Overexpression of Fgfr2c causes craniofacial bone hypoplasia and ameliorates craniosynostosis in the Crouzon mouse

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ABSTRACT

FGFR2c regulates many aspects of craniofacial and skeletal development. Mutations in the FGFR2 gene are causative of multiple forms of syndromic craniosynostosis, including Crouzon syndrome. Paradoxically, mouse studies have shown that the activation (Fgfr2cC342Y; a mouse model for human Crouzon syndrome), as well as the removal (Fgfr2cnull), of the FGFR2c isoform can drive suture abolution. This study aims to address the downstream effects of pathogenic FGFR2c signalling by studying the effects of Fgfr2c overexpression. Conditional overexpression of Fgfr2c (R26RFgfr2c;act) results in craniofacial hypoplasia as well as microtia and cleft palate. Contrary to Fgfr2cnull and Fgfr2cC342Y, Fgfr2c overexpression is insufficient to drive onset of craniosynostosis. Examination of the MAPK/ERK pathway in the embryonic sutures of Fgfr2cC342Y and R26RFgfr2c;act mice reveals that both mutants have increased pERK expression. The contrasting phenotypes between Fgfr2cC342Y and R26RFgfr2c;act mice prompt us to assess the impact of the Fgfr2c overexpression allele on the Crouzon mouse (Fgfr2cC342Y), in particular its effects on the coronal suture. Our results demonstrate that Fgfr2c overexpression is sufficient to partially rescue craniosynostosis through increased proliferation and reduced osteogenic activity in E18.5 Fgfr2cC342Y embryos. This study demonstrates the intricate balance of FGF signalling required for correct calvarial bone and suture morphogenesis, and that increasing the expression of the wild-type FGFR2c isoform could be a way to prevent or delay craniosynostosis progression.

KEY WORDS: FGFR2, FGF, Craniosynostosis, Cleft palate, Crouzon, ERK

INTRODUCTION

Normal craniofacial development is a precisely coordinated process that involves the modelling of a framework supporting the soft tissues of the head, in particular the brain. During normal development, growth of the brain is possible because suture tissue separates the calvarial bones and allows for skull vault expansion. Craniosynostosis, a common birth defect with an incidence of 1:2500, is characterised by the loss of suture tissue followed by premature fusion of calvarial bones. This results in the restriction of brain growth and is often associated with dramatic dysmorphology of the skull and face. A significant number of craniosynostosis cases are syndromic and associated with additional skeletal phenotypic features. Many of these syndromes are caused by mutations in FGF pathway genes (Johnson and Wilkie, 2011).

FGF signalling is important for cellular proliferation, differentiation and survival. Receptor activation allows signals to be conveyed through the RAS-MAPK, P13K-AKT and PLCγ-PKC cascades via a series of protein intermediates (Ornitz and Itoh, 2015; Eswarakumar et al., 2005). FGF receptors (FGFRs) are highly conserved receptor tyrosine kinases (RTKs) located on the cell membrane with intracellular and extracellular domains. Tissue specific isoforms are produced by alternative splicing, affecting the third extracellular immunoglobulin-like loop (DIII) of FGFR1-3. The FGF ‘IIb’ and ‘IIc’ isoforms differ only in the C-terminal half of DIII, which is encoded by exons 8 or 9 for the IIb and IIc splice forms, respectively (Eswarakumar et al., 2005; Ornitz and Itoh, 2015). Furthermore, the expression of the isoforms are tissue specific and critical for establishing paracrine reciprocal signalling loops: while the IIb isoform is commonly expressed in epithelial cells and receives FGF ligand from the mesenchyme, the IIc is expressed in mesenchymal cells and receives FGF ligand from the neighbouring epithelium (Orr-Urtreger et al., 1993).

Mutations affecting the FGFR2 gene can cause a spectrum of craniofacial phenotypes commonly associated with growth dysplasia, mid-facial hypoplasia, coronal synostosis, orbit dysmorphology and cleft palate (Wilkie, 1997). Notably, synostosis of the coronal suture is a hallmark of human Crouzon and Pfeiffer syndromes (Johnson and Wilkie, 2011). Crouzon syndrome is most commonly caused by a point mutation in exon 9 of the FGFR2 gene, is autosomal dominantly inherited and exclusively affects the IIc isoform (Reardon et al., 1994). The substitution of a cysteine to a tyrosine residue (p.C342Y) results in the stabilisation of intermolecular disulphide bonds in the receptor extracellular domains that lead towards ligand independent receptor activation, and is often referred to as a gain-of-function (GOF) mutation (Reardon et al., 1994).

In the developing mouse cranial vault, Fgfr2 is expressed in the osteogenic fronts of the calvarial bones, but the specific cellular localisation of the different FGFR2 isoforms remains elusive due to their high sequence homology (Johnson et al., 2000; Iseki et al., 1997). FGFR2c function is commonly associated with craniofacial and skeletal development, as genetic mutation in mouse models leads to a series of craniofacial malformations and additional skeletal dysmorphology (Lee et al., in press). Deletion of the Fgfr2c isoform (Fgfr2cnull) results in skeletal hypoplasia and craniosynostosis owing to a series of craniofacial malformations and additional skeletal dysmorphology (Lee et al., in press). Deletion of the Fgfr2c isoform (Fgfr2cnull) results in skeletal hypoplasia and craniosynostosis owing...
to imbalances in osteoprogenitor proliferation and differentiation in the endochondral and intramembranous skeleton (Eswarakumar et al., 2002). The most common coding mutation responsible for human Crouzon syndrome has been introduced into the mouse (\textit{Fgfr2c}\textsuperscript{C342Y}) to study the pathogenesis of the disease in more detail (Eswarakumar et al., 2004). The craniofacial phenotype in these mice includes brachycephaly due to postnatal coronal craniosynostosis, as well as a short snout caused by mid-facial hypoplasia (Eswarakumar et al., 2004). Contrary to \textit{Fgfr2c}\textsuperscript{null} mice, increased numbers of osteoprogenitor cells were observed but, paradoxically, synostosis of the coronal suture was also present (Eswarakumar et al., 2004). Recently, it was shown that increased levels of MAPK/ERK signalling downstream of FGFR2c are present in suture osteoprogenitors in \textit{Fgfr2c}\textsuperscript{C342Y}, but not \textit{Fgfr2c}\textsuperscript{null}, mice (Pfaff et al., 2016). In addition, hemizygous mutants (\textit{Fgfr2c}\textsuperscript{C342Y/−}) show a more severe craniofacial phenotype, demonstrating that lowering the \textit{Fgfr2c} expression levels does not alleviate the features associated with FGFR2c activation (Pfaff et al., 2016). Altogether, the mechanism behind pathogenic FGFR2c signalling in the coronal suture is complex, as indicated by the observation that coronal synostosis is a notable phenotype to both activation (\textit{Fgfr2c}\textsuperscript{C342Y}) and loss (\textit{Fgfr2c}\textsuperscript{null}) of FGFR2 signalling (Eswarakumar et al., 2002, 2004). Furthermore, analysis of the cleft palate phenotype in \textit{Fgfr2c}\textsuperscript{C342Y} mice demonstrates that activation, as well as inhibition, of GF receptor signalling causes delayed palatal shelf growth and elevation (Snyder-Warwick et al., 2010). These results imply that the downstream interpretation of FGFR2c signalling can be different from the activity of the receptor itself, and the phenotype elicited might not be a direct translation of an overactive FGFR2c signalling pathway.

The complexities of FGFR2c signalling prompted us to delineate the molecular basis for signalling misregulation further by studying \textit{Fgfr2c} receptor overexpression using a conditional allele that allows tissue-specific induction (i.e. \textit{R26R\textit{Fgfr2c}\textsuperscript{lox/lox}}). This study reports that the phenotypic consequences of \textit{Fgfr2c} overexpression show similarities as well as differences to those found in \textit{Fgfr2c}\textsuperscript{C342Y} and \textit{Fgfr2c}\textsuperscript{null} mice. Here, we interrogated the biochemical and transcriptional FGFR2 pathway activity in the coronal sutures prior to the onset of synostosis, and show that both constitutive activation and overexpression of FGFR2c results in increased levels of ERK phosphorylation \textit{in vivo}. Furthermore, we provide evidence that increasing the expression of a wild-type (WT) \textit{Fgfr2c} allele can ameliorate the craniosynostosis phenotype in \textit{Fgfr2c}\textsuperscript{C342Y} Crouzon mice.

**RESULTS**

**\textit{Fgfr2c} overexpression causes growth restriction, microtia and cleft palate**

To assess which tissues are sensitive to increased FGFR2c signalling, \textit{R26R\textit{Fgfr2c}\textsuperscript{lox/lox}} mice were crossed with $\text{β}\text{actin}\text{CRE}$ (also known as $\text{Actb}\text{CRE}$) mice to drive ubiquitous \textit{Fgfr2c} overexpression (Fig. 1A). Ubiquitous overexpression of the \textit{R26R\textit{Fgfr2c}} allele (i.e. \textit{R26R\textit{Fgfr2c}\textsuperscript{lox/lox}}, $\text{β}\text{actin}\text{CRE}$, abbreviated to \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}}) led to a total upregulation of \textit{Fgfr2c} transcripts close to 2-fold ($n=3$) as shown by quantitative PCR (Fig. S1A) at embryonic day (E)12.5. Transgenic FGFR2c-V5 protein expression was validated at E12.5 by immunoblotting showing transgenic protein in the CRE-positive animals only (Fig. S1B). Subsequently, \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} embryos were examined at E18.5 for their size [head length and crown-rump length (CRL)] and weight (Fig. 1C-E). Results were expressed as an average percentage change (Av. Δ%) relative to controls (\textit{R26R\textit{Fgfr2c}\textsuperscript{lox/lox}}). Ubiquitous \textit{Fgfr2c} overexpression ($n=9$) led to a significant reduction in the head length [Av. Δ 7.19%; $P<0.0001$; t(17.78)=7.74], CRL [Av. Δ 4.83%; $P=0.0018$; t(13.23)=3.90] and weight [Av. Δ 12.26%; $P=0.0001$; t(22.16)=5.39] compared with that of controls (\textit{R26R\textit{Fgfr2c}\textsuperscript{lox/lox}}, $n=17$) (Fig. 1C-E). Additionally, \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} mice displayed microtia characterised by a smaller or absent pinna ($n=6/6$) (Fig. 1B). To assess whether decreased size was caused by generalised defects of the whole skeleton, we analysed limb length as a proxy for this (Fig. S2). No difference in the size of the limbs ($n=13$) was detected, suggesting that the overall size reduction was mostly due to reduced head size. Whole-mount skeletal staining of the head showed that mutants had craniofacial dysmorphology, showing notable disruptions to the tympanic ring of the middle ear ($n=9/10$) and a cleft palate ($n=4/10$) (Fig. 2A). As different parts of the calvaria are derived from either the neural crest,

![Fig. 1: Gross phenotype of Fgfr2c overexpression in E18.5 mutants.](image-url)

- **(A)** Breeding strategy for conditional Fgfr2c overexpression.
- **(B)** Microtia (arrowhead+inset) was observed in \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} only.
- **(C)** Skeletal hypoplasia in \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} ($P<0.0001$).
- **(D)** Craniofacial hypoplasia in \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} ($P<0.0001$).
- **(E)** \textit{R26R\textit{Fgfr2c}\textsuperscript{loc/loc}} and \textit{R26R\textit{Fgfr2c}\textsuperscript{Wnt1}} display significant reductions in weight ($P<0.0001$ and $P=0.0028$, respectively). Statistics: Student’s t-test with Welch’s correction. Scale bars: 5 mm.
As all observed phenotypic features in R26R<sup>Fgfr2c</sup>-flox animals were subsequently crossed with Wnt1<sup>CRE</sup> or Mesp1<sup>CRE</sup> to ask which cell types are responsible for the observed phenotypes, and to eliminate any potential ectopic effects generated by the ubiquitous β<sup>act</sup>CRE line. To assess the promoter activity of the different CRE lines specifically in calvarial tissues, we investigated conditional fluorescent alleles (i.e. mTmG and YFP). We found that R26RF<sup>Fgfr2c</sup>β<sup>act</sup> embryos displayed palatal shelf hypoplasia (data not shown) (Fig. 2A). In one of six embryos exhibited an overt cleft, whilst three of six R26RF<sup>Fgfr2c</sup>β<sup>act</sup> embryos. Tympanic ring hypoplasia is exclusive to R26RF<sup>Fgfr2c</sup>β<sup>act</sup> embryos (red arrowhead). No apparent phenotype was observed in R26RF<sup>Fgfr2c</sup>β<sup>act</sup>Mesp1 mice. (B,C) Quantitative analysis of the frontal and parietal bones. Significant reductions of the frontal bone were observed in both R26RF<sup>Fgfr2c</sup>β<sup>act</sup> and R26RF<sup>Fgfr2c</sup>Wnt1 embryos. Student’s t-test with Welch’s correction. F, frontal bone; P, parietal bone. Scale bars: 1 mm.

**Fgfr2c overexpression causes anterior, NC-derived bone hypoplasia**

As all observed phenotypic features in R26R<sup>Fgfr2c</sup> mice seem to affect tissues in the head, we assessed the craniofacial morphology in more detail at E18.5 using Alcian Blue/Alizarin Red whole-mount skeletal staining, and quantified the size and shape of individual craniofacial bones. The results were expressed as an average percentage change in the mutants relative to the controls (R26RF<sup>Fgfr2c</sup>β<sup>act</sup>flox<sup>+</sup>), normalised to 100% (Av.Δ%). Frontal bones of R26RF<sup>Fgfr2c</sup>β<sup>act</sup> skulls were significantly smaller [Av.Δ 11.4%; P<0.0001; t(22.98)=6.99; n=9] (Fig. 2B,C), as were other NC derivatives, such as nasal bone [Av.Δ 12.90%; P<0.0001; t(22.06)=7.91; n=10] and mandible [Av.Δ 5.77%; P<0.0001; t(20.64)=7.30; n=9] (Fig. S4). No significant reduction was observed in the mesoderm-derived parietal bone. Similarly, R26RF<sup>Fgfr2c</sup>β<sup>act</sup>;Wnt1 embryos followed a comparable trend, with significant decreases in NC derivatives: frontal bone [Av.Δ 9.12%; P=0.0131; t(6.89)=3.32; n=6], nasal bone [Av.Δ 7.91%; P=0.0024; t(9.077)=4.17; n=6] and mandible [Av.Δ 3.71%; P<0.0007; t(9.96)=4.82; n=6]. Again, no size difference was seen in the mesoderm-derived parietal bone (n=6). As expected, R26RF<sup>Fgfr2c</sup>β<sup>act</sup>Mesp1 mice, calvarial bone sizes were unaffected. In summary, Fgfr2c overexpression causes bone hypoplasia of anterior, NC-derived bones, explaining the overall reduction of craniofacial dimensions.

**Fgfr2c overexpression does not cause coronal craniosynostosis**

Coronal synostosis is a hallmark of Crouzon and Pfeiffer syndromes, and can be observed in the Fgfr2c<sup>C342Y</sup> mouse model as well as in Fgfr2c<sup>null</sup> mice. Although subtle changes in the Fgfr2c<sup>C342Y</sup> heterozygous coronal suture morphology are visible from E17.5, full fusion of frontal and parietal bones is not visible until 3 weeks after birth. Owing to the presence of a cleft palate in R26RF<sup>Fgfr2c</sup>β<sup>act</sup> embryos, mice do not survive after birth, making it...
impossible to assess the postnatal synostosis phenotype. *Ex vivo* explant cultures were adopted to overcome this problem. Calvarial explant cultures in our laboratory routinely show that coronal synostosis can be achieved *in vitro* after 1-2 weeks of culture in Fgfr2c*C342Y* heterozygote E17.5 explants. In R26R*Fgfr2c*;βact calvaria, coronal synostosis was not observed (n=6), similar to WT and R26R*Fgfr2c*-floxed controls (n=7). At the same time, Fgfr2c*C342Y* heterozygous coronal sutures are reduced in size and signs of suture fusion can be observed after 15 days in culture (Fig. 3). Additionally, we examined the effect of FGF2 perturbation on osteoblast maturation at a pre-synostosis embryonic stage (E16.5) by analysing alkaline phosphatase (ALP) activity. In all cases, Fgfr2c*C342Y* and pFGFR2c mutations display increased suture overlap and ectopic ALP in the sutureal mesenchyme (n=3), whilst R26R*Fgfr2c*;βact frontal and parietal bones are spaced normally and resemble controls (n=3) (Fig. 4). These data imply that Fgfr2c overexpression does not cause coronal craniosynostosis and does not mimic FGFR2c activation in the Fgfr2c*C342Y* suture.

**Fgfr2c overexpression and Fgfr2c*C342Y* mutation activate MAPK/ERK signalling in the suture**

To assess whether Fgfr2c overexpression leads to a similar activation of the MAPK/ERK pathway as found in the Fgfr2c*C342Y* mouse, we looked at levels of phosphorylated (p)ERK in the suture *in vivo* and *in vitro*. We sought to visualise the expression of pERK in E16.5 coronal sutures using immunohistochemistry at a stage at which the sutures were morphologically similar, i.e. prior to the onset of synostosis (R26R*Fgfr2c*;βact, n=3; Fgfr2c*C342Y/+; n=4; control, n=4). Interestingly, both Fgfr2c*C342Y/+* and R26R*Fgfr2c*;βact mutants showed upregulated pERK in the osteogenic fronts of frontal and parietal bones flanking the suture (Fig. 5A). We also modelled pERK activity *in vitro* using the human embryonic kidney (HEK293T) cell line (n=4 independent transfections), pERK1 and pERK2 (also known as pMAPK3 and pMAPK1, respectively) were activated upon transfection of pFGFR2c(WT)V5 (encoding the WT FGFR2-IIIc isoform) and pFGFR2c(C342Y)V5 (encoding the mutated receptor) relative to mock transfected cells (Fig. 5B). Quantification of the blots using densitometry revealed significant upregulation of pERK in both FGFR2c*V5* transfected conditions (Fig. 5C). Specifically, there was significantly more pERK activity in the C342Y-V5 (P<0.0001) and WT-V5 (P=0.0101) transfected cells relative to the pcDNA3.1 control cells, as expected from the western blot results. Cells transfected with C342Y-V5 had an increased pERK output, by 8.1 units, compared with that of the WT-V5 transfected group (P=0.0005), likely due to the constitutive activation of the mutant receptor. Although both models show an activated RAS-MAPK pathway in the suture, the fact that only Fgfr2c*C342Y* activation results in craniosynostosis suggests that FGFR2c overexpression cascade activation is functionally distinct from that of the mutant receptor.

**Introduction of the Fgfr2c overexpression allele into Fgfr2c*C342Y* mice delays craniosynostosis**

As FGFR2c overexpression does not cause coronal suture synostosis, we hypothesised that the addition of this allele to the Fgfr2c*C342Y* mice would modify the craniofacial phenotype. To assess the impact of the overexpression allele, we generated a double mutant (i.e. R26R*Fgfr2c*;βact; Fgfr2c*C342Y*) and performed quantitative analysis on the calvaria as before (Fig. 6). The most apparent external anomaly resulting from Fgfr2c overexpression alone was microtia. This was exacerbated in double mutants, in which anotia was present in 90% of mice (Fig. 6A) (n=10/11). External ear development was normal in all Fgfr2c*C342Y* single mutants (n=15). Double mutants showed cleft palate with an increased penetrance (n=7/7). Examination of the craniofacial skeleton reveals partial rescue of the Crouzon phenotype: an ectopic interfrontal Wormian bone is characteristic of Fgfr2c*C342Y* heterozygotes (n=6/7), and these were generally smaller in double mutants (n=4/5), combined with an enlarged widening of the posterior interfrontal suture (Fig. 6B). In general, quantitative analyses of calvarial bones show that R26R*Fgfr2c*;βact; Fgfr2c*C342Y* frontal bones were smaller than those of controls (Av.Δ 11.86%; P<0.001; control, n=8; double mutant, n=6) (Fig. 6C). Significance size reduction was present when R26R*Fgfr2c*;βact frontal bones (n=6)
were compared with Fgfr2c\(^{C342Y}\) frontal bones \((n=10)\) (Av.\(\Delta\) 9.19\%; \(P<0.001\)). Also, there was a significant decrease in frontal bone size in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\) compared with Fgfr2c\(^{C342Y}\) (Av.\(\Delta\) 14.80\%; \(P<0.001\)). Quantitative analysis of the parietal bones indicated a significant increase in the parietal bone of Fgfr2c\(^{C342Y}\) \((n=10)\) compared with all other genotypes (control, Av.\(\Delta\) 6.54\%, \(P=0.001\), \(n=8\); R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Av.\(\Delta\) 6.08\%, \(P=0.006\), \(n=6\); R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\), Av.\(\Delta\) 5.44\%, \(P=0.016\), \(n=6\)) (Fig. 6D). Most strikingly, coronal sutures in double mutants appeared more patent than those in Fgfr2c\(^{C342Y}\) alone. Frontal and parietal bone overlap was decreased in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\) coronal sutures, as shown by Alizarin Red staining and ALP activity (Fig. 6B) (control, \(n=2\), Fgfr2c\(^{C342Y}\), \(n=2\); R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\), \(n=3\)). However, the observed ectopic ALP in the sutter mesenchyme of Fgfr2c\(^{C342Y}\) animals (Fig. 4) was not decreased in the majority of double mutant sutures (Fig. 6B). This suggests that the overexpression allele is potentially only delaying the synostosis process, possibly through calvarial bone hypoplasia caused by decreased osteogenic differentiation or increased mesenchymal proliferation.

**Increased proliferation in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\) double mutants**

To test the hypothesis that altered proliferation in the suture underlies the delayed onset of craniosynostosis in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\), Fgfr2c\(^{C342Y}\) double mutants, we performed phosphohistone-3 (pHH3) immunohistochemistry to assess mitotic index (Fig. 7A). Although levels of proliferation were unchanged in the peristomeum overlying the coronal suture, a statistically significant \((P<0.05)\) increase was detected in the sutter mesenchyme of double mutants compared with Fgfr2c\(^{C342Y}\) mutants alone (Fig. 7B). This implies that the addition of the R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\) allele increases sutural proliferation, which, in turn, delays osteogenic differentiation. This leads to bone hypoplasia and rescues the craniosynostosis phenotype.

**DISCUSSION**

The paradox of FGFR2c signalling was first implicated when it was found that craniosynostosis can be caused by deletion of the IIc isoform (Fgfr2\(^c\)\(^{\text{null}}\)), as well as by introducing an activating mutation linked to human Crouzon syndrome (Fgfr2\(^c\)\(^{C342Y}\)) (Eswarakumar et al., 2002, 2004). Fgfr2\(^c\)\(^{C342Y}\) heterozygotes display an early postnatal craniosynostosis phenotype, whereas Fgfr2\(^c\)\(^{\text{null}}\) mice show late postnatal onset (Eswarakumar et al., 2004, 2002). We sought to increase our understanding of FGFR2c signalling misregulation through Fgfr2c overexpression and comparison with Fgfr2c\(^{C342Y}\). Our data show that global Fgfr2c overexpression yields craniofacial hypoplasia, microtia and cleft palate. Strikingly, this cohort of mutants did not develop coronal synostosis, as opposed to Fgfr2c\(^{C342Y}\) and Fgfr2c\(^{\text{null}}\) mutants.

There are no reports of external ear defects related to human Crouzon syndrome or in Fgfr2c\(^{C342Y}\) mice. This study describes microtia with hypoplasia of the tympanic ring in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\) that has similarities to lacrimo-auriculo-dento-digital (LADD) syndrome, which can be caused by mutations in FGFR10 or FGFR2 (Rohmann et al., 2006). However, the external ear phenotype observed in the R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\) is more likely to be a consequence of ectopic expression of FGFR2c in the ectoderm, potentially activated in an autocrine way by ectodermal FGF ligands with a high affinity for the IIc isoform (Wright and Mansour, 2003). The lack of an external ear phenotype observed in other mouse models of Fgfr2c supports this (Eswarakumar et al., 2002, 2004). Nonetheless, a clear hypoplastic tympanic ring was noted in R26\(^{R}\)Fgfr2c\(^{\beta\text{act}}\) mutants, a phenotype similar to Fgfr2c\(^{\text{null}}\) embryos at E18.5, implying the importance of FGFR2c in the osteogenesis of the auditory bulla (Eswarakumar et al., 2002).
Murine palatogenesis commences at E11.5 and is complete by E17.5 (Bush and Jiang, 2012). The prevalence of cleft palate in human Crouzon syndrome is less than that in Apert patients, which may be due to the mutation affecting both isoforms in the latter (Stanier and Pauws, 2012). The overt cleft phenotype in both R26R^Fgfr2c^βact and R26R^Fgfr2c^Wnt1 was not fully penetrant: 40% (4/10) of R26R^Fgfr2c^βact embryos and 16% (1/6) of R26R^Fgfr2c^Wnt1 embryos, respectively. As Fgfr-2b is a major player for palatal shelf
elevation, the partial penetrance implicating the IIIc isoform is less critical to this process, which is supported by the observation that Fgfr2\textsuperscript{C342Y} null mice do not have a cleft palate phenotype (Rice et al., 2004; Eswarakumar et al., 2002). However, in the double mutants (i.e. R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y}; Fgfr2\textsuperscript{C342Y}), the penetrance of cleft palate was 100% (n=7/7), indicating that the combination of the constitutively active receptor with Fgfr2\textsuperscript{C342Y} overexpression exacerbates the cleft phenotype, resembling the Fgfr2\textsuperscript{C342Y} homozygotes (Peskett et al., 2017).

Our data support the conclusion that craniofacial hypoplasia is likely to be a result of conditional Fgfr2\textsuperscript{C342Y} overexpression in the NC lineage. A previous quantitative analysis of the adult Fgfr2\textsuperscript{C342Y} reports smaller frontal and nasal bones, accompanied by a short anterior cranial base (Liu et al., 2013). Diminished calvarial bone volume was also reported in other mouse models for syndromic craniosynostosis, most notably in Fgfr2\textsuperscript{S252W} and Fgfr3\textsuperscript{P244R} (Muenke syndrome) (Twigg et al., 2009; Chen et al., 2003). This is consistent with our finding that hypoplasia of NC-derived bones is present in E18.5 R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y} mice. Instead of a specific effect on NC-derived tissues, it is also possible that ectopic expression of FGFR2 in the ectoderm is the likely explanation for the microtia phenotype. Similarly, ectopic expression in any other non-NC cells, or increased expression of FGRFR2 in NC and mesoderm concomitantly during embryonic development of the head might contribute to the different craniofacial phenotype found in R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y} mice compared with R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y}; Wnt1 and R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y}; Mesp1 mice. Interestingly, Fgfr2\textsuperscript{C342Y} embryos at E18.5 do not show similar signs of hypoplasia at this stage. This is likely due to the nature of the C342Y mutation, which plays different roles in both early and late stages of development (Liu et al., 2013; Mansukhani et al., 2000). The C342Y mutation favours premature osteoblast commitment, but inhibits bone mineralisation and facilitates cellular apoptosis during late gestation (Liu et al., 2013; Mansukhani et al., 2000; Rice et al., 1999). Despite similarities observed in the anterior bone phenotype of Fgfr2\textsuperscript{C342Y} and R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y}, it is likely that the mechanism behind the phenotype is different. We propose that the hypoplasia found in R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y} mutants is mainly a result of insufficient osteogenesis, but we cannot exclude an ectopic effect at this stage.

A major difference between the R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y} mice and Fgfr2\textsuperscript{C342Y} mutants is the absence of coronal synostosis in the overexpression model; R26\textsuperscript{R}Fgfr2\textsuperscript{C342Y} mutants do not mimic the coronal synostosis phenotype of Fgfr2\textsuperscript{C342Y} mice. It is likely that
the overall signalling disruption by receptor overexpression is less extreme than that of complete signalling removal (Fgfr-2c\textsuperscript{null}) or a constitutively active receptor (Fgfr-2c\textsuperscript{C342Y}). This is reflected by the relatively subtle phenotypic spectrum, even with ubiquitous receptor overexpression under the control of the \(\beta\text{actin}\) promoter (R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}). MAPK/ERK signalling is confined within a specific spatial domain along the membranous bones and the osteogenic fronts of the coronal suture. This was expected, as expression of Spry gene downstream targets coincides with periosteoblast cells known to be expressing Fgfr2 (Deckelbaum et al., 2005; Johnson et al., 2000). Also, the relevant FGF ligands are confined to the osteogenic front. For example, Fgf18 and Fgf20 transcripts were detected in the osteogenic fronts of the coronal suture, coinciding with those genes involved in Fgfr2 signalling, pointing towards potential autocrine interactions (Hajihosseini and Heath, 2002; Ornitz and Itoh, 2015).

However, owing to the nature of ubiquitous overexpression and the morphogenic nature of FGFs, we expected ectopic expression to affect the R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}} suture mesenchyme. The lack of phenotype in these overexpressing mutants suggests that, although pERK is upregulated in both Fgfr2\textsuperscript{C342Y} and R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}, only Fgfr2\textsuperscript{C342Y} sutures show increased differentiation as revealed by increased levels of ALP, hence the craniosynostosis phenotype. Together, these results suggest that augmentation of MAPK/ERK signalling in the suture alone is not sufficient to derive a coronal synostosis phenotype in the craniofacial skeleton (Eswarakumar et al., 2004; Pfaff et al., 2016; Li et al., 2007).

Phenotypic rescue of the coronal suture could be generally achieved at the level of the receptor, such as by uncoupling Frs2 or through ERK knockdown (Eswarakumar et al., 2006; Shukla et al., 2007). Ultimately, the aim is to ameliorate the Crouzon phenotype through FGFR2c signalling attenuation. We sought to address whether Fgfr2c overexpression is sufficient to delay ossification in Fgfr2\textsuperscript{C342Y}, given the biochemical properties reported in vitro (Miraoui et al., 2009; Baddoo et al., 2003). Previous studies attempted to elucidate the paradoxical nature of FGFR2c signalling, where the perception of a GOF mutation leads towards signalling activity attenuation (Snyder-Warwick et al., 2010; Pfaff et al., 2016; Bagheri-Fam et al., 2015). Specifically, two studies attempted to ameliorate phenotypes caused by the C342Y allele, by introducing the C342Y allele into the Fgfr2\textsuperscript{null} background, but yielded a more severe phenotypic spectrum (Pfaff et al., 2016; Bagheri-Fam et al., 2015). Our data suggest that the Fgfr2c overexpression allele was sufficient to delay synostosis. In particular, the extent of suture overlap in R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}, Fgfr2c\textsuperscript{C342Y} mutants was decreased compared with control or Fgfr2\textsuperscript{C342Y} mice. However, suture abolition remains apparent due to the presence of the Fgfr2\textsuperscript{C342Y} allele. As FGFR2 is critical for cell renewal, one speculation for this rescue is shifting the balance from osteoblast differentiation to proliferation through ‘scavenging activity’ of endogenous FGF ligands by excess FGFR2c. Our results show that there is a distinct increase in proliferation in the sutural mesenchyme of R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}, Fgfr2c\textsuperscript{C342Y} double mutants, which would indicate a shift from differentiation to proliferation, explaining the osteogenic hypoplasia and the absence of craniosynostosis. This hypothesis is also supported by in vitro culture experiments with mesenchymal stem cells (MSCs), as exposure to FGFR2 promotes stemness in the presence of osteoblast differentiation media (Baddoo et al., 2003). Another possibility is that the addition of an extra WT Fgfr2c allele into the Fgfr2\textsuperscript{C342Y} heterozygous mice disrupts the receptor turnover at the membrane and/or disturbs the dimerisation equilibrium, favouring WT homodimers over WT/C342Y heterodimers. Altogether, the delayed synostosis in double mutants suggests that Fgfr2c overexpression partially rescues reduced premature osteoblast commitment caused by the C342Y allele (Eswarakumar et al., 2004; Peskett et al., 2017).

We propose the FGFR2c paradox should not be based solely on the signalling amplitude but, rather, the cascade that FGFR2c transactivates. This is due to activation of TRK receptors, such as FGFR2, which triggers signal transduction through three major cascades (PI3K-AKT, RAS-MAPK and PLC\(\gamma\)-PKC) (Ornitz and Itoh, 2015). It was previously reported that GOF mutations in the FGFR2c receptor elicit dissimilar cascade activation to that of WT-FGFR2c (Kim et al., 2003; Miraoui et al., 2009). Miraoui et al. (2009) compared the differences in cascade activation and concluded that Apert-FGFR2c-S252W transactivates the PLC\(\gamma\)-PKC pathway to drive osteoblast differentiation and mineralisation, whereas WT-FGFR2c predominantly signals through the RAS-MAPK pathway (Miraoui et al., 2009). Other growth factor pathways involving TRK receptor misregulation behave similarly; for example, PDGFR\(\alpha\), for which sustained activation of the receptor is responsible for complex craniosynostosis through PLC\(\gamma\)-PKC cascade (Moennig et al., 2009). Generally speaking, augmentation of RAS-MAPK signalling promotes proliferation, whilst cellular differentiation is a consequence of cascade suppression (Dinsmore and Soriano, 2018). This cellular consequence is comparable to embryonic stem cells maintaining pluripotency (Yamanaka et al., 2010). For example, in the murine palate, it is understood that proliferation of palatal mesenchyme cells is driven by activated ERK through exposure to FGFs in culture, and that its inhibition led to the downregulation of ‘stemness’ genes (Vasudevan et al., 2015). In the same study, genetic ablation of Fgfr1 (Fgfr1\textsuperscript{null}) led to ectopic osteoblast differentiation in the palatal shelves in vivo (Vasudevan et al., 2015). Unpublished data from this laboratory (K.K.L.L., E. Pauws) also support this, as oncogenic activation of Kras (i.e. Kras-G12D), an effector of the MAPK/ERK pathway in the NC lineage (Kras\textsuperscript{G12D};Wnt1), led to severe craniofacial hypoplasia. Oncogenic Kras is known to cause terminal differentiation defects, suggesting that upregulation of MAPK/ERK signalling alone resulted in insufficient osteoblast differentiation (Tuveson et al., 2004; Haston et al., 2017). Therefore, the pathogenic mechanism for craniosynostosis cannot be attributed to RAS/MAPK misregulation alone.

In summary, this study has demonstrated that FGFR2c overexpression yields craniofacial hypoplasia without a craniosynostosis phenotype. The most striking observation is the phenotypic variation between R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}, and Fgfr2c\textsuperscript{C342Y}, despite similarities in signalling dynamics. This implies that receptor overexpression and GOF mutations are mechanistically different, and require a different downstream interpretation to the WT. This is well characterised in vitro with preferential signal transduction, and our results relate these findings to an in vivo context. Maintaining the correct balance between proliferation and differentiation is crucial for osteogenesis and suture patency. Elucidating the role of FGFR2c signalling will improve the understanding of normal craniofacial development and its related pathologies, while providing a framework for the development of novel therapeutic strategies.

**MATERIALS AND METHODS**

**Animals**

Fgfr2c overexpression mouse [Gt(Rosa)26Sor\textsuperscript{tm1(Fgfr2-\textit{I10f};\textit{Pauw})}; also known as R26\textsuperscript{R}\textsuperscript{Fgfr2c}\textsuperscript{\textit{\textit{\beta}\text{act}}}, MGI:6150825] to target the Rosa26 genomic locus [Gt(Rosa)26\textsuperscript{I10f};\textit{Sor}], an Fgfr2c complementary DNA (cDNA) expression construct containing a V5 epitope on the C-terminal end of the protein was inserted into a targeting vector carrying the loxP-PGK-neo-\textit{Ipa}-loxP cassette (Soriano, 1999), followed by...
homologous recombination and the creation of chimera. Germline mice carrying the R26R^Fgfr2c^loxP allele were backcrossed onto a CD1 background and maintained as homozygotes. To generate Fgfr2c overexpressing mutants, mice carrying the R26R^Fgfr2c^loxP allele were crossed with animals carrying a CRE recombinase allele (i.e. Wnt1^CRE^; R26R, Fgfr2c^Cre allele was crossed with mTmG/+; Fgfr2c^mTmG/+; Fgfr2c^V5/+) upon CRE deletion.

### Crouzon mouse model (Fgfr2c^mTmG/+; also known as Fgfr2c^C342Y, MGI:3053095)

Fgfr2c^C342Y were derived through the European Mouse Mutant Archive (EMMA) at MRC Harwell as previously described (Peskett et al., 2017).

Generation of double mutants was performed by crossing R26R^Fgfr2c^loxP animals with Fgfr2c^C342Y heterozygotes, followed by genotyping for both alleles. Fgfr2c^C342Y, R26R^Fgfr2c^loxP animals were subsequently crossed with Wnt1^CRE^ to drive ubiquitous overexpression in the Crouzon mouse (i.e. R26R^Fgfr2c^loxP; Fgfr2c^C342Y).

The Wnt1^CRE^; R26R^YFP/+ reporter is described (Freem et al., 2010). CRE recombinase is driven under the control of the Wnt1 promoter to generate conditional expression in the NC cell lineage. Wnt1^CRE^ mice were crossed with R26R^YFP/YFP reporter to generate Wnt1^CRE^+/YFP/+ offspring. Cells positive with the CRE allele will express the reporter protein, thus labelling cells from the NC cell lineage.

R26R^YFP/TmG (Muzumdar et al., 2007). The mTmG allele was crossed with R26R^Fgfr2c^V5/; Fgfr2c^V5^ to generate a double-conditioned mouse that overexpresses both mTmG and Fgfr2c^V5 (R26R^mTmG^cre; R26R^Fgfr2c^V5^) upon CRE deletion.

All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 (project license number 70/8817). Animals were maintained by UCL Biological Services.

### Cells

HEK293T cells were cultured in minimum essential medium (MEM) alpha culture medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cells were transfected with pCDNA3.1 (Invitrogen) plasmids containing an Fgfr2c ORF (WT and C342Y) with a V5 tag on the C-terminal end, as well as control pcDNA3.1 without insert, when they had reached 50% confluence. (i.e. R26R^Fgfr2c^loxP, Fgfr2c^C342Y). Embryos were genotyped for CRE recombinase and loxP excision. Genotyping primers are available upon request.

**ImmunobLOTS**

E12.5 embryos were dissected under ice-cold PBS and homogenised in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (Sigma-Aldrich), 0.1% SDS (Sigma-Aldrich), 50 mM Tris-pH 8.0 (Fisher)) with MINI protease inhibitor cocktail (Sigma-Aldrich) and centrifuged to obtain the lysates. Protein concentration was determined using Bradford reagent (Bio-Rad) and spectrophotometry. A special cell lysis buffer [50 mM pH 7.6 Tris-Borate; 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 1 mM Mini protease inhibitor (Roche), 1 mM sodium orthovanadate, 25 mM sodium fluoride] was adopted for cells. Lysates were resolved, transferred and blotted using standard protocols. Anti-V5 antibody (Invitrogen) was used at a concentration of 1:1000 for E12.5 embryos and 1:3000 for cell lysates. Anti-pERK (1:2000, Cell Technology) was used to measure the phosphorylation state of ERK. For paraffin embedding, E16.5 embryo heads were skinned, eviscerated and fixed in 90% EtOH overnight in 80% glycerol and flat mounted onto frosted slides (Fisher). Images were taken for surface area measurements of frontal, parietal and nasal bones using ImageJ. Two measurements were made from both hemispheres of the bone, and the results were expressed as an average value. The quantification was performed blindly without knowledge of the embryo’s genotype. The mandibles were quantified in the same manner with the length measured instead. Quantified data were normalised to limb length to provide an endogenous control that was unaffected by increased FGFR2c signalling (Fig. S2).

**Quantitative RT-qPCR**

RNA was extracted using the Trizol method (Invitrogen). Any genomic DNA was subsequently eliminated using the DNA-free DNA Removal Kit (Ambion) prior to the reverse transcription reaction. A Quantitect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis. qDNA was used for RT-qPCR reactions using Taqman assays (Applied Biosystems) according to the manufacturer’s protocol. Fgfr2c assay (Applied Biosystems; Mm01269938_m1) was used to determine Fgfr2c overexpression. The collected dataset was analysed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification efficiencies were checked for target genes and controls prior to data analysis using the ΔΔCt method.

**Ex vivo calvarial explants**

A calvarial explant protocol was performed as previously described (Lana-Eloa et al., 2007). Embryos were harvested at E17.5 and dissected in PBS. The calvaria was removed from the skin and brain and cultured in Dulbecco’s modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and penicillin-streptomycin. The medium, with or without the addition of 10 ng/ml FGF2, was refreshed every other day and cultured for 10-15 days at 37°C, 5% CO2. Calvaria were fixed in 90% ethanol and processed for Alizarin Red staining.

**Gross phenotypic analysis**

Embryos were weighed using a fine balance. An electronic caliper (Fisher) was used to measure the CRL and head length of the embryo.

**Whole-mount skeletal staining**

The procedure for whole-mount skeleton histology was as previously described (Peskett et al., 2017). E18.5 embryos were skinned, eviscerated and fixed in 90% EtOH overnight at 4°C and stained with Alcian Blue (Sigma-Aldrich) working reagent (0.01% Alcian Blue; 20% acetic acid; 80% of 75% EOH) overnight at room temperature. Embryos were washed in 75% EtOH for a further 24 h and cleared in 1% potassium hydroxide (KOH) the following day. After sufficient clearing, 0.01% Alizarin Red (Sigma-Aldrich) working solution (0.01% Alizarin Red in 1% KOH) was added to the embryos. The embryos were stained overnight and washed in 1% KOH the following day. Graded glycerol (Sigma- Aldrich) was used to prepare samples for imaging and long-term storage in 80% glycerol.

**Quantitative analysis of the calvaria**

Stained E18.5 calvaria were subjected to ‘region of interest’ (ROI) analysis using ImageJ 2.0 software (National Institutes of Health). The craniofacial skeleton was dissected for the frontal, parietal and nasal bones in 80% glycerol and flat mounted onto frosted slides (Fisher). Images were taken for surface area measurements of frontal, parietal and nasal bones using ImageJ. Two measurements were made from both hemispheres of the bone, and the results were expressed as an average value. The quantification was performed blindly without knowledge of the embryo’s genotype. The mandibles were quantified in the same manner with the length measured instead. Quantified data were normalised to limb length to provide an endogenous control that was unaffected by increased FGFR2c signalling (Fig. S2).

**Statistical analysis**

SPSS Statistics 22 (IBM) software was used as the primary statistical package for data analysis. First, the data were tested for normality using Shapiro–Wilk test to determine the use of parametric or non-parametric tests. Independent sample Student’s t-test with Welch’s correction was used to compare the difference of means between the control and mutant groups for quantification of gross phenotype and craniofacial skeleton. One-way ANOVA with Tukey post hoc or non-parametric Kruskal–Wallis with Dunn-Bonferroni post hoc test was adopted for analysis of three or more groups. A P-value <0.05 was considered significant. The analysed data were plotted using Prism 6.0 software (GraphPad).

**Embryo embedding and histology**

For paraffin embedding, E16.5 embryo heads were skinned and fixed in 10% formalin overnight before graded dehydration in ethanol. Embryos were cleared in analytical grade xylene (Fisher) before paraffin wax displacement in a 60°C oven. The samples were embedded and sectioned between 8 and 10 μm on the axial plane using a microtome (Leica). For frozen sections, E16.5 or E18.5 heads were embedded in OCT compound and snap frozen using the −80°C isopentane method. Samples were sectioned between 15 and 20 μm on the cryostat (Bright).
Immunofluorescence
Paraffin sections were dewaxed in HistoClear (National Diagnostics) before graded EtOH rehydration. Antigen retrieval was executed in a decloaking chamber (BioCare Medical) at 110°C for 10 min in 10 mM sodium citrate pH 6.5 buffer. Sections were permeabilised in 0.1% PBST and blocked in 10% sheep serum (Sigma-Aldrich) and blocking buffer (0.15% glycine, 2 mg/ml bovine serum albumin in 0.1% PBST). Primary antibodies were incubated on the sections overnight in 1% sheep serum (Sigma-Aldrich) and blocking buffer. pERK (rabbit mlgG, Cell Signaling Technology) was used at 1:200, and appropriate secondary antibodies were incubated for 1 h the following day. Biotin goat-anti-rabbit Alexa Fluor 488 secondary antibodies (Dako) were used against pERK. The pERK signals were amplified using Streptavidin 555 conjugates (Life Technologies) at 1:500. Sudan Black (0.1%) was applied onto tissue sections for 5 min to quench any autofluorescence, and the sections were rinsed briefly in PBST to relieve any excess staining. Finally, tissue sections were stained in 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) at 1:10,000 in PBS before mounting in Mowiol mounting medium (Sigma-Aldrich).

pHH3 immunofluorescence
Standard immunofluorescence was performed on E18.5 cryosectioned heads. Briefly, cryosections were thawed at room temperature in a humidified chamber and rehydrated in PBS before fixation in 4% paraformaldehyde (PFA), 0.1% PBST permeabilisation and blocking of non-specific binding in sheep serum as described above. Anti-pHH3 (rabbit polyclonal IgG, Millipore) was incubated overnight at a concentration of 1:100 in blocking buffer prior to Alexa Fluor 488 (Invitrogen) secondary antibody detection at a concentration of 1:250 against the host species. Further washes in 0.1% PBST were carried out prior to DAPI-PBS nuclei staining as described above. Sections were mounted in Mowiol mounting medium (Sigma-Aldrich) as described above. Quantification of pHH3 cells was achieved using a macro written for FIJI software. The mitotic index was expressed as a percentage of the total pHH3 cells with respect to the total number of nuclei. The output was subsequently processed for statistical analysis using SPSS (IBM).

ALP assay
Cryosectioned embryos were thawed and immediately fixed in 4% PFA before permeabilisation in 0.1% TBST. Samples were equilibrated in NTMT before developing in NBT-BCIP solution. Developed samples were counterstained with Nuclear Fast Red (Sigma-Aldrich) and mounted in Mowiol mounting medium (Sigma-Aldrich).

Acknowledgements
We thank Albert Basson (King’s College London) for the C342Y mouse colony was derived from the EMMA Consortium.

Competing interests
The authors declare no competing or financial interests.

Author contributions

Supplementary information
Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.035311.supplemental

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