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A New Mode of RAF Autoregulation: A Further Complication in the Inhibitor Paradox

Fiona Hey[1] and Catrin Pritchard[1,*]
[1]Department of Biochemistry, University of Leicester, Lancaster Road, Leicester LE1 9HN, UK
*Correspondence: cap8@le.ac.uk
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ERK pathway activation in cells expressing wild-type BRAF is a well-reported, clinically-relevant adverse effect of the otherwise impressive response of $BRAF^{V600E}$-mutated melanomas to RAF inhibitors. In this issue of *Cancer Cell*, Holderfield and colleagues show that RAF autoinhibition underpins this paradox, further complicating therapeutic strategies centered around RAF.

The addiction of cancers to driver oncoproteins has been exploited in the design of novel therapies for cancer treatment; there have been numerous success stories in translating this approach to the clinic. $BRAF^{V600E}$ is a founder, oncogenic driver mutation detected in ~50% of human malignant melanomas. Tumors bearing this mutation are remarkably sensitive to ATP competitive RAF kinase inhibitors, notably vemurafenib (PLX4032) and dabrafenib (GSK2118436) (Flaherty et al., 2010). Resistance to many targeted therapies is associated with longer-term treatment, and, although the initial response to the drug is impressive, certainly in the case of vemurafenib, resistant tumors re-emerge after 2–18 months of treatment.

Apart from acquired drug resistance, the use of RAF inhibitors, unlike other targeted agents, introduces an additional confounding issue that arises due to unique complexities of RAF regulation: ERK pathway activation in cells bearing oncogenic or normally activated RAS. This phenomenon of "paradoxical activation" was initially reported in cancer cell lines bearing *RAS* mutations and was supported by data utilizing a mouse model expressing kinase inactive BRAF (Heidorn et al., 2010). Subsequently, the phenomenon has been shown to account for the emergence of squamous cell carcinomas (SCCs), many of which bear *RAS* mutations, in 15%–30% of vemurafenib-treated human melanoma patients (Su et al., 2012). SCCs are not considered to be a threat to patients as they are well differentiated, nonmetastatic, and relatively easy to remove because they arise on the skin. However, there is always the concern (although not yet proven) that drug treatment may accelerate progression of *RAS*-driven internal lesions that are more difficult to detect. Consequently, there has been a drive to understand the mechanisms underpinning ERK reactivation and to design novel therapies that may circumvent this problem. Paradoxical activation mechanisms have also been proposed to explain drug resistance acquired in $BRAF^{V600E}$ mutant melanomas in situations where a spliced truncated variant of $BRAF^{V600E}$ lacking a RAS-binding domain is expressed (Poulikakos et al., 2011).

Inhibitor-induced ERK pathway activation in cells expressing $BRAF^{WT}$ requires RAF dimerization and transactivation promoted by active RAS (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Initial reports proposed that inhibitor-bound BRAF acts as a scaffold to enhance CRAF activity at the plasma membrane following RAS-induced BRAF-CRAF heterodimer formation. However, in a separate study, Poulikakos et al. (2010) demonstrated RAF homodimer and heterodimer formation in response to inhibitor and proposed a model whereby inhibitor binding to one protomer of the RAF homo- or heterodimer leads to its inhibition but causes transactivation of the other inhibitor-free protomer. Furthermore, transactivation was not diminished in BRAF null cells, highlighting a dominant role of CRAF homodimers. In $BRAF^{V600E}$ tumors, RAS is inactive and BRAF remains a monomer, transactivation of RAF^{WT} is not possible, and inhibition of $BRAF^{V600E}$ becomes dominant. The importance of dimerization was supported by analysis of dimerization mutants ($CRAF^{R401A}$ and $BRAF^{R509H}$) that prevented inhibitor-induced transactivation (Hatzivassiliou et al., 2010). Overall, these studies led to the hypothesis that development of non-ATP competitive inhibitors that prevent RAF dimerization would prevent the adverse transactivation effects.

A novel mechanism involving RAF autoregulation has now been identified by Holderfield et al. (2013), in this issue of *Cancer Cell*, which changes the therapeutic approach significantly. This group developed nine compounds with varying

potencies against RAF, all of which inhibited the ERK pathway in $BRAF^{V600E}$-bearing human melanoma cells but induced ERK activation in KRAS mutant ($BRAF^{WT}$) cells. Further analysis using purified proteins showed the activities of $BRAF^{WT}$ and $CRAF^{WT}$ kinase domains could be blocked by the compounds but only at sub-physiological concentrations of ATP (1 μM). Furthermore, ATP concentration negatively correlated with RAF activity and, at ATP concentrations resembling cellular conditions (1–5 mM), $BRAF^{WT}$ and $CRAF^{WT}$ activities greatly diminished, while that of $BRAF^{V600E}$ increased. The authors suggest ATP-dependent RAF inhibition and the stable nature of the inhibition supported a covalent modification such as phosphorylation. The work demonstrated the need for RAF catalytic activity for ATP-dependent inhibition, and the authors postulated that inhibitor activation of RAF^{WT} was through the prevention of RAF autoinhibition (Figure 1).

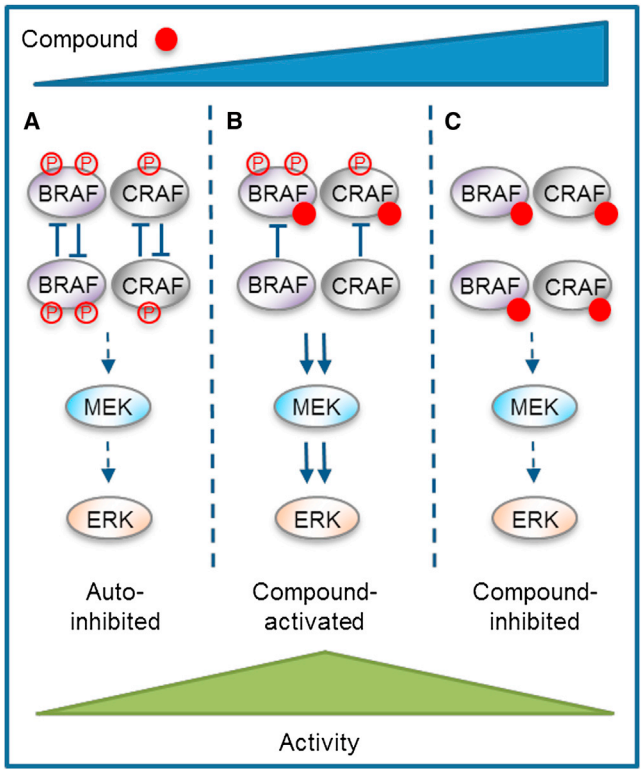

Figure 1. Novel Role of RAF Autophosphorylation and Autoinhibition in Paradoxical ERK Activation

(A) At physiological ATP concentrations, RAF is autoinhibited by trans-P loop phosphorylation of one RAF protomer by the other protomer within a dimer (residues S359 in CRAF and S465/467 in BRAF). These events render RAF inactive and downstream ERK signaling low.

(B) Low to intermediate levels of compound prevent transphosphorylation of one RAF protomer. The nonphosphorylated protomer becomes active, resulting in high ERK signaling.

(C) At high compound concentrations, RAF protomers are inhibited and transphosphorylation is prevented. Although RAF protomers are no longer autoinhibited, compound levels are sufficiently high to displace ATP from the catalytic clefts and inactivate all RAF protomers, blocking downstream ERK activation.

To explore the mechanisms of autoinhibition, Holderfield et al. (2013) performed mass spectrometry of $BRAF^{WT}$ and $CRAF^{WT}$ in the presence of 1 mM ATP. Serine 359 in CRAF and serines 465 and 467 in BRAF, within the glycine-rich phosphate-binding (P) loops, were identified as being important for inhibitory phosphorylation. In HEK293 cells, $CRAF^{S359A}$ was unable to increase phospho-ERK levels in response to RAF inhibitors; BRAF mutants were not tested in this system as overexpression of $BRAF^{WT}$ was unable to induce compound-dependent ERK phosphorylation. As expected, $BRAF^{V600E}$ was found not to be regulated by autoinhibition; consequently, the authors investigated several other frequently occurring non-V600E mutations in cancer, namely glycines 464, 466, and 469 within the P loop, and found G464 mutations also disrupted BRAF autoinhibition and compound-induced activation in vitro. The authors suggest that P loop autophosphorylation may stabilize the inactive "DFG-out" conformation of RAF, leading to autoinhibition, and propose that P loop mutations of BRAF detected in human cancers may render the enzyme unresponsive to P loop autophosphorylation.

To examine the relationship between RAF autoinhibition and dimerization, cell lines with coincident $KRAS^{G12/13D}$ and $BRAF^{G464E/V}$ mutations were analyzed. In cells with P loop mutations, the presence of activated RAS could not drive compound-dependent ERK pathway activation, even though BRAF-CRAF heterodimerization was demonstrated. Furthermore, mutation of the inhibitory serine (S359A) rescued the kinase activity of the dimerization incompetent $CRAF^{R401A}$ mutant, suggesting that monomeric CRAF can become active through the relief of autoinhibition by blocking P loop phosphorylation. These data led the authors to speculate that P loop autophosphorylation is the dominant event over RAF dimerization in compound-induced RAF activation.

RAF regulation is an incredibly complex process involving a multitude of tightly regulated events including RAS interaction, membrane localization, phosphorylation, dimerization, and binding of adaptor proteins (Wellbrock et al., 2004). Autoinhibition of the RAF catalytic domain by the N-terminal domain has been known for many years, and this inhibition is relieved by binding to activated RAS. Sites of phosphorylation in RAF, particularly CRAF, are numerous, but studies of the known regulatory phosphorylation sites have so far only identified S621 in CRAF as an autoregulated event (Noble et al., 2008). The RAF P loop phosphorylations identified by Holderfield et al. (2013) have not been previously documented, despite extensive searches for regulatory RAF phosphorylation events, possibly because ATP concentrations utilized in previous studies were too low. The new data are important as they shed light on the mechanisms of RAF autoregulation (Figure 1), provide an explanation for P loop *BRAF* mutations in human cancers, and question strategies for overcoming paradoxical activation of the ERK pathway in drug-treated situations.

An ideal RAF inhibitor would be one that preserves potency but does not hyperactivate RAF. These new data question whether this can ever be a reality, as a more potent drug will be more potent at relieving RAF autoinhibition (Figure 1). A dimer interface peptide that inhibits RAF dimerization has been shown to suppress ERK pathway activation in cells expressing lower activity RAF mutants (Freeman et al., 2013), but the recent data suggest

this may not be the answer. Administering higher doses of drug to patients is not an option because of off-target effects and toxicities. Due to its unique complexities, perhaps it is time to look beyond RAF? MEK inhibitors are showing promise in the clinic while we all eagerly await clinical data on inhibitors targeting ERK.

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EZH2: An Epigenetic Gatekeeper Promoting Lymphomagenesis

Holger Heyn[1] and Manel Esteller[1,2,3,*]
[1]Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, 08908 Barcelona, Catalonia, Spain
[2]Department of Physiological Sciences II, School of Medicine, University of Barcelona, 08036 Barcelona, Catalonia, Spain
[3]Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Catalonia, Spain
*Correspondence: mesteller@idibell.cat
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In this issue of *Cancer Cell*, Béguelin and colleagues highlight EZH2 as an essential regulator for B cell activation and report an addiction of germinal center-derived neoplasms to EZH2 activity. This reversible process is specifically targetable and hence presents high translational value for lymphoma therapy.

The *enhancer of zeste homolog 2* (EZH2) is a SET domain containing methyltransferase catalyzing the methylation of histone H3, forming the transcriptional repressive epigenetic mark H3K27me3. EZH2 is a subunit of a multi-enzyme complex known as polycomb repressive complex 2 and is involved in chromatin compaction and gene repression. EZH2 is expressed in undifferentiated stem and progenitor cell types but predominantly silenced in somatic cells. Despite its repressive function through H3K27 tri-methylation, it frequently co-localizes with the activating histone modification H3K4me3. These bivalently marked genes present minimal expression level in undifferentiated cells, but upon differentiation initiation, lose H3K27me3 and are transcriptionally activated.

Lymphogenesis represents a special case wherein EZH2 is repressed in resting naive B cells, but is highly upregulated in primary lymphoid follicles during B cell activation and germinal center (GC) formation (Velichutina et al., 2010). Herein, EZH2 defines a GC-specific repression profile including silencing of cell cycle checkpoints and differentiation factors. This epigenetic setting allows rapid B cell proliferation, an important step during the maturation process in germinal centers. Consistently, EZH2 silencing results in cell cycle arrest at G_1/s transition (Velichutina et al., 2010). In line with its proliferation promoting function, EZH2 was shown to be highly expressed in GC-derived lymphomas, such as diffuse large B cell lymphomas (DLBCLs) (van Kemenade et al., 2001). Moreover, mutations in the SET domain, favoring the formation of trimethylated H3K27, have recently been reported as frequent events in DLBCL (Morin et al., 2011). EZH2 mutant tumor cells are almost exclusively detected in the GC-derived subtype, affecting about 20% of GCB-DLBCL patients and suggesting a subtype-specific function of the alteration. Clinically, mutant EZH2 can be specifically targeted using small molecule inhibitors, such as GSK126 (McCabe et al., 2012). Following drug application, EZH2 mutant lymphoma cells revealed reduced levels of H3K27me3 and, most importantly, presented a highly impaired proliferative potential in vitro and in mouse DLBCL xenograft models.

Although there is clear evidence for the contribution of EZH2 to B cell maturation and neoplastic transformation in GCB-DLBCLs, the underlying molecular