Dissecting the roles of Raf- and PI3K-signalling pathways in melanoma formation and progression in a zebrafish model

Christina Michailidou1, Mary Jones1, Paul Walker1, Jivko Kamarashev2, Amanda Kelly1 and Adam F. L. Hurlstone1,*

SUMMARY
Deregulated Ras signalling is implicated in most human neoplasia, exemplified by melanoma. Whereas Raf activation occurs almost ubiquitously in benign and malignant melanocytic neoplasms, implying an involvement in tumour initiation, phosphoinositide 3-kinase (PI3K) activation occurs predominantly in malignant neoplasms, implying an involvement in malignant progression. Here, we dissect the contributions of these two pathways to tumourigenesis in vivo, by modulating their activities in zebrafish melanocytes. Misexpression of oncogenic Ras (V12RAS) in founder fish induced frequent melanoma, beginning at larval stages, with concomitant activation of Raf-Mek-Erk and PI3K-Akt signalling. Misexpression of effector-domain mutants of V12RAS, or of various downstream effectors, confirmed a selective role for the Raf-Mek-Erk pathway in initiating neoplasia, but highlighted the requirement for additional Ras effector pathways for malignancy. The phenotype of animals with germ-line transmission of V12RAS resembled familial atypical mole and melanoma (FAMM) syndrome: melanocytes displayed hyperplasia, dysplasia, altered terminal differentiation and spontaneously progressed to invasive melanoma. Co-expressing a dominant-interfering form of PI3K abolished V12RAS-induced malignancy, demonstrating a direct role for PI3K signalling in the malignant progression of melanoma in vivo, and highlighting PI3K as a promising target for melanoma therapy.

INTRODUCTION
Cutaneous melanoma, an aggressive cancer arising from epidermal melanocytes, is a disease with an unmet clinical need. Its incidence in Northern Europe, North America and Australia has risen dramatically over the last few decades, faster than any other cancer (Cancer Research UK, 2006). In the majority of cases, the disease is still localised at diagnosis and can be cured by surgical excision. However, metastatic disease is largely refractory to all current forms of therapy and affected individuals have a median survival time of only 6 months. Approximately 20% of people diagnosed with melanoma succumb to their disease (Cancer Research UK, 2006). Thus, there is an urgent need to understand the molecular basis of this disease in order to develop better treatments.

Melanoma progresses through a series of histological steps. The first step is proliferation of cytologically normal, nested melanocytes and subsequent senescence, resulting in a benign naevus. The second step is more deranged growth with scattered cytological atypia, which may occur in a pre-existing benign naevus or, more typically, at a de novo site, generating a dysplastic naevus. At the third step, dysplastic melanocytes proliferate and invade the epidermis and papillary dermis, giving rise to a superficially spreading lesion with continuous atypia, commonly referred to as a radial-growth phase (RGP) melanoma, but also as melanoma in situ. At the fourth step, vertical-growth phase (VGP) melanoma, malignant cells acquire the ability to invade the dermis and subcutaneous tissues, resulting in an expansile nodule. At the fifth and final step, metastatic melanoma, malignant cells disseminate through the vasculature and lymphatic system to secondary sites. The transition from RGP to VGP is accompanied by a dramatic deterioration in prognosis.

In common with other cancer progression models, the above transitions are assumed to reflect a stepwise transformation of melanocytes by the acquisition of mutations in select oncogenes and tumour suppressor genes, a number of which have now been identified (Haluska et al., 2006; Dahl and Guldberg, 2007). Activating BRAF mutations (particularly the valine for glutamate substitution at position 600) are believed to initiate the sequence of melanocyte neoplasia, since both common naevi and malignancies frequently (approximately 80% of cases) harbour such mutations (Pollock et al., 2003). Clearly, BRAF mutation alone does not trigger malignant progression. Raf serine/threonine kinases that act in the Mek-Erk signal transduction pathway are downstream effectors of Ras oncoproteins. Activating mutations in NRAS (typically a lysine for glutamine substitution at position 61) occur in 15-25% of naevi and melanomas, but never occur together with BRAF mutations, suggesting that activation of NRAS can play an equivalent role to BRAF activation in initiating melanocyte neoplasia (Haluska et al., 2006; Dahl and Guldberg, 2007).

Class I phosphoinositide 3-kinases (PI3Ks) are also effectors of Ras signalling and are activated in a significant fraction of melanomas, largely through loss of function of the tumour suppressor phosphatase and tensin homolog (PTEN) (Wu et al., 2003). PTEN is a phosphatase which removes the 3’ phosphate from the secondary messenger molecule phosphatidylinositol (3,4,5)-trisphosphate [PIP3, also known as PtdIns(3,4,5)P3], which is generated by PI3K. Akt serine/threonine kinases that are stimulated upon PIP3 binding are found constitutively activated in cells that lack PTEN (Birck et al., 2000). Activated Akt promotes cell survival...
by inhibiting apoptosis (Manning and Cantley, 2007), and increased Akt activity, which can arise through either amplification of the gene encoding AKT3 or inactivation of PTEN, may occur in 40-60% of melanomas (Stahl et al., 2004). Loss of function of PTEN is not observed in naevi (Tsao et al., 2004) and is observed mostly as a late-stage event in melanoma (more than 60% of metastatic melanomas have PTEN inactivation, in contrast to only one-third of primary melanomas) (Guldberg et al., 1997; Birck et al., 2000; Whiteman et al., 2002). Similarly, AKT3 activity levels also increase with advancing melanoma stage (Stahl et al., 2004). These findings implicate PI3K activation in the malignant progression of melanoma. Intriguingly, NRAS and PTEN mutations appear to be mutually exclusive in melanoma. By contrast, BRAF and PTEN mutations coincide frequently (Haluska et al., 2006). Thus, the human genetic data suggest that the biochemical functions of Ras can be partitioned in melanoma cells into a Raf-signalling arm and a PI3K-signalling arm, with discrete functions that synergise to induce malignancy.

Studies investigating the susceptibility of hybrids of different Xiphophorus species to develop melanocytic neoplasms were the first to link genetic alterations with melanoma (Gordon, 1931). Fish and mammalian melanocytes are highly analogous; in both taxa, melanocyte precursors (melanoblasts), which are derived from a subset of neural crest cells that arise in the dorsum, migrate to their final destination in the integument of the animal. The proteins regulating melanoblast specification, proliferation, survival, migration and subsequent differentiation to melanocytes are highly conserved in sequence and function (Bennett and Lamoreux, 2003; Kelsh, 2004). Similarly, the biochemical changes observed in melanoma cells in Xiphophorus reveal striking similarities with human melanoma, including activation of Ras, Erk and PI3K signalling modules (Meierjohann and Scharl, 2006). Notably, expression of oncogenic BRAFV600E in zebrafish melanocytes leads to benign melanocyte neoplasia, the first demonstration in vivo that activation of Raf-Mek-Erk signalling is sufficient to initiate melanocyte neoplasia. Moreover, misexpression of BRAFV600E in a p53-deficient background resulted in progression of zebrafish naevi to melanoma (Patton et al., 2005).

In the present study, we have generated a novel melanoma model by targeting expression of oncogenic Ras (HRASG12V) to zebrafish melanocytes. Unlike BRAFV600E, oncogenic Ras alone was sufficient to induce malignancy, prompting us to explore which Ras effector pathways are implicated in initiation and which are implicated in malignant progression of melanocyte neoplasia. We establish that the Raf-Mek-Erk pathway is distinct among Ras effector pathways in being able to initiate melanocyte neoplasia and demonstrate, in this autochthonous melanoma model, a requirement for PI3K in promoting the malignant progression of melanocytes.

RESULTS

**Zebrafish melanocytes expressing oncogenic Ras are malignantly transformed**

The pigment pattern of zebrafish larvae is largely complete by 60 hours post-fertilisation (hpf), and comprises approximately 460 post-mitotic melanocytes arrayed in four longitudinal stripes (control, Fig. 1A) (Kelsh et al., 1996). This pattern is gradually replaced by the adult pigment pattern during the larval-to-adult metamorphosis [2-4 weeks post-fertilisation (wpf)], and new stripes are added gradually as the animal grows (control, Fig. 1B) (Johnson et al., 1995).

To generate a zebrafish model of melanoma, a promoter fragment from the zebrafish microphthalmia-associated transcription factor a (mitfa) gene, which has previously been

![Fig. 1. Generation of a melanoma model.](image-url)
reported to direct exogenous protein expression to cells of the melanocyte lineage (Dorsky et al., 2000) and has been used to misexpress BRAF\(^{V600E}\) and generate zebrafish naevi (Patton et al., 2005), was coupled to a cDNA encoding human HRAS with an oncogenic valine for glycine substitution at position 12 (HRAS\(^{G12V}\), hereafter referred to as V12RAS). Whereas in humans oncogenic valine for glycine substitution at position 12 (HRAS\(^{G12V}\)) was coupled to a cDNA encoding human HRAS with an oncogenic valine for glycine substitution at position 12 (HRAS\(^{G12V}\)), hereafter referred to as V12RAS). Whereas in humans oncogenic HRAS (Chin et al., 1997; Broome Powell et al., 1999) or NRAS (Ackermann et al., 2005) in mouse melanocytes induces melanoma formation. To aid identification of transgenic cells and organisms, the transgene plasmid used to induce melanoma in our model also contained a second expression cassette comprising green fluorescent protein (GFP) cDNA coupled to the mitfa promoter fragment (Fig. 1C).

From 96 hpf onward, and in contrast to control animals injected with only a GFP-encoding plasmid, mosaic \(G_3\) animals injected with the V12RAS plasmid developed one or more clones of ectopic melanocytes that continued to expand radially, generating superficial spreading lesions with irregular borders that were frequently associated with scattered melanocytes [a representative lesion at 10 days post-fertilisation (dpf) is shown in Fig. 1A and another at 4 wpf in Fig. 1B]. Ectopic patches of xanthophores were also observed, consistent with Mitf being expressed in a subset of xanthoblasts (xanthophore precursors) (Parichy et al., 2000). Within a few weeks, a fraction of these lesions progressed to give expansile nodules (arrowhead in Fig. 1D). Overall, we determined that, by 12 weeks, 60% of transgenic animals possessed one or more superficial lesion, whereas 30% possessed nodules (\(n=200\) injected animals); tumour nodules arose most frequently on the fins, but could also arise on the flanks and, occasionally, the uvea (see Fig. 4A). By examining malignant cells in living hosts by exploiting their fluorescence, we consistently (\(n=12/12\)) observed an advancing front of superficially located tumour cells that were GFP-positive but unpigmented. This advancing front was continuous, albeit with an irregular margin, and we observed numerous pseudopodial extensions (Fig. 1D). Fluorescence time-lapse videos of up to 8 hours did not reveal significant translocation of tumour cells, although frequent retraction and extension of pseudopodia were observed (data not shown). These types of dynamic changes in cell shape are associated typically with a migratory and invasive cellular behaviour (Yamaguchi et al., 2005).

Fish integument consists of a stratified squamous epidermis and an underlying dermis, itself comprising an outer looser network of connective tissue (the stratum spongiosum) and an inner collagen-rich layer (the stratum compactum) (Ostrander, 2000). In teleost (ray-finned) fish, the trunk epidermis is layered over mineralised collagenous plates called elasmoid scales, which overlap in zebrafish. Squamation begins at around day 20 or when fish reach a standard length of 8 mm (Sire et al., 1997). The disposition of pigment cells in the zebrafish integument has been detailed previously (Hirata et al., 2003; Hirata et al., 2005). The majority of melanocytes (typified by those cells comprising the horizontal stripes of the trunk) reside in ribbons that are four to six cells wide and one cell thick, sandwiched between sheets of iridophores (metallic-appearing pigment cells) and xanthophores. This sandwich of pigment cells is located between the stratum compactum and the muscle layer, in a layer known as the hypodermis (Hirata et al., 2003; Hirata et al., 2005). Occasional pigment cells are also dispersed in the stratum spongiosum of the dermis. This is particularly evident in the scales over the dorsum.

Histological examination of radially expanding lesions induced by V12RAS revealed a malignant spread of atypical melanocytes beneath the dermis, as well as into the loose connective tissue surrounding the muscle fascicles of the trunk musculature and, occasionally, into the epidermis (Fig. 1E–F). We classified these neoplastic lesions as zebrafish RGP melanoma. This contrasts with the presentation of benign melanocyte neoplasia induced by oncogenic BRAF, which has been reported previously in zebrafish (Patton et al., 2005), where hyperplasia alone was observed, resulting in nests of melanocytes in their correct location and with normal morphology. Moreover, histological examination of tumour nodules and the surrounding structures was consistent with a classification of VGP melanoma. V12RAS-expressing tumour cells were found to have dispersed extensively (Fig. 2A,B), infiltrating bone, muscle and spinal chord (Fig. 2C,D), but not to have invaded the peritoneum or to have formed visceral metastases (Fig. 2B). The lack of metastasis to visceral organs was common to all V12RAS-expressing animals that were examined histologically (\(n=22\), three sections per animal, encompassing its breadth). Similar to human melanoma (Massi and LeBoit, 2004), zebrafish melanoma cells within nodules most frequently displayed epithelioid morphology, nuclear pleiomorphism with prominent nucleoli, frequent mitotic figures, and were unpigmented, although a significant fraction of melanin-containing dendritic cells were typically present, leading to an overall melanotic appearance (Fig. 2E). Malignant cells were hyperproliferative as indicated by the induction of proliferating cell nuclear antigen (PCNA) expression (Fig. 2F).

As further confirmation that the expansile nodules were largely comprised of melanoma cells, Schmor histochemical staining was performed to detect reducing substances such as melanin. This revealed the presence of melanin throughout the malignant cells, even in the absence of visible pigment granules (Fig. 3A). Transmission electron microscopy (TEM) also confirmed the existence of premelanosomes and mature melanosomes within cancer cells (Fig. 3B). Furthermore, using immunohistochemistry, fine granular tyrosinase expression could be detected in malignant cells, again even in the absence of visible pigment granules (Fig. 3C). Since tyrosinase is required for the synthesis of melanin but not pteridine or carotenoid pigment, its expression is consistent with these cells belonging to the melanocyte lineage rather than the xanthophore lineage. Previously, it has been demonstrated that 94% of human melanoma specimens displayed positive immunoreactivity for tyrosinase (Hofbauer et al., 1998). We did occasionally (<5% of cases) also observe malignant neoplasms comprising mainly pigmented cells with spindle morphology (supplementary material Fig. S2D,E), as well as nodules where the predominant pigmented cell fraction contained yellow pigment (these neoplasms perhaps arise from cells committed to the xanthophore lineage) (data not shown).
Only Raf-Mek-Erk activation is sufficient to initiate melanocyte neoplasia

Zebrafish melanoma cells stained positively with anti-phosphoErk and anti-phosphoAkt polyclonal antibodies following immunohistochemistry (Fig. 3D and 3E, respectively). The anti-phosphoErk antibody detects Erk1 and Erk2 [p44 and p42 mitogen-activated protein kinase (Mapk)] when phosphorylated by Mek1 or Mek2 at threonine 202 and tyrosine 204 (these residues are conserved in zebrafish Erk1 and Erk2) (supplementary material Fig. S3A,B). Phosphorylation at these sites (in combination with phosphorylation at threonine 308 of Akt1, or its equivalent in Akt2 or Akt3) activates Akt (Alessi et al., 1996). Thus, again, positive staining with this reagent indicated that V12RAS could efficiently activate zebrafish PI3K-Akt signalling. Immunoblotting and immunocytochemistry performed on insulin-like growth factor 1 (IGF-1)-treated zebrafish fibroblasts (AB9) also demonstrated the suitability of the above-mentioned antibodies in detecting activated zebrafish Erk and Akt (supplementary material Fig. S3D).
To address which Ras effector pathways were sufficient for initiating melanocyte neoplasia, we adopted two approaches: in the first, V12RAS effector-domain mutants were substituted for V12RAS; in the second, deregulated versions of Ras effector molecules were substituted for V12RAS. A point mutation in the effector domain of Ras can disrupt its interaction with a subset of effectors (Rodriguez-Viciana et al., 1997). In this study, we used V12RAST35S that retains signalling through Raf but is compromised in signalling through, among other effectors, PI3K; V12RAS Y40C that retains signalling through the p110 catalytic subunit of PI3K but is compromised in signalling through, among other effectors, Raf; and V12RASE37G that is compromised in signalling through both Raf and PI3K but retains signalling through Raf guanine nucleotide dissociation stimulators (RalGDsSs), which are another class of Ras effectors. Again, transgene vectors were tagged with a GFP expression cassette that allowed us to confirm comparable uptake of the constructs (not shown). Expression of the Ras effector-domain dual cassettes was confirmed by transient expression in cultured cells and by immunoblotting (supplementary material Fig. S4A-C). Of these three effector-domain mutants, V12RAST35S induced melanocyte neoplasia (11% at 12 wpf, n=81) (Fig. 4B), whereas V12RASY40C (Fig. 4C) and V12RASE37G (not shown) did not (see Table 1 for neoplasia incidences per transgenic construct). All lesions were, however, benign naevi that took in excess of 8 weeks to materialise and whose size was limited over >24 months. Histologically, lesions were indistinguishable from those induced by BRAFV600E (Patton et al., 2005), comprising only hyperplastic melanocytes (supplementary material Fig. S2B). The finding that lesions were obtained with V12RAST35S alone suggests that only Raf-Mek-Erk signalling is sufficient to initiate neoplasia. Furthermore, the absence of any lesions with V12RASY40C or V12RASE37G (which fail to activate Raf) is consistent with Raf-Mek-Erk signalling being necessary for melanocyte neoplasia. Finally, the finding that benign lesions only were obtained with V12RAST35S with impaired PI3K signalling ability, whereas V12RAS with a wild-type effector domain induced malignancy, is consistent with a role for PI3K in the malignant progression of melanocytic neoplasms.

To further validate the selective role of Raf-Mek-Erk signalling in initiating melanocytic neoplasia, we also constructed transgenes coupling the mitfA promoter fragment with cDNA encoding human BRAFV600E (this oncogenic mutation introduces a phosphomimetic conformational change in the activation domain of BRAF, resulting in a large increase in basal kinase activity) (Wan et al., 2004); p110αCAAX (p110α is rendered constitutively active by membrane tethering through fusion to an exogenous CAAX motif) (Rodriguez-Viciana et al., 1997); AktDD (Akt1, in which two regulatory amino acids threonine 308 and serine 473 have been replaced with phosphomimetic aspartic residues, rendering the kinase constitutively active) (Govindarajan et al., 2007); RalCAAX (a constitutively active membrane-tethered form of the RalGDS Rif, also known as Rgl2, which also lacks its auto-inhibitory Ras-association domain) (Wolthuis et al., 1997); or N-terminally truncated (C1199)Tiam1 (a markedly stabilised version of Tiam1) (Habets et al., 1994), a Rac guanine nucleotide exchange factor that is also a Ras-interacting protein and downstream effector (Lambert et al., 2002). The proteins encoded by these cDNAs are highly conserved in zebrafish (supplementary material Fig. S5A-E). Likewise, the zebrafish Ral and Rac isoforms, which interact with Rif and Tiam1, respectively, are >95% identical to their mammalian counterparts (supplementary material Fig. S5F,G).

Successful cloning of all the above constructs was verified by transient transfection and immunoblotting (supplementary material Fig. S4D-F). Each of the above constructs was injected into zebrastin zygotes. GFP expression from an accompanying expression cassette confirmed equal uptake of all transgene constructs (not shown). Only BRAFV600E induced naevi (Fig. 4D), with an incidence (15%, n=87) and latency that were comparable to those observed by V12RAST35S (see also Table 1), as described previously (Patton et al., 2005). Histological examination of BRAFV600E-induced neoplasms confirmed their benign status

<table>
<thead>
<tr>
<th>Transgenic G0 animals</th>
<th>Lesions at 12 wpf</th>
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<tbody>
<tr>
<td>V12RAS::GFP</td>
<td>90% (n=251)*</td>
</tr>
<tr>
<td>V12RAS T35S::GFP</td>
<td>11% (n=81)†</td>
</tr>
<tr>
<td>V12RAS Y40C::GFP</td>
<td>0 (n=93)</td>
</tr>
<tr>
<td>V12RAS E37G::GFP</td>
<td>0 (n=78)</td>
</tr>
<tr>
<td>BRAF V600E::GFP</td>
<td>15% (n=87)†</td>
</tr>
<tr>
<td>p110αCAAX::GFP</td>
<td>0 (n=126)</td>
</tr>
<tr>
<td>RifCAAX::GFP</td>
<td>0 (n=122)</td>
</tr>
<tr>
<td>(C1199)Tiam1::GFP</td>
<td>0 (n=87)</td>
</tr>
<tr>
<td>BRAF V600E:p110αCAAX</td>
<td>14% (n=84)†</td>
</tr>
<tr>
<td>AktDD::GFP</td>
<td>0 (n=121)</td>
</tr>
<tr>
<td>BRAF V600E::AktDD</td>
<td>0 (n=167)</td>
</tr>
</tbody>
</table>

Table 1 summarises the incidence of neoplasia for each transgenic construct. *Both superficial spreading lesions and expansile nodules. †Benign naevi.

Fig. 4. Raf-Mek-Erk selectively initiates melanocyte neoplasia. (A) G0 animals expressing V12RAS often exhibit multiple lesions along the trunk, fins and, less frequently, in the uvea (arrowheads). (B) By comparison, the effector-domain mutant V12RAS T35S (which signals through Raf) induced formation of benign naevi only (arrowhead). (C) V12RAS Y40C (an effector-domain mutant that signals through PI3K) did not induce neoplasia. (D) In agreement with B, expression of BRAF V600E alone in melanocytes was sufficient to induce benign neoplasias only (arrowhead). The fish depicted are representative for each construct. Bars, 0.5 cm (A); 1 cm (B-D).
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V12RAS with Δp85 (V12RAS::Δp85) were scored positive if they displayed one or more melanin-expressing V12RAS and Δp85 compared with those expressing V12RAS alone. This result suggests that stimulation of the PI3K-signalling module by V12RAS and its mutational targeting in human melanoma prompted us to examine whether PI3K-Akt signalling is required for efficient melanomagenesis by oncogenic Ras. To address this, we co-expressed V12RAS with an inhibitor of the PI3K pathway, a mutant form of the PI3K regulatory domain p85 (referred to elsewhere, and also herein, as Δp85) in which a 35 amino acid in-frame deletion in the interSH2 domain deletes the p110 interaction domain (Rodriguez-Viciana et al., 1997) (supplementary material Fig. S4G; Fig. S5H). Expression of Δp85 alone produced no overt effect on pigmentation in G0 animals (data not shown). G0 animals expressing V12RAS alone or co-expressing V12RAS with Δp85 (V12RAS::Δp85) were followed until 12 wpf and were scored positive if they displayed one or more melanin-pigmented neoplasms, either superficial or nodular. Co-expression of Δp85 with V12RAS (Fig. 5A; supplementary material Fig. S4G) significantly impaired V12RAS-induced tumourigenicity: we observed an 80% reduction in neoplasia-positive animals co-expressing V12RAS and Δp85 compared with those expressing V12RAS alone (Fig. 5B) (a typical lesion induced by the Ras::Δp85 construct is shown in Fig. 5C). Furthermore, nodular lesions were only observed in animals expressing V12RAS alone. This result suggests that stimulation of the PI3K-signalling module is required for efficient initiation of neoplastic transformation by V12RAS. Indeed, the frequency of neoplasia induced by the V12RAS::Δp85 construct was comparable to the frequency induced by V12RAS and BRAFV600E, consistent with the above finding that Raf-Mek-Erk signalling is inefficient at inducing neoplasia.

Efficient melanomagenesis requires an intact PI3K-Akt signalling pathway

The relative inefficiency of V12RAS\(^{T35S}\) and BRAF\(^{V600E}\) at inducing neoplasia (11% and 15%, respectively) when compared with V12RAS (90%), suggested that additional ‘hits’ (genetic or epigenetic) other than activation of Raf signalling are required for initiation of melanoma. Together, activation of the PI3K-Akt signalling module by V12RAS and its mutational targeting in human melanoma prompted us to examine whether PI3K-Akt signalling is required for efficient melanomagenesis by oncogenic Ras. To address this, we co-expressed V12RAS with an inhibitor of the PI3K pathway, a mutant form of the PI3K regulatory domain p85 (referred to elsewhere, and also herein, as Δp85) in which a 35 amino acid in-frame deletion in the interSH2 domain deletes the p110 interaction domain (Rodriguez-Viciana et al., 1997) (supplementary material Fig. S4G; Fig. S5H). Expression of Δp85 alone produced no overt effect on pigmentation in G0 animals (data not shown). G0 animals expressing V12RAS alone or co-expressing V12RAS with Δp85 (V12RAS::Δp85) were followed until 12 wpf and were scored positive if they displayed one or more melanin-pigmented neoplasms, either superficial or nodular. Co-expression of Δp85 with V12RAS (Fig. 5A; supplementary material Fig. S4G) significantly impaired V12RAS-induced tumourigenicity: we observed an 80% reduction in neoplasia-positive animals co-expressing V12RAS and Δp85 compared with those expressing V12RAS alone (Fig. 5B) (a typical lesion induced by the Ras::Δp85 construct is shown in Fig. 5C). Furthermore, nodular lesions were only observed in animals expressing V12RAS alone. This result suggests that stimulation of the PI3K-signalling module is required for efficient initiation of neoplastic transformation by V12RAS. Indeed, the frequency of neoplasia induced by the V12RAS::Δp85 construct was comparable to the frequency induced by V12RAS and BRAFV600E, consistent with the above finding that Raf-Mek-Erk signalling is inefficient at inducing neoplasia.

Raf-Mek-Erk and PI3K-Akt signalling fail to synergise to induce malignancy

From the above data, we inferred that although an input from BRaf is essential for neoplasia, it is not sufficient for malignancy. Since expression of oncogenic Ras alone can induce malignancy, we hypothesised that a combination of Ras effectors, which should include Brf, is required to reconstitute the full effect of V12RAS. For reasons stated above, co-activation of the PI3K-Akt pathway seemed the most obvious candidate. To test this, we constructed plasmids allowing co-expression of BRaf\(^{V600E}\) with either p110αCAAX or Akt\(^{DD}\) (see supplementary material Fig. S4D and S4F, respectively). Neither of these constructs could induce melanoma (see Table 1). Rather, with co-expression of p110αCAAX, we observed benign naevi at a similar frequency and latency as for BRaf alone (14%, n=84) (Fig. 5D), whereas co-expression of Akt\(^{DD}\) resulted in no apparent lesions (n=167) (Table 1). Co-expression of RlfCAAX or (C1199)Tiam1 also failed to augment the tumourigenic effect of BRaf\(^{V600E}\) (data not shown). In all cases, a benign histology was confirmed (data not shown). Thus, combined Raf-Mek-Erk and PI3K-Akt-signalling was not sufficient to induce malignancy, suggesting that, in addition to activation of these two pathways, one or more additional V12RAS-dependent signals are required to reconstitute the full effect of V12RAS.

F0 animals demonstrate the role of PI3K signalling in the benign-to-malignant transition

G0 animals display mosaic expression of transgenic constructs, which results in phenotypic variation both between transgenic clones of cells within an organism, as well as inter-individual variation. To generate transgenic animals with a uniform phenotype and, thus, to simplify analysis, G0 fish were used to found stable transgenic lines. F1 transgenic animals expressing V12RAS in all melanocytes (from four independent lines) had a common phenotype that entailed both hyperplasia and dysplasia, resulting in complete loss of the stripe pattern over the body owing to random assortment of pigment cells (compare the wild-type fish in Fig. 6A with the V12RAS F1 fish in Fig. 6B). Frequent progression to nodular melanoma was observed in the fish expressing V12RAS (37% of animals had one or more nodules by 16 weeks).
Intriguingly, the scales over the dorsum of these animals contain almost half the number of pigmented melanocytes when compared with wild-type scales from the equivalent region (supplementary material Fig. S7). However, these melanocytes were more than four times larger than melanocytes found on wild-type scales (supplementary material Fig. S7). In contrast to V12RAS transgenic animals, and in agreement with a previous study (Patton et al., 2005), F1 animals expressing human BRAFV600E (from two independent lines) displayed only melanocytic hyperplasia (Fig. 6C), as apparent from broader fused stripes and more populous hypertrophic, but correctly positioned, melanocytes associated with the scales over the dorsum (Fig. 6C; supplementary material Fig. S7). We also generated F1 animals co-expressing V12RAS and Δp85 (V12RAS::Δp85) (three independent lines), all with a consistent phenotype [semi-quantitative reverse transcriptase (RT)-PCR was used to confirm Δp85 expression] (supplementary material Fig. S6C). These animals also displayed an overall benign phenotype that was analogous to the BRAFV600E-expressing line (Fig. 6D), retaining melanocyte hyperplasia but, unlike the lines expressing V12RAS alone, no marked dysplasia. Thus, animals co-expressing V12RAS and Δp85 possessed broader fused stripes (although interstripes were still apparent ventrally) and more populous, correctly positioned melanocytes associated with the scales over the dorsum (Fig. 6D; supplementary material Fig. S7). These melanocytes were the most similar in size and distribution to the wild-type melanocytes (compare scales in Fig. 6A with those in Fig. 6D; see also supplementary material Fig. S7), further confirming their benign nature. Neither BRAFV600E-expressing animals, nor V12RAS::Δp85 animals developed expansile nodules (no nodules observed in >200 animals after >15 months). However, localised regions of progression to a pigment pattern like the one seen in fish expressing V12RAS alone were noted in V12RAS::Δp85 animals that, nevertheless, failed to progress to VGP melanoma.

Fig. 6. PI3K-signalling is required for benign-to-malignant transition. The panels illustrate representative animals with an accompanying scale from the dorsum of wild-type zebrafish (A) and of each of the transgenic line series that were established (B-D). (B) V12RAS::GFP animals display melanocytic hyperplasia and dysplasia that results in total aberration of the pigment pattern. The melanocytes associated with the dorsal scales exhibit a more chaotic distribution compared with in other genotypes. (C) BRAFV600E animals only display melanocytic hyperplasia where the dorsal and lateral pigment lines are fused together; melanocytes associated with the dorsal scales are more populous in these fish. (D) V12RAS::Δp85 animals also exhibit the features described in C, consistent with a benign neoplastic phenotype; however, stripes are generally less uniform. Bars, 1 cm (fish); 200 μm (scales).

Fig. 7. Evidence that attenuated PI3K signalling affects the invasiveness of transformed cells. (A-D) H&E staining on transverse sections of 6-month-old animals. (A) Wild type: melanocytes are situated only in the hypodermis (arrowheads). (B) V12RAS::GFP F1: note the reduction of pigmented cells in the hypodermis and their replacement by a hyperplastic layer of unpigmented cells (arrows). Large pigmented stellate melanocytes have invaded the interstitial space between the muscle fascicles (asterisks) and can also be observed in hyperplastic areas of the epidermis covering the scale (arrowhead). (C) BRAFV600E F1: note the thicker pigmented layer in the hypodermis; as in wild-type animals, no melanocytes are present in the muscle. (D) V12RAS::Δp85 F1: analogous to the benign BRAFV600E F1 animals. (E-J) Ras-expressing cells are significantly reduced in the V12RAS::Δp85 line. (E) Immunofluorescence on a transverse section of a V12RAS::GFP F1 animal reveals unpigmented Ras-expressing cells (red) in the hypodermis. Ras-transformed cells can be observed invading between the muscle fascicles throughout the section (E and F). (G) A small nodule (vertical growth phase melanoma) has started to form showing marked invasion of the lower skin layers. (H-J) Ras staining on a transverse section of a V12RAS::Δp85 F1 transgenic fish reveals a thin layer of Ras-expressing cells in the hypodermis (H and arrowheads in I) and a few sporadic Ras-transformed cells between the muscle fascicles (arrowhead in J). Immunofluorescence stainings were performed in parallel and the images, captured using the same settings, are representative of sections from three animals. Bars, 50 μm (A-D); 100 μm (E,H); 20 μm (F,G,J). Abbreviations: ep, epidermis; de, dermis.
These macroscopic observations gave a first indication that attenuation of PI3K signalling had suppressed the malignant phenotype induced by oncogenic Ras. This was subsequently confirmed by histology. As stated above, melanocytes in wild-type animals are located largely in the hypodermis (i.e. between the skin dermis and muscle) (Fig. 7A). In V12RAS-expressing lines, melanocytes had expanded in the dermis and hypodermis, and were now accompanied by a population of unpigmented cells in the hypodermis (Fig. 7B). Strikingly, pigmented stellate melanocytes were also observed in the interstitial spaces between the muscle fascicles. Occasional melanocyte invasion into epidermal layers was also apparent (Fig. 7B). This presentation can be equated to RGP melanoma encompassing the entire body surface. In both BRAFV600E-expressing lines (Fig. 7C) and V12RAS::Δp85 lines (Fig. 7D), melanocytes were positioned correctly in the hypodermis. Significantly, both the presence of stellate melanocytes in between muscle fascicles and invasion of the epidermis were completely absent in BRAFV600E-expressing lines (Fig. 7C) and largely absent in V12RAS::Δp85 lines (Fig. 7D and see below). This was analysed further using immunofluorescence to detect V12RAS expression, revealing the full extent of invasion of oncogenic Ras-transformed cells in animals expressing V12RAS alone compared with animals co-expressing V12RAS and Δp85. In animals expressing V12RAS alone, the new population of unpigmented cells generated in the hypodermis was shown to be V12RAS positive (Fig. 7E-G). Frequent V12RAS-positive cells were also identified between skeletal muscle fascicles of the trunk and in the overlying dermis (Fig. 7F,G). The presence of V12RAS-expressing cells in each of these locations in V12RAS::Δp85 animals was generally reduced greatly (Fig. 7H), with only a few sporadic Ras-expressing cells observed between muscle fascicles (Fig. 7I,J).

**Attenuated Akt activation in V12RAS::Δp85 animals is not accompanied by decreased cell survival**

The V12RAS::Δp85 phenotype was accompanied by reduced Akt activation (Fig. 8A), demonstrating clearly that endogenous PI3K signalling was compromised. Furthermore, immunoblotting revealed equivalent expression of V12RAS in animals co-expressing V12RAS and Δp85 with compared animals with V12RAS alone, excluding the possibility that reduced V12RAS expression was responsible for the benign phenotype (Fig. 8A). Immunohistochemistry on transverse sections of adult animals expressing V12RAS alone, V12RAS and Δp85, or BRAFV600E alone revealed, in all cases, only very infrequent cells that were positive for PCNA in the integument, although PCNA positivity was detected readily in nodular lesions arising in F1 animals expressing V12RAS alone (data not shown). Thus, qualitatively, melanocyte proliferation appeared similar in all genotypes. Akt is believed to play a significant role in suppressing oncogene-induced apoptosis and thus promoting tumour cell survival (Manning and Cantley, 2007). However, we detected an equivalent number of cells with TdT-mediated dUTP nick-end labelling (TUNEL), signifying apoptosis, in the integument of animals expressing either V12RAS alone or V12RAS and Δp85 (Fig. 8B). Thus, changes in proliferation rates or apoptosis rates seem an unlikely cause of the reduced malignancy observed in V12RAS::Δp85 transgenic fish.

**DISCUSSION**

Using transgenic zebrafish models, we have demonstrated that Raf-Mek-Erk signalling is distinct among Ras effector pathways in being both sufficient and necessary for initiating melanocyte neoplasia. Both mutant BRAFV600E and the Ras effector-domain mutant V12RAS(Δ355), which maintains an ability to signal through Raf, induced benign melanocyte neoplasia (Fig. 4). By contrast, Ras effector-domain mutants that are inefficient at activating Raf (V12RAS(Δ40E) and V12RAS(INDG)) did not induce neoplasia; nor did misexpression of p110αCAAX, AktDD, RICAAX or (C1199)Tiam1 (summarised in Table 1). However, through co-expression of V12RAS and Δp85, we identified a requirement for PI3K-signalling in the initiation of melanoma by V12RAS (Fig. 5B). This is in agreement with Gupta et al. who found that recombinant mice, in which the p110α-Ras interaction was abolished, were resistant to Ras-induced tumourigenesis (Gupta et al., 2007).

Co-expression of BRAFV600E with either p110αCAAX or AktDD in melanocytes did not result in more malignant lesions than expression of BRAFV600E alone (Fig. 5D). Thus, simultaneous...
activation of Raf-Mek-Erk and PI3K-Akt signalling does not fully reconstitute oncogenic Ras signalling. This may explain why naevi in individuals with germ-line loss-of-function mutations in the PTEN gene are not reported to be at increased risk of progressing to melanoma. Both the Raf-Mek-Erk and PI3K-Akt signalling pathways individually induce senescence in primary cells (Chen et al., 2005; Michaloglou et al., 2005; Courtois-Cox et al., 2006), and this effect could even be additive resulting, potentially, in the complete suppression of neoplasia that we observed with the BRAF-AktΔp85 dual construct (Table 1). We propose that other Ras-induced signalling pathways uncouple the Raf-Mek-Erk and PI3K-Akt signalling pathways from the induction of senescence. Recombinant mouse models have indicated essential roles for RalGDS, Tiam1 and phospholipase Cε in Ras-induced skin tumour formation (Malliri et al., 2002; Bai et al., 2004; Gonzalez-Garcia et al., 2005), suggesting that multiple Ras pathways synergise to elicit neoplasia.

Although F1 transgenic zebrafish expressing BRAFV600E developed only melanocyte hyperplasia, F1 animals expressing V12RAS displayed melanocyte hyperplasia, dysplasia and invasion of loose connective tissue (RGP melanoma) (Fig. 6B; Fig. 7B), progressing spontaneously to deeply invasive (VGP) melanoma (supplementary material Fig. S6B). As such, these animals may serve as a model for familial atypical mole and melanoma (FAMM) syndrome (formerly dysplastic nevus syndrome), although germ-line mutations in the genes encoding the Ras proteins have not been linked to FAMM (Pho et al., 2006). Because PTEN inactivation or Akt gain of function are observed mainly in advanced melanoma (Haluska et al., 2006; Dahl and Guldberg, 2007), we tested whether deregulated PI3K signalling was required for the malignant conversion of V12RAS-expressing melanocytes. Again in the F1 setting, we were able to demonstrate in our forward genetic power, and amenability to transgenesis and small molecule treatment, promise to make this model a valuable tool for gaining further insight into disease mechanism and for developing new treatments.

**METHODS**

**Reagents**

HA-epitope-tagged human V12HRAS, V12HRAS effector-domain mutants (V12RAST33S, V12RASG37G, V12RASV40C), and HA-epitope-tagged murine (C1199) Tiam1 were all gifts from Angeliki Malliri (Paterson Institute for Cancer Research, Manchester, UK). Human BRAFV600E was a kind gift from Richard Marais (Institute for Cancer Research, London, UK). HA-epitope-tagged murine RlIcAAX was donated by Channing Der (University of North Carolina, NC). Myc-epitope-tagged bovine p110αCAAX was contributed by Andrew Gilmore (The University of Manchester, Manchester, UK). HA-epitope-tagged bovine AktΔp85 was donated by Jack Arbiser (Emory University, Atlanta, GA). Bovine Ap85 was a kind gift from Julian Downward (London Research Institute, London, UK). A modified pBlueScript SK+ vector, in which the multiple cloning site had been flanked by I-SceI meganuclease restriction sites, was kindly donated by Jochen Wittbrodt (EMBL, Heidelberg, Germany).

**Constructs and generation of transgenic lines**

A 744-base-pair DNA fragment, proximal to the translation initiation codon of the zebrafish homologue (isoform a) of the mammalian MITF gene, was cloned by PCR and subsequently inserted into a pGEM-T cloning vector (Promega) (sequence available upon request). Transgenes of interest, combining cDNAs with the mitfa promoter, were assembled in a modified pBlueScript vector using standard cloning techniques. To test combinations of
Ras effectors, dual expression cassettes were assembled combining cDNAs for two Ras effectors (e.g. BRAF<sup>V600E</sup> and p110αCAAX). To assess the effect of antagonising PI3K, a V12RAS expression cassette was combined with a Δp85 expression cassette. Successful cloning of transgene constructs was verified in each case by (1) restriction digest analysis (data not shown), (2) sequencing (data not shown) and (3) by transient transfection of constructs into HEK293 cells, followed by protein extraction and immunoblotting to confirm that exogenous protein(s) were generated (see supplementary material Fig. S4).

Transgenic animals were generated by co-injection of 1 nl of plasmid DNA (25 ng/μl) with 10 U I-Scel meganuclease into one-cell stage AB-strain zebrafish zygotes, using a PLI-90 picoinjector microinjection station as described previously (Thermes et al., 2002). Subsequently, animals were raised under standard conditions (28.5°C ambient temperature, 14 hour-10 hour dark-light cycle, a diet of brine shrimp and flake food) at the Biological Services Facility at The University of Manchester. Individual G<sub>0</sub> founders were backcrossed to AB animals to generate stable transgenic lines. Animals anaesthetised with MS222 (Sigma) were photographed using either an unmounted Canon Digital Ixus 80 IS camera or an Axiocam MR digital camera mounted on a Zeiss StereoLumar stereodissecting microscope. Images were processed in Adobe Photosho or using Axiovision software. Fin tissue and scales were also isolated from animals under general anaesthesia. All animal procedures were subject to local ethical review and performed under a Home Office Licence.

**Histology and immunostaining**

Adult fish euthanised by exposure to excess anaesthesia were fixed in 4% paraformaldehyde (PFA), and paraffin-embedded tissue sections (5 μm) were used for H&E staining, immunofluorescence and immunohistochemistry. H&E staining was carried out using standard methods (Thermo Shandon staining machine). For immunofluorescence, the primary antibodies used were mouse anti-Ras (1:100 dilution; BD Transduction Laboratories) and mouse anti-PCNA (1:100 dilution; Euro-Diagnostica). The secondary antibody was Vectastain biotinylated horse anti-pan-IgG. Following incubation with the secondary antibody, the sections were incubated with Cy3-labeled streptavidin (1:100 dilution; Sigma) and mounted using Vectashield with DAPI (Vectorlabs) in order to visualise nuclei. For immunohistochemistry, tissue sections were stained with mouse anti-tyrosinase (1:50 dilution; Upstate 05-647), rabbit anti-phosphoErk (1:100 dilution; Cell Signalling Technology) and rabbit anti-phosphoAkt (1:100 dilution; Cell Signalling Technology) antibodies, as per the manufacturer’s instructions. Images were captured using a Zeiss Axioplan 2 compound fluorescent microscope and Axiocam MR camera, and processed using Axiovision software. Alternatively, sections were scanned, and images were captured and processed using a Zeiss Mirax Scan system with a ×20 objective lens and a Marlin f14 6C camera. All histological samples were evaluated by a clinical pathologist (Jivko Kamarashev).

**Schmorl stain**

Paraffin sections of fish with neoplastic lesions were deparaffinised in xylene and rehydrated in descending concentrations of ethanol. The sections were incubated for 10 minutes with a working solution comprising 0.75% ferric chloride and (freshly prepared) 0.1% potassium ferricyanide in distilled water, and washed in distilled water. Melanin has the ability to reduce ferricyanide to ferrocyanide, which in the presence of ferric ions forms Prussian Blue and, therefore, cells containing melanin are stained blue. Nuclear Fast Red was used as a counterstain (2 minutes) before sections were dehydrated in ascending concentrations of ethanol. Images were captured using a Zeiss Axioplan 2 compound microscope and Axiocam MR camera, and processed using Axiovision software.

**Transmission electron microscopy**

TEM was carried out in the EM facility of the Faculty of Life Sciences at The University of Manchester. Specimen processing was performed as described previously (Toma et al., 1999). Fish with tumour nodules were sacrificed by excess anaesthetisation, and the tumours were excised and immersed in a mixture of 3% PFA and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hours at room temperature. The specimens were then cut into small pieces, immersed in the same fixative overnight at 4°C, and post-fixed in 2% osmium tetraoxide for 2 hours at 0°C. The specimens were block stained with 0.5% uranyl acetate in 50% ethanol overnight and embedded in Epon 812 resin after dehydration in a graded series of ethanol. Ultra-thin sections (70-80 nm) were cut using a Reichert Jung Ultramicrotome. TEM observations were carried out using a FEI Tecnai12 Biotwin microscope and photographs were taken using a CCD camera (Eagle, FEI Company). Images were acquired using an Imacon scanner and FlexColor software.

**Immunoblotting**

For immunoblotting, protein was extracted from caudal fin clips (n=5 for each genotype), resolved in 4-15% gradient SDS-polyacrylamide gels (Biorad), transferred to PVDF membranes (Millipore), and incubated with the following antibodies: anti-Ras (BD Transduction Laboratories), anti-phosphoAkt (Cell Signaling Technology), anti-total Akt (Cell Signaling Technology) and anti-MITF (DakoCytomation). The secondary antibody was either anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Amersham Biosciences). Proteins were visualised with ECL (enhanced chemiluminescence) plus (Amersham Biosciences), as per the manufacturer’s guidelines. All immunoblotting procedures were performed in triplicate and generated equivalent results.

**Fluorescent (F)-TUNEL assay**

Adult fish (n=3) from each of the V12RAS::GFP and V12RAS::Δp85 transgenic lines, sacrificed by excess anaesthetisation, were fixed in 4% PFA and embedded in paraffin. Transverse sections (5 μm) were used for the F-TUNEL assay. The assay was performed using the ApopTag Red in situ apoptosis detection kit (Chemicon International), as per the manufacturer's instructions. Apoptotic cells in the integument (i.e. superficial to the muscle) were counted from three sections of each animal (i.e. nine sections in total for each genotype) and the perimeter of each section was measured using Axiovision software, in order to determine the number of apoptotic cells per millimetre of integument. The measurements were analysed for statistical significance using an independent samples t-test.
Bioinformatics
Protein sequences for human and murine Ras isoforms; human ERK1 and ERK2; human AKT1, AKT2 and AKT3; human BRAF; bovine p110α; bovine Akt; murine Raf; murine Tiam1; murine RaA and RaB; murine Rac1; and bovine p85 were used to identify homologues in the zebrafish genome Zv7 sequence build (http://www.ensembl.org/Danio_rerio/index.html) by performing TBLASTN or BLASTP searches. Reciprocal best-BLAST and synteny were used to confirm orthology. Peptide sequences were aligned using ClustalW multiple alignment application of BioEdit software.

Cell culture
HEK293 cells were maintained in DMEM/F12 Glutamax (GIBCO) supplemented with 10% foetal calf serum (Lonza) and 1% penicillin-streptomycin (Lonza). The cells were transfected with 1 μg of the plasmid of interest using FuGene6 (Roche) transfection reagent, as per the manufacturer’s instructions. AB9 zebrafish fibroblasts were maintained in DMEM/F12 Glutamax supplemented with 15% foetal calf serum (Lonza), 1% penicillin-streptomycin (Lonza) and 0.6% Fungizone (GIBCO). Cells were maintained at 28°C with 5% CO2.

Cell culture immunoblotting
For immunoblotting, protein was extracted from transfected HEK293 cells, on the third day after transfection, resolved in 4-15% gradient SDS-polyacrylamide gels (Biorad), transferred to PVDF membranes (Millipore) and incubated with the following antibodies: anti-HA high affinity (Roche), anti-Ras (BD Transduction Laboratories), anti-Akt (Cell Signaling Technology), anti-Erk (Cell Signaling Technology), anti-BRAF (BD Transduction Laboratories), anti-P13K p110α (Upstate Biotechnology, Inc) and anti-β-actin (Sigma). The secondary antibody was anti-rat IgG, anti-mouse IgG or anti-rabbit IgG, all of which were conjugated to HRP (Amersham Biosciences). Proteins were visualised with ECL plus (Amersham Biosciences) according to the manufacturer’s guidelines. All immunoblotting procedures were performed in triplicate and generated equivalent results.

AB9 cells
AB9 cells were starved of serum for 24 hours before treatment with IGF-1 (Gro-Pep) for 10 minutes at a final concentration of 100 ng/μl. Negative control cells were left untreated. Immediately after the IGF-1 treatment, cells were fixed for 30 minutes at 4°C with 4% PFA, and stained with mouse anti-phosphoErk (1:400 dilution; Cell Signalling Technology) and rabbit anti-phosphoAkt (1:25 dilution; Cell Signalling Technology) antibodies, as per the manufacturer’s instructions. In order to reduce non-specific antibody binding, an extra step of avidin-biotin block (Vectorlabs) was added just before incubation with the primary antibody. Images were captured using a Zeiss Axioplan 2 compound fluorescent microscope and Axioacam MR camera, and processed using Axiovision software. The above immunocytochemical stainings were performed in triplicate and generated equivalent results.

Immunocytochemistry
AB9 zebrafish fibroblasts grown on glass coverslips were starved of serum for 24 hours before treatment with IGF-1 (Gro-Pep) for 10 minutes at a final concentration of 100 ng/μl. Negative control cells were left untreated. Immediately after the IGF-1 treatment, cells were fixed for 30 minutes at 4°C with 4% PFA, and stained with mouse anti-phosphoErk (1:400 dilution; Cell Signalling Technology) and rabbit anti-phosphoAkt (1:25 dilution; Cell Signalling Technology) antibodies, as per the manufacturer’s instructions. In order to reduce non-specific antibody binding, an extra step of avidin-biotin block (Vectorlabs) was added just before incubation with the primary antibody. Images were captured using a Zeiss Axioplan 2 compound fluorescent microscope and Axioacam MR camera, and processed using Axiovision software. The above immunocytochemical stainings were performed in triplicate and generated equivalent results.

Semi-quantitative RT-coupled PCR
Total RNA was isolated and pooled from caudal fin clips (n=3 for each genotype) using the RNAeasy mini kit (Qiagen), and reverse transcribed with an Omniscript reverse transcription kit (Qiagen) to produce cDNA. cDNA was amplified through RT-PCR using GoTaq polymerase (Promega) and primers that flank the wild-type p85 sequence of both bovine and zebrafish p85. Primer sequences are available upon request.

Clinical issue
Deregulated Ras signalling is observed in the vast majority of human solid neoplasias, exemplified by cutaneous melanoma. Mutational profiling suggests that activation of Raf-Mek-Erk signalling downstream of Ras may be crucial for initiating melanocyte neoplasia, whereas phosphoinositide 3-kinase (PI3K)-Akt signalling is involved in malignant progression. These hypotheses have not yet been fully tested in in vivo models. Treatment options for patients with advanced (metastatic) melanoma are very limited and their prognosis remains poor. Understanding the molecular basis of melanoma formation and progression should identify potential targets for the development of more effective medicines.

Results
The authors generate several transgenic zebrafish melanoma models by expressing oncogenic Ras, attenuated versions of Ras, and activated Ras effectors in their melanocytes. The ability of Ras and Ras effector mutants to induce melanocyte neoplasia confirms an important role for Raf-Mek-Erk signalling in melanoma initiation. Germline transmission of oncogenic Ras produced fish that were covered with dysplastic melanocytes, which frequently progress to melanoma, reminiscent of familial atypical mole and melanoma (FAMM) syndrome seen in humans. Malignancy was abrogated in these animals by co-expressing a P13K inhibitor, confirming a role for PI3K in melanoma progression.

Implications and future directions
The authors created and characterise zebrafish models of melanoma through disruption of Ras signalling. These fish display high penetrance and a rapid onset of melanoma. These findings and other characteristics of zebrafish, including optical clarity, forward genetic power, and amenability to transgenesis and small molecule treatment, suggest the future utility of this model in understanding and treating human cancer.

doi:10.1242/dmm.003574
Scale melanocyte number and size measurements

Adult animals representative of the genotypes of interest (wild-type, V12RAS::GFP, BRAFV600E and V12RAS::Δp85) were anaesthetised with MS222 (Sigma), and scales were then removed from their dorsum, behind the head, using fine forceps (n=6 scales for each of three animals per genotype). The isolated scales were photographed using a Zeiss StereoLumar stereo dissecting microscope and the obtained images were analysed using Axiomvision Automeasure software. This application enables the measurement of areas with a specified density (in this case the pigment in melanocytes). The results are given as pigmented areas in μm². The scales were then treated with 1 mg/ml epinephrine (Sigma) for 5 minutes and photographed using the same microscope with Axiomvision software. Epinephrine aggregates the melanosomes in the melanocytes and facilitates cell counting by making individual cells distinct (Rawls and Johnson, 2001). For each scale: (1) the melanocytes were counted and (2) the measurement of pigmented area (as measured with Axiomvision Automeasure) was divided by the number of melanocytes on the scale, and a measurement of μm²/melanocyte was obtained. A mean measurement was generated for each genotype and analysed for statistical significance using a one-way ANOVA test.

ACKNOWLEDGEMENTS

This work has been funded by a Cancer Research UK Career Development Fellowship and a Cancer Research UK Phd studentship awarded to A.H. We thank Martin Humphries, Angeliki Malliri, Claudia Wellbrock and Stephen Taylor for critically reading the manuscript. We also thank Robert Kelsh, James Lister, Stephen Johnson and Keith Hoek for helpful comments and suggestions, and Jack Arbiser for advice on phosphoErk and phosphoAkt immunohistochemical staining. The authors also thank the staff in the EM facility of the Faculty of Life Sciences at The University of Manchester for their assistance, and thank the Wellcome Trust for equipment grant support to the EM facility. In addition, we thank Steve Bagley, of the Paterson Institute Advanced Imaging Facility, for help in acquiring histology images, and thank the staff of The University of Manchester BSL for maintaining the zebrafish facility.

COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

C.M. performed the majority of experiments, analysed data and assisted in the preparation of the manuscript; M.J. generated Ras transgenic lines and certain DNA constructs; P.W. generated certain DNA constructs and provided bioinformatics support; J.K. is a dermatopathologist with expertise in melanoma and performed histological analyses of zebrafish melanocytic neoplasms; A.K. first generated and tested dual expression cassettes; A.F.L.H. instigated and devised the study and contributed with MS222 (Sigma), and scales were then removed from their dorsum, behind the head, using fine forceps (n=6 scales for each of three animals per genotype). The isolated scales were photographed using a Zeiss StereoLumar stereo dissecting microscope and the obtained images were analysed using Axiomvision Automeasure software. This application enables the measurement of areas with a specified density (in this case the pigment in melanocytes). The results are given as pigmented areas in μm². The scales were then treated with 1 mg/ml epinephrine (Sigma) for 5 minutes and photographed using the same microscope with Axiomvision software. Epinephrine aggregates the melanosomes in the melanocytes and facilitates cell counting by making individual cells distinct (Rawls and Johnson, 2001). For each scale: (1) the melanocytes were counted and (2) the measurement of pigmented area (as measured with Axiomvision Automeasure) was divided by the number of melanocytes on the scale, and a measurement of μm²/melanocyte was obtained. A mean measurement was generated for each genotype and analysed for statistical significance using a one-way ANOVA test.

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