The EMBO Journal (2012) 31, 2629–2647 | © 2012 European Molecular Biology Organization | All Rights Reserved 0261-4189/12
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THE EMBO JOURNAL

Distinct requirement for an intact dimer interface in wild-type, V600E and kinase-dead B-Raf signalling

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The dimerisation of Raf kinases involves a central cluster within the kinase domain, the dimer interface (DIF). Yet, the importance of the DIF for the signalling potential of wild-type B-Raf (B-Raf^{wt}) and its oncogenic counterparts remains unknown. Here, we show that the DIF plays a pivotal role for the activity of B-Raf^{wt} and several of its gain-of-function (g-o-f) mutants. In contrast, the B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} oncoproteins are remarkably resistant to mutations in the DIF. However, compared with B-Raf^{wt}, B-Raf^{V600E} displays extended protomer contacts, increased homodimerisation and incorporation into larger protein complexes. In contrast, B-Raf^{wt} and Raf-1^{wt} mediated signalling triggered by oncogenic Ras as well as the paradoxical activation of Raf-1 by kinase-inactivated B-Raf require an intact DIF. Surprisingly, the B-Raf DIF is not required for dimerisation between Raf-1 and B-Raf, which was inactivated by the D594A mutation, sorafenib or PLX4720. This suggests that paradoxical MEK/ERK activation represents a two-step mechanism consisting of dimerisation and DIF-dependent transactivation. Our data further implicate the Raf DIF as a potential target against Ras-driven Raf-mediated (paradoxical) ERK activation.

The EMBO Journal (2012) **31,** 2629–2647. doi:10.1038/emboj.2012.100; Published online 17 April 2012

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Received: 1 November 2011; accepted: 23 March 2012; published online 17 April 2012

Subject Categories: molecular biology of disease; signal transduction
Keywords: A-Raf; *BRAF*; PLX 4032/4720; sorafenib; V600E-specific antibody

Introduction

The Ras/Raf/MEK/ERK pathway controls proliferation and differentiation and is often dysregulated in cancer. Owing to their role as gatekeepers of this pathway, Raf kinases are attractive therapeutic targets (Michaloglou *et al*, 2008; Wimmer and Baccarini, 2010). The Raf family is found in all metazoans. Studied members include the A-Raf, B-Raf and Raf-1/C-Raf isoforms in vertebrates as well as D-Raf and LIN-45 in *Drosophila* and *Caenorhabditis*, respectively. B-Raf is the major MEK/ERK activator and represents the most frequently mutated kinase in human tumours with particularly high frequencies in melanoma, various tumour entities of the nervous system, hairy cell leukaemia, Langerhans cell histiocytosis as well as in thyroid, biliary tract and colorectal carcinoma (Garnett and Marais, 2004; Michaloglou *et al*, 2008; Kamata and Pritchard, 2011; Schindler *et al*, 2011 and references therein). In addition, germ-line *BRAF* mutations are found in cardio-facio-cutaneous (CFC) syndrome patients (Niihori *et al*, 2006; Rodriguez-Viciana, 2006a; Sarkozy *et al*, 2009). Since B-Raf is frequently mutated in some tumour entities for which no or only limited therapies are available, a lot of hope has been placed on inhibiting its activity. However, this attempt requires a solid understanding of its structure, regulation and interaction network. This notion is corroborated by recent publications reporting paradoxical ERK pathway activation by Raf inhibitors (Cox and Der, 2010; Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010; Poulikakos *et al*, 2010).

B-Raf shares three highly conserved regions (CRs) with other family members (Figure 1A). The N-terminal CR1 contains the Ras–guanine 5′-triphosphate (GTP)-binding domain (RBD) and Cysteine-rich domain (CRD). The RBD mediates the interaction with Ras-GTP. A conserved arginine residue (R188 in B-Raf) in the RBD is required for the recruitment and activation of Raf at the plasma membrane as well as for dimerisation with Raf-1 (Marais *et al*, 1997; Heidorn *et al*, 2010). Substitution of this residue by leucine prevents the Ras/Raf interaction and renders Raf unresponsive to most extracellular signals. The CR2 harbours phosphorylation sites of which S365 recruits 14-3-3 proteins. Displacement of 14-3-3 from the CR2 and subsequent dephosphorylation of S365 (or its equivalent) is a key step in Raf activation (Abraham *et al*, 2000; Jaumot and Hancock, 2001; Dhillon *et al*, 2002; Rodriguez-Viciana *et al*, 2006b). Consequently, disruption of 14-3-3 binding by the

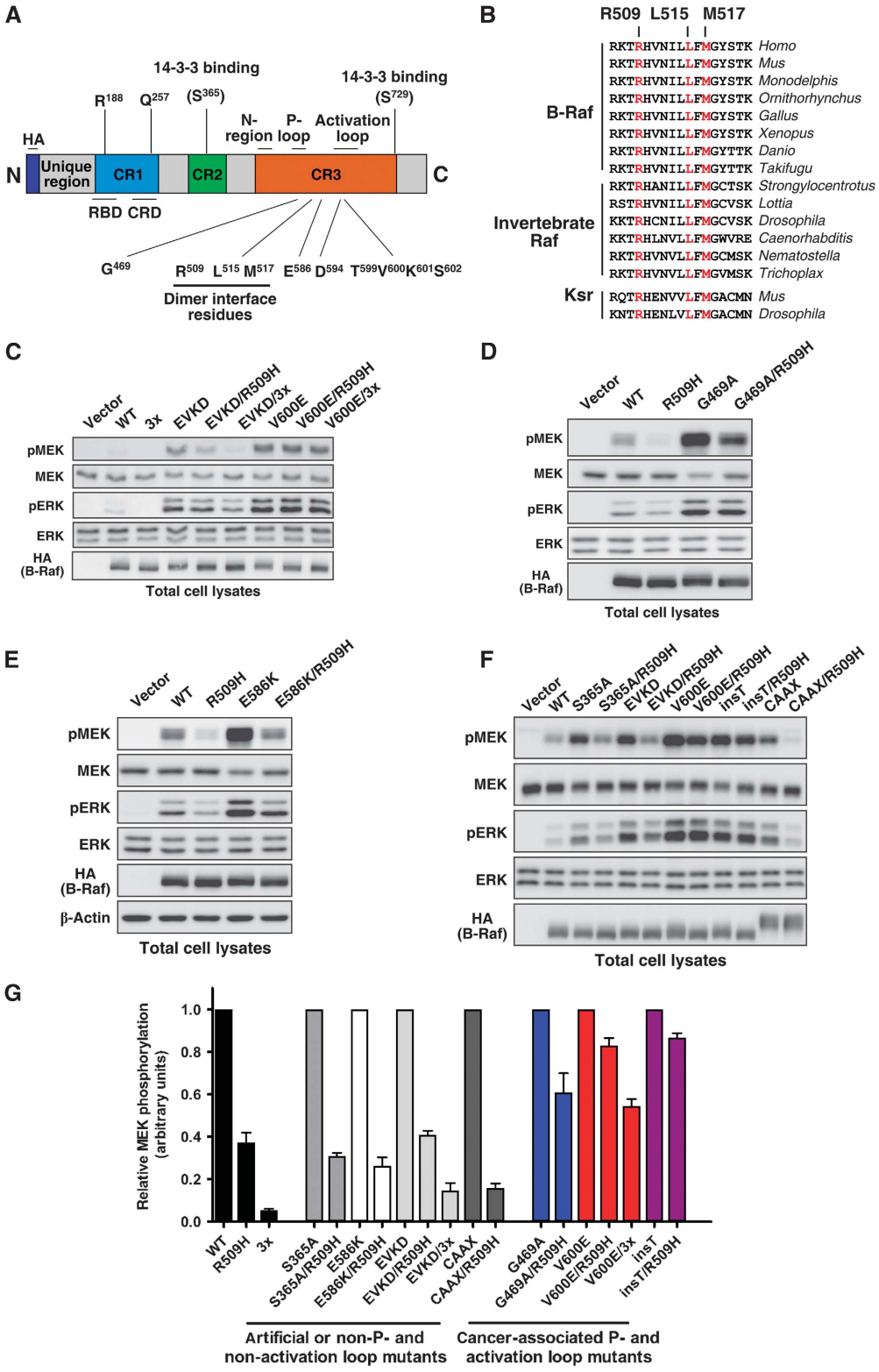


Figure 1 B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} are much lesser affected by DIF mutations than B-Raf^{wt} and several of its gain-of-function mutants. (**A**) Overview of the primary structure of the human B-Raf protein (HA–B-Raf) used in this study. See text for details. For discrimination against endogenous B-Raf, the analysed proteins were expressed with an N-terminal hemagglutinin (HA) tag. (**B**) The equivalents of R509, L515 and M517 are conserved throughout metazoan evolution. See Supplementary Table S1 for further details. (**C**–**F**) The MEK/ERK activation potential of the indicated human B-Raf proteins was analysed by western blotting using total cell lysates of transiently transfected Plat-E cells. (**G**) The chemiluminescence signal elicited by the individual reference proteins (B-Raf^{wt}, B-Raf^{S365A}, B-Raf^{E586K}, B-Raf^{EVKD} B-Raf^{CAAX}, B-Raf^{V600E}, B-Raf^{G469A} and B-Raf^{insT}) was set in each analysis to 100%. Data represent the mean ± s.e.m. from at least three independent transfections. See Supplementary Table S2 for further statistics. Figure source data can be found with the Supplementary data.

S365A substitution leads to enhanced B-Raf activity (Guan *et al*, 2000; Brummer *et al*, 2006; Rodriguez-Viciana *et al*, 2006). The catalytic domain (CR3) harbours important regulatory phosphorylation sites within two segments, the N-region and the activation loop (Mason *et al*, 1999; Zhang and Guan, 2000). Furthermore, Raf isoforms contain between their αC-helix and the β4-sheet in their CR3s a highly conserved 'side to side' dimer interface (DIF; Figure 1B) that is supposed to impinge on several aspects of Raf regulation such as dimerisation, but also N-region function and membrane localisation, for some aspects in an isoform-specific manner (Rajakulendran *et al*, 2009; Baljuls *et al*, 2011). Several basic residues within this motif have been directly implicated in Raf homodimerisation and heterodimerisation (Figure 1B; Rajakulendran *et al*, 2009; Baljuls *et al*, 2011). From here on, we will refer to this cluster of contact points as the DIF, although multiple residues distributed in other regions of the CR3 have been also implicated as contact points between Raf protomers (Rajakulendran *et al*, 2009). Of note, an RH*x*NΦΦLFMG motif (with $x = $ V, C or E and $\Phi = $ I, L or V) in the C-terminal portion of the DIF has been conserved not only in Raf, but also in the related KSR proteins (Figure 1B). Furthermore, all Raf proteins carry a second 14-3-3 binding motif at the C-terminal end of the CR3. Mutation of its key residue (S729 in B-Raf) abrogates the cellular activity of Raf (MacNicol *et al*, 2000; Light *et al*, 2002; Brummer *et al*, 2006; Noble *et al*, 2008).

Raf activation is accompanied by changes of its phosphorylation status, which is often reflected by its altered electrophoretic mobility. Numerous, mostly ill-defined kinases mediate feed-forward and feed-back phosphorylations by upstream activators and downstream effectors, respectively (Mason *et al*, 1999; Brummer *et al*, 2003; Ritt *et al*, 2010). For example, wild-type B-Raf (B-Raf^{wt}) is stringently controlled by the Ras-dependent phosphorylation of its activation loop ($T^{599}VKS^{602}$ motif; Zhang and Guan, 2000). This supposedly restructures the catalytic centre and induces kinase activity (Wan *et al*, 2004). By far the most prevalent B-Raf mutation, V600E, mimics activation loop phosphorylation and locks the kinase in an active conformation thereby leading to chronic MEK/ERK signalling and transformation (Wan *et al*, 2004). This mutation bypasses several steps in B-Raf activation and also renders the oncoprotein resistant to negative regulation (Emuss *et al*, 2005; Brummer *et al*, 2006; Brady *et al*, 2009; Fischer *et al*, 2009). Likewise, substitution of the phospho-acceptor sites of the TVKS motif by negatively charged residues (EVKD mutation) confers high and constitutive activity to D-Raf, LIN-45, Raf-1 and B-Raf (Zhang and Guan, 2000; Chong *et al*, 2001).

Another layer of complexity is added to the regulation of Raf proteins by their homo- or heterodimerisation (Weber *et al*, 2001; Garnett *et al*, 2005). Rushworth *et al* (2006) have identified B-Raf/Raf-1 heterodimers as a particularly potent MEK activator. Recently, Raf heterodimers and homodimers have been implicated in the paradoxical ERK activation elicited by kinase-inhibited Raf molecules. This phenomenon occurs in B-Raf^{wt} expressing tumours with excessive Ras-GTP levels and has been made responsible for drug resistance and side effects (Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010; Nazarian *et al*, 2010; Poulikakos *et al*, 2010; Ribas and Flaherty, 2011). Detailed structural insights into Raf dimers were provided by Rajakulendran *et al* (2009), who reported that D-Raf and B-Raf have an intrinsic property for dimerisation, a prerequisite for allosteric Raf activation and MEK phosphorylation in *Drosophila* S2 cells. Enforced dimerisation is also a mechanism likely to be exploited by some oncoproteins as two tumour-associated mutants with elevated signalling potential, B-Raf^{E586K} and Raf-1^{E478K}, bear an amino-acid substitution known to promote the dimerisation of kinase domains (Emuss *et al*, 2005; Rajakulendran *et al*, 2009). Importantly, the latter study identified a highly conserved residue within the DIF (R481/R509 in D- and B-Raf, respectively), which plays a critical role in Raf activation by promoting dimerisation. Strikingly, the R481H mutation completely ablates the MEK phosphorylation potential of D-Raf and its artificial g-o-f mutant D-Raf^{EAKD}, in which the phosphorylatable residues of the TVKS motif (TAKT in D-Raf) are replaced by a glutamate and aspartate residue. As the equivalent EVKD mutation confers constitutive activity to human B-Raf (Zhang and Guan, 2000) and is believed to have very similar functional consequences as V600E (Davies *et al*, 2002), Rajakulendran *et al* (2009) suggested that strategies directed at targeting the DIF could act as a therapeutic for Raf-dependent tumours. Given the aforementioned paradoxical ERK activation by existing Raf inhibitors, such a strategy appears very attractive: First, this paradoxical activation relies on the homodimerisation or heterodimerisation of Raf isoforms (Cox and Der, 2010; Wimmer and Baccarini, 2010). Thus, understanding the mechanisms underlying Raf dimerisation may help to circumvent the paradoxical action of current inhibitors as it was also pointed out recently (Lavoie and Therrien, 2011). Second, inhibiting dimerisation itself could even represent a novel stand-alone approach to block aberrant Raf signalling triggered by mutations, for example, V600E, or oncogenic Ras proteins. However, both clinically important concepts were not tested on human B-Raf proteins so far. Here, we have evaluated the DIF in the context of full-length B-Raf and Raf-1 for various signalling aspects. We demonstrate that normal, oncogenic Ras-driven and paradoxical MEK/ERK activation rely on an intact DIF, while the B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} oncoproteins are particularly recalcitrant to DIF mutations. We also demonstrate that the formation of drug-induced Raf complexes relies on distinct structural features of the DIF and the individual inhibitor.

Results

B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} do not require an intact DIF for efficient signalling

A recent study demonstrated that the R481H substitution (R509H in B-Raf) ablates the cellular MEK kinase activity of D-Raf^{EAKD} (Rajakulendran *et al*, 2009). As the equivalent B-Raf^{EVKD} mutant is supposed to have very similar functional properties as the B-Raf^{V600E} oncoprotein (Davies *et al*, 2002), it has been suggested that strategies directed at targeting the DIF could act as a therapeutic for Raf-dependent tumours (Rajakulendran *et al*, 2009). However, this attractive concept was not tested on human B-Raf and in particular not for any of the naturally occurring, oncogenic high activity mutants including B-Raf^{V600E}. Given the clinical significance of the V600E mutation, we tested whether the R509H substitution

blunts the MEK/ERK activation potential of B-Raf^{wt}, B-Raf^{EVKD} and B-Raf^{V600E}. We also combined the R509H mutation with the L515G and M517W substitutions (3x; see Supplementary Table S1) as the individual substitution of the conserved L515 and M517 residues by glycin and tryptophan, respectively, has comparable consequences on D-Raf as the R509H single mutation (Rajakulendran *et al*, 2009). In agreement with the aforementioned results on D-Raf, the R509H and 3x mutations reduce the cellular MEK phosphorylation potential of B-Raf^{wt} by >60 and 90%, respectively (Figure 1C–E and G; Supplementary Figure S1). However, the signalling capacity of the artificial B-Raf^{EVKD} mutant is only reduced by 60% by the R509H mutation, (Figure 1C, F and G; Supplementary Figure S1), but not completely abrogated as it was observed for the equivalent D-Raf mutant (Rajakulendran *et al*, 2009). Most importantly, the cellular MEK/ERK phosphorylation potential of the B-Raf^{V600E} oncoprotein, which is found in about 7% of human tumours, is only slightly affected by the R509H mutation and even the B-Raf$^{V600E/3x}$ mutant retains >50% of the activity of B-Raf^{V600E} (Figure 1C, F and G; Supplementary Figure S1).

The majority of B-Raf g-o-f mutants cluster in either the activation loop, for example, V600E, or the glycine-rich P-loop (Garnett and Marais, 2004). According to a model based on crystal structures of the B-Raf kinase domain (Wan *et al*, 2004), both loops interact with each other *via* a hydrophobic interaction mediated primarily by V600 and F468. Disruption of this interaction either by TVKS motif phosphorylation or by mutations in the activation or P-loop results in a conformational change within the kinase domain and full activation of B-Raf. Consequently, as P-loop mutations such as G469A have a similar effect on the signalling potential of B-Raf as the V600E substitution (Davies *et al*, 2002; Wan *et al*, 2004; Ritt *et al*, 2010), we addressed the importance of R509 for the signalling potential of the B-Raf^{G469A} oncoprotein, the most common P-loop mutant found in cancer (see COSMIC database for details). This analysis revealed that the MEK phosphorylation potential of this oncoprotein is reduced by <40% by the R509H substitution (Figure 1D and G).

Astonished by the unexpected resilience of the B-Raf^{V600E} and B-Raf^{G469A} oncoproteins against DIF mutations, we addressed the role of R509 in a panel of additional, structurally distinct g-o-f mutants. First, given that the E586K substitution has been observed in the B-Raf and Raf-1 proteins in human tumours (Emuss *et al*, 2005), we introduced this g-o-f mutation into B-Raf^{R509H} as well. This mutant was of particular interest as the E586K substitution increases the self-dimerisation potential of the isolated B-Raf kinase domain *in vitro* (Rajakulendran *et al*, 2009). Interestingly, the R509H mutation reduced the cellular activity of B-Raf^{E586K} by 73±7%, indicating that the dimer promoting effect of the E586K substitution still relies on an intact DIF (Figure 1E and G).

As representatives for a g-o-f mutant that bears no mutation in the kinase domain, we used B-Raf^{Q257R} (Supplementary Figure S2), the most frequent g-o-f mutation in CFC syndrome (Rodriguez-Viciana, 2006a), and the artificial B-Raf^{S365A} lacking the N-terminal 14-3-3 binding motif (Figure 1F). Both mutants escape the auto-inhibition imposed by the CRD and CR2 domains and therefore display increased activity (Brummer *et al*, 2006; Niihori *et al*, 2006; Rodriguez-Viciana *et al*, 2006b). We also included the recently discovered activation loop mutant B-Raf^{insT}, which was identified in three cohorts of pilocytic astrocytoma patients with a frequency of ~2% and has been also found at low frequencies in melanomas, thyroid and pancreatic carcinoma (Jones *et al*, 2009; Kubo *et al*, 2009; Yu *et al*, 2009; Eisenhardt *et al*, 2011; Gauchotte *et al*, 2011; Long *et al*, 2011). This oncoprotein is generated by triplet insertions leading to the duplication of T599. B-Raf^{insT} was also chosen as it displays a very comparable signalling and transforming potential as B-Raf^{V600E} (Eisenhardt *et al*, 2011). Lastly, we included B-Raf^{CAAX}, which bears no mutations in the B-Raf sequence, but is extended by the polybasic region and isoprenylation motif of K-Ras at its C-terminus. Consequently, B-Raf^{CAAX} is tethered to the membrane and displays transforming activity (Papin *et al*, 1998) as it had been originally demonstrated for Raf-1 (Leevers *et al*, 1994).

All g-o-f mutants induce strong MEK/ERK phosphorylation, which is strongly reduced upon introduction of the R509H mutation in the artificial mutants B-Raf^{S365A} and B-Raf^{CAAX} and the naturally occurring mutants B-Raf^{E586K} and B-Raf^{Q257R} (Figure 1E–G; Supplementary Figure S2). However, similarly to B-Raf^{V600E}, the cellular MEK phosphorylation potential of the B-Raf^{insT} mutant is reduced by only 13±4% (Figure 1F and G). This suggests that these cancer-associated activation loop mutants are somewhat exempted from the requirement for an intact DIF.

We also titrated B-Raf^{V600E} expression levels by reducing the amount of transfected plasmid DNA to rule out that our model system is saturated by the expression of ectopic B-Raf^{V600E}. Using a lower amount of plasmid DNA, we detected a significant reduction of pERK levels by >70% compared with those observed by transfecting the pre-established amounts of the pMIG/HAh*BRAF*^{V600E} construct (Supplementary Figure S3A). Importantly, however, the differential in the MEK phosphorylation potential of B-Raf^{V600E}, B-Raf$^{V600E/R509H}$ and B-Raf$^{V600E/3x}$ was very similar under both conditions (compare Figure 1G and Supplementary Figure S3B). This indicates that the minor differential between B-Raf^{V600E} and B-Raf$^{V600E/R509H}$ does not represent an artefact caused by system saturation. These data are also in agreement with the growing notion in the field that the analysis of pMEK levels represents a better read-out for Raf activity as pERK levels are the outcome of multiple amplification steps, in particular between MEK and ERK (Schilling *et al*, 2009; Birtwistle and Kolch, 2011). Indeed, the reduction in MEK phosphorylation potential caused by the R509H mutation observed in the high activity mutants B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} is hardly translated to the level of ERK (Figure 1C, D and F). This represents a key finding considering that ERK is a major effector in B-Raf-driven malignant transformation (Old *et al*, 2009) and that clinical responses in B-Raf^{V600E}-driven melanoma require at least 80% reduction of pERK levels (Bollag *et al*, 2010).

So far, we have analysed the *steady-state* MEK/ERK activation potential of all B-Raf mutants in the presence of 10% serum. However, we also show that efficient epidermal growth factor (EGF)-induced, B-Raf-mediated MEK/ERK activation in murine embryonic fibroblasts (MEFs) requires an intact DIF (Figure 2A).

We also considered the possibility that the unexpected resilience of B-Raf^{V600E} against DIF mutations might be

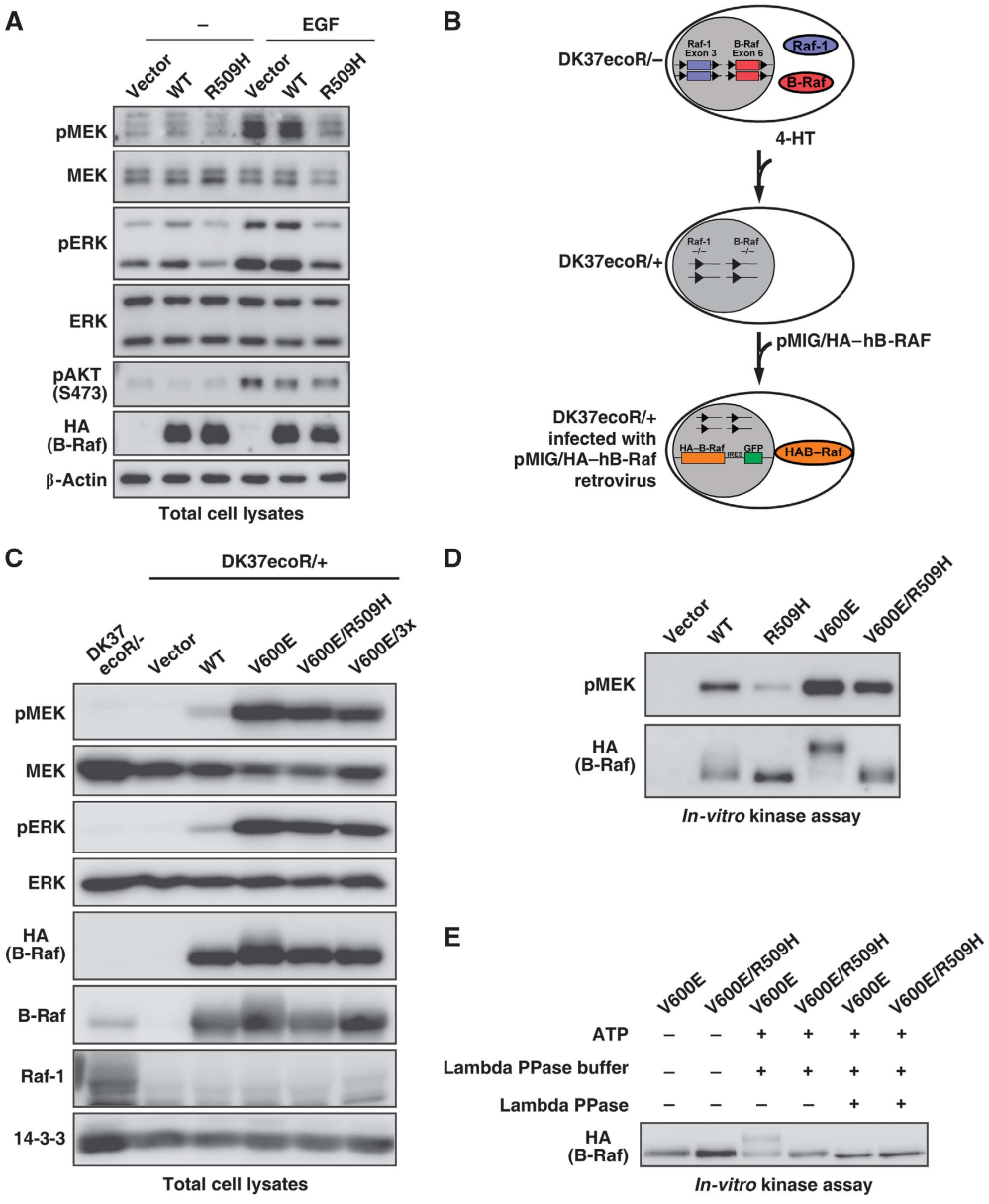


Figure 2 EGF-induced activation of B-Raf^{wt} requires an intact DIF while B-Raf^{V600E} proteins with DIF mutations activate the ERK pathway independently of endogenous Raf proteins. (**A**) The indicated B-Raf proteins were expressed in serum-starved MEFs and stimulated with 100 ng/ml epidermal growth factor (EGF) for 5 min. Total cell lysates were analysed by western blotting with the indicated antibodies. (**B**) Scheme of the B-Raf complementation system in Raf-1/B-Raf double-deficient DT40 B cells (subclone DK37ecoR). See main text and Supplementary data for details. (**C**) DK37ecoR/+ cells were infected with the indicated HA–B-Raf constructs and total cell lysates were analysed by western blotting with the indicated antibodies. (**D**) The indicated HA–B-Raf proteins were purified by anti-HA immunoprecipitation from Plat-E cells and subject to an *in-vitro* kinase (IVK) assay using recombinant GST–MEK1 as a substrate. (**E**) The electrophoretic mobility shift of B-Raf^{V600E} is an outcome of the IVK reaction. The kinase reaction was set up as described in (**D**) except that the samples in lanes 1 and 2 were incubated in the absence of ATP. Subsequently, the kinase reaction was split in two halves, one being treated with Lambda-Phosphatase (Lambda-PPase), the other only treated with Lambda-PPase buffer. Following incubation at 30°C for 30 min, the samples were analysed by western blotting. Figure source data can be found with the Supplementary data.

mediated by endogenous Raf isoforms present in the Plat-E cells and MEFs. Thus, we used our previously established chicken DT40 B-cell system in which the loci for B-*raf* and *raf*-1, the only *raf* genes in chicken, can be efficiently inactivated by Cre-mediated recombination. Importantly, these cells are not impaired in their viability and their defective ERK activation can be rescued by re-expression of B-Raf (Brummer *et al*, 2002). Consequently, we infected Raf-1/B-Raf double-deficient DT40 cells with retroviral expression vectors encoding human B-Raf^{wt}, B-Raf^{V600E}, B-Raf$^{V600E/R509H}$ and B-Raf$^{V600E/3x}$ (Figure 2B) and analysed cellular MEK/ERK phosphorylation (Figure 2C). Expression of B-Raf^{V600E} and its DIF mutants leads to a strong upregulation of pMEK and pERK levels 48 h post infection, indicating that all these proteins can signal to MEK independently of endogenous Raf kinases. Taken together, our data demonstrate for the first time the importance of the DIF for full-length human B-Raf under *steady-state* conditions, growth

factor stimulation and in the context of various g-o-f mutations. Remarkably, the high activity, cancer-associated mutants B-Raf^{G469A}, B-Raf^{insT} and B-Raf^{V600E} are far less affected by DIF mutations than B-Raf^{wt}.

Next, we addressed the impact of the R509H mutation on the *in-vitro* kinase (IVK) activity of B-Raf^{wt} and B-Raf^{V600E}. As shown in Figure 2D, the R509H mutation reduces the IVK activity of B-Raf^{wt} and B-Raf^{V600E}. However, the B-Raf^{R509H} and B-Raf$^{V600E/R509H}$ mutants still retain IVK activity, indicating that this DIF mutation does not abolish catalysis. Likewise, a recent report showed that the B-Raf^{R509A} mutant retains some catalytic activity (Baljuls *et al*, 2011). This further supports that H or A substitutions of R509, which is closely located to the catalytically relevant αC-helix, are not completely incompatible with kinase activity.

Interestingly, we observed that a substantial portion of B-Raf^{V600E} undergoes a prominent electrophoretic mobility shift (EMS) during the IVK reaction (Figure 2D). As omission of ATP from the reaction or the subsequent incubation of the IVK reactions with Lambda-phosphatase prevents this EMS, we attribute this phenomenon to phosphorylation events on B-Raf occurring during the IVK assay (Figure 2E). Importantly, this EMS is completely abrogated upon introduction of the R509H mutation, indicating that these phosphorylation events require an intact DIF and are potentially caused by *trans*- rather than auto-phosphorylation *in cis*.

B-Raf^{V600E} and B-Raf^{G469A} form more stable homodimers than B-Raf^{wt}

Our observation that the signalling output of B-Raf^{V600E} is hardly affected by mutations in the DIF could be explained by various possibilities: First, B-Raf^{V600E} is not strictly reliant on dimerisation and can signal as a monomer. Second, B-Raf^{V600E} could homodimerise independently of an intact DIF. Indeed, B-Raf contains several potential dimerisation contacts outside of its CR3 (Rushworth *et al*, 2006; Terai and Matsuda, 2006). Consequently, we co-transfected Plat-E cells with various pMIG/HAhB-Raf mutants and pMITom/hB-Raf^{wt}–Myc/His or pMITom/hB-Raf^{V600E}–Myc/His vectors, which encode for B-Raf proteins with a combined C-terminal His/Myc epitope tag (abbreviated as B-Raf–Myc in the following; Figure 3A). As expected, Myc-tagged B-Raf^{wt} and Myc-tagged B-Raf^{V600E} co-immunoprecipitated HA-tagged B-Raf^{wt} or B-Raf^{V600E} proteins, respectively. Importantly, however, our analysis shows for the first time that B-Raf^{V600E} has potent homodimerising capacity, while HA–B-Raf^{wt}/B-Raf^{wt}–Myc complexes are less efficiently isolated (Figure 3B). A significant increase in stable homodimers was also observed for the P-loop mutant B-Raf^{G469A} (Figure 3C), suggesting that both B-Raf oncoproteins possess increased dimerisation potential. In all combinations, however, we observed that homodimers of B-Raf^{wt}, B-Raf^{V600E} and B-Raf^{G469A} were drastically reduced, but not completely absent in the presence of the R509H and 3× mutations

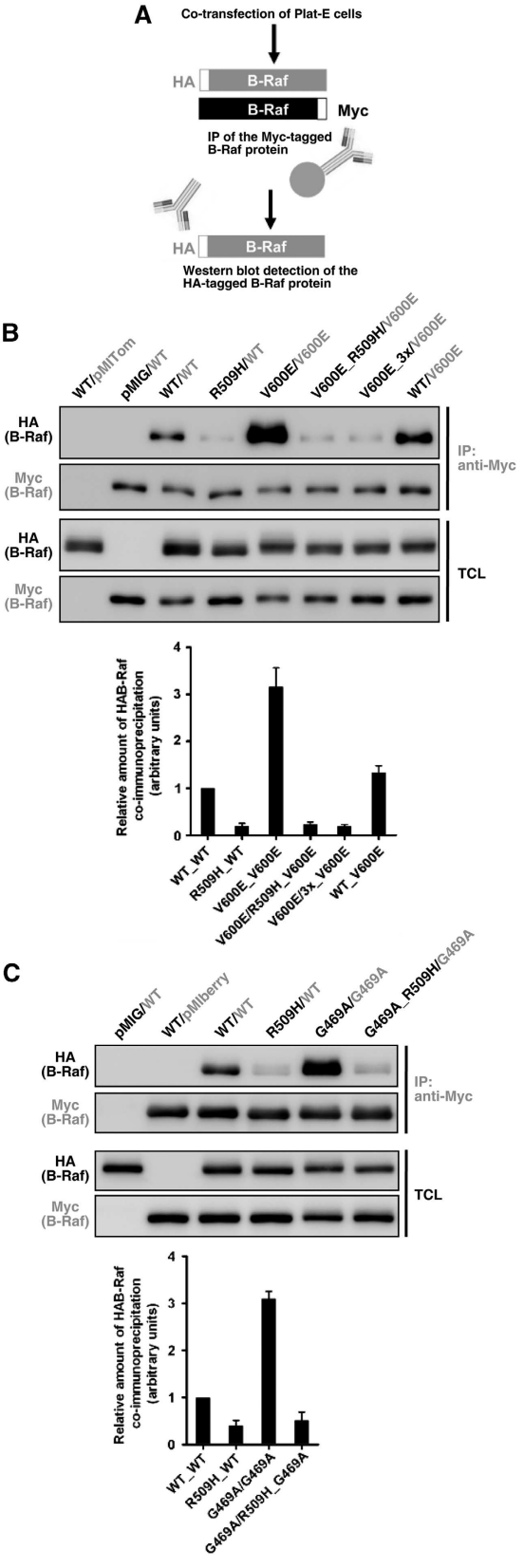


Figure 3 Compared with wild-type B-Raf, B-Raf^{V600E} and B-Raf^{G469A} form particularly stable homodimers in a DIF-dependent manner. (**A**) Schematic of the experimental procedures relevant for this figure. The increased homodimerisation of B-Raf^{V600E} (**B**) and B-Raf^{G469A} (**C**) relies on an intact DIF. The indicated HA- or Myc-tagged B-Raf proteins were co-expressed in Plat-E cells and purified with anti-Myc antibodies. Immunecomplexes and total cell lysates were analysed by western blotting. The ratio of co-purified HA–B-Raf/precipitated B-Raf–Myc is shown in the graphs below. Data represent the mean ± s.e.m. from at least three independent transfections. See Supplementary Table S2 for further statistics. Figure source data can be found with the Supplementary data.

(Figure 3B and C). Thus, stable B-Raf homodimers are predominantly formed *via* the DIF.

The V600E mutation has a profound effect on the formation of B-Raf complexes

The efficiency by which this oncoprotein forms homodimers prompted us to examine native protein complexes containing HA-B-Raf by Blue Native-polyacrylamide gel electrophoresis (BN-PAGE) (Camacho-Carvajal *et al*, 2004). To this end, lysates from Plat-E cells transfected with empty vector or those expressing B-Raf^{wt}, B-Raf^{3x}, B-Raf^{V600E} or B-Raf$^{V600E/3x}$ were analysed. Here, we demonstrate for the first time that B-Raf^{V600E} differs significantly from B-Raf^{wt} in that a large proportion of the oncoprotein exists in complexes with an apparent size >440 kDa (Figure 4A). In contrast, the bulk of B-Raf^{wt} is found in smaller complexes, while only a minor fraction occurs in larger complexes. The unprecedented size difference between the predominant B-Raf^{wt} and B-Raf^{V600E} containing complexes implies that the active conformation of B-Raf^{V600E} has a profound effect on the abundance of the large protein complexes. Interestingly, the DIF plays an important role in the formation of the larger complexes assembled by both B-Raf^{wt} and B-Raf^{V600E}, as the 3x mutation reduces their abundance by ~90%. Upon longer exposure, however, some larger complexes are still detectable in lysates from B-Raf$^{V600E/3x}$-transfected cells, suggesting that large B-Raf^{V600E} containing complexes can occur to some degree in the absence of an intact DIF. This is in agreement with our co-immunoprecipitation (co-IP) studies (Figure 3A).

Next, we extended our BN-PAGE analyses to the human colon carcinoma cell line HT29, which harbours an endogenous *BRAF*^{V600E} allele (Little *et al*, 2011). In order to discriminate between B-Raf^{wt} and B-Raf^{V600E}, we infected these cells with a doxycycline (dox)-inducible shRNAmir construct expressing a V600E-specific hairpin based on a previously published siRNA (Hingorani *et al*, 2003). Commensurate with the bi-allelic expression of B-Raf in HT29 cells (Little *et al*, 2011), our direct sequence analysis of RT–PCR amplicons from untreated HT29 cells infected with the aforementioned lentiviral construct revealed the presence of wild-type (GTG peaks) and V600E (GAG peaks) encoding transcripts. Following exposure with dox for 96 h, the signal of the *BRAF*^{V600E} encoded transcript is reduced to background levels (Figure 4B). Using a novel B-Raf^{V600E}-specific antibody (Capper *et al*, 2011), we confirm the complete loss of B-Raf^{V600E} protein in HT29 cells expressing the V600E-specific shRNA, while B-Raf^{wt} expression is maintained (Figure 4C). This selective B-Raf^{V600E} depletion was also correlated with a strong reduction in MEK/ERK phosphorylation. Importantly, we show by BN-PAGE that the allele-specific knockdown of B-Raf^{V600E} results in a disproportionate reduction of the large B-Raf containing complexes compared with the smaller ones (Figure 4D). Thus, both ectopically and endogenously expressed B-Raf^{V600E} are more likely to appear in larger protein complexes than B-Raf^{wt}.

The increased dimerisation of B-Raf^{V600E} and its assembly into large complexes suggested that the V600E substitution might have profound effects on the overall conformation of the kinase domain. This assumption led us to revisit the naturally occurring dimers within the crystal structures of the kinase domains of B-Raf^{wt} and B-Raf^{V600E} (Wan *et al*, 2004; Bollag *et al*, 2010). Interestingly, our analysis of kinase domains complexed with sorafenib shows that the structure of the B-Raf^{V600E} kinase domain displays a larger contact zone than that of B-Raf^{wt}. Likewise, we observe a similar protomer contact area in the kinase domain of B-Raf^{V600E} in complex with PLX4032. However, as a B-Raf^{wt} kinase domain in complex with PLX4032 has not been published to our knowledge, we cannot make a similar comparison as for sorafenib. In particular, the models reveal an increased dimer contact around the αE-helix in B-Raf^{V600E} compared with B-Raf^{wt} (Figure 4E). Thus, B-Raf^{V600E} seems to exhibit additional contact points that could contribute to increased dimer stability of B-Raf^{V600E} proteins in a DIF-dependent manner.

Mutations in the DIF of B-Raf^{V600E} hardly affect its transformation potential

As it was suggested that targeting the DIF could provide a novel therapeutic strategy for Raf-dependent tumours (Rajakulendran *et al*, 2009; Lavoie and Therrien, 2011), we analysed the transformation potential of B-Raf^{V600E} with mutations in the DIF in two cellular model systems. First, we infected MEFs derived from conditional B-*raf*-deficient mice (Chen *et al*, 2006) with the aforementioned pMIG/HAh*BRAF* constructs and analysed their cellular behaviour. As shown in Figure 5A, B-*raf*$^{-/-}$ MEFs infected with the empty pMIG vector or expressing B-Raf^{wt} retain their normal flat morphology and form a regular monolayer. In sharp contrast, cells expressing B-Raf^{V600E} or B-Raf$^{V600E/3x}$ display a transformed phenotype with elongated, refractile shape, criss-cross growth and reduced adhesion to neighbouring cells and the substratum. Importantly, B-Raf proficient MEFs expressing B-Raf^{V600E} and B-Raf$^{V600E/3x}$ continue to proliferate under anchorage-independent conditions, while cells expressing ectopic B-Raf^{wt} or empty vector-infected cells cannot thrive under these conditions (Figure 5B).

Second, we evaluated the transforming potential of B-Raf^{V600E} and B-Raf$^{V600E/3x}$ in a cell type, in which aberrant B-Raf signalling has been implicated as a driver of carcinogenesis. Therefore, we turned to Caco-2 cells, a human intestinal cell line, which retains many features of colon epithelium. Importantly, Caco-2 cells lack mutations in the *KRAS* and *BRAF* genes, but express a non-functional version of the tumour suppressor APC (Matos *et al*, 2008; Oikonomou *et al*, 2009). As loss of APC function is considered to be an early event preceding *BRAF* mutations (Walther *et al*, 2009), we deemed this as an ideal experimental system to model the effects of B-Raf^{V600E} on colon epithelium. Consequently, we have recently established a Caco-2 cell line allowing the dox-regulated expression of a bi-cistronic B-Raf-IRES-GFP transcript (Fritsche-Guenther *et al*, 2011; Herr *et al*, 2011). As observed in Plat-E and DT40 cells, induction of B-Raf^{V600E} and B-Raf$^{V600E/3x}$ led to a marked increase in ERK pathway activation (Figure 5C). Commensurate with the retention of an epithelial phenotype, Caco-2 cells form polarised epithelial cysts in three-dimensional (3D) matrigel cultures, which become considerably enlarged upon exposure to cholera toxin and maintain a hollow lumen thereafter (Jaffe *et al*, 2008). We reasoned that this 3D culture system would allow us to assess the transforming properties of the B-Raf proteins in question in a more physiological setting and to study the impact of oncoproteins in established cysts. Indeed, the

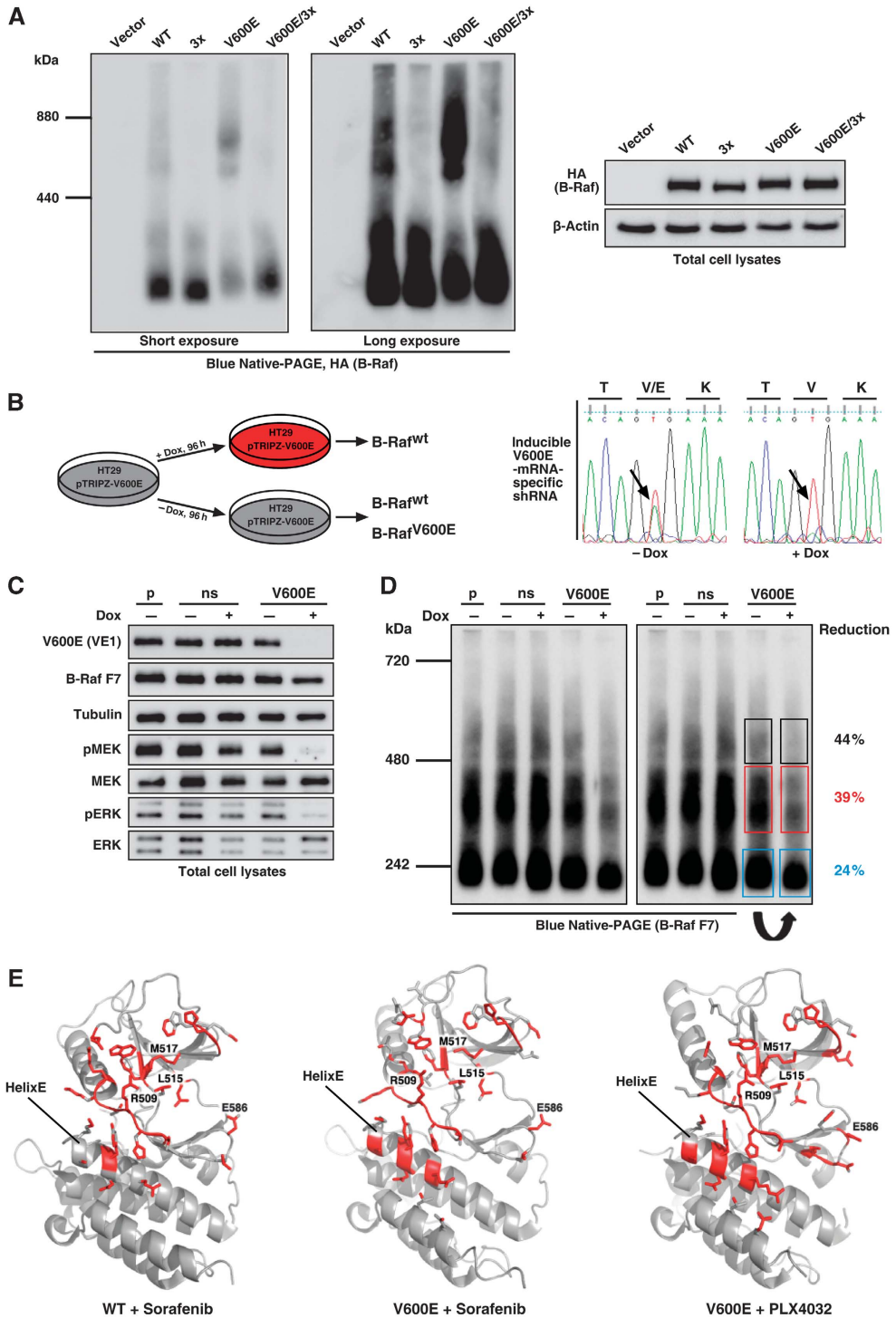


Figure 4 The V600E mutation induces larger B-Raf complexes in Plat-E as well as in HT29 colon carcinoma cells and increases the contact zones between two B-Raf protomers. (**A**) BN-PAGE analysis of HA–B-RAF containing protein complexes using the anti-HA (3F10) antibody with lysates of Plat-E cells expressing the indicated HA-tagged proteins (left). Conventional western blot analysis showing comparable expression of HA–B-Raf proteins in the Plat-E cell lysates used for the BN-PAGE analysis (right). (**B**) Schematic description of the experimental workflow for specific ablation of endogenous B-Raf^{V600E} expression in HT29 cells (left). Sequencing of RT–PCR products confirms that doxycycline (dox) treatment of pTRIPZ-V600E-infected human HT29 cells induces knockdown of the B-Raf^{V600E}-encoding mRNA (right). (**C**) Immunoblotting of pTRIPZ-V600E-infected HT29 cell lysates confirms complete loss of B-Raf^{V600E} after knockdown induction with dox. Note that the total B-Raf antibody (F7) still detects B-Raf^{wt} protein. (p: parental HT29 cells; ns: non-silencing). (**D**) Blue Native-PAGE analysis with the anti-B-Raf (F7) antibody showing that selective knockdown of the endogenous B-Raf^{V600E} allele preferentially reduces larger B-Raf containing protein complexes in human HT29 colon carcinoma cells. Please note that the shown pictures are identical except for the quantification of the right blot. (**E**) Ribbon diagrams based on crystal structures of the kinase domains of human B-Raf^{wt} (left; 1uwh) and B-Raf^{V600E} (middle; 1uwj) in complex with sorafenib (Wan *et al*, 2004). For comparison, the structure of the B-Raf^{V600E} kinase domain is shown in complex with PLX4032 (Bollag *et al*, 2010). Contact zones between the protomers are shown in red. Figure source data can be found with the Supplementary data.

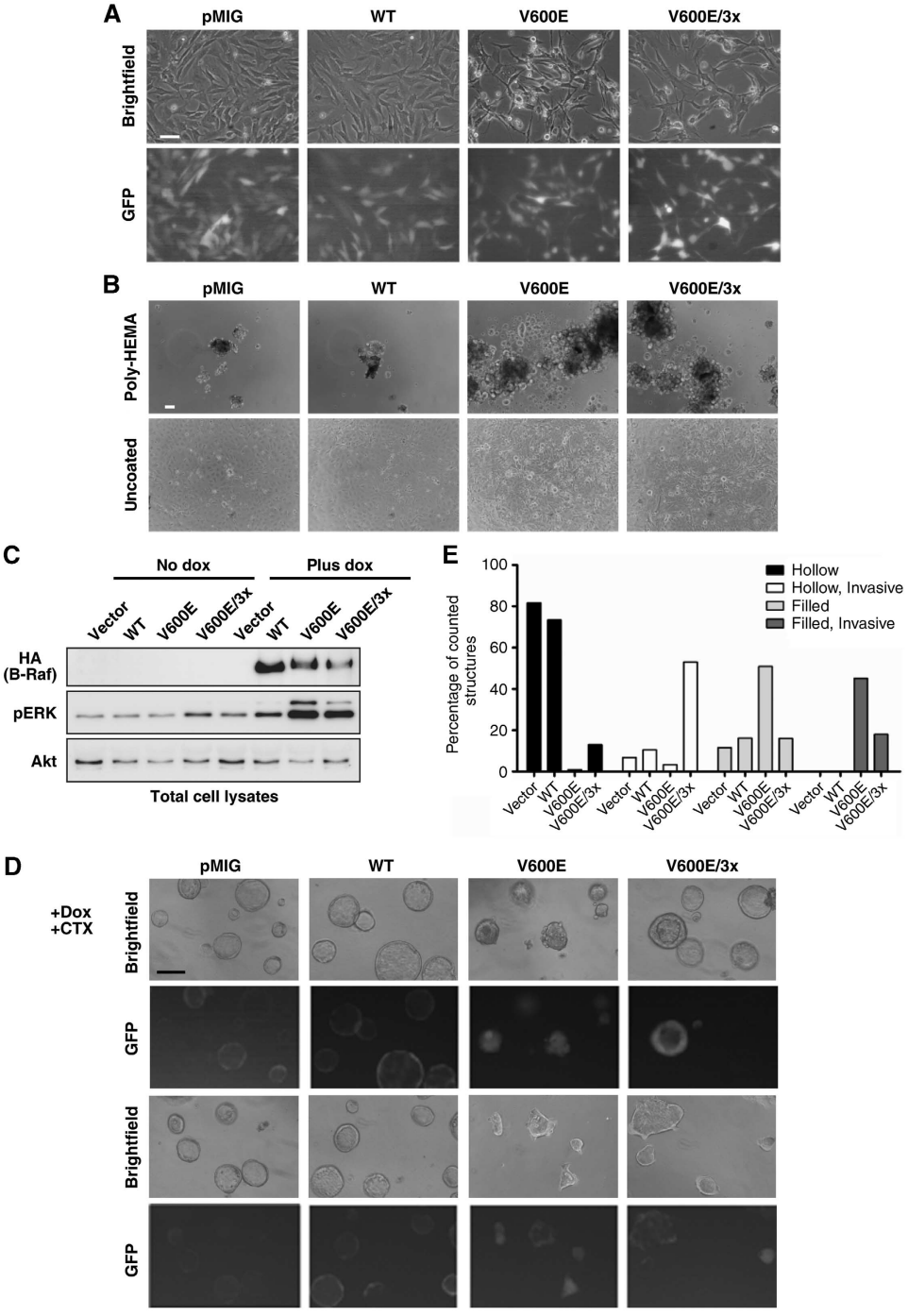


Figure 5 B-Raf^{V600E} proteins with a defect DIF still display transforming activities. (**A**) B-Raf-deficient MEFs were transduced with the indicated pMIG constructs encoding HA-tagged B-Raf and GFP. Morphological changes were monitored using phase contrast microscopy. Note that MEFs expressing B-Raf^{V600E} and B-Raf$^{V600E/3x}$ display criss-cross growth and the refractile morphology of Raf-transformed fibroblasts. The length of the white scale bar equals 200 μm. (**B**) MEFs expressing B-Raf^{V600E} or B-Raf$^{V600E/3x}$ grow under anchorage-independent conditions. The efficient growth of all cell pools on non-coated plates (lower row) indicates a comparable plating fitness. The length of the white scale bar equals 200 μm. (**C**) Inducible expression of B-Raf^{V600E} and B-Raf$^{V600E/3x}$ induces ERK phosphorylation in human Caco-2 cells. Cells fitted with an inducible expression system, which encodes a bi-cistronic transcript, the indicated HA-tagged B-Raf proteins and GFP, were plated and treated with 2 μg/ml doxycycline (dox) for 24 h to induce transgene expression. (**D**) B-Raf^{V600E} and B-Raf$^{V600E/3x}$ counteract cyst formation of Caco-2 cells. Cells harbouring dox-inducible B-Raf^{wt}, B-Raf^{V600E}, B-Raf$^{V600E/3x}$ and vector control constructs were seeded into matrigel (5000 cells per chamber). At day 3, the cultures were treated with 100 ng/ml choleratoxin (CTX) to induce lumen expansion. The following day, B-Raf transgene expression was switched on by addition of 2 μg/ml dox. Representative micrographs taken at day 7 are shown. Note the presence of normal, but GFP-negative cysts in B-Raf$^{V600E/3x}$ cultures, which serve as an internal control. The length of the black scale bar equals 500 μm. (**E**) Quantification of normal and aberrant Caco-2 cysts. A representative result from two independent experiments is shown. At least 100 structures were counted for each category and scored by a person blinded to the genotype.

induction of B-Raf^{V600E} and B-Raf$^{V600E/3x}$ had a profound effect on cyst morphology (Figure 5D). In most cases, cells expressing oncogenic B-Raf grew into the established lumen (filled structures) and/or disintegrated due to the invasive properties of the cells (invasive structures). Although these phenomena were occasionally observed in cysts expressing B-Raf^{wt}, they were a hallmark of cysts formed by B-Raf^{V600E} and B-Raf$^{V600E/3x}$ expressing cells. Furthermore, hollow, symmetrical cysts, which predominated the B-Raf^{wt} expressing culture, were hardly observed in the B-Raf^{V600E} and B-Raf$^{V600E/3x}$ expressing populations (Figure 5E). This indicates that B-Raf^{V600E} and B-Raf$^{V600E/3x}$ induce aberrant morphologies in Caco-2 cysts, although the latter mutant seems to provoke a less severe abnormal phenotype. Taken together, our transformation assays in MEFs and human Caco-2 cells demonstrate that mutations within the DIF have little or no effects on the transformation potential of B-Raf^{V600E}.

The role of the DIF for heterodimer formation and paradoxical ERK activation

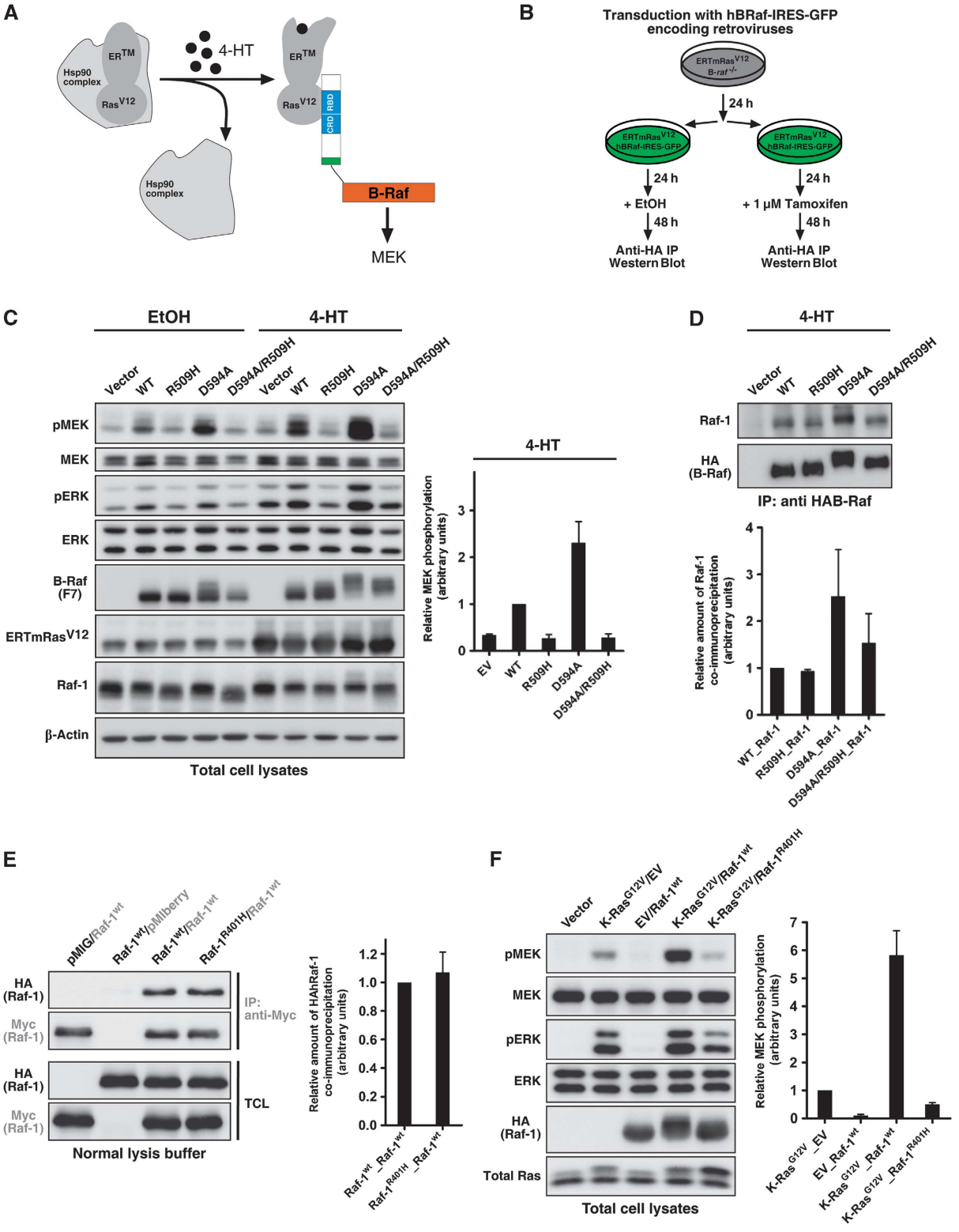
The signalling and oncogenic potential of B-Raf^{wt} and B-Raf^{V600E} is also modulated by heterodimer formation with Raf-1 and KSR (Brennan *et al*, 2011; Karreth *et al*, 2009; McKay *et al*, 2011). Consequently, we addressed the importance of the DIF in spontaneous B-Raf heterodimer formation using the same experimental set-up as for the study of homodimer formation. First, we co-expressed HA–B-Raf and mutants thereof with N-terminally Myc-tagged murine KSR1 (mKSR1), human KSR1 (hKSR1) and human KSR2 (hKSR2). Surprisingly, all HA–B-Raf proteins interacted with the myc-tagged KSR proteins (Supplementary Figure S4A and B). Likewise, Raf-1 was efficiently co-immunoprecipitated with the DIF triple mutant B-Raf$^{V600E/3x}$ (Supplementary Figure S4A). Thus, the DIF is far more important for the homodimerisation of B-Raf than for its heterodimerisation with Raf-1 and KSR. This observation is also in agreement with previous publications reporting that heterotypic contacts between full-length B-Raf, Raf-1 and KSR proteins are mediated by various regions (Rushworth *et al*, 2006; Claperon and Therrien, 2007). Furthermore, a recent report demonstrated that Venus–Raf-1 and mCherry–B-Raf fusion proteins, which carry the R401H and R509H mutation, respectively, still interact with each other albeit with reduced efficiency (Hatzivassiliou *et al*, 2010).

The formation of heterodimers also underlies the paradoxical ERK activation elicited by kinase-dead B-Raf molecules, which were rendered catalytically inactive either by mutation (D594A) or by binding of clinically relevant drugs (Heidorn *et al*, 2010). This phenomenon is observed in tumour cells lacking *BRAF* mutations, but displaying aberrant activity of Ras or receptor tyrosine kinases (RTKs) and has been made responsible for drug resistance and side effects (Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010; Nazarian *et al*, 2010; Poulikakos *et al*, 2010; Ribas and Flaherty, 2011; Su *et al*, 2012). However, the role of the DIF in B-Raf^{D594A} signalling has not been investigated so far. Thus, we addressed this question by generating a unique reconstitution system in which B-Raf$^{-/-}$ MEFs were infected with a retroviral construct encoding a 4-hydroxy-tamoxifen (4-HT)-regulated H-RAS^{G12V} protein (ERTmRAS^{V12}) (Dajee *et al*, 2002). Upon addition of 4-HT, ERTmRAS^{V12} becomes 'uncaged' and is then able to recruit Raf kinases and to trigger ERK pathway activation (Figure 6A). We re-introduced B-Raf or mutants thereof and analysed their signalling and dimerisation potential in the presence of oncogenic Ras signalling (Figure 6B).

First, the MEK/ERK phosphorylation potential of B-Raf^{wt} and B-Raf^{D594A} was analysed in the absence and presence of ERTmRAS^{V12} signalling. MEFs re-expressing B-Raf^{wt} and B-Raf^{D594A} displayed a slight and prominent elevation of MEK/ERK phosphorylation levels in vehicle-treated cells, respectively. As expected, MEK and ERK phosphorylation was further increased in 4-HT-treated MEFs expressing B-Raf^{wt} and B-Raf^{D594A} (Figure 6C). In contrast, B-Raf^{R509H} expressing MEFs behaved like their counterparts reconstituted with the empty control vector indicating that this protein behaves rather inert or slightly dominant negative in the absence and presence of ERTmRAS^{V12} signalling. This is also in agreement with the behaviour of this mutant in EGF receptor (EGFR) signalling (Figure 2A). Most importantly, introduction of the R509H mutation reduced the strong MEK/ERK phosphorylation elicited by the kinase-dead B-Raf^{D594A} mutant, which has been shown to cooperate with oncogenic Ras in paradoxical ERK activation and tumourigenesis (Heidorn *et al*, 2010), to the level of that in MEFs infected with the empty control vector (Figure 6C). This observation

Figure 6 The DIF is required for activation of wild-type B-Raf and Raf-1 by oncogenic Ras^{V12} as well as for paradoxical ERK pathway activation by kinase-dead B-Raf^{D594A}. (**A**) Scheme of the ERTmRAS^{V12} system. In the absence of 4-hydroxy-tamoxifen (4-HT), the ERTmRAS^{V12} fusion protein is sequestered in heat-shock protein (HSP) complexes. Upon administration of 4-HT, this ligand binds to the oestrogen receptor (ER) moiety of the fusion protein leading to a conformational change and exposure of the Ras^{V12} moiety, which in turn recruits B-Raf *via* its Ras-binding domain (Dajee *et al*, 2002). (**B**) Workflow for complementation of B-Raf-deficient MEFs with retroviral B-Raf expression vectors and subsequent release of oncogenic Ras^{V12}. (**C**) The indicated HA-tagged B-Raf proteins were expressed in the B-Raf-deficient MEFs described in (**A** and **B**), treated with 1 μM 4-HT or vehicle (EtOH) and total cell lysates were analysed by western blotting with the indicated antibodies. A bar graph showing the effects of the R509H dimer interface mutation on the MEK phosphorylation potential of B-Raf^{wt} and B-Raf^{D594A} is shown on the right. (**D**) The indicated HA–B-Raf constructs were expressed in the MEFs described in (**A** and **B**) and purified with anti-HA (3F10) antibodies from the lysates shown in (**C**). Immunecomplexes were analysed by western blotting for endogenous Raf-1. The ratio of co-purified endogenous Raf-1/HA-B-Raf is shown in the graph below. Note that all B-Raf proteins interact with Raf-1. Data represent the mean ± s.e.m. from three independent transductions. See Supplementary Table S2 for further statistics. (**E**) The indicated HA- and Myc-tagged Raf-1 constructs were co-expressed in Plat-E cells and purified using anti-Myc (9E10) antibodies. Immunecomplexes and total cell lysates were analysed by western blotting (left). Bar graph showing the ratio of co-purified HA-Raf-1/precipitated Myc–Raf-1 (right). Data represent the mean ± s.e.m. from three independent transfections. Please note that the R401H mutant represents the Raf-1 equivalent of B-Raf^{R509H}. (**F**) The indicated HA-tagged Raf-1 constructs were co-expressed with K-Ras^{G12V} or empty vector (EV) in Plat-E cells and total cell lysates were analysed by western blotting (left). Bar graph representing the mean MEK phosphorylation ± s.e.m. from three independent transfections (right). See Supplementary Table S2 for further statistics. Figure source data can be found with the Supplementary data.

prompted us to address the interaction of B-Raf^{wt} and B-Raf^{D594A} proteins with an intact or mutated DIF with endogenous Raf-1 as this isoform phosphorylates MEK in the presence of inactive B-Raf (Hatzivassiliou *et al*, 2010; Heidorn *et al*, 2010; Poulikakos and Solit, 2011). As shown in Figure 6D, all four B-Raf proteins co-purified with Raf-1 indicating that the loss of paradoxical MEK/ERK activity observed in the MEFs expressing B-Raf$^{D594A/R509H}$ cannot be explained by the absence of Raf-1 in the isolated immune-complexes. This finding led us then to re-visit the requirements of Raf-1 for an intact DIF in terms of its homodimerisation and MEK phosphorylation potential. As shown in Figure 6E, introduction of the R401H mutation, which represents the equivalent to R509H in B-Raf, did not



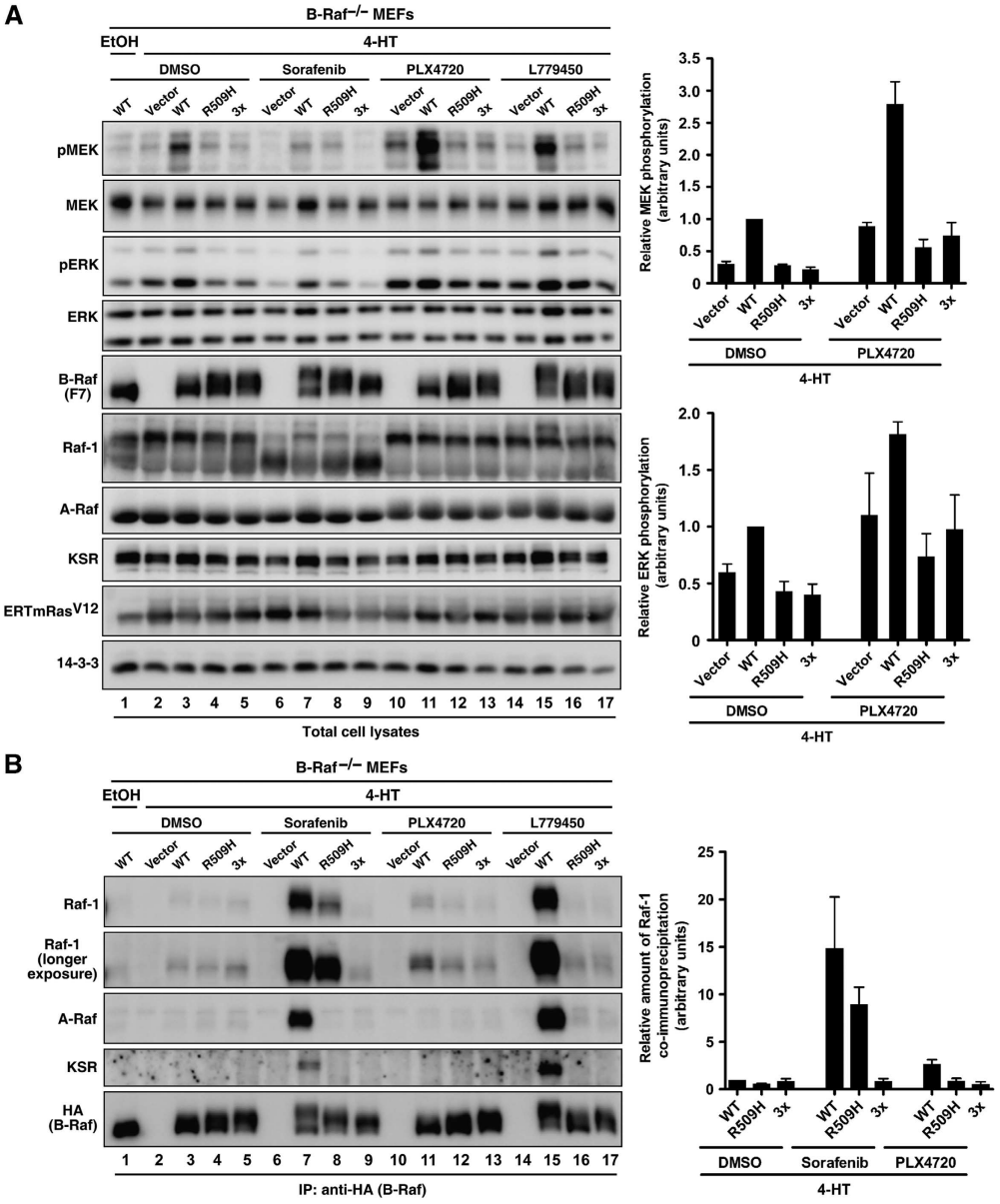


Figure 7 Raf inhibitor-induced paradoxical ERK activation is DIF dependent as is concurrent protein complex formation of HA–B-Raf with endogenous Raf-1, A-Raf and KSR. The indicated HA–B-Raf proteins were expressed in the MEFs described in (Figure 6A) and purified with anti-HA (3F10) antibodies. Prior to lysis, the MEFs were treated with 1 μM 4-HT or vehicle (EtOH) followed by sorafenib (10 μM), PLX4720 (1 μM), L779450 (1 μM) or vehicle (DMSO) for 4 h. Total cell lysates (**A**) and immunecomplexes (**B**) were analysed by western blotting with the indicated antibodies. Shown bar graphs represent the mean ± s.e.m. from three independent transductions. Figure source data can be found with the Supplementary data.

reduce the homodimerisation of Raf-1. Furthermore, while MEK phosphorylation was strongly enhanced by ectopically expressed wild-type Raf-1 in combination with oncogenic K-Ras^{G12V}, expression of Raf-1^{R401H} significantly quenched the MEK phosphorylation elicited by K-Ras^{G12V} below that of cells expressing this GTPase mutant alone (Figure 6F). This dominant-negative effect is in full agreement with the behaviour of B-Raf^{R509H} in MEFs either stimulated with EGF (Figure 2A) or containing 4-HT released ERTmRAS^{V12} (Figure 6C).

As kinase-dead mutants such as B-Raf^{D594A} mimic the effect of B-Raf inhibitors in cells with active Ras (Heidorn *et al*, 2010), we next asked whether an intact DIF is required for drug-induced heterodimerisation (Supplementary Figure S5A). To this end, we used sorafenib that inhibits several kinases including all Raf isoforms and therefore also ERK pathway activation (Figure 7A; Wilhelm *et al*, 2004; Heidorn *et al*, 2010). As a result, B-Raf/Raf-1 heterodimers, which are disrupted by ERK-mediated feedback phosphorylation (Rushworth *et al*, 2006; Heidorn *et al*, 2010; Ritt *et al*, 2010), are preserved and particularly abundant even in the absence of 4-HT triggered ERTmRAS^{V12} signalling (Figure 7B; Supplementary Figure S5B). Importantly, this experiment revealed for the first time distinct structural requirements within the DIF for the formation of sorafenib-induced B-Raf containing heterodimers. We observed low levels of heterodimers between Raf-1 and A-Raf with B-Raf including its DIF mutants in vehicle (DMSO)-treated cells with active ERTmRAS^{V12} signalling. Upon sorafenib treatment, however, the R509H mutation in B-Raf prevents the strong drug-in-

duced increase in A-Raf and KSR1 containing heterodimers, while the B-Raf/Raf-1 interaction is less affected (Figure 7B; Supplementary Figure S5B). Nevertheless, the 3x mutation prevents sorafenib-induced B-Raf/Raf-1 heterodimerisation indicating an important contribution of L515 and M517 in this process.

We also analysed the behaviour of our DIF mutants in SW480ecoR cells, a subline of the human colon carcinoma cell line SW480, which harbours an endogenous *KRAS*^{G12V} oncogene (Capon *et al*, 1983) and expresses the receptor for murine retroviruses (ecoR; Albritton *et al*, 1989). Again, as we have observed in our MEF system, the sorafenib-induced increase of the B-Raf/Raf-1 interaction was completely blunted by the 3x, but not the R509H mutation (Supplementary Figure S6A). Very similar findings were obtained using an ecoR subline of the human colon carcinoma cell line HCT116 (Supplementary Figure S6B), which is driven by a *KRAS*^{G13D} oncogene (Shirasawa *et al*, 1993). For an additional comparison, we also analysed the heterodimerisation behaviour of the Ras binding-deficient mutant B-Raf^{R188L}, which failed to interact with Raf-1 in both colon carcinoma cell lines as it has already been shown by Heidorn *et al* (2010) for melanoma cell lines.

Although our experiments are based on experiments using drug concentrations comparable to the plasma levels of sorafenib in patients (Strumberg *et al*, 2005; Houben *et al*, 2009; Huber *et al*, 2011) and are therefore of potential clinical interest, we next sought to analyse the dimerisation and signalling potential of the DIF mutants for two additional Raf inhibitors, L779450 and PLX4720. The latter represents the tool compound for the clinically applied drug vemurafinib/PLX4032 (Bollag *et al*, 2010). Treatment of MEFs re-expressing B-Raf^{wt} with PLX4720 and L779450 causes a pronounced paradoxical MEK/ERK activation as described by others (Figure 7A, compare lanes 2, 3 with 10, 11). Thus, although PLX4720 is able to induce MEK/ERK phosphorylation in B-Raf-deficient MEFs as described previously (Poulikakos *et al*, 2010), our complementation approach demonstrates that the extent of drug-induced paradoxical MEK/ERK phosphorylation is correlated with the B-Raf expression levels. Importantly, we show for the first time that paradoxical MEK phosphorylation induced by PLX4720 and L779450 is almost completely abrogated in MEFs complemented with B-Raf^{R509H} or B-Raf^{3x} (Figure 7A, lanes 11–13 and 15–17). Likewise, pERK levels in drug-treated MEFs expressing these DIF mutants are comparable to MEFs infected with the empty vector control construct, which remain B-Raf deficient. Furthermore, B-Raf^{R509H} quenched PLX4720-induced paradoxical MEK/ERK phosphorylation driven by the endogenous N-RAS^{Q61K} oncoprotein in SBcl2 melanoma cells (Supplementary Figure S7), which were previously shown to exhibit paradoxical ERK activation upon PLX4720 treatment (Kaplan *et al*, 2011).

In agreement with Heidorn *et al* (2010), but in contrast to others (Hatzivassiliou *et al*, 2010; Poulikakos *et al*, 2010), we also observe an increase in B-Raf/Raf-1 heterodimers in PLX4720-treated MEFs, although the degree of heterodimerisation is by far not as pronounced as with sorafenib or L779450 (Figure 7B). This observation can be explained by recent findings showing that binding of PLX4720 as well as PLX4032 causes a reorientation of the Cα helix (Tsai *et al*, 2008; Bollag *et al*, 2010), which in turn could affect DIF function. This drug-induced reorientation might also explain the absence of B-Raf/A-Raf and B-Raf/KSR1 heterodimers. In contrast, Raf-1 was still present in B-Raf^{R509H} immunecomplexes from PLX4720-treated cells. Interestingly, while the R509H substitution has only a minor effect on sorafenib-induced B-Raf/Raf-1 heterodimerisation, the same mutation abrogates the prominent formation of stable L779450-induced B-Raf/Raf-1 and B-Raf/A-Raf complexes (Figure 7B). Furthermore, the drug-induced formation of B-Raf/KSR1 complexes was also prevented by the R509H mutation as it was recently reported (McKay *et al*, 2011). Taking all this into account, our data indicate that the formation of B-Raf homodimers and heterodimers is based on distinct structural requirements with respect to the DIF. Furthermore, our data suggest that the various Raf inhibitors induce the formation of drug-specific dimers that differ in their requirement for R509.

Discussion

Dimerisation emerges as an important regulatory layer of the ERK signalling pathway (Wimmer and Baccarini, 2010; Matallanas *et al*, 2011). However, our knowledge about the formation, regulation, dynamics and (patho-)physiological significance of these dimers is still superficial, in particular in relation to oncogenic B-Raf. Indeed, a series of recently published commentaries use working models describing B-Raf^{V600E} acting either as a monomeric enzyme (Solit and Sawyers, 2010; Poulikakos and Rosen, 2011; Poulikakos and Solit, 2011; Solit and Rosen, 2011) or as a dimer (Cichowski and Janne, 2010; Downward, 2011). However, no experimental data that support one or the other model were published by the time of manuscript submission. Using full-length human B-Raf proteins, we provide now a detailed analysis of the importance of the DIF for B-Raf signalling. Entirely consistent with the data of Rajakulendran *et al* (2009) on D-Raf, we demonstrate that the DIF plays a critical role for the dimerisation and signalling potential of B-Raf^{wt} and several of its g-o-f mutants, including some of clinical relevance. This was not necessarily expected as the unique N-terminal region of B-Raf and several other areas in the N-terminal moiety of the molecule have also been implicated in homodimerisation and heterodimerisation (Rushworth *et al*, 2006; Terai and Matsuda, 2006). However, in sharp contrast to the artificial D-Raf^{EAKD} mutant (Rajakulendran *et al*, 2009), which is considered to closely mimic B-Raf^{V600E}, we demonstrate that DIF mutations are not sufficient to inhibit the cellular and transforming activity of the most frequently occurring B-Raf oncoprotein. We found that the cellular MEK phosphorylation potential of B-Raf^{V600E} in Plat-E cells was only reduced to 80 and 50% by the R509H and 3× mutations, respectively, while pERK levels were hardly affected at all. While our manuscript was under review, Poulikakos *et al* (2011) also reported that B-Raf$^{V600E/R509H}$ retains its full ability to phosphorylate MEK and ERK, although detailed quantitative analyses and transformation assays were not conducted in their study. Given that activated ERK is accountable for most, if not for all, hallmarks of B-Raf-driven tumours (Old *et al*, 2009) and that a clinical response of melanoma towards the B-Raf inhibitor PLX4032 requires at least >80% inhibition of ERK phosphorylation (Bollag *et al*, 2010), the results from both

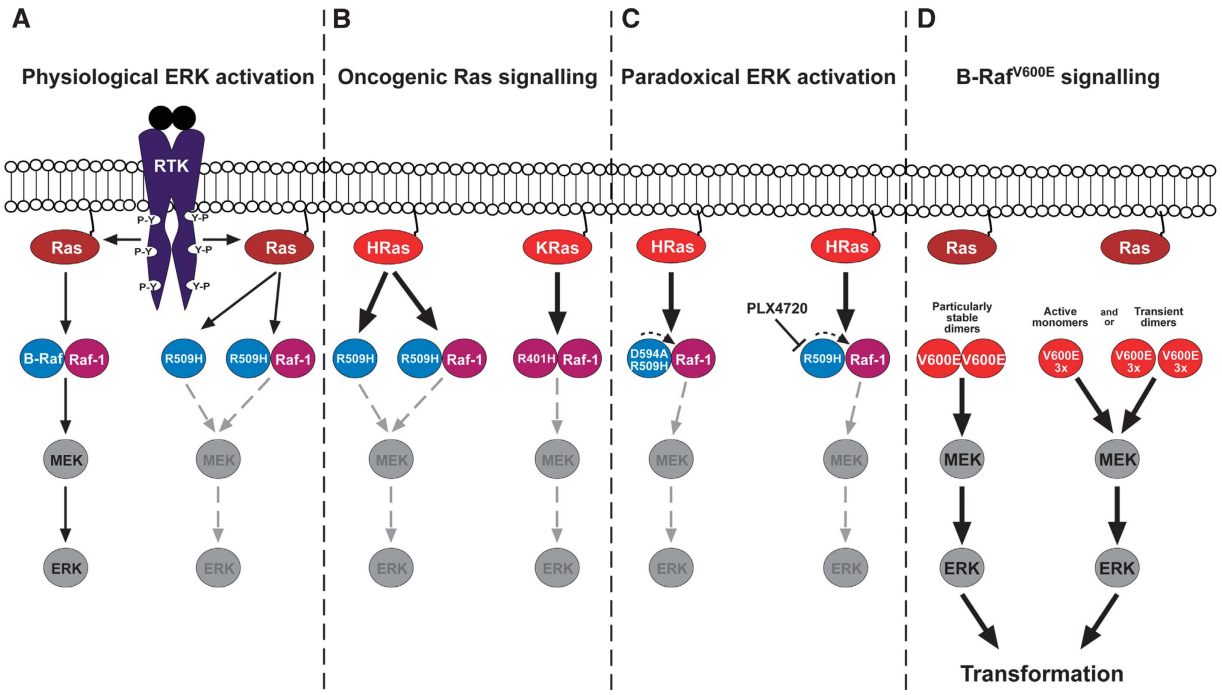


Figure 8 The DIF is a major determinant of normal and oncogenic Ras-induced Raf signalling as well as paradoxical ERK activation, but is dispensable for B-Raf^{V600E}. (**A**) Expression of ectopic B-Raf^{R509H} in EGF stimulated murine embryonic fibroblasts (MEFs) has a dominant-negative effect on MEK/ERK phosphorylation levels. (**B**) Wild-type B-Raf or Raf-1 mediated signalling to MEK/ERK induced by active ERtmHRas^{G12V} (HRas) or K-Ras^{G12V} (KRas), respectively, is abrogated upon introduction of dimer interface mutations (R509H in B-Raf, R401H in Raf-1). Importantly, introduction of the R509H and R401H DIF mutations into one protomer of a respective dimer does not abolish B-Raf/Raf-1 heterodimer and Raf-1/Raf-1 homodimer formation. (**C**) Introduction of the R509H DIF mutation blunts B-Raf mediated paradoxical Raf-1 transactivation (dashed black arrow) as well as the resulting ERK phosphorylation triggered by B-Raf^{D594A} and PLX4720 treatment. Nevertheless, substantial amounts of Raf-1 can still be purified in complex with B-Raf$^{D594A/R509H}$ and B-Raf^{R509H}, respectively. (**D**) Compared with B-Raf^{wt}, B-Raf^{V600E} forms particularly stable dimers and transforms MEFs and Caco2 cells despite the presence of the R509H, L515G and M517W DIF mutations (3x).

independent studies are quite sobering. Furthermore, we report here a similar resilience for the less frequent B-Raf^{G469A} and B-Raf^{insT} mutant suggesting that these oncoproteins can also signal efficiently despite their lack of an intact DIF. Although B-Raf$^{V600E/3x}$ can still interact with Raf-1, our complementation analysis in B-Raf/Raf-1-deficient DT40 cells indicates that these proteins are capable to signal in an Raf-1- and A-Raf-independent manner and potentially even as a monomer (Figure 8D). The latter notion is supported by our finding that DIF mutations led to a marked reduction of B-Raf^{V600E} homodimers in our co-IP and BN-PAGE analyses. Furthermore, earlier observations demonstrated that mutation of the C-terminal 14-3-3 binding site, which also promotes Raf dimerisation (Weber *et al*, 2001; Garnett *et al*, 2005; Rushworth *et al*, 2006; Rajakulendran *et al*, 2009), has no effect on the signalling and transforming potential of the high activity oncogenic mutants B-Raf^{V600E} and B-Raf^{G469A}, while that of B-Raf^{wt} and intermediate g-o-f mutants such as B-Raf^{S365A} are severely affected (Brummer *et al*, 2006; Fischer *et al*, 2009; Ritt *et al*, 2010). However, as 14-3-3 proteins clearly contribute to Raf dimerisation, we reasoned that the loss of DIF function could be potentially compensated by the intact C-terminal 14-3-3 binding motif around S729. Therefore, we generated B-Raf$^{V600E/R509H/S729A}$ and B-Raf$^{V600E/3x/S729A}$ proteins. Surprisingly, these mutants still display >50% of the MEK-phosphorylation potential of B-Raf^{V600E} (Supplementary Figure S8). This implies that a B-Raf^{V600E} protein, in which these key dimerisation mechanisms have been inactivated, retains remarkable biological activity. Still, we cannot completely rule out that transient dimer formation between B-Raf^{V600E} protomers occurs in a DIF- and 14-3-3-independent manner leading to MEK phosphorylation (Figure 8D).

However, the resilience of B-Raf^{V600E} against DIF and 14-3-3 mutations could be simply explained by the impact of this mutation on the conformation of the B-Raf kinase domain. Indeed, the V600E substitution, but also the insT and G469A mutations, is supposed to imitate the consequences of activation loop phosphorylation (Wan *et al*, 2004; Eisenhardt *et al*, 2011). This event disrupts the aforementioned self-inactivating interaction between P-loop and activation loop and presumably represents the last step to induce full B-Raf activity (Wan *et al*, 2004). As proposed by Rajakulendran *et al* (2009), dimerisation of Raf with itself or with KSR is accompanied by a conformational change of its kinase domain leading to its activation in an allosteric manner. Consequently, one could envisage that this conformational change, which requires an intact DIF, contributes to the disruption of the self-inactivating interaction between P-loop and activation loop and ultimately B-Raf activity. However, as this hydrophobic interaction is already and constitutively disrupted in B-Raf^{V600E} (Wan *et al*, 2004), it can be assumed that the oncoprotein is locked in its active conformation and thus the postulated allosteric activation mechanism, and hence dimerisation, would become superfluous. In contrast, artificial and non-P-loop and non-activation loop B-Raf mutants, for example, CAAX, E586K, S365A or Q257R, would still require this allosteric activation and are therefore strongly impaired by the R509H mutation (Figure 1G; Supplementary Figure S2). We also notice

that the activation loop double-mutant B-Raf^{EVKD} is strongly affected by R509H mutation as well. In strong contrast to the B-Raf^{V600E}, B-Raf^{insT} and B-Raf^{G469A} oncoproteins, however, B-Raf^{EVKD} is an artificial double mutant and not an oncogene product positively selected as a driver of a human tumour. This might explain the observed discrepancy with respect to the DIF.

Although our data suggest that B-Raf^{V600E} can signal as a monomer, we do not posit that oncogenic B-Raf mutants necessarily occur as monomers *in vivo*. In fact, our BN-PAGE and co-IP experiments revealed that B-Raf^{V600E} forms very stable and abundant homodimers that also tend to occur in high molecular mass complexes. Surprisingly, both novel properties were strongly dependent on an intact DIF. But does this increased homodimerisation represent a cause or consequence of the active conformation? We suggest that the latter scenario is more likely since a recent report demonstrated that V600E and other activation and P-loop mutations induce a conformational change of the kinase domain, which in turn prevents its interaction with Sprouty, a negative regulator binding to the CR3 of Raf kinases (Brady *et al*, 2009). This indicates that the kinase domains of B-Raf^{wt} and B-Raf^{V600E} differ in their overall conformation and it is therefore conceivable that the V600E-induced conformational change confers properties that result in an increased dimerisation potential. Indeed, our structural comparison of dimers formed between the kinase domains of either B-Raf^{wt} or B-Raf^{V600E} demonstrated that the latter displays more extended protomer contacts, suggesting that the V600E-induced conformation is better suited for dimerisation. In that regard, it would be interesting to obtain crystal structures of the P-loop mutant B-Raf^{G469A}, which also displays increased homodimerisation (Figure 3C).

Although oncogenic mutants such as B-Raf^{G469A} or B-Raf^{V600E} form strong and abundant homodimers, they are at the same time mildly influenced in their signalling potential by mutations impairing dimerisation. This raises the question whether the observed increase in homodimerisation represents merely a side-effect of the altered kinase domain or leads to additional functional consequences contributing to tumourigenesis and affecting drug efficacy. Based on recent reports, several scenarios could be envisaged: First, the enhanced dimerisation potential of B-Raf^{V600E} and B-Raf^{G469A} (Figure 3) is of particular interest in the light of the recent findings by Poulikakos *et al* (2011). This study showed that a B-Raf^{V600E} splice variant, which lacks large parts of CR1 and CR2, displays a greater dimerisation potential than full-length B-Raf^{V600E} and possesses an intrinsic resistance against PLX4032. It should be noted that this study did not compare the homodimerisation potential between B-Raf^{wt} and B-Raf^{V600E} and therefore the already increased dimerisation potential of these oncoproteins identified in our study was not reported there. Nevertheless, although detailed titration experiments have not been conducted yet, it is tempting to speculate that this enhanced dimerisation potential of full-length B-Raf^{V600E} might influence its affinity towards Raf inhibitors. In any case, although the mechanism by which a B-Raf^{V600E} dimer is protected against vemurafinib remains obscure, it is conceivable that blocking DIF function might sensitise B-Raf^{V600E} towards vemurafinib. Second, the dimer could be more efficient in assembling an entire Raf/MEK/ERK module and would thereby improve the efficiency of signal transduction. Indeed, despite not having an effect on biological end points such as pERK levels or cellular transformation, B-Raf^{V600E} proteins with DIF mutations display a small but discernible reduction in their MEK phosphorylation potential (Figure 1G; Supplementary Table S2). This assumption is further supported by our BN-PAGE analyses showing that introducing DIF mutations is associated with a very strong reduction of larger protein complexes. As we have also shown that B-Raf^{V600E} is more efficiently purified with itself than with B-Raf^{wt}, we propose that dimers consisting of two B-Raf^{V600E} protomers are particularly stable and potentially protected against negative regulators such as Raf-1, which has been shown to inhibit the oncoprotein through the formation of B-Raf^{V600E}/Raf-1 complexes (Karreth *et al*, 2009). Moreover, two independent reports using distinct colon carcinoma lines have reported the amplification and overexpression of the *BRAF*^{V600E} allele in response to pathway inhibition (Corcoran *et al*, 2010; Little *et al*, 2011). Thus, one could envisage that the increased expression and homodimerisation of the oncoprotein alters the equilibrium of Raf homodimers and heterodimers and therefore signalling output and robustness of the ERK pathway. This represents an area for further investigations, which are beyond the scope of this study. Nevertheless, because of the inefficiency of mutations within the DIF and/or the C-terminal 14-3-3 binding motif to abrogate B-Raf^{V600E} signalling, we argue that interfering with the DIF cannot be easily employed on its own to target the cellular activity of this clinically relevant oncoprotein. This assumption is corroborated by our transformation assays in MEFs and Caco-2 cells.

However, targeting the B-Raf DIF might be a successful approach to attenuate MEK/ERK activation in a setting in which tumourigenesis is driven by oncogenic Ras or aberrantly activated or expressed RTKs. This concept is supported by our observation that B-Raf^{R509H} fails to elicit a prominent MEK/ERK phosphorylation in response to EGFR stimulation or oncogenic Ras (Figure 8A and B). These data also suggest that the B-Raf DIF is critical for the function of the Raf-1/B-Raf heterodimers that were described as the most potent form of Raf activity in EGF-treated cells (Rushworth *et al*, 2006). Furthermore, blocking the DIF could be useful to control some disease-associated mutants such as B-Raf^{E586K} or B-Raf^{Q257R}. Most importantly, our finding that a single mutation in the DIF of B-Raf^{D594A} does not prevent its interaction with Raf-1, but blocks paradoxical MEK/ERK activation in cells with excessive Ras signalling, suggests that the DIF could be exploited therapeutically to minimise the risks of side effects and drug resistance (Nazarian *et al*, 2010; Chapman *et al*, 2011; Arnault *et al*, 2012; Su *et al*, 2012). Indeed, using two distinct Raf inhibitors, L779450 and PLX4720 (vemurafinib tool compound), we show that the R509H mutation is sufficient to blunt the Ras-driven paradoxical MEK/ERK phosphorylation evoked by B-Raf^{wt} in this system (Figure 8C).

In addition to the potential therapeutic implications of our findings, our data pinpoint to a dual function of the B-Raf DIF: First, mediating dimerisation itself potentially allows Raf dimers to be more efficient in the formation and fine-tuning of Raf/MEK/ERK signalosomes as it has been discussed above and by others (Wimmer and Baccarini, 2010; Matallanas *et al*, 2011). Second, we show for the first time

that kinase-dead B-Raf$^{D594A/R509H}$ still forms heterodimers with Raf-1 but yet fails to induce paradoxical MEK/ERK phosphorylation. Likewise, although Raf-1^{R401H} is still able to form homodimers, it strictly quenches K-Ras^{G12V}-mediated MEK phosphorylation. Hence, besides mediating dimerisation, the DIF seems to have a second function, such as facilitating the allosteric mechanism proposed for D-Raf homodimers and D-Raf/KSR heterodimers (Rajakulendran *et al*, 2009). Our observation that a single substitution of the DIF residue R509 within the B-Raf protomer is sufficient to prevent B-Raf^{D594A}-dependent paradoxical ERK activation further supports this concept with functional data involving full-length proteins. So far, the concept of allosteric transactivation has been based on structural studies using isolated, dephosphorylated and truncated Raf kinase domains bound to inhibitors, their ligand enforced dimerisation or experimental set-ups in which DIF mutations were introduced into both protomers simultaneously (Rajakulendran *et al*, 2009; Hatzivassiliou *et al*, 2010; Poulikakos *et al*, 2010, 2011). As the latter approach also impairs dimer formation, it is impossible to discern whether the reduction in pMEK levels observed in this setting was caused by the loss of dimers in general or by the lack of allosteric activation. Thus, our observation that kinase-dead B-Raf$^{D594A/R509H}$ cannot induce paradoxical ERK activation, despite being similarly complexed with endogenous Raf-1 as B-Raf^{D594A}, supports the notion from structural studies that R509 plays a crucial role in mediating transactivation.

Lastly, our observations indicate that distinct mechanisms and requirements underlie Raf homodimerisation and heterodimerisation. While the R509H DIF mutation largely reduces B-Raf homodimers, spontaneous heterodimerisation with Raf-1 and KSR proteins was less affected. However, drug-induced heterodimerisation between B-Raf and the Raf-1, A-Raf and KSR1 proteins is differentially affected by the R509H and 3x DIF mutations and also depends on the individual inhibitor. These findings contribute to our understanding of the action of sorafenib and vemurafinib, which are applied and trialled for an increasing number of tumour entities. Similarly, they could represent useful information to develop drugs, which selectively target B-Raf and avoid drug-induced dimerisation and paradoxical ERK activation at the same time.

Materials and methods

Detailed information about expression vectors, cell line generation and cultivation as well as information on kinase assays and BN-PAGE analyses can be found in Supplementary data.

Antibodies and reagents

B-Raf proteins were detected using anti-Raf-B (F-7) antibody (Santa Cruz Biotechnology, CA, USA). Other antibodies used in this study were anti-pan14-3-3 (H-8), anti-β-Actin (C-4), anti-A-Raf (C-20), anti-Tubulin (B-5-1-2) (Santa Cruz Biotechnology), anti-KSR-1 (BD Biosciences, NJ, USA) anti-HA 3F10 (Roche Applied Science, Mannheim, Germany) and anti-phospho-MEK1/2 (pS217/221), anti-MEK1/2, anti-p42/p44 MAPK, phospho-MAPK (pT202/pY204;ERK1/2), anti-Ras (recognising all isoforms), anti-Akt, anti-phospho-Akt (S473) (Cell Signaling Technology, MA, USA). Anti-Myc 9E10 monoclonal antibodies were produced from the 9E10 hybridoma (Evan *et al*, 1985) in-house in protein-free medium (Panserin 4000; Pan Biotech) and purified using protein G Sepharose. Sorafenib and 4-HT were purchased from Santa Cruz Biotechnology and Sigma-Aldrich (MO, USA), respectively.

Cell lysis and western blotting

TCLs were generated and analysed by western blotting as described previously (Brummer *et al*, 2002). As indicated, the cells were lysed in either normal lysis buffers (NLB: 50 mM Tris/HCl, pH 7.5; 1% Triton X-100; 137 mM sodium chloride; 1% glycerine; 1 mM sodium orthovanadate; 0.5 mM EDTA; 0.01 μg/μl leupeptin, 0.1 μg/μl aprotinin, 1 mM AEBSF) or RIPA buffer, which is NLB supplemented with 0.5% sodium deoxycholate and 0.1% sodium dodecylsulphate. Blotted proteins were visualised with horseradish peroxidase-conjugated secondary antibodies (Roche) using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and the LAS-4000 reader (FujiFilm). Densitometry measurements were performed using MultiGauge software (FujiFilm).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We would like to thank Franziska Wöhrle, Kate Jeffrey, Elmar Dolezal and Sebastian Herzog for discussions. MR and GJF are fellows of the German Research Foundation (DFG) funded Spemann Graduate School of Biology and Medicine. TB is supported by the DFG *via* the *Emmy-Noether-Program*, the Collaborative Research Center 850 and the Center for Biological Signalling Studies (*BIOSS*) funded by the Excellence Initiative of the German federal and state governments (EXC294). WWS is supported by the Freiburg Initiative in Systems Biology FRISYS (FKZ0313921) of the BMBF through the FORSYS program. DNS is a fellow of Cancer Institute New South Wales.

Author contributions: MR, RH, GJF, KH, SB, AEE, SB and TB performed all experiments. DNS conducted the structural analyses. AvD and DC provided the B-Raf^{V600E}-specific antibody. All authors contributed to the design, analysis and discussion of experiments. TB and MR directed the study and wrote the manuscript with input from DNS. All authors reviewed the manuscript and provided additional input.

Conflict of interest

The authors declare that they have no conflict of interest.

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