TRANSGELIN EXPRESSING MYOFIBROBLASTS ORCHESTRATE VENTRAL MIDLINE CLOSURE THROUGH TGF-β SIGNALLING

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Summary statement:

A specific population of migratory myofibroblasts at the leading edge of the closing ventral body wall respond to epithelial TGF-β gradient to drive midline closure.

Abstract:

Ventral body wall (VBW) defects are among the most common congenital malformations, yet their embryonic origin and underlying molecular mechanisms have remained poorly characterized. Although transforming growth factor beta (TGF-β) signalling is essential for VBW closure the responding cells are not known. Here we identify a population of migratory myofibroblasts at the leading edge of the closing VBW that express the actin-binding protein Transgelin (TAGLN) and TGF-β receptor (TGFβR). These cells respond to a temporally regulated TGF-β2 gradient originating from the epithelium of the primary body wall. Targeted elimination of TGFβR2 in TAGLN+ cells impairs midline closure and prevents the correct subsequent patterning of the musculature and skeletal components. Remarkably, deletion of TGFβR2 in myogenic or chondrogenic progenitor cells does not manifest in midline defects. Our results thus indicate a pivotal significance of VBW myofibroblast in orchestrating ventral midline closure by mediating the response to TGF-β gradient. Altogether, our data enables us to distinguish a highly regulated epithelial-mesenchymal signalling and successive cellular migration events in VBW closure that explain early morphological changes underlying the development of congenital VBW defects.
Introduction:

Abdominal wall defects are common in humans and bear significant morbidity and mortality (Wilson and Johnson, 2004). They show varied phenotype anomalies that differ not only in their anatomy, but also in their mode of development, organs involvement and short and long term outcomes (Carnaghan et al., 2013; Christison-Lagay et al., 2011; Gamba and Midrio, 2014; Sadler, 2010; Sadler and Feldkamp, 2008). Nevertheless, little is known on the mechanisms that drive ventral midline closure in mammals. The VBW in mice arises as a result of the turning process that transforms the “cup shaped” embryo proper. During this morphogenetic process initial ectodermal-mesodermal layer, known as the primary body wall, provide an initial thin cover to the embryonic endoderm at around E8.5-E9.5 (Kaufman, 1992). Starting from around E12 differentiated secondary mesenchymal components arising laterally from the flanks follow the primary body wall. This later cellular migration process continues through late embryonic stages and complete migration and fusion of the secondary body wall elements is fully achieved by E15.5 in the thorax and E16.5 in the abdomen (Kaufman and Bard, 1999). Yet, the mechanisms that drive these large scale morphogenetic movements and the cell types that are involved have remained largely unknown. Similarly, a possible role of the cells of the primary body wall in facilitating ventral midline closure remains to be investigated. In diaphragm development, fibroblasts pilot the way for muscle cell migration and it is defect in these connective tissue fibroblasts that result in the development of congenital diaphragmatic hernia rather than a primary muscle cells defect (Merrell et al., 2015). In addition, recent evidence suggests a role of epithelial-mesenchymal signalling not only in epithelium patterning, but also as a major regulator of secondary body wall elements migration (Brewer and Williams, 2004; Budnick et al., 2016; Candille et al., 2004; Eng et al., 2012; Zhang et al., 2014). Nonetheless, the cellular and morphogenic components of the epithelial-mesenchymal pathway in ventral midline closure are still largely obscure.

TGF-β (transforming growth factor β) signalling was proven to play a pivotal role in facilitating closure of the midline in various tissues and body districts (Dünker and Kriegstein, 2002; Kaartinen et al., 1995; Sanford et al., 1997a). Interestingly, TGF-β2/3 double knockout mouse showed severe midline closure defects confirming the role of TGFβ signalling in VBW closure (Dünker and Kriegstein, 2002). Similarly, total knockout of different members of the Homeobox gene, AP-2α, ACLP (aortic carboxypeptidase-like protein) transcription factors and Wnt signalling pathway cause different midline closure defects including that of the VBW (Brewer and Williams, 2004; Layne et al., 2001; Snowball et al., 2015; Zhang et al., 1996; Zhang et al., 2014). However, due to the complete loss of function nature of all of these models, it was impossible to identify specific cellular players in the closure process.

It has been shown that TGF-β signalling possesses distinct roles on specific target cells and tissues which are mediated by the TGF-β receptors (Massagué, 2012). During embryogenesis, TGF-β superfamily ligands including Dpp (decapentaplegic), BMP and Activin act as dose dependant morphogens in a variety of fundamental embryonic processes such as left to right asymmetry and anterior-posterior patterning (Belenkaya et al., 2004; Entchev et al., 2000; Meno et al., 1996; Teleman
and Cohen, 2000; Wu and Hill, 2009). While all TGF-β morphogens signal via common receptors (TGFβR1/2 complex) their expression varies between tissues, explaining the differences in knockout mouse phenotypes. Furthermore, partial compensation may exist between TGF-β morphogens, leading to variable penetrance of the defect in individual morphogen knockout models. Cleft palate and defects in diverse midline components are evident in all individual Tgf-β knockouts, suggesting their common involvement in midline closure (Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997b). These analyses of the TGF-β1/2/3 morphogen knockout models have provided invaluable insights into their role in embryonic development, but left open the question of the cell type responding to their signals.

TGF-β signalling was shown to enhance cell motility by inducing re-organization of the actin cytoskeleton (Boland et al., 1996; Edlund et al., 2002). The TGF-β induced transcriptional changes, mediated by SMAD2/3 transcription factors, control the actomyosin cytoskeleton by up regulating CITED1 (Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1) and thereby promoting cell migration (Cantelli et al., 2015). TGF-β is also known to induce tagln in vitro and in vivo (Adam et al., 2000; Hirschi et al., 1998; Yu et al., 2008) through SMAD binding on tagln promoter (Chen et al., 2003). TAGLN, on the other hand, is an actin binding cytoskeleton protein that is linked to increased cell motility and migration (Assinder et al., 2009; Elsafadi et al., 2016; Lin et al., 2009; Yu et al., 2008; Zhou et al., 2016).

Here we show that ventral body wall closure relies on polarized migration of TAGLN+ myofibroblasts towards TGF-β morphogen gradient originating from the epithelium of the primary body wall. The progeny of these embryonic myofibroblasts is maintained as a narrow line at the closed midline. Specific knockout of TGFβR2, the common receptor to all TGF-β morphogens, from TAGLN+ cells leads to complete failure of ventral body wall closure. In contrast, no such defects are evident when TGFβR2 is deleted from developing skeletal muscles. Our data reveals a principal role for myofibroblasts in mediating TGF-β signalling in ventral body wall morphogenesis.
Results:

Ventral midline develops from convergent movement of TAGLN transgene marked cells

We noticed high expression of TAGLN in the primary body wall area from early stages of VBW development (Fig. 1 A-B). In order to follow the fate of TAGLN expressing cells in primary body wall, we crossed Taglin-Cre mouse strain to Rosa26-NGZ (Gt(Rosa)26Sortm1(CAG-lacZ,-EGFP)Glh) reporter strain and performed whole mount β-galactosidase staining at various embryonic stages. At E11.5 we detected lacZ marked cells across the developing VBW and in the ventral aspect of the myotomes (Fig. 1C). Over the course of development the lacZ labelled ventral area became progressively more restricted. At E13.5 the lacZ labelled area takes a triangular shape wide at the caudal end. At E15.5, by the time the VBW has almost completely closed, only a narrow line of cells remains visible, besides blood vessels which are also clearly labelled (Fig. 1C). We also crossed Tagln-Cre to Rosa26-tdTomato (tdTom) reporter mouse and analysed VBW closure in embryo sections. The wide expression of TAGLN reporter can be seen in the primary body wall in early embryonic stages (E11.5), whereas over a 4 day time-window it became limited to a small area both at the thoracic and abdominal midline (Fig. 1E-F) and the closure followed the cranio-caudal axis similar to the lacZ whole mount staining. Interestingly, the tdTom signal in the midline continues to be present into juvenile postnatal growth phase and even into adulthood (Fig. S1 A). This suggests that primary VBW cells are derived from TAGLN+ cells that continue to exist as resident cells in the midline of adult mice. We next analysed whether the spatial narrowing of the tdTom+ cell population was due to cell death in the body wall or convergent migration of cells towards the midline. We found that the number of apoptotic cells in the ventral midline is low and we did not observe any difference in TUNEL labelling between tdTom+ and tdTom- cells at E15.5 (Fig. 1G). The only noticeable apoptosis we observed was in the periphery of thoracic ribs at E15.5 and E16.5 where TAGLN derived cells (tdTom+) were also initially present, but eventually died (Fig. S1 B,C). On the other hand, TAGLN derived cells (tdTom+) in the midline (ML) area showed much less mitotic activity when compared to adjacent para-midline (PML) tdTom- secondary body wall component cells, as revealed by KI67 staining (Fig. 1 H). The average relative expression of nuclear KI67 signal in the midline area was 50% of that of the adjacent para-midline in the abdominal and thoracic regions (Fig. 1I). This excludes apoptosis as a mechanism behind the spatial narrowing of the tdTom signal and suggests that the secondary body wall of the ventral midline develops from proliferating lateral flank cells.

TAGLN cells migrate towards the ventral midline

We next set to test whether TAGLN expressing cells of the primary VBW actively migrate from the somite region towards the midline. We used ex-vivo body wall explants from E11.5 Tagln-Cre:Rosa26-tdTom: PAX3-GFP embryos (Fig. S2 A). At this stage GFP expression marks myoblasts in the somite and TAGLN cells of the VBW (tdTom+) are seen ventral to the somite (Fig. 2A). Over nine hour period the tdTom+ cells at the flanks of the body wall showed active directional migration
towards the midline (Fig. 2 B-F) and (Movie 1). The migrating cells showed active locomotion signs expressed by cell protrusions and lamellipodia formation (Movie 2). On the other hand, the more dorsally located tdTom$^+$ resident cells (Fig. S2 B) retained their localization (arrow in Fig. 2 A-F).

Using Tagln-Cre:Rosa26-tdTom model we performed topographical characterization of the primary body wall at E14.5 and time lapse confocal analysis of these cells in an ex vivo body wall explant culture. At the thoraco-abdominal junction at E14.5 tdTom$^+$ cells formed a cone shaped mass that is wider caudally (Fig. S2 C). Analysis over 10-hour period time-lapse showed dynamic constriction of tdTom$^+$ cells in a dorso-ventral fashion, alongside the cranio-caudal axis (Movie 3). This proves that TAGLN cells of the primary wall migrate from the somite region and continue to migrate towards the midline during VBW closure.

TAGLN is downregulated when VBW closure is complete

In order to verify Tagln-Cre transgene specificity and distinguish between TAGLN expressing cells and their daughter cells we next analysed the expression of native TAGLN protein. In the early stages of VBW closure (E12.5-E13.5) we found complete overlap between the Tagln-Cre:Rosa26-tdTom and TAGLN antibody expression in the primary body wall both in the thoracic and abdominal regions (Fig. 3A,D,E). This was still the case till E14.5 where nearly full overlap was noted between the two signals in the primary ventral wall (Fig. 3B,F). However, from E15.5 onwards TAGLN signal in the closed midline diminished in the thoracic (Fig. 3C) and abdominal (Fig. 3G) midline regions and using confocal microscopy we noticed that TAGLN is no longer expressed in the residual Tagln-Cre area (Fig. 3C1,G1). This indicates that mature midline cells, progeny of TAGLN positive cells, progressively switch off TAGLN gene expression when their migration is complete and the ventral midline has fused.

TAGLN expressing myofibroblasts drive midline closure

We further characterised the ventral midline cells during the closure process. The TAGLN expressing cells of the ventral midline express the mesodermal and fibroblast marker Vimentin throughout the studied time points (Fig. 4A,B,H). The expression of smooth muscle cell markers, however, was gestation dependant. In the thorax αSMA (smooth muscle alpha actin) was expressed more abundantly at early time points (Fig. 4A), while, when the ventral midline was fully closed by E16.5, αSMA expression was more limited to a small number of midline cells ventral to the closed sternum (Fig. 4D). Similarly, early in the closure process Desmin is expressed in the TAGLN$^+$ VBW cells both in the thoracic and abdominal areas (Fig. 4B,H respectively). These data suggest that at the time of midline closure the migrating cells express several contractile and cytoskeletal proteins (αSMA, TAGLN, Vimentin and Desmin) some of which are downregulated in the mature midline. The TAGLN positive cells of the midline also co-expressed ER-TR7 (anti-reticular fibroblasts and reticular fibres antibody 7) confirming their fibroblast-like nature. This expression was evident at late time points in the closure process both in the thoracic and abdominal areas (Fig. 4C,4I respectively). Of note, the
cells of the midline did not express tendon cell markers. Tenascin (an extracellular matrix glycoprotein) expression at E13.5 and E14.5 was strong just lateral to the primary VBW and cells expressing Tenascin-C appeared to be encapsulating the TAGLN derived midline cells (Fig. 4F-G). Moreover, when the ventral closure process is complete at E16.5 we did not detect Tenascin-C expression in the resident TAGLN positive cells strictly in the midline (Fig. 4J).

**TGF-β epithelial-mesenchymal signalling in the primary VBW**

We found high expression of TGFβR2 in the ventral midline at E13.5 and 14.5 that localises to the primary body wall area labelled with tdTom (Fig. 5A). Using confocal microscopy we noticed that TGFβR2 is abundant, in the primary VBW, on tdTom positive cells just beneath the epithelial layer (Fig. 5A1). In a similar fashion, TGF-β2 protein expression is profuse in the primary VBW from E12.5 compared to the secondary body wall area (Fig. 5B). This TGF-β2 signal is noticeably strong in the epithelium and is expressed to a lower degree in the subdermal layer, as shown by confocal microscopy (Fig. 5B1). We detected far less TGF-β2 in the secondary body wall, where it was restricted primarily to the subdermal layer (Fig. 5B2). In order to prove the presence of TGF-β2 gradient in the VBW during the closure, we dissected the midline (ML) and para-midline (PML) VBWs from wild type mouse embryos (as shown in Fig. 5C) at different time points and tested the expression of Tgfβ2 using RT-qPCR. We found an anatomical and temporal TGF-β2 gradient towards the midline during VBW closure time points. The TGFβ2 RNA expression in the midline dramatically increases at E12.5, peaks at E13.5 and then starts to tail off at E15.5 when the VBW is almost fully closed (Fig. 5D). Furthermore, to prove the high expression of TGFβR2 in TAGLN+ cells we dissected the VBW as shown (Fig. 5E1) from Tagln-Cre:Rosa26-tdTom embryos, isolated tdTom+ and tdTom- cells by FACS and tested Tgfbr2 expression using RT-qPCR. Interestingly, tdTom+ cells only accounted for an average of ~15% (from all experiments) of the total ventral midline cells population at E14.5 (Fig. 5E2). Yet, Tgfbr2 expression is significantly higher in Tagln-Cre:tdTom+ cells compared to tdTom- VBW cells (Fig. 5F).

**TGF-β signalling in TAGLN+ cells is required for midline closure**

TGFβ signalling plays an important role in midline closure (Dünker and Krieglstein, 2002). However it is largely unknown which specific cell types mediate the response to TGF-β. We knocked out Tgfbr2 selectively in TAGLN+ cells and observed a dramatic VBW closure defect in 100% of mutant mice (n=10). In the Tagln-Cre:Tgfbr2flx/flx embryos a thin primary body wall covers the ventral midline and secondary body wall elements fail to migrate towards the midline after E13.5 (Fig. 6A). At E14.5 it is obvious that the thoracic and abdominal cavities are only covered with a thin and membranous primary body wall (Fig. 6B) that measures less than one-third the thickness of the primary body wall of that of the wild type. We characterised this mutant model by serial sectioning of the thoracic and abdominal regions. Secondary body wall components fail to migrate beyond two thirds of the lateral wall. This failure of migration is most evident at E14.5; in the thoracic region the ribs are mal-aligned,
fail to progress ventrally and the sternal primordium is completely absent. Whilst, wild type embryos show formed sternebrae separated by a narrow residual primary body wall in the midline (Fig. 6C). Similarly, in the abdominal region we detected large herniation of the abdominal contents at E14.5. In contrast, the wild type embryo shows only a small physiological umbilical hernia at this stage and the small intestine has largely returned to the abdominal cavity (Fig. 6D). Equivalently, all \textit{Tagln-Cre:}Tgfβr2^{flx/ft} (n=20) have normal developmental milestones similar to that of the wild type and did not show any VBW closure defect (Fig. S3 A). Using immunohistochemistry we show that there is little progression of the lateral ribs and muscles in \textit{Tagln-Cre:}Tgfβr2^{flx/flx} mice. At E13.5 we noticed a condensation of chondrogenic cells (SOX9$^+$) and skeletal muscle cells (expressing sarcomeric myosin) at the most rostral part of the secondary body wall in the thoracic and abdominal regions respectively (Fig. 6E). We detected similar pattern of expression at E14.5, where very limited progression of secondary elements has taken place over one gestational day (Fig. 6F). We propose that in \textit{Tagln-Cre:}Tgfβr2^{flx/flx} mice secondary wall component cells have lost the signalling required for their patterning and hence condensate at the junction between primary and secondary body walls. The immature primary body wall is made of epithelium and a thin layer of mesodermal cells expressing Vimentin (Fig. S3B). Still, this thin membrane maintains TAGLN expression (Fig. 6G) and preserves its TGFβ2 secretory activity (Fig. S3D). Epithelial cover of the VBW appears to be preserved in the mutant and resembles that of the wild type. In \textit{Tagln-Cre:}Tgfβr2^{flx/flx} embryos immature single layer of squamous epithelium covers the thin primary ventral wall, while, multi-layered cuboid-shaped epithelial cells cover the secondary body wall in the flanks (Fig. 6I). Similar patterning of epithelial cells is seen in the wild type embryo (Fig. S3C). In the knockout model TGFβR2 can be detected in the secondary elements areas laterally, but very little and scarce TGFβR2 is seen in the primary areas in the thoracic (Fig. S3E) and abdominal (Fig. S3F) regions. This further confirms that selective elimination of TGFβR2 in our knockout model and indicates that a minority subset of cells in the primary body wall drives ventral midline closure through TGF-β signalling. Furthermore, this highly suggests that different mechanisms and cell types are involved in the closure of the lateral and ventral body walls. Quite clearly the secondary body wall elements in the dorsal half of the body wall looked anatomically normal with distinct layers of intercostal muscles and all four lateral abdominal wall muscles layers (Figs. 6C-F). Of note, the more caudal VBW of the pelvic region closed normally and we did not observe any bladder exstrophy type of anomaly (Fig. S3G). The knockout model expressed severe cardiac and major vessels defects, as often observed in other cases of midline closure defects. The Aortic arch showed aneurysmal changes (Fig. 6B) and cardiac septal defects were also evident (Fig. 6F). Nevertheless, all mutant embryos collected until E14.5 of gestation were viable and showed active cardiac function (Movie 4). These defects most likely contributed to late embryonic fatality. The embryos did not survive beyond E15.5 and show an obvious VBW defect and intra-embryonic bleeding (Fig. S3I).
TGFβ signalling in myogenic and chondrogenic progenitors is not essential for VBW closure

We next analysed whether, in addition to myofibroblasts, the main components of the body wall – myoblasts and chondrocytes – respond to the TGF-β gradient during midline closure. Taglin is also expressed in embryonic myoblasts (Li et al., 1996) raising the possibility that our observed midline defect might arise due to TGFβR2 targeting in myogenic cells. To rule this out we crossed TgfβR2<sup>flx/flx</sup> strain to MyoD-Cre mouse line (Chen et al., 2005). MyoD is abundantly expressed in myoblasts before and at the time of midline closure in intercostal and abdominal wall myoblasts (Chen et al., 2001). Importantly, we observed no midline closure defects in the MyoD–Cre:<sup>Tgfβr2</sup> <sup>flx/flx</sup> embryos (Fig. 7A,B) and their body wall musculature was indistinguishable from wild type mice (Fig. 6C,D). Postnatally these mice had fully closed ventral midline and normally developed ventral skeletal muscles (Fig. 7C). To assess the importance of TGF-β signalling in cartilage primordium we used NG2-Cre-<sup>ER<sup>TM</sup></sup> mouse line (Zhu et al., 2011) crossed to <sup>TgfβR2</sup> <sup>flx/flx</sup> strain and administered tamoxifen at E12.5. Similarly, NG2-Cre<sup>ER<sup>TM</sup></sup>:<sup>Tgfβr2</sup> <sup>flx/flx</sup> embryos did not express any defects in midline closure (Fig. 7D-E) and the foetuses had normally developed rib cage and fused sternum (Fig 7. F). We can thus conclude that TGF-β signalling in myoblasts and chondrocytes is not necessary for ventral body wall formation.
Discussion:

We describe a population of cells in the primary VBW that orchestrates the closure of the ventral midline in mice. These pioneering myofibroblasts respond to spatio-temporal TGF-β gradient originating from the primary body wall and migrate towards the midline. We show that specific ablation of TGF-β receptor in myofibroblasts but not in myoblasts or chondrogenic cells leads to severe midline closure defect.

Tagln is expressed in a variety of developing tissues and is not a specific smooth muscle marker during embryogenesis. It is expressed in the myotome as early as E9.5 and maintained till nearly E11.5 (Li et al., 1996). At E11.5 we detected Tagln expression in the hypaxial myotome region that extends to cover the primary VBW. Later on during embryogenesis Tagln expression is confined to a narrower area in the primary body wall, besides being expressed in the blood vessels and gut wall. In situ hybridization studies at E14.5 shows confined Tagln expression in the primary midline mesenchyme in a similar pattern to our results (Diez-Roux et al., 2011). These data imply that the primary body wall originates from convergent migration of lateral somitic TAGLN⁺ mesodermal cells. In line with the migratory status of the TAGLN⁺ myofibroblasts, these cells express a number of cytoskeletal regulators that are normally found in migratory cells. This is also supported by recent studies that showed that TAGLN expression enhances migration of metastatic cells (Elsafadi et al., 2016; Yu et al., 2008; Zhou et al., 2016).

TGF-β is a known regulator of midline closure (Dünker and Krieglstein, 2002) and its localized signalling gradient is important in the development of a number of embryonic tissues (Massagué, 2012). TGFβ2 RNA is expressed in the somites from E9.5 and is later confined to the dermatome and VBW at E12.5 (Dickson et al., 1993). At later stages Tgfβ2 is expressed in the primary midline at E14.5, but not in the para midline regions (Diez-Roux et al., 2011). We show a TGFβ2 gradient in the VBW during the closure process, which peaks at E13.5 when the majority of secondary elements patterning occurs. Importantly, we detected the highest expression of the ligand in the epithelial cells, suggesting a paracrine effect in recruiting somite-derived TAGLN⁺ TGFβR2⁺ cells to the ventral midline. A similar mechanism involving TGF-β superfamily and cell motility was demonstrated in dorsal closure in the drosophila embryo. Jun-amino-terminal-kinase (JNK) activated Dpp was shown to rearrange the cytoskeleton and generate morphogenic cell changes leading to dorsal closure (Hou et al., 1997; Sluss and Davis, 1997).

TGFβR2 knockout in Tagln expressing cells leads to a thinner primary body wall and subsequent complete failure of midline closure. However, this thin primary body wall in the Tagln-Cre:Tgfbr2 flox/flox is made of ectodermal and mesodermal components and preserves TAGLN and TGFβ2 expression. In contrast, the phenotype and cellular component in other VBW closure defects, like thoracoabdominoschesis, are anatomically different and sometime lack any tissue cover to the endoderm. Considering the various complexities and severities of VBW closure defects and the effects of different genetic mutations in generating them we propose a model of successive cell-movements during ventral body wall closure. In the first cell migration wave, following embryo turning,
at E9 mesodermal cells originating from the dermatome and epithelial cells provide the first tissue cover to the embryonic endoderm. These early cells secret TGF-β and initiate a second wave of migration of myofibroblasts expressing TAGLN and TGF-β receptors. In the third wave of migration the secondary body wall elements, including progenitors of skeletal muscles and ribs, develop.

We provide here several lines of evidence to support active migration of these myofibroblasts towards the midline: we demonstrate their directed migration in an ex vivo culture model; show their elevated expression of cytoskeletal components (TAGLN, αSMA and Desmin); and reveal their high expression of TGFβR2, making them responsive to morphogen gradient. We propose that the pre-patterning of the body wall by myofibroblasts is required for the final cellular wave in the body wall development in which skeletal and myogenic progenitors reach their correct anatomical location. Indeed, elimination of TGFβR2 in myofibroblasts results in failed VBW closure, encompassing both skeletal and myogenic components. In contrast, TGFβR2 knockout in neither chondrogenic nor in myogenic cells themselves results in such defect.

Our proposed model of VBW closure may provide a logical explanation to the different anatomical configurations in different VBW defects. In thoracoabdominoschesis defects there is complete absence of any ventral tissue cover, while in our and other models of exomphalos a thin ‘sac’ covers the ventral midline (Brewer and Williams, 2004; Carnaghan et al., 2013; Dünker and Krieglstein, 2002; Eng et al., 2012). The anatomy of the defect may reflect the stage of cellular wave failure. The first cell wave is probably not TGF-β dependant, but rather more linked to epithelial factors. Knockout of pathways involved in epithelial patterning (AP-2α, Wls or β-catenin) lead to thoracoabdominoschesis defects where the ventral body cavities lack any cover and are directly exposed to the amniotic fluid (Brewer and Williams, 2004; Zhang et al., 1996; Zhang et al., 2014). Whilst in exomphalos, initial tissue cover is present and the defect results from failure of advancement of later cell waves. At the other end of the spectrum, milder defects are rather due to failure of the third cell wave. In Prune belly syndrome there is lack of skeletal muscle cover to the ventral midline and a thicker sac covers the abdominal cavity (Jennings, 2000). This indicates that several, temporally regulated, mechanisms derive ventral midline closure and the type of the VBW defect depends on the stage at which the insult took place.

Collectively, we demonstrate here that ventral midline closure relies on a dynamic TGF-β dependent recruitment of myofibroblast. Our data allow proposing a model to describe the sequential waves of cellular movements in VBW development that may explain the diversity of phenotypes in VBW closure defects.
Materials and methods:

Animals:

All mice used were housed and bread in the University of Manchester animal facility. All mice have been previously published. We used Tagln-Cre (Li et al., 1996), Pax\textsuperscript{GFP} (Relaix et al., 2005), MyoD-Cre (Chen et al., 2005) and NG2-CreERT\textsuperscript{TM} (Zhu et al., 2011) mice models. Tagln-cre mice were crossed with C57BL Rosa26 tdTom (Madisen et al., 2010) and CD1 Rosa NGZ/ LacZ (Soriano, 1999) reporter mice. Tagln-Cre: Tgfβr2\textsuperscript{flx/flx}, MyoD-Cre: Tgfβr2\textsuperscript{flx/flx} and NG2-CreERT\textsuperscript{TM}: Tgfβr2\textsuperscript{flx/flx} were obtained by crossing the Tagln-Cre, MyoD-Cre and NG2-CreERT\textsuperscript{TM} respectively to Tgfβr2\textsuperscript{flx/wt} mice (Chytil et al., 2002) and the offspring was crossbred to obtain homozygous embryos confirmed by genotyping. Recombination in NG2-CreERT: Tgfβr2\textsuperscript{flx/flx} was triggered at E12.5 by single intraperitoneal dose of tamoxifen-progesterone at (1 mg & 0.5 mg per 10g of body weight respectively). All animal work was conducted according to the Home office regulations and was approved under license no. 707435.

Immunofluorescence staining:

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS solution between 2-4 hours (depending on their gestational stage) and dehydrated in a sucrose gradient overnight. All embryos were embedded in an OCT mould snap-frozen in liquid nitrogen. 10 μm cryosections were cut with Leica cryostat (CM3050). Slides were washed in PBS, PBS with 0.2% tween (3 times 5 minutes) and then blocked in an incubation buffer (10% normal donkey serum, 1% bovine serum albumin (BSA) and 0.2% tween in PBS) for 4 hours. All slides were incubated with primary antibody overnight at 4\textdegree C. On day 2 the slides were washed with PBS-Tween and blocked with a second incubation buffer (1% BSA and 0.2% tween in PBS) for 1 hour at room temperature before adding fluorescent labelled secondary antibodies. Slides were incubated for 1 hour at room temperature with secondary antibodies specific to the primary antibody host species. Slides were washed as described above and mounted in Vectashield mounting media with DAPI (Vectorlabs). Slides were imaged using Zeiss Axio Imager M2. Zeiss Zen software was used for image analysis (tiling, counting). Whole mount IHC were described elsewhere (Merrell et al., 2015)

Antibodies:

A full list of all antibodies used is provided in supplemental Table 1.

Whole mount β-gal staining:

Fixed embryos (as above) were permeabilised in (1% Triton X and 0.4% NP40 in PBS) solution for 4 hours and incubated overnight with β-Galactosidase solution at 37\textdegree C. Embryos were imaged using Zeiss Axio Zoom microscope and Zeiss Zen software was used for image analysis.
**Alcian blue and nuclear fast red staining**

Cryosection slides (as above) were washed in PBS, PBS with 0.2% tween (3 times 5 minutes) and 3% Acetic acid (3 times 5 minutes). Alcian Blue (Sigma-Aldrich, 1% solution in 3% Acetic acid) was then added for 20 minutes. The slides were then washed off with 3% Acetic acid followed by PBS (5 minutes each) and counter stained with nuclear fast red (Sigma-Aldrich, Nuclear fast red 0.1% in 5% aluminium sulphate) for 5 minutes.

**TUNEL apoptosis staining:**

Cryosection slides (as above) underwent antigen retrieval using sodium citrate solution (1M, pH 6; boiling for 45 seconds). Cooled slides were then washed in PBS (3 times 5 minutes) and peroxidase block (3% H2O2 in PBS) for 10 minute. TUNEL (Roche) assay continued as per manufacturer protocol.

**Whole mount Alcian blue and Alizarin red staining:**

The protocol is described elsewhere (McLeod, 1980). Briefly, embryos were fixed in 95% ethanol for 24 hours, placed in acetone for 24 hours and then in staining solution for 24 hours at 37°C. The tissue was then cleared in KOH 20% glycerine for one week and stored in pure glycerine.

**Cell proliferation**

Cell proliferation of ventral body wall cells at E14.5 embryo sections was analysed using KI67 antibody. Comparison was made between Tagln-Cre-tdTom midline cells and non TAGLN expressing para-midline cells both in the abdominal and thoracic regions as shown in Figure1 (B2). Nuclei of tdTom positive and negative cells were marked in the Zen software per each image field at 20x (without KI67 staining overlay). A minimum of 100 cells from each group per section were labelled and the relative KI67 signal intensity (calculated in Zeiss Zen software) was exported into Excel for statistical analysis.

**Tissue dissociation, Fluorescence-activated cell sorting (FACS) and RNA extraction**

In Tgfβ2 gradient experiment ventral body wall area from wild type embryo was dissected as described in figure (4C) and ML and PML tissue samples were placed on dry ice during dissection. Once tissue from ≥ 5 embryos collected, TRIZOL® (Life Technologies, Railey, UK) was added and RNA extraction was conducted according to the kit protocol. In Tgfβr2 expression experiment ventral body wall area from Tagln-Cre:Rosa26-TdTom embryo was dissected as described in figure (4E). The tissue was incubated in Krebs-Ringer-Hepes (KHR) containing 2 mgml⁻¹ collagenase type 2 (Worthington), 50 µgml⁻¹ Dnase 1, 2.5 mM glucose and 5% FBS at 37°C in a shaking water bath. Dissociation of 30-minute cycles continued until the tissue was digested into homogenous cell suspension. Cells were diluted (1:5) in ice cold HBSS with 20% FBS, centrifuged (600g, 5 minutes), re-suspended in HBSS with 2% FBS and passed through 50 μm cell strainer. Cells were then sorted using BD Bioscience FACSAria for tdTom signal into tdTom positive and negative groups. Cells were...
collected directly into TRIZOL® LS (Life Technologies, Railey, UK) and RNA extraction was conducted according to the kit protocol.

**RT-qPCR:**

Extracted RNA (as above) was re-suspended and quantified with Nanodrop 2000. cDNA was synthesized using Superscript IV kit (ThermoFisher) and 20 ng of cDNA were used for PCR amplification. Primers used were: 5'-GAACGACAAGAACATTACTCTGGAG-3' (forward) and 5'-GATGTCCTTCTCTGTGTTCACGA-3' (reverse) for Tgfβr2 amplification. 5'-TCGACATGGATCAGTTATGCG-3' (forward) and 5'-CCCTGGTACTGTTGTAGATGGA-3' (reverse) for Tgfβ2 amplification. Roche Lightcycler 96 and FastStart Essential DNA Green Master were used for all qPCR experiments (in biological and technical triplicate). Absolute quantification of each target was performed using a standard curved as a reference in Roche LightCycler software version 1.5.

**Time-lapse confocal laser scanning:**

E11.5-E14.5 Tagln-Cre:Rosa26-tdTom or Tagln-Cre: Rosa26-tdTom::PAX3-GFP embryos were beheaded and the trunk full circumference was maintained. The body wall explant was placed on a glass bottom 35 mm tissue culture disc (WPI, FD3510-100), stabilised with phenol red free growth factor reduced matrigel (Corning®, 356231) standard cell culture media (DMEM & 10% fetal bovine serum) was added and then cultured between 6-16 hours. The Explants were imaged in a laser confocal microscope system (Leica SP8 Inverted gSTED) maintained at 37°C and 5% CO2 with a humidifier. Z slices were acquired using ×10 objective every 10-20 minutes. Four-dimensional data sets were analysed with Leica confocal and Bitplane/ Imaris software.

**Statistics**

Quantitative data are presented as mean and standard error. Two-tailed Student T-test was used for statistical analysis and p value <0.05 was set significant.
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Competing interests:

The authors declare no conflict of interest regarding this manuscript.

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Figure 1: *Tagln*-Cre expression in the ventral midline and mitotic activity of TAGLN+ cells. Cross section levels are shown in D. A: Cross-section at a thoracic level in an E12.5 WT embryo stained for TAGLN showing gross expression in the primary VBW (area between arrows). B: Cross-
section at an abdominal level in an E13.5 WT embryo stained for TAGLN showing expression in the primary abdominal wall (area between arrows) that is circulating the umbilical hernia. C: Whole mount β-gal staining in Tagln-Cre: R26-NGZ at three embryonic stages. The expression of TAGLN is evident in the somite at E11.5 and localises to the midline area when ventral body wall closure is complete. D: D1: The lines (1, 2 & 3) indicate the cross section level in (A, B, E, F, G & H). 1: A, E & H; 2: B, F and 3: G. D2:) schematic of midline (red) and para-midline (grey) areas presented in Ki67 analysis in H. E-F: Expression of Tagln-Cre:Rosa26-tdTom in the thoracic (E) and abdominal (F) ventral midline over 4-day time window during the closure process and at postnatal day 20 (P20). TAGLN expression becomes restricted to the midline area with advanced gestation and this expression is maintained postnatally. Inset in E15.5 shows high magnification of midline, (^): internal mammary/ superior epigastric vessels and (‘) xiphisternum. G: TUNEL apoptosis assay in the ventral midline at E15.5. No obvious pattern of apoptosis in Tagln derived cells in the midline. Boxes show examples of individual cells expressing TUNEL in the midline and para-midline areas. H: Ki67 staining of the ventral midline at E14.5. Primary body wall remnant at this stage shows limited mitotic activity evident in the Ki67 individual channel. I: Comparison of Ki67 expression between midline primary VBW cells (tdTom+) and para midline secondary body wall cells (tdTom−) in the thoracic and abdominal regions. Comparison made on 200 cells from three different sections at each level, data presented as (mean±s.e.m.), asterisks above connected lines illustrate two-tailed t-test between primary and secondary body wall elements: ** p<0.001. H: heart, L: lungs, LV: liver, UH: umbilical hernia. Scale bars: A, B, F, 500 µm; C & P20 in (E-F) 1000 µm; E, G, H 200 µm.
Figure 2: Directional migration of the TAGLN$^+$ cells towards the ventral midline. Snapshot panels from 9 hours time lapse of ex vivo body wall explant culture. The VBW is located at the right side of the panel and dorsally located tdTom$^+$ cells (arrow) are seen in the left top corner of the panel. 

A: still image at time zero. B: Start of time lapse, each tdTom$^+$ cell centre is labelled with a grey square and the path and time course of the journey is marked with a colour coded line. C: At 3 hours VBW cells show directional migration towards the ventral midline, while dorsal cells show little change in position. D, E: 6 and 9 hours respectively, midline directional migration continues in VBW cells. F: Trajectories and journey length in the analysed cells, the arrow points towards the migration pathway and its length reflects the travelled space. VBW cells show consistent directional migration towards the midline compared to the dorsal cells. Scale bars: 100 µm, time of each panel is shown in the right lower corner.
Figure 3: TAGLN protein expression in the ventral midline during the closure process, in the thoracic (A-C) and abdominal (D-G) VBWs. All panels are from Tagln-Cre:Rosa26-tdTom embryo cross sections stained with TAGLN antibody; individual channels as named. A: Cross section at E12.5 showing the left primary body wall, complete overlap between Tagln-Cre (tdTom) and TAGLN signals is seen. B: At E14.5 there is still near complete overlap between the tdTom and TAGLN
signals. **B1**: Magnified view of the closing midline (boxed area in B) and individual channels.  **C**: At E16.5 the thoracic midline has completely closed. The tdTom signal is still seen as a narrow line in the midline, but TAGLN signal cannot be identified. **C1**: Confocal image of the midline (boxed area in C) showing fine line of tdTom cells that have now become completely negative for TAGLN. **D**: The abdominal ventral midline at E12.5, Tagln-Cre and TAGLN signals show complete overlap. **E**: At E13.5 the Tagln-Cre derived cells (tdTom⁺) of the primary body wall still express TAGLN. **F**: At E14.5 the TAGLN signal area in the primary ventral midline is restricted compared to the tdTom signal area of the Tagln-Cre cells. **G**: The ventral midline, labelled by tdTom, at E15.5 (same level of F) has largely downregulated TAGLN. **G1**: Confocal image of the midline (boxed area in G) showing tdTom cells of the midline have now become completely negative for TAGLN. **H**: heart, LV: Liver, IN: intestine. Scale bars: 200 µm- C1 & G1 (25 µm).
Figure 4: Characterisation of the ventral midline cells in *Tagln-Cre:Rosa26-tdTom* throughout VBW closure time points. Cross-section levels are shown in E. Expression of smooth muscle contractile proteins (A-D, H) in the primary wall is more evident at early stages of midline closure. A: αSMA and Vimentin are grossly expressed in the thoracic primary body wall at E12.5 and correlate with tdTom signal. Insets are magnified view (at cellular level) of the boxed area showing merged and individual channels. B: At E14.5 primary body wall cells labelled by tdTom are still strongly positive for
Vimentin and express smooth muscle intermediate filament protein Desmin. Insets are magnified view (at cellular level) of the boxed area showing merged and individual channels. C: E15.5 midline cells express fibroblast marker ER-TR7, inset represents a magnification of the ventral midline in the box. D: When the thoracic midline is fully closed at E16.5 the residual primary midline cells still labelled by tdTom have now downregulated αSMA. Inset illustrates magnification (at cell level) of the midline in the box, only a small number of cells (arrow) of the midline show expression of αSMA. E: The lines (1 & 2) indicate the cross section level. 1: A-D, F-G. 2: H-J. Tendon markers are absent in the primary body wall (F-G). F: Tendon marker Tenascin-C is expressed at E13.5 around rib primordium and just lateral to primary elements (bottom box), while sporadic low-expression is seen in the primary body wall (top box). G: At E14.5 no Tenascin-C expression is seen in the primary body wall in the midline. Sternal primordium cells express Tenascin-C and are seen encircling the primary body wall cells. Insets are magnified view (at cellular level) of the boxed area showing merged and individual channels. Abdominal primary body wall is made of myofibroblasts (H-J). H: Cross-section in the abdominal midline at E14.5, primary body wall cells express Vimentin and Desmin. Insets are magnified view (at cellular level) of the boxed area showing merged and individual channels. I: At E15.5 the cells of the abdominal midline express fibroblast marker ER-TR7, magnification in inset. J: At E16.5 the ventral midline has fully closed and resident tdTom+ cells are seen in the midline. Tenascin-C expression can be detected in the edges of the falciform ligament, but not at the midline (magnification in inset). Scale bars: 100 µm.
Figure 5: TGF-β2 and TGFβR2 in the VBW. A: Cross-section in the abdominal VBW at E14.5 showing wide expression of TGFβR2 focused in the primary body wall area (labelled by tdTom) in the ventral midline. A1: Confocal image from the box area in (A) showing high TGFβR2 expression in tdTom+ cells beneath the epithelium. B: Cross-section in the mid thoracic area at E12.5 Tagln-Cre:Rosa26-tdTom mouse embryo stained for TGFβ2 and E-cadherin to label epithelium. TGFβ2 protein is abundant in the midline area of the primary body wall (tdTom channel is removed to elucidate TGFβ2 signal). B1: confocal image of the primary body wall area (box P) showing strong TGFβ2 expression in the epithelium (arrows) and less strong signalling in the subdermal layer (arrowhead). B2: confocal image of the secondary body wall area (box S) showing weak TGFβ2 signal in the subdermal layer (arrows). C: Schematic figure of midline (ML) and para-midline (PML) ventral wall dissection in an E12.5 WT mouse embryo. C1: The embryo was decapitated and the tail
was excised. **C2**: The dorsal body wall was opened para-sagittal and the thoracic and abdominal organs were exposed. **C3**: The embryo was eviscerated taking care to preserve the thin primary body wall. **C4**: The thin primary (midline) body wall was carefully dissected from the secondary (para-midline body) wall and safe margins were removed from both segments to avoid transitional areas. **D**: RT-qPCR comparing *Tgfβ2* expression in the ML and PML of WT mouse embryos between E11.5 to E15.5. There is an anatomical and temporal *Tgfβ2* gradient in the midline during the closure period. Error bars presented as (s.e.m.), each time point presented is from at least 3 biological replicates each containing tissue from ≥5 embryos. **E**: *Tagln*-Cre:Rosa26-tdTom cell sorting and RT-qPCR for *Tgfβr2*. **E1**: Schematic of E14.5 embryo, the VBW shown in the dotted box was dissected from *Tagln*-Cre:Rosa26-tdTom embryos and FACS sorted for tdTom signal. **E2**: The FACS sorted cohort; tdTom+ cells only accounted for an average of 15% of the total cell population of the VBW (as shown in E1). **F**: RT-qPCR on the FACS sorted cells showed higher expression of *Tgfβr2* in *Tagln* Cre-tdTom ventral midline cells. Error bars presented as (s.e.m.), data presented is from 3 biological replicates each containing cells from tissue derived from ≥7 embryos. Asterisks (in D-F) above connected lines illustrate two tailed t-test, ** p<0.001, * p<0.05, NS: non-significant. H: heart, P: primary and S: secondary body walls. Scale bars: 200 µm & 10 µm in insets. Each experiment shown above was repeated at least three times.
Figure 6: Tagln-Cre:Tgfβr2^flx/flx develops VBW closure defect. Morphological comparison between 
Tagln-Cre:Tgfβr2^flx/flx and Tagln-Cre:Tgfβr2^flx/wt (A-B). A: E13.5 Tagln-Cre:Tgfβr2^flx/flx embryos shows 
translucent ventral midline, more lateral limit of secondary body wall (arrow) and absence of midline 
rhaphe (arrow head) when compared to Tagln-Cre:Tgfβr2^flx/wt. B: The ventral midline closure defect in 
Tagln-Cre:Tgfβr2^flx/flx is apparent. A thin membrane covers the ventral body wall cavities compared to 
the nearly closed thoracic midline in the WT (arrow) and the embryos show a large exomphalos
compared to the physiological umbilical hernia in the WT (arrowhead). C: Cross-section in mid-thorax at E14.5 in WT (left) and Tagln-Cre: Tgfβr2\textsuperscript{flx/flx} (right). Alcian blue staining to delineate ribs and counter staining with nuclear fast red. The VBW is made of a thin sac in the mutant, while the two lateral sternebrae are nearly meeting in the midline in the WT. D: Cross-section at level of umbilical hernia at E14.5 in WT (left) and Tagln-Cre: Tgfβr2\textsuperscript{flx/flx} (right), Alcian blue staining to delineate ribs and counter staining with acid fast red. In the WT only a small physiological umbilical hernia is present and the small intestine are returning to the abdominal cavity, while the mutant shows a large exomphalos defect and very few bowel loops are present in the abdominal cavity. Characterisation of cell type in Tagln-Cre: Tgfβr2\textsuperscript{flx/flx} thoracic (right panel) and abdominal (left panel) body wall using IHC (E-H). E: E13.5 mutant embryos show normal lateral body wall muscles (MF20\textsuperscript{+}) and ribs (SOX9\textsuperscript{+}), whilst the ventral midline is made of a thin sac. Condensations of SOX9\textsuperscript{+} and MF20\textsuperscript{+} cells (arrow) are seen just lateral to the VBW in the thoracic and abdominal areas respectively. F: E14.5 mutant embryo show very little progression in secondary elements migration and the condensation of chondrocyte and myocyte (arrow) is still seen lateral to the ventral body wall in the thoracic and abdominal compartments respectively. G: The VBW of Tagln-Cre: Tgfβr2\textsuperscript{flx/flx} still expresses TAGLN. I: The skin covering the premature VBW in Tagln-Cre: Tgfβr2\textsuperscript{flx/flx} is made of a single layer of squamous epithelial cells (insets P), while in secondary elements multi-layered cuboid epithelium covers the lateral body wall (insets S). H: Heart, L: Lungs, LV: Liver, IN: intestine, P: primary body wall, S: secondary body wall, TA: transverses abdominis, IO: internal oblique, EO: external oblique, PC: panniculus carnosus, IC: intercostal muscles, R: Rib. Scale bars: A-B, 1000 µm; C,-G 500 µm; H, 200 µm & insets 50 µm.
Figure 7: TGFβR2 knockout in myogenic and chondrogenic cells does not affect midline closure. A,B: Cross-section in the thoracic and abdominal region of an E14.5 MyoD-Cre: Tgfβr2^{flx/flx} embryo. A: Alcian blue (AB) and nuclear fast red (NFR) staining show normal developmental milestone comparable to that of the WT (Fig. 6C,D). B: Normal muscle (MF20^+) and chondrocyte (NG2^+) development in the midline area of the mutant mouse. C: Whole mount MF20 staining of a 2 day old pup. Normal muscle development in the midline postnatally, umbilicus site is marked with a dotted circle. D,E: Cross-section in the thoracic and abdominal region of an E15.5 NG2-CreER:Tgfβr2^{flx/flx} embryo. D: Alcian blue (AB) and nuclear fast red (NFR) staining show normal developmental milestone comparable to that of the WT. E: Normal muscle (MF20^+) and chondrocyte (NG2^+) development in the midline area of the mutant mouse. F: Whole mount Alizarin red and Alcian blue showing normal rib cage development and fused sternum in the midline at the fetal stage. Scale bars: A, B, D, E 500 µm; C, F 1000 µm.
Supplemental figure 1: Tagln-Cre expression in the ventral midline and apoptosis of TAGLN<sup>+</sup> cells.  
A: Abdominal ventral wall of Tagln-Cre:Rosa26-tdTom adult mouse, fixed perfused with 4% PFA, dissected and scanned in the stereomicroscope. The ventral midline evidently expresses tdTom even at adult life, umbilical cicatrix is marked with an arrow. B: TUNEL apoptosis staining of a cross section of the lateral body wall of E16.5 Tagln-Cre:Rosa26-tdTom mouse embryo, the only apparent pattern of apoptosis in tdTom<sup>+</sup> cells is noted in the periphery of the ribs (arrows), individual channels are shown. C: Higher magnification of cross section of the rib from E15.5 Tagln-Cre:Rosa26-tdTom mouse embryo showing apoptosis in tdTom<sup>+</sup> cells in the peripheral area of the rib (arrows), individual channels are shown. S: skin, D: dermis, IC: intercostal space, P: pleural space. Scale bars: A, 1000 µm; C 100 µm; D, 50 µm.
Supplemental figure 2: The spatial narrowing of the tdTom signal is due to TAGLN cells migration.

A: E11.5 Tagln-Cre:Rosa26-tdTom:PAX3-GFP mouse embryo, the dotted box represents the area of the time lapse. B: Confocal microscopy 3D reconstruction of the time lapse area (in A) showing the dorsal neural tube as well. The neural tube and the somite are labelled by GFP and tdTom signal is present in the ventral body wall area and the area dorsal to the somite. C: Ex-vivo confocal microscope scanning of the embryonic primary ventral midline. C1: The trunk of E14.5 Tagln-Cre:Rosa26-tdTom mouse embryos were isolated in full circumference and eviscerated, the area marked in the dotted box were scanned in a confocal microscope. C2: Higher magnification of the confocal scanned area, the residual primary midline borders are represented in the dotted white shape. C3: 3D reconstruction of the tdTom+ cells of the ventral primary body wall (shown in C2). These cells take a pyramidal shape wider at the caudal end and have a maximum depth of 200 microns. C4: Caudal view of the 3D reconstruct from C3 showing again a pyramidal shaped cell mass along the dorso-ventral axis. Scale bars: A, 500 µm; B, 200 µm; C1, 1000 µm; C2-4, 200 µm.
Figure S3

A
Tagln-Cre:Tgfβr2^{flx/wt}\ E14.5

B
Tagln-Cre:Tgfβr2^{flx/flx}\ E14.5

Vimentin / Claudin-1 / DAPI

C
E-Cadherin / DAPI

D
E-CAD / TGFβ2 / DAPI

E

F

G
Tagln-Cre:Tgfβr2^{flx/flx}\ Wild Type

H

I

E13.5

E12.5

E14.5

E15.5

Development 144: doi:10.1242/dev.152843: Supplementary information
Supplemental figure 3: Characterization of the ventral midline defect in *Tagln-Cre:Tgfβr2<sup>flx/flx</sup>*. A: Cross-sections of E14.5 *Tagln-Cre:Tgfβr2<sup>flx/wt</sup>* thoracic (top panel) and abdominal (lower panel) regions stained with Alcian blue and counter stained with nuclear fast red. *Tagln-Cre:Tgfβr2<sup>flx/wt</sup>* littermates do not express ventral midline defect phenotype and has similar anatomical configuration of the WT. At E14.5 the sternal primordial is nearly closing in the midline and only a small physiological umbilical hernia is seen in the abdomen. B: Sagittal section of E14.5 mutant mouse and magnified inset with individual channels. The thin primary ventral body wall expresses mesodermal and fibroblast marker Vimentin. However, the epithelium (arrow in inset) of this primary wall is immature and does not express the tight junction protein Claudin-1. C: Cross-section at lower thoracic level of E13.5 wild type embryo stained for E-Cadherin. Magnified insets from a primary (P), secondary (S) and junctional (J) ventral body wall areas marked in the boxes and separate channel for E-Cadherin is shown to the right. The epithelium covering the primary body wall (P) is made of a single layer of squamous epithelium, while the flank areas show multi-layered cuboid epithelium. The junction between the primary and secondary areas (J) shows a transition pattern between the two made of two to three layers of epithelial cells. D: Cross section at thoracic level of E12.5 *Tagln-Cre:Tgfβr2<sup>flx/flx</sup>* embryo stained for TGFβ2 and E-cadherin showing preserved TGFβ2 expression in the primary body wall of the mutant. E-F: Cross section of the thoracic (C) and abdominal (D) body wall of E13.5 *Tagln-Cre:Tgfβr2<sup>flx/flx</sup>* embryo stained for TGFβR2. Magnified insets from primary (P) and secondary (S) ventral body wall areas marked in the boxes and the oblique line resembles the border between the primary and secondary body walls. Individual TGFβR2 channel is shown on the right. TGFβR2 is present in the secondary body wall area, but no signal is seen in the primary body wall confirming the selective knock out in our model. G: Cross-sections in the lower abdominal regions at E14.5 in mutant and WT embryos stained with Alcian blue and counter stained with nuclear fast red. The lower abdominal body wall in the mutant is normally developed and no signs of bladder extrophy type of anomaly are seen. I: E15.5 *Tagln-Cre:Tgfβr2<sup>flx/flx</sup>* embryo looks small for gestation and shows signs of subcutaneous haemorrhage in the back, limbs and genital tubercle. Large exomphalous anomaly is apparent with the umbilical cord situated at the centre of the defect (arrow). H: heart, L: lung, LV: liver, R: rib, IN: intestine, K: kidney, UB: urinary bladder. Scale bars: A,B,F,G 500 µm; C,D,E 200 µm; insets in (B,C,D,E): 50 µm.
Movie 1: VBW TAGLN cells migrate towards the ventral midline. Nine hours time-lapse of E11.5 Tagln-Cre:Rosa26-tdTom;PAX3-GFP body wall explant. The location corresponding to the video is marked in (Fig. S2A). Td-Tom$^+$ cells of the primary body wall migrate ventrally towards the midline, while dorsal tdTom$^+$ cells showed little movement and on a different axis. The myofibroblast of the primary body wall are located more ventrally to the PAX3 GFP$^+$ cells of the somite. Many of the primary body wall myofibroblasts (ventral to the somite) are showing weak tdTom signal (due to short time since Cre activation and hence little accumulation of the tdTom protein) but can be seen displacing ventrally as well. The centre of each tracked cell is labelled with a grey sphere and the path and time course of the migration track is shown in a “dragon tail” style. Each cell drags the migration path behind, the migration track is shown in a gradient rainbow-coloured line where blue represents position at the start of the time-lapse and “red” the end position. Time between frames is 10 minutes and movie played at 6 frames per second.
Movie 2: VBW TAGLN cells show active locomotion. Cell surface rendering of the TdTom* cells during E11.5 Tagln-Cre:Rosa26-tdTom ex-vivo body wall explant time-lapse. The cells are migrating towards the ventral midline (lower left to upper right in the video) and display cell protrusions and lamellipodia formation. Time between frames is 10 minutes and movie played at 4 frames per second.
Movie 3: VBW TAGLN cells continue convergent migration. Confocal microscopy time-lapse depth-analysis 3D-reconstruction of the thoraco-abdominal junction at E14.5 of Tagln-Cre:Rosa26tdTom ex-vivo body wall culture. The primary VBW labelled by TdTom was topographically analysed and time-lapsed over 10 hours period. Ventral cells (closer to the microscope objective) are labelled by the software in red colour and the deeper the cell the more “cold colour” is given, as shown in the scale bar. The software reflects change in cell position along the dorso ventral axis by colour change from the blue to the red spectrums. The primary VBW in this area takes a conical shape wider at the caudal end and during time-lapse the tdTom cells are moving in a dorso ventral fashion along the cranio-caudal axis. Time between frames is 20 minutes and movie played at 6 frames per second. Scale bar: 200 µm.
Movie 4: *Tagln-Cre:Tgfβr2^flx/flx* mutant embryos are viable at E14.5. Stereo microscope time lapse of E14.5 *Tagln-Cre:Tgfβr2^flx/flx* embryo freshly collected showing active cardiac output and filled peripheral vascular space.
| Antibody     | Host   | Source        | Conc   | Product No. | Lot No.       | Dilution | Ref                                      |
|--------------|--------|---------------|--------|-------------|---------------|----------|------------------------------------------|
| TAGLN (αSM22) | Rabbit | Abcam         | 1 mg/ml| AB14106     | GR274712-2   | 1:100    | (Shang et al., 2008)                     |
| TAGLN (αSM22) | Goat   | Abcam         | 0.5 mg/ml| AB10135    | GR1926-4   | 1:50     | (Speer et al., 2010)                    |
| αSMA         | Goat   | Abcam         | 0.5 mg/ml| AB21027    | GR230733-4 | 1:200    | (van Gils et al., 2012)                 |
| Claudin-1    | Rabbit | Life technologies | 100 ug/400ul | 717800   | PI208774   | 1:200    | (Xu et al., 2005)                       |
| Desmin       | Rabbit | Sigma         | -      | D8281       | 101M478     | 1:30     | (Matar et al., 2015)                    |
| E-cadherin   | Rat    | Abcam         | 100 ug | AB11512     | GR272810-2  | 1:300    | (Tang et al., 1994)                     |
| ER-TR7       | Rat    | Abcam         | 100 ug | AB51824     | GR212403    | 1:100    | (Hu et al., 2011)                       |
| KI67         | Rabbit | Abcam         | 100 ug | AB15580     | GR92689-1   | 1:200    | (Adolph et al., 2013)                   |
| MF20         | Mouse  | DSHB          | -      | AB21477     | 81          | 1:3      | (Bader et al., 1982)                    |
| NG2          | Rabbit | Millipore     | 100 ug | AB 5320     | 2669913     | 1:300    | (Bondjers et al., 2003)                 |
| RFP          | Rabbit | Rockland      | 100 ug | 600-401-379 | 34944       | 1:500    | (Achim et al., 2012)                    |
| SOX-9        | Rabbit | Millipore     | 100 ug | AB5535      | 1969702     | 1:200    | (da Silva et al., 1996)                 |
| Tenascin-C   | Rabbit | Millipore     | 50 ug  | AB19013     | 2828619     | 1:150    | (Fei et al., 2014)                      |
| TGFβ2        | Rabbit | Abcam         | 0.5 mg/ml| AB 66045   | GR129421-1 | 1:100    | (Florea et al., 2013)                   |
| TGFβR2       | Rabbit | Novus Bio     | 0.1 mg/ml| NB100-91994| GN1151     | 1:100    | (Phatnani et al., 2013)                 |
| Vimentin     | Chicken| Novus Bio     | -      | NB300-223  | 1-0104     | 1:300    | (Okawa et al., 2007)                    |
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