Ventral closure, headfold fusion and definitive endoderm migration defects in mouse embryos lacking the fibronectin leucine-rich transmembrane protein FLRT3

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A B S T R A C T
The three fibronectin leucine-rich-repeat transmembrane (FLRT) proteins contain 10 leucine-rich repeats (LRR), a type III fibronectin (FN) domain, followed by the transmembrane region, and a short cytoplasmic tail. XFLRT3, a Nodal/TGFβ target, regulates cell adhesion and modulates FGF signalling during Xenopus gastrulation. The present study describes the onset and pattern of FLRT3 expression in the early mouse embryo. FLRT3 expression is activated in the anterior visceral endoderm (AVE), and during gastrulation appears in anterior streak derivatives namely the node, notochord and the emerging definitive endoderm. To explore FLRT function we generated a null allele via gene targeting. Early Nodal activities required for anterior–posterior (A–P) patterning, primitive streak formation and left–right (L–R) axis determination were unperturbed. However, FLRT3 mutant embryos display defects in headfold fusion, definitive endoderm migration and a failure of the lateral edges of the ventral body wall to fuse, leading to cardiac bifida. Surprisingly, the mutation has no effect on FGF signalling. Collectively these experiments demonstrate that FLRT3 plays a key role in controlling cell adhesion and tissue morphogenesis in the developing mouse embryo.

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I N T R O D U C T I O N

Several well known families of transmembrane glycoproteins including the integrins and cadherins control dynamic cell adhesion events and activate downstream signalling pathways at diverse tissue sites in the developing vertebrate embryo. In the early post-implantation mouse embryo, the process of gastrulation converts the epiblast into the three primary germ layers. Efficient downregulation of the epithelial cell adhesion molecule E-cadherin that is robustly expressed throughout the epiblast is a prerequisite for the delamination of nascent mesoderm on the posterior side of the embryo (Ciruna and Rossant, 2001). The mesoderm constitutes a loosely adherent cell population programmed to execute a complex set of migratory behaviours. By contrast the definitive endoderm forms a tightly adherent sheet of cells. The cell surface molecules responsible for coordinating regulation of proliferation and migration in the definitive endodermal cell lineage remain ill defined.

The fibronectin leucine-rich-repeat transmembrane protein FLRT3 was originally identified in a screen for extracellular matrix proteins expressed in human skeletal muscle (Lacy et al., 1999). Three closely related FLRT1–3 family members expressed in humans, rodents, chick and frog all share conserved structural features, namely 10 leucine rich repeats (LRR) flanked by cysteine rich regions, a type III fibronectin (FN) domain followed by the transmembrane segment, and a short intracellular tail. The LRR module forms a characteristic “horseshoe” shaped structure with specific ligand binding capabilities that mediate a wide range of recognition processes by numerous diverse proteins (reviewed in Bell et al., 2003; Kobe and Kajava, 2001). Besides the FLRTs, several families of surface molecules containing LRR modules are expressed in the developing mouse embryo (Haines et al., 2005, 2006; Haines and Rigby, 2007). Interestingly the three NLRR family members contain 12 LRRs, a type III FN domain and an Ig domain, and as for FLRT1–3, are encoded by a single exon (Haines et al., 2005). Consistent with the idea that these surface molecules mediate cell recognition and signalling at diverse tissue sites, highly dynamic and developmentally regulated patterns of expression have been described (Haines et al., 2005, 2006; Haines and Rigby, 2007; Robinson et al., 2004). However, the functional roles played by these conserved families of LRR transmembrane proteins in the early mouse embryo have yet to be elucidated.

Considerable evidence suggests that FLRT3 modulates FGF signalling and guides cell movements in Xenopus embryos during gastrulation (Bottcher et al., 2004). XFLRT3 plays a key role in cell de-adhesion during gastrulation via interactions with its cytoplasmic partner the small GTPase Rnd1 (Ogata et al., 2007). XFLRT3 and Rnd1 co-expression in the involuting cells of the marginal zone promote localized downregulation of the cell surface adhesion molecule C-cadherin causing...
cells to internalize and migrate along the inner surface of the blastocoe1 cavity. Interestingly, both FLRT3 and Rnd1 were identified in a Xenopus microarray screen as downstream targets of TGFβ/Nodal signalling (Ogata et al., 2007). In the early mouse embryo Nodal signals are essential for establishment of the A–P and L–R body axes, mesodermal patterning and definitive endoderm specification (Brennan et al., 2001; Collignon et al., 1996; Dunn et al., 2004; Vincent et al., 2003). An interesting possibility is that FLRT3, as a conserved Nodal target, might also play a key role in mouse development.

The present report describes the onset of FLRT3 expression in the early mouse embryo. FLRT3 expression is initially restricted to the extra-embryonic ectoderm and the AVE, and during gastrulation appears in derivatives of the anterior primitive streak (APS), namely the node, the notochord, and nascent definitive endoderm. To disrupt FLRT3 function we generated a null allele via gene targeting. Homozygous mutant embryos gastrulate normally but mostly die at around E10.5. Loss of FLRT3 causes defects in ventral closure, headfold fusion and definitive endoderm migration. Surprisingly FGF signalling is unaffected in FLRT3 mutant embryos and inhibition of FGF signalling has no effect on FLRT3 expression. Collectively these experiments demonstrate FLRT3 requirements at three distinct tissue sites in the developing mouse embryo and strongly suggest that FLRT3-mediated cell interactions are independent of FGF signalling.

Materials and methods

Generation of targeted ES cell clones and mutant mice

A 10.7 kb BglII–ClaI genomic fragment containing exons 2 and 3 of the FLRT3 locus was isolated from a 129BAC (bMQ35b07; Sanger, Cambridge) and subcloned into pBSKII (BamHI–ClaI). A 4.7 kb LacZ-neo-stop-polyA exon trapping reporter cassette (a kind gift from Bill Skarnes) containing an ATG translational start site flanked by an upstream splice acceptor site and multiple in-frame translational stop codons followed by a polyadenylation signal was introduced into the EcoRV site immediately upstream of exon 3. Linearized vector was electroporated into CCE ES cells. DNA from drug resistant clones was digested with BglII and screened by Southern blot hybridization with a 5′ external probe. Stul-digests were subsequently analyzed with an internal probe derived from exon 3. Forty-eight out of 212 clones were correctly targeted. Three clones injected into C57BL/6J blastocysts gave rise to germline chimeras. The mutation was maintained on an inbred 129/C57BL/6J genetic background. Genotyping was performed by PCR with 3 primers: FLRT3-FW 5′-ATGAGCCTGCTTCACAGCC-3′, FLRT3-RV 5′-AATGCTGTTCTGATGACACCC-3′, FLRT3-tGeo 5′-ATTTCACTTACCCACCCGGG-3′.

Following 35 cycles of 94 °C for 15 s, 67 °C for 1 min, 72 °C extension for 1 min, 72°C final extension for 7 min 328 bp (wt) and 725 bp (null) allele products were resolved on a 1% agarose gel.

Production of FLRT3 antibodies, immunoprecipitations and Western blots

The segment encoding the FLRT3 extracellular domain corresponding to amino acids 1–525 was cloned in-frame into EcoRI and KpnI sites of the pHSec mammalian expression vector containing the hexahistidine (His6) tag (Ariescu et al., 2006b). Numbering corresponds to GenBank entry NP_848469. C-terminally His6-tagged FLRT3 protein was expressed in transiently transfected HEK-293T cells, as previously described (Ariescu et al., 2006a). Immediately after transfection, the fetal calf serum (FCS) concentration was lowered to 2%. After 4 days, conditioned media was harvested, debris removed by centrifugation and 0.2 μm filtration, two volumes of PBS added, and the pH adjusted to 8.0 with 10 mM Tris–HCl. His6-tagged FLRT3 purified on NP2- charged chelating Sepharose (GE Healthcare) was then subjected to gel filtration. The fractions judged to have >95% purity by SDS-PAGE were pooled and injected into rabbits. Rabbit polyclonal antibodies were also raised against the peptide VTKQPD1NKPLIKDisclosure—Conflicts of interest: The authors declare no conflicts of interest. 185

Fig. 1. Developmentally regulated expression of three fibronectin leucine-rich transmembrane genes FLRT1–3 at early post-implantation stages. (A–P) Whole mount in situ hybridization analysis of FLRT1–3 expression. FLRT3 mRNA is initially expressed at early streak stages in the AVE and the chorion (A and B). At early headfold stages FLRT3 expression is maintained in the AVE and appears in the node and nascent definitive endoderm, and is down-regulated in the chorion (C). At E8.5 FLRT3 is up-regulated in the headfolds, the somites and the proepicardial organ (D). From E9.5 onwards, FLRT3 mRNA is present in the telencephalic vesicles, limb buds and the somites (E). At E10.5 FLRT3 transcripts are detectable in the pharyngeal arches, the apical ectodermal ridge (AER) of the limb buds and the somites (F). FLRT2 transcripts initially appear at mid-streak stages in the AVE and ADE tissue (arrowhead) (G). At E8.25 FLRT2 expression is up-regulated in the most anterior somites and the allantois (H). Slightly later, at E8.5, FLRT2 transcripts are strongly expressed in the trunk mesenchyme immediately caudal to the heart (I). At E9.0 FLRT2 expression is detectable in the midbrain mesenchyme and the anterior somites (J). At E10.5 FLRT2 is expressed in the pharyngeal arches and the somites (K). FLRT1 initially shows ubiquitous weak expression at streak stages (L). At early headfold stages FLRT1 is induced in the anterior neuroectoderm (M). Between E8.5 and E9.0 strong expression is strictly confined to the forming midbrain (N, O). Slightly later at around E10.5, FLRT1 expression is activated at additional sites, including the dorsal root ganglia and the ZPA of the limb buds (P). Abbreviations: AER, apical ectodermal ridge; al, allantois; AVE, anterior visceral endoderm; ch, chorion; drg, dorsal root ganglia; lb, limb buds; mb, midbrain; me, mesenchyme; n, node; ne, neural ectoderm; pa, pharyngeal arches; pc, proepicardial organ; peo, proepicardial organ; pc, pericardium; s, somites; t, telencephalon; ve, visceral endoderm; ZPA, zone of polarizing activity.
In situ hybridization, X-gal staining and Scanning Electron Microscopy

Whole mount in situ hybridization was performed as described (Hogan et al., 1994) with the following probes: FLRT1, FLRT2 and FLRT3 full-length cDNAs, AFP (Waldrip et al., 1998), Cer-I (Belo et al., 1997), Fgf8 (Crossley and Martin, 1995), GATA4 (Arceci et al., 1993), MLCV (Lyons et al., 1995), Otx2 (Ang et al., 1994), Shh (Echelard et al., 1993), Snail, Spry2, Tbx5 and Tbx18 (Kraus et al., 2001). Embryos were fixed in 4% paraformaldehyde, followed by dehydration through a graded ethanol series, cleared in Histoclear, embedded in wax and sectioned. Slides were stained with Hematoxylin and Eosin using standard protocols. LacZ activity was visualized by whole mount X-gal staining as described (Hogan et al., 1994). For scanning electron microscopy (SEM) embryos were dissected in DMEM plus 10% FCS, were transferred to DMEM supplemented with 50% rat serum, glutamine and pen/strep. The FGFR inhibitor SU5402 (Calbiochem), dissolved in DMSO (40 μM), or DMSO alone was added. Embryos cultured for 20 h at 37 °C in 5% CO2, were fixed and processed for in situ hybridization.

Results

Expression of the three FLRT family members at early stages of mouse development

To investigate whether FLRT3 plays a conserved role as a TGFβ/ Nodal downstream target in the developing mouse embryo, we initially assessed the onset of expression of the three FLRT family members by RT-PCR. FLRT3 is the predominant family member, most strongly expressed at E6.5. FLRT2 is weakly expressed and up-regulated by E7.5. By contrast FLRT1 expression is only detectable starting at E7.5 (data not shown). To describe tissue specific expression patterns of the three FLRT family members, we performed whole mount in situ hybridization (WISH) analysis (Fig. 1). FLRT3 transcripts are initially detected at pre-streak stages (E6.0) in the extra-embryonic lineages, namely the AVE and the chorion, a derivative of the extra-embryonic ectoderm (Figs. 1A, B). By early headfold stages, FLRT3 expression becomes visible in anterior streak derivatives namely the node, notochord and nascent definitive endoderm, and in the anterior ectoderm (Fig. 1C). FLRT3 expression subsequently expands and by E8.5 is present in the neuroectoderm and the somites. FLRT3 is also strongly expressed in tissues ventral and caudal to the heart (Fig. 1D). Consistent with previous findings (Haines et al., 2006; Robinson et al., 2004), at E9.5 robust FLRT3 expression is seen in the...
telencephalic vesicles and the apical ectodermal ridge (AER) of the developing limb buds (Figs. 1E, F). Finally, by E10.5 FLRT3 expression extends to the pharyngeal arches and the somites (Fig. 1F).

FLRT2 is co-expressed with FLRT3 in the anterior endoderm domain but shows a delayed onset (E7.5) (Fig. 1G). One day later, FLRT2 mRNA delineates the most anterior somites and is very strongly expressed in both the allantois and the mesenchymal tissue beneath the forming heart (Figs. 1H, I). At E9.0 FLRT2 expression is confined to the midbrain and the tissues dorsal to the heart (Fig. 1J), but at E10.5 expression expands to include the first and second pharyngeal arches, the somites and cells forming the outer lining of the heart (Fig. 1K). Thus by E10.5, FLRT2 and FLRT3 expression domains are broadly overlapping in the pharyngeal mesenchyme and somites, but also display distinctive expression patterns in the heart wall and limb buds. By contrast, FLRT1 shows tightly restricted expression at E7.5 exclusively in the neurectoderm. Between E8.5 and E9.0, FLRT1 expression is confined to the midbrain (Figs. 1N, O) but by E10.5, becomes detectable throughout the brain, in the mesenchyme of the zone of polarizing activity of the limb buds and the dorsal root ganglia (Fig. 1P).

Targeted inactivation of FLRT3 expression by a gene trap strategy

FLRT3 is encoded by a single exon, exon 3. We initially designed a targeting construct to conditionally excise this portion of the locus. However the frequency of homologous recombination was disap-

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**Fig. 3.** The FLRT3 β-geo reporter allele faithfully recapitulates endogenous expression patterns. Beginning at around E6.5, LacZ expression is detectable in the AVE and the chorion (A), but not the ectoderm (B, B'). As gastrulation proceeds, expression is maintained in the AVE and is activated in the node and definitive endoderm (C, C'). By E8.5, LacZ staining becomes visible at numerous sites including the midbrain, somites and foregut pocket (D, D'). Tissue sections (D' and E') reveal strong expression in the dorsal tip of the neural folds (D'), in the forming otic vesicles, and the notochord, as well as the surface ectoderm and within myocardial wall of the heart (E'). At E9.5 the cells of the forming proepicardial organ (F') and hepatic diverticulum (F'') strongly express LacZ. Expression is also detectable in the somites and the endoderm lining the gut (F'''). Abbreviations: am, amnion; AVE, anterior visceral endoderm; ect, ectoderm; end, endoderm; fg, foregut; ge, gut endoderm; hd, hepatic diverticulum; mb, midbrain; myo, myocardium; n, notochord; ne, neural ectoderm; opv, optic vesicle; otv, otic vesicle; peo, proepicardial organ; s, somites; se, surface ectoderm; sv, sinus venosus.
pointingly low and none of the few targeted clones recovered incorporated all three loxP sites. Since FLRT3 is strongly expressed in ES cells (data not shown), as an alternative strategy we employed an exon trapping vector. A β-geo cassette containing an upstream splice acceptor site, its own ATG translational start site followed by multiple in-frame translational STOP codons and a poly adenylation signal was introduced immediately upstream of exon 3 (Fig. 2A). As expected from earlier studies describing exon trapping strategies (Friedel et al., 2005), correctly targeted ES cells clones were recovered at a relatively high frequency (approximately 25%). Three independent clones were subsequently used to generate germ line chimeras.

Heterozygous animals were healthy and fertile. As shown in Fig. 3, the FLRT3fl/fl/−/− reporter allele faithfully recapitulates endogenous expression patterns. Thus LacZ activity is detectable at E6.5 specifically in the AVE and chorion (Figs. 3A, B, B′) and by E7.5 staining is present in nascent definitive endoderm underlying the former headfolds and lateral edges of the anterior neuroectoderm (Figs. 3C, C′). LacZ staining is observed slightly later in the dorsal edges of the neuroectoderm, the otic vesicles and the notochord. The ventral body wall and the myocardial layer of the heart clearly show the strongest signal (Figs. 3D–E′). By E9.5 β-gal activity was also detectable in the midbrain neuroectoderm, the optic vesicles and the gut tube. Cells within the proepicardial organ lying caudal to the heart, the sinus venosus and the hepatic diverticulum also strongly express LacZ activity (Figs. 3F–P′).

At E7.5–E8.5 homozygous FLRT3 mutant embryos were recovered from intercross matings at the expected Mendelian ratios. With the exception of a small fraction of mutants (~3%) that complete development and are viable and fertile as adults, almost all FLRT3 deficient embryos die by E10.5 (Table 1). To confirm that the targeted mutation encodes a null allele, we analyzed FLRT3 expression in adult brain samples (Figs. 2D–E). Rabbit polyclonal antibodies raised against three distinct FLRT3 epitopes were used in Western blot and immunoprecipitation experiments. Results shown in Figs. 2D–E demonstrate that the insertion of the β-geo reporter cassette upstream of exon 3 eliminates FLRT3 expression.

**Table 1**

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Weanlings 219 (38.2) 339 (59.2) 15 (2.6) 573

The table shows total numbers and corresponding percentages of embryos at the indicated stages and viable offspring recovered from FLRT3 heterozygous intercross matings.

**FLRT3 mutant embryos display ventral closure defects**

Ventral morphogenesis involves a striking movement of cells at the lateral edges of the body wall that subsequently fuse at the midline, bringing together both sides of the primary heart field to initiate formation of the primitive linear heart tube. Concomitantly the lateral edges of definitive endoderm fuse ventrally to form the foregut pocket (Kaufman, 1992). FLRT3 is strongly expressed in the lateral edges of the definitive endoderm, and in the lateral mesoderm cells that intercalate across the midline (Figs. 4A–D). Homozygous mutant embryos are phenotypically normal at E7.5, but by E8.5 display a spectrum of morphological abnormalities. Roughly 30% of FLRT3 deficient embryos present with a pronounced ventral closure defect, invariably associated with cardiac bifida (Figs. 4F, H, J, L, N, P).

The transcription factor GATA4 plays an essential role in heart tube formation and ventral morphogenesis (Molkentin et al., 1997). GATA4 mutants display a cardiac bifida phenotype as described here for embryos lacking FLRT3. However at E8.5 GATA4 is normally expressed in the heart tube and the anterior intestinal portal (Figs. 4G, H). Thus FLRT3 ventral closure defects are not caused by GATA4 misexpression. To further characterise these tissue disturbances we assessed additional markers. We used Tbx18 to identify the proepicardial organ, MLCV to delineate the developing heart, AFP to mark the extra-embryonic yolk sac endoderm, and Shh the midline structures (Figs. 4I–P′). The two wings of splanchnic mesoderm caudo-lateral to the heart tube normally migrate towards the midline and intercalate to become the proepicardial organ. The proepicardium gives rise to both the epicardium of the heart and the coronary vasculature (for review see Manner et al., 2001). Interestingly these “grape-like cells” which cluster just ventral and caudal to the forming heart tube, strongly express FLRT3 (Fig. 3F). As judged by Tbx18 expression, at E8.5 in both wild type and mutant embryos proepicardial cells are correctly specified. Thus FLRT3 is not required for proepicardial cell specification, however in FLRT3 mutants, proepicardial cells do not migrate towards the midline and intercalate as expected (Figs. 4I, J). As shown by MLCV expression the cardiac progenitor cells can correctly form a heart tube which appear to be correctly patterned and undergo chamber formation (Figs. 4O, P). Thus loss of FLRT3 has no noticeable effect on specification or morphogenesis of the heart tubes, or on the process of looping morphogenesis (Fig. 4P).

By early headfold stages an anterior invagination, or portal, develops to form the so termed foregut pocket. AFP expression marks the visceral yolk sac (VYS) that normally extends across the front of the foregut pocket (anterior intestinal portal). AFP expression along the lateral edges of the VYS was unperturbed in FLRT3 mutant embryos. However mutant embryos lack a contiguous domain of AFP expression across the anterior ventral midline suggesting development of the foregut pocket is selectively disrupted (Figs. 4K, L). Shh expression in the notochord and floorplate of the neural tube is unperturbed in FLRT3 mutant embryos (Figs. 4M, N). Thus the failure to undergo ventral closure is not associated with gross tissue disturbances, and all the cell lineages involved in ventral closure are correctly specified. Rather, loss of FLRT3 disrupts fusion of the lateral edges of the body wall.

**FLRT3 functional loss disrupts headfold fusion**

An essential process for formation of the anterior CNS is the fusion of the lateral edges of the neural plate. By E8.5 in wild type embryos the headfolds acquire regional patterning, become elevated, and fuse along the dorsal midline. In contrast, approximately one-third of the
mutants display abnormal morphogenesis of the neuroepithelium and headfold fusion defects (Figs. 5B, C). Fgf8 expression domains in the most rostral neuroepithelium, the isthmus separating the mid and hindbrain, and the pharyngeal arches (Fig. 5A) are unperturbed in FLRT3 mutant embryos (Fig. 5B). Similarly Otx2 expression in the anterior CNS is maintained despite the absence of headfold fusion
As for ventral closure defects described above, all of the cell lineages involved in the formation of the CNS are correctly specified. These results suggest that the robust expression of FLRT3 in the tips of the neurectoderm (Fig. 3D′) is required for a correct fusion of the lateral edges of the anterior neural epithelium at the midline of the embryo.

Loss of FLRT3 function compromises definitive endoderm migration and causes anterior axis truncations

The nascent definitive endoderm that migrates anteriorly along the midline of the embryo is the source of essential signalling cues that maintain and pattern the emerging overlying neural plate. Anterior truncations associated with defects in the formation and/or migration of the definitive endoderm have been extensively described in the literature (reviewed in Lu et al., 2001). For example, reduced Nodal signalling in the primitive streak compromises definitive endoderm specification and secondarily leads to anterior CNS patterning defects (Dunn et al., 2004; Roebroek et al., 1998; Vincent et al., 2003). Interestingly, a final category of FLRT3 mutant embryos (approximately one-third) show pronounced anterior truncations (Fig. 5D).

(D, D′) Fgf8 expression shows that the anterior neural tissues are missing in a mutant embryo where ventral closure has occurred. Otx2 expression is drastically reduced in embryos presenting anterior truncations (compare panel E with F). (E) Both the left and right heart fields fuse to form the primitive heart tube in wild type embryos whereas the mutant embryo (F) displays cardia bifida (red asterisks) in addition to severely reduced anterior neural tissue. (G–K) Definitive endoderm migration defects in FLRT3 null embryos. (G) In wild type E7.5 embryos Cerl positive definitive endoderm cells have migrated to the anterior proximal region. (H–K) Cerl in situ hybridization in stage-matched mutant embryos. (H) Lateral and frontal (I) views show a marked reduction in the number of Cerl positive definitive endoderm cells, and a marked delay in their migration (J, K).

Abbreviations: A, anterior part of the embryo; ave, anterior visceral endoderm; aip, anterior intestinal portal; de, definitive endoderm; is, isthmus; nep, neuroepithelium; P, posterior side of the embryo; pa, pharyngeal arches; R, right side of the embryo; L, left side of the embryo.

Fig. 5. Defective headfold fusion and anterior truncations in FLRT3 mutant embryos. Fgf8 expression in wild type (A) and mutant E9.0 embryos (B, B′) demonstrates that FLRT3 is required for correct fusion of the anterior neural region. (C) The headfold fusion phenotype is shown in an unstained mutant embryo. The black asterisk marks the open neural folds. (D, D′) Fgf8 expression shows that the anterior neural tissues are missing in a mutant embryo where ventral closure has occurred. Otx2 expression is drastically reduced in embryos presenting anterior truncations (compare panel E with F). (E) Both the left and right heart fields fuse to form the primitive heart tube in wild type embryos whereas the mutant embryo (F) displays cardia bifida (red asterisks) in addition to severely reduced anterior neural tissue. (G–K) Definitive endoderm migration defects in FLRT3 null embryos. (G) In wild type E7.5 embryos Cerl positive definitive endoderm cells have migrated to the anterior proximal region. (H–K) Cerl in situ hybridization in stage-matched mutant embryos. (H) Lateral and frontal (I) views show a marked reduction in the number of Cerl positive definitive endoderm cells, and a marked delay in their migration (J, K).

Abbreviations: A, anterior part of the embryo; ave, anterior visceral endoderm; aip, anterior intestinal portal; de, definitive endoderm; is, isthmus; nep, neuroepithelium; P, posterior side of the embryo; pa, pharyngeal arches; R, right side of the embryo; L, left side of the embryo.
Collectively our experiments demonstrate FLRT3 controls cell behaviour during ventral closure, headfold fusion and definitive endoderm migration. The penetrance of each phenotype is approximately 30–40%, and many mutant embryos present with a spectrum of defects. A very small number (<3%) of embryos successfully bypass FLRT3 requirements and develop into viable fertile adults.

**Fig. 6.** FGF signalling is unperturbed in FLRT3 deficient embryos. Normal Spry2 (A, D), Tbx6 (B, E) and Snail (C, F) expression patterns in wild type (A–C) and mutant (E–F) embryos at E8.5. (G–L) FLRT3 expression is maintained in the absence of FGF signalling. E7.75 embryos were cultured for 20 h in medium alone (G–I) or in the presence of the FGFR inhibitor SU5402 (J–L). Shh (G, J) and Tbx6 (H, K) expression patterns in control (G, H) or treated embryos (J, K) demonstrate that SU5402 specifically disrupts FGF signalling. (L, L) Under identical conditions FLRT3 expression is unaffected. Abbreviations: aip, anterior intestinal portal; al, allantois; ch, chorion; fp, floor plate; psm, pre-somitic mesoderm; nc, neural crest; ps, primitive streak; s, somites.
FLRT3 expression is independent of FGF signalling

Considerable evidence suggests that FLRT3 modulates the strength of FGF signalling via a regulatory feedback loop (Bottcher et al., 2004; Haines et al., 2006). To assess FGF signalling activity in FLRT3 mutant embryos, we next examined expression of three well known FGF target genes Spry2, Tbx6, and Snail (Ciruna and Rossant, 2001). At E8.0 Spry2 marks the primitive streak. At E8.5 Tbx6 is specifically expressed in the pre-somatic mesoderm, whereas Snail marks the cephalic neural crest cells, the migratory mesodermal cell populations exiting the primitive streak and the allantoic bud (Figs. 6A, B, C). As shown in Fig. 6, Spry2, Tbx6, and Snail expression patterns in wild type and FLRT3 mutant embryos were indistinguishable (Figs. 6D, E, F). Thus the striking phenotypes caused by loss of FLRT3 expression are not associated with FGF signalling defects.

To test whether FGF signalling regulates FLRT3 expression, E7.5 embryos were cultured in the presence of the FGFR-specific inhibitor SU5402 (Mohammadi et al., 1997) and FLRT3 expression was analysed by WISH 24 h later. As expected the expression of the control marker Shh was unaffected in treated embryos (Figs. 6G, J). Expression of Tbx6, a downstream target of the FGF pathway (Ciruna and Rossant, 2001), is selectively abolished in the presence of SU5402 (Figs. 6H, K). In contrast there was no noticeable effect on FLRT3 expression (Figs. 6I, L). Thus we conclude that FLRT3 expression in the early mouse embryo is independent of FGF signalling.

Discussion

The three fibronectin leucine-rich transmembrane proteins are strongly conserved across vertebrate evolution. In humans, FLRT3 is broadly expressed in adult somatic tissues including pancreas, skeletal muscle, heart and brain. FLRT2 expression is more restricted and FLRT1 expression is confined to the adult brain (Lacy et al., 1999). Similarly here we demonstrate in the developing mouse embryo that FLRT3 is the predominant and earliest family member expressed, FLRT2 expression is intermediate, and FLRT1 has a tightly restricted pattern of expression. In the early Xenopus embryo XFLRT3 expression is localized to the forming dorsal blastopore lip and expands around the entire equatorial zone as gastrulation proceeds, becoming confined to the deep mesoderm. XFLRT3 has been identified as a direct target of the Activin/Nodal signalling pathway (Ogata et al., 2008). Consistent with the idea that FLRT3 potentially represents a conserved Nodal target playing a key role in axis formation, our WISH demonstrate that FLRT3 is strongly expressed in the AVE, a key signalling centre that is responsible for establishment of the A–P axis (Brennan et al., 2001; Waldrip et al., 1998). Dose-dependent Nodal/Smad2 signals also govern the specification of the APS derivatives (Dunn et al., 2004; Vincent et al., 2003), and these tissues contribute to the definitive endoderm, node and notochord, also strongly express FLRT3.

To assess FLRT3 activities in the developing mouse embryo, we generated a loss of function allele. FLRT3 mutant embryos develop a normal A–P axis. Similarly Nodal activities required in the posterior epiblast for primitive streak formation and in the node for establishment of the L–R axis are unaffected (Brennan et al., 2002; Norris et al., 2002). The present report describes three distinct phenotypes associated with loss of FLRT3 expression. Defects in ventral closure, fusion of the anterior headfolds, and definitive endoderm migration associated with anterior truncations of the CNS were observed. FLRT3 thus possibly acts downstream of Nodal/Smad signals in the emerging nascent definitive endoderm.

The definitive endoderm initially appears as a discrete cell layer at the most distal end of the primitive streak (Lawnson et al., 1986; Lawson and Pedersen, 1987), and migrates medially and anteriorly, displacing primitive visceral endoderm to form the foregut tissue (Lawson and Pedersen, 1987; Tam and Beddington, 1992; Thomas and Beddington, 1996). As gastrulation proceeds, anterior mesendodermal cell populations emerging from the streak are laid down along the axis in an anterior to posterior fashion. By late headfold stages morphogenetic movement and cell growth towards the midline, in concert with the process of embryonic turning, results in closure of the gut tube along the ventral midline. Genetic studies have provided considerable insights into the molecular pathways that regulate endoderm formation and migration in vertebrates (reviewed in Rossant and Tam, 2002; Stainier, 2002). High levels of Nodal/Smad2 signalling are necessary to specify APS derivatives (Dunn et al., 2004; Vincent et al., 2003). Importantly, specification of the definitive endoderm progenitors also depends on transcriptional partnerships with Smad4 (Chu et al., 2004) and FoxH1 (Hoodless et al., 2001; Yamamoto et al., 2001). Mouse embryos mutant for the Mix1 homolog Mix1I lack definitive endoderm, fail to form a heart tube, and exhibit anterior CNS patterning defects (Hart et al., 2002). In frogs and zebrafish, Mix genes activate Sox17 homologs to specify endodermal cell fates (reviewed in Stainier, 2002). A mouse Sox17 homolog is required for survival and proliferation of definitive endoderm (Kanai-Azuma et al., 2002). Several genes controlling the process of ventral closure have been identified. For example, loss of the proconvertase furin/SFPC1 disrupts definitive endoderm specification and migration, possibly as a secondary consequence of TGFβ/Nodal signalling defects (Roebroeck et al., 1998). The present study identifies FLRT3 as the first cell surface adhesion molecule controlling development of the definitive endoderm cell lineage.

FLRT3 expression is upregulated in response to FGF signalling (Bottcher et al., 2004; Haines et al., 2006) and FLRT3 modulates the strength of FGF signalling during Xenopus gastrulation. FGF signals are also essential for guiding correct cell movements in the early mouse embryo. For example Fgf8 expression in the primitive streak is required for ingression of nascent mesoderm (Sun et al., 1999). Inactivation of the FGFR1 disrupts mesoderm migration due to the failure to down-regulate the epithelial cell adhesion molecule E-cadherin at adherens junctions (Ciruna and Rossant, 2001; Ciruna et al., 1997). Surprisingly, under physiological conditions, loss of FLRT3 has no effect on FGF target gene expression during gastrulation stages. Similarly inhibition of FGF signalling fails to perturb FLRT3 expression in embryos cultured to the headfold stage. Moreover in contrast to the situation in Xenopus (Bottcher et al., 2004), there is no overlap between FLRT3 and Fgf8 expression domains in the early mouse embryo. These results strongly suggest that FLRT3 feedback regulation of the FGF signalling pathway is not conserved between frogs and mice. However, due to the early lethality of FLRT3 mutants we cannot exclude a function for FLRT3 in modulating FGF signalling in tissues of the later embryo or adult where the expression domains overlap.

In Xenopus, XFLRT3 interacts with the small GTPase Rnd1 in the involuting equatorial cells to promote cell de-adhesion (Ogata et al., 2007; Wunnenberg-Stapleton et al., 1999). The present results suggest the opposite may potentially hold true in the mouse. Thus FLRT3 expression seems to strengthen cell adhesion necessary for headfold fusion, ventral closure and definitive endoderm migration. Both ventral closure and fusion of the anterior headfolds depend on cell interactions over a long distance. The simplest idea is that surface FLRT3 expressed on opposing cells promotes homotypic cell sorting. These complex changes in tissue architecture may also involve additional FLRT3 partnerships. FLRT3 recognition motifs are potentially provided by the concave LRR ligand binding surface (Jin et al., 2007), the FN type III domain and its cytoplasmic tail. Two closely related Drosophila LRR transmembrane proteins, Tartan and Capricious, establish affinity boundaries that distinguish the dorsal and ventral compartments in the developing wing disc (Milan et al., 2001). Biochemical experiments suggest that both the extracellular LRR domains as well as the intracellular tail are required for cell sorting behaviour (Milan et al., 2005). Recent structural studies of human Toll-like receptors (TLRs) demonstrate ligand-induced dimerization (Jin et al., 2007). It will be interesting to learn more about higher order FLRT3 structures that cooperatively govern complex cell behaviours during
ventral closure, neural fold fusion and definitive endoderm migration in the developing mouse embryo.

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