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## Mouse models for *BRAF*-induced cancers

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### Abstract

Oncogenic mutations in the *BRAF* gene are detected in ~7% of human cancer samples with a particularly high frequency of mutation in malignant melanomas. Over 40 different missense *BRAF* mutations have been found, but the vast majority (>90%) represent a single nucleotide change resulting in a valine → glutamate mutation at residue 600 (*V600E**BRAF*). In cells cultured *in vitro*, *V600E**BRAF* is able to stimulate endogenous MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] and ERK phosphorylation leading to an increase in cell proliferation, cell survival, transformation, tumorigenicity, invasion and vascular development. Many of these hallmarks of cancer can be reversed by treatment of cells with siRNA (small interfering RNA) to *BRAF* or by inhibiting MEK, indicating that *BRAF* and MEK are attractive therapeutic targets in cancer samples with *BRAF* mutations. In order to fully understand the role of oncogenic *BRAF* in cancer development *in vivo* as well as to test the *in vivo* efficacy of anti-*BRAF* or anti-MEK therapies, GEMMs (genetically engineered mouse models) have been generated in which expression of oncogenic *BRaf* is conditionally dependent on the Cre recombinase. The delivery/activation of the Cre recombinase can be regulated in both a temporal and spatial manner and therefore these mouse models can be used to recapitulate the somatic mutation of *BRAF* that occurs in different tissues in the development of human cancer. The data so far obtained following Cre-mediated activation in haemopoietic tissue and the lung indicate that *V600E**BRAF* mutation can drive tumour initiation and that its primary effect is to induce high levels of cyclin D1-mediated cell proliferation. However, hallmarks of OIS (oncogene-induced senescence) are evident that restrain further development of the tumour.

### Keywords

*BRAF*; cancer; Cre-Lox; genetically engineered mouse model; proliferation; senescence

### Introduction

Cell growth is controlled by a number of key genes and, as a general rule of thumb, some of these genes need to be mutated before a cell becomes cancerous. Although more than 350 such 'cancer genes' have been identified to date, many others are thought to have been overlooked [1]. With the advent of whole genome sequencing, it is now possible to provide a molecular signature of individual cancers and this has opened up the possibility of identifying the full complement of cancer genes. One of the main efforts has centred on the sequencing of protein kinase genes [2]. In the first large-scale sequencing project of this type, the *BRAF* protein kinase gene was identified as a key growth control gene that is frequently mutated in human cancers [3]. Although *BRAF* mutations are present in only

~7% of human cancers as a whole, importantly, *BRAF* mutations are preponderant in malignant melanoma, being detected in up to 70% of all samples tested. They are also detected at a high frequency in papillary thyroid cancer (36-53%), serous ovarian cancer (~30%) and colorectal cancer (5-22%). The vast majority of the *BRAF* mutations (>90%) represent a single nucleotide change of T to A at nucleotide 1799 resulting in a valine → glutamate mutation at residue 600 (V600E).

Over 40 different missense *BRAF* mutations have been detected in human cancer samples and these have been found to cluster in either the glycine-rich P loop or the activation segment of the *BRAF* kinase domain [4]. Wan et al. [5] analysed the kinase activity of a panel of 22 *BRAF* mutants and classified them as having either high, intermediate or impaired activity (Table 1). A unifying feature of the high and intermediate activity mutants is that they disrupt the hydrophobic interaction between the P loop and the activation segment of the kinase domain. This results in destabilization of the inactive conformation of *BRAF*, thus stimulating its kinase activity leading to increased ERK (extracellular-signal-regulated kinase) activation. However, the impaired activity mutants have less activity than the inactive WT (wild-type) kinase and thus do not fall into this neat model. Three of the four impaired activity mutants are still able to stimulate ERK and they appear to do this by activating *CRAF* through mechanisms that are not yet fully understood. The one remaining impaired activity mutant, *D594V BRAF*, behaves similarly to kinase-defective *BRAF* and does not stimulate ERK or *CRAF* activity (Table 1). How this mutant is oncogenic is currently not clear, but it is certainly intriguing and further studies of it may well lead to the unravelling of novel functions for *BRAF*.

Data established from sequencing human tumour samples has led to some insight into the role of *BRAF* in the cancer phenotype. First, the data show that oncogenic *BRAF* and *RAS* mutations are mutually exclusive, rarely being present in the same cancer samples. This has been taken as evidence that *BRAF* and *RAS* contribute to cancer progression through similar mechanisms, most notably through their shared ability to activate the downstream MEK [MAPK (mitogen-activated protein kinase)/ERK]/ERK pathway, which is known to influence many cancer hallmarks [6]. Secondly, mutations in other *raf* genes are extremely rare and recent studies have indicated that *CRAF* requires mutation within the N-region of the kinase domain in addition to *V492E CRAF* mutation (the equivalent of a *V600E BRAF* mutation) in order to be converted into an oncogene [7]. Thirdly, *BRAF* mutations are present in benign naevi [8] that express senescent markers and *V600E BRAF* can induce senescence in melanocytes [9]. These observations are consistent with the view that OIS (oncogene-induced senescence) is a *bona fide* tumour-suppressive mechanism in the development of human cancers [10] and suggests that inactivation of key tumour suppressor genes such as *CDKN2A* may be necessary to unleash the full tumorigenic effects of *V600E BRAF*.

## Mouse models for *Raf*-induced cancers

As elucidated above, studies of human cancer samples have not only led to the identification of *BRAF* as a human oncogene but also have provided mechanistic insight into how this oncogene operates. Further progress is hampered by the limited accessibility of human cancer material, particularly from early cancer lesions that may allow assessment of the progression of the disease. A major effort therefore has focused on the generation of GEMMs (genetically engineered mouse models) for cancer. Although there are many physiological differences between laboratory mice and humans, some of which have a big impact on cancer [11], it is now possible to make the mouse more human-like and turn on/off oncogenic or tumour suppressor gene mutations at the right time in the right tissue. The availability of such accurate models is making a big impact on our understanding of the

progression of cancer at the molecular level. GEMMs are also being applied to the development of anticancer therapies, including their use for target validation, toxicity studies and biomarker discovery [12].

The first mouse models for *Raf*-induced cancer were generated by expressing either the full-length *craf* gene or constitutively active *craf-BxB* under the control of the human surfactant protein-C promoter that directs expression to the type II epithelial cells lining the lung alveoli [13]. Both transgenics developed multifocal lung adenomas associated with high levels of phosphorylated ERK and high rates of cell proliferation that could be reduced by treatment with the MEK inhibitor CI-1040 [14]. Development of the tumours was also suppressed by *bcl-2* deficiency [15] or by *bag1* (Bcl-2-associated athanogene 1) haploinsufficiency [16], suggesting that suppression of apoptosis co-operates in lung tumour induction. A similar approach was used to drive <sup>V600E</sup>*BRaf* expression from the bovine thyroglobulin promoter that directs expression to thyroid follicular cells [17]. This oncogene was observed to initiate multifocal tumours with evidence of progression to poorly differentiated carcinomas.

More recently, conditional knockin GEMMs using the Cre-lox system have been developed by modification of the endogenous *BRaf* locus using homologous recombination in ES cells [18,19]. The model developed in our laboratory is illustrated in Figure 1. In this model, the endogenous *BRaf* locus was modified such that an LSL (Lox-Stop-Lox) cassette was placed in intron 14 consisting of three *loxP* sites, a mini-cDNA encoding exons 15-18 of *BRaf* and a *neo<sup>R</sup>* cassette. Thus, in the absence of Cre recombinase, the WT BRaf protein is expressed with exons 15-18 being encoded by the mini-cDNA, whereas, in the presence of the Cre recombinase, deletion of the mini-cDNA and *neo<sup>R</sup>* cassette occurs such that <sup>V600E</sup>BRaf is expressed. The advantage of this system over those previously described is that the mutant BRaf is expressed at physiological levels and, by controlling the activity of the Cre recombinase, it is possible to mimic the situation that occurs in human cells where one copy of the normal *BRAF* gene is mutated somatically to <sup>V600E</sup>*BRAF*. So far this system has been used to induce somatic <sup>V600E</sup>BRaf expression in MEFs (mouse embryonic fibroblasts), the haemopoietic system using the inducible *Mx1-Cre* strain [18] and in the lung by intranasal instillation of adenoviral Cre [19]. Current efforts are being directed towards induction of <sup>V600E</sup>BRaf expression in other tissue types, particularly melanocytes and colonocytes as well as the development of mouse models for other *BRAF* oncogenic mutations.

## What the data currently show

In the systems so far analysed, <sup>V600E</sup>BRaf is able to induce high levels of proliferation and this is associated with the induction of high levels of phosphorylated MEK/ERK and a large increase in cyclin D1 expression. Changes in other cell cycle proteins have not yet been reported although <sup>V600E</sup>BRaf has been shown to suppress p21<sup>CIP1</sup> expression in MEFs that may also contribute to increased S-phase progression [18]. In the haemopoietic system, <sup>V600E</sup>BRaf induces a rapidly fatal acute myeloid leukaemia reminiscent of histiocytic sarcoma in which there is amplification of circulating cells of the macrophage lineage [18]. In the lung, <sup>V600E</sup>BRAF induces the formation of mixed papillary and solid adenomas that arise from amplification of alveolar type II pneumocytes within 6-8 weeks of Cre infection [19]. This phenotype can be reversed by treatment of mice with PD0325901, a MEK inhibitor structurally similar to CI-1040. However, at later times post-infection, fewer proliferating cells are observed in the lung adenomas, even though the MEK/ERK cascade remains active, and the expression of senescent markers including p19<sup>ARF</sup> and Dec1 is induced. This result has been taken as evidence that, although <sup>V600E</sup>BRaf can promote a period of rapid cell proliferation, this is followed by OIS that restrains the further development of the tumours. Few of the adenomas spontaneously progress to

adenocarcinomas although concomitant loss of *TP53* or *CDKN2A* leads to more rapid cancer progression, presumably by overcoming OIS.

The phenotypes observed in <sup>V600E</sup>BRAF-expressing mice are similar in a number of ways to conditional knockin mice expressing oncogenic *KRas* but also demonstrate some differences. Expression of <sup>G12D</sup>*KRas* using the *Mx1-Cre* strain induces a different myeloproliferative disorder to <sup>V600E</sup>*BRAF* [20], indicating that the two oncogenes have similar proliferative effects but target different populations of haemopoietic cells. Expression of oncogenic *KRas* in the lung leads to the similar development of adenomas that also express senescent markers [21], but these progress more rapidly to adenocarcinomas than tumours induced by <sup>V600E</sup>BRAF [22], suggesting that additional *KRas* effector pathways contribute to further tumour progression.

These initial studies have led to a model of how <sup>V600E</sup>BRAF may be involved in tumour development (Figure 2). In the first stage, the oncogene is able to initiate tumour development by promoting an initial dramatic wave of cell proliferation driven by activation of the MEK/ERK cascade and high levels of cyclin D1. In the case of certain haemopoietic neoplasias such as histiocytic sarcoma, this level of proliferation may be sufficient to give severe clinical symptoms. In the case of solid cancers, however, a second stage ensues involving the induction of at least some markers of OIS that constrain the further development of the tumours. In the third stage, inactivation of key tumour suppressors such as p16<sup>INK4a</sup> or p19<sup>ARF</sup> is required to overcome the senescent phenotype to further progress the disease. How <sup>V600E</sup>BRAF can promote striking cell proliferation on the one hand and cell cycle arrest on the other is a key quandary of this model and the role of the MEK/ERK pathway in both phenotypes will require the further analysis of GEMMs combined with cell culture model systems to resolve.

## Conclusion

GEMMs are being used to recapitulate *BRAF*-induced cancers in humans. These mice are proving vital in dissecting how oncogenic *BRAF* contributes to cancer and so far have shown that mutation of *BRAF* can be an initiating event, that oncogenic *BRAF* acts similarly to oncogenic *RAS* and that OIS may be an important step in development of the disease, at least in the lung. Further studies of these and other mouse models for *BRAF*-induced cancers using a wider range of Cre delivery methods to activate the oncogene in different tissues will undoubtedly help provide a complete molecular picture of events leading to metastatic disease.

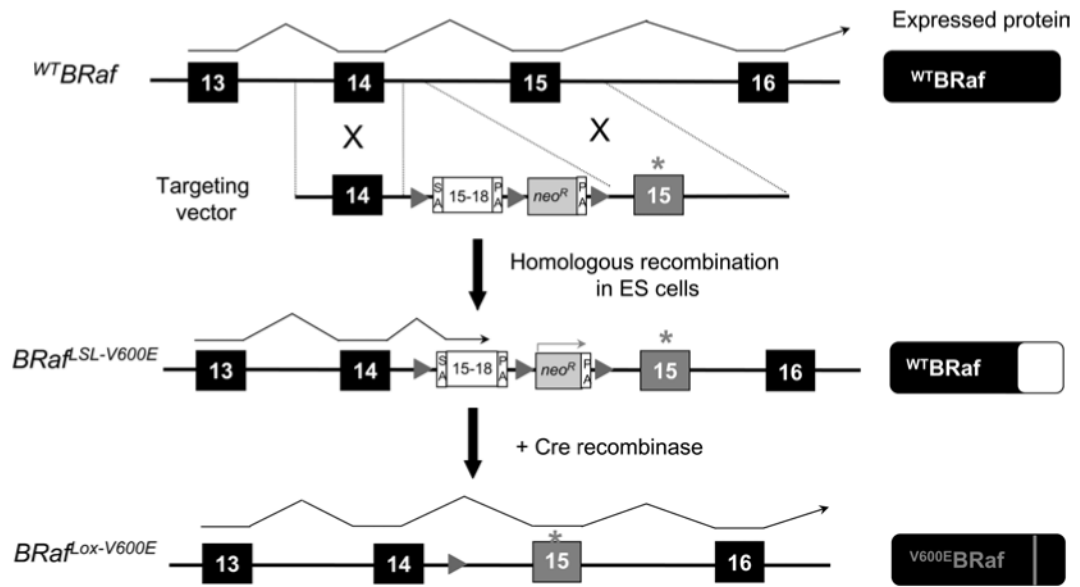
## Abbreviations used

<b>ERK</b>	extracellular-signal-regulated kinase
<b>GEMM</b>	genetically engineered mouse model
<b>LSL</b>	Lox-Stop-Lox
<b>MAPK</b>	mitogen-activated protein kinase
<b>MEF</b>	mouse embryonic fibroblast
<b>MEK</b>	MAPK/ERK kinase
<b>OIS</b>	oncogene-induced senescence
<b>PA</b>	polyadenylation
<b>siRNA</b>	small interfering RNA

WT wild-type

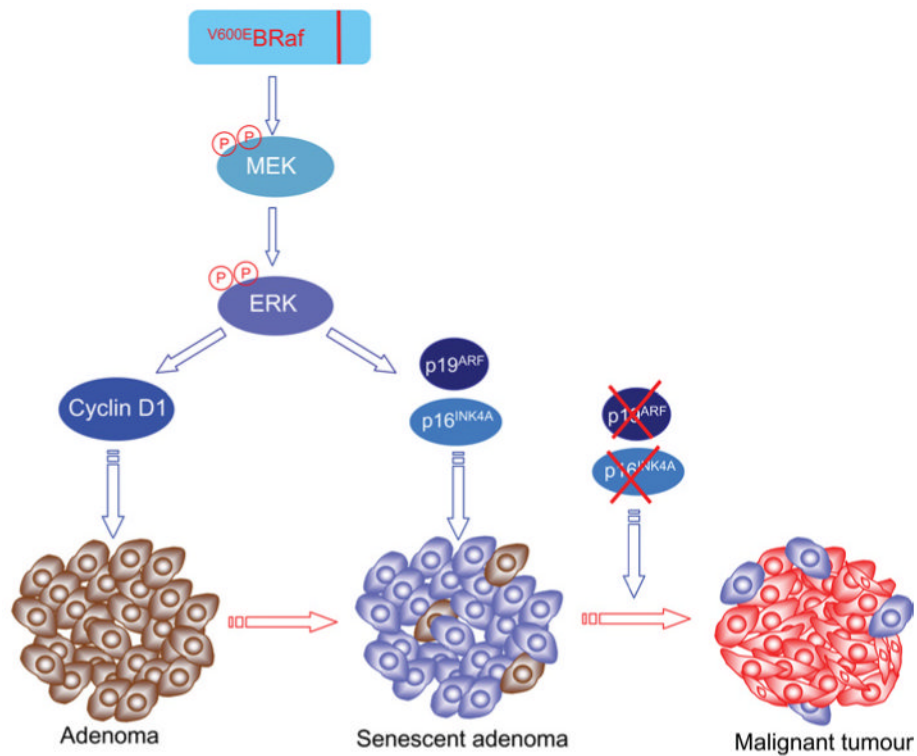
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**Figure 1. Schematic representation of a conditional knockin allele for *V600E* BRAF in mice**

In this allele, the endogenous *BRAF* locus was modified such that an LSL cassette was placed in intron 14. The LSL cassette consists of a mini-cDNA encoding exons 15-18 of the *BRAF* gene with a  $\beta$ -globin splice acceptor sequence at the 5'-end of the mini-cDNA and a polyadenylation (PA) sequence at the 3'-end. The cassette also has a *neo<sup>R</sup>* gene driven by the PGK (phosphoglycerate kinase) promoter and a PA sequence at the 3'-end. On the 3'-end of the LSL cassette, the endogenous exon 15 has been modified to include the T1799A mutation. Three loxP sites are present in the LSL cassette; the first is between exon 14 and the mini-cDNA, the second between the mini-cDNA and the *neo<sup>R</sup>* cassette and the third between the *neo<sup>R</sup>* cassette and modified exon 15. Expression of the Cre recombinase converts the *BRaf<sup>LSL-V600E</sup>* allele into the *BRaf<sup>Lox-V600E</sup>* allele that expresses *V600E* BRAF. A similar model was described by Dankort et al. [19], although in this model only two loxP sites were utilized: one placed between exon 14 and the mini-cDNA and the other between the *neo<sup>R</sup>* cassette and the modified exon 15. Adapted from [18] with permission.



**Figure 2. Current model for role of  $V600E$ BRAF in tumour development and progression**  
 $V600E$ BRAF is able to initiate the development of pre-malignant adenomas by inducing a rapid phase of cell proliferation through up-regulation of cyclin D1 expression.  $V600E$ BRAF can also induce the up-regulation of senescent markers including p16<sup>INK4A</sup> and p19<sup>ARF</sup>, through the MEK/ERK pathway. As a result, many of the cells within the adenoma are cell cycle arrested and the tumour cannot progress further. What controls the switch from the proliferative to senescent stage is currently not clear but there is evidence that both phenotypes are MEK/ERK-mediated. Further progression of the tumours requires a second hit within tumour suppressor genes such as *CDKN2A*, resulting in inactivation of p16<sup>INK4A</sup> and/or p19<sup>ARF</sup>. Cells with such an additional mutation have a proliferative advantage and allow further progression of the tumour. This model proposes that mutation of  $V600E$ BRAF is an initiating event and multiple genetic hits are required for development of the malignant disease. Proliferating  $V600E$ BRAF mutant cells are shown in brown, senescent  $V600E$ BRAF mutant cells in blue and malignant cells with  $V600E$ BRAF and *CDKN2A* mutations are shown in red.

**Table 1**  
**Classification of 22 missense BRAF mutations detected in human cancer samples as tested by Wan et al. [5]**

Mutants were expressed as c-Myc-tagged fusion proteins in COS cells and classified as having either high, intermediate or impaired levels of BRAF kinase activity. The high and intermediate activity mutants have greater kinase activity than <sup>WT</sup>BRAF and stimulate ERK activation directly. Although these mutants also stimulate CRAF activity, siRNA (small interfering RNA) to CRAF indicates that this isoform does not account for the increased ERK activation. The impaired activity mutants have less activity than <sup>WT</sup>BRAF. In the case of three or four of these mutants, ERK activation is greater than that mediated by <sup>WT</sup>BRAF and this is thought to be mediated by CRAF. The fourth impaired mutant, D594V, does not stimulate CRAF or ERK activation and has a similar level of BRAF kinase activity as kinase-defective BRAF. The mechanism by which this mutant operates in cancer development is currently unclear

Class of mutant	BRAF activity	CRAF activity	ERK activity	Mutant
High	> <sup>G12V</sup> RAS-induced BRAF;	> <sup>G12V</sup> RAS-induced CRAF	Similar levels as <sup>G12V</sup> RAS-induced	G487A
	~130-700-fold greater than <sup>WT</sup> BRAF		ERK activation (~4-fold)	E586K V600D V600E V600K V600R K601E
Intermediate	< <sup>G12V</sup> RAS-induced BRAF but > <sup>WT</sup> BRAF;	> <sup>G12V</sup> RAS-induced CRAF	< <sup>G12V</sup> RAS-induced ERK activation	R462I
	1.3-64-fold more than <sup>WT</sup> BRAF		but > <sup>WT</sup> BRAF-induced ERK activation	I463S G464E G464V
Impaired	0.6-0.8-fold less than <sup>WT</sup> BRAF	> <sup>G12V</sup> RAS-induced CRAF	< <sup>G12V</sup> RAS-induced ERK activation	G466A G469E N581S F595L L597V T599I A727V
			but > <sup>WT</sup> BRAF-induced ERK activation	G466E G466V G596R
			Inhibited by CRAF siRNA	



<b>Class of mutant</b>	<b>BRAF activity</b>	<b>CRAF activity</b>	<b>ERK activity</b>	<b>Mutant</b>
Impaired	0.3-fold less than <sup>WT</sup> BRAF	No activation	No activation	D594V