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Sfrp1 and *Sfrp2* are required for normal male sexual development in mice

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ABSTRACT

Secreted frizzled-related proteins (Sfrps) are antagonists of WNT signalling implicated in a variety of biological processes. However, there are no reports of a direct role for Sfrps in embryonic organogenesis in mammals. Using *in vivo* loss-of-function studies we report here for the first time a redundant role for *Sfrp1* and *Sfrp2* in embryonic sexual development of the mouse. At 16.5 dpc, male embryos lacking both genes exhibit multiple defects in gonad morphology, reproductive tract maturation and gonad positioning. Abnormal positioning of the testis appears to be due to failed gubernaculum development and an unusually close association between the cranial end of the reproductive tract and the kidney. The testes of double homozygotes are smaller than controls, contain fewer cords from the earliest stages, but still express *Insl3*, which encodes the hormone required for gubernacular masculinisation. *Lgr8*, which encodes the Insl3 receptor, is also expressed in the mutant gubernaculum, suggesting that Sfrp1/Sfrp2 signalling is not required for expression of the ligand or receptor that controls transabdominal testicular descent. Similarities between the abnormalities of embryonic sexual development in $Sfrp1^{-/-}$ $Sfrp2^{-/-}$ embryos with those exhibited by the Looptail and Wnt5a mutants suggest that disrupted non-canonical Wnt signalling may cause these defects.

Introduction

Secreted frizzled-related proteins (Sfrps) are a family of secreted glycoproteins containing a cysteine-rich domain (CRD) at their N-terminus that is homologous to the CRD of the Wnt receptor, Frizzled (Fz) (Jones and Jomary, 2002; Kawano and Kypta, 2003). Several reports describe evidence of Sfrps antagonizing Wnt-mediated signalling by direct competitive interaction with Wnt ligands via the CRD (Wang et al., 1997; Leyns et al., 1997; Dann et al., 2001) or by formation of non-signalling complexes with Frizzled proteins (Bafico et al., 1999), with different Sfrps exhibiting differential specificities with respect to their inhibitory potential (Galli et al., 2006). However, despite this growing body of knowledge concerning molecular interactions of Sfrps, there is still a relative paucity of data describing their specific physiological roles.

In the mouse, five members of the *Sfrp* gene family have been identified using a variety of approaches (Rattner et al., 1997; Shirozu et al., 1996; Finch et al., 1997; Melkonyan et al., 1997). Phylogenetic analysis using protein sequence comparisons indicates that Sfrp1, Sfrp2 and Sfrp5 are the most closely related members of the family. We have previously analysed expression of *Sfrp2* and *Sfrp5* in the developing mouse reproductive organs and utilised ENU mutagenesis to generate point mutations in these genes that are predicted to severely disrupt function (Quwailid et al., 2004; Cox et al., 2006). Both *Sfrp2* and *Sfrp5* are expressed from early stages in the developing mesonephros, which contains the primordia of the male and female reproductive tracts. Both exhibit sexually dimorphic patterns of expression in the Müllerian duct, the female reproductive tract primordium. Although these sex-specific profiles suggest a possible role in the sexually dimorphic fate of the mesonephros, homozygosity for these ENU-induced point mutations in *Sfrp2* and *Sfrp5* does not cause any overt abnormalities in development or reduction in viability (Cox et al., 2006). This is consistent with reports of other recent genetic studies utilising targeted null alleles (Leaf et al., 2006; Satoh et al., 2008).

An analysis of *Sfrp1* and *Sfrp2* during mouse embryogenesis reveals widespread and overlapping expression patterns (Leimeister et al., 1998). Such expression profiles raise the possibility of functional redundancy between closely related family members. Genetic proof of such redundancy was obtained recently when the genes encoding Sfrp1 and Sfrp2 were inactivated in the mouse by gene targeting (Satoh et al., 2006). Mice lacking *Sfrp1* appear to be viable and fertile, and the great majority of those lacking *Sfrp2* are also normal apart from very occasional syndactyly. Mice of the genotype $Sfrp1^{-/-}$ $Sfrp2^{+/-}$ also appeared viable and fertile, but embryos homozygous for both targeted mutations ($Sfrp1^{-/-}$ $Sfrp2^{-/-}$) die after 16.5 days *post coitum*

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(dpc) and exhibit occasional oedema, craniofacial defects and extra digits. The anteroposterior body axis is also shortened, primarily in the thoracic region, and this is attributed to defects in cell migration in the paraxial mesoderm. Aberrant somitogenesis also contributes to this dysmorphology and is correlated with perturbed Notch function.

Wnt signalling is known to play an important role in various aspects of embryonic sexual development (Carroll et al., 2005; Jeays-Ward et al., 2004; Kim et al., 2006), but there have been no previous mutational studies describing a direct role for Sfrps in embryonic organogenesis or embryonic sexual development. In order to address the function of *Sfrp* genes in mouse sexual development we have adopted a loss-of-function approach, using targeted null alleles, and analysed embryos lacking both *Sfrp1* and *Sfrp2* at later stages of gestation. This is the unique pairwise combination of *Sfrp* null alleles known to lead to phenotypic abnormalities in the embryo (Satoh et al., 2006, 2008). Here we report abnormalities in male reproductive organ development, most notably in the morphology of the developing testis and its failure to undergo the first transabdominal phase of descent, and attribute this to a failure of the reproductive tract and associated gubernaculum to masculinise appropriately and an unusually close physical association between the developing reproductive tract and kidney. A comparison is made between these abnormalities and those found in embryos lacking key non-canonical Wnt signalling molecules, Vangl2 and Wnt5a. Strong similarities between *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ and *Wnt5a*$^{-/-}$ embryos suggest that Sfrp1 and Sfrp2 regulate non-canonical Wnt signalling during sexual development. We discuss these comparative data and the possible molecular mechanisms by which Sfrps modulate Wnt signalling during development of the reproductive organs.

Materials and methods

Generation of mutant embryos and expression analyses

Sfrp1$^{-/-}$ *Sfrp2*$^{-/-}$ embryos were generated by timed matings of *Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$ females and males bred on a mixed 129J/C57BL/6J background (Satoh et al., 2006). Noon on the day of the copulatory plug was counted as 0.5 dpc. Embryos were staged accurately based on the number of tail somites or limb and gonad morphology. Dead or dying embryos late in gestation (after 16.5 dpc) were discarded. Wholemount *in situ* hybridization (WMISH) analysis of embryonic tissues was performed as previously described (Cox et al., 2006; Grimmond et al., 2000). Probes for *Sox9* (Wright et al., 1995), *Lim1* (Kobayashi et al., 2004), *Oct4* and 3β*HSD* (Siggers et al., 2002) have been previously described. An *Lgr8* probe was generated from IMAGE clone 40129664. Probes for *Insl3*, *Jag1* and *AR* were generated by reverse transcription polymerase chain reaction (RT-PCR) from embryonic gonad RNA samples (13.5 dpc) using the following primers: 5′-AGCTGCTGCAGTGGCTAGA-3′ and 5′-GGGACACAGGGAGGAGGT-3′ (*Insl3*); 5′-AATGGTGATGGCAGCCTTAG-3′ and 5′-GCCTGCCTGTCTCTTTTCAA-3′ (*Jag1*); 5′-CACCTTGTTCCCTTTCCAGA-3′ and 5′-TGGGGTCAACCTGCTCTTTA-3′ (*AR*).

Detection of the *Sfrp1* KI *lacZ* reporter was performed using a protocol based on (Whiting et al., 1991). Embryos were dissected in PBS to expose the developing reproductive organs/tracts, fixed on ice (1% PFA, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40 in PBS) and then washed in PBS/0.02% NP-40. Staining was carried out in the dark, at room temperature for 16 h or until blue colour fully developed in X-gal stain (PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% deoxycholate, 0.02% NP-40, 1 mg/ml X-Gal). Samples were post-fixed in 4% PFA/PBS.

Mouse mutants utilised and genotyping

Genotyping for the targeted alleles of *Sfrp1* and *Sfrp2* was performed as previously described (Satoh et al., 2006). The identification and analysis of the *Sfrp2*^{HC50F} and *Sfrp2*^{I153N} mutations has been described previously (Quwailid et al., 2004; Cox et al., 2006). Mice heterozygous for these mutations were maintained on a C57BL/6J background. Genotyping for both alleles was performed by an allelic discrimination PCR (AD-PCR) performed using an ABI Prism 7000 Sequence Detection System according to manufacturer's guidelines as previously described (Cox et al., 2006).

Looptail mice (*Vangl2*$^{Lp/+}$) were maintained on the C3H/HeH background and identified by the presence of a prominent tail loop (Murdoch et al., 2001). Homozygous embryos were identified by the occurrence of severe neural tube defects characteristic of this mutant.

Mice expressing green fluorescent protein (GFP) ubiquitously (Tg(GFPU)5Nagy/J) were purchased from The Jackson Laboratory and carriers identified by neonatal fluorescence.

Embryos were sexed by a PCR assay that simultaneously amplifies the *Ube1y1* and *Ube1x* genes, using the following primer pair: 5′-TGGATGGTGTGGCCAATG-3′ and 5′-CACCTGCACGTTGCCCTT-3′. Y- and X-linked amplicons give products of 335 bp and 253 bp, respectively.

Testosterone assay

Whole embryos (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ and control littermates) were collected at 14.5 dpc and homogenised in PBS. Testosterone levels were measured using a previously validated ELISA kit from Neogen (# 402510) according to the manufacturer's instructions. Three embryos in each class were measured in quadruplicate. Given in the text are the mean values for each class of embryo.

Exogenous administration of testosterone

Androgens were administered to embryos using a protocol adapted from (Hammes et al., 2005). Pregnant mice were injected subcutaneously with a daily dose of 2 mg of dihydrotestosterone (DHT: 5α-androstan-17β-OH-ol-3-one, Sigma-Aldrich) in a 1:9 mix of ethanol/sunflower oil from days 10.5 to 17.5 dpc (or with just ethanol/oil as control) and embryos were harvested at 17.5/18.5 dpc.

Organ culture

Culturing of embryonic gonads and recombination experiments between sub-dissected gonads and marked mesonephroi were performed based on methodologies described in (Martineau et al., 1997). XY urogenital ridges (UGRs), consisting of gonad and attached mesonephros, were collected at 11.5 dpc and initially cultured to establish conditions under which testis cords formed reliably after 48 h culture. Samples were incubated on 1.5% agar blocks at 37 °C/5% CO_2 in Dulbecco's Minimal Eagle's Medium (DMEM)/10% fetal calf serum (FCS)/50 μg/ml ampicillin/200 mM L-glutamine. For recombination cultures, 11.5 dpc XY male UGRs from mutant embryos (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) which also carried a ubiquitously expressed GFP transgene (Tg(GFPU)5Nagy/J)) and littermate controls were sub-dissected into component gonad and mesonephros in PBS. These were then reassembled in appropriate combinations and cultured for 48 h, as above. Migration from the marked mesonephros into the attached gonad was imaged using a Leica TCS SP5 confocal microscope. No migration was observed into control XX gonads during these experiments.

Immunohistochemistry

The following antibodies were utilised in this study: PECAM-1 (BD Bioscience, #553708); phospho-histone H3 (pHH3) (Upstate/Millipore, #06-570); anti-cleaved caspase 3 (Cell Signalling, #9661); active (non-phosphorylated) β-catenin (ABC) antibody (Upstate/Millipore, #05-665). Immunostaining was performed on sectioned material, apart from PECAM-1, which was performed on wholemounts and visualised using confocal microscopy. For quantitative analysis of cell proliferation in the gonad mesonephros, cryosections were

immunostained with anti-pHH3 antibody. After immunostaining, sections were counterstained with DAPI and cells were visualised using a Zeiss Axiophot2.

Results

Sfrp1 is expressed in the developing reproductive tracts and associated ligaments

We have previously described the expression profile of *Sfrp2* in the developing gonads and reproductive tracts between 11.5 and 14.5 dpc (Cox et al., 2006). Expression is detectable in both the developing testis cords and ovary during this period, whilst expression in the mesonephros is sexually dimorphic. In males, expression is prominent in the epithelium of the Müllerian duct at 13.5 dpc, whilst in females it is most prominent in the periductal mesenchyme. Lower levels of expression are also found throughout the mesonephric mesenchyme during this time.

In order to analyse the expression of *Sfrp1* over several days of mouse sexual development we exploited a *lacZ* knock-in that replaces exon 1 to generate a targeted null allele of *Sfrp1* (called *Sfrp1* KI) as previously described (Satoh et al., 2006). Analyses of the embryonic gonads and reproductive tracts from *Sfrp1* KI homozygotes ($Sfrp1^{-/-}$ $Sfrp2^{+/-}$ and $Sfrp1^{-/-}$ $Sfrp2^{+/+}$ embryos) revealed no overt abnormalities of morphology, as anticipated given the normal reproductive health of adult individuals. Staining for *lacZ* expression between 10.5 and 16.5 dpc revealed prominent expression at several sites in the urogenital organs, but no significant expression in the male or female gonads. In contrast, expression in the mesonephros was visible at 10.5 dpc (data not shown) and became prominent in both males and female embryos at 11.5 dpc in the mesenchyme surrounding the Wolffian duct, in the region of the mesonephros proximal to the gonad

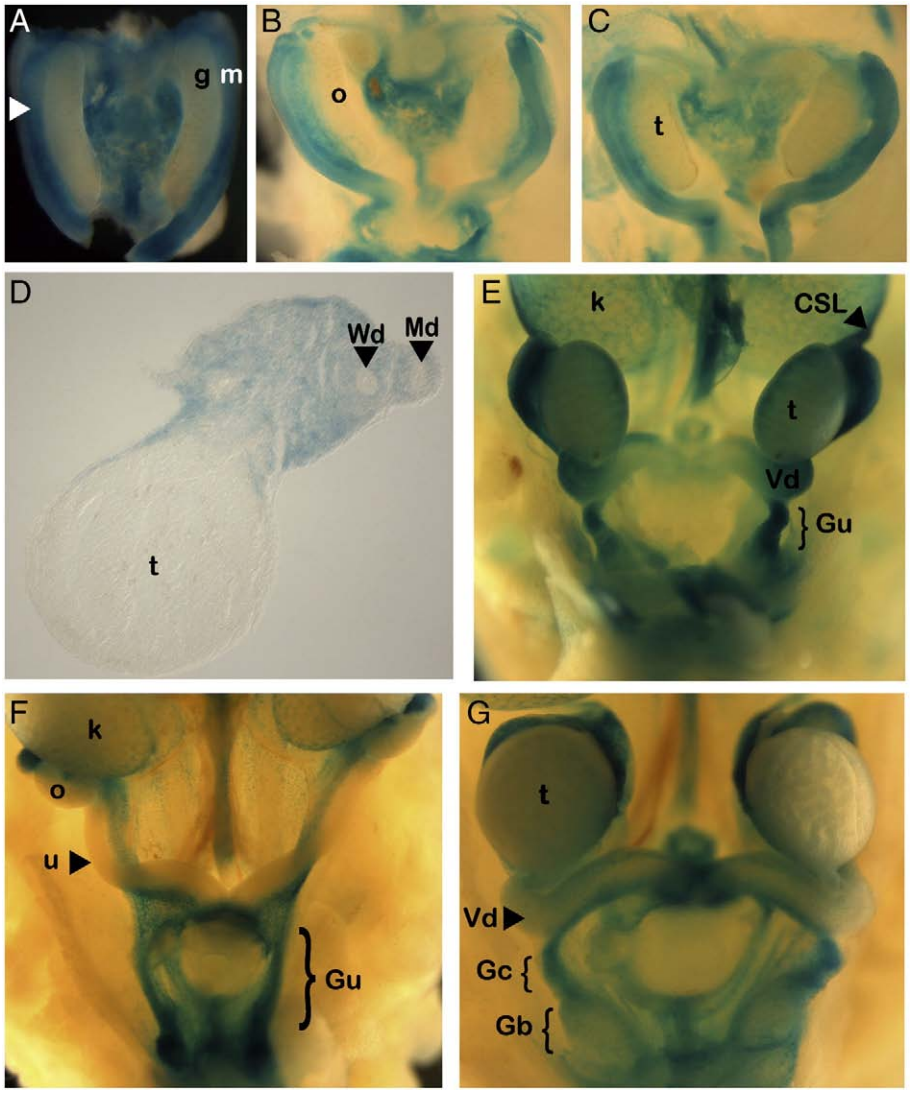


Fig. 1. *Sfrp1* is strongly expressed in the developing reproductive tracts and associated ligaments. (A) Examination of the *Sfrp1* KI reporter at 11.5 dpc reveals prominent expression in the mesonephros in the region of the Wolffian duct (arrowhead), adjacent to the gonad. No differences between males (XY) and females (XX) were observed. By 12.5 dpc expression is observed throughout the mesonephros in females (B) and males (C). (D) Section data at 13.5 dpc reveal that expression is restricted to the mesonephric mesenchyme and is absent from the Wolffian duct and Müllerian duct. (E) By 15.5 dpc expression is restricting to the segment of the mesonephros adjacent to the testis in males. Strong expression is now observed in the gubernaculum, which at this stage in males is a thickened cord and wider bulb at the caudal end. Expression is also seen in the cranial suspensory ligament (CSL). (F) Expression in females at 16.5 dpc reveals a continuous line of expression along the cranial and caudal ligaments associated with the reproductive tract. The vestigial gubernaculum is long and thin in females at this stage. No expression is observed in the uterus. Low levels of expression are observed in the kidney. (G) In males at 17.5 dpc expression is still strong in the gubernacular cord but much reduced in the gubernacular bulb. Expression extends from the gubernacular cord along the vas deferens. M, mesonephros; g, gonad; WD, Wolffian duct; MD, Müllerian duct; Gu, gubernaculum; t, testis; o, ovary; k, kidney; Vd, vas deferens; u, uterus; Gc, gubernacular cord; Gb, gubernacular bulb; CSL, cranial suspensory ligament.

(Fig. 1A). At these early stages no expression was detected in the lateral portion of the mesonephros in the region of the future Müllerian duct. From approximately 12.5 dpc onwards the mesenchymal expression in the mesonephros becomes more widespread and includes the Müllerian duct and Wolffian duct regions (Figs. 1B–D). Sectioning of the reproductive tracts at 13.5 dpc indicates that epithelial cells of the reproductive tracts themselves do not express *Sfrp1* (Fig. 1D). From 15.5 days onwards expression in the male and female reproductive tracts begins to be restricted to the segment adjacent to the developing gonad (Figs. 1F, G). By 15.5 dpc the most prominent expression in males is observed in the developing gubernaculum (Fig. 1E), the caudal ligament required for the transabdominal phase of testicular descent (Nef and Parada, 2000). Expression is detected in both the gubernacular bulb and cord and also extends into the body wall itself, adjacent to the ligament. By 16.5 dpc the gubernacular expression in males is most prominent in the cord but is beginning to diminish (Fig. 1G). Similarly, expression is also prominent in the female gubernaculum at 16.5 dpc (Fig. 1F), where the absence of male hormones results in a longer, thinner structure that does not support gonadal descent. *Sfrp1* expression in females at this stage extends in an unbroken fashion along the genital mesentery, from the gubernaculum, along the edge of the uterus, through to the connective tissue associated with the cranial suspensory ligament adjacent to the ovary (Fig. 1F and data not shown). Comparison of the *Sfrp1 lacZ* reporter with a known gubernacular marker, *Hoxa10* (Nightingale et al., 2008; Satokata et al., 1995), confirmed *Sfrp1* expression in the gubernaculum (data not shown). Given the prominent expression of *Sfrp1* in the developing gubernaculum, we examined expression of *Sfrp2* in this structure at 13.5 and 14.5 dpc using WMISH and also detected expression (data not shown). WMISH analysis of *Sfrp1* at earlier stages (10.5 and 11.5 dpc (data not shown)) also revealed no significant differences between endogenous gene expression and the *Sfrp1* KI reporter. Thus, we conclude that the profile of *Sfrp1* KI *lacZ* expression is a reliable indicator of endogenous *Sfrp1* expression in the embryonic reproductive organs.

Analysis of the morphology of the developing reproductive organs of embryos lacking Sfrp1 and Sfrp2

Of the three Sfrps that fall into *Sfrp1*, *Sfrp2* and *Sfrp5* phylogenetic cluster (Jones and Jomary, 2002), only the pair-wise combination of homozygosity for null alleles of *Sfrp1* and *Sfrp2* results in overt abnormalities of embryogenesis (Cox et al., 2006; Satoh et al., 2006, 2008). Embryos lacking all three genes die at 11.5 dpc, which is too early to examine anything but the very beginnings of sexual development. Embryos lacking *Sfrp1* and *Sfrp2* ($Sfrp1^{-/-}$ $Sfrp2^{-/-}$) have been shown to die late in gestation, after 16.5 dpc (Satoh et al., 2006). Embryonic lethality at 16.5 dpc (or later) permits an almost

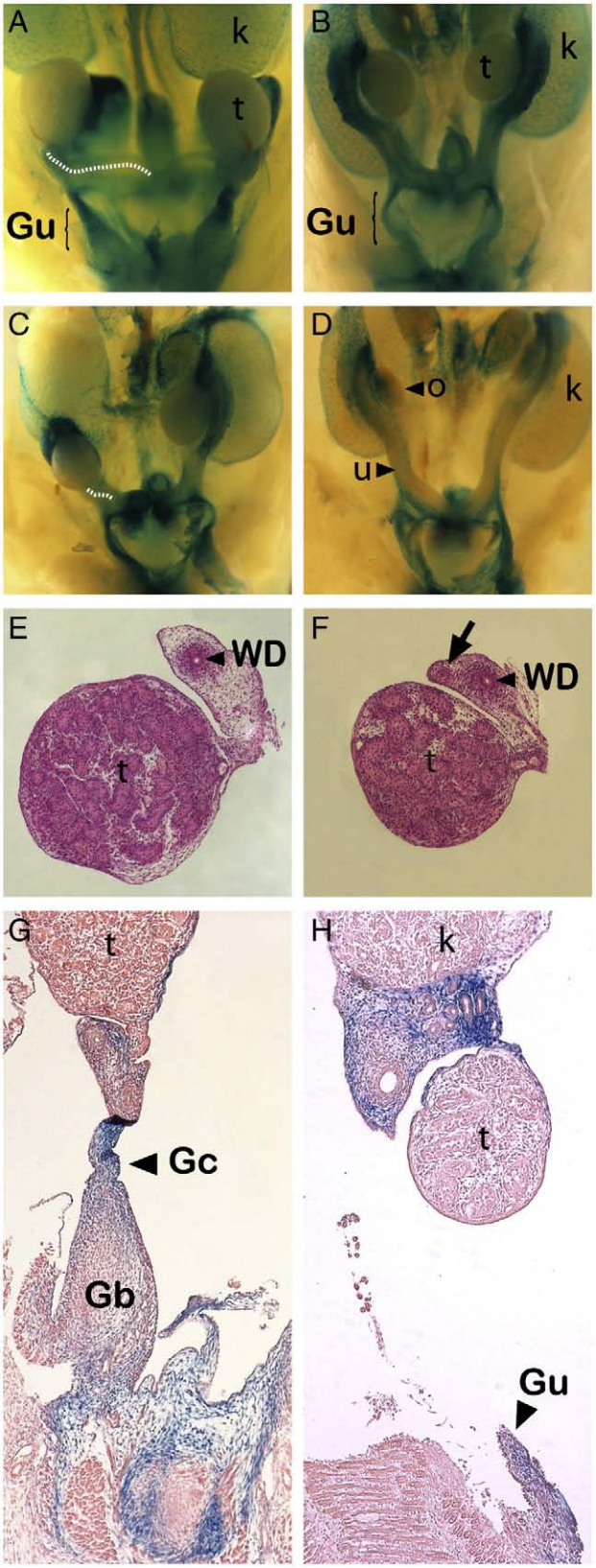


Fig. 2. $Sfrp1^{-/-}$ $Sfrp2^{-/-}$ (double homozygote) embryos exhibit abnormalities of gonad size, morphology and position at 16.5 dpc. (A) Control ($Sfrp1^{-/-}$ $Sfrp2^{+/-}$) male embryo showing the testis, vas deferens and gubernaculum at 16.5 dpc after staining for *Sfrp1 KI* reporter expression. Note the thickened gubernaculum and long vas deferens (dotted line). (B) A double homozygote male embryo showing the position of the testes in the abdominal cavity at 16.5 dpc. Note the thin, vestigial gubernaculum that resembles that found in wild-type females at the same stage (see Fig. 1F) and the hypoplastic testes. (C) This double homozygote has greatly reduced length of the vas deferens (dotted line), which is approximately the same length as the testis. (D) A double homozygote female at 16.5 dpc showing abnormal position of the ovaries on the ventral surface of the kidney. (E, F) Histological (H&E stained) section through double homozygote testis at 16.5 dpc (F) showing overall reduced testis size and reduced number of seminiferous cords when compared with control testis in (E). The Wolffian duct (WD) is visible in both sections. The arrow in (F) points to the Müllerian duct remnant, which in this embryo at this position in the mesonephros is still visible as a clump of disorganised cells. This may indicate some delay in the completion of Müllerian duct regression in double homozygote males. Complete regression is, however, observed in embryos at later stages (data not shown). (G) Coronal section of 16.5 dpc control embryo reveals the thickened gubernacular bulb and cord. Note the strong *Sfrp1* reporter gene expression in the cord and cranial end of the bulb and tissue beneath and medial to the gubernaculae. (H) Histological section through double homozygote embryo at 16.5 dpc showing the close association between the cranial reproductive tract and the kidney and absence of a thick, masculinised gubernaculum. WD, Wolffian duct; MD, Müllerian duct; Gu, gubernaculum; t, testis; o, ovary; k, kidney; Vd, vas deferens; u, uterus; Gc, gubernacular cord; Gb, gubernacular bulb.

comprehensive survey of pre-natal sexual development (Wilhelm and Koopman, 2006). The embryonic gonads are morphologically distinct in the two sexes by 12.5 dpc and highly differentiated at the cellular level by 13.5 dpc (Brennan and Capel, 2004; Lovell-Badge et al., 2002). Male-specific Müllerian duct regression, initiated by testicular anti-Müllerian hormone (AMH), is complete by 16.5 dpc (Jamin et al., 2002; Josso and Clemente, 2003; Roberts et al., 1999; Kobayashi and Behringer, 2003) and the transabdominal phase of testicular descent is completed by 17.5 dpc, though close to completion at 16.5 dpc (Nef and Parada, 2000; Adham and Agoulnik, 2004; Klonisch et al., 2004).

The morphology of the gonads and reproductive tracts was first analysed in doubly homozygous (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) male embryos at 16.5 dpc and compared to littermate controls (*Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$ or *Sfrp1*$^{-/-}$ *Sfrp2*$^{+/+}$). When doubly homozygous mutant male embryos were dissected so as to expose the urogenital system, the most striking abnormality was the position of the testes (Figs. 2B, C). Instead of being in their normal position either side of the bladder, as in control male embryos (Fig. 2A), they were closely associated with the kidney higher in the abdominal cavity (Figs. 2B, C, H). This position of the testis is due to a close physical association between the cranial end of the male reproductive tract and the kidney, revealed by histological examination of sections (Fig. 2H). The vasa deferentia were often very short and simply projected up into the abdominal cavity, frequently appearing asymmetric in length and position (Fig. 2C). No obvious persistence of the Müllerian duct was observed after histological analysis of doubly homozygous male embryos at 16.5 dpc (Fig. 2E and data not shown), indicating that the Müllerian duct regresses as usual in affected male embryos under the influence of testicular AMH.

In doubly homozygous mutant female embryos the ovaries are misshapen and are also positioned abnormally (Fig. 2D), residing on the ventral surface of the kidneys rather than the lower dorsal position observed in wild-type female fetuses (Fig. 1F), and they exhibit asymmetric positioning. A detailed description of female sexual development in the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryo will be described elsewhere (Warr et al., manuscript in preparation).

In addition to this abnormal morphology of the reproductive tract and position of the male gonad, the mutant testes are smaller and have fewer seminiferous cords when compared to control littermates (Figs. 2A, B). Histological analysis of sections from affected testes confirmed this reduction in size and number of cords (Figs. 2E, F).

We also examined embryos lacking *Sfrp1* that were also homozygous for two ENU-induced *Sfrp2* mutations previously described (Quwailid et al., 2004; Cox et al., 2006). The aim of these studies was to determine whether these point mutations, *Sfrp2*^{HC50F} and *Sfrp2*^{HI153N}, were hypomorphic and would thus permit the study of *Sfrp* gene function in neonate or adult physiology. However, mice with the genotypes *Sfrp1*$^{-/-}$ *Sfrp2*$^{HC50F/HC50F}$ and *Sfrp1*$^{-/-}$ *Sfrp2*$^{HI153N/HI153N}$ died before birth. Examination of fetuses at 16.5 dpc revealed abnormalities similar to those lacking both *Sfrp1* and *Sfrp2*, including a decrease in anteroposterior axis length, reduced outgrowth of facial structures and limbs and abnormal reproductive organ development (data not shown). However, the severity of these abnormalities was reduced in *Sfrp1*$^{-/-}$ *Sfrp2*$^{HI153N/HI153N}$ embryos. From these genetic studies we conclude that *Sfrp2*^{HC50F} is a null allele and *Sfrp2*^{HI153N} is hypomorphic i.e. a partial loss-of-function allele.

Defects of gubernaculum development in affected male embryos are not caused by the absence of genes required for gubernacular masculinisation

Failure of normal testicular descent, known as cryptorchidism, affects approximately 1–4% of live male births in the human population (Ivell and Hartung, 2003). Descent of the testes has two phases: transabdominal and inguinoscrotal. In the first, transabdominal, phase the testis descends from its embryonic pararenal position to the inguinal region at the base of the abdomen. The second, or inguinoscrotal phase, involves the descent of the testis down through the inguinal canal and into the scrotum and is controlled by androgens and the genitofemoral nerve through their affects on the gubernaculum (Momose et al., 1993). In the mouse, the first phase is completed by 17.5 dpc and is regulated by hormonal products of the developing testis, namely androgens and insulin-like factor 3 (Insl3) (Adham et al., 2000). Mice lacking Insl3 fail to undergo transabdominal descent and exhibit bilateral cryptorchidism (Nef and Parada, 1999; Zimmermann et al., 1999), primarily due to a failure of the fetal gubernaculum to grow in size, differentiate and promote testicular descent. Given the prominent expression of *Sfrp1* in the developing gubernaculum and the role that this structure plays in determining the position of the testes, we examined the gubernaculum in doubly homozygous mutants and littermate controls. Control male embryos exhibited thick gubernacular bulbs and cords at 16.5 dpc (Figs. 1G and 2A, G). In contrast, examination of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male embryos revealed the presence of a thin, elongated gubernaculum, similar to that found in control female embryos (Figs. 1F and 2B, C). The vestigial gubernaculum of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male embryos was confirmed by histological analysis (Fig. 2H).

We then examined the mutant testes in order to determine whether they contained Leydig cells that expressed *Insl3*, since absence of this hormone might explain the vestigial gubernaculum of mutant males. WMISH analysis of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos using a marker of embryonic Leydig cells, 3β*HSD*, revealed the presence of these steroidogenic cells at 13.5 dpc (Fig. 3A). Affected embryonic testes at the same stage were then used to examine *Insl3* expression by WMISH. This revealed normal levels of expression when compared to littermate controls. We also observed a reduction in the length of the affected male gonads at 13.5 dpc, which, whilst variable on such a mixed genetic background, were on average 76% of the length of control gonads (n=21 stage-matched pairwise comparisons SD=11.3%). The reduced size of the mutant testis might be expected to result in a decreased number of *Insl3*-positive Leydig cells (Fig. 3B). From this we conclude that Sfrp1 and Sfrp2 are not required for expression of *Insl3*.

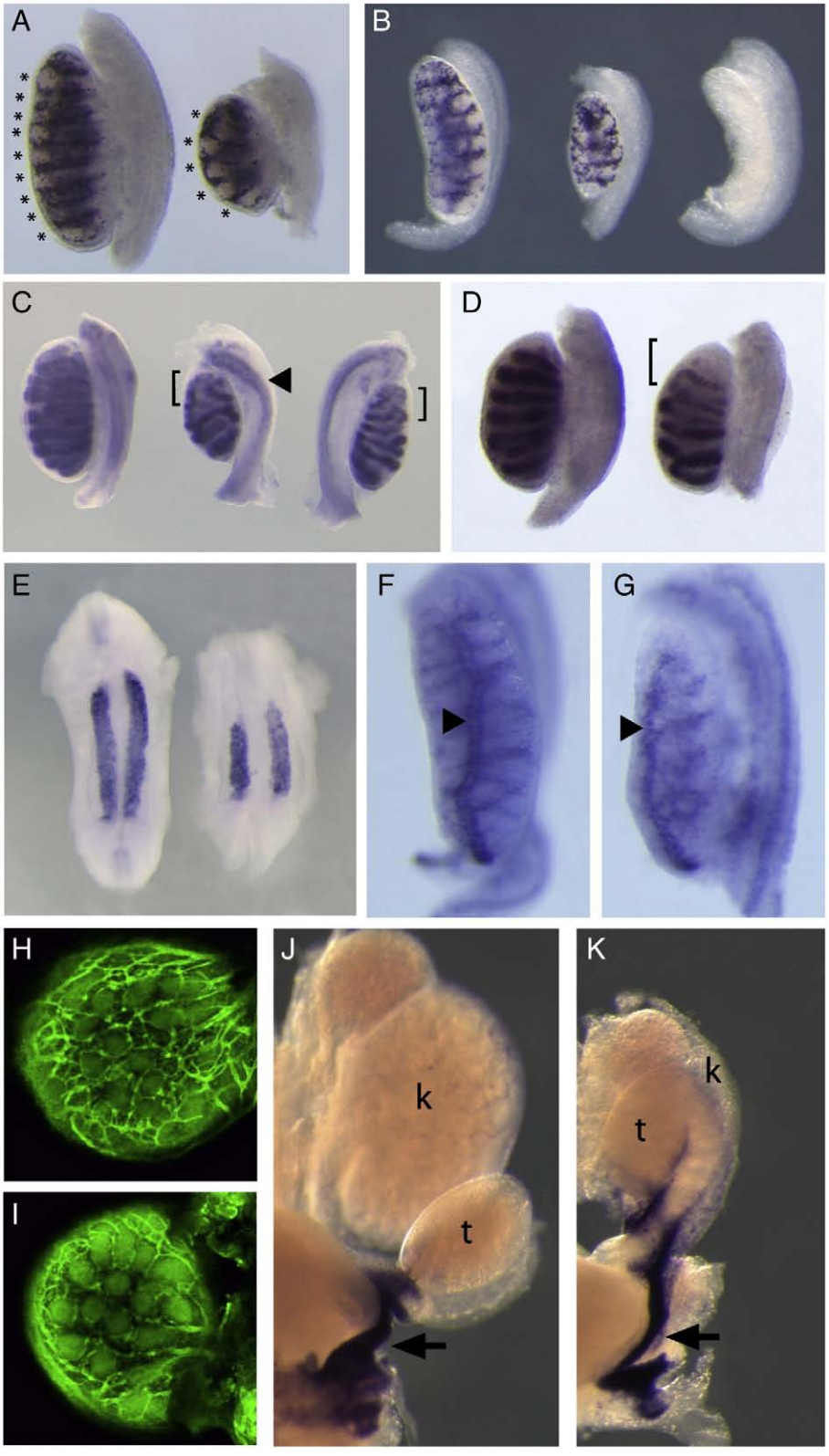
A variety of other markers of testis development were also examined between 10.5 and 15.5 dpc. Analysis of *Sox9* expression at 13.5 dpc, a marker of Sertoli cells, revealed a reduced number of testis cords with an irregular pattern (Fig. 3C). On average, the number of testis cords in affected gonads was 71% that of controls (n=16 stage-matched pairwise comparisons SD=12.1%). Expression analysis of *Oct4* (germ cells), *Jag1* (coelomic vessel) and PECAM (endothelial cell and germ cells) confirmed that whilst the overall size of the gonad was reduced, its shape irregular and the number of testis cords reduced, differentiation of constituent testicular cell types occurs in an overtly normal fashion and no significant loss of markers occurs (Figs. 3D–F). Reduction in the length of the male gonad was observed at the earliest stages examined, as evidenced by *Sf1* expression (Fig. 3E).

We next examined the expression of *Lgr8*, the gene encoding the receptor for Insl3 (Kumagai et al., 2002; Overbeek et al., 2001), in affected and control embryos using WMISH. Mouse genetics has shown Lgr8 to be the sole Insl3 receptor (Bogatcheva et al., 2003) and in the rat it is expressed in the developing gubernaculum and germ cells of the testis (Anand-Ivell et al., 2006; Scott et al., 2005). WMISH analysis of male embryos at 14.5 dpc, when Lgr8 is actively regulating gubernacular development, revealed expression of *Lgr8* in the gubernaculum of both double homozygotes and controls (Figs. 3J, K). We conclude that signalling by Sfrp1 and Sfrp2 in the developing gubernaculum is not required for *Lgr8* expression.

Examination of the role of androgen signalling in the aetiology of the mutant phenotype

Testosterone is responsible for several steps in male sexual development, including the maturation of the embryonic Wolffian

duct into the epididymis, vas deferens and seminal vesicles and the masculinisation of the external genitalia. The shortened vasa deferentia observed in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male mutant embryos raises the possibility of a deficit in androgen signalling. Such abnormalities might be due to androgen production, or transduction of its signal through the androgen receptor (AR). To examine these possibilities, we first measured testosterone levels in affected and control fetuses at 14.5 dpc. Average testosterone levels in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male embryos (0.51 ± 0.15 ng/mg) were not significantly different (based on *t*-test (*p* value = 0.7)) from those observed in littermate control male embryos (0.47 ± 0.14 ng/mg). Next, we assayed the responsiveness of doubly homozygous and littermate control embryos to exogenous dihydrotestosterone (DHT) administered *in utero*. Female embryos (control and *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) in the experimental group showed signs of



testosterone-mediated events at 16.5–18.5 dpc, namely, regression of the cranial suspensory ligament (CSL) and coiling of the oviduct, as previously described (Hammes et al., 2005). These observations confirmed the efficacy of the DHT administrations. Administration of DHT to *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male embryos did not result in any changes to morphology (data not shown). Together, these experimental data indicate that the abnormalities in affected mutant male embryos are not caused by insufficient production of testosterone from the testes, but may reflect insensitivity of some target tissues to androgens. An examination of other androgen-dependent events in sexual development, such as differentiation of the prostate gland and sexual differentiation of the external genitalia, could not be performed in the doubly homozygous mutants because these occur in the mouse embryo after 16.5 dpc (Berman et al., 2004; Yamada et al., 2006). However, examination of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ male embryos at 17.5 dpc indicates that the CSL regresses in these individuals and epididymal coiling is observed, although not to the same extent as controls, suggesting that there is no widespread androgen insensitivity (data not shown). Examination of a number of developing reproductive tract markers, including *Lim1*, *Wnt7a*, *Wnt4*, *Wnt9b* and the gene encoding the androgen receptor, *AR*, also revealed no overt defects in differentiation that distinguished doubly homozygous embryos from littermate controls (data not shown).

The cause of abnormal testis development in Sfrp1$^{-/-}$ *Sfrp2*$^{-/-}$ *embryos*

One puzzling aspect of the phenotype of affected males concerns the morphological abnormalities of the gonad from early stages, including reduction in size from 10.5 dpc and the irregular pattern and reduced number of testis cords from 12.5 dpc. No significant expression of *Sfrp1* was observed in the testis at these stages and testicular expression of *Sfrp2* is prominent only in germ cells of the developing testis cords ((Cox et al., 2006) and unpublished data). Germ cells are not required for the normal morphological development of the testis, including the formation of testes cords or associated vascularisation (McLaren, 1998). Thus, the absence of *Sfrp2* alone from the testes of doubly homozygous embryos does not appear to be a compelling explanation of the abnormal early development of the organ.

One alternative explanation of these testicular abnormalities is that the mesonephros in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos is functionally compromised at early stages (from 10.0 dpc onwards). Both Sfrp1 and Sfrp2 are prominently expressed in the mesonephros at this time. The gonad forms on the ventromedial aspect of the mesonephros at approximately 10.0 dpc and subsequently acts as a source of cells for the growing gonad (Swain and Lovell-Badge, 1999). By 11.5 dpc the mesonephros is an important source of cells for the developing XY gonad, providing endothelial cells and other connective tissue cell-types by a process of male-specific migration (Martineau et al., 1997; Tilmann and Capel, 1999; Brennan et al., 2002; Ross and Capel, 2005). The mesonephric cell migration assays described in the above studies require marked mesonephroi, often derived from mice ubiquitously expressing fluorescent reporters. We employed this approach in order to assess whether the mesonephros from *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos at 11.5 dpc was capable of supporting cell migration into a wild-type gonad after recombination and culturing *in vitro*. A breeding scheme was devised to allow generation of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos expressing green fluorescent protein (GFP) ubiquitously. When mesonephroi from such embryos were isolated at 11.5 dpc and cultured adjacent to a wild-type (CD1 strain) embryonic gonad of the same stage, levels of migration into the gonad after 48 h were comparable to control co-cultures in which *Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$ mesonephroi were used (Figs. 4A, B). These data suggest that any deficit in the ability of the mesonephros in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos to support gonad development is likely to affect earlier events in gonad differentiation i.e. prior to 11.5 dpc. Of course, we cannot rule out the possibility that a combination of mesonephric and gonadal deficits in cellular functions results in the aberrant morphology of the testis observed in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos.

We also examined proliferation in the developing gonad and mesonephros in doubly homozygous mutant and control gonads and mesonephroi at 11.5 dpc. Reduced cell proliferation at these stages in the XY gonad is known to disrupt testis development (Schmahl and Capel, 2003), and a recent study of mice lacking just *Sfrp1* suggested a pro-proliferative role for this gene in post-natal development of the prostate gland (Joesting et al., 2008). Analysis using an antibody to phospho-histone H3 (pHH3) revealed no differences in cell proliferation between XY *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos and controls in either the gonad or mesonephros at 11.5 dpc (Figs. 4C–E). Nor were differences observed in the frequency of apoptotic cells in the same tissue samples, as evidence by analysis with an anti-cleaved caspase 3 antibody (data not shown). No apoptotic cells were detected in either group at these stages despite validation of the assay's sensitivity and specificity with a positive control.

Two recent papers have shown disruption of testis development resulting from artificial stabilisation of β-catenin levels in the developing organ (Chang et al., 2008; Maatouk et al., 2008). It is thought that increased levels of stabilised, non-phosphorylated β-catenin in the nucleus of Sertoli cells antagonizes the male pathway of development and promotes the ovarian pathway. The possibility exists that absence of Sfrp1 and Sfrp2, putative Wnt/β-catenin signalling antagonists, increases nuclear β-catenin levels in testicular cells. We compared levels of stabilised, non-phosphorylated β-catenin in doubly homozygous mutant and control testes and mesonephroi at 14.5 dpc using an antibody (ABC) reported to detect the stable form of the protein (van Noort et al., 2002; Satoh et al., 2006). Prominent expression was only associated with the cell periphery of epithelial cells of the reproductive tract of the mesonephros and coelomic epithelium and no significant nuclear signals were observed in any cells (data not shown). However, the levels of stabilised, nuclear β-catenin in the control or mutant reproductive organs at this stage may be too low to be detected by the sensitivity of the immunohistochemistry protocol employed, and yet still be biologically significant. Thus,

Fig. 3. Abnormal testis and gubernaculum development in double homozygotes (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) is not associated with failure of differentiation of constituent cell-types. (A) Expression of 3β*HSD*, a Leydig cell marker, in the interstitium of control (left) and double homozygote testes at 13.5 dpc. The asterisks are placed next to individual testis cords in order to show the reduced number in double homozygotes. (B) The gene encoding the hormone Insl3, required for gubernacular masculinisation, is expressed in control male testes (left) and double homozygotes (centre), but is absent from control ovaries at 13.5 dpc (right). (C) Analysis of *Sox9* expression, a marker of Sertoli cells, in control (left) and double homozygote (centre and right) testes at 13.5 dpc, showing the reduction in size of the testis and its abnormal shape in double homozygotes. The number of testis cords is reduced and their arrangement is irregular, when compared to controls, especially at the cranial end of the testis (square brackets). Cords are frequently too short to span the entire width of the testis and occasionally form "horse shoe"-shapes (square bracket). Expression of *Sox9* in the Müllerian duct mesenchyme (arrowhead) is normal in double homozygotes. (D) *Oct4* expression in 13.5 dpc testes from control (left) and double homozygotes (right). Germ cells are present as normal in double homozygotes although disruption to testis cord formation, especially at the cranial pole, is apparent (square bracket). (E) *Sf1* expression at 10.5 dpc shows reduced length of gonadal region is already apparent in double homozygotes (right) when compared to controls (left). (F, G) *Jag1* expression reveals presence of the coelomic vessel in testes from control (F) and double homozygote (G) embryos. Note the irregular shape and diffuseness of interstitial vessels in double homozygote. (H, I) Immunostaining of 16.5 dpc testes from control (H) and double homozygote (I) embryos with anti-PECAM antibody, revealing presence of branching blood vessels in double homozygotes, but in reduced number. The fainter green patches in both samples correspond to germ cell expression in the seminiferous cords. (J, K) *Lgr8* expression in the developing gubernaculum of control (J) and double homozygote (K) embryos at 14.5 dpc. *Lgr8* expression is robust in the double homozygote (arrow in K), but the gubernaculum is under-developed in comparison to the control at the same stage, which has already grown into a short, thick, bulbous structure (arrow in J). t, testis; k, kidney.

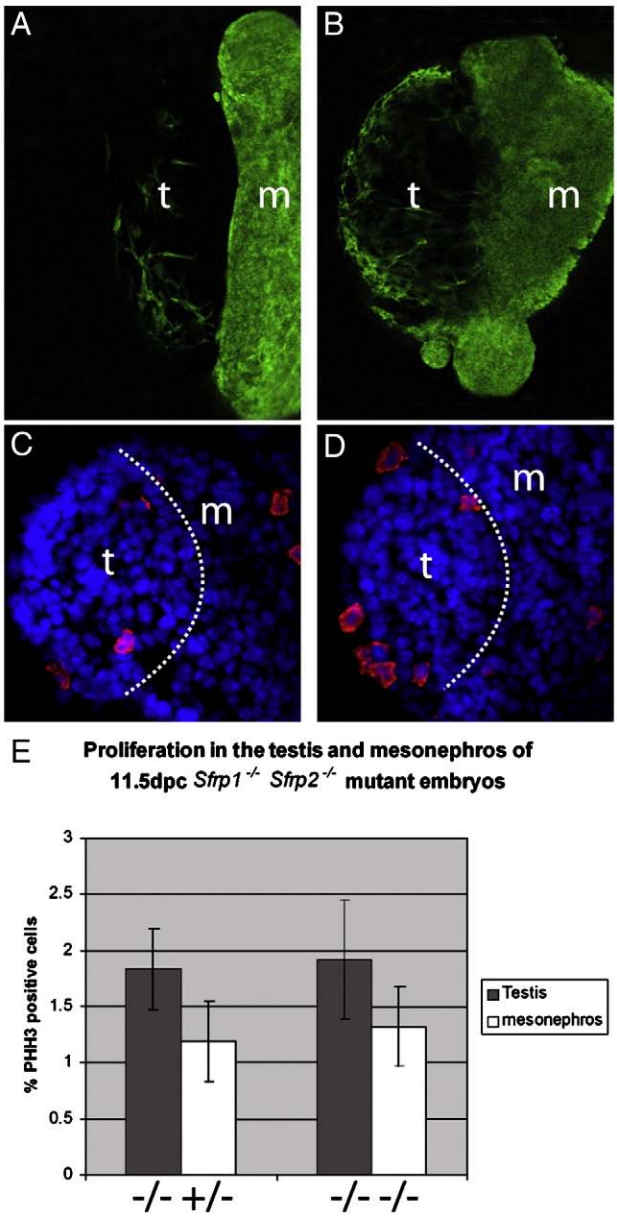


Fig. 4. Studies on the causes of abnormal testis development in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos. (A–B) Confocal images of organ co-culture experiments. Control CD1 testes were co-cultured with mesonephroi which ubiquitously express GFP and were of the following control and mutant genotypes: (A) *Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$ (B) *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$. GFP-positive cells in the gonad are clearly identifiable in both classes of culture. (C–D) pHH3 expression at 11.5 dpc in (C) control (*Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$) and (D) mutant (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) testis/mesonephros. Anti-pHH3 staining is red and nuclei are counter-stained blue with DAPI. The dashed line indicates the extent of the gonad. (E) Proliferation was quantified by scoring the percentage of pHH3-positive cells in total counts of approximately 1000 cells per embryo. Graph shows mean percentage (N=3) and error bars indicate±SEM (standard error mean). These data indicate no significant alteration to proliferation in the mutants. t, testis; m, mesonephros.

whilst these data suggest that the disruption to testis development in the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos is not readily explained by elevated Wnt/β-catenin signalling at these stages, they should not be considered definitive.

Absence of Sfrp1 and Sfrp2 results in abnormalities that phenocopy defects in sexual development in the Looptail (Lp) and Wnt5a mutants

Genetic evidence suggests that Sfrps modulate the activity of Wnt molecules acting through both the canonical and/or non-canonical pathways (Satoh et al., 2006, 2008). To assess whether defective non-canonical Wnt signalling might also contribute to the abnormal sexual development of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos we compared their phenotype with that observed in the case of embryos homozygous for two mutations disrupting non-canonical Wnt signalling. *Vangl2* (Looptail, *Lp*) functions in the non-canonical Wnt/planar cell polarity (PCP) signalling pathway (Murdoch et al., 2001; Ybot-Gonzalez et al., 2007). Disruption of the process of convergent extension has previously been observed in embryos harbouring compound mutations in *Sfrps* and *Vangl2*, indicating genetic interaction between these loci (Satoh et al., 2008). Given this genetic interaction between *Sfrps* and *Vangl2* in early embryos, we examined *Vangl2*$^{Lp/Lp}$ embryos at 16.5 dpc for evidence of previously unreported abnormalities of sexual development. *Vangl2*$^{Lp/Lp}$ embryos have testes of normal size and morphology, vasa deferentia of normal length and masculinised gubernaculae (compare Figs. 5A, B). However, the cranial reproductive tract is in very close association with the kidney, higher in the abdominal cavity, in a manner reminiscent of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos (compare Figs. 5D, E and 2G).

We also examined embryos lacking Wnt5a, another molecule associated with non-canonical Wnt signalling through activation of JNK via the Ror2 receptor (Oishi et al., 2003; Schambony and Wedlich, 2007). Embryos lacking Wnt5a (*Wnt5a*$^{-/-}$) exhibit a variety of outgrowth defects, although defects in male sexual development have not been reported (Yamaguchi et al., 1999). At 15.5 dpc, the morphology of the developing reproductive organs in males appears to phenocopy that found in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos at the same stage (compare Figs. 5C and 2K). The testes are reduced in size and are again in close association with kidneys via the cranial reproductive tract (Figs. 5C, F). Moreover, the gubernaculum is vestigial in appearance as evidenced by WMISH analysis with the marker *Lgr8* (Fig. 5C). These observations on *Vangl2*$^{Lp/Lp}$ and *Wnt5a*-deficient embryos suggest that the pathways and processes in male sexual development disrupted by the absence of Sfrp1 and Sfrp2 may be the same as those disrupted by mutations in the non-canonical Wnt, especially Wnt5a-mediated, signalling pathway.

Discussion

We describe here the first direct evidence that secreted frizzled-related proteins are required for the normal embryonic organogenesis in mammals. Absence of overt abnormalities in fetuses lacking either *Sfrp1* or *Sfrp2* individually suggests that they act redundantly during development of the reproductive organs. Male embryos lacking both genes exhibit multiple defects in gonad morphology, reproductive tract maturation and gonad positioning. Abnormal positioning of the testis appears to be due to abnormalities in both gubernaculum development and an unusually close association between the cranial end of the reproductive tract and the kidney. The abnormal morphology of the mutant testis itself is not easily explained by the absence of *Sfrp1* or *Sfrp2* given that neither gene is expressed prominently in the supporting (Sertoli) cell lineage that is most likely to result in such abnormalities if disrupted. The possibility remains that *Sfrp* genes are expressed at low levels in this or other gonadal cell lineages. However, it is also possible that the testis abnormalities are explained by a defective contribution from the closely associated mesonephros. Our cell migration assay suggests that this contribution is not impaired at 11.5 dpc when endothelial cells migrate into the XY gonad from the mesonephros. However, given that the gonad of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos is small when examined as early as 10.5 dpc, it might be that gonad development is compromised from its earliest stages due to its association with the mutant mesonephros. Assessing mesonephric function at such early stages is not currently feasible using organ culture techniques. It is worth noting that, given the importance of gonadal growth in testis determination, disruption to the early

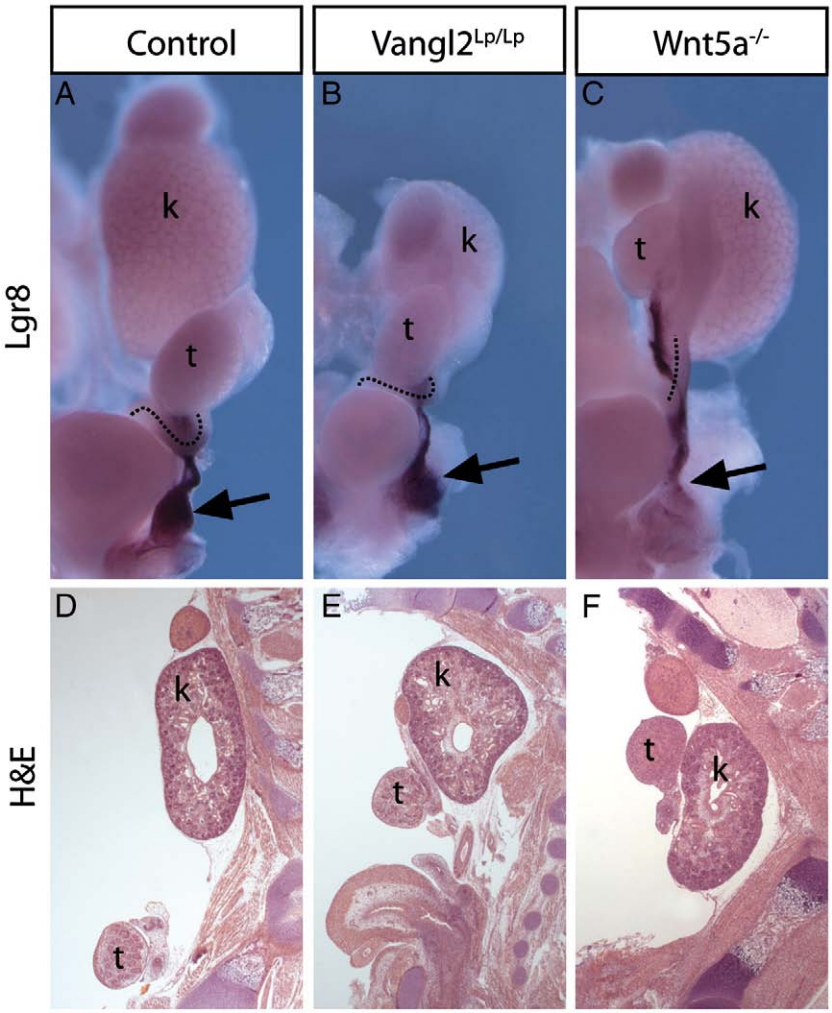


Fig. 5. The reproductive organs of Vangl2$^{Lp/Lp}$ and Wnt5a$^{-/-}$ in late gestation phenocopy those of the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ mutant. (A–C) WMISH of 15.5 dpc reproductive tracts showing expression of *Lgr8*. Dotted lines highlight vasa deferentia. Arrows indicate gubernaculum. (A) In control embryos the Wolffian duct lengthens and remodels and the gubernacular bulb develops and thickens. (B) In *Vangl2*$^{Lp/Lp}$ embryos the Wolffian duct remodels and the gubernacular bulb develops to a lesser extent. (C) In *Wnt5a*$^{-/-}$ embryos both the Wolffian duct and the gubernaculum fail to develop normally and phenocopy abnormalities observed in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos (see Fig. 3K). Note that the vestigial gubernaculum of the *Wnt5a*$^{-/-}$ embryo continues to express *Lgr8*. (D–F) H&E staining of sagittal sections of 17.5 dpc reproductive tracts. (D) In control fetuses there is a marked distance between the testis and the kidney at this stage. (E–F) In *Vangl2*$^{Lp/Lp}$ and *Wnt5a*$^{-/-}$ mutants the testis remains closely associated with the kidney. k, kidney; t, testis.

growth of the gonad might be predicted to have more severe consequences for males (Brennan and Capel, 2004).

Data described here suggest that putative signalling events disrupted by the absence of both Sfrp1 and Sfrp2 are not required for the production of major hormone receptors, Lgr8 and AR, or their ligands. Although we were unable to assay directly for the presence of Insl3 in affected fetuses, the expression of the gene encoding this hormone was clearly unaltered, notwithstanding the reduced testis size in those individuals. Thus, growth of the male reproductive tract and masculinisation of the caudal ligament (gubernaculum) require both Sfrp1 and Sfrp2, but the disrupted events are most likely to be downstream of hormone-dependent activities. Alternatively, *Sfrp1* and *Sfrp2* might be required for the normal activity of those receptors or ligands, rather than events initiated downstream of those activities.

Examination of the expression of *Sfrp1* using the lacZ knock-in allele (*Sfrp1 KI*) provides a striking molecular paint of the developing reproductive tract ligaments. Markers of the cranial and caudal suspensory ligaments are rare. Markers of the caudal ligament, or gubernaculum, include *Lgr8* and *Hoxa10* (Satokata et al., 1995). No similar cranial suspensory ligament markers have been reported. The continuity of this ligamenture is revealed most clearly in the female embryo (Fig. 1). However, failure of appropriate development of the gubernaculum contributes to a phenotype that is most apparent in the male, namely failure of the testes to descend to the inguinal region of the lower abdomen. The identification of *Sfrp1*, and the related molecule *Sfrp2*, as markers of the gubernaculum that are required for its normal development in XY embryos may allow the molecular pathway of Insl3-mediated masculinisation to be characterised. At present, the nature of the molecules induced by Lgr8 activation is unknown. The close association observed between the testes and the kidneys in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos may itself be an impediment to transabdominal descent, as might the reduced length of the vas deferens in some individuals. However, it is worth noting that failure of testicular descent cannot, in itself, be the cause of abnormal gubernaculum development in males. This is clear from observations of the testicular feminisation (*tfm*) mouse mutant, which lacks androgen receptor (AR). In these mice, lack of an androgenic effect results in retention of the CSL, but wild-type levels of Insl3 result in normal gubernaculum masculinisation. The testes are retained in the peritoneal cavity as if on a taut bowstring (Adham et al., 2000). Interestingly, given the outgrowth defects apparent in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ limbs (Satoh et al., 2006), the development of the gubernaculum has been likened to limb

development, with the gubernacular tip proposed to grow like a limb bud (Nightingale et al., 2008).

Defective development of the gubernaculum in male mouse embryos lacking Sfrp1 and Sfrp2 suggests a possible role for these genes in the aetiology of cryptorchidism, or failure of testicular descent, in humans. Cryptorchidism is the most common ailment of newborn males, affecting approximately 1–4% of live births (Hutson et al., 1994; Toppari and Kaleva, 1999). Studies on two mouse mutants have recently shed light on the genetic control of testicular descent. Mice lacking the testicular hormone insulin-like 3 (Insl3) fail to undergo the first phase of testis descent and as a consequence the testes are found high in the abdomen of adult males (Nef and Parada, 1999; Zimmermann et al., 1999). Mice lacking the receptor for Insl3, Lgr8, also exhibit a similar cryptorchid phenotype (Overbeek et al., 2001; Tomiyama et al., 2003). Two transcription factors, Hoxa10 and Hoxa11, also cause failure of the first phase of descent when deleted by gene targeting (Hsieh-Li et al., 1995; Rijli et al., 1995; Satokata et al., 1995). This suggests that a complex network of signalling molecules and transcriptional regulators are required to mediate sexually dimorphic development of the gubernaculum and subsequent transabdominal descent. The inguinoscrotal phase of descent is thought to be mediated by androgen activity, as evidenced by failure of this process in mice harbouring specific genetic defects in the function of the hypothalamo-pituitary axis, such as LuRKO knockout mice (Zhang et al., 2001) and hypogonadal (*hpg*) mice (Charlton et al., 1983). It may, therefore, be profitable to screen for mutations in *SFRP1* and *SFRP2* in humans exhibiting cryptorchidism.

We have described similarities between the sexual development phenotype of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos and that of the Looptail (*Vangl2*$^{Lp/Lp}$) mutant embryo and embryos lacking Wnt5a. This is most notable in the case of Wnt5a-deficient embryos, which have previously been noted for their phenotypic similarities to *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos at earlier stages of development (Satoh et al., 2006, 2008). This phenocopying indicates a positive regulatory role for Sfrps in non-canonical Wnt signalling. However, it remains unclear whether any functional connection between Sfrp function and non-canonical Wnt signalling in sexual development is direct or indirect. Previous studies of early embryogenesis in the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos utilised here have revealed disruptions to both canonical and non-canonical Wnt signalling (Satoh et al., 2006, 2008). Doubly mutant embryos exhibit defects in somitogenesis from 8.5 dpc onwards, demonstrating that Sfrp1 and Sfrp2 are required for anteroposterior axis elongation in the thoracic region. Analysis with an antibody to the stable non-phosphorylated form of β-catenin reveals activation of the canonical (Wnt/β-catenin) pathway in specific regions of doubly homozygous embryos at 8.5 dpc, consistent with the putative inhibitory role of Sfrps during canonical Wnt signalling (Satoh et al., 2006). The involvement of disruption to the canonical Wnt pathway in the somitogenesis phenotype of embryos lacking Sfrp1 and Sfrp2 was subsequently established using genetic approaches (Satoh et al., 2008). Compound mutant embryos harbouring different combinations of null alleles of *Sfrp1*, *Sfrp2* and Dickkopf (*Dkk1*) were generated and exhibited somite segmentation defects. Given that Dkk1 is thought to inhibit only the Wnt/β-catenin pathway (Niehrs, 2006), the observed genetic interaction between *Sfrps* and *Dkk1* suggest that Sfrp-mediated inhibition of the Wnt/β-catenin pathway regulates somitogenesis. Similar approaches revealed genetic interactions between *Sfrps* and *Vangl2*, a molecule which functions in the planar cell polarity (PCP) pathway. These suggest that disruption to non-canonical (PCP) WNT signalling also accounts for aspects of the phenotype of *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos. However, the interpretation of such data is complicated by reports indicating that non-canonical Wnt signalling can antagonize canonical Wnt signalling (Weidinger and Moon, 2003).

It is also worth noting that functions other than Wnt signal inhibition have been attributed to Sfrps in different model systems (Bovolenta et al., 2008). These include the ability to antagonize BMP signalling by acting as proteinase inhibitors (Lee et al., 2006), regulation of axon growth by activation of Fz receptor and regulation of intracellular cGMP (Rodriguez et al., 2005), interaction with other receptors or matrix molecules (Chuman et al., 2004) and the ability to antagonize the activity of other Sfrps (Yoshino et al., 2001). This latter example, of potential mutual antagonism between Sfrps, is especially pertinent because it has been reported that Sfrp2 can block Sfrp1-mediated events during kidney development *in vitro* (Yoshino et al., 2001).

Given the above observations, the precise contributions of Sfrp1 and Sfrp2 to the phenotype of the doubly homozygous embryos remain difficult to disentangle due to the complexity of the system that has been disrupted. One approach to circumventing some of this complexity is to identify single-gene defects that phenocopy aspects of the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryo. In this context, it is important to note the similarity in phenotype between embryos lacking the *Wnt5a* gene and *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos. An examination of *Wnt5a* expression in the developing male and female reproductive organs reveals significant expression in several structures, including the mesonephros and gubernaculum. This *Wnt5a* expression persists in *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ embryos (N.W., A.G., unpublished data). Although this *Wnt5a* expression profile suggests a direct role for this gene in gubernacular development, it is worth noting that axis defects, which are common to the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$, *Wnt5a*$^{-/-}$ and *Vangl2*$^{Lp/Lp}$ mutants examined here, may also contribute to aspects of their sexual development phenotypes by some unknown mechanism.

The phenocopy data presented here raise the possibility that Sfrp1/Sfrp2 and Wnt5a may act in converging parallel pathways to influence sexual development or interact in the same pathway, perhaps by antagonism of non-canonical or canonical Wnt signalling. Interaction in the same pathway suggests the possibility of a physical interaction between Sfrp1/Sfrp2 and Wnt5a, in a manner often associated with Sfrps and Wnts. The most commonly attributed consequence of such an interaction is inhibition of Wnt activity by Sfrps. However, it is not clear how to reconcile the similarities in the sexual development phenotypes of the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ and *Wnt5a*$^{-/-}$ embryos with such an inhibitory interaction. Two possibilities exist: firstly, that the interaction *in vivo* is positive and Sfrp1/2 do promote Wnt5a activity; secondly, that the phenotype of the *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ double homozygote is caused by hyperactivity of Wnt5a, due to loss of inhibition. However, this second scenario is only plausible if Wnt5a-deficient embryos exhibit the same, or a very similar, sexual development phenotype as embryos carrying a gain-of-function *Wnt5a* mutation. A precedent for this general scenario exists: overexpression of Wnt5a in *Xenopus* and loss of Wnt5 in zebrafish both disrupt convergent extension cell movements during gastrulation (Moon et al., 1993; Kilian et al., 2003). Of course, it is also possible that both positively reinforcing and inhibitory interactions occur within an organism, and these may be regulated in a tissue- and stage-specific fashion during development. Future studies will focus on the *in vivo* molecular relationship between Sfrps and Wnt5a signalling during sexual development.

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