstrated that this variant occurred at the level of sequencing errors (1 of 156 reads) in the relapse sample (Figure S6D). This implies that a rare subclone in the de novo AML sample containing this *IDH1* variant preferentially engrafted in 6/6 mice, but did not contribute to the relapse in this patient; this pattern contrasts with what was observed for AML31, where a rare subclone with enhanced engraftment properties emerged at relapse. Lastly, only one xenograft (AML94, NSG3) contained a xenograft-specific mutation (*TP53*, e8-1; splice site mutation) that may have pathologic significance.

In sum, xenotransplantation of de novo AML samples resulted in skewing of the subclonal architecture in all samples, implying that genetically defined subclones usually represent the dominant engrafting cell population in an individual mouse. We detected subclones in 38 of the 52 xenografts from the eight AML samples. Most commonly, we observed engraftment by a single subclone (27 of 38 xenografts had only a single subclone with a mean VAF >5%). Of the 27 xenografts with monoclonal engraftment, 12 demonstrated engraftment and outgrowth of a minor subclone in the primary sample.

DISCUSSION

Studies of genetic heterogeneity in cancer thus far have focused on identifying the somatic variants that mark tumor subclones as a way to understand the origin, population dynamics, and evolutionary history of a tumor. However, it is not yet clear whether genetically defined tumor subclones possess unique phenotypic and/or functional properties that may explain some aspects of a tumor's history and perhaps predict its future potential for relapse or resistance to therapy. Here, we used WGS followed by capture-based targeted deep sequencing to define the clonal architecture of unfractionated bone marrow cells of AML patients and then to follow these subclones after experimental manipulation. We purified individual cell populations with well-established cellular phenotypes and found that most myeloid cells in the peripheral blood at the time of AML diagnosis (even those that were morphologically nonblastic) were derived from the AML founding clone; in some cases, genetically defined subclones corresponded to distinct cell populations that could be identified by cell-surface markers. We also used xenotransplantation of unmanipulated tumor samples in immunodeficient mice (a mainstay in the experimental characterization of primary cancer samples) to understand the functional heterogeneity of tumor subclones and found that only one subclone engrafted in most mice even though multiple subclones were present in the sample that was injected. In some instances, the engrafting subclone represented only a small fraction of the injected cells (<10%), implying that some subclones have a cell-intrinsic advantage (due to increased engraftment potential, excess proliferation, and/or other factors) after transplantation. These observations, based on tracking tumor subclones with hundreds of somatic variants in primary AML samples with a variety of different initiating mutations, show that functional and phenotypic



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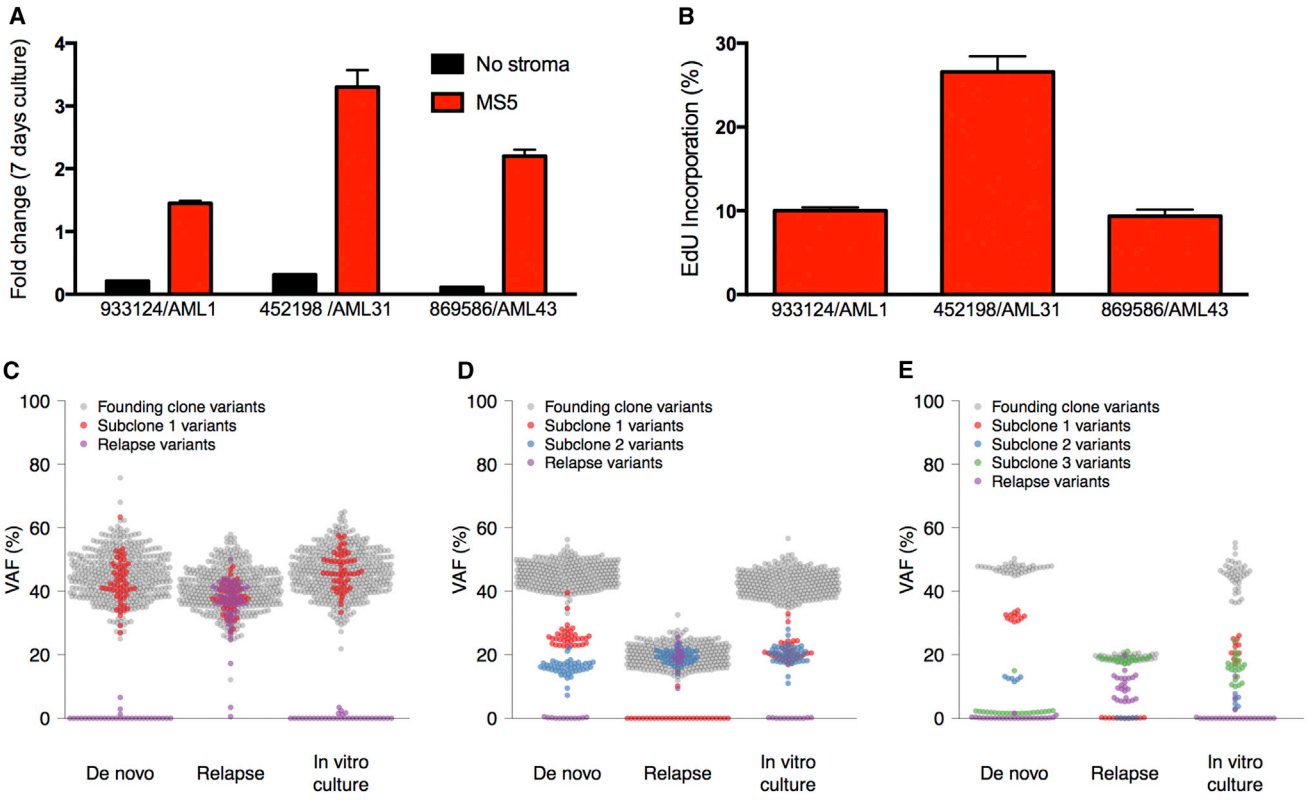


Figure 5. Rare Subclones Can Have Unique In Vitro Growth Properties

Cells were grown in human hematopoietic cytokines in the presence or absence of MS5 stromal cells for 7 days.

(A) Fold change in cell number after 7 days.

(B) Percentage of 5-ethynyl-2′-deoxyuridine (EdU)-positive cells; cells were incubated with EdU for the last 18 hr of culture. Mean values (n = 3) are shown; error bars represent SD.

(C–E) Subclonal architecture of AML1 (C), AML43 (D), and AML31 (E) at day 0 (label: de novo), relapse (Ding et al., 2012), and day 7 of culture (label: in vitro). Each column shows the VAFs (indicated on the y axis) of founding clone and subclonal variants for each case.

See also Figure S5.

heterogeneity of subclones are manifest not only in experimental systems, but also in primary tumor samples at disease presentation.

Many studies of functional heterogeneity in leukemia have used xenotransplantation to characterize leukemia stem cells (LSCs; also known as leukemia-initiating cells), which are rare cells that are functionally defined by unique cell-surface markers as well as potential for engraftment and prolonged self-renewal in immunodeficient mice (Bonnet and Dick, 1997; Jaiswal et al., 2009; Jin et al., 2009; Lapidot et al., 1994). More recent studies have found that engraftment is not necessarily restricted to specific cellular phenotypes, and models have been proposed where the cancer stem cell phenotype is stochastic and subject to equilibrium within the tumor cell population (Gupta et al., 2011;

Figure 4. Single-Cell Genotyping of Primary AML Samples

Individual cells from AML samples 28 and 31 were isolated by flow cytometry, and single-cell genotypes were determined by whole-genome amplification and amplicon-based sequencing.

(A) Flow-sorting strategy for AML28, in which individual myeloid cells (excluding lymphocytes) or maturing myelomonocytic cells were collected.

(B–G) Single-cell genotyping results. (B), (C), and (F) show the proportion of cells harboring leukemia-associated variants predicted from the VAFs in unfractionated cells (in blue) and observed in individual cells (in red) for individual myeloid cells (B) and maturing myelomonocytic cells (C) from AML28 and peripheral blood from AML31 (F). For each comparison, the predictions from unfractionated cells used VAFs obtained directly from deep-sequencing read counts (and multiplied by two to correct for heterozygosity), and the observed single-cell proportions were obtained from single-cell genotyping experiments. Error bars show the 95% binomial confidence interval for each point estimate. (D), (E), and (G) show single-cell genotype frequencies from sorted myeloid cells (D) and maturing myelomonocytic cells (E) from AML28 and peripheral blood from AML31 (G). The bottom panel in each figure shows the observed single-cell genotypes, with each column representing a single observed genotype that consists of at least one of the founding clone or subclonal variants in each subclone (indicated in red). The height of the vertical bars and corresponding numbers show the frequency and absolute number of cells with the indicated genotype, respectively. Only genotypes observed in more than two cells in each single-cell experiment are shown; additional genotypes were also observed in low numbers of cells that are likely due to allele "dropout" (due to unequal amplification of the two alleles), which we estimated to be 30% using control data from heterozygous SNPs (see Supplemental Experimental Procedures).

See also Figure S4.

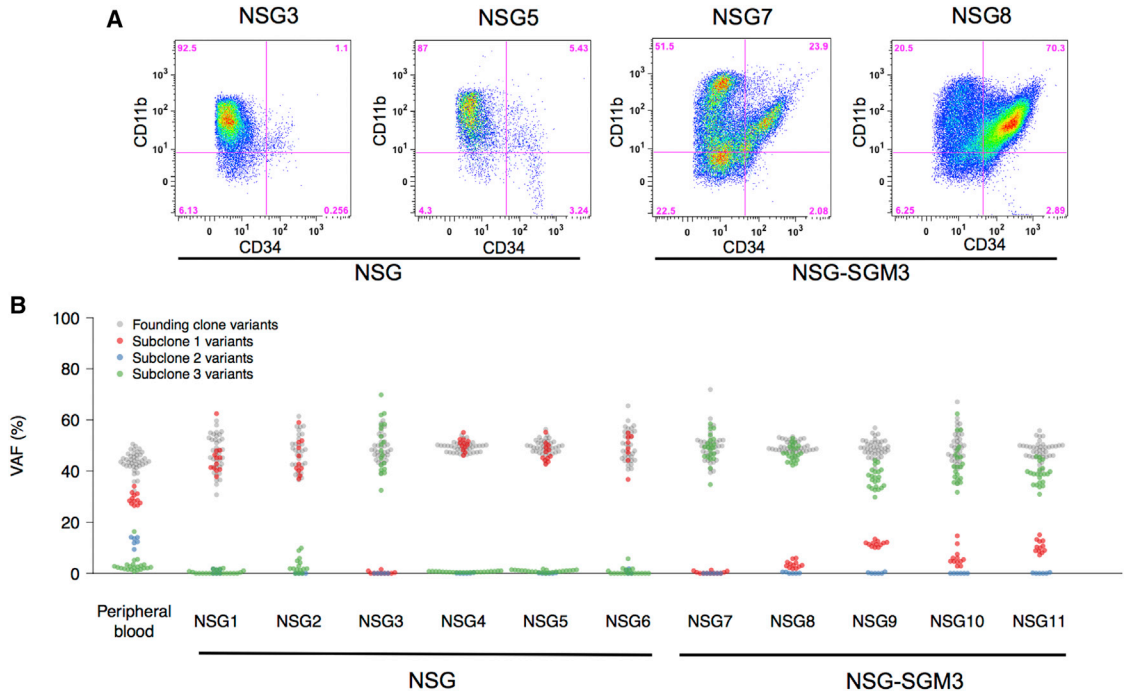


Figure 6. Phenotype and Subclonal Composition of AML31 Xenografts

Mice were injected with unfractionated peripheral blood leukemia cells and followed for 12–16 weeks, at which time cells were harvested from the bone marrow. (A) Representative examples of the immunophenotypic properties of AML31 xenografts in individual NSG and NSG-SGM3 mice. Shown are expression patterns of human CD34 and CD11b in the human myeloid leukemia cells (mCD45$^-$, hCD45$^+$, and CD33$^+$).

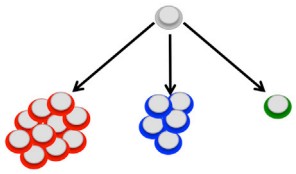
(B) Human CD45$^+$CD33$^+$ cells were purified by cell sorting, and DNA was analyzed by targeted deep sequencing of all known somatic variants in the sample. The VAFs for all founding clone and subclonal variants are shown for each individual AML31 xenograft and the peripheral blood sample (input, far left).

See also Figure S6 and Table S7.

Sarry et al., 2011). Our study adds another layer of complexity by showing that discreet subclones (not the founding clone) generally define the engrafting population in individual mice and that these subclones do not have equal engraftment and/or outgrowth potential. This argues against a purely stochastic model of functional diversity, because specific subclones preferentially engrafted in multiple experiments despite their low frequency in the injected sample. However, in some samples, the engrafting subclone differed across experiments and between immunodeficient mouse strains (e.g., AML31), demonstrating that different initiating populations with unique subclonal mutations can exist within a single AML sample. There was also no consistent relationship between subclone engraftment potential and the propensity of a subclone to emerge at relapse (see AMLs 62 and 71; Figure S6C). This implies that functional and phenotypic properties of LSCs (such as engraftment, self-renewal, and chemoresistance) are experimentally variable and not directly related to the evolutionary history of the tumor, suggesting that the selective pressures imparted during xenotransplantation may not be equivalent to those imparted in vivo. In contrast to our findings, Clappier et al. (2011) established a relationship between xenotransplantation potential and T lymphoblastic leukemia/lymphoma relapse. This discrepancy may reflect inherent biological differences between these leukemias, including their disparate patterns of mutations, as well as experimental differences, such as animal conditioning, engraftment method, and cell dose. Additional studies will be needed to better define the relationship between engraftment potential and relapse for hematologic malignancies.

We anticipate that different xenotransplantation and cell manipulation approaches (i.e., extent of preconditioning, cell dose, level of immunodeficiency of the recipient mouse, transplantation route/procedure, number of passages, and timing of analysis posttransplant) could also alter the subclonal architecture of the engrafting tumor (Kelly et al., 2007; McDermott et al., 2010; Taussig et al., 2008; Wunderlich et al., 2013). In particular, the number of injected cells may alter the competitive balance of subclones (Notta et al., 2011), because the frequency of initiating cells may be variable among different subclones. It is also possible that more permissive xenotransplantation conditions (such as intrafemoral injections into irradiated recipients) may provide less selective pressure and allow for engraftment of multiple subclones. Ultimately, these different experimental approaches will need to be formally tested in light of the data presented in this study. Regardless of these uncertainties, our results do not invalidate the use of xenotransplantation models to study cancer. However, they do highlight the need for genomic characterization of tumors both before and after xenotransplantation. In fact, this study suggests that xenotransplantation may also be exploited as a means to isolate subclones for further study.

We were also able to integrate both technical and functional assays to demonstrate that subclones are unique genetic entities derived from a common ancestral cell (Figure 7). Although



	Founding Clone	Subclone 1	Subclone 2	Subclone 3
Mutations	*DNMT3A* R882H *NPMc*	*DNMT3A* R882H *NPMc* *FLT3* D835H *IDH1* R132H	*DNMT3A* R882H *NPMc* *FLT3*-ITD	*DNMT3A* R882H *NPMc* *IDH2* R140Q
% of de novo sample	-	70%	24%	2%
% of relapse sample	-	0%	0%	100%
Morphology	?	Monocytic	Likely monocytic	Blastic
Engraftment in NSG		5/6	0/6	1/6
Engraftment in NSG-SGM3		0/5	0/5	5/5
Predicted response to FLT3 inhibitors	-	+	+	-
Predicted response to IDH1/IDH2 inhibitors	-	+	-	+

Figure 7. Model of AML31 Subclonal Architecture and Predicted Phenotypes

Schematic representation of the implications of AML clonal heterogeneity, based on the integrated analysis of AML31. The "% of de novo sample" values were calculated from sequencing the unfractionated AML samples and are consistent with data obtained from the interrogation of individual cells.

previous studies of AML and MDS have inferred the clonal architecture in unfractionated bone marrow samples through the identification of clusters of mutations with similar VAFs (Ding et al., 2012; Walter et al., 2012; Welch et al., 2012) and others have demonstrated the hierarchical nature of cancer at the single-cell level (Potter et al., 2013), we employed multiple orthogonal approaches using the same AML samples to verify the identity and stability of imputed subclones. We observed that subclonal variants were present at the expected fractions among a population of single cells and mutations with similar VAFs in the unfractionated tumor were present in the same individual cells. Similarly, individual subclones that engrafted in immunodeficient mice contained the expected group of variants for that subclone, in addition to all founding clone mutations. Lastly, purified cell populations with well-established morphologic and immunophenotypic features corresponded to distinct subclones in some cases. Some of these resembled normal differentiating hematopoietic cells, suggesting that some AML subclones are capable of myeloid differentiation despite harboring known AML driver mutations.

In this study, we were not able to define specific genetic determinants that explain the functional heterogeneity among tumor subclones. The patterns of subclone engraftment we observed in immunodeficient mice were inconsistent—some leukemias (e.g., AML31 and AML94) show variable retention (or loss) of different subclones, whereas others (e.g., AML88 and AML63) consistently engrafted a single subclone. We did not find that specific mutations consistently conferred preferential engraftment, nor did we identify new mutations in known leukemia-associated genes that were clearly responsible for enhanced engraftment, survival, or proliferation. For example, subclones with canonical mutations in the *FLT3* tyrosine kinase did not preferentially engraft, despite the fact that this mutation causes potent activation of this signaling kinase and has been reported to result in higher engraftment in NSG mice (Sanchez et al., 2009). The variable presence of key mutations in engrafting subclones would influence results from trials designed to test targeted chemotherapeutic agents in xenografted mice, such as *FLT3* inhibitors (Smith et al., 2012; Williams et al., 2013), or drugs targeting other mutations that often occur in subclones, such as those in *RAS* genes, or *IDH1*/*IDH2* (Losman et al., 2013; Rohle et al., 2013); clearly, the identity of the engrafting subclone (and its mutations) will be required to understand the response to a targeted drug (see Figure 7). However, the association of genetically defined subclones with enhanced engraftment potential suggests that some stable feature within these subclones may be responsible for their altered function. Although these genetic (or epigenetic) factors remain to be discovered, it is clear that subclones are discrete entities with important functional differences that may be genetically determined.

Although this study focused on AML, these observations likely extend to the experimental study of cancer in general, as well as its diagnosis and treatment. The presence of cancer-associated somatic mutations in cellular populations that are morphologically benign has implications for diagnostic testing of cancer samples and the use of phenotypically normal tissue for research studies. Our finding that mouse xenografts can have a skewed clonal architecture when compared to the parental tumor means that functional data obtained from these models, such as the capacity for self-renewal and chemoresistance, should not be generalized to the entire tumor or to other subclones that may contain different mutations without a rigorous genomic analysis of the xenograft. Integration of xenotransplantation and genomic data also demonstrates that the LSCs (defined functionally in immunodeficient mice) and the founding clone of a patient's tumor (defined genetically) are not the same; in fact, there does not appear to be a consistent relationship between the cells that engraft in mice and the tumor's evolutionary hierarchy. There is already some evidence that these conclusions generalize to other cancer models, because preferential engraftment of rare subclones has been demonstrated in xenotransplantation studies of ALL and breast cancer samples (Anderson et al., 2011; Ding et al., 2010; Notta et al., 2011), although this observation has not been true in all studies using solid tumor models (Kreso et al., 2013; Li et al., 2013). In this study, we observed engraftment of a rare, previously undetected subclone in one case (AML62), which was only identified through unbiased sequencing of the xenograft. It is possible that this approach, along with expression and epigenetic profiling of xenografts, may provide a mechanism for understanding the mutations and phenotypes that are under selective pressure in these model systems. The fact that primary AML samples (both from marrow and blood) contain the entire subclonal repertoire of the tumor and are therefore not subject to the same sampling biases of solid tumors suggests that this disease provides a very powerful platform for understanding the functional and genetic heterogeneity in cancer. Ultimately, functional and genetic data will have to be integrated for experimental systems to accurately model cancer and to develop therapeutic strategies to effectively treat it.

EXPERIMENTAL PROCEDURES

Primary AML Samples

All cryopreserved AML samples were collected as part of a study approved by the Human Research Protection Office at Washington University School of Medicine after patients provided informed consent in accordance with the Declaration of Helsinki. Informed consent explicit for whole-genome sequencing was obtained for all patients in this study on a protocol approved by the Washington University Medical School Institutional Review Board.

Flow Cytometry

Cryopreserved AML cells were thawed as previously described (Klco et al., 2013). The following human antibodies were used: anti-CD45 PerCP-Cy5.5 (eBioscience; clone 2D1), anti-CD33 phycoerythrin (PE) or antigen-presenting cell (APC) (eBioscience; clone WM-53), anti-CD19 APC (BD Biosciences; clone HIB19), anti-CD34 (PE-pool; Beckman Coulter Genomics; PN IM1459U), anti-CD15 fluorescein isothiocyanate (BD Biosciences; clone HI98), anti-CD11b BV421 (BD Biosciences; ICRF44), and CD3 V450 (eBioscience; clone OKT3). Live cell populations were discriminated initially via CD45/SSC scatterplots, and different subpopulations were defined as follows: blasts, $CD45^{dim}/SSC^{low}$, CD33+; lymphocytes, $CD45^{bright}/SSC^{low}$, CD33−; monocytes, $CD45^{int/bright}$, SSC^{inter}, CD33+; and maturing myelomonocytic cells, $CD45^{int}$, SS^{high}, $CD33^{dim/neg}$. When available in sufficient numbers, lymphocytes were further sorted into CD19+ and/or CD3+ populations. For NSG experiments, human hematopoietic cells (human CD45 positive) from the bone marrow were separated from murine cells (murine CD45) via flow cytometry; human cells were further isolated by expression of either CD33 and/or CD34. Cells were sorted on a modified Beckman Coulter MoFlo into PBS; genomic DNA (gDNA) was prepared using a QIAamp DNA mini kit (QIAGEN). For capture experiments, a minimum of 100 ng of gDNA was required for sequencing. Cytospins were performed using a Shandon Cytospin 3, and cells were stained using Wright-Giemsa (Sigma). Images were obtained with an Olympus BX51 microscope equipped with an Olympus DP26 camera. Morphologic studies were performed by a board-certified hematopathologist (J.M.K.).

Xenotransplantation Studies

Animals were used in accordance to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Washington University. Mice were produced at Washington University School of Medicine using breeders obtained from the Jackson Laboratory (NSG stock 005557; NSG-SGM3 stock 013062). Six- to ten-week-old unconditioned mice were injected with one million viable cells via lateral tail vein route. Mice were treated with antibiotics for 2 weeks after injection and then followed for 12–16 weeks. For these studies, engraftment was defined >1% human CD45 and CD33 (or CD34) positivity at the time of sacrifice. This threshold was determined rather than the standard of 0.1% (Sarry et al., 2011) to ensure that sufficient material would be present for downstream analyses. For all NSG/NSG-SGM3 comparisons, cells from a single cryovial were injected at the same time into age-matched animals.

Variant Discovery and VAF Measurements

Whole-genome sequencing and capture validation was performed as described in the Supplemental Experimental Procedures. All variant count data were obtained from raw BAM files using bam-readcount (https://github.com/genome/bam-readcount) following filtering of reads for low mapping quality (<10), low base quality (<10), and reads with more than four mismatches. Indel variant counts were obtained separately using a custom script to align overlapping reads from the raw BAM file to short sequences with either the reference or alternate indel allele using cross_match (http://www.phrap.org/phredphrap/phrap.html) and tabulate variant counts based on the highest scoring alignment to these two sequences. All variant counts and fractions were manipulated and visualized in R.

ACCESSION NUMBERS

The database of Genotypes and Phenotypes accession number for the AML tumor sequence variants is phs000159.

See Supplemental Experimental Procedures for additional information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.01.031.

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Cancer Cell
Article

Genome Sequencing of SHH Medulloblastoma Predicts Genotype-Related Response to Smoothened Inhibition

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SUMMARY

Smoothened (SMO) inhibitors recently entered clinical trials for sonic-hedgehog-driven medulloblastoma (SHH-MB). Clinical response is highly variable. To understand the mechanism(s) of primary resistance and identify pathways cooperating with aberrant SHH signaling, we sequenced and profiled a large cohort of SHH-MBs (n = 133). SHH pathway mutations involved *PTCH1* (across all age groups), *SUFU* (infants, including germline), and *SMO* (adults). Children >3 years old harbored an excess of downstream *MYCN* and *GLI2* amplifications and frequent *TP53* mutations, often in the germline, all of which were rare in infants and adults. Functional assays in different SHH-MB xenograft models demonstrated that SHH-MBs harboring a *PTCH1* mutation were responsive to SMO inhibition, whereas tumors harboring an *SUFU* mutation or *MYCN* amplification were primarily resistant.

INTRODUCTION

Medulloblastoma (MB) comprises a collection of clinically and molecularly distinct tumor subgroups that arise either in the cerebellum or brainstem (Grammel et al., 2012; Louis et al., 2007; Taylor et al., 2012). In children, they comprise the most frequent embryonal brain tumor, whereas in adults the disease is relatively rare, accounting for less than 1% of all intracranial malignancies (Louis et al., 2007). Current therapy regimens including surgery, cranio-spinal radiotherapy, and chemotherapy, may cure 70%–80% of patients with MB. Most survivors, however, suffer from long-term sequelae because of the intensive treatment, demonstrating that less toxic treatments are urgently needed. Molecular analyses have shown that there are four major MB subgroups (WNT, Sonic Hedgehog [SHH], Group 3, and Group 4; Taylor et al., 2012). They are highly distinct in tumor cell histology and biology, and in addition show divergent clinical phenotypes such as patient demographics, tumor dissemination, and patient outcome (Kool et al., 2012; Northcott et al., 2012a; Taylor et al., 2012). Recent studies, largely focusing on pediatric MB, have utilized next-generation sequencing technologies to map the genomic landscape of MB and to identify novel driver mutations in each molecular subgroup (Jones et al., 2012; Northcott et al., 2012a, 2012b; Parsons et al., 2011; Pugh et al., 2012; Rausch et al., 2012; Robinson et al., 2012). Due to the infrequent occurrence of this disease in adulthood, little is known about the biology and genetics of MB in adults. This also explains why there are few prospective phase III trials for this age group. Most centers treat adult patients with MB either using glioblastoma protocols (which are largely ineffective) or, alternatively, using pediatric MB protocols, although toxicity profiles differ greatly between children and adults, leading to dose-limiting toxicity in a high proportion of adults treated on pediatric protocols (Brandes et al., 2009; Padovani et al., 2007; Spreafico et al., 2005).

Targeted therapy as an alternative treatment option for patients with MB is especially interesting for SHH-MBs. SHH pathway antagonists, primarily those inhibiting at the level of smoothened (SMO), are currently a major area of interest in the pharmaceutical industry because they can potentially be applied in multiple cancers with activated SHH signaling (Lin and Matsui, 2012). Some of these drugs are already in clinical trials for MB (Low and de Sauvage, 2010; Ng and Curran, 2011). SHH-MBs with alterations in downstream SHH pathway genes, however, such as *SUFU*, *GLI2*, or *MYCN*, may demonstrate primary resistance to SMO inhibition (Lee et al., 2007). Furthermore, as has been shown in both humans and mice, tumors may also rapidly acquire secondary resistance to treatment (Dijkgraaf et al., 2011; Rudin et al., 2009; Yauch et al., 2009), suggesting that such inhibitors might be ineffective as a curative option when administered as monotherapy. SHH-MBs present the most common subgroup in infants ($\leq$3 years old) and adults ($\geq$18 years old),

Significance

Our data show that most adults, but only half of the pediatric patients, with SHH-MB will likely respond to SMO inhibition as predicted by molecular analysis of the primary tumor and tested in the SHH xenografts, demonstrating that the next generation of SMO inhibitor trials should be based on these predictive biomarkers. Recurrent mutations in additional pathways suggest rational combination therapies including epigenetic modifiers and PI3K/AKT inhibitors, especially in adults. We also show that tumor predisposition (Gorlin syndrome and Li-Fraumeni syndrome) is highly prevalent in patients with SHH-MB. Each patient with SHH-MB, especially those 4–17 years old with LCA histology, should be tested for germline *TP53* mutations. Separate LFS-MB trials should be considered, sparing radiotherapy and excluding alkylating drugs.

whereas in children (4–17 years old) other subgroups are more prevalent (Kool et al., 2012). Transcriptome analyses and whole genome sequencing have already shown that SHH-MBs are quite heterogeneous (Northcott et al., 2011a; Rausch et al., 2012). Childhood SHH-MBs, for instance, are genetically distinct from those in infants, because they frequently harbor *TP53* mutations and as a result of chromothripsis, their genomes are often dramatically rearranged (Rausch et al., 2012). To preselect patients who might qualify for clinical trials using SMO antagonists or future combination therapies, a better understanding of the biology of SHH-MBs across different age groups is required. We have therefore sequenced the genomes of 133 cases of SHH-MB, including 50 adult and 83 pediatric cases. In addition, we analyzed the tumors for DNA methylation and gene expression.

RESULTS

SHH-MBs in Infants, Children, and Adults Are Genomically Distinct

Unsupervised *k*-means consensus cluster analysis of DNA methylation data (n = 129) identified two major clusters, mainly separating infant from childhood and adult SHH-MB tumors (Figure 1A, left panel). Unsupervised cluster analysis of gene expression data (n = 103) showed similar results, with the infant cases again being the most distinct (Figure 1A, right panel). GISTIC2 analysis of somatic copy number aberrations in all SHH-MB cases (n = 266) reported by MAGIC (Northcott et al., 2012b), however, showed that childhood SHH-MBs are very different from both infant and adult SHH-MBs (Figure 1B). Childhood SHH-MBs typically show much greater genomic instability and are characterized by frequent amplifications of oncogenes including *GLI2*, *MYCN*, and *PPM1D*, most likely due to underlying chromosome shattering (chromothripsis; Rausch et al., 2012).

Next-Generation Sequencing of SHH-MB

To determine the mutational landscape of SHH-MBs across age groups, we sequenced a large series of SHH-MB tumors from infants ($\leq$3 years old; n = 50), children (4–17 years old; n = 33), and adults ($\geq$18 years old; n = 50; Table 1; Table S1 available online). In the discovery cohort of 67 SHH-MBs, analyzed by whole genome or whole exome sequencing, we identified 1,090 nonsynonymous somatic single nucleotide variants (SNVs) and 155 small insertions or deletions (indels), 89 of which introduced translational frameshifts and 9 affected splice sites. In total, 1,054 genes were found to be somatically mutated in this discovery cohort, including 78 with alterations in more than one tumor. In the two replication cohorts (43 pediatric and 23 adults), we identified another 666 nonsynonymous SNVs and 76 indels. For the combined 133 SHH-MBs, we found mutations in 1,156 genes, 215 of which were recurrently altered. All coding somatic SNVs/indels identified are listed in Table S2.

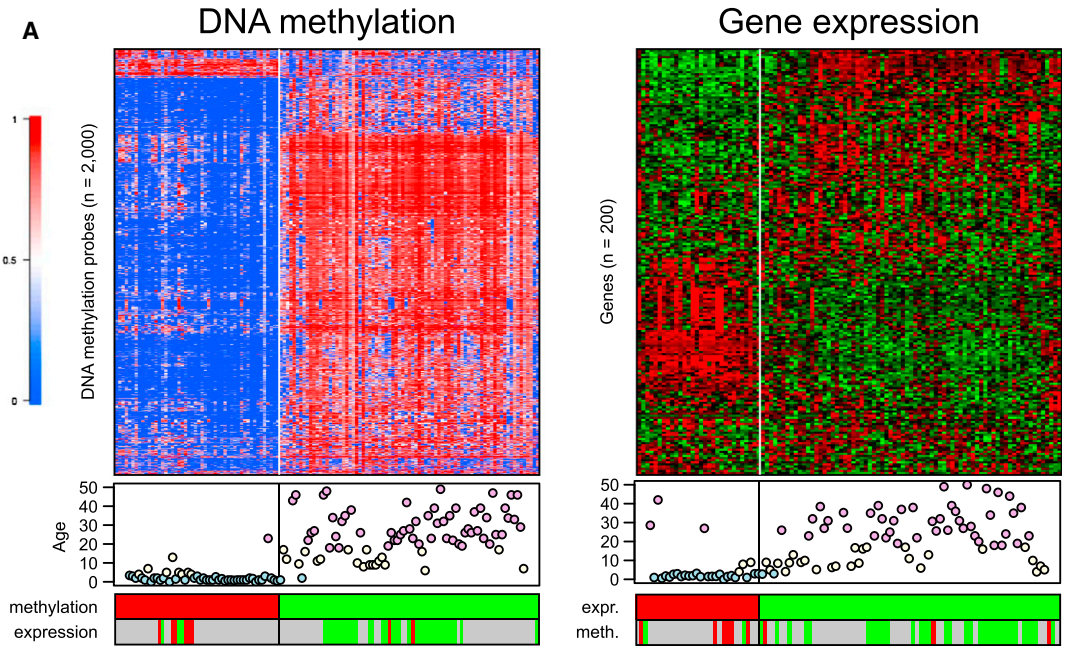
As previously reported (Jones et al., 2012), pediatric SHH-MBs harbored very few nonsynonymous SNVs (infants, 0–13, median 3.0; children [*TP53* wild-type], 1–26, median 9.5; Table S2; Figures 2A and 2B). Exceptions were the eight *TP53* mutated tumors in children, in this discovery cohort all between 9.5 and 14 years old, which harbored on average many more mutations (7–29, median 19.5). WGS data showed that adult SHH-MBs also contained many more nonsynonymous SNVs (9–48, median 25.0), in line with other adult solid tumors. The average number of small indels was also higher in adults (0–10, median 3.0) than in children (0–4, median 1.0) and infants (0–3, median 1.0). Interestingly, there was a much stronger correlation between somatic mutation rate and patient age, both genome-wide (r^2 = 0.58, p = 1.6 × 10^{-9}, Pearson's product moment correlation), and for coding mutations (r^2 = 0.62, p = 2.2 × 10^{-15}), than previously reported across all MB subgroups (Figures 2A and 2B; Jones et al., 2012). Assessment of mutation classes revealed a predominance of cytosine to thymine (C > T) transitions in a CpG context (likely due to deamination of methylated cytosines), as expected for an age-related background mutation pattern (Figures 2C and 2D; Welch et al., 2012). Interestingly, the C > T fraction in the *TP53* mutated cases appeared to be much lower, with a relatively higher proportion of cytosine to adenosine (C > A) transitions. Whether this can be explained by the *TP53* mutation itself remains elusive.

Mutations in the SHH Pathway

Overall, we detected mutations in known SHH pathway genes (116/133 cases; 87%), further substantiating the tumor-driving role of the SHH pathway in this medulloblastoma subgroup (Table S3). As expected, among the most frequently mutated genes were *PTCH1* (60 cases), *SMO* (19 cases), and *SUFU* (10 cases), all mutually exclusive (Figure 3A; Figures S1A–S1C). In addition, we found two *PTCH1* and six *SUFU* mutations in the germlines of eight pediatric patients, including two twin brothers with an identical small indel in *SUFU* (Table S3). The second replication cohort (for which germline controls were unavailable) contained another two cases from twin brothers both with the same inactivating *SUFU* mutation, strongly suggesting that this was also a germline event. For all other samples in this replication cohort, it remains unknown whether any of the identified *PTCH1* or *SUFU* mutations were germline events. Interestingly, while *PTCH1* mutations were found at roughly equal frequency in infants (42.0%), children (36.4%), and adults (54.0%), *SMO* mutations were highly enriched in adult patients (15/19 mutations; p = 1.8 × 10^{-4}), while *SUFU* mutations were almost exclusively found in infants $\leq$3 years old (16/18 mutations; p = 8.4 × 10^{-6}). Mutations in *SMO* and *SUFU* were absent or extremely rare in children (4–17 years old; Figures 3A and 3B). Instead, they frequently harbored *TP53* mutations (16/33 children; p = 1.2 × 10^{-11}), all found in children between 8 and 17 years old. The *TP53* mutations were mutually exclusive with *PTCH1* mutations but often co-occurred with amplifications of *GLI2* (p = 2.5 × 10^{-6}) and *MYCN* (p = 2.8 × 10^{-8}), three events that were rare in infants, young children, and adults (Figures 3A and 3B). In addition, we identified four cases, including three children with a *TP53* mutation, with an amplification of the *SHH* gene. These results show that activating mutations in the SHH pathway are detectable in almost all SHH-MBs, but the type of mutation and targeted genes are largely variable in the different age groups (Figure 3C).

Large Cell/Anaplastic Histology and 17p Loss Are Strongly Associated with TP53 Mutated SHH-MBs

Losses of 9q, 10q, and/or 17p are the most common copy number aberrations associated with SHH-MBs (Kool et al., 2012). All



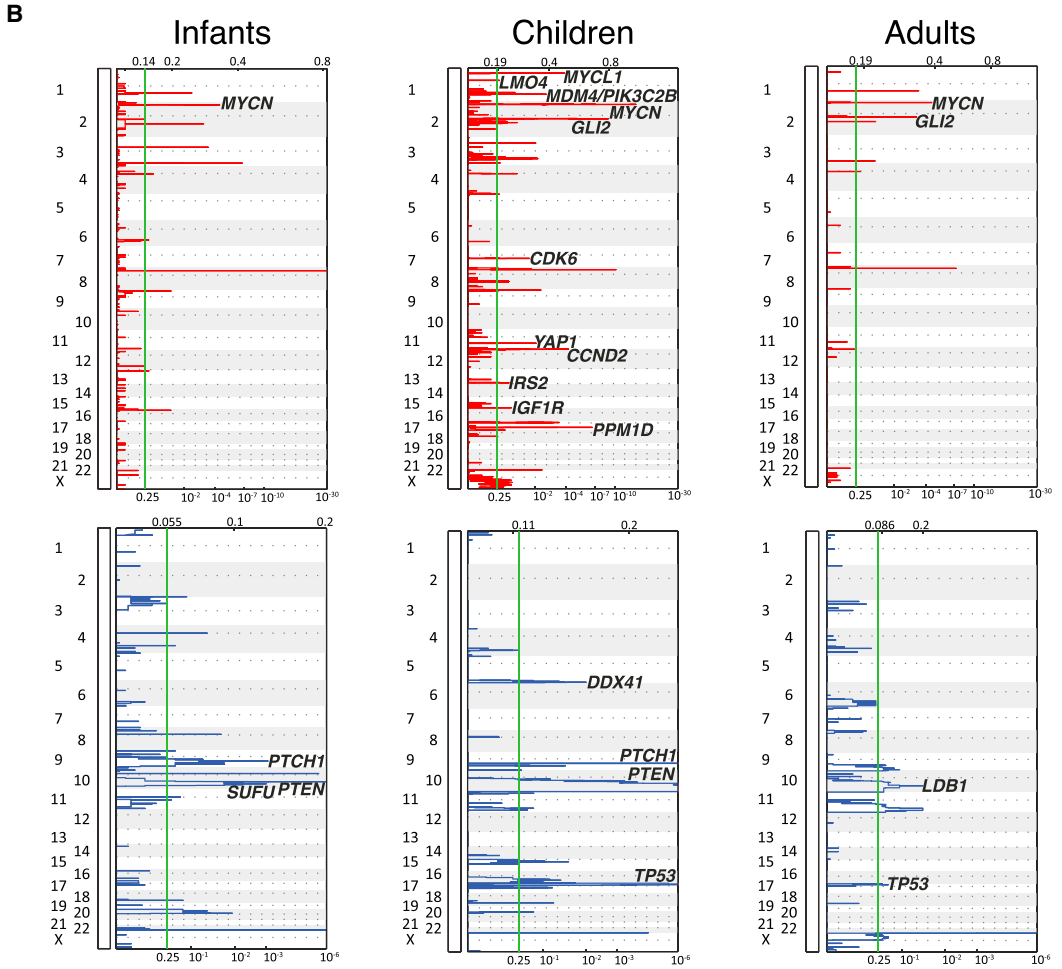


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Table 1. SHH-MB Patient Cohorts

Cohort	Number of Patients
Whole genome sequencing[a]	n = 45
Infants[b]	5
Children[c]	13
Adults[d]	27
Whole exome sequencing[a]	n = 22
Infants	13
Children	9
Adults	0
Targeted sequencing 2734 genes[a] 12	n = 12
Infants	7
Children	5
Adults	0
Targeted sequencing 400 genes[e]	n = 54
Infants	25
Children	6
Adults	23
Immunohistochemistry	n = 155
Infants	31
Children	54
Adults	70

See also Tables S1, S2, S3, and S4.
[a]Tumor-normal pairs were sequenced.
[b]Infants: 0–3 years of age.
[c]Children: 4–17 years of age.
[d]Adults: ≥ 18 years of age.
[e]Only tumors were sequenced.

three were most frequent in childhood cases, with 17p loss highly enriched in *TP53* mutant cases (14/17 had 17p loss; $p = 7.8 \times 10^{-8}$; Figures 3A and 3B). Histology was also unequally distributed between the three age groups, with most large cell/anaplastic (LCA) cases found in childhood (15/21; $p = 4.1 \times 10^{-9}$). Thirteen of these 15 had a *TP53* mutation. Nodular/desmoplastic MB variants were most prevalent in infant cases. Moreover, all four MBs with extensive nodularity (MBEN) were found in infants (Figures 3A and 3B). In contrast to a recent report (Brugières et al., 2012), which was, however, reporting on a larger number of MBEN MBs, only 1/4 MBEN cases in our series had an *SUFU* mutation, while two harbored a *PTCH1*, and one displayed an *SMO* mutation (Figure 3A).

TERT Promoter Mutations Are Highly Recurrent in Adult SHH-MBs

Recently, several groups have reported that *TERT* promoter mutations that drive telomerase activity are frequently found in various cancers, including medulloblastoma, of mainly adult patients (Killela et al., 2013; Koelsche et al., 2013; Remke et al., 2013). Two mutually exclusive hotspot mutations in the promoter region have been reported: C228T and the less frequent C250T. Using our WGS data and data from the replication cohort in which the *TERT* promoter region was analyzed by PCR and Sanger sequencing (Remke et al., 2013), we found that indeed these mutations almost exclusively and with high frequency occur in adult SHH-MBs (Table S1). Strikingly, almost all adult patients for which we had data available had a somatic *TERT* promoter mutation (43/44, 98%; 40 had the C228T mutation and 3 had the C250T mutation). In contrast, in infants and children, only 3/24 (13%) and 3/14 (21%) SHH-MBs, respectively, had a *TERT* mutation (five C228T and one C250T).

DDX3X and Chromatin Modifiers Are Frequently Mutated in Adult SHH-MBs

Other genes previously reported as being recurrently mutated in pediatric SHH-MBs (*MLL2*, *BCOR*, and *LDB1*) were also found in adult SHH-MBs (Figures 4A–4C). Interestingly, however, we identified several recurrent mutations in adult SHH-MBs that were completely absent or very rare in pediatric SHH-MBs, including *BRPF1*, *KIAA0182*, *TCF4*, *CREBBP*, *NEB*, *LRP1B*, *PIK3CA*, *FBXW7*, *KDM3B*, *XPO1*, *PRKAR1A*, and *PDE4D* (Figures 4A–4C; Figures S1D–S1I). Another striking example is the gene encoding the RNA helicase DDX3X, which was mutated in 27 adult SHH-MBs (54%) and only 6 pediatric MBs (7.2%, $p = 4.5 \times 10^{-9}$). *DDX3X* was among the new genes identified in recent sequencing studies of pediatric MB (Jones et al., 2012; Pugh et al., 2012; Robinson et al., 2012). Notably, whereas mutations were found in 50% of WNT-MBs in children (Northcott et al., 2012a), few *DDX3X* mutations were seen in SHH-MBs in these studies (Pugh et al., 2012; Robinson et al., 2012). All identified mutations affected one of the two helicase domains with no difference in their distribution between WNT- and SHH-MBs (Figure S1D). Interestingly, mutations affecting the SWI-SNF complex, also mainly found in the WNT-MBs in children (Jones et al., 2012; Northcott et al., 2012a; Pugh et al., 2012; Robinson et al., 2012), were also frequently seen in adult SHH-MBs.

Pathway analyses, performed separately for the three age groups, showed marked differences in altered processes. In infant cases, developmental processes and DNA/histone methylation are prominently affected. Both in children and in adults, chromatin organization is also affected, but especially in adults many more chromatin modifiers and/or transcription regulators were additionally altered, as well as different and larger gene sets involved in brain development (Figure S2 and Table S4). Remarkably, most of the mutations in chromatin modifiers in adults were found to be mutually exclusive (Figure 4D). Interestingly, some of these mutations in chromatin

Figure 1. Genetic and Epigenetic Differences between SHH-MBs from Infants, Children, and Adults

(A) Cluster analysis of DNA methylation and gene expression data of SHH-MB. Both methylation profiling (left; n = 129) and gene expression profiling (right; n = 103) reveal two SHH-MB subgroups identified by unsupervised *k*-means consensus clustering. Each row represents a methylation probe/expression probeset, each column represents a sample. The level of DNA methylation (b value) is represented with a color scale as depicted. For each sample patient age (blue, infants; yellow, children; and pink, adults) and clustering according to expression data or methylation data (when available) is shown. Grey indicates that no data were available.

(B) GISTIC2 significance plots of amplifications (red) and deletions (blue) observed in SHH-MB infants, children, and adults. Candidate genes mapping significant regions have been indicated.

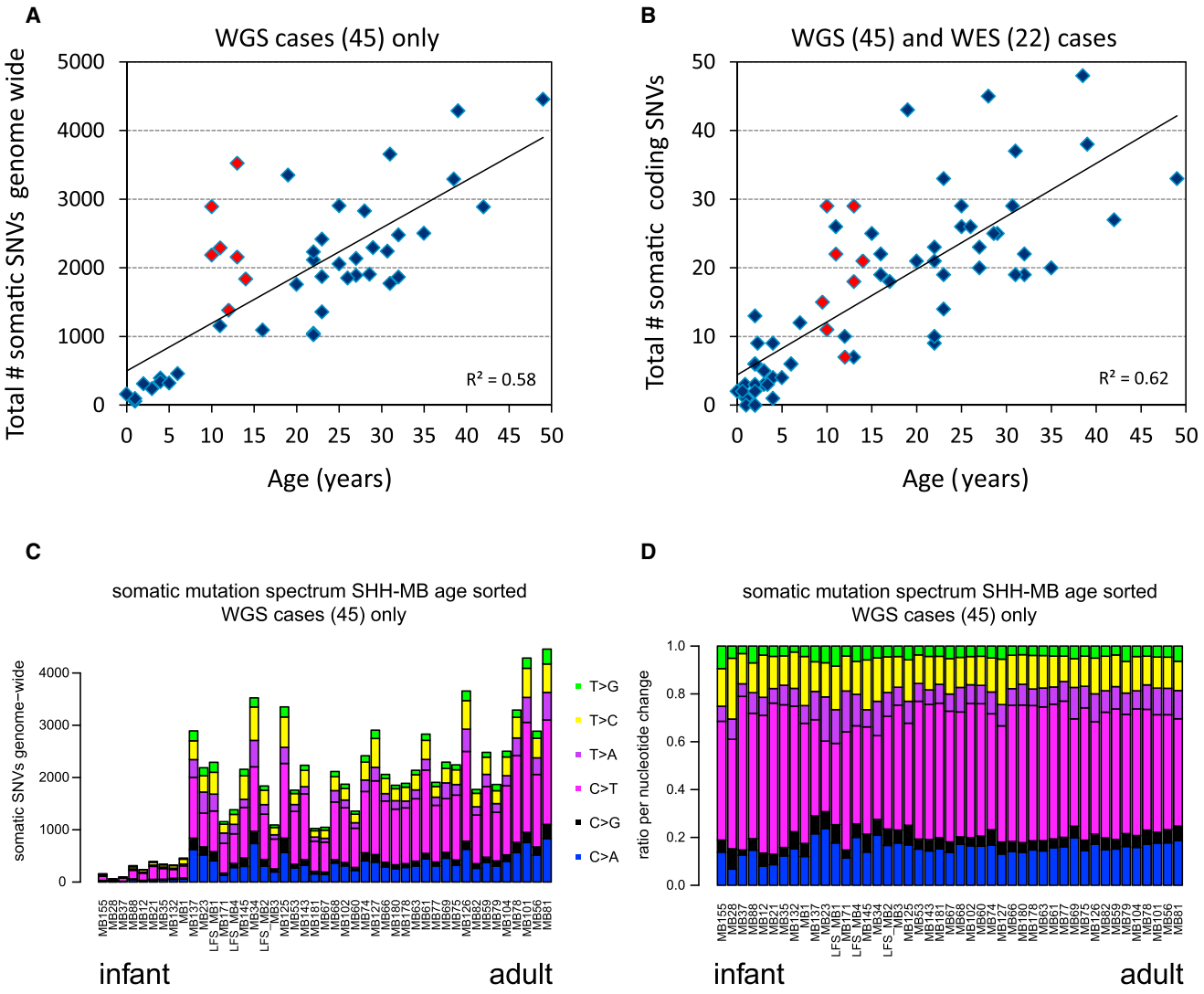

Figure 2. Number and Type of Somatic Mutations in Medulloblastoma Tumors in Relation to the Age of the Patient

(A) Total number of somatic mutations genome wide correlates with age of the patient. Plotted are the total number of somatic SNVs identified genome wide versus age of the patient for all cases for which we performed whole genome sequencing (WGS; n = 45). Red indicates patients harboring a *TP53* mutation.

(B) Same as in (A), but only the total number of coding SNVs is plotted versus age for all cases for which we performed either whole genome or whole exome sequencing (WGS and WES, n = 67).

(C) Mutation signatures. Plotted are the total numbers of somatic mutations genome wide sorted by age of the patient. Coloring of bars represents the ratio of the six possible nucleotide changes (C > A, C > G, C > T, T > A, T > C, and T > G) for each sample.

(D) Normalized mutation signatures sorted by age.

modifiers were more closely associated with *SMO* mutations, like the ones in *BRPF1/3*, while mutations in *CREBBP* or *KDM3B* were more often found in *PTCH1*-mutated tumors.

PI3K/AKT Signaling Activated in Adult SHH-MB Associates with Poor Outcome

As we identified recurrent mutations affecting the PI3K/AKT/mTOR-pathway in SHH-MBs (*PIK3CA*, *PTEN*, and *PIK3C2G* are all mutated in >5% of SHH-MBs; Figures 4A–4C), which could lead to GLI activation independent of SMO (Wang et al., 2012), targeting this pathway could be an option for combination therapies. To investigate which SHH patients would be most suitable for targeting PI3K/AKT/mTOR-signaling, we examined activation of the pathway in a large series of SHH-MBs (n = 155) by immunohistochemistry using antibodies for p-AKT and p-S6. p-AKT and p-S6 positivity were each detected in 17% of cases, with 12% positive for both (Figures 5A–5F). Surprisingly, the vast majority of positive cases were tumors from adult patients, with 31% and 30% of the adult SHH-MBs staining positive for p-AKT or p-S6, respectively. Moreover, survival analysis showed that both p-AKT and p-S6 positivity were strongly associated with a poor outcome in adult patients with SHH-MB (Figure 5G). Other factors shown to be associated with a poor outcome in SHH-MB patients, like

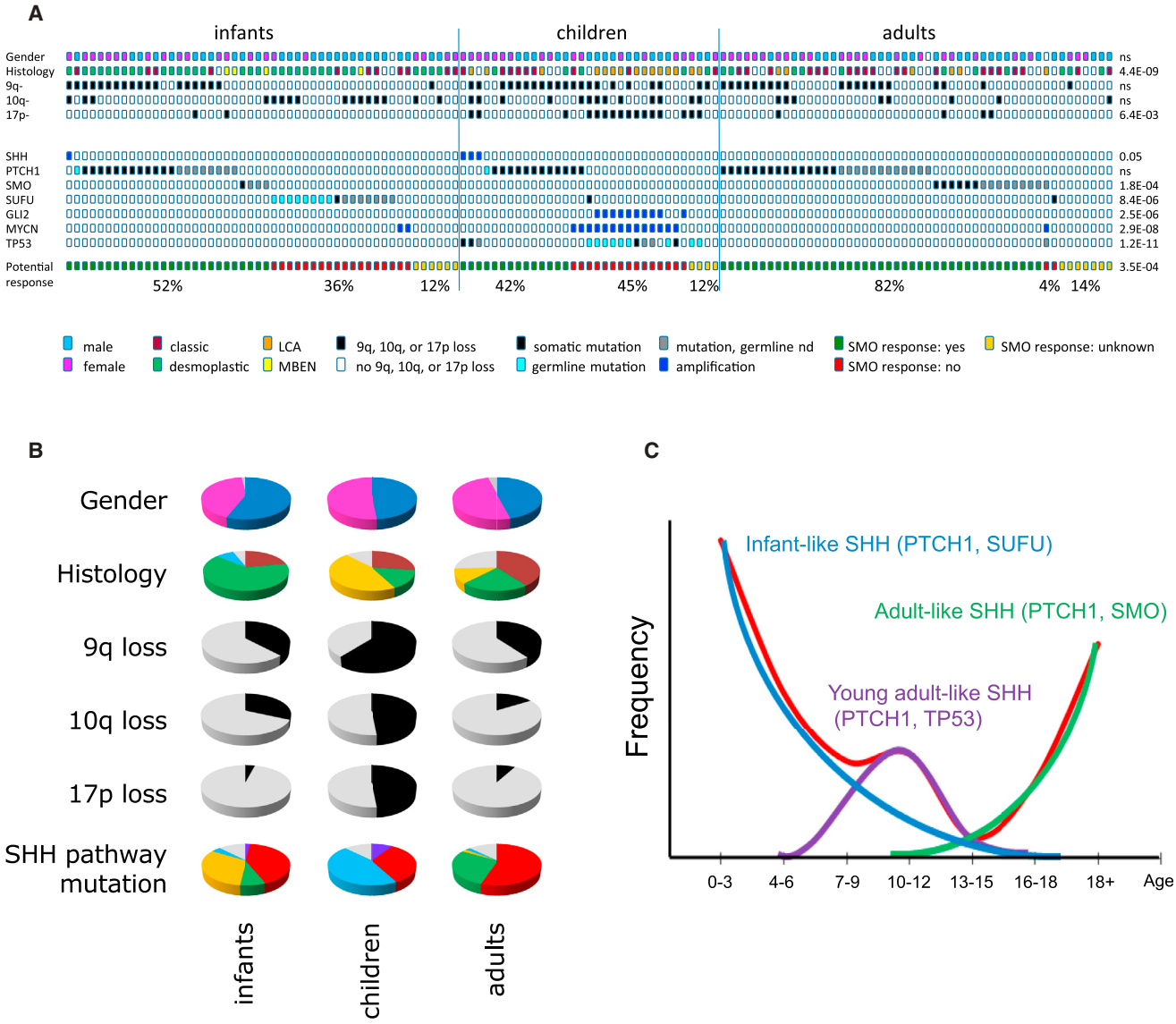

Figure 3. Genetic and Histological Differences between SHH-MBs from Infants, Children, and Adults

(A) SHH pathway mutations, gender, histology and 9q/10q/17p aberrations in all sequenced 133 SHH-MB. Cases have been split up in infants, children and adults, and are sorted based on type of mutation in the SHH-pathway. Potential response to SMO inhibition: cases with *SHH* amplifications, *PTCH1* mutations, or *SMO* mutations will likely respond to SMO inhibition (indicated in green). Cases with *SUFU* mutations or *MYCN* or *GLI2* amplifications will likely not respond to SMO inhibition (indicated in red). In cases for which no mutations in the SHH pathway were detected, it is not clear whether they will respond to SMO inhibitors (indicated in yellow). Percentages indicate fraction of infants, children, or adults, respectively, of each category. p Values indicate whether distributions are significantly different among infants, children, and adults.

(B) Pie charts showing in infants, children, and adults with SHH the distribution of gender (male, blue; female, pink; unknown, gray), histology (classic, dark red; nodular/desmoplastic, green; large cell/anaplastic LCA, orange; MBEN, yellow; and unknown, gray), 9q loss (yes, black; no, gray), 10q loss (yes, black; no, gray), 17p loss (yes, black; no, gray), and type of SHH pathway mutation (*SHH* amp, purple; *PTCH1* mut, red; *SMO* mut, green; *SUFU* mut, orange; *GLI2*/*MYCN* amp, blue; and unknown, gray).

(C) Trimodal age distribution of patients with SHH-MB. Red line indicates age distribution of all patients with SHH-MB. Three subgroups make up this age distribution: young children with *PTCH1* and *SUFU* mutations (blue line), older children with *PTCH1* and *TP53* mutations (purple line), and adults who mostly have *PTCH1* or *SMO* mutations (green line).

See also Figure S1.

MYCN or GLI2 amplification, LCA histology or metastasis at diagnosis, are all exceptionally rare in adult SHH-MB patients (Figures 1C and 3A; Kool et al., 2012), and could therefore not explain the poor outcome of these p-AKT/p-S6-positive subgroup of patients. Our results suggest that adult patients with SHH-MB may be the best group to benefit from combination therapies of SMO inhibitors with PI3K/AKT/mTOR inhibition.

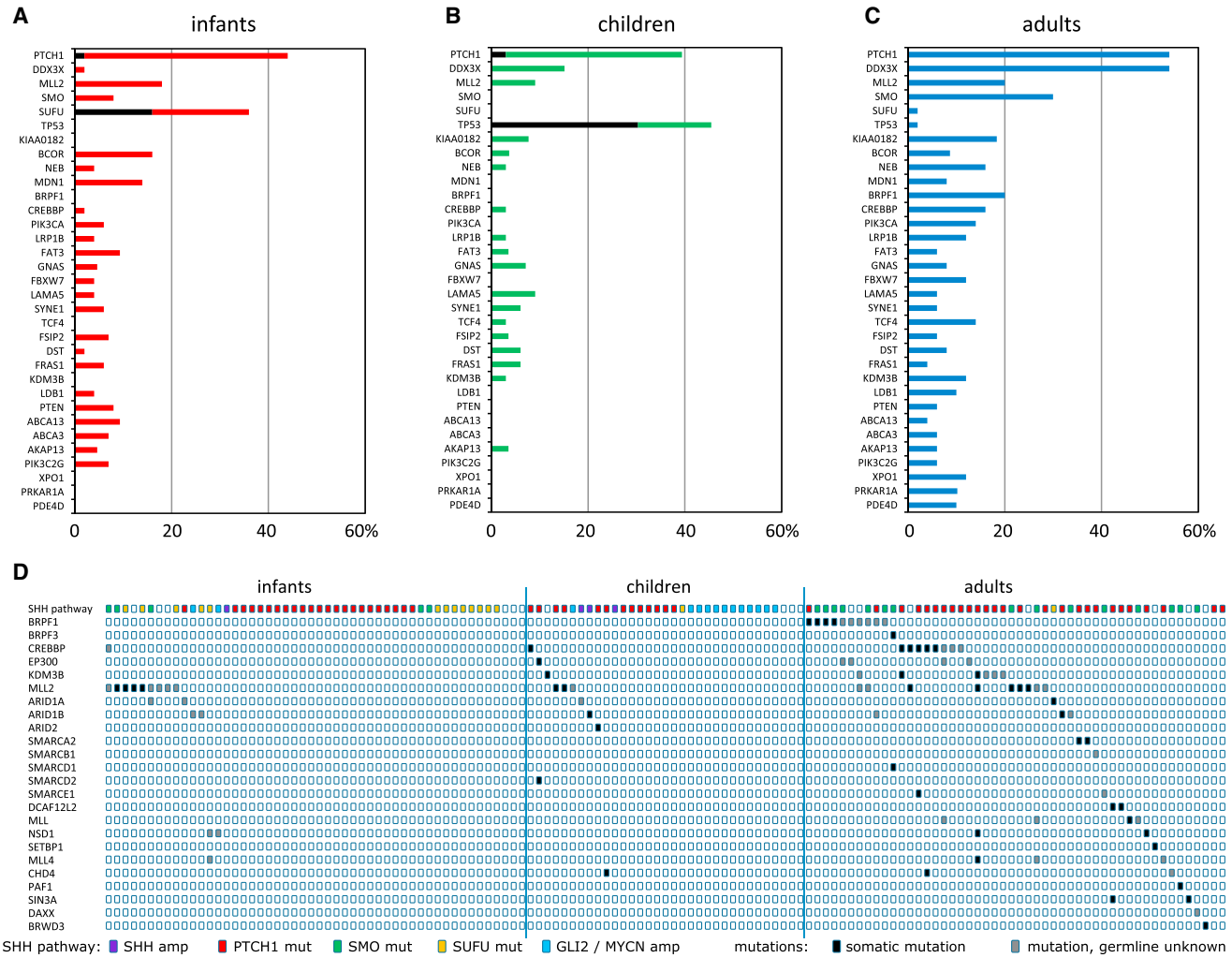

Figure 4. Most Frequently Mutated Genes in SHH-MB and the Mutual Exclusivity of Mutations in Chromatin Modifier Genes

(A–C) Mutation frequencies of 33 genes that are mutated either in ≥5% of all SHH-MB cases or in ≥10% of SHH-MB cases in one of the age categories. Mutation frequencies for these 33 genes are shown in infants (A), children (B), and adults (C). Black indicates the fraction of mutations that is found in the germline.

(D) Mutations in chromatin modifiers in infants, children, and adults with SHH-MB. The top line shows the mutations in the SHH pathway for each case.

See also Figure S2.

SHH Medulloblastomas with Mutations Downstream of SMO Are Resistant to LDE-225

Assuming a linear pathway, we anticipate that patients with mutations in the SHH pathway downstream of SMO (e.g., *SUFU*, *GLI2*, and *MYCN*) show primary resistance to targeted SMO inhibition. To test this hypothesis, we used xenografts from three SHH-associated MBs (DMB-012, RCMB-018, and RCMB-025; Yeh-Nayre et al., 2012). These xenografts were generated by stereotaxic orthotopic xenotransplantation of cells immediately after surgical resection, maintained by serial intracranial transplantation, and harvested only for use in short-term experiments, allowing them to maintain the characteristics of the original tumors (Shu et al., 2008; Zhao et al., 2012). WES showed that each xenograft harbored a different alteration in the SHH pathway (Figure 6A). Cells from each xenograft line were treated in vitro with NVP-LDE225, an SMO inhibitor that is currently being applied in phase III clinical trials for relapsed childhood and adult SHH-MB (Geoerger et al., 2012). Proliferation was measured based on incorporation of tritiated thymidine. Treatment with LDE225 significantly inhibited the proliferation of DMB-012 cells (*PTCH1* mutant), but did not affect the proliferation of RCMB-018 (*MYCN* amplification) or RCMB-025 cells (*SUFU* deletion; Figures 6B–6D). Preclinical testing in vivo also demonstrated a strong inhibition of tumor growth by LDE225 in DMB-012 (Figure 6E), but not RCMB-018 (Figure 6F and Figure S3), confirming the in vitro data. Survival analyses indeed show that mice with DMB-012 tumors live longer when treated with LDE-225 (Figure 6G), but mice with RCMB-018 tumors do not (Figure 6H). Finally, we have tested whether RCMB-018 cells, resistant to LDE-225, are responsive to arsenic trioxide (ATO) targeting cells at the level of GLI (Beauchamp et al., 2011). Figure 6I illustrates that RCMB-018 cells are responsive to ATO. At concentrations of 5–10 μM, cells are markedly inhibited in growth. Our data

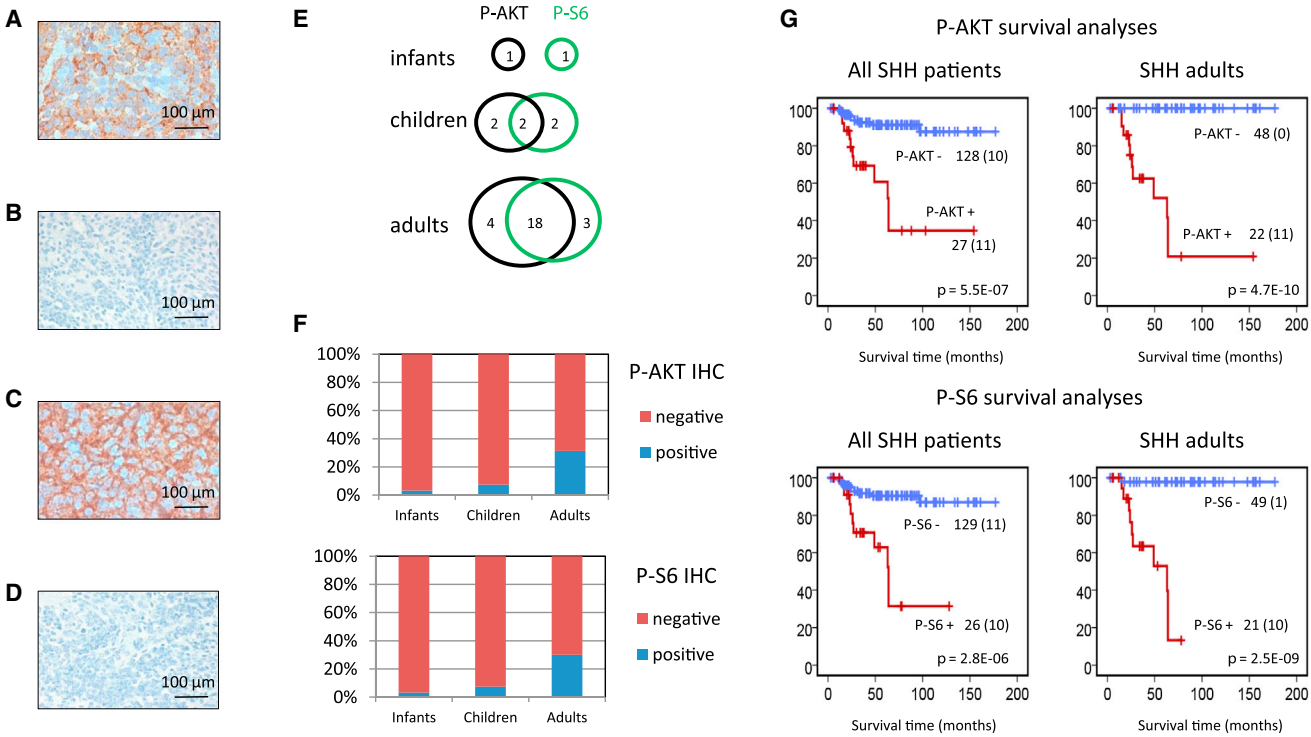

Figure 5. Immunohistochemical Staining of MB Tissue Arrays for p-AKT and p-S6

(A) Example of positive p-AKT MB.
(B) Example of negative p-AKT MB.
(C) Example of positive p-S6 MB.
(D) Example of negative p-S6 MB.
(E) Overlap in staining results between p-AKT and p-S6.
(F) Frequencies of p-AKT and p-S6 staining in infants, children, and adults.
(G) Survival analysis for p-AKT and p-S6 in all SHH patients and in adults only. Numbers on the y-axis indicate the fraction of surviving patients. Numbers on the x-axis indicate the follow-up time in months. The number of patients per group is indicated next to the graphs plus the number of events within that group (between brackets). For infants and children, the number of patients staining positive was too low to draw conclusions from separate survival analyses.

show that classification as an SHH-MB using a five-gene expression signature currently being applied in clinical trials is not sufficient as a predictive biomarker for response to SMO antagonists, because all SHH-MBs are detected by this signature, regardless of their underlying genetic makeup (Amakye et al., 2012).

DISCUSSION

Herein we have shown that genetic hits in SHH-MBs are very heterogeneous. Tumors in infants, children, and adults strongly differ in transcriptome, methylome, and copy-number aberrations as well as in number and type of mutations they contain. Hereditary predisposition syndromes involving germline mutations of *SUFU* (or rarely *PTCH1*; Gorlin syndrome) are highly prevalent in infant (0–3 years old) SHH-MBs, while germline *TP53* mutations (Li-Fraumeni syndrome) are common in older children (>3 years old), especially in children between 8 and 17 years old. Strikingly, almost all adults harbored somatic mutations in the *TERT* promoter, whereas they were much less common in pediatric patients. Our data show that three groups of SHH-MBs should be considered: young children with mostly *PTCH1* or *SUFU* mutations, older children with frequent germline *TP53* mutations and chromothripsis-associated amplifications of SHH pathway genes, and adults harboring mostly *PTCH1* and *SMO* mutations (Figure 3C). Recent data showing that SHH-MBs can arise from different precursor cells in the cerebellum or brainstem (Grammel et al., 2012) suggest that infant SHH-MBs may have a different cellular origin or hit the same progenitor cell at a different stage of differentiation than childhood or adult SHH-MBs (which were more similar at the transcriptome/methylome levels).

Most importantly, our results show that patients with different underlying SHH mutations should be stratified accordingly. We have demonstrated that targeting the SHH pathway in SHH-MB using SMO antagonists will most likely give the best results in adult patients. A vast majority (82%) of adult patients harbor tumors with mutations in either *PTCH1* or *SMO*, rendering them likely responsive to these drugs. In contrast, infant (36%) and childhood (45%) SHH-MBs frequently have mutations downstream of SMO, which makes these tumors intrinsically resistant to drugs targeting SMO. Indeed, SHH-MB xenografts harboring these downstream mutations did not respond to SMO antagonists. The impact of bone developmental toxicity may additionally limit the use of SMO inhibitors in infants (Kimura et al., 2008).

A

case #	MB subgroup	patient age	gender	tumor histology	mutation
DMB-012	SHH	3	female	desmoplastic	*PTCH1* (4 bp del, hemizygous)
RCMB-018	SHH	7	male	anaplastic	*TP53* (R175H, hemizygous) *MYCN* amplification
RCMB-025	SHH	0	female	MBEN	*SUFU* deletion (heterozygous)

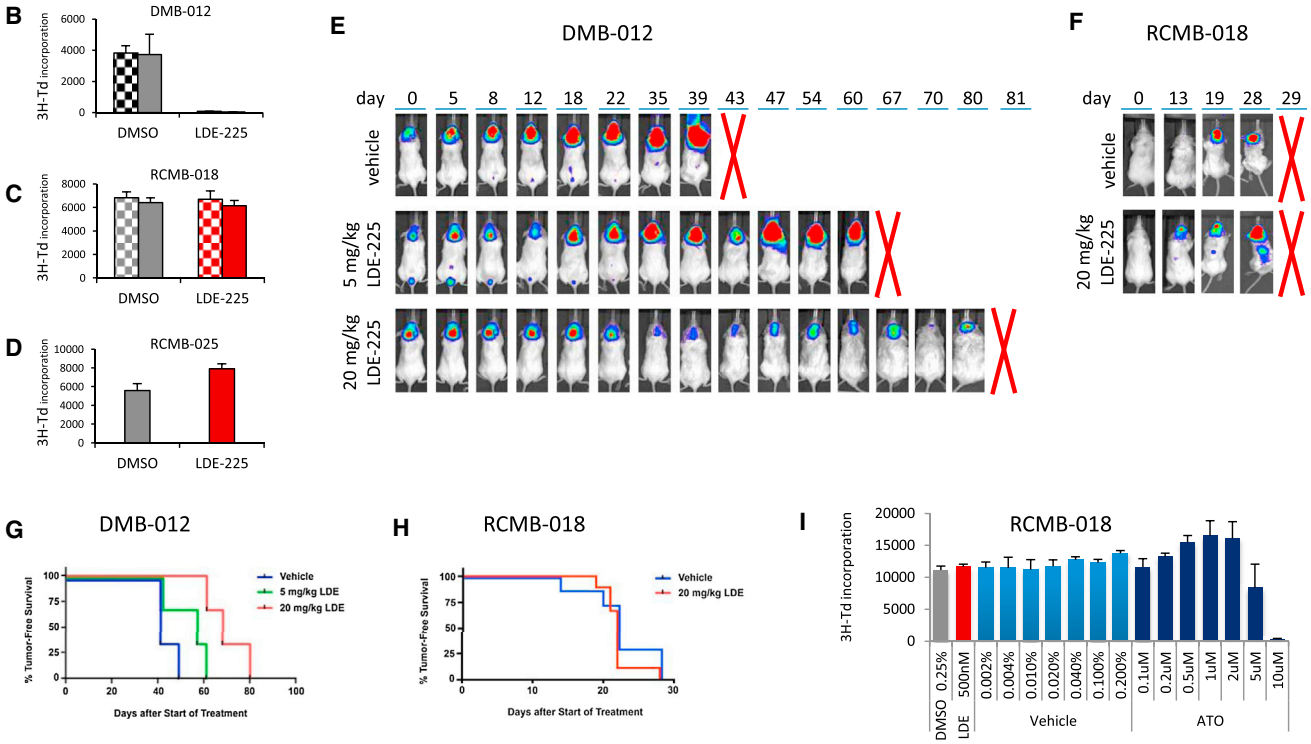

Figure 6. SMO Antagonists Do Not Suppress Proliferation of All SHH-Associated MB Tumors

(A) Characteristics of SHH-MB models treated with LDE225.

(B–D) Cells from patient-derived xenografts of SHH-associated MB were treated with DMSO (0.05% [hatched bars] or 0.25% [solid bars]) or LDE-225 (100 [hatched bars] or 500 nM [solid bars]). Cells were pulsed with [methyl-^{3}H]thymidine (^{3}H-Td) after 48 hr and harvested for analysis of ^{3}H-Td incorporation at 66 hr. In DMB-012 (B), LDE-225 significantly inhibited ^{3}H incorporation compared to DMSO control ($p < 0.01$ based on paired two-tailed t test). In RCMB-018 (C) and RCMB-025 (D), LDE-225 did not significantly inhibit ^{3}H incorporation ($p > 0.5$ and $p > 0.1$, respectively). Data represent means of triplicate samples ± SD.

(E and F) Cells from MB xenograft DMB-012 (E) or RCMB-018 (F) were infected with luciferase virus and transplanted into NSG mice. Bioluminescence images were taken pretreatment (day 0) and at different time points after daily treatment with vehicle or SHH antagonist (LDE-225, 5 or 20 mg/kg/day). Five mice per group were used. Representative examples from each group are shown. Other examples are shown in Figure S3. A red cross indicates when mice were sacrificed.

(G and H) Kaplan-Meier survival plots for the mice harboring DMB-012 tumors (G) or RCMB-018 tumors (H) and treated with vehicle or LDE-225.

(I) RCMB-018 cells were treated with DMSO (0.25%; gray bar), LDE-225 (500 nM; red bar), vehicle (PBS + 0.01 N NaOH; light blue bars), or increasing concentrations of ATO (dark blue bars). Cells were pulsed with [methyl-^{3}H]thymidine (^{3}H-Td) after 48 hr and harvested for analysis of ^{3}H-Td incorporation at 66 hr. LDE-225 did not inhibit ^{3}H incorporation compared to DMSO control, but ATO did at 5 and 10 μM concentrations. Data represent means of triplicate samples ± SD.

See also Figure S3.

Furthermore, our results strongly suggest that each patient with a SHH-MB, but especially those between 4 and 17 years of age with LCA histology, should be tested for germline *TP53* mutations. Currently, these patients with Li-Fraumeni syndrome (LFS)-MB are often not recognized and therefore treated with standard protocols, including ionizing radiotherapy and alkylating chemotherapy. Moreover, as almost all patients with germline *TP53* mutations have tumors with LCA histology, they are often stratified as high risk and will therefore get even higher doses of radiotherapy and chemotherapy. It seems that these patients are often cured of their MB, but frequently die of secondary malignancies induced by previous radio-chemotherapy. This may partly explain why *TP53* mutations in SHH-MBs are associated with a particularly poor outcome (Zhukova et al., 2013), and is also in line with the finding that *MYCN* amplification in SHH-MBs is associated with an inferior prognosis (Kool et al., 2012; Korshunov et al., 2012; Ryan et al., 2012). We therefore strongly suggest that separate LFS-MB trials

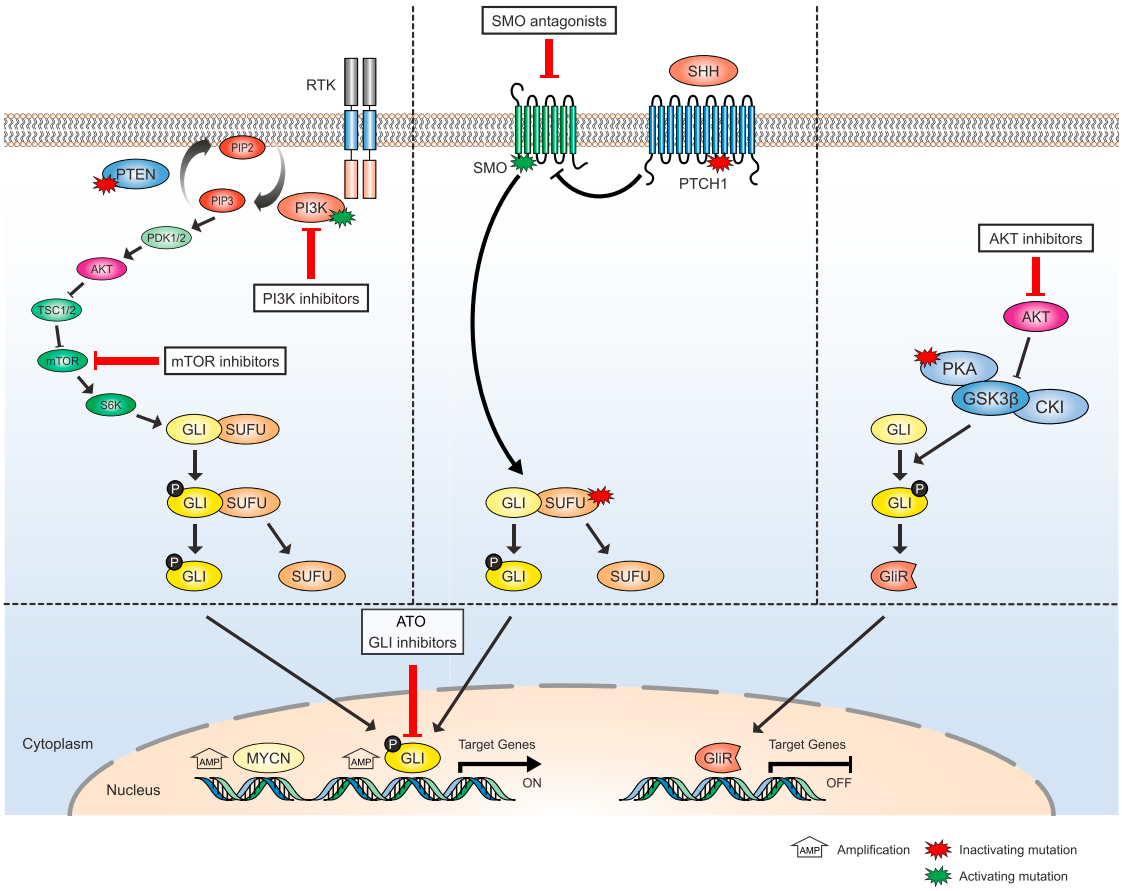

Figure 7. Schematic Overview of SHH-, PI3K/AKT/mTOR-, and PKA Pathways and How They Interact

Genes that were found in the genomic analyses of SHH-MBs to harbor activating mutations (green stars), inactivating mutations (red stars), or were found to be amplified (*MYCN* and *GLI*) are indicated. All these mutations lead to activation of GLI proteins and their downstream pathways. Options for targeted treatment are indicated. Patients harboring mutations in either *PTCH1* or *SMO* should be responsive to SMO inhibitors, whereas patients harboring mutations more downstream in the SHH pathway (*SUFU*, *MYCN*, and *GLI*) or in the PI3K/AKT/mTOR and/or PKA-pathways may be treated using arsenic trioxide (ATO) or other more specific GLI-inhibitors or PI3K/AKT/mTOR inhibitors.

should be developed using chemotherapy-only protocols and excluding alkylating drugs.

We further strongly advocate that the next generation of SMO inhibitor trials should be based on underlying tumor genetics because many patients with SHH-MB will not respond to these inhibitors. Alternative treatment options could include arsenic trioxide (ATO) targeting GLI transcription factors by degrading the protein (Figure 7; Kim et al., 2010, 2013). ATO and the antifungal agent itraconazole (which acts on SMO) have also been suggested in preclinical experiments for use in SHH-MBs that become resistant after treatment with SMO antagonists (Kim et al., 2013) or in combination with SMO inhibitors upfront knowing that *GLI2* amplifications comprise a common mechanism of secondary resistance to SMO inhibition in preclinical models (Buonamici et al., 2010; Dijkgraaf et al., 2011). Other options for combination therapies to avoid or delay the development of resistance include drugs targeting PI3K/AKT/mTOR- or PKA-signaling pathways (Figure 7), both mutated in a subset of patients with SHH and both also leading to GLI activation (Metcalfe et al., 2013; Milenkovic and Scott, 2010; Wang et al., 2012), or epigenetic drugs.

EXPERIMENTAL PROCEDURES

Patient Samples

Patient materials were collected after receiving informed consent according to International Cancer Genome Consortium guidelines (http://www.icgc.org) and as approved by the institutional review board of contributing centers. DNA derived from SHH-MBs and matched normal blood from 45 patients was subjected to whole genome sequencing (WGS) using Illumina technologies. Two additional tumor-normal pairs were sequenced by whole exome sequencing (WES). WGS data for 13/45 and WES data for another 20 pediatric tumor-normal pairs were previously reported (Jones et al., 2012; Pugh et al., 2012). All patients in this discovery cohort (n = 67) were confirmed to have a MB of the SHH subtype by either gene expression profiling, DNA methylation, or immunohistochemistry (SFRP1 Northcott et al., 2011b and GAB1 Ellison et al., 2011). In addition, we used data from 12 pediatric SHH-MB tumor-normal pairs that were sequenced for 2,734 genes as part of a previously reported replication cohort (Jones et al., 2012). Finally, a set of 400 genes, including those identified as recurrently mutated in SHH-MBs in our discovery cohort, was investigated in another independent set of pediatric (31) and adult

(23) SHH-MBs, for which only tumor DNA was available. In total, sequencing data for 133 (83 pediatric and 50 adult) SHH-MBs are presented in this study. Patient details are listed in Table S1.

Animals

Immunocompromised (NOD-scid IL2Rgammanull or NSG) mice used for transplantation were purchased from Jackson Labs. Mice were maintained in the Animal Facility at Sanford-Burnham. All experiments were performed in accordance with national guidelines and regulations, and with the approval of the animal care and use committee at Sanford-Burnham.

The experimental procedures used in this study are described in more detail in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The Gene Expression Omnibus accession numbers for the complete CpG methylation values are GSE49576 and GSE49377; for the complete gene expression values, the number is GSE49243. The European Genome-phenome Archive accession number for the sequencing data is EGAS00001000607.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.02.004.

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Erratum

A Peptide Mimicking VGLL4 Function Acts as a YAP Antagonist Therapy against Gastric Cancer

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Following the publication of this paper, the authors noticed that Table S7 in the Supplemental Information contained an input error. The value of "red blood cell" for the super-TDU-treated group should be "8.0 ± 0.3," but was inadvertently typed as "0.8 ± 0.3." The Supplemental Information has been replaced with a corrected document online.

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