Available online at www.sciencedirect.com
ScienceDirect
Genomics 88 (2006) 600–609
GENOMICS
www.elsevier.com/locate/ygeno

Defective splicing of *Megf7/Lrp4,* a regulator of distal limb development, in autosomal recessive mulefoot disease

Eric B. Johnson [a], David J. Steffen [b], Kristen W. Lynch [c], Joachim Herz [a,*]

[a] *Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75390-9046, USA*
[b] *Veterinary Diagnostic Center, University of Nebraska–Lincoln, Lincoln, NE 68588, USA*
[c] *Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390-9038, USA*

Received 23 May 2006; accepted 3 August 2006
Available online 11 September 2006

Abstract

Mulefoot disease (MFD) is an autosomal recessive disorder of phenotypically variable expression that causes syndactyly in certain strains of cows. MFD maps to a narrow interval on bovine chromosome 15 that is syntenic to human chromosome 11p12–p11.2. This region contains *MEGF7/LRP4* (approved gene symbol *LRP4*), a gene that encodes a member of the multifunctional low-density lipoprotein receptor gene family. Targeted and naturally occurring mutations in the murine *Megf7/Lrp4* gene, a putative coreceptor in the Wnt signaling pathway, cause polysyndactyly in the rodent. Thus, *Megf7/Lrp4* is a strong candidate for the MFD mutation. Using PCR analysis of tissue samples and sperm from confirmed homozygous MFD carriers, we have identified a functional single base pair mutation in the affected animals. We show that a G → A transition at the first nucleotide in the splice donor site of intron 37 completely disables this splice site. The abnormal splicing that is caused by this mutation predicts the generation of a dysfunctional membrane-anchored receptor lacking the normal cytoplasmic domain. These findings confirm that autosomal recessive loss-of-function mutations in *Megf7/Lrp4* result in phenotypically similar forms of syndactyly in different mammalian species and that such mutations are the cause of MFD in bovines.

Keywords: Apical ectodermal ridge; LDL receptor family; Wnt; Syndactyly; Lipoprotein receptor

Syndactylies and polysyndactylies, malformations of the distal limbs that result in fusion with or without the presence of supernumerary digits, are not uncommon and occur in a number of etiologically distinct genetic syndromes in humans [1]. A number of naturally occurring mutations that cause similar syndromes have also been identified in rodents and other species [2]. In particular the advent of gene knockout technology has yielded an increasing number of mutant mouse strains that comprise the entire spectrum of limb deformities [3,4].

A naturally occurring form of autosomal recessive syndactyly with variable penetrance in the bovine species, termed mulefoot disease (MFD), results in the painful fusion of the hooves and reduced mobility. It occurs in several valuable strains known for meat or milk production, including Angus, Holsteins, and Friesians [5–7]. As few as one and up to all four hooves may be abnormally fused in homozygous carriers of the disease, suggesting that the syndrome is caused by one or more hypomorphic mutations in a single gene and that the range of phenotypic expression may be modulated by other genetic or epigenetic factors. Heterozygous female carriers of MFD have been reported to show increased milk production, which could explain the selection for and persistence of the mutant allele in the cow population [6–8].

Through identity-by-descent analysis, and owing to the detailed records of pedigrees maintained by commercial breeders for the Angus, Holstein, and Dutch black-and-white Friesian populations, the monogenic autosomal recessive MFD trait has been mapped to bovine chromosome 15 [5,8]. This chromosome is almost entirely contained within human chromosome 11 [9], making it highly likely that the human ortholog to the bovine MFD gene resides on this chromosome.

We and others have previously reported that loss-of-function mutations in the *Megf7/Lrp4* gene (approved gene symbol *Lrp4*) result in an autosomal recessive form of polysyndactyly

* Corresponding author.
E-mail address: joachim.herz@utsouthwestern.edu (J. Herz).

0888-7543/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2006.08.005

in the mouse [10,11]. *Megf7/Lrp4* encodes a member of the multifunctional low-density lipoprotein (LDL) receptor gene family [12,13]. Expression analysis of misregulated or abnormally expressed genes involved in the control of limb development combined with in vitro reporter gene experiments suggested that *Megf7/Lrp4* can regulate Wnt signaling and that it may also be involved in the control of bone morphogenetic protein (Bmp), fibroblast-like growth factor, and sonic hedgehog (Shh) signals. Potential roles in Bmp [14] and Shh signaling [15] have previously been proposed for Megalin, another LDL receptor family member, and the highly homologous Lrp1 can antagonize Wnt signaling in vitro [16]. Moreover, the structural organization of the extracellular domain of Megf7/Lrp4 closely resembles that of Lrp5/6, two related receptors that function as coreceptors for Wnt in the canonical Wnt signaling pathway [17–19]. Taken together, these findings make it likely that *Megf7/Lrp4* controls the formation of the mammalian limbs by serving as a coreceptor or modulator of one or more of these morphogenic signals.

MFD maps to a narrow interval on bovine chromosome 15 that is syntenic to human chromosome 11p12–p11.2 and mouse chromosome 2. A lod score of 24.3 links the chromosomal marker BM848 to the MFD locus [8]. *Megf7/Lrp4* lies within this region, making it a strong candidate for the MFD mutation. Using PCR analysis of tissue samples and sperm from confirmed homozygous MFD carriers, we have identified a single functional base pair mutation in two affected animals. We show that a G → A transition at the first nucleotide in the splice donor site of intron 37 of *Megf7/Lrp4* disrupts this splice site. The abnormal splicing that is caused by this mutation predicts the generation of a dysfunctional membrane-anchored receptor that lacks its normal cytoplasmic domain. Low residual functional activity of this truncated receptor, or variable generation of small amounts of a normal splice product, might explain the individual phenotypic variability of MFD.

Results

Megf7/Lrp4 resides within the MFD region

Naturally occurring [11] and experimentally induced [10] loss-of-function mutations in the murine *Megf7/Lrp4* gene result in an autosomal recessive phenotype that includes a fully penetrant form of polysyndactyly of fore- and hind limbs and incompletely penetrant facial dysmorphisms that include defects in tooth development [10]. Syndactyly syndromes in humans are common and genetically heterogeneous, and none of the known human autosomal recessive syndactyly syndromes have been mapped to chromosome 11p12–p11.2, where *Megf7/Lrp4* resides. By contrast, an autosomal recessive form of syndactyly in the cow, known as MFD, has been mapped to bovine chromosome 15, which is almost in its entirety syntenic to human chromosome 11 [9]. The MFD locus has been mapped to the vicinity of the chromosomal marker BM848, which is in linkage disequilibrium with MFD with an extremely high lod score of 24.3 [8]. *Megf7/Lrp4* resides within 2.1 Mb of BM848 (Fig. 1), and in light of its known biological functions and genetic properties it is an obvious and prime candidate for MFD.

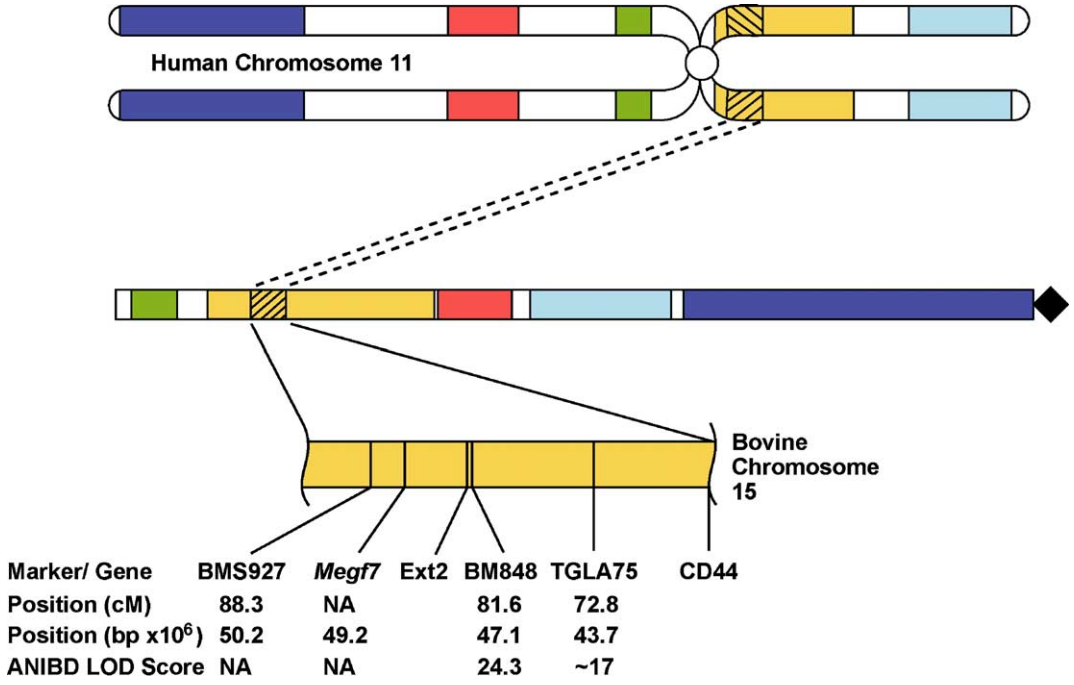

Fig. 1. *Megf7/Lrp4* is closely linked to marker BM848 on bovine chromosome 15. Comparative schematic of human chromosome 11 and bovine chromosome 15 from data collected from the NCBI (http://www.ncbi.nih.gov), Ensembl (http://www.ensembl.org), and USDA MARC (http://www.marc.usda.gov) databases and from Charlier et al. [8] and Gautier et al. [9]. Bovine *Megf7/Lrp4* is separated by 2.1 Mb from BM848, the marker linked to MFD with the highest lod score (24.3). TGLA75 and BM848 are markers shown by Charlier at al. [8] to be linked to MFD.

Homozyous G → A splice site mutation in MFD

To determine whether genetic mutations that disrupt the function of the bovine *Megf7/Lrp4* gene are present in MFD carriers we have analyzed the genomic sequence at the *Megf7/Lrp4* locus of two known homozygous MFD carriers (Fig. 2), using tissue samples obtained from one of the affected animals (MF1) and frozen sperm from another (MF2). PCR primers were designed and used to amplify most of the *Megf7/Lrp4* coding region, including flanking intronic sequences (described under Experimental procedures). Several independently obtained wild-type samples were used as controls. No functionally predictable sequence differences were found between MFD and wild-type samples, except at a single nucleotide position at the exon/intron boundary 37 (Fig. 2A). Both MFD samples (MF1 and MF2) differed from wild type at the first nucleotide of the splice donor site, where the normally occurring "GT" sequence was changed to "AT". "GT" at this position is highly conserved among splice sites. Moreover, G → A mutations in splice donor sites at this position have been shown to severely cripple or completely abrogate splicing [20]. This finding suggests that in the two affected disease carriers we have examined MFD is caused by the disruption of *Megf7/Lrp4* pre-mRNA splicing. The site of the mutation at exon/intron boundary 37 is located immediately downstream of the first exon encoding cytoplasmic domain sequences of *Megf7/Lrp4* (Fig. 2B), suggesting that the MFD phenotype is caused either by destabilization of the transcript or by exon skipping and abnormal truncation of the translation product, or both.

The G → A splice site mutation prevents normal splicing

To demonstrate that the single point mutation we have found in exon/intron boundary 37 does indeed abrogate the normal splicing of *Megf7/Lrp4,* and that the resulting transcript would thus result in an abnormal and dysfunctional translation product, we cloned exon 37 including approximately 200 bp of flanking upstream and downstream intronic sequences into the splicing test vector pcAT7. This vector is an artificial minigene consisting of the β-actin promoter and a fragment of the β-globin gene that has been engineered to facilitate fusion to heterologous intronic and exonic sequences [21]. The structure of the test vector/minigene is shown in Fig. 3A. Two splicing test vectors were generated, one that contained the indicated wild-type bovine sequences (cloned between the *Nde*I and the *Hin*dIII sites) and another one that was identical except for the single point mutation at the exon/intron 37 boundary. Wild-type (pcAT7-WT), mutant (pcAT7-MF), and empty (pcAT7-SC12) vectors

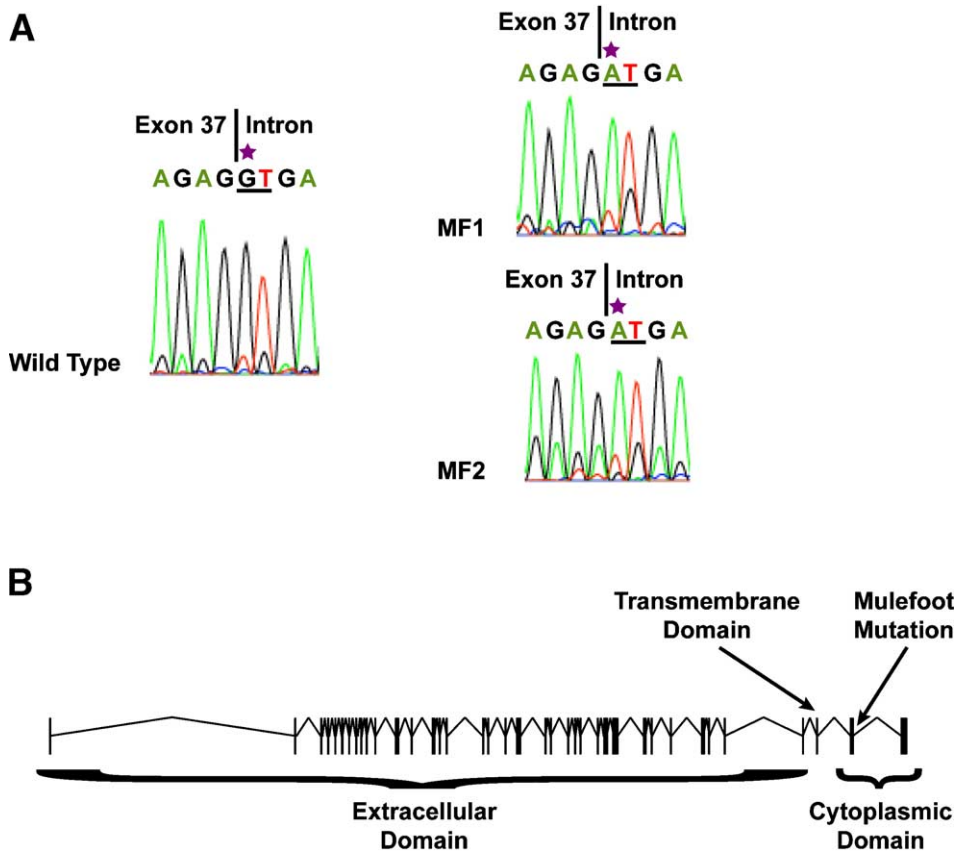

Fig. 2. A G → A mutation disrupts the 5′ splice site of intron 37 in the Angus mulefoot allele of bovine *Megf7/Lrp4*. (A) The sequence of the 3′ exon/intron border of bovine exon 37. In wild type (left) the first base of intron 37 is G. In the mulefoot samples (right) this, in splice sites, highly conserved G is mutated to A. The purple star indicates the mutated base. The underlined nucleotides are the intronic bases that bind to U1 RNA. (B) Graphic representation of the bovine *Megf7/Lrp4* sequence. Vertical lines represent exons. The first 35 exons encode the extracellular domain. Exon 36 encodes the transmembrane domain. Exons 37 and 38 encode the cytoplasmic domain of *Megf7/Lrp4*. The mulefoot mutation occurs at the first base of intron 37.

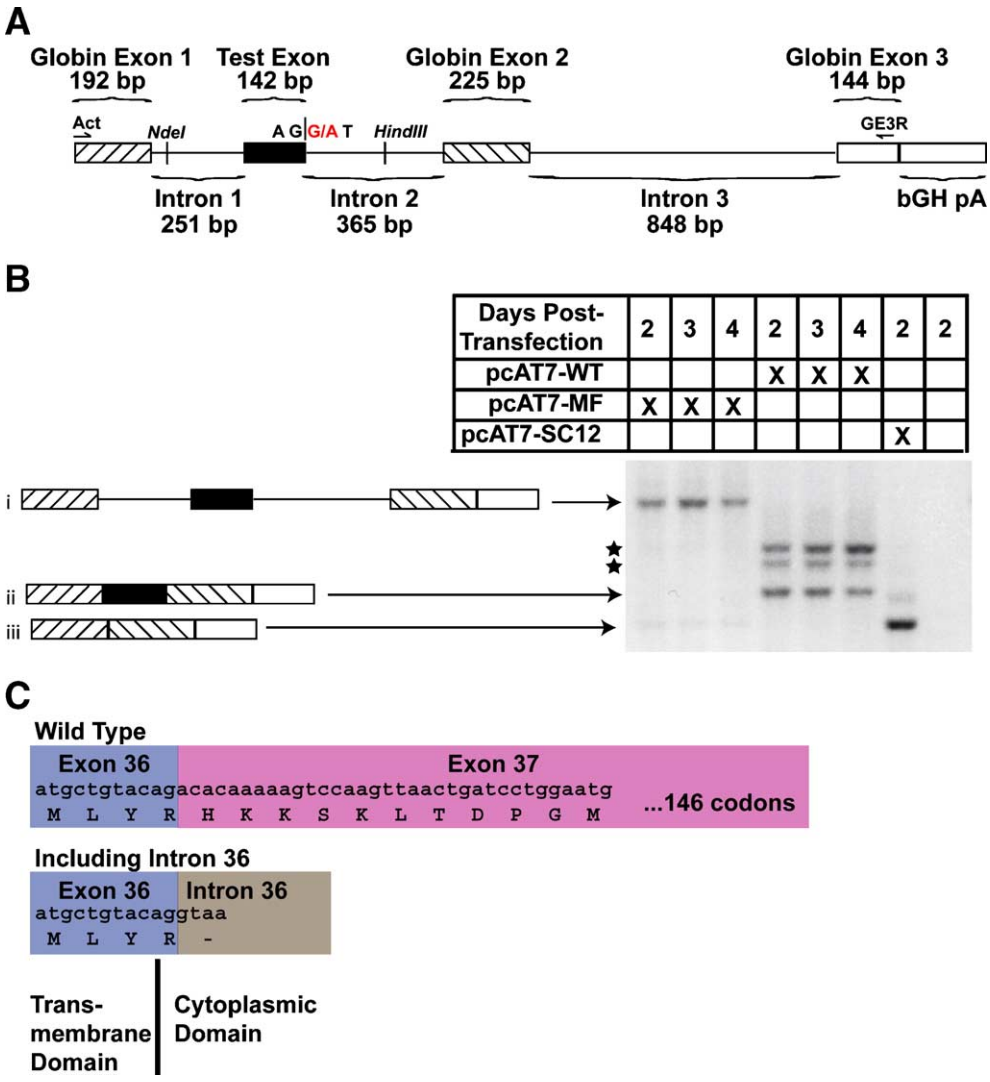

Fig. 3. Minigene splicing analysis of the mulefoot mutation. (A) Diagram of the primary unspliced product of the minigene. Bovine exon 37 and 200 bp of flanking intronic sequence was cloned between the first and the second exon of the β-globin minigene using the restriction enzymes *Nde*I and *Hin*dIII. The only difference between the wild-type and mulefoot minigene constructs is the G-to-A mulefoot mutation (red) at the first residue of intron 37. (B) Both exon 37 and flanking introns fail to splice in the mulefoot minigene RNA. Transcripts from the transfected minigene constructs were reverse-transcribed and amplified by PCR using the indicated Act and Ge3R primers. The regions of the minigene that generated the observed products are indicated on the left and these splice products were verified by sequencing. (Lanes 1–3) Mulefoot sequence (MF), (lanes 4–6) wild-type sequence (WT), (lane 7) parental pcAT7-SC12 plasmid, (lane 8) untransfected cells. (C) Expected MF-*Megf7/Lrp4* translation product. Wild-type sequence is shown at the top, MF sequence (including intron 36) below.

were transfected into HEK293 cells, and the resulting transcripts were analyzed by RT-PCR using the indicated primers Act (forward) and GE3R (reverse), as described previously [22]. Fig. 3B shows the RT-PCR products that were isolated from multiple independently transfected culture dishes at different days after transfection and subjected to agarose gel analysis and sequencing. Splicing was not affected by the time at which the cells were harvested after transfection. As shown in lanes 1–3, MF sequences did not undergo productive splicing and instead yielded a single high-molecular-weight transcript, which resulted from the inclusion of the upstream as well as downstream introns together with exon 37 (uppermost splice product i, present in lanes 1 and 3). Splicing of the pre-mRNA did occur, however, since the normal globin intron present in this construct (intron 3) was quantitatively removed and absent from all products, as confirmed by sequencing of the PCR products. By contrast, the wild-type test construct (lanes 4–6) was efficiently spliced to the expected product (ii, middle), with no detectable long form (i) remaining. Two cryptic or incompletely spliced intermediate products (indicated by the asterisks) were also detected, as often occurs in such minigene assays [22]. Transfection with the control vector (lane 7) yielded the expected product (iii, lower band).

Aberrant splicing of intron 36 in MFD

Megf7/Lrp4 is expressed postnatally in various tissues, including the brain, kidney, lung, stomach, etc. (our own unpublished results). However, *Megf7/Lrp4* transcripts were not detectable in the sperm samples (sperm generally does not

contain mRNA), and only very low levels were detectable by RT-PCR in the skin. To investigate whether the *Megf7/Lrp4* transcript is misspliced in vivo, as predicted by our in vitro splicing assay, we performed RT-PCR on wild-type and MFD RNA isolated from the skin sample using the primers indicated in Fig. 4. Primers located in exons 30 (forward) and 36 (reverse) were used as controls to demonstrate normal splicing of exons upstream of the MFD mutation and yielded the expected product, which was identical in the wild type and in the MFD samples (Fig. 4B). To detect unspliced intron 36 sequences we used another primer pair, consisting of a forward primer in exon 35 and a reverse primer in intron 37. The latter was chosen instead of a primer in exon 38, since intron 37 is too large for effective amplification and thus did not allow us to demonstrate the presence of aberrantly spliced products containing this intron in MFD. We thus had to rely instead on the presence of nuclear hnRNA in which intron 37 had not yet been spliced in wild-type and MFD samples. Fig. 4B reveals the presence of a single RT-PCR product in the wild-type RNA and a single longer product in MFD. Sequencing of both bands revealed a wild-type product from which introns 35 and 36 had been normally spliced. By contrast, intron 36 was aberrantly spliced in the MFD sample, resulting in the use of a cryptic splice acceptor site and partial inclusion of this intron. This in vivo result confirms our findings in the in vitro splicing assay and shows that the single point mutation in the splice donor site of intron 37 interferes with the normal splicing of the upstream intron 36.

Taken together, the results from this in vitro splicing analysis show that splicing of the primary *Megf7/Lrp4* transcript at intron 36/exon 37 as well as exon 37/intron 37 is completely prevented by the single nucleotide substitution that is present in the MFD alleles, with the normal splice product being completely undetectable. This splicing defect results in the partial inclusion of intron 36 in the final transcript (Fig. 4C).

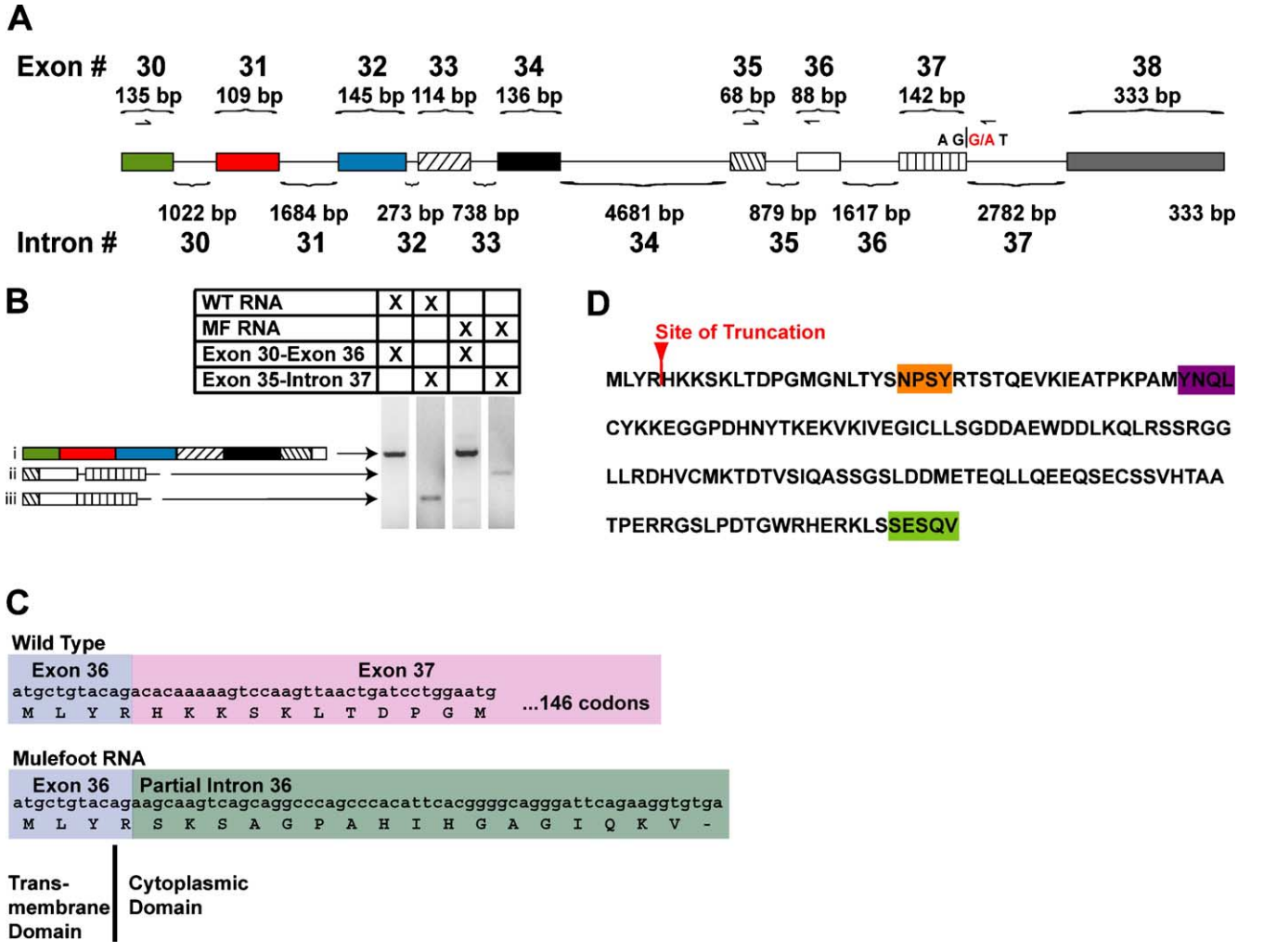

Fig. 4. RT-PCR analysis of wild-type and MFD *Megf7/Lrp4* RNA. (A) Structure of the *Megf7/Lrp4* gene ranging from exon 30 to 38. The location of the splice site mutation is shown in red. Forward primers in exons 30 and 35 and reverse primers in exon 36 and intron 37 are indicated by arrows. (B) Normal and equivalent RT-PCR products including exon 30–36 sequences are obtained from wild-type and MFD mRNA, while a longer product, indicating incomplete splicing of the MFD sample, is obtained when exon 35 and intron 37 primers are used. (C) Incomplete splicing of intron 36 in MFD *Megf7/Lrp4* mRNA was confirmed by sequencing. Partial inclusion of intron 36 is predicted to replace the wild-type cytoplasmic domain of Megf7 with 17 missense amino acids followed by a premature stop codon. (D) Sequence of the cytoplasmic domain of bovine *Megf7/Lrp4*. The NPXY motif, which is important for the function of other LDL receptor gene family members [23], is highlighted in orange. A YXXL endocytosis motif [25] is highlighted in purple. The PDZ-domain binding consensus sequence is highlighted in green.

This truncated translation product would be predicted to be anchored in the plasma membrane through its normal transmembrane segment, which is encoded in its entirety by exon 36. However, the entire normal cytoplasmic domain of *Megf7/Lrp4* would be missing, since the sequences encoding this domain would be replaced by 17 missense amino acids resulting from the inclusion of the partially unspliced intron 36.

Fig. 4D shows the amino acid sequence of the cytoplasmic domain of bovine *Megf7/Lrp4* and the predicted site of truncation in MFD. Highlighted are predicted functional sequence motifs that would be missing in the mutant protein. The "NPSY" sequence (orange) can serve as an endocytosis sequence [23] as well as a docking site for phosphotyrosine-binding (PTB) domain adapter proteins [24]. "YXXL" motifs

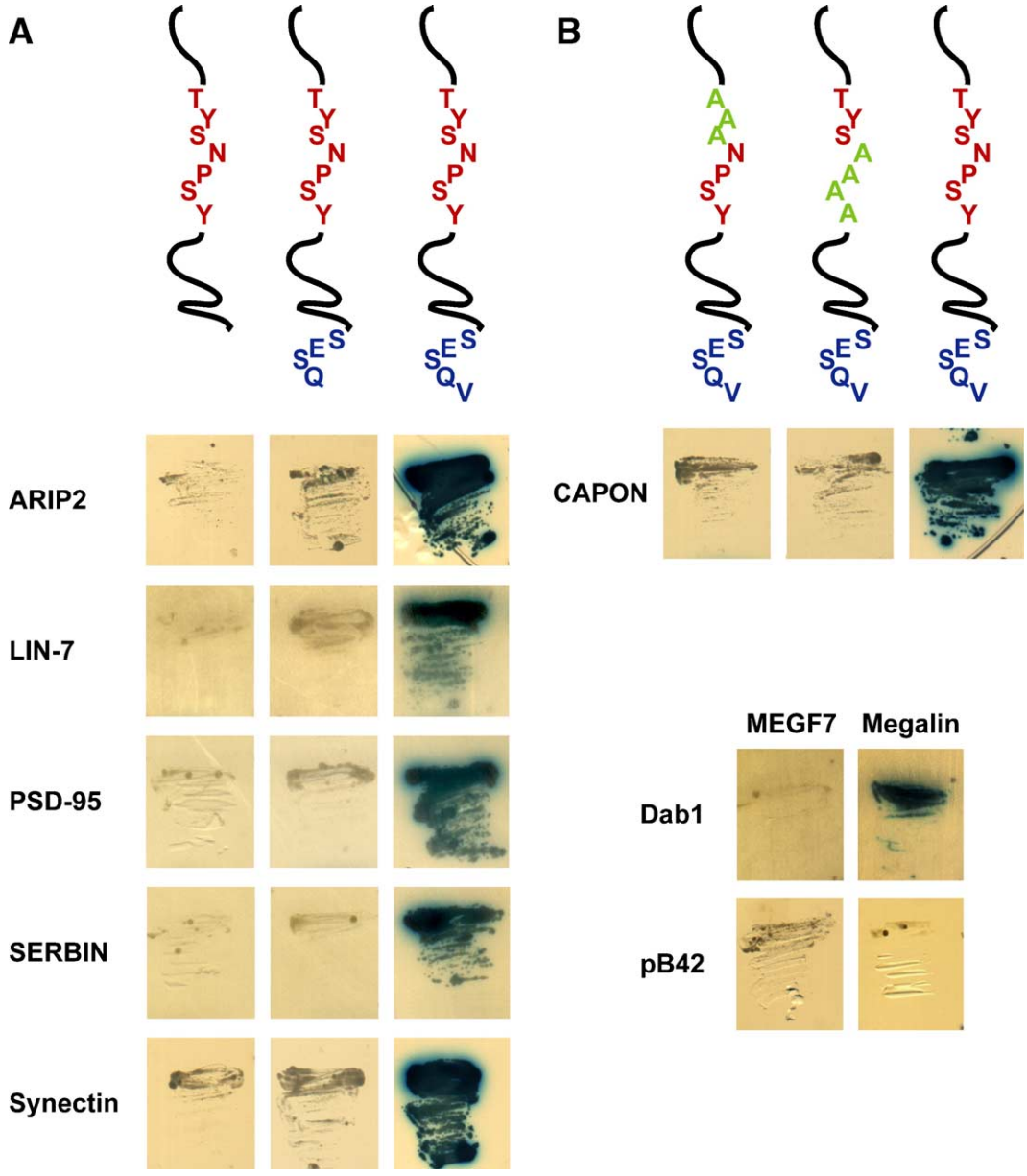

Fig. 5. Yeast two-hybrid clones interact with functional domains in the cytoplasmic tail of *Megf7/Lrp4.* A yeast two-hybrid screen was performed using the full cytoplasmic domain of Megf7 as the bait plasmid against a brain cDNA library (prey library). Mutational analysis shows that clones identified in this screen bind to at least two functional domains within the Megf7 cytoplasmic tail. (A) Clones containing PDZ domains bind to the carboxyl-terminal amino acids of the *Megf7/Lrp4* cytoplasmic domain. Deletion of the entire PDZ binding consensus sequence (left) or only the carboxyl-terminal valine (middle) disrupts PDZ domain-dependent interactions (right). (B) CAPON is a PTB domain-containing protein that binds to the NPXY motif of several LDL receptor gene family members [27]. Substitution of either the NPSY motif (middle) or the three upstream amino acids (TYS) (left) with alanines disrupts the binding of CAPON to wild-type *Megf7/Lrp4* (right). Dab1, a PTB domain adapter protein that binds to several members of the LDL receptor gene family [27], interacts with the Megalin cytoplasmic domain (right) but does not bind to *Megf7/Lrp4* (left) in a yeast two-hybrid mating experiment. Interactions are revealed by the growth of yeast on minimal medium, as well as the expression of β-galactosidase, which converts X-gal to a blue dye. ARIP2, activin receptor-interacting protein 2 (gb|AF414433.1); LIN-7, mouse homolog of *Caenorhabditis elegans* Lin-7 type 2 (gb|BC031780.1); PSD-95, presynaptic density protein 95 (sp|Q62108); SERBIN, similar to Erbb2-interacting protein (gb|AAH28256.1); Synectin, SemaF cytoplasmic domain-associated protein 1 (gb|BC003490.1); CAPON, C-terminal PDZ domain ligand of neuronal nitric oxide synthase (gb|AAC4065.1).

(purple) can also serve as endocytosis motifs that couple cell-surface receptors of the LDL receptor gene family to the endocytosis machinery [25], and the SESQV sequence meets the consensus for a PDZ domain protein docking motif.

Functional interaction motifs in the cytoplasmic domain of Megf7/Lrp4

Using a yeast two-hybrid approach we have shown that the "NPSY" and "SESQV" motifs can indeed specifically interact with intracellular adaptor and scaffolding proteins that contain PDZ (ARIP2, LIN-7, PSD-95, SERBIN, Synectin) or PTB domains (CAPON) (Fig. 5). Mutations of critical residues within the consensus motif, e.g., complete deletion of the SESQV motif or deletion of only the carboxyl-terminal valine (SESQ) in the PDZ domain binding motif, eliminated binding of the identified PDZ motif binding proteins (Fig. 5A). Similarly, mutations of Megf7/Lrp4 cytoplasmic domain residues that interact with critical side chains within the PTB domain binding groove [26] eliminated the ability of the tail sequences to interact with the PTB domain protein CAPON (Fig. 5B). PTB domain proteins do not interact indiscriminately with "NPXY" motifs [24]. The PTB domain protein Dab1 interacts specifically with Megalin, another LDL receptor family member [27]. However, Dab1 did not interact with *Megf7/Lrp4* sequences (bottom).

Discussion

We have found a single point mutation in a splice site of *Megf7/Lrp4* in homozygous carriers of MFD, a syndactyly disorder in cows and an economic concern for breeders and meat producers. We have shown that the G → A substitution in the first nucleotide of the highly conserved "GT" core that constitutes splice donor sites in most mammalian exon/intron boundaries prevents the generation of a normal *Megf7/Lrp4* mRNA. As a result of either transcript instability or translation of an abnormally truncated product, or a combination of both, the production of a normal *Megf7/Lrp4* gene product would be virtually abolished. The resulting phenotype closely matches that seen in naturally occurring or experimentally induced mutations in this gene and thus strongly supports our conclusion that MFD in the cow is also caused by loss-of-function mutations in the *Megf7/Lrp4* gene.

Samples from two known affected homozygous MFD carriers were available to us for direct genomic sequence analysis of *Megf7/Lrp4:* a skin sample from a recently deceased and clinically confirmed case of MFD and frozen sperm from another clinically confirmed animal, both belonging to the Angus strain of cattle. PCR analysis of exons and flanking intronic sequences was achieved using primers located within the introns, based on the bovine genomic sequence in the Ensembl database. Comparison of the exon sequences with those obtained from wild-type control samples or the Ensembl database did not reveal any differences. However, when we analyzed the exon/intron boundaries, we discovered a single point mutation in the first nucleotide of the donor splice site of intron 37. Similar G → A mutations have been described in other genetic diseases in which they have been shown to be responsible for the genetic defects by disrupting the normal splicing of the gene [20,28].

To demonstrate that the primary *Megf7/Lrp4* transcript is indeed misspliced in the homozygous carriers we used a minigene-based splicing assay that is routinely used to study the functionality and efficiency of splice sites to determine how this single nucleotide substitution might alter the splicing of the transcript. The substitution of A for G at the first nucleotide of an intron abolishes the interaction of the splicing machinery with the 5′ end of the intron and prevents use of this splice site [29]. Moreover, splicing to the upstream 3′ splice site on the other side of the exon is also typically inhibited by such mutations due to a loss of exon definition [20,30]. Based on these earlier studies, the G → A substitution in *Megf7/Lrp4* would be predicted to cause either skipping of exon 37 (i.e., splicing of exon 36 to 38) or retention of introns 36 and 37. The results from our minigene splicing experiment unambiguously show that in the *Megf7/Lrp4* case the G → A substitution causes retention of both introns 36 and 37 (Fig. 3B). This observation is consistent with studies that have suggested that certain exons contain sequences that inhibit their skipping [31] and provides a naturally occurring and biomedically relevant example of such a phenomenon. Aberrant splicing resulting in the partial retention of intron 36 was also confirmed by RT-PCR analysis of the MFD skin sample. The mRNA that would result from retention of introns 36 and 37 in *Megf7/Lrp4* would be predicted to encode a truncated Megf7 protein that remains anchored in the plasma membrane, but that completely lacks its normal cytoplasmic domain. This domain contains several sequence motifs that are known to activate intracellular signaling pathways through interaction with cytoplasmic adapter proteins [32], as well as endocytosis signals that can mediate the internalization of the normal protein and its recycling back to the cell surface.

This finding reveals two potential mechanisms, both of which on their own or in combination could severely cripple or completely abrogate *Megf7* function and thus disrupt normal limb development in MFD carriers: First, the truncated product would likely have no or only reduced functions on its own, since a functionally essential domain of the protein is missing. Second, the missplicing could reduce the stability of the truncated *Megf7/Lrp4* mRNA, resulting in less protein overall being produced. Either way, the data show that Megf7/Lrp4 functions would likely be severely impaired. Taken together with the distinct phenotype, these findings indicate that MFD is almost certainly caused by loss-of-function mutations in the *Megf7/Lrp4* gene.

Although we have found only a single point mutation that is identical in both MFD cases belonging to the Angus cattle strain we have been able to examine and that fully explains the phenotype, it is possible that mutations in other *Megf7* exons or introns can also cause MFD in other strains. Such mutations may be responsible for other cases that have been described. Over 700 independent mutations that impair the function of the LDL receptor, a close relative and family member of *Megf7* [33], have been found. However, in contrast to MFD, LDL receptor mutations are phenotypically codominant, inasmuch as they already manifest themselves in a milder form in heterozygotes, in which they occur with a frequency of approximately 1 in 500

in the general population. The availability of an excellent and simple assay that is widely used in humans further facilitates the identification of human patients with LDL receptor defects [33]. If loss-of-function mutations occur at a similar rate in the *Megf7* gene, one would thus predict a frequency of 1 case of MFD in 250,000 calves.

The nature of the mutation we have described here may explain the variability of the phenotype among homozygous carriers of MFD, which can range from only one to all four hooves being affected as well as including the absence of craniofacial dysmorphisms, which occur with incomplete penetrance in the *Megf7/Lrp4*-deficient mouse [10]. The mere loss of the cytoplasmic domain may not completely destroy the biological function of this receptor, if the primary role of Megf7 is to antagonize the function of one or more morphogens, such as Wnt's or Bmp's, at the plasma membrane. Although we did not detect any normally spliced product in our in vitro splicing assay (Fig. 3) or in the skin sample (Fig. 4B), individual variations in splicing efficiency in the apical ectodermal ridge during development could account for small amounts of functionally spliced products. Similarly, since limb development is a complex process in which numerous genes participate in an intricate interplay, it is highly likely that many, if not most of these genes can act as modifiers, i.e., they function at different levels or are regulated slightly differently between individuals, or their function may be epigenetically modulated to some extent. Alone or in combination, these mechanisms may account for the observed individual variability of MFD by effectively generating a hypomorphic phenotype in which not all four limbs are equally malformed.

The precise mechanisms by which Megf7/Lrp4 regulates signaling in the developing limb are now beginning to emerge. It is clear that this LDL receptor family member can function as a coreceptor and modulator in one or more of the morphogenic signaling pathways we have discussed above. However, exactly how Megf7/Lrp4 modulates these signals on the molecular level, i.e., the precise nature of the biochemical interactions in which it is involved, remains to be elucidated. Almost certainly the cytoplasmic domain harbors molecular interaction motifs that are critical for its function. The results of our yeast two-hybrid approach (Fig. 5) will help us to define these essential interactions and ultimately allow us to demonstrate their importance using knock-in approaches in the mouse.

The results we have reported in this paper have identified a splice site mutation in *Megf7/Lrp4* as the cause for autosomal recessive MFD in bovines. This is the second species after the mouse in which naturally occurring or experimentally induced mutations in this gene have been shown to cause syndactyly. These findings will enable breeders to test for the presence of MFD in their animal populations.

Experimental procedures

Genetic marker and lod scores

Positions (in cM) on bovine chromosome 15 of BMS927 and BM848 are from the Ensembl cow database (http://www.ensembl.org). The position of TGLA75 is from the NCBI database map viewer of bovine chromosome 15. Positions (in Mb) for the different gene markers are from the Ensembl cow database. Lod scores of BM848 and TGLA75 for the SY (mulefoot) locus are reported in [8] using the ANIBD program.

Megf7/Lrp4 sequencing

Wild-type and mulefoot DNA samples (Angus strain) were prepared using standard genomic DNA extraction protocols. The MF sperm sample was obtained from bull 16AN E829 on April 12, 1982. Briefly, approximately 200 mg tissue was digested in 1 ml of SNET buffer (20 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, 400 mM NaCl) with 200 μg of proteinase K (Roche). Tissues were digested overnight at 55°C with shaking. Five hundred microliters of the digested material was extracted with phenol/chloroform/isoamyl alcohol, precipitated with 500 μl isopropanol, and redissolved in 200 μl TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5). Primers were designed to amplify the bovine exon sequences including approximately 200 bp of flanking intronic sequences. Exons 4, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 19, 20, 21, 22, 23, 24, 26, 27, 28, 30, 31, 33, 34, 36, 37, and 38 of the MFD allele were successfully amplified, sequenced, and compared to the reference bovine *Megf7* gene sequence in the Ensembl database. No successful PCRs were obtained from the remaining exons.

Plasmid constructs

Construction of pcAT7-SC12 was essentially as described previously for SC6 [21]. pcAT7-SC12 is a β-globin minigene expression vector with CD45 exon 4 and introns inserted between exon 1 and exon 2 of the β-globin gene using *Nde*I and *Hin*dIII restriction sites. Wild-type and mulefoot exon 37 and approximately 200 bp upstream and downstream flanking sequences were amplified by PCR using primers MEJ415, 5′-GTGTGTCATATGATCCCACACTCTTCTATG-TTCTGTTCCAC-3′, and MEJ416, 5′-GTGTGTAAGCTTAGGGCCCCTCA-AGCTGGTCCCTCGCAAGCTG-3′. PCR products were digested with *Nde*I and *Hin*dIII and cloned into pcAT7-SC12.

Transfections and RT-PCR

HEK 293 cells were cultured in six-well plates and transfected with the indicated plasmids at 60–80% confluency. Other cell types produced similar results (data not shown). One microgram of plasmid DNA was transfected using Fugene-6 transfection reagent (Roche) and the manufacturer's recommended protocol. At the indicated times total RNA was isolated with RNA STAT-60 (Tel-Test, Inc.) from the cells using the manufacturer's recommended protocol. RT-PCR was performed as described previously [34]. Reactions were separated on 1.5% agarose gels. DNA bands were cut out, isolated from the gel, and cloned into pCR4 using the TOPO Cloning Kit for Sequencing (Invitrogen) and the manufacturer's recommended protocol.

RT-PCR analysis of MFD skin mRNA

Total RNA was extracted from wild-type and MFD tissue samples as described above. The RT reaction was performed as recommended for the M-MLV reverse transcriptase kit (Invitrogen). The RNA was mixed with 500 ng of poly(dT) oligomers (Roche) and nuclease-free water. The mixture was heated to 65°C for 5 min, cooled on ice, and then mixed with 5× First Strand Buffer, dNTPs, recombinant RNase inhibitor, and DTT. M-MLV reverse transcriptase was added and reactions were incubated at 37°C for 50 min and then at 70°C for 15 min. RNase H (2.5 U) was added to the reaction and incubated for 20 min at 37°C. PCR products were amplified using the following primers: Oligonucleotides MEJ444, 5′-CGCTGAGGAGTCCACCGATGATGTAGCTGG-3′, and MEJ445 5′-GGC-CACATTGCCAAGATCGAACGGGCGAAC-3′, were used to amplify the exon 30 to exon 36 fragment. Oligonucleotides MEJ446, 5′-GGCCTCTGTGCGCAT-TCCAATGAGGCCGTG-3′, and MEJ448, 5′-AGAAACAAACTCTCAGATG-CAAGTCTTCAC-3′, were used to amplify the exon 35 to intron 37 fragment.

Yeast two-hybrid constructs

The *Megf7/Lrp4* bait plasmid 2-3-4 was cloned by PCR amplification using mouse brain cDNA and the primers MEJ9, 5′-CCATGGAGACACAGGAAATC-CAAGTTCACGG-3′, and MEJ10, 5′-CTCGAGTTAGACCTGGCTTTCT-GAGGAGAGCTTG-3′. The PCR product was digested with *Nco*I and *Xho*I

and cloned into the pLexA bait plasmid. Truncated bait plasmid constructs were constructed with the same strategy using MEJ9 and MEJ118, 5′-GTGTGTCTCGAGCTACTGGCTTTCTGAGGAGAGCTTGCGTTCGTG-3′, for the –V truncation and MEJ9 and MEJ43, 5′-CTCGAGCTAGGA-GAGCTTGCGTTCGTGTTTCCAGCCTGTG-3′, for the –SESQV truncation. Oligonucleotides MEJ107 5′-GGAAGTTCGGTAGGACGGGTTTGCA-GCTGCCAGGTTTCCCATTCCAGGATCCGTGAACTTG-3′ and MEJ9, and MEJ108, 5′-CCTGGAATGGGAAACCTGGCAGCTGCAAACCCCTCC-TACCGAACTTCCACTCAGGAAG-3′ and MEJ10, respectively, were used for PCR mutagenesis of the TYS>AAA mutant bait insert. Oligonucleotides MEJ109 5′-CACTTCCTGAGTGGAAGTTCGTGCTGCAGCTGCGCTA-TAGGTCAGGTTTCCCATTCCAGGATCC-3′ and MEJ120 5′-GTGT-GTCCATGGAGACACAGGAAATCCAAGTTCACGG-3′, and MEJ110, 5′-GGAATGGGAAACCTGACCTATAGCGCAGCTGCAGCACGAACTTC-CACTCAGGAAGTGAAACTGGA-3′ and MEJ119, 5′-GTGTGTCTCGAGT-TAGACCTGGCTTTCTGAGGAGAGCTTG-3′, respectively, were used for PCR mutagenesis of the NPSY>AAAA mutant bait insert. The partial products for each mutation were combined and amplified in another PCR reaction. The resulting PCR products were then cloned into the NcoI and XhoI sites of pLexA.The Dab1 prey plasmid was described previously [27].

Yeast two-hybrid screen

The manufacturer's recommended protocol for the Matchmaker system (Clontech) was followed with minor alterations and as described [27]. The yeast strain EGY48 [p8op Lac-Z] was transformed with the wild-type *Megf7/Lrp4* cytoplasmic tail bait plasmid 2-3-4 using the standard LiAc method. Cells were grown on −Ura, −His dropout agar plates to select for transformed colonies. Yeast were then transformed with a mouse brain cDNA library cloned into the pB42 prey plasmid [27] using the Yeast Maker 2 system (Clontech) and plated onto −Ura, −His, −Trp dropout agar plates to select for transformed colonies. Colonies were pooled and plated on −Ura, −His, −Trp, −Leu dropout agar plates containing X-gal to select for transformed colonies that expressed prey proteins that interact with the wild-type cytoplasmic domain of mouse *Megf7/Lrp4.* Positive clones were replated on −Ura, −His, −Trp, −Leu dropout agar plates containing X-gal to confirm interactions. Prey plasmids from positive clones were isolated as described [27], transformed into KC8 bacteria (Clontech), and selected on −Trp dropout agar plates. Plasmids isolated from the bacterial clones were tested for insert by restriction digest and sequencing. Interactions were confirmed by retransforming EGY48 [p8op Lac-Z] with the wild-type *Megf7/Lrp4* bait and prey plasmids. Interactions were also confirmed by mating yeast clone EGY48 [p8op Lac-Z] transformed with wild-type *Megf7/Lrp4* bait plasmid and yeast clone YM4271 transformed with the prey plasmid. All bait plasmids showed minimal self-activation. Expression of all bait plasmid proteins was confirmed by Western blot using an anti-LexA antibody.

Note added in proof

While this paper was under review, Duchesne et al. (Genomics 88 (2006) 610–621) reported a two amino acid substitution in exon 33 of the Megf7/Lrp4 gene in Holstein cattle. Based on prior extensive functional analysis of the LDL receptor gene [reviewed in 33], this mutation in an epidermal growth factor repeat would be predicted to interfere with the transport of the mutant Megf7/Lrp4 protein through the secretory pathway to the cell surface, thereby severely crippling its normal cellular functions. Taken together with the data we have presented in this paper, these findings now provide unequivocal evidence that mutations in Megf7/Lrp4 cause MFD in cattle.

Acknowledgments

We are indebted to Wen-Ling Niu, Huichuan Reyna, Karen Brown, Debbie Morgan, Priscilla Rodriguez, Isaac Rocha, and Elizabeth Lummus for excellent technical assistance and to Keith Wharton, Tom Wilkie, Jon Graff, Mike Brown, and Joe Goldstein for discussions and helpful comments. This work was supported by National Institutes of Health Grants HL20948, HL63762, and NS43408; the Alzheimer's Association; the Wolfgang-Paul Award of the Humboldt Foundation; and the Perot Family Foundation.

References

[1] A. Hamosh, A.F. Scott, J.S. Amberger, C.A. Bocchini, V.A. McKusick, Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders, Nucleic Acids Res. 33 (2005) D514–D517.
[2] F.W. Nicholas, Online Mendelian Inheritance in Animals (OMIA): a comparative knowledgebase of genetic disorders and other familial traits in non-laboratory animals, Nucleic Acids Res. 31 (2003) 275–277.
[3] J. Capdevila, J.C. Izpisua Belmonte, Patterning mechanisms controlling vertebrate limb development, Annu. Rev. Cell Dev. Biol. 17 (2001) 87–132.
[4] C. Tickle, Patterning systems—From one end of the limb to the other, Dev. Cell 4 (2003) 449–458.
[5] C. Drogemuller, O. Distl, Genetic analysis of syndactyly in German Holstein cattle, Vet. J. 171 (2006) 120–125.
[6] H.W. Leipold, G.L. Schmidt, D.J. Steffen, J.G. Vestweber, K. Huston, Hereditary syndactyly in Angus cattle, J. Vet. Diagn. Invest. 10 (1998) 247–254.
[7] S.A. Ojo, H.W. Leipold, M.M. Guffy, C.M. Hibbs, Syndactyly in Holstein–Friesian, Hereford, and crossbred Chianina cattle, J. Am. Vet. Med. Assoc. 166 (1975) 607–609.
[8] C. Charlier, F. Farnir, P. Berzi, P. Vanmanshoven, B. Brouwers, H. Vromans, M. Georges, Identity-by-descent mapping of recessive traits in livestock: application to map the bovine syndactyly locus to chromosome 15, Genome Res. 6 (1996) 580–589.
[9] M. Gautier, H. Hayes, A. Eggen, An extensive and comprehensive radiation hybrid map of bovine chromosome 15: comparison with human chromosome 11, Mamm. Genome 13 (2002) 316–319.
[10] E.B. Johnson, R.E. Hammer, J. Herz, Abnormal development of the apical ectodermal ridge and polysyndactyly in Megf7-deficient mice, Hum. Mol. Genet. 14 (2005) 3523–3538.
[11] D. Simon-Chazottes, S. Tutois, M. Kuehn, M. Evans, F. Bourgade, S. Cook, M.T. Davisson, J.L. Guenet, Mutations in the gene encoding the low-density lipoprotein receptor LRP4 cause abnormal limb development in the mouse, Genomics 87 (2006) 673–677.
[12] J. Herz, H.H. Bock, Lipoprotein receptors in the nervous system, Annu. Rev. Biochem. 71 (2002) 405–434.
[13] A. Nykjaer, T.E. Willnow, The low-density lipoprotein receptor gene family: a cellular Swiss army knife? Trends Cell Biol. 12 (2002) 273–280.
[14] R. Spoelgen, A. Hammes, U. Anzenberger, D. Zechner, O.M. Andersen, B. Jerchow, T.E. Willnow, LRP2/megalin is required for patterning of the ventral telencephalon, Development 132 (2005) 405–414.
[15] R.A. McCarthy, J.L. Barth, M.R. Chintalapudi, C. Knaak, W.S. Argraves, Megalin functions as an endocytic sonic hedgehog receptor, J. Biol. Chem. 277 (2002) 25660–25667.
[16] A. Zilberberg, A. Yaniv, A. Gazit, The low density lipoprotein receptor-1, LRP1, interacts with the human frizzled-1 (HFz1) and down-regulates the canonical Wnt signaling pathway, J. Biol. Chem. 279 (2004) 17535–17542.
[17] K.I. Pinson, J. Brennan, S. Monkley, B.J. Avery, W.C. Skarnes, An LDL-receptor-related protein mediates Wnt signalling in mice, Nature 407 (2000) 535–538.
[18] K. Tamai, M. Semenov, Y. Kato, R. Spokony, C. Liu, Y. Katsuyama, F. Hess, J.P. Saint-Jeannet, X. He, LDL-receptor-related proteins in Wnt signal transduction, Nature 407 (2000) 530–535.
[19] M. Wehrli, S.T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz, D. Vaizel-Ohayon, E. Schejter, A. Tomlinson, S. DiNardo, Arrow encodes an LDL-

receptor-related protein essential for Wingless signalling, Nature 407 (2000) 527–530.
[20] M. Krawczak, J. Reiss, D.N. Cooper, The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences, Hum. Genet. 90 (1992) 41–54.
[21] A. Tong, J. Nguyen, K.W. Lynch, Differential expression of CD45 isoforms is controlled by the combined activity of basal and inducible splicing-regulatory elements in each of the variable exons, J. Biol. Chem. 280 (2005) 38297–38304.
[22] C. Rothrock, B. Cannon, B. Hahm, K.W. Lynch, A conserved signal-responsive sequence mediates activation-induced alternative splicing of CD45, Mol. Cell 12 (2003) 1317–1324.
[23] W.J. Chen, J.L. Goldstein, M.S. Brown, NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor, J. Biol. Chem. 265 (1990) 3116–3123.
[24] M. Trommsdorff, J.P. Borg, B. Margolis, J. Herz, Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein, J. Biol. Chem. 273 (1998) 33556–33560.
[25] Y. Li, M.P. Marzolo, P. van Kerkhof, G.J. Strous, G. Bu, The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein, J. Biol. Chem. 275 (2000) 17187–17194.
[26] P.C. Stolt, H. Jeon, H.K. Song, J. Herz, M.J. Eck, S.C. Blacklow, Origins of peptide selectivity and phosphoinositide binding revealed by structures of disabled-1 PTB domain complexes, Structure 11 (2003) 569–579.
[27] M. Gotthardt, M. Trommsdorff, M.F. Nevitt, J. Shelton, J.A. Richardson, W. Stockinger, J. Nimpf, J. Herz, Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction, J. Biol. Chem. 275 (2000) 25616–25624.
[28] N.A. Faustino, T.A. Cooper, Pre-mRNA splicing and human disease, Genes Dev. 17 (2003) 419–437.
[29] M.J. Moore, C.C. Query, P.A. Sharp, Splicing of precursors to mRNA by the spliceosome, in: R.F. Gesteland, J.F. Atkins (Eds.), The RNA World, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993, pp. 303–357.
[30] S.M. Berget, Exon recognition in vertebrate splicing, J. Biol. Chem. 270 (1995) 2411–2414.
[31] C. Ibrahim el, T.D. Schaal, K.J. Hertel, R. Reed, T. Maniatis, Serine/arginine-rich protein-dependent suppression of exon skipping by exonic splicing enhancers, Proc. Natl. Acad. Sci. USA 102 (2005) 5002–5007.
[32] P. May, J. Herz, H.H. Bock, Molecular mechanisms of lipoprotein receptor signalling, Cell. Mol. Life Sci. 62 (2005) 2325–2338.
[33] J.L. Goldstein, H.H. Hobbs, M.S. Brown, Familial hypercholesterolemia, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, B. Childs, K.W. Kinzler, B. Vogelstein (Eds.), Metabolic and Molecular Bases of Inherited Disease, McGraw–Hill, New York, 2001, pp. 2863–2913.
[34] K.W. Lynch, A. Weiss, A model system for activation-induced alternative splicing of CD45 pre-mRNA in T cells implicates protein kinase C and Ras, Mol. Cell. Biol. 20 (2000) 70–80.