Tissue-specific requirements for the proprotein convertase Furin/SPC1 during embryonic turning and heart looping

Daniel B. Constam and Elizabeth J. Robertson*

Harvard University, Department of Molecular and Cellular Biology, 16 Divinity Ave, Cambridge, MA 02138, USA

*Author for correspondence (e-mail: ejrobert@fas.harvard.edu)

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SUMMARY

Furin, the mammalian prototype of a family of serine proteases, is required for ventral closure and axial rotation, and formation of the yolk sac vasculature. Here we show additionally that left-sided expression of pitx2 and lefty-2 are also perturbed in Furin-deficient embryos. These tissue abnormalities are preceded by a marked delay in the expansion of the definitive endoderm during gastrulation. Using a chimera approach, we show that Furin activity is required in epiblast derivatives, including the primitive heart, gut and extraembryonic mesoderm, whereas it is nonessential in the visceral endoderm. Thus, chimeric embryos, derived by injecting wild-type embryonic stem (ES) cells into fur−/− blastocysts, develop normally until at least 9.5 d.p.c. In contrast, Furin-deficient chimeras developing in the context of wild-type visceral endoderm fail to undergo ventral closure, axial rotation and yolk sac vascularization. Fur−/− cells are recruited into all tissues examined, including the yolk sac vasculature and the midgut, even though these structures fail to form in fur mutants. The presence of wild-type cells in the gut strikingly correlates with the ability of chimeric embryos to undergo turning. Overall, we conclude that Furin activity is essential in both extraembryonic and precardiac mesoderm, and in definitive endoderm derivatives.

Key words: Convertase, Proprotein processing, Axial rotation, Ventral morphogenesis, Endoderm, TGFβ

INTRODUCTION

Mammalian embryos set aside specialized cells early in development to form the extraembryonic tissues essential for maternal-fetal interactions. Following implantation, polar trophoderm cells invading the uterus form the chorion and placenta, whereas primitive endoderm cells delaminating from the inner cell mass give rise to the parietal and visceral endoderm lineages. The embryo proper initially forms as a cup-shaped single layer of primitive ectoderm, the epiblast, which is subsequently converted into the three definitive germ layers during gastrulation. Thus, the process of gastrulation results in the ingress of epiblast cells through the primitive streak to form mesoderm and the definitive endoderm. Mesodermal cells emerging from the most posterior primitive streak give rise to extraembryonic structures, including the amnion and the allantois, or migrate along the visceral endoderm to line the exocoelomic cavity, eventually forming blood islands and the vasculature of the visceral yolk sac. Cells ingressing through the medial primitive streak spread laterally to form the lateral plate mesoderm. Descendants of the anteriormost streak are found in the axial mesoderm and in endodermal derivatives (Lawson et al., 1986; Tam and Beddington, 1987; Beddington, 1994; Sulik et al., 1994).

While mesodermal cell movements have been extensively studied, the migratory route of endodermal precursors remains ill-defined. Fate-mapping experiments have shown that a subset of endodermal cells emanating from the distal streak transit the notochordal plate, a derivative of the ventral layer of the node, and eventually colonize the dorsal primitive gut (Beddington, 1994). Concurrently, a sheet of definitive endoderm contiguous with the notochordal plate expands anteriorly, displacing the visceral endoderm to a more proximal position (Lawson and Pedersen, 1987; Thomas and Beddington, 1996). By the end of gastrulation, this sheet of cells is already regionalized, since individual endoderm precursors are incorporated preferentially into the foregut, midgut or hindgut, depending on their position along the antero-posterior axis (Lawson et al., 1986). However, the time when these cells become committed to distinct fates is unknown, and the molecular mechanisms regulating their morphogenetic movements are poorly understood.

The tissue movements initiated during gastrulation eventually establish the distinct left-right orientation of the developing viscera. This asymmetry first becomes visible after the formation of 8-10 somite pairs when the primitive heart tube loops to the right side, followed shortly thereafter by a rightward 180° rotation of the entire embryo along its antero-posterior axis (Kaufman, 1992). Concurrently, the lateral edges of the definitive gut endoderm and the embryonic body wall fold ventrally and fuse along the midline in a process termed
ventral closure. The coincidence of axial rotation and ventral closure suggests that these probably interdependent processes may be regulated by the same molecular signals. Interestingly, axial rotation and ventral closure are preceded by the asymmetric expression of several genes, including the TGFβ family members lefty-1, lefty-2 and nodal. Lefty-1 is exclusively transcribed on the left side of the ventral neural tube (Meno et al., 1997), whereas nodal and lefty-2 are specifically expressed in left lateral plate mesoderm (Levin et al., 1995; Collignon et al., 1996; Lowe et al., 1996; Meno et al., 1996). Mice lacking Lefty-1 exhibit bilateral expression of nodal and lefty-2, associated with thoracic left isomerism, suggesting that lefty-1-dependent repression of lefty-2 and nodal in the right lateral plate mesoderm is required for normal left-right axis formation (Meno et al., 1998). In keeping with this conclusion, ectopic expression of nodal and lefty-2 in the right lateral plate of inv/inv and iv/iv mice lacking inversin or left-right dynein, respectively, correlates with situs alterations (Collignon et al., 1996; Lowe et al., 1996). Studies in chick similarly demonstrate that ectopic expression of Nodal or Lefties in the right lateral plate randomizes the direction of heart looping (Levin et al., 1997), an effect that appears to be mediated via the activation of the bicoid-related homeobox transcription factor Pitx2 (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). Together, these observations strongly suggest that the establishment of left-right asymmetry critically depends on Nodal and Lefty activities.

We recently generated a loss-of-function mutation in the fur gene to disrupt Furin expression. Furin is the mammalian prototype of a family of serine proteases that specifically...
Furin chimera analysis

activate various prohormones and growth factors via cleavage at a cluster of basic residues (for a review, see Nakayama, 1997). Furin-deficient embryos fail to undergo axial rotation and develop severe ventral closure defects of the gut and heart, leading to cardiac insufficiency and developmental arrest by 10.5 d.p.c. (Roebroek et al., 1998). The heart primordia of fur mutants either fail to fuse at the ventral midline, or only form a short, unlooped heart tube marked by a reduction in the number of cardiomyocytes. In addition, vascular...
morphogenesis is abolished in the visceral yolk sac. Yolk sac angiogenesis, cardiac fates and axial rotation are regulated in part by TGFβ3 and related signaling molecules of the BMP subfamily, respectively, which are candidate Furin substrates. It is possible, therefore, that Furin controls the maturation of several TGFβ-related activities (Roebroek et al., 1998). In keeping with this suggestion, recent studies demonstrate that Furin cleaves TGFβ3 and related proteins, including BMP4 and Nodal precursors (Dubois et al., 1995; Cui et al., 1998; Constam and Robertson, 1999).

Here, we further characterize the complex phenotype of fur plus mutants by analyzing the expression of specific components of the left-right molecular pathway, and of specific genes marking early endodermal cell populations. In addition, we generated chimeric embryos in which extraembryonic tissues exclusively comprise wild-type or mutant ES cell derivatives, in order to determine whether the primary defect reflects the loss of Furin activity in extraembryonic tissues or within the embryo proper, or both. We found that Furin activity is essential in both the extraembryonic and cardiogenic mesoderm to promote yolk sac vascular morphogenesis and heart looping, respectively. Furthermore, we show that axial rotation depends on Furin activity in the definitive endoderm.

**MATERIALS AND METHODS**

**Mouse strains and ES cells**

Animals carrying the fur<sup>R026</sup> allele on a C57Bl/6 background were genotyped as described (Roebroek et al., 1998). The ROSA26 gene trap line Tg(Rosa26)R26.1(RSor) ubiquitously expressing lacZ (Friedrich and Soriano, 1991) was obtained from Jackson Laboratories (Bar Harbor) and maintained on a C57Bl/6J hybrid background. Heterozygous ROSA26 mice were genotyped by X-Gal staining of ear tissue. Heterozygous ROSA26 mice were genotyped by X-Gal staining of ear tissue biopsies as described (Hogan et al., 1994).

Furin-deficient ES cell lines were derived from delayed blastocysts (Robertson, 1987) obtained from heterozygous females crossed to fur<sup>+/−</sup>: ROSA26/+ males. The genotype of individual ES cell lines was determined by Southern blot analysis of genomic DNA digested with KpnI. Thus, a 580 bp KpnI fragment corresponding to nucleotides 176-756 of murine fur cDNA hybridized to a 4.8 kb wild-type and 11 kb mutant fragment (Roebroek et al., 1998). The genotype with respect to the ROSA26 locus was analyzed by X-Gal staining. R26.1 ES cells carrying the ROSA26 allele were as described (Varlet et al., 1997).

**Chimera analysis**

Chimeric embryos (wt→fur chimeras) were generated by injecting 12-14 R26.1 ES cells into blastocysts obtained from fur<sup>+/−</sup> intercrosses. Manipulated embryos were transferred into pseudopregnant (C57Bl/6xB6CBA/JFl) females (Jackson Laboratory, Bar Harbor) and recovered 7-8 days later, corresponding to 9.5 or 10.5 d.p.c. (days post coitum), respectively. The genotype of a proportion of host embryos was analyzed retrospectively by PCR analysis of DNA from visceral yolk sac endoderm isolated by pancreatin/trypsin digestion (Hogan et al., 1994). A second category of chimeras (fur→wt) was generated by injecting 12-14 cells obtained from two independent fur<sup>+/−</sup> ES cell lines into host embryos that were either wild type or heterozygous for the ROSA26 allele, respectively. Thus, mutant cells were distinguished in chimeric embryos by the presence or absence of the ROSA26 allele. The ratio of mutant and wild-type ES cell derivatives was estimated by examining serial sections of individual embryos.

**Whole-mount in situ hybridization and X-Gal staining**

LacZ expression was visualized by X-Gal whole-mount staining overnight as described (Hogan et al., 1994). After post-fixation in 4% paraformaldehyde and dehydration in ethanol and xylenes, embryos were embedded and sectioned at 8 μm.

Whole-mount mRNA in situ hybridization using digoxigenin-labelled antisense RNA probes specific for mouse cerberus-like (Thomas et al., 1997), α-fetoprotein (Waldrip et al., 1998) and pitx2 (Ryan et al., 1998) was as described (Wilkinson and Nieto, 1993). A PsI-XhoI fragment corresponding to nucleotides 986-1387 of the Lefty-1 cDNA, and a BglII-EcoRI 0.5 kb cDNA fragment comprising the 3′ untranslated region of Lefty-2 (kind gifts of H. Hamada) were used as templates to synthesize antisense probes specific for lefty-1 or lefty-2 mRNA, respectively. Genotypes of 8-8.5 d.p.c. embryos collected from fur<sup>+/−</sup> intercrosses were determined by PCR using DNA extracted from yolk sac tissue. At earlier stages, homozygous fur mutants were recognized based on the frequency of abnormal expression patterns for cer-1 and α-fetoprotein mRNAs in 25% of the embryos (n=6/25 and 7/27, respectively).

**RESULTS**

**Ectopic expression of left-right asymmetry markers**

In the node and lateral plate mesoderm, fur transcripts colocalize with several BMPs, and with Nodal and Lefty-2, respectively. In tissue culture cells transfected with a Furin expression vector, the proteolytic processing of these proteins is significantly enhanced (Constam and Robertson, 1999; D. B. C and E. J. R., unpublished). Thus, impaired signaling by these ligands may contribute to the turning and heart looping defects of fur mutants (Roebroek et al., 1998). Since ectopic expression of Nodal or Lefty in the right lateral plate of chick embryos induces the bicoid-related homeobox transcription factor Pitx2, Pitx2 is thought to act downstream in the left-right signaling pathway (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). It was therefore of interest to test whether the loss of Furin activity interferes with the induction of pitx2 expression in the left lateral plate. Unexpectedly, we found that pitx2 mRNA levels were generally increased in fur mutants (n=6) as compared to stage-matched control embryos (n=10; Fig. 1A,B). Although expression of a nodal lacZ reporter allele is unperturbed in Furin-deficient embryos (Roebroek et al., 1998), pitx2 is occasionally expressed bilaterally (n=2/6; Fig. 1B), as is lefty-2 (n=1/5; Fig. 1D). We conclude that Furin-dependent signals normally inhibit pitx2 and lefty-2 on the right.

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**Table 1. wt→fur chimeras generated by injecting Rosa26 ES cells into blastocysts obtained from fur<sup>+/−</sup> intercrosses**

<table>
<thead>
<tr>
<th>Transfer number</th>
<th>n</th>
<th>Recovered</th>
<th>Mutant phenotype</th>
<th>Partial rescue&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Leftward turning</th>
<th>Normal</th>
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<td>1</td>
<td>30</td>
<td>21</td>
<td>2</td>
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<td>2</td>
<td>5</td>
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<td>2</td>
<td></td>
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<td>8</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td></td>
<td>3</td>
<td></td>
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<td>153</td>
<td>8</td>
<td>10</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>83</td>
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</tr>
</tbody>
</table>

<sup>*</sup>i.e. embryo turned, but heart tube failed to loop.

n, number of blastocysts.
Endoderm patterning defects in fur mutants

Fur mutants show a marked delay in hindgut formation and a failure of the midgut to undergo ventral closure (Roebroek et al., 1998). Consistent with this, fur mRNA is abundantly expressed in the anterior and posterior intestinal portals where the lateral edges of the endodermal cell layer fuse along the ventral midline. In addition, fur transcripts are detected in the ventral layer of the node, suggesting that Furin activity at an earlier stage may regulate the formation or function of definitive endoderm. To examine this possibility, we assessed the expression of cer-l, the homologue of the Xenopus gene cerberus, as a marker specific for early endodermal cells. Gain-of-function experiments in frog embryos demonstrate that Cerberus can inhibit endoderm formation, possibly by antagonizing Nodal and/or BMP signals (Piccolo et al., 1999). As shown in Fig. 2A, a contiguous sheet of epiblast-derived definitive endoderm expressing cer-l mRNA normally displaces the visceral endoderm to a more proximal position by 7.5 d.p.c. In contrast, stage-matched fur mutants (n=6) contain two distinct populations of cer-l-positive endodermal cells (Fig. 2B). The more anterior patch of cells (red circle) likely corresponds to anterior visceral endoderm, or possibly the most anterior extension of the axial mesendoderm. Due to the absence of specific markers, we cannot distinguish these distinct cell lineages at present. However, α-fetoprotein mRNA, a marker of lateral and posterior visceral endoderm (Dziadek and Adamson, 1978), is expressed in a complementary pattern (n=7; Fig. 2C,D), as expected if the expansion of definitive endoderm is delayed in fur mutants. Thus, Furin may regulate the formation or survival of endodermal cell subpopulations.

Furin activity in the visceral endoderm is not essential for cardiac and vascular morphogenesis

From gastrulation onwards, fur mRNA is expressed in the visceral endoderm and extraembryonic mesoderm, as well as in cardiogenic mesoderm, the allantois, the notochordal plate and prospective gut endoderm (Roebroek et al., 1998). To examine the function of Furin in the visceral endoderm and in epiblast derivatives, we took advantage of the inability of ES cells to colonize the extraembryonic endoderm and ectoderm cell lineages in injection chimeras (Beddington and Robertson, 1989). If Furin is essential in the visceral endoderm, wild-type ES cells should be unable to rescue the defects of fur+/− host embryos. To test this possibility, we injected wild-type lacZ-marked R26.1 donor cells (Varlet et al., 1997) into blastocysts from fur+/− intercrosses. Regardless of the host genotype, the resulting wt→fur chimeras developed normally if they contained more than approximately 80% wild-type cells (Fig. 3A; Table 1). Thus, Furin activity is not required in visceral endoderm for ventral closure, axial rotation or heart looping morphogenesis. Furthermore, the yolk sac vasculature developed normally in these chimeras (Fig. 3B), indicating that the vascular defects of fur mutants cannot be attributed to the absence of Furin in the visceral endoderm. In contrast, mutant embryos containing less than a 30% wild-type contribution were not rescued (Fig. 3C; Table 1). Their yolk sacs appeared ruffled and, like fur−/− embryos, lacked large blood vessels (Fig. 3D). In addition, we recovered several partially rescued embryos (n=10; Table 1) that had turned, although their heart tubes were abnormally short and incompletely looped. Interestingly, these rudimentary hearts contained a low percentage (i.e. <50%) of wild-type ES cell descendants (Fig. 3E), as to be expected if cardiac morphogenesis critically depends on Furin expression in the heart tube or in cardiogenic precursor cells.

Wild-type visceral endoderm fails to rescue fur mutants

To test whether Furin expression in extraembryonic tissues is sufficient to promote normal development, we also generated reciprocal chimeras (referred to as fur→wt chimeras). Fur−/− ES cells were derived from intercrosses of fur+/− animals carrying the ubiquitous ROSA26 lacZ reporter allele (Fig. 4A). When injected into wild-type blastocysts, fur−/− ES cells gave rise to a proportion of embryos that phenocopied the ventral closure, turning and yolk sac defects of fur mutants (n=9; Fig. 4B,C), demonstrating that Furin activity in the visceral endoderm is not sufficient to promote normal development. Identical results were obtained by injecting two independent mutant ES cell lines marked by the presence or absence of lacZ expression into wild-type or ROSA26 blastocysts, respectively (Table 2). However, this severe phenotype is observed only in rare chimeras that are entirely colonized by mutant cells (>95%). In contrast, fur→wt chimeras containing as little as 5-10% residual wild-type cells frequently turn or form a looping heart tube (n=18; Fig. 4D), or both (n=87; Table 2), although other features including pericardial swelling, disorganization of the allantois, failure of umbilical vein formation and poor yolk sac vascularization are reminiscent of fur mutants (Figs

### Table 2. fur→wt chimeras generated by injecting mutant ES cells into wild-type (wt) or ROSA26 (R26) host blastocysts

<table>
<thead>
<tr>
<th>ES cell line (fur−/−)</th>
<th>Host</th>
<th>n</th>
<th>Recovered</th>
<th>Mutant phenotype</th>
<th>Partially mutant phenotype*</th>
<th>Leftward turning</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF11‡</td>
<td>wt</td>
<td>101</td>
<td>70</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>62</td>
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<tr>
<td>DF9</td>
<td>R26</td>
<td>54</td>
<td>43</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
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<td>113</td>
<td>9</td>
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<td></td>
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<td>8</td>
<td>16</td>
<td>2</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

* i.e. unturned, or failure of heart looping morphogenesis.
‡DF11, but not DF9, cells carry the ubiquitous lacZ reporter allele ROSA26.
Overall, these chimera experiments demonstrate that Furin activity in the visceral endoderm is neither required nor sufficient for normal development to occur. However, the proportion of wild-type cells necessary for normal ventral closure and turning was significantly higher (up to 80%) in wt→fur chimeras than in fur→wt chimeras (20-30%), pointing to a supportive role for Furin expression in the visceral endoderm or other extraembryonic tissues. In addition, we cannot rule out the possibility that Furin activity in extraembryonic tissues may also influence the direction of embryonic turning since it was reversed in several wt→fur chimeras (n=7; Table 1).

In both classes of chimeras, fur mutant cells were recruited into all tissues examined, including the endocardial and myocardial layer of the primitive heart, somites, mesodermal and endodermal derivatives of the axial mesoderm, the lateral plate mesoderm, the endothelia of the umbilical veins and the yolk sac vasculature (Fig. 5). Indeed, fur→wt chimeras composed of up to 80% mutant cells were remarkably normal (Figs 5A,F, 6C), showing only a minor delay during yolk sac vascularization (Fig. 5D-G), and in some instances, a poorly organized allantois (Fig. 5F). Hence, it seems most likely that the critical Furin substrates are secreted and act at a distance on neighbouring cells. In the developing heart, where Furin has been implicated to control the number of cardiomyocytes (Roebroek et al., 1998), its ability to act non-cell autonomously potentially also explains why looping morphogenesis in fur→wt chimeras requires fewer wild-type cells (10-20%, Fig. 5A), compared to wt→fur chimeras (>50%, Fig. 3E), since wild-type visceral endoderm abuts the cardiogenic field only in fur→wt chimeras.

**Correlation between axial rotation and wild-type contributions to the definitive endoderm**

In both classes of chimeras, the overall ratio of wild-type to...
mutant cells had surprisingly little impact on whether or not the embryos underwent axial rotation. Thus, we recovered wt→fur chimeras containing more than 50% wild-type cells that failed to turn \((n=3; \text{Fig. 6A})\), as well as fur→wt chimeras almost entirely composed of mutant cells that turn normally \((n=4; \text{Fig. 6C,D})\). Interestingly, serial sections of these embryos revealed a remarkable correlation between the ability to turn and the presence of wild-type cells in the primitive gut. Thus, wt→fur chimeras that failed to turn lacked wild-type cells in the midgut region \((\text{Fig. 6A1,2, black arrowhead})\). In contrast, if significant proportions of the mid- and hindgut region consisted of wild-type cells, chimeras underwent turning \((\text{Fig. 6B1,2, red arrowheads})\). Similarly, fur→wt chimeras able to turn consistently contained a residual population of wild-type cells in the mid- and hindgut regions \((n=4; \text{Fig. 6C,D})\), whereas all fur→wt chimeras lacking wild-type cells in the midgut failed to turn \((n=3; \text{Fig. 6E})\). These data strongly argue that Furin-dependent signals are required in the prospective gut endoderm to promote embryonic turning.

**DISCUSSION**

Ventral closure is initiated shortly after gastrulation by the
coordinated expansion of mesodermal and endodermal cell populations forming the heart, lateral plate and the primitive gut tube. In Furin-deficient embryos, severe perturbations of these morphogenetic movements abolish axial rotation and fusion of the heart primordia at the ventral midline. Moreover, expression levels of the homeobox transcription factor Nkx2.5, an early cardiac marker, are significantly reduced, suggesting that failure of heart looping reflects an impairment in the specification, and perhaps migration of cardiomyocytes (Roebroek et al., 1998). The present chimera experiments demonstrate that Furin is required in epiblast derivatives. In both wt→fur and fur→wt chimeras, we find that the primitive heart tube is abnormally short, and hence poorly looped if entirely comprising mutant cells. In contrast, hearts containing a significant proportion of wild-type cells are remarkably normal, as to be expected if mutant cardiac progenitor populations are rescued by neighbouring wild-type cells. Thus, in precardiac mesoderm Furin appears to act non-cell autonomously, possibly by activating BMP signals that specify cardiomyocytes (Schultheiss et al., 1997). Likewise, a wild-type contribution to the hearts of fur→wt chimeras is sufficient to prevent ballooning of the pericardium. These observations uncover a requirement for Furin-dependent signals in mesodermal cell lineages which probably accounts for the heart defects observed in fur mutants.

Previous studies have shown that specification of cardiomyocytes in part depends on signals provided by endodermal lineages. For example in Xenopus, extirpation of embryonic endoderm populations adversely affects cardiogenesis (Nascone and Mercola, 1995). Tissue recombination experiments show that endoderm explants induce cardiac fates in paraxial chick mesoderm, strongly arguing that endodermal lineages promote heart morphogenesis (Schultheiss et al., 1995, and references therein). In addition, studies of the fork head family transcription factor HNF3β suggest that endodermal lineages are also critical for orchestrating ventral morphogenesis. Thus, tetraploid aggregation chimeras lacking HNF3β activity in the definitive endoderm fail to undergo heart looping and axial rotation, presumably because the definitive endoderm fails to expand and displace the visceral endoderm (Dufort et al., 1998). Extensive cell death in the definitive endoderm may also account for the severe ventral closure and turning defects observed in embryos lacking Hrs, an endosomal FYVE finger protein involved in vesicular trafficking (Komada and Soriano, 1999). Severe ventral closure defects and cardiobifida are also observed in mouse embryos lacking GATA4 (Kuo et al., 1997; Molkentin et al., 1997), a zinc finger transcription factor expressed in early precardiac splanchnic mesoderm, the adjacent visceral endoderm and definitive endoderm (Arcetti et al., 1993; Heikinheimo et al., 1994). GATA4 regulates cardiac gene expression (Grepin et al., 1994; Molkentin et al., 1994; Jiang and Evans, 1996), and potentially plays an important role in specifying cardiac fates (Grepin et al., 1995). However, GATA4-deficient embryos develop normally in the context of wild-type visceral endoderm, demonstrating that while GATA4 is non-essential in cardiac lineages, it is required in the visceral endoderm (Narita et al., 1997).

Here we demonstrate that unlike GATA4, Furin activity is neither required nor sufficient in the visceral endoderm. Thus, Furin-deficient blastocysts injected with wild-type ES cells give rise to chimeric embryos that turn and form a looping heart, provided they contain a majority of wild-type cells. Conversely, reciprocal fur→wt chimeras derived from fur ES cells phenocopy the heart and turning defects of fur mutants and fail to be rescued by wild-type visceral endoderm. Nevertheless, Furin-dependent signals derived from wild-type visceral endoderm apparently support heart morphogenesis since in fur→wt chimeras, the proportion of wild-type cells required within the developing heart is significantly lower (20-30%) compared to wt→fur chimeras (50-80%). Interestingly, fur→wt chimeras entirely derived from fur ES cells also mimic the yolk sac vascularization defect documented in fur mutants (Roebroek et al., 1998), demonstrating that wild-type visceral endoderm is not sufficient to rescue the formation of large blood vessels. By contrast, mesodermal wild-type cells promote the incorporation of mutant cells into vitelline vessels. Thus, endothelial precursors that form normally in the absence of Furin (Roebroek et al., 1998) appear to be induced by Furin-dependent signals to undergo terminal differentiation. Alternatively, Furin activity may be required to promote the differentiation of other mesodermal derivatives that are critical for angiogenesis. For example, Furin may be responsible for activating the ligand(s) of the type III TGFβ receptor endoglin, which is required for smooth muscle cell differentiation (Li et al., 1999).

Interestingly, the presence of wild-type cells within the definitive gut strongly correlates with the ability of both fur→wt and wt→fur chimeras to turn and undergo gut closure, suggesting that these processes depend on Furin activity in endodermal progenitor populations or their derivatives. How does Furin act in the definitive endoderm to promote turning? One possibility is that Furin functions relatively late during closure of the gut tube, for example by regulating cell proliferation or migration in the anterior and/or posterior intestinal portals. Consistent with this, the intestinal portals abundantly express fur mRNA, and the formation of the hindgut diverticulum is significantly delayed in fur mutants (Roebroek et al., 1998). Hence, the direction of axial rotation may be determined by lateral asymmetries in cell proliferation rates, combined with the physical constraints imposed on the embryo by the surrounding extra-embryonic layers of the amnion and visceral yolk sac. Indeed, proliferation indices along the left splanchnopleure and somatopleure, i.e. the mesodermal tissues located at the lateral edges of the embryo abutting the visceral yolk sac, are higher than those on the right side (Miller and Runner, 1978; Miller and White, 1998), being regulated perhaps by signals derived from adjacent definitive endoderm. Alternatively, Furin-dependent signals acting at an earlier stage may control the formation or migration of nascent definitive endoderm, possibly by regulating activation of Nodal and/or other TGFβ-related signal(s) in the node and midline. Indeed, endoderm formation is inhibited in Xenopus by overexpression of the Nodal antagonist Cerberus (Piccolo et al., 1999), or a dominant negative form of the Nodal-related Xnr-2 (Osada and Wright, 1999). Likewise, fish embryos doubly homozygous for loss-of-function mutations in cyclops and sqt, two nodal-related genes, fail to form endoderm (Feldman et al., 1998). Interestingly, the proximal expansion of the definitive endoderm, marked by the expression of cer-l, and the concomitant displacement of visceral endoderm, are severely perturbed in fur mutants, suggesting a delay in the
formation or migration of the definitive endoderm. Alternatively, as in hrs mutants (Komada and Soriano, 1999), the survival of endodermal subpopulations may be impaired. Moreover, we found that fur−/− ES cells inefficiently colonize endodermal derivatives, as expected if Furin activity is required at early stages during the formation of the definitive endoderm. Furin-dependent signals thus seem to play an important role in coordinating the movements of endodermal cell populations, which probably generate the mechanical force driving axial rotation.

Furin may be one of several convertases contributing to the activation of partially overlapping TGFβ-related activities, including Nodal and Lefty proteins (Roebroek et al., 1998; Constam and Robertson, 1999). Nodal expression is unperturbed in fur mutants, as expected if Furin regulates the maturation of Nodal protein (Roebroek et al., 1998). However, pitx2 expression, a likely target of Nodal signaling (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998), is not downregulated in Furin-deficient embryos. Instead, we unexpectedly found that both pitx2 and lefty-2 are expressed bilaterally in a proportion of fur mutants. These results parallel the recent finding that pitx2, but not nodal, is bilaterally expressed in a proportion of embryos transheterozygous for a hypomorphic and a null allele of fgf8 (Meyers and Martin, 1999). Similarly lefty-2, but not nodal, is bilaterally expressed in tetraploid HNF3β aggregation chimeras (Dufort et al., 1998). Given that HNF3β is known to

Fig. 7. Model for the regulation of asymmetric pitx2 and lefty-2 expression by Furin-dependent activities. A schematized ventral view of a gastrulation stage mouse embryo (anterior is up, right is to the left) indicates the left-sided induction of pitx2 and lefty-2 expression (red arrows), which is thought to be mediated by asymmetrically distributed, node-derived signals (Nonaka et al., 1998; Pagan-Westphal and Tabin, 1998). In this model, induction is normally repressed (broken arrow in A–C) by Furin-dependent asymmetric signals (purple) derived from (A) the node (n) or definitive endoderm (e), (B) visceral endoderm (ys) and/or (C) lateral plate mesoderm (m). (A–C) The loss of Furin activity alleviates the right-sided repression of pitx2 and/or lefty-2 (full arrow) by decreasing these inhibitory signals below the necessary threshold concentration.

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