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Autophosphorylation on S614 inhibits the activity and the transforming potential of BRAF

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Abstract

The BRAF Proto-Oncogene Serine/Threonine-Protein Kinase, known as BRAF, belongs to the RAF kinase family. It regulates the MAPK/ERK signaling pathway affecting several cellular processes such as growth, survival, differentiation, and cellular transformation. BRAF is mutated in ~8% of all human cancers with the V600E mutation constituting ~90% of mutations. Here, we have used quantitative mass spectrometry to map and compare phosphorylation site patterns between BRAF and BRAF V600E. We identified sites that are shared as well as several quantitative differences in phosphorylation abundance. The highest difference is phosphorylation of S614 in the activation loop which is ~5fold enhanced in BRAF V600E. Mutation of S614 increases the kinase activity of both BRAF and BRAF V600E and the transforming ability of BRAF V600E. The phosphorylation of S614 is mitogen inducible and the result of autophosphorylation. These data suggest that phosphorylation at this site is inhibitory, and part of the physiological shut-down mechanism of BRAF signaling.

Keywords: BRAF; S614 autophosphorylation; inhibition of kinase activity and transformation

1. Introduction

BRAF, a protein encoded by the *BRAF* gene, belongs to the RAF family of serine-threonine protein kinases. The three RAF proteins, ARAF, BRAF and CRAF, are key components of the ERK pathway, which regulates fundamental cellular processes such as proliferation, survival, and differentiation [1, 2]. RAF kinases activate ERK by phosphorylating and activating MEK, which is the only known upstream activator kinase of ERK. Although the function of RAF family proteins overlaps, they have different capacities to activate the ERK pathway with BRAF being the most potent MEK kinase [3, 4]. BRAF mutations have been described in about 8% of all human cancers [5], particularly in malignant melanoma, thyroid, ovarian and colorectal cancer. Hence, BRAF has emerged as an important drug target, especially in malignant melanoma where BRAF mutations occur with frequencies of 40-60% [6]. Interestingly, several germ line mutations of the *BRAF* gene have also been described in cardio-facio-cutaneous syndrome patients [7] emphasizing the important role that BRAF plays in the pathogenesis of diseases that are hallmarked by the hyper-activation of ERK signaling.

Although the regulation of RAF kinases, and BRAF in particular, has been extensively studied over the last two decades, many important aspects are still incompletely understood [1, 2]. Physiologically, in response to growth factors RAF activation ensues when its regulatory domain binds to activated RAS proteins at the cell membrane. RAS binding requires the de-phosphorylation of S259 in CRAF (S365 in BRAF) [8], which then triggers a series of phosphorylation events in the N-terminal acidic region (SS338/9 in CRAF) and in the activation loop residues (T491 and S494 in CRAF, T598 and S601 in BRAF) [9]. BRAF already features a constitutively phosphorylated N-terminal acidic region (SS446/7) [3, 9], and the negative effect of S365 phosphorylation seems to be much weaker than the strong inhibition exerted by pS259 on CRAF [9]. Hence, BRAF is already primed for activation just requiring activation loop phosphorylation for full activity. In fact, the most common BRAF mutation in cancer is V600E [10], which introduces a negative charge into the activation loop thereby mimicking activation loop phosphorylation and breaking intra-molecular interactions that normally restrain BRAF catalytic activity [11]. In addition to activation loop phosphorylation, hetero-dimerization with CRAF plays an

important role in BRAF signalling. Hetero-dimerization vastly enhances RAF kinase activity towards MEK, and is part of the physiological RAF activation process [12, 13].

Much less is known about mechanisms that inhibit BRAF signalling. Feedback phosphorylation by ERK can inhibit both, CRAF [14, 15] and BRAF [12, 16], albeit by different mechanisms. While ERK feedback phosphorylation can directly suppress CRAF kinase activity [14, 15], phosphorylation of BRAF inhibits dimerization with CRAF rather than catalytic activity [12, 15, 16]. Here, we describe a novel negative regulatory mechanism that involves the phosphorylation of S614 by autophosphorylation, leading to the inhibition of the kinase activity and tranformation potential of oncogenic BRAF.

2. Materials and methods

2.1. Cell culture and transfection: HEK293T (ATCC no. CRL-1573), PC12, and NIH 3T3 cells were grown in a humidified atmosphere at 37®C with 5% CO_2, in Dulbecco's modified minimal Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Sigma) and 0.1% penicillin/streptomycin (Invitrogen). Before use, the cells were tested free of mycoplasma. For transfection, freshly seeded cells were grown overnight, and then transfected with the adequate amount of DNA using polyethylenimine (PEI) at a ratio of 1:6 DNA to PEI. When necessary, cells were starved in DMEM without FBS 12 hours prior to harvesting.

2.2. Generation of BRAF mutants: All BRAF mutants were fused with a double StrepII-FLAG tag [17], in a pcDNA3.0 expression plasmid containing a CMV promoter. The generation of the different mutants was achieved by site directed mutagenesis using the QuickChange II (Agilent). To ensure the success of the mutagenesis, the clones were sequenced by Eurofins genomics. For mCITRINE-tagged versions, the coding sequence of the corresponding mutant was sub-cloned into mCITRINE-N1, a gift from Philippe Bastiaens.

2.3 Lysates, immunoprecipitation, and* in vitro kinase *assay: Cells were transfected and treated as indicated. Stimulation with epidermal growth factor (EGF, Sigma) was carried out at 50nM of EGF for the indicated times. Total lysates for western blotting and co-immunoprecipitation experiments were prepared on ice in TBS (30mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.5% Nonidet-P40 (Sigma-Aldrich). For *in vitro* kinase assay, cells were lysed on ice using RIPA buffer (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 0.5% deoxycholate, 0.1% SDS). All lysis buffers were supplemented with protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Immunoprecipitation was done using anti-Flag (M2) affinity gel (A2220) from Sigma-Aldrich. BRAF kinase activity was measured by *in vitro* kinase assay as previously described [18]. After immunoprecipitation, beads were washed 2x with RIPA, 3x with kinase assay buffer (50mM Tris, pH 7.5, 75 mM NaCl, 5mM EGTA, 5 mM $MgCl_2$) and subsequently incubated in 30ul assay buffer containing 300ng recombinant MEK1 (Millipore #14-420) and 500uM ATP for 20 minutes at 30 degrees (shaking).

2.4. Western blotting: Cleared lysates were adjusted to equal protein concentrations after protein quantification using the Bradford assay (Biorad), separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk in TBS containing 0.1% Tween20 (Sigma-Aldrich) and incubated with the following primary antibodies overnight: The rabbit polyclonal α-phospho-S614 antibody which was produced by Eurogentec against the phosphorylated version of the HQFEQLS(P)GSILW peptide

and affinity purified against this peptide, α-FLAG M2 (Sigma Aldrich), α-phospho-Erk1/2, α-Erk1/2, α-phospho-MEK, α-MEK (Cell Signalling), α-GAPDH (Merck Millipore), α-14-3-3ε and α-CRAF C-12 (Santa Cruz Biotechnology). Membranes were washed and then incubated one hour at ambient temperature with species-macthed secondary antibodies (Jackson Immunoresearch). Protein bands were visualized by ECL plus (GE Healthcare).

2.5. Focus assay: NIH 3T3 cells were transfected as described under 2.1., seeded into a fresh plate, and grown to confluency while feeding them every 3 to 4 days for three weeks. Cells were then fixed with ice-cold methanol for 10 minutes, stained with 0.5% crystal violet prepared in 26% methanol, and foci were counted [19].

2.6. SILAC labelling and affinity purification: SF-TAP-tagged BRAF and BRAF V600E constructs were transiently expressed in HEK293T cells. Stable isotope labeling by amino acids in cell culture (SILAC) was used to quantitify phosphorylation changes between BRAF and BRAF V600E and was carried out as described [20]. Cells were grown for 48 hours after transfection, and were starved 12 hours prior to harvesting. To capture SF-TAP fusion proteins equal amounts of cell lysates were incubated with Strep-Tactin-Superflow beads (IBA) for one hour at 4°C. Beads were washed three times with TBS containing 0.1% Nonidet-P40, phosphatase inhibitor cocktail I and II. Proteins were eluted from the beads by incubation with Strep-elution buffer (IBA) for 10 minutes, and then concentrated using 10 kDa cut-off VivaSpin 500 centrifugal devices (Sartorius Stedim Biotech). The concentrated samples were separated by SDS-PAGE, followed by in-gel tryptic cleavage as described previoulsy [21] for MS analysis

2.7. Mass-spectrometry (MS): LC-MS/MS analysis was performed using a NanoRSLC3000 HPLC system (Dionex), coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) by a nano-spray ion source. Tryptic peptide mixtures were automatically injected at a flow rate of 6 µl/min in 98% buffer C (0.1% trifluoroacetic acid in HPLC-grade water) and 2% buffer B (80% actetonitrile and 0.08% formic acid in HPLC-grade water) onto a nano trap column (75 µm i.d. × 2 cm, packed with Acclaim PepMap100 C18, 3 µm, 100 Å; Thermo Scientific). After 5 minutes, peptides were eluted and separated on the analytical column (75 µm i.d. × 25 cm, Acclaim PepMap RSLC C18, 2µm, 100 Å; Thermo Scientific) by a linear gradient from 2% to 35% of buffer B in buffer A (2% acetonitrile and 0.1% formic acid in HPLC-grade water) followed by a short gradient from 35% to 95% buffer B. The eluted peptides were analyzed by using a LTQ Orbitrap XL mass spectrometer. From the high-resolution MS pre-scan with a mass range of 300–1,500, the 10 most intense peptide ions were selected for fragmentation in the linear ion trap, if they exceeded an intensity of at least 200 counts and if they were at least doubly charged. The normalized collision energy was set to a value of 35, and the resulting fragments were detected with normal resolution in the linear ion trap. The lock mass option was activated and set to a background signal with a mass of 445.1200264. Every ion selected for fragmentation was excluded for 20 seconds by dynamic exclusion. For quantitative analysis, MS raw data were processed using the MaxQuant software (version 1.5.2.8) [22]. Trypsin/P was set as cleaving enzyme. Cysteine carbamidomethylation was selected as fixed modification. Serine, threonine, tyrosine phosphorylation, methionine oxidation and protein acetylation were allowed as variable modifications. Two missed cleavages per peptide were allowed. The peptide and protein false discovery rates were set to 1%. The initial mass tolerance for precursor ions was set to 6 ppm, and the first search option was enabled with 10 ppm precursor mass tolerance. The fragment ion mass tolerance was set to 0.5 Da. The human sub-set of the human proteome reference set provided by SwissProt (selected for Homo sapiens, version 03/2015, 20203 entries) was used for

peptide and protein identification. Contaminants like keratins were automatically detected by enabling the MaxQuant contaminant database search. A minimum number of 2 unique peptides with a minimum length of 7 amino acids needed to be detected to perform protein quantification. Only unique peptides were selected for quantification. For data analysis using Mascot, tandem mass spectra were extracted by extract_msn. Charge state deconvolution and de-isotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science; version 2.4). Mascot was set up to search the SwissProt (selected for Homo sapiens, version 03/2015, 20203 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 1,00 Da and a parent ion tolerance of 10,0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Serine and threonine phosphorylation was specified in Mascot as variable modifications. Scaffold (version Scaffold_3.6.3, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm [23]. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [24]. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3. Results

3.1. Identification of a BRAF phosphorylation site on S614

Phosphorylation plays an important role in the regulation of RAF kinases exerting both activating as well as inhibitory functions [1, 2]. In order to assess whether the V600E mutation changes the phosphorylation pattern of BRAF, we compared the phosphorylation of wildtype (wt) BRAF and mutant BRAF V600E using mass spectrometry (MS). For this purpose, the respective cDNAs were fused to a double StrepII-FLAG tag and transiently expressed in HEK293T cells. To enable quantitative comparisons the cells were SILAC labelled [25]. BRAF proteins were enriched by affinity purification, separated by SDS-PAGE, and eluted from the gel by tryptic digestion.

Analysis of the tryptic peptides by MS identified a number of known and unknown phosphorylation sites (Table 1). Phosphorylation of the following phosphopetides was not changed significantly (<1.5fold) between BRAF and BRAF V600E: (i) pS365, a negative regulatory phosphorylation site in the regulatory domain of BRAF [26]; (ii) pS394/pT401 and pS399/pS409 doubly phosphorylated peptides, which contain sites of unknown regulatory relevance (pS394, pS399, pS409) in the linker region between the regulatory and kinase domains; (iii) pT401 (single phosphorylated peptide), a negative ERK feedback phosphorylation site [27, 28]; (iv) pS446 and p447 in the N-terminal acidic region (NtA), which are required for BRAF activity [3]. The lack of change in pS446/7 is not surprising as phosphorylation of these residues seems to be less critical for mutant BRAF as their mutation does not impair the catalytic activity of BRAF V600E nor its transformation potential [29-31]; (v) pS605, which corresponds to a putative activating site (pS497) in CRAF [32], and which is also found phosphorylated in rapamycin treated cells [33].

Phosphorylation of BRAF V600E was upregulated on the following sites: (i) pS614 (4.7-fold), which is characterised further in this paper; (ii) pT373 (2.7fold), which is a major BRAF in vitro autophosphorylation site of unknown function significance [34]; (iii) pS405 in combination with T401 (2.4 fold) which are involved in regulation of BRAF V600E protein stability [35], (iv) pS409 in combination with T401 (2.5 fold), and (v) pS750 (1.5 fold), which occupy similar positions as

inhibitory ERK feedback phosphorylation sites in CRAF [14]. S750 was previously identified as an ERK phosphorylation site mediating negative feedback [16, 28]. This phosphorylation is consistent with observations that V600E constitutively activates the ERK pathway and consequently ERK dependent feedback phosphorylations [16, 28]. However, the BRAF residues S405 and S409 lack the proline at position +1 that is typical for ERK phosphorylation sites [36], and therefore are unlikely to be ERK feedback sites. S409 corresponds to S262 in ARAF, a site which participates in ARAF activation in response to EGF stimulation [37]. This pattern of phosphorylation sites selectively increased in BRAF V600E is not revealing any obvious functions based on previous knowledge.

Thus, we further investigated the role of pS614, which is located within the activation segment of the BRAF kinase domain and showed the highest change, i.e. a 4.7fold up-regulation in BRAF V600E versus BRAF (Table 1). The localization of phosphorylation at S614 was substantiated by Mascot, MaxQuant as well as by manual inspection of the fragment spectrum (Fig.1A). The identification of this site has been reported before in two phosphoproteomics studies [38, 39] but without investigating its function. To study this phosphorylation further, we generated a phospho-specific pS614 antibody. The specificity of the antibody was confirmed by expression of BRAF, BRAF V600E and corresponding variants, carrying neutral alanine or charged aspartic acid substitutions at position 614. While phosphorylation of S614 was easily detected in wildtype BRAF and, confirming the MS-based quantification, to a higher extent in BRAF V600E, no signal could be detected for both the S614A and S614D mutants (Fig. 1B). Thus, these data confirmed the specificity of the phospho-specific S614 antibody, and corroborated the MS data that pS614 is a genuine BRAF phosphorylation site that is highly increased in BRAF V600E.

3.2. S614 phosphorylation inhibits BRAF activity and BRAF V600E transforming activity

Because of the prominent change of pS614 in BRAF V600E, we investigated the impact of this phosphorylation on BRAF activity by substituting serine 614 to alanine or the phospho-mimetic aspartic acid in both, BRAF and BRAF V600E. These mutants and the corresponding parental proteins were expressed in HEK293T and PC12 cells in order to account for possible cell specific differences (Fig. 2A). The S614A mutation enhanced the ability of BRAF to activate ERK in both cell lines. The S614D mutation had no effect in PC12 cells, but increased ERK activation in HEK293T cells albeit to a slightly lesser extent than the S614A mutation. BRAF V600E caused a higher ERK activation, which was strongly enhanced by the S614A mutation in both cell lines, while the S614D mutation yielded a clear, but lower activation than S614A in HEK293T cells. These results suggest that S614 phosphorylation suppresses the activity of BRAF and BRAF V600E in particular in the cellular context. In order to confirm a direct effect of the phosphorylation on BRAF kinase activity, we performed *In vitro* kinase assay on *wildtype* BRAF and the S614 mutants, isolated from HEK293T cells. Both S614D and S614A mutations increased the activity over the *wildtype* protein (Figure 2B). We furthermore investigated whether pS614 phosphorylation affects the ability of BRAF to transform cells using the classic NIH 3T3 focus forming assay, which has been widely used to identify oncogenes and quantify their oncogenic potency [19]. BRAF was inactive in this assay, even when S614 was mutated (Fig. 2C) However, the S614A mutation significantly increased the transforming potential of BRAF V600E, while the S614D mutation had a less prominent and statistically non-significant effect. These data indicate that phosphorylation of this site not only impairs kinase activity but also biological activity. While the S>D mutation provides a negative charge at position 614, it does not fully compensate for other effects caused by S614 phosphorylation.

3.3. S614 phosphorylation results from autophosphorylation

Given the inhibitory effect of pS614, we investigated the regulation of this phosphorylation and tried to identify the kinase responsible. As RAF kinases are regulated by feedback phosphorylation through ERK [12, 14-16], we tested the effects of RAF and MEK inhibitors on S614 phosphorylation (Fig. 3A). All inhibitors reduced ERK1/2 activation, showing that they all were used at efficacious concentrations. Interestingly, all RAF inhibitors tested (Sorafenib, Vemurafenib, PLX-4720, SB590885) reduced S614 phosphorylation, whereas MEK inhibitors (RO5126766, PD184352, UO0126) were ineffective. MEK inhibition even enhanced S614 phosphorylation in WT BRAF (Fig. 3B). These results suggest that S614 phosphorylation is due to autophosphorylation. To corroborate this hypothesis we tested whether S614 phosphorylation correlates with BRAF kinase activity. For this purpose, we used BRAF mutants found in cancer and RASopathies that have a range of catalytic activities (Fig. 3C). High activity BRAF mutants featured high levels of S614 phosphorylation while medium and low activity BRAF mutants exhibited a corresponding decline in S614 phosphorylation. In addition, we were able to confirm that activation loop mutations like V600E by themselves do not increase S614 phosphorylation, but they need to be associated with an increase in BRAF kinase activity, as the kinase-inactive V600E/K483M variant displays no S614 phosphorylation (Figure 3D). Thus, S614 phosphorylation is highly correlated with BRAF activity, sensitive to RAF inhibitors but insensitive to MEK inhibitors, suggesting that pS614 is an inhibitory autophosphorylation site. This conclusion was supported by assessing the time-course of S614 phosphorylation (Fig. 3E). Phosphorylation of S614 was mitogen-inducible peaking after ERK activation reached its maximum. This behaviour is consistent with the hypothesis that S614 is a BRAF autophosphorylation site that is induced after BRAF has stimulated the ERK pathway and that physiologically limits BRAF signalling. While the former results strongly suggest that the presence of active BRAF is necessary for S614 phosphorylation, we also wanted to investigate whether it is sufficient. We therefore co-transfected hyperactive (V600E) and kinase-dead (K483M) BRAF mutants that were tagged with either SF-TAP or mCITRINE, resulting in different molecular weights (Fig. 3F). While BRAF V600E was highly phosphorylated at S614, no phosphorylation was detectable for the K483M mutant, thus indicating an intramolecular phosphorylation mechanism.

3.4. Inhibitory mechanism of S614 phosphorylation

To investigate the potential inhibitory mechanism of S614 phosphorylation, we analysed the BRAF-CRAF heterodimer formation as well as binding of MEK, the main downstream effector of BRAF. To this end, we expressed all SF-TAP tagged variants in HEK293T cells, purified the complexes by FLAG purification and analysed the binding pattern of CRAF and MEK by western blotting (Fig. 4). CRAF binding was not affected by S614 modifications. The same was true for 14-3-3, which did not show any alteration in binding due to S614 modification. By contrast, we observed reduced MEK binding to V600E compared to WT BRAF, consistent with an acceleration of the MEK binding, phosphorylation and dissociation cycle in the more active BRAF V600E mutant. However, the phosphomimetic S614E mutant showed a strong reduction of MEK binding in both WT and V600E compared to the non-modified residue. In addition, the S614D mutation reduced MEK phosphorylation especially by BRAF V600E (Fig. 4A) suggesting that in this case the reduction in MEK binding does not reflect enhanced substrate turnover but interference with substrate binding. The localisation of an inhibitory phosphorylation site in the activation loop is unusual, as activation loop phosphorylation is normally linked to the activation of kinase activity. Interestingly, S614 forms part of the interaction interface of BRAF with MEK [40] (Fig. 4B) suggesting that phosphorylation of this residue could impair MEK

binding and subsequent MEK activation. This mechanism of inhibition for pS614 is consistent with our observation that replacing S614 by a phosphomimetic aspartic acid reduces MEK binding, whereas replacement by alanine slightly enhances MEK binding (Fig. 4A). However, both types of S614 mutations increase ERK activation (Fig. 2A) indicating that S614 phosphorylation inhibits BRAF by a complex mechanism where the regulation of MEK binding may be part of. Nevertheless, the physiological relevance and inhibitory nature of S614 phosphorylation is also supported by the fact that BRAF S614 mutations are observed in cancer [41].

4. Discussion

The activity of RAF kinases is finely regulated in order to specify biological effects [1, 2]. This requirement likely is responsible for the complexity of RAF kinase regulation and the multiple layers of regulation. Evidence suggests that negative regulation is as important as positive regulation. Negative regulation includes both feedback from ERK [14-16, 28, 42] but also autophosphorylation [39, 43, 44]. Our results suggest that S614 phosphorylation is an auto-regulatory mechanism that adjusts the activity of WT and mutated BRAF proteins. The localisation of an inhibitory phosphorylation site in the activation loop is unusual, as activation loop phosphorylation is normally linked to the activation of kinase activity. However, the physiological relevance and inhibitory nature of S614 phosphorylation is also supported by the fact that BRAF S614 mutations are observed in cancer [41]. Interestingly, BRAF with V600E and S614P mutations was observed in a lymph node metastasis of a malignant melanoma patient [45].

We have previously shown that CRAF can inhibit its own activity by autophosphorylation of S621, which initiates when CRAF runs out of its substrate MEK [43]. This phosphorylation regulates ATP binding and its inhibitory effect can be reversed by 14-3-3 binding to pS621 [46]. Thus, S621 phosphorylation acts as a 14-3-3 dependent toggle switch for CRAF kinase activity. Both CRAF and BRAF kinases have recently been shown to inhibit their own activity by autophosphorylation in the ATP binding P-loop [44]. This mechanism can contribute to the paradoxical activation of the ERK pathway caused by most RAF inhibitors as they interfere with the inhibitory autophosphorylation of the P-loop [44]. The authors further suggest that P-loop autophosphorylation keeps RAF kinases in an inactive state in unstimulated cells. Importantly, the regulation and role of S614 phosphorylation seems to be quite different. Whereas P-loop autophosphorylation is ATP-dependent and only inhibits wildtype but not mutant BRAF [44], S614 phosphorylation is mitogen induced and can downregulate both wildtype and mutant BRAF activity. Interestingly, autophosphorylation of a patch of amino acids surrounding T401 was recently reported to inhibit the transforming potential of BRAF without having a strong effect on the in vitro kinase activity [39]. However, the phosphorylation of T401 and S405 shorten the half-life of both wildtype and mutant BRAF proteins. These results suggest that different forms of autophosphorylation may regulate different aspects of BRAF signalling.

An intriguing possibility is that S614 phosphorylation affects dynamic properties of BRAF signalling. For instance, the inhibition of CRAF by direct feedback phosphorylation by ERK [14] not only limits its kinase activity, but also conveys robustness to the overall output of the ERK pathway [15, 42]. The amplification of input signals through the three-tiered RAF-MEK-ERK kinase cascade combined with the negative feedback from ERK to CRAF forms a negative feedback amplifier motif. This motif reduces the overall gain of the amplifier but renders the output resistant to interference, as any up or down changes in the amplifier will change the negative feedback in the opposite direction, thereby stabilising the output and allowing smooth linear response curves to input signals. Breaking

the feedback leads to switch-like, bi-stable response patterns, which cause cellular heterogeneity, where some cells respond while other do not. Importantly, the negative feedback amplifier also conveys robustness against MEK inhibitors, which as a consequence need to inhibit MEK almost completely to have an effect on ERK activity. Weakening the negative feedback by RAF inhibition sensitises the amplifier to MEK inhibition explaining the counterintuitive synergism between RAF and MEK inhibitors [15]. Thus, the auto-inhibition exerted by S614 phosphorylation may contribute to the enhanced sensitivity of BRAF V600E mutant cells to RAF and MEK inhibitors, and especially to the combination of these inhibitors, which is now used for the treatment of melanoma [47]. This hypothesis remains to be tested, but the co-occurrence of BRAF V600E and S614P mutations in RAF inhibitor resistant melanoma lends initial support to it [45].

5. Conclusions

We here identify autophosphorylation of S614 in BRAF as a mechanism that, in contrast to previously reported autophosphorylation events, constrains the activity of both wildtype and mutant BRAF. This phosphorylation is mitogen-inducible suggesting that it is part of the physiological regulation cycle of BRAF. The occurrence of S614 mutations in cancer, and especially preliminary links to drug resistance, support the notion that S614 phosphorylation also constitutes a process of pathophysiological significance.

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Table and figure legends

Table 1. Phosphorylation sites identified and quantified in BRAF and BRAF V600E. WT and V600E BRAF proteins were affinity purified from SILAC labelled HEK293 cells and analysed by mass spectrometry. Identification, quantification and determination of the localization probability for all potential phosphorylation sites were achieved by MaxQuant. For each peptide, the sequence for the identified tryptic peptide, including the site localization probability, the sites with best localization probabilities and the V600E to WT ratio, as well as the standard deviation for the ratio are shown.

Figure 1. S614 is a genuine BRAF phosphorylation site. (A) Mass spectrum, identified by Mascot and annotated using Scaffold, showing allocation of the S614 phosphorylation. **(B)** HEK293T cells were transfected with BRAF and the indicated mutants as well as the SF-TAP tag as negative control. BRAF was enriched from lysates by FLAG purification and immunoblotted with an antibody against pS614 or the FLAG tag. For pS614, two exposure times are shown (upper panel: 2 minutes, lower panel: 15 minutes).

Figure 2. S614 phosphorylation inhibits BRAF kinase and transforming activity. (A) ERK phosphorylation in PC12 and HEK293T cells transfected with BRAF WT, BRAF V600E or the corresponding S614A/D mutants. After lysis, samples from each cell line were separated on the same gel and Western blotted. The blots are shown in separate panels as irrelevant lanes were removed. **(B)** Kinase activity of *wildtype* BRAF and S614D/A mutants was measured by *in vitro* kinase assay. Transfected proteins from HEK293T cells were isolated by FLAG-IP and analysed for their ability to phosphorylate recombinant MEK1 *in vitro*. Shown is one representative experiment from three replicates. **(C)** Focus forming transformation assay in NIH3T3 cells expressing BRAF, BRAF V600E or the corresponding S614A/D mutants. Non-transfected cells were used as control. Shown is the ratio of foci in the sample versus the number of foci in the control. The graph summarises the results of three independent experiments. The error bars represent SD, and p values were calculated by Graphs denote the mean ± standard deviation (SD) from three independent experiments. P-values were calculated using paired Student's t-test.

Figure 3. S614 is an autophosphorylation site. HEK293T cells were transfected with a variety of different BRAF mutants. Following serum starvation overnight, cells were treated as indicated (if applicable) and total lysates were collected and analysed by western blotting. **(A)** HEK293T cells were transfected with BRAF V600E, serum starved and treated with different RAF inhibitors (S, Sorafenib; V, Vemurafenib; X, PLX4020; A, 885-A) or MEK inhibitors (R, RO5126766; PD184352; U, U0126) for 1 hour. **(B)** Cells transfected with FLAG-tagged BRAF or BRAF V600E were treated with the MEK inhibitor U0126 for 1 hour and were assessed for pS614 phosphorylation and ERK1/2 activation. **(C)** HEK293T cells were transfected with BRAF mutants found in cancer and RASopathies featuring different kinase activities. Total lysates from serum starved cells were analysed for transfected BRAF (FLAG), pS614, ppERK (kinase activity) and ERK 1/2. **(D)** Total lysates from serum-starved HEK293T cells expressing FLAG-tagged BRAF V600E as well as its kinase-dead variant V600E/K483M were collected and analysed by western blotting, (*) represents non-specific bands. **(E)** HEK293T cells were transfected with FLAG-tagged BRAF and, following serum-starvation, were stimulated for the indicated time with EGF. BRAF S614 and ERK1/2 phosphorylation were analysed. **(F)** Cells were transfected with hyperactive (V600E) or kinase-dead (K483M) BRAF, either tagged with SF-TAP (resulting in lower MW band) or mCITRINE (higher MW band). Total lysates from serum starved cells were analysed for transfected BRAF(F-7), pS614, ppERK, and ERK1/2, (*) represents non-specific bands.

Figure 4. S614 affects MEK binding. (A) HEK293T cells were transfected with WT and V600E BRAF or with the corresponding S614A and S614D variants. Following serum starvation, transfected BRAF proteins were immunoprecipitated by FLAG affinity purification and analysed by Western blotting. **(B)** S614 is located close to the interface between BRAF and MEK. The location of S614 (shown as cylinders) was mapped onto the crystal structure of BRAF (light blue ribbon) and MEK1 (grey surface). The figure was prepared from PDB-ID 4MNE [40].

References

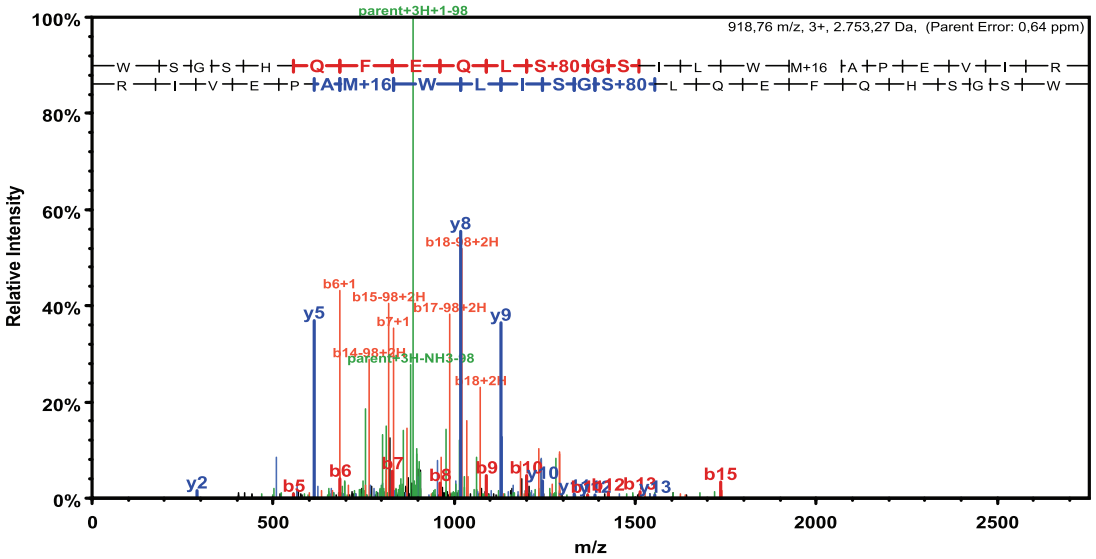
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Figure 1

A


parent+3H+1-98
918,76 m/z, 3+, 2.753,27 Da, (Parent Error: 0,64 ppm)
W S G S H Q F E Q L S+80 G S I L W M+16 A P E V I R
R I V E P A M+16 W L I S G S+80 L Q E F Q H S G S W
Relative Intensity
100%
80%
60%
40%
20%
0%
0
500
1000
1500
2000
2500
m/z
y8
b18-98+2H
b6+1
b15-98+2H
b17-98+2H
y5
y9
b7+1
b14-98+2H
parent+3H-NH3-98
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b12
b13
y13
b15

B

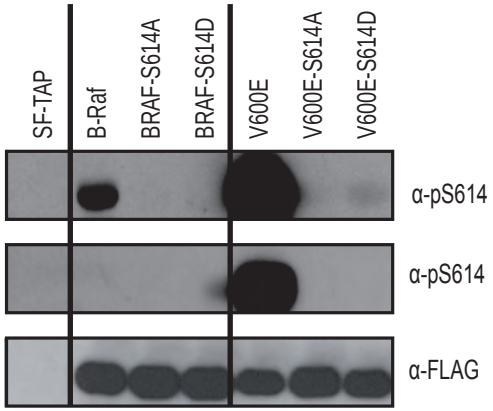
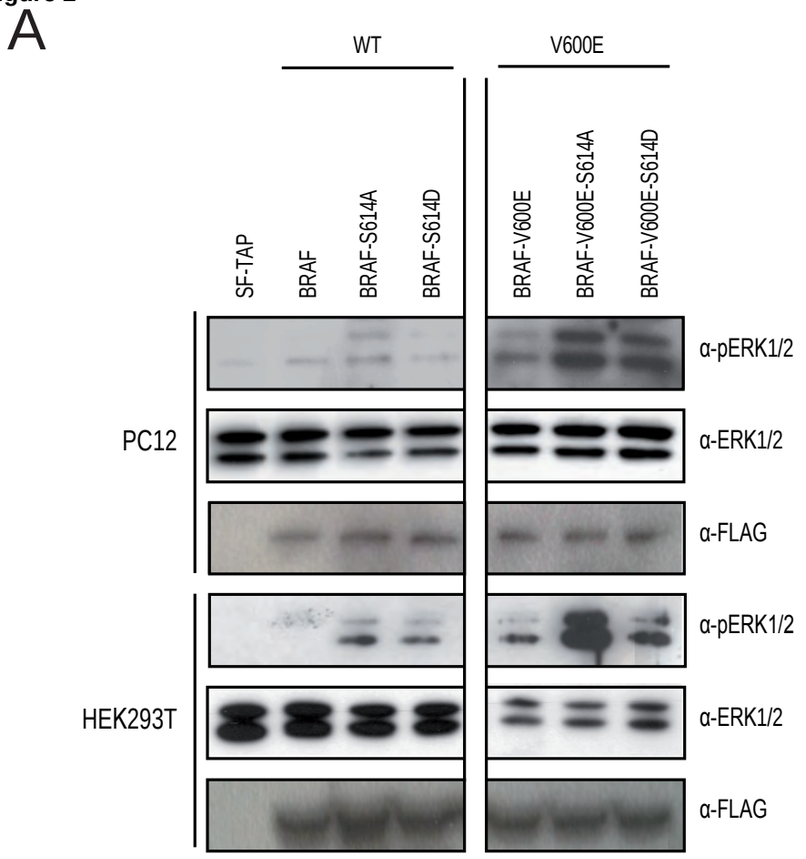
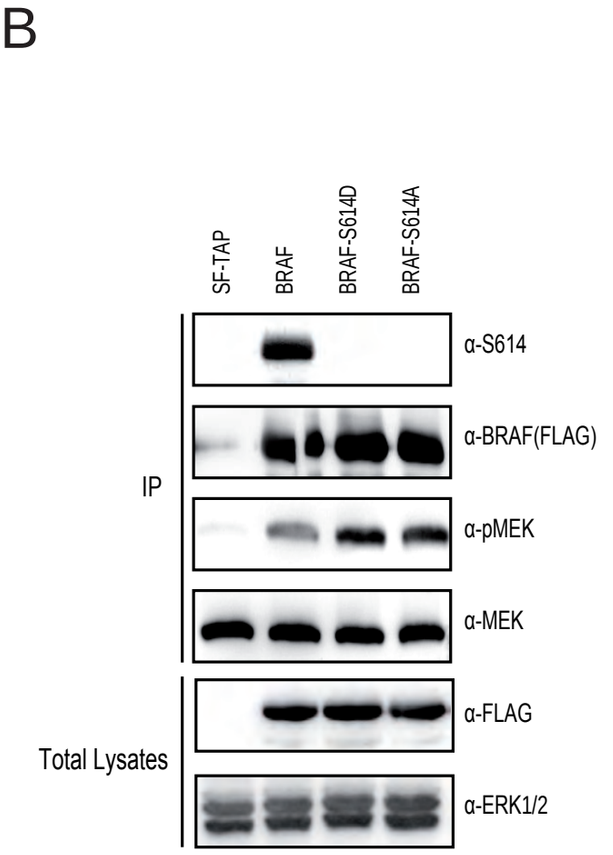

SF-TAP
B-Raf
BRAF-S614A
BRAF-S614D
V600E
V600E-S614A
V600E-S614D
α-pS614
α-pS614
α-FLAG

Figure 2


A
WT
V600E
SF-TAP
BRAF
BRAF-S614A
BRAF-S614D
BRAF-V600E
BRAF-V600E-S614A
BRAF-V600E-S614D
PC12
HEK293T
α-pERK1/2
α-ERK1/2
α-FLAG
α-pERK1/2
α-ERK1/2
α-FLAG


B
SF-TAP
BRAF
BRAF-S614D
BRAF-S614A
IP
Total Lysates
α-S614
α-BRAF(FLAG)
α-pMEK
α-MEK
α-FLAG
α-ERK1/2

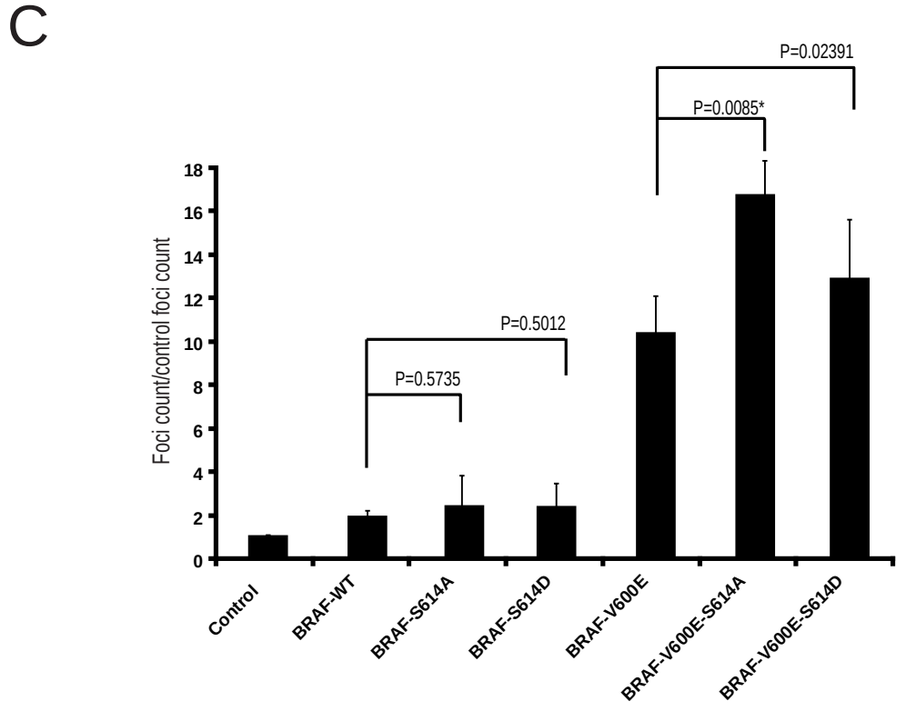

C
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P=0.0085*
P=0.5012
P=0.5735
Foci count/control foci count
0
2
4
6
8
10
12
14
16
18
Control
BRAF-WT
BRAF-S614A
BRAF-S614D
BRAF-V600E
BRAF-V600E-S614A
BRAF-V600E-S614D

Figure 3

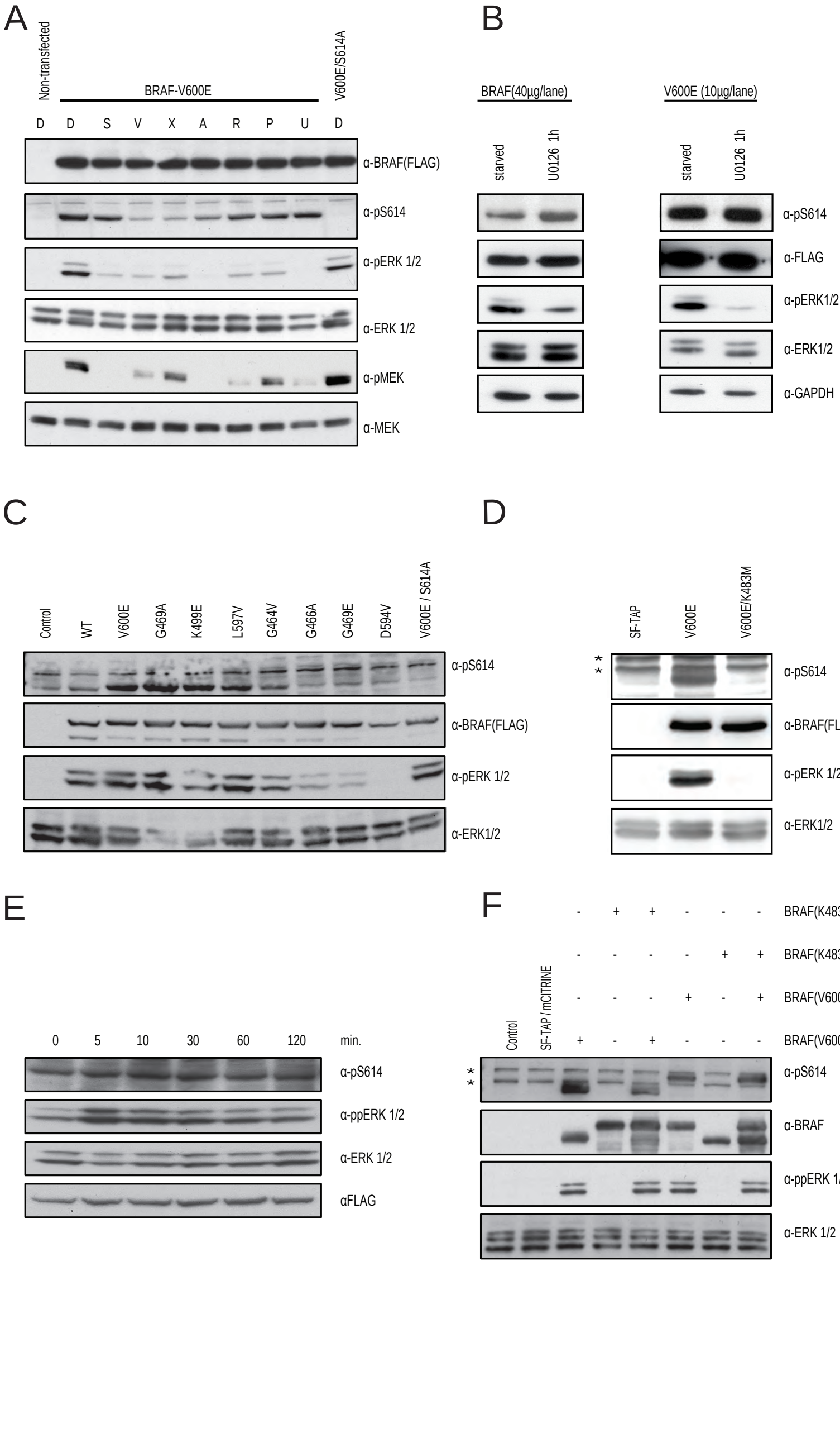
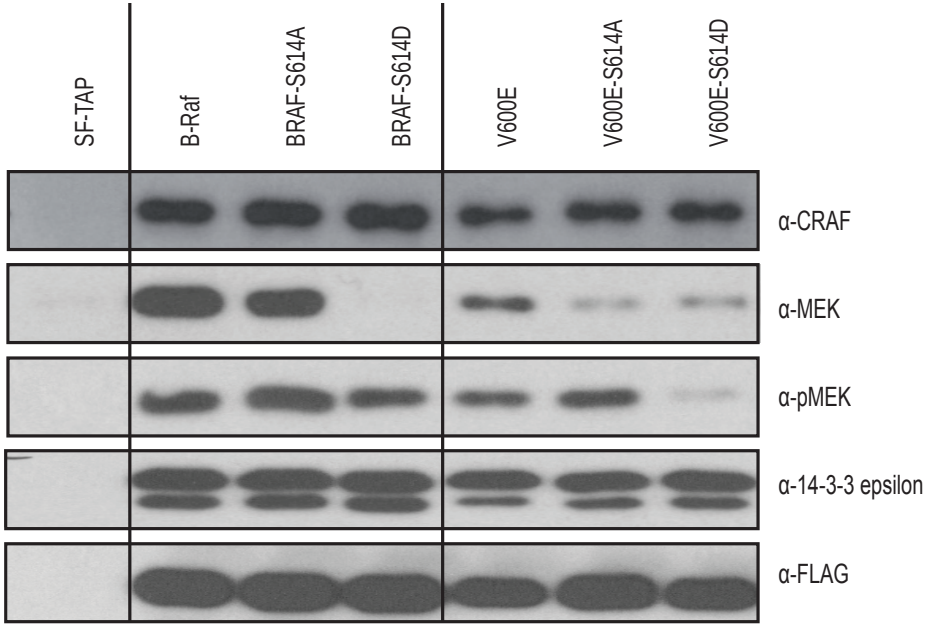

A
Non-transfected
BRAF-V600E
V600E/S614A
D D S V X A R P U D
α-BRAF(FLAG)
α-pS614
α-pERK 1/2
α-ERK 1/2
α-pMEK
α-MEK
B
BRAF(40µg/lane)
V600E (10µg/lane)
starved
U0126 1h
α-pS614
α-FLAG
α-pERK1/2
α-ERK1/2
α-GAPDH
C
Control
WT
V600E
G469A
K499E
L597V
G464V
G466A
G469E
D594V
V600E / S614A
α-pS614
α-BRAF(FLAG)
α-pERK 1/2
α-ERK1/2
D
SF-TAP
V600E
V600E/K483M
α-pS614
α-BRAF(FLAG)
α-pERK 1/2
α-ERK1/2
E
0 5 10 30 60 120 min.
α-pS614
α-ppERK 1/2
α-ERK 1/2
αFLAG
F
BRAF(K483M)-mCITRINE
BRAF(K483M)-FLAG
BRAF(V600E)-mCITRINE
BRAF(V600E)-FLAG
Control
SF-TAP / mCITRINE
α-pS614
α-BRAF
α-ppERK 1/2
α-ERK 1/2

Figure 4

A


SF-TAP
B-Raf
BRAF-S614A
BRAF-S614D
V600E
V600E-S614A
V600E-S614D
α-CRAF
α-MEK
α-pMEK
α-14-3-3 epsilon
α-FLAG

B

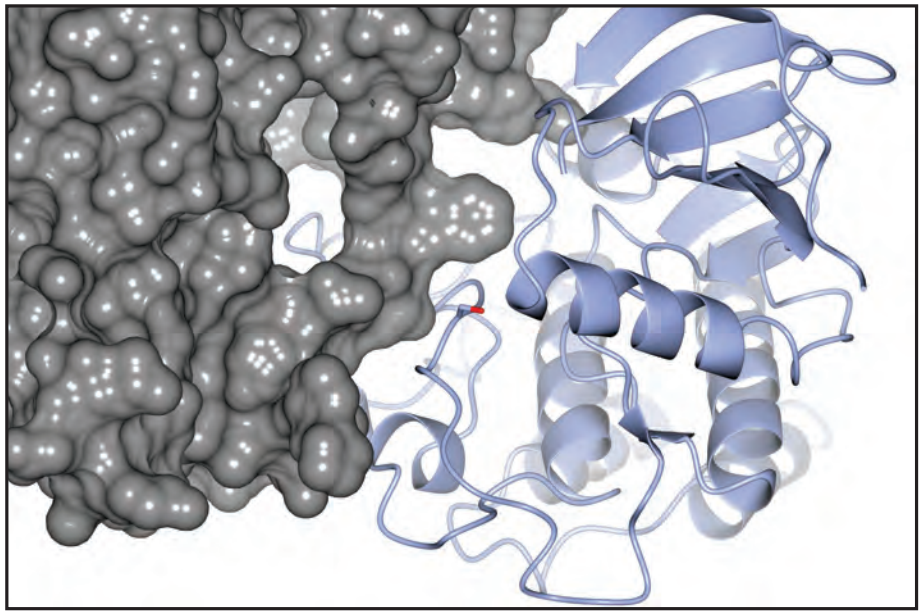


Table 1

Protein name	Gene name	Sequence	Best localization probability	Ratio V600E/WT	% standard deviation
Serine/threonine-protein kinase B-raf	BRAF	WSGSHQFEQLS(99.6%)GS(0.4%)ILWMAPEVIR	S614	4.67	0.18
Serine/threonine-protein kinase B-raf	BRAF	SSSAPNVHINT(100%)IEPVNIDDLIR	T373	2.70	0.56
Serine/threonine-protein kinase B-raf	BRAF	GDGGSTTGLS(0.3%)AT(99.7%)PPASLPGS(99.9%)LT(0.1%)NVK	T401, S409	2.54	0.17
Serine/threonine-protein kinase B-raf	BRAF	GDGGSTTGLS(0.7%)AT(99.3%)PPAS(99.4%)LPGS(0.6%)LTNVK	T401, S405	2.43	0.51
Serine/threonine-protein kinase B-raf	BRAF	AGFQTEDFSLYACAS(100%)PK	S750	1.52	0.18
Serine/threonine-protein kinase B-raf	BRAF	GDGGS(75.5%)T(23.6%)T(0.9%)GLS(0.1%)AT(98.7%)PPAS(1.2%)LPGS(0.1%)LTNVK	S394, T401	1.33	0.29
Serine/threonine-protein kinase B-raf	BRAF	GDGGSTT(0.1%)GLS(97.3%)AT(2.5%)PPAS(0.1%)LPGS(92.5%)LT(7.5%)NVK	S399, S409	1.22	0.48
Serine/threonine-protein kinase B-raf	BRAF	S(11.4%)S(11.4%)S(77.2%)APNVHINTIEPVNIDDLIR	S365	1.00	0.16
Serine/threonine-protein kinase B-raf	BRAF	RDS(3%)S(97%)DDWEIPDGQITVGQR	S447	1.00	0.22
Serine/threonine-protein kinase B-raf	BRAF	GDGGSTTGLSAT(100%)PPASLPGSLTNVK	T401	0.93	0.23
Serine/threonine-protein kinase B-raf	BRAF	RDS(100%)SDDWEIPDGQITVGQR	S446	0.92	0.19
Serine/threonine-protein kinase B-raf	BRAF	S(5.5%)RWS(91.5%)GS(3%)HQFEQLSGSILWMAPEVIR	S605	0.83	0.52