Mutation of the *LUNATIC FRINGE* Gene in Humans Causes Spondylocostal Dysostosis with a Severe Vertebral Phenotype

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The spondylocostal dysostoses (SCDs) are a heterogeneous group of vertebral malsegmentation disorders that arise during embryonic development by a disruption of somitogenesis. Previously, we had identified two genes that cause a subset of autosomal recessive forms of this disease: *DLL3* (SCD1) and *MESP2* (SCD2). These genes are important components of the Notch signaling pathway, which has multiple roles in development and disease. Here, we have used a candidate-gene approach to identify a mutation in a third Notch pathway gene, *LUNATIC FRINGE* (*LFNG*), in a family with autosomal recessive SCD. *LFNG* encodes a glycosyltransferase that modifies the Notch family of cell-surface receptors, a key step in the regulation of this signaling pathway. A missense mutation was identified in a highly conserved phenylalanine close to the active site of the enzyme. Functional analysis revealed that the mutant LFNG was not localized to the correct compartment of the cell, was unable to modulate Notch signaling in a cell-based assay, and was enzymatically inactive. This represents the first known mutation in the human *LFNG* gene and reinforces the hypothesis that proper regulation of the Notch signaling pathway is an absolute requirement for the correct patterning of the axial skeleton.

The spondylocostal dysostoses (SCDs) are a group of disorders characterized by multiple vertebral segmentation defects and rib anomalies (Turnpenny et al. 2003). SCD is most frequently seen in sporadic cases and with diverse radiological phenotypes, which are difficult both to classify and to investigate. Nevertheless, the developmental mechanisms that produce all these defects in the embryo are likely to involve abnormal formation of somites (precursors of vertebra and associated musculature). In vertebrates, all skeletal muscle of the body, the axial skeleton, the tendons, and the dorsal dermis are derived from somites. These are paired blocks of mesoderm located on either side of the neural tube that form by a regular wave of segmentation in a rostral-to-caudal direction throughout the trunk (reviewed by Pourquié [2001]). Somite formation is a reiterative process—for example, in the mouse, somites segment from the presomitic mesoderm every 2 h for 8–10.5 d *post coitum* (equivalent to human Carnegie stages 9–13 or days 19–29). The Notch signaling pathway is central to somite formation, and, in the mouse, mutations in several components of the pathway (*Notch1, Dll3, Dll1, Lfng, Psen1,* and *CSL*) and a downstream target gene (*Hes7*) result in abnormal somitogenesis (reviewed by Weinmaster and Kintner [2003]).

The Notch signaling pathway is an evolutionarily conserved signal-transduction pathway that has important roles throughout embryonic development, as well as in the onset of diseases such as cancer (Harper et al. 2003). Mechanistically, the pathway is relatively simple, with ligand-activated signaling mediated by the nuclear translocation of the intracellular portion of the receptor to form part of a transcriptional complex. Notch is a heterodimeric one-pass transmembrane receptor. The epidermal growth factor (EGF)–like repeat containing extracellular fragments mediates interactions with DSL (Delta, Serrate, and Lag-2) ligands such as Delta-like–1 (Dll1) and Jagged1. The second fragment of Notch spans the membrane and contains an intracellular domain (ICD) that mediates Notch signaling. Binding to a ligand leads to cleavage by a disintegrin and metalloproteinase and release of the extracellular domain of Notch. A second cleavage by γ-secretase releases the ICD. The ICD translocates to the nucleus, forms a complex with the CSL (CBF1/Su(H)/Lag-1) DNA-binding protein, and converts it from a repressor to an activator of transcrip-

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Table 1

Primer Sequences

Exon(s)	Primer Sequence (5′→3′)		Internal Sequencing
	Forward	Reverse	
1:			
Pair 1	cgacgggcttcgggtcggtg	tcttgacagcgatgaagacg	…
Pair 2	agtctgtccgagtacttcag	ctggcgtcgcccacagatgg	…
2	cacgagtggggaaaccaagg	ggcggggcatggagcaaacc	…
3	ggagggtccacaggcccaag	gggcccgcaggttgacgtag	…
4 and 5	tcatcgagtccggcaggaag	tgaaacccagagggaagtgg	caggcgctgctgcccctcac; cgagaacaaggtggtgagtg
6	tgaggagtgcagcgcctttg	tgtttagaacagtgccccac	…
7	ccagtttgggaccttattcc	catttgcagtcccacaacac	…
8	gtgttgtgggactgcaaatg	ccccttcacctgtgtgcctc	tcttaagccacagcgtccag

tion. Known direct targets of Notch include members of the hairy/enhancer-of-split (HES) and HES-related family of basic helix-loop-helix transcription factors (Iso et al. 2003) and the glycosyltransferase Lunatic fringe (Morales et al. 2002).

Elsewhere, we identified two genes that cause a subset of autosomal recessive forms of SCD: *DLL3* (SCD1 [MIM 277300]) (Bulman et al. 2000; Sparrow et al. 2002; Turnpenny et al. 2003; Whittock et al. 2004*a*) and *MESP2* (SCD2 [MIM 608681]) (Whittock et al. 2004*b*). Mutations in either of these genes account for ~20%–25% of all cases of this disorder, with 24 distinct mutations detected in *DLL3* (Bonafé et al. 2003; Turnpenny et al. 2003; Whittock et al. 2004*a*; authors' unpublished data) and a single mutation detected in *MESP2* (Whittock et al. 2004*b*). In this article, we describe a patient with a skeletal phenotype more severe than that of previously reported cases of SCD1 and SCD2. We show that this patient is homozygous for a missense mutation in the *LFNG* gene. LFNG is a fucose-specific β 1,3 *N*-acetylglucosaminyltransferase that adds *N*-acetylglucosamine (GlcNAc) residues to *O*-fucose on the EGF-like repeats of Notch receptors (Bruckner et al. 2000; Moloney et al. 2000). LFNG localizes to the Golgi, where the modification of Notch receptors is believed to occur (Haines and Irvine 2003). LFNG enhances Notch1's ability to be activated by Dll1 and reduces Notch1 signaling when Jagged1 is the activating ligand. Functional analysis revealed that the mutant LFNG was not localized to the correct compartment of the cell, was unable to modify Notch signaling in vitro, and was enzymatically inactive. Therefore, we conclude that this missense mutation is likely to be causative of SCD in this patient.

Material and Methods

Human Subjects

Appropriate informed consent was obtained from the proband and family for this study.

DNA Sequencing

Genomic DNA was sequenced as described elsewhere (Sparrow et al. 2002), by use of primers listed in table 1.

RFLP Analysis

The F188L mutation produces an *Mse*I restriction site. Control samples were tested by amplification with primers 5′-GGAGGGTCCACAGGCCCAAG-3′ (forward) and 5′-GGGCCCGCAGGTTGACGTAG-3′ (reverse) and were digested with *Mse*I to yield fragments of 413 bp for the wild-type allele and 251 bp and 162 bp for the mutant allele.

In Vitro GlcNAc-Transferase Assays

HEK 293T cells were transfected with expression plasmids encoding full-length mouse Lfng cDNAs (wild-type and mutants) fused to human IgG1 Fc (at the carboxy terminus). Protein was affinity purified from either concentrated medium or whole-cell extract by use of Protein-G-Sepharose beads (Sigma), as described elsewhere (Moloney et al. 2000). GlcNAc-transferase activity was determined as described elsewhere (Moloney et al. 2000).

Cell Lines and Coculture Assays

C2C12 and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. C2C12 cells, which overexpress full-length Notch1, are described elsewhere (G. Chapman, L. Liu, C. Dahlqvist, and U. Lendahl, unpublished data). Control cells, cells overexpressing mouse Dll1, and cells overexpressing mouse Jagged1 were created by stably transfecting NIH3T3 cells with pCAGIRESpuro, pCAG-mDll1-IRESpuro, and pCAG-mJagged1-IRESpuro, respectively. Cultures were grown in 1.5 μg/ml puromycin for 10 d. Individual mDll1 or mJagged1 stable clones were screened for expression by western blotting and immunocytochemistry with rabbit anti-Dll1 or anti-Jagged1 antibodies (Santa Cruz). Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen) in 12-well trays with 14 ng of pCMV-Renilla, 350 ng of p6 × TP1-Luc (CSL reporter [Kato et al. 1997]), and 350 ng of each expression plasmid or pCMV-CAT control plasmid. Cocultures were performed in triplicate by addition of 2×10^5 NIH3T3 cells stably transfected with vector (con-

trol cells), mDll1, or mJagged1. Cocultures were harvested in 200 μl of Passive Lysis Buffer (Promega) 24 h after transfection. Firefly and Renilla luciferase activities were assayed using the Dual-Luciferase reporter system (Promega) and were measured on a FLUOstar Optima Luminometer (BMG). Firefly luciferase counts were normalized against Renilla luciferase counts, to account for differences in transfection efficiency. Fold activation of ligand-expressing cells over control NIH3T3 cells was expressed relative to that of chloramphenicol transferase (CAT)–transfected cocultures. One-way analysis of variance was performed on data from four independent experiments. Significance was determined using Tukey's post hoc test.

Western Blots and Immunocytochemistry

Western blotting was performed as described elsewhere (G. Chapman, L. Liu, C. Dahlqvist, and U. Lendahl, unpublished data). Confocal immunofluorescence of hemagglutinin (HA)–tagged Lfng and mutants was done as described elsewhere (Dahlqvist et al. 2003), by use of a TCS SP confocal microscope (Leica Microsystems). Antibodies used were obtained from Santa Cruz (Lfng), Clontech (rabbit anti-HA), Covance (mouse anti-HA), and BD transduction labs (GM130).

Results

The proband was of Lebanese background and presented with extensive congenital vertebral anomalies; long, slender fingers; and camptodactyly of the left index finger. X-ray (fig. 1*A*) and magnetic resonance imaging (MRI) scans (fig. 1*B*) showed multiple vertebral ossification centers in the thoracic spine, which showed fitted angular shapes similar to those seen in the patient with SCD2 who was described elsewhere (Whittock et al. 2004*b*). Severe foreshortening of the spine is emphasized by the comparison of the patient's arm span (186.5 cm) with adult height (155 cm; lower segment 92.5 cm). The patient had nonprogressive scoliosis of the cervical and thoracic spine. However, in contrast to SCD2, vertebral anomalies were also present in the cervical and lumbar spine (fig. 1*A* and 1*C*). Vertebral anomalies along the whole length of the spine are a feature of SCD1; however, in SCD1, the vertebrae have a more rounded shape ("pebble beach" sign [Turnpenny et al. 2003]) than in this case. Although vertebral shape is known to change with increasing age—with vertebrae becoming less rounded and more angular—the proband clearly had a spine more severely disorganized (throughout all the vertebral bodies) than that in the majority of SCD1 cases seen so far and much more severe than the singly reported sibling cases with an SCD2 mutation. Analysis of the entire coding region and splice sites of *DLL3* revealed no deviations from the published sequence. Likewise, no mutations were found in the *MESP2* coding region for this patient.

We therefore sought to identify other candidate genes for sequencing in this patient. Using the *Dll3* null mouse as a model for SCD, we have shown that SCD is caused by a defect in the formation of embryonic structures called "somites," which are the precursors of the axial skeleton, tendons, muscle, and the dorsal dermis (Dunwoodie et al. 2002). The Notch signaling pathway is central to the regulation of this process (reviewed by Weinmaster and Kintner [2003]). Indeed, both of the genes thus far implicated in causing SCD encode components of this signaling pathway: DLL3 is a ligand for the Notch family of signaling receptors, and MESP2 is a downstream target of Notch signaling. Deletion of

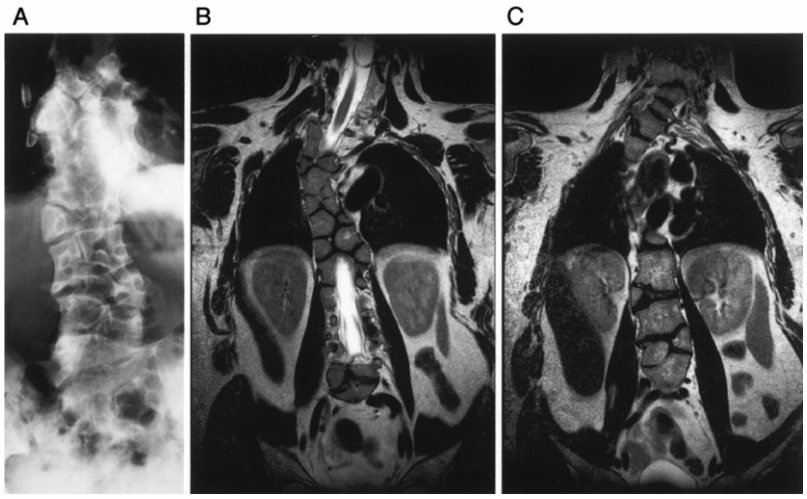

Figure 1 Radiograph (*A*) and T2-weighted coronal MRI images (*B* and *C*) in the vertebral plane of the proband. *A*, Severe vertebral segmentation anomalies throughout the vertebral column. *B*, Thoracic spine, showing vertebral centers with a fitted angular shape. *C*, Cervical and lumbar spine, showing similar segmentation anomalies.

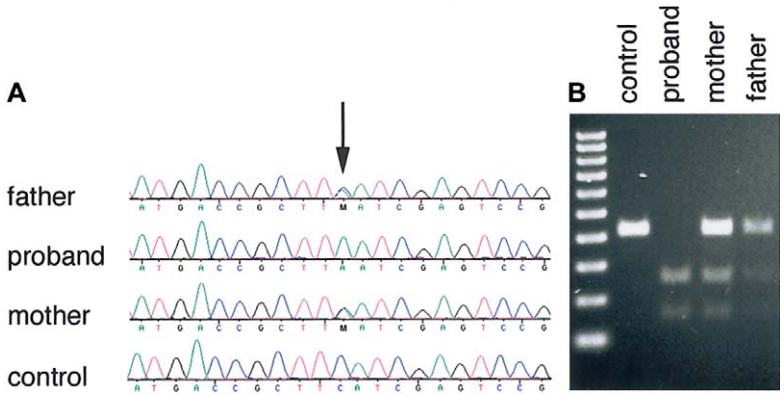

Figure 2 Detection of the c.564C→A mutation. *A,* Electropherograms documenting the affection status of the proband and parents. *B,* Confirmation of the presence of the c.564C→A mutation by *Mse*I RFLP in genomic DNA isolated from an unrelated control individual, the proband, and the proband's mother and father.

other components of the Notch signaling pathway in the mouse also causes somite dysmorphology, and these mutants represent the majority of defects in somitogenesis reported thus far (see Sparrow et al. 2002). We therefore examined the phenotypes of such mouse lines in detail, in an effort to identify further candidate genes for sequencing in our patient. The phenotypes of *Dll3* (pudgy) and *Lfng* null mice were virtually identical (Zhang et al. 2002), indicating that murine somitogenesis is similarly dependent on each gene. LFNG is a fucose-specific β 1,3 *N*-acetylglucosaminyltransferase (Bruckner et al. 2000; Moloney et al. 2000) that functions in the Golgi to post-translationally modify the Notch receptors, altering their signaling properties (Haines and Irvine 2003). Furthermore, our previous analysis revealed that *Lfng* gene expression is severely disregulated in *Dll3* null mice, suggesting that *Lfng* expression is dependent on Dll3 function (Dunwoodie et al. 2002; Kusumi et al. 2004). We sequenced the entire coding region and splice sites of the *LFNG* gene in the proband (fig. 2*A*), using primers listed in table 1. A homozygous missense mutation (c.564C→A) in exon 3 was detected that results in substitution of leucine for phenylalanine (F188L). Sequencing of DNA from the proband's parents, who had normal spinal and hand anatomy, confirmed that they were both heterozygous for the mutant allele. A number of approaches were taken to demonstrate that this base change was not a common polymorphism unassociated with the SCD phenotype. The mutation created a novel *Mse*I restriction enzyme site, and the resultant RFLP was used to confirm the sequencing results in the pedigree (fig. 2*B*). The *Mse*I polymorphism was not found in 125 control subjects (250 chromosomes), giving ~80% power to distinguish a normal sequence variant from a mutation (Collins and Schwartz 2002). In addition, the underlying base substitution was not present in the National Center for Biotechnology Information SNP database (dbSNP Web site).

Structural Consequences of the F188L LFNG Mutation

The mutated phenylalanine is absolutely conserved in all known fringe proteins, from *Drosophila melanogaster* to humans (Correia et al. 2003). Examination of the mutation within an LFNG model based on solved gly-

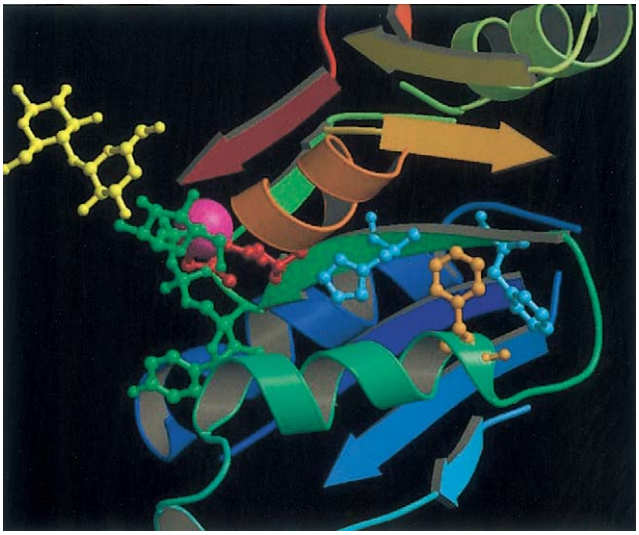

Figure 3 Structural model of LFNG, showing the proximity of the mutated phenylalanine residue (*orange*) to the Mn^{2+} binding site (Mn^{2+} in *purple*). Directly interacting residues F196 and H198 are shown in cyan, and the nearby Mn^{2+}-ligating residues D202 and D203 are in red. The position of the UDP-sugar donor group (*green*) and an acceptor sugar (*yellow*) are shown for reference. α-helices and β-sheets are colored in a gradient from dark blue (amino terminus) to red (carboxy terminus).

cosyltransferase structures suggests that the conserved phenylalanine residue (F188) is not directly involved in uridine diphosphate (UDP)–GlcNAc (donor) or protein binding (fig. 3). Rather, it is likely to reside in a helix that packs against the strand containing the Mn^{2+}-ligating residues D200 and the nearby D202. F188 is predicted to form an aromatic cluster with residues F196 and H198, and thus the F188L mutation may either cause steric perturbation of the Mn^{2+} binding site by altered packing of the smaller mutant (leucine) residue or cause electronic disruption of the enzymatic reaction by removal of required π-π interactions associated with the aromatic ring. Further details of structural modeling techniques are given in appendix A (online only).

Functional Analysis of the F188L LFNG Mutation

To provide evidence that this was, indeed, an SCD-causative mutation, we undertook a range of assays to assess the function of the LFNG mutant. Since no full-length human cDNA was available, we created two F187L mutations in mouse Lfng that correspond to F188L in human LFNG: a c.564C→A mutation encoding the rare leucine codon (TTA) observed in the proband and the c.562T→C + c.567C→G mutation encoding the most common human leucine codon (CTG). In addition, a previously characterized, enzymatically inactive form of Lfng (D202A) was created that disrupts the conserved DDD Mn^{2+} binding active site (Chen et al. 2001). We initially examined whether the mutant protein was stable and efficiently expressed, since, in addition to the potential destabilization of the protein by amino acid substitution, the presence of low-frequency codons in transcripts has been shown to cause stalling of translation machinery, transcript degradation, and lowered levels of protein expression (Lemm and Ross 2002). HA-tagged versions of Lfng were transiently transfected into C2C12 cells, and protein levels were determined by western blot (fig. 4*A*). Both F187L mutant Lfng proteins were expressed at higher levels than the wild-type or D202A Lfng forms, indicating that both translation efficiency and protein stability were not adversely affected by the F187L amino acid change or the presence of a low-frequency codon in the transcript. In all subsequent assays, the TTA and CTG mutants behaved identically (figs. 4*A,* 5*A,* and 5*B* and data not shown).

Lfng protein is normally present in the Golgi apparatus. To determine whether the F187L mutation affected the targeting of Lfng, intracellular protein localization was examined using immunofluorescence (fig. 4*B*–4*D*). Wild-type and D202A mutant Lfng were localized predominantly to the Golgi, as assessed by co-staining with the Golgi-specific marker GM130 (fig. 4*B* and 4*C*). By contrast, the F187L mutant Lfng did not colocalize with GM130 (fig. 4*D*). Therefore, we conclude that the F187L mutant form of Lfng is expressed but is mislocalized within the cell.

To assess the effect of the F187L mutation on Lfng function, we used a cell coculture assay described elsewhere (Hicks et al. 2000; G. Chapman, L. Liu, C. Dahlqvist, and U. Lendahl, unpublished data). In this system, C2C12 myoblasts expressing the mouse Notch1 receptor (i.e., responding cells) were cocultured with NIH3T3 cells expressing either mouse Dll1 or Jagged1 ligands. Prior to coculture, responding cells were transiently transfected with a luciferase reporter gene driven by multiple copies of the Notch signaling–responsive CSL binding site (Kato et al. 1997). After coculture for 24 h, luciferase levels were determined, to measure the extent of Notch signaling in the responding cells. Expression of wild-type Lfng in responding cells acted to potentiate Dll1 signaling (fig. 6*A*) and to inhibit Jagged1 signaling (fig. 6*B*), as reported elsewhere (Hicks et al. 2000; Shimizu et al. 2001). However, when either the F187L or the D202A mutant forms of Lfng were used in these assays, they had little or no effect on the levels of Notch signaling induced by Dll1 or Jagged1 in the responding cells. These data indicate that the F187L mutation renders Lfng functionally inactive in these Notch signaling assays.

The incorrect subcellular localization of the F187L Lfng could be responsible for the failure of this mutant to function in Notch signaling, and thus it is possible that this form of Lfng is still capable of modifying the Notch receptors. Therefore, to assess the enzymatic activity of the wild-type and mutant Lfng proteins, we performed a GlcNAc-transferase assay in vitro. Carboxy-terminal Fc-tagged versions of wild-type or mutated forms of Lfng were expressed in 293T cells and were affinity purified from medium, as described elsewhere (Moloney et al. 2000), and protein levels were analyzed by immunoblot with an Lfng-specific antibody (fig. 5*A*). Although Lfng is normally considered to be localized to and function in the Golgi apparatus in vivo (Bruckner et al. 2000; Munro and Freeman 2000), when it is overexpressed in cultured cells, significant amounts of protein are secreted into the medium. This is a common feature when Golgi glycosyltransferases are overexpressed, but it is not known whether the secreted form serves any biological function, although evidence from the *Drosophila* homologue (Fringe) suggests that the major function of the protein occurs within the Golgi (Bruckner et al. 2000; Munro and Freeman 2000). In keeping with our subcellular localization results, wild-type and D202A mutant forms of Lfng were efficiently secreted from the cell, but only small amounts of F187L Lfng were present in conditioned medium (2%–3% of normal protein levels) (fig. 5*A*). This is likely to be the result of a defect in protein secretion, since the F187L mutation does not affect protein translation and stability

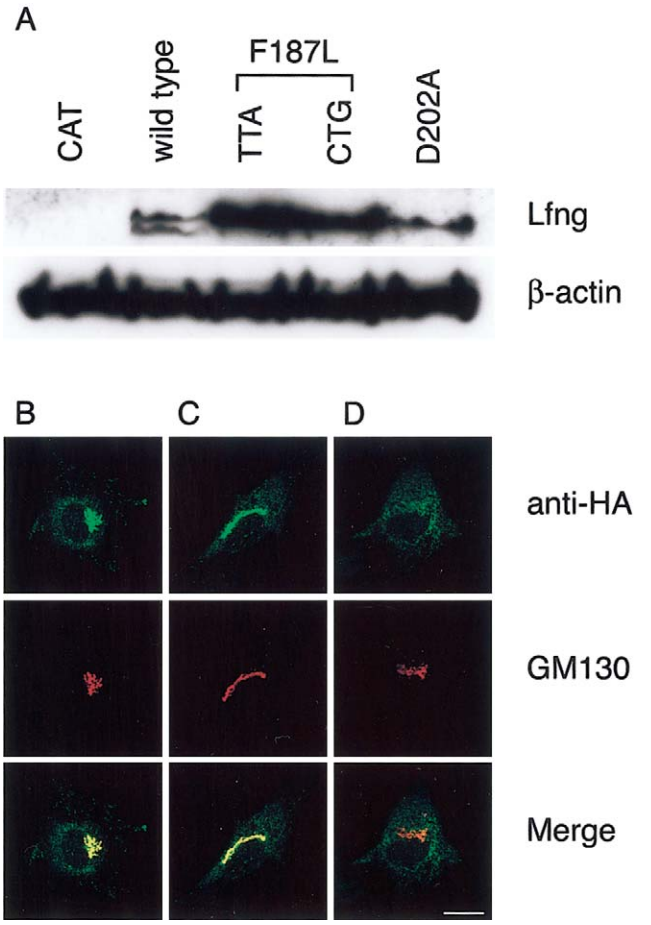

Figure 4 *A,* Relative expression of transfected wild-type and mutant Lfng proteins. Shown is western blot detection of Lfng in lysates from C2C12 cells transfected with HA-tagged wild-type, TTA F187L, CTG F187L, and D202A Lfng. A total of 50 μg of each lysate was run on a 4%–12% PAGE gel and was blotted. Lfng proteins were detected using a mouse anti-HA antibody. Western blot detection of β-actin was performed to control for loading. *B–D,* Immunofluorescence analysis of wild-type and mutant Lfng proteins in cultured cells. F187L Lfng does not localize to the Golgi. C2C12 myoblasts were transiently transfected with constructs encoding HA-tagged wild-type Lfng (*B*), D202A mutant Lfng (*C*), and F187L Lfng (*D*). In each case, the top panel shows a confocal image of immunofluorescence with a rabbit anti-HA antibody, and the middle panel with the Golgi-specific antibody (GM130); the bottom panel shows a merged image. The scale bar represents 25 μm.

within the cell (fig. 4*A*). Therefore, the protein purification was repeated using a whole-cell protein extract. As expected, all four proteins were detectable (fig. 5*A*). Affinity-purified protein derived from whole-cell extract was then used in an in vitro GlcNAc-transferase assay with the use of *p*-nitrophenyl-α-L-fucose (which structurally mimics *O*-linked fucose) as an acceptor and UDP-[3H]GlcNAc as a donor substrate (Moloney et al. 2000). The difference in size between protein purified from conditioned medium and protein purified from whole-cell extract may be indicative of the absence of pro-protein processing. However, there was no discernible difference in the relative in vitro enzymatic activity of wild-type protein purified from either source (data not shown). As shown in figure *5B,* the F187L (TTA and CTG) and D202A mutants showed no enzymatic activity above background, whereas wild-type Lfng showed activity similar to that described elsewhere (Chen et al. 2001). This demonstrates that F187 is necessary for GlcNAc-transferase activity and that substitution of leucine at this site renders the enzyme inactive.

Discussion

Our data strongly support the hypothesis that the F188L missense mutation of LFNG is causative of SCD in the proband, because it is not localized to the correct compartment of the cell, is unable to modulate Notch signaling in the same manner as the wild-type protein, and is enzymatically inactive. We propose that this form of SCD be referred to as "SCD3" because mutation in *LFNG* demonstrates that this case is genetically distinct from SCD1 and SCD2. The proband has a more severely disorganized spine, compared with the majority of SCD1 cases seen so far, and certainly compared with the SCD2 cases; however, clear differentiation in phenotype between cases of SCD caused by mutation in *DLL3, MESP2,* and *LFNG* cannot be confirmed until more patients with mutations in *LFNG* (and *MESP2*) are uncovered. It is unclear how frequent such mutations are within the group of patients with costovertebral defects, since we have, to date, only sequenced the *LFNG* gene for 28 patients with SCD who lack mutations in both *DLL3* and *MESP2*. Discovery that mutation in a third gene causes a discernible SCD phenotype emphasizes the importance of detailed examination of the vertebral anomalies of patients with SCD to assist diagnosis of the underlying genetic defect in this group of vertebral segmentation disorders.

In contrast to the apparent situation in humans, close analysis of the vertebral phenotypes in mice lacking *Dll3* (Dunwoodie et al. 2002), *Mesp2* (Saga et al. 1997), or *Lfng* (Zhang and Gridley 1998) shows no obvious differences in either the appearance of vertebrae or the extent of malformation along the rostral-caudal axis. Therefore, it is possible that differences are observed in the human phenotypes because the expression of these genes in humans is different from the expression in mice. In the mouse, detailed expression profiles during somitogenesis have been established for *Dll3* (Dunwoodie et al. 1997), *Mesp2* (Saga et al. 1997), and *Lfng* (Forsberg et al. 1998; Aulehla and Johnson 1999). In contrast, nothing is known about the expression of these genes during somite formation in humans. It is also possible that the differences between mouse and human pheno-

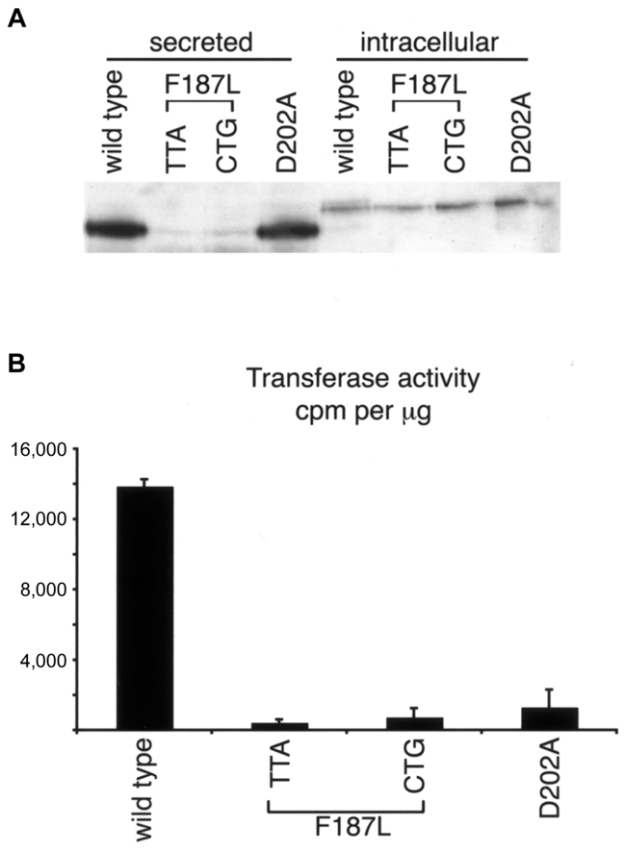

Figure 5 Results showing that F187 Lfng is enzymatically inactive. *A*, Western blot using an Lfng-specific antibody on Lfng-Fc fusion protein that was affinity purified from conditioned medium ("secreted") or whole-cell extract ("intracellular") derived from transiently transfected HEK 293T cells. *B*, GlcNAc-transferase assays performed on Lfng that was affinity purified from whole-cell extract by use of pNp-fucose (4 mM) as an acceptor and UDP-[3H]GlcNAc as a donor. Assays were performed in duplicate, and error bars represent SDs of the mean.

types are the result of the nature of the causative mutations, rather than being intrinsic to the genes themselves. Here, we have shown that the *LFNG* F188L mutation is equivalent to a null allele. Likewise, the majority of *DLL3* mutations are probably also null alleles (Turnpenny et al. 2003). In contrast, the only known *MESP2* mutant allele produces a truncated protein that retains its DNA-binding domain and may possess some of its normal function. This may explain the lesser extent of the phenotype along the vertebral column in the patients with the *MESP2* mutation than that in the patients with *DLL3* and *LFNG* mutations.

In addition to vertebral anomalies, the proband has long, slender fingers and camptodactyly of the left index finger. We feel that this is unlikely to be a consequence of the mutation in *LFNG,* since mouse embryos lacking *Lfng* expression do not show limb defects (Zhang et al. 2002) and, within the developing vertebrate limb, *Lfng* expression is limited to hemangioblasts, the precursors of blood vessels. However, the exact expression pattern of *LFNG* in the human embryo remains to be investigated, and it is formally possible that human limb development differs from that in the mouse. It should also be noted that another member of the fringe family, *Radical fringe* (*Rfng*), is expressed in the apical ectodermal ridge in both mouse and chick. This is a key organizing center in the developing vertebrate limb that has several distinct roles in embryonic limb formation (Capdevila and Izpisua Belmonte 2001). The role of Rfng within the developing limb is controversial because studies in the chick and mouse show differences. In the chick, ectopic overexpression of *Rfng* in the developing limb causes a disruption of normal limb formation (Laufer et al. 1997; Rodriguez-Esteban et al. 1997). However, in mouse embryos lacking *Rfng,* no overt phenotype is seen in the limb or elsewhere (Moran et al. 1999; Zhang and Gridley 1999; Zhang et al. 2002). Furthermore, mouse embryos lacking both *Lfng* and *Rfng* show an identical phenotype to *Lfng* null embryos, with no overt limb deformity (Zhang et al. 2002). These discrepancies may be caused by differences between chick and mouse limb formation or by differences in experimental approach (i.e., overexpression vs. gene deletion). Additional patients with mutation in *LFNG* will show whether there is a correlation between the mutation and the presence of camptodactyly; however, an anomaly of a single finger could possibly be a secondary consequence of the cervical segmentation phenotype through nerve entrapment.

Lfng is expressed in a number of other locations in the developing mouse embryo, including the segmenting hindbrain, neural crest migrating into the branchial arches, olfactory placode, inner ear, lung, kidney, thymus, ovary, and teeth (Cohen et al. 1997; Johnston et al. 1997; Zhang et al. 2000; Koch et al. 2001; Mustonen et al. 2002; Leimeister et al. 2003; Hahn et al. 2005; van Tuyl et al. 2005). Because of a reduced viability of the *Lfng* null mouse line, phenotypic assessment of these organs in null mice has been limited to the inner ear (Zhang et al. 2000) and the ovary (Hahn et al. 2005). In the latter case, female null mice have been shown to be infertile because of defects in the meiotic maturation of oocytes. The availability of a null *LFNG* allele in humans provides a valuable opportunity to explore the consequence of a loss of *LFNG* beyond the axial skeleton. In particular, *LFNG* is expressed in restricted regions of the developing brain and nervous system, and thus clinical neurological investigation of the proband may be useful to further elucidate the importance of modulation of Notch signaling in the development of the brain.

It is not clear how the F188L mutation leads to a loss

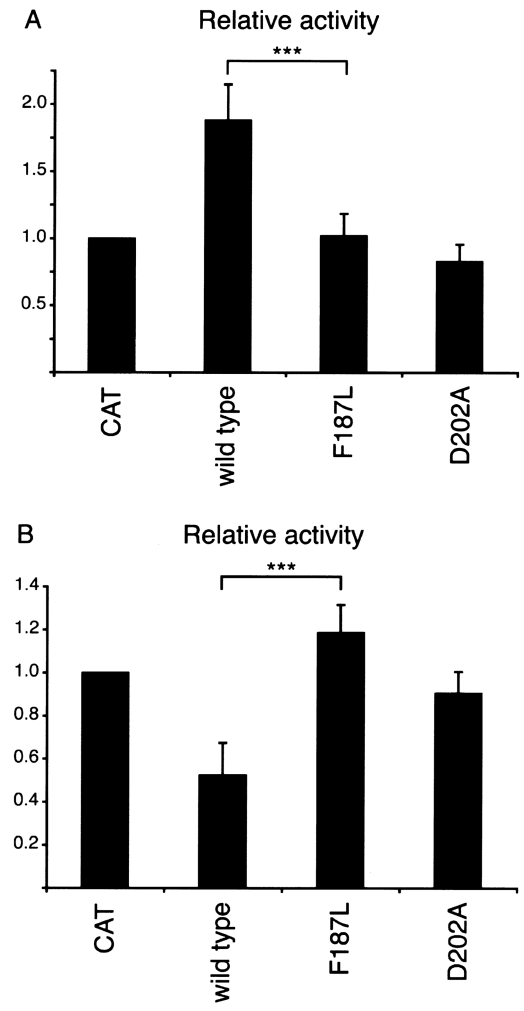

Figure 6 Results showing that F187L Lfng does not modulate ligand-induced Notch signaling. *A,* Wild-type Lfng significantly potentiates activation of Notch1 by coculture with Dll1-expressing cells, whereas F187L and D202A mutants do not alter levels of Notch1 activation. Three asterisks (***) denote $P < .001$ for wild-type versus F187L Lfng. *B,* Wild-type Lfng significantly inhibits activation of Notch1 by coculture with Jagged1-expressing cells, whereas F187L and D202A mutants do not alter levels of Notch1 activation. Three asterisks (***) denote $P < .001$ for wild-type versus F187L Lfng. Notch1 cells were cotransfected with plasmids encoding wild-type Lfng, mutant Lfng, or CAT (as a negative control) and a CSL reporter. Transfected cells were cocultured with either control NIH3T3, Dll1, or Jagged1 cells and were assayed for luciferase activity. Fold activation of ligand-expressing cells over control NIH3T3 cells is expressed relative to that of CAT-transfected cocultures. Notch signaling was activated 4–10-fold in these experiments. Error bars represent SDs of four independent experiments.

of subcellular localization of Lfng in the Golgi, especially since the D202A mutant (which also lacks enzymatic activity) is localized correctly. It is possible that this mutation has uncovered a new nonenzymatic functional domain in the Lfng protein that is required to bind another protein or chaperone to achieve proper subcellular localization. Such a dual role for a glycosyltransferase has been recently described for the *Drosophila* protein *O*-fucosyltransferase (Okajima et al. 2005). This enzyme adds an *O*-fucose residue to the Notch receptor to create the substrate for the fringe family of glycosyltransferases. However, this protein is likely to possess a nonenzymatic ability to facilitate folding of the Notch receptor and subsequent trafficking out of the endoplasmic reticulum to the Golgi.

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Web Resources

The URLs for data presented herein are as follows:

dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SCD1 and SCD2)

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