Saturation mutagenesis defines novel mouse models of severe spine deformity

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SUMMARY STATEMENT
We report selected mouse models of spine deformity following mutagenesis across 30% of autosomal genes, results of which are made publicly available to advance understanding of spine development and disease.

ABSTRACT
Embryonic formation and patterning of the vertebrate spinal column requires coordination of many molecular cues. After birth, the integrity of the spine is impacted by developmental abnormalities of the skeletal, muscular, and nervous systems, which may result in deformities such as kyphosis and scoliosis. We sought to identify novel genetic mouse models of severe spine deformity by implementing in vivo skeletal radiography as part of a high-throughput saturation mutagenesis screen. We report selected examples of genetic mouse models following radiographic screening of 54,497 mice from 1,275 pedigrees. An estimated 30.44% of autosomal genes harbored predicted damaging alleles examined twice or more in the homozygous state. Of the 1,275 pedigrees screened, 7.4% presented with severe spine deformity developing in multiple mice, and of these, meiotic mapping implicated ENU alleles in 21% of pedigrees. Our study provides proof-of-concept that saturation mutagenesis is capable of discovering novel mouse models of human disease, including conditions with skeletal, neural, and neuromuscular pathologies. Furthermore, we report a mouse model of skeletal disease, including severe spine deformity, caused by recessive mutation in Scube3. By integrating results with a human clinical exome database, we identified a patient with undiagnosed skeletal disease who harbored recessive mutations in SCUBE3, and we demonstrated that disease-associated mutations are associated with reduced trans-activation of Smad signaling in vitro. All radiographic results and mouse models are made publicly available through the Mutagenetix online database with the goal of advancing understanding of spine development and discovering novel mouse models of human disease.
INTRODUCTION

Development of the spinal column is a highly regulated process that begins early in embryogenesis. Post-natal growth and maintenance of a structurally sound spine requires coordinated integration of the vertebrae (skeleton), inter-vertebral discs, nerves, and muscles. Disruption of any of these components may lead to spinal malformations. In humans, scoliosis, defined as a lateral curvature of the spine, is the most common spine deformity and is characterized as idiopathic, congenital, neuromuscular, or syndromic (Herring, 2013).

Alternatively, kyphosis manifests as an anterior-posterior curvature of the spine, sometimes in combination with scoliosis (so-called kyphoscoliosis). Unlike idiopathic scoliosis, congenital scoliosis can be associated with skeletal malformations or segmentation defects of the vertebrae, and neuromuscular scoliosis is associated with neurological or muscular disease etiologies. Each of these disease types present with different scoliotic deformities and may require different treatment paradigms.

Population-based genome-wide association studies have identified several loci associated with idiopathic scoliosis, while next-generation sequencing studies have elucidated rare mutations underlying congenital scoliosis (Chen et al., 2020; Gao et al., 2007; Khanshour et al., 2018; Kou et al., 2013; Sharma et al., 2011; Sharma et al., 2015; Takahashi et al., 2011; Wu et al., 2015). Despite the success of human genetic studies, modeling spine deformities for these loci in mice has proven challenging. For example, the correlation of human scoliosis with non-genetic factors, such as the adolescent growth spurt, hormonal influences during adolescence, and the distribution of mechanical forces of a bipedal spine, may be difficult to recapitulate in mice or other model systems. Moreover, certain genes implicated in severe forms of scoliosis, such as \( \text{TBX6} \), may be sub-viable in genetically engineered knockout mouse models (Chapman and Papaioannou, 1998; Wu et al., 2015). Alternative strategies, such as using Cre-inducible conditional knockout mouse models or engineering hypomorphic alleles, have been used successfully to investigate scoliosis-associated genes \textit{in vivo} (Karner et al., 2015; Yang et al., 2019). These strategies, however, are limited due to a lack of knowing \textit{a priori} the relevant cell type for conditional models or which alleles may be hypomorphic and allow for viable mouse models of spine deformity in essential genes.
To facilitate discovery of genes required for proper spine development, we performed live-animal radiography to detect severe spine deformity as part of a saturation mutagenesis skeletal screen in mice (Rios et al., 2021; Wang et al., 2015). As proof-of-concept, we report selected results from 54,497 mice from 1,275 pedigrees. As previously described (Wang et al., 2018), estimates of saturation based on the standard of detecting lethal effects in a curated collection of essential genes suggests that 30.44% of all protein encoding autosomal genes were mutated to a state of detectable hypomorphism and examined in the homozygous state twice or more in this sample of mice. Among the mutations were 5,746 putative null alleles in 4,320 genes examined twice or more in the homozygous state. Mutations in genes regulating proper skeletal, muscular, and nervous system development are all implicated in scoliotic manifestations discovered through our screen. Results from our study may inform human genetic studies, provide novel mouse models of human disease, and advance knowledge of spine development and deformity. As proof-of-principle, we cross-referenced results from our study with a database of undiagnosed subjects who underwent clinical exome sequencing, and we report a patient with a skeletal syndrome caused by recessive mutations in the \textit{SCUBE3} gene that resulted in reduced hetero-dimerization and reduced Smad signal transduction. All results and mouse models, including others not reported here, are made publicly available through the online Mutagenetix database (mutagenetix.utsouthwestern.edu).

**RESULTS**

**Radiographic screen for spine deformity**

The breeding scheme for generating and screening mice harboring ENU-induced alleles is shown in Fig. 1. C57BL/6J male mice are mutagenized with ENU and out-crossed to non-mutagenized C57BL/6J females, resulting in male pups (termed the G1 generation) heterozygous for germline ENU-induced alleles. G1 male mice are out-crossed to C57BL/6J female mice, and G2 pups are subsequently back-crossed to their G1 sire to produce G3 mice homozygous for the reference allele, heterozygous for the ENU allele, or homozygous for the ENU allele. All G3 mice undergo radiographic screening, including both dorsal and lateral radiography (Fig. 1). Phenotypic scoring is performed from radiographs as either presence or absence of any rigid spine deformity or malformation, such as kyphosis, scoliosis, or kinked/curl tail.
ENU-induced alleles are detected by exome sequencing G1 male mice, as previously described (Wang et al., 2015). Nonsynonymous variants identified relative to the C57BL/6J reference genome are genotyped by targeted capture and sequencing of all G2 and G3 mice. Automated meiotic mapping tests the null hypothesis that ENU-induced alleles segregating within the pedigree are not associated with risk for spine deformity. Significantly associated loci are identified following Bonferroni correction for the number of ENU-induced alleles in the pedigree.

Of the 1,275 pedigrees evaluated, 94 (7.3%) pedigrees were identified with at least 2 mice scored with a spine deformity phenotype. Of these, 20 (20.6%) were mapped to ENU-induced alleles (Fig. 1, Table S1), including 19 recessive and 1 dominant allele. We sought to evaluate variables limiting detection of significantly associated loci, such as the total number of mice in the pedigree and the total number of affected mice in the pedigree. Pedigrees with mapped alleles had significantly higher numbers of affected mice (Wilcoxon p=1.59e^-5) compared to unmapped pedigrees (Fig. S1A), and this remained significant among only recessive alleles (Wilcoxon p=4.41e^-5). Total numbers of mice were unchanged between pedigrees with mapped and unmapped alleles (Fig. S1B). Most (12/19; 63%) pedigrees with mapped recessive alleles had at least 80% penetrance of the spine deformity phenotype among homozygous G3 mice (Table S1).

**Altered skeletal development leading to spine deformity in mice**

*Mouse model and case report of SCUBE3-associated skeletal disease*

We identified the *Scube3^C301Y* allele associated with recessive spine deformity, including severe kyphosis and kinked tail (Fig. 2A). While the homozygous *Scube3^C301Y* allele co-segregated with homozygous *Rhot2^V604A* and *Nrde2^D607E* alleles, we considered these to be less likely candidates based on the presence of skeletal phenotypes in mice homozygous for a previously-reported *Scube3^N294K* allele (Fuchs et al., 2016). Additionally, the *Scube3^C301Y* allele was predicted to be more damaging by PolyPhen analysis than the *Rhot2^V604A* and *Nrde2^D607E* alleles.
The human SCUBE3 gene, encoding the Signal peptide, CUB domain, and EGF like domain containing 3 protein, is highly expressed in osteoblasts (Wu et al., 2004). The SCUBE3 protein was identified as a transforming growth factor-beta (TGF-β) receptor ligand and activator of Smad signaling (Wu et al., 2004; Wu et al., 2011). Proper regulation of the TGF-β signaling cascade is essential for normal skeletal development (Crane and Cao, 2014). Disruption of TGF-β signal transduction leads to defects in endochondral ossification via dysregulation of osteogenesis, which has been studied extensively using germ-line and conditional mouse models (Wu et al., 2016). Furthermore, disruption of TGF-β signaling affects the cross-talk between bone forming osteoblasts and bone resorbing osteoclasts during bone remodeling (Erlebacher and Derynck, 1996; Filvaroff et al., 1999). Mice homozygous for a Scube3\(^N294K\) allele developed mild phenotypic abnormalities, including shorter hindlimbs, rib defects, and auditory abnormalities, while Scube3\(^-/-\) mice were recently shown to develop a post-natal dwarfism that was not apparent during embryonic development (Fuchs et al., 2016; Lin et al., 2021; Xavier et al., 2013).

Although all three Scube genes (Scube1-3) are implicated in regulating proper skeletal development in mice (Fuchs et al., 2016; Lin et al., 2015; Tu et al., 2008), mutations in any one of three human SCUBE gene (SCUBE1-3) have yet to be associated with human disease. However, during the course of this study, multiple subjects with recessive SCUBE3 mutations were reported with short stature, oral-facial abnormalities, skeletal abnormalities, and, although variable, spine deformity, suggesting a novel SCUBE3-associated skeletal disease (Lin et al., 2021). To identify additional patients with skeletal disease potentially attributable to mutations in SCUBE3, we queried the Baylor Genetics Laboratory database that includes nonsynonymous variants identified from clinical exome sequencing and clinician-provided clinical synopses. We sought patients described with skeletal abnormalities yet remaining without a genetic diagnosis following evaluation by clinical exome sequencing among whom rare nonsynonymous variants in SCUBE3 were identified. We identified a now 16-year-old male patient presenting with GI malrotation, hearing loss, short stature, joint hyperextensibility, dilated aortic root with abnormal aortic valve, and oral-facial differences including cupped ears, high-arched palate, and micrognathia (Table 1). Skeletal abnormalities included asymmetric fusion of a pseudoepiphysis of the 2\(^{nd}\) metacarpal bilaterally (Fig. 2B). His feet were notable for an unusual fusion of the 2\(^{nd}\) metatarsal to the 2\(^{nd}\) cuneiform (Fig. 2B). The patient’s parents were unaffected; however, an
affected sibling was also noted with similar clinical findings, consistent with a recessive disease presentation in this family (Table 1, Fig. 2C). The male patient previously underwent extensive genetic testing, including microarray analysis, testing for congenital disorders of glycosylation, and targeted genetic testing, including *COL1A1*, *COL2A1*, *COL3A1*, *COL11A1*, *COL11A2*, *CDH7*, *TGFBR1*, and *TGFBR2*; however, all testing, including subsequent clinical exome sequencing, was negative. Through the database query, the patient was identified with two novel *SCUBE3* variants, including a predicted loss-of-function frameshift mutation (NM_152753:c.1521_1522insGC, p.(Ile508AlafsTer74)) and a missense mutation (NM_152753:c.2785C>G, p.(Arg929Gly)). Sanger sequencing performed in the patient and both parents confirmed compound heterozygous inheritance in the affected son; additional testing in the siblings was not available (Figs. 2C, S2A-C).

*SCUBE3* was previously shown to heterodimerize with *SCUBE1* (Wu et al., 2004), though it remains unclear whether this interaction is required for *SCUBE3* function. We performed co-immunoprecipitation experiments to test potential deleterious effects of the novel *SCUBE3* p.(Arg929Gly) variant on *SCUBE1*-*SCUBE3* heterodimerization. Results were compared to an allelic series including recently reported *SCUBE3* variants (Lin et al., 2021), to a common *SCUBE3* p.(Thr231Ala) polymorphism (rs79753406) predicted to have normal function, and to the *SCUBE3* p.(Cys301Tyr) variant orthologous to our mouse ENU allele associated with severe spine deformity (Fig. 2D). Compared to wild-type and *SCUBE3* p.(Thr231Ala) proteins, *SCUBE3* containing disease-associated variants showed reduced interaction with *SCUBE1* (Fig. 2E, S2D). These results suggest disease-associated mutations alter formation of the *SCUBE1*-*SCUBE3* heterodimer.

*SCUBE3* was also previously shown to activate Smad signaling via direct interaction with the TGF-β type II receptor (*TGFBR2*) (Wu et al., 2011). Consistent with this, clinical features of the index patient reported here resembled Loeys-Dietz syndrome type 2 caused by mutations in *TGFBR2* (Mizuguchi et al., 2004). We tested trans-activation of Smad signaling by treating MDA-scp28 cells with conditioned media from cells expressing recombinant *SCUBE3* protein. Conditioned media from cells expressing the wild-type or *SCUBE3* p.(Thr231Ala) protein readily activated Smad signaling, while conditioned media from cells expressing disease-associated *SCUBE3* variants failed to activate Smad signaling (Fig. 2F). All samples showed similar levels of *SCUBE3* secretion into the conditioned media (ANOVA P=0.32).
differences in trans-activation were not due to differences in SCUBE3 secretion (Fig. 2G, S2D). These results demonstrate disease-associated SCUBE3 missense mutations are loss-of-function alleles causing SCUBE3-associated skeletal disease.

Mouse model of Hes7-associated spine deformity

During early embryonic development, the paraxial mesoderm gives rise to segmented bodies within the pre-somitic mesoderm and, ultimately, to the components of the vertebrate spinal column. The segmented bodies are formed through an intricate cyclical wave of gene regulation (i.e. segmentation clock), including the hairy and enhancer of split (HES)/HES-related (HER) family of transcription factors (Hubaud and Pourquie, 2014). Proper timing of the segmentation clock proceeds through feedback inhibition that controls Notch signaling periodicity. Disruption of the segmentation clock during early development causes various recognizable phenotypes, such as the spondylocostal dysostoses in humans. Spondylocostal dysostosis type 4, in which patients present with a variety of skeletal abnormalities, including spine and vertebral malformations, is caused by recessive loss-of-function mutations in the HES7 gene (Sparrow et al., 2008; Sparrow et al., 2010). Hes7 knockout mice died shortly after birth, while some heterozygous Hes7+/+ mice displayed kinked tails but no other significant skeletal abnormalities (Bessho et al., 2001). We detected significant association of severe spine deformities with the heterozygous Hes7V20E allele, which co-segregated with a synonymous mutation in Slfn4 (Fig. 3A,B). A total of 15/21 (71%) heterozygous mice developed severe kyphoscoliosis with a sharp vertebral angulation resembling human patients with spine deformity associated with segmentation defects, and, of these, 6 also developed kinked tails. The Hes7V20E variant is located at the beginning of the basic-helix-loop-helix domain of the protein and may disrupt DNA binding, which is necessary for transcriptional feedback repression as part of the segmentation clock during mouse somitogenesis (Bessho et al., 2001). Interestingly, no additional skeletal abnormalities were observed in Hes7+/V20E mice. As the mice described here develop a more severe spine deformity compared to Hes7+/+ mice (Bessho et al., 2001), it remains to be determined whether the Hes7V20E allele is loss-of-function or whether it has a dominant-negative effect on Hes7 transcription factor feedback signaling in vivo.
Neurogenic spine deformities in mice

Disruption of peripheral nervous system development

Some inborn errors of metabolism, such as lysosomal storage diseases, may manifest as neurodegenerative disease (Onyenwoke and Brenman, 2015). Krabbe disease is caused by recessive mutations in the \textit{GALC} gene, which encodes galactosylceramidase, a lysosomal enzyme that hydrolyzes galactolipids prevalent in myelin of the central and peripheral nervous systems (Sakai et al., 1994; Wenger et al., 2000). Though the age of onset may vary, the majority of patients with Krabbe disease present during infancy with spasticity and developmental delay, leading to death by 2 years of age (Tappino et al., 2010). Among patients with infantile-onset Krabbe disease, most develop scoliosis by 2 years of age (Beltran-Quintero et al., 2019). Loss of Galc activity has been described in the “\textit{twitcher}” mouse line, which develops early-onset tremor, weakness, late-onset kyphosis, and wasting with death by 3 months of age (Duchen et al., 1980; Kobayashi et al., 1980). We identified significant association between the \textit{Galc}^{K207R} allele and recessive spine deformity (Fig. 4A). All six homozygous mice displayed rigid cervico-thoracic kyphosis (Fig. 4B) and wasting. The \textit{Galc}^{K207R} variant is located within an alpha-helix of the central triosephosphate isomerase (TIM) barrel domain located at the inner core of the Galc protein, near the Galc$^{E198}$ catalytic residue involved in binding galactose (Deane et al., 2011). These results suggest the Galc$^{K207R}$ variant results in loss of Galc protein function, possibly through altering substrate binding.

Proteasome dysfunction

The 20S proteasome is a multimeric complex composed of seven alpha (PSMA1-7) and seven beta (PSMB1-7) subunits (Demartino and Gillette, 2007). Together with the PA700 regulatory component, the 20S protease degrades polyubiquitinated proteins. The genes encoding alpha and beta subunits of the 20S proteasome are highly conserved in humans, with high intolerance to loss-of-function mutations (Karczewski et al., 2020). Recently, a homozygous missense mutation in \textit{PSMB1} (\textit{PSMB1}^{Y103H}) was identified in a consanguineous family presenting with short stature, microcephaly, neurocognitive delay, and other manifestations (Ansar et al., 2020). The \textit{PSMB1}^{Y103} residue was predicted to interact with the PSMA5 subunit, and the \textit{PSMB1}^{Y103H} variant disrupted formation and activity of the 20S proteasome in transient transfection assays. In mice, 20S proteasome subunits are largely unstudied; however, we
detected significant association with spine deformity in mice homozygous for the Psma5^{V146G} allele (Fig. 4C,D). These mice also variably developed ataxia and a wobbling gait (P_{REC}=1.15\times10^{-6}; Supplementary Movie), suggestive of a neurologic deficit, and were significantly smaller (Fig. S3). It remains to be determined whether the Psma5^{V146G} allele reported here is a complete loss-of-function or hypomorphic, as Psma5 is likely an essential gene, and knockout mice have not been characterized.

**Mouse models of neuromuscular disease**

*Myotonic dystrophy*

Neuromuscular conditions, such as muscular or myotonic dystrophies, present increased risk for spine deformities. For example Becker-type myotonic dystrophy is a skeletal muscle disorder caused by recessive mutations in the CLCN1 gene, which encodes the chloride voltage-gated channel 1 protein (Lorenz et al., 1994). In contrast, dominant-negative mutations in the same gene cause Thomsen-type myotonic dystrophy, though the recessive Becker-type is more common and more severe. Both diagnoses present with a general inability to relax skeletal muscles throughout the body, and some patients with CLCN1 deficiency have been noted to develop spine deformity (Skalova et al., 2013). The CLCN1 homodimer functions as a chloride ion channel chiefly responsible for opposing excitatory signals in resting skeletal muscle. In mice, a spontaneous Clcn1 mutant line (Clcn1^{adr}) was described with myotonia, muscle weakness, reduced growth, and brittle bones (Watkins and Watts, 1984). We identified significant association of the homozgygous Clcn1^{V292A} allele with spine deformity (Fig. 5A,B). The Clcn1^{V292A} allele co-segregated with a missense allele in the olfactory receptor 13 gene (Olfr13^{Y59H}); however, the phenotypic resemblance of these mice with the Clcn1^{adr} line and the lack of any published characterization of this olfactory receptor led us to implicate the Clcn1^{V292A} allele in these mice. Similar to Clcn1^{adr} mice, homozygous Clcn1^{V292A} mice were noticeably smaller. It is notable that a human mutation (CLCN1^{E291K}) in the residue adjacent to the orthologous human CLCN1^{V292} position was reported in three unrelated families with recessive myotonic dystrophy (Meyer-Kleine et al., 1995). To validate this mouse phenotype, we engineered Clcn1 knockout mice using CRISPR/Cas9. Knockout mice homozygous for a predicted frameshift mutation p.(Phe337GlyfsTer4) developed significant spine deformity and reduced growth (Fig. 5C; Table S1), suggesting the Clcn1^{V292A} allele is loss-of-function.
Muscular dystrophy

Dystroglycanopathies represent a subset of congenital muscular dystrophies including Walker-Warburg syndrome and muscle-eye-brain (MEB) disease. Both are among the most severe of the dystroglycanopathies and are caused by recessive mutations in LARGE1, encoding the LARGE xylosyl- and glucuronyltransferase 1 protein (Clement et al., 2008; van Reeuwijk et al., 2007). LARGE1 functions to glycosylate α-dystroglycan, an integral component of the skeletal muscle extracellular matrix (Inamori et al., 2012). α-dystroglycan binds to β-dystroglycan, a myofiber membrane protein that itself binds intracellular dystrophin (Martin, 2003). Disruption of different components of this complex results in several muscular dystrophies. Mice lacking Large1, as in the spontaneous myd mouse line, show reduced survival, reduced growth, and kyphoscoliosis (Grewal et al., 2001; Lane et al., 1976). We identified significant association of the Large1Q359X nonsense allele with recessive spine deformity (Fig. 5D,E). Interestingly, the Large1Q359X allele was not significantly associated with post-natal lethality. The variant is located in the middle of the first glycosyltransferase domain and is predicted to result in complete loss of function.

DISCUSSION

Discovering the genetic causes of human spine deformities has been elusive until recently, due, in part, to technological advances such as genome-wide association studies and next-generation sequencing (Grauers et al., 2016; Wise et al., 2020). Understanding the genetic and mechanistic basis of human spinal disorders may be accelerated by forward genetic screens in model systems. To identify novel genetic mouse models of spine deformity, we performed a live-animal radiography screen in 54,497 mice collectively harboring predicted damaging or loss-of-function N-ethyl-N-nitrosourea (ENU)-induced mutations in at least two homozygous mice across 30.44% of autosomal genes. ENU mutagenesis in mice provides unique advantages. First, the saturation mutagenesis approach employed here enables high-throughput generation and testing for associated phenovariance with predicted deleterious and loss-of-function alleles throughout the mouse genome (Wang et al., 2018; Wang et al., 2015). Second, ENU mutagenesis introduces single-base variants typically resulting in missense alterations that may discover hypomorphic effects (Arnold et al., 2012). Third, though less frequently, introduction of
missense alleles may disrupt specific protein functions that result in detectable phenotypes not
evident in knock-out mouse models. Our results demonstrate the feasibility of detecting severe
spine deformities by radiographic imaging within a high-throughput saturation mutagenesis
screen in mice. While our approach enables detection of spine deformities that may be missed
with observation alone (i.e., kinked or curly tails), the relatively low percentage of pedigrees
presenting with spine deformity suggest that challenges remain. For example, limitations from
subjective radiographic evaluations may necessitate larger pedigrees to confidently map disease-
causing ENU alleles or sexually dimorphic alleles. A recent saturation mutagenesis screen in
zebrafish reported similarly low occurrence of pedigrees with spine deformity and with mapped
alleles (Gray et al., 2020), suggesting such challenges may not be unique to mouse or other
quadruped species. While selected examples are presented here, results from the entire skeletal
screen are deposited into the public online Mutagenetix database
(mutagenetix.utsouthwestern.edu), which complements ongoing efforts of the IMPC
(mousephenotype.org) to advance discovery of genes required for proper spine development in
mice and, potentially, novel mouse models of human disease.

By integrating results with a clinical exome database of undiagnosed patients, we report a
patient with bi-allelic SCUBE3 mutations presenting with skeletal abnormalities. The implication
of SCUBE3 as a skeletal disease-causing gene was supported by our identification of mice
homozygous for the Scube3^{C301Y} allele developing severe skeletal defects. In the course of this
study, multiple patients were reported with recessive SCUBE3 mutations presenting with skeletal
and non-skeletal manifestations similar to the index patient reported here (Lin et al., 2021).
Using two independent experimental assays, we show that missense variants identified in our
mouse model and the undiagnosed patient reported here, as well as other missense variants
recently reported (Lin et al., 2021), abrogated the interaction between SCUBE3 and SCUBE1
and reduced trans-activation of Smad signaling. These results demonstrate that the
characterization of novel mouse models may inform as-yet undiscovered rare human genetic
diagnoses when integrated with patient-based exome sequencing studies.

Results from our study also suggest novel mouse models of human Mendelian skeletal
diseases may be readily identified from the Mutagenetix database. As proof-of-concept, we
present novel mouse models for multiple known human diseases representing different
developmental etiologies, including segmentation defects (Hes7), leukodystrophy (Galc), and
neuromuscular disease (Clcn1 and Large1). Though selected examples of severe spine deformity are presented here, the Mutagenetix database facilitates discovery of, and public access to, ENU-induced alleles and CRISPR-generated mouse lines associated with a spectrum of severe and mild (i.e., kinked tail) spine deformity phenotypes.

Other mutagenesis efforts have been undertaken to identify alleles associated with spine deformity in non-rodent model systems, such as zebrafish (Gray et al., 2020; Henke et al., 2017). Such large-scale mutagenesis efforts can rapidly resolve causal alleles with the integration of next-generation sequencing (Andrews et al., 2012; Wang et al., 2015). Furthermore, in vivo gene editing using CRISPR/Cas9 to simultaneously engineer knockout and allele-specific knock-in models provides for rapid confirmation of suspected disease-causing alleles. By integrating with ongoing large-scale human genome sequencing efforts, in vivo saturation mutagenesis screens have potential to advance discovery of novel rare and ultra-rare human disease-causing genes.

MATERIALS AND METHODS

Skeletal mouse screen

The mutagenesis and breeding strategy are previously described (Wang et al., 2018; Wang et al., 2015). Briefly, male mice (designated G0 generation) are mutagenized with N-ethyl-N-nitrosourea (ENU) and out-crossed to C57BL/6J female mice. Transmitting ENU-induced alleles are detected in the resulting G1 male mice by exome sequencing. G1 male mice are out-crossed to C57BL/6J females, resulting in G2 mice heterozygous for ENU-induced alleles. Female G2 mice are back-crossed to their G1 sire, and all G2 and resulting G3 mice are genotyped by high-throughput targeted capture and next-generation sequencing.

All G3 mice are screened for spine deformity by live-animal radiography. Mice are anesthetized using inhaled isoflurane and imaged using a Faxitron UltraFocusDXA instrument. Spine deformities are scored as a binary variable by visualizing dorsal and lateral X-ray images. Following phenotype analysis, automated meiotic mapping identifies ENU-induced alleles associated with the spine deformity phenotype using dominant and recessive inheritance models (Wang et al., 2015).
All procedures were approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

**Automated meiotic mapping of ENU alleles**

ENU alleles are genotyped in G2 and G3 mice using a targeted capture and massively parallel sequencing approach. Genotypes of G2 and G3 mice are integrated with skeletal phenotypes, and the Linkage Analyzer tool tests the null hypothesis that each ENU allele within a pedigree has no association with the spine deformity phenotype using dominant and recessive models, as previously described (Wang et al., 2015). Statistical significance is assessed using logistic regression and p-values are reported using Wald tests. Results are visualized with Manhattan plots, and significantly associated alleles are those exceeding Bonferroni correction for the number of ENU alleles tested in the pedigree.

**Generation of Clcn1 knockout mice**

*Clcn1* mutant mice were engineered using CRISPR/Cas9 as previously described (Ran et al., 2013). Female C57BL/6J mice were super-ovulated by injection of 6.5 U pregnant mare serum gonadotropin (Millipore), followed by injection of 6.5 U human chorionic gonadotropin (Sigma-Aldrich) 48 h later. The super-ovulated mice were subsequently mated overnight with C57BL/6J male mice. The following day, fertilized eggs were collected from the oviducts and *in vitro*-transcribed Cas9 mRNA (50 ng/μL) and *Clcn1* small base-pairing guide RNA (50 ng/μL; 5′-GTTCAGAACGAATTTCCGAA-3′) were injected into the cytoplasm or pronucleus of the embryos. The injected embryos were cultured in M16 medium (Sigma-Aldrich) at 37 °C in 5% CO₂. For the production of mutant mice, two-cell-stage embryos were transferred into the ampulla of the oviduct (10–20 embryos per oviduct) of pseudo-pregnant Hsd:ICR (CD-1) female mice (Harlan Laboratories).

Founders were genotyped by Sanger sequencing. Mice carrying a predicted loss-of-function allele p.(Phe337GlyfsTer4) were mated to C57BL/6J mice. Subsequent pups harboring heterozygous loss-of-function alleles were inter-crossed, and resulting pups underwent skeletal screening. All mice were genotyped by Sanger sequencing by amplifying genomic DNA with the following primers: F: 5′-GTCTTGGGTAGGATGATGTAATGAAA-3′ and R: 5′-ATGACTTTGGCGATGCAGATAAAC-3′. PCR amplicons were sequenced with the following
primers: F: 5’-AAATAGGGTGATGGGAAGG -3’ and R: 5’- CGAAACACAGCTCCCAAGAAC -3’ (Fig. S4).

Exome sequencing and analysis

Clinical exome sequencing was performed as previously described (Yang et al., 2014). Briefly, sequencing libraries were generated following capture using the VCRome version 2.1 capture system. Paired-end sequencing was performed using the Illumina HiSeq system with 100 basepair reads, resulting in mean >100X coverage of targeted bases. Sequence reads were analyzed using software designed and implemented in the clinical laboratory. Variants in SCUBE3 were confirmed by Sanger sequencing (Fig. S2, Table S2).

Per Institutional guidelines, informed consent was not obtained nor required for the case report included here.

Co-immunoprecipitation

The SCUBE1 coding sequence (NM_173050.3) was cloned into pcDNA3.1 introducing an N-terminal hemagglutinin (HA) tag, and the SCUBE3 coding sequence (NM_152753.2) was cloned into pcDNA3.1 introducing a C-terminal MYC tag. Site directed mutagenesis (Takara, Mountain View, CA) was used to introduce the following point mutations: SCUBE3 p.(Thr231Ala), SCUBE3 p.(Cys97Trp), SCUBE3 p.(Gly240Asp), SCUBE3 p.(Cys301Tyr), SCUBE3 p.(Ile815Thr), SCUBE3 p.(Arg929Gly). HA-tagged SCUBE1 was co-transfected with MYC-tagged SCUBE3 using FuGene HD (Promega, Madison, WI) into HEK-293T cells cultured in DMEM with 10% fetal bovine serum and antibiotic. After 48h, cells were incubated with RIPA buffer (Santa Cruz Biotechnology, Dallas, TX), scraped from the well, incubated on ice, then pelleted at 12,000xg. 10µl of cell lysate was used for western blotting (input).

Pure Proteome Protein A/G Mix Magnetic Beads (Sigma, St. Louis, MO) were centrifuged at 1,000xg for 30 seconds at 4°C then incubated with 900µl cell lysate and 10 µg HA-antibody (Sigma, St. Louis, MO) at 4°C overnight with mixing. After incubation, the beads were pelleted by centrifugation at 1,000xg for 30 seconds at 4°C. The pellet was washed 3 times with RIPA buffer. After, the pellet was resuspended in 20µl Electrophoresis Sample Buffer (Santa Cruz Biotechnology, Dallas, TX). The beads were boiled for 5m, centrifuged at 1,000xg for 30s, and used for western blot analysis. Primary antibodies used for western blotting were
anti-HA (1:1,000; polyclonal, Sigma, St. Louis, MO, cat# H6908) and anti-Myc (1:1,000 clone 9E10; Sigma, cat# OP10). Secondary antibodies were IRDye goat anti-mouse (1:5,000; Li-Cor) and goat anti-rabbit (1:5,000; Li-Cor).

Western blots from three replicate experiments were quantified using ImageJ. For each experiment, the amount of SCUBE3 and SCUBE1 protein used for immunoprecipitation (by volume) was calculated based on the INPUT sample, and the proportion of protein recovered following immunoprecipitation was calculated. The proportion of SCUBE3 protein recovered after immunoprecipitation was normalized to the proportion of SCUBE1 protein recovered after immunoprecipitation. These values were evaluated for statistically significant differences using ANOVA, and, to account for inter-experiment variation, the experimental replicate was included as covariate. ANOVA p-values for each SCUBE3 variant are shown. For plotting, each variant SCUBE3 was then normalized to the SCUBE3 WT control from the same experiment, and the mean and standard error of the mean across the three replicate experiments were plotted.

**Smad activation assays**

The coding sequence of human SCUBE3 (NM_152753.2) was cloned into pcDNA3.1 introducing an N-terminal hemagglutinin (HA) tag. Site-directed mutagenesis (Takara, Mountain View, CA) was used to introduce the following point mutations: SCUBE3 p.(Thr231Ala), SCUBE3 p.(Cys97Trp), SCUBE3 p.(Gly240Asp), SCUBE3 p.(Cys301Tyr), SCUBE3 p.(Ile815Thr), SCUBE3 p.(Arg929Gly). Each clone was transfected into HEK-293T cells, and 48h later, conditioned media (CM) was harvested and concentrated. The Smad reporter cell line MDA-scp28 was treated with CM to quantify trans-activation of Smad signaling (Korpali et al., 2009). Briefly, 50,000 MDA-scp28 cells/well were plated in a 96-mutli well plate in high glucose DMEM supplemented with 10% FBS and 100 U/ml Penicillin/Streptomycin. Cells were not tested for contamination. MDA-scp28 cells were serum-starved 24 h then treated with CM for 20h. Luciferase activity was detected by the Dual Luciferase kit (Promega, Madison, WI; E1910), following the manufacturer instructions. Firefly luciferase activity was normalized by the Renilla luciferase activity (ratio F-Luc/R-Luc). Statistically significant differences were determined by one-way ANOVA and pairwise differences compared to NTC were determined by Dunnett’s test.
For testing SCUBE3 secretion, 5mL of media was concentrated to 200µl by centrifugation in Amicon Ultra-4 filters (Sigma) then treated with Aurum serum protein mini kit (Bio-Rad). For each sample, 60µl was evaluated by Western blotting using an anti-Myc antibody (1:1,000 clone 9E10; Sigma, cat# OP10). Western blots from three replicate experiments were quantified using ImageJ. These values were evaluated for statistically significant differences using ANOVA, and, to account for inter-experiment variation, the experimental replicate was included as covariate. For plotting, each clone was normalized to the SCUBE3WT control from the same experiment, and the mean and standard error of the mean across the three replicate experiments were plotted.

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COMPETING INTERESTS

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DATA AVAILABILITY

Results and mouse models are available online via the Mutagenetix website (mutagenetix.utsouthwestern.edu) and the Mutant Mouse Resource and Research Center (mmrrc.org).
AUTHOR CONTRIBUTIONS

Conceptualization: J.J.R, C.A.W, B.B; Methodology: J.J.R, B.B; Validation: J.J.R; Formal analysis: J.J.R; Investigation: J.J.R., K.D., H.Y., K.M., S.G., J.R., S.L., J.A.R., P.L., J.M., D.J.S., C.A.W.; Resources: J.J.R., J.R., B.B.; Data curation: J.J.R., K.D.; Writing - original draft preparation: J.J.R.; Writing – review and editing: J.J.R., K.D., K.M., J.A.R., B.B., C.A.W.; Supervision: J.J.R., B.B., C.A.W.; Project administration: J.J.R., B.B.; Funding acquisition: J.J.R., B.B.

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Fig. 1. Schematic of the mutagenesis, breeding, and screening strategy. Mutagenized G0 mice (ENU) are out-crossed to C57BL/6J (BL6) females. G1-generation pups are outcrossed, and subsequent G2-generation mice are back-crossed to their G1 sire to produce pedigrees of G3 mice for screening. Dorsal and lateral radiographs are obtained for all G3 mice, and mice are visually scored as affected or unaffected. Arrows indicate a spine deformity. Scale bar, 1cm. Automated meiotic mapping tests whether ENU alleles are associated with the spine deformity phenotype after correction for the number of ENU alleles tested in the pedigree.
Fig. 2. Mutations in SCUBE3 are associated with skeletal deformity. (A) Representative radiographic imaging of spine deformity in 2-month old female mice homozygous for the Scube3^{C301Y} allele compared to a gender-matched littermate homozygous for the reference allele (Scube3^{WT}). Enlarged radiographs of the kinked tail and spine deformities are shown (inset). Arrows indicate location of the deformity. Scale bar, 1cm. (B) Clinical radiographs of the hand (left) and feet (right) of a male patient with bi-allelic SCUBE3 mutations. Arrows indicate bone fusions that were evident in both the hands and feet. (C) Pedigree of a family with recessive skeletal disease. Affected individuals are shown with filled symbols. Two mutations were identified by exome sequencing, and genotype results for each mutation are shown for available subjects. Both mutations were confirmed compound heterozygous in the affected child by Sanger sequencing (Fig. S2). Square, male; Circle, female; WT, no mutation; HET, heterozygous. (D)
Schematic of the human SCUBE3 protein, including relative positions of annotated domains. Recently published variants identified in patients with skeletal disease (Lin et al., 2021) are shown in bold black. The human orthologue of the mouse ENU variant and the novel human variant reported here are shown in red. Location of the SCUBE3 p.(Thr231Ala) polymorphism is also shown. (E) Relative normalized quantification of SCUBE3 protein co-immunoprecipitated with SCUBE1. Data are represented as mean and standard error of the mean from three independent experiments. Statistically significant differences were evaluated by ANOVA (P=0.01; see Methods) (F) The Smad signaling luciferase reporter MDA-scp28 cells were treated with conditioned media from HEK293T cells expressing wild-type SCUBE3 (WT), and SCUBE3 containing the common polymorphism (T231A) or disease-associated mutations (as indicated in panel D). An untransfected no template control (NTC) sample is shown as negative control. Data are represented as mean and standard error of the mean from three independent experiments. Statistically significant differences were determined by one-way ANOVA (p=2.695e-12) and pairwise differences compared to NTC were determined by Dunnett’s test. (G) Relative secretion of Myc-tagged SCUBE3 was evaluated in media from HEK293 cells transiently expressing wild-type SCUBE3 (WT), and SCUBE3 containing the common polymorphism (T231A) or disease-associated mutations (as indicated in panel D). Data are represented as mean and standard error of the mean from three independent experiments. Statistically significant differences were evaluated by ANOVA (P=0.32; see Methods)
**Fig. 3.** Mouse model of a dominant *Hes7*-associated spine deformity. (A) Manhattan plot showing statistical significance following automated meiotic mapping of ENU-induced alleles with a dominant spine deformity phenotype. The *Hes7^{V20E}* allele was significantly associated with spine deformity in heterozygous mice. No homozygous mice were detected. (B) Representative radiographic image of the severe spine deformity in 3-month old female mice heterozygous for the ENU allele (*Hes7^{V20E}*E) compared to a gender-matched littermate homozygous for the reference allele (*Hes7^{WT}*). Arrows indicate location of the deformity. Scale bar, 1cm.
**Fig. 4.** Neurogenic spine deformity. (A) Manhattan plot showing statistical significance of the Galc\(^{K207R}\) allele following automated meiotic mapping of ENU-induced alleles with a recessive spine deformity phenotype. (B) Representative radiographic image of 2-month old female mice heterozygous for the ENU allele (Galc\(^{+/K207R}\)) or homozygous for the ENU allele (Galc\(^{K207R/K207R}\)). Homozygous mice were smaller and developed a rigid thoracic kyphosis phenotype. Arrows indicate location of the deformity. Scale bar, 1cm. (C) Manhattan plot showing statistical significance of the Psma5\(^{V146G}\) allele following automated meiotic mapping of ENU-induced alleles with a recessive spine deformity phenotype. (D) Representative radiograph of 5-month old female mice heterozygous for the ENU allele (Psma5\(^{+/V146G}\)) or homozygous for the ENU allele (Psma5\(^{V146G/V146G}\)). Arrows indicate location of the deformity. Scale bar, 1cm.
Fig. 5. Neuromuscular spine deformity. (A) Manhattan plot showing statistical significance of the $Clcn1^{V292A}$ allele following automated meiotic mapping of ENU-induced alleles with a recessive spine deformity phenotype. (B) Representative radiograph of 2-month old male mice heterozygous for the ENU allele ($Clcn1^{+/V292A}$) or homozygous for the ENU allele ($Clcn1^{V292A/V292A}$). Arrows indicate location of the deformity. Scale bar, 1 cm. (C) Representative radiograph of 5-month old male CRISPR-engineered mice heterozygous for the p.(Phe337GlyfsTer4) mutation ($Clcn1^{+/ko}$) or homozygous for the mutation ($Clcn1^{ko/ko}$). Arrows indicate location of the deformity. Scale bar, 1 cm. (D) Manhattan plot showing statistical significance of the $Large1^{Q359X}$ allele following automated meiotic mapping of ENU-induced alleles with a recessive spine deformity phenotype. (E) Representative radiographs of 4-month old male mice heterozygous for the ENU allele ($Large1^{+/Q359X}$) or homozygous for the allele ($Large1^{Q359X/Q359X}$). Arrows indicate location of the deformity. Scale bar, 1 cm.
Table 1. Clinical characteristics of affected siblings. DD, Developmental delay; ID, Intellectual disability; N, Not noted

|                         | Sibling 1 (male)          | Sibling 2 (female)          |
|-------------------------|---------------------------|-----------------------------|
| Cognition               | DD/ID                     | DD/ID                       |
| Craniofacial            | Micrognathia              | Pierre-Robin sequence       |
|                         |                           | Cleft palate                |
| Skeletal                | Short stature             | Short stature               |
|                         | Hand and foot fusions     | Hand malformations          |
|                         |                           | Foot fusions                |
| Auditory                | Conductive hearing loss   | Hearing loss                |
| Cardiac                 | Dilated aortic root       | N                           |
| Gastrointestinal        | Intestinal malrotation    | Intestinal malrotation      |
| Genital-urinary         | Hypospadias               | N                           |
Fig. S1. Total number of affected mice is associated with successful meiotic mapping to ENU alleles. (A,B) The (A) total number of affected G3 mice in the pedigree and (B) total number of G3 mice in the pedigree were evaluated among 20 pedigrees with alleles mapped to a spine deformity phenotype compared to 74 pedigrees with at least two affected mice but for which no allele was mapped. The dominant mutations in Hes7 is indicated. Statistically significant differences were determined using Wilcoxon test.
Fig. S2

A. 

**Unaffected Father**

GCGAATACCA
GCAGAAGCATCA

ATGTGGCAGAT
ATGTGGCAGAT

G R I/V T/S

B. 

**Unaffected Mother**

GCGAATACCA
GCAGAAGCATCA

ATGTGGCAGAT
ATGTGGCAGAT

G R I T

C. 

**Affected Child**

GCGAATACCA
GCAGAAGCATCA

ATGTGGCAGAT
ATGTGGCAGAT

G R I/V T/S

D. 

**IP: HA-SCUBE1**

**IB: MYC-SCUBE3**

**IB: HA-SCUBE1**

**INPUT**

**IB: MYC-SCUBE3**

**IB: HA-SCUBE1**

**SECRETED**

**IB: MYC-SCUBE3**
**Fig. S2.** Sanger sequence confirmation of *SCUBE3* mutations. (A-C) Sequence chromatograms testing presence of the *(left)* NM_152753.4: c.1521_1522insGC/p.(Ile508AlafsTer74) and *(right)* NM_152753.4: c.2785C>G/p.(Arg929Gly) mutations in the *(A)* unaffected father, *(B)* unaffected mother, and *(C)* affected patient. Resulting transcript and protein sequence is shown below, with mutant sequence shown in red. *(D)* Representative immunoblot (IB) of recombinant HA-tagged SCUBE1 and Myc-tagged SCUBE3 following immunoprecipitation (IP) of SCUBE1. Normal (WT) SCUBE3 and SCUBE3 harboring the common polymorphism (SCUBE3^{T231A}) are shown as positive controls. Input cell lysate is shown for both SCUBE1 and SCUBE3, and secreted SCUBE3 is also shown.
Fig. S3. The \( Psma_{5}^{V146G} \) allele is associated with reduced body weight. Boxplot demonstrating the reduced body weight (adjusted Z-score) of mice homozygous for the \( Psma_{5}^{V146G} \) allele (VAR) compared to mice heterozygous for the allele (HET) or homozygous for the reference allele (REF). Male mice are shown in blue and female mice shown in pink. Numbers of mice for each genotype are shown below. Statistical significance was determined by automated meiotic mapping for recessive alleles in the pedigree.
Fig. S4. Sequence confirmation of a loss-of-function allele in CRISPR-engineered Clcn1 mice. (A) Representative chromatogram from a mouse homozygous for the wild-type allele. (B) Representative chromatogram from a mouse homozygous for the p.(Phe337GlyfsTer4) frameshift mutation. The deletion results in premature truncation, encoding a predicted protein of 336 amino acids. The wild-type protein has a length of 994 amino acids.
Tables S1 and S2.

Click here to download Tables S1 and S2

**Movie 1.** Altered gait in *Psma5*-mutant mice. Representative video of a 3-month old female mouse homozygous for the *Psma5*<sup>V146G</sup> allele demonstrating the altered hindlimb gait and ataxia.