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Manic fringe is not required for embryonic development, and fringe family members do not exhibit redundant functions in the axial skeleton, limb, or hindbrain

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Abstract

Tight regulation of Notch pathway signaling is important in many aspects of embryonic development. Notch signaling can be modulated by expression of *fringe* genes, encoding glycosyltransferases that modify EGF repeats in the Notch receptor. Although Lunatic fringe (*Lfng*) has been shown to play important roles in vertebrate segmentation, comparatively little is known regarding the developmental functions of the other vertebrate fringe genes, Radical fringe (*Rfng*) and Manic fringe (*Mfng*). Here we report that *Mfng* expression is not required for embryonic development. Further, we find that despite significant overlap in expression patterns, we detect no obvious synergistic defects in mice in the absence of two, or all three, *fringe* genes during development of the axial skeleton, limbs, hindbrain and cranial nerves.

Notch signaling is a highly conserved pathway with roles in numerous developmental decisions. Notch genes encode single pass transmembrane receptors that are cleaved in the Golgi and presented on the cell surface as a mature heterodimer of the extracellular region and the transmembrane/intracellular region. Upon binding of a Jagged- or Deltalike ligand (collectively termed DSL or Delta, Serrate and Lag2), further protein cleavages take place, including a presenillin (*Psen*) dependent cleavage that releases the intracellular region of Notch (NICD). The NICD translocates to the nucleus where it forms a complex with the CSL (CBF, Suppressor of Hairless, LAG1) transcription factor and transcriptional coactivators of the mastermind-like (*Maml*) family. This complex associates with DNA and activates the transcription of target genes including a number of hairy-enhancer of split related (Hes) genes, encoding basic helix-loop-helix (bHLH) transcription factors (reviewed in Weinmaster and Kintner, 2003).

Notch signaling is modulated though a number of different post-transcriptional mechanisms, one of which is modification through glycosylation by the fringe family of proteins. Fringe genes encode ß1,3 N-acetylglucosaminyltransferases that elongate O-linked fucose on EGF repeats (Bruckner *et al.*, 2000; Moloney *et al.*, 2000). These proteins modify both Notch receptors and the Delta ligand, leading to either enhanced or reduced signaling in a context dependent manner (reviewed in Weinmaster and Kintner, 2003; Luther and Haltiwanger,

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2008). Thus, co-expression of FRINGE proteins with NOTCH receptors can serve to spatially and temporally fine-tune Notch signaling during development.

The vertebrate *fringe* family comprises three members: Lunatic fringe (*Lfng*), Manic fringe (*Mfng*), and Radical fringe (*Rfng*) (formally the LFNG, MFNG and RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferases). During embryonic development, these genes are expressed at widespread and overlapping sites throughout the embryo, suggesting possible roles in many developmental decisions (Cohen et al., 1997; Johnston et al., 1997). Mice with loss of *Lfng* function have skeletal defects that results from the loss of *Lfng* in the segmentation clock and during somite patterning (Evrard et al., 1998; Zhang and Gridley, 1998; Shifley et al., 2008). *Lfng* null animals also exhibit reduced fertility, which is suggested to arise, at least in part, from defects in oogenesis in *Lfng* null females (Hahn *et al.*, 2005), and from defects in the rete testis in *Lfng* null males (Hahn *et al.*, 2008). In contrast, *Rfng* null animals are reported to be viable and fertile with no overt defects (Moran et al., 1999; Zhang et al., 2002), and no synergistic effects were observed in *Rfng*−/−;*Lfng*−/− double mutants (Zhang *et al.*, 2002).

The lack of developmental phenotypes in *Rfng* null mice, combined with the overlapping expression patterns observed among family members, raises the possibility that FRINGE proteins may be functionally redundant during development. However, this possibility is argued against by functional and biochemical analysis of fringe activity. In mammalian systems, different FRINGE proteins are reported to have distinct effects on Notch signaling, depending on receptor, ligand and context (Hicks *et al.*, 2000; Shimizu *et al.*, 2001; Yang *et al.*, 2005). Work in *Drosophila* indicates that no single modified site on the Notch receptor can fully account for the effects of FRINGE activity on Notch signaling, implying that the level and pattern of glycosylation across the Notch extracellular domain will influence Notch activation (Xu et al., 2005). In vertebrate systems, recent work demonstrates that fringe proteins preferentially modify distinct EGF repeats, recognizing amino acids surrounding the O-fucose (Shao et al., 2003; Rampal et al., 2005). Further, different fringe proteins modify specific EGF repeats with distinct efficiencies (Rampal et al., 2005) indicating that Notch receptor modified by different FRINGE proteins will be modulated in distinct ways. Finally, analysis of the post-translational turnover of LFNG suggests that the FRINGE proteins may have different functional half lives as well as different specificities (Shifley and Cole, 2008). Taken together, these findings argue that FRINGE proteins may be unlikely to be functionally redundant.

The importance of proper spatial and temporal regulation of Notch signaling during embryonic development is clear. Understanding the modulation of Notch signaling by the fringe proteins is a key aspect in understanding this process. Thus, it is critical to understand the expression and function of fringe genes during embryonic development. Here we describe a targeted deletion of the mouse *Mfng* locus, and conclude that *Mfng* is not essential for embryonic development, fertility or viability aspects of adult homeostasis. To examine the question of functional redundancy among FRINGE proteins, we examine mice with loss-of-function mutations for two or all three fringe family members. In these compound mutants we do not find evidence for functional overlap among the three genes during embryogenesis. In particular, a key role for radical fringe in limb development and AER positioning was proposed on the basis of gain-of-function studies performed in chicken embryos (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). In contrast to these findings, we observe that mouse embryos with loss-of-function mutations in all three fringe genes reveal no defects in limb development. These findings support a view that loss of modulation of Notch signaling by the fringe family members does not cause profound phenotypes that are observed with loss or gain of Notch signaling, with the exception of the functions of *Lfng* during somitogenesis.

Materials and Methods

Mouse strains and genotyping

Lfng null mice (*Lfng*^{tm1Rjo}) were obtained from Dr. Randy Johnson (Evrard *et al.*, 1998). *Rfng* null mice (*Rfng*^{tm1Tfv}) have been described (Moran et al., 1999). *En1* null mice (*En1*^{tm1Alj}) (Wurst *et al.*, 1994) were obtained from The Jackson Laboratory Induced Mutant Resource. EIIA-Cre mice were obtained from Dr. Heiner Westphal (Lakso *et al.*, 1996). Fringe mutant strains were generally maintained on a mixed 129/Ola × C57Bl6/J background. However, to assess postnatal viability of mice with deletions of multiple fringe genes, mice were crossed one generation onto FVB/NJ before intercrossing to generate null animals, as the viability of *Lfng* null mice is increased on this mixed background . Mice were maintained in SPF facilities initially under the care of the Princeton University IACUC and subsequently by the Ohio State University IACUC.

Genomic DNA was prepared from tail clips via proteinase K saltout or from yolk sac fragments via the HOTSHOT procedure (Truett *et al.*, 2000) and animals were genotyped by PCR or Southern blot. Detailed genotyping procedures are available upon request.

Generation and analysis of *Mfng* targeted deletion

The *Mfng*$^{\Delta 1neo}$ targeting vector was constructed in ploxPNT (Shalaby *et al.*, 1995). The 5′ arm extends 1260 bp upstream from a point 87 nt upstream from the translation start site. The 3′ arm was a 5.5 kb BglII fragment containing exons 2 and 3. The final allele deletes 898 bp of *Mfng* sequence including 87 bp of 5′ UTR, the coding sequences in exon 1 and 556 bp of intron 1. After electroporation into E14TG2A cells, G418 resistant colonies were screened by Southern blot with external probes. Targeted clones were injected into blastocysts. Chimeras were bred to C57BL6/J mice to generate F1 mice, and mice were maintained on a mixed 129/Ola × C57BL6/J background. To generate *Mfng*$^{\Delta 1}$ mice, *Mfng*$^{\Delta 1neo}$ mice were bred to EIIA-cre mice (maintained on the FVB/N background) and offspring were screened by Southern blotting. for the loss of the PGK neo cassette.

5′ RACE

5′ RACE was performed using the BD SMART-RACE kit (Clontech). First strand synthesis was performed on polyA+ mRNA isolated from adult brain or on total RNA isolated from whole embryos primed with oligo dT in the presence of the BD SmartII oligo. 5′ RACE products were amplified using the UPM primer (Clontech) and *Mfng* specific primer SC-231 (5′ GCTTGCCCACATAGACATCA). RACE products were TA cloned (Invitrogen) and sequenced.

RNA expression analysis

Total RNA was isolated from adult tissues or embryos using Trizol reagent (Invitrogen), run on a denaturing formaldehyde gel, and transferred to nylon membrane, Blots were probed with the full length open reading frame of the *Mfng* cDNA.

For RT-PCR analysis, total RNA was isolated from 10.5 d.p.c. embryos, and first strand cDNA was made using SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed with primers SC-244 (5′- CATGGCCAGCCATTTGGT) and SC-229 (5′- TGGGCTGTCAGTGAAGATGA) to examine splicing between sequences in intron 1 of *Mfng* and exon 2 of *Mfng*.

Whole mount RNA in situ hybridization

Embryos were collected from timed pregnancies with noon of the day of plug identification designated as 0.5 d.p.c. RNA in situ hybridization using digoxigenin-labeled probes was performed essentially as described (Riddle et al., 1993). Probes utilized include *Mfng* and *Rfng* (Johnston *et al.*, 1997), *Krox20* (Wilkinson *et al.*, 1989), and *Fgf8* (Crossley and Martin, 1995).

Skeletal analysis and neurofilament staining

Alizarin red/ Alcian blue stained skeletal preparations of neonates or 18.5 d.p.c embryos were performed essentially as described (Kessel and Gruss, 1991). Neurofilament staining was performed using the 2H3 antibody (Developmental Studies Hybridoma Bank), using standard protocols.

Results and Discussion

Manic fringe mutant mice are viable and fertile

To examine the roles of *Mfng* during mouse development we utilized gene targeted mutation approach to disrupt the endogenous *Mfng* gene. The targeting vector replaces 898 nt surrounding the first coding exon with a floxed PGK neo cassette, resulting in the allele $Mfng^{\Delta 1neo}$ (Fig 1A). The deletion of the first exon in the Mfng parallels the design of the presumed null mutations of *Lfng* which delete exon 1(Evrard et al., 1998;Zhang and Gridley, 1998). To produce homozygous $Mfng^{\Delta 1neo/\Delta 1neo}$ mice, intercrosses of heterozygous $Mfng^{\Delta 1neo/+}$ mice were performed on a mixed 129Ola/C57 background, and homozygous $Mfng^{\Delta 1neo/\Delta 1neo}$ mice were born at the expected frequency, and appeared normal and fertile (Table 1).

We then mated $Mfng^{\Delta 1neo}$ mice with EIIAcre (Lakso *et al.*, 1996) mice to remove the floxed neo cassette, and deletion was confirmed by Southern analysis. Mice heterozygous for the resulting allele, $Mfng^{\Delta 1}$, were interbred to analyze the homozygous offspring, which were again viable and fertile and found at the expected Mendelian ratios (Table 1), suggesting that *Mfng* is not required for viability on either the mixed 129/Ola × C57Bl6/J background or following outcrossing to the FVB/N strain (the background of the EIIAcre mice).

To determine whether the $Mfng^{\Delta 1}$ allele represents a null allele of *Mfng* we first analyzed the RNA expression from the targeted allele. We find that in these embryos, the 1.8 kb endogenous *Mfng* RNA is absent, but we detect a 2.0 kb band in the $Mfng^{\Delta 1/\Delta 1}$ embryos indicating that an aberrant *Mfng* RNA is being expressed in these embryos, although at lower levels than the endogenous RNA (Fig 1C). RT-PCR analysis indicates that this allele contains exons 2-8 of the endogenous gene, and whole mount RNA in situ analysis demonstrates that it is expressed during embryogenesis, in the expected *Mfng* expression pattern (data not shown).

To assess the molecular nature of the $Mfng^{\Delta 1}$ allele we performed 5′RACE analysis to identify the upstream regions of the mRNA produced from this allele. In the majority of sequenced RACE products, a region from the first intron of *Mfng* (ending at nucleotide 2323) is spliced to the endogenous *Mfng* exon2 in the mRNA of $Mfng^{\Delta 1/\Delta 1}$ embryos, (Fig. 1D). The 3′ splice site at nucleotide 2323 is also utilized in a single EST in the database (GenBank Accession number BG085496), and this splice junction can be detected in wild type embryos by RT-PCR (Fig. 1E), and thus may represent the usage of strong cryptic splice sites present in intron 1 of the *Mfng* gene.

Given the presence of an *Mfng* transcript, we attempted to use Western Blot analysis to examine protein expression from the $Mfng^{\Delta 1}$ allele. Two different MFNG antibodies (Genex Bioscience and Abcam) were unable to detect endogenous levels of MFNG protein in embryos or cell lines. To our knowledge, no commercially available antibodies have been used to detect endogenous levels of MFNG with the exception of sc-8238 (Santa Cruz Biotechnology), which is raised against the region deleted in the $Mfng^{\Delta 1}$ allele, and is thus inadequate for our purposes.

In the absence of suitable antibody reagents to direct evidence of protein expression, we assessed the potential nature of any proteins produced by the $Mfng^{\Delta 1}$ allele. The imputed $Mfng^{\Delta 1}$ RNA does not contain any in-frame methionines upstream of the endogenous *Mfng* exon2 sequences, but the MFNG protein contains an internal methionine at amino acid 122 that could potentially serve as a start site for translation. We predict that if this methionine is utilized as a translation start site, a non-functional protein would be produced, as the resulting polypeptide is not predicted to have a signal sequence, and fringe proteins are thought to be functional only in the Golgi. Cell expression analysis indicates that no stable protein is produced from the imputed $Mfng^{\Delta 1}$ RNA (Supplemental fig. 1). Thus, our results from 5′ RACE analysis, protein prediction, and protein expression in cell culture indicate that the $Mfng^{\Delta 1}$ allele represents a null allele of Manic fringe. The $Mfng^{\Delta 1}$ allele reported here was recently utilized to examine *Mfng* function in the liver, and the finding of increased bile duct proliferation in $Jag1^{+/-}$;$Mfng^{+/\Delta 1}$ mice (Ryan *et al.*, 2008), supports the hypothesis that *Mfng* expression is in fact perturbed in $Mfng^{\Delta 1}$ mice, and that loss of *Mfng* expression may contribute to adult phenotypes. The lack of overt phenotypes in either $Mfng^{\Delta 1}$ or $Mfng^{\Delta 1neo}$ mice supports our hypothesis that *Mfng* expression is not critical for embryonic development.

During the course of preparation of this manuscript Svennsonn et al. reported a deletion of exon 4 of the *Mfng* locus. These mice are viable and without a detected phenotype (Svensson et al., 2009). In their paper the authors raise the possibility that a *Mfng* NH2 protein translated from exons 1-3 could provide a unexpected function. Taken together the findings that our deletion of exon 1 and Svensson's deletion of exon 4 both result in viable and fertile progeny is consistent with the interpretation that the two best described *Mfng* deletions encode null alleles and that *Mfng* null mice are viable and do not manifest an overt phenotype.

Mice with deletions of all three *fringe* genes are viable

One possible explanation for the lack of embryonic phenotypes seen in *Mfng* mutant mice is that the *fringe* family genes are functionally redundant during mouse embryogenesis when FRINGE proteins are co-expressed. For instance, in the developing embryo hindbrain both *Mfng* and *Lfng* are expressed in rhombomeres 3 and 5, creating boundaries of fringe-expressing and fringe-nonexpressing cells during hindbrain segmentation. Similarly, more detailed expression of *Rfng* expression utilizing a previously described *Rfng* Lac Z knockin allele ($Rfng^{tm1Tfv}$) (Moran et al., 1999) identifies *Rfng* expression at sites where other *fringe* genes are expressed, including in the sclerotome of epithelial somites, at the midbrain/hindbrain junction, and in the neural tube, as well as at numerous other sites, providing additional examples of co-expression of fringe family members (Supplemental figures 2 and 3).

To examine the possibility that fringe genes are functionally redundant during embryogenesis, *Mfng* mutant mice were crossed with *Lfng* null ($Lfng^{tmRjo1}$, hereafter referred to as *Lfng* null or *Lfng*-) and *Rfng* null ($Rfng^{tm1Tfv}$, hereafter referred to as *Rfng* null or *Rfng*-) mice, to create *Mfng;Lfng* mutant mice and *Rfng;Mfng;Lfng* mutant mice. While we routinely maintain *fringe* mutant mice on a mixed 129SvOla/C57BL6 background, we

find that on this background *Lfng*−/− mice rarely survive postnatally. Therefore, to assess whether the additional loss of *Mfng* and/or *Rfng* affects development and survival in *Lfng*−/− mice, *fringe* null mice were outbred one generation to FVB/NJ mice before intercrossing to increase the survival of *Lfng*−/− mice. In crosses between *Lfng* null and $Mfng^{\Delta 1}$ mice, $Mfng^{\Delta 1/\Delta 1}$;*Lfng*−/− mice are born and survive to weaning (Table 2). Although they are observed at a lower rate than expected, this loss is not statistically significant, and a similar reduction is seen in all *Lfng*−/− genotypes, regardless of the genotype at the *Mfng* locus.

Similarly, in crosses involving mutations in all three *fringe* genes, triple knockouts (*Rfng*−/−; $Mfng^{\Delta 1/\Delta 1}$;*Lfng*−/−) mice are observed, and at least two females were found to be fertile. In these crosses, the recovery rate of all genotypes including *Lfng* −/− alleles are further reduced, and are not seen at Mendelian ratios (Table 2), but all possible allele combinations are observed at weaning, and all genotypes incorporating *Lfng*−/− alleles are reduced similar amounts. We suggest that the increased postnatal loss of *Lfng* null mice in the *fringe* triple knockouts reflects the additional generations of inbreeding required to bring all three alleles to homozygosity. Together, these results indicate that the loss of all three *fringe* genes does not decrease the viability of mice below that observed in mice lacking only *Lfng*, indicating that any novel phenotypes do not affect survival up to weaning.

No synergistic phenotypes are observed in skeletons of mice lacking multiple fringe genes

$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$ mice and $Rfng^{-/-}$;$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$ mice appear outwardly similar to *Lfng*−/− mice. To examine whether the loss of other *fringe* genes exacerbates the skeletal phenotypes observed in *Lfng*−/− mice, skeletal preparations were observed across fringe genotypes. We observe no overt differences in the skeletal phenotypes in mice lacking multiple fringe genes (Fig. 2A). To assess whether loss of *Mfng* and/or *Rfng* has more subtle effects on the *Lfng* null phenotype, the number of rib abnormalities and the total number of tail vertebrae were quantified across genotypes. No dramatic differences were observed among $Rfng^{+/?}$; $Mfng^{+/?}$;$Lfng^{-/-}$, $Rfng^{+/?}$;$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$, $Rfng^{-/-}$;$Mfng^{+/?}$;$Lfng^{-/-}$, and $Rfng^{-/-}$; $Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$ mice (Fig. 2B, as no differences were observed between homozygous wildtype and heterozygous embryos, these were pooled and referred to as "+/?"). Thus, the loss of additional *fringe* genes does not exacerbate the skeletal defects observed in *Lfng*−/− mice. These findings recapitulate and expand findings from other groups indicating that *Rfng*−/−;*Lfng*−/− mice have similar skeletal phenotypes to those found in *Lfng* null mice (Zhang et al., 2002)

Loss of multiple fringe genes does not perturb limb development

Analysis of the skeletons of embryos with losses of multiple fringe genes allowed us to revisit the function of fringe genes during limb development. Previous experiments including expression analysis in chick mutants and gain-of-function experiments support a role for *Rfng* in positioning the AER (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). Although we observe *Rfng* expression in the developing limb bud (Moran et al., 1999), none of the described *Rfng* mouse mutants affects limb development, even if combined with mutations in *Lfng* (Moran et al., 1999; Zhang et al., 2002). Similarly, we find that mice lacking all three fringe gene function do not appear to have significant perturbations of limb development (Fig. 3A). The lack of limb phenotypes in fringe mutant mice strengthens the possibilities either that the regulation of limb development by RFNG may reflect true biological differences between mouse and chick, or that the retroviral gain-of-function studies do not reflect the function of RFNG in normal development.

In gain-of-function experiments in chick, expression of *En1* in the dorsal ectoderm represses *Rfng* expression (Laufer *et al.*, 1997; Rodriguez-Esteban *et al.*, 1997). In chick *limbless* mutants, *En1* expression in absent, *Rfng* is expressed throughout the limb bud, and no AER

forms (Grieshammer *et al.*, 1996; Laufer *et al.*, 1997). To address this issue we examined the regulatory relationship between *Rfng* and *En1* in the developing mouse embryo. $Rfng^{lacz}$ mice were mated to *En1* mutant mice (Wurst *et al.*, 1994), and *Rfng* expression was monitored by ßgal staining. As previously described, the AER of *En1* mutants is flattened and ventrally expanded (Loomis *et al.*, 1996). *Rfng* expression in 9.5-11.5 d.p.c. embryos is not altered in the absence of *En1*, thus our results do not support a role for *En1* repression of *Rfng* expression in the ventral ectoderm (Fig. 3).

En1 mutant limbs form ectopic ventral AERs, which occasionally lead to the growth of ectopic ventral digits (Loomis *et al.*, 1996; Loomis *et al.*, 1998). To assess the requirement for *Rfng* in the formation of ectopic AERs, we examined limb development in $Rfng^{lacZ/lacZ}$; $En1^{-/-}$ embryos. These double knockout embryos were indistinguishable from $Rfng^{lacZ/+}$; $En1^{-/-}$ embryos at all stages examined. At 10.5 d.p.c. AERs appear flattened and ventrally expanded, and by 11.5 d.p.c. clefts and bifurcations are evident, along with ventral anterior nubs (Fig. 3). Staining of *En1* null limb buds with *Fgf8* (a marker of the AER) reveals two distinct ectodermal ridges separated by an epithelial area, regardless of whether *Rfng* is expressed (Fig. 3F). Thus, we find that *Rfng* is not required for the formation of ectopic AERs in *En1* embryos.

These results suggest that not all *En1* functions are conserved between chick and mouse limb development. Indeed, other pathways involved in AER formation differ between mouse and chick. In chick, ectopic expression of *Wnt3a* induces the expression of AER markers and ectopic AERs, and endogenous *Wnt3a* expression is observed in the limb field and in the AER (Kengaku *et al.*, 1998). In contrast, mouse *Wnt3a* is not expressed in the limb, and *Wnt3a* mutants do not exhibit limb defects (Parr *et al.*, 1993; Takada *et al.*, 1994). Thus, our data support the idea that there may be differences in the mechanisms utilized during growth and patterning of tetrapod limbs (reviewed in Stopper and Wagner, 2005). It will be necessary to perform loss-of-function analysis of fringe genes in the developing chick to address these issues.

Fringe genes are not required in vertebrate hindbrain segmentation

Expression studies suggest a possible role for *fringe* genes in the segmentation of the hindbrain. *Mfng* and *Lfng* are co-expressed in rhombomeres 3 and 5, creating a juxtaposition of fringe-expressing and fringe-nonexpressing cells at sites where developmental boundaries are formed. In addition, morpholino-mediated knockdown of *Rfng* in zebrafish leads to a loss of Wnt1 expression at hindbrain boundaries (Cheng et al., 2004). We examined hindbrain segmentation by assessing the expression of *Krox20* (formally *Egr2)*, a zinc finger transcription factor that is expressed in presumptive r3 and r5 (Wilkinson *et al.*, 1989) and is required for the maintenance of these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1997). *Krox20* expression in r3 and r5 of $Rfng^{+/?}$; $Mfng^{\Delta 1/\Delta 1}$;$Lfng^{+/?}$, $Rfng^{+/?}$;$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$, and $Rfng^{-/-}$;$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$ embryos is indistinguishable from that observed in wildtype embryos (Fig. 4A), indicating that the r3 and r5 rhombomeres are intact in mice lacking two or all three fringe genes.

The segmental nature of the hindbrain is also reflected in the organization of associated cranial nerves, each of which have characteristic positions within, and migration through, the hindbrain. Cranial nerve organization was examined in fringe mutant mice through whole mount immunohistochemistry with an anti-neurofilament antibody. No abnormal positioning or migration was observed in $Rfng^{+/?}$; $Mfng^{\Delta 1/\Delta 1}$;$Lfng^{+/?}$, $Rfng^{+/?}$;$Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$, and $Rfng^{-/-}$; $Mfng^{\Delta 1/\Delta 1}$;$Lfng^{-/-}$ embryos at 10.5 d.p.c. (Fig. 4B) or at 11.5 d.p.c. (data not shown). These data support the idea that the hindbrain segments normally in mice lacking two or all three fringe genes.

Embryonic functions of fringe genes?

The overlapping expression patterns of the *fringe* family members suggests the possibility of functional redundancies, while protein function and biochemical data may suggest that different FRINGE proteins have distinct functions. We find that even in areas of overlapping expression, like the developing hindbrain, no overt phenotypes are seen in embryos with deletion of multiple fringe genes. However, the analyses reported here are not comprehensive, thus these studies leave open questions regarding the extent to which the functions of *Lfng*, *Rfng* and *Mfng* may overlap during embryonic development and adult life.

It is clearly possible that defects in mice with loss of multiple fringe genes remain to be identified as other systems are examined. Recent data using a novel *Mfng:*ßgeo fusion allele indicates that *Lfng* and *Mfng* act cooperatively to promote the development of marginal zone B-cells (Tan et al., 2009). In addition, many reports have suggested functions for Lunatic fringe in T-cell development (Koch et al., 2001; Visan et al., 2006; Besseyrias et al., 2007). For instance, *Lfng* null T-cell progenitors produce fewer thymocytes than wild type progenitors in competition assays (Visan et al., 2006), although adult *Lfng* null mice are reported to have normal numbers of peripheral T-cells (Besseyrias et al., 2007). T-cell differentiation and development may therefore provide a fertile system to examine potential synergistic functions of fringe family members. A detailed analysis of T-cell development in mice with mutations in one or more fringe genes is currently underway utilizing the alleles described in this manuscript (P. Stanley, unpublished data). Finally, some effects of *fringe* genes may be revealed through analyses in the postnatal period. For instance, mice that are haploinsufficient for both *Jagged1* and one *fringe* gene exhibit subtle alterations in bile duct proliferation in the adult liver, which are not observed in the newborn mice (Ryan *et al.*, 2008)

Together, these findings raise interesting questions regarding the potential of redundant functions among fringe family members. The three fringe family members are evolutionarily conserved across diverse organisms. Thus, it is likely that positive selective forces are operating to retain the family in diverse taxa. Although duplicate genes in the mouse are commonly suggested to be functionally redundant, some analyses suggest that duplicate genes are not less likely than singleton genes to be essential (Liao and Zhang, 2007). Thus, situations in which duplicate genes play truly redundant functions may be rarer than has previously been assumed. In fact, in many developmental systems the concept of distributed robustness may play a more important role than that of classical genetic redundancy (Wagner, 2005). In this model, when one part of a genetic system fails due to loss or mutation, the system compensates without relying on a redundant replacement. *Fringe* genes may act in complex networks of systems that modulate Notch signaling, and thus the loss of one or more fringes may be compensated at other levels of pathway control. This distributed robustness is potentially revealed in numerous developmental systems where the loss of multiple distinct Notch pathway members uncovers novel phenotypes, for example the liver phenotypes observed in *Jag1*+/−;*Lfng*+/− mice or the hepatic, cardiac and kidney defects observed in *Jag2*+/−;*Notch2*+/− mice (McCright et al., 2002).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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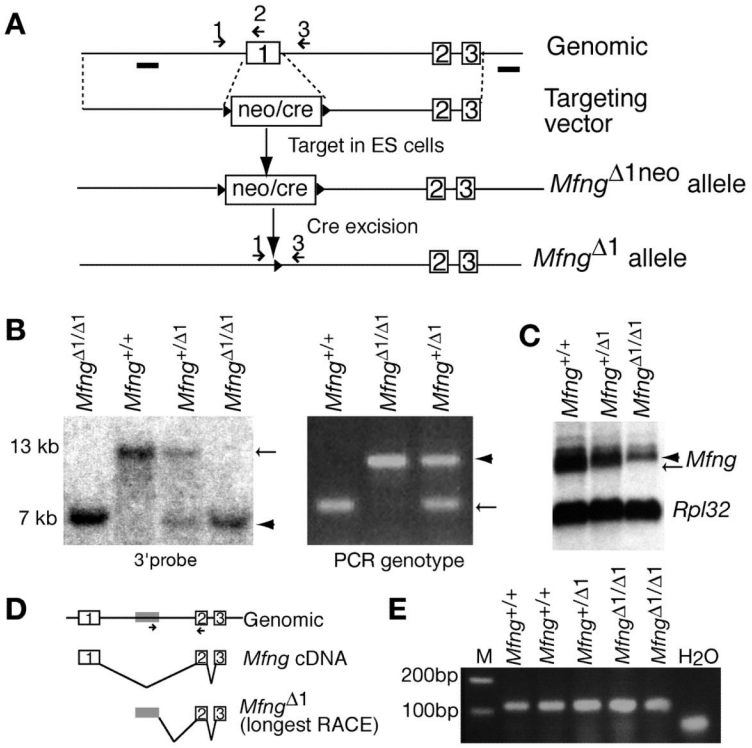

Figure 1.

Targeted deletion of *Mfng*. **A)** The *Mfng* endogenous locus, (numbered boxes = exons), the targeting vector and the structure of the targeted locus before and after cre recombination are shown. Locations of probe (solid lines) and PCR primers used in genotyping (arrows) are indicated. In the final $Mfng^{\Delta 1}$ allele, 899 bp surrounding exon 1 are replaced with a loxP site. **B)** After mating to EIIA cre mice, $Mfng^{\Delta 1}$ mice were genotyped by Southern blot using the 5′ probe to detect a 13 kb wildtype EcoRI band (arrow) or a 7 kb mutant EcoRI band (arrowhead). A representative PCR genotype is shown Arrow: endogenous band. Arrowhead: targeted band. **C)** Northern blot analysis of polyA+ mRNA isolated from brains of mice of the indicated genotypes. Blots were probed with a full length open reading frame cDNA probe to detect a 1.8kb *Mfng* mRNA. The blot was additionally probed with *Rpl32* as a loading control. A 2.0 kb *Mfng* band is visible in $Mfng^{+/\Delta 1}$ and $Mfng^{\Delta 1/\Delta 1}$ RNA. **D)** The 5′ end of the *Mfng* locus is shown with numbered exons shown as boxes, and he 5′ end of the *Mfng* mRNA is shown. The longest identified 5′RACE product from $Mfng^{\Delta 1}$ mice is shown below with the spliced intro sequences indicated as a grey box. **E)** RT-PCR using indicated primers (small arrows) demonstrates that this splicing event can be detected in wild type RNA, as well as $Mfng^{\Delta 1}$ RNA.

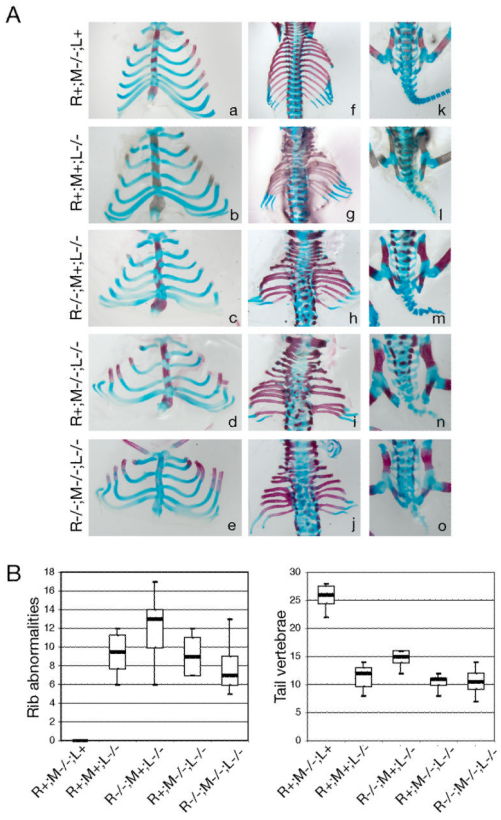

Figure 2.

No synergistic effects on skeletal development are observed in mice with deletions of multiple fringe genes. A) Skeletal preparations of mice of indicated genotypes stained with Alizarin red and Alcian Blue. Ventral (a-e) and dorsal (f-j) views of the ribs and dorsal views of the sacral spine and tail (k-o) are shown. Similar levels of skeletal disorganization are observed in all *Lfng* null genotypes, regardless of the genotype of other fringe family members. As no differences were observed between homozygous wildtype and heterozygous embryos, these were pooled and referred to as either "+" or "+/Δ". **B)** The total number of rib abnormalities (left) and the total number of tail vertebrae (right) were quantified in *Rfng*+/?;*Mfng*−/−;*Lfng*+/? (n= 7), *Rfng*+/?;*Mfng*+/?;*Lfng*−/− (n=8) *Rfng*−/−;*Mfng*+/?;*Lfng*−/− (n=5), *Rfng*+/?;*Mfng*−/−;*Lfng*−/− (n=5) and *Rfng*−/−;*Mfng*−/−;*Lfng*−/− (n=14) animals. Results are shown as bar and whisker graphs (solid horizontal dash indicates the mean). In both analyses, all *Lfng*−/− genotypes were highly significantly different from the *Rfng*+/?;*Mfng*−/−;*Lfng*+/? group ($p<0.001$, ANOVA followed by Tukey post hoc).

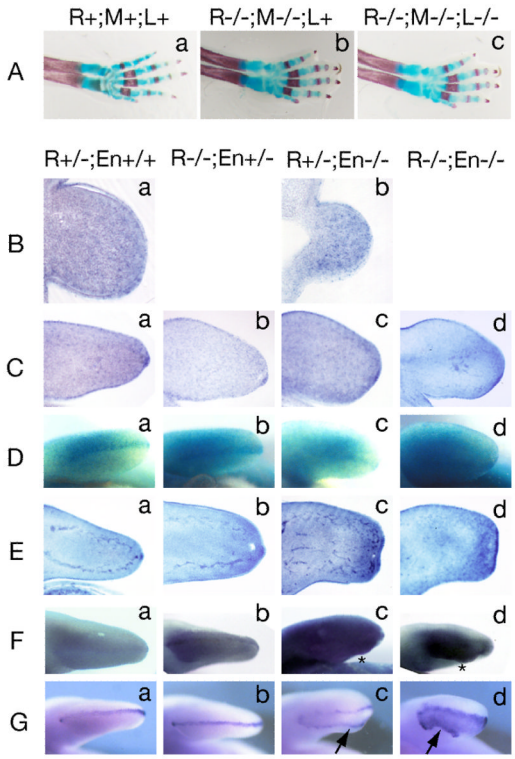

Figure 3.

Loss of multiple fringe genes does not perturb limb development, and *Rfng* expression is not regulated by *En1*. A) Alizarin red/ Alcian blue stained preparations of forelimbs of neonates of the indicated genotypes do not reveal overt limb patterning defects in the absence of two or all three *fringe* genes. C-F) ßgal staining of *Rfng*;*En1* forelimbs of embryos of the indicated genotypes, with dorsal to the top. B,C, and E are 70um sections, while D and F are whole mount limb buds. B) *Rfng* expression is unchanged in 9.5 d.p.c. limb buds regardless of *En1* genotype. C,D) *Rfng* is expressed in the dorsal ectoderm, ventral ectoderm and mesoderm of the limb bud at 10.5 d.p.c. AERs are visible as thickenings of the ectoderm at the limb apex (C) and along the distal margin of limb buds (D) in $En1^{+/+}$ and $En1^{+/-}$ limbs. In $En1^{-/-}$ limbs the bud is broader and the AER is ventrally expanded and flattened. No phenotypic differences are observed between $Rfng^{lacZ/+}$;$En1^{-/-}$ and $Rfng^{lacZ/lacZ}$;$En1^{-/-}$ embryos. E,F) At 11.5 d.p.c. $En1^{-/-}$ limbs are wider than controls and exhibit flattened, expanded AERs, with frequent bifurcations, as well as nubs on the ventral anterior surface (*). Again, no phenotypic differences are observed between $Rfng^{lacZ/+}$;$En1^{-/-}$ and $Rfng^{lacZ/lacZ}$;$En1^{-/-}$ embryos. G) *Fgf8* expression reveals that $Rfng^{lacZ/+}$;$En1^{+/+}$ and $Rfng^{lacZ/lacZ}$;$En1^{+/-}$, limb buds exhibit a single rim of *Fgf8* staining, while both $Rfng^{lacZ/+}$;$En1^{-/-}$ and $Rfng^{lacZ/lacZ}$;$En1^{-/-}$ embryos exhibit an additional ventral rim of *Fgf8* staining (arrow).

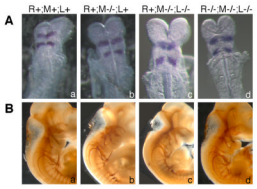

Figure 4.

Fringe genes are not required for mouse hindbrain segmentation. **A)** Whole mount in situ hybridization with a probe against *Krox20* was performed on 8.5 d.p.c. embryos of the indicated genotypes. *Krox20* expression was observed in rhombomeres 3 and 5, and no overt differences were seen among the genotypes (n= 8 *Rfng*+/+;*Mfng*+/+;*Lfng*+/+, n=7 *Rfng* +/?;*Mfng*−/−;*Lfng*+/+, n=6 *Rfng*+/?;*Mfng*−/−;*Lfng*−/−, and n= 4 *Rfng*−/−;*Mfng*−/−;*Lfng*−/ −). **B)** Whole mount immunohistochemistry was performed with a neurofilament antibody (2H3, Developmental Studies Hybridoma Bank) on 10.5 d.p.c. embryos of the indicated genotypes. Cranial nerve organization and positioning appears unaffected regardless of genotype. In the trunk irregular axon projections are observed in all *Lfng*−/− mice, as expected. (n= 20 *Rfng*+/+;*Mfng*+/+;*Lfng*+/+, n=15 *Rfng*+/?;*Mfng*−/−;*Lfng*+/+, n=2 *Rfng* +/?;*Mfng*−/−;*Lfng*−/−, and n= 6 *Rfng*−/−;*Mfng*−/−;*Lfng*−/−)

Table 1

Viability of *Mfng* knockout mice

Allele	Parental cross	Total at Weaning	Genotype % observed (% expected)				
			$M^{+/+}$	$M^{+/-}$	$M^{-/-}$	χ^2	P
Mfng ^{neo1}	$M^{+/-} \times M^{+/-}$	135	30 (25)	51 (50)	19 (25)	3.33	0.26
Mfng $^{\Delta 1}$	$M^{+/-} \times M^{+/-}$	57	18 (25)	56 (50)	26 (25)	1.75	0.45

Table 2

Viability of fringe multiple knockout mice

Parental Cross	Total at weaning	Genotype % observed (% expected)											
		$M^{+/-};L^{+/+}$	$M^{+/-};L^{+/-}$	$M^{+/-};L^{-/-}$	$M^{-/-};L^{+/+}$	$M^{-/-};L^{+/-}$	$M^{-/-};L^{-/-}$	χ^2	P
$M^{-/-};L^{+/-}$ × $M^{+/-};L^{+/-}$	72	12.5 (12.5)	33.3 (25)	5.5 (12.5)	13.8 (12.5)	29.2 (25)	5.5 (12.5)	8.2	.15
		$M^{+/+};L^{+/+}$	$M^{+/+};L^{+/-}$	$M^{+/+};L^{-/-}$	$M^{+/-};L^{+/+}$	$M^{+/-};L^{+/-}$	$M^{+/-};L^{-/-}$	$M^{-/-};L^{+/+}$	$M^{-/-};L^{+/-}$	$M^{-/-};L^{-/-}$	χ^2	P
$M^{+/-};L^{+/-}$ × $M^{+/-};L^{+/-}$	56	10.7 (6.25)	10.7 (12.5)	3.6 (6.25)	16.1 (12.5)	32.1 (25)	8.9 (12.5)	7.1 (6.25)	8.9 (12.5)	1.8 (6.25)	7.3	.50
		$R^{+/-};M^{-/-};L^{+/+}$	$R^{+/-};M^{-/-};L^{+/-}$	$R^{+/-};M^{-/-};L^{-/-}$	$R^{-/-};M^{-/-};L^{+/+}$	$R^{-/-};M^{-/-};L^{+/-}$	$R^{-/-};M^{-/-};L^{-/-}$	χ^2	P
$R^{-/-};M^{-/-};L^{+/-}$ × $R^{+/-};M^{-/-};L^{+/-}$	36	30.5 (12.5)	22.2 (25)	2.8 (12.5)	16.7 (12.5)	27.8 (25)	0 (12.5)	17.3	<0.01
		$R^{-/-};M^{+/-};L^{+/+}$	$R^{-/-};M^{+/-};L^{+/-}$	$R^{-/-};M^{+/-};L^{-/-}$	$R^{-/-};M^{-/-};L^{+/+}$	$R^{-/-};M^{-/-};L^{+/-}$	$R^{-/-};M^{-/-};L^{-/-}$	χ^2	P
$R^{-/-};M^{-/-};L^{+/-}$ × $R^{-/-};M^{+/-};L^{+/-}$	71	7.0 (12.5)	45.1 (25)	0 (12.5)	14.1 (12.5)	29.6 (25)	4.2 (12.5)	26.6	<0.01
		$R^{-/-};M^{+/-};L^{+/+}$	$R^{-/-};M^{+/-};L^{+/-}$	$R^{-/-};M^{+/-};L^{-/-}$	$R^{-/-};M^{-/-};L^{+/+}$	$R^{-/-};M^{-/-};L^{+/-}$	$R^{-/-};M^{-/-};L^{-/-}$		
$R^{-/-};M^{-/-};L^{+/-}$ × $R^{+/-};M^{+/-};L^{+/-}$	67	8.9 (6.25)	14.9 (12.5)	4.5 (6.25)	8.9 (6.25)	7.5 (12.5)	1.5 (6.25)		
		$R^{+/-};M^{+/-};L^{+/+}$	$R^{-/-};M^{+/-};L^{+/-}$	$R^{+/-};M^{+/-};L^{-/-}$	$R^{+/-};M^{-/-};L^{+/+}$	$R^{+/-};M^{-/-};L^{+/-}$	$R^{+/-};M^{-/-};L^{-/-}$	χ^2	P
		6.0 (6.25)	13.4 (12.5)	3.0 (6.25)	10.4 (6.25)	19.4 (12.5)	1.5 (6.25)	14.1	.23