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B-Raf activation loop phosphorylation revisited

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The Ras/Raf/MEK/ERK pathway plays a crucial role in physiological processes and its aberrant activity drives various tumor entities.[1] The three RAF isoforms (A-Raf, B-Raf and Raf-1) are major ERK pathway regulators. Commensurate with its role as a signaling hub, B-Raf, the most potent MEK activator, represents the most frequently mutated protein kinase in cancer. *Knock-out/knock-in* approaches delivered critical insights into the unique and overlapping functions of Raf isoforms. If *Braf* deficiency is introduced *via* the germline, $Braf^{-/-}$ mice display a lethal placental phenotype and, if deficiency is limited to the nervous system, a myelination defect restricts viability to the first 5 postnatal weeks (see Refs. [2,3] for discussion and references).

Tight regulation of B-Raf activity is achieved by the incompletely understood protein-protein interaction and (de)phosphorylation events guiding B-Raf through its activation cycle.[1,4] In its inactive state, B-Raf resides in an auto-inhibited conformation in the cytoplasm that is stabilized by 14-3-3 proteins binding to phospho-S365 in the N-terminal regulatory moiety and the C-terminus of the kinase (Fig. 1). Upon interaction with Ras, 14-3-3 is displaced from phospho-S365, thereby exposing the kinase domain of B-Raf, which now forms either homo- or heterodimers.[1] In addition, Ras promotes the *in cis* auto-phosphorylation of T599 and S602 in the evolutionary conserved TVKS-motif of the B-Raf activation loop (AL).[1,5] These phosphorylations induce conformational changes in the kinase domain, leading to the alignment of the so-called C- and R-spine residues, ATP uptake and ultimately MEK phosphorylation.[1] These conformational changes expose, the dimer interface (DIF), a set of amino acid residues forming a contact zone essential for the allosteric activation of the receiver by the activator protomer.[1,4] The relevance of TVKS-motif phosphorylation was demonstrated by experiments showing that substitution of T599/S602 by alanine (AVKA) and phosphomimetic (EVKD) residues impairs Ras-induced activity and confers transforming properties to B-Raf, respectively.[3,5] The dominance of V600 substitutions in tumor-associated *BRAF* mutations further underscores that AL phosphorylation mimicking mutations induce conformational changes that cut the B-Raf activation cycle short. Indeed, $B\text{-}Raf^{V600E}$ signals independently of RAS, 14-3-3 binding, critical phosphorylation sites and, although it forms particularly stable dimers in its normal state, an intact DIF.[3,4] Thus, various lines of evidence suggest that AL phosphorylation induces conformational changes in the B-Raf kinase domain, promoting both dimerization and kinase activity (Fig. 1) and that, once this conformation is stabilized by V600E mutation-specific effects,[1,4] AL phosphorylation becomes redundant.

In addition to point mutations like $BRAF^{V600E}$, chromosomal rearrangements represent alternative tumor-associated *BRAF* alterations. The resulting fusion oncoproteins lack the N-terminal regulatory moiety, but expose an intact kinase domain with increased homo-dimerization potential.[1,6] Hence, they are regarded as constitutively active, although little is known about their regulatory requirements, e.g. whether they require AL phosphorylation.

To further investigate the relevance of the TVKS-motif for B-Raf signaling in development and physiology, we generated a conditional *knock-in* allele allowing the production a B-Raf mutant with alanine substitutions of T599 and S602.[3] Although this $Braf^{AVKA}$ allele produces a kinase with significantly impaired activity, mice homozygous for this allele were surprisingly viable, fertile and had a normal life span. Nevertheless, $Braf^{AVKA}$ mice presented with mild abnormalities in the haematopoietic system, a distinct facial morphology, reduced MEK/ERK phosphorylation in the brain and slight gait abnormalities. Thus, genetic impairment of AL phosphorylation does not phenocopy the lethality of $Braf^{-/-}$ mice, further supporting a scaffolding role for B-Raf. This concept is supported by (pre) clinical observations showing that drug-bound or kinase-dead B-Raf provokes paradoxical ERK-pathway activation, a phenomenon that underlies therapy resistance, inhibitor promoted secondary neoplasms and restricts the application of clinically approved B-Raf inhibitors to $BRAF^{V600E/K}$ mutant tumors.[1,7] Interestingly, however, $B\text{-}Raf^{AVKA}$ did not provoke paradoxical

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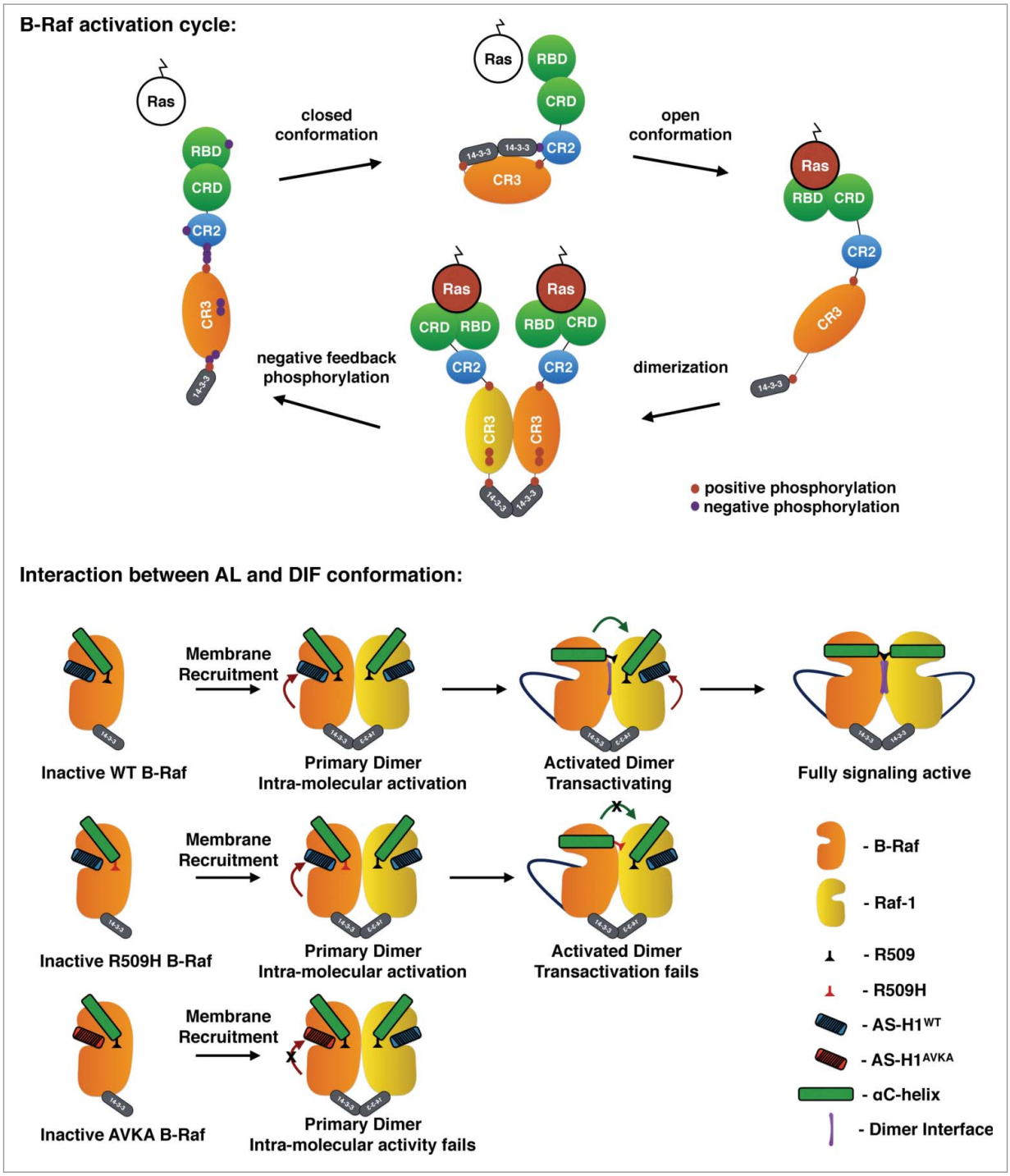

Figure 1. Upper sketch: Model of the B-Raf activation cycle. Negative feedback phosphorylations disrupt the B-Raf/Raf-1 hetero-dimer, leading to re-formation of the closed B-Raf monomer.[1] The regulatory moiety consists of the Ras-binding domain, the cysteine-rich domain and the conserved region 2 encompassing S365. Lower sketch: Interplay between AL phosphorylation, dimerization and transactivation. Top: Dimerization of B-Raf with Raf-1 induces AL phosphorylation (red arrow) followed by conformational changes promoting DIF formation and Raf-1 transactivation. Middle: The R509H mutation prevents DIF formation and Raf-1 transactivation. Bottom: The AVKA mutation in B-Raf precludes the conformational change leading to DIF formation and Raf-1 transactivation.

ERK activation like the kinase-dead B-Raf^{D594A} mutant,[3,4,7] suggesting that the inability of B-Raf^{AVKA} to undergo AL phosphorylation precludes a proper DIF conformation in which it can trans-activate another protomer, e.g., Raf-1 (Fig. 1). Consequently, Raf−1, which still interacts with B−Raf^{AVKA}, cannot be trans-activated, ultimately resulting in impaired MEK activation (Fig. 1). Interestingly, this phenotype mimics the DIF mutant B-Raf^{R509H} that also interacts with Raf-1, but fails to trans-activate its dimerization partner.[4] These related phenotypes further illustrate the tight relation between AL phosphorylation and dimerization, as also proposed by structural studies (see Refs. [1,3] for discussion and references).

Given the mild phenotype of *Braf*^{AVKA} mice, a pharmacological strategy mimicking the effects of the AVKA mutation might allow quenching B-Raf activity without inducing debilitating side-effects, incl. paradoxical ERK

activation. Therefore, we analyzed the requirement for the TVKS-motif in B-Raf oncoproteins. As expected from structural studies showing that B-Raf^{V600E} is locked in an active conformation[1], alanine substitution of T599/S602 did not affect its signaling potential. In contrast, the AVKA mutation reduced the transformation potential of tumor-associated B-Raf oncoproteins such as the dimer promoting B-Raf^{E586K} mutant or the FAM131B-B-Raf fusion protein. These findings have two implications. Firstly, they highlight a strategy to target tumors with non-V600E mutants that might resist V600E/K selective drugs, as it has been observed for the vemurafenib-insensitive fusion proteins.[3,6] Secondly, our findings illustrate that B-Raf fusions and oncogenic non-V600E point mutants are not as constitutively active as previously thought, but still retain a certain level of regulation that could be exploited pharmacologically.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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