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Supplementary discussion part 1

Our model suggests that, in tumours that express Class 3 BRAF mutants and activated RAS, both $BRAF^{MUT}/RAF^{WT}$ heterodimers and WT RAF dimers are expressed (Extended Data Figure 2b). RAF inhibitors will paradoxically activate the WT RAF dimers in these tumours. We found that RAF inhibitors can also inhibit the activity of class 3 $BRAF^{MUT}/CRAF^{WT}$ heterodimers when they bind to CRAF, but have no effect when bound to the mutant BRAF protomer (Figure S1). This model is supported by Figure S2. When high levels of class 3 BRAF mutants were co-expressed in the CRAF knockout MEFs with low levels of $CRAF^{WT}$ or of the $CRAF^{T421N}$ gatekeeper mutant, vemurafenib only inhibited p-MEK in the cells expressing $CRAF^{WT}$ without the gatekeeper mutation. It had no effect on ERK signaling driven by the Class 3 $BRAF/CRAF^{T421N}$ heterodimer. Thus binding of drug to the Class 3 mutant will not affect kinase activity of the heterodimer and binding of drug to wild type CRAF in the dimer will inhibit activity. In sum, the drugs cause 50% inhibition of class 3 $BRAF^{MUT}$/CRAF heterodimers and paradoxically activate WT dimers, resulting in little effect on total ERK signaling.

This model suggests that the degree of inhibition of ERK driven by Class 3 BRAF mutants will be a function of the ratio of expression of mutant BRAF to wild type RAF in the cell. At low levels of mutant expression, effect of RAF inhibitor on WT RAF heterodimers will predominate and ERK will be paradoxically activated by drug. As expression of the mutant increases, the proportion of mutant BRAF/WT RAF dimers increases. The activity of the heterodimers can be inhibited 50% by the drug, so at high levels of mutant expression, the drug can be moderately effective. In support of this idea, when we expressed low levels of class 3 BRAF mutants in NIH-3T3 cells, vemurafenib had almost no effect on pMEK and pERK (Figure 3b). This is also the case in the class 3 BRAF mutant tumour cell lines we tested (Figure 3c). However, when these mutants are expressed at higher levels, partial inhibition of pMEK/pERK by vemurafenib was observed (Figure S3).

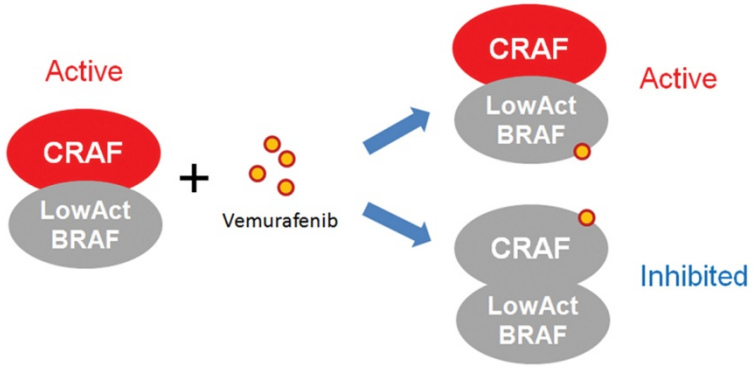

Figure S1. Model shows that the binding of vemurafenib to different partners of the mutant BRAF dimer determines the biochemical consequences of drug treatment.

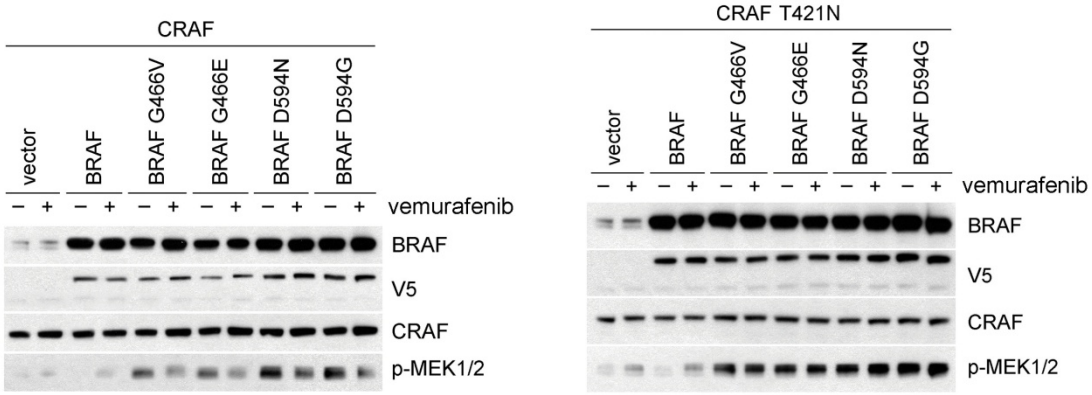

Figure S2. V5-tagged WT or mutant BRAF were co-expressed with lower level V5-tagged WT (left panel) or T421N (right panel) CRAF in CRAF KO MEF cells, followed by treatment with or without vemurafenib (1 uM, 1 hr). The ERK signaling of treated cells was assayed by Western blot.

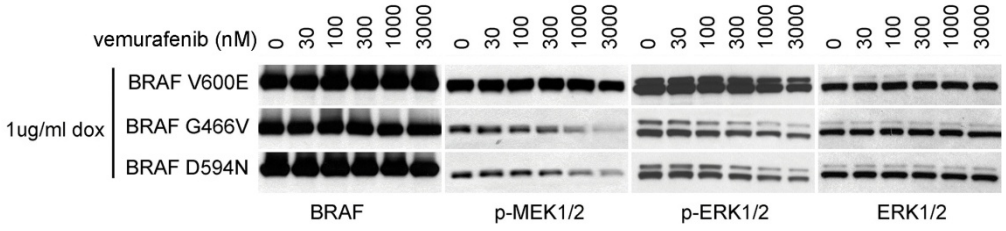

Figure S3. NIH-3T3 cells inducibly expressing the indicated BRAF proteins were exposed to 1 ug/ml (high dose) doxycycline for 24 hrs, followed by vemurafenib treatment at the indicated concentrations for 1 hr. Cells were collected and cell lysates were examined by Western blot.

Supplementary discussion part 2

A functional analysis of tumour-associated BRAF mutants was first reported in 2004 by Wan, Marais and colleagues[1], who made the seminal observation that whereas the catalytic activity of many tumour associated BRAF mutants is hyperactive, a subset are hypoactive or kinase dead.

In Garnet et al. (2005)[2], Marais and colleagues examined the mechanism of activation of ERK by hypoactive RAF mutants. In that work, they showed that these hypoactive mutants bind to and directly activate CRAF in a RAS-independent manner in the cytosol, as opposed to WT BRAF which activates CRAF after binding to active RAS at the membrane.

This view of activation of signaling by hypoactive mutants is not supported by our work. As shown in this paper, these mutants are RAS-dependent. They failed to activate CRAF in a RAS-less cells when they were expressed at a physiological level. This difference has extremely important mechanistic and translational consequences.

In Heidorn et al. (2010)[3], the mechanism of activation of ERK signaling by the kinase dead mutant BRAF D594A mutant was examined. First, they studied the mechanism whereby BRAF inhibitors paradoxically activate BRAF/CRAF dimers in tumours with NRAS mutation. They relied on their previous work showing that BRAF is not active in these tumours and on a presumption that the RAF inhibitors they used were BRAF selective, They posited that in these cells, WT BRAF is active enough to inhibit CRAF. The selective pharmacologic inhibition of BRAF therefore activates CRAF and stimulates signaling. To test this model they introduced the D594A kinase dead BRAF mutant into RAS mutant cells and found that it also induces ERK activation. Moreover, the kinase dead mutant synergized with mutant KRAS G12D to cause melanomas in GEMM models, whereas neither did by itself. The potential clinical importance of this finding was supported by their observation that D594 mutations in cancer coexisted with RAS mutations in 11.8% of the cases, suggesting a functional interaction.

This paper reports several important observations, including the RAS dependence of activation of signaling by the kinase dead mutant and that kinase dead BRAF drives signaling by activating WT CRAF in mutant/WT CRAF heterodimers. However, the purported mechanism, inhibition of WT CRAF kinase by BRAF kinase and relief of this inhibition by selective BRAF inhibitors or by kinase dead BRAF, has been refuted by a variety of findings. First, the RAF inhibitors used in the paper are not selective BRAF inhibitors, they bind to and inhibit all 3 RAF family members in the proper context and they induce paradoxical activation of ERK in cell lacking BRAF[4] (in BRAF knockout cells). Second, RAF inhibitors paradoxically activate CRAF homodimers which are RAS-independent[4]. Third, selective inhibition of BRAF by most RAF inhibitors is not the proximal mechanism for paradoxical activation by these drugs, which is, instead, a result of three effects of binding of these cells to RAF: induction of dimerization of RAF monomers in a RAS-dependent fashion[5], allosteric activation of the second protomer in the RAF dimer after drug binds to the first[4] and, induction of negative cooperativity of binding to the second RAF protomer in the dimer by of drug to the first protomer.[6] Lastly, CRAF kinase dead mutants also activate signaling[7] and cause tumours (our unpublished data).

This summarizes the state of our understanding of the mechanism of activation of signaling by these mutants prior to our current work. The previous work[2,3] states that hypoactive mutants and kinase dead RAS mutants activate signaling by different mechanisms, the former RAS independent, the latter RAS dependent, it does not adequately explain the mechanism of activation of either class of mutant, nor the reason that hypoactive mutants are found to coexist with RAS or NF1 mutations

The previous work[1, 2, 3] was extremely important, but, unsurprisingly, did not answer all questions and came to some erroneous conclusions. The outlined in this paper reports the following advances in understanding these mutants and oncogenic RAF mutants in general.

1) It shows that both hypoactive and kinase dead RAF mutants signaled in a RAS-dependent manner.

2) It shows that ERK-dependent feedback is a powerful determinant of the types of RAF mutants found in cancer and is key for understanding how these mutants activate output. Activation of ERK potently feedback inhibits activation of WT RAS, therefore, in order to hyperactivate ERK output, oncogenic RAF mutants must be either RAS independent (Class 1 and 2 mutants) or coexist with mechanisms that maintain RAS activation despite induction of feedback (Class 3 mutants).

3) Consistent with this general model, we showed that activation of ERK output by Class 3 mutants depended on coexistent mutational or physiologic activation of RAS signaling to overcome feedback and that in melanoma, both hypoactive and kinase dead mutants coexist with mutants that served to activate RAS (NF1 inactivating or KRAS activating mutants).

4) We showed that both types of mutant activate signaling by the same proximal mechanism-increased binding to active RAS, which caused them to recruit and activate wild type CRAF. We further show that this is likely to be the proximate mechanism, since introducing a mutation that disables dimerization of these mutants with CRAF abolishes their activation of CRAF, but not their tighter binding to RAS.

5) Since hypoactive and kinase dead BRAF mutants have common properties ((a common mechanism of activating the ERK signaling (increased binding to active RAS), RAS dependence, sensitivity to ERK-dependent feedback inhibition of RAS, and requirement for co-existent mutational or physiologic activation of RAS)), we operationally define them as a single group of Class 3 BRAF mutants.

6) We recognize the biologic and translational implications of this model. Tumour cells with Class 3 mutants can be distinguished by the mechanism by which they activate RAS: RAS or NF1 mutation versus RTK activation. In the former, we have no current means of inhibiting RAS activation, in the latter, we do. This directly leads to a currently feasible means of effectively treating the latter tumours—combining an inhibitor of the RTK that dominantly drives RAS with a MEK or an ERK inhibitor.

There are a significant number of patients with such tumours. This method of treatment would be contraindicated if the Garnet model[2] is followed, since it asserts that hypoactive mutants are RAS-independent (and therefore likely to be insensitive to upstream inhibitors). The data in the Heidorn paper[3] is consistent with possibility that tumours with kinase dead BRAF mutations and WT RAS/NF1 would be sensitive to upstream inhibition, but this idea was not mentioned or subsequently pursued. To our knowledge, this paper is the first to suggest this mechanism based strategy for therapy and the first to demonstrate its efficacy in model systems and patients.

7) Mass sequencing of human tumours now reveals many different BRAF mutants, 16 of which we have characterized and identified as Class 3. They occur at significant frequency in cancer, and may constitute 3% of adenocarcinomas of the lungs. There is no published data on the great majority of these and those not in significant hotspots are often called passengers. There is no specific knowledge about their treatment. Previous work on these mutations has not been helpful in these regards. In this and in another paper[6] we present experimental algorithms which together allow classifying unknown BRAF mutations as belonging to Class 1, 2 or 3 or as potential passengers and testing class specific therapeutic strategies for each.

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Oligos for mutagenesis of Class 3 BRAF mutants

D287H-F:

5′- TATGACCAACTTCATTTGCTGTTTG-3′

D287H-R:

5′- TTGGAGACAAACAGCAAATGAAGTTGGTC-3′

V459L-F:

5′-AGATTACACTGGGACAAAGAATTGGA-3′

V459L-R:

5′-CTTTGTCCCAGTGTAATCTGCCCATC-3′

G466V-F:

5′-AGAATTGGATCTGTATCATTTGGA-3′

G466V-R:

5′- TGTTCCAAATGATACAGATCCAAT -3′

G466E-F:

5′-AGAATTGGATCTGAATCATTTGGA-3′

G466E-R:

5′-TGTTCCAAATGATTCAGATCCAAT-3′

G466A-F:

5′-AGAATTGGATCTGCATCATTTGGA-3′

G466A-R:

5′- TGTTCCAAATGATGCAGATCCAAT -3′

S467L-F:

5′-GGATCTGGATTATTTGGAACAGTCTA-3′

S467L-R:

5′- TGTTCCAAATAATCCAGATCCAATTCT-3′

G469E-F:

5′-GGATCTGGATCATTTGAAACAGTCTAC-3′

G469E-R:

5′-CCTTGTAGACTGTTTCAAATGATCC-3′

N581S-F:

5′-GACCTCAAGAGTAATAGTATATTTCTT-3′

N581S-R:

5′- TTCATGAAGAAATATACTATTACTCTT-3′

N581I-F:

5′-GACCTCAAGAGTAATATTATATTTCTT-3′

N581I-R:

5′- TTCATGAAGAAATATAATATTACTCTT-3′

D594N-F:

5′-ATAGGTAATTTTGGTCTAGCTACAGTG-3′

D594N-R:

5′-TAGACCAAAATTACCTATTTTTACTGTGA-3′

D594G-F:

5′-CTCACAGTAAAAATAGGTGGTTTTGGTCTAGC-3′

D594G-R:

5′-CACTGTAGCTAGACCAAAACCACCTATTTTTAC-3′

D594A-F:

5′-ATAGGTGCTTTTGGTCTAGCTACAGTG-3′

D594A-R:

5′-TAGACCAAAAGCACCTATTTTTACTGTGA-3′

D594H-F:

5′-ATAGGTCATTTGGTCTAGCTACAGTG-3′

D594H-R:

5′-TAGACCAAAATGACCTATTTTTACTGTGA-3′

F595L-F:

5′-AATAGGTGATTTAGGTCTAGCTACA-3′

F595L-R:

5′- TAGACCTAAATCACCTATTTTTACTGTGA-3′

G596D-F:

5′-ATAGGTGATTTTGATCTAGCTACAGTGA-3′

G596D-R:

5′-GTAGCTAGATCAAAATCACCTATTTTTACTGTGAGGT-3′

G596R-F:

5′-ATAGGTGATTTTCGTCTAGCTACAGTGA-3′

G596R-R:

5′-GTAGCTAGACGAAAATCACCTATTTTTACTGTGAGGT-3′