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Extra-embryonic *Wnt3* regulates the establishment of the primitive streak in mice

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Abstract

The establishment of the head to tail axis at early stages of development is a fundamental aspect of vertebrate embryogenesis. In mice, experimental embryology, genetics and expression studies have suggested that the visceral endoderm, an extra-embryonic tissue, plays an important role in anteroposterior axial development. Here we show that absence of *Wnt3* in the posterior visceral endoderm leads to delayed formation of the primitive streak and that interplay between anterior and posterior visceral endoderm restricts the position of the primitive streak. Embryos lacking *Wnt3* in the visceral endoderm, however, appear normal by E9.5. Our results suggest a model for axial development in which multiple signals are required for anteroposterior axial development in mammals.

Introduction

The establishment of the head to tail axis is a fundamental step in generating the basic body plan of vertebrates (Stern et al., 2006). In amniote embryos, like those of birds and mammals, this axis is marked at its caudal end by the primitive streak, a region of epithelial to mesenchymal transition that serves as a conduit for the generation of mesoderm and endoderm during gastrulation (Stern, 2004). In mammals, understanding how the primitive

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streak forms has special significance because deciphering why the primitive streak forms where it does can explain how the anteroposterior axis is established during embryogenesis (Beddington and Robertson, 1998).

In mice, two components of the early post-implantation embryo, the extra-embryonic ectoderm and the posterior visceral endoderm, have been proposed to induce the formation of the primitive streak (Bachvarova, 1996; Beddington and Robertson, 1999; Conlon and Beddington, 1995). The extra-embryonic ectoderm is a trophectoderm derivative that abuts the epiblast, the tissue that gives rise to the primitive streak. Several lines of evidence suggest that Bmp4 emanating from the extra-embryonic ectoderm signals to the adjacent epiblast to activate primitive streak markers (Arnold and Robertson, 2009; Beddington and Robertson, 1999; Ben-Haim et al., 2006). The induced epiblast then undergoes an epithelial to mesenchymal transition to become the primitive streak, allowing gastrulation and the subsequent generation of mesoderm and endoderm.

A less studied potential signaling center is the posterior visceral endoderm, an extra-embryonic component derived from primitive endoderm located adjacent to the region of epiblast that becomes the primitive streak. Tissue recombination experiments have shown that the posterior visceral endoderm has the capacity to re-specify anterior ectoderm into a posterior mesodermal fate and that this reprograming is effected by a diffusible signal (Belaoussoff et al., 1998). A candidate molecule is Wnt3; *Wnt3* knockout embryos lack a primitive streak and fail to gastrulate (Liu et al., 1999). In addition, *Wnt3* expression is first observed in the posterior visceral endoderm of embryos dissected at embryonic day 5.5 and expands to the adjacent epiblast tissue a few hours later (Rivera-Perez and Magnuson, 2005). In support of the hypothesis that Wnt3 derived from visceral endoderm has an inductive role in primitive streak formation, embryos in which *Wnt3* was conditionally inactivated in the epiblast were able to establish the primitive streak and initiate gastrulation although they failed to thrive soon afterwards (Tortelote et al., 2013). Intriguingly, in *Wnt3*-epiblast mutant embryos, *Wnt3* mRNA is present transiently in the posterior visceral endoderm suggesting that Wnt3 signaling from the posterior visceral endoderm is responsible for the initiation of gastrulation (Tortelote et al., 2013).

In order to determine if *Wnt3* function in the posterior visceral endoderm is required for the formation of the primitive streak, we inactivated *Wnt3* in the posterior visceral endoderm using *Transthyretin-Cre* mice. Our results show that embryos lacking *Wnt3* solely in the visceral endoderm specify the primitive streak later than control littermates. This effect is exacerbated if a copy of *Wnt3* is additionally inactivated in the epiblast. Despite these defects, *Wnt3* visceral endoderm mutant embryos are able to gastrulate and develop normally. From these results, we conclude that *Wnt3* function in the posterior visceral endoderm regulates the formation of the primitive streak and gastrulation but its function is dispensable for embryo development. Our results also indicate that the anterior visceral endoderm plays a role in preventing the expansion of Wnt3 signaling to the anterior side of the embryo restricting the formation of the primitive streak to the opposite side of the epiblast. We propose a model in which a series of coordinated events involving the visceral endoderm, extra-embryonic ectoderm and epiblast control the development of the primitive streak in mammals.

Materials and Methods

Embryo staging and mice

Embryos were staged based on morphological landmarks as previously described (Downs and Davies, 1993; Rivera-Perez et al., 2010) or described in terms of dissection time. Noon of the day that a mating plug was observed was considered embryonic day 0.5 (E0.5) of gestation. *Ttr*^{Cre} mice were previously described (Kwon and Hadjantonakis, 2009). *Wnt3*^{c} mice were obtained from Dr. Jeff Barrow (Barrow et al., 2003). *Cripto* mutant mice (*Cripto*^{lacZ}) were provided by Dr. Michael Shen (Ding et al., 1998). *Hex*^{GFP} mice were provided by Dr. Tristan Rodriguez (Rodriguez et al., 2001). All *Wnt3* mutant alleles, *Ttr*^{Cre}, *Cripto*^{lacZ} and *Hex*^{GFP} mice were maintained on a CD-1 outbred genetic background.

Generation of *Wnt3*^{lacZ} knock-in mice

To target the *Wnt3* locus, we designed a targeting vector containing a 6 kb NotI-BamHI genomic DNA fragment (129S6/SvEvBrd) that included exons 3 and 4. A *lacZ* cassette and a floxed PGK*neo*bpA cassette were inserted into the ClaI site in exon 4. The *IacZ* cassette contained an internal ribosomal entry site (IRES) and an SV40 polyA signal sequence. An HSV-*tk*pA cassette was added to the 5′-homologous arm. Gene targeting was conducted in AB1 ES cells as previously described (Mishina et al., 1995). A total of 192 G418; FIAU double-resistant ES cell colonies were screened for homologous recombination by Southern blotting using a *Wnt3* 3′ UTR probe (Liu et al., 1999). Twenty-seven lines were positive in the initial screening, and two lines gave rise to germ-line chimeras. We obtained *Wnt3*^{lacZneo} heterozygous mice after crossing chimeras to C57BL/6 mice. *Wnt3*^{lacZneo} heterozygous mice were crossed to *Sox2*^{Cre} mice (Hayashi et al., 2002) to remove the neo cassette and generate *Wnt3*^{lacZ} heterozygous mice. Heterozygous mice for both alleles were normal and fertile. To determine whether the *Wnt3*^{lacZneo} and *Wnt3*^{lacZ} alleles are null alleles, we generated *Wnt3*$^{lacZneo/lacZneo}$ and *Wnt3*$^{lacZ/lacZ}$ homozygous embryos or crossed *Wnt3*^{lacZneo} and *Wnt3*^{lacZ} heterozygous mice with mice carrying the *Wnt3*$^{\Delta 3,4}$ null allele (Barrow et al., 2003) to generate *Wnt3*$^{lacZneo/\Delta 3,4}$ and *Wnt3*$^{lacZ/\Delta 3,4}$ mutant embryos. All types of mutants phenocopy the previously reported phenotype of *Wnt3* null embryos (Barrow et al., 2007; Liu et al., 1999), that is, they lack a primitive streak, fail to elongate the anteroposterior axis and are resorbed by ~E9.5 indicating that they are null alleles. The *Wnt3*^{lacZneo} strain has been registered with the Mouse Genome Informatics international database under the identifier MGI:5439832 (Wnt3tm1Bhr).

Germ layer isolation

To separate the epiblast from the visceral endoderm, we followed the germ layer separation protocol described previously (Nagy, 2003). Briefly, embryos were cut just below the epiblast –extra-embryonic ectoderm boundary, incubated in a solution containing a mixture of pancreatin and trypsin and pipetted up and down using a pulled Pasteur pipette. Germ layer separation was performed retrospectively after wholemount *in situ* hybridization was conducted.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (Rivera-Perez and Magnuson, 2005) using the following probes: *Wnt3* (full length cDNA, 2,084 bp). *Brachyury* (full length cDNA, 1,784 bp) (Herrmann, 1991). All riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Cat. No. 1175025).

β-Galactosidase staining protocol

We performed β-galactosidase assays as described by Sundararajan and co-workers (Sundararajan et al., 2012). After β-galactosidase staining, embryos were rinsed in wash solution re-fixed in 4% paraformaldehyde at room temperature for 20 min., cleared in glycerol and imaged.

Genotyping

The genotype of the embryos was determined retrospectively after wholemount *in situ* hybridization or after β-galactosidase assays. Each litter was imaged before genotyping to allow unique identification of each embryo. Embryos were placed in 15–20 μl of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl pH8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% IGEPAL (Sigma, Cat. No. 18896) and 0.45% Tween 20) containing 100 mg/ml Proteinase K and incubated overnight at 56°C. After lysis, the proteinase K was inactivated at 95°C for 5 min and 1 μl of the lysate was used for the PCR reaction. Mice were genotyped at postnatal day 10 using a 2 mm tail tip piece.

PCR reactions were carried out using the following oligonucleotides: *Wnt3* wild-type allele, ptmw1 GAC TTC CTC AAG GAC AAG TAC G and ptmw2 GAA GAC GCA ATG GCA TTT CTC (309 bp); Wild-type and $Wnt3^{c}$ alleles, Wnt3F3 5′ TGG CTT CAG CAT CTG TTA CCT TC 3′ and Wnt3R6 5′ AAG ATC CCC ATA CTG CCA TCA C 3′ (389 bp and 546 bp, respectively); $Wnt3^{\Delta 3,4}$ allele, Wnt3F10 5′ GGG AGC CGC CCG TTG CTA 3′ and Wnt3R6 5′ AAG ATC CCC ATA CTG CCA TCA C 3′ (388 bp); $Wnt3^{lacZ}$ allele, LacZF 5′TGG CGT TAC CCA ACT TAA TCG 3′ and LacZR 5′ATG TGA GCG AGT AAC AAC CCG 3′ (324 bp); $Wnt3^{lacZneo}$ allele, NeoF2 TGG CTA CCC GTG ATA TTG CTG and ptmw2 GAA GAC GCA ATG GCA TTT CTC (~500 bp); Ttr^{Cre} transgene, qCreF1 5′ GAA CCT CAT GGA CAT GTT CAG G 3′ and qCreR1 5′ AGT GCG TTC GAA CGC TAG AGC CTG T 3′ (320 bp); *Cripto* wild type allele, Cripto5′UTRf CCT CCG AAG TCC TCA ATC AC and CriptoR3 TCC GAA GTG GCT ATC TCC AG (333 bp); $Cripto^{lacZ}$ Cripto5′UTRf CCT CCG AAG TCC TCA ATC AC and CriptolacZ GAT TAA GTT GGG TAA CGC CAG (~300 bp).

Statistical analysis

We used the Student *t*-test to determine if there was significant difference between the extent of *T* expression relative to epiblast length among *Wnt3*-VE mutants ($Wnt3^{c/c};Ttr^{Cre/0}$) and control ($Wnt3^{c/c}$) littermates.

Results

Visceral endoderm *Wnt3* regulates its own expression in the epiblast

Previous experiments have shown that expression of *Wnt3* in the posterior visceral endoderm appears at ~E5.5 and then in the adjacent epiblast six hours later at ~E5.75 (Rivera-Perez and Magnuson, 2005). In addition, embryos in which *Wnt3* was inactivated in the epiblast but not in the visceral endoderm were able to form a primitive streak, suggesting a signaling role for visceral endoderm-derived Wnt3 in this process (Tortelote et al., 2013). To explore the ability of visceral endoderm-derived Wnt3 to signal to the epiblast we took advantage of $Wnt3^{lacZ}$ mice. $Wnt3^{lacZ}$ mice have the *lacZ* gene inserted in exon 4 of the *Wnt3* locus (Fig. 1A), making it possible to monitor the activity of the *Wnt3* locus using β-galactosidase (β-gal) assays. We reasoned that if *Wnt3* is first activated in the visceral endoderm and it is required for its own expression in the epiblast, $Wnt3^{lacZ/lacZ}$ mutant embryos should show β-galactosidase activity in the visceral endoderm but not in the epiblast.

We demonstrated that the $Wnt3^{lacZ}$ allele is a null allele of *Wnt3* by showing absence of *Brachyury* (*T*) expression in the epiblast of $Wnt3^{lacZ/lacZ}$ mutant embryos and by failure to undergo elongation of the anteroposterior axis (Fig. 1B, C). This phenotype was reiterated in mutants obtained from crosses between $Wnt3^{lacZ}$ mice and mice carrying the $Wnt3^{\Delta 3,4}$ null allele (Barrow et al., 2007) (not shown).

Analysis of E6.5 litters obtained from heterozygous $Wnt3^{lacZ}$ crosses revealed embryos with strong, weak or no β-gal activity (Fig. 1D). Retrospective embryo genotyping showed that $Wnt3^{lacZ}$ heterozygous embryos (n=7) had strong staining at the epiblast/extra-embryonic ectoderm boundary on one side of the embryo (Fig. 1D, E), mimicking the pattern of *Wnt3* expression (Rivera-Perez and Magnuson, 2005; Sundararajan et al., 2012). $Wnt3^{lacZ}$ homozygous embryos (n=7), on the other hand, showed a weak area of staining restricted to half the circumference of the proximal posterior region of the embryo and extended distally only about one fourth of the length of the epiblast region (Fig. 1D, F). As expected, no staining was observed in wild-type embryos (Fig. 1D). Histological analysis revealed that heterozygous embryos exhibited strong β-gal activity in both the posterior visceral endoderm and adjacent epiblast (Fig. 1G), while staining in mutant embryos was restricted to the visceral endoderm layer (Fig. 1H).

The observed β-gal activity in both the epiblast and visceral endoderm of heterozygous embryos but only in the posterior visceral endoderm of mutant embryos suggests that *Wnt3* signaling derived from the posterior visceral endoderm is required for its own expression in the adjacent epiblast at E6.5.

Wnt3 in the visceral endoderm is dispensable for primitive streak formation

To determine the requirement for visceral endoderm-derived *Wnt3* in the establishment of the primitive streak, we inactivated *Wnt3* in the visceral endoderm using mice carrying a conditional allele of *Wnt3* ($Wnt3^{c}$) (Barrow et al., 2003) and Ttr^{Cre} transgenic mice (Kwon and Hadjantonakis, 2009). The $Wnt3^{c}$ allele has loxP sites flanking exons 3 and 4 and generates the $Wnt3^{\Delta 3,4}$ null allele after Cre-mediated recombination (Fig. 2A) (Barrow et al.,

2003). *Ttr*^{Cre} transgenic mice express Cre recombinase under the control of the *Transthyretin* (*Ttr*) promoter in the visceral endoderm of early post-implantation embryos at ~E5.0 (Kwon and Hadjantonakis, 2009).

To generate *Wnt3* visceral endoderm knockout (VE-KO) embryos we initially crossed males heterozygous for the conditional *Wnt3*^{c} allele and hemizygous for the *Ttr*^{Cre} transgene (*Wnt3*$^{c/+}$;*Ttr*$^{Cre/0}$) with females homozygous for the *Wnt3*^{c} allele (*Wnt3*$^{c/c}$). However, we observed embryos that carried a recombined *Wnt3* allele (*Wnt3*$^{\Delta 3,4}$) even in the absence of the *Ttr*^{Cre} transgene. This phenomenon was confirmed by crossing *Wnt3*$^{c/+}$;*Ttr*$^{Cre/0}$ males with CD-1 wild-type females and genotyping of E9.5 embryos. The genotype was determined using only the head of the embryos to avoid contamination with visceral endoderm derivatives from the visceral yolk sac or the gut (Kwon et al., 2008). Approximately 50% (50/85) of embryos obtained from crosses of *Wnt3*$^{c/+}$;*Ttr*$^{Cre/0}$ males and wild-type females were heterozygous for the *Wnt3*$^{\Delta 3,4}$ allele while none of them carried the *Wnt3*^{c} allele indicating complete recombination in the paternal germline. We also tested whether recombination of the conditional allele occurred in the female germline. Crosses between *Wnt3*$^{c/+}$;*Ttr*$^{Cre/0}$ females with wild-type males revealed recombination in a subset of oocytes. From a total of 37 E9.5 embryos analyzed from these crosses, we observed that 18 embryos had inherited the *Wnt3*^{c} allele and 4 of them had undergone recombination to generate the *Wnt3*$^{\Delta 3,4}$ allele, corresponding to a frequency of 22% maternal germline recombination. Despite these drawbacks we were able to generate *Wnt3*-VE mutant embryos from *Wnt3*$^{c/+}$;*Ttr*$^{Cre/0}$ female by *Wnt3*$^{c/c}$ male crosses (Fig. 2B) that were unequivocally identified by genotyping the epiblast and visceral endoderm components of the conceptus (Fig. 2C, D).

Embryos were first hybridized with a *Wnt3* probe to confirm ablation of *Wnt3* in the visceral endoderm. Analysis of embryos at E6.25 revealed absence of *Wnt3* expression in both the epiblast and the visceral endoderm in mutant embryos (n=3) (Fig. 2E). At E6.5, however, weak *Wnt3* expression became evident in the epiblast of *Wnt3*-VE mutant embryos (n=5) (Fig. 2F–I). These results show that *Wnt3* expression was abolished in the visceral endoderm of *Wnt3*-VE mutant embryos. At the same time, the presence of *Wnt3* transcripts in the epiblast of *Wnt3*-VE mutants suggests that Wnt3 activity emanating from the posterior visceral endoderm is not solely responsible for inducing *Wnt3* expression in the epiblast. We confirmed these results by analyzing the expression of the *Wnt3*^{lacZ} transgene in *Wnt3*$^{lacZ/lacZ}$ mutant embryos at E7.5 (n=19). In these embryos, we were able to detect weak β-galactosidase activity in the epiblast (Fig. 2J–M). This data suggests that Wnt3 and other factors control the activity of *Wnt3* in the epiblast.

Inactivation of *Wnt3* in the visceral endoderm leads to delayed formation of the primitive streak

To further characterize the phenotype of *Wnt3*-VE mutant embryos, we performed wholemount *in situ* hybridization using a *T* probe. *T* marks the primitive streak and its precursors in the epiblast (Rivera-Perez and Magnuson, 2005; Wilkinson et al., 1990). We found that *Wnt3*-VE mutant embryos exhibited *T* expression at E6.5, however, this expression tended to be weak and occupied a smaller area that in control littermates (Fig.

3A, B). To quantify the level of *T* expression in *Wnt3*-VE mutant embryos, we measured the extent of *T* expression in the posterior epiblast relative to epiblast length (T/Epi ratio) and compared it to control littermates using a *t*-test (Fig. 3C, Supp. Fig. 1). We found that *Wnt3*-VE null embryos ($Wnt3^{c/c};Ttr^{Cre/0}$, n=10) had a smaller area of *T* expression compared to controls ($Wnt3^{c/c}$, n=18), indicative of delayed development ($p<0.001$). Analysis of embryos at E.9.5 showed that *Wnt3*-VE null embryos ($Wnt3^{c/c};Ttr^{Cre/0}$) were indistinguishable from control littermates (not shown) suggesting that *Wnt3*-VE mutant embryos reach normal development soon after gastrulation. From these results, we conclude that *Wnt3* function in the visceral endoderm regulates the timing of primitive streak formation but it is dispensable for further embryonic development.

To independently confirm that *Wnt3* function in the visceral endoderm is not essential for primitive streak formation, we used tetraploid complementation chimeras. We generated tetraploid embryos from crosses between mice carrying two different *Wnt3* null alleles ($Wnt3^{lacZneo/+}$ X $Wnt3^{\Delta 3,4/+}$). In this way, mutant chimeras are unequivocally identified by the presence of two mutant *Wnt3* alleles in the yolk sac. Tetraploid blastocysts were injected with ES cells carrying the $R26^{lacZ}$ allele (Tremblay et al., 2000), chimeras were recovered at E9.5 and assayed for β-galactosidase activity. We obtained six chimeras of which two of them were derived from mutant $Wnt3^{lacZneo/\Delta 3,4}$ tetraploid blastocysts. These embryos were able to undergo gastrulation and reached the initial stages of organogenesis (Fig. 3D), supporting our genetic results.

Reduced *Wnt3* activity in the epiblast of *Wnt3*-VE null mutants leads to gastrulation defects

Recombination of the conditional *Wnt3* allele in the maternal germline sometimes led to the generation of *Wnt3*-VE null embryos that carried one *Wnt3* mutant allele in the epiblast. This resulted in embryos lacking *Wnt3* in the visceral endoderm ($Wnt3^{\Delta 3,4/\Delta 3,4}$) with a reduced dose of *Wnt3* function in the epiblast ($Wnt3^{\Delta 3,4/c}$). We noticed that these embryos exhibited a more severe phenotype than *Wnt3*-VE null mutants containing two $Wnt3^{c}$ alleles in the epiblast ($Wnt3^{c/c}$). To further explore this phenotype, we crossed $Wnt3^{\Delta 3,4/+};Ttr^{Cre}$ males and $Wnt3^{c/c}$ females to generate $Wnt3^{\Delta 3,4/c};Ttr^{Cre}$ embryos. At E6.5 (n=5), these embryos showed delayed development with failure to elongate the epiblast and incipient *T* expression (Fig. 3E, F). Morphological defects were more pronounced at E7.5 (n=6). At this stage, embryos showed a bulging in the primitive streak, failure to close the proamniotic canal and failure to generate axial mesoderm (Fig. 3G). This phenotype is similar to the phenotype observed in *Wnt3* epiblast knockout embryos (Tortelote et al., 2013). These results indicate that a combination of Wnt3 visceral endoderm signaling and proper *Wnt3* dosage in the epiblast is necessary for gastrulation.

The anterior visceral endoderm restricts expression of *Wnt3* to the posterior side of the embryo

Analysis of *Wnt3* expression relative to the AVE marked by *Hex*-GFP revealed that expression of *Wnt3* is complementary to the AVE (Fig. 4A–C). This observation led us to postulate that the AVE prevents Wnt3 activity in the anterior side of the embryo. To address this question, we took advantage of *Cripto* mutant embryos. In *Cripto* mutants, the AVE

fails to localize to the anterior side of the epiblast and remains at the distal tip of the conceptus (Ding et al., 1998). This misallocation of the AVE leads to radialization of the primitive streak in the proximal epiblast as indicated by the expression of *T* and other markers. Since *T* expression in the epiblast depends on *Wnt3* (Barrow et al., 2007; Liu et al., 1999; Tortelote et al., 2013), we wondered if *Wnt3* expression was radialized in *Cripto* mutant embryos.

Analysis of *Wnt3* expression in *Cripto* null embryos (n=11) revealed that *Wnt3* expression was indeed radialized in mutant embryos forming a ring of expression around the proximal epiblast region (Fig. 4D). Surprisingly, histological analysis showed that *Wnt3* expression was restricted to the visceral endoderm layer and was absent from the epiblast (Fig. 4E). These results indicate that the AVE prevents the expansion of Wnt3 signaling to the anterior side of the embryo and that *Wnt3* expression is downstream of *Cripto* in the epiblast.

To determine if the ectopic expression of *Wnt3* in the visceral endoderm of *Cripto* mutants was responsible for the expression of *T* in the anterior epiblast, we removed *Wnt3* activity from *Cripto* null embryos by generating *Cripto/Wnt3* double mutant embryos. We generated 54 embryos from double heterozygous ($Wnt3^{lacZ/+};Cripto^{+/-}$) crosses and obtained 5 *Cripto/Wnt3* double mutant embryos (Fig. 4F–H). We did not detect *T* expression in these double mutant embryos (Fig. 4H). Therefore, the radialized expression of *Wnt3* in the proximal visceral endoderm of *Cripto* mutants leads to ectopic activation of *T* in the anterior epiblast. These results suggest that Wnt3 is sufficient to induce primitive streak markers in a non-cell autonomous manner at an ectopic location.

Discussion

Previous studies showed that a diffusible signal derived from the visceral endoderm was able to reprogram anterior epiblast to a posterior mesodermal fate to produce hematopoietic tissues (Belaoussoff et al., 1998). Recent evidence further implicated the visceral endoderm as the source of an inductive signal that directs the formation of mesoderm during gastrulation and pointed to Wnt3 as the main regulator acting through the canonical Wnt pathway (Rivera-Perez and Magnuson, 2005; Tortelote et al., 2013). To address this possibility, we inactivated *Wnt3* in the visceral endoderm of mouse embryos while leaving two functional copies of *Wnt3* in the rest of the embryo. Our results show that embryos lacking *Wnt3* in the posterior visceral endoderm have delayed formation of the primitive streak but are able to continue with subsequent development. Hence, Wnt3 activity derived from the posterior visceral endoderm has a temporal role in establishing the primitive streak but its function is dispensable for embryo development. We confirmed these results using tetraploid complementation chimeras. Our data support previous chimera studies conducted by Barrow and co-workers that showed that *Wnt3* in the visceral endoderm was dispensable for development of E9.5 embryos (Barrow et al., 2007). At the same time, it reveals a role for visceral endoderm-derived Wnt3 in the establishment of the primitive streak that was not evident in tetraploid complementation experiments.

Based on the data presented here and the observations from previous studies (Rivera-Perez and Magnuson, 2005; Tortelote et al., 2013), we propose the following model for

specification of the primitive streak and gastrulation in mouse embryos (Fig. 5): First, *Wnt3* is initially active in the posterior visceral endoderm of embryos at the distal visceral endoderm (DVE) stage (~E5.5). This is supported by expression studies (Rivera-Perez and Magnuson, 2005) and by our current results showing that E6.5 $Wnt3^{lacZ/lacZ}$ mutant embryos exhibit β-gal activity in the posterior visceral endoderm but not the epiblast. Second, *Wnt3* emanating from the posterior visceral endoderm induces its own expression and that of other markers for the primitive streak such as *T* in the epiblast. This process is underway in AVE stage (~E5.75-E6.0) embryos. Evidence for this assertion is provided by analysis of *Cripto* mutant embryos. *Cripto* mutants have radialized expression of *Wnt3* only in the visceral endoderm yet they express *T* in the adjacent epiblast. Since *T* expression in the epiblast is downstream of *Wnt3* (Liu, 1999, Barrow, 2007), in *Cripto* mutant embryos expression of *T* in the epiblast must depend on the activity of *Wnt3* derived from the visceral endoderm. An additional source of support is provided by embryos that can initiate gastrulation despite lacking *Wnt3* in the epiblast but that express *Wnt3* in the posterior visceral endoderm (Tortelote et al., 2013). Third, Wnt3 activity is restricted to the posterior side of the embryo by the AVE, the source of multiple antagonists of Wnt signaling that include *Sfrp1*, *Sfrp5* and *Dkk1* (Pfister et al., 2007). This is supported by our analysis of *Cripto* mutant embryos. In *Cripto* mutants the AVE remains at the distal tip of the epiblast and *T* expression becomes radialized in the proximal epiblast region alongside radialization of *Wnt3* in the proximal visceral endoderm. *Wnt3* had previously been shown to be radialized in the proximal region of *Cripto* mutants, however, these studies did not determine if *Wnt3* expression was restricted to the epiblast or visceral endoderm or both tissue layers of the embryo (Kimura et al., 2001). Markers of the primitive streak such as *T*, *Fgf8* and *nodal* have been shown to be radialized in the proximal region of several mutants with distal AVE. This phenomenon occurs in *Otx2* (Kimura et al., 2000; Perea-Gomez et al., 2001) *Lpp3* (Escalante-Alcalde et al., 2003), *Pten* (Bloomekatz et al., 2012) and $Nodal^{D600/--}$ (Robertson et al., 2003) mutants. In all of these mutants, radialized expression is also accompanied by radialization of *Wnt3* expression.

The fact that *Wnt3*-VE mutant embryos can form a primitive streak, albeit at a delayed time, suggests that additional signaling molecules work in concert with Wnt3 to direct the formation of the primitive streak. An obvious candidate is Bmp4, a signaling molecule expressed in the extra-embryonic ectoderm abutting the proximal epiblast. Bmp4 loss of function mutations lead to gastrulation defects although some mutants can undergo gastrulation and reach somite stages (Lawson et al., 1999; Winnier et al., 1995). A role of the extra-embryonic ectoderm in primitive streak induction is also supported by the location of radialized *T* expression only in the proximal epiblast of *Cripto* mutants. Thus, signaling from the extra-embryonic ectoderm may be the source of a signal that allows Wnt3 activity derived from the visceral endoderm to activate the canonical Wnt signaling pathway in the adjacent epiblast leading to expression of primitive streak markers in the epiblast. Another possibility is that additional signaling molecules are at play in the visceral endoderm and could compensate for the absence of *Wnt3*. In fact, *Wnt2b* is expressed in the posterior visceral endoderm and epiblast of E6.5 embryos (Kemp et al., 2005). It is also interesting to note that in *Otx2/Cripto* double mutant embryos, *T* and *Fgf8*, are expressed in the whole epiblast even though *Wnt3* expression is not detected in these embryos (Kimura et al., 2001).

These studies suggest that a molecule other than Wnt3 activates these genes. Alternatively, absence of *Otx2* and *Cripto* may lead to de-repression of these genes in the epiblast.

In conclusion, our study indicates that establishment of the primitive streak and gastrulation is a complex process that involves multiple tissue layers and multiple molecules. This view of mammalian gastrulation may help integrate the role that members of different signaling pathways such as Wnt, Tgfβ and Fgf pathways play during gastrulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We inactivated *Wnt3* in the visceral endoderm using genetics and tetraploid complementation chimeras.
- *Wnt3* ablation in the visceral endoderm leads to delayed development of the primitive streak.
- *Wnt3* function in the visceral endoderm is dispensable for embryo development.
- Interplay between anterior and posterior visceral endoderm controls the position of the primitive streak.
- We provide a new model to explain axial development in mammals.

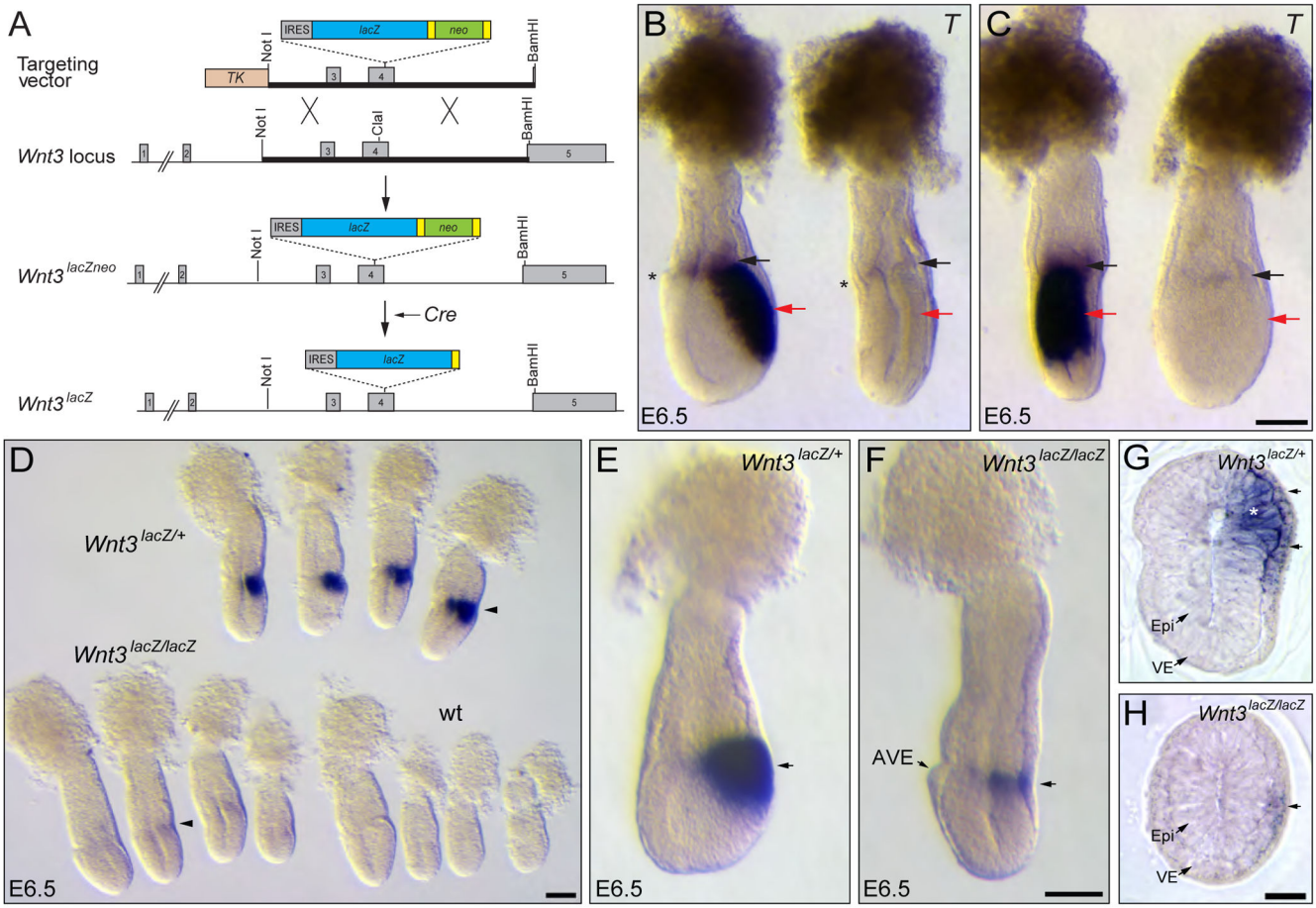

Figure 1. Visceral endoderm *Wnt3* regulates its own expression in the epiblast

A. Schematic representation of the strategy for the generation of $Wnt3^{lacZNeo}$ and $Wnt3^{lacZ}$ alleles. A replacement vector carrying an IRES-*lacZ*-loxP-Neo-loxP cassette inserted into the ClaI site of exon 4 was used to generate $Wnt3^{lacZNeo/+}$ mice. Heterozygous $Wnt3^{lacZNeo/+}$ mice were crossed to $Sox2^{Cre}$ mice to remove the Neo cassette and generate $Wnt3^{lacZ/+}$ animals. **B, C**. Lateral (B) and posterior (C) view of the same control and $Wnt3^{lacZ/lacZ}$ mutant embryos hybridized with *T* The $Wnt3^{lacZ/lacZ}$ mutant embryo lacks expression of *T* in the epiblast (red arrows). Faint *T* expression is observed in the extra-embryonic ectoderm of the mutant embryo (black arrows). The mutant embryo shows the characteristic flattened anteroposterior morphology of *Wnt3* null embryos, indicating that $Wnt3^{lacZ}$ is a null allele. The anterior visceral endoderm (AVE), indicating the anterior side of the embryo, is marked with an asterisk. **D.** Analysis of β-galactosidase activity in embryos derived from $Wnt3^{lacZ/+}$ heterozygous crosses at E6.5. Strong staining is observed in the posterior region of heterozygous ($Wnt3^{lacZ/+}$) embryos, while only weak staining is observed in mutant ($Wnt3^{lacZ/lacZ}$) embryos (arrowheads) and no staining in wild-type (wt) embryos. **E, F**. Wholemount β-galactosidase activity assay of heterozygous (E) or homozygous (F) embryos for $Wnt3^{lacZ}$ alleles dissected at a slightly older stage than those shown in D. In the homozygous embryo, β-galactosidase activity is restricted to a narrow band on the posterior side (arrow) opposite to the AVE. **G, H.** Cross sections of

heterozygous (G) and mutant (H) *Wnt3*^{lacZ} embryos assayed for β-galactosidase activity. Heterozygous embryos show β-galactosidase activity in the epiblast (asterisk) and visceral endoderm (arrows), whereas mutant embryos exhibit staining only in the visceral endoderm (arrow). IRES, internal ribosomal entry site. TK, Thymidine Kinase cassette; Yellow rectangles represent loxP sites. Epi, epiblast; VE, visceral endoderm. Panels B and C, E and F and G and H are shown at the same scale. Scale bars, 100 μm in C, D, F and H.

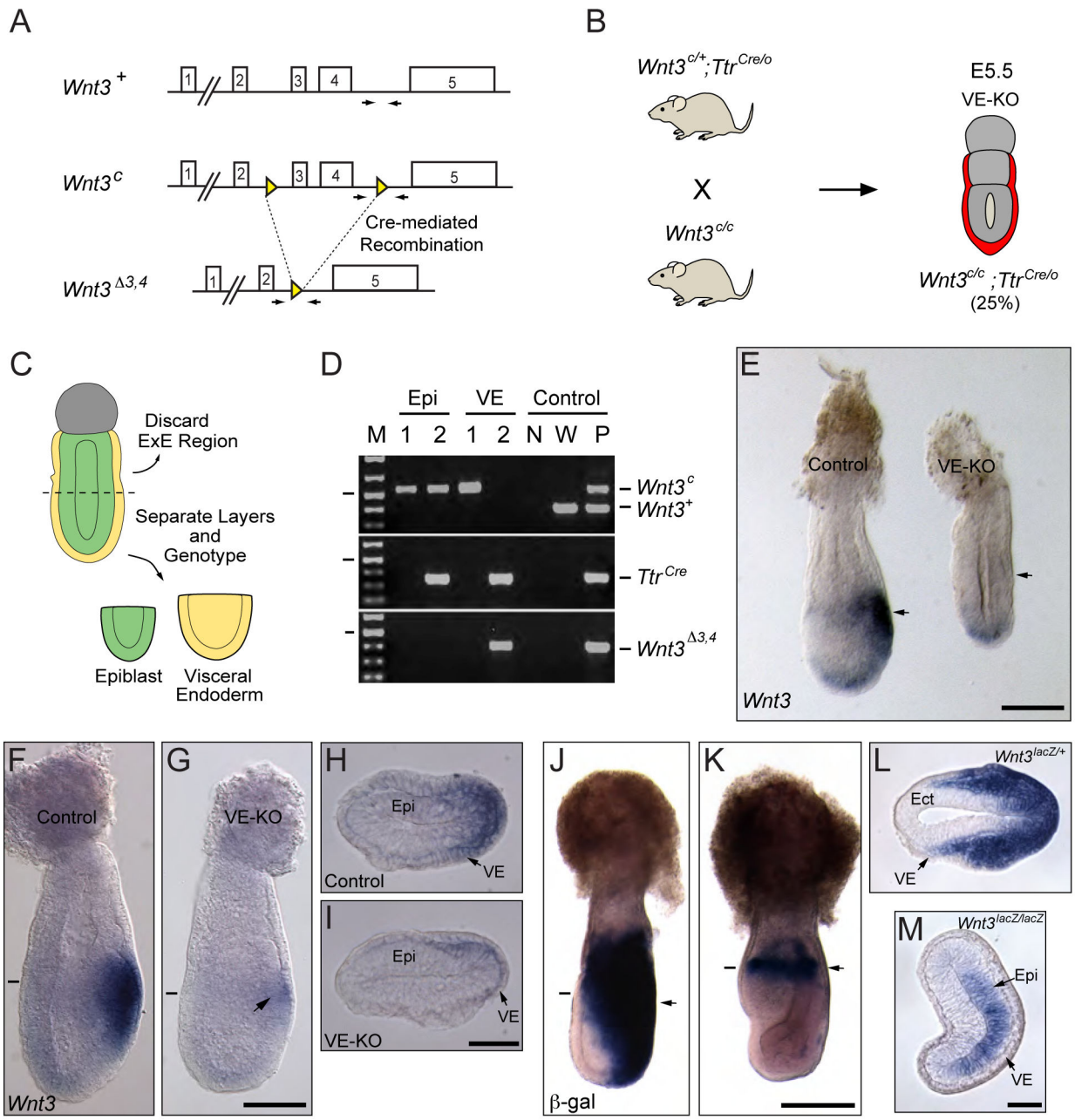

Fig. 2. *Wnt3* function in the visceral endoderm is dispensable for *Wnt3* expression in the epiblast

A. Schematic representation of *Wnt3* wild-type (*Wnt3*$^{+}$), floxed (*Wnt3*^{c}) and null (*Wnt3*$^{\Delta 3,4}$) alleles. Arrows indicate the position of the oligos used for genotyping. **B.** Mating strategy for generating embryos with conditional ablation of *Wnt3* in the visceral endoderm. *Wnt3*-VE null embryos are mutant for *Wnt3* in the visceral endoderm (red) but retain two functional alleles of *Wnt3* (*Wnt3*^{c}) in the rest of the conceptus. **C.** Schematic representation of the strategy for isolating the epiblast and visceral endoderm layers from control and *Wnt3*-VE mutant embryos. **D.** PCR-genotyping of the epiblast and visceral endoderm from control and *Wnt3*-VE embryos. Genotyping for Cre indicates that only sample 2 is positive for the *Ttr*^{Cre} transgene (320 bp). Therefore, sample 1 corresponds to a *Wnt3*$^{c/c}$ and sample

2 to a *Wnt3*$^{c/c}$;*Ttr*$^{Cre/0}$ embryo. Both samples retain the non-recombined *Wnt3*^{c} allele (546 bp) but not the wild-type (381 bp) or recombined *Wnt3*$^{\Delta 3,4}$ (388 bp) alleles in the epiblast. The presence of the *Wnt3*$^{\Delta 3,4}$ allele and the absence of the *Wnt3*^{c} allele in the visceral endoderm of sample 2 indicates that the *Ttr*^{Cre} transgene induced a visceral endoderm-specific recombination of the *Wnt3*^{c} allele in this tissue. The oligos used to amplify the *Wnt3*^{c} allele also amplify the *Wnt3*$^{+}$ allele, as seen in wild type (W) and positive (P) control samples. M, 1kb plus DNA ladder. The lines on the left indicate the 500 bp band. N, no template control. **E.** E6.25 control and *Wnt3*-VE mutant (VE-KO) embryos hybridized with a *Wnt3* probe. The VE-KO embryo lacks *Wnt3* expression whereas the control embryo exhibits the *Wnt3* expression in the posterior region (arrows). Background staining is evident in the distal region of both embryos. **F, G.** E6.5 control and *Wnt3*-VE mutant embryos hybridized with *Wnt3* probe. The control embryo exhibits expression of *Wnt3* in the posterior epiblast and visceral endoderm. The VE-KO embryo has weak *Wnt3* expression in the epiblast (arrow). **H, I.** Histological sections from control (H) and VE-KO mutant (I) embryos shown in F and G, respectively. The approximate position of the sections is indicated by a small line. **J, K.** E7.25 heterozygous (*Wnt3*$^{lacZ/+}$) or homozygous (*Wnt3*$^{lacZ/lacZ}$) embryos assayed for β-galactosidase activity. The heterozygous embryo exhibits β-galactosidase staining in the primitive streak and its mesendoderm descendants while the homozygous embryo has β-galactosidase activity restricted to a small area of the epiblast (arrows). **L, M**. Histological sections of the embryos shown in Panels J and K. The approximate location of the sections is indicated by a small line. Panels F and G, H and I, J and K and L and M are shown at the same scale. Scale bars, 100 μm in E, G, I and M; 200 μm in K.

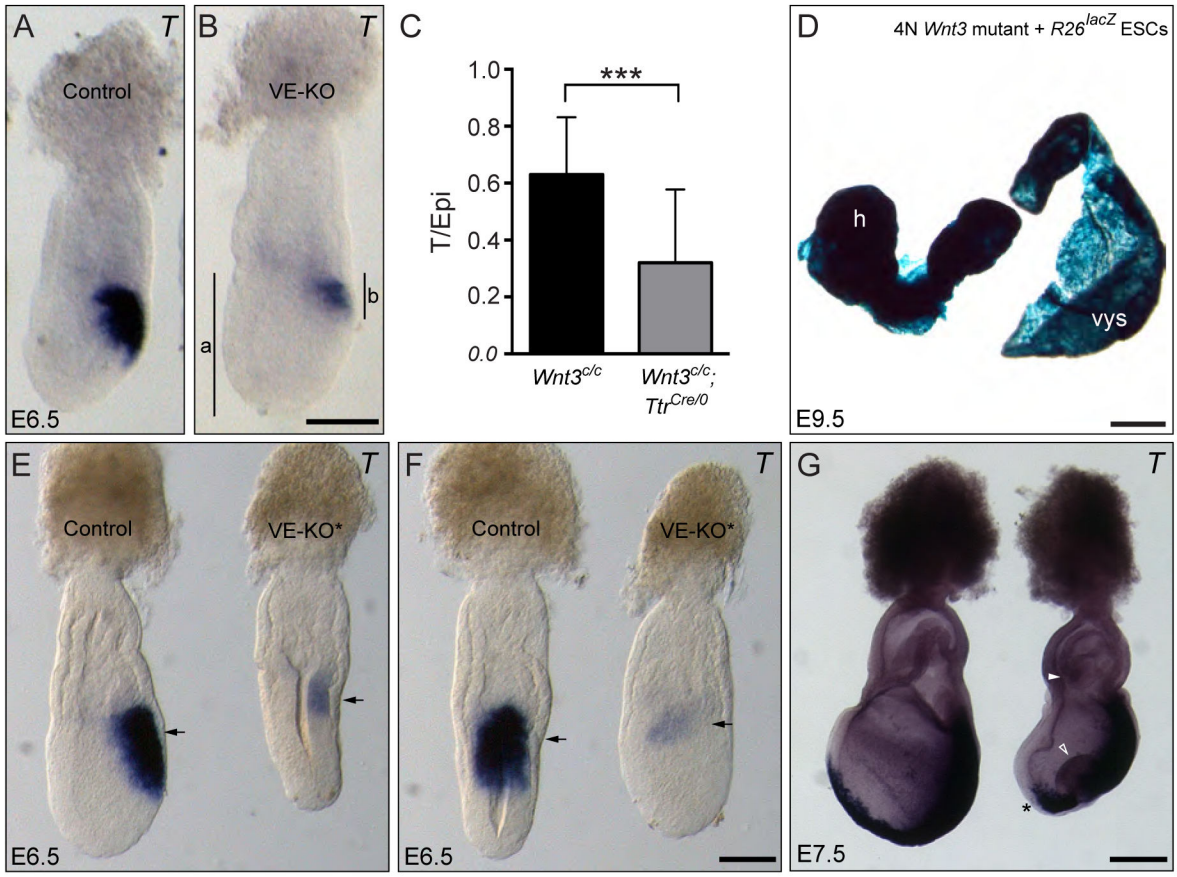

Fig. 3. Inactivation of *Wnt3* in the visceral endoderm leads to delayed formation of the primitive streak

A, B. Lateral views of control (A) and *Wnt3* VE-null (VE-KO) (B) embryos hybridized with *T*. The vertical lines in B, indicate the length of the epiblast (a) and primitive streak (b) as indicated by *T* expression. **C.** Analysis of the extent of primitive streak development between control ($Wnt3^{c/c}$) and VE-KO ($Wnt3^{c/c};Ttr^{Cre/0}$) embryos. A comparison of the ratio of the extent of *T* expression relative to the proximodistal length of the epiblast (as indicated in B) reveals significant delay in the development of the primitive streak in VE-KO embryos relative to controls ($p<0.001$). **D.** Chimera derived from a tetraploid *Wnt3* null ($Wnt3^{lacZNeo/\Delta 3,4}$) blastocyst injected with $R26^{lacZ}$ ES cells. Embryo is composed solely of ES cells and has undergone gastrulation, indicating that *Wnt3* function in the visceral endoderm is dispensable for gastrulation. The yolk sac appears blue due to the presence of β-gal positive extra-embryonic mesoderm cells derived from the $R26^{lacZ}$ ES cells. h, head; vys, visceral yolk sac. **E – G.** Lateral (E) and posterior (F) views of control ($Wnt3^{c/\Delta 3,4}$) and *Wnt3*-VE mutant embryos heterozygous for *Wnt3* in the epiblast (VE-KO*; $Wnt3^{c/\Delta 3,4};Ttr^{Cre/0}$) hybridized with *T* at E6.5 (E and F) and E7.5 (G). The $Wnt3^{c/\Delta 3,4};Ttr^{Cre/0}$ embryo fails to elongate the anteroposterior axis at E6.5 and has abnormal gastrulation at E7.5 as shown by the presence of a bulge of cells in the primitive streak (arrow), failure to close the proamniotic canal (arrowhead) and absence of *T* staining in axial mesoderm (asterisk). Panels A and B and E and F are shown at the same scale. Scale bars, 100 μm in B and F, 500 μm in D, and 200 μm in G.

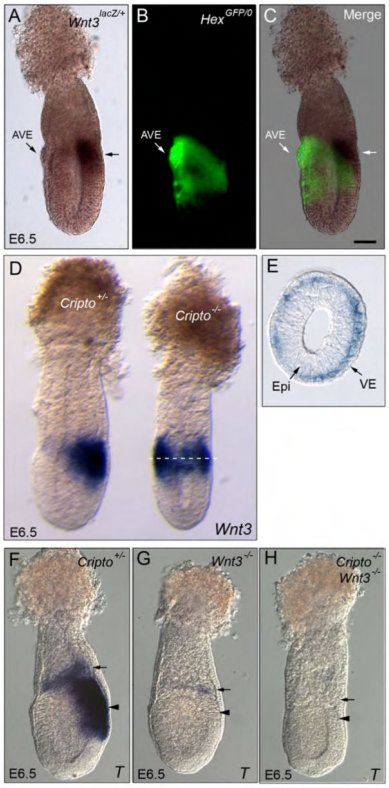

Fig. 4. The AVE restricts *Wnt3* expression to the posterior side of the embryo

A–C. E6.5 embryos double heterozygous for Hex^{GFP} and $Wnt3^{lacZ}$ assayed for β-galactosidase activity. *Wnt3* expression (arrows) is complementary to the AVE (marked by Hex^{GFP}). **D.** Heterozygous and *Cripto* mutant embryos dissected at E6.5 and hybridized with a *Wnt3* probe. In the *Cripto* mutant embryo, *Wnt3* expression is radialized in the proximal epiblast region. **E.** Transverse section of *Cripto* mutant embryo shown in D. The approximate location of the section is marked by a dotted line. *Cripto* expression is radialized but restricted to the visceral endoderm layer. **F–H.** *Cripto* Heterozygous (F), *Wnt3* mutant (G) and double *Wnt3/Cripto* mutant (H) embryos dissected at E6.5 and hybridized with *T. T* is expressed in the extra-embryonic ectoderm (arrow) and epiblast (arrowhead) of heterozygous embryos and weakly in the extra-embryonic ectoderm of *Wnt3* mutants. The double mutant embryo completely lacks *T* expression showing that in *Cripto* mutant embryos, the radialization of *T* in the epiblast depends on radialized *Wnt3* activity in the visceral endoderm.

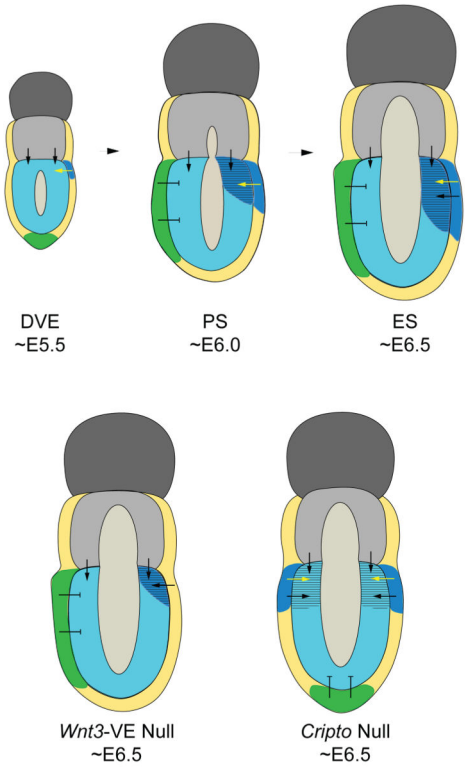

Fig. 5. Proposed model for primitive streak formation in the mouse

At approximately E5.5, the DVE (green) becomes evident at the distal tip of the epiblast concomitantly with the appearance of *Wnt3* expression (blue) in the posterior visceral endoderm. At ~E5.75-E6.0, Wnt3 emanating from the posterior visceral endoderm (yellow arrows) activates its own expression and that of other markers of the primitive streak, such as *T* (striped), in the adjacent epiblast. The antagonistic activity of the AVE prevents the spread of Wnt3 activity in the anterior epiblast. In *Wnt3*-VE mutants, expression of primitive streak markers is delayed appearing at late E6.5 stages. In *Cripto* mutants, the AVE remains at the tip of the epiblast; this allows expansion of *Wnt3* expression to the anterior visceral endoderm leading to radialization of the primitive streak. The black arrows indicate potential signaling events derived from extra-embryonic ectoderm and/or posterior visceral endoderm that may work in concert with Wnt3 in the establishment of the primitive streak and maintenance of gastrulation. Abbreviations: DVE, distal visceral endoderm stage; PS, Pre-streak stage; ES, early streak stage.