

Disruption of early proximodistal patterning and AVE formation in *Apc* mutants

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In the postimplantation mouse embryo, axial patterning begins with the restriction of expression of a set of genes to the distal visceral endoderm (DVE). This proximodistal (PD) axis is subsequently transformed into an anteroposterior axis as the VE migrates anteriorly to form the anterior visceral endoderm (AVE). Both Nodal and Wnt signaling pathways are involved in these events. We show here that loss of function in the adenomatous polyposis coli gene (*Apc*) leads to constitutive β -catenin activity that induces a proximalization of the epiblast with the activation of a subset of posterior mesendodermal genes, and loss of ability to induce the DVE. The loss of some DVE genes such as *Hex* and *gooseoid* is rescued in chimeras where only the epiblast was wild type; however, these DVE markers were no longer restricted distally but covered the entire epiblast. Thus, the *Apc* gene is needed in both embryonic and extra-embryonic lineages for normal PD patterning around implantation, suggesting that early restricted activation of the Wnt pathway may be important for initiating axial asymmetries. In addition, we found that nuclear β -catenin and other molecular markers are asymmetrically expressed by 4.5 days.

Key words: Mouse embryogenesis, Axis formation, Wnt, Chimera, Asymmetry, Mouse

INTRODUCTION

In mice, the anteroposterior (AP) axis is morphologically identified by the formation of the primitive streak (PS) on the posterior side of the embryo. The ‘early gastrula organizer’ (EGO), just anterior to the streak, and then the node behave as classic organizers, as shown by grafting studies (Beddington, 1994; Tam and Steiner, 1999) and by expression of organizer-related genes, such as *Gsc* and *Foxa2* (Blum et al., 1992; Ang and Rossant, 1994; Filosa et al., 1997). However, axis induction by the EGO or node is incomplete with no anterior structures being formed (Beddington, 1994; Tam and Steiner, 1999). More recently, the anterior visceral endoderm (AVE) has been shown to be important for head induction in the embryo (Thomas and Beddington, 1996; Beddington and Robertson, 1999; Tam and Steiner, 1999), and interactions between AVE, the node and PS signals are required to establish the full AP axis (Tam and Steiner, 1999). Markers of the AVE, such as *Hex*, are first expressed in the distal part of the visceral endoderm (DVE) at 5.5 dpc (days post coitum) (Thomas et al., 1998; Rivera-Perez et al., 2003; Srinivas et al., 2004). Thus, by 5.5 dpc, the first molecular asymmetries are established in the embryo, setting up a proximodistal (PD) axis. Subsequently, the cells of the DVE move towards the anterior side (Thomas and Beddington, 1996; Thomas et al., 1998; Kimura et al., 2000; Rivera-Perez et al., 2003; Srinivas et al., 2004), becoming the AVE. The AVE produces molecules, such as *Cer1* and *Lefty1*, that act to inhibit posterior fate and establish the AP axis (Kimura et al., 2000; Perea-Gomez et al., 2001). After the AVE has moved, genes such as *brachyury* (*T*) are expressed proximally in the epiblast and the extra-embryonic ectoderm (ExE) (Liu et al., 1999; Brennan et al., 2001; Kimura et al., 2001; Beck et al., 2002; Perea-Gomez et al.,

2004; Thomas et al., 1998). Multiple signaling pathways are involved in these events of molecular asymmetry and axis formation. Most notably, the activity of the Nodal/CFC signaling pathway has been shown to be crucial for several aspects of mesendoderm development and axial patterning in mice, including formation and migration of the AVE (Ding et al., 1998; Brennan et al., 2001) with *Cer1* and *Lefty1* acting as localized Nodal antagonists ensuring repression of anterior fates (Perea-Gomez et al., 2002).

Wnt/ β -catenin signaling has also been shown to be involved in the earliest asymmetries that are associated with axis development in different vertebrates (Harland and Gerhart, 1997; Yamanaka et al., 1998; Fekany et al., 1999; Roeser et al., 1999). In the canonical Wnt/Wingless signaling pathway, Wnt/Wingless activates Frizzled receptors, which then act through Dishevelled to increase free β -catenin levels by preventing its degradation by the proteasome pathway (Cadigan and Nusse, 1997). β -Catenin is then translocated to the nucleus, where it can cooperate with members of the *Lef/Tcf* transcription factor family to activate gene transcription (Huelsken and Behrens, 2002). In the absence of a Wnt/Wingless signal, β -catenin is phosphorylated by Gsk3 β (Rubinfeld et al., 1996; van Es et al., 2003) and released from a complex that promotes its ubiquitination and degradation. The tumor suppressor protein adenomatous polyposis coli (*Apc*) is a key component of this complex, acting as a scaffold to which β -catenin, Axin1 and Axin2 can bind (Polakis, 2001; van Es et al., 2003), and can also behave as a shuttle between the nucleus and cytoplasm for β -catenin (Bienz, 2002). Given this mode of action of the Wnt signaling pathway, mutations in different components can lead to both gain and loss of signaling activity, providing different insights into the role of Wnt/ β -catenin signaling in development. Loss-of-function mutations in Wnt proteins, Frizzled proteins, β -catenin and the *Lef/Tcf* genes should lead to reduced Wnt signaling, while mutations in *Apc* or axin will lead to nuclear accumulation of β -catenin and to constitutive activation of the Wnt signaling pathway (van Es et al., 2003).

A number of different mutations implicate Wnt/ β -catenin signaling in patterning around gastrulation in the mouse. Of the different Wnt proteins expressed around gastrulation, mutation of

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Wnt3 results in the earliest phenotype. Embryos mutant for *Wnt3* completely lack a primitive streak and mesendoderm formation (Liu et al., 1999), demonstrating a clear role for Wnt signaling in mesendoderm development. However, genes expressed in the AVE are correctly localized (Liu et al., 1999), suggesting that *Wnt3*^{-/-} embryos initiate AP axis formation and that *Wnt3* acts downstream of an earlier AP induction signal. β -Catenin mutant embryos (Haegel et al., 1995; Huelsken et al., 2000) also fail to form mesendoderm and they completely lack expression of mesoderm markers such as *T* and gooseoid (*Gsc*). However, they also show defects in the AVE not shown by *Wnt3* mutants. They fail to express *Hex* in the visceral endoderm (VE) while other AVE markers, such as *Cer1* and *Lhx1*, remain distal. β -Catenin signaling has indeed recently been shown to have a role in AVE migration (Kimura-Yoshida et al., 2005). Thus, β -catenin is required for both mesendoderm induction and transformation of the PD to AP axis via movement of the AVE. Recent studies on microarray profiling of β -catenin mutant embryos have identified *cripto*, a Nodal co-receptor, as a likely β -catenin target gene (Morkel et al., 2003). As *cripto* is known to be required for movement of the AVE (Ding et al., 1998), this suggests that the loss of AVE movement in β -catenin mutants may be a secondary effect of reduced Nodal/*cripto* signaling.

Activation of Wnt signaling in the embryo also implicates Wnt pathways in axis formation in mice. Misexpression of chick *Wnt8c* in transgenic lines produces a range of defects including ectopic embryonic axes (Popperl et al., 1997). *Axin1* mutations (Zeng et al., 1997) and a hypomorphic mutation in *Apc* (Ishikawa et al., 2003) also lead to embryos with ectopic posterior axis development, phenocopying the chick *Wnt8c* transgenic embryos. Thus, ectopic activation of the Wnt pathway can interfere with axis development around gastrulation. However, in both the *Axin1* mutation and the hypomorphic *Apc* allele, β -catenin activity is not completely deregulated (Zeng et al., 1997; Ishikawa et al., 2003). In other words, the earliest effects of constitutive activation of the Wnt- β -catenin pathway are not being assessed. Several different *Apc* mutations have been made by gene targeting in mice (Shibata et al., 1997; Kielman et al., 2002; Ishikawa et al., 2003) with different levels of activity. However, the original spontaneous *Apc*^{Min} mutation (Moser et al., 1990; Su et al., 1992) appears to be the most severe (Kielman et al., 2002). The *Apc*^{Min} mutation was first identified by its heterozygous effect in promoting intestinal neoplasia (Su et al., 1992). It produces a stable truncated protein of 850 amino acids that lacks all β -catenin binding and other regulatory motifs (Su et al., 1992), leading to complete inability to bind and downregulate β -catenin, thus producing constitutively active β -catenin signaling. Complete loss of function of the *Apc* gene in *Apc*^{Min/Min} embryos results in block of development at the 5.5-6.5 dpc stage and mutant embryos fail to form a pro-amniotic cavity or a primitive streak (Moser et al., 1995). This suggests that the patterning of the earliest phases of PD axis formation may also be susceptible to elevated β -catenin activity. *Apc*^{Min/Min} ES cells fail to differentiate into teratomas in vivo (Kielman et al., 2002), suggesting that elevated β -catenin could lead to a general block in differentiation. This has been postulated to result from induction of cell death by high levels of nuclear β -catenin (Kim et al., 2000), rather than a specific result of disturbed Wnt signaling.

We have re-examined the phenotype of *Apc*^{Min/Min} embryos to determine whether non-specific induction of cell death or specific effects on embryo patterning are the cause of early lethality. We see no obvious induction of cell death but find very specific effects on expression of genes involved in early PD and AP patterning. Known Wnt target genes, normally proximally restricted, such as *T*, are

expressed throughout the epiblast. In addition, the DVE fails to form, as assessed by expression of genes such as *Hex*. These effects of activated β -catenin signaling are autonomous to the epiblast, as some DVE marker expression can be rescued in mutant VE when the epiblast is wild type. However, these DVE markers are no longer restricted to the distal tip of the embryo, suggesting that β -catenin activity in the extra-embryonic lineages may drive expansion of the DVE. We suggest that localized Wnt- β -catenin signaling is required initially to establish proximodistal identity, restricting the formation of the DVE to the distal tip, prior to its known role in posteriorizing the proximal epiblast.

MATERIALS AND METHODS

Mice

Heterozygous *Apc*^{Min/+} males (Jackson Laboratory, C57BL/6J-*Apc*^{Min}) were outcrossed with ICR females to maintain a stock colony. Noon of the vaginal plug was considered as 0.5 dpc. Embryos were staged according to their dissection time, their morphology (Smith, 1985) and markers staining as follows: 3.75 dpc, late blastocyst; 4.0 dpc, just before implantation (smooth trophoblast); 4.5 dpc, implanted embryo with PrE epithelium; 4.75 dpc, induction of ExE (thickening of the polar trophectoderm, induction of *Pem* ExE expression), PrE epithelium is rounder; 5.0 dpc, bowl-shaped epiblast and PrE, just before amniotic cavitation; 5.5 dpc, DVE induction distally; 5.75 dpc, AVE has moved anteriorly; 5.75-6.0 dpc, onset of mesendoderm induction (*T* expression); 6.25-6.5 dpc, onset of gastrulation movements.

Litters from heterozygous crosses were processed for in situ hybridization or immunohistology and finally genotyped by PCR. The whole embryo was lysed in PCR tissue homogenizing buffer (Nagy et al., 2003) and 10% of the extract was used for PCR reactions: wild-type allele, 5'-TAAAGAC-CAGGAAGCCTGT-3' with 5'-AATACCTGCTCTCTCTCCA-3', the annealing temperature was 67°C; *Apc*^{Min} allele, 5'-TGAGAAAGACAG-AAGTTA-3' with 5'-TTCCACTTTGGCATAAGGC-3', the annealing temperature was 50°C. In figures, +/+ embryos are either wild type or heterozygous as there was no difference in the phenotype.

Whole-mount fluorescent in situ hybridization

Double whole-mount fluorescent in situ hybridization was adapted from Ciruna and Rossant (Ciruna and Rossant, 2001) and was performed according to Chazaud et al. (Chazaud et al., 2006) (see also www.sickkids.ca/rossant/protocols/doubleFluor.asp).

Immunohistology

After dissection in PBS, embryos were fixed in 4% paraformaldehyde for 3 hours to overnight. They were washed in PBST (PBS, 0.1% Triton), twice for 5 minutes. Embryos were preblocked in PBST with 10% FBS and incubated overnight with the antibodies (rabbit anti- β -catenin, 1/500, Sigma; rat anti-uvomorulin, 1/500, Sigma; mouse anti-Dab-2, 1/400, BD Transduction Laboratories). After five 20-minute washes in PBST they were incubated with the second antibody coupled to a fluorophore (Jackson Laboratories). After two washes in PBST, nuclei were stained for 10-30 minutes with YOYO-1 (Molecular Probes, 1/1000 in PBST with 200 μ g/ml RNase A) or propidium iodide (Molecular Probes, 1 μ g/ml) and washed three times for 20 minutes in PBST. Embryos were observed under Zeiss confocal microscope (LSM510, 20 \times air-objective with NE 0.75). In combined staining with in situ hybridization, the immunolocalisation was processed after the probe hybridization. *Lefty1* was hybridized with a probe recognizing both *Lefty1* and *Lefty2*.

Chimeras

Eight-cell embryos were collected from heterozygous *Apc*^{Min/+} crosses and aggregated to ES cells constitutively expressing YFP, using the standard morula aggregation technique (Nagy et al., 2003). Chimeras were transferred to pseudopregnant recipients and embryos were dissected out at different stages and processed for in situ hybridization or/and immunostaining as described above. Wild-type cells were visualized with a riboprobe for EGFP. Mutant embryos were identified either by *T* expression, by PCR genotyping of the extra-embryonic ectoderm or by analyzing nuclear β -catenin immunostaining after in situ hybridization.

RESULTS

Nuclear localization of β -catenin in *Apc*^{Min/Min} mutants

We first looked at β -catenin cellular localization in *Apc*^{Min/Min} mutants to determine whether loss of APC led to nuclear accumulation of β -catenin, as predicted. *Apc*^{Min/Min} mutant embryos were flushed or dissected from the uterus from 3.5 to 6.5 dpc and did not show any morphological changes until 4.75 dpc (Fig. 1D,E). By 5.5-5.75 dpc, *Apc*^{Min/Min} embryos could be easily distinguished by their round unpolarized shape (Fig. 1A-C) and reduced or absent ExE. They consisted of a mass of epiblast-like cells surrounded by a thick primitive endoderm layer (PrE). Some embryos had a more elongated shape (compare Fig. 1B with 1C), suggestive of development of a proximodistal axis but very few had an amniotic cavity ($n=3/73$).

Immunostaining at 5.75 dpc showed high levels of β -catenin in both the PrE and the epiblast of *Apc*^{Min/Min} mutants (Fig. 1G). The staining in PrE cells was clearly localized to the cell membrane and the nucleus whereas the epiblast cells accumulated β -catenin throughout the cell, including the nucleus, with variable expression levels among cells. The remaining ExE cells did not show obvious nuclear accumulation of β -catenin. As early as 4.75 dpc, when no morphological phenotype was apparent, nuclear accumulation of β -catenin was observed in the epiblast and PrE of most mutant embryos (Fig. 1H-J, $n=4/5$). At 3.5 dpc, patterns of β -catenin localization were similar in wild-type and mutant embryos, with only membrane localized β -catenin apparent (data not shown, $n=3$). Staining with the nuclear dye, YOYO-1, did not reveal any obvious

apoptotic or pycnotic nuclei in mutants at any stage. TUNEL staining did not show any increase in cell death even in the ExE between 5.0 and 5.5 ($n=3$, data not shown).

Mis patterning of epiblast and PrE in *Apc*^{Min/Min} mutants

Given the clear accumulation and nuclear translocation of β -catenin in the early epiblast of mutant embryos, we asked whether genes associated with later domains of Wnt activity were ectopically activated in *Apc*^{Min/Min} mutants. We first analyzed the PS/mesendoderm marker brachyury (*T*), as it has been shown to be a direct target of the Wnt pathway (Yamaguchi et al., 1999; Arnold et al., 2000). In *Apc*^{Min/Min} mutants, the entire epiblast expressed *T* by 5.75 dpc, a stage when it could not be detected in the wild-type embryos with our fluorescent procedure (Fig. 2A-C). Despite *T* expression, *Oct4* (Rosner et al., 1990) (Fig. 2A,B, $n=4$) was still expressed in the epiblast. We then examined earlier stages and observed that *T* was co-expressed with *Oct4* in the entire epiblast as early as 4.75 dpc (Fig. 2D,E, $n=10$) before any morphological phenotype could be seen. At late blastocyst stage (3.75 dpc), ectopic expression of *T* was seen only in a subset of embryos (Fig. 2F,G), suggesting that the onset of ectopic gene activation is around 4.0 dpc.

Other markers of PD and later AP patterning of the epiblast were affected in *Apc* mutants. Eomesodermin (*Eomes*) (Ciruna and Rossant, 1999), a marker of the middle streak, was expressed ectopically in a few epiblast cells in a transient manner (Fig. 3D, $n=2$). *Snail* (*Snail1*), which is involved in epithelium-mesenchyme transition (Nieto et al., 1992; Carver et al., 2001), was also expressed ectopically in the mutant epiblast (Fig. 3I, $n=3$), demonstrating that the epiblast acquired a mesendoderm identity. Expression of *Otx2*, a marker of distal and then anterior ectoderm (Ang et al., 1994), was absent in mutant epiblast (Fig. 3E, $n=3$), consistent with a respecification of the fate of the epiblast from distal/anterior to proximal/posterior. Interestingly, *Lhx1* and *Cer1* (Barnes et al., 1994;

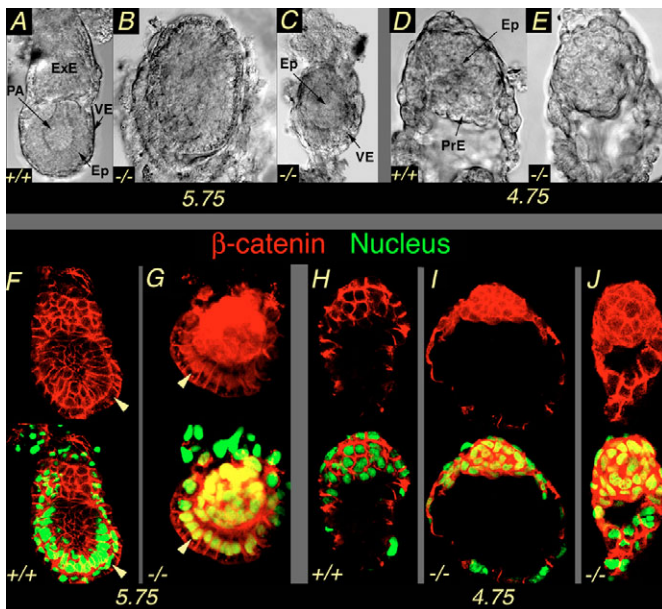


Fig. 1. Morphology and β -catenin expression in *Apc*^{Min/Min} embryos. (A-E) Bright-field images of wild-type and mutant embryos at 5.75 (A-C) and 4.75 (D,E) dpc embryos. (F-J) Immunohistochemistry of β -catenin (red) with YOYO-1 nuclear staining (green) (bottom panels) in 5.75 (F,G) and 4.75 dpc embryos (H-J). Arrowheads indicate nuclei of visceral endoderm cells. Co-expression of β -catenin and nuclear stain is apparent in the epiblast and visceral endoderm at 5.75 dpc and in the epiblast and PrE at 4.75 dpc. Nuclear β -catenin is not obviously seen in the trophoblast lineage. Ep, epiblast; ExE, extra-embryonic ectoderm; PA, proamniotic cavity; VE, visceral endoderm; PrE, primitive endoderm.

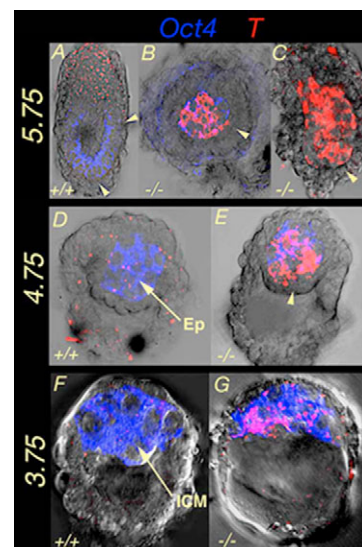


Fig. 2. Ectopic *T* expression in *Apc*^{Min/Min} epiblast, detected by in situ hybridization with *T* and *Oct4*. (A-C) Ectopic expression of *T* in the entire epiblast at 5.75 dpc. Wild-type and mutant VE differ in thickness (arrowheads). (C) Only *T* probe was hybridized. (D-G) *T* and *Oct4* expression in wild-type and mutant embryos at 4.75 and 3.75 dpc. The arrowhead indicates the unlabelled PrE. Ep, epiblast; ICM, inner cell mass; *Oct4*, blue; *T*, red.

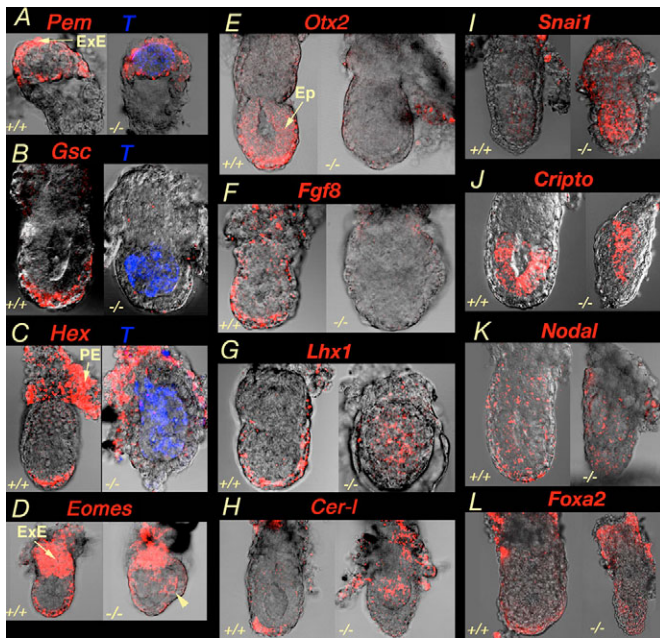


Fig. 3. Expression of primitive streak and AVE markers in wild-type and *Apc*^{Min/Min} embryos. In situ hybridization in 4.75 (A), and 5.5 (B-L) dpc embryos. Double labeling with *T* and (A) *Pem*, (B) *Gsc* or (C) *Hex*. (D-H) Single labeling of *Eomes* (D), *Otx2* (E), *Fgf8* (F), *Lhx1* (G), *Cer1* (H), *Snai1* (I), *cripto* (J), *Nodal* (K) and *Foxa2* (L). The arrowhead in D indicates a labeled epiblast cell. Ep, epiblast; PE, parietal endoderm; ExE, extra-embryonic ectoderm.

Shawlot and Behringer, 1995), which mark later AVE and organizer tissues, were ectopically expressed in the epiblast at 5.5 dpc (Fig. 3G,H; $n=5$ and $n=3$, respectively). Although *T* expression was induced widely in the epiblast, this did not reflect a broad induction of PS markers. Levels and patterns of expression of other PS/mesendoderm markers such as *Fgf8* (Crossley and Martin, 1995) (Fig. 3F, $n=3$), *Mml* (Pearce and Evans, 1999; Robb et al., 2000) ($n=2$ and data not shown) and *Wnt3* (Liu et al., 1999) remained unchanged in the epiblast of mutant embryos. *Cripto*, a proposed Wnt target gene (Morkel et al., 2003), is normally expressed throughout the epiblast at 5.5 dpc and was not obviously altered in expression pattern at this stage in mutants (Fig. 3J, $n=2$). The epiblast-wide weak expression of *Nodal* at this stage was also not affected (Fig. 3K, $n=3$).

The PrE/VE was present in *Apc*^{Min/Min} embryos as illustrated by *Rhox5* (*Pem*) expression (Lin et al., 1994) (Fig. 3A, $n=3$) and *Foxa2* (Ang et al., 1993; Belo et al., 1997) (Fig. 3L, $n=4$), but it was not patterned in a PD manner. Indeed, all the genes normally expressed in the DVE/AVE that we have tested, such as *Gsc* (Fig. 3B, $n=6$), *Fgf8* (Fig. 3F, $n=3$), *Lhx1* (Fig. 3G, $n=5$), *Eomes* (Fig. 3D, $n=2$), *Cer1* (Fig. 3H, $n=3$), *Otx2* (Fig. 3E, $n=3$) and *Hex* (Fig. 3C, $n=2$), failed to be expressed at all in the PrE of *Apc* mutants at 5.5 dpc. These results suggest that the main effect of activated β -catenin signaling in *Apc*^{Min/Min} mutants is a proximalization of the epiblast and concomitant failure to induce the DVE, such that the PD axis of the early egg cylinder fails to be specified.

Chimeric analysis of *Apc*^{Min/Min} cells

To determine whether the effect of activated β -catenin on PD axis formation was intrinsic to embryonic or extra-embryonic lineages, wild-type ES cells constitutively expressing YFP were aggregated

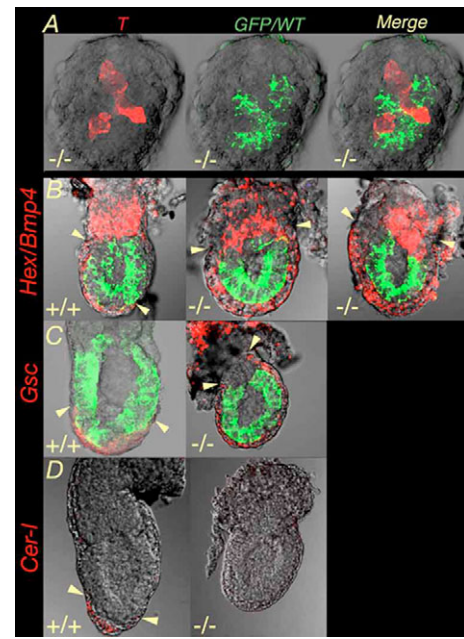


Fig. 4. Chimeric analysis of *Apc*^{Min/Min} mutants. (A) *T* (red) and EGFP (green) in situ hybridization on chimeras with low epiblast contribution from wild-type YFP-labeled ES cells in *Apc*^{Min/Min} mutant embryos at 5.5 dpc. (B-D) Chimeras with high epiblast contributions from wild-type ES cells in wild-type and *Apc*^{Min/Min} mutant embryos. Chimeras (5.5-6.0 dpc) were hybridized with probes against EGFP (B,C, green) and (in red) *Hex/Bmp4* (marks the ExE; B), *Gsc* or *Cer1* alone (C,D). The arrowheads indicate boundaries of the DVE/AVE.

with embryos from heterozygous *Apc*^{Min/+} matings. Chimeras with contributions from homozygous mutant embryos were identified by the presence of cells with high β -catenin immunolocalization in the VE or ectopic *T* expression in the epiblast. Low and high contribution of ES cells produced chimeras with a mix of wild-type and mutant cells in the epiblast or a fully wild-type epiblast respectively. In all cases, the extra-embryonic tissues (VE and ExE) were derived from the mutant embryo as ES cells cannot differentiate into these tissues in vivo (Beddington and Robertson, 1989). In mixed wild-type and mutant epiblasts, *T* expression was seen only in the mutant cells, indicating that the ectopic activation of this gene in epiblast is cell-autonomous (Fig. 4A).

In chimeras in which the entire epiblast was wild type, no ectopic *T* or *Gsc* expression was observed in the epiblast (not shown), and the pro-amniotic cavity was rescued (Fig. 4B-D). Thus, the *Apc* mutation does not affect the ability of the VE to induce cavitation in the epiblast (Coucovanis and Martin, 1995; Coucovanis and Martin, 1999), but rather it interferes with the ability of the epiblast to respond to the cavitation-inducing signals. As the egg cylinder looked more normal than in *Apc*^{Min/Min} mutants alone, we asked whether wild-type epiblast could rescue the proximodistal patterning of the *Apc*^{Min/Min} VE. Markers of the DVE/AVE, which were not expressed at all in *Apc*^{Min/Min} mutants, were examined in chimeras where the epiblast was wild type. *Gsc* and *Hex* expression were rescued in the VE of the 5.5 dpc mutant chimeras (Fig. 4B,C; $n=4$). This chimera analysis showed that the *Apc*^{Min/Min} mutant VE is still competent to express some DVE genes, once the epiblast is wild type. However, expression of these DVE markers was not restricted distally, but was induced in the whole VE. By contrast, *Cer1* and *Lefty1*, which are known to regulate DVE size (Perea-Gomez et al.,

2002; Yamamoto et al., 2004), were not expressed in the mutant VE in the chimeras (Fig. 4D, $n=3/3$). Although the epiblast appeared more normal in the rescued chimeras, they could not proceed to gastrulate and showed no expression of *T* at 6.5 dpc ($n=3$, not shown).

Molecular asymmetry in implanting embryo

The analysis of the early development of the *Apc^{Min/Min}* mutants suggests that highly restricted activation of β -catenin is normally required to initiate PD axis development. Expression patterns of the components of the Wnt pathway do not necessarily indicate actual sites of active Wnt signaling. In other species, localized nuclear β -catenin has been observed in association with active sites of signaling (Lemaire and Kodjabachian, 1996; Harland and Gerhart, 1997). We have sought evidence of early asymmetric activation of β -catenin in the normal embryo to identify possible sites of Wnt signaling. We have used fluorescent antibody staining and confocal imaging on all stages from 3.5 to 5.5 dpc and have been unable to detect any nuclear β -catenin with the exception of a transient phase around 4.5 dpc, during implantation and PrE differentiation. β -Catenin nuclear localization was detected in one cell of the trophoblast facing the PrE (Fig. 5A,B,E,G) in about 15% of the embryos examined ($n=13/86$). We noticed that β -catenin was not restricted to the nucleus but was found throughout the cell, as reported previously in other tissues (Merrill et al., 2001). E-cadherin, its binding partner in the plasma membrane, remained at the cell surface (Fig. 5B). A few hours later, by 4.75 dpc, non-membrane-bound localization of β -catenin was never observed.

If the nuclear localization of β -catenin observed transiently at the blastocyst is causally involved in patterning, we would predict that other genes would be expressed asymmetrically at the same time or subsequent to β -catenin nuclear localization. The homeobox gene *Hex* has been reported to be expressed in the whole PrE at 4.5 dpc and later to be restricted to the DVE (Thomas et al., 1998). *Pem* is another homeobox gene expressed in the PrE and ExE during implantation (Lin et al., 1994) (and data not shown). *Hex* and *Pem* were perfectly co-expressed in the PrE, with stronger expression in one or a few cells adjacent to the trophoblast (Fig. 5C,H). This asymmetric expression was found in 75% ($n>25$) of the embryos analyzed, but was transient: a few hours later, by 4.75 dpc, *Hex* was expressed uniformly in all the cells at the periphery of the PrE, in proximity to the trophoblast (Fig. 5D), and *Pem* was expressed throughout the PrE. By analyzing triple staining for β -catenin, *Hex* and the nucleus, we found that *Hex* and β -catenin asymmetric expression do not colocalize but are rather on opposite sides (Fig. 5E-H; $n=4/4$).

A recent report described that *Lefty1* is asymmetrically expressed at implantation (Takaoka et al., 2006). In an attempt to compare *Lefty1* expression with the asymmetric markers described above, we carried out in situ hybridization in 4.5 to 5.0 dpc embryos. To identify all the PrE cells we co-stained the embryos with Dab-2 antibody (Yang et al., 2002). In 4.5-4.75 dpc embryos, *Lefty1* was always expressed symmetrically within the PrE compared with Dab-2 expression (Fig. 5I,J). Interestingly, owing to different levels of the sensitivity of the experiment, varying degrees of *Lefty1* expression could be observed ranging from no staining ($n=3/16$), one cell expression at the center of the PrE (Fig. 5I, $n=5/16$), two cells expression at the center of the PrE ($n=1/16$), to a fully stained PrE ($n=7/16$), with a gradual decreasing expression from the center towards the periphery in some cases (Fig. 5J). Thus, *Lefty1* is expressed in a gradient with expression higher at the center of the PrE and lower at the periphery. We were not able to detect any *Lefty1*

RNA in 4.75-5.0 dpc embryos, just before amniotic cavitation (Fig. 5K). This contradiction with the results of Takaoka and collaborators could be due to differing interpretation of morphology and differing staining techniques. Indeed, between 4.5 and 5.0 dpc, the embryo goes through morphological changes probably resulting from ExE asymmetrical growth and anchoring (Smith, 1985; Rossant and Tam, 2004). Thus, the center of the PrE might appear asymmetric (arrowhead in Fig. 5K) when analyzing embryos with transmission light. Moreover, β -galactosidase protein activity lasts longer than native mRNA and could explain why Takaoka and collaborators

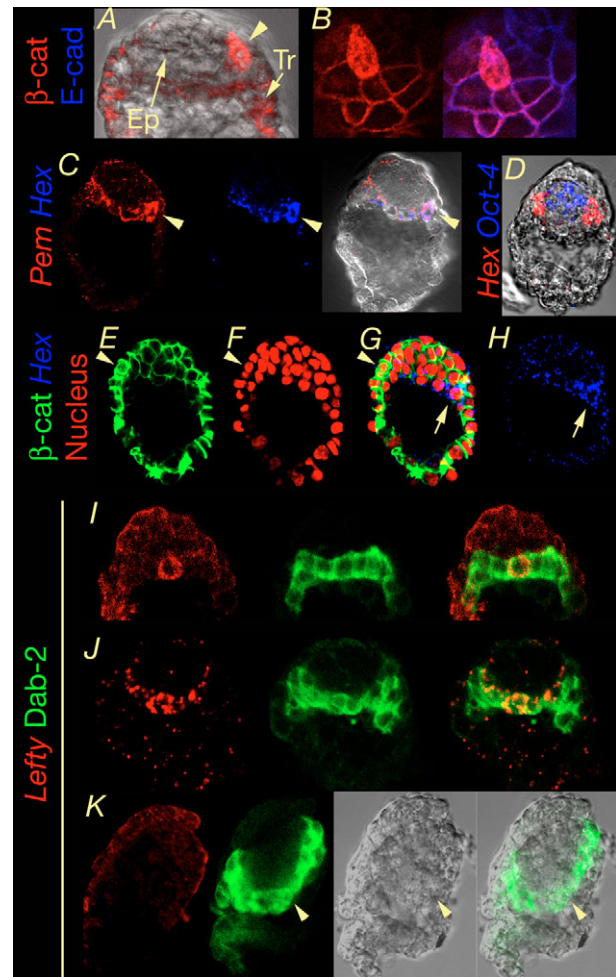


Fig. 5. Asymmetric expression of molecular markers.

(A, B) Immunohistochemistry of β -catenin (red) and E-cadherin (blue, B only) on 4.5 dpc embryos. B is a magnified right lateral view of A. The arrowheads indicate the trophoblast expressing β -catenin throughout the cell. (C) In situ hybridization of *Hex* and *Pem* at 4.5 dpc. Arrowheads indicate the enhanced expression of *Hex* and *Pem*. (D) In situ hybridization of *Hex* (red) and *Oct4* (blue) at 4.75 dpc. (E-H) Embryo (4.5 dpc) labeled with β -catenin antibody (green), with propidium iodide for nuclei (red) and with *Hex* probe (blue). E and F are at the same z section, whereas H is in a different plane of the same embryo. G is an overlay of E,F,H. The arrowheads indicate the same trophoblast cell bearing nuclear β -catenin. The arrow in G,H indicates the enhanced expression of *Hex*. (I, J) In situ hybridization of *Lefty1* (red) and immunolocalisation of Dab-2 (green) at 4.5 dpc. (K) In situ hybridization of *Lefty1* (red) and immunolocalisation of Dab-2 (green) at 4.75-5.0 dpc. The arrowhead indicates the center of Dab-2-expressing cells. Ep, epiblast; Tr, trophoblast.

detected *lacZ* staining after 4.75 dpc; it could also reflect the clonal growth of previously *Lefty1*-expressing cells, shortly after symmetrical expression of *Lefty1* mRNA. Accordingly, it is interesting that such clonal growth is asymmetric, perhaps depending on morphological changes in the embryo during ExE growth (Rossant and Tam, 2004).

DISCUSSION

The *Apc* gene product is required for correct initiation of embryonic patterning in the mouse embryo, demonstrating the importance of precise regulation of β -catenin signaling in axis development. Previous studies on a hypomorphic allele of *Apc* had shown that deregulated β -catenin activity could lead to disruption of anteroposterior axis development (Ishikawa et al., 2003) and a series of *Apc* alleles of differing severity was shown to affect differentiation capacity of ES cells in a dose-dependent manner (Kielman et al., 2002). The *Apc*^{Min} mutation leads to a truncation of the APC protein so that no binding to β -catenin is possible and other regulatory domains are deleted. Homozygous *Apc*^{Min/Min} ES cells show the highest levels of activation of β -catenin reporter constructs and nuclear accumulation of β -catenin (Kielman et al., 2002). They were also severely impaired in their differentiation capacity in vitro and were unable to form teratomas. As all other alleles produced some teratomas, it was suggested that excess nuclear β -catenin in the mutant ES cells might be promoting cell death (Kielman et al., 2002), as observed in other cell types (Kim et al., 2000). The reported phenotype of *Apc*^{Min/Min} mutant embryos was a block in development prior to gastrulation and failure to form a normal elongated egg cylinder (Moser et al., 1995). This early block in development could imply non-specific apoptotic effects of excess β -catenin. Here, we show that *Apc*^{Min/Min} embryos do not show any early evidence of excess apoptosis. Rather, they show a complex series of alterations in expression of embryonic patterning genes that can explain the phenotypes observed and are consistent with a role for APC in regulating the activity of the Wnt/ β -catenin pathway in the early embryo.

The early mouse embryo, even as early as the blastocyst stage, has been shown to express most components of the downstream canonical Wnt signaling response (Hamatani et al., 2004; Wang et al., 2004), although there is no previous evidence that the pathway can actually be activated at these stages. Disruption of the complex that normally shunts β -catenin to the proteasome for degradation by mutation of *Apc* demonstrates clearly that the pathway can be activated in both embryonic and extra-embryonic lineages, and that the *Apc* complex is necessary to prevent ectopic Wnt/ β -catenin signaling from disrupting development. When APC is lost, cells of the epiblast and PrE show accumulation of non-membrane-bound β -catenin in the cytoplasm and the nucleus from 4.75 dpc. Consistent with the appearance of nuclear β -catenin, some direct Wnt target genes were ectopically activated in *Apc*^{Min/Min} embryos. Notably, brachyury (*T*), which has been shown to be a direct Wnt target in embryos and ES cells (Yamaguchi et al., 1999; Arnold et al., 2000), is ectopically expressed throughout the epiblast in *Apc*^{Min/Min} mutants and is even expressed in the late blastocyst stage, long before its usual expression. This ectopic expression is cell-autonomous, as only mutant cells misexpress *T* in chimeric embryos. However, *T* expression is not seen in the trophoblast or VE of *Apc*^{Min/Min} mutants. *T* is normally turned on in the ExE and proximal epiblast region (Perea-Gomez et al., 2004) (C.C. and J.R., unpublished), and then moves to the PS, where its expression is known to be regulated by Wnt signaling (Liu et al., 1999). Thus, constitutive activation of the Wnt signaling pathway in the epiblast

seems to lead to proximalization of epiblast identity. Consistent with this, *Otx2*, which is expressed distally and then later anteriorly (Simeone et al., 1993; Rhinn et al., 1998; Kimura et al., 2001), is repressed in *Apc*^{Min/Min} mutants. *Snail*, which is involved in epithelial-mesenchyme transition and required for mesendoderm formation (Nieto et al., 1992; Carver et al., 2001), as well as *Eomes*, another marker of the primitive streak, are also ectopically expressed. Not all markers of later primitive streak show ectopic expression in the mutant epiblast, and expression of the key signaling molecule Nodal and its co-receptor Cripto is not altered – both are expressed throughout the early epiblast of wild-type and mutant embryos.

Activation of β -catenin throughout the embryo is sufficient to block the normal induction of patterning in the overlying VE. No VE expression of DVE markers, such as *Hex*, *Cer1* and *Gsc*, was observed in *Apc*^{Min/Min} mutants. This effect is cell-autonomous to the epiblast, as some DVE gene expression can be rescued in chimeras where the epiblast is wild type. This suggests that proximalization of the epiblast by activated β -catenin overrides the Nodal signal that would normally induce DVE marker gene expression (Fig. 6B). Nodal signal from the epiblast is required for DVE induction, as no DVE marker gene expression is seen in Nodal mutants (Brennan et al., 2001).

In chimeras where the epiblast was wild type and the trophoblast and PrE lineages were *Apc*^{Min/Min} mutant, embryonic development was superficially more normal. The epiblast showed normal epithelial morphology with a proamniotic cavity and the extra-embryonic ectoderm was partially rescued, as expression of DVE markers such as *Gsc* and *Hex* (Blum et al., 1992; Thomas et al., 1998) was turned on in the mutant VE. However, expression of these markers was no longer restricted to the distal tip but was found throughout the VE overlying the epiblast. Thus, rescue of the mutant

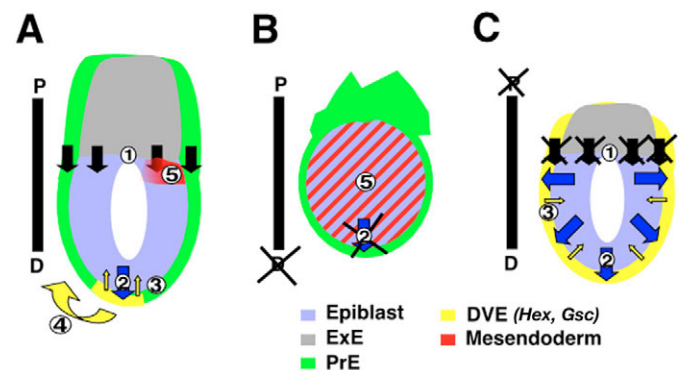


Fig. 6. Representation of signaling events in early egg cylinder.

Wild-type (A), *Apc*^{Min/Min} mutants (B) and chimeras with mutant extra-embryonic lineages and wild-type epiblast (C). (A) (1) ExE induces proximodistal axis in epiblast and PrE/VE. (2) Through Nodal, epiblast induces the DVE that is restricted distally by *Lefty1* and *Cer1*. (3) DVE distalizes the epiblast by restricting *Nodal*, *cripto* and later mesendoderm markers proximally. (4) DVE moves to the anterior side to become AVE. (5) Mesendoderm (7) is induced proximally and afterwards gastrulation movements begin on the posterior side. (B) In *Apc*^{Min/Min} mutants, the epiblast is unable to induce the DVE (2). *T* is not restricted proximally but is induced throughout the epiblast (5). The embryo is proximalized. (C) In chimeras, the extra-embryonic tissues ExE and VE (or both) are unable to set up the proximodistal axis (1). Without distal pole, the DVE (*Gsc*, *Hex*) is induced throughout the PrE (2). DVE represses mesendoderm induction (3) in the whole epiblast. The embryo is distalized. D, distal; P, proximal.

epiblast reveals a second role for β -catenin signaling in the extra-embryonic lineages. Unlike the mutant embryos, where no DVE forms at all, the rescued epiblast allows the induction of some DVE markers, but constitutive activation of β -catenin in the extra-embryonic lineages leads to expansion of these markers over the entire epiblast (Fig. 6C). Although these AVE markers, such as *Hex*, were present and expanded in the mutant VE of these chimeras, expression of the Nodal inhibitors *Lefty1* and *Cer1* was absent. *Lefty1* and *Cer1* are known to regulate DVE migration (Yamamoto et al., 2004) and size, because, in *Cer1/Lefty1* double mutants, *Gsc* and *Hex* expression domains are expanded (Perea-Gomez et al., 2002). Thus, the absence of *Cer1* and *Lefty1* in the mutant VE in the *Apc^{Min/Min}* chimeras is consistent with the expansion of the *Gsc* and *Hex* domains.

It is not clear exactly how such expansion occurs. β -Catenin overexpression in the extra-embryonic lineages could inhibit *Lefty1* and *Cer1* expression in the VE directly. Indeed, some microarray data have shown an increase of *Cer1* expression in β -catenin 6.0 dpc mutant embryos (Morkel et al., 2003). It is also possible that β -catenin overexpression in the ExE disrupts the normal signals to the epiblast and VE that establish proximal identity. Indeed, embryos with surgically removed ExE can show an expansion of expression of some AVE genes (Rodriguez et al., 2005; Richardson et al., 2006), but only under specific culture conditions (Georgiades and Rossant, 2006), suggesting that ExE could be involved in the restriction of DVE genes expression distally. In chimeras, Nodal signaling would then not be restricted in its action to the distal region and DVE markers would be induced throughout the overlying VE (Fig. 6C) (Ang and Constam, 2004). Whatever the mechanism, the expansion of the DVE leads to an anteriorization of the epiblast, as judged by the absence of *T* expression and normal gastrulation in the rescued chimeras. Thus, the loss of *Apc* in the epiblast and the extra-embryonic lineages separately give essentially opposite phenotypes, indicating the complex interactions between β -catenin signaling activity in the different components of the embryo.

Loss of *Apc* in both epiblast and extra-embryonic lineages can influence the development of the PD axis before 5.5 dpc, suggesting that localized Wnt/ β -catenin activity may be important for the events that initiate the PD axis. Loss-of-function *Wnt* mutations or β -catenin mutations have not previously indicated such early roles for the Wnt signaling pathway. In addition, a mutation in β -catenin that should lead to constitutive activation by removing its ability to be phosphorylated leads to disruption of patterning at later stages than observed with *Apc^{Min/Min}* mutants (Morkel et al., 2003), as does mutation in *Axin1* (Zeng et al., 1997). Another mutation stabilizing β -catenin leads to defects in the epiblast before gastrulation, although less severe than the *Apc^{Min/Min}* mutants (Kemler et al., 2004). This mutation does not impair ExE early development, as seen in *Apc^{Min/Min}* mutants. This might suggest that some of *Apc^{Min/Min}* phenotypes may result from some β -catenin-independent roles of *Apc* such as in cell adhesion or in microtubule function (Bienz, 2002). Although this cannot be completely ruled out, the phenotype and gene expression patterns in *Apc^{Min/Min}* mutants are largely consistent with an ectopic activation of the canonical Wnt signaling pathway. Presence of maternal β -catenin (Haegel et al., 1995) and redundant action of multiple Wnts and Axins could obscure early roles for regulated β -catenin activity in other Wnt pathway mutants.

Clearly, the mouse embryo is primed for canonical Wnt signaling as early as the blastocyst stage and removing the restraint imposed by APC has major effects on embryonic patterning. As the β -catenin pathway can be activated as early as the blastocyst stage, this leaves

open the possibility that localized Wnt activity could be important at these early stages to initiate the events that lead to induction of the PD and then the AP axis. We have found some tantalizing evidence of localized nuclear β -catenin in restricted cells in the trophoblast of 4.5 dpc embryos, in a region in close proximity to the PrE. We have also shown that the PrE shows morphological and molecular asymmetries by the same stage. These data are circumstantial evidence that Wnt signaling and asymmetries leading to later axis development may begin as early as 4.5 dpc and might be driven by extra-embryonic tissues. Combined with recent evidence that the growth of the PrE from the blastocyst is asymmetric with relation to the PD axis of the postimplantation embryo (Weber et al., 1999) and that the DVE is asymmetric before moving anteriorly (Yamamoto et al., 2004) (C.C. and J.R., unpublished), this suggests that there may be complex interactions between embryonic and extra-embryonic lineages by the late blastocyst stage leading to later axial asymmetries (Rossant and Tam, 2004; Ang and Constam, 2004).

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References

- Ang, S. L. and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.
- Ang, S. L. and Constam, D. B. (2004). A gene network establishing polarity in the early mouse embryo. *Semin. Cell Dev. Biol.* **15**, 555-561.
- Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**, 1301-1315.
- Ang, S. L., Conlon, R. A., Jin, O. and Rossant, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* **120**, 2979-2989.
- Arnold, S. J., Stappert, J., Bauer, A., Kispert, A., Herrmann, B. G. and Kemler, R. (2000). Brachyury is a target gene of the Wnt/ β -catenin signaling pathway. *Mech. Dev.* **91**, 249-258.
- Barnes, J. D., Crosby, J. L., Jones, C. M., Wright, C. V. and Hogan, B. L. (1994). Embryonic expression of *Lim-1*, the mouse homolog of *Xenopus Xlim-1*, suggests a role in lateral mesoderm differentiation and neurogenesis. *Dev. Biol.* **161**, 168-178.
- Beck, S., Le Good, J. A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F. and Constam, D. B. (2002). Extraembryonic proteases regulate Nodal signalling during gastrulation. *Nat. Cell Biol.* **4**, 981-985.
- Beddington, R. S. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-620.
- Beddington, R. S. and Robertson, E. J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105**, 733-737.
- Beddington, R. S. and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96**, 195-209.
- Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M. and De Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68**, 45-57.
- Bienz, M. (2002). The subcellular destinations of APC proteins. *Nat. Rev. Mol. Cell Biol.* **3**, 328-338.
- Blum, M., Gaunt, S. J., Cho, K. W., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene goosecoid. *Cell* **69**, 1097-1106.
- Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S. and Robertson, E. J. (2001). Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* **411**, 965-969.
- Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* **11**, 3286-3305.
- Carver, E. A., Jiang, R., Lan, Y., Oram, K. F. and Gridley, T. (2001). The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell. Biol.* **21**, 8184-8188.
- Chazaud, C., Yamanaka, Y., Pawson, T. and Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Dev. Cell* **10**, 615-624.

- Ciruna, B. G. and Rossant, J. (1999). Expression of the T-box gene *Eomesodermin* during early mouse development. *Mech. Dev.* **81**, 199-203.
- Ciruna, B. and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* **1**, 37-49.
- Coucouvanis, E. and Martin, G. R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.
- Coucouvanis, E. and Martin, G. R. (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. *Development* **126**, 535-546.
- Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.
- Ding, J., Yang, L., Yan, Y. T., Chen, A., Desai, N., Wynshaw-Boris, A. and Shen, M. M. (1998). *Cripto* is required for correct orientation of the anterior-posterior axis in the mouse embryo. *Nature* **395**, 702-707.
- Fekany, K., Yamanaka, Y., Leung, T., Sirotkin, H. I., Topczewski, J., Gates, M. A., Hibi, M., Renucci, A., Stemple, D., Radbill, A. et al. (1999). The zebrafish *bozozok* locus encodes *Dharma*, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development* **126**, 1427-1438.
- Flosa, S., Rivera-Perez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R. and Ang, S. L. (1997). Goosecoid and HNF-3beta genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124**, 2843-2854.
- Georgiades, P. and Rossant, J. (2006). *Ets2* is necessary in trophoblast for normal embryonic anteroposterior axis development. *Development* **133**, 1059-1068.
- Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K. and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. *Development* **121**, 3529-3537.
- Hamatani, T., Carter, M. G., Sharov, A. A. and Ko, M. S. (2004). Dynamics of global gene expression changes during mouse preimplantation development. *Dev. Cell* **6**, 117-131.
- Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell Dev. Biol.* **13**, 611-667.
- Huelsken, J. and Behrens, J. (2002). The Wnt signalling pathway. *J. Cell Sci.* **115**, 3977-3978.
- Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C. and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* **148**, 567-578.
- Ishikawa, T. O., Tamai, Y., Li, Q., Oshima, M. and Taketo, M. M. (2003). Requirement for tumor suppressor *Apc* in the morphogenesis of anterior and ventral mouse embryo. *Dev. Biol.* **253**, 230-246.
- Kemler, R., Hierholzer, A., Kanzler, B., Kuppig, S., Hansen, K., Taketo, M. M., de Vries, W. N., Knowles, B. B. and Solter, D. (2004). Stabilization of beta-catenin in the mouse zygote leads to premature epithelial-mesenchymal transition in the epiblast. *Development* **131**, 5817-5824.
- Kielman, M. F., Rindapaa, M., Gaspar, C., van Poppel, N., Breukel, C., van Leeuwen, S., Taketo, M. M., Roberts, S., Smits, R. and Fodde, R. (2002). *Apc* modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. *Nat. Genet.* **32**, 594-605.
- Kim, K., Pang, K. M., Evans, M. and Hay, E. D. (2000). Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. *Mol. Biol. Cell* **11**, 3509-3523.
- Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S. and Matsuo, I. (2000). Visceral endoderm mediates forebrain development by suppressing posteriorizing signals. *Dev. Biol.* **225**, 304-321.
- Kimura, C., Shen, M. M., Takeda, N., Aizawa, S. and Matsuo, I. (2001). Complementary functions of *Otx2* and *Cripto* in initial patterning of mouse epiblast. *Dev. Biol.* **235**, 12-32.
- Kimura-Yoshida, C., Nakano, H., Okamura, D., Nakao, K., Yonemura, S., Belo, J. A., Aizawa, S., Matsui, Y. and Matsuo, I. (2005). Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. *Dev. Cell* **9**, 639-650.
- Lemaire, P. and Kodjabachian, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet.* **12**, 525-531.
- Lin, T. P., Labosky, P. A., Grabel, L. B., Kozak, C. A., Pitman, J. L., Kleeman, J. and MacLeod, C. L. (1994). The *Pem* homeobox gene is X-linked and exclusively expressed in extraembryonic tissues during early murine development. *Dev. Biol.* **166**, 170-179.
- Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for *Wnt3* in vertebrate axis formation. *Nat. Genet.* **22**, 361-365.
- Merrill, B. J., Gat, U., DasGupta, R. and Fuchs, E. (2001). *Tcf3* and *Lef1* regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev.* **15**, 1688-1705.
- Morkel, M., Huelsken, J., Wakamiya, M., Ding, J., van de Wetering, M., Clevers, H., Taketo, M. M., Behringer, R. R., Shen, M. M. and Birchmeier, W. (2003). Beta-catenin regulates *Cripto*- and *Wnt3*-dependent gene expression programs in mouse axis and mesoderm formation. *Development* **130**, 6283-6294.
- Moser, A. R., Pitot, H. C. and Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**, 322-324.
- Moser, A. R., Shoemaker, A. R., Connelly, C. S., Clipson, L., Gould, K. A., Luongo, C., Dove, W. F., Siggers, P. H. and Gardner, R. L. (1995). Homozygosity for the *Min* allele of *Apc* results in disruption of mouse development prior to gastrulation. *Dev. Dyn.* **203**, 422-433.
- Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, K. (2003). *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Press.
- Nieto, M. A., Bennett, M. F., Sargent, M. G. and Wilkinson, D. G. (1992). Cloning and developmental expression of *Sna*, a murine homologue of the *Drosophila* *snail* gene. *Development* **116**, 227-237.
- Pearce, J. J. and Evans, M. J. (1999). *Mml*, a mouse Mix-like gene expressed in the primitive streak. *Mech. Dev.* **87**, 189-192.
- Perea-Gomez, A., Rhinn, M. and Ang, S. L. (2001). Role of the anterior visceral endoderm in restricting posterior signals in the mouse embryo. *Int. J. Dev. Biol.* **45**, 311-320.
- Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H. et al. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev. Cell* **3**, 745-756.
- Perea-Gomez, A., Camus, A., Moreau, A., Grieve, K., Moneron, G., Dubois, A., Cibert, C. and Collignon, J. (2004). Initiation of gastrulation in the mouse embryo is preceded by an apparent shift in the orientation of the anterior-posterior axis. *Curr. Biol.* **14**, 197-207.
- Polakis, P. (2001). More than one way to skin a catenin. *Cell* **105**, 563-566.
- Popperl, H., Schmidt, C., Wilson, V., Hume, C. R., Dodd, J., Krumlauf, R. and Beddington, R. S. (1997). Misexpression of *Cwnt8C* in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. *Development* **124**, 2997-3005.
- Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M. and Ang, S. L. (1998). Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**, 845-856.
- Richardson, L., Torres-Padilla, M. E. and Zernicka-Goetz, M. (2006). Regionalised signalling within the extraembryonic ectoderm regulates anterior visceral endoderm positioning in the mouse embryo. *Mech. Dev.* **123**, 288-296.
- Rivera-Perez, J. A., Mager, J. and Magnuson, T. (2003). Dynamic morphogenetic events characterize the mouse visceral endoderm. *Dev. Biol.* **261**, 470-487.
- Robb, L., Hartley, L., Begley, C. G., Brodnicki, T. C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Elefanty, A. G. (2000). Cloning, expression analysis, and chromosomal localization of murine and human homologues of a *Xenopus* mix gene. *Dev. Dyn.* **219**, 497-504.
- Rodriguez, T. A., Srinivas, S., Clements, M. P., Smith, J. C. and Beddington, R. S. (2005). Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. *Development* **132**, 2513-2520.
- Roeser, T., Stein, S. and Kessel, M. (1999). Nuclear beta-catenin and the development of bilateral symmetry in normal and LiCl-exposed chick embryos. *Development* **126**, 2955-2965.
- Rosner, M. H., Viganò, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. and Staudt, L. M. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686-692.
- Rossant, J. and Tam, P. P. (2004). Emerging asymmetry and embryonic patterning in early mouse development. *Dev. Cell* **7**, 155-164.
- Rubinfield, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.
- Shawlot, W. and Behringer, R. R. (1995). Requirement for *Lim1* in head-organizer function. *Nature* **374**, 425-430.
- Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K. et al. (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the *Apc* gene. *Science* **278**, 120-123.
- Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M. R., Nigro, V. and Boncinelli, E. (1993). A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* **12**, 2735-2747.
- Smith, L. J. (1985). Embryonic axis orientation in the mouse and its correlation with blastocyst relationships to the uterus. II. Relationships from 1/4 to 9/12 days. *J. Embryol. Exp. Morphol.* **89**, 15-35.
- Srinivas, S., Rodriguez, T., Clements, M., Smith, J. C. and Beddington, R. S. P. (2004). Active cell migration drives the unilateral movements of the anterior visceral endoderm. *Development* **131**, 1157-1164.
- Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A. and Dove, W. F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homologue of the APC gene. *Science* **256**, 668-670.

- Takaoka, K., Yamamoto, M., Shiratori, H., Meno, C., Rossant, J., Saijoh, Y. and Hamada, H.** (2006). The mouse embryo autonomously acquires anterior-posterior polarity at implantation. *Dev. Cell* **10**, 451-459.
- Tam, P. P. and Steiner, K. A.** (1999). Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissues of the mouse embryo. *Development* **126**, 5171-5179.
- Thomas, P. and Beddington, R.** (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr. Biol.* **6**, 1487-1496.
- Thomas, P. Q., Brown, A. and Beddington, R. S.** (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**, 85-94.
- van Es, J. H., Barker, N. and Clevers, H.** (2003). You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. *Curr. Opin. Genet. Dev.* **13**, 28-33.
- Wang, Q. T., Piotrowska, K., Ciemerych, M. A., Milenkovic, L., Scott, M. P., Davis, R. W. and Zernicka-Goetz, M.** (2004). A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev. Cell* **6**, 133-144.
- Weber, R. J., Pedersen, R. A., Wianny, F., Evans, M. J. and Zernicka-Goetz, M.** (1999). Polarity of the mouse embryo is anticipated before implantation. *Development* **126**, 5591-5598.
- Yamaguchi, T. P., Takada, S., Yoshikawa, Y., Wu, N. and McMahon, A. P.** (1999). T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. *Genes Dev.* **13**, 3185-3190.
- Yamamoto, M., Saijoh, Y., Perea-Gomez, A., Shawlot, W., Behringer, R. R., Ang, S. L., Hamada, H. and Meno, C.** (2004). Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. *Nature* **428**, 387-392.
- Yamanaka, Y., Mizuno, T., Sasai, Y., Kishi, M., Takeda, H., Kim, C. H., Hibi, M. and Hirano, T.** (1998). A novel homeobox gene, dharmia, can induce the organizer in a non-cell-autonomous manner. *Genes Dev.* **12**, 2345-2353.
- Yang, D. H., Smith, E. R., Roland, I. H., Sheng, Z., He, J., Martin, W. D., Hamilton, T. C., Lambeth, J. D. and Xu, X. X.** (2002). Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. *Dev. Biol.* **251**, 27-44.
- Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., 3rd, Lee, J. J., Tilghman, S. M., Gumbiner, B. M. and Costantini, F.** (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. *Cell* **90**, 181-192.