Kinase-activating and kinase-impaired cardio-facio-cutaneous syndrome alleles have activity during zebrafish development and are sensitive to small molecule inhibitors

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The Ras/MAPK pathway is critical for human development and plays a central role in the formation and progression of most cancers. Children born with germ-line mutations in BRAF, MEK1 or MEK2 develop cardio-facio-cutaneous (CFC) syndrome, an autosomal dominant syndrome characterized by a distinctive facial appearance, heart defects, skin and hair abnormalities and mental retardation. CFC syndrome mutations in BRAF promote both kinase-activating and kinase-impaired variants. CFC syndrome has a progressive phenotype, and the availability of clinically active inhibitors of the MAPK pathway prompts the important question as to whether such inhibitors might be therapeutically effective in the treatment of CFC syndrome. To study the developmental effects of CFC mutant alleles *in vivo*, we have expressed a panel of 28 BRAF and MEK alleles in zebrafish embryos to assess the function of human disease alleles and available chemical inhibitors of this pathway. We find that both kinase-activating and kinase-impaired CFC mutant alleles promote the equivalent developmental outcome when expressed during early development and that treatment of CFC-zebrafish embryos with inhibitors of the FGF-MAPK pathway can restore normal early development. Importantly, we find a developmental window in which treatment with a MEK inhibitor can restore the normal early development of the embryo, without the additional, unwanted developmental effects of the drug.

INTRODUCTION

Perception of the RAS-RAF-MEK-ERK mitogen-activated protein kinase (Ras/MAPK) signalling components, known for their role in signalling and cancer, has been altered by the discovery that germ-line mutations underlie a series of syndromes with overlapping features. These Ras/MAPK syndromes include the genetic disorders neurofibromatosis Type I, LEOPARD syndrome, Noonan syndrome, Costello syndrome, capillary malformation arteriovenous malformation syndrome and cardio-facio-cutaneous (CFC) syndrome. The overlapping clinical features, including heart defects, distinctive facial appearances, skin and hair abnormalities, short stature and mental retardation, propelled the discovery that these syndromes are caused by germ-line mutations in the same genetic pathway, and reflect a common underlying molecular pathogenesis through mutation of core components of the Ras/MAPK signalling pathway. Characteristics that distinguish between the syndromes coupled with the now available sequence-based genetic testing, has been an important

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step forward in diagnosis and characterization of this group of disorders (1).

With refined diagnostic tools in hand, there is a need to understand the nature of the CFC mutations *in vivo* and develop therapeutic approaches. Activated in most cancers, the Ras/MAPK signalling pathway is among the key drug targets for anti-cancer therapies (2). The pathway is activated in tumour cells through many different ways, including mutation of the components themselves, often through gain-of-function mutations. For example, the BRAF oncogene is mutated in over 60% of melanomas (3). The 'addiction' of a broad spectrum of tumours to the continued activation of Ras/MAPK signalling has made it a prime target for pharmacological intervention, and specific BRAF and MEK inhibitors are currently in clinical trails (2,4–6). Although patients with Costello syndrome are prone to developing neural crest malignancies, it is not clear if CFC patients have an elevated risk of developing cancer, with only a few individuals developing neoplasms in different tissues (1). CFC BRAF mutations have a wider mutation spectrum than BRAF-nevi/cancer mutations, yet two notable similarities emerge. First, the spectrum of CFC and nevi/cancer mutations overlap, some with identical mutations. Second, CFC and nevi/cancer disease BRAF alleles result in both kinase-activating and kinase-impaired activities *in vitro* (7–10). Although *in vitro* functional assays have established the effects of the CFC mutation on kinase activity, an outstanding question is how both activating and inactivating BRAF mutations give rise to the same clinical phenotype. We wanted to establish if CFC allele mutations promote the same phenotypic outcome *in vivo*, and which mutations are sensitive to currently available MAPK-pathway inhibitors (11,12).

Animal models play an important role in furthering our understanding of the pathogenesis associated with Ras/MAPK syndromes disease alleles. Studies in transgenic *Drosophila* have shown that both loss-of-function and gain-of-function LEOPARD and Noonan syndrome *PTPN11* mutations can give rise to similar developmental phenotypes in the eye and wing veins of the fly, suggesting a rationale for how different *PTPN11* mutations can give rise to syndromes with clinically overlapping phenotypes (13,14). In zebrafish, loss of *PTPN11* (SHP-2) and expression of Noonan and LEOPARD syndrome alleles cause early cell movement phenotypes and developmental features that overlap with the principal features of the syndromes, including growth and heart defects, craniofacial abnormalities and ocular hypertelorism (15). Adult zebrafish and mouse models of H-Ras^{G12V} reveal overlapping phenotypes with Costello syndrome patients (16,17). Both models provide insight into the syndrome. The zebrafish H-Ras^{G12V} model suggests that oncogene-induced senescence contributes to the pathogenesis of Costello syndrome (16), and the H-Ras^{G12V}-induced cardiomyopathies in the mouse can be prevented with treatment with standard anti-hypertensive therapies (17). Thus, animal models can rapidly reveal the nature of the human genetic mutation *in vivo*, their impact upon development, and provide insight into evaluating new therapeutic opportunities.

Cell culture studies have shown that CFC MEK mutant alleles are sensitive to the widely used MEK inhibitor,

U0126 (12). Most CFC patients have mutations in BRAF, and it has been predicted that BRAF CFC alleles might also be sensitive to MEK inhibition (12). Given that MAPK signalling plays an important role in cell movement in zebrafish gastrulation (18), we wanted to establish the effects of BRAF and MEK CFC mutant alleles in early development. Here, we use the zebrafish system to explore the function of a panel of 28 BRAF and MEK mutant alleles in development, and to assess the potential of using small molecule inhibitors to prevent these defects. We show that the expression of both BRAF and MEK kinase-activating and kinase-impaired CFC and melanoma alleles cause similar phenotypes with significant cell movement defects in early embryogenesis. In addition, we find that the cell movement phenotypes of both the kinase-activating and kinase-impaired CFC alleles can be prevented by treatment with specific MEK inhibitors. Treatment is most effective within a developmental time window: a 1 h treatment at the start of significant convergence–extension cell movements is necessary and sufficient to prevent the CFC induced developmental effects. FGF-MAPK signalling is active during early embryogenesis, and therefore we hypothesized that the inhibition of endogenous FGFR signalling might partially prevent the developmental effects of CFC alleles in gastrulation. We show inhibition of upstream signalling is also able to restore normal development for CFC, except for the most active BRAF melanoma allele, demonstrating the importance of overall activation of the MAPK signalling during development. With sequencing-based genetic tests available to identify individuals with mutations, our work provides a rationale for varied kinase activity in the CFC allele spectrum and will contribute to the clinical discussion about the treatment strategy for individuals with CFC syndrome using currently available MAPK-pathway inhibitors.

RESULTS

CFC allele activity in zebrafish development

The Ras/MAPK pathway is highly conserved in vertebrates, and we have used the zebrafish system to examine the functional activity of CFC alleles and their response to chemical inhibition (Fig. 1), based on the important role of the FGF-MAPK pathway during early embryonic development. Within the past two decades, the zebrafish system has become established as a useful model for vertebrate developmental biology and disease (19). Organogenesis in the transparent embryos can be followed *in vivo* under the light microscope, and specific genetic and chemical models of human developmental syndromes have advanced our understanding of human disease and treatment (19–21).

Vertebrates share a conserved body plan that is established through gastrulation, the critical process that involves extensive cell movement and shapes the relatively unstructured early embryo into a gastrula with conserved germ layers (18). FGF-MAPK signalling contributes to the establishment of the dorsoventral axis, in which the highest concentration of FGF-signalling specifies the dorsal most part of the embryo and acts as a local attractive centre for convergence–extension movements within the gastrula (18). Expression of activated
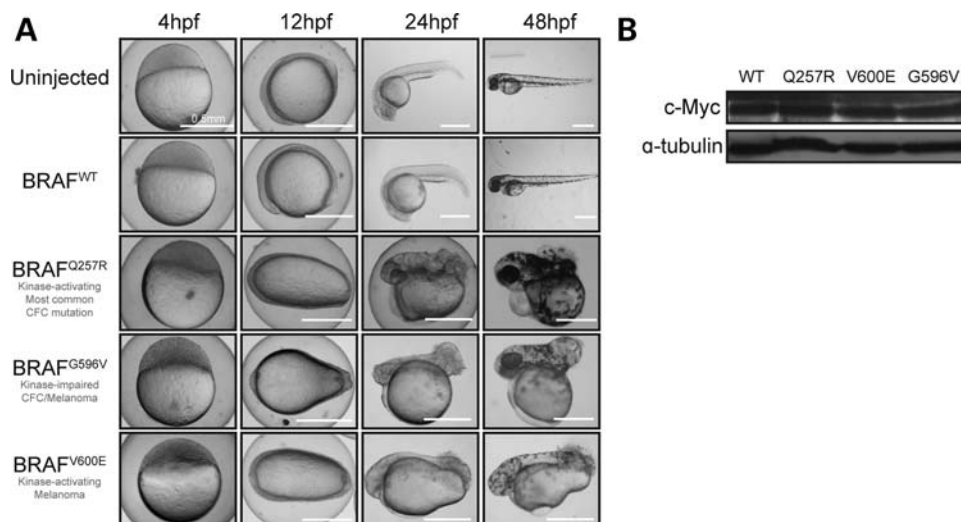
Figure 1. CFC syndrome alleles promote developmental changes during early embryogenesis. (**A**) RNA expression of CFC and melanoma variants BRAF^{Q257R} (kinase-activating, CFC), BRAF^{G596V} (kinase-impaired, CFC and melanoma) and BRAF^{V600E} (very high-kinase, melanoma) cause elongation of the developing zebrafish embryo at 12 hpf, and severe developmental abnormalities at 24 and 48 hpf. In contrast, embryos expressing BRAF^{WT} undergo normal development at all stages. No differences were detected in WT or disease allele expressing 4 hpf embryos. (**B**) Western blotting of zebrafish extracts reveals expression of the myc-tagged BRAF variants with the 9E10 antibody, and α-tubulin is a loading control.

FGF-RAS-RAF-MEK signalling in zebrafish embryo causes a loss of localized FGF-concentration that would normally promote convergence of cells towards the dorsal midline, but does not affect the continued epiboly movements and thereby results in an elongated embryo (22–24). Loss of the downstream ERK1 or ERK2 kinases in zebrafish also results in distinct convergence–extension cell migration defects during gastrulation (25), as does the expression of Noonan and LEOPARD syndrome SHP-2 alleles (15).

Building on these observations and coupled with the tractability of the zebrafish system, we reasoned that the expression of CFC mRNA in zebrafish embryos would allow us to rapidly assess the functional significance of BRAF, MEK1 and MEK2 kinase-active and kinase-impaired variants within a developmental context, and test the action of currently available FGF-MAPK-pathway inhibitors on the CFC allele phenotypic outcome (Fig. 1 and Table 1). We began by injecting mRNA into the one-cell zebrafish embryo and closely monitoring development of the effects of the high-kinase, most common variant in melanoma, BRAF^{V600E}, the kinase-impaired CFC/melanoma variant, BRAF^{G596V} and the most common kinase-activating variant in CFC syndrome, BRAF^{Q257R}. Early cell-cleavage was not affected, and initial gastrulation appeared normal. However, by 12 h post-fertilization (hpf), the embryos were highly elongated (Fig. 1A). Later stages of development showed that anterior embryonic structures still formed, but that there was a lack of tail formation, similar to ectopic MAPK signalling (22–24). In addition, the embryos expressing the high-kinase BRAF^{V600E} had a complete loss of eye development (Fig. 1A). Importantly, injection of normal human BRAF (wild-type, WT) into the embryo caused no evidence of elongation, suggesting that the expression of BRAF^{WT} does not alter normal development, even when ectopically expressed (Fig. 1A). Western blotting confirmed expression of the myc-tagged BRAF^{WT} and BRAF disease alleles (Fig. 1B).

Analysis of kinase-active and kinase-impaired CFC and melanoma alleles

The Ras/MAPK signalling pathway is highly conserved in humans and zebrafish. As in nevi/melanoma, the BRAF-CFC mutations result in both kinase-activating and kinase-impaired activities (2,7–9,26). Notably, all MEK1 and MEK2 CFC mutations are kinase active (7,27) (K.A.R., unpublished data). To assess the effects of the kinase-active and kinase-impaired melanoma and CFC alleles *in vivo*, we generated a panel of 20 BRAF and MEK disease variants in addition to the normal and engineered BRAF and MEK alleles (7,27,28) (Figs 1, 2 and Table 1), and expressed each individually by mRNA injection in the zebrafish embryo. First, we tested a panel of BRAF CFC and melanoma alleles, and found all BRAF variants promote an elongated embryonic phenotype, suggesting that *in vivo*, kinase-active and kinase-impaired BRAF alleles can promote the same developmental outcome (Fig. 2A, B and Table 1). To further test our system, we expressed normal and CFC syndrome activating MEK1 and MEK2 alleles, and also found that the disease alleles, but not the normal MEKs (MEK1^{WT}, MEK2^{WT}), promoted an elongated embryonic phenotype (Fig. 2C and Table 1). Western blotting of zebrafish embryonic lysates for total ERK protein and phospho-ERK confirmed that all BRAF and MEK alleles caused ERK activation in the zebrafish embryo (Fig. 2B, D, E). Because the BRAF^{WT}, MEK1^{WT} and MEK2^{WT} expressing embryos developed normally, but both kinase-active and kinase-impaired alleles caused altered embryonic development, this suggested to us that the *in vitro* biochemical kinase activity might not predict the potential for disease development. We found both constitutively kinase-active and kinase-inactive BRAF and MEK alleles to also promote an elongated embryonic phenotype (Fig. 2 and Table 1), suggesting that additional

Table 1. Summary of the BRAF and MEK variants expressed in zebrafish

Gene	Amino acid change	Predicted activity	Disease	Developmental phenotype in zebrafish (n)	Respond to treatment
BRAF	Wild-type	Wild-type	NA	No (0/40)	NA
	A246P	ND	CFC	Yes (31/57)	Yes
	Q257R	Kinase-activating	CFC	Yes (83/95)	Yes
	G464V	Kinase-activating	CFC	Yes (25/45)	Yes
	S467A	Kinase-activating	CFC	Yes (49/57)	Yes
	K483M	Kinase-inactivating	Engineered	Yes (34/44)	Yes
	K499E	Kinase-activating	CFC	Yes (23/44)	Yes
	G534R	Kinase-activating	CFC	Yes (33/38)	Yes
	N581D	ND	CFC	Yes (23/51)	Yes
	D594V	Kinase-impaired	Melanoma	yes (43/56)	yes
	G596V	Kinase-impaired	CFC and Melanoma	Yes (86/104)	Yes
	T599E/S602D	Constitutively active	Engineered	Yes (36/73)	Yes
	V600E	Kinase-activating	Melanoma	Yes (68/84)	Yes CI-1040 no SU-5402
	D638E	ND	CFC	Yes (66/89)	Yes
MEK1	Wild-type	Wild-type	NA	No (0/40)	NA
	F53L	Constitutively active	Engineered	Yes (16/33)	Yes
	F53S	Kinase-activating	CFC	Yes (20/33)	Yes
	T55P	Kinase-activating	CFC	Yes (26/45)	Yes
	K97M	Kinase-inactivating	Engineered	Yes (45/62)	Yes
	G128V	Kinase-activating	CFC	Yes (22/35)	Yes
	Y130C	Kinase-activating	CFC	Yes (30/45)	Yes
	S218D/S222D	Constitutively active	Engineered	Yes (45/55)	Yes
	ΔN3DD	Constitutively active	Engineered	Yes (41/60)	Yes
MEK2	Wild-type	Wild-type	NA	No (0/40)	N/A
	F57C	Kinase-activating	CFC	Yes (31/42)	Yes
	A62P	Kinase-activating	CFC	Yes (25/39)	Yes
	K101M	Kinase-inactivating	Engineered	Yes (39/52)	Yes
	G132V	Kinase-activating	CFC	Yes (45/72)	Yes
	Y134C	Kinase-activating	CFC	Yes (30/54)	Yes
	S222D/S226D	Constitutively active	Engineered	Yes (33/49)	Yes
	K273R	Kinase-activating	CFC	Yes (42/63)	Yes

NA, not applicable; ND, not determined; n, number.

interactions *in vivo*, possibly with endogenous WT BRAF and MEK, may be capable of altering normal development.

We observed control and CFC BRAF^{Q257R} expressing embryos in detail and noted initial changes to embryo shape at 7.5–8.5 hpf (Fig. 3A). As FGF-MAPK signalling is critical for cell movements during gastrulation, and Noonan and LEOPARD mutant Shp2 alleles promote defective convergence and extension cell movements (15), we examined whether early gastrulation movements were affected by the expression of BRAF and MEK disease alleles using *in situ* hybridization markers (15,25). *HggI* (hatching gland, marker of anterior–posterior axis) expression remained unaffected, whereas *dlx3* (edge neural plate marker, convergence marker) was widely modified in the embryos injected with the disease, but not normal, alleles. This indicates that the CFC and melanoma alleles disrupt cell movements during gastrulation (Fig. 3B and C), similar to expression of activated FGF-MAPK signalling in zebrafish development (22–24). The convergence phenotypes appear more severe than the Shp2 Noonan and LEOPARD syndromes alleles (15), suggesting Shp2 mutations may act differently than BRAF and MEK CFC syndrome alleles during gastrulation. The ability to detect a clear phenotypic readout that could distinguish between BRAF^{WT} and BRAF disease alleles, allowed us to use the zebrafish as the basis for further investigation of the CFC-pathway allele function.

BRAF kinase-active and kinase-impaired alleles can promote an additive effect during development

Our findings, that CFC kinase-impaired mutant alleles behave similar to kinase-active mutant alleles *in vivo*, are reminiscent of the action of both gain-of-function Noonan and loss-of-function LEOPARD Shp2 disease alleles to promote ectopic wing vein growth in *Drosophila* (13,14), and cell movement phenotypes in zebrafish (15). In zebrafish, Shp2 Noonan and LEOPARD mutant alleles are not additive, and do not lead to an increase in the number of embryos with a phenotype in early development (15). This suggests that Noonan and LEOPARD Shp2 mutations induce the same phenotype by activating or inhibiting pathway signalling. We investigated how BRAF kinase-activating and kinase-impaired alleles promote similar phenotypes during embryogenesis. We co-injected suboptimal levels of the kinase-active BRAF^{Q257R} allele, the kinase-active BRAF^{S467A} or the kinase-impaired BRAF^{G596V} allele alone or with the BRAF^{WT} allele (Fig. 4). Co-injection of BRAF CFC mutant alleles with BRAF^{WT} did not significantly affect the number of embryos with a phenotype compared with the injection of the suboptimal BRAF CFC allele alone. Only 60–66% of the embryos have an early embryonic phenotype when expressing only one BRAF CFC allele, or in combination with BRAF^{WT}. In contrast, co-injection of kinase-active BRAF^{Q257R} with kinase-active
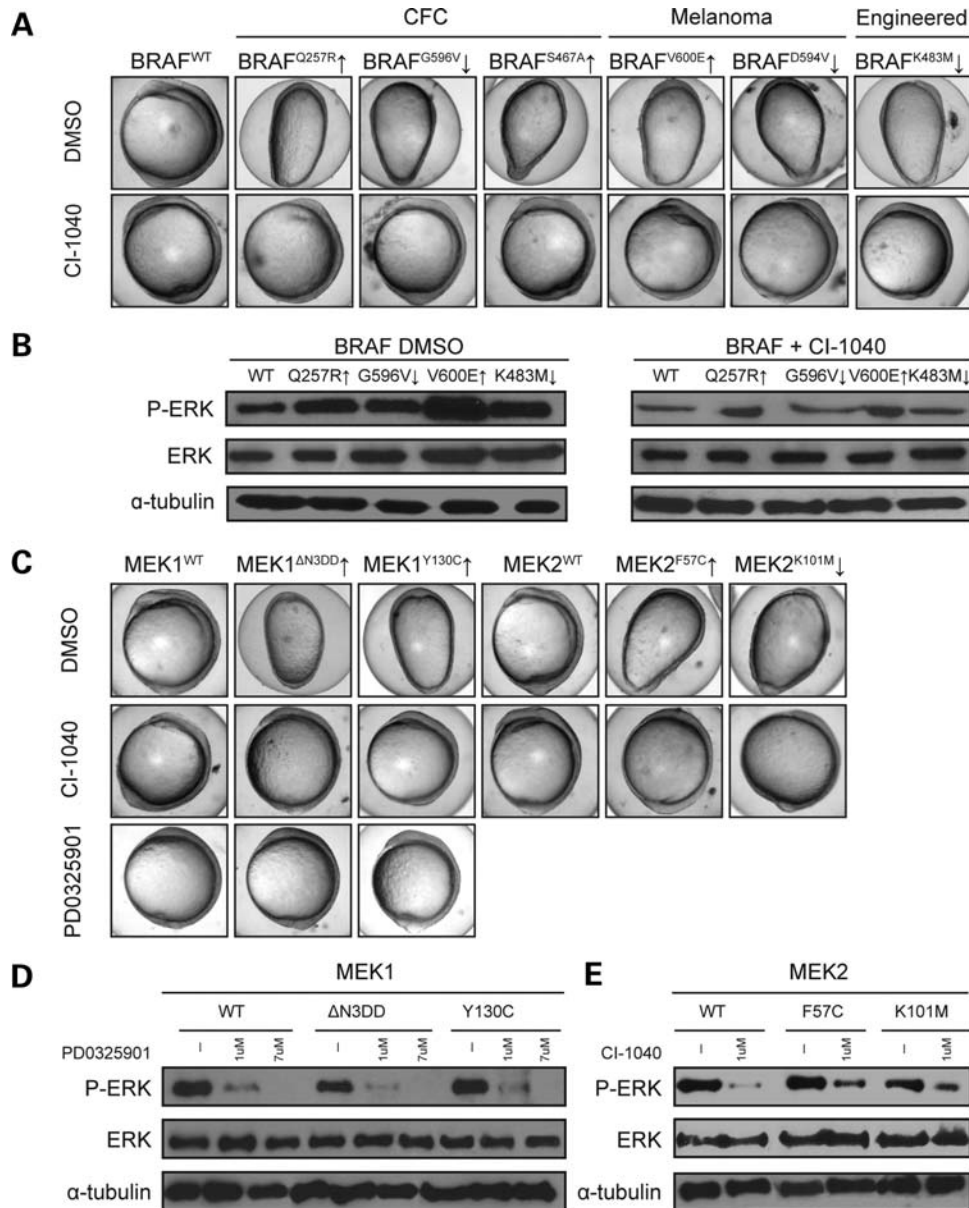
Figure 2. Analysis and treatment of kinase-activating and kinase-impaired BRAF and MEK disease variants. (**A**) RNA expression of BRAF CFC and melanoma variants, as well as engineered BRAF mutations, promotes an elongated embryo phenotype. RNA expression of wild-type BRAF has no effect on development. The developmental phenotypes caused by the BRAF variants are prevented by treatment with the MEK inhibitor, CI-1040 and the embryo has a normal rounded shape. (**B**) Western blotting of zebrafish lysates with anti-ERK and anti-phospho-ERK antibodies reveals a reduction in the ratio of phospho-ERK to total ERK protein in treated embryos, and α-tubulin is a loading control. (**C**) RNA expression of CFC MEK variants, the MEK1 constitutively activating (ΔN3DD) and the MEK2 kinase-inactivating K101M mutations promotes elongation in the developing embryo. Expression of wild-type MEK1 and MEK2 do not affect development. The developmental phenotypes caused by the MEK alleles are prevented by treatment with the MEK inhibitors, CI-1040 and PD0325901. (**D** and **E**) Western blotting of zebrafish lysates with anti-ERK and anti-phospho-ERK antibodies reveals downstream activity of phospho-ERK and confirms the potency of the MEK inhibitors, and α-tubulin is a loading control.

BRAF^{S467A} resulted in a significant enhancement of the number of embryos with an elongated phenotype. In addition, co-injection of kinase-active BRAF^{Q257R} with kinase-impaired BRAF^{G596V} also resulted in a significant increase in the number of elongated embryos. The number of embryos with a phenotype induced by BRAF$^{Q257R/S467A}$ (91.9%) or BRAF$^{Q257R/G596V}$ (88.2%) is consistent with the 82–87% of embryos that develop a developmental phenotype when the optimal dose is used (Table 1). Our results are consistent

with both the kinase-active and the kinase-impaired BRAF CFC mutations acting as gain-of-function mutations during development.

MEK inhibitors can prevent the effects of CFC alleles

We wanted to assess whether currently available MAPK-pathway inhibitors might prevent the effects of CFC disease alleles in development. Previous studies have shown that

Figure 3. BRAF and MEK disease alleles promote cell movement phenotypes. (**A**) The most common CFC varient, BRAF^{Q257R}, was expressed in developing embryos, and the embryos imaged every hour. Development of the embryos begins to become affected by BRAF^{Q257R} expression between 7.5 and 8.5 hpf. (**B**) BRAF and (**C**) MEK disease variants promote significant changes in cell movement, as revealed by *in situ* hybridization. The gene expression domain of *dlx3* is altered for both expression pattern and intensity of signal, such that there is no *dlx3* detected in the BRAF^{V600E} expressing embryos. *HggI* expression remains unaffected. The bottom panel shows the lateral view of the same embryos.

cancers with activated BRAF are highly sensitive to MEK inhibitors (29) and that CFC MEK alleles in cell culture are sensitive to the widely used MEK inhibitor, U0126 (12). However, it was unknown whether the CFC kinase-activating and kinase-impaired BRAF and MEK variants would be sensitive to the inhibition of MEK during development. We expressed BRAF CFC and melanoma alleles in developing embryos as before, and at 4 hpf added CI-1040, a clinically active MEK inhibitor that is the basis for new second-generation MEK inhibitors (5,6) to the embryo medium. We found that chemical inhibition of MEK was able to restore normal development until 10.5 hpf in all embryos expressing BRAF CFC and melanoma disease alleles (Fig. 2A). We found a similar result when we expressed CFC and engineered MEK1 and MEK2 alleles in zebrafish embryos, and treated with CI-1040 or PD0325901, a derivative of CI-1040 (Fig. 2C). With both BRAF and MEK variants, western blotting confirmed that the ratio of phosphorylated ERK to total ERK protein was reduced after chemical inhibition of MEK (Fig. 2B, D, E).

MEK signalling is critical for development, and we have previously shown that prolonged treatment (up to day 4 pf)

with the pharmacological inhibition of MEK using CI-1040 causes severe axis, heart and craniofacial developmental abnormalities (30), and we find a similar effect using PD0325901 (C.A., E.E.P., unpublished data). Although we were able to restore normal gastrulation in CFC embryos with CI-1040 and PD0325901 (Fig. 2A and C), treated embryos subsequently developed axis abnormalities associated with the effects of the inhibitor later in development (30). To circumvent this problem, we exposed the most common BRAF^{Q257R} CFC allele expressing embryos to the MEK inhibitor for 12 different treatments that varied for the time of exposure. We found that although all experimental treatments that involved adding the inhibitor early in development prevented embryo elongation (10.5 hpf) (Fig. 5A treatments A–F), a 1 h treatment within a 4.5–5.5 hpf developmental window was sufficient to restore normal development at 24 hpf (Fig. 5A treatment A), without the additional, subsequent unwanted abnormalities caused by the inhibitor (Fig. 5A treatments C–F). Treatment within this early developmental window was necessary, as a 1 h treatment later in development (Fig. 5B treatment F) was unable to prevent CFC mutant allele phenotypes, as were the treatments
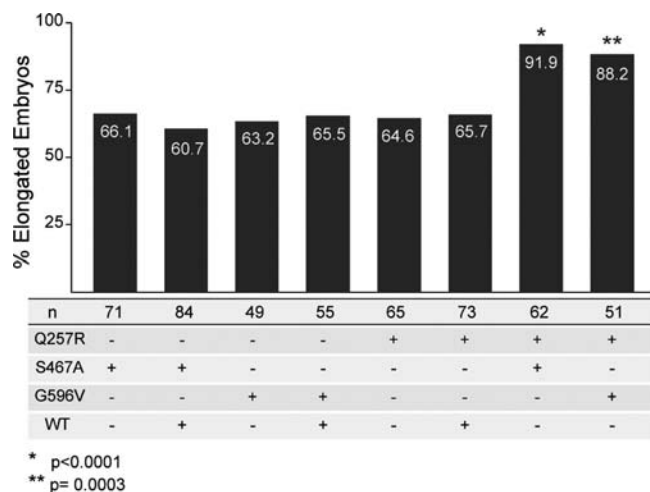
Figure 4. BRAF kinase-active and kinase-impaired alleles can promote an additive effect during development. Embryos co-expressing combinations of suboptimal doses (15 pg) of kinase-active ($BRAF^{Q257R}$, $BRAF^{S467A}$) and kinase-impaired ($BRAF^{G596V}$) CFC alleles or $BRAF^{WT}$ mRNA were assessed for the elongation phenotype at 10 hpf. The number of elongated embryos did not change significantly upon expression of a single BRAF CFC allele (15 pg), or in combination with $BRAF^{WT}$ (for a total of 30 pg). A significant increase in the mutant phenotype was induced by co-injections of $BRAF^{Q257R}$ with $BRAF^{S467A}$ ($P < 0.0001$) or $BRAF^{Q257R}$ with $BRAF^{G596V}$ ($P = 0.0003$) compared with the BRAF CFC allele co-injected with $BRAF^{WT}$ as indicated by χ^2 tests. The numbers in the bars indicates the percentages of elongated embryos; n is the number of injected embryos.

that had CI-1040 throughout the experiment, with the exception of the 4.5–5.5 hpf developmental window (Fig. 5B treatment B).

Distinguishing between BRAF alleles for sensitivity to pathway inhibition

The ability to treat kinase-active and kinase-impaired CFC embryos suggests correction of signalling downstream of the CFC mutation is sufficient to restore normal development. This is consistent with the sensitivity of cancers expressing RAS and RAF high-kinase oncogenes to MEK inhibitors (5,6,29), and the sensitivity of CFC MEK alleles to MEK inhibition in cell culture (12). FGF-MAPK signalling plays an important role during embryonic cell movements in zebrafish. Unlike in a cancer cell with a BRAF mutation, a developing animal with a BRAF or MEK CFC mutation also has endogenous FGFR signalling. We hypothesized that total MAPK signalling may underlie CFC syndrome development and that suppression of endogenous FGFR signalling could partially suppress the activity of the effects of the downstream CFC alleles. As with the MEK inhibitor, we treated 4 hpf embryos expressing BRAF (Fig. 6A) and MEK (Fig. 6B) CFC and melanoma mutant alleles with SU-5402, an inhibitor of the FGF receptor 1 (FGFR1). Western blotting confirmed reduction of phosphorylated ERK after SU-5402 treatment (Fig. 6C). We found the development to be normal for all treated embryos expressing BRAF and MEK disease alleles at 10.5 hpf, with the exception of the very high-kinase activity melanoma mutant $BRAF^{V600E}$ (Fig. 6A and B). The most common mutation in melanoma and nevi, $BRAF^{V600E}$ is one

of the highest kinase activity mutants, has transforming activity and is sufficient to promote nevi and melanoma development, as well as other cancers, in animal models (3,26,31–34). This suggests that total levels of MAPK signalling may be responsible for the action of the CFC alleles, and reduction of either endogenous FGF signalling or downstream MEK signalling can prevent some of the pathological function of the alleles.

DISCUSSION

Our study addresses the *in vivo* action of CFC mutant alleles and may point to a potential therapeutic approach for individuals with CFC syndrome. First, we have demonstrated that CFC mutant alleles cause similar developmental phenotypes in an *in vivo* zebrafish model system, despite their *in vitro* kinase activity. Second, we have used our model system to explore the therapeutic potential of small molecule inhibitors to prevent the *in vivo* activity of CFC mutations during early development. We have evaluated both the developmental activity and the therapeutic potential of 18 human CFC and three melanoma disease alleles, as well as three different small molecule inhibitors, in 12 treatment conditions. In this work, zebrafish embryos are injected at the single cell stage with RNA of the human disease allele, or with control RNA, and the phenotype of the embryo assessed by 10 h (Figs 1, 3 and 7A). Embryos normally express FGF-MAPK signalling during development in a localized manner to shape the development of the embryos during gastrulation (18). We found BRAF and MEK kinase-active and kinase-impaired disease variants interfere with convergence–extension cell movements during gastrulation (Fig. 1), providing insight into how similar clinical CFC phenotypes are caused by kinase-activating and kinase-impaired alleles. Future studies will reveal how the effects on early cell movement (Fig. 1) correlate with disease allele penetrance and disease presentation in humans.

In our *in vivo* animal system, and in the context of endogenous signalling, we find CFC alleles with kinase-inactivating mutations, as defined *in vitro*, promote the same phenotype as kinase-active alleles. One possibility is that BRAF kinase-impaired proteins interact with CRAF to stimulate MEK-ERK signalling (9,10,35). Kinases frequently act through dimerization, including BRAF and CRAF (10,36), and crystal structures of MEK predict MEK1 and MEK2 self associate via a homodimerization interface to form stable dimers (37). Such mechanisms may be at work in our zebrafish studies, providing the molecular context for kinase-impaired BRAF and MEK alleles to be able to promote active signalling of the pathway, including the engineered kinase-inactive alleles (36) as determined by *in vitro* kinase assays (Fig. 3). Another possibility is that dysregulation of Ras/MAPK signalling through gain-of-function or loss-of-function mutations may cause similar disease phenotypes (38). As an important example, the disease spectrum associated with varying SHP-2 mutations in Noonan syndrome and cancer argue against SHP-2 activity as the defining predictor of disease outcome (39). Both loss-of-function and gain-of-function mutations in SHP-2 lead to the clinically
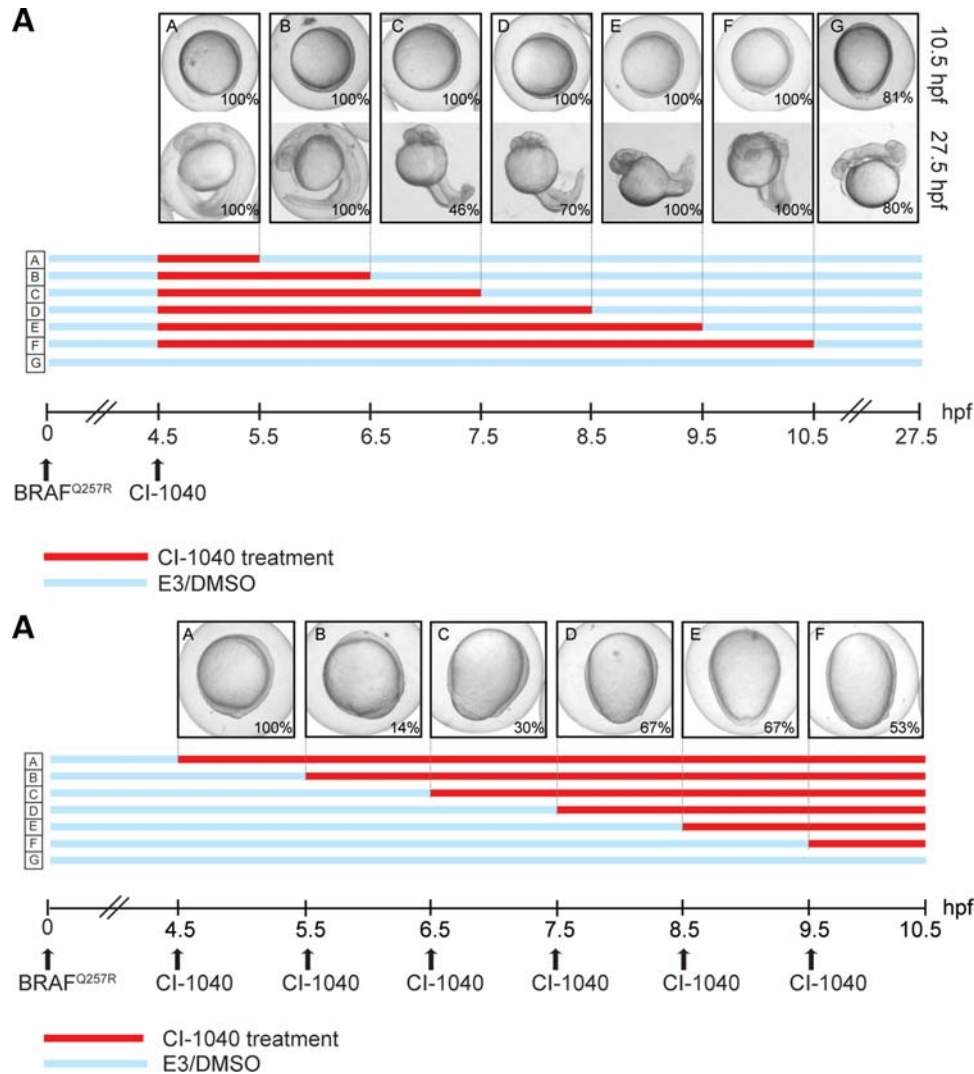
Figure 5. Identification of a zebrafish-CFC treatment window. (**A**) Six CI-1040 treatments (A−F) and a control treatment (G) for zebrafish embryos expressing CFC variant BRAF^{Q257R} reveal that all drug treatments restore normal development at 10.5 hpf (treatments A−F), whereas only treatments A and B restore normal development both at 10.5 and 27.5 hpf. Other drug treatments (C−F) cause additional developmental defects at 27.5 hpf, most notably, reduced posterior development. (**B**) Six additional drug treatments (A−F) and a control treatment (G) for zebrafish-CFC variant BRAF^{Q257R} show that the 4.5−5.5 hpf developmental window is necessary for the prevention of zebrafish-CFC early phenotypes. The percentage of embryos displaying the phenotype is given at the lower right of each image (a minimal of $n = 30$/experiment). Blue bars represent embryo medium, whereas red bars represent CI-1040 treatment.

similar LEOPARD and Noonan syndromes, and expression of LEOPARD and Noonan syndrome alleles in zebrafish and *Drosophila* produce equivalent developmental phenotypes (15–17). Our work suggests that both kinase-active and kinase-impaired CFC alleles are effectively gain-of-function mutations and activate the pathway because combinations of active and impaired BRAF mutant alleles can promote an additive effect during early development (Fig. 4).

Designing new therapies for rare birth disorders is problematic due to the great costs and research efforts of drug development, and the required clinical safety and efficacy testing for new therapeutics (40). Since the Ras/MAPK pathway has been a prime target for cancer therapeutics, application of these small molecule inhibitors presents a possible therapeutic avenue, since the underlying molecular dysfunction is common. Previously, the activity of CFC

MEK alleles has been shown to be sensitive to MEK inhibitors in cells (12). Direct testing of the effects of anti-cancer therapeutics on BRAF and MEK CFC characteristics in zebrafish is an important next step in exploring the therapeutic potential for CFC syndrome. Using our model, we have tested the ability of FGF-MAPK inhibitors to prevent the developmental effects of CFC and melanoma disease alleles (Fig. 7B). We found that MEK inhibitors prevent the cell migration defects caused by the disease alleles, and also that additional developmental side-effects of the drug could be avoided by treating the embryos within a specific developmental time-window (Fig. 5A). These results suggest that future studies in pre-clinical models of CFC should explore if similar drug treatment time windows may help ease the developmental abnormalities and symptoms associated with CFC progression. However,
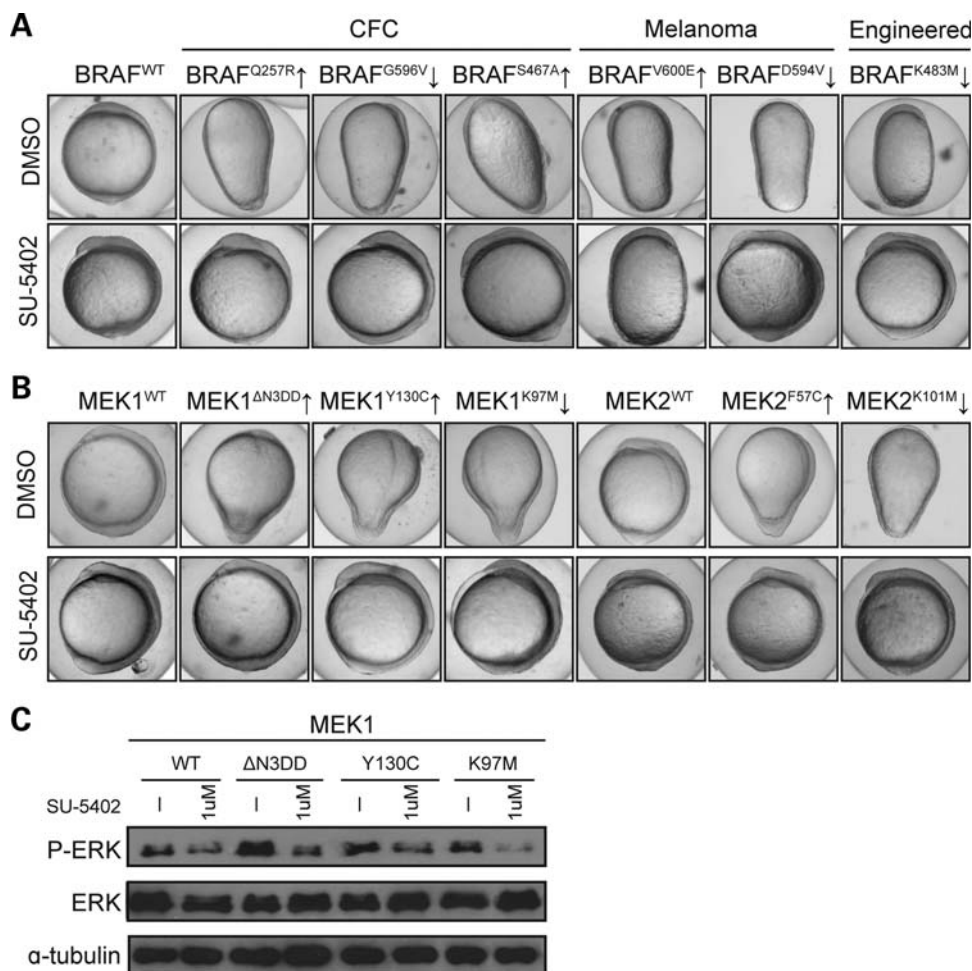
Figure 6. Treatment of BRAF, MEK1 and MEK2 variants with SU-5402. (**A**) The mutant phenotypes promoted by the RNA expression of BRAF variants are prevented by pharmacological treatment with the FGFR1 inhibitor SU-5402, with the exception of the developmental phenotype caused by BRAF^{V600E}. (**B**) Similarly, the elongation promoted by MEK1 and MEK2 disease variants is prevented by SU-5402 treatment. (**C**) Western blotting of total zebrafish lysates for ERK and phospho-ERK protein shows that SU-5402 treatment causes reduction of ERK phosphorylation, with α-tubulin as a loading control.

because CFC mutations affect gastrulation (Fig. 1–3), and have an early developmental treatment window (Fig. 5), application of MEK inhibitors for CFC syndrome patients may be severely limited. Nonetheless, because CFC syndrome has a progressive phenotype, and many of the phenotypic effects develop post-natally, patients may still be helped by systemic therapies after birth (1).

We also provide evidence that the developmental effects of the disease alleles can be prevented by the inhibition of endogenous FGFR-signalling, with the exception of one of the highest kinase-activating melanoma mutations, BRAF^{V600E} (Fig. 6). We reason that as normal gastrulation involves endogenous FGFR signalling, FGFR inhibition reduces the total level of defective CFC BRAF or MEK signalling, thereby preventing the altered cell movement phenotype. This supports the idea that total MAPK signalling is important in CFC development (Fig. 4), and also emphasizes the importance of testing the action of developmental syndrome mutant alleles and inhibitors in a developing animal. *In vitro*, the CFC BRAF^{Q257R} and melanoma BRAF^{V600E} mutant alleles both promote similar high-kinase activity (7), and yet no individual

with CFC syndrome has been identified with a BRAF^{V600E} mutation. This demonstrates, for the first time, that the BRAF^{V600E} mutation is probably stronger *in vivo* than the CFC mutations.

The high conservation of the MAPK signalling pathway means that our CFC chemical-genetic studies in zebrafish embryos will be relevant to the development of future preclinical models of CFC. For example, mice exhibiting Apert-like syndrome from dominant mutations in fibroblast growth factor receptor-2 can be treated pre- and post-natally with the small molecule MEK inhibitor, U0126 (41). We note, however, that similar comprehensive CFC allele comparisons, coupled with multiple treatment testing, within the short-time span described here, is not currently feasible in mouse models. This makes the zebrafish system a tractable tool for medical and research geneticists to explore allele activity and therapeutic potential. This work establishes a foundation to propel forward the clinical discussion and scientific strategy for assessing the suitability of using currently available cancer drugs to treat the progressive phenotypes of CFC in children.
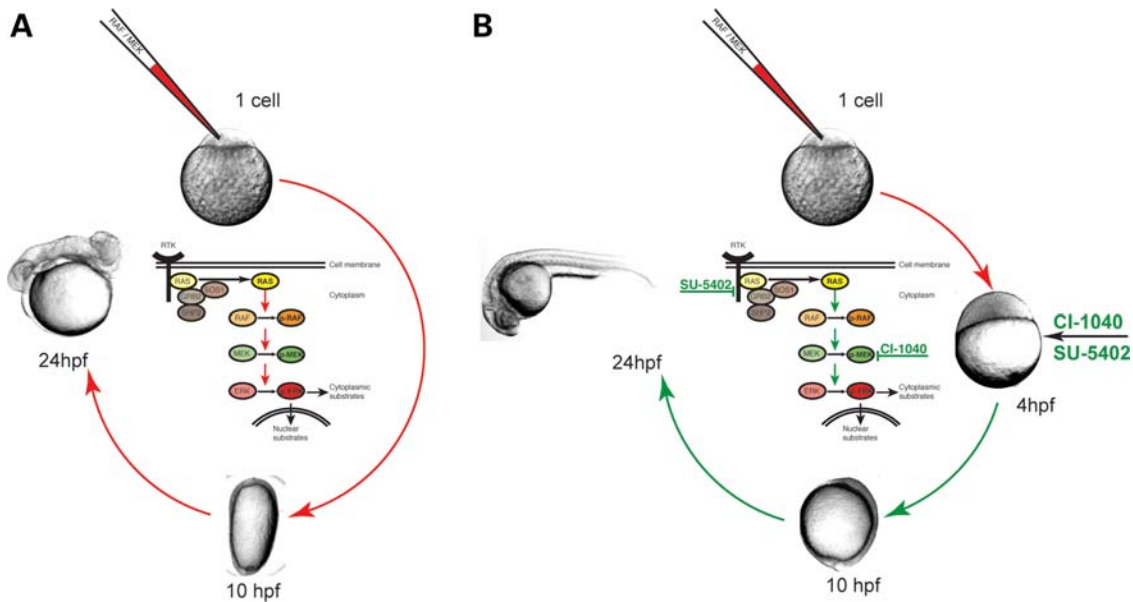
Figure 7. Evaluation of CFC-disease variant *in vivo* activity and potential treatment. Schematic representation of the zebrafish-based approach designed to examine the *in vivo* significance of BRAF and MEK CFC disease mutations. (**A**) Microinjection of BRAF or MEK CFC variant mRNA into the single-cell zebrafish embryo promoted an elongated zebrafish embryo at 10 hpf that gives rise to an animal with severe development defects, including axis formation, and heart defects at 24 hpf (red arrow). (**B**) Treatment of CFC-microinjected embryos with inhibitors of the FGF-MAPK signalling pathway (green) restores normal development to the CFC-zebrafish embryo, possibly by restoring appropriate total levels of MAPK-signalling (green arrows).

MATERIALS AND METHODS

Animal husbandry

Adults and zebrafish embryos were raised and maintained at 28.5°C. Embryos were acquired by pair matings of AB* and TL zebrafish lines.

Cloning and RNA production

Patient and engineered BRAF, MEK1 and MEK2 DNA were cloned into pENTR 3C (Invitrogen), and using the Gateway® (Invitrogen) technology the DNA sequences were subcloned into the pDEST17 (Invitrogen) vector. Expression vectors were linearized, and *in vitro* transcription of synthetic capped mRNA was performed using the T7 RNA polymerase mMESSAGE mMACHINE Kit (Ambion).

Microinjection of embryos

Injections were performed on WT zebrafish embryos using a nitrogen-powered Picospritzer III microinjector (Intracel) conjugated to a Nikon SMZ 1000 stereomicroscope. One-cell stage embryos were injected with 35 pg (optimal) or 15 pg (suboptimal) of capped mRNA and were monitored until throughout the first 24 h of development.

Pharmacological inhibition of FGF and MAPK signalling

To test the prevention of the mRNA-promoted phenotype, 4 hpf embryos injected with mRNA were treated with small molecule inhibitors. To inhibit FGFR1 activity, embryos were incubated in SU5402 (Calbiochem) at 1 µM in E3 embryo medium (42) at 28.5°C in the dark. To inhibit MEK1/2, embryos were treated with 1 µM CI-1040 and 1or 7 µM PD-0325901 (University of Dundee) in E3 embryo medium at 28.5°C as previously described (30).

Whole-mount RNA in situ hybridization

Embryos collected at the tail-bud stage were fixed overnight in 4% parafolmaldehyde/PBS at 4°C, were hand-dechorionated and dehydrated overnight in methanol at −20°C. *In vitro* transcribed digoxigenin-labelled antisense RNA probes were synthesised (Roche). *Dlx3* and *HggI* riboprobes (15) and whole-mount *in situ* hybridization were carried out following previously described protocols (43). Anti-digoxigenin anti-serum alkaline phosphatase was incubated in a 1:5000 dilution overnight and the samples were washed in BCL3 solution (1 M Tris pH 9.5, 5 M NaCl, 0.5 M $MgCl_2$, 20% Tween 20). The embryos were, subsequently, stained in 500 µl of BM Purple alkaline phosphatase (Roche) for 30–45 min and the reaction was stopped in 20 mM EDTA/PBS. Processed embryos were imaged using a Nikon SMZ1500 stereomicroscope in conjunction with a Nikon Coolpix 5400 camera.

Protein blotting

Embryo buffer was removed, and tail-bud stage embryos were frozen at −80°C. Samples were ribolyzed for 5 s in protein extraction buffer [2 M Tris pH 7.5, 5 M NaCl, 1% NP40, Na deoxycholate, 10% SDS, 0.5 M NaF, 1 M β-glycosyl phosphate, protease inhibitor cocktail tablet (Roche)]. The protein content was measured and the samples were normalized. Total protein extracts were analyzed by western blotting, probed with antibodies raised in rabbit [p44/42 MAPK (1:2000) (Cell Signaling)] and in mouse [phospho p44/42

MAPK (E10) (1:2000), c-myc (9E10) (1:2000) (Sigma), α-tubulin B-5-1-2 (1:50000) (Santa Cruz)]. Secondary antibodies conjugated to horseradish peroxidase were used to detect the proteins.

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