Activation loop phosphorylation regulates B-Raf *in vivo* and transformation by B-Raf mutants

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Abstract

Despite being mutated in cancer and RASopathies, the role of the activation segment (AS) has not been addressed for B-Raf signaling *in vivo*. Here, we generated a conditional knock-in mouse allowing the expression of the B-Raf^{AVKA} mutant in which the AS phospho-acceptor sites T599 and S602 are replaced by alanine residues. Surprisingly, despite producing a kinase-impaired protein, the B-Raf^{AVKA} allele does not phenocopy the lethality of B-Raf-knockout or paradoxically acting knock-in alleles. However, B-Raf^{AVKA} mice display abnormalities in the hematopoietic system, a distinct facial morphology, reduced ERK pathway activity in the brain, and an abnormal gait. This phenotype suggests that maximum B-Raf activity is required for the proper development, function, and maintenance of certain cell populations. By establishing conditional murine embryonic fibroblast cultures, we further show that MEK/ERK phosphorylation and the immediate early gene response toward growth factors are impaired in the presence of B-Raf^{AVKA}. Importantly, alanine substitution of T599/S602 impairs the transformation potential of oncogenic non-V600E B-Raf mutants and a fusion protein, suggesting that blocking their phosphorylation could represent an alternative strategy to ATP-competitive inhibitors.

Keywords BRAF fusion; Cre/loxP system; gene targeting; MAPK pathway; Ras

Subject Categories Cancer; Signal Transduction

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Introduction

The Ras/Raf/MEK/ERK pathway plays a pivotal role in controlling proliferation, survival, and differentiation. As this pathway is often deregulated in various diseases, in particular cancer and RASopathies, its components are pursued as targets for pharmacological intervention (Holderfield *et al*, 2014; Samatar & Poulikakos, 2014). Raf kinases represent a particularly important node as they are subject to a complex and tight regulation (Cseh *et al*, 2014). The Raf family comprises A-Raf, B-Raf, and Raf-1 in vertebrates and single *raf* genes in invertebrates such as D-Raf and LIN-45 in *Drosophila* and *Caenorhabditis*, respectively. Distant relatives are the KSR proteins, which promote MEK activation as scaffolds, allosteric Raf activators, and potentially by their own kinase activity (Brennan *et al*, 2011). As described below, the presence of three Raf and two KSR isoforms allows fine-tuning of Raf activity by forming homo- and heterodimers with distinct signaling potential (Cseh *et al*, 2014; Mooz *et al*, 2014). For example, B-Raf/Raf-1 heterodimers represent the most potent MEK activator (Rushworth *et al*, 2006; Freeman *et al*, 2013).

Genetic analyses demonstrated that Raf isoforms possess unique and overlapping functions. B-Raf is required for maximum ERK activation and for placental development as it is illustrated by the embryonic lethality of B-Raf-deficient mice (Wojnowski *et al*, 2000; Brummer *et al*, 2002; Galabova-Kovacs *et al*, 2006, 2008; Zhong *et al*, 2007). B-Raf displays the most potent transforming activity among the three isoforms and is often activated by somatic alterations in cancer (Pritchard *et al*, 1995; Holderfield *et al*, 2014). Consequently, B-Raf appears as an attractive therapeutic target and inhibitors such as vemurafenib yield unprecedented response rates

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in melanoma (Samatar & Poulikakos, 2014). However, the use of current B-Raf selective inhibitors is restricted to *BRAF* mutant tumors, because binding of these ATP-competitive compounds to wild-type B-Raf (B-Raf^{WT}) provokes the so-called paradoxical activation of the ERK pathway. This phenomenon involves active Ras and the allosteric activation of a drug free by an inhibitor-bound or kinase-dead Raf protomer *via* the dimer interface (DIF) (Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010; Poulikakos *et al*, 2010; Röring *et al*, 2012). This paradoxical action of B-Raf inhibitors contributes to drug resistance and promotes secondary neoplasms ((Yaktapour *et al*, 2014) and references therein). Therefore, current B-Raf inhibitors must not be used in tumors with aberrant Ras activity. Thus, alternative strategies are urgently needed for the inhibition of Raf kinases and are likely to emerge from a better understanding of B-Raf regulation.

All Rafs share three highly conserved regions (CRs): the N-terminal CR1 mediates the interaction with Ras-GTP via R188 (amino acid positions refer to human B-Raf). The CR2 recruits 14-3-3 proteins and is critical for autoinhibition. The CR3 harbors the kinase domain and phosphorylation sites within two areas, the N-region and the activation segment (AS) consisting of the activation and P + 1 loops (Zhang & Guan, 2000; Nolen *et al*, 2004; Röring & Brummer, 2012). The CR3 also includes key residues controlling dimerization such as the DIF, including R509 in vicinity to the α C helix, and the C-terminal 14-3-3 binding motif (Rajakulendran *et al*, 2009; Röring *et al*, 2012). The DIF also mediates the allosteric trans-activation between Raf protomers (Röring *et al*, 2012; Hu *et al*, 2013).

Upon membrane recruitment by Ras-GTP, B-Raf undergoes conformational changes associated with increased dimerization, phosphorylation, and activity (Lavoie & Therrien, 2015). The Ras-GTP- and dimerization-triggered autophosphorylation of the AS at T599 and S602 in human B-Raf is supposed to represent a key event in Raf activation (Zhang & Guan, 2000). Based on crystal structures, V600 of the AS engages in a hydrophobic interaction with the P-loop that stabilizes the closed inactive conformation of the kinase domain (Wan *et al*, 2004). Phosphorylation of the T⁵⁹⁹VKS⁶⁰² motif is supposed to disrupt this interaction and initiates the subsequent phosphotransferase reaction by ATP uptake (Thevakumaran *et al*, 2015). Alternatively, AS phosphorylation might contribute to conformational changes in the kinase domain, leading to catalysis and allosteric activation (Hu *et al*, 2015; Thevakumaran *et al*, 2015). The precise mechanism leading to AS phosphorylation remains elusive, although recent data on Raf-1 suggest a dimerization-induced autophosphorylation *in cis* (Hu *et al*, 2013).

The high frequency of point mutations and in-frame insertion/deletions in the AS further underscore its critical role in B-Raf regulation. Most alterations affect the N-terminal portion of the AS, the activation segment helix 1 (AS-H1). The dominating AS-H1 mutation, the V600E substitution, occurs in about 7% of tumors and generates a constitutively active oncoprotein that is uncoupled from many regulatory processes controlling wild-type B-Raf activity (Garnett & Marais, 2004; Röring *et al*, 2012). Albeit at lower frequency, mutations of T599 and S602 are also found in a variety of cancers ((Eisenhardt *et al*, 2011; Thevakumaran *et al*, 2015) and the COSMIC database). Moreover, various *in vitro* studies using ectopically expressed proteins with T599 and S602 (or their equivalents) replaced by phosphomimetic residues further underscore the

essential role of the TVKS motif for the activation of B-Raf, Raf-1, LIN-45, and D-Raf (Zhang & Guan, 2000; Chong *et al*, 2001; Rajakulendran *et al*, 2009; Röring *et al*, 2012). On the other hand, conversion of the TVKS into an AVKA motif by alanine substitution prevents the Ras-induced increase in B-Raf *in vitro* kinase activity (Zhang & Guan, 2000). However, the phosphoacceptor sites of the TVKS motif have not been assessed for their importance in oncogenic B-Raf mutants and most importantly, as it was also pointed out recently (Lavoie & Therrien, 2015), not for the activation of B-Raf *in vivo*. Furthermore, although crystallography provides essential insights into the regulation of the kinase domain, these studies all rely on truncated, dephosphorylated, and drug-bound proteins in which the precise orientation of the complete AS remains unresolved (Hu *et al*, 2015; Thevakumaran *et al*, 2015). Thus, structural studies are best complemented by biochemical and genetic approaches interrogating full-length B-Raf in its cytoplasmic environment.

Here, we evaluated the relevance of TVKS motif phosphorylation for normal and oncogenic B-Raf signaling. By using a knock-in approach, we show that a B-Raf mutant lacking the two phosphoacceptor residues of the TVKS motif permits long-term postnatal development. Nevertheless, TVKS motif phosphorylation is essential for optimal signaling output of wild-type B-Raf and several non-V600E oncoproteins. Thus, blocking TVKS motif phosphorylation could represent an alternative strategy to the ATP-competitive compounds that are currently in use for B-Raf inhibition.

Results

The TVKS motif plays an important role for normal and aberrant B-Raf signaling

As little functional characterization of the TVKS motif has been conducted since its first description (Zhang & Guan, 2000), we revisited the function of its phosphoacceptor sites in more detail and in the context of the advances of the field. First, we asked how much this sequence motif has been conserved during evolution. Indeed, the AS, which spans the region between the DFG and APE motifs in subdomains VII and VIII, respectively, has been highly conserved in Raf orthologues, in particular if the AS-H1 is considered. Importantly, the phosphoacceptor sites of the TVKS motif are not only found in eumetazoans, but also in the sponge *Amphimedon* suggesting a conserved function from the emergence of multicellular animals onwards (Fig 1A). Interestingly, while the V600 equivalent is substituted by other aliphatic residues such as alanine or isoleucine in some species, the threonine and lysine residues were maintained throughout evolution and the S602 equivalent has been subject to a conservative exchange to threonine in Raf proteins of protostomic invertebrates and in A-Raf.

So far, a role of the phosphoacceptor sites in the TVKS motif has been demonstrated for the activation of A-Raf, B-Raf, and Raf-1 in the context of oncogenic Ras, phorbol esters, EGF, and the GPCR agonist carbachol (Barnard *et al*, 1998; Zhang & Guan, 2000; Chong *et al*, 2001; Tsukamoto *et al*, 2004; Baljuls *et al*, 2008). These studies, which differ in design and experimental approaches, identified distinct requirements of the individual isoforms for an intact TVKS motif, for example, in response to EGF (Baljuls *et al*, 2008).

Figure 1. Loss of the phosphoacceptor sites of the TVKS motif impairs the signaling potential of wild-type B-Raf and some of its gain-of-function mutants.

A The TVKS/T motif has been conserved throughout metazoan evolution. The phosphoacceptor sites are indicated in red, and the DFG and APE motif flanking the activation loop (AL) are indicated in blue. Residues differing from human B-Raf are shown in gray or in red/blue with reduced intensity.

B B-Raf^{E586K} signals independently from Ras-GTP, but requires an intact AL. The MEK-ERK activation potential of the indicated HA-tagged B-Raf mutants was assessed by Western blotting using TCLs from transiently transfected Plat-E cells.

C B-Raf^{CAAX} but not the B-Raf^{V600E} oncoprotein signals independently from Ras-GTP, but requires an intact AL. Same experimental setup as in (B).

D Quantification of experiments shown in (B) and (C). The signal elicited by the individual reference proteins (B-Raf^{WT}, B-Raf^{E586K}, B-Raf^{V600E}, and B-Raf^{CAAX}) was set in each analysis to 100%. $n = 3$, mean + SEM, t-test, ** $P < 0.01$, *** $P < 0.001$.

However, the question whether oncogenic B-Raf mutants require the phosphoacceptor residues of the AS has not been addressed so far. Therefore, we introduced alanine substitutions of T599 and S602 of the TVKS motif, termed as AVKA mutation in the following, into wild-type B-Raf (B-Raf^{WT}) and three of its gain-of-function mutants. We first turned to B-Raf^{E586K}, which occurs in human tumors and displays oncogenic activity due to its enhanced dimerization potential and ability to activate other Raf protomers *in trans* (Emuss *et al*, 2005; Rajakulendran *et al*, 2009; Röring *et al*, 2012). Interestingly, while blocking Ras/B-Raf interaction with the R188L substitution had no effect on B-Raf^{E586K}, the AVKA mutation reduced the MEK/ERK phosphorylation potential of this B-Raf oncoprotein by 70% (Fig 1B and D). In light of its enhanced dimerization

behavior (Rajakulendran *et al*, 2009; Freeman *et al*, 2013) and the notion that B-Raf dimerization is strongly promoted by Ras (Heidorn *et al*, 2010), the impaired signaling potential of B-Raf^{E586K/AVKA} further supports the recent model in which Ras promotes full Raf activity by dimerization and subsequent AS phosphorylation (Hu *et al*, 2013). The AVKA mutation also reduced the signaling potential of B-Raf^{WT} and B-Raf^{CAAX} (Fig 1B–D). The latter displays transforming activity due to its constitutive membrane localization conferred by the polybasic region and CAAX-box of K-Ras (Papin *et al*, 1998; Röring *et al*, 2012). In sharp contrast, a B-Raf mutant introducing the V600E mutation into B-Raf^{AVKA} (B-Raf^{AEKA}) displayed a comparable MEK/ERK phosphorylation potential as B-Raf^{V600E} (Fig 1C and D). This demonstrates that the AVKA

mutation does not inactivate the kinase, but prevents its activation following membrane recruitment and dimerization.

As truly kinase-dead B-Raf mutants like B-Raf^{D594A} or B-Raf^{K483M} act as allosteric activators for wild-type Raf proteins and thereby provoke paradoxical activation of the MEK/ERK pathway in the context of oncogenic Ras (Heidorn *et al*, 2010; Röring *et al*, 2012), we next compared them with B-Raf^{AVKA} in this setting. To this end, we expressed these B-Raf mutants in a *Braf*-deficient murine embryonic fibroblast (MEF) reconstitution system (*Braf*^{-/-};ERTmH-Ras^{G12V} MEFs) in which oncogenic H-Ras signaling can be initiated by 4-hydroxy-tamoxifen (4-HT)-mediated release of an ERTmH-Ras^{G12V} fusion protein from its inhibitory chaperone complex (Röring *et al*, 2012). As shown in Fig 2A and B, B-Raf^{WT} and its kinase-dead mutants augment MEK and ERK phosphorylation in B-Raf-deficient MEFs, which is further increased by 4-HT-induced Ras signaling. These data are in full agreement with the paradoxical MEK/ERK activation triggered by kinase-dead B-Raf (Heidorn *et al*, 2010; Röring *et al*, 2012). In contrast, complementation of *Braf*^{-/-} MEFs with B-Raf^{AVKA} leads only to a slight improvement of steady-state MEK/ERK phosphorylation compared to empty vector infected cells, indicating again that it lacks the full signaling potential of B-Raf^{WT}. Most importantly, B-Raf^{AVKA}, despite its impaired signaling potential, does not provoke paradoxical MEK/ERK phosphorylation as the B-Raf^{D594A} mutant (Fig 2A and B).

Next, we asked whether the AVKA mutation was able to reduce the paradoxical MEK/ERK phosphorylation of B-Raf^{D594A}. As shown in Fig 2C and D, the AVKA mutation had only a marginal effect on the paradoxical effect of B-Raf^{D594A}. This result is in sharp contrast to the effect of the R509H mutation on B-Raf^{D594A} (Röring *et al*, 2012) and indicates that this kinase-dead mutant does not require AS phosphorylation but an intact DIF for allosteric transactivation of Raf-1. Accordingly, the B-Raf^{D594A/AVKA} double mutant still recruited more Raf-1 than B-Raf^{WT}, although there was a significant reduction compared to B-Raf^{D594A} proper (Fig 2E). Interestingly, the AVKA mutation also reduced the prominent phosphorylation-dependent electrophoretic mobility shift of B-Raf^{D594A} (Figs 2C and E, and EV1A), suggesting that TVKS motif phosphorylation either directly or indirectly contributes to this phenomenon.

Generation of a conditional *Braf*^{AVKA}-knock-in mouse model

So far, the role of the phosphoacceptor residues of the AS has been only investigated in tissue culture experiments involving the ectopic expression of mutant Raf kinases with alanine (AVKA) or phosphomimetic (EVKD) substitutions (Zhang & Guan, 2000; Chong *et al*, 2001; Baljuls *et al*, 2008; Rajakulendran *et al*, 2009; Röring *et al*,

2012), but not by *in vivo* approaches analyzing endogenous B-Raf. Therefore, we addressed the role of the phosphoacceptor sites of the TVKS motif by a knock-in approach producing a B-Raf protein in which T599 and S602 were replaced by alanine residues. As B-Raf expression and activity are subject to a tight spatiotemporal control and are regulated by alternative splicing and presumably two alternative promoters (Barnier *et al*, 1995), a conditional mutagenesis allowing the expression of B-Raf^{AVKA} from its endogenous locus appeared necessary (Fig 3A). This strategy maintains B-Raf^{WT} expression until Cre excises the loxP-site-flanked (floxed) minigene cassette. This places the mutated exon 15^{AVKA} downstream of exon 14, thereby allowing the production of a transcript encoding B-Raf^{AVKA}.

Using the targeting vector shown in Fig 3A, we obtained several correctly targeted ES cell clones (Fig 3B and C) of which two (#215, #273) successfully contributed to germline chimera (see Appendix for further details). Their progeny was then mated with Flp-e transgenic mice to remove the neo^R cassette and to generate the *Braf*^{loxAVKA} allele (Fig 3A).

For initial characterization of the targeted locus, we crossed homozygous *Braf*^{loxAVKA/loxAVKA} mice of a mixed 129SVxC57/Bl6 background with CMV::Cre deleter mice. Depending on inheritance of the Cre transgene, this generates either *Braf*^{+/loxAVKA} or *Braf*^{+/AVKA} mice, as confirmed by genotyping PCR (Fig 3D). Using RNA from a *Braf*^{+/AVKA}; Cre⁻ mouse and *Braf*^{+/loxAVKA}; Cre⁺ littermate, we confirmed the co-expression of *Braf*^{AVKA} and *Braf*^{WT} transcripts in the latter (Fig 3E). We also crossed homozygous *Braf*^{loxAVKA/loxAVKA} animals with Sox2::Cre transgenic mice (Hayashi *et al*, 2002). Mating *Braf*^{loxAVKA/loxAVKA} mice with both Cre deleter strains yielded normal sized litters with the expected allele frequencies. Thus, in contrast to *Braf*^{V600E/+}, *Braf*^{Q257R/+}, *Braf*^{D594A/+}, and *Braf*^{L597V/+}-knock-in mice (Mercer *et al*, 2005; Kamata *et al*, 2010; Andreadi *et al*, 2012; Inoue *et al*, 2014), which all display a severe phenotype with pre- or postnatal lethality, heterozygous *Braf*^{+/AVKA} mice appear normal, are fertile, and were kept without any obvious abnormalities.

Next, we assessed the phenotype of homozygous *Braf*^{AVKA} mice. To this end, we exploited the cytoplasmic Cre expression in Sox2::Cre oocytes to generate *Braf*^{+/AVKA} animals without a Cre transgene in the F₁ generation. Subsequently, Cre-negative *Braf*^{+/AVKA} mice were mated with each other to generate *Braf*^{+/+}, *Braf*^{+/AVKA}, and *Braf*^{AVKA/AVKA} mice. As shown in Table 1, these matings produced litters with genotype frequencies at the expected Mendelian ratios in progenies derived from ES cell clones #215 and #273. At weaning, there was no significant difference in weights (Fig 4A), although we observed a tendency for *Braf*^{AVKA/AVKA} mice to be lighter. In sharp contrast, when *Braf*^{+/+} animals (also

Figure 2. Release of oncogenic H-Ras leads to hyperphosphorylation of MEK by kinase-dead B-Raf mutants, but not by B-Raf^{AVKA}.

- Braf*^{-/-};ERTmHRas^{G12V} MEFs were reconstituted with HA-tagged hBRAF constructs or empty vector (EV). Infected cells were either treated with 4-HT (1 μM) to induce oncogenic Ras activation or with ethanol (OH) as a control.
- Quantification of pMEK (left) and pERK (right) normed to WT level. *n* = 3, mean + SEM, *t*-test, ***P* < 0.01.
- The AVKA mutation has no discernible influence on Ras-induced paradoxical ERK activation triggered by the D594A mutation. Experimental setup as in (A), shown are lysates from 4-HT-treated MEFs.
- Quantification of pMEK and pERK levels normed to HA expression. *n* = 3, mean + SEM, *t*-test, ***P* < 0.01.
- Top: *Braf*^{-/-};ERTmHRas^{G12V} MEFs were reconstituted with the indicated B-Raf constructs. Following induction of Ras signaling, HA-B-Raf proteins were purified using anti-HA antibodies and subjected to Western blotting to analyze immunoprecipitation of Raf-1. Bottom: Quantification of co-immunoprecipitated Raf-1 normed to HA-B-Raf levels. *n* = 3, mean + SEM, *t*-test, **P* < 0.05.

Figure 2.

Figure 3. Generation of the conditional *Braf*^{AVKA}-knock-in mouse.

- A** Targeting strategy. Top: Targeting vector pSC-floxAVKA and a part of the murine *Braf* locus containing exons E14–E16 (not drawn to scale). Middle: *Braf* locus after homologous recombination with the targeting vector replacing E15 with a loxP-flanked (floxed) minigene (MG; E15–20) segment. pA, polyadenylation signals; SA, splice acceptor. LoxP and Frt sites are indicated by red triangles and blue hexagons, respectively. Bottom: The modified *Braf* locus after Flp-e and Cre-mediated recombination.
- B** Genomic PCR with the primers indicated as red arrows in (A) showing 3 out of 9 clones being positive for homologous recombination.
- C** Southern blot analysis of genomic DNA of parental W4 ES cells, wild-type MEFs, and the ES cell clones #286 and #273 (with recombined *Braf*^{floxAVKA} allele). Genomic DNA was digested by Asel and analyzed using the external probe indicated in (A).
- D** Genomic PCR showing Cre-mediated recombination of the *Braf*^{floxAVKA} allele *in vivo* using the primer pair E14fwd and E15rev (indicated as blue arrows in A).
- E** Electropherograms of sequenced RT–PCR amplicons generated using splenic RNA of a *Braf*^{+/floxAVKA} (left) and a *Braf*^{+/AVKA;Cre} mouse (right). RT–PCR amplicons were generated using the primers E14fwd and E16revNEW. Note the double peaks at the first position corresponding to the threonine/serine and alanine codons (indicated by red arrows).

Table 1. Litter genotype distribution from *Braf*^{+/AVKA} intercrossed derived the two different founder knock-in strains # 215 and #273.

	Observed	Observed (%)	Expected	Expected (%)
#215				
+/+	49	26.63	46	25
+/AVKA	91	49.46	92	50
AVKA/AVKA	44	23.91	46	25
Total	184	100	184	100
#273				
+/+	111	23.57	117.8	25
+/AVKA	232	49.26	235.5	50
AVKA/AVKA	128	27.18	117.8	25
Total	471	100	471	100

generated by the Sox2::Cre strategy), were mated with each other, we observed among the 73 weaned pups 42% *Braf*^{+/+} and 58% *Braf*^{+/-}, but no *Braf*^{-/-} animals. This is in full agreement with the embryonic lethal phenotype reported for three independently generated *Braf*-knockout lines (Wojnowski *et al*, 1997; Galabova-Kovacs *et al*, 2006; Zhong *et al*, 2007). Thus, despite producing a B-Raf protein with impaired MEK phosphorylation potential (Fig 1B and C), the *Braf*^{AVKA}-knock-in allele does not phenocopy the effects of the knockout allele. Homozygous *Braf*^{AVKA} mice did not display any shortened life span, and both males and females were fertile and able to raise pups.

Phenotypic characterization of *Braf*^{AVKA} mice

As loss of B-Raf expression impairs the development, activation, and effector functions of cells of the immune and hematopoietic systems (Brummer *et al*, 2002; Kamata *et al*, 2005; Tsukamoto *et al*, 2008; Dillon *et al*, 2013), we next analyzed the cellular composition of lymphatic organs. First, we noticed that spleens of homozygous *Braf*^{AVKA} mice contained significantly lower amounts of cells (Fig 4B). Although all major hematopoietic subsets were present (Fig EV2A–E), certain subpopulations differed in size between wild-type and knock-in mice. The number of naive CD8⁺ T cells was significantly increased in B-Raf^{AVKA} mice compared to wild-type littermates. This observation was in line with a significant reduction in CD8⁺ T effector memory cells (TEM) and CD8⁺ T regulatory cells (Tregs) and suggests that differentiation into effector populations requires B-Raf activity (Fig 4C–E). To evaluate whether this maturation defect is directly linked to B-Raf signaling output, we isolated splenic T cells, stimulated them with a combination of anti-CD3 and anti-CD28 antibodies, and analyzed pERK levels by flow cytometry. We observed a slight but significant reduction in pERK levels in CD8⁺ cells 15 min after addition of the stimulus (Fig EV2F). Consistent with their normal population size in *Braf*^{AVKA} mice, pERK levels were not significantly affected CD4⁺ T cells (Fig EV2G). Furthermore, *Braf*^{AVKA} B cells were impaired in their activation *ex vivo* upon B-cell antigen receptor (BCR) and Toll-like receptor 4 (TLR4) stimulation as measured by surface expression of the activation marker and ERK target gene product CD69 (Minguet *et al*, 2005) (Fig 4F). Also, impaired LPS responses were associated with the failure to induce proliferation upon stimulation (Fig 4G).

With increasing age, we noticed two further abnormalities. Firstly, homozygous *Braf*^{AVKA} mice presented with shorter snouts and a more upward orientation of the eyes (Fig 5A). Secondly, homozygous *Braf*^{AVKA} animals could be readily detected in cages by their distinct gait accompanied by a hunchback posture that is often displayed by mice with ataxia (Video EV1).

The later observation, together with the long-known fact that B-Raf is highly expressed in the brain and fulfills multiple functions in neurons and macroglia (Chen *et al*, 2006; Zhong *et al*, 2007; Galabova-Kovacs *et al*, 2008; Tien *et al*, 2012; Pfeiffer *et al*, 2013), prompted us to compare the B-Raf signaling output in total brain extracts of *Braf*^{WT} and *Braf*^{AVKA} littermates. Firstly, we performed *in vitro* kinase (IVK) assays and could confirm for the first time that endogenously expressed B-Raf^{AVKA} possesses < 50% of the MEK phosphorylation potential of B-Raf^{WT}, irrespective of its purification under mild (NLB; 0.5% NP-40) or harsh (RIPA) buffer conditions (Fig 5B). This decrease in IVK activity was also reflected *in vivo* as MEK and ERK phosphorylation was consistently reduced in *Braf*^{AVKA} brain lysates (Figs 5C and EV3A and B). This reduction was not affected by sex or age. To obtain further insights into the brains of *Braf*^{AVKA} mice, we performed immunohistochemistry. Macroscopic and histological examination did not reveal gross differences between brains from *Braf*^{WT} and *Braf*^{AVKA} littermates. However, Purkinje cells of *Braf*^{AVKA} mice presented with reduced phospho-ERK staining (Fig 5D), a phenotype that was also reported for *Braf*^{-/-} mice (Galabova-Kovacs *et al*, 2008). As *Braf*^{-/-} mice display myelination defects, we analyzed the expression of myelin basic protein (MBP), whose expression was strongly reduced in *Braf*^{-/-} animals (Galabova-Kovacs *et al*, 2008). Interestingly, however, we could not observe significant differences in MBP levels in brain extracts and the staining intensity of myelinated fibers in cerebellar sections from *Braf*^{AVKA} and *Braf*^{+/+} mice (Fig EV3C–E). This lack of an obvious myelination defect could explain why *Braf*^{AVKA}-knock-in mice show a less severe and delayed neurological phenotype than *Braf*^{-/-} animals.

As B-Raf^{AVKA} is potentially expressed in homozygous knock-in animals from the zygotic stage onwards, it was conceivable that other Raf isoforms could be up-regulated to compensate for the impaired signaling output by B-Raf^{AVKA}. Nevertheless, B-Raf, Raf-1, A-Raf, or KSR1 expression levels were not different between *Braf*^{WT} and *Braf*^{AVKA} mice (Figs 5C and EV4A and B). Interestingly, however, we observed a significant reduction in KSR1 by 40% in B-Raf^{AVKA} compared to B-Raf^{WT} immunoprecipitates, while the amount of co-immunoprecipitated 14-3-3 proteins did not differ between B-Raf^{WT} and B-Raf^{AVKA} (Fig 6A). This suggests that the interaction of B-Raf with KSR1 is promoted by AS phosphorylation. In contrast, the heterodimerization between B-Raf and Raf-1 was not affected by the AVKA mutation in mouse brain lysates (Fig 6A) or in a co-immunoprecipitation assay (Fig 6B). By co-expressing HA-tagged and Myc-tagged B-Raf^{AVKA} proteins, we could also show that an intact TVKS motif is not essential for the formation of B-Raf homodimers (Fig 6C). Interestingly, however, the phosphomimetic substitutions of T599 and S602 (EVKD) enhanced the homodimerization potential of B-Raf to a similar degree as the E586K mutation, which promotes homodimerization (Rajakulendran *et al*, 2009; Freeman *et al*, 2013), although this effect was not as pronounced as observed for B-Raf^{V600E} (Fig 6C).

Figure 4. Basic comparison and immune phenotyping between homozygous *Braf* WT and AVKA mice.

- A Weight at weaning of *Braf*^{AVKA} mice from two different founders showed no significant differences. 215: *n* = 24 (WT), 32 (Heterozygous), 19 (AVKA); 273: *n* = 114 (WT), 117 (Heterozygous), 52, *t*-test.
- B Homozygous *Braf*^{AVKA} mice show reduced splenic cellularity. *n* = 7, *t*-test.
- C–E Relative number of CD8⁺ T cells. FACS analysis shows an increase in naïve cells (C), whereas differentiated TEM (D) and Tregs (E) reveal a reduction in *Braf*^{AVKA} mice compared to WT. *n* = 8, *t*-test.
- F Primary B cells of *Braf*^{AVKA} mice show reduced CD69 surface expression upon LPS and anti-IgM stimulation. *n* = 3, *t*-test.
- G Primary B cells also show reduced proliferation upon stimulation compared with WT B cells. *n* = 3, *t*-test.

Data information: Mean + SEM, **P* < 0.05, ***P* < 0.01.

Figure 5. MEK and ERK activity is drastically impaired in the brain of AVKA-knock-in mice.

A Facial appearance of *Braf*^{AVKA} mice.

B Top: *In vitro* kinase (IVK) assay of B-Raf complexes purified from either *Braf*^{WT} or *Braf*^{AVKA}-knock-in brains from both founder strains revealed a reduced MEK phosphorylation potential regardless of their purification from NLB or RIPA lysates. Bottom: Quantification of IVK differentials: #215 samples indicated in black, #273 samples in gray. $n = 5$, mean + SEM, t -test, $^{**}P < 0.01$.

C Western blot analysis showing reduced pMEK and pERK levels in *Braf*^{AVKA} brains.

D Immunohistochemistry showing reduced pERK levels in Purkinje cells of homozygous *Braf*^{AVKA} mice. Brain sections imaged at 10 $\times$ magnification.

Figure 6. Characterization of the homo- and heterodimerization potential of B-Raf^{AVKA}.

- A** Total brain lysates of WT and *Braf*^{AVKA} mice were subject to immunoprecipitation (IP) using either anti-B-Raf antibodies or non-specific IgG as a negative control. The co-purified proteins were visualized using the indicated antibodies and quantified. Ksr1 is significantly decreased in B-Raf^{AVKA} complexes, while the other proteins are equally bound to B-Raf^{WT} or B-Raf^{AVKA}. *n* = 3, mean + SEM, t-test, **P* < 0.05.
- B** Anti-Myc IP of Plat-E cells transfected with Myc-tagged Raf-1 and different HA-B-Raf constructs. Western blot analysis showed that B-Raf^{AVKA} is not impaired in its ability to interact with Raf-1.
- C** Anti-Myc IP of Plat-E cells expressing different Myc- and HA-B-Raf proteins. Note that B-Raf^{AVKA} is not impaired in its ability to interact with other B-Raf proteins, while homodimerization of B-Raf^{EVKD} is enhanced.
- D** Top: Exemplary Western blot analysis of EGF-stimulated primary astrocytes. Mutant astrocytes show drastic reduction in basic and stimulated levels of pMEK and pERK. Bottom: Quantification of all Western blot samples. WT: *n* = 7, AVKA: *n* = 6, mean + SEM, t-test, **P* < 0.05, ***P* < 0.01.

As astrocytes represent the most abundant cell lineage in the brain and because B-Raf signaling is of growing importance for our understanding of normal and neoplastic macroglia (Eisenhardt *et al*, 2011; Tien *et al*, 2012), we compared MEK and ERK phosphorylation in short-term cultured primary astrocytes isolated from newborn *Braf*^{WT} and *Braf*^{AVKA} animals (Fig 6D). This revealed that both pMEK and pERK levels were significantly reduced under normal growth conditions and in the presence of EGF.

TVKS motif phosphorylation is required for efficient ERK signaling

To obtain more detailed biochemical insights into the signaling properties of B-Raf^{AVKA}-expressing cells, we required an experimental isogenic system allowing the generation and analysis of large quantities of homogenous cells. To this end, we generated fibroblasts from homozygous *Braf*^{floxAVKA} (#273) embryos (murine embryonic fibroblast = MEF) and immortalized them by retroviral transduction with an expression vector for the SV40 large T antigen (TAG). Subsequently, two pools of these MEFs (#5 and #6) were transduced with the expression vector pMIBerry/CreERT2 encoding the 4-HT-regulated Cre recombinase CreERT2 and the fluorescent dsRed2 protein (Röding *et al*, 2012). Infected cells were sorted to homogeneity by flow cytometry, expanded, and then either treated with ethanol (OH) or 4-HT to induce recombination of the *Braf*^{floxAVKA} locus. As shown in Fig EV5A, a pulse of 30–100 nM 4-HT for 48 h was sufficient to induce recombination at the DNA level and an exchange of the wild-type against the mutant transcript. Based on data from an analogous MEF system generated from *Braf*^{floxE12} mice in which we observed the loss of B-Raf protein within 3–5 days (Fig EV5B), we estimated that 5 days of culture after 4-HT addition was sufficient to exchange B-Raf^{WT} against B-Raf^{AVKA}.

Firstly, we stimulated MEFs retaining B-Raf^{WT} or expressing B-Raf^{AVKA} with EGF for various time points (Fig 7A). In both populations, EGF-induced MEK/ERK phosphorylation, although pERK returned faster to baseline levels in B-Raf^{AVKA}-expressing MEFs than in their B-Raf^{WT}-expressing counterparts. Importantly, expression of the immediate early gene (IEG) product c-Fos was completely blocked in B-Raf^{AVKA}-expressing MEFs. Similarly, expression of Egr1, another IEG product, was reduced in B-Raf^{AVKA}-expressing MEFs. Similar findings for pMEK/pERK were obtained for MEFs stimulated with PDGF or NGF (Fig EV5C and D). Interestingly, the reduction in Egr1 induction of the B-Raf^{AVKA}-expressing MEFs is reminiscent of our observations with TAG-immortalized *Braf*-deficient MEFs (Fig EV5E and F). This indicates that AS phosphorylation and hence

full B-Raf activity is required for optimal downstream signaling and an IEG response toward activated growth factor receptors.

The signaling defect of B-Raf^{AVKA}-expressing MEFs prompted us to investigate the heterodimerization potential of the activation loop mutant in EGF-stimulated MEFs (Fig 7B), either in the absence or presence of sorafenib, which is a potent inducer of B-Raf heterodimers compared to other Raf inhibitors (Röding *et al*, 2012). Although we failed to detect heterodimers between endogenous B-Raf and Raf-1 or KSR1, probably due to their low abundance under these conditions, we could detect them for both B-Raf^{WT} and B-Raf^{AVKA} in the presence of sorafenib, indicating again that the latter is able to interact with Raf-1 or KSR1.

The *Braf*^{AVKA} allele impairs the fitness of immortalized MEFs

Next, we measured the effect of the *Braf*^{AVKA} allele on the proliferation in the aforementioned TAG-immortalized MEF pools originating from *Braf*^{floxAVKA/floxAVKA} embryos (Fig 7C). As another control, we used MEFs from a heterozygous *Braf*^{floxAVKA/+} animal (Fig EV5G). Although we noticed a significant twofold reduction in *Braf*^{AVKA/+} MEFs numbers compared to their isogenic counterpart, this difference was by far more pronounced in homozygous *Braf*^{AVKA/AVKA} MEFs (4- to 10-fold reduction) (Fig 7C). In the course of these experiments, we noticed changes in the morphology of *Braf*^{AVKA/AVKA} MEFs, which we further evaluated by microscopy using the cytoplasmic expression of dsRed2 introduced with the CreERT2 construct and the DNA stain TOPRO-3 as markers for overall cell shape and nuclear morphology, respectively. This analysis revealed that B-Raf^{AVKA}-expressing MEFs were often enlarged and poly-nucleated (Fig 7D). This aberrant morphology was accompanied by an increase in caspase-3 activity (Fig 7E). These observations suggest that the inducible expression of B-Raf^{AVKA} induces endomitotic events in MEFs accompanied with the induction of cell death. Given the relatively good fitness of *Braf*^{AVKA} mice, we reasoned that B-Raf^{AVKA}-expressing MEFs only display this phenotype in the presence of TAG, which enforces cell cycle progression by the inactivation of E2F and p53 (Ahuja *et al*, 2005). Therefore, we took an early passage aliquot of primary *Braf*^{floxAVKA/floxAVKA} MEF pool #5 and infected them with a doxycycline (dox) inducible TAG expression system. This experimental setup allows the expansion of MEFs in the presence of dox and TAG and the subsequent withdrawal of the immortalizing oncoprotein by dox withdrawal (Fig 7F). Indeed, B-Raf^{AVKA}-expressing MEFs presented with aberrant nuclei only in the presence of TAG, while B-Raf^{AVKA} MEFs in which dox was withdrawn prior to CreERT2-mediated recombination continued to display smooth regularly shaped nuclei as they were observed in

Figure 7. Phenotypic characterization of conditional *Braf*^{AVKA} MEFs.

- A MEFs expressing B-Raf^{WT} or B-Raf^{AVKA} were stimulated with EGF for the indicated times (min) and analyzed by Western blotting.
- B IP of EGF-stimulated MEFs expressing B-Raf^{WT} or B-Raf^{AVKA}. Sorafenib was used to increase complex build up.
- C Proliferation of two different MEF pools expressing either B-Raf^{WT} or B-Raf^{AVKA}. MEFs expressing B-Raf^{AVKA} shows a strong reduction in growth rate. *n* = 3, mean + SEM, t-test, ***P* < 0.01, ****P* < 0.001.
- D Cell morphology of B-Raf^{AVKA}-expressing MEFs is enlarged and present with abnormal nuclei.
- E MEFs expressing B-Raf^{AVKA} show a significant increase in caspase-3 activity. *n* = 3, mean + SEM, t-test, ***P* < 0.01.
- F Decay of TAG expression in MEFs following dox withdrawal.
- G Morphology of MEFs expressing B-Raf^{WT} or B-Raf^{AVKA} in the presence or absence of TAG.

Figure 7.

B-Raf^{WT}-expressing MEFs, regardless whether they have been exposed to dox or not (Fig 7G). Taken together, these data suggest that the coordination of events permitting cellular proliferation enforced by TAG requires maximum B-Raf signaling.

The AVKA mutation impairs transformation by non-V600E B-Raf mutants

As we observed that the AVKA mutation reduces cellular proliferation and is able to disrupt the signaling potential of transforming non-V600E B-Raf mutants (Fig 1B–D), we tested whether the lack of T599 and S602 also affected their transformation potential. To this end, we transduced the mutants described in Fig 1 into TAG-immortalized MEFs and conducted focus-forming assays. We also included the *FAM131B-BRAF* fusion oncogene that has been recurrently found in low-grade astrocytomas (Fig EV5A) (Cin *et al*, 2011; Roth *et al*, 2015). This oncoprotein was chosen as a representative of the growing spectrum of oncogenic B-Raf fusion proteins, which have been discovered in various tumor entities and all share the possession of an intact kinase domain and the loss of the autoinhibitory N-terminal moiety.

As shown in Fig 8A and B, MEFs expressing B-Raf^{V600E} and B-Raf^{E586K}, but not those transduced with an empty control vector or expressing B-Raf^{WT}, generated multiple foci, indicative of impaired contact inhibition. Transformation efficiency was further enhanced in MEFs expressing B-Raf^{EVKD}, B-Raf^{CAAX}, or FAM131B-B-Raf. This finding can be explained by our previous observations that these mutants trigger lower MEK/ERK phosphorylation levels than B-Raf^{V600E} (Fig 1; Cin *et al*, 2011; Röring *et al*, 2012), which might be more compatible with proliferation. Strikingly, the AVKA mutation drastically reduced the transformation potential of all mutants except B-Raf^{V600E}. These findings further underscore the relevance of the AS for the signaling output of non-V600E B-Raf mutants and identify it as a promising target for the therapy of these oncoproteins.

Discussion

Since its first description 15 years ago, the role of the TVKS motif phosphorylation sites in the activation of Raf isoforms has been predominantly deduced from *in vitro* kinase assays (Zhang & Guan, 2000; Baljuls *et al*, 2008; Holderfield *et al*, 2013) and from the large number of mutations affecting the AS in cancer, most notably V600E, and in RASopathies (Garnett & Marais, 2004; Andreadi *et al*, 2012) and references therein or in the COSMIC database. However, very little is known about its role for B-Raf signaling in intact cells and no evaluation of the TVKS motif has been conducted *in vivo*. Here, we report for the first time the phenotype of mice expressing a B-Raf protein lacking the highly conserved AS phosphorylation sites T599 and S602. Surprisingly and in sharp contrast to mice with *Braf*-knock-out (Wojnowski *et al*, 1997; Galabova-Kovacs *et al*, 2006; Zhong *et al*, 2007) and *Braf*-knock-in alleles causing increased or paradoxical B-Raf activity (Mercer *et al*, 2005; Kamata *et al*, 2010; Andreadi *et al*, 2012; Inoue *et al*, 2014), B-Raf^{AVKA}-expressing mice are viable, despite a significant reduction in MEK/ERK signaling output *in* and *ex vivo*. As we could neither observe a paradoxical behavior of B-Raf^{AVKA} in our *Braf*^{-/-} MEF complementation assay nor in tissues from *Braf*^{AVKA} mice, we conclude that mutation of the

TVKS motif and the associated reduction in kinase activity does not provoke a paradoxical action like it is observed for the kinase-dead B-Raf^{D594A} mutant in tissue culture (Fig 2 and Röring *et al*, 2012) and *in vivo* (Heidorn *et al*, 2010; Kamata *et al*, 2010). Moreover, our observation that *Braf*^{AVKA} mice do not represent a phenocopy of *Braf*-deficient animals supports the growing notion that B-Raf fulfills critical biological functions that are not directly proportional to its enzymatic activity, for example, a scaffolding role independent of AS phosphorylation.

However, the signaling deficits and phenotypic alterations observed in B-Raf^{AVKA} expressing MEFs, primary astrocytes, and mice clearly demonstrate the relevance of the TVKS motif. Indeed, by comparing the growth factor-induced *Egr1* expression in conditional *Braf*^{AVKA/AVKA} MEFs with *Braf*^{-/-} MEFs (Figs 7A and EV5C–F), it became evident that the AVKA knock-in might confer similar effects like B-Raf deficiency on growth factor-induced IEG expression. Furthermore, the differential in pERK phosphorylation observed in brain lysates of *Braf*^{AVKA} animals (Figs 5C and EV3A and B) was similar to that reported for 18-d-old *Braf*^{-/-} mice (Galabova-Kovacs *et al*, 2008). In further analogy to the study by Galabova-Kovacs *et al* (2008) in mice with *Nestin::Cre*-mediated *Braf* deletion, we observed a reduction in pERK staining intensity in the Purkinje cells in cerebella of *Braf*^{AVKA} mice. This defect or other potential functional impairments caused by reduced ERK activity in macroglial cell types could also account for the ataxia observed in older *Braf*^{AVKA} mice. However, it should be noted that the neurological deficits observed in *Braf*^{AVKA} mice were never as severe as reported for *Braf*^{-/-} animals, which, depending on the Cre transgene, did not live beyond P21–P35 (Zhong *et al*, 2007; Galabova-Kovacs *et al*, 2008; Pfeiffer *et al*, 2013). This could be explained by the observation that *Braf*^{AVKA} animals show no obvious effects in myelination levels compared to wild-type mice. This is in strong contrast to *Braf*-deficient mice, suggesting that the loss of the B-Raf protein shapes the knockout phenotype by MEK/ERK-dependent and MEK/ERK-independent mechanisms. Thus, *Braf*^{AVKA} mice will be useful to study the role of B-Raf in the development, function, and maintenance of the nervous system at stages that are not reached by *Braf*^{-/-} animals.

Given the well-documented role of B-Raf in normal and malignant hematopoiesis and immune cell function (Brummer *et al*, 2002; Tsukamoto *et al*, 2004; Kamata *et al*, 2005; Röring & Brummer, 2012; Yaktapour *et al*, 2014), we initially characterized the cell populations in peripheral blood and lymphatic organs. These analyses revealed a significant reduction in CD8⁺ T effector memory cells (TEM) and T regulatory cells (Tregs) in *Braf*^{AVKA} mice suggesting that optimal B-Raf signaling is required to differentiate progenitors into these CD8⁺ T-cell subpopulations. These findings could be explained by the critical role of ERK signaling in the differentiation of T-cell effector lineages and the emerging role of B-Raf in T-cell antigen receptor (TCR) signaling (Tsukamoto *et al*, 2004, 2008; Deswal *et al*, 2013; Dillon *et al*, 2013). However, our flow cytometry analyses revealed only a very subtle reduction in ERK phosphorylation in CD8⁺ T cells raising the question for its biological significance. Among several possibilities, this could be caused by feedback loops in the mutant cells conferring robustness to pathway perturbation (Fritsche-Guenther *et al*, 2011). In any case, our *in vivo* data further extend the experiments by Tsukamoto *et al* (2004), who showed that overexpression of B-Raf^{AVKA} in the Jurkat

Figure 8. The AVKA mutation is able to block transformation potential of B-Raf mutants and model of B-Raf activation incorporating the roles of the activation segment and the DIF.

- A** MEFs were then infected with retroviral vectors encoding the indicated B-Raf proteins. Giemsa staining revealed transformed and hyper-proliferated cells by dark purple color.
- B** Quantification of focus formation potential of the B-Raf mutants used in (A). $n = 3$, mean + SEM, t -test, *** $P < 0.001$.
- C** Following recruitment by Ras-GTP, B-Raf forms homo- or heterodimers with the assistance of 14-3-3 proteins. This loosely dimerized and membrane-bound state is initiated by the B-Raf^{E586K} and B-Raf^{CAAX} mutants, which still require an intact DIF and AL phosphorylation. Within the dimer, one protomer becomes phosphorylated at its TVKS motif *in cis* (depicted by black bent arrow) leading to reorientation of the DIF and induction of a conformational change in the other protomer (blue bent arrow) followed by phosphorylation of the AS in the other protomer *in cis*. B-Raf^{V600E} is locked in the highly active state and thus signals independently of an intact TVKS motif or DIF. See text for further details.

T-cell line impaired the sustained phase of TCR-induced MEK/ERK phosphorylation and IL-2 production. Likewise, B lymphocytes from *Braf*^{AVKA} mice responded with reduced proliferation and impaired CD69 induction toward BCR or TLR4 stimulation. In summary, *Braf*^{AVKA} mice show no obvious signs of immune deficiency. Thus,

it remains to be investigated whether the observed changes in immune cell population size and signaling impact on the functionality of the immune system.

Our data demonstrate that endogenously expressed B-Raf^{AVKA} displays significantly reduced kinase activity and impairs the ERK

signaling pathway *in vivo* and in various *ex vivo* systems. This raises the question whether these signaling defects are caused by impaired B-Raf activity, thereby mimicking the functional loss of an important MEK/ERK activator, like it is observed in B-Raf deficiency (Brummer *et al*, 2002; Galabova-Kovacs *et al*, 2006, 2008), or whether B-Raf^{AVKA} exerts a dominant-negative effect, for example, by being incorporated into Raf heterodimers. Although both possibilities are not mutually exclusive, the latter scenario appears quite realistic. Firstly, B-Raf^{AVKA} entertains interactions with itself, Raf-1 and KSR1, albeit the number of stable B-Raf/KSR1 complexes was significantly reduced. Based on these findings and our previous observation that B-Raf^{R509H} is still able to interact with but fails to transactivate Raf-1 (Röring *et al*, 2012), a dominant-negative action of B-Raf^{AVKA} appears feasible, in particular given the recent structural insights into the role of the AS-H1 (Thevakumaran *et al*, 2015). Here, Thevakumaran *et al* (2015) show in B-Raf^{V600E} crystals that the salt bridge between E600 in the mutant AS-H1 and K507 strengthens the nearby “ α C helix-in” conformation and thereby promotes phosphotransferase activity and dimerization. They also emphasize that, due to the close positioning of T599 and S602 position to V600, a similar conformational change is induced by TVKS motif phosphorylation. This concept is supported by our observation that the B-Raf^{E586K} and B-Raf^{CAAX} gain-of-function mutants, but not the B-Raf^{V600E} oncoprotein, which can form its mutation-specific E600-K507 salt bridge and is thus exempted from AS-H1 phosphorylation, require an intact TVKS motif (Figs 1 and 8A and B). Moreover, B-Raf^{EVKD}, which carries phosphomimetic substitutions of T599 and S602, displayed enhanced homodimerization compared to B-Raf^{WT} (Fig 6C). Importantly, the model by Thevakumaran *et al* further suggests that dimerization and thereby allosteric transactivation *via* R509 is promoted by phosphorylation-induced reorientation of the AS-H1. Thus, it appears very unlikely that the aforementioned interaction between the AS-H1 and the α C helix can be formed in B-Raf^{AVKA} as it lacks the negative charges provided either by pT599/pS602, their phosphomimetic substitutions in the B-Raf^{EVKD} mutant, or the glutamic acid in B-Raf^{V600E}. Consequently, enzymatic activity of B-Raf^{AVKA} cannot be increased and the α C helix stays in the outward orientation leaving R509 in a position in which it cannot mediate efficient dimerization with KSR1 and/or transactivation of Raf-1. According to this model, Raf-1, which we observed in B-Raf^{AVKA} complexes in MEFs and in brain lysates, cannot be transactivated and remains trapped in a functionally compromised complex, ultimately resulting in impaired downstream signaling. Thus, residual ERK signaling in *Braf*^{AVKA} mice is probably driven by Raf-1 homo- and Raf-1/A-Raf heterodimers (Mooz *et al*, 2014), while Ras-mediated recruitment of B-Raf^{AVKA} containing homo- or heterodimers represents a futile approach. This would lower the capacity of the cell to respond to extracellular signals with maximum MEK/ERK activation and IEG induction, but would still allow enough pathway activity to promote a relatively normal development as displayed by *Braf*^{AVKA} mice. Certain cell types, however, in particular those with a high expression or dependence of the B-Raf isoform, could still be impaired in their development, function, or maintenance as it is suggested by the phenotype of *Braf*^{AVKA} mice.

Our approach to introduce the AVKA substitution into various B-Raf mutants with increased kinase activity provides new

insights into the B-Raf activation cycle (Fig 8C). For example, we investigated the tumor-associated B-Raf^{E586K} mutant that displays elevated MEK phosphorylation and transforming potential (Emuss *et al*, 2005), most likely due to its increased dimerization potential (Rajakulendran *et al*, 2009; Freeman *et al*, 2013). In agreement with the model that Raf dimerization follows recruitment by Ras (Lavoie & Therrien, 2015), the R188L substitution had little to no effect on the MEK phosphorylation potential of B-Raf^{E586K} (Fig 1B). However, the activity of this oncoprotein is sensitive to N-region neutralization (Emuss *et al*, 2005), the R509H (Röring *et al*, 2012), and AVKA mutations (Figs 1B and 8A and B). Based on the aforementioned concept that AS-H1 phosphorylation leads to DIF reorientation and the model by Hu *et al* (2013) incorporating the charged N-region into the extended DIF, these observations suggest that dimer initiation by Ras and the phosphorylation events in the N region and AS cooperate in reorientating the DIF in the activator to induce optimal transactivation and trans-phosphorylation of the receiver protomer. The concept that TVKS motif phosphorylation occurs after membrane recruitment by Ras is also supported by our observation that the MEK phosphorylation and focus formation provoked by B-Raf^{CAAX} is reduced by 80% and 90% by the AVKA mutation, respectively.

Lastly, our observation that the AVKA mutation reduces the signaling output and transforming potential of B-Raf and some of its gain-of-function mutants identifies AS phosphorylation as a potential therapeutic target, for example, for tumors expressing B-Raf oncoproteins, which are activated by mutations outside of the AS or by N-terminal truncation. Since B-Raf^{AVKA} does not behave paradoxically, a therapeutic approach blocking AS phosphorylation could represent an alternative strategy to the current ATP-competitive inhibitors that must not be used in the context of dysregulated Ras signaling and that also disappoint in the context of oncogenic B-Raf fusion proteins (Hutchinson *et al*, 2013; Sievert *et al*, 2013). Indeed, we demonstrate here that the AVKA mutation strongly impairs transformation by the FAM131B-B-Raf fusion, which, like all other oncogenic B-Raf fusions reported so far, possesses a non-mutated kinase domain and exhibits oncogenic properties due to the loss of the CR1/CR2 and its inability for autoinhibition (Cin *et al*, 2011). The drastic effect of the AVKA mutation on FAM131B-B-Raf demonstrates the relevance of the AS for the signaling output of N-terminally truncated B-Raf oncoproteins. As FAM131B-B-Raf lacks an RBD, our data indicate that Ras-mediated displacement of the autoinhibitory N-terminal moiety is not sufficient for full B-Raf activation, but requires the aforementioned conformational changes within the kinase domain that are induced by AL phosphorylation (Thevakumaran *et al*, 2015). Thus, even the kinase domain of oncogenic B-Raf fusion proteins appears to toggle between an inactive and active conformation, depending on the degree of AL phosphorylation. This concept might contribute to our understanding how existing B-Raf inhibitors bind to oncogenic B-Raf fusions.

In summary, our study shows that T599 and S602 are dispensable for the development and normal life span and cycle of laboratory mice, while being essential for oncogenic signaling by non-V600E B-Raf mutants. These findings appear as ideal prerequisites to design therapies exploiting oncogene addiction while minimizing the risk for side effects. The conditional *Braf*^{AVKA}-knock-in

model will be invaluable to further address this concept in future studies.

Materials and Methods

Mouse strains

Mice were housed in the specific pathogen-free barrier facility of the University Medical Center Freiburg in accordance with the institutional guidelines and approved by the local animal ethics committee (G-10/51). The generation and genotyping of *Braf*^{floxAVKA} mice is described in detail in the Appendix. *Braf*^{floxE12} mice (Chen *et al*, 2006) were kindly provided by Dr. Manuela Baccarini (Vienna) and backcrossed onto a C57/Bl6 background. *Sox2::Cre* deleters were kindly provided by Dr. Sebastian Arnold (Freiburg) and backcrossed on a C57/Bl6 background. Mice were kept under standard conditions (12-h light/dark cycle) with food and water *ad libitum*. Littermates were used throughout the experiments, and investigators were blinded for genotypes previous to data analysis except for Western blot and video analyses.

Tissue culture

The cultivation and transfection of Plat-E cells as well as the generation and cultivation of TAG-immortalized *Braf*^{floxE12/floxE12} MEFs was described previously (Röding *et al*, 2012). *Braf*^{floxAVKA/floxAVKA} MEFs were generated using the same procedures. Primary astrocytes were isolated from brains of 4-d-old mice and dissociated mechanically in DMEM. Cells were washed and plated in Plat-E culture medium on poly-L-ornithine-coated dishes. When cells reached confluency, EGF was added for 5 min and cells were lysed.

Plasmids

The pMIG/HA-B-Raf, pMIG/FAM131B-B-Raf, and pMIBerry/B-raf-Myc/His expression vectors and the pMIBerry/CreERT2 plasmid have been described previously (Cin *et al*, 2011; Röding *et al*, 2012). Mutations were introduced using standard site-directed mutagenesis (oligonucleotides sequences can be provided upon request). For construction of the dox-inducible TAG expression vector, the TAG cDNA from pQCXIH/TAG (Röding *et al*, 2012) was subcloned into the retroviral vector SH570, which combines the elements of a tightly regulated dox-inducible cDNA expression system (Herr *et al*, 2011) and which was similarly constructed as the retroviral two-component system for dox-inducible cDNA expression (Haug *et al*, 2015).

Antibodies

Antibodies used in this study were as follows: anti-Egr1 (C-19), anti-Raf-A (C-20), anti-Raf-B (F-7 or H-145), anti-Raf-1 (C-12 or E-10), anti-pan 14-3-3 (H-8), anti-β-actin (C-4), anti-tubulin (B-5-1-2; all from Santa Cruz Biotechnology, USA), anti-HA 3F10 (Roche, Germany), anti-c-FOS (9F6), anti-phospho-MEK1/2 (pS217/221), anti-phospho-MEK1/2 (pS221), anti-MEK1/2, anti-p42/p44 MAPK, anti-phospho-MAPK (pT202/pY204; ERK1/2; all from Cell Signaling Technologies, USA), and anti-KSR-1 (BD, USA). Detailed

information on antibodies used for immune phenotyping is provided in the Appendix.

Western blot analysis of tissue culture cells

Total cell lysates (TCLs) were generated and analyzed as described previously (Röding *et al*, 2012). In brief, cells were lysed in either normal lysis buffers (NLB: 50 mM Tris/HCl, pH 7.5; 1% Triton X-100; 137 mM NaCl; 1% glycerol; 1 mM sodium orthovanadate; 0.5 mM EDTA; 0.01 μg/μl leupeptin, 0.1 μg/μl aprotinin, 1 mM AEBSF) or RIPA buffer (NLB supplemented with 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate). Blotted proteins were visualized with a Fusion Solo chemiluminescence reader and quantified using FusionCapt software (Vilber Lourmat, Germany).

B-cell stimulation and proliferation assay

Magnetic cell sorting-sorted (Miltenyi Biotec, Germany) CD19⁺ B cells were stimulated with indicated concentration of IgM or LPS (Southern Biotech, USA). CD69 expression was measured 12 h later by FACS analysis. For proliferation, B cells were labeled with 1 μM CFSE (Molecular Probes) and stimulated with LPS (10 μM). Cells were cultured for 96 h and cell proliferation was determined by the extent of CFSE dye dilution, as measured by flow cytometry (Stickel *et al*, 2014).

T-cell stimulation and pERK phosphoflow

Magnetic cell sorting-sorted (Miltenyi Biotec, Germany) untouched T cells were stimulated with 5 μg/ml anti-CD3/anti-CD-28 for indicated time points. Cell were fixed with 4% formaldehyde solution for 30 min and stained with anti-CD4/CD8 antibodies. Afterward, cells were permeabilized, stained for pERK, and measured by flow cytometry.

Brain lysates and *in vitro* kinase assays

Individual brains were lysed in 2 ml ice-cold lysis buffer (NLB in which 1% Triton X-100 was replaced by 0.5% NP-40 or RIPA buffer as indicated) using a Dounce homogenizer. Following clearance by centrifugation, brain lysates were mixed with 2× sample buffer and boiled or incubated with either 2 μg anti-B-Raf H-145 antibodies or rabbit control IgG for 30 min followed by addition of protein G-sepharose beads (approx. 60 μl bead volume; GE Healthcare). After incubation on a rotator at 4° C for 3 h, the beads were washed 6 times with lysis buffer. *In vitro* kinase assays using recombinant GST-MEK1 were performed with the anti-B-Raf H-145 immunoprecipitates as described previously (Röding *et al*, 2012) with some minor modifications. In brief, after four washes with kinase assay buffer (KAB; 20 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.2; 25 mM β-glycerol phosphate; 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol), the beads were resuspended in 150 μl KAB. Then, 10 μl of this suspension were mixed with 2 μg recombinant GST-MEK1 and 5 mM ATP and 18.75 mM MgCl₂ in a total reaction volume of 40 μl. GST-MEK1 was expressed in *E. coli* Rosetta cells using the pGEX-MEK1 plasmid (Coles & Shaw, 2002) and purified using standard procedures (Brummer *et al*, 2004). The IVK reaction was incubated at 30°C for 60 min and immediately stopped by addition of sample buffer and boiling for 5 min.

Subsequently, IVK reactions were separated by SDS–PAGE and blotted with phospho-MEK antibodies as a readout for the MEK kinase activity of the purified B-Raf proteins.

Histology

Brains were fixed in 10% formaldehyde solution (Rotifix, Roth, Germany) and embedded into paraffin blocks. Embedded brain tissue was sectioned and rabbit anti-pERK antibody (Cell signaling technology; 1:50 dilution) was used for detecting the phosphorylation in the cerebellum as described previously (Galabova-Kovacs *et al*, 2008). Specificity was confirmed using rabbit control IgG (Santa Cruz, Biotechnology). Myelination was assessed by Luxol fast blue staining (Abcam) following the instructions by the manufacturer.

cDNA synthesis and sequencing

Murine embryonic fibroblasts were trypsinized and washed once with ice-cold PBS. The cell pellet was used for RNA isolation (RNeasy kit, Qiagen, Germany) according to the manufacturer's recommendation. cDNA was generated using oligo-dT primer and the RevertAid First-strand cDNA synthesis kit (Fermentas, USA) employing the provided standard protocol. Following a PCR with the indicated oligonucleotides, the obtained amplicons were sequenced at GATC biotech (Konstanz, Germany).

Stimulation with growth factors

Murine embryonic fibroblasts were treated with 200 nM 4-hydroxy-tamoxifen (4-HT) or ethanol (OH) for 72 h. Cells were starved for 24 h in medium containing 0.5% FCS. Indicated growth factors were purchased from R&D Systems and added to a final concentration in the starvation medium for the indicated time points.

MEF proliferation assay

Murine embryonic fibroblasts were induced with 4-HT or OH as control for 5 days. At day zero, cells were trypsinized and plated in 24-well plates at a density of 10,000 cells per well. At indicated time points, cells were trypsinized and counted.

Caspase-3 activity assay

Total cell lysates of the desired cell population were lysed and adjusted to the same protein concentration by BCA assay (Thermo Scientific, USA). For the reaction, 80 μ l of reaction buffer (100 mM HEPES, 1 mM DTT) was mixed with 20 μ l cell lysate and supplemented to a final concentration of 6 μ M fluorogenic caspase-3 substrate (Ac-DEVD-AMC, Enzo Life Science, Switzerland). Fluorescence measurement (excitation: 380 nm, emission: 460 nm) was performed every minute over a one hour time course in a plate reader (Infinite m200, Tecan, Switzerland).

Transformation Assay

TAg-immortalized MEFs were infected with diluted virus supernatant of expression vectors of above-described B-Raf wild-type and

mutant proteins. Cells were cultured for 20 days with medium exchange every two to three days and stained with Giemsa to visualize foci of transformed cells.

Statistical analysis

Quantitative data are presented as mean $\pm$ SE. Pairwise comparisons were performed by Student's *t*-test (two-tailed), respectively. If data did not conform to a normal distribution, the Mann–Whitney *U*-test was utilized. A *P* value of ≤ 0.05 was considered statistically significant ($***P < 0.001$; $**P < 0.01$; and $*P < 0.05$). Unless noted otherwise, bar diagrams report the mean of three independent experiments.

Further Materials and Methods are detailed in the Appendix.

Expanded View for this article is available online.

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Author contributions

MK, MR, and TB designed and conceived experiments, performed experimental work and data interpretation, and wrote the manuscript. BS, KH, NS, LCS, SB, GJF, SE, FMU, TK, and FW performed experimental work and data interpretation. SH, RZ, WWS, and HJ contributed to experimental design and data interpretation. TB supervised research and project planning.

Conflict of interest

The authors declare that they have no conflict of interest.

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