ORIGINAL ARTICLE

MBTPS1/SKI-1/S1P proprotein convertase is required for ECM signaling and axial elongation during somitogenesis and vertebral development†

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Abstract

Caudal regression syndrome (sacral agenesis), which impairs development of the caudal region of the body, occurs with a frequency of about 2 live births per 100 000 newborns although this incidence rises to 1 in 350 infants born to mothers with gestational diabetes. The lower back and limbs can be affected as well as the genitourinary and gastrointestinal tracts. The axial skeleton is formed during embryogenesis through the process of somitogenesis in which the paraxial mesoderm periodically segments into bilateral tissue blocks, called somites. Somites are the precursors of vertebrae and associated muscle, tendons and dorsal dermis. Vertebral anomalies in caudal regression syndrome may arise through perturbation of somitogenesis or, alternatively, could result from defective bone formation and patterning. We discovered that MBTPS1/SKI-1/S1P, which proteolytically activates a class of transmembrane transcription factors, plays a critical role in somitogenesis and the pathogenesis of lumbar/sacral vertebral anomalies. Conditional deletion of Mbp1 yields a viable mouse with misshapen, fused and reduced number of lumbar and sacral vertebrae, under-developed hind limb bones and a kinky, shortened tail. We show that Mbp1 is required to (i) maintain the Fgf8 ‘wavefront’ in the presomitic mesoderm that underpins axial elongation, (ii) sustain the Lfng oscillatory ‘clock’ activity that governs the periodicity of somite formation and (iii) preserve the composition and character of the somitic extracellular matrix containing fibronectin, fibrillin2 and laminin. Based on this spinal phenotype and known functions of MBTPS1, we reason that loss-of-function mutations in Mbp1 may cause the etiology of caudal regression syndrome.

†Formatting of protein and gene names follows recommendations of the International Committee on Standardized Genetic Nomenclature for Mice (http://www.informatics.jax.org/nomen).

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Introduction

The spine provides structural support for the body, flexibility for movement and protection for the spinal cord and nerves. The vertebral column consists of bone segments separated by intervertebral disk joints supported by ligaments and axial muscles. Collectively this constitutes the musculoskeletal component of the spine. The precise integration of bone, cartilage, nerves, muscles and tendons is essential for proper spine function and birth defects or degenerative conditions that perturb these tissues and their developmental integration result in congenital vertebral malformations. Caudal regression syndrome (sacral agenesis) is one such birth defect, and occurs with a frequency of about 2 in 100,000 live births, although the frequency rises to 1 in 350 in mothers with gestational diabetes (1,2). While presentation can be variable, multiple vertebrae of the lower spine are absent or misshapen in this syndrome that can also exhibit underdeveloped lower limb bones in a frog-leg position.

The vertebral column is formed through a process known as somitogenesis (3). During embryogenesis, the paraxial mesoderm periodically segments into bilateral blocks of tissue called somites, which are the precursors of the vertebrae and their associated muscle, tendons, ligaments and dorsal dermis. The periodicity of somite formation is governed by a molecular oscillator termed the segmentation clock which is evident in the form of cyclic patterns of FGF8, WNT and NOTCH activity in the presomitic mesoderm (PSM). The actual process of somite boundary formation and thus axial segmentation involves a mesenchymal to epithelial transformation (MET) of the PSM and this coincides with intersection of oscillatory gene activity with the determination front. The determination front is thought to be specified primarily by a gradient of FGF8 and WNT signaling in the caudal PSM that diminishes rostrally. However, in contrast to this established model, recently it was proposed that somites may have the capacity for self-organization independent of any clock and wavefront mechanism (4).

Vertebral anomalies may result from perturbation of somitogenesis or alternatively via defective bone formation and patterning. Mutations causing autosomal-recessive spondylocostal dysostosis which affects the spinal vertebrae and ribs have been found in LFNG (5), HES7 (6), DLL3 (7) and MESP2 (8), which play a critical role in regulating different steps of somitogenesis. In contrast, the cause of caudal regression syndrome, which predominantly affects the lower spine and hind limbs, is unclear. However, exposure to retinoic acid (RA) (9), telomere dysfunction (10), Brachyury (T) loss-of-function (11), double transgenic overexpression of flael-1 under a Hoxc8 promoter (12) and inactivation of PCK5 (13) produce these symptoms in mice. Since known mutations only account for a minor fraction of congenital vertebral anomaly cases, there is a need to identify additional causative loci to clarify their etiology and pathogenesis.

Here we describe a novel role for MBTPS1/SKI-1/S1P (membrane bound transcription factor protease, subtilisin kexin isozyme-1, or site 1 protease) in somite segmentation and in the pathogenesis of vertebral anomalies. MBTPS1 is an auto-catalytically activatable member of the proprotein convertase family of serine proteases (14). MBTPS1 activates a small family of transmembrane spanning b-ZIP transcription factors (15). MBTPS1, also known as site 1 protease (SKI-1, or site 1 protease) in somite segmentation and in the pathogenesis of vertebral anomalies (16,17) is expressed ubiquitously in the post-implantation and mid-gestation mouse embryos.

We discovered that this conditional Mbtps1 loss-of-function mouse model exhibits phenotypic changes localized to the lumbar/sacral vertebral region (decreased vertebral number, vertebral fusion and kinky tail) which mimic those in caudal regression syndrome. Consistent with this phenotype, we show that Mbtps1 plays critical roles in regulating somitogenesis.

Results

Mbtps1 is expressed ubiquitously in the post-implantation and mid-gestation mouse embryo

As a first step in determining the developmental function of MBTPS1, we characterized its spatiotemporal activity in embryonic day (E) 7.5–11.5 embryos via section in situ hybridization. At E7.5, Mbtps1 mRNA was detected throughout the embryo, the ectoplacental cone and in the decidua (Fig. 1A–C). In E8.5 embryos, Mbtps1 was highly expressed in the neural tube and in the newly formed somites (Fig. 1D–F). These domains of expression continue from E9.5 until E11.5 (Fig. 1G–O). Although the level of activity varies between different tissues, Mbtps1 is expressed ubiquitously throughout post-implantation and mid-gestation mouse embryos.

Col1-Cre mediated Mbtps1 knockout mice survive until at least P10 but exhibit severe vertebral defects

Mbtps1 was previously conditionally deleted in cartilage by using the Col2-Cre recombinase (22). These conditional mice have a shortened body axis, severe chondrodysplasia and die during or shortly after birth. Since we wanted to investigate the role of Mbtps1 both embryonically and postnatally during osteogenesis, we decided to conditionally excise it using the Col1-Cre. In order to do that we crossed heterozygous male Mbtps1 floxed mice carrying one copy of the 3.6 Col1-Cre transgene (Mbtps1fl/+; 3.6 Col1-Cre−/−) with female mice homozygous for the Mbtps1 floxed allele (Mbtps1fl/fl). Offspring genotypes were consistent with expected Mendelian ratios. P0 heterozygous (Mbtps1fl/fl; 3.6 Col1-Cre−/−) pups were visibly indistinguishable from wild-type littermates. In contrast, P0 homozygous conditional knockout mice (Mbtps1fl/fl; 3.6 Col1-Cre−/−), which we call Mbtps1ΔΔ, were all postnatal viable, but were smaller than their control siblings with a short kinky tail and smaller hind limbs (Fig. 2A). By P10 the difference in size was particularly evident [an average body weight of 6.5 gm for wild-type males versus 4.7 gm for male Mbtps1ΔΔ (P = 0.001) littermates] along with severe hypertrophy and paralysis of the hind limbs (Fig. 2B and C). Signifying the severity of the phenotype, about 60% of the Mbtps1ΔΔ pups were cannibalized by their mothers shortly after birth. MicroCT scans of spines from P10 Mbtps1ΔΔ mice revealed extensive developmental anomalies in vertebral bones compared with normal littermate controls. Figure 2D and E depict a representative sampling of two Mbtps1ΔΔ mice which display abnormal lumbar and sacral vertebrae. Abnormal bone patterning was consistently observed on the posterior faces of lumbar (L) 4 to 6 vertebrae (Fig. 2D and E). This ectopic mineralized bone obscured the boundaries of individual vertebrae, demonstrating that these abnormal vertebrae are effectively fused. However, cross-sectional views at this location did not show an obstruction of the spinal column (Fig. 2E). These spinal defects resemble congenital vertebral anomalies such as caudal dysgenesis and spondylocostal dysostosis, which are, respectively, characterized by severe malformations of the vertebral column including a shortened trunk.
due to loss of vertebral segments and fusion of vertebrae during development (23).

Expression of Cre recombinase under the control of the 3.6 Col1 promoter

Vertebral anomalies observed in conditional Mbtps1KO mutant newborn pups were surprising given the reported expression of 3.6 Col1-Cre in adult calvaria, long bone, tendon and skin (24). This suggested firstly that there may be an earlier, as yet unappreciated, 3.6 Col1-Cre activity during embryogenesis and secondly that MBTPS1 may play a critical role in somitogenesis, the process in which the paraxial mesoderm is segmented into somites. Therefore, we crossed 3.6 Col1-Cre transgenic mice to ROSA26 reporter mice (25). The latter expresses β-galactosidase in the presence of Cre recombinase after excision of a floxed

Figure 1. Mbtps1 is expressed ubiquitously in the post-implantation and mid-gestation mouse embryo. Left column shows embryo sections stained with Cresyl Violet; middle column shows the same sections with comparative antisense hybridization labeling seen as bright under darkfield illumination (arrows) and right column shows control sense hybridization. (A–C) Comparison of in situ hybridization with sense and antisense probes reveals a broad expression of Mbtps1 in the uterine decidua and in the embryo at E7.5 (arrow). E7.5 early embryo sagittal section seen within uterine cavity near site of implantation. Presence of Mbtps1 mRNA labeling is evident within embryo, ectoplacental cone and decidua. (D–F) E8.5 embryo coronal section. (G–I) E9.5 embryo sagittal section. (J–L) E10.5 embryo sagittal section. (M–O) E11.5 embryo sagittal section. AC, amniotic cavity; AV, atrioventricular canal; DA, dorsal aorta; primordium; De, decidua; EEc, embryonic ectoderm; EEM, extraembryonic membranes; EEn, embryonic endoderm; Em, embryo; EMe, embryonic mesenchyme; EPCa, ectoplacental cavity; EPCo, ectoplacental cone; FBr, forebrain; H, heart; HBr, hindbrain; HP, hepatic primordium; HR, hindbrain roof; M, mandible; NE, neuroepithelium; NP, nasal pit; NT, neural tube; S, somite; SV, sinus venosum; T, tail; and Tr, trophoblasts. Magnifications: (A–C) ×36; (D–F) ×24; (G–I) ×15 and (J–O) ×10. Scale bar: 1 mm.
STOP cassette that inhibits the expression of the reporter gene. We observed expression of 3.6 Coll1-Cre as determined by β-galactosidase activity in whole-mount embryos beginning around E8.5 (Fig. 3A and B). Interestingly, β-galactosidase expression was highly enriched in the caudal region of the embryo, particularly in the PSM (Fig. 3A and D). Transverse sectioning of labeled embryos revealed widespread recombination among tissues that had not been previously appreciated (Fig. 3E and F) (24). In particular, there are high levels of expression in the ectoderm, the neuroepithelium and hind limb buds. Therefore, these findings indicate that 3.6 Coll1-Cre-mediated excision of the floxed Mbp1 allele initially takes place around E8.5 in the caudal region of the embryo. Beginning in the PSM at an axial level posterior to the future forelimb, the labeled cells contribute to a variety of tissues including the paraxial mesoderm that will give rise to the embryonic somites and consequently to the adult spine.

**Skeletal preparations support vertebral abnormalities**

Consistent with the early expression of 3.6 Coll1-Cre in the PSM at E8.5, we observed a caudal axial truncation in Mbp1 cKO embryos as early as E10.5 compared with control littermates (Fig. 3G and H). Immunostaining for fibrillin2 (FBN2), which is a structural component of myofibrils that demarcates individual somites, demonstrated that the axial truncation is consistent with a significantly (P<0.012) reduced number of somites (26.4 ± 2.1 STD) in E10.5 Mbp1 cKO embryos compared with each control genotypic group [30.1 ± 1.5 STD (Mbp1fl/fl: 3.6ColCre+); 30.5 ± 0.9 (Mbp1fl/fl); 28.9 ± 1.1 (Mbp1fl/fl)] (Fig. 3I). Caudal axial truncation was more prevalent by E12.0 and progressively worsened throughout embryogenesis (Fig. 3H). Consistent with Mbp1 expression and 3.6 Coll1-Cre activity in the neuroepithelium, we also observed a striking neuronal phenotype of E11.5 Mbp1 cKO embryos. Immunostaining with neuron-specific antibodies against type III β-tubulin revealed a lack of neuronal projections from the spinal cord into the limb in contrast to controls (Fig. 3J and K). This embryonic neuronal phenotype likely presages the hind limb paralysis exhibited by postnatal Mbp1 cKO mice.

To investigate the developmental cause of the axial truncation phenotype and its correlation with postnatal malformations, we characterized bone and cartilage differentiation via Alizarin red and Alcian blue staining, respectively, in E15.5 to P13 individuals. At E15.5, we observed defects in the developing spine of Mbp1 cKO embryos in the form of hypoplastic cartilage pre-vertebrae particularly in the sacral and coccygeal regions (Fig. 4A and B). A day later at E16.5, we observed a general delay in ossification in association with vertebral irregularities in Mbp1 cKO embryos compared with wild-type littermates (Fig. 4C).
and D). By P0, more severe defects were consistently observed in the form of fused vertebrae in the sacral and the coccygeal regions together with infrequently altered anterior–posterior vertebral identity, particularly at the lumbar-sacral boundary (Fig. 4G and H, arrows). Thus, the irregular formation or ‘stacking’ of the developing cartilaginous vertebrae in the coccyx observed at E15.5 presages the fused vertebrate subsequently observed in that same region in P13 Mbtps1cKO mice (Fig. 4B’ and J, brackets).

In summary, conditional knockout of Mbtps1 using the 3.6 Col1-Cre recombinase results in axial truncation, vertebral fusions and occasional unilateral fate changes of sacral and lumbar vertebrae. These defects demonstrate that MBTPS1 is required during embryogenesis for proper vertebral segmentation and development.

**MBTPS1 is required for somitogenesis during development**

Based on the expression of Mbtps1, and the activity of 3.6 Col1-Cre, we hypothesized that the axial truncation phenotype observed as early as E10.5 in Mbtps1cKO embryos was consistent with a...
precursors that resemble
This pattern of disorganized somite segmentation is consistent of-function results in irregular and indistinguishable somite and coccygeal (C) vertebrae. (L) Fusion of L6 and S1 vertebrae in Mbtps1KO embryos is observed at P0 (arrow). (I and J) Tails of Mbtps1KO mice routinely exhibited multiple fused vertebrae (compare bracketed regions). (I’ and J’) Some S1 vertebrae in Mbtps1KO spines displayed asymmetric abnormal differentiation (see arrow) resembling that for L6. Scale bar: 2 mm.

perturbation of somitogenesis. To test this idea we characterized the expression of Uncx4.1 which demarcates the posterior boundary of newly formed somites and provides a readout of the regularity and bilateral symmetry of somite formation as well as proper polarity or anterior–posterior patterning (26,27). In E11.5 control embryos, in situ hybridization revealed strong segmental expression of Uncx4.1 in the caudal region of each newly formed and maturing somite (Fig.5A). In contrast, in Mbtps1KO embryos, the segmental pattern was perturbed (Fig.5B). Thus Mbtps1 loss-of-function results in irregular and indistinguishable somite boundaries together with abnormally spaced and fused somites. This pattern of disorganized somite segmentation is consistent with the vertebral fusions and axial observation in Mbtps1KO embryos and postnatal mice.

Several models have been proposed to describe the periodicity of somitogenesis and integral to each of these models is an oscillator that drives segmentation (28–32). A negative feedback loop mechanism involving the NOTCH, WNT and FGF signaling pathways constitutes the core of this oscillatory clock. One of the genes whose oscillatory expression is required for proper progression of the clock is Lunatic fringe (Lfng) (33–37). LFNG is a glycosyltransferase which is both a transcriptional target and a negative regulator of the NOTCH signaling pathway. Lfng is normally expressed in distinct phases that progress dynamically from the tail bud through the PSM to the boundary of the newly forming somite (Fig. 5G–I). In contrast, representative E10.5 Mbtps1KO embryos exhibited reduced levels and diminished domains of Lfng activity in the PSM (Fig.5J–L). Additionally, Lfng was only infrequently expressed at the boundary of any newly formed somite. Collectively, these results illustrate a perturbation of cyclic expression of Lfng in Mbtps1cKO embryos which could subsequently impact somite segmentation. Consistent with these observations, it was shown that the segmentation clock and consequently posterior skeletal development are sensitive to LFNG dosage during embryogenesis (38). Furthermore, these results are indicative of a disruption of the NOTCH signaling pathway. NOTCH signaling is a key component of the molecular oscillator, the perturbation of which disrupts the cyclic activity of genes such as Lfng and Hes7, resulting in perturbed somite segmentation (39). Thus an alteration of Lfng expression in Mbtps1KO embryos demonstrates that Mbtps1 loss of function disrupts the segmentation clock.

To further validate this idea, we examined the activity of Mesp2, a basic helix-loop–helix transcription factor that suppresses NOTCH activity through induction of Lfng and which is required for the initiation of somite formation (40–42). In control embryos at E11.5, Mesp2 is expressed in bilateral single bands that correspond with the anterior half or border of the next presumptive somite to form (Fig. 5M–O). By comparison, in Mbtps1KO siblings, expression of Mesp2 is substantially reduced in two out of three representative samples and slightly reduced in the third (Fig.5P–R). Overall, all embryos showed a disruption in Mesp2 activity which is consistent with abnormal somite segmentation and the subsequent vertebral fusions and anomalies observed in Mbtps1KO mice.

The translation of the temporal periodicity of cyclic gene expression, as driven by the segmentation clock, into the spatial periodicity of somite formation is mediated by two dynamic antagonizing gradients: a caudorostral FGF8/WNT gradient and a rostrocaudal RA gradient (3,43). The interface between these gradients is thought to define a threshold level of FGF8 signaling activity that establishes the position of the ‘determination’ or ‘maturational’ wavefront. Cells in the PSM are competent to respond to the segmentation program during axis elongation only when exposed to a specific threshold of FGF8 activity. Fgf8 is expressed in the posterior region of the PSM and is critical for the spatial positioning of the determination front (44–46). In E9.5 and E10.5 control embryos, we observed strong expression of Fgf8 in the caudal PSM (Fig. 5C, D and D’) which is consistent with previous reports (44). In contrast, Mbtps1KO embryos fail to exhibit any measurable expression of Fgf8 in the PSM at E10.5 (Fig. 5F and F’). The FGF8 gradient is thought to be required for body axis elongation by controlling cell motility in the PSM (47). The
Figure 5. Expression of key markers of somitogenesis including Fgf8 and Lunatic Fringe (Lfng) are dramatically decreased in the presomitic mesoderm of Mbtps1cKO embryos. Whole mount in situ hybridization was performed at E9.5 (C and E), E10.5 (D and G-L) or E11.5 (A, B and M-R) in control and Mbtps1cKO embryos. (A and B) Expression of Uncx4.1 reveals a general disorganization of the somites including loss of somite polarity in Mbtps1cKO embryos, 3 days after the onset of the 3.6 Col1-Cre recombinase. (C-F) Expression of Fgf8, normally restricted to the posterior-most region of the PSM at E9.5 and E10.5 (brackets) (C and D), is absent in Mbtps1cKO embryos at E10.5 (F), but still present a day earlier in Mbtps1cKO embryos at E9.5 (E). (G-L) Lfng expression is greatly reduced and its cyclic expression disturbed in E10.5 Mbtps1cKO embryos as demonstrated by dorsal (G-L) and lateral (G'-L') views of the non-segmented PSM. (M-R) As illustrated by a representative sampling of three embryos, Mesp2 expression displays a range of expression in the determination front of Mbtps1cKO embryos which is consistently less than that for controls. Scale bar: 100 µm.
loss of Fgf8 expression as a consequence of Mbtps1 loss-of-function is consistent with the truncated posterior body axis observed in Mbtps1<sup>−/−</sup> mice. Consistent with this rationale, conditional knockout of the FGF receptor 1 (Fgfr1), the only FGF receptor expressed in the paraxial mesoderm, results in abnormal somite segmentation and vertebral development (48,49) similar to Mbtps1<sup>−/−</sup> mice. These similarities in phenotype suggest that an absence of FGF8 signaling in the PSM mechanistically underpins much of the Mbtps1<sup>−/−</sup> phenotype. Furthermore, a day earlier, at E9.5, Fgf8 expression in the PSM of Mbtps1<sup>−/−</sup> mice is unchanged compared with control littermates (Fig. 5E). This is consistent with normal somite development observed at this stage and correlates with the latency of effect of Cre recombinase on the spatiotemporal excision of Mbtps1. This further supports the idea of altered Fgf8 expression being a cause of the phenotype. In summary, vertebral defects observed in Mbtps1<sup>−/−</sup> embryos and newborn mice are due to perturbation of somitogenesis during development as evidenced by the defects in the segmentation clock, the determination front, and the spatiotemporal lack of Fgf8 expression in the PSM.

**Cell death is increased in the PSM in Mbtps1<sup>−/−</sup> embryos**

Since FGF8 is known to be required for the proliferative maintenance of the PSM (46), we explored whether there were alterations in cell survival in Mbtps1<sup>−/−</sup> mice that correlate with the axial truncation phenotype. TUNEL staining of E10.5 mouse embryos revealed a comparable basal level of cell death in the caudal trunk of control and Mbtps1<sup>−/−</sup> embryos (Fig. 6A and B). However, by E11.5, Mbtps1<sup>−/−</sup> embryos showed a dramatic increase in cell death throughout the PSM and in the newly formed somites compared with their control littermates (Fig. 6C and D). The loss of Fgf8 expression is consistent with elevated apoptosis in the PSM, which may negatively impact the proliferation of cells during axial elongation and somite development.

**Extracellular matrix components are down-regulated and are highly disorganized in the caudal region of Mbtps1<sup>−/−</sup> embryos**

In order for a somite to form, mesenchymal cells in the PSM move rostrally and, upon reaching the determination front, undergo an MT (23). These types of cell movements and MT require a properly organized extracellular matrix (ECM). We have previously shown that inactivation of MBTPS1 protease in osteoblastic cells resulted in down-regulation of ECM genes including Fn1, Fbn2 and Lama (19). Interestingly, Fn1 knockout mice form no somites (50,51) and the importance of the fibronectin matrix in somite formation has also been demonstrated during zebrafish, chick and frog embryogenesis (52–55). Therefore, we examined the ECM in the PSM of Mbtps1<sup>−/−</sup> mouse embryos for perturbations of fibronectin, consistent with disrupted somitogenesis.

In wild-type embryos, fibronectin localizes to the medial interface of the non-segmented PSM and neural tube, while more rostrally, fibronectin surrounds each formed somite (Fig. 7A). In contrast, Mbtps1<sup>−/−</sup> embryos exhibit a considerable reduction in fibronectin in the PSM validating our previous micro-array and qPCR results obtained after MBTPS1 inactivation in cultures of osteoblast-like cells (Fig. 7B) (19). Additionally, we observed a reduction in the fibronectin content of the matrix surrounding each somite (see arrowheads, Fig. 7B) along with fibronectin-containing fibrils forming abnormally near the determination front. Our results are consistent with a role for MBTPS1 protease in regulation of fibronectin expression and suggest that insufficient ECM proteins in Mbtps1<sup>−/−</sup> embryos limit assembly of the highly organized fibronectin matrix resulting in the formation of ectopic fibrils in the rostral PSM.

To further validate the association of perturbed ECM with somitogenesis defects, we examined fibrillin2, which is one of the major structural components of extracellular microfibrils that serve as a scaffold for deposition of elastin. In control E10.5 mouse embryos, fibrillin2 demarcates the boundaries of each somite, which is consistent with what has been shown previously in avian embryos (Fig. 7C) (56). Unlike fibronectin, however, fibrillin2 does not accumulate in the non-segmented PSM. In sibling Mbtps1<sup>−/−</sup> embryos, fibrillin2 containing microfibrils are highly disorganized in the anterior PSM from where the newly forming somites will emerge (see arrowheads, Fig. 7D). In addition, fibrillin2 localizes in the posterior PSM in a similar highly disorganized fashion. The disorganization of the ECM is even more evident at E11.5 (compare Fig. 7E and F). Also, the levels of fibrillin2 in the somites may also be reduced in Mbtps1<sup>−/−</sup> embryos compared with the control siblings.

![Figure 6](https://academic.oup.com/hmg/article-abstract/24/10/2884/623615) Cell death is evident in the caudal region of Mbtps1<sup>−/−</sup> embryos. (A–D) Whole mount TUNEL staining reveals increased cell death in the caudal region of the Mbtps1<sup>−/−</sup> embryos at E11.5 (C and D), but no change at E10.5 (A and B). Scale bar: 200 µm.
Lastly, we examined the distribution of laminins as they are major components of the basal lamina, one of the ECM layers. The distribution of laminin in sections of E11.5 wild-type mouse embryos resembled that of fibrillin2 and fibronectin, in that it demarcates the boundaries of each somite (Fig. 7G). In Mbp1cKO littermate embryos, however, this restricted localization is absent, replaced by a disorganized distribution of extracellular laminins in the non-segmented PSM as well as in the newly formed somites (see arrowheads, Fig. 7H). Overall, the ECM in the PSM of Mbp1cKO embryos is highly disorganized with aberrant localization of key components, e.g. fibronectin, fibrillin2, and laminin. The level of expression of fibronectin protein is also lower than controls which is consistent with results in cultured osteoblastic cells showing that Fn1 transcription is MBTPS1 dependent (19) and furthermore suggests that proper fibronectin deposition is critical for normal somite formation.

Discussion

We previously discovered that MBTPS1 is essential for bone mineralization in vitro (19). However, the developmental requirement for MBTPS1 in vivo remains unknown. Here we showed that Mbp1 is broadly expressed as early as E7.5 during mouse embryogenesis. Since Mbp1 null mice die during embryogenesis prior to implantation (20,21), we conditionally deleted Mbp1...
using Cre recombinase under the control of a 3.6 kb fragment of the rat Col1a1 gene promoter (3.6 Col1 Cre). In postnatal mice, this promoter is active predominantly in bone and tail tendon, but is also expressed in non-osseous tissues (24,57). However, we discovered that Cre recombinase expression under the 3.6kb Col1a1 promoter occurs earlier than previously thought (24) and is spatially restricted primarily to the posterior region of the embryo. Importantly this also coincides with domains of enriched Mbtps1 expression in the posterior region of young embryos identified here by in situ hybridization and with the lumbar/sacral spinal defects we observe in Mbtps1\(^{−/−}\) mice. After excision of Mbtps1 starting at about E8.5, all homozygous Mbtps1\(^{−/−}\) mice survive until birth and consistently exhibit short, kinky tails and para-zyed hind limbs. Skeletal preparations and micro-CT scans revealed fewer than normal lumbar, sacral and tail vertebrae along the anterior/posterior axis with a general truncation of the tail. Histological analyses of embryonic development indicated the early onset of these defects was concentrated specifically in the PSM and somites.

**Defects in somitogenesis lead to embryonic and postnatal lumbar/sacral vertebral and hind limb defects in Mbtps1\(^{−/−}\) mice**

To date, no definitive cause for causal regression syndrome has been identified; however, environmental influences such as hyperglycemia, vascular malformation and environmental toxins have been proposed to underlie its pathogenesis (13). Interestingly, Mbtps1\(^{−/−}\) mice exhibit skeletal defects that are localized to the lower lumbar, sacral and caudal vertebrae resembling causal regression syndrome. Mbtps1\(^{−/−}\) mice consistently display axial truncation of their spinal columns together with misshapen and fused lower lumbar vertebrae.

Although somitogenesis initiates normally, the onset of 3.6 Col1-Cre-mediated Mbtps1 deletion within the caudal region of Mbtps1\(^{−/−}\) embryos around E8.5 results in the loss of Fg\(\beta\)8 in the caudal PSM by E10.5 (Fig. 5D and F). It is well known that a gradient of Fg\(\beta\)8 is critical for establishing the determination front and maintaining cell proliferation in the PSM, and consistent with this role, TUNEL staining revealed a dramatic increase in cell death in the PSM of Mbtps1\(^{−/−}\) embryos in association with the loss of Fg\(\beta\)8. Mechanistically this helps to account for the axial shortening observed in Mbtps1\(^{−/−}\) mice.

Fg\(\beta\)8 may also represent a common target of environmental factors known to increase the incidence of causal agenesis, e.g. RA and gestational diabetes. Intriguingly, RA is known to directly repress the expression of Fg\(\beta\)8 through a specific RA receptor binding site element (RARE) in its promoter (58). Furthermore, causal regression syndrome phenotypes similar to that observed in Mbtps1\(^{−/−}\) mice are associated with elevated RA exposure supplied from the paraxial mesoderm in the caudal region of the tail bud. The phenotype of Mbtps1\(^{−/−}\) mice resembles that of the Pudgy mutant which has a point mutation in Dll3 which disrupts NOTCH signaling, giving rise to a similarly disrupted pattern of Uncx4.1 expression (62). The randomized polarity of somites in Pudgy mutants leads to misshapen vertebral bodies which resemble those in the Mbtps1\(^{−/−}\) mice. Embryonic manipulation and gene expression studies have shown that rostrocaudal polarity is established in the anterior PSM before the initiation of segmentation.

The axial skeleton (vertebral bodies and intervertebral discs) develops from a mesenchymal cell mass derived from the ventral halves of the somites while the lateral regions of the sclerotome form the vertebral arches and ribs. Uncx4.1 expression is restricted to the caudal half of each newly formed somite and future sclerotome (63), and is required for maintenance and differentiation of particular elements of the axial skeleton. Mutations in Uncx4.1 result in the absence of pedicles, transverse processes and proximal ribs, along the entire vertebral column (27). In Mbtps1\(^{−/−}\) embryos, Uncx4.1 expression was dramatically disrupted and lacked its normal polarity. This implies that Mbtps1 deficiency may cause the formation of the vertebral arch and contributes to the abnormally shaped sacral S1 and lumbar L6 processes observed in Mbtps1\(^{−/−}\) embryos.

In addition to formation of the bony vertebrae, somitogenesis also produces the organized development and segmental organization of associated muscles, nerves and blood vessels of the body. Fibronectin, fibrillin2 and laminin all play important developmental roles in the formation of these tissues. Fibronectin is essential for proper development of embryonic mesenchymal tissues and is required for development of the notochord and formation of somites (51,64). Fibrillin2 surrounds the developing notochord, the somites and the neural tube, and has been suggested to regulate spinal patterning via long-range mechanical tension fields via its microfibrils (65). Consistent with this idea, Fbn2 null mice exhibit bone and tendon defects (66,67). Laminin, a major component of the basal lamina, is localized at both the notochord/somite and the intersomitic boundaries (68). In the frog, inhibition of dystroglycan, a cell adhesion receptor for laminin, with morpholino antisense oligonucleotides or via dominant-negative approaches affects the number, size and integrity of the somites (69). In this work we have shown that Mbtps1\(^{−/−}\) embryos exhibit perturbed fibronectin, fibrillin2 association between in utero RA exposure and the risk for causal regression has not been established for humans.

During the formation of a new somite every 2 h in the mouse, Lfng is normally expressed in a dynamic and cyclic fashion throughout the PSM that proceeds caudororally through the PSM (35,36). However, in Mbtps1\(^{−/−}\) embryos, the expression of Lfng was reduced and its cyclic expression was perturbed. Additionally, expression of Mesp2, a gene that defines the segmental boundary and the rostrocaudal identity of each somite as it forms, was also considerably reduced. Subsequently, the perturbed expression of Uncx4.1 as well as of fibronectin, fibrillin-2 and laminin proteins within the prospective lumbar and sacral vertebral regions of Mbtps1\(^{−/−}\) embryos, provides a clear temporal record of the progression of somitogenesis defects as a result of Mbtps1 loss-of-function.

**Relationship of Mbtps1 deficiency to other mutant or transgenic strains with similar spinal phenotypes**

Somites bud off sequentially from the rostral end of the PSM while more undifferentiated presomitic cells are continuously supplied from the paraxial mesoderm in the caudal region of the tail bud. The phenotype of Mbtps1\(^{−/−}\) mice resembles that of the Pudgy mutant which has a point mutation in Dll3 which disrupts NOTCH signaling, giving rise to a similarly disrupted pattern of Uncx4.1 expression (62). The randomized polarity of somites in Pudgy mutants leads to misshapen vertebral bodies which resemble those in the Mbtps1\(^{−/−}\) mice. Embryonic manipulation and gene expression studies have shown that rostrocaudal polarity is established in the anterior PSM before the initiation of segmentation.

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and laminin patterns in the trunk region. Therefore, we conclude that the phenotypes observed in Mbtps1<sup>−/−</sup> mice are due in part to defects in the ECM during somitogenesis. Not only is the clock and wavefront mechanism underpinning the spatiotemporal periodicity of somitogenesis affected as a consequence of Mbtps1<sup>−/−</sup> loss-of-function, but so too is the ECM which is critical for proper signaling and tissue morphogenesis during somitogenesis (70–72).

How can MBTPS1 act as a new upstream mediator of somitogenesis?

A major impediment to unraveling the signaling networks which control the clock mechanism has been the apparent individuality with which key genes are regulated in a cyclical and positional manner. We hypothesize that MBTPS1 protease could function as an upstream transcriptional regulator for a number of genes that govern somitogenesis.

Mechanistically, proteolytically active MBTPS1 is required for activation of SREBP-1, SREBP-2 and CREB/ATF family transcription factors and we have previously shown that transcription of Fn1, Fbn2, six Lama isoforms, and ~140 genes in osteoblastic cells requires activation and nuclear import of CREB-H, SREBP-1 and SREBP-2 (19). Importantly, MBTPS1 activated CREB/ATF family members CREB-H, CREB4, CREB3L2 (OASIS), ATF-6 as well as non-membrane derived CREB and ATF-4 recognize the cyclic AMP response element sequence (73, 74). We speculate that once MBTPS1 activated CREB/H, SREBP-2 and CREB/ATF factors reach the nucleus they regulate the transcription of responsive genes such as Fn1, Fbn2, Lama and others by binding to CRE and/or SRE enhancer sequences within the respective promoters. Consistent with this paradigm, it was recently shown that dynamic CREB activity drives segmentation and posterior polarity specification during mammalian somitogenesis (75). Furthermore, CREB loss-of-function results in perturbation of oscillatory (Lfg), segmentation (Mesp2) and ECM (laminin) activity which resembles the phenotype observed in Mbtps1<sup>−/−</sup> mice. Similarly, mutations in the zebrafish CREB homolog result in skeletal defects due to defective chondrocyte development (76, 77). While there remains much to be learned about how CREB co-ordinates segmentation, it is known that CREB is associated with regulating Notch signaling and that CREB confers maturation to the PSM at the determination front (75). Given that it has been well established that MBTPS1 is required to activate transmembrane forms of CREB, MBTPS1 appears to lie upstream of CREB in the regulation of somitogenesis. Thus, we propose that MBTPS1 protease functions as a new upstream mediator of somitogenesis by regulating the transcription of Fgf8, Lfg, Fn1, Fbn2 and Lama within the PSM.

While a complete loss of Mbtps1 in humans would likely be embryonically lethal, coding mutations that generate hypomorphic alleles or cis-regulatory mutations that affect the spatiotemporal activity of MBTPS1 may indeed elicit a causal regression phenotype. Additionally, expression of Mbtps1 may be further regulated by a complex mix of environmental factors such as fatty acids, cholesterol, polyunsaturated fatty acids, hyperglycemia, oxysterols and insulin. However, a causative role for Mbtps1 in human caudal regression syndrome remains speculative at this time.

We believe the paralytic phenotype observed in Mbtps1<sup>−/−</sup> mice is a consequence of the combined transcriptional effects of blocking Mbtps1 expression, which leads to the failure of axons to project into the hind limbs. Furthermore, we envision that the absence of an FGF8 gradient within the PSM inhibits the proliferation of mesenchymal cells and perturbs the determination front. Subsequently, disorganization of the ECM in the anterior PSM in Mbtps1<sup>−/−</sup> embryos disrupts the process of segmentation. The organization of cables containing fibrillin2, which normally coalesce near the midline before somite formation, is considerably disrupted. Fibrillin2 containing cables have been proposed to participate in mechanical tension fields linked to patterning events such as somitogenesis (65).

Materials and Methods

Skeletal preparations

E15.5–E18.5 embryos and P0–P13 postnatal mice were fixed in 99% ethanol and stained for bone and cartilage as previously described (78). A total of five E15.5–E16.5 Mbtps1<sup>−/−</sup> embryos, five E18.5–P0 Mbtps1<sup>−/−</sup> embryos and two P10 Mbtps1<sup>−/−</sup> mice were analyzed after skeletal preparation.

Immunohistochemistry and apoptosis assay

E10.5–E11.5 mouse embryos were fixed with 4% paraformaldehyde in PBS overnight at 4°C and dehydrated in a graded series of methanol solutions. Dehydrated embryos were then bleached in MeOH:DMSO:30%H<sub>2</sub>O (4:1:1, Dents Bleach) for 2 h at room temperature and rehydrated sequentially in 75% MeOH/PBS, 50% MeOH/PBS and in 25% MeOH/PBS for 10 min each followed by two final washes in PBS for 5 min each. Embryos were then blocked in 3% BSA for 2 h at room temperature before adding the primary antibody (fibrillin2, Rabbit, Sigma, Cat. #HPA012853, 1:50), fibronectin, (Rabbit, Sigma, Cat. #F3648, 1:100), or Laminin (Chicken, Abcam, Cat. #ab14055, 1:200). Embryos were then incubated in primary antibody at 4°C overnight and the next day were washed 5 times for 1 h each in PBS. Rabbit secondary AlexaFluor 488 conjugated antibody (Invitrogen, 1:500) or chicken secondary AlexaFluor conjugated 488 antibody (Invitrogen, 1:500) was then added and incubated overnight at 4°C in the dark. Both primary and secondary antibodies were diluted in 3% BSA. Stained embryos were then washed in PBS 3 × 5 min and 3 × 1 h at room temperature and were either counterstained with DAPI (1 µg/ml) for 20 min at room temperature or immersed in Vectashield with DAPI to label individual nuclei. To analyze cell death, E11.5 embryos were subjected to TUNEL staining using the FITC Cell Death Detection Kit (Roche) according to the manufacturer’s protocol after the dehydration and rehydration step.

For the sections, we fixed E10.5–E11.5 mouse embryos with 4% paraformaldehyde in PBS for 2 h at room temperature and then immersed them in 15% sucrose in PBS at 4°C overnight, followed by immersion in 30% sucrose in PBS at 4°C overnight. Embryos were then embedded in OCT and stored at −80°C until used. Cryosections of 10 µm were then generated, washed in PBS for 10 min, blocked in PBS containing 3% BSA, 0.15% glycerine and 0.1% Triton X100 for 30 min. Primary antibodies were then used as mentioned above and washes were performed in PBS 3 × 10 min the next day. Secondary antibodies (as above) were then applied and incubated for 2 h at room temperature. After 3 × 10 min washes, sections were mounted in Vectashield with DAPI. Fluorescence microscopy was performed on an LSM5 PASCAL confocal microscope (Carl Zeiss) using 2.5×, 5× and 10× objective lenses. Confocal optical slices were collected and maximum-intensity projection stacks were made with Zeiss LSM5 software. A total of two Mbtps1<sup>−/−</sup> embryos were analyzed for each antibody staining, with the exception of the E12.0 TUJ1 staining for which one embryo was analyzed. For the TUNEL staining, a total of four Mbtps1<sup>−/−</sup> mice were analyzed.
In situ hybridization

E7.5–E11.5 embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C and dehydrated in a graded series of methanol. In situ hybridization on whole mount embryos was performed as previously described using digoxigenin-labeled probes for Lfng, Mesp2, Uncx4.1 (gift of Dr Olivier Pourquie), Fgfl (gift of Dr Ivor Mason), and NBT-BCIP detection (79). A total of three Mbtps1KO embryos were analyzed for Fgfl and Uncx4.1 at E10.5, a total of four Mbtps1KO embryos were analyzed for Fgfl at E9.5, a total of five Mbtps1KO embryos were analyzed for Lfng and a total of five Mbtps1KO embryos were analyzed for Mesp2.

For analysis of Mbtps1, embryo stages E7.5 to E11.5 were examined. Tissues were frozen and sectioned with cryostat into 8–10 μm sections and mounted on microscope slides. Tissues were fixed in 4% formaldehyde and hybridized with 35S-labeled-Mbtps1 cRNA antisense and sense probes overnight at 55°C as previously described (80). After hybridization, slides were exposed to X-ray film for 4 days, then dipped in Kodak NTB nuclear track emulsion, and exposed for 14 days in light-tight boxes with desiccant at 4°C. Photographic development was undertaken with Kodak D-19. Slides were lightly counterstained with Cresyl Violet and analyzed under both light- and darkfield optics. Antisense probes that are complimentary to mRNAs generate ISH labeling while control sense RNA probes that are identical to mRNAs always gave background levels of the hybridization signal. Values obtained by subtraction of sense labeling from antisense labeling were considered as specific.

Mating and animal strains

All animals were maintained in the UMKC Laboratory Animal Research Center under an approved protocol, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Mbtps1fl/fl mice were obtained from Dr. Jay Horton, Southwest Medical School and Dr. Linda Sandell, Washington University (21,22); Mesp2x/fl mice were obtained from the Jackson Laboratory.

Females [L221, 5′-CTTGTGATCCGGCCTCGAGATATT-3′; L238, 5′-CGGGCGGCGTGTTAAGTGCAGTTACGT-3′; and R316 5′-GGAGGGGAGAAATGAGATATG-3′] to generate 186 bp (wild-type) and 400 bp (LacZ allele) bands.

Statistics

Numbers of somites in E10.5 Mbtps1KO and control embryos were counted and subjected to a one-way ANOVA analysis using SigmaPlot (version 12.5). Data passed both a normality and an equal variance test. Pairwise comparisons revealed somite numbers for Mbtps1KO embryos were significantly different from the three control genotypes (P < 0.012).

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Conflict of Interest statement. None declared.

Embryo collection

Females were checked daily for semen plugs and separated from male mice once a plug was detected. Pregnant mice were euthanized on embryonic day 7.5, 8.5, 9.5, 10.5, 11.5, 12, and P0 to P14 using carbon dioxide gas and embryos were immediately removed for genotyping and analysis.
References