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Impaired intermediate formation in mouse embryos expressing reduced levels of Tbx6

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Abstract

Intermediate mesoderm (IM) is the strip of tissue lying between the paraxial mesoderm (PAM) and the lateral plate mesoderm that gives rise to the kidneys and gonads. Chick fate mapping studies suggest that IM is specified shortly after cells leave the primitive streak and that these cells do not require external signals to express IM-specific genes (James and Schultheiss, 2003). Surgical manipulations of the chick embryo, however, revealed that PAM-specific signals are required for IM differentiation into pronephros – the first kidney (Mauch *et al.*, 2000). Here, we use a genetic approach in mice to examine the dependency of IM on proper PAM formation. In *Tbx6* null mutant embryos, which form 7–9 improperly patterned anterior somites, IM formation is severely compromised, while in *Tbx6* hypomorphic embryos, where somites form but are improperly patterned along the axis, the impact to IM formation is lessened. These results suggest that IM and its derivatives, the kidneys and the gonads, are directly or indirectly dependent on proper PAM formation. This has implications for humans harboring *Tbx6* mutations which are known to have somite-derived defects including congenital scoliosis (Sparrow *et al.*, 2013; Wu *et al.*, 2015).

INTRODUCTION

Intermediate mesoderm (IM) is the strip of tissue lying between the paraxial mesoderm (PAM) and the lateral plate mesoderm (LPM) that gives rise to the kidneys and gonads. Fate mapping studies in the mouse show that cells emerging from the mid-streak level of the primitive streak (PS) will give rise to the LPM, which includes the IM (Lawson *et al.*, 1991; Smith *et al.*, 1994; Tam and Beddington, 1987; Wilson and Beddington, 1996). Despite these cell fate assignments, transplantation experiments have demonstrated that cells in the PS display a high degree of plasticity or developmental potential (Tsang *et al.*, 2000). Therefore at these early stages cell fates are not yet fixed and thus cells can respond to signals from surrounding tissues. Identifying the tissues that provide these signals and ultimately the nature of these signals is an important next step in understanding IM formation.

Chick fate mapping results are consistent with data from the mouse, namely that cells from the more caudal regions of the PS are fated to form IM and that PS cells have a higher developmental potential than their fate map suggests (Garcia-Martinez and Schoenwolf, 1992; James and Schultheiss, 2003; Psychoyos and Stern, 1996; Schoenwolf *et al.*, 1992).

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By definition, specification of a tissue is said to occur when cells removed from the embryo and cultured in a minimal media can still form that tissue or express markers of that tissue. James and Schultheiss (2003) examined the timing of chick IM specification and found that at stage 6, soon after emerging from the caudal region of the PS, cells express *Pax-2*, a marker of IM. They further showed that similar to the mouse studies described above, IM cell fate is not determined until after the cells have left the streak and occupy a more lateral position, which occurs at stage 8 in the chick. Interestingly, others have shown that PAM cells contribute to the stomal cells of the developing kidney (Guillaume *et al.*, 2009).

Once the IM cell fate is established, cells undergo differentiation to form the pronephros, or the first kidney, and later will differentiate to form the mesonephros and finally the metanephric kidney through inductive interactions between the ureteric bud and metanephric mesenchyme, which is also derived from the IM (reviewed in (Saxen, 1987). Lineage tracing experiments in the chick demonstrated that chick IM (pronephros) descendants of cells at stage 10 adjacent to somite contribute to the nephric duct epithelium caudal to the site of labeling. Therefore extension of the IM along the axis in the chick occurs by caudal extension of this pronephros population and not by the addition of cells from the PS (Obara-Ishihara *et al.*, 1999). Similar studies in the mouse have not been reported.

In the mouse, IM formation in terms of kidney development has been primarily studied through gene knock out experiments. *Pax2* and *Pax8*, related paired homeobox-containing transcription factors, are the earliest known markers of IM with *Pax8* being expressed slightly earlier than *Pax2* in the pronephric duct (Bouchard *et al.*, 2000; Pfeffer *et al.*, 1998; Plachov *et al.*, 1990). These factors are required in a redundant fashion to specify the pronephros (Bouchard *et al.*, 2002). Interestingly the forkhead transcription factors, *Foxc1* and *Foxc2*, function to regulate PAM versus IM formation; double homozygous mutant embryos fail to form somites and instead exhibit expanded IM, while their misexpression in IM leads to a PAM fate (Wilm *et al.*, 2004). The transcription factor *lim1* is expressed at all stages of IM formation – the nascent IM at embryonic day (e) 7.5 and in the pro-, meso- and metanephric kidney (Barnes *et al.*, 1994; Tsang *et al.*, 2000). *lim1*^{-/-} mouse embryos form only rudimentary pronephric ducts that never express *Pax2* (Shawlot and Behringer, 1995; Tsang *et al.*, 2000). Later IM differentiation includes formation of the metanephric mesenchyme, which is under the control of *Eya1* (Sajithlal *et al.*, 2005; Xu and Xu, 2015) and *Osr1* (James *et al.*, 2006), and branching of the ureteric bud, which is controlled through *ret9*-*GDNF* signaling. *ret9* encodes a receptor tyrosine kinase and is expressed along the entire length of the nephric duct at e10–11 and later at the tips of the ureteric bud branches, while the *ret-9* ligand, GDNF, is expressed in the metanephric mesenchyme surrounding the ureteric bud. Both, Ret and GDNF are critical for ureteric bud outgrowth, such that in the absence of either factor renal agenesis occurs (reviewed in (Costantini and Shakya, 2006)).

A great deal is known about the proteins necessary for the cell autonomous events controlling IM formation, however less is known about the non-cell autonomous signals, i.e. signals emerging from the surrounding tissue, that control IM formation and differentiation. Midline signaling, specifically through Shh, is required for bilateral mesonephros formation as more medial positioning of the metanephric mesenchyme and later kidney fusions are observed in embryos lacking midline *Shh* (Tripathi *et al.*, 2010). Later midline signaling, but

not Shh from IM, is needed to regulate the number of mesonephric tubules, such that conditional knock out of *Shh* in the notochord and floorplate at later stages of development results in ectopic mesonephric tubules formation (Murashima *et al.*, 2014). Because Shh-responsive cells were found in the PAM but not the IM, it was concluded that Shh working through the PAM was critical to regulate these processes. Work in the chick further suggests that signals from the PAM are required for the differentiation of IM into pronephros (James and Schultheiss, 2003; Mauch *et al.*, 2000). Using classical surgical manipulations combined with molecular marker analysis, Mauch *et al.* examined the effect of physically separating perspective IM from the surrounding tissues at different times during development (Mauch *et al.*, 2000). Separation of PAM from the perspective IM resulted in the cessation of IM extension posteriorly, while similar surgical separations that prevented signals from the axial and LPM had no effect. They therefore concluded that PAM signals were necessary for IM induction. James and Schultheiss showed that IM is specified shortly after the cells move through the caudal PS, but also found that axial/paraxial signals promoted IM formation (James and Schultheiss, 2003). From these and other results, the authors proposed a two-step model for patterning the IM: first, IM specification occurs shortly after cells emerge from the PS, thereafter, competing signals from the axial tissues and LPM are required to determine the number of cells committed to an IM fate. It is not clear whether this two-step model is complete, as it does not address prior results implicating PAM signals for posterior extension of the IM.

Here, we use a genetic approach in mice to analyze the PAM requirement for IM formation. We find that IM formation is compromised in mouse embryos that are deficient in PAM formation, specifically those null for *Tbx6* or those expressing hypomorphic levels of *Tbx6*. Interestingly, either a reduction in PAM formation or mispatterning of the PAM results in the absence or malformation of the IM. Altogether our results show a dependency of IM on properly formed and patterned PAM.

RESULTS

IM formation in *Tbx6* mutant embryos

We became interested in the requirement for PAM in the formation and patterning of surrounding tissues during our studies of the *Tbx6* mouse mutant embryo, in which posterior paraxial tissue is replaced by ectopic neural tissue from the forelimb bud caudally (Chapman and Papaioannou, 1998). We reasoned that if PAM-specific signals are required for IM formation, patterning, or elaboration of pattern, then the *Tbx6* mutant would be a useful genetic tool to explore this. In addition to the *Tbx6* null allele, we could also examine IM formation in the partially rescued *Tbx6*^{-/-} *Tg46*^{+/+} embryos (*Tg46*-rescued) in which PAM forms but is mispatterned, with somites adopting entirely posterior identity (White *et al.*, 2003). As demonstrated by *myogenin* expression in the myotome of the somite, the *Tbx6* null embryo forms 9–11 irregular anterior somites, while the *Tg46*-rescued embryo forms irregular somites along the entire axis (Fig. 1a-f). Ectopic neural tissue forms in both the *Tbx6* and *Tg46*-rescued embryos beginning at the level of the forelimb bud and extending posteriorly as shown by *Sox2* expression (Fig. 1a-c, g-i). Initially we examined the expression of genes known to be active during the early stages of IM formation and

elaboration; *pax2*, *lim1*, *ret9* and *wnt-11* are all markers of the pro- and mesonephros. As many of these markers are also expressed in the developing neural tissue, and since both *Tbx6* null and *Tg46*-rescued embryos have lateral ectopic neural tissue, we examined these marker genes at e9.5 during pronephros development and prior to their expression in neural tissue (Fig. 1j-k). In wild-type embryos at e9.5, *Pax2* expression extends from the forelimb bud region to the cloaca. In *Tbx6*^{-/-} embryos, *Pax2* expression in the IM is observed only in the forelimb bud region, while posterior to this *Pax2* IM expression truncated prematurely or became discontinuous, and thus never reached the cloaca (Fig. 1j-k). Expression patterns of *lim1* and *ret-9* confirmed this observation (data not shown).

Histological analysis of the *Tbx6* null embryo confirmed marker gene studies, specifically the absence of mesonephros posterior to the forelimb bud (Fig. 2). In contrast, mesonephric tubules have clearly formed in the forelimb region of the e10.5 *Tg46*-rescued embryos (Fig. 2). By e13.5, the metanephric kidney has formed in the normal embryo, with ureteric bud and kidney tubules developing within a condensed mesenchyme (Fig. 3). Although the *Tbx6* null embryos do not survive past e12.5, the *Tg46*-rescued embryos are viable, thus permitting histological analysis of the metanephric kidney and surrounding tissue that might be influencing its formation. Metanephric kidney formation was highly variable in the *Tg46*-rescued embryos as was the extent of ectopic neural tissue. In some embryos, tubules formed within a condensed mesenchyme, however the tissue was reduced in size compared to the normal embryo (Fig. 3b). On the other extreme, tubules formed within a loose mesenchyme with no clear kidney capsule (Fig. 3c). In some embryos, ectopic neural tissue formation was extensive and displaced the developing organs (including the kidneys) more anteriorly. A clear correlation between the extent of kidney formation and the amount of ectopic neural tissue, however, could not be made. Indeed, in at least one embryo, metanephric tubules were observed adjacent to ectopic neural tissue (Fig. 3b).

As previously mentioned, *pax2*, *lim1* and *ret9* are all expressed in the neural tube at later stages (e10.5 and older) of development (Barnes *et al.*, 1994; Nornes *et al.*, 1990; Pachnis *et al.*, 1993; Shawlot and Behringer, 1995). Consequently, these were also expressed in the lateral regions of e10.5 *Tbx6* null and *Tg46*-rescued embryos and thus limited our marker gene analyses. To circumvent this problem we examined the expression of the *hoxb7-GFP* reporter transgene that is expressed in the developing pro-, meso- and metanephros (Srinivas *et al.*, 1999). These results confirmed both marker gene and histological studies and showed that IM is severely disrupted from the forelimb bud caudally in the *Tbx6* mutant and variably forms in the *Tg46*-rescued embryos (Fig. 4).

We and others have previously identified *rib-vertebrae* as a hypomorphic allele of *Tbx6* (Watabe-Rudolph *et al.*, 2002; White *et al.*, 2003). *Tbx6*^{rv/rv} embryos express less than heterozygous levels of *Tbx6* and the resulting mice have mispatterned somites, which results in rib and vertebrae fusions that are reminiscent of the *Tg46*-rescued embryos (White *et al.*, 2003). We therefore examined e15.5 *Tbx6*^{rv/rv} kidneys using the *hoxb7-GFP* transgene. Kidney formation in these embryos was again variable – some embryos formed two normal kidneys, while others formed only one with no preference for right or left side (Fig. 4). In the most severely affected embryos, GFP expression was observed in the ureteric buds, but these failed to undergo branching morphogenesis (Fig. 4p).

Impaired IM formation in *wnt3a*^{-/-} and *Tbx6*^{-/-} *wnt3a*^{-/-} embryos

Our lab has been taking a genetic approach to look for interactions between *Tbx6* and genes known to be required for PAM formation and patterning. *wnt3a* functions in a number of different processes during development, but we are primarily interested in its role in PAM formation. *wnt3a*^{-/-} embryos form the first 9+ anterior somites, thereafter ectopic neural tissue forms at the expense of PAM, with a single large ectopic neural tube found ventral to the axial neural tube (Yoshikawa *et al.*, 1997). *Tbx6* is initially expressed in the *wnt3a* mutants, but expression is not maintained (Yamaguchi *et al.*, 1999). Conversely, *wnt3a* is expressed in the *Tbx6* mutant embryos (Chapman and Papaioannou, 1998). Based on this, we hypothesized that *wnt3a* lies upstream of *Tbx6* in the PAM pathway. To test this, we generated *Tbx6 wnt3a* double heterozygous animals, which are viable and fertile, and crossed these to generate the *Tbx6*^{-/-} *wnt3a*^{-/-} embryos (Fig. 5). Morphologically, the double mutants are similar to the *wnt3a* mutant, possessing a truncated axis and dysmorphic tail region. Interestingly, *myogenin* expression revealed only 4–5 somites in the double mutants as opposed to the 9+ *myogenin* positive somites in the *wnt3a* mutant. This more severe phenotype in double mutant embryos suggests that *wnt3a* and *Tbx6* may be functioning in parallel pathways to control anterior somite formation and not in a direct pathway as we had previously thought.

IM formation is initiated but its posterior extension is interrupted in *Tbx6*^{-/-} embryos, which form 9–11 irregular anterior somites. We also examined *wnt3a* mutant embryos to determine whether the IM phenotype was specific to *Tbx6*, or resulted from a loss of PAM tissue and hence a PAM-specific signal. The *wnt3a* mutant embryos also displayed truncated IM as revealed by *Pax2* expression patterns (Fig. 5 h-i). If IM relies on signals from the PAM, then the initial formation of IM in the *wnt3a* and *Tbx6* mutant embryos would result from signals arising from the anterior somites. Since the *Tbx6*^{-/-} *wnt3a*^{-/-} embryos form only 4–5 anterior somites, we reasoned that IM formation would be more severely impaired and therefore examined *Pax2* expression in the double homozygous mutant embryos. Interestingly, *Pax2* expression was absent in *Tbx6*^{-/-} *wnt3a*^{-/-} embryos (Fig. 5k-l) indicating a complete loss of IM with further loss of PAM.

DISCUSSION

We have investigated the role of PAM in the formation and differentiation of IM in the mouse using a genetic approach. Embryos harboring mutations in *Tbx6* or *wnt3a* form only 9–11 anterior somites. In these embryos, IM formation initiates but fails to extend caudally past the forelimb bud region, often becoming discontinuous. Caudal migration of the pronephric duct could be blocked by the ectopic neural tissue that forms in these mutants, however this does not explain the discontinuous nature of IM marker gene or *hoxb7-GFP* transgene expression. Instead IM may be specified anew, perhaps receiving a PAM signal from the anterior PAM that forms in these mutants. Interesting, our studies revealed that the *Tbx6 wnt3a* double mutant embryos are more severely affected than either mutant alone, forming only 4–5 irregular anterior somites. These double mutants fail to express the IM marker *Pax2*. Altogether these results suggest that the anterior 9–11 somites which form in *Tbx6* and *wnt3a* single mutants are sufficient for the initiation of IM formation, however

further reduction of anterior PAM leads to the loss of IM derivatives. *wnt3a*^{-/-} and *Tbx6*^{-/-} embryos exhibit axial truncations later in development. Although axis extension in these mutants appears unaffected at the time IM is predicted to be migrating through the PS, we cannot rule out that migration defects through the streak contribute to the cessation of IM formation.

Previously, we examined the cell autonomous requirement for *Tbx6* in various tissues during development (Chapman *et al.*, 2003). For these studies, *Tbx6* mutant ES cells were injected into wildtype blastocysts and the resulting embryos were dissected at e9.5–10.5 and examined for the contribution of *Tbx6*^{-/-} cells to various tissues. These studies revealed that *Tbx6* is required for the formation of posterior somites, but not for IM as *Tbx6* mutant cells could contribute to IM. This, together with the absence of *Tbx6* expression in the IM, support the idea that the loss of IM in the *Tbx6* mutant is due to cell-non-autonomous defects resulting from a loss of PAM, as opposed to a direct effect of *Tbx6* in the IM tissue. In the chick, lineage-tracing studies showed that PAM contributes to the renal stromal cells of the meso- and metanephros (Guillaume *et al.*, 2009). More recent studies used a *Tbx6*-*Cre-ERT2* line to lineage trace cells that expressed *Tbx6* revealing that timed-induction starting at e7.5 to e8.5 resulted in labeled cells in the mesonephric tubules and metanephric primordia (Concepcion *et al.*, 2017). This is perhaps not surprising since this would label cells in the primitive streak, which are known to express *Tbx6* (Chapman *et al.*, 1996). However, because cells that once expressed *Tbx6* contribute to IM derivatives, we cannot rule out that this contributes to the disruption in IM formation observed in the *Tbx6*^{-/-} embryo.

We also examined IM formation and differentiation in embryos with disruptions in somite patterning. Although somites form in both the *Tg46*-rescued and *Tbx6*^{tv/rv} embryos, there is a loss of rostral-caudal patterning which ultimately leads to the fusions of ribs and vertebrae (White *et al.*, 2003). In addition to these improperly patterned somites, *Tg46*-rescued embryos also produce ectopic neural tissue in the paraxial position. Kidney formation in the *Tg46*-rescued embryo was highly variable, and this variability may reflect the amount of ectopic neural tissue that forms. It is possible that signals from neural tissue inhibit IM formation, or that the ectopic neural tissue displaces the PAM away from the developing IM. The former situation is unlikely as James and Schultheiss (2003) found that grafts of PAM and neural tissue tended to attract the IM, such that IM wrapped around the graft. Indeed our histological analysis revealed the formation of kidney tubules adjacent to ectopic neural tissue (Fig. 3b). We cannot rule out that the ectopic neural tissue is inhibiting caudal migration of the pronephros in more severely affected mutants, if this is indeed the mechanism by which IM extends along the axis in the mouse. It is equally possible that the displacement of tissues anteriorly by the ectopic neural tissue in the *Tg46*-rescued embryos contributes to the observed kidney malformations.

Abnormal kidney formation has previously been reported for the *Tbx6*^{tv/rv} mice, which possess a hypomorphic *Tbx6* allele and have a less severe phenotype than the *Tg46*-rescued embryos (Nacke *et al.*, 2000; White *et al.*, 2003). Homozygosity for the *Tbx6*^{tv} allele is viable, however these mice possess fusions of ribs and vertebrae that result in short stature and kinky tail phenotypes. *Tbx6*^{tv/rv} mice frequently develop one bilateral horseshoe shaped

kidney or a single kidney located on one side of the axis (Nacke *et al.*, 2000; Theiler and Gluecksohn-Waelsch, 1956). Using the *hoxb7-GFP* transgene to track kidney formation in the *Tbx6^{tv/rv}* embryos, we showed that one possible cause for the defects observed in the mice was the failure of the ureteric bud to branch. Interestingly, the *Tbx6^{tv/rv}* vertebral, rib and kidney phenotypes are similar to human scoliosis patients, who exhibit curvature of the spine often associated with fusions of ribs and vertebrae (MacEwen *et al.*, 2005).

Results from the chick and the results from the mouse presented here are in stark contrast to that found in the zebrafish (Griffin and Kimelman, 2002). *Spadetail (spt)* encodes a T-box protein and is required for trunk somite formation. *One-eyed pinhead (oep)* encodes a nodal-receptor co-factor, which is required for endoderm and prechordal mesoderm. Interestingly, mutations in both genes result in synergistic effects, with double mutant embryos failing to form all somitic and cardiac mesoderm. Examination *pax2.1* expression in these mutants revealed a relatively normal IM-staining pattern and thus normal pronephros formation, despite the absence of PAM. The reason for this discrepancy between zebrafish and mouse/chick IM formation is unclear and requires further investigation.

Altogether, our results support the findings of Mauch *et al.* (2000) in the chick, namely that a PAM-specific signal is required for initiation and elaboration of the IM. Failure of nephric duct extension in the *Tbx6* and *wnt3a* single mutants and the more severe loss of IM derivatives in *Tbx6 wnt3a* double mutant embryos further support a role for PAM in IM formation and differentiation. We further demonstrate that proper formation of the PAM is required for correct morphogenesis of the kidney. Whether this is a direct effect of improper somite formation or the consequence of ectopic neural tissue is not clear and would require the analysis of mutant embryos that do not form neural tissue when PAM fails to form.

EXPERIMENTAL PROCEDURES

Mice:

Tbx6^{tm1Pa} mice have previously been described and are maintained on a mixed C57Bl6/J 129Sv/Ev genetic background (Chapman and Papaioannou, 1998). These mice were crossed to the *Tbx6^{Tg46}* transgenic mice, which are maintained on a FVB/N background. *wnt3a^{tm1Amc}* were obtained from Jackson Laboratories and are maintained on a mixed C57Bl6/J 129S1/Sv background (Takada *et al.*, 1994). *Hoxb7-GFP* transgenic mice, which have green fluorescent protein (GFP) under the control of the *Hoxb7* enhancer, were ultimately obtained from G. Martin (UCSF) (Srinivas *et al.*, 1999). Genotyping of the *Tbx6^{tm1Pa}* and *Tbx6^{Tg46}* transgenic mice and embryos was performed by Southern blot analysis using a *Tbx6*-specific probe as previously described (Chapman and Papaioannou, 1998; White *et al.*, 2003). The *hoxb7-GFP* transgene was genotyped according to (Srinivas *et al.*, 1999). *wnt3a* mice were genotyped using the following PCR primers: forward: 5'-CCAACTCCGTGTAAGACCTGAAAC-3', reverse: 5'-GGTGAAAAAGCAGCCCTTGC-3', mutant forward: 5'-GGTGGATGTGGAATGTGTGCG-3', which generated a 450 bp wild-type and 620 bp mutant bands.

Whole mount *in situ* hybridization:

Whole mount *in situ* hybridization was performed as previously described by Wilkinson (1992) using antisense riboprobes for *myogenin*, *Pax2*, *sox2*, *Lim1* and *ret9*. Hybridization and washes were performed at 63°C.

Histology:

Embryos were dissected at the designated times in PBS with 0.2% BSA, fixed overnight in Bouin's fixative, dehydrated through an ethanol series and embedded in 1:1 Paraplast Xtra:Tissue Prep2 paraffin according to standard techniques. Sections were cut at 6–8 µm, placed on Superfrost slides and stained with Mason's trichrome or hematoxylin and eosin.

Immunofluorescence:

GFP embryos and dissected tissue were visualized in Vectashield mounting media in whole mount using a Leica DMR microscope.

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Figure 1. Marker gene analysis of *Tbx6* null and *Tg46*-rescued embryos.

(a-c) Schematic of transverse sections through normal, *Tbx6* null and *Tg46*-rescued (*Tg46R*) embryos showing the position of paraxial somites (pink) or ectopic neural tubes (blue) lateral to the axial neural tube (blue). Whole mount *in situ* hybridization of e10.5 (d-f) or e9.5 (j-k) embryos for *myogenin* expression in the myotome (d-f); *Sox2* in neural tissue (g-i); and *Pax2* in the IM (j-k). The regularly spaced stripes of *myogenin* expression in the normal embryo (d) is altered in the *Tbx6*^{-/-} anterior somites and absent posteriorly (e). *myogenin* expression is disordered along the entire A-P axis of *Tg46R* embryos (f).

compared to the normal embryo (**d**). Both *Tbx6*^{-/-} (**h**) and *Tg46R* (**i**) embryos have ectopic neural tissue in the paraxial position, as seen by *Sox2* expression from the forelimb bud caudally. Neural staining is highlighted by blue arrows and arcs. (**j**) *Pax2* expression in the normal embryo extends from the forelimb bud caudally to the cloaca (red and blue arrowheads indicate the anterior and posterior of *Pax2* expression, respectively, in panels **j-k**). (**j'**) High magnification of a side view of the tail shown in panel **j**. *Pax2* is expressed at the forelimb bud level, but never reaches the cloaca in the *Tbx6*^{-/-} embryos (**k**). (**k'**) High magnification of a dorsal view of the tail shown in panel **k**. Red asterisks mark the forelimb bud.

Figure 2. Histological analysis of mesonephros formation in *Tbx6* null and *Tg46*-rescued embryos.

Transverse sections of e10.5 normal (**a, d, e**), *Tbx6* null (**b, c**) and *Tg46*-rescued (**f, g**) embryos stained with H&E (**a-c**) or Mason's trichrome (**d-g**). Mesonephric tubules have formed in the normal embryo (**a**, red arrowheads), whereas tubules are absent in the *Tbx6* mutant (**b, c**; blue arrowheads denote the region of normal IM formation). Secondary ectopic neural tissue is denoted by 2°. Low (**d**) and high (**d'**) magnification of a section along the length of a normal mesonephros (red arrowhead). Mesonephric tubules form in the forelimb

region of the *Tg46*-rescued embryos (red arrowheads in f, high magnifications of the tubules are shown in **f'** and **f''**). Evenly-spaced somites are found in the normal embryo (**e**) compared to the irregular segmentation of the PAM in the *Tg46*-rescued embryo (**g**). Boundaries between the somites are denoted by black arrowheads (**e**, **g**).

Figure 3. Histological analysis of kidney formation in *Tg46*-rescued embryos.

Transverse sections of e13.5 normal and *Tg46*-rescued embryos stained with Mason's trichrome. Primed panels are high magnifications of the respective panels. **(a)** At e13.5, the normal embryo has well-organized kidneys (red arrowheads) with tubules forming within distinct kidney mesenchyme. The kidney (k) and urogenital ridge (ugr) are shown in the high magnification **(a')**. **(b-d)** The *Tg46*-rescued embryos display variable kidney formation and secondary neural tissue (2°). The primary/axial neural tube is denoted by 1° in panel **b**. **(b)** In some *Tg46*-rescued embryos, kidney tubules (red arrowheads) form within condensed mesenchyme that is indicative of kidney mesenchyme. These tubules are immediately adjacent to ectopic neural tissue as shown in the neighboring sections **(b', b'')**. In other *Tg46*-rescued embryos, kidney tubules form (red arrowheads) but are not found within condensed mesenchyme **(c-c')** or within a distinct kidney capsule **(d-d'')**.

Figure 4. IM formation is disrupted in *Tbx6* null and in embryos expressing reduced levels of *Tbx6*.

The *hoxb7-GFP* transgene, which is expressed in the pro, meso- and metanephric kidney, was used to examine the extent of IM formation and differentiation in the various *Tbx6* mutant embryos. Embryos and dissected tissue were flat mounted and examined by fluorescence microscopy. Embryos were dissected at e9.5 (**a-b**); e10.5 (**c-f**); e11.5 (**g-i**); e13.5 (**j-n**); and e15.5 (**o-p**). (**a, c, d**) Normal (n) embryos show GFP in the IM from the forelimb bud to the cloaca at e9.5 and in the formed ureteric bud (ub) by e10.5. GFP expression in the *Tbx6*^{-/-} embryo truncates prematurely or is disrupted at e9.5 (**b', b''** show higher magnifications) and by e11.5 (**h**, dorsal view) expression is limited to a short segment in the forelimb region. Red and blue arrowheads mark the anterior and posterior, respectively, of the IM segments in all panels so marked. White arrows in **b''** show islands of GFP expressing cells. At e10.5 and e11.5, GFP expression in *Tg46R* embryos extends along the axis similar to the normal embryos, however, it appears to be more disorganized (**e, f, i**). At e13.5, kidney morphogenesis is highly variable in the *Tg46R* embryos (**k, m, n**): a branching ureteric bud observed in one embryo (**k**) resembles the normal littermate (**j**), whereas bilateral failure or decreased tubule branching is seen in another *Tg46R* embryo (**m, n**). (**p**) The *Tbx6*^{rv/rv} embryo shows ureteric buds (arrows) that have failed to undergo branching morphogenesis (white arrows mark the tip of the ureter), in comparison to the highly developed kidney tubules in the normal embryo (**o**). This is a rare event in the *Tbx6*^{rv/rv} embryos that usually form at least one kidney. Abbreviations used: pronephros, pn; ureteric bud, ub; gonad, g.

Figure 5. Genetic interaction between *Tbx6* and *wnt3a* reveals a loss of IM.

Embryos from intercrosses of *Tbx6*^{+/-} *wnt3a*^{+/-} mice were dissected at e9.5. (a-f) Whole mount *in situ* hybridization of *myogenin* expression in normal (n) and mutant embryos (genotypes are indicated). (a-c) and (d-f) represent embryos from separate litters. (a-c) Disruption of somite-derived myotomes in the *Tbx6*^{-/-} and *wnt3a*^{-/-} embryos (~11 irregular stripes) compared to normal embryos (~27 stripes). *Tbx6*^{-/-} *wnt3a*^{-/-} embryos (e, f) show only 4-5 *myogenin*-positive stripes compared to ~17 in the normal littermate (d). (g-l) *Pax2* whole mount *in situ* hybridization in normal and mutant embryos. *Pax2* expression

extends from the forelimb bud (red asterisk) to the cloaca in normal embryos (anterior and posterior of IM are designated with red and blue arrowheads, respectively, or by red arrowheads where there are only spots). In *wnt3a*^{-/-} (**h-i**) and *Tbx6*^{-/-} embryos (**j**), *Pax2* expression is discontinuous, while in *Tbx6*^{-/-} *wnt3a*^{-/-} embryos no *Pax2* expression is detected (**k-l**). Panels labeled with prime are high magnifications of their respective panel. Red asterisks indicate the position of the forelimb bud.