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Traffic Jam in the Primitive Streak: The Role of Defective Mesoderm Migration in Birth Defects

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Abstract

Gastrulation is the process in which the three germ layers are formed that contribute to the formation of all major tissues in the developing embryo. We here review mouse genetic models in which defective gastrulation leads to mesoderm insufficiencies in the embryo. Depending on severity of the abnormalities, the outcomes range from incompatible with embryonic survival to structural birth defects, such as heart defects, spina bifida, or caudal dysgenesis. The combined evidence from the mutant models supports the notion that these congenital anomalies can originate from perturbations of mesoderm specification, epithelial–mesenchymal transition, and mesodermal cell migration. Knowledge about the molecular pathways involved may help to improve strategies for the prevention of major structural birth defects.

Keywords

gastrulation; mouse mutant; cell migration; epithelial-mesenchymal transition; cell accumulation; basement membrane; Fgf signaling; Wnt signaling; cell adhesion; endoderm; neuroepithelium; spinal cord; neural tube defect; caudal regression; VACTERL; maternal diabetes

Introduction

The most frequent neural tube defect in humans is spina bifida, the failure of the neural tube to close in regions of the trunk. Mutant mouse strains have been used extensively as experimental animal models for human neural tube defects. In contrast to humans, however, the fraction of mouse mutants that exhibit spina bifida is small (Harris and Juriloff, 2007). Among some 200 mutant strains, Harris and Juriloff identified ~5% with only spina bifida, and ~20% that can have spina bifida and exencephaly, or both (Harris and Juriloff, 2007). We noticed that in some mutant models with neural tube defects, the closure defect was preceded by abnormalities in mesoderm development that, in severe cases, manifested as ectopic accumulation of cells in the primitive streak. Examples are mutants for FGF receptor 1 (*Fgfr1*$^{-/-}$) (Deng et al., 1994; Yamaguchi et al., 1994; Guo and Li, 2007) and mutants

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for Protein tyrosine phosphatase 11 (*Shp2*$^{-/-}$) (Saxton et al., 1997), as well as chimeras of *Fgfr1*$^{-/-}$ (Ciruna et al., 1997) or *Shp2*$^{-/-}$ (Saxton and Pawson, 1999) mutant with wild-type cells. Because we observed related phenotypes in embryos from diabetic pregnancies (Salbaum et al., manuscript submitted), we sought to identify additional mouse mutants with similar gastrulation defects. Of those mutants that survive long enough, many exhibit neural tube defects, implicating abnormal gastrulation as a cause of neural tube defects (NTDs) in the mouse. Other malformations common to these mutants include heart defects and caudal growth defects. The evidence from these mutants implicates particular pathogenic cellular and molecular pathways and highlights the importance of gastrulation processes for the pathogenesis and potential prevention of neural tube and other structural birth defects.

Gastrulation

Gastrulation in vertebrate embryos leads to the generation of the three primary germ layers ectoderm, mesoderm, and endoderm. It is a process that is highly dynamic as well as complex in terms of space, time, cellularity, and the interplay of molecular factors. In the mouse, gastrulation begins with the induction of the primitive streak in the posterior region of the embryo (Tam and Gad, 2004), which also coincides with final determination of the anterior–posterior axis and initial establishment of the body plan. Induction and localization of the primitive streak are influenced by signals from the anterior visceral endoderm (Ramkumar and Anderson, 2011). The primitive streak begins to extend in an anterior direction, culminating with the formation of the node, which then retreats together with the primitive streak toward the caudal pole of the embryo. During gastrulation, epiblast cells move toward the organizing center, i.e., either the primitive streak or the node, where they ingress. With ingression comes fate specification; the classical view was that mesoderm fate was the result, yet, in the mouse it is now accepted that a large part of the definitive endoderm is also derived from gastrulating cells (Lickert et al., 2002). When and where cells ingress into the organizing center affects their fate: movement into the early primitive streak results in heart mesoderm, prechordal mesoderm, and cranial mesoderm; subsequent ingression creates lateral plate mesoderm and paraxial mesoderm, whereas ingression in the node results in formation of the axial mesoderm of the notochord (Tam et al., 1997). Similar temporal dependencies exist for region-specific endodermal fate (Zorn and Wells, 2009).

In the mouse, cells move individually toward and into the primitive streak (Williams et al., 2012). Cells lose contact with the basal lamina as they reach the primitive streak. As they ingress, cells undergo epithelial–mesenchymal transition (EMT) (Chuai et al., 2012). Altered adhesion properties together with cytoskeletal rearrangements facilitate movement through and eventually out of the primitive streak (Ichikawa et al., 2013). As node and primitive streak recede toward the posterior end of the embryo, they leave behind a germ layer organization that consists of (i) an ectodermal layer set to enter neurulation along the midline of the embryo, (ii) axial mesoderm in the form of the notochord underlying the midline, and wings of paraxial and lateral mesoderm, and (iii) a layer of definitive endoderm. In this manner, gastrulation sets the stage for the next big step in development: formation of the central nervous system through neurulation.

Mutants with Perturbed Gastrulation

The common feature of the mutants we review here is the appearance of ectopic cell accumulations in the primitive streak that protrude from the streak as a bulge (Fig. 1). The identity of the accumulated cells, in the vast majority of cases, has been determined to be mesodermal, although they are often covered with a layer of epithelial cells. Tables 1 and 2 shows that such primitive streak abnormalities during gastrulation are associated with manifestation of spina bifida, heart defects, and caudal growth defects at later stages of development. The collection of mutants that display the ectopic cell accumulations allows us to identify the disrupted molecular pathways that cause defects in the formation of mesoderm, epithelial to mesenchymal transition (EMT), and cell migration.

WNT-ß-CATENIN SIGNAL TRANSDUCTION PATHWAY—The appearance of the primitive streak is marked by expression of *Nodal* and *Wnt3* in the posterior region of the embryo. The restricted localization is due to inhibitory signaling from the anterior visceral endoderm (Perea-Gomez et al., 2002; Ben-Haim et al., 2006; Egea et al., 2008; Stuckey et al., 2011). Ablation of Nodal activity is associated with loss of primitive streak formation, although some cells with expression of mesodermal markers have been observed in these mutants (Conlon et al., 1994; Robertson, 2014). Similarly, loss of Wnt3 results in absence of the primitive streak (Liu et al., 1999) and of *Nodal* expression. The requirement of the canonical Wnt/ß-catenin signaling pathway for primitive streak formation is also highlighted by absence of the primitive streak in ß-catenin null mutants (Huelsken et al., 2000), and in mutants with combined disruption of *Lrp5* and *Lrp6* (Kelly et al., 2004). Thus, the Wnt3 and Nodal signal transduction pathways are critically involved in the early stages of gastrulation, in formation of the primitive streak.

Furthermore, Wnt3 null mutants lack expression of *T/Brachyury*, a marker for nascent mesoderm in the primitive streak (Liu et al., 1999), suggesting that the induction of mesodermal cell fate is dependent on Wnt3. This was shown by epiblast-specific knockout of *Wnt3*, through the use of the epiblast-specific *Sox2-cre* transgene. In this way, the contribution of signaling from the posterior visceral endoderm, which is also a site of *Wnt3* expression (Rivera-Perez and Magnuson, 2005), can be distinguished from signaling in epiblast cells, the precursors for the embryo proper. Embryos which lack Wnt3 specifically in the epiblast were reported to phenocopy the Wnt3 null mutants at E6.5 and to lack mesodermal derivatives (Barrow et al., 2007). Intriguingly, they display accumulation of cells that bulge into the amniotic cavity; beyond being of epiblast origin, the identity of these cells has not been established. Such ectopic cell accumulations were also found by another laboratory (Tortelote et al., 2013), which demonstrated that the cells did not express *T/Brachyury*, and thus were unlikely to be of mesodermal nature. In contrast to the earlier report, however, these authors detected *T*-expressing cells in what appeared to be shortened primitive streaks in epiblast-deleted *Wnt3* mutant embryos at E7.5, calling into question whether induction of mesoderm requires epiblast-derived Wnt3, or is simply delayed in these mutants. Yet, the inability to complete gastrulation, and the consequent lethality of these embryos by E9.5, indicate that potentially remaining extraembryonic sources for Wnt3 are not sufficient to maintain proper mesoderm formation. This is underscored by the

absence, in these Wnt3 mutants (Tortelote et al., 2013), of expression of *Axin2*, a direct target and known inhibitor of Wnt signaling (Jho et al., 2002).

In *canopus* mutants, the Axin2 protein is altered near the N-terminus, resulting in increased protein stability (Qian et al., 2011). This would be predicted to result in greater inhibition of Wnt signaling (Zeng et al., 1997; Yamamoto et al., 1999; Huang et al., 2009). Consistent with this expectation, activity of the TOPGAL reporter, which responds to Wnt wignaling (Maretto et al., 2003), was reduced at E7.25, and expression of *Tbx6*, a marker of nascent mesoderm, was reduced by E7.5. Yet, the mutants exhibited close to normal numbers of somites, indicating that presomitic mesoderm was formed. Intriguingly, $Axin2^{canp}$ mutants displayed ectopic protrusions from the midline of the neural plate. Mesodermal identity of these cells was established by expression of mesodermal markers *T* and *Meox1*. Pharmacological stabilization of Axins was associated with increased phosphorylation of Lrp6 in the posterior region of the embryo (Qian et al., 2011).

Intriguingly, cell accumulations in the primitive streak are also found in embryos with homozygous disruption of *Lrp6* in the presence of a single functional allele for *Lrp5* in heterozygous configuration ($Lrp5^{+/-}$;$Lrp6^{-/-}$) (Kelly et al., 2004). *T* expression was present in some accumulated cells, but *Tbx6* was absent, as were somites, indicating that paraxial mesoderm, which is derived from the posterior primitive streak, is nevertheless deficient in these embryos; in some mutant embryos, the posterior region remained undifferentiated. Embryos that are only deficient in Lrp6 ($Lrp6^{-/-}$) exhibit heart defects (Song et al., 2010), posterior mesoderm is reduced or absent (Pinson et al., 2000), and the neural tube fails to close anteriorly (Bryja et al., 2009) or posteriorly (Pinson et al., 2000). When the *ringelschwanz Lrp6* allele was combined with a *Lrp6* null allele, offspring displayed spina bifida (Kokubu et al., 2004); combination of the *crooked tail Lrp6* allele with the null allele yielded exencephaly with incomplete penetrance (Carter et al., 2005). It was recently shown that Lrp6 can also affect noncanonical pathways during neurulation (Gray et al., 2013) in addition to its role in canonical Wnt signaling.

In mutants deficient for Tcf3, a known transcriptional effector of Wnt signaling, several mesodermal defects were found (Merrill et al., 2004): in severely affected embryos, the anterior region was truncated, and somites and heart were absent at E8.5, while they were present in more mildly affected embryos. In addition, duplicated primitive streaks, and supernumerary nodes and notochords, or split notochords were found. Because anterior visceral endoderm (AVE) markers are expressed normally in mutant embryos, the axis duplications are likely not due to AVE abnormalities (Merrill et al., 2004). Thus, $Tcf3^{-/-}$ embryos form excess axial mesoderm, as evidenced by an expanded expression of *T/Brachyury*.

The *T/Brachyury* gene is a direct target of Wnt signaling (Yamaguchi et al., 1999; Arnold et al., 2000). Deletion of the *T* gene leads to defective primitive streaks, defective notochords and failure of axis elongation (Wilkinson et al., 1990). In chimeras with wild-type cells, *T/T* cells accumulate preferentially in the posterior primitive streak, the site of strongest *T* expression. Because the accumulated *T/T* cells adhere to one another rather than mix with wild-type cells, the authors conclude that the defect is one of adhesion (Wilson et al., 1995),

and that it occurs in cell-autonomous manner during epithelial to mesenchymal transition. In some of the chimeric embryos, open neural tubes were found in the cephalic region, as well as delayed closure of posterior neural folds (Wilson et al., 1993; Wilson and Beddington, 1997). The authors attribute these defects to the failure of *T/T* cells to exit from the midline, forming a "wedge" that inhibits posterior neuropore closure (Wilson et al., 1995). In human families affected by neural tube defects, biased transmission of a variant *T* allele has been reported (Morrison et al., 1996; Shields et al., 2000; Jensen et al., 2004), but this was not confirmed in all populations (Papapetrou et al., 1999; Trembath et al., 1999; Speer et al., 2002). The *T* gene was also identified in a single nucleotide polymorphism screen for candidate NTD susceptibility risk genes (Pangilinan et al., 2012).

The T protein has been shown to interact with the *paired*-like homeodomain transcription factor Mixl1 (Pereira et al., 2011). In $Mixl1^{-/-}$ mutant embryos, development stops at E9.0, head folds are abnormally formed, heart tube and gut are missing, and paraxial mesoderm is underdeveloped (Hart et al., 2002). Instead of the notochord and node, mutant embryos accumulate *T*-expressing tissue that protrudes from the ventral side of the trunk of the embryo, like a branched embryonic axis, or forms a thick tail bud at the caudal end. The expanded domains of *T* expression in Mixl1-deficient embryos suggest that Mixl1 normally represses *T* (Hart et al., 2002).

Taken together, these experimental models provide ample evidence to implicate defective Wnt signaling and its targets in ectopic cell accumulation in the primitive streak. What is less well understood is whether this involves increased or decreased output from the canonical ß-catenin pathway. The possibility exists that, as highlighted by the $Axin2^{canp}$ mutants (Qian et al., 2011), activity may be modulated differently at early and late stages of mesoderm development. There is also indication from the $Tcf3^{-/-}$ mutants that some Tcf3 functions may be independent of ß-catenin activation (Merrill et al., 2004). In addition, participation of elements of canonical Wnt signaling in the noncanonical Wnt pathway has been observed, such as in genetic interactions between Lrp6 and Wnt5a (Bryja et al., 2009; Andersson et al., 2010) and in $Lrp6^{cd}$ mutants (Gray et al., 2013). The variable phenotypes of the Wnt signaling mutants reviewed here also suggest that early and late descendants from the primitive streak respond differently to changes in the levels of Wnt signaling.

MESP1 AND 2, EOMESODERMIN, AND CRIPTO—An indication of differential requirements in subpopulation of mesodermal derivatives comes from findings in embryos with ablation of Mesp1 and Mesp2. Mesp1-deficiency results in embryos with defective heart formation, characterized by the presence of two heart tubes (Saga et al., 1999). *Mesp1* is normally expressed in nascent mesoderm in the primitive streak, and in null mutants, migration of mesodermal cardiac precursors is delayed, but axial mesoderm formation was unaffected. Because this could potentially be due to compensatory upregulation of the neighboring *Mesp2* gene, double knockout mutants were constructed ($Mesp1^{-/-}$;$Mesp2^{-/-}$) (Kitajima et al., 2000). These embryos lacked heart and somite formation but exhibited expression of mesodermal markers, and displayed accumulation of mesodermal cells in the primitive streak. Notably, although some axial mesodermal cells were detected, by virtue of *T* expression, rostral extension of the axis did not proceed. Chimera analysis showed that the inability of double mutant cells to contribute mesoderm to the heart was cell-autonomous,

and that defects in paraxial mesoderm, i.e. somite formation, were non–cell-autonomous. The authors suggest that reduced expression of *Fgf4* in the mutants could be responsible for the inability of the accumulated mesoderm to exit from the primitive streak (Kitajima et al., 2000).

Upstream of Mesp1 in the formation of cardiac mesoderm is the T-box transcription factor Eomesodermin (Eomes) (Costello et al., 2011). Eomes (also known as Tbr2) can bind to T-box sites in the *Mesp1* locus; absence of expression of *Mesp1* in *Eomes*$^{-/-}$ mutants at E7.0 indicates that Eomes normally activates *Mesp1* transcription. Eomes also acts in trophectoderm (Russ et al., 2000) and in extraembryonic mesoderm formation (Wardle and Papaioannou, 2008), and it is required in visceral endoderm for correct positioning of the primitive streak (Nowotschin et al., 2013). Embryos with epiblast-specific ablation of *Eomes*, by virtue of *Sox2-cre*-mediated recombination (Arnold et al., 2008), display a thickened primitive streak with accumulation of mesenchymal cells. *T* expression is increased in the posterior region of the mutant embryo, indicating that these mutants are able to generate mesodermal cells, but they get stuck at the primitive streak. E-cadherin expression is maintained in mesodermal cells, despite expression of *Snail*, which normally downregulates E-cadherin (Cano et al., 2000). Interestingly, explants from Eomes-deficient embryos are able to downregulate E-cadherin and migrate in culture, demonstrating that, in the mutant embryo, they are not receiving the signals for proper migration behavior.

Teratoma-derived growth factor (Cripto), a member of the EGF-CFC family, serves as a ligand and co-receptor in Nodal signaling (Ding et al., 1998), and as recently discovered, for Wnt signaling, through binding to Lrp5 and Lrp6 (Nagaoka et al., 2013). Before gastrulation, *Cripto* expression is found in the epiblast. During gastrulation, *Cripto* is strongly expressed in the primitive streak, the node, axial mesendoderm and migrating mesoderm. Mutants with epiblast-specific ablation of *Cripto* accumulate mesenchymal cells in the primitive streak (Jin and Ding, 2013). Although these cells appear to have undergone EMT, their differentiation seems impaired, as evidenced by the absence of *Tbx6, Mixl1* and *Mesp1* expression. Interestingly, mesodermal *Fgfr1* expression was reduced in epiblast-deleted *Cripto* mutants, suggesting that Fgf signaling could be affected in these embryos (Jin and Ding, 2013).

FGF SIGNAL TRANSDUCTION PATHWAY—Fibroblast growth factor signaling has been implicated in cell migration out of the primitive streak since it was found that embryos with targeted disruption of the Fgf receptor 1 gene (*Fgfr1*) exhibited cell accumulations in the primitive streak (Deng et al., 1994; Yamaguchi et al., 1994). These accumulations included epiblast and mesodermal cells and were also protruding from the midline in the anterior neural folds of one mutant embryo (Yamaguchi et al., 1994). Expression of *T* was found in both cell types in the protrusion and was generally expanded in *Fgfr1* mutants, indicating that, although disorganized, mesoderm was formed. However, the accumulation of mutant cells in the primitive streak suggested migration defects that impaired movement of mutant paraxial mesoderm out of the streak (Deng et al., 1994). In chimeras of *Fgfr1*$^{-/-}$ mutant with wild-type cells, depending on the extent of contribution from the mutant cells, embryos exhibited failure to close the anterior neural tube, abnormal heart development, posterior truncations, and posterior neural tube duplications (Ciruna et al., 1997). Mutant

cells preferentially accumulated in the primitive streak, but were underrepresented in the mesodermal wings, confirming the impaired capacity for migration. Mutant cells contributed to the posterior mesenchyme, but were also found in embryonic ectoderm, suggesting a possible failure to undergo epithelial to mesenchymal transition. This might explain the appearance of secondary neural tubes in many of these chimeras at later stages. The successful colonization of limb bud and lateral mesoderm was interpreted as a possible differential requirement for Fgf signaling in different mesoderm derivatives (Ciruna et al., 1997). Notably, those mutant cells that ingressed at the streak maintained expression of E-cadherin (Ciruna and Rossant, 2001), which would be expected to alter their adhesive properties. Furthermore, loss of Fgfr1 resulted in reduced expression of *T/Brachyury*, indicating that the output from the Wnt signaling pathway in the primitive streak is modulated by Fgf signaling.

Both *Fgf8* and *Fgf4* are expressed in the primitive streak during gastrulation. Fgf4 is required in the postimplantation embryo even before formation of the primitive streak (Feldman et al., 1995). Embryos deficient for Fgf8 displayed thicker primitive streaks, with protrusion of a “mass of cells” of apparently mesenchymal character (Sun et al., 1999). Mesodermal derivatives, however, such as somites and heart, and endodermal derivatives like the gut, were missing by E8.5. While the epithelial layer covering the bulge of cells in the primitive streak exhibited *T* expression, the cells interior to the bulge did not express *T*; *Lim1* expression indicated that these cells were nascent mesodermal cells. Intriguingly, *Fgf4* was not expressed in the *Fgf8* mutant embryos, suggesting that *Fgf4* expression could be dependent on Fgf8, and potentially implicating Fgf4 in the failure of cells to exit the primitive streak. Ectopic cell accumulations in the primitive streak at E7.5 were also obtained with another *Fgf8* mutant allele (Guo and Li, 2007), and less pronounced in embryos with specific ablation of the *Fgf8b* splice form. Only the latter embryos displayed *Fgf4* expression, and generally less severe phenotypes at E8.5, indicating that the residual expression of the *Fgf8a* splice form, possibly together with Fgf4, ameliorated some of the mesoderm migration deficiencies (Guo and Li, 2007).

Defective mesoderm migration was also observed in embryos with an ENU-induced mutation in the gene encoding UDP-glucose dehydrogenase (*Ugdh*), that is hypothesized to disrupt the structure or function of the enzyme involved in the synthesis of glucosaminoglycans and proteoglycans. In embryos homozygous for the *lazy mesoderm* mutant allele ($Ugdh^{lzme/lzme}$) (Garcia-Garcia and Anderson, 2003), a bulge of mesenchymal cells was found contiguous with the mesodermal wings, indicating failure of cell migration away from the streak region. *Tbx6*, a marker of nascent mesoderm (Chapman et al., 1996), was not expressed, similar to the findings in Fgf8-deficient mutants (Sun et al., 1999). Because the defects are similar in *Fgf8* and $Ugdh^{lzme/lzme}$ mutant embryos, and expression of *Wnt3* and *Nodal* is detected in $Ugdh^{lzme/lzme}$ mutants, the authors hypothesize that the $Ugdh^{lzme}$ mutation does not affect Nodal or Wnt signaling, but interferes specifically with Fgf signaling; this would likely be downstream of ligand, because *Fgf8* expression can be detected in $Ugdh^{lzme/lzme}$ mutant embryos (Garcia-Garcia and Anderson, 2003). In these mutants, products of Ugdh activity, such as heparan sulfate and chondroitin sulfate, could not be found in embryonic tissues, confirming that the mutation is associated with defects in glycosaminoglycan synthesis, which thus is required for Fgf signaling.

Ptpn11 (also known as Shp2) is a SH2-domain containing tyrosine phosphatase that acts downstream of Fgf receptor stimulation. Null mutants for *Ptpn11* (*Shp2*$^{-/-}$) have defects in node, somitogenesis and axis elongation, and they form ectopic notochord material (Saxton et al., 1997). In chimeras of *Shp2*$^{-/-}$ and wild-type cells, mutant cells accumulate in the posterior region of the embryo (Saxton and Pawson, 1999). The accumulated mutant cells are mostly of neuroectoderm identity, and form secondary neural tubes. Mutant cells populated the mesodermal wings to a lesser extent than wild-type cells did, indicating that the presence of Ptpn11 is required for migration of mesodermal cells away from the streak. Consistent with a role downstream of Fgf signaling, *Shp2*$^{-/-}$ mutant cells were unable to respond to FGF in a chemotaxis assay, when their response to PDGF was normal (Saxton and Pawson, 1999). Intriguingly, the dominantly inherited Noonan Syndrome, which is characterized by short stature and heart defects, is associated with mutations in the *Ptpn11* gene that increase signaling activity (Tartaglia et al., 2001).

It was recently reported for osteoblasts (Lammi and Aarnisalo, 2008) that Fgf8 signaling induces expression of genes in the nuclear receptor NR4A family, one of which encodes the nuclear receptor transcription factor Nor1. *Nor1*-deficient mutants exhibit reduced growth and accumulation of cells in the primitive streak that were deemed to be mesoderm (DeYoung et al., 2003). *T* expression appeared normal in *Nor1*-deficient embryos, but *Tbx6* expression was substantially reduced. Although anterior mesoderm was not produced in *Nor1*$^{-/-}$ mutant embryos, the expression of *Lim1* indicated that lateral movement of mesoderm can occur. Intriguingly, modulation of *Nor1* expression in monocytes affected cell adhesion properties (Zhao et al., 2010), providing a possible explanation for the cell migration defects in the absence of Nor1.

Snail is a transcription factor affected by Fgf signaling in the primitive streak (Ciruna and Rossant, 2001). Embryos devoid of *Snail* expression die by E8.5 (Carver et al., 2001) but are able to form mesoderm, as evidenced by expression of *T/Brachyury* and *Lim1*. But the cells that had egressed from the primitive streak displayed epithelial morphology, suggesting a failure in epithelial–mesenchymal transition. Concomitantly, E-cadherin expression was maintained, at the protein and transcriptional level. Epiblast-restricted conditional ablation of Snail (Murray and Gridley, 2006) caused accumulation of cells in the primitive streak region; the cells were specified as mesoderm, as indicated by *Tbx6* expression. Ectopic expression of E-cadherin was also present. Because *Fgfr1* mutant embryos lack *Snail* expression (Ciruna and Rossant, 2001), these results provide the link between Fgf signaling and the regulation of cell adhesion and migration.

REGULATORS OF CELL MIGRATION AND ADHESION—Mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4, also known as Nik) is a serine-threonine kinase that binds to SH3 domains of the SH2/SH3 adapter of NCK1 and activates the JNK pathway. Map4k4-deficient *(Nik*$^{-/-}$) mutant embryos are truncated posteriorly and fail to form somites or a hindgut (Xue et al., 2001). At E8.5, protrusions of cells were found in the posterior primitive streak. These cells expressed *Tbx6*, indicating that mesoderm was formed, and *Lim1* expression showed that epithelial–mesenchymal transition was achieved; however, the nascent mesoderm failed to migrate away from the primitive streak. In chimeric embryos with moderate contribution from *Nik*$^{-/-}$ mutant cells, migration of mesodermal

cells appeared normal, demonstrating a non–cell-autonomous rescue of the migration defect. Wild type cells in this assay possibly provided factors that stimulated migration by mutant cells. Because *Fgf4* expression was normal in mutant embryos, the cellular defect is likely independent or downstream of Fgf signaling (Xue et al., 2001). Phosphorylated p38 was not detected in primitive streak or the accumulated mesodermal cells in $Nik^{-/-}$ mutant embryos (Zohn et al., 2006), suggesting that Map4k4 can stimulate activation of p38, which is also known as Mapk14.

Another protein required for p38/Mapk14 activation is p38-interacting protein (p38IP, also known as Supt20). A gene trap allele for p38IP ($p38IP^{RRK}$) deletes the C-terminal domain, which interacts with p38α (Zohn et al., 2006); the ENU-induced *droopy eye* mutation also removes this domain. Consequently, activation of p38 by phosphorylation is absent in $p38IP^{RRK/RRK}$ mutants. At gastrulation, the mutant embryos exhibit a cell mass in the primitive streak. These cells express *Tbx6* and *Lim1*, indicating that mesoderm is specified. However, activated/phosphorylated p38 was absent, and E-cadherin protein remained expressed in the accumulated cells, possibly affecting their cell adhesion properties. On the other hand, *E-cadherin* transcripts were absent from the accumulated mesoderm, presumably due to the expression of *Snail*. The presence of *Snail* expression indicates that the failure of mesoderm migration in these mutants is independent of Fgf signaling, and rather involves processes regulated by Map kinase signaling. Of interest, at later stages, mutants carrying *p38IP* mutant alleles displayed neural tube defects, manifesting as exencephaly with both alleles, and as spina bifida in a fraction of *droopy eye* mutants (Zohn et al., 2006). Both mutants also suffer posterior truncations.

The reorganization of the cellular actin cytoskeleton during cell migration requires the activity of proteins in the Wiskott-Aldrich syndrome protein family (WASP/WAVE) (Yamazaki et al., 2003; Yan et al., 2003). Wave1 is not detectable in $Nap1^{khlo/khlo}$ mutants, which carry an ENU-induced mutation in the gene encoding Nap1, the NCK-associated protein (Rakeman and Anderson, 2006). The $Nap1^{khlo/khlo}$ mutant embryos display cell accumulations at the primitive streak that were identified as mesenchymal. Although E-cadherin expression was reduced in these cells, they did not efficiently migrate away from the primitive streak. Cells isolated from $Nap1^{khlo/khlo}$ mutants had smaller lamellopodia and did not display the polarization needed for migration. The migration of cells forming the anterior visceral endoderm was also impaired in mutant embryos (Rakeman and Anderson, 2006).

The small GTPase Rac1 belongs to the family of Rho-GTPases that act in reorganization of the cytoskeleton during cell migration. Rac1-deficient embryos at E7.5 display abnormal folding of ectoderm, and cell death in areas where newly formed mesoderm would be expected (Sugihara et al., 1998). Mutant epiblast cells in culture migrated at slower speed, lacked lamellopodia and died within 2 days, likely due to altered cell adhesion. When Rac1 was specifically ablated in epiblast cells (Migeotte et al., 2011), the greatest effect was on migration of mesoderm: mutant embryos accumulated a bulge of mesoderm in the primitive streak, as evidenced by expression of *T/Brachyury*. Wnt and Fgf signaling appeared normal in mutant embryos at E7.5, and E-cadherin was decreased in the accumulated cells, indicating that epithelial–mesenchymal transition had occurred. In

contrast to *Nap1*$^{khlo/khlo}$ mutants, in which the WAVE complex was absent, it was detectable in Rac1 epiblast-deleted embryos (Migeotte et al., 2011). Yet, cells from these mutants did not migrate in explant cultures. Intriguingly, the presence of one deletion allele for *Pten* in *Rac1*$^{-/C}$;*Sox2*^{Cre};*Pten*$^{+/-}$ mutant embryos rescued cell death, but the compound mutants still exhibited cell accumulations, demonstrating that these are not caused by cell death.

Phosphatase and tensin homolog deleted from chromosome 10 (Pten) is a phosphatase that removes a phosphate group from phosphatidylinositol triphosphate (PIP_3) and regulates cell proliferation and survival (Stambolic et al., 1998). Embryos lacking Pten have improperly specified anterior–posterior body axes, due to defects in migration of cells normally destined to form the anterior visceral endoderm (Bloomekatz et al., 2012). Epiblast-specific ablation of *Pten* produces accumulation of cells in the mesodermal wings, with protrusion into the amniotic cavity. Thus, Pten is also required for migration of mesodermal cells in the primitive streak (Bloomekatz et al., 2012). The authors propose that in the absence of Pten, PIP_3 accumulates, and because PIP_3 activates the WAVE complex, the excess WAVE activity may account for the migration defects in *Pten*$^{-/-}$ mutant cells.

Talin is a cytoskeletal protein that provides the links between cytoplasmic domains of cell adhesion molecules at the plasma membrane, particularly integrins, to the actin cytoskeleton and the actomyosin contractile complex. Mutant embryos homozygous for a disrupted *Talin* gene (Monkley et al., 2000) exhibit a mass of mesodermal cells that accumulate in the primitive streak, likely due to migration failure. Expression of *T/Brachyury* was reduced in mutant embryos, indicating that only a small amount of axial mesoderm was produced. Mutant blastocysts displayed altered cell adhesion in culture, so it is possible that both cell adhesion and migration are affected by loss of functional Talin.

Summary

We here reviewed 28 mouse models that exhibit ectopic cell accumulation in the primitive streak. In 2 models, *T/T* chimeras and *Wnt3* mutants, the identity of the accumulated cells was not positively defined, although there was a clear absence of mesodermal markers in embryos with epiblast-specific deletion of Wnt3. In mutants with epiblast-specific ablation of Snail1, the accumulated cells, while expressing mesodermal markers, retained epithelial morphology, indicating that epithelial–mesenchymal transition was defective, similar to findings in *Fgfr1*$^{\Delta tmk/\Delta tmk}$;*Rosa26*$^{LacZ/+}$ chimeras (Ciruna et al., 1997). In 22 models, the cells in the protrusions were determined to be mesoderm. These evidences show that in the majority of cases, ingression of some precursors into the streak and their specification toward mesoderm had occurred. Where it has been investigated, there was no evidence of excess cell proliferation, consistent with the interpretation that the mesodermal cells accumulated because they got stuck at the streak due to a failure to migrate away from the streak (Ciruna et al., 1997). In most of the models, the accumulating cell bulges were covered by a layer of epithelial cells, indicating that migration of epiblast cells toward the streak was not perturbed to the same degree. Theoretically, in analogy to vehicular traffic, it is also possible that the accumulation of cells in the primitive streak could be the result of an increased rate of movement toward and cell ingression into the streak, causing accumulations because the rate of exit is not increased. The deficient contribution of

primitive streak mesoderm to various mesodermal derivatives, however, would argue against this possibility.

Altered cell adhesion was implicated in many models, owing to the retention of E-cadherin expression in the accumulated cells, such as for example in the conditional mutants for Snail and Eomesodermin (Murray and Gridley, 2006; Arnold et al., 2008) and the *p38IP*$^{RRK/RRK}$ mutants (Zohn et al., 2006). Yet, defective migration was observed even in the absence of E-cadherin protein (Rakeman and Anderson, 2006; Migeotte et al., 2011), implicating additional pathways in the cellular defects. Basal lamina components present within the cell accumulations, such as found in the mutants with epiblast-specific ablation of Rac1 (Migeotte et al., 2011), could also impede cell migration. How extracellular matrix is degraded as cells ingress into the streak is currently unknown (Chuai and Weijer, 2009).

Cell migration also requires reorganization of the cytoskeleton. Consistent with this, cell accumulations were found in mutants for regulators of cytoskeletal rearrangements, such as Rac1 and Nap1. However, in mutants for these molecules, additional defects in cell migration were present in nonmesodermal tissues, such as the AVE (Migeotte et al., 2010), which is also affected in null mutants for Cripto, Pten, and Fgf8. In this regard, the presence of both mesodermal and epithelial cells contributing to the cell accumulations in Talin-deficient and some Fgfr1-deficient embryos point toward more general failures in cell migration that are not restricted to mesodermal precursors or derivatives. In 4 models (*Wnt3*$^{-/-}$, *Lrp5*$^{-/-}$;*Lrp6*$^{-/-}$, *Eomes*$^{-/-}$, and *Cripto*$^{-/-}$ mutants), mesoderm was not specified, with the primitive streak being absent as well in 3 of these (*Wnt3*$^{-/-}$, *Lrp5*$^{-/-}$;*Lrp6*$^{-/-}$, *Eomes*$^{-/-}$).

The variable size, anterior–posterior location within the primitive streak, appearance in time (Bloomekatz et al., 2012), and incomplete penetrance of the cell accumulations/bulges implicates interactions of multiple factors that are involved in (i) the positioning of the primitive streak, (ii) specification of mesoderm, (iii) epithelial–mesenchymal transition during gastrulation, and (iv) cell migration and cell adhesion/communication. The major molecular pathways involved are the canonical Wnt pathway and Fgf signaling, with additional input coming from MAP kinase and Akt pathways, as well as ECM/cell adhesion molecule signaling. Although these pathways are active in many cell types in the embryo, epithelial–mesenchymal transition and cell migration at the primitive streak stage of embryogenesis appear to be particularly sensitive to perturbation.

Perturbations of the gastrulation process, as represented by the models reviewed here, are associated with major developmental defects that are incompatible with survival beyond midgestation in many cases (Table 2). Among the 24 models with mesodermal cell accumulations, defects in heart development were noted in 12 models, ranging in severity from absence of formation of a heart tube altogether to failures of fusion of the cardiac anlagen causing cardia bifida, to left–right asymmetry and other heart defects. In the majority of models, there was an insufficient contribution of cardiac mesoderm, highlighting the impact of impaired mesoderm migration on heart development. In 12 out of the 24 models, axial extension was defective (concurrent with heart defects in 7 models), resulting in a spectrum of caudal truncations and reductions. It remains to be determined to what

extent the position of the cell bulge along the anterior–posterior axis restricts the generation of posterior mesoderm as the node regresses, or whether there are additional defects in cell proliferation in the posterior region.

As with the other structural anomalies, considerable variation in severity was evident for defects affecting the neural tube, which were present in 10 out of 24 models with presence of mesodermal cell accumulations in the primitive streak at earlier stages. In Fgf8 null mutants, no neural tube is formed, whereas duplicated neural tubes were found in chimeras with *Fgfr1* mutant and *Eomes* mutant cells. In $Tcf3^{-/-}$ null mutants, neural tubes and notochords were duplicated. Open neural tubes were present in $Eomes^{-/-}$ chimeras, in some $Talin^{hyg/hyg}$, $Nap1^{khlo/khlo}$ and conditional Rac1 mutants; closure defects were also observed in the posterior of the $Axin^{canp/canp}$ mutant, and as exencephaly in $p38IP^{RRK/RRK}$ mutants. Chimeras with *T/T* mutant cells also had defects in closure of the posterior neuropore and, occasionally, of the neural tube in the cephalic region (Wilson et al., 1993). In this regard, it is noteworthy that the authors envision the cell accumulations in the primitive streak to form a “wedge” that prevents closure more caudally (Wilson et al., 1995). In the case of spina bifida, multiple scenarios can be envisioned: (i) the cell accumulations could physically hinder neural tube closure, due to widening of the neural plate, or (ii) impaired mesoderm migration may not provide sufficient support for elevation of the neural folds, leaving the tube open. Cardiac defects and caudal growth defects have also been interpreted to result from insufficient contribution of mesodermal cells into the target organ, heart, axial or paraxial mesoderm, and caudal development.

The embryonic lethality of many of the mutants reviewed here precludes an assessment whether these neural tube closure defects could have contributed to spina bifida, exencephaly or anencephaly at later stages. Similarly, the severe heart defects are incompatible with survival beyond midgestation. However, it is important to note that many of the alleles are null or loss-of-function alleles and recessive, revealing their deleterious effects only when all wild-type alleles are absent. In humans, embryonic lethal alleles in homozygous configuration likely go undetected, as candidate gene screens are typically performed with samples collected after birth. Even less severe mutations, such as in the *canopus* (*Axin2*), *kahlo* (*Nap1*), *droopy eye* (*p38IP*), and *lazy mesoderm* (*Ugdh*) alleles, typically produce defects only in homozygotes. On the other hand, the reduction of *Lrp5* gene dosage in the $Lrp5^{+/-};Lrp6^{-/-}$ mutants provides evidence that protein expression levels can also be important. Further support for quantitative effects comes from the observation that some $Pten^{+/-}$ mutants display open neural tubes (Cully et al., 2004). Of interest, a mutation in the *Pten* gene (in heterozygous configuration) has been identified in a patient with macrocephaly and VATER association (Reardon et al., 2001), which also originates during gastrulation and includes mesoderm deficiencies (Stevenson and Hunter, 2013).

Heart defects, neural tube defects, and caudal growth defects are characteristic structural birth defects in human pregnancies complicated by maternal diabetes. Association of VATER/VACTERL, a spectrum of vertebral, anorectal, cardiac, tracheo-esophageal, renal and limb (VACTERL) defects, with maternal diabetes has also been reported (Loffredo et al., 2001). This comparison to mouse mutant phenotypes suggests that impaired mesoderm formation and migration could be the unifying etiology for the human neural tube, heart,

and caudal anomalies in offspring from diabetic pregnancies. What is currently unclear is whether in such pregnancies the same molecular mechanisms are perturbed that were manipulated in the mouse mutants reviewed here. The combined evidence suggests that the cellular and molecular pathways involved in mesoderm development, and especially mesoderm migration, could be attractive targets for interventions aimed at preventing structural birth defects that originate from defective gastrulation.

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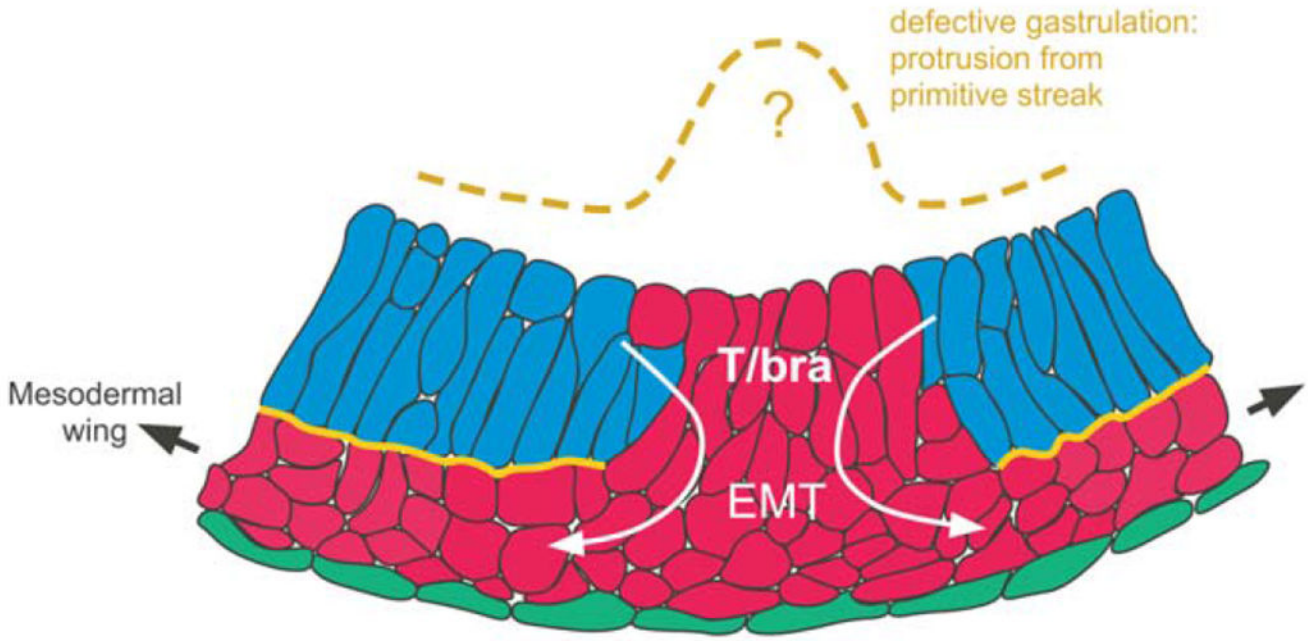

FIGURE 1.

Generation of mesoderm in the primitive streak through epithelial-mesenchymal transition and cell migration. Epiblast cells are organized in a columnar epithelium (blue). With onset of expression of T/Brachyury (red) and as cells approach the primitive streak, epiblast cells undergo epithelial–mesenchymal transition (EMT) and ingress through the primitive streak (white arrows), ultimately migrating away anteriorly (not pictured) and laterally (black arrows) into the mesodermal wings between the epithelium and the endoderm (green). Abnormalities in the process are evidenced by cell accumulation in the primitive streak, morphologically obvious as a protrusion into the amniotic cavity (brown line). Mutations involved in this phenotype (question mark) and the outcomes from cell accumulation at the primitive streak are the focus of this review.

TABLE 1.

Genes Associated with Mouse Models That Display Ectopic Cell Accumulation in the Primitive Streak

Gene Name	Expression in gastrulating embryo	Protein Product	Authors
Axin2	E7.5: throughout posterior region, PS, mesoderm and posterior embryonic ectoderm E8.5: rostral and caudal ends of neural ectoderm.	Negative regulator of canonical Wnt signaling pathway	Jho et al., 2002
Eomes	During gastrulation: PS and embryonic mesoderm; visceral endoderm; later restricted to anterior PS.	T-box transcription factor	Ciruna and Rossant, 1999; Hancock et al., 1999; Arnold et al., 2009
Fgf8	Shortly before onset of PS formation: expression in visceral endoderm and epiblast cells on the posterior of the embryo, with a sharp boundary at the node. Cells within the PS express Fgf8, but downregulate Fgf8 after exiting the streak.	Fibroblast growth factor	Crossley and Martin, 1995; Mahmood et al., 1995; Maruoka et al., 1998
FgfR1	E7.5: primitive ectodermal-neural plate and presomitic mesoderm. Expression in migrating mesodermal wings, and in embryonic ectoderm.	Fibroblast growth factor receptor	Orr-Urtreger et al., 1991; Yamaguchi et al., 1992
Lrp5	E6.5: throughout ectoderm; E7.5: visceral endoderm overlaying the extraembryonic region, embryonic ectoderm, but not in nascent mesoderm or definitive endoderm emerging from PS.	co-receptor for Wnt signal transduction	Kelly et al., 2004
Lrp6	ubiquitously expressed	co-receptor for Wnt signal transduction	Pinson et al., 2000
MesP1; MesP2	Mesp1 and Mesp2 : posterior of embryo at E6.5–6.75. Mesp2 is downregulated before E7.5, Mesp1 after E7.5.	b-HLH transcription factors	Kitajima et al., 2000
Mixl1	Expression in PS and emerging mesoderm. By head-fold stage expression becomes restricted to the posterior PS and midline visceral endoderm. E9.5: expressed in tailbud only.	Homeodomain transcription factor, target of TGF-ß pathway	Pearce and Evans, 1999; Robb et al., 2000; Wolfe and Downs, 2014
Nckap1 (Nap1)	E6.5: epiblast and visceral endoderm. E7.5: mesoderm, endoderm, ectoderm. E8.0: expression in all embryonic structures and tissues. Enriched in aplical and basal regions of cells.	Nck-associated protein, regulatory component of the WAVE complex	Rakeman and Anderson, 2006
Map4k4 (Nik)	E7.5-E8.5: broadly expressed.	NCK-interacting kinase	Xue et al., 2001
Nr4a3 (Nor1)	E7.5: ubiquitous expression.	Transcription factor, member of the nuclear receptor family	DeYoung et al., 2003
Supt20 (p38IP)	E7.5-E12.5: ubiquitous expression.	p38-interacting protein	Zohn et al., 2006
Pten	E6.5: epiblast, visceral endoderm, extraembryonic ectoderm.	Lipid Phosphatase, tumor suppressor	Bloomekatz et al., 2012
Rac1	Ubiquitously expressed.	Member of Rho GTPase family	Sugihara et al., 1998; Wang and Zheng, 2007
Ptpn11 (Shp2)	Broadly expressed.	SH2-domain-containing tyrosine phosphatase	Saxton et al., 1997
Snail	E7.5: ectoderm, primitive streak, mesoderm E8.5: not in ectoderm, but present in mesoderm and migrating cells; cephalic mesenchyme	Zinc-finger transcription factor	Nieto et al., 1992; Smith et al., 1992
T/Brachyury	E6.5-E12.5: PS, node and notochord.	T-box transcription factor	Wilkinson et al., 1990; Herrmann, 1991; Wilson et al., 1995
Tcf3	E6.5: broadly expressed E7.5: expression reduced in posterior, but intense in the ectoderm and mesoderm anterior to the node.	Member of Lef/Tcf protein family, transcription factor	Korinek et al., 1998; Merrill et al., 2004

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Gene Name	Expression in gastrulating embryo	Protein Product	Authors
Tdgf (Cripto)	During gastrulation: PS, nascent mesoderm, node and axial mesendoderm. E7.5: not in neurectoderm	Ligand and co-receptor in Nodal signaling	Ding et al., 1998; Chu et al., 2005; Wang and Zheng, 2007
Tln	Ubiquitously expressed throughout development.	Cytoskeletal protein	Monkley et al., 2000
Ugdh	E7.5: ubiquitously expressed in the epiblast, but absent from the endoderm.	UDP-glucose dehydrogenase	Garcia-Garcia and Anderson, 2003
Wnt3	Expression in the posterior visceral endodem and posterior epiblast prior to PS formation.	Wnt signaling molecule	Rivera-Perez and Magnuson, 2005

Genes are listed in alphabetical order; gene names follow the current MGI nomenclature, with synonyms given in brackets.

TABLE 2.

Genetic Manipulations Associated with Appearance of Ectopic Cell Accumulation in the Mouse Primitive Streak

Gene / mutant	Allele	Defects						Lethality	Authors
		Cell acc.	AVE	Notochord	NTD	Heart	Caudal		
Axin2$^{canp/canp}$	ENU	M			✓ open in posterior	✓ bifid	✓	E10.5	Qian et al., 2011
Cripto$^{LacZ/LacZ}$	N	no M	✓					by E10.5	Ding et al., 1998
Cripto$^{LacZ/C}$;*Sox2*^{Cre}	C	M				✓ bifid	✓	post-gastrulation	Jin and Ding, 2013
Eomes$^{-/-}$	N	no PS, no M						implantation	Russ et al., 2000; Strumpf et al., 2005
Eomes$^{-/-}$	CH	M			✓ open, duplicated	✓		n.d.	Arnold et al., 2008
Eomes$^{-/C}$;*Sox2*^{Cre}	C	M	no defect	no node				n.d.	Arnold et al., 2008
Fgf8$^{-/-}$	N	M	✓	no node	no neural tube			E9.5	Sun et al., 1999
Fgf8$^{\Delta b\text{-}neo/\Delta b\text{-}neo}$	N	M		no node				n.d.	Guo and Li, 2007
Fgfr1$^{ex4/ex4}$	DEL	M		✓			✓	E6.0-E9.5	Deng et al., 1994
Fgfr1$^{\Delta tmk/\Delta tmk}$	DEL	M+E						E7.5-E9.5	Yamaguchi et al., 1994
Fgfr1$^{\Delta tmk/\Delta tmk}$;*Rosa26*$^{LacZ/+}$	CH	M			✓ duplicated	✓	✓	after E16.5	Ciruna et al., 1997; Ciruna and Rossant, 2001
Lrp5$^{-/-}$	N							viable	Kato et al., 2002
Lrp6$^{-/-}$	N				✓ EX+SB		✓	n.d.	Zhou et al., 2010
Lrp6$^{-/-}$	GT				✓ EX+SB	✓	✓/	at birth	Pinson et al., 2000; Bryja et al., 2009; Song et al., 2010
Lrp5$^{-/-}$;*Lrp6*$^{-/-}$	N/GT	no PS, no M						mid-gestation	Kelly et al., 2004
Lrp5$^{+/-}$;*Lrp6*$^{-/-}$	N/GT	M		✓				E10.5	Kelly et al., 2004
Mesp1$^{-/-}$	N					✓ bifid		E9.5	Saga et al., 1999
Mesp2$^{-/-}$	N						somitogenesis	at birth	Saga et al., 1997
Mesp1$^{-/-}$;*Mesp2*$^{-/-}$	N	M				✓ no tube	✓	E9.5	Kitajima et al., 2000
Mixl1$^{-/-}$	N	M		✓ split		✓ no tube	✓	E10.5	Hart et al., 2002
Nap1$^{khlo/khlo}$	ENU	M, dupl. PS	✓	✓ dupl.	✓ open	✓ bifid		E9.0	Rakeman and Anderson, 2006
Nik$^{-/-}$	N	M					✓	E9.5 - E10.5	Xue et al., 2001

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Gene / mutant	Allele				Defects			Lethality	Authors
Nor1$^{-/-}$	N	M					✓	E8.5 - E9.5	DeYoung et al., 2003
p38IP^{drey}	ENU				✓ EX+SB		✓	variable	Zohn et al., 2006
p38IP$^{RRK/RRK}$	GT	M			✓ EX		✓	n.d.	Zohn et al., 2006
Pten$^{+/-}$	N				✓ open	✓		after E9.5	Cully et al., 2004
Pten$^{ex5/ex5}$	DEL				✓ cephalic	✓		E9.5 - E10.5	Freeman et al., 2006
Pten$^{-/-}$	N	M, dupl. PS	✓	✓				n.d.	Bloomekatz et al., 2012
Pten$^{-/C}$;*Sox2*^{Cre}	C	M				✓ bifid		E10.5	Bloomekatz et al., 2012
Rac1$^{-/-}$	N		✓					before E9.5	Sugihara et al., 1998; Migeotte et al., 2010
Rac1$^{-/C}$;*Sox2*^{Cre}	C	M			✓ open	✓ bifid	✓	E8.5	Migeotte et al., 2011
Shp2$^{ex3/ex3}$	DEL	M		✓ split			✓	E10.5	Saxton et al., 1997
Shp2$^{ex3/ex3}$; *Rosa26*$^{LacZ/+}$	CH	E			✓ SB, duplicated			n.d.	Saxton and Pawson, 1999
Snai1$^{-/-}$	N	E						E8.5	Carver et al., 2001
Snai1$^{-/-}$;*Meox2*^{Cre}	C	M				− left-right		after E9.5	Murray and Gridley, 2006
T/T;LacZ	CH	Q		− node	− posterior	−	−	n.d.	Wilson et al., 1993; Wilson et al., 1995
Tcf3$^{-/-}$	N	M, dupl. PS	no defect	− dupl.	− duplicated	− large		E11.5	Merrill et al., 2004
Tln$^{hyg/hyg}$	GT	M+E			✓ in some chimeras	✓	✓	E8.5 - E9.5	Monkley et al., 2000
Ugdh$^{lzme/lzme}$	ENU	M						after E7.5	Garcia-Garcia and Anderson, 2003
Wnt3$^{-/-}$	N	no PS, no M		no node				E10.5	Liu et al., 1999
Wnt3$^{-/C}$;*Sox2*$^{Cre/0}$	C	Q, not M	no defect	no node				E9.5	Barrow et al., 2007; Tortelote et al., 2013

Mutants are listed in alphabetical order; names were taken as published. Alleles are denoted as ENU for ENU-induced mutation, N for null allele, C for conditional allele, CH for chimera with wild-type cells, DEL for deletion, GT for gene trap. PS, primitive streak; M, embryonic mesoderm. Identity of cells present in the mass bulging from the primitive streak is denoted by M for mesodermal cells, E for epithelial cells (not including the epithelial layer that most often covers a mesodermal bulge), M/E for cells that express mesoderm markers but exhibit epithelial morphology, and Q for undetermined cell type. EX, exencephaly; SB, spina bifida; dupl., duplicated. Time point of lethality was taken as reported (n.d., not determined).

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