

# Expression of Notch signaling pathway genes in mouse embryos lacking $\beta$ 4galactosyltransferase-1

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Received 14 September 2005; accepted 20 September 2005

Available online 18 January 2006

## Abstract

A requirement for  $\beta$ 4galactosyltransferase-1 ( $\beta$ 4GalT-1) activity in the modulation of Notch signaling by the glycosyltransferase Fringe was previously identified in a mammalian co-culture assay. Notch signaling is necessary for the formation of somites in mammals. We therefore investigated the expression of eleven Notch pathway and somitogenic genes in E9.5 mouse embryos lacking  $\beta$ 4GalT-1. Four of these genes were altered in expression pattern or expression level. The Notch target genes *Hes5* and *Mesp2* were affected to some degree in all mutant embryos. The Notch ligand genes *Dll1* and *Dll3* were reduced or altered in expression in a significant proportion of mutants. While there were no differences in the number or morphology of somites in E9.5 *B4galt1* null embryos, the number of lumbar vertebrae in mutant embryos differed from control littermates ( $P \leq 0.01$ ). The subtlety of the *in vivo* phenotype may be due to redundancy since several *B4galt* genes related to *B4galt1* are expressed during embryogenesis.

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**Keywords:** Notch; Fringe; Galactosyltransferase; Somitogenesis; Vertebrae; B4galt1; Lfng; Mesp2; Delta1; Delta3; Jagged1; Hes5; Hes7; Myogenin; Uncx4.1; Hox; Lumbar

## 1. Results and discussion

Notch receptors at the cell surface transmit signals critical for the determination of cell fate in the metazoa (Lai, 2004; Schweisguth, 2004). Many of the epidermal growth factor-like (EGF) repeats in the extracellular domain of Notch receptors are modified by *O*-linked fucose (Moloney et al., 2000b; Haines and Irvine, 2003). Inactivation of protein *O*-fucosyltransferase 1 that transfers fucose to Notch receptors causes severe, cell-autonomous Notch signaling defects that are embryonic lethal in *Drosophila* (Okajima and Irvine, 2002; Okajima et al., 2003; Sasamura et al., 2003) and mouse (Shi and Stanley, 2003). Notch ligands (Jagged and Delta in mammals) are cell surface glycoproteins that induce signal transduction when they bind to Notch receptors. Ligand binding does not occur when *Drosophila* Notch lacks *O*-fucose

(Okajima et al., 2003), and similar results have been obtained for mouse Notch receptors (K. Uemura and P. Stanley, unpublished observations).

*O*-fucose (*O*-Fuc) on Notch is extended to the disaccharide *O*-Fuc-GlcNAc by the action of Fringe, a  $\beta$ 3N-acetylglucosaminyltransferase (Bruckner et al., 2000; Moloney et al., 2000a). Fringe modulates Notch signaling in *Drosophila* (Irvine and Wieschaus, 1994; Panin et al., 1997; Fleming et al., 1997; Haines and Irvine, 2003), in the mouse (Evrard et al., 1998; Zhang and Gridley, 1998; Zhang et al., 2002), and in mammalian cell-based signaling assays (Chen et al., 2001; Hicks et al., 2000; Moloney et al., 2000a; Shimizu et al., 2001; Yang et al., 2005). Notch signaling is either enhanced or inhibited by Fringe, depending on the Notch ligand (Haines and Irvine, 2003; Haltiwanger and Stanley, 2002). Inactivation of the *Lfng* gene or constitutive expression of *Lfng* in the presomitic mesoderm (PSM) causes severe defects in somitogenesis with altered expression of several Notch pathway genes and severe perturbation of axial skeleton formation (Evrard et al., 1998; Zhang and Gridley, 1998; Zhang et al., 2002). In mammalian cells, the *O*-Fuc-GlcNAc disaccharide formed by Fringe on Notch EGF repeats may be further extended by the addition of galactose (Gal) followed by sialic acid (Chen et al., 2001; Moloney et al., 2000a; Shao et al., 2002). Using Chinese hamster ovary (CHO) glycosylation

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mutants in a co-culture assay, we showed that Fringe modulation of Jagged1-induced Notch signaling in CHO cells requires the addition of Gal to form the trisaccharide O-Fuc-GlcNAc-Gal on Notch (Chen et al., 2001). The galactosyl-transferase  $\beta$ 4GalT-1 was shown to be necessary to obtain the Fringe-mediated effects on Notch signaling in this assay (Chen et al., 2001). Here we characterize mouse embryos lacking  $\beta$ 4GalT-1 (Lu et al., 1997) encoded by the *B4galt1* gene for expression of a subset of somitogenic and Notch signaling pathway genes (*Notch1*, *Jag1*, *Dll1*, *Dll3*, *Hes5*, *Hes7*, *Mesp2*, *Lfng*, *Tbx18*, *Uncx4.1* and *myogenin*), and for skeletal development during embryogenesis. While mice lacking  $\beta$ 4GalT-1 do not have skeletal defects at birth (Asano et al., 1997; Lu et al., 1997), they have not previously been examined during embryogenesis.

*Hes5* is expressed during somitogenesis in the prospective somite, the PSM and the neural tube (Barrantes et al., 1999; de la Pompa et al., 1997). Although *Hes1*, *Hes3* and *Hes5* Notch target genes are expressed at E8.5 to E9.0, only *Hes5* is severely downregulated in the PSM of *Notch1* and *Dll1* mutant embryos (Barrantes et al., 1999). In *Lfng* mutant embryos, *Hes5* expression is greatly reduced or completely lost in the posterior half of the prospective somite and in the PSM (Evrard et al., 1998). Similarly, *B4galt1* mutant embryos ( $n=6$ ) showed either no expression or only faint expression of *Hes5* in the posterior half of the forming somite (Fig. 1;  $P \leq 0.01$  by the exact Chi square test for association). Expression of *Hes5* remained strong in the neural tube of mutant embryos (Fig. 1). Wild type and *B4galt1* heterozygous embryos also had strong *Hes5* expression in neural tube ( $n=19$ ), and the majority (14/19) showed expression in the posterior half of the forming somite and/or the PSM (Fig. 1).

The *Mesp2* gene encodes a bHLH protein expressed in the anterior region of the PSM (Saga et al., 1997). *Mesp2* expression demarcates the anterior compartment of the second

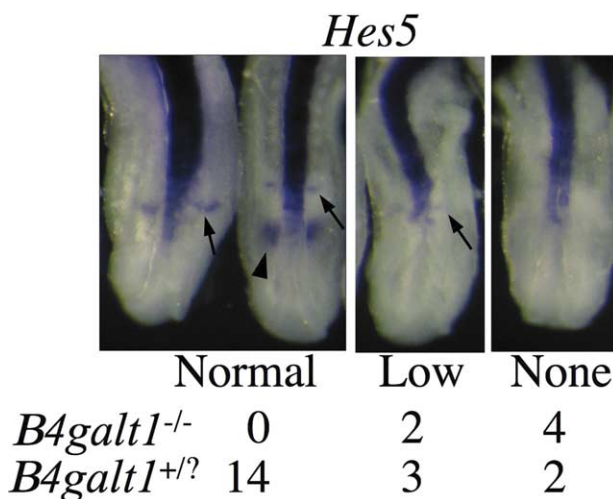


Fig. 1. Expression of *Hes5* in *B4galt1* mutant embryos. Whole-mount in situ hybridization with a *Hes5* probe performed on control and *B4galt1* mutant embryos at E9.5. *Hes5* expression was decreased or lost in the prospective somite (arrow head) and/or in the PSM (arrow) in *B4galt1* mutant embryos. *Hes5* was strongly expressed in the neural tube of all embryos (vertical stripe).

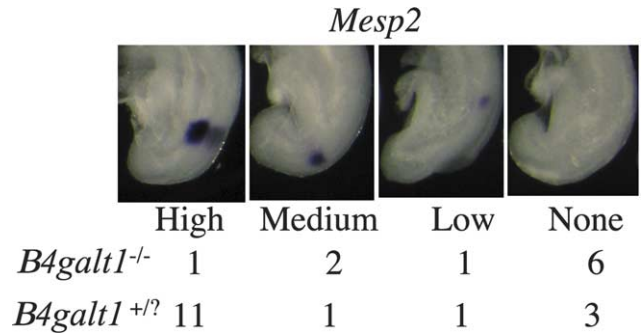


Fig. 2. Expression of *Mesp2* in *B4galt1* mutant embryos. Whole-mount in situ hybridization with a *Mesp2* probe was performed on control and mutant embryos at E9.5. *Mesp2* expression in the prospective somite was lost or reduced in  $\beta$ 4GalT-1 mutant embryos.

presumptive somite in the PSM. Mice with a homozygous mutation of the *Mesp2* gene die shortly after birth and have delayed somite segmentation and severe skeletal malformations (Saga et al., 1997; Takahashi et al., 2003; Takahashi et al., 2005). In *Lfng* mutant embryos, *Mesp2* expression is expanded and more diffuse compared to controls (Zhang et al., 2002). Altered *Mesp2* expression was also observed in mice lacking *B4galt1* (Fig. 2). In *B4galt1* mutant embryos ( $n=10$ ), *Mesp2* expression was reduced or completely lost in the posterior half of the prospective somite compared to the expected expression of *Mesp2* in wild type and heterozygous embryos ( $n=16$ ) (Fig. 2;  $P=0.03$  by the exact Chi square test for association).

The mouse *Dll1* gene is expressed in the PSM as well as the posterior half of formed somites (Bettenhausen et al., 1995). Double label in situ hybridization showed that *Dll1* overlaps with *Jag1* expression in the posterior half of the forming somite (Barrantes et al., 1999; Dunwoodie et al., 1997). Mice with an inactivated *Dll1* gene have a severe patterning defect in paraxial mesoderm, segments have no cranio-caudal polarity and epithelial somites do not form (Hrabe de Angelis et al., 1997). In *Lfng* mutant embryos, *Dll1* expression in the posterior half of the formed somites is lost or becomes diffuse in most somites (Evrard et al., 1998; Zhang and Gridley, 1998). While strong expression of *Dll1* remains in the PSM of *Lfng* mutant embryos, the pattern is slightly more diffuse than in controls. When in situ hybridization with a *Dll1* probe was performed in *B4galt1* mutant embryos, 4 out of 10 had somewhat irregular and more diffuse *Dll1* expression in the posterior half of formed somites (Fig. 3A). However, all the wild type and heterozygous embryos ( $n=18$ ) had the expected expression of *Dll1* in both the PSM and the caudal half of somites (Fig. 3A;  $P=0.02$  by the exact Chi square test for association).

*Dll3* is expressed in the PSM, in the prospective somite and in the rostral compartment of formed somites (Dunwoodie et al., 1997). Mice with a targeted loss-of-function mutation in the *Dll3* gene have a shortened body and truncated tail similar to *Lfng* mutant mice and die within 10 days of birth (Dunwoodie et al., 2002). The Pudgy mouse mutation is in the *Dll3* gene (Bulman et al., 2000; Kusumi et al., 1998) and

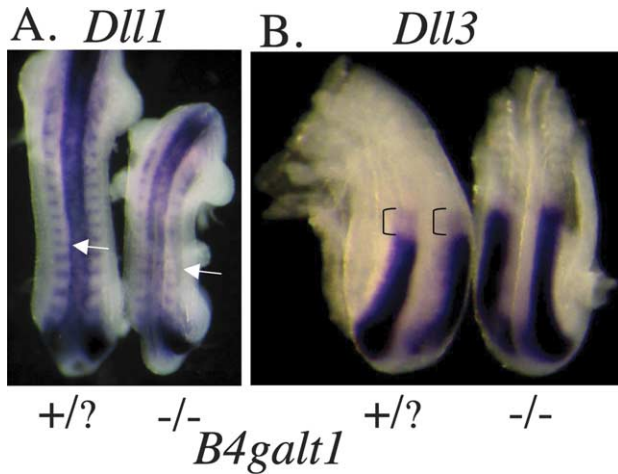


Fig. 3. Expression of *Dll1* and *Dll3* in *B4galt1* mutant embryos. Whole-mount in situ hybridization with a *Dll1* or *Dll3* probe performed on control and mutant embryos at E9.5. *Dll1* expression in the posterior half of formed somites (arrows) was decreased and diffuse in some *B4galt1* mutant embryos. Expression of *Dll3* in the prospective somite (bracket) was reduced and the PSM border was not so sharply demarcated in some *B4galt1* mutant embryos.

alters the proper formation of morphological borders in early somite formation as well as the anterior–posterior polarity within somites (Kusumi et al., 2004). In *Lfng* mutant embryos, *Dll3* expression in the prospective somite is also reduced, the boundary with the PSM is not sharply demarcated, and strong expression in the PSM is observed (Evrard et al., 1998; Zhang and Gridley, 1998). All wild type and heterozygous *B4galt1* embryos expressed *Dll3* strongly in the PSM, and in a faint band through the prospective somite above the clearly demarcated boundary with the PSM (Fig. 3B). A faint band in the anterior region of the formed somite was observed but is not visible in Fig. 3B. Three of seven *B4galt1* mutant embryos showed a marked loss of expression of *Dll3* in the prospective somite but maintained strong expression of *Dll3* in the PSM and a well-demarcated border (Fig. 3B;  $P=0.03$  by the exact Chi square test for association).

*Notch1* is expressed strongly in the prospective somite and weakly throughout the PSM and *Notch1* null embryos have somitogenic defects (Conlon et al., 1995; Swiatek et al., 1994). While one group found that the boundary of *Notch1* expression in the most recently formed somite was more diffuse in E9.5 *Lfng* mutant embryos (Zhang and Gridley, 1998), another group did not observe this difference (Evrard et al., 1998). In *B4galt1* null ( $n=4$ ) and control ( $n=12$ ) embryos *Notch1* expression was sharply demarcated at the boundary of the prospective somite and was expressed similarly in the PSM of *B4galt1* mutant and control embryos (Fig. 4A).

The Notch ligand *Jag1* is expressed in the posterior region of the prospective somite and in the tail bud (Xue et al., 1999). *Jag1* expression in the prospective somite is severely reduced in the Notch pathway mutants *Dll1* (Barrantes et al., 1999), *Notch1* (Conlon et al., 1995; Swiatek et al., 1994), *Rbp-Jκ* (Oka et al., 1995) and *Pofut1* (Shi and Stanley, 2003), suggesting that *Jag1* expression depends on Notch pathway activation, although *Jag1* null embryos have no apparent defects in

somitogenesis (Xue et al., 1999). In *Lfng* null embryos at E9.5, the stripe of *Jag1* expression at the border of the forming somite is not affected and *Jag1* is expressed at low levels in the posterior half of the prospective somite as in wild type (Zhang and Gridley, 1998). In *B4galt1* null ( $n=4$ ) embryos, expression of *Jag1* was also found in the posterior half of the prospective somite and in the tail bud to be similar in mutant ( $n=4$ ) and control embryos ( $n\sim 12$ ; Fig. 4B).

Of the three mammalian Fringes, *Lfng* is the only one expressed in the PSM (Cohen et al., 1997; Johnston et al., 1997) in a dynamic pattern cycling every 2 h in a wave through the PSM and across the forming somite in a manner similar to the periodicity of somite formation (Aulehla and Johnson, 1999; Cole et al., 2002; Morales et al., 2002). Mice with a targeted mutation of *Lfng* have defects in somite formation and anterior–posterior polarity of somites (Evrard et al., 1998; Zhang and Gridley, 1998). In several of the *B4galt1* mutant embryos ( $n=4$ ), *Lfng* was expressed continuously throughout the PSM (Fig. 4C), while in others ( $n=3$ ) *Lfng* was expressed in the prospective somite. In control embryos, *Lfng* was also expressed either in the prospective somite ( $n=5$ ) or in the PSM ( $n=3$ ). Therefore, *Lfng* displays a dynamic expression pattern in *B4galt1* mutant embryos.

*Hes7* is a bHLH gene that is also a downstream target of the Notch signaling pathway (Bessho et al., 2001a,b). *Hes7* is expressed in a dynamic pattern in the PSM in the same oscillatory expression pattern as *Lfng*. *Hes7* mutant mice have a short trunk and tail, somites are not properly segmented and their anterior–posterior polarity is lost in embryos (Bessho et al., 2001b). While the expression of *Notch1* and its ligands is not affected significantly in *Hes7* mutant embryos, *Lfng* is expressed throughout the PSM and its expression does not oscillate in 2 h cycles (Bessho et al., 2001b). *Hes7* functions as a repressor that can block transcription from E-box containing promoters and it plays an important role in the maintenance of *Lfng* oscillation in the PSM (Cole et al., 2002; Morales et al., 2002). In some of the *B4galt1* mutant embryos ( $n=3$ ), *Hes7* was strongly expressed throughout the PSM (Fig. 4D). In other mutant embryos ( $n=3$ ), *Hes7* expression was weakly expressed at the prospective somite as well as in the tail bud. *Hes7* was also expressed only in the tail bud in one mutant embryo. In control embryos, *Hes7* was expressed in the tail bud ( $n=2$ ), or in both the prospective somite and the tail bud ( $n=3$ ) and in the PSM ( $n=10$ ). These results indicate that *Hes7* displays a dynamic expression pattern in *B4galt1* mutant embryos.

To evaluate the anterior–posterior expression pattern of somitogenic genes in *B4galt1* mutant embryos the expression of *Uncx4.1*, *Myogenin* and *Tbx18* were examined at E9.5. *Uncx4.1* encodes a paired homeodomain protein and is expressed in a metamer pattern in the posterior half of formed somites at E9.5 (Mansouri et al., 1997). In *Lfng* mutant embryos, posterior *Uncx4.1* expression is reduced and the expression pattern is disorganized (Evrard et al., 1998; Zhang and Gridley, 1998). However, in *B4galt1* null embryos ( $n=4$ ), *Uncx4.1* was expressed in a clear and regular pattern in the posterior half of formed somites (Fig. 4E). *Myogenin*, a bHLH

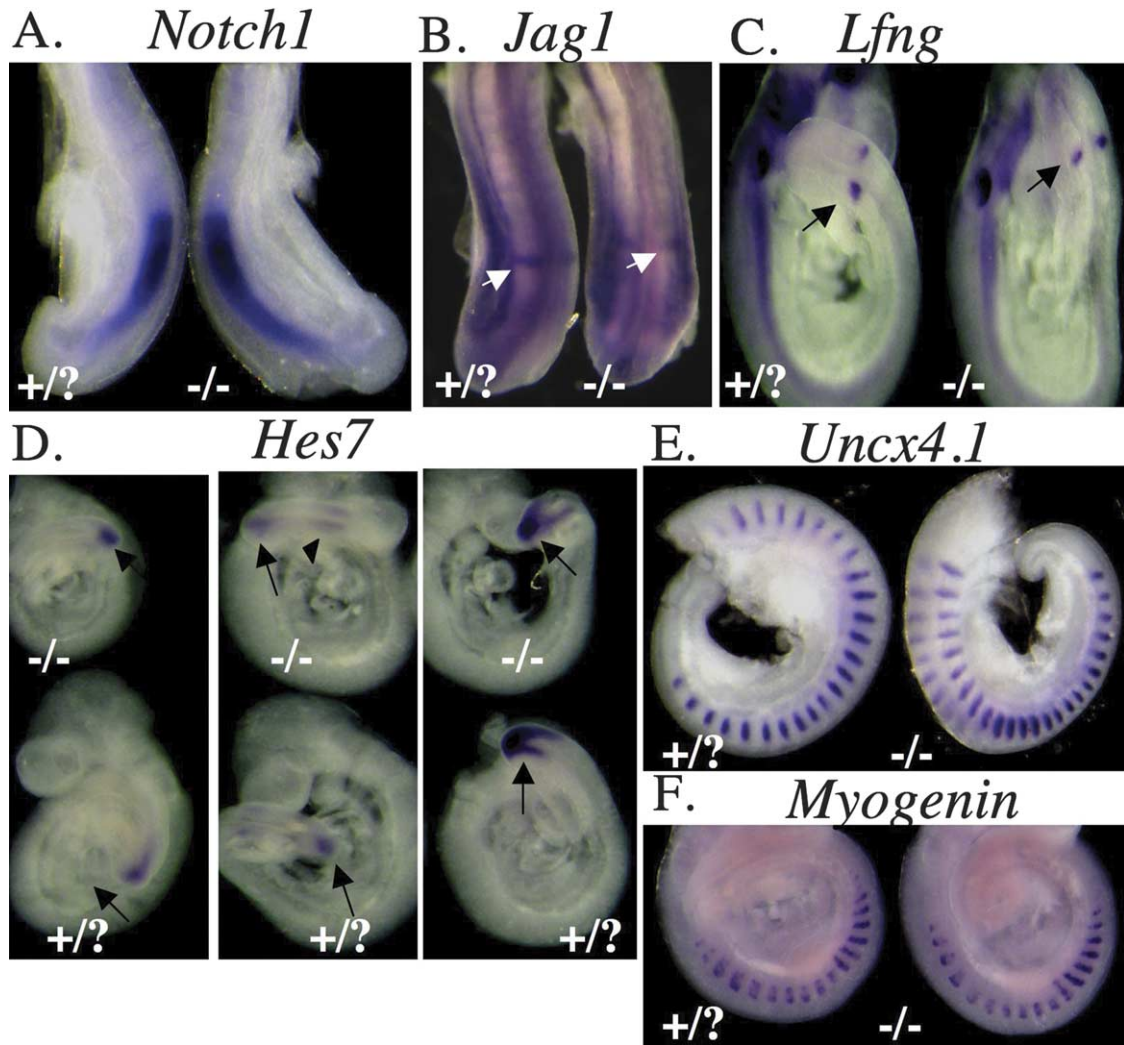


Fig. 4. Expression of Notch pathway and somitogenic genes in *B4galt1* mutant embryos. Whole-mount in situ hybridization of control and *B4galt1* mutant embryos isolated at E9.5 with the following probes. (A) *Notch1*; (B) *Jag1* is expressed at low levels in the posterior half of the prospective somite in both control and *B4galt1* mutant embryos (arrow); (C) *Lfng* was dynamically expressed in the posterior half of the prospective somite (arrow) or in the PSM (not shown) in both wild type and mutant embryos; (D) dynamic expression pattern of *Hes7* in both control and mutant embryos. *Hes7* was expressed either in the tail bud (left panel, arrow), in both the prospective somite (middle panel, arrow head) and the tail bud (middle panel, arrow) or in PSM (right panel, arrow). (E) *Uncx4.1*; (F) *Myogenin*.

transcription factor specific to the myogenic lineage, is expressed in the myotomes in a repeating pattern along the craniocaudal axis (Montarras et al., 1991). In *Lfng* mutant embryos, the *Myogenin* gene is expressed in a metameric pattern, but the stripes of expression are often fused and not evenly spaced, especially in caudal somites (Evrard et al., 1998; Zhang and Gridley, 1998). *B4galt1* null embryos ( $n=4$ ) had a normal pattern of *Myogenin* expression with evenly spaced stripes that were not diffuse (Fig. 4F). These results indicate that there are no apparent defects of anterior–posterior somite patterning in *B4galt1* null embryos at stage E9.5. Consistent with this, there were no significant differences in *B4galt1* mutant embryos ( $n=8$ ) in the expression of the *Tbx18* gene that is required for the maintenance of anterior–posterior somite polarity (Bussen et al., 2004) compared to expression in controls ( $n=25$ ; data not shown).

In summary, the expression of the Notch target genes *Hes5* and *Mesp2* and the Notch ligand genes *Dll1* and *Dll3* were

altered in a significant proportion of E9.5 *B4galt1* mutant embryos. The expression of each of these genes is changed in a similar manner in *Lfng* null embryos (Evrard et al., 1998; Zhang and Gridley, 1998; Zhang et al., 2002). However, *Lfng* mutant embryos and mutants in other Notch pathway genes also show altered expression of the *Notch1*, *Myogenin*, *Uncx4.1* and *Tbx18* genes (Barrantes et al., 1999; Bussen et al., 2004; Weinmaster and Kintner, 2003) that were not apparently changed in *B4galt1* mutant embryos. It appears that the defects in gene expression observed in *B4galt1* mutants at E9.5 were transient as they did not lead to changes in the number, polarity or segmentation of somites. However, there was a significant difference in the number of lumbar vertebrae in older embryos (Fig. 5). When skeletons were compared at E18.5 or older, *B4galt1* null embryos were most likely to have 6 lumbar vertebrae (Table 1). By contrast control embryos were more likely to have 5 lumbar vertebrae. Three heterozygote embryos had an additional A1 vertebra that was

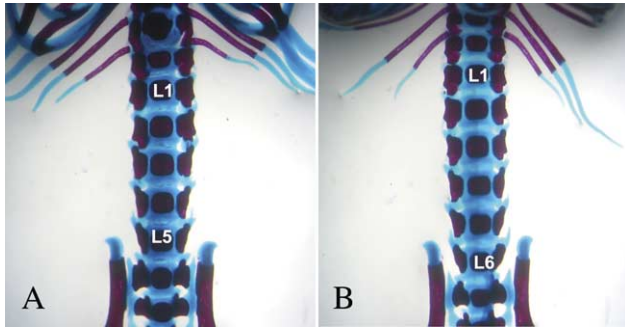


Fig. 5. *B4galt1* mutant embryos have an extra lumbar vertebra. Skeletons were prepared from embryos at E18.5 and stained for bone and cartilage as described in methods. L marks the lumbar vertebrae. (A) Control embryo with five lumbar vertebrae. (B) *B4galt1* null mutant with 6 lumbar vertebrae.

of mixed lumbar/sacral symmetry. This difference between mutant and control *B4galt1* embryos is significant ( $P \leq 0.01$  based on the Chi squared test). The number of lumbar vertebrae is regulated by Notch signaling via an influence on *Hox* gene expression. Thus, *Rbp-Jk* null embryos with globally defective Notch signaling exhibit reduced *Hoxd-1* and *Hoxd-3* gene expression in the PSM (Zakany et al., 2001). In *Dll1* and *Lfng* heterozygotes, or transgenic mice expressing a dominant negative *Dll1* or a constitutive *Lfng* transgene in the PSM, structural transformations in cervical, thoracic and lumbar vertebrae are observed (Cordes et al., 2004). An increase from 6 to 7 lumbar vertebrae occurs with variable penetrance in *Hoxd-11* mouse mutants (Davis and Capecchi, 1994). Mice doubly mutant for *Hoxa-11* and *Hoxd-11* have as many as 8 lumbar vertebrae (Zakany et al., 1996) whereas overexpression of *Hoxd-11* causes a reduction to as few as 4 lumbar vertebrae (Boulet and Capecchi, 2002; Zakany et al., 1996). Deletion of whole clusters of *Hox* genes has shown that the clusters of *Hox10* and *Hox11* genes are necessary for the development of a normal skeleton and that many of these *Hox* genes function redundantly (Boulet and Capecchi, 2004; Wellik and Capecchi, 2003).

The comparatively mild differences between *B4galt1* mutant and control embryos may be due to compensation by several factors. Thus, fibroblast growth factor pathways (Dubrulle et al., 2001; Sawada et al., 2001; Yamaguchi et al., 1994) and Wnt pathways (Aulehla et al., 2003; Galceran et al.,

2004; Takada et al., 1994; Yoshikawa et al., 1997) function in somitogenesis and may partially rescue *B4galt1* mutant embryos. It is also known that modifier genes exist because, depending on genetic background, *B4galt1* null mice may survive to adulthood (Asano et al., 2003; Mori et al., 2004; Nishie et al., 2004). However, perhaps the most likely compensation is from the expression of other *B4galt* genes (Almeida et al., 1997; Lo et al., 1998). In situ hybridization analyses showed that the *B4galt1* and *B4galt2* genes are ubiquitously expressed in wild type E9.5 embryos (J. Chen and P. Stanley, unpublished observations). Microarray analyses reveal expression of 5 genes related to *B4galt1* during embryogenesis and increased expression of *B4galt1* and *B4galt3* genes at E6.5 and E7.5 (Su et al., 2004). The  $\beta 4\text{GalT-1}$ ,  $\beta 4\text{GalT-2}$  and  $\beta 4\text{GalT-3}$  galactosyltransferases have a similar substrate specificity (Almeida et al., 1997). Thus, more than one of the *B4galt* genes may need to be inactivated to observe a strong phenotype during somitogenesis and skeleton formation.

## 2. Experimental procedures

### 2.1. Mice

Mice heterozygous for a targeted mutation in the *B4galt1* gene encoding the  $\beta 4\text{GalT-1}$  glycosyltransferase (Lu et al., 1997) were obtained from Barry Shur, Emory University School of Medicine, Atlanta, Georgia. The *B4galt1* mutant strain was maintained as heterozygotes by cousin matings because homozygous *B4galt1* mutants generally died at birth. The genetic background of the mice was mixed 129<sup>Sv</sup> and C57Bl/6 (Lu et al., 1997). Timed matings were performed to obtain embryos at different stages of development with day 0.5 being the morning after females were found to be plugged. All animal experiments were approved by the Animal Institute Committee of the Albert Einstein College of Medicine.

### 2.2. Whole-mount embryo in situ hybridization

All RNA probes were from mouse gene coding sequences: Notch1 extracellular fragment, (4.7 kb) from Jeffrey Nye, Northwestern University, Chicago, Illinois; Jag1 (1.8 kb) from Tim Mitsiadis, King's College, London, UK; *Dll1* (2.1 kb) and *Tbx18* (2.0 kb) from Achim Gossler, Institut für Molekularbiologie, Hannover, Germany; Lunatic fringe (700 bp) from Thomas Vogt, Merck Research Laboratories, West Point, PA; *Dll3* (729 bp), *Uncx4.1* (1.7 kb), *Hes5* (1.3 kb) and *Hes7* (790 bp) from Ryoichiro Kageyama, Kyoto University, Japan; Myogenin (1.5 kb) from Hanh Nguyen, Albert Einstein College of Medicine, New York; and *Mesp2* (1.1 kb) from Yumiko Saga, National Institute of Genetics, Mishima, Japan. Probes were transcribed and labeled with the digoxigenin RNA labeling kit (Roche) as previously described (Shi and Stanley, 2003).

### 2.3. Alcian Blue and Alizarin Red staining of bone and cartilage

Embryos were fixed in 95% ethanol overnight at room temperature and stained with 150  $\mu\text{g/ml}$  Alcian Blue in 1:4 mixture of acetic acid and 95% ethanol at RT for 24–48 h. After washing with 95% ethanol for 1 h, the embryos were treated with 2% KOH for 6–24 h. Embryos were subsequently stained with 75  $\mu\text{g/ml}$  Alizarin Red S in 1% KOH solution for 12–24 h and cleared in a solution of 20% glycerol and 1% KOH for a week with daily changes. Samples were transferred to 50% glycerol, 50% ethanol for photography and storage.

Table 1  
Skeletal vertebrae of *B4galt1* and control embryos

Embryos <sup>a</sup>	Cervical	Thoracic	Lumbar <sup>b</sup>	
			5	6
Control (n=30)	7	13	17	13 <sup>c</sup>
<i>B4galt1</i> <sup>-/-</sup> (n=13)	7	13	1	12

<sup>a</sup> Embryos were at E18.5, E19.5 or postnatal P1. Control embryos were *B4galt1*<sup>+/-</sup> or *B4galt1*<sup>+/+</sup>.

<sup>b</sup> Difference between control and *B4galt1*<sup>-/-</sup> is  $P \leq 0.01$  based on the exact Chi square test.

<sup>c</sup> Three heterozygotes had five lumbar and one asymmetric lumbar/sacral A1 vertebra.

## Acknowledgements

We are most grateful to Barry Shur for *B4galt1* mice and to all those who supplied plasmids for the generation of in situ probes (see Methods). We also thank Achim Gossler, Thomas Vogt, Robert Haltiwanger and Ken Irvine for helpful comments on the manuscript. This work was supported by NIH CA 95022 to PS and by the core facilities and biostatistics unit of the Albert Einstein Cancer Center grant PO1 13330.

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