Dimerization contributes to oncogenic activation of MLL chimeras in acute leukemias

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Summary

MLL is a histone methyltransferase that can be converted into an oncoprotein by acquisition of transcriptional effector domains following heterologous protein fusions with a variety of nuclear transcription factors, cofactors, or chromatin remodeling proteins in acute leukemias. Here we demonstrate an alternative mechanism for activation of MLL following fusions with proteins (AF1p/Eps15 and GAS7) that normally reside in the cytoplasm. The coiled-coil oligomerization domains of these proteins are necessary and sufficient for leukemogenic transformation induced by the respective MLL fusion proteins. Furthermore, homodimerization of MLL by synthetic dimerization modules mimics bona fide MLL fusion proteins resulting in *Hox* gene activation and enhanced self-renewal of hematopoietic progenitors. Our studies support an oligomerization-dependent mechanism for oncogenic conversion of MLL, presumably in part by recruitment of accessory factors through the dimerized MLL moiety of the chimeric protein.

Introduction

Generation of chimeric transcription factors by chromosomal translocations is a common pathogenetic mechanism in various human malignances, particularly acute leukemias (Cleary, 1991; Look, 1997; Rabbitts, 1994). A common target for chromosomal rearrangements is the mixed lineage leukemia gene, *MLL*, which is mutated in 80% of infant and 10% of adult acute leukemias (Djabali et al., 1992; Gu et al., 1992; Tkachuk et al., 1992; Zeminvan der Poel et al., 1991). It codes for a histone methyltransferase that has been reported to assemble in a supercomplex containing several chromatin-modifying components and activities (Nakamura et al., 2002), suggesting a multifunctional role in gene regulation. Remarkably, as a result of chromosomal translocations, MLL is fused with up to 50 different partner proteins, which may be of either nuclear or cytoplasmic origin (DiMartino and Cleary, 1999). There are no known unifying properties shared by this vast array of MLL fusion proteins in human leukemias. Thus, the diverse nature of MLL mutations suggests that multiple molecular mechanisms may activate the oncogenic potential of this multifunctional epigenetic regulator.

Although diverse mutations in leukemias could abrogate a possible tumor suppressor role for MLL, several lines of evidence support an important role for fusion partners in activating transdominant gene regulatory and leukemic potentials of MLL (Ayton and Cleary, 2001). Simple truncation of MLL at the site of translocation-induced fusion does not lead to development of leukemias in gene-targeted mice (Corral et al., 1996) or in retroviral transduction/transplantation models of MLL-associated myeloid leukemogenesis (Lavau et al., 1997). MLL mutant proteins resulting from chromosomal translocations are always in-frame chimeras that reside in the nucleus, regardless of whether the fusion partner is normally nuclear or cytoplasmic in origin (DiMartino and Cleary, 1999). Several MLL fusion partners function as DNA binding transcription factors (forkhead proteins AFX and FKHRL1) (Borkhardt et al., 1997; Hillion et al., 1997), transcriptional coactivators (CBP and p300) (Ida et al., 1997; Sobulo et al., 1997; Taki et al., 1997), or components of chromatin remodeling machines (ENL is a component of EBAF) (Nie et al., 2003). Structure/function studies of the respective fusion proteins revealed that the minimal domains required for oncogenesis display transactivation potentials that vary from strong (AFX, FKHRL1) (So and Cleary, 2002, 2003) to weak (ENL, ELL, AF10, CBP) (DiMartino et al., 2000, 2002; Lavau et al., 2000; Luo et al., 2001; Slany et al., 1998). Thus, acquisition of heterologous transcriptional effector domains by MLL may represent a common oncogenic pathway for deregulating its transcriptional functions by some, if not all, of its nuclear fusion partners.

SIGNFICANCE

A remarkable number and variety of proteins undergo in-frame fusions with MLL as a consequence of chromosomal translocations in acute leukemias, suggesting a diversity of mechanisms for the oncogenic activation of MLL, which normally functions as a histone methyltransferase. Although MLL can be activated by acquisition of heterologous transcriptional effector domains following fusions with various nuclear factors, most MLL fusion partners are cytoplasmic proteins with unknown effects on its function. Here we demonstrate that simple dimerization of MLL by bona fide cytoplasmic or synthetic fusion partners activates its transcriptional and oncogenic properties. These studies reveal a novel mechanism for oncogenic conversion of MLL and demonstrate that oligomerization of a chimeric transcription factor leads to cellular transformation and myeloid leukemias.

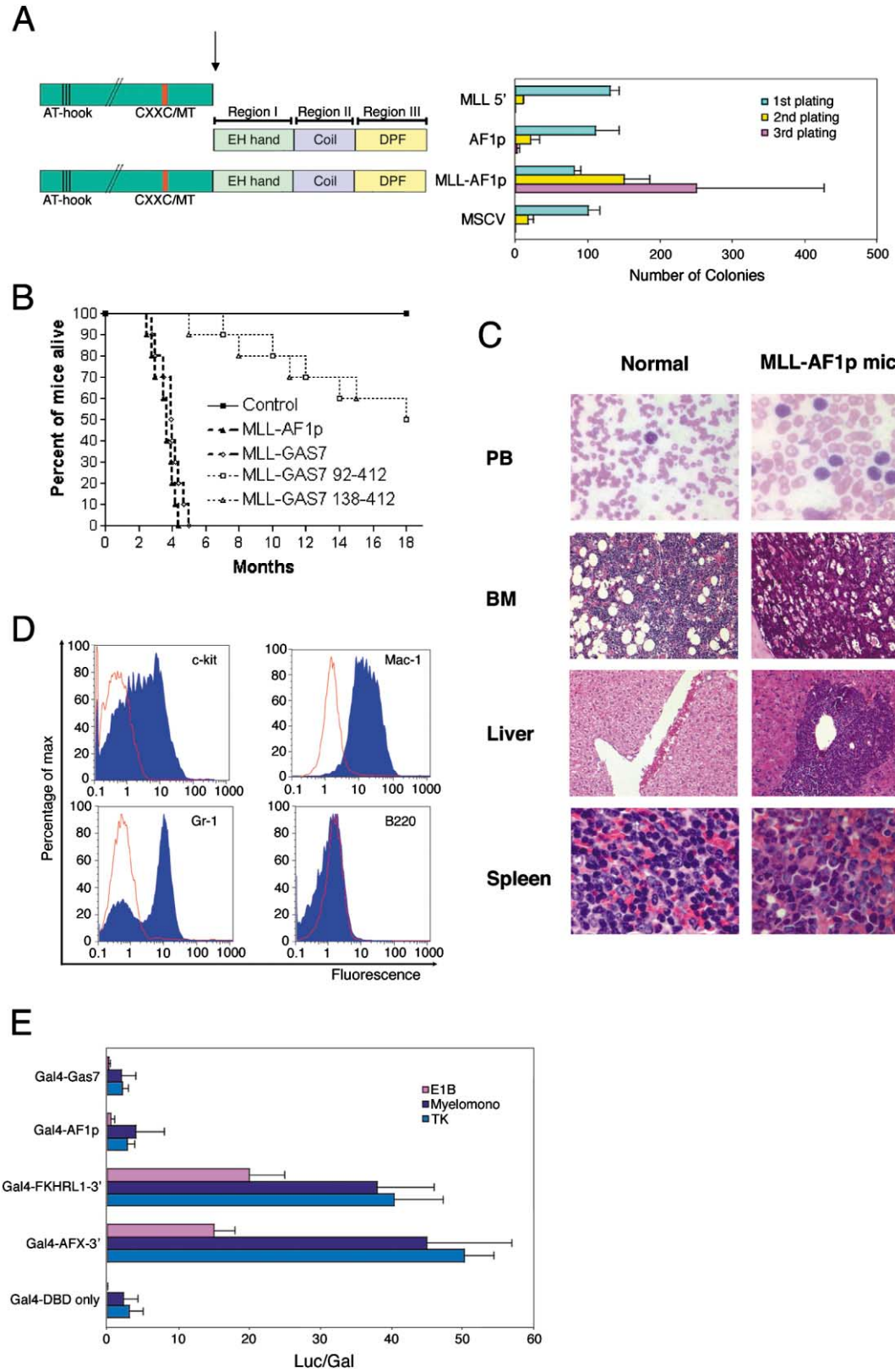


Figure 1. Cytoplasmic proteins AF1p and GAS7 activate the oncogenic properties of MLL but lack intrinsic transcriptional activation potential

A: Murine bone marrow cells were enriched for stem/progenitor cells by c-Kit-positive selection and transduced with retroviral constructs expressing proteins that are schematically shown on the left. Bar graph (right) indicates numbers of colonies obtained at each round of serial replating in methylcellulose cultures.

B: Survival curves are shown for cohorts ($n \geq 10$) of sublethally irradiated C57BL/6 mice that were injected with cell lines immortalized by various MLL fusion proteins or mock injected (Control).

The majority of MLL fusion partners, however, are cytoplasmic proteins with no known roles in transcriptional regulation (DiMartino and Cleary, 1999). Most attempts to establish disease models for studying their leukemogenic mechanisms have been unsuccessful (Fuchs et al., 2001; Strehl et al., 2003). Thus, little is known about the oncogenic properties of the respective MLL fusion proteins or how a protein that normally functions in the cytoplasm results in oncogenic activation of MLL. The potential roles of MLL fusion proteins are further complicated by observations that expression of an MLL-βGAL fusion protein in a knockin mouse model results in development of acute myeloid leukemias, albeit with reduced penetrance and long latencies (Dobson et al., 2000).

In this study, we demonstrate that homo-oligomerization (formation of complexes with two or more identical subunits) mediated by two different cytoplasmic proteins contributes to the oncogenic activation of MLL. One of these partners, AF1p, was originally discovered as a substrate of the EGF receptor tyrosine kinase (Fazioli et al., 1993); the other, GAS7, functions in undefined growth arrest pathways (Ju et al., 1998). Neither protein possesses inherent transcriptional effector properties, but both contain coiled-coil domains that mediate homo-oligomerization and are necessary and sufficient for activation of MLL. Furthermore, the transcriptional and growth-altering properties of MLL could be induced by fusion with synthetic dimerization modules. These studies reveal a novel mechanism for oncogenic conversion of MLL and demonstrate that oligomerization of a chimeric transcription factor results in cellular transformation and myeloid leukemia.

Results

Fusion with the EGF pathway substrate AF1p activates the leukemogenic properties of MLL

To assess the oncogenic ability of MLL-AF1p (Bernard et al., 1994), it was expressed under control of the long terminal repeat (LTR) of the murine stem cell virus (MSCV) following transduction into murine hematopoietic progenitors and stem cells. MSCV-neo vector alone and retroviruses expressing the respective portions of MLL (MLL-5′) or AF1p (AF1p-3′) were also tested as controls (Figure 1A). Initial plating of transduced cells in methylcellulose media containing myeloid cytokines showed similar numbers and morphologies of colonies for all constructs, indicating comparable transduction efficiencies. In subsequent platings, MLL-AF1p-transduced cells yielded increasing numbers of colonies with blast-type morphology, a phenotype consistent with the enhanced self-renewal typically induced by other MLL oncogenes. In contrast, cells transduced with control constructs exhausted their self-renewal potentials by the second plating. These results demonstrated that fusion with the EGF pathway substrate AF1p activates the in vitro oncogenic potential of MLL.

Table 1. Features of leukemic mice injected with MLL-GAS7 and MLL-AF1p transformed cells

	Control	MLL-GAS7	MLL-AF1p
Disease latency (days)*	n.a.	106 ± 45	93 ± 31
WBC ($10^3/\mu l$)*	6.9 ± 1.2	61 ± 48	50 ± 41
Blasts in peripheral blood (%)*	0	42 ± 21	42 ± 33
Liver weight (mg)*	0.45 ± 0.12	3.2 ± 2.4	2.8 ± 1.4
Spleen weight (mg)*	0.12 ± 0.03	0.69 ± 0.35	0.62 ± 0.28

*Values shown as mean ± standard deviation.
n.a., not applicable.

Cells transduced with MLL-AF1p also induced leukemias in sublethally irradiated mice with short latencies (≤5 months) (Figure 1B). In pre-terminal leukemic mice, greater than 30% of BM cells were comprised of leukemic blasts, which were also present in the peripheral blood and infiltrated the spleen and liver (Figure 1C). Blasts consistently displayed a surface phenotype characteristic of myeloid progenitors (Figure 1D). The latencies and presentations of leukemias arising in MLL-AF1p mice were similar to leukemias induced by MLL-GAS7 (So et al., 2003), another MLL fusion involving a cytoplasmic partner protein (Megonigal et al., 2000). Their comparative properties are summarized in Table 1. Taken together, these results indicated that fusion of MLL with two different cytoplasmic proteins (AF1p or GAS7) results in its oncogenic activation in vitro and in vivo.

AF1p and GAS7 lack intrinsic transcriptional activation potential

Previous studies have consistently demonstrated that fusion partners of MLL that normally reside in the nucleus possess intrinsic transcriptional effector properties, which are required for oncogenic activation of MLL (DiMartino et al., 2000, 2002; Lavau et al., 2000; Luo et al., 2001; Slany et al., 1998; So and Cleary, 2002, 2003). Thus, AF1p and GAS7 were assessed for potential transcriptional effector properties in transient transcriptional assays following fusion to the Gal4 DBD. Under conditions in which two nuclear fusion partners of MLL (AFX and FKHRL1) strongly activated expression of three different promoters, Gal4-AF1p and Gal4-GAS7 did not exhibit any significant transcriptional properties in transiently transfected 293 (Figure 1E), COS7, or REH cells (data not shown). These data suggested that the pathogenic contributions of AF1p and GAS7, in contrast to those demonstrated for nuclear fusion partners of MLL, are unlikely to be based on an intrinsic ability to function as transcriptional activators.

C: Representative histology from leukemic MLL-AF1p mice. Paraffin sections were stained with H&E; blood smears were stained with May-Grünwald-Giemsa (MGG). In MLL-AF1p mice, the spleen and liver were heavily infiltrated with leukemic blasts. Bone marrow was densely packed with a homogeneous population of blasts. Leukemic blasts were present in the peripheral blood and infiltrate the portal veins of liver of moribund MLL-AF1p mice.
D: FACS analysis shows that leukemic cells from MLL-AF1p mice display an early myeloid precursor phenotype. Shadow profiles represent FACS staining obtained with antibodies specific for the indicated cell surface antigens. Red lines represent staining obtained with isotype control antibodies.
E: Gal4-AF1p and Gal4-GAS7 fusion proteins lack transactivation properties in transient transactivation assays. Normalized luciferase values are shown for the average of three experiments using reporter genes under control of promoters for either the HSV thymidine kinase, adenoviral E1b, or myelomonocytic growth factor receptor genes. Comparable expression levels of the Gal4 constructs were confirmed by Western blot analysis (data not shown).

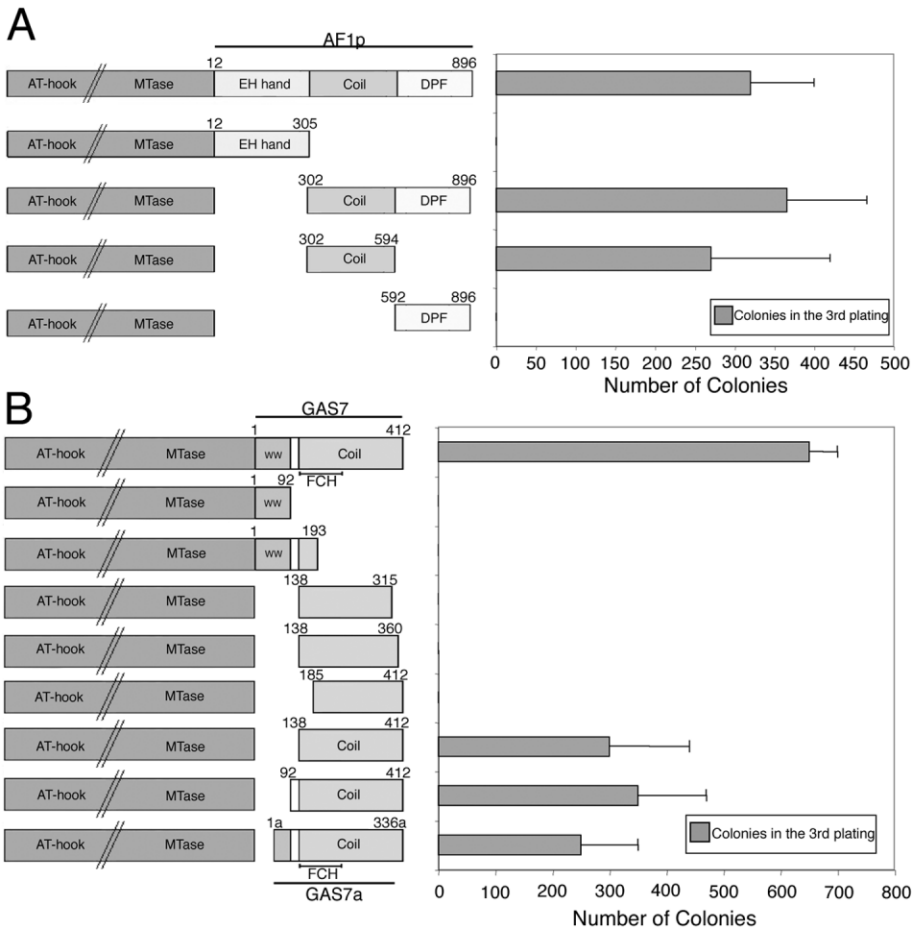


Figure 2. The coiled-coil domains of cytoplasmic fusion partners AF1p and GAS7 are necessary and sufficient for activation of MLL oncogenic potential

A and **B:** Schematic diagrams on the left illustrate various mutant forms of MLL-AF1p and MLL-GAS7 used for structure/function studies to map domains required for in vitro transformation. Bar graphs on the right indicate the number of colonies obtained at the third round of serial replating in methylcellulose cultures. EH, EH hands; Coil, coiled-coil domain; DPF, DPF repeats; WW, WW domain; FCH, Fer-CIP4 homology domain, which overlaps with part of the coiled-coil domain of GAS7.

The coiled-coil domains of MLL-AF1p and MLL-GAS7 are required for transformation

Structure/function studies were conducted to identify the specific domains in AF1p and GAS7 required for cellular transformation. Mutants of AF1p (Figure 2A) targeted three well-defined functional motifs designated as EH hands, coiled-coil, and DPF domains. Methylcellulose replating assays showed that the EH hands were neither necessary nor sufficient for in vitro transformation since a construct containing only this region (MLL-AF1p$^{12\text{-}305}$) yielded no third round colonies whereas a construct lacking this region (MLL-AF1p$^{302\text{-}896}$) induced robust transformation (Figure 2A). Similar findings applied to the DPF domain. Conversely, constructs lacking the coiled-coil domain were unable to enhance the clonogenic potential of progenitors in vitro whereas all constructs containing this region were oncogenic including MLL-AF1p$^{302\text{-}594}$, which consisted exclusively of the coiled-coil fused to MLL. These data indicated that the AF1p coiled-coil domain is critical for MLL-AF1p-mediated transformation.

Mutants of MLL-GAS7 targeted the WW domain, FCH (Fer-CIP4 Homology) domain, and a C-terminal coiled-coil domain (Figure 2B). The WW domain, which mediates binding to proline-rich sequences, was dispensable for in vitro transformation (Figure 2B) since a mutant lacking this domain (MLL-GAS7$^{92\text{-}412}$) was active in the assay. Furthermore, a synthetic construct (MLL-GAS7a) encoding an alternatively spliced form of GAS7 lacking the WW domain was transformation competent (Figure 2B). Cells transduced with mutants containing only the FCH domain (MLL-GAS7$^{138\text{-}315}$) or with deletion of part of the coiled-coil domain (MLL-GAS7$^{138\text{-}360}$ and MLL-GAS7$^{185\text{-}412}$) exhausted their proliferative capability after the first plating. Only constructs containing the complete coiled-coil domain (MLL-GAS7$^{92\text{-}412}$ and MLL-GAS7$^{138\text{-}412}$) were able to sustain continued replating into the third round of culture. Cells transduced by these latter mutants could be adapted to growth in liquid medium and induced acute leukemias in sublethally irradiated syngeneic recipient mice, albeit with longer latencies (mean 12 months) and reduced penetrance (50% at 18 months) compared to MLL-GAS7 (Figure 1B). Thus, structurally similar domains (coiled-coil) of the unrelated AF1p and GAS7 proteins are sufficient to activate the oncogenic properties of MLL.

MLL-AF1p and MLL-GAS7 homo-oligomerize through their coiled-coil domains

The coiled-coil domain of AF1p has been shown to mediate its homodimerization (Cupers et al., 1997; Tebar et al., 1997), whereas homo-oligomerization of GAS7 has been proposed (She et al., 2002) but not yet demonstrated. Therefore, a GST pulldown assay was used to assess the dimerization potential of GAS7 in vitro. Full-length ^{35}S-labeled GAS7 interacted with GST-GAS7 but not GST alone, indicating that GAS7 is capable of forming homo-oligomers in vitro (Figure 3A). Analysis of vari-

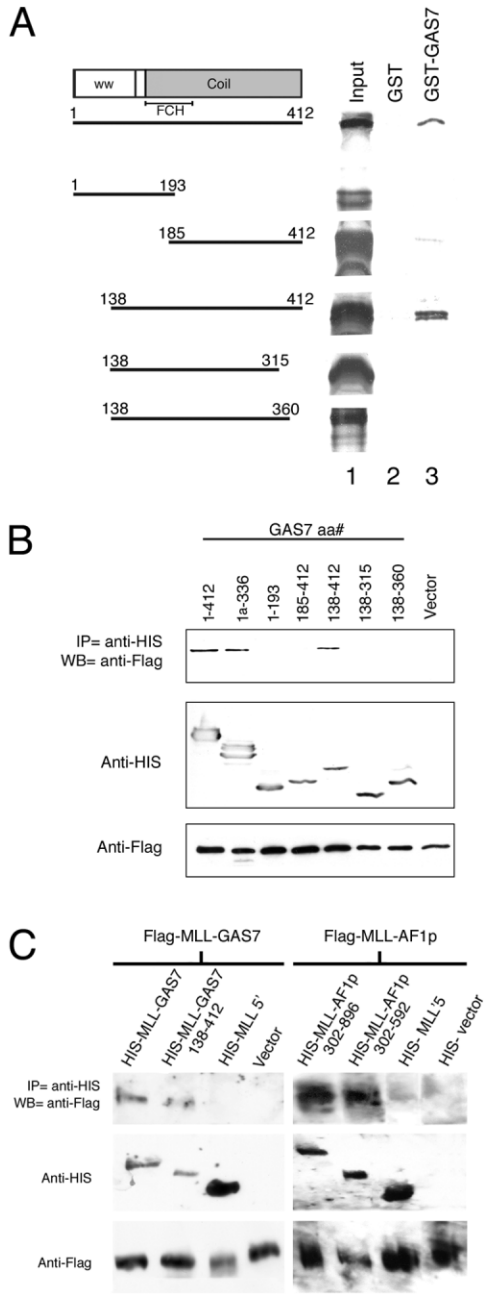


Figure 3. The coiled-coil domains of AF1p and GAS7 mediate homo-oligomerization of MLL fusion proteins

A: GST pulldown assays were performed with various portions of GAS7 (indicated by horizontal bars on left), which were translated in vitro and incubated with GST (lane 2) or GST-GAS7$^{1-412}$ (lane 3). Bound proteins (generally 1%–2% of input) were washed, eluted, and subjected to SDS-PAGE and autoradiography. Radiolabeled input proteins are shown in lane 1.

B: In vivo homodimerization of GAS7. Anti-His tag immunoprecipitations were performed on lysates of cells coexpressing Flag-tagged GAS7 and His-tagged mutant forms of GAS7 (indicated at top of lanes). Anti-Flag Western blot analysis revealed that only constructs containing intact coiled-coil domains coprecipitated with full-length GAS7. Lower panels demonstrate presence of HIS- and Flag-tagged proteins in transfected cell lysates.

C: In vivo homodimerization of MLL-GAS7 and MLL-AF1p mediated by the coiled-coil domains. Anti-His tag immunoprecipitations were performed on lysates of cells coexpressing Flag-tagged MLL-GAS7 and His-tagged mutants (indicated at the top of left panel) or Flag-tagged MLL-AF1p and His-tagged mutants (indicated at the top of right panel). Anti-Flag Western blot analysis revealed that only constructs containing intact coiled-coil domains coprecipitated with MLL-GAS7 or MLL-AF1p. Lower panels demonstrate presence of HIS- and Flag-tagged proteins in transfected cell lysates.

ous GAS7 mutants indicated that an intact coiled-coil domain was required and appeared sufficient for the observed interaction. Similar results were obtained using an immunoprecipitation/Western blot assay of COS7 cells that were cotransfected with Flag-tagged full-length GAS7 and various His-tagged GAS7 mutants. Coprecipitation of Flag-tagged GAS7 was only observed to occur with His-tagged GAS7 proteins containing an intact coiled-coil (GAS7$^{1-412}$, GAS7$^{1a-336a}$, and GAS7$^{138-412}$). His-tagged mutants with complete or partial deletion of the coiled-coil (GAS7$^{1-193}$, GAS7$^{185-412}$, GAS7$^{138-315}$, and GAS7$^{138-360}$) failed to coprecipitate Flag-tagged GAS7 (Figure 3B). Therefore, similar to AF1p, the coiled-coil of GAS7 is capable of mediating homo-oligomerization.

Immunoprecipitation/Western blot analysis was also used to assess whether MLL-AF1p and MLL-GAS7 homo-oligomerize in living cells. Lysates from 293 cells coexpressing Flag-tagged and His-tagged MLL fusion proteins were immunoprecipitated with anti-HIS antibodies, and the pellets subjected to Western blot analysis with anti-Flag antibodies. Flag-tagged MLL-GAS7 coprecipitated with His-tagged MLL-GAS7 but not with MLL lacking GAS7 sequences (His-MLL 5′) (Figure 3C). Similarly, Flag-tagged MLL-AF1p coprecipitated with His-tagged MLL-AF1p if the coiled-coil domain was present in both constructs. Taken together, our results indicate that MLL-AF1p and MLL-GAS7 self-associate through their coiled-coil domains, which are also necessary and sufficient for myeloid transformation in vitro.

Dimerization of amino-terminal MLL is sufficient to enhance the self-renewal of hematopoietic progenitors

To test whether homodimerization may be sufficient to activate MLL, synthetic dimerization modules were used to construct a set of novel MLL fusion proteins (Figures 4A and 4B). Conditional dimerization modules consisted of FKBP and FRB$_{2098L}$, which form heterodimers in the presence of the synthetic dimerizing agent AP21967 (Chen et al., 1995; Choi et al., 1996). Transduction of either construct alone was unable to sustain the clonogenic potential of myeloid progenitors in serial replating assays in the presence of AP21967 (Figure 4A). However, cotransduction of MLL-FKBP with MLL-FRB resulted in enhanced generation of CFU-GM-like colonies in the third round of replating in the presence but not absence of AP21967. To further address the role of dimerization on its oncogenic potential, MLL was separately fused with short (~40 amino acid) amphipathic acidic or basic leucine zippers, respectively, which have been shown to mediate constitutive heterodimerization (Scott et al., 1996). Primary myeloid progenitors transduced with either construct alone were unable to generate colonies beyond the first round of plating, while progenitors cotransduced with both constructs formed compact colonies in the third plating (Figure 4C). However, the colonies displayed reduced plating efficiencies compared to MLL-GAS7 or MLL-AF1p transduced progenitors (Figure 4B versus Figure 1) and could not be sustained indefinitely, although all constructs expressed at similar levels in transduced

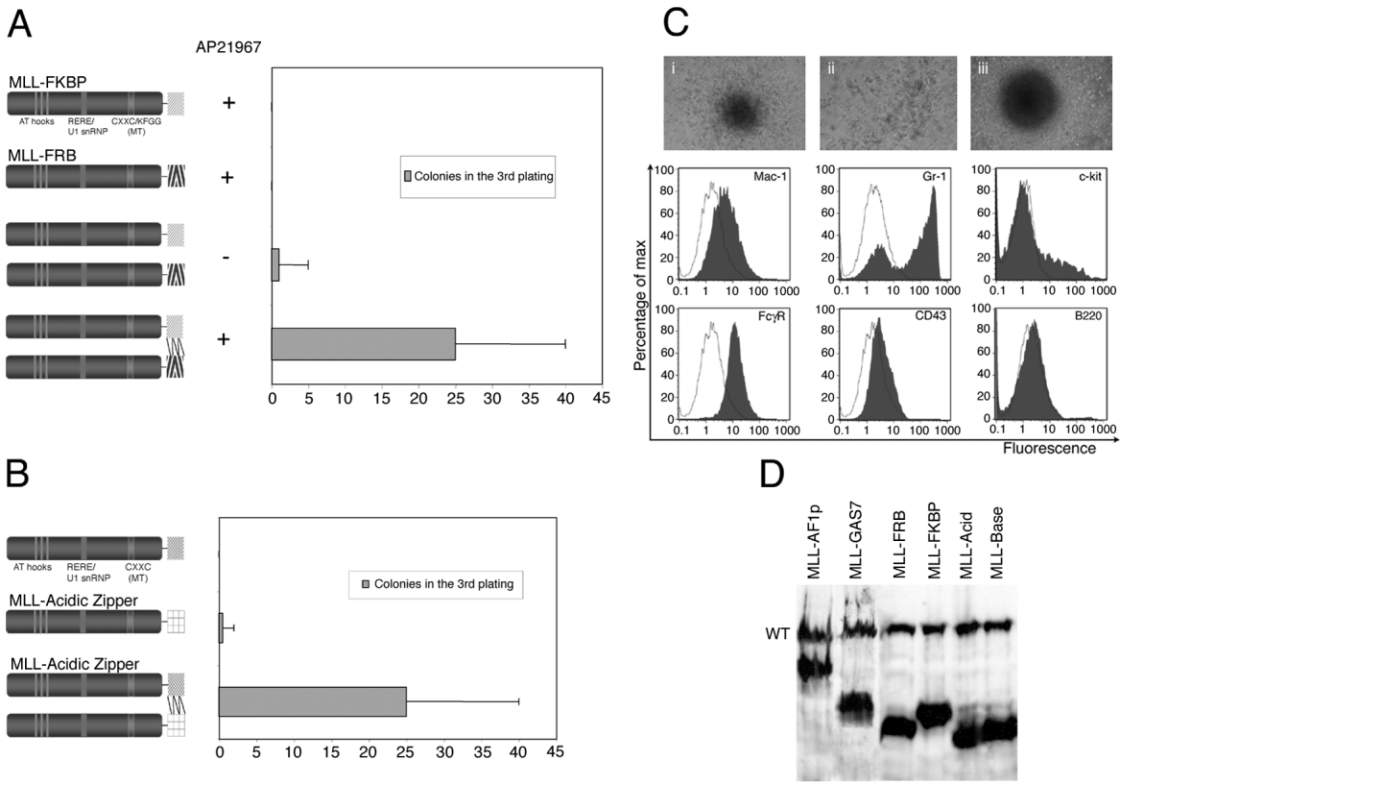


Figure 4. Forced dimerization of amino-terminal MLL enhances the self-renewal properties of myeloid progenitors

A: Methylcellulose replating assays were performed on myeloid progenitors transduced with constructs encoding MLL-FKBP or MLL-FRB fusion proteins in the presence (+) or absence (−) of dimerizing agent AP21967. Bar graph (right) indicates numbers of colonies obtained at the third round of replating in methylcellulose cultures.

B: Results are shown for similar analysis of myeloid progenitors transduced with constructs encoding MLL fusion proteins containing acidic or basic leucine zippers at their C termini (schematically illustrated on left).

C: Typical morphology is shown for methylcellulose colonies generated from progenitors cotransduced with retroviruses expressing MLL-FKBP and MLL-FRB, in the presence (i) or absence (ii) of dimerizer AP21967, or MLL-zipper constructs (iii). Phenotypic analysis of cells cotransduced with MLL-zipper constructs indicates a myeloid precursor phenotype. Shadow profiles represent FACS staining obtained with antibodies specific for the indicated cell surface antigens. Black lines represent staining obtained with isotype control antibodies.

D: Detection of MLL fusion protein expression in Phoenix cells by Western blot analysis. WT = wild-type MLL proteins.

Phoenix cells (Figure 4D). FACS analysis of cells from these cultures showed features of myeloid precursors, which were more mature than those of MLL-AF1p-transformed cells (Figure 4B and data not shown). These results indicated that dimerization of MLL per se is capable of enhancing the self-renewal of hematopoietic progenitors in vitro.

Distinct MLL functional domains are required for enhanced self-renewal induced by homodimerization

To investigate the mechanism of dimer-mediated transformation, various mutations that targeted known structural or functional motifs were introduced into the MLL acidic zipper construct (Figure 5). These constructs were then cotransduced with an intact MLL basic zipper construct into primary myeloid progenitors whose growth properties were evaluated in methylcellulose serial replating assays. Point mutation or deletion (CXXC or KFGG) that disrupted the DNA binding properties of the methyltransferase homology domain (Chen et al., 2002) abrogated the ability of MLL homodimers to enhance progenitor self-renewal (Figure 5), suggesting that DNA binding through this domain by both members of the MLL dimer is critical for transformation. Conversely, deletion of the AT-hook motifs did not eliminate clonogenic potential, indicating that these minor groove DNA binding motifs are not absolutely required for MLL-dimer oncogenic function. Deletion of the conserved but functionally obscure RERE/snRNP and Ala/Ser-rich (AS-rich) domains abolished clonogenic growth, suggesting that these domains also are critical for each member of the dimer. The essential MLL domains revealed by these structure/function studies are similar to those required by MLL-ENL (Chen et al., 2002), indicating that MLL contributions to myeloid transformation are similar for oncogenic pathways that are dependent on MLL dimerization and those that appear to be dimerization independent.

MLL-GAS7 and MLL-AF1p activate *Hox* gene expression

Although AF1p and GAS7 do not exhibit intrinsic transactivation properties, we investigated the possibility that their respective MLL fusion proteins may activate transcription as a consequence of dimerization. Transient transactivation assays were

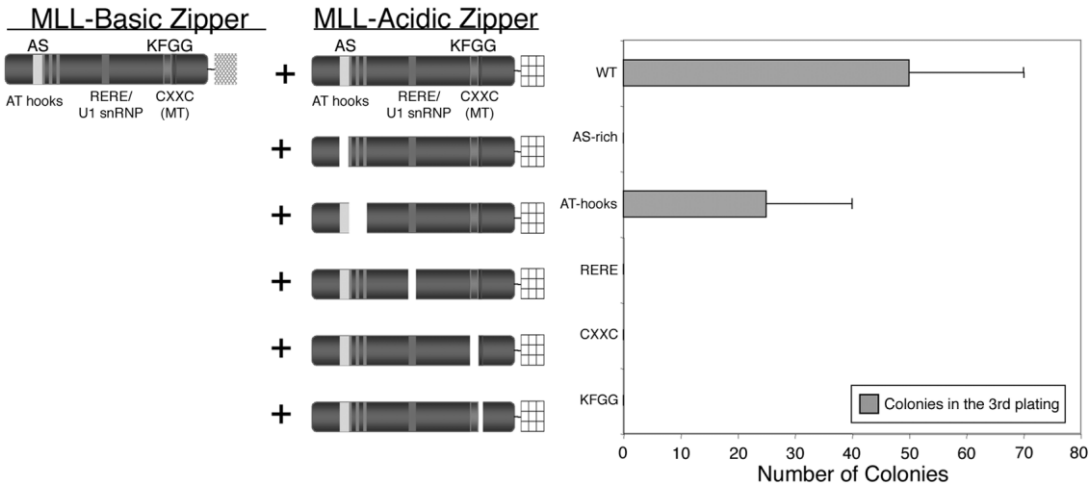


Figure 5. Mapping of MLL domains required for enhanced self-renewal ability induced by dimerization

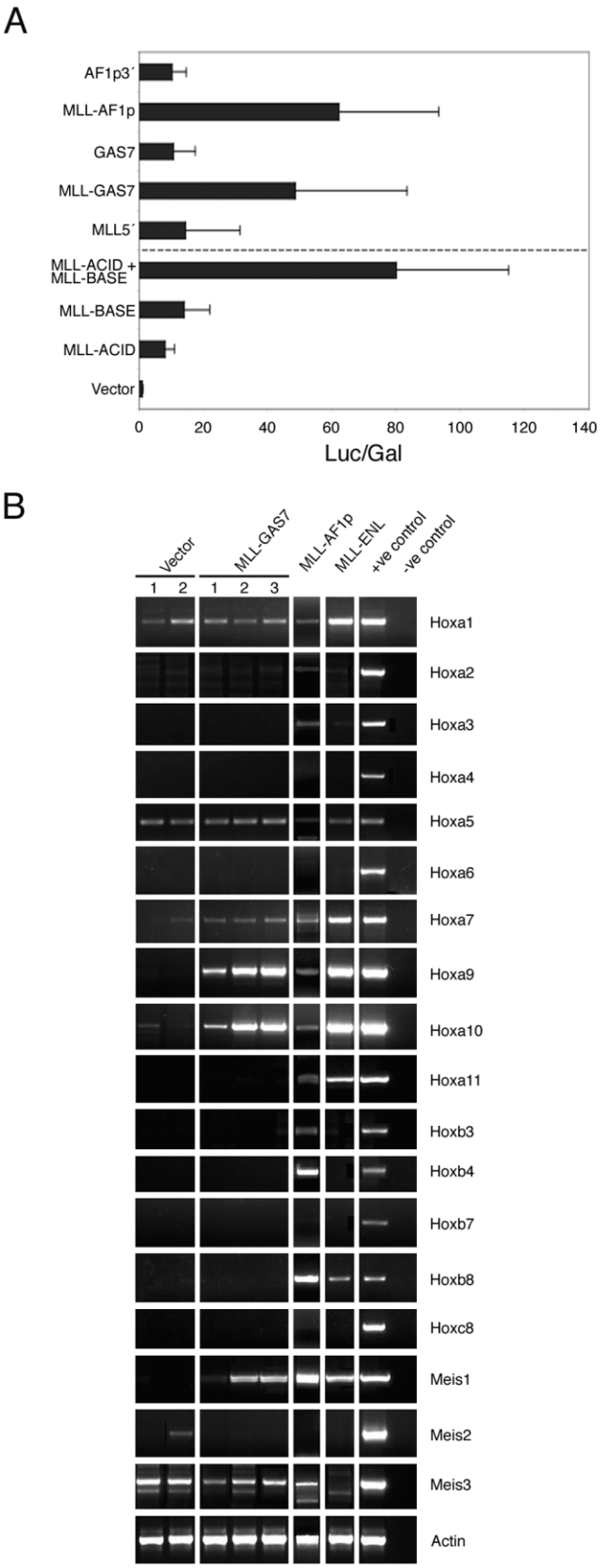
Transformation assays were performed on progenitors cotransduced with constructs encoding synthetic MLL fusion proteins containing acidic or basic C-terminal leucine zippers. Fusion proteins with acidic zippers contained mutations of various MLL motifs as indicated schematically on the left. Bar graph (right) indicates numbers of colonies obtained at the third round of replating in methylcellulose cultures.

performed using 293 or REH cells coexpressing MLL-GAS7 or MLL-AF1p together with a reporter gene under control of the *HoxA7* promoter, which is a downstream target of MLL (Kawagoe et al., 2001; Schreiner et al., 1999; Yu et al., 1995). Both fusion proteins activated transcription by more than 50-fold (Figure 6A), whereas control constructs (MLL-5′, AF1p-3′, GAS7) had much more modest effects. Similarly, cotransfection of MLL acidic and basic zipper constructs led to strong transactivation compared to minimal activation by each alone (Figure 6A). Taken together, these results suggested that forced dimerization created a transcriptional activator complex, presumably through recruitment of unknown cofactors by the dimerized MLL moiety, analogous to dimerization-mediated alterations of transcriptional properties of other chimeric oncoproteins (Lin and Evans, 2000; Minucci et al., 2000).

To investigate the effects of MLL dimerization on target gene expression in transformed cells, we assessed the expression of select *Hox* cluster genes and *Hox* cofactors, which are known downstream targets of wild-type MLL (Yu et al., 1995) and/or are involved in human leukemia (Owens and Hawley, 2002; van Oostveen et al., 1999). Hematopoietic progenitors transduced by MLL-GAS7 or vector alone were analyzed for *Hox* gene expression by RT-PCR at the end of each round of plating in methylcellulose cultures. Although similar numbers of colonies were observed in the first round of plating, cells transduced by MLL-GAS7 or vector alone exhibited very different *Hox* gene expression profiles. 5′ *HoxA* genes, which have been implicated in expansion of early hematopoietic progenitors, were mostly downregulated in the vector-transduced cells. Conversely, MLL-GAS7-transduced cells expressed a broad array of *Hox* genes (including *HoxA5*, *A7*, *A9*, and *A10*) and *Hox* cofactors Meis1 and Meis3. Expression of these genes was maintained in the second and subsequent platings (Figure 6B). The *Hox* gene expression profiles in MLL-GAS7 and MLL-AF1p cultures were similar to those of cultures initiated by transduction of MLL-ENL, whose fusion partner ENL is a component of the EBAF mammalian SWI/SNF complex (Nie et al., 2003) and displays inherent transcriptional effector activity (Rubnitz et al., 1994; Schreiner et al., 1999). These results suggested that MLL fusions with cytoplasmic partners that mediate dimerization have similar effects on downstream *Hox* gene expression in transformed cells as MLL oncoproteins that recruit transcriptional cofactors directly through the fusion partner moiety.

Discussion

A distinguishing feature of MLL-associated leukemias is the remarkable number and variety of partner proteins that undergo in-frame fusions with MLL as a consequence of chromosomal translocations. This is likely to reflect a diversity of mechanisms for oncogenic activation of MLL leading to different pathways of acute leukemogenesis. Some MLL fusion partners are nuclear proteins that normally function as transcription factors, cofactors, or components of chromatin remodeling machines, and deregulation of transcriptional activity through these partner moieties is critical for a subset of MLL oncoproteins (DiMartino et al., 2000, 2002; Lavau et al., 2000; Luo et al., 2001; Slany et al., 1998; So and Cleary, 2002, 2003). Here we provide evidence for an alternative mechanism involving two different MLL fusion partners that are normally cytoplasmic proteins, one of which (Eps15/AF1p) was initially identified as an EGF receptor substrate. While these cytoplasmic proteins do not display inherent transactivation properties, they are nonetheless required for their respective MLL fusion proteins to transform hematopoietic progenitors and induce leukemias in mice. AF1p and GAS7 belong to different families of proteins, which have not been reported to involve common signaling pathways. Their only shared features are coiled-coil domains, which are required by the respective MLL-AF1p and MLL-GAS7 oncoproteins for leukemogenesis. Biochemically, the coiled-coil domains mediate oligomerization of the respective MLL fusion proteins, which directly correlates with their transformation abilities. In addition, forced dimerization of MLL by synthetic dimerization modules mimics bona fide MLL fusion proteins in altering the growth



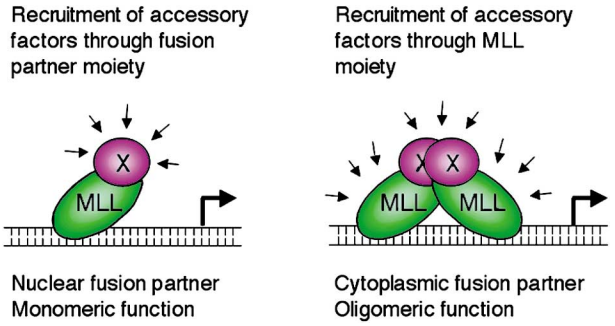


Figure 7. Alternative mechanisms for activating the oncogenic potential of MLL

MLL fusion proteins are schematically shown bound to DNA through their MLL moieties (MLL). Some nuclear fusion proteins with nuclear partner proteins may function as monomers to recruit accessory factors (arrows) through the partner protein moiety (X). A subset of cytoplasmic partner proteins mediates constitutive dimerization/oligomerization to facilitate recruitment of accessory factors in part through the MLL moiety.

properties of myeloid progenitors in vitro. Thus, we conclude that oligomerization of MLL is a novel mechanism for activating its oncogenic potential.

Forced homo-oligomerization of MLL by either bona fide fusion partners or synthetic dimerizers resulted in transcriptional activation of the *HoxA7* promoter, which is likely to be a physiologically relevant target for the MLL oncoprotein. Similar transactivation readouts have also been observed using reporter genes with different promoters (Galoian et al., 2000). These findings are consistent with our observations that expression of MLL-GAS7 and MLL-AF1p results in maintained expression of specific *Hox* genes, which are otherwise repressed in differentiating primary myeloid progenitors. Since similar *Hox* gene expression profiles are observed in progenitors transduced with MLL fusion proteins in which the partner contains a transcriptional effector domain (Ayton and Cleary, 2003), our studies strongly suggest that oligomerization of MLL may lead to oncogenic transformation by aberrant recruitment of coactivator complexes to target genes through the MLL moiety (Figure 7). This mechanism is consistent with the proposed role of oligomerization in recruiting transcriptional repression machinery by myeloid oncoproteins (Scandura et al., 2002). The oligomerization domains of PML (Grignani et al., 1996), PLZF (Dong et al., 1996), NPM (So et al., 2000), Stat5 (Dong and Tweardy, 2002) and NuMA (Dong et al., 2003) are essential for transcriptional repression mediated by the respective RARα fusion proteins.

Figure 6. Transcriptional activation mediated by MLL dimerization

A: MLL-GAS7, MLL-AF1p, or cotransfected MLL-zipper constructs activated transcription from the *HoxA7* promoter in transient transfection assays. 293 cells were cotransfected with *HoxA7* luciferase reporter and various MLL fusion constructs (indicated on left) for luciferase assays. Luciferase values were normalized by LacZ internal control; normalized luciferase values are shown for the average of three experiments.

B: *Hox* gene and cofactor expression profiles of cells transduced by vector alone, MLL-GAS7, or MLL-ENL. RT-PCR analyses were performed on transduced hematopoietic cells harvested at the end of each round of plating (as indicated at top) in methylcellulose culture or from liquid cultures. Total RNA from E15.5 embryos served as a positive control.

Moreover, forced dimerization of RARα by synthetic fusion with oligomerization modules of p53 or p50 NFκB results in aberrant recruitment of transcriptional repression complexes (Lin and Evans, 2000; Minucci et al., 2000). Similarly, the oligomerization domain of ETO in the chimeric transcription factor AML1-ETO is necessary to recruit transcriptional repressor complexes (Minucci et al., 2000). For neither RARα nor AML1, however, has oligomerization been shown to be a requirement for cellular transformation. Our studies extend the role of oligomerization to potential recruitment of activation-associated machinery and demonstrate the essential role of oligomerization in myeloid transformation mediated by chimeric oncoproteins.

Oligomerization may be a critical although not exclusive role for cytoplasmic fusion partners of MLL. MLL fusion proteins containing synthetic dimerization modules only modestly altered the in vitro growth properties of myeloid progenitors, as evidenced by enhanced plating efficiencies that could not be sustained indefinitely, in contrast to the immortalization of progenitors typically induced by bona fide MLL fusion proteins. This may reflect inefficient oligomerization properties of the synthetic leucine zipper and FKBP/FRB dimerization modules in vivo. However, we also observed that mutants of MLL-GAS7 and MLL-AF1p that contained only their coiled-coil domains displayed reduced oncogenic potency in vitro and in vivo compared with the respective intact fusion proteins. This suggests that additional domains present in AF1p and GAS7 may contribute to the oncogenic process, possibly by assisting in the recruitment of cofactors to promote the assembly of stable transcriptional complexes mediated by oligomerization of the MLL moiety. GAS7 contains a WW domain, which in PCIF1 and the histone methyltransferase Set2 has been implicated in transcriptional elongation owing to its specific binding to phosphorylated serines of the carboxy-terminal domain of RNA polymerase II (Fan et al., 2003; Li et al., 2002). In AF1p, the EH hands and DPF repeats are well-characterized protein-protein interaction domains that are capable of recruiting various functionally important proteins (Salcini et al., 1999). The C terminus of AF1p also contains two functionally conserved ubiquitin interaction motifs (UIMs) (Polo et al., 2002), which are important for mediating ubiquitination that has been recently implicated in epigenetic regulation of gene expression (Sun and Allis, 2002). Another possibility is that these domains in the context of MLL fusion proteins may have transdominant antagonistic effects on growth control pathways normally regulated by the partner protein and, thus, facilitate the transformation process. Indeed, disturbances of AF1p and GAS7 expression have been implicated in deregulated cell growth and differentiation. Forced expression of AF1p transforms mouse fibroblasts (Fazioli et al., 1993) while elevated levels of GAS7 induce growth arrest and cell differentiation (Ju et al., 1998). Intact MLL-AF1p or MLL-GAS7, therefore, may have the capability to interfere with the normal functions of the respective partner protein to accelerate leukemogenesis, which has been reported for other MLL fusion proteins (Simone et al., 2001; So and Cleary, 2002). Lack of these properties in the MLL-oligomerization constructs may account for their reduced oncogenic potency. Oligomerization may also account for the leukemias that arise in MLL-βGAL knockin mice with low penetrance and long latency (Dobson et al., 2000) since βGAL is a metabolic enzyme that lacks known transcriptional effector properties but contains a tetramerization domain.

A structure/function analysis demonstrated that several conserved motifs in MLL are required for dimerization-mediated enhancement of the growth properties of myeloid progenitors in vitro. One of these domains, CXXC, shares similarity with DNA methyltransferase and has been shown to mediate binding to unmethylated, but not methylated, CpG DNA (Birke et al., 2002). A second set of DNA binding motifs, the AT hooks, were not necessary. All other conserved motifs targeted by mutations were required for dimer-induced transformation, although their biochemical functions have yet to be determined. Similar MLL requirements were determined recently in a refined structure/function analysis of MLL-ENL (Chen et al., 2002), which is not known to function as a dimer. Rather, ENL is a component of EBAF, a mammalian SWI/SNF chromatin remodeling complex, and MLL-ENL associates with the EBAF complex in leukemia cells (Nie et al., 2003). Our results suggest that dimerized MLL needs to interact with DNA through both halves of the MLL moiety, each displaying similar structural requirements as presumed monomeric fusion proteins such as MLL-ENL. Although wild-type MLL is not known to dimerize in vivo, the third PHD finger of MLL is capable of self-interaction in vitro (Fair et al., 2001), raising the interesting possibility that MLL transcriptional activity may normally be regulated by dimerization. Our structure/function studies may also have implications for the subset of myeloid leukemias containing MLL self-fusions, which result in partial tandem duplication of MLL 5′ sequences (Schichman et al., 1995). The region of duplication typically spans the MLL domains (AS-rich to CXXC) that we found to be required for the growth-altering actions of dimerized MLL. Thus, we hypothesize that MLL self-fusion may be functionally equivalent to forced MLL dimerization leading to sustained expression of *Hox* and other subordinate genes due in part to inappropriate recruitment of accessory factors through the intramolecularly dimerized MLL moiety.

In summary, our studies demonstrate a novel mechanism by which a subset of fusion partners activates the oncogenic properties of MLL. The consequences of forced, constitutive MLL dimerization on subordinate *Hox* genes associated with myeloid leukemic transformation appear similar to those displayed by MLL fusion with nuclear partners. Since coiled-coil or dimerization domains are present in other MLL fusion partners (Prasad et al., 1994; Chaplin et al., 1995; So et al., 1997; Kuefer et al., 2003), oligomerization of MLL may be a general oncogenic mechanism that merits further studies to establish its prevalence in human leukemias.

Experimental procedures

DNA constructs

Retroviral constructs were made by cloning of various cDNA fragments of GAS7 (accession number AB007854), AF1p (accession number U07707), 2×FKBP, FRB_{T2098L} (Ariad Pharmaceuticals, Inc) (www.ariad.com/regulationkits) (Chen et al., 1995; Choi et al., 1996), Acid Zipper, and Base Zipper (Scott et al., 1996) into the NruI and XhoI sites of the MSCV Flag-tagged MLL 5′ cloning vector, which encodes MLL amino acids 1–1396 as previously described (So and Cleary, 2002). MLL 5′ mutant retroviral constructs were made by PCR or site-directed mutagenesis as previously described (Chen et al., 2002). For dimerization studies, cDNA fragments were fused in-frame with the HIS-epitope tag in pcDNA4B cloning vector (Invitrogen); Flag-epitope tagged fusion proteins were expressed using the pcDNA3.1 expression vector (Invitrogen). A GST-GAS7 construct was made by in-frame cloning of GAS7 cDNA into the pGEX vector (Pharmacia). Gal4 fusion constructs consisted of GAS7 (aa 1–412) and AF1p (aa 72–896) cloned into the EcoRI sites of pSG424, which encodes the Gal4 DNA binding domain (aa 1–147)

(DBD). All constructs were sequenced to exclude mutations introduced by PCR.

Hematopoietic progenitor transformation and tumorigenicity assays

Hematopoietic progenitor transformation assays were performed as previously described (So et al., 2003). Briefly, viral supernatants were collected 60 hr after transfection of Phoenix cells and used to infect hematopoietic progenitors and stem cells that were positively selected for c-Kit expression by magnetic activated cell sorting (MACS). Donor cells were harvested from the bone marrows of 4- to 10-week-old wild-type C57BL/6 mice. After spinoculation by centrifugation at 500 × g for 2 hr at 32°C, transduced cells were cultured overnight in RPMI supplemented with 10% FCS and 20 ng/ml stem cell factor (SCF), and 10 ng/ml each of IL-3 and IL-6 (R&D Systems, Minneapolis, Minnesota). Transduced cells were then plated in 1% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with the same cytokines plus 10 ng/ml GM-CSF (R&D Systems) in the presence or absence of appropriate drug selection (i.e., 1 mg/ml G418, 1 μg/ml puromycin, or 1 mg/ml hygromycin). After 7 days culture, colonies were counted to calculate the transduction efficiency. Single-cell suspensions (10^4 cells) of drug-resistant colonies were then replated in methylcellulose media supplemented with the same growth factors. Plating was repeated every 7 days. Transformation results were determined from at least two independent experiments. For tumorigenicity assays, single cell suspensions from each round of replating were expanded in RPMI liquid culture containing 20% FCS plus 20% WEHI-conditioned medium. 10^6 immortalized cells were injected into the retro-orbital venous sinus of 6-week-old syngeneic C57BL/6 mice, which had received a sublethal dose of 5.25 Gy total body γ irradiation (^{135}Cs). Mice were maintained on antibiotic water to avoid infection and monitored for development of leukemia by complete blood count, blood smear, and FACS analysis. Tissues were fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis.

Phenotype analysis

Immunophenotypic analysis was performed by FACS using fluorochrome-conjugated monoclonal antibodies to Sca-1 (D7 clone), c-Kit (2B8 clone), Mac-1 (M1/70 clone), Gr-1 (RB6-8C5 clone), B220 (RA3-6B2 clone), and CD19 (1D3 clone) (Pharmingen Inc, San Diego, California), respectively. Staining was generally preformed on ice for 15 min. Cells were washed twice in staining medium and resuspended in 1 μg/ml propidium iodine (PI) before analysis using a Moflops (a modified triple laser Cytomation/Becton Dickinson hybrid FACS). Dead cells were gated out by high PI staining and forward light scatter.

RT-PCR and Western blot analysis

Cells harvested from liquid or methylcellulose cultures were lysed in Trizol (Life Technologies Ltd) for RNA extraction. cDNAs were synthesized using random hexamer primers and Superscript II (Life Technologies Ltd). RT-PCR was performed using primers specific for *Hox* transcripts or β-actin (control). Details of primer sequences and PCR conditions are available on request. Western blotting was performed using an anti-MLL monoclonal antibody (N4.4) as previously described (So and Cleary, 2002, 2003).

Transcriptional transactivation assays

293 or COS7 cells (5×10^4) were seeded overnight in 24-well plates before transfection of DNA constructs using Fugene (Roche Molecular Biochemicals). Gal4 fusion constructs (0.1 μg) were cotransfected with pcDNA3.1/*Lac*Z internal control plasmid (0.2 μg) and a luciferase reporter construct (0.2 μg), which contained two tandem copies of Gal4 consensus binding sites and the luciferase gene driven by either a herpes simplex virus thymidine kinase (TK), adenovirus E1b, or myelomonocytic growth factor promoter (So and Cleary, 2002, 2003). REH cells (5×10^6) were electroporated with 0.8 μg of each Gal4-DBD fusion construct and luciferase reporter construct together with 0.4 μg of pcDNA3.1/LacZ in RPMI containing 10 μg of DEAE-dextran at 300 V and 960 μF in a 0.4 mm cuvette (Bio-Rad electroporator). Twenty-four hours after transfection, luciferase and β-galactosidase activities were analyzed using commercially prepared reagents. Similar procedures were employed for transactivation studies using the *HoxA7* Luc-reporters (DiMartino et al., 2002; Schreiner et al., 1999) except that 0.3 μg of MLL fusion construct was cotransfected with 0.1 μg of each pcDNA3.1/*Lac*Z control plasmid and the luciferase reporter construct. Luciferase activities were normalized based on β-galactosidase levels. Means and standard deviations were determined from at least two independent experiments performed in duplicate.

In vitro and in vivo interaction studies

GST pulldowns and immunoprecipitation-Western blot (IP-WB) assays were performed as previously described (So and Cleary, 2002). Briefly, [^{35}S] methionine-labeled GAS7 proteins were generated by in vitro transcription and translation using the TNT-coupled reticulocyte lysate system according to the manufacturer's instructions (Promega). GST fusion protein (1 μg) was preincubated with glutathionine-Sepharose beads (Sigma) in NETN buffer (0.5% [v/v] Nonidet P-40, 20 mM Tris/HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) for 60 min at 4°C before [^{35}S] methionine-labeled proteins were added. After 90 min incubation at 4°C, the beads were washed five times in buffer H (20 mM Hepes [pH 7.7], 50 mM KCl, 20% [v/v] glycerol, 0.1% [v/v] Nonidet P40, 0.007% β-mercaptoethanol). Bound proteins were eluted by boiling in SDS/PAGE loading buffer, resolved by electrophoresis, and detected by autoradiography. For IP-WB, 293 cells were transfected in 6-well plates with 1 μg of each His-tagged and Flag-tagged construct. Cells harvested 48 hr posttransfection were subjected to IP-WB using either Catch and Release system (Upstate Biotechnologies) according to the manufacturer's instructions or conventional IP-WB procedures as previously described (So and Cleary, 2002). Briefly, transfected cells were lysed in buffer B (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) for 20 min incubation at 4°C. Cell lysates were recovered by centrifugation at 12,000 g for 15 min and brought to 20% glycerol for immunoprecipitation. three micorgrams D8 anti-His antibody (Santa Cruz Biotechnologies) were incubated with the cell lysates at 4°C for 3 hr. Twenty microliters of protein G sepharose beads (Pharmacia) were then added to the lysates and incubated for additional 2 hr at 4°C before they were washed five times in buffer D (0.5 M KCl, 0.1% Tween 20, 0.5 mM DTT, 0.2 mM EDTA). Precipitated proteins were then denatured at 100°C for 5 min and separated by polyacrylamide gel electrophoresis before transfer to ECL membranes (Amersham) and blotting with M2 anti-Flag antibody (Sigma).

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