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Original Article

Analysis of a new allele of *limb deformity (ld)* reveals tissue- and age-specific transcriptional effects of the *Ld* Global Control Region

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ABSTRACT The mouse *limb deformity (ld)* phenotype is characterized by developmental failure of distal limb structures often associated with renal anomalies. It is caused by loss of the BMP-antagonist Gremlin in the limb buds, either through mutation of *Grem1*, or by loss of a transcriptional global control region (GCR) located in the neighboring *Fmn1* gene. In this report, we describe a new allele of *ld* due to complete deletion of *Fmn1,* including its GCR. Unlike many other *ld* strains, these mice are viable and fertile as homozygotes. As expected, this genomic deletion causes loss of Gremlin in the developing limb buds, but effects in other tissues are variable. Specifically, *Grem1* expression is retained in the developing lung and kidney, whereas expression is lost from the corresponding adult tissues. In contrast, expression in the brain appears to be unaffected by loss of the GCR. To provide information about long-range transcriptional effects of this region, effects of the deletion on the transcription of neighboring genes were also investigated. This analysis revealed that alterations in neighboring genes do occur, but only in a limited fashion. These data indicate that the predominant effect of the *Ld* GCR is to activate the expression of *Grem1* in the developing limb buds, although it may serve a minor role in long-range transcriptional effects that extend beyond *Fmn1* and *Grem1*.

KEY WORDS: *limb development, global control region, gremlin, formin, transcriptional control*

Introduction

The formation of the vertebrate limb illustrates an important paradigm in development, as it involves distinct transcriptional and signaling programs along each of the three axes, proximal-distal, dorsal-ventral and anterior-posterior (Niswander, 2000). Detailed characterization of this process has been carried out at multiple levels, including genetic, biochemical and functional; these studies have focused on multiple systems, including predominantly the mouse and chick (for reviews, see Gurrieri *et al.*, 2002, Mariani and Martin, 2003, Tickle, 2003, Stopper and Wagner, 2005). From a genetic standpoint, there are a small number of mouse single gene mutants that have proved suitable for the analysis of mouse limb development, including mutants such as *extra-toes* (*Gli3), brachypodism* (*Gdf5), syndactylism (Serrate2)* and mutations affecting members of the *Hox* genes such as *synpolydactyly (Hoxd13)* (reviewed in Gurrieri *et al.*, 2002, Zelzer and Olsen, 2003). There are also other genes shown to be crucial for limb development such as *Trp63* or *Sonic hedgehog (Shh)*, although mice lacking these genes are not viable (Mills *et al.*, 1999, Yang *et al.*, 1999, Niedermaier *et al.*, 2005). Tissue specific knockouts such as those generated using *Msx2*-cre (Sun *et al.*, 2000) have provided another means for investigation and confirmation of important signaling pathways affecting limb development, as these mice can often circumvent embryonic lethality caused by global gene disruption (Pan *et al.*, 2005).

Abbreviations used in this paper: BMP, bone morphogenic protein; FGF, fibroblast growth factor; GCR, global control region; ld, limb deformity; qRT-PCR, quantitative real-time PCR; Shh, sonic hedgehog.

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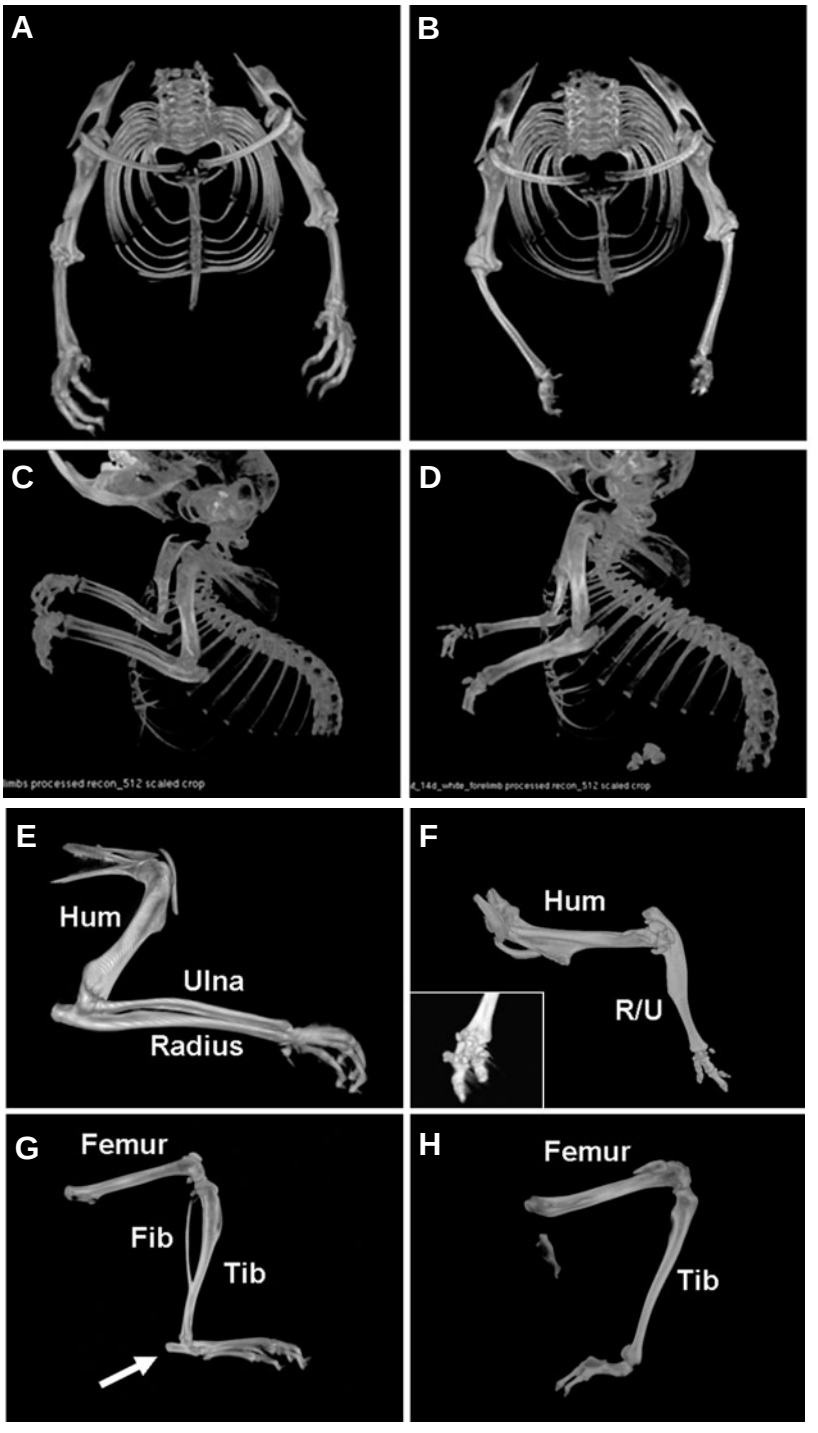


Fig. 1. MicroCT imaging of *Id*^{1LSK} mice. (A,B) *Images of the upper torso of 6-wk old heterozygote and mutant animals taken from a superior view after removal of the head.* **(C,D)** *Lateral images of the upper body of P14 heterozygote and mutant animals. Note that the bony abnormalities are confined to the distal limb and paw structures. (E-H) Images of upper* **(E, F)** *and lower* **(G, H)** *extremities from 6 month old heterozygote (E,G) and mutant (F,H) animals. In the upper limb, note the fusion of the radius and ulna (R/U) and failure of development of the distal paw structures. The inset in panel (F) shows a typical paw structure, with 3 rudimentary digit structures. In the lower limb, note the absence of the fibula and the calcaneus (arrow in Panel G) in the mutant, as well as the malrotation of the paw.*

One of the single gene mutations whose alleles have been studied extensively is the *limb deformity* (*ld*) phenotype, which was initially described in the 1960's as a spontaneously arising autosomal recessive mutation. Since its initial description, several alleles have been isolated, either spontaneously or through insertional or radiation-induced mutagenesis (reviewed in Wang *et al.*, 1997). These mice have foreshortening of the distal appendages, both upper and lower, including fusion of distal limb bones and malformation of the paws. Through genetic mapping of the induced mutations, the genetic locus for *ld* was localized to mouse chromosome 2 and eventually mutations were detected within the *Fmn1* gene (Maas *et al.*, 1990, Woychik *et al.*, 1990). This gene, spanning almost 400 kb, encodes Formin1, a member of a small family of large proteins thought to be important for cell polarity and motility (Zeller *et al.*, 1999). In addition to the limb defects, *ld* mice also variably exhibited defects in renal formation, so that many strains die after birth from failure of kidney development (Maas *et al.*, 1994).

Although the genetic lesions were found to reside within the *Fmn1* structural gene, follow-up studies, including targeted knockout of *Fmn1*, did not recapitulate the *ld* limb phenotype (Zeller *et al.*, 1999). Around the same time, careful analysis of distal limb specification revealed the need for the establishment of a feedback loop involving fibroblast growth factor (FGF) and Sonic hedgehog (Shh) in the proper development of distal limb structures and that the *ld* mutants displayed a disruption in this process (Haramis *et al.*, 1995). The role of bone morphogenic proteins (BMPs) and the Gremlin protein, a BMP antagonist of the Cerberus/Dan family (Topol *et al.*, 1997, Pearce *et al.*, 1999) was noted to be crucial in these interactions (Capdevila *et al.*, 1999, Zuniga *et al.*, 1999). Knockout of *Grem1* (Khokha *et al.*, 2003, Michos *et al.*, 2004) demonstrated that mice exhibited a limb and kidney phenotype similar to that of the *ld* mice. It was subsequently shown in an elegant series of experiments (Zuniga *et al.*, 2004) that the *Fmn1* gene mutations in *ld* mice led to a failure of Gremlin1 expression in embryonic limb buds. Furthermore, two of the previously uncharacterized *ld* mutants were found to be mutations directly affecting *Grem1* through other means. These findings suggested that the mutations in *Fmn1* caused deletion of a Global control region (GCR) affecting limb development, leading to a failure of *Grem1* expression and the *ld* phenotype.

In this manuscript, we present data on a new, spontaneous allele of *ld* and show that this allele is, in fact, a deletion of the entire *Fmn1* gene. In order to understand the effects of loss of the *Ld* GCR, we have tested the expression of Gremlin1 in other tissues, as well as studied the effects of this mutation on the expression of neighboring genes. These data reveal that the major role of the *Ld* GCR is to regulate the expression of the *Grem1* gene in the developing limb,

although it also exerts subtle effects on other genes within this chromosomal environment.

Results

Characterization of the phenotype

During the course of a transgenic project, we noticed that a small number of pups were born with obvious limb deformities (Fig. 1). Because the number of mouse mutants with similar deformities is quite small (Gurrieri *et al.*, 2002), we decided to further characterize the phenotype and to identify the causative genetic locus. Through breeding studies, we determined that the locus was inherited as an autosomal recessive trait. Although the mutant pups were smaller than non-mutant littermates, the distribution of the offspring from mutant x heterozygote crosses was not different from predicted by Mendelian inheritance (data not shown).

In order to characterize the phenotype better, we observed the mice for over 18 months, with no excess mortality detected (data not shown). MicroCT of mutants of varying ages revealed that the developmental anomaly was confined to the distal limbs, but that development of the axial skeleton, the limb girdle and proximal long bones was unremarkable (Fig. 1 A-D). In the upper extremity, there was fusion of the radius and ulna, with variably formed paw structures, typically comprised of 3 rudimentary digits (Fig. 1F). In the lower limb, there was shortening of the tibia and complete absence of the fibula in all mice examined. It was frequently observed that the paws of the lower extremities were malrotated (Fig. 1H). These changes were detectable in the embryos by e12.5, but mutants were not distinguishable by morphologic changes at e10.5 (data not shown).

Identification of the mutation as an allele of the ld *phenotype*

At this juncture in our studies, we became aware that a similar phenotype had previously been described in the 1960's and named the *limb deformity (ld)* mutant (see Kleinebrecht *et al.*, 1982). Previous genetic mapping of other *ld* strains obtained through insertion mutagenesis (Woychik *et al.*, 1985, Messing *et al.*, 1990) or radiation-induced chromosomal breaks (Woychik *et al.*, 1990) had localized these causative mutations to the *Fmn1* gene on mouse chromosome 2 (Wang *et al.*, 1997). However, recent analysis of the *ld* phenotype had indicated that loss of Formin1 itself was not the causative event in the phenotype; rather, the limb mutant phenotype was due to loss of expression of the nearby *Grem1* gene, encoding the BMP antagonist Gremlin/Drm (Zuniga *et al.*, 2004). This was confirmed by the generation of a truncated deletion mutation of Formin1 or by identification of a failure of Grem1 expression in two other alleles of *ld, ld*^{1} and *ld*^{OR} (Zuniga *et al.*, 2004)

To determine if our spontaneous mutant exhibited a decrease in *Grem1* expression, we isolated limb buds from e12.5 embryos and performed semi-quantitative RT-PCR for Fmn1 and Grem1 expression. These studies showed that neither gene transcript was detectable in embryonic limb buds, although Grem1 was clearly detected by this method in samples from the brain (Fig. 2). We took these data to indicate that our mutant was a new, spontaneously-arising allele of the *ld* phenotype, which we name *ld*^{1LSK}.

Because *ld* has frequently been associated with renal agenesis (Maas *et al.*, 1994), we undertook a survey of mice to determine the frequency of this anomaly in our strain. Of 20 mice examined, we observed unilateral renal agenesis in 1 mouse, indicating that this aspect of the phenotype is less common in our

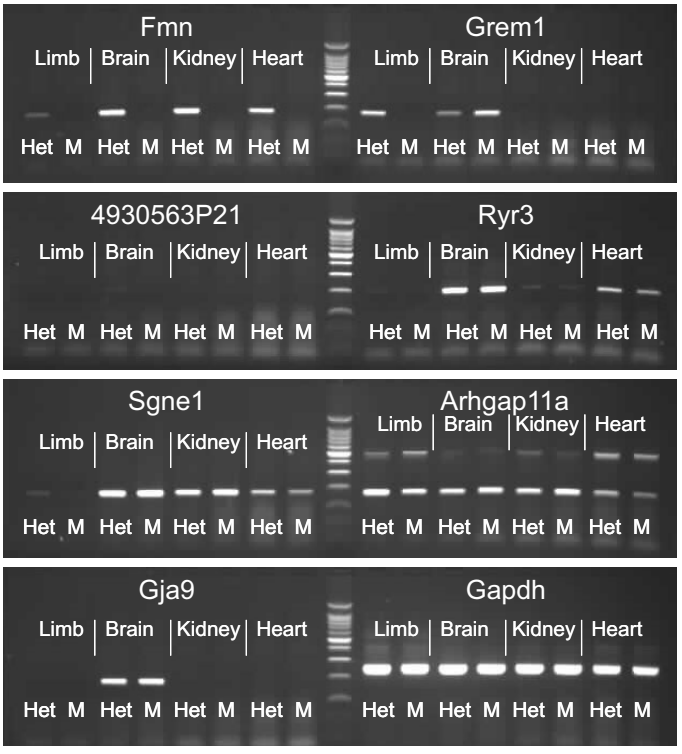


Fig. 2. Semi-quantitative RT-PCR analysis of transcriptional changes in *ld*^{1LSK} mice. *mRNA was isolated from heterozygote* **(Het)** *or mutant* **(M)** *e12.5 limb buds and from adult brain, kidney and heart and analyzed for expression of 7 genes in the region of the* Fmn1-Grem1 *locus on chromosome 2. Note that expression of* Formin *is lost in all tissues tested, whereas expression of* Gremlin *is lost in the limb buds, but not the brain. Gene expression for the other transcripts is variably affected in this semi-quantitative assay.*

TABLE 1

PRIMERS FOR GENOMIC AND cDNA AMPLIFICATION

	Left	Right	Amplicon size
Genomic Primers			
Ryr3	GCGAGGATGAGATCCAGTTC	GAGTGTGAGGTGGCAGCAC	239
4930563p21	CTCTGTCCCCATCCCCTAAG	CAGGGTGTCCACGAGAAAGT	242
FMN 40M from 3'	GTTACACGTGGCACCCTTTT	GCACTTTCCTGGCTCAGACT	181
FMN 3' end	GGACCCTGGGATAGATGGTT	TTTGTTTGCTTGCTTCGTTG	157
Grem1	TCATTGTGCTGAGCCTTGTC	GAATCGCACCGCATACACT	126
Sgne1	GCTGGTGAATGACTGGGTTT	CTTAAGCTGGGGTGGTGGTA	188
cDNA primers			
Fmn1	GACTCCAGGCAGACTCCAAG	CCGGATGTGAAATGTCTTGA	180
Grem1	ACTCGTCCACAGCGAAGAAC	TCATTGTGCTGAGCCTTGTC	172
4930563p21	CCGGGATCAACTCTGGTAGA	CAGGGTGTCCACGAGAAAGT	188
Ryr3	GCGAGGATGAGATCCAGTTC	GGACTGTTCCAGCACGAAAT	197
Gja9	ACTGCCCAGTCTTTGTCTGC	TCGTACACCGTCTCCCCTAC	179
Sgne1	GAGTTCAGCCGAGAATTCCA	TTGCCACAACATTGTCCAAC	177
Arhfgap11a	GAGTTCAGCCGAGAATTCCA	TTGCCACAACATTGTCCAAC	171
Gapdh	GCAAATTCAACGGCACAGTCAAG	GTTCACACCCATCACAAACATGG	158

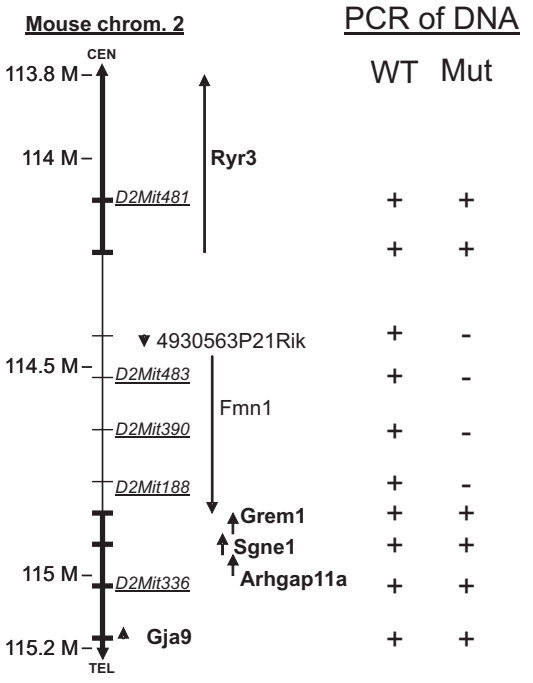


Fig. 3. Analysis of the gene deletion in *Id*^{1LSK} mice. *A schematic diagram of chromosome 2 is shown to scale* **(left)**, *with the location of tested markers and genes indicated. The right side shows a summary of the results of genomic PCR from comparing phenotypically normal to mutant animals* **(right)**. *All samples were run in duplicate. The scale to the left indicates sequence distance from the centromere of chromosome 2. The thin portion of the line indicates the region of the deletion. Note that 2 additional* Fmn1 *intragenic markers between D2Mit188 and Grem1 were also tested (Fmn 3' and Fmn 40K from 3', see Table 1) and found to be missing in the mutants. These data indicate that the* Id^{1LSK} *mice carry a deletion of the entire* Fmn1 *gene and the* 4930563P21 *Riken gene prediction.*

allele than in others. Examination of the kidneys from adult mice revealed no abnormality at the histologic level (data not shown). The mutation was initially detected on a mixed genetic background, which included contributions from129/SvJ, C57Bl/6 and FVB. The mutation was backcrossed for 5 generations into a BALB/c background, with no significant change in the phenotype, from which it was maintained by sibling intercrosses.

Genetic characterization of the mutation

Prior to this report, there were 7 other reported alleles of the *Id* phenotype (see Wang *et al.*, 1997, Ward-Bailey *et al.*, 2006). Four of these arose spontaneously (*Id*^{OR}, *Id*^{0}, *Id*^{2J} and *Id*^{8J}), whereas two were induced by transgene insertion (*Id*^{TgBri137} and *Id*^{TgHd}) and one was a radiation-induced rearrangement (*Id*^{In2}). Of the 4 spontaneous alleles, the *Id*^{OR} allele was found to be due to a spontaneous 12.7 kb deletion encompassing *Grem1,* whereas the *Id*^{0} allele was found to be a point mutation affecting *Grem1* splicing (Zuniga *et al.*, 2004). In our mice, because we did not observe transcripts of either Fmn1 or Grem1 in the limb buds, we suspected that our allele would involve the *Fmn1* locus. To evaluate this possibility, genomic DNA from mutants and control animals was subject to PCR amplification using primers from the *Fmn1* and *Grem1* genes. Our initial analysis demonstrated that both exons of *Grem1* were intact, but that polymorphic markers located with the *Fmn1* gene were missing from the mutants (Fig. 3). In order to determine the extent of this deletion, additional primer sets were generated both proximal and distal to the *Fmn1* gene (Table 1). This analysis demonstrated that the deletion in the *Id*^{1LSK} allele encompassed the entire *Fmn1* gene, as well as the anonymous EST *4930563P21*. The next gene on the centromeric side, *Ryr3* (Ryanodine receptor 3) appeared intact, as did all genes telomeric to *Grem1.* In order to define if there were any residual portions of the *Fmn1* gene present, we designed additional primer sets at the 3' end of the gene. Neither primers 40 kb from the 3' end nor primers located in the most 3' exon amplified a fragment from mutant mice. Comparison of this data to the mouse genomic sequence map indicates that the deletion spans at least 400 kb of DNA and includes the entirety of the *Fmn1* gene. The telomeric breakpoint between *Fmn1* and *Grem1* is localized to an area of approximately 40 kb, although the exact location has not been determined.

Expression changes associated with loss of the Fmn1 *gene*

The 3' of the *Fmn1* gene has been shown to contain a locus that controls expression of the *Fmn1* and *Grem1* gene in the limb buds, despite the fact that this region is located >200 kb from the *Fmn1* promoter and >100 kb from the *Grem1* promoter (Zuniga, 2005). In order to determine if loss of this region affects the transcription of other genes in the area, as is commonly observed in long-range control elements (Kleinjan and van Heyningen, 2005) we performed RT-PCR analysis on the genes located in this region. For these studies, the mice analyzed were littermates from a mutant x heterozygote cross, so that mice are either homo- or heterozygotes for the mutation.

As a first step, we performed semi-quantitative RT-PCR using a pooled sample of e12.5 limb buds and mRNA from the adult brain, kidney and heart. As shown in Fig. 2, no expression of Formin1 message was detected in any tissue. Although Gremlin expression was notably absent from the limb buds, expression in the brain was retained. This assay did not show significant expression of Gremlin in the adult kidney or the heart. Genes centromeric to *Fmn1* included the deleted EST *4930563P21*, whose expression was not detected in the heterozygotes or mutants and *Ryr3*, which was detected in the brain and heart at levels unchanged between mutants and heterozygotes. Of the genes tested telomeric to *Grem1*, *Sgne1* (recently renamed *Scg5)* expression appeared to be diminished only in the limbs, but the levels were quite low. No changes were observed in the expression or tissue distribution of *Arhgap11a* (Rho GTPase activating protein 11A, previously known as 6530401L4) or *Gja9*, the gap junction membrane channel protein α9.

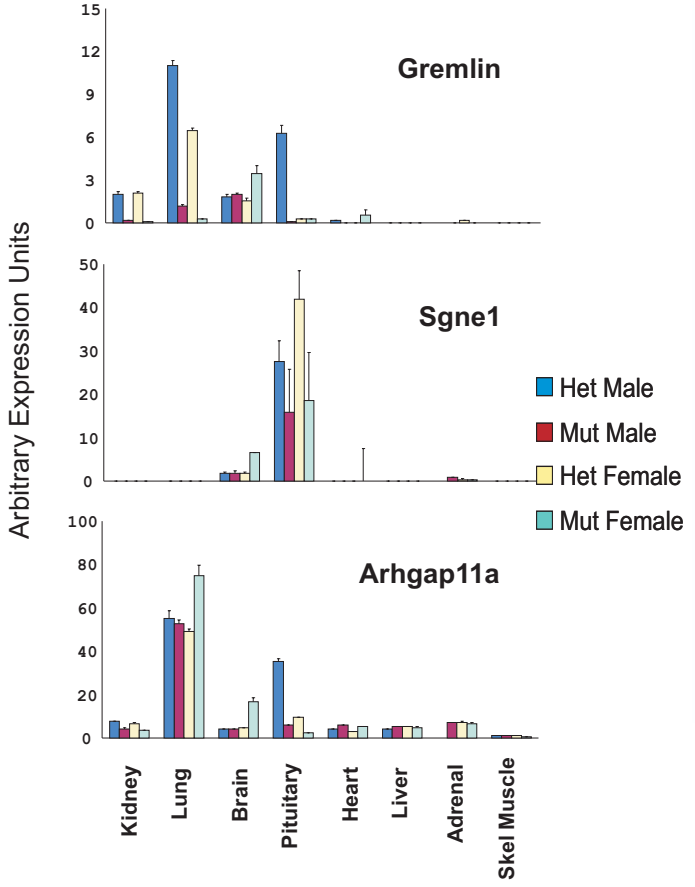
To obtain a better understanding of organism-wide alterations in gene expression, we next performed quantitative RT-PCR (qRT-PCR) on tissues from a pair of adult male and female animals. Because we had not detected any expression changes in genes centromeric to *Fmn1* (specifically *Ryr3*) by semi-quantitative analysis, we decided to focus on *Grem1* and the two genes telomeric to it, *Sgne1* and *Arhgap11a*. As shown in Fig. 4, Grem1 expression was notably absent in the kidneys from both male and female mutant animals, despite the lack of a renal phenotype. Gremlin has also been reported to be expressed in the lungs and

to be required for lung branching morphogenesis (Lu *et al.*, 2001, Shi *et al.*, 2001). Although levels were easily detected in the heterozygotes, no expression was detected in the mutant lungs, despite the fact that no lung abnormalities were observed (data not shown). Similar to previous reports (Topol *et al.*, 1997), we also detected expression of Grem1 in the brain and kidney, but not in the heart or liver. We also detected a discrepancy in the expression of Grem1 in the pituitary glands of male mice, with easily detectable levels in heterozygotes but not in the mutants. Female mice did not express Grem1 in the pituitary.

Sgne1, the gene telomeric to *Grem1*, encodes a protein thought to be important for neuroendocrine peptide hormone processing (Westphal *et al.*, 1999). This gene has previously been shown to be expressed in the pituitary and other tissues, with minimal expression in other tissues (Iguchi *et al.*, 1984). In agreement with these prior studies, we found that this gene was highly expressed in the pituitary and there appeared to be a loss of expression in the mutant animals. Although expression was detectable in whole brain, adrenal gland and at low levels in the kidney, no differences were observed between mutants and heterozygote mice.

We continued our analysis to include *Arhgap11a*, which lies adjacent to *Sgne1* on the distal side from *Grem1*. Expression of this gene was detectable in all tissues, although levels were very low in skeletal muscle. Similar to Grem1, we noted a decreased expression of this gene in the pituitary glands of mice of both sexes. For all other tissues tested, there did not appear to be significant differences in expression in the mutants compared to the controls.

In order to eliminate effects caused by variations in gene expression in individual mice, qRT-PCR was performed from an additional 4 mice per group (5 mice total per group). Because our initial data indicated that Grem1 was expressed at significant levels in only 4 tissues (kidney, lung, brain, pituitary) and these



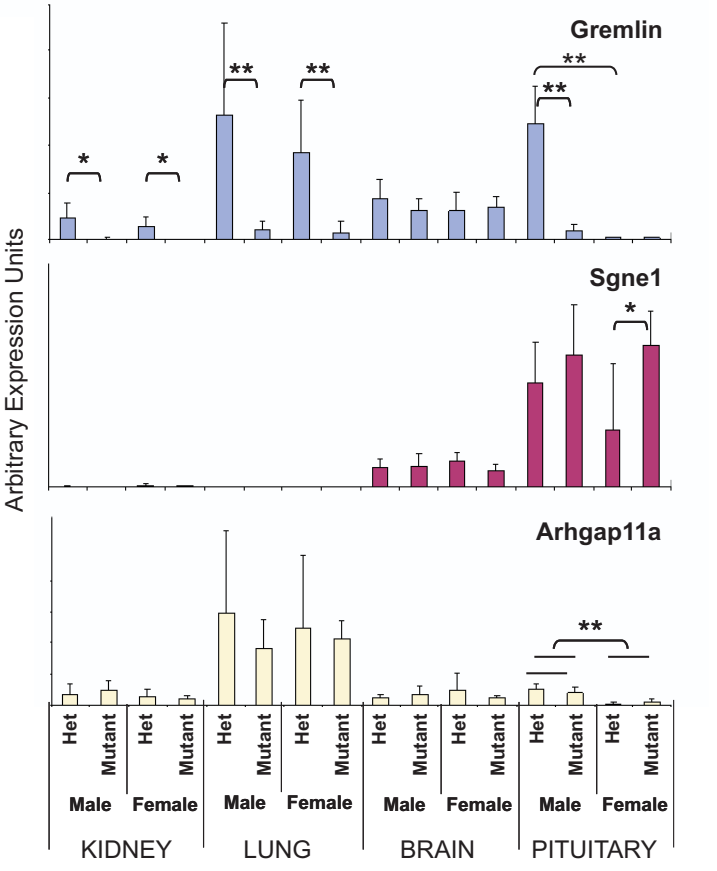


Fig. 4 (Left). Survey analysis of quantitative RT-PCR of *Grem1, Sgne1* and *Arhgap11a* in *Id*^{1LSK} mice. *mRNA was isolated from a heterozygote (Het) or mutant (Mut) adult male and adult female from the tissues indicated and analyzed by qRT-PCR as described in Materials and Methods. All data is presented as expression relative to* Gapdh, *which was normalized to* 10^5 *expression units. Note that the* Sgne1 *data is in thousands of units.* Gremlin *is expressed only in 4 tissues (kidney, lung, brain and pituitary) with clear reduction in both kidney and lung, but no alterations in brain and potentially sex-specific changes in pituitary.*

Fig. 5 (Right). Quantitative RT-PCR analysis of *Grem1, Sgne1 and Arhgap11a* in *Id*^{1LSK} mice in kidney, lung, brain and pituitary. *mRNA was isolated from heterozygote (Het) or mutant (Mut) adult males (N=5 for each group) and adult females (N=5 for each group) from the tissues indicated and analyzed by qRT-PCR as described in Fig. 4. *: p<0.05 between groups. **: p<0.01 between groups, by unpaired 2-tailed Student t-test.* Gremlin *expression is retained in mutant brains, but is lost in kidney and lung; reduction in the pituitary occurs only in male mice. Also note the sexually dimorphic changes in the expression of* Sgne1 *and* Arhgap11a *observed in the pituitary gland.*

same tissues appeared to exhibit changes in the other transcripts, we focused the in-depth analysis on these 4 tissues, studying the expression of *Grem1, Sgne1 and Arhgap11a* (and *Gapdh* as a normalization control). This study confirmed that Gremlin is essentially absent from mutant kidney, lung and pituitary, whereas expression in brain is unaffected (Fig. 5, top). This analysis also confirmed the sex-specific expression of *Grem1* in the pituitary; it is present in moderate levels in of males but is essentially absent from this gland in females. Interestingly, we also found that *Arghap11a* exhibits the same pattern of sex-specific expression, although there is no effect of the mutation in this phenomenon. In contrast to the initial set of mice, we observed in this larger group of mice that expression of *Sgne1* appeared to be increased in the mutant animals. This reached statistical significance in the females ($p<0.05$), but not the males, making the biological significance unclear.

As shown above, the data indicated that the mutant mice did not express Grem1 in the adult kidney or lung, despite the lack of a phenotype affecting these organs (data not shown). To determine if Gremlin expression was present during embryogenesis, mRNA was prepared from the limb buds, upper body (containing the developing lung) and lower body (containing the developing kidneys) of heterozygote or mutant animals at e11.5. Although we did not prepare mRNA from isolated embryonic kidneys or lungs, our data (Fig. 4) and that of others (Topol *et al.*, 1997, Michos *et al.*, 2004) suggest that the kidneys and lungs are the only organs (outside of the limb buds) that express Gremlin in the lower and upper body halves, respectively. Expression of *Fmn1* and *Grem1* were assayed by semi-quantitative PCR from these tissues (Fig. 6). As expected, *Fmn1* transcripts were not detected in any tissue. As in Fig. 2, no *Grem1* message was amplified from limb buds, although an easily detected signal was seen in both the lower and upper body halves, which we interpret as showing that *Grem1* expression was retained in the embryonic kidneys and lungs.

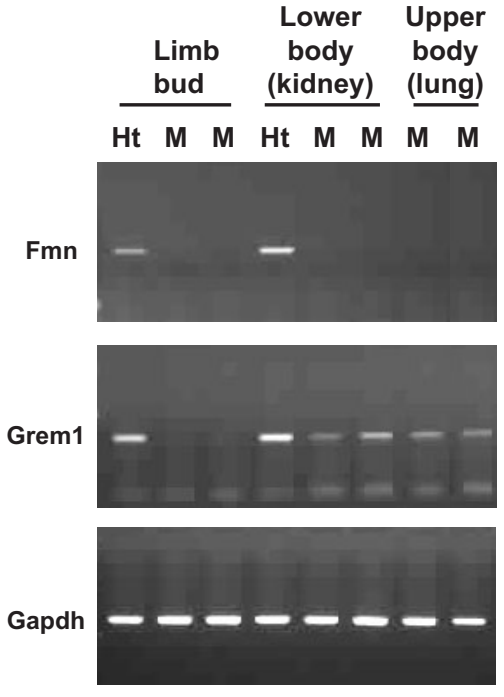


Fig. 6. Semi-quantitative RT-PCR analysis of *Fmn1* and *Grem1* in developing kidney and lung in *Id*^{1LSK} mice. *mRNA was isolated from e11.5 embryos from heterozygote* **(Ht)** *or mutant* **(M)** *mice and analyzed by RT-PCR as described in Materials and Methods. These data indicate that expression of* Gremlin *is retained in the developing kidney and lungs.*

Discussion

In this report, we present the isolation and clinical characterization of a new allele of the *Id* phenotype, which we designate *Id*^{1LSK} or *Fmn1*$^{Id\text{-}1LSK.}$ The limb phenotype which we observe is identical to other previously described *Id* mutants, consisting of defects of the distal portions of both the upper and lower limbs. The renal phenotype of the various *Id* alleles is quite variable (Maas *et al.*, 1994) and renal involvement was only seen rarely in our mice. Although we have not performed a quantitative assessment of the ability of these mice to breed, we have had no trouble maintaining the line as homozygotes, indicating reproductive suitability of both male and female mutants. Additionally, when we have performed heterozygote x homozygote crosses, the affected offspring did not seem to have excess mortality compared to their phenotypically normal littermates. Our initial consideration was that this might be due to mixed strain vitality, although we have continued to observe the same viability characteristics in the mice backcrossed for 5 generations into a Balb/c background.

As has been previously observed, the *Fmn1* gene does not appear to be essential, since our mice lack this gene in its entirety, spanning from upstream of the gene and including the final exon. In contrast to our observations, two knockout alleles targeting the 5' half of the Fmn1 gene (Wynshaw-Boris *et al.*, 1997, Chao *et al.*, 1998) developed renal anomalies, with no limb phenotype. In contrast, an allele leading to deletion of the entire 3' half of the gene (exons 10-24) recapitulated the *Id* phenotype with much less renal involvement (Zuniga *et al.*, 2004). The findings here and in Zuniga *et al.* suggest that the renal anomalies observed in the targeted KOs as well as some of the prior *Id* alleles may result from a dominant negative effect of the mutant *Fmn1* alleles. Because Formin1 itself has a complex transcriptional unit with multiple splice variants (Wang *et al.*, 1997, Chao *et al.*, 1998,), the deletion of portions of the gene might alter the production of downstream isoforms and this may specifically affect the embryonic development of the kidney.

As a side issue, we also note that the predicted gene *4930563P21* is similarly not essential, since it is also completely deleted in our mice. We did not detect expression of this gene in the 4 tissues tested (even in the heterozygous mice), so the validity of this gene prediction is uncertain, despite the fact that it is predicted to encode a 12 exon gene of 307 amino acids. It is possible that this gene is expressed in other tissues or at other times than those examined in this study.

The more interesting finding reported here relates to the effects of the deletion of the *Grem1* GCR on the expression of Gremlin1 and, to a lesser extent, on its neighboring genes. As has been observed in many other *Id* alleles, loss of this GCR leads to a failure of *Grem1* expression in the developing limb buds (Fig. 2). Loss of Gremlin production causes failure to establish the Fgf4-Shh signaling feedback loop in the developing limb, as well as outgrowth failure (Haramis *et al.*, 1995, Merino *et al.*, 1999, Zuniga *et al.*, 1999). Our data also suggests that loss of this GCR causes loss of

Gremlin expression in the kidney, lung and pituitary. However, the transcriptional effects of this GCR are specific for post-natal life, as embryonic expression, at least in the kidney and lung, appears intact. These observations imply that the GCR functions in a tissue- and stage-restricted fashion to ensure proper expression in the developing limb bud. The basal promoter for the gene, which has not been characterized, most likely resides in the 5' of the gene and controls expression of Gremlin in the brain. The location of promoter/enhancer elements responsible for embryonic expression of Gremlin in the lungs and kidney may also reside at the 5' end of the gene, although they have not been mapped. This hypothesis is supported by the observation that the *ld*^{In2} allele, which disrupts the relationship between the GCR and the *Grem1* gene without affecting the 5' end, causes only the limb phenotype (Maas *et al.*, 1994).

As a corollary, it is also worthwhile to note that the role of Gremlin in kidney and lung development must be limited to embryonic development, since our mice retain expression in embryos but not in adults. As Gremlin has been reported to be required for branching morphogenesis of the lungs (Shi *et al.*, 2001), it is possible that a similar role is necessary in kidney tubule development; however, once formed, Gremlin function becomes dispensable. This finding has not been previously noted because *Grem1* KO mice die at birth (Khokha *et al.*, 2003, Michos *et al.*, 2004), with lung failure as part of the reason for mortality (Michos *et al.*, 2004). In addition to the tissue-specific effects of the GCR, our data demonstrate that Grem1 exhibits sexually dimorphic expression in the pituitary, with high levels of expression only in male mice, which was lost in the mutants. The loss of Grem1 from the male pituitary does not lead to compromise of the pituitary-gonadal axis, as male mutants are fertile and also does not appear to affect other pituitary functions (e.g., growth).

This study also suggests that loss of the GCR led to changes in the expression of *Sgne1* in the pituitary, with female mutant mice exhibiting an increase in expression compared to their phenotypically normal littermates. This observation suggests that the *Fmn1-Grem1* GCR may have the ability to function as a tissue-specific transcriptional repressor, although the magnitude of the effect was only on the order of 2.5-fold. The mechanism of this effect has not been elucidated. It is likely that the changes in message level of *Sgne1* are due to the effect of an enhancer acting at a distance. However, we have not excluded the possibility that the effect could occur in trans, e.g., by the production of a microRNA affecting *Sgne1* steady state mRNA levels. Expression of this gene in the brain, or in other tissues with much lower levels of expression, did not appear to be affected by this mutation. We also observe the sexually dimorphic expression of the *Arhgap11a* gene, which is also expressed only in male mice.

In a broader sense, long-range transcriptional control appears to be a common element in limb development. Not only is expression of Gremlin controlled by a distant GCR, but similar findings have long been known for the control of the *Hox* genes, which are expressed in a graded spatial fashion throughout the body, including the limb (Deschamps and van Nes, 2005). Further, transcriptional control of *Shh* has also been shown to be dependent on an enhancer element located nearly 1 Mb away in the *Limb region 1* (*Lmbr1*) gene and that disruption at this locus causes the pre-axial polydactyly and the *sasquatch* phenotype (Lim *et al.*, 2003). This sequence is highly conserved and appears to play a role in limb development from teleost fins up through vertebrate limbs (Sagai *et al.*, 2004) Another transgene-induced limb mutant, *doubleridge,* characterized by syn- and polydactyly of the forelimb had a similar mechanism of limb defects, as the insertion caused reduced expression of the Wnt pathway inhibitor *Dickkopf-1 (Dkk1)* (Adamska *et al.*, 2003, MacDonald *et al.*, 2004). Finally, the spontaneous mouse mutation *doublefoot* exhibits pre-axial polydactyly and head defects (Lyon *et al.*, 1996). Although the causative mutation has not yet been identified, careful genetic mapping has demonstrated that the mutation maps in the region of *Indian hedgehog (Ihh)* (Hayes *et al.*, 2001); mice with this phenotype have increased activation of Ihh targets (Crick *et al.*, 2003) and it would seem likely that the mutation will be found in a region that affects the expression of *Ihh*, in analogy to many of the mutations described above.

The difficulty in identifying the *doublefoot* mutation highlights the problematic nature of identifying these mutations in the absence of transgene insertions or chromosomal abnormalities. Since the mutation is not located in a coding portion of the gene or in a clearly identifiable promoter region, the absence of a mutation in a structural gene does not rule out a role in causing the genetic defect. The *FMN1-GREM1* locus is located at 15q13-q14 in humans, where there are no known limb or renal syndromes mapped (Grzeschik, 2002). Such clinical phenotypes are extremely rare, so that genetic linkage studies are not possible. Many human syndromes might be considered analogous to the *ld* mutation in mice, including syndromes such as Renal Dysplasia-Limb Defects Syndrome (OMIM 266910), Fibuloulnar Aplasia/Hypoplasia With Renal Abnormalities (OMIM 228940), or Brachydactyly-Ectrodactyly With Fibular Aplasia/Hypoplasia (OMIM 113310) (McKusick, 2003). In fact, any of the human syndromes associated with fibular dysplasia (Lewin and Opitz, 1986), one of the striking features of the *ld* mice, might be an analogous human syndrome. The identification of one of these (or other) syndromes as an *ld* analog would await the identification of a *FMN1* or *GREM1* mutation in one of these extremely rare patients.

In conclusion, we have identified and characterized a new allele of the *ld* phenotype. In contrast to other *ld* alleles and to targeted alleles of *Fmn1,* the mice described here have a complete deletion of the *Fmn1* gene along with a neighboring EST of unknown function. These data help to clarify the functional effect of the *Fmn1* GCR, indicating that it is essential for the control of Gremlin expression in the developing limb, but that expression in other organs is under the control of other regulatory elements that have yet to be characterized. Unexpectedly, Gremlin exhibits sexually dimorphic expression in the pituitary under the control of the same GCR, but the functional role in this gland is unclear at present. Functionally, Gremlin appears to be required during organogenesis of the kidneys and lungs, but appears to be dispensable for adult function, at least under homeostatic conditions. Finally, although most other genes in the region were unaffected by loss of the GCR, there may be subtle long range effects involving tissue-specific expression of the neighboring *Sgne1* gene, although functional consequences are as yet unknown.

Materials and Methods

Identification of mutant strain

The *ld*^{4LSK} strain arose spontaneously during mating of two unrelated heterozygous knockout strains, one carrying a null allele of *Pten* (Cao *et al.*, 2004) and the other an allele of *Prkar1a* (Kirschner *et al.*, 2005), neither of which is located on chromosome 2 near the *Fmn1-Grem1* locus.

After identification of the phenotype, mice were backcrossed to wild-type littermates to separate the mutation from the other targeted alleles. Once the distinct identity of the phenotype was confirmed, the mutants were backcrossed x 5 generations to BALB/c mice (Harlan). All mice described in this paper were housed in sterile microisolators and handled in accordance with the highest ILACUC standards.

PCR and RT-PCR

DNA was collected by tail snips from mice and analyzed by PCR using standardized conditions (Kirschner *et al.*, 1999). Polymorphic mouse genomic primers used in the analysis were obtained from the Mouse Genome Informatics website (http://www.informatics.jax.org/). Other primers used for genomic or cDNA amplification are listed in Table 1. For analysis of mRNA samples, tissue was collected from e12.5 embryos (limb buds) or adult mice into Trizol (Invitrogen, Carlsbad, CA). mRNA was prepared according to the manufacturer's recommendations and repurified over RNAeasy columns (Qiagen, Valencia, CA). RNA was reverse transcribed using iScript cDNA synthesis reagents using conditions suggested by the manufacturer (Bio-Rad, Hercules, CA). For semi-quantitative experiments, PCR reactions using 1 ul of cDNA were run under standard conditions and resolved in 1.8% agarose gels. For quantitative experiments, cDNA samples were analyzed in an iCycler using iCycler SYBR green reagents (Bio-Rad). Samples were run in triplicate for each assay and assays were repeated 1-3 times for each sample analyzed. Expression data were pooled for all experiments to derive the data presented. To provide a 'real' expression value for each message and tissue, data were normalized to a relative expression of Gapdh set at 10,000; thus, a relative expression of 1 indicates an expression level 10^5 times lower that than for Gapdh for any given sample. Statistical analysis of the data in Fig. 5 was performed using an unpaired 2-tailed Student's t-test.

Radiology

For microCT imaging, mice were imaged essentially as described (Powell *et al.*, 2005) by collecting 360 2048 x 2048 12-bit projection radiographs at 1° intervals around the entire specimen (circular data acquisition). These images were collected at 34 kV, 450 μA and 1†s exposure time with the image intensifier operating in 7-in. mode. Rendering of the data was performed using VolSuite v3.3 (http://www.osc.edu/~jbryan/VolSuite/index.shtml) using custom adjusted transfer and segmentation functions. For scanning electron micrographs, samples were prepared according to standard methods (Bozzola and Russell, 1999) and imaged using a Philips XL 30 scanning electron microscope equipped with secondary and back-scatter detectors.

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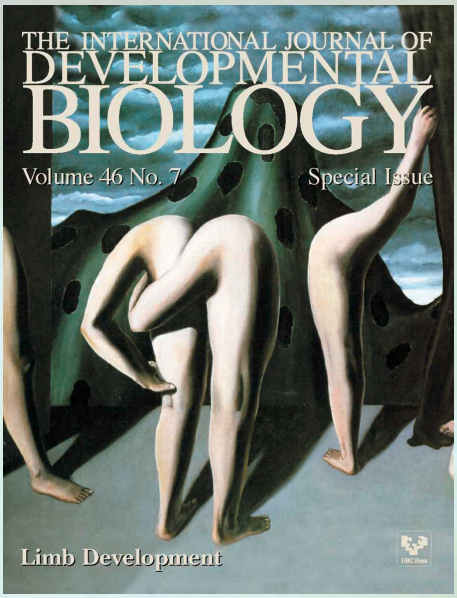

Central motif of a magic realist painting portraying animate, isolated limbs as individuals. This picture entitled "Intermission" (1928) was painted by the Belgian surrealist René Magritte (1898-1967). Oil on canvas. Private collection.

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