A family of mammalian *Fringe* genes implicated in boundary determination and the *Notch* pathway

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SUMMARY

The formation of boundaries between groups of cells is a universal feature of metazoan development. *Drosophila fringe* modulates the activation of the *Notch* signal transduction pathway at the dorsal-ventral boundary of the wing imaginal disc. Three mammalian *fringe*-related family members have been cloned and characterized: *Manic, Radical* and *Lunatic Fringe*. Expression studies in mouse embryos support a conserved role for mammalian *Fringe* family members in participation in the *Notch* signaling pathway leading to boundary determination during segmentation. In mammalian cells, *Drosophila* fringe and the mouse Fringe proteins are subject to post-translational regulation at the levels of differential secretion and proteolytic processing. When misexpressed in the developing *Drosophila* wing imaginal disc the mouse Fringe genes exhibit conserved and differential effects on boundary determination.

Key words: *Fringe*, boundaries, *Notch*, hindbrain, presomatic mesoderm, neurogenesis, mouse, *Drosophila*

INTRODUCTION

Intercellular signaling is a fundamental feature underlying the establishment of pattern during embryonic development (Jessell and Melton, 1992). Theoretical models predict that an important aspect of embryonic patterning is likely to be the establishment of boundaries (Meinhardt, 1991). These boundaries are expected to demarcate key regions of the embryo, and to pattern those regions by acting as organizers (Lawrence and Struhl, 1996).

Such models have recently received direct experimental support from studies of patterning in the *Drosophila* wing imaginal disc. This embryonic field is subdivided by cell lineage restrictions into anterior, posterior, dorsal and ventral cell populations termed compartments (Garcia-Bellido, 1975; Blair, 1993). It was recently shown that the dorsal-ventral (D-V) boundary is established through the activities of the *Drosophila* genes *fringe (D-fng)*, *Serrate (Ser)*, *Delta (Dl)*, and *Notch* (Irvine and Wieschaus, 1994; Speicher et al., 1994; de Celis et al., 1996; Kim et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). Molecular cloning of *D-fng* indicated that it encodes a putative secreted signaling protein. Genetic mosaic analysis and ectopic expression studies demonstrated that it is the juxtaposition of *D-fng*-expressing cells (dorsal) with *D-fng* non-expressing cells (ventral) that results in *fringe*-dependent cell signaling. *D-fng* is thus unique among known signaling molecules in that it is not *D-fng* expression itself that acts as a signal, but rather its expression boundaries.

The boundary-specific nature of fringe activity suggests that *D-fng* not only influences signaling to *fringe* non-expressing neighboring cells, but it also determines a *fringe*-expressing cell’s inability to respond to *fringe*-dependent signals.

Although *Notch* is expressed throughout the entire imaginal disc, at the time of boundary determination it is activated specifically in the dorsal and ventral cells just adjacent to the compartment boundary (de Celis et al., 1996). *D-fng* activity modulates the ability of *Ser* and *Dl* to activate *Notch* (V. Panin, V. Papayannopoulos, R. W. and K. D. I., unpublished data). This influence of *D-fng* results in a positive feedback loop between the two Notch ligands at the D-V boundary such that *Dl* expressed in ventral cells binds and activates Notch in dorsal cells, and *Ser* expressed in dorsal cells binds and activates Notch in ventral cells. An important consequence of *D-fng* boundary-mediated signaling for the formation of the wing is that the initially asymmetric boundary between dorsal and ventral cells is translated by reciprocal activation of *Notch* at the D-V compartment boundary into symmetrical expression of downstream targets and the expression of wingless (wg) as the organizing signaling molecule (de Celis et al., 1996; Doherty et al., 1996; Kim et al., 1995, 1996; Rulifson and Blair, 1995; Diaz-Benjumea and Cohen, 1995; Zecca et al., 1996).

Given the evolutionary conservation of the *Notch* pathway (Artavanis-Tsakonas et al., 1995), we searched for and have identified three *Fringe* genes in mouse and human. Our characterization of their coincident expression with *Notch* ligands at developmental boundaries, their differential protein secretion...
and processing, and their ability to participate in boundary determination in the *Drosophila* wing disc provides evidence for both conserved and novel aspects of restricting fringe activities.

**MATERIALS AND METHODS**

**Cloning and sequencing**

Ten human cDNAs with homology to *Drosophila fringe* were initially identified in a screen of The Institute for Genome Research (T.I.G.R.) EST database and additional human and mouse cDNAs were identified by reiteratively screening the Database ofExpressed Sequence Tags at the National Center for Biotechnology Information (N.C.B.I.) using the BLAST network service (Altschul et al., 1990). The following human clones were obtained and sequenced: cDNA clone 40887 (GenBank accession number: R56561; IMAGE Consortium) was designated LUNATIC FRINGE, clones HCEOB60 (TIGR) and HSC3MD091 (GenBank accession number: F13368; Genexpress cDNA program) were designated RADICAL FRINGE and clone HNBA16 (TIGR) was designated MANIC FRINGE. Clones 40887, HCEOB60 and HNBA16 were used separately to screen a 8.5 d.p.c. mouse embryonic cDNA library (Fahrner et al., 1987). Mouse Lunatic, Radical and Manic cDNAs were identified. Mouse Manic and Radical cDNAs were extended 5' using nested RACE PCR with the Marathon cDNA Amplification Kit (Clontech) on an adult mouse brain linker ligated cDNA library (gift from T. Vasichek). Primer sequences are available on our laboratory homepage (www.molbio.princeton.edu/vogt/vogt.html).

**In situ hybridization**

Embryos were obtained from pregnant female Swiss mice; 0.5 d.p.c. corresponding to the morning the vaginal plug was detected. RNA in situ hybridizations on serial 8-μm paraffin tissue sections were performed essentially as described by Wilkinson and Nieto (1993). Whole-mount in situ hybridizations using digoxigenin-labeled fringe riboprobes were performed according to the protocol of Riddle et al. (1993). In double-label experiments, the other probe was fluorescein-labeled and detected with anti-fluorescein-AP using FastRed (Sigma) as a substrate. The other probes were Notch1 (Amo et al., 1992), Dll1 (Bettenhausen et al., 1995), Jagged (Lindsell et al., 1995), Sek-I (Nieto et al., 1992) and Mox-I (Candia et al., 1992).

**Fringe-AP fusion constructs and expression analysis of Fringe-AP fusion proteins**

The ORFs of each cDNA were PCR amplified and cloned into AP-tag-2 (Cheng et al., 1995). Primer sequences are available on our laboratory homepage (www.molbio.princeton.edu/vogt/vogt.html). COS 7 and Hep3B cells were grown in 100 mm plates in DMEM with 10% bovine calf serum and were transiently transfected using Lipofectamine (GIBCO BRL). The media and lysed cells were collected and AP activities measured essentially as described by Flanagan and Leder (1990) and Cheng et al. (1995).

[^35]Smethionine ([^35]S]Met) labeling of COS cells and immunoprecipitations of labeled media or cell fractions with anti-human alkaline phosphatase (Genzyme Diagnostics) were performed essentially as described by Flanagan and Leder (1990). A number of controls were performed to diminish the likelihood that the absence of Radical-AP protein in the media fractions was due to regulatory home pag e (www.molbio.princeton.edu/vogt/vogt.html).

**N-terminal sequence analysis of Fringe-AP and Lunatic-AP**

N-terminal protein sequencing was performed on an Applied Biosystems 476A Protein Sequencer System. The N-terminal sequence obtained for D-fringe-AP was YPGIQ which corresponds to amino acids 40-45 and the N-terminal sequence obtained for mouse Lunatic-AP was DADPP which corresponds to amino acids 86-90.

**Misexpression in the *Drosophila* wing**

cDNAs encoding each respective mouse Fringe were cloned into pUAST (Brand and Perrimon, 1993). Constructs were transformed into *Drosophila* (Rubin and Spradling, 1982), and several independent lines were established for each transgene. Flies carrying Fringe transgenes were crossed to the ptc-Gal4 driver strain (GAL4 559.1; Hinz et al., 1994) and the wings and wing discs of their ptc-Gal4 UAS-Mfng progeny were examined, mounted and stained for wg protein expression as described previously (Irvine and Wieschaus, 1994). Lunatic expression along the A-P boundary of the wing imaginal disc was confirmed by in situ hybridization and DNA sequencing confirmed that no mutations had been introduced during cloning.

**RESULTS**

**Identification of a mammalian Fringe gene family**

We used the D-fng protein sequence to interrogate human expressed sequence tagged (EST) databases and identified three classes of fringe-related sequences. Using the human FRINGE-related ESTs as probes, we identified full length open reading frames for three orthologous mouse genes (Fig. 1). We have named the three mammalian fringe-related family members: Lunatic Fringe, Radical Fringe and Manic Fringe.

**Fringe expression in mouse embryos**

Examination of the mammalian fringe genes' sequences suggests conservation of several features consistent with secreted proteins: predicted amino-terminal signal peptides, potential N-linked glycosylation sites, distribution of cysteine residues and conservation of an internal proteolytic processing site in Lunatic (Fig. 1 and Irvine and Wieschaus, 1994). The presence of the conserved proteolytic processing site among D-fng, mouse Lunatic, and *Xenopus* Lunatic (Wu et al., 1996), suggests they may be synthesized as inactive pre-pro-protein precursors that would require proteolytic processing for full activity. Interestingly, the mouse Manic and Radical predicted signal sequence cleavage sites align at the same relative position as the pre-processing site in D-fng and Lunatic (Fig. 1A). Therefore, all mature Fringe proteins are predicted to be of similar size and pI (Fig. 1 legend). The existence of three mammalian family members could allow for differential regulation and the deployment of a spectrum of overlapping and specialized activities.

**Manic and Lunatic Fringe expression demarcates segment boundaries during mammalian development**

Northern analysis shows that each individual Fringe family member is expressed in the embryo and in adult tissues derived from all three germ layers (data not shown). Analysis of their expression in mouse embryos at 8.0-10.5 d.p.c. by whole-mount RNA in situ hybridization reveals that the Fringe genes are dynamically expressed in many different cell types undergoing cell fate decisions (Fig. 2). Strikingly, Manic and Lunatic expression is suggestive of a role for mammalian Fringe genes in the demarcation of segmental boundaries observed during anterior-posterior patterning of the hindbrain and somites (Lumsden and Krumlauf, 1996; Tam and Trainor, 1994).
Amino acid comparisons of D-fringe to mouse and human Lunatic, Radical and Manic Fringe. (A) PILEUP alignment of the Drosophila, mouse and human Fringe amino acid sequences (accession numbers for mouse Manic Fringe, U349349; Radical Fringe, U94350; Lunatic Fringe, U94351 and human MANIC FRINGE, U94352, RADICAL FRINGE, U94353; and LUNATIC FRINGE, U94354). Human genes are in upper case. The predicted pre regions/signal sequences are underlined (Nielsen et al., 1996). A conserved dibasic processing site between D-fng and Lunatic Fringe is boxed. Conserved cysteines are denoted by arrowheads and predicted N-linked glycosylation sites are double underlined. Amino acids identical among 50% of the sequences are shaded in black and amino acids with a high degree of similarity among 50% of the sequences are shaded in gray. Numbers represent the amino acid number of D-fng. (B) Schematic representation of the Fringe molecules. D-fng and Lunatic are predicted to be pre-pro-protein precursors while Manic and Radical are predicted to be pre-protein precursors. Conserved dibasic processing sites are indicated by arrows. Predicted N-linked glycosylation sites are denoted by branches. Numbers to right indicate amino acid length of the predicted ORFs. The conserved mature domain among Fringe family members, indicated by black boxes, have similar relative molecular masses: D-fng 33.4, Lunatic 32.9, Manic 32.7 and Radical 32.7 (×10³); and p’s: D-fng 7.5, Lunatic 7.76, Manic 7.88 and Radical 7.89. (C) Pairwise comparisons of percentage amino acid identity in the conserved domain (beginning at Asp 149 of D-fng) among D-fng and the mouse Fringe family members. Values in parentheses represent similarities when allowing for conservative amino acid substitutions. Based on their primary amino acid sequences two recently identified Xenopus Fringe genes (Wu et al., 1996) are most closely related to the mammalian Radical and Lunatic genes. However, in contrast to mouse Radical, Xenopus Radical predicts a pre-pro protein precursor.

Fig. 1.
Vertebrate hindbrain development proceeds by a process of internal subdivision resulting in seven metameric units termed rhombomeres (reviewed by Lumsden and Krumlauf, 1996). Both Manic and Lunatic are expressed in anterior-posterior stripes in the hindbrain prior to the formation of morphologically recognizable rhombomeric boundaries. In embryos at the 5 somite stage, Manic is expressed in pre-r3, while Lunatic is expressed in pre-r3 and pre-r5 (Fig. 3A,B). In embryos with 12-13 somites, r3 and r5 expression of both Manic and Lunatic is observed (Fig. 2A,D). The restricted pattern of Manic and Lunatic Fringe to r3 and r5 creates a juxtaposition of Fringe-expressing and non-expressing cells between even and odd numbered rhombomeres, an observation consistent with the demarcation and establishment of boundaries. As development proceeds, expression of both genes is eventually observed within all rhombomeres, although the relative levels of expression between rhombomeres varies (Fig. 2C,D; data not shown).

Segmentation of the vertebrate body following gastrulation involves the progressive anterior-posterior arrangement of paraxial and tail bud mesoderm into metameric units called somites. Of the three mouse Fringe genes, Lunatic is solely expressed in the presomitic mesoderm in a striking pattern that appears to demarcate the formation of intersomitic boundaries and the appearance of separated somites (Fig. 2A). The expression of Lunatic is dynamic, reflecting the rostral-caudal wave of somitic developmental progression (Fig. 3C,D; Tam and Trainor, 1994). In the posterior presomitic mesoderm, Lunatic is expressed in a broad swath of cells. As somitogenesis proceeds, this band of expression becomes narrower, paralleling the cell condensation observed within the anterior presomitic mesoderm as it organizes into clusters of cells recognizable in scanning electron micrographs as segmental units termed somitomeres (Fig. 3C,D). Stripes of Lunatic expression are observed in the anterior two presumptive somitomeres that are about to form somites. These stripes of expression appear to correspond to what will form the posterior half of the somite (Fig. 3D). Once the somite has formed, transcript levels of Lunatic decline rapidly, such that expression is seldom evident in the newly formed somites. However, Lunatic expression is later observed in the dermamyotome of the mature somites (Fig. 2C; data not shown).

Fringe may participate in a conserved Notch pathway to determine somitic boundaries

In the fly wing D-fng acts upstream of Notch (Kim et al., 1995;
Mouse \textit{Notch1} is expressed throughout the presomitic mesoderm and somite segmentation is perturbed in \textit{Notch1} mutant embryos (Conlon et al., 1995). The candidate vertebrate \textit{Notch} ligand, \textit{Delta-like 1} (Dll1), is also expressed in a broad domain throughout the presomitic mesoderm (Fig. 3E; Bettenhausen et al., 1995). By performing double label RNA in situ we observe that the initial broad posterior band of \textit{Lunatic} expression is detected in pre-r3 (arrow) in embryos with 5 somites. (B) \textit{Lunatic} Fringe is expressed in pre-r3 and -r5 (arrows) and anterior within the neural folds in embryos with 8 somites and in embryos with 5 somites (data not shown). Inset shows a double-label in situ hybridization with \textit{Lunatic} (blue) and \textit{Sek-1} (red); both are expressed in pre-r3 and -r5. Note that their expression patterns are coincident. (C) Expression of \textit{Lunatic} (blue; arrowheads) in the presomitic mesoderm of a 6 somite embryo as compared to that of \textit{Mox-1} (red), which is expressed in mature somites and the next somite about to form (presumptive somitomere 1; arrow). Strong \textit{Lunatic} expression is observed in presumptive somitomere 2, with broader, weaker expression in the more posterior presomitic mesoderm. (D) A 10 somite embryo with anterior to the left; less differentiated presomitic mesoderm is to the right. \textit{Lunatic} expression precedes the formation of intersomitic boundaries (arrows). Expression is weak in presumptive somitomere 1 and strong in presumptive somitomere 2; the posterior edge of the \textit{Lunatic} expression domain (arrowheads) is consistent with the eventual position of the morphological somite boundary. (E) Expression of \textit{Lunatic} (blue) and \textit{Dll1} (red) in an 8 somite embryo. Their expression domains overlap at the posterior edge of the anterior \textit{Lunatic} stripe (arrowheads), while more caudally they overlap entirely. (F) As somitogenesis proceeds and the anterior \textit{Lunatic} stripe begins to fade, presomitic cells not expressing either \textit{Lunatic} (blue), nor \textit{Dll1} (red), are apparent (arrow). Anterior is up in this panel.

\textit{Fringe} expression in the neural tube and its relationship to the \textit{Notch} pathway. (A-F) RNA in situ hybridizations of \textit{Manic} (A,D), \textit{Lunatic} (B,E) and \textit{Radical} (C,F) on transverse sections of 10.5 d.p.c. mouse embryo anterior (A-C) and posterior (D-F) neural tube. The plane of section is indicated on the drawing in A. \textit{Manic} and \textit{Lunatic} expression is shown on adjacent sections and \textit{Radical} expression is shown on a section 40 \textmu m caudal to the \textit{Lunatic} section. (G,H) Double label RNA in situ hybridizations of \textit{Manic} (blue) and \textit{Jagged} (G; red) and \textit{Dll1}(H; red) on transverse sections of 10.5 d.p.c. mouse embryo neural tube. Arrowheads indicate domains of \textit{Jagged} expression. The plane of section is indicated on the drawing in G. The sections are separated by 48 \textmu m. (I) \textit{Notch1} expression in a transverse section of an 11 d.p.c. mouse embryo.

\textit{Fringe expression suggests a role in vertebrate neurogenesis} Mammalian \textit{Fringe} genes are expressed in the three contexts in which vertebrate neurogenesis occurs: within the central neuroepithelium, during migration of neural crest cells, and in expression is found in the midst of \textit{Dll1}-expressing cells. However, as the somitomere matures, \textit{Lunatic} and \textit{Dll1} expressions refine. \textit{Lunatic} expression is localized to the posterior half of presumptive somitomere 2, while \textit{Dll1} expression is localized to the posterior border of this same somitomere (Fig. 3E). \textit{Dll1} expression also extends caudally throughout the adjoining presomitic mesoderm. As somitogenesis continues and transcript levels of \textit{Lunatic} diminish, cells that do not express either gene, the prospective anterior half of the somite, are observed between the \textit{Lunatic} and \textit{Dll1} expression domains (Fig. 3F).
cranial placodes (Figs 2, 4). At 10.5 d.p.c. Fringe family members are expressed in a series of distinct longitudinal stripes within the ventricular region of the hindbrain and the neural tube (Fig. 2C; data not shown). Examination of their expression in cross sections of the neural tube at different rostral-caudal levels reveals Lunatic and Manic Fringe in bilateral symmetrical stripes at different dorsal-ventral levels: a ventral stripe that appears to border the floor plate, a narrow medial stripe and a broad domain that includes most of the alar plate (Fig. 4). As the rostral-caudal development of the spinal cord progresses the dorsal domains of Manic and Lunatic expression refine into two stripes, with the dorsal limit bordering the roof plate. At this developmental stage, Radical does not appear to be expressed in the caudal region and its rostral expression appears overall to be weaker and more diffuse (Figs 2H, 4C,F). At later stages Radical expression is associated with the appearance of ventral motor neurons (data not shown). Therefore, Radical expression is correlated with the rostral-caudal maturation of the spinal cord and the differentiation of neuronal populations.

The vertebrate Notch pathway has been proposed to mediate the maintenance of a population of dividing uncommitted cells in the ventricular zone of the neural epithelium by lateral specification (reviewed in Lewis, 1996; Tanabe and Jessel, 1996; Chitnis et al., 1995). To determine the relationship of Fringe gene expression to Notch ligands within the neural tube, we examined the expression of Manic Fringe relative to Dll1 and to Jagged/Serrate-1. We observe that Manic and Dll1 expression are largely coincident, whereas Manic and Jagged expression appear to be complementary (Fig. 4G,H). Therefore, although within the neural tube there is a juxta-position of Fringe and Notch ligands, their relative expression is complementary to the expression of D-fng and Ser and Dl during wing boundary determination (Kim et al., 1995; Doherty et al., 1996).

Fringe family members are also expressed at other sites and times of neurogenesis. Fringe family members exhibit restricted expression patterns in cranial placodes (e.g. olfactory placode and the otic vesicle) that will give rise to sensory structures (Fig. 2B,C,E,F; data not shown). The development of sensory structures also appears to be associated with the expression of Notch and regionalized expression of its ligands (Lewis, 1996). Lastly, the expression of Fringe family members is evident in the neural crest derived dorsal root ganglia and sensory ganglia (Figs 2, 4).

**Fringe family member activities may be regulated at the level of secretion and proteolytic processing**

The mammalian Fringe gene family expression profiles provide evidence for the acquisition of different regulatory elements controlling spatial-temporal transcription. The structures of the Fringe precursor proteins suggests an opportunity for posttranslational regulation (Fig. 1). Expression of an epitope-tagged D-fng protein in fly cell lines (S2 and clone-8) and in the wing imaginal disc show that the protein can be secreted (V. Panin, V. Papayannopoulos, R. W. and K. D. I., unpublished data). To determine if Lunatic, Radical and Manic proteins are secreted and processed we generated Fringe-epitope-tagged protein fusion constructs (AP-tag) and assayed their expression in COS (monkey kidney) and Hep3B (human liver) cell lines. For comparison, we also examined the secretion and processing of D-fng-AP in COS cells. Protein expression was determined in media and cell lysates by measuring alkaline phosphatase (AP) activity and by immunoprecipitation of [35S]Met labeled Fringe AP-tagged proteins (Flanagan and Leder, 1990).

Significant levels of AP activity in the media were observed for Lunatic-AP, Manic-AP and D-fng-AP constructs, indicative
of efficient secretion (Fig. 5A; data not shown). In contrast, Radical-AP produced significantly lower AP activity in the media when compared to the other Fringe proteins and to the secreted alkaline phosphatase (Seap) control (Fig. 5A; data not shown). Pulse-chase labeling in COS cells demonstrates that Radical-AP exhibits a similar rate of synthesis and intracellular stability to both Manic-AP and Lunatic-AP; however, over the time period of the chase, Manic-AP and Lunatic-AP appear in the media, whereas Radical-AP does not (data not shown).

In addition, D-fng-AP, Lunatic-AP and Manic-AP are immunoprecipitated from the media of [35S]Met labeled cells while Radical-AP is not (Fig. 5B; Materials and Methods). These observations suggest that although Radical enters the secretory pathway and is processed to the mature form, it is not found as a stable extracellular protein.

We also examined whether the Fringe proteins are processed. Both in [35S]Met labeled COS cell lysates and media, the fastest migrating immunoprecipitated bands correspond to the predicted sizes of the mature proteins for Manic-AP and Radical-AP (Fig. 5B). The slower bands are indicative of glycosylation known to occur on AP. By contrast in COS cell lysates we observe bands corresponding to the predicted sizes of the pro-proteins for Lunatic-AP and D-fng-AP (Fig. 5B). However, in the media we observe two bands for Lunatic-AP that migrate at relative positions consistent with their representing the pro- and mature forms (Fig. 5B). The identity of the major secreted Lunatic-AP band was determined by N-terminal sequencing to represent the fully processed mature form. In contrast to Lunatic, D-fng is observed in the media as a single major band. The identity of the secreted D-fng-AP protein recovered from the media was determined by N-terminal sequencing to represent its pro-form (Fig. 5B). In summary, although individual Fringe family members are evolutionarily conserved within the mature protein region and they all appear to enter the secretory pathway, they differ in their efficiency of secretion and in their requirement for posttranslational proteolytic processing.

Expression of mammalian Fringe genes in the Drosophila wing affects boundary determination

The Drosophila wing disc offers an established context in which to investigate the potential boundary-determining activities of mouse Fringe family members. We misexpressed Manic, Radical and Lunatic in the wing disc using the UAS-Gal4 system and compared their activity to that of D-fng (Kim et al., 1995). The patched (ptc) promoter driving Gal4 directs UAS-Fringe expression in a broad stripe along the anterior side of the A-P compartment boundary, perpendicular to endogenous, dorsally restricted D-fng expression (schematized in Fig. 6F). Misexpression of D-fng using the ptc-Gal4 driver has two effects on Drosophila wing development (Kim et al., 1995). First, wing margin tissue is lost at the D-V compartment border where misexpression has eliminated a fringe expression and non-expression boundary (Fig. 6B). This loss of wing margin tissue can also be visualized in the developing wing imaginal disc as a loss of wg protein expression (Fig. 6G). Second, an ectopic wing margin is induced in ventral cells where misexpression has created a novel fringe expression boundary (Fig. 6B). Induction of ectopic wing margin can be visualized in the developing wing imaginal disc as ectopic wg protein expression (Fig. 6G).

Misexpression of mouse Manic using the ptc-Gal4 driver eliminates wing margin tissue at the D-V compartment border (Fig. 6C) and causes a localized loss of wg protein expression (Fig. 6H). Misexpression of Manic in the ventral compartment is able to induce ectopic wg protein expression but is unable to induce ectopic wing margin formation (Fig. 6C,H). Thus, in comparison to D-fng, Manic’s ability to induce ectopic margin in the ventral compartment, whether assessed by marginal bristle formation or wg protein expression, is compromised (compare Figs 6B,G to 6C,H). Similar to Manic, misexpressed Radical also retains an ability to prevent normal margin formation and causes a localized loss of wg protein expression in the wing imaginal disc (Fig. 6D,J). However, in contrast to D-fng and Manic, this does not result in ectopic wg protein expression or margin formation (Fig. 6I). Lunatic expression does not affect the margin or wg protein expression (Fig. 6E,J). Lunatic’s inactivity may result from an inability of wing imaginal disc cells to process it to the mature form (see Materials and Methods and Discussion). Overall, misexpression of mouse Fringe genes in the fly wing suggests a conserved ability to participate in aspects of boundary-determination via the Notch pathway, leading to wg expression at the D-V organizing center (Fig. 6K).

DISCUSSION

We have identified three mammalian genes (Manic, Radical, and Lunatic Fringe) that constitute a gene family related to Drosophila fringe (D-fng). The expansion and divergence of the Fringe family members has allowed for the evolution of differential regulation at the level of sites of their expression, protein secretion and proteolytic processing. Assessment of mammalian Fringe genes in the context of the Drosophila wing disc demonstrates that the mouse Manic and Radical genes exhibit a conserved role in the Notch pathway leading to expression of wingless at the D-V compartment boundary. The coordinated expression in mammals of Fringe genes with Notch and its ligands, Delta-like 1 and Jagged/Serrate-1, further suggests that Fringe genes operate in this pathway to determine boundaries during segmentation and in cell fate decisions occurring during neurogenesis.

Fringe gene family members are subject to posttranslational regulation that restrict activities

Regulation of secreted signaling molecules at the level of protein processing is an important feature of embryonic development and organismal homeostasis (Barr, 1991; Lee et al., 1994; Thomsen and Melton, 1993; Morisato and Anderson, 1995). The divergence of Fringe family protein precursor structure allows for differential regulation and a restriction of activities amongst individual family members. The differential processing and secretion of D-fng and mouse Fringe family members in mammalian cell lines and their distinct phenotypes when expressed in the Drosophila wing lead us to infer these posttranslational controls have biological consequences. The evolutionary conservation of a dibasic processing site amino terminal to the conserved regions of D-fng and vertebrate Lunatic Fringe proteins suggests that proteolytic cleavage plays an important regulatory role in Fringe function (Wu et al., 1996). The processing sites in D-fng (RRSR) and the ver-
D-fng compartment boundary, but does not induce ectopic wing compared to and eliminates wing margin tissue and wg expression at the Radial margin in ventral cells. Radial margin loss is consistently weaker in multiple independent UAS lines expression. (K) Summary of mouse wing. (B,G) Misexpression of Gal4 by cross-hatching. (A) Wild-type ptc-Lunatic of induces ectopic wg in ventral cells (H; arrow). (E,J) Mis-expression than the effect observed following eliminates wing margin tissue (arrow) and ectopic wg expression (arrow) in ventral cells and eliminates wing margin tissue. (C,D,H,I) Misexpression of Manic and Radical eliminates wing margin tissue and wg expression at the D-V compartment boundary, but does not induce ectopic wing margin in ventral cells. Radical’s effect on morphological wing margin loss is consistently weaker in multiple independent UAS lines than the effect observed following Manic misexpression. Manic induces ectopic wg in ventral cells (H; arrow). (E,J) Mis-expression of Lunatic has no observable effect on wing development or wg expression. (K) Summary of mouse Fringe gene activities as compared to D-fng when misexpressed in the Drosophila wing disc.

Fig. 6. Misexpression of mammalian Fringe genes differentially affects boundary determination in the Drosophila wing. (A–J) Misexpression of D-fng, Manic, Radical and Lunatic in a stripe along the anterior side of the A-P compartment boundary by ptc-Gal4. (A–E) Wings are mounted with anterior up and distal to the right. (G–J) Immunohistochemical staining of wing pouches with anti-wg antibody (van den Heuvel et al., 1989). Wing pouches are oriented with ventral up and anterior to the left. (F) A schematic diagram of the wing pouch. The dorsal (D) and ventral (V) compartments are labeled. The position of the future wing margin is indicated by a black solid line, D-fng expression by gray shading and ectopic expression from ptc-Gal4 by cross-hatching. (A) Wild-type wing. (B,G) Misexpression of D-fng induces ectopic wing margin tissue (arrow) and ectopic wg expression (arrow) in ventral cells and eliminates wing margin tissue. (C,D,H,I) Misexpression of Manic and Radical eliminates wing margin tissue and wg expression at the D-V compartment boundary, but does not induce ectopic wing margin in ventral cells. Radical’s effect on morphological wing margin loss is consistently weaker in multiple independent UAS lines than the effect observed following Manic misexpression. Manic induces ectopic wg in ventral cells (H; arrow). (E,J) Mis-expression of Lunatic has no observable effect on wing development or wg expression. (K) Summary of mouse Fringe gene activities as compared to D-fng when misexpressed in the Drosophila wing disc.

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pathway, while fringe-dependent signaling requires extracellular secretion of a mature Fringe protein. Alternatively, the reduced extent of Manic’s (and formally Radical’s) induction of ectopic wg expression and of a morphological margin may reflect either a quantitative difference in the amount of secreted protein or a qualitative difference in mouse Fringe protein interaction with Drosophila proteins.

The expansion and divergence of mammalian Fringe family members has allowed for the acquisition of different regulatory elements controlling the localized expression of the different precursor proteins. This combination of transcriptional and posttranslational control could enable cells expressing different combinations of Fringe family members to fine-tune their overall reception and transmission of Fringe-dependent signaling activities. For example, we envision that it may be important during certain stages of either boundary determination or cellular differentiation to have cells acquire a state where they are refractory to Fringe-mediated signaling, yet are unable to send a Fringe signal. Expression of Radical would achieve this state.

**Fringe expression demarcates segmental boundaries in the mammalian embryo and may act through the Notch pathway**

Our observation that mammalian Fringe family members can affect boundary determination in the fly wing imaginal disc suggests that they operate through the Notch pathway. Furthermore, this result indicates that aspects of boundary organizing functions may be conserved from flies to man. This notion is supported by the observations that Fringe-expressing and non-expressing cells are juxtaposed prior to the formation of morphological segment boundaries during somitogenesis and in the hindbrain.

In mammals the clearest parallel between D-fng and its relationship to Notch in the wing is the probable participation of Lunatic in this pathway during somitogenesis. Absence of Notch1 in the presomitic mesoderm leads to a defect in the transition between presomitic mesoderm and somites (Conlon et al., 1995). Although Notch signaling is essential for somitogenesis, it is still unclear what patterning mechanisms provide the positional information to locate and establish segmental boundaries (Keynes and Stern, 1988). Our expression analysis suggests that the relationship of Lunatic to Dll1 is reminiscent of their expression during Drosophila wing margin boundary formation (Doherty et al., 1996). The expression of Lunatic in presumptive somitomeres suggests it may be a critical factor in determining segmental boundaries by acting to modulate the expression and/or signaling activities of Notch ligands to establish boundary-specific gene expression and possibly differences in cell adhesion and morphogenesis.

Although the role of the Notch pathway in the determination of hindbrain boundaries is unclear, the expression of Manic and Lunatic, as well as Jagged/Serrate-1, in pre-rhombomere 3 and pre-rhombomere 5 (C. R. and T. F. V., unpublished) are consistent with their playing a role in the segmentation of this structure (Lumsden and Krumlauf, 1996).

**Fringe expression during neurogenesis suggests a role in developmental decisions mediated by the Notch pathway**

*D-fng*, in addition to its role in boundary determination at the wing margin, is also involved in other cell fate decisions that interface with the Notch pathway (K. D. I., unpublished). Similarly, mammalian Fringe family members are expressed along with Notch and its ligands where obvious boundaries do not exist and this expression may be indicative of a role in interactions that specify cell differentiation. The regionalized expression of Fringe genes, the Notch ligands, Delta and Serrate, and the multiple Notch receptors in the neural tube, cranial placodes and neural crest is highly suggestive of a role for Fringe family members in Notch signaling in the selection of neural fates (Lewis, 1996; Tanabe and Jessell, 1996, Chitnis et al., 1995). We suggest that integration of Fringe family activities with the Notch pathway may be required for generation of the complex set of neurons and support cells that originate along the D-V and A-P axis of the neural tube.

The dynamic and localized expression of individual Fringe family members suggest that they may function independently and in concert to influence cell fate decisions that occur during embryonic development and in adult tissue homeostasis. Furthermore, as has been found for deregulated Notch in mammals (Ellisen et al., 1991), we anticipate that alterations in Fringe expression and function may result in developmental defects and disease.

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