ZNRF3 functions in mammalian sex determination by inhibiting canonical WNT signaling

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Mammalian sex determination is controlled by the antagonistic interactions of two genetic pathways: The SRY-SOX9-FGF9 network promotes testis determination partly by opposing proovarian pathways, while RSPO1/WNT-β-catenin/FOXL2 signals control ovary development by inhibiting SRY-SOX9-FGF9. The molecular basis of this mutual antagonism is unclear. Here we show that ZNRF3, a WNT signaling antagonist and direct target of RSPO1-mediated inhibition, is required for sex determination in mice. XY mice lacking ZNRF3 exhibit complete or partial gonadal sex reversal, or related defects. These abnormalities are associated with ectopic WNT/β-catenin activity and reduced *Sox9* expression during fetal sex determination. Using exome sequencing of individuals with 46,XY disorders of sex development, we identified three human *ZNRF3* variants in very rare cases of XY female presentation. We tested two missense variants and show that these disrupt ZNRF3 activity in both human cell lines and zebrafish embryo assays. Our data identify a testis-determining function for ZNRF3 and indicate a mechanism of direct molecular interaction between two mutually antagonistic organogenetic pathways.

sex determination | ZNRF3 | DSD | WNT signaling | organogenesis

Mammalian sex determination involves the sexually dimorphic development of a gonadal primordium. In the presence of *SRY* on the Y chromosome, supporting cell precursors of the developing gonad differentiate into Sertoli cells, and this somatic lineage orchestrates morphological events required for testis determination (reviewed in refs. 1 and 2). Thus, the "decision" as to whether supporting cells develop as Sertoli (testicular) or granulosa (ovarian) cells is pivotal to sex determination, and understanding the molecular events that result in fate specification of this lineage remains critical to our understanding of gonadogenesis.

SRY acts to up-regulate the expression of the testis-determining gene *SOX9* (3), a transcription factor that initiates a program of gene activity that directs Sertoli cell differentiation (4, 5). The timing of these protestis events is crucial: Any delay in the expression of *Sry* can result in sex reversal or ovotestis development in XY mice (6). Studies have shown that the testis-determining genetic pathway is important for the inhibition of the equivalent ovarian-determining pathway. Indeed, the two pathways, most notably FGF signaling in the testis and canonical WNT signaling in the ovary, act in a mutually antagonistic fashion (7). This mutual antagonism persists in the adult gonad: Postnatal deletion of genes such as *DMRT1* (8) and *FOXL2* (9) can result in reprogramming of cells of the adult testis and ovary, respectively, to the alternative sexual fate.

Canonical WNT/β-catenin signals are required for normal ovarian development from the embryonic XX gonad: Loss of WNT4 or Rspondin-1 (RSPO1), which effect such signals through stabilization of β-catenin, can result in partial XX gonadal sex reversal in mice and 46,XX testicular disorders of sex development (DSD) or virilization in humans (10–14). Mechanistically, R-spondins, in association with LGR4/5 cell surface receptors, promote WNT signaling by binding to and sequestering the transmembrane E3 ubiquitin ligases ZNRF3 and RNF43; these two molecules, in turn, inhibit WNT signaling by targeting Frizzled receptor for degradation by ubiquitination and increased membrane turnover (15–19). Loss of function genetic studies show that testis determination requires the inhibition of proovarian canonical WNT/β-catenin signals (7, 20), and these observations are consistent with the report that ectopic stabilization of β-catenin in transgenic XY mice can disrupt testis development (21). However, the molecular effectors of this inhibition of WNT/β-catenin during testis determination have not been identified. Components of the

Significance

Sex determination involves antagonistic interactions between the testis-determining (SRY-SOX9-FGF9) and ovary-promoting (RSPO1-WNT/β-catenin-FOXL2) pathways, but the underlying molecular mechanisms remain unclear. We show that ZNRF3, an E3 ubiquitin ligase that inhibits WNT signaling and is a direct target of RSPO1-mediated membrane clearance, is testis-determining in mice. Testis determination defects in the absence of ZNRF3 arise due to ectopic canonical WNT signaling in XY gonads at the sex-determining stage. We identify human *ZNRF3* sequence variants in cases of 46,XY disorders of sex development with XY female presentation. In vitro functional assays show that these variants disrupt ZNRF3 function. Our data reveal a sex-determining role for ZNRF3 and indicate that interactions between ZNRF3 and RSPO1 regulate mammalian sex determination.

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core sex-determining pathways are only very rarely identified, but, given their inhibition by RSPO1 in other contexts, ZNRF3 and RNF43 are excellent candidates for gene products that act to inhibit canonical WNT signaling during sex determination to tilt the balance toward the testicular fate.

Here, we report testis determination defects, including gonadal sex reversal, in mice lacking ZNRF3. In contrast, RNF43 is not required for testis determination. Loss of ZNRF3 results in ectopic canonical WNT signaling in XY gonads at the sex-determining stage of 11.5 days post coitum (dpc) and a consequent reduction in *Sox9* expression. We also report variants in human *ZNRF3* associated with 46, XY DSD and show that two missense substitutions identified can disrupt ZNRF3's anti-WNT activity in a cell line and zebrafish embryos. Our data reveal an antagonistic molecular interaction, between RSPO1 and ZNRF3, at the heart of the sex-determining mechanism.

Results

We examined *Znrf3* expression in somatic (*Sf1(Nr5a1)*-positive) cells of the developing XY and XX gonads between 10.5 and 16.5 dpc using single-cell RNA sequencing (sc-RNAseq) (*SI Appendix*, Fig. S1). This revealed early *Znrf3* expression in progenitor cells and supporting cells in both sexes, but with no significant sexual dimorphism before 13.5 dpc, by which time primary sex determination is complete. In contrast, *Sox9* expression was enhanced in XY supporting cells and *Rspo1* expression was enhanced in XX supporting cells from around 11.5 dpc (*SI Appendix*, Fig. S1 *B* and *C*), as previously described (12). Quantitative RT-PCR (qRT-PCR) analyses of *Znrf3* expression in XY and XX *Sf1*-positive cells and whole gonads at 11.5 dpc also revealed no sexual dimorphism (*SI Appendix*, Fig. S1 *D* and *E*). These data indicate that ZNRF3 activity in developing gonadal somatic cells may be regulated in a sexually dimorphic fashion primarily due to the enhanced expression of *Rspo1* in XX supporting cells: ZNRF3 activity is predicted to be higher in XY cells than in XX cells due to RSPO1-mediated membrane clearance in XX cells.

We then tested directly whether loss of ZNRF3 disrupted testis determination in the mouse. XY fetal gonads homozygous for a *Znrf3* null allele (16) on the C57BL/6J (B6) background exhibited pronounced defects in testis determination with variable severity at 14.5 dpc (Fig. 1). The most severely affected appeared sex-reversed, with an ovarian morphology characterized by the absence of *Sox9* expression and patchy *Stra8* expression throughout (Fig. 1 *A* and *B*). *Stra8* expression is indicative of germ cell meiotic entry, normally only detectable in XX gonads at this stage. Other mutants developed ovotestes, with only particular regions of the gonad expressing significant *Stra8* or *Sox9*. The least affected class exhibited disruption to morphology with irregular testis cords and restricted areas containing *Stra8*-positive cells. XY mutant gonads had significant numbers of FOXL2-positive cells, depending on the degree of sex reversal, indicating ovarian granulosa cell differentiation (Fig. 1 *A* and *B*). Significantly, phenotypic variability in the degree of sex reversal was observed between gonads of an individual mutant fetus at 14.5 dpc (Fig. 1*C*), indicating innate phenotypic stochasticity in the absence of ZNRF3, in addition to any contributions made by residual genetic background differences.

We also examined the consequences of loss of ZNRF3 for XY gonad development on the B6.Y^{AKR} genetic background, which is highly sensitized to disruptions to testis determination due to the presence of the *Mus domesticus* AKR Y chromosome (22, 23). Embryos lacking ZNRF3 on B6.Y^{AKR} consistently exhibited complete gonadal sex reversal, having an ovarian morphology and lacking *Sox9* expression and often containing large numbers of *Stra8*-positive cells (Fig. 2*A*). Heterozygous embryos lacking just a single copy of *Znrf3* also had severe gonadal defects, but with variable severity (Fig. 2*B*). Some developed ovotestes, with central gonadal *Sox9* expression, some exhibited *Sox9* expression but lacked testis cords, and others had an ovarian morphology with no detectable *Sox9* expression. Adult B6.Y^{AKR} heterozygous mice are viable, unlike homozygotes, which die at around birth (15). B6.Y^{AKR} adult heterozygotes exhibited a range of

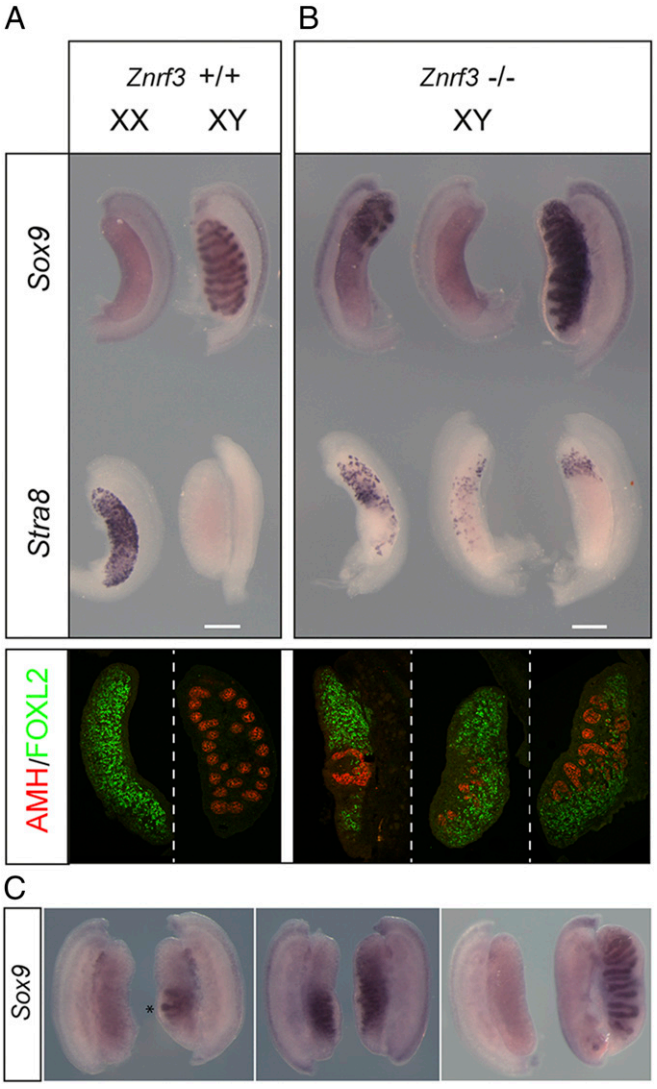

Fig. 1. Testis determination defects in XY gonads lacking ZNRF3. (*A*) Wild-type controls. (*B*) XY gonads (14.5 dpc) lacking ZNRF3 exhibit severe morphological abnormalities in comparison with wild-type controls. WMISH with *Sox9* (*Top*, upper row) reveals mutant gonads with a sex-reversed (ovarian) morphology (left and center) and either a major reduction in levels of *Sox9* or no detectable expression. Less affected gonads have higher *Sox9* levels but abnormal testis cords (right). WMISH with *Stra8* (*Top*, lower row) reveals a significant number of meiotic germ cells in ZNRF3-deficient gonads. Gonadal sex reversal in XY gonads lacking ZNRF3 is confirmed by immunostaining of 14.5 dpc gonadal sections for AMH (red), a Sertoli cell marker, and FOXL2 (green), a granulosa cell marker (*Bottom*). (*C*) Pairs of gonads dissected from individual *Znrf3* fetuses (14.5 dpc) exhibit asymmetry. Note rudimentary testis cord formation in one gonad (asterisk), but not the other (*Left*). *Center* shows distinct *Sox9* expression profiles in two fetal gonads. *Right* shows, remarkably, an ovary on one side and a well-formed testis on the other. (Scale bar, 250 μm.)

gonadal abnormalities; remarkably, a minority (2/5) developed as phenotypic XY females with internal genitalia and anogenital distances grossly similar to XX controls (Fig. 2*C* and *SI Appendix*, Fig. S2). Others either contained an ovary-like gonad on one side and a very small contralateral testis or contained two small testes (*SI Appendix*, Fig. S3). These data establish ZNRF3 as testis-determining in the mouse. In contrast, B6 XY embryos lacking the functionally related paralogue RNF43 (16, 18, 24) develop testes as normal (*SI Appendix*, Fig. S4).

To determine the basis of the observed testis determination defects at 14.5 dpc, we performed molecular analyses around the

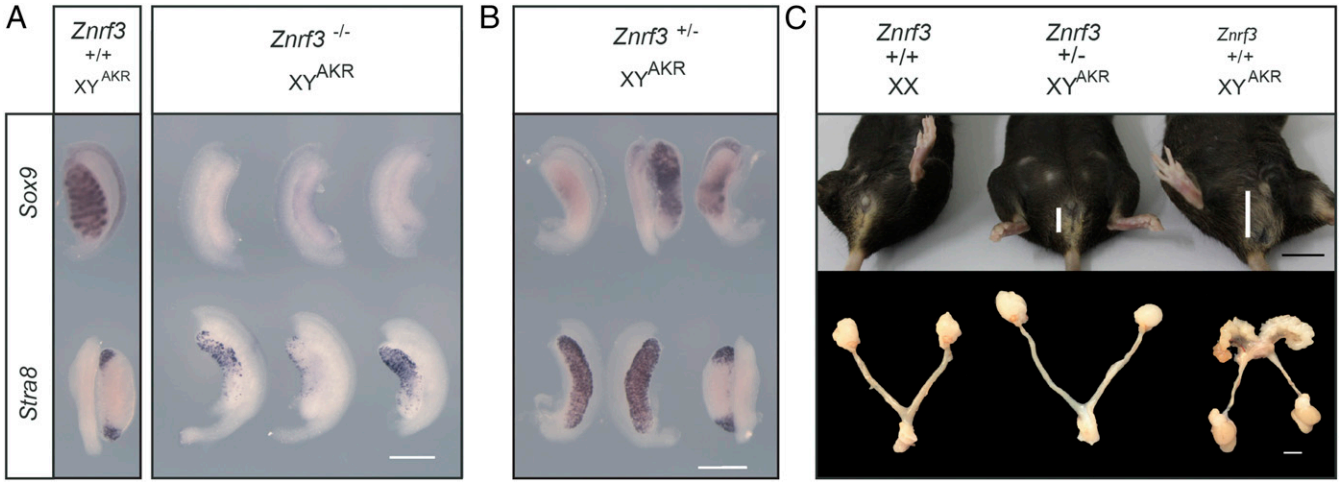

Fig. 2. *Znrf3* mutants exhibit gonadal sex reversal phenotypes on B6.Y^{AKR}. (*A*) B6.Y^{AKR} embryonic gonads lacking ZNRF3 at 13.5 (*Right*, upper row) and 14.5 dpc (*Right*, lower row) have an ovarian morphology. They lack *Sox9* expression and have variable numbers of *Stra8*-positive germ cells. *Stra8* is also expressed in a more restricted and transient fashion at the poles of the B6.Y^{AKR} wild-type controls (*Left*, lower row). *Sox9* expression is expressed in testis cords of controls (*Left*, upper row). (*B*) Fetal XY gonads lacking a single functional copy of *Znrf3* exhibit variable degrees of sex reversal, revealed by *Sox9* WMISH (upper row) and *Stra8* WMISH (lower row). (*C*) Female phenotypic sex in an adult B6.Y^{AKR} mouse lacking one copy of *Znrf3* (*Top*, center). Sex-reversed animals have an anogenital distance (vertical white bar) usually found in XX controls. Mammary gland pigmentation is also visible in the XY female mouse (center). Examination of internal genitalia in XY heterozygotes reveals uterine horns and bilateral ovaries (*Bottom*, center). [Scale bar, 500 μm (*A* and *B*); 1 cm (*C*, *Top*, in black); 0.5 cm (*C*, *Bottom*, in white).]

time of sex determination, from 11.5 dpc to 12.5 dpc, in XY *Znrf3* mutant gonads and controls. No overt disruption to *Sry* expression was detected by qRT-PCR (Fig. 3*A*). In contrast, *Sox9* expression was either reduced or absent compared with XY controls (Fig. 3 *B* and *C*). These data suggest that disruption to *Sox9*, rather than *Sry*, accounts for the failure to undergo normal testis determination in homozygous XY embryos. Gonadal somatic cell proliferation was also reduced in *Znrf3* mutants at 11.5 dpc, consistent with their feminization (*SI Appendix*, Fig. S5). We then tested whether disruption to *Sox9* expression is caused by elevated canonical WNT signaling in XY mutant gonads due to the absence of ZNRF3-mediated clearance of Frizzled receptor. *Lef1*, which is positively regulated by WNT/β-catenin signals, was detected at higher levels in XY mutant homozygotes than XY controls at both 11.5 and 12.5 dpc (Fig. 3 *D* and *F*). Similarly, *Axin2* levels were also elevated in XY gonads lacking ZNRF3 (Fig. 3*E*). By contrast, expression of the somatic cell marker *Sf1* and the primordial germ cell marker *Oct4* were not affected at 12.5 dpc (*SI Appendix*, Fig. S6). Interestingly, proovarian *Wnt4* expression was enhanced by loss of ZNRF3, both at 11.5 and 12.5 dpc, with noticeable variability in expression levels between distinct mutant XY gonads (Fig. 3*G* and *SI Appendix*, Fig. S6). These data suggest that testis determination defects in XY gonads lacking ZNRF3 are caused by ectopic canonical WNT signaling resulting in significant reduction in levels of *Sox9* in supporting cell precursors at 11.5 dpc. The inhibitory effect of β-catenin on gonadal *Sox9* expression has been previously reported and explained by disruption to SF-1–mediated activation of its gonad-specific enhancer, testis-specific enhancer core (TESCO) (25), although disruption to other *Sox9* enhancers is conceivable (26). SRY-dependent promotion of *SOX9* transcription may sometimes tilt the balance toward the testicular fate in XY mutants, generating an inherently unstable sex-determining network, accounting for some of the phenotypic variability observed.

Finally, we tested whether *ZNRF3* mutations might disrupt human sexual development, by performing next-generation sequencing of whole exomes from a cohort of patients with very rare 46,XY DSD (Table 1 and *SI Appendix*, *Supplementary Methods*). We identified two novel *ZNRF3* variants and two known variants in five individuals, including four with 46,XY female presentation (Table 1). No other DSD-associated variants were present in these patients. We then assessed the functional consequences of two of the missense variants. Human cell line transfection assays (Fig. 4*A*) and zebrafish embryo assays (Fig. 4*B*) indicate that the Ser554Asn amino acid substitution (patient 2, 46,XY complete gonadal dysgenesis) disrupts the ability of ZNRF3 to inhibit canonical WNT signals compared with wild-type ZNRF3. Both assays also demonstrated that the Arg768Gly substitution (patients 3 and 4, 46,XY DSD of unknown gonadal phenotype) acts to enhance WNT/β-catenin signaling (Fig. 4). Thus, both human variants tested can disrupt ZNRF3's role in inhibiting canonical WNT signals in both a cellular and organismal context.

Discussion

Here we report detailed analysis of a developmental role for ZNRF3, a known tumor suppressor (27, 28). The testis determination defects described here in ZNRF3-deficient fetuses are associated with a clear elevation of canonical WNT signaling in the developing XY gonad, consistent with the known role of ZNRF3 in increasing membrane turnover of Frizzled receptor and thereby inhibiting WNT signaling (15, 16). The basis of the sex reversal phenotypes observed at 14.5 dpc is the reduction of *Sox9* seen at 11.5 dpc, when commitment to either the testicular or ovarian fate is decided. The simplest explanation of this loss of *Sox9* expression is the elevation in canonical WNT signals: In other genetic contexts, forcing stabilization of β-catenin can disrupt testis determination (21). Phenotypic variability in the severity of the XY gonadal defects in mutants indicates a degree of instability in the sex-determining mechanism in the absence of ZNRF3; however, while the *Znrf3* mutant line was maintained on C57BL/6J for several generations, we cannot exclude residual genetic background differences as a contributory factor. It is worth noting here too that large-scale phenotypic studies of mouse knockouts reveal that incomplete penetrance and variable expressivity are common even on pure genetic backgrounds (29). This phenomenon, like pronounced phenotypic variability between the gonads of a single fetus (Fig. 3*C*), cannot clearly be explained by genetic mechanisms.

The role of ZNRF3 in testis determination and that of RSPO1 in ovary development (12–14) indicates an antagonistic molecular interaction at the heart of mammalian gonad development (*SI Appendix*, Fig. S7). Our studies suggest that *Znrf3* is expressed at equivalent levels in supporting cells of the XY and XX gonads at the time of sex determination. This suggests that *Znrf3* is not an obvious target for transcriptional up-regulation by testis-determining factors such as SRY, SOX9, or FGF9, despite the known role of

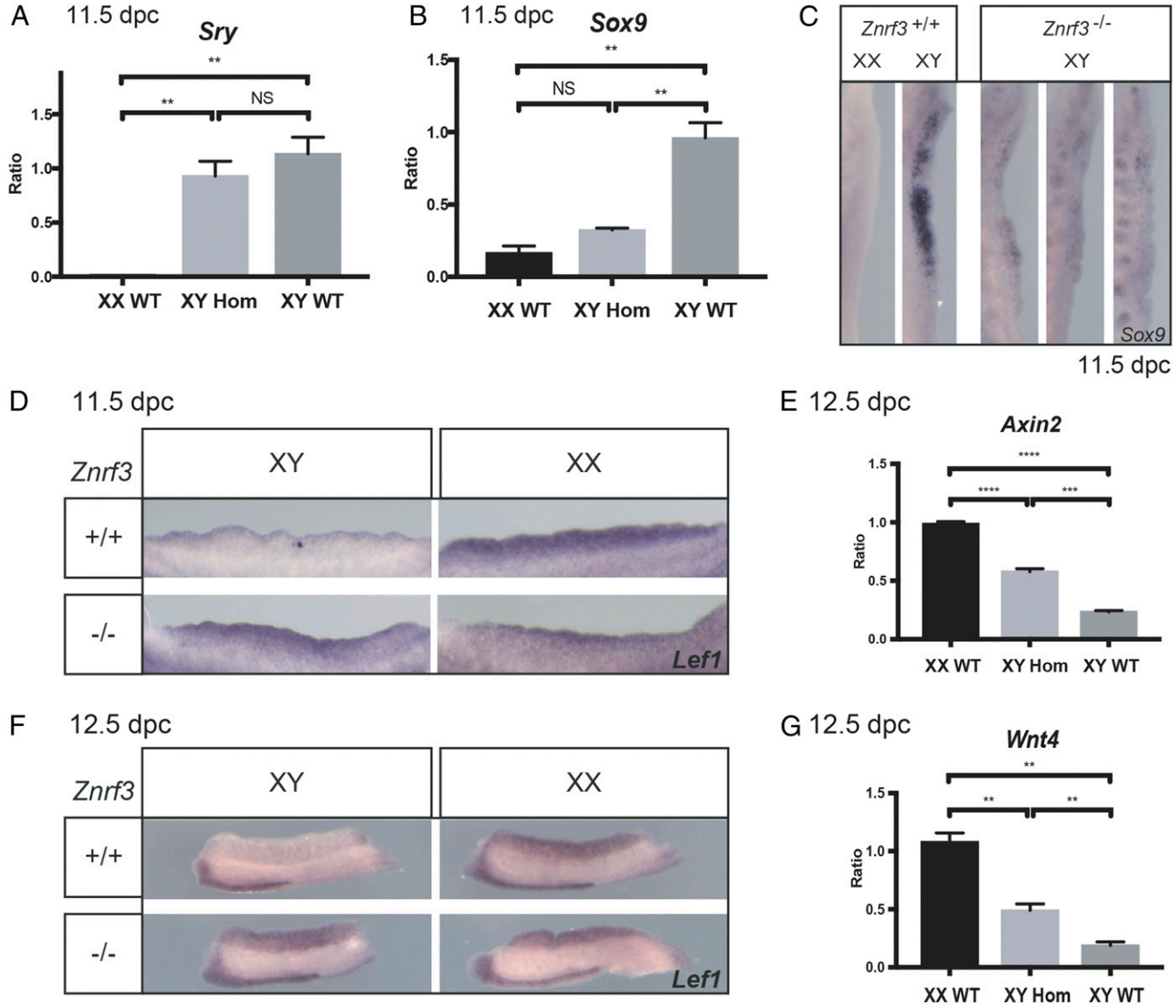

Fig. 3. Ectopic activation of canonical WNT signaling and disruption to *Sox9* expression during sex determination in XY *Znrf3* homozygotes. (*A*) The qRT-PCR shows that *Sry* is expressed in *Znrf3* homozygous mutant embryos at 11.5 dpc with no significant difference to controls. (*B*) The qRT-PCR showing *Sox9* expression is significantly reduced in mutants. (*C*) Reduced *Sox9* expression is observed in some mutant gonads (left-hand mutant) following WMISH, while expression is negligible in others. *Sox9* expression in XY control gonads is strong. (*D*) WMISH shows *Lef1* expression is elevated in XY homozygous mutant gonads at 11.5 dpc. (*E*) Expression of *Axin2* is also elevated in XY homozygous mutant gonads at 12.5 dpc, although not to XX control levels. (*F*) *Lef1* expression is also clearly detectable in XY homozygotes at 12.5 dpc, in contrast to controls. (*G*) Expression of *Wnt4* is also elevated in XY homozygotes at 12.5 dpc, although, again, not to XX control levels. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NS, not significant.

FGF signals in opposing WNT signals in the mouse (7, 20). We do not, of course, rule out posttranscriptional mechanisms of activation. RSPO1 promotes WNT signaling by inducing membrane clearance of ZNRF3, and the related molecule RNF43 (17), and given the elevated levels of *Rspo1* in the XX gonad at the sex-determining stage, we conclude that ZNRF3 activity is reduced in XX supporting cells and therefore elevated in XY cells. In XY cells, ZNRF3 acts to inhibit canonical WNT signaling by increasing membrane turnover of Frizzled receptor, and the consequences of removing that inhibition in the XY gonad are the testis determination defects described here, mediated by suppression of *Sox9* expression. Thus, during gonad development, RSPO1 acts to inhibit an inhibitor of canonical WNT signaling, a double-negative operation that is familiar in models of sex determination (30). While loss of RSPO1 results in masculinization of the XX supporting cell lineage only in the postnatal period, this may be due to a number of factors, including the availability of proovarian FOXL2 and/or other pro-WNT signaling factors, the complexity of the ovarian somatic cell lineages, or the contribution of germ cells to ovary development (13, 31, 32). It will be interesting to determine the consequences of removal of both ZNRF3 and RSPO1 on XX and XY gonad development. Likewise, it will be interesting to determine the consequences of *Znrf3* overexpression on XX gonad development.

We report that two human *ZNRF3* variants, associated with 46,XY DSD and causing amino acid substitutions, can disrupt the role of ZNRF3 in inhibiting canonical WNT signaling. All variants detected map to the long intracellular domain of ZNRF3, the function of which is unclear (24). Affected individuals are heterozygous carriers of each variant identified, and not all are novel. However, in this context, it is very important to reconsider the role played by genetic background in the penetrance and expressivity of sex determination phenotypes. Sex reversal in some

Table 1. *ZNRF3* variants detected by exome sequencing of DSD patients

Variable	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Ancestry	Tunisian	North African	Indian	Indian	North African
Karyotype	46,XY	46,XY	46,XY	46,XY	46,XY
Age at presentation	17 y	16 y	19 y	17 y	Birth
External genitalia	Female	Female	Female	Female	Perineal hypospadias
Internal genitalia	Hypoplastic uterus	Uterus present	Absent uterus	Absent uterus	Male
Gonads	Not seen by US	"streak" gonad	Not seen by US	Not seen by US	Intrascrotal testis
Endocrine data (reference values)	T, 0.13 ng/mL (2.7 to 9); FSH, 132 U/L (ND to 13.5); LH, 35.9 U/L (2.4 to 13)	T, 0.2 ng/mL (2.7 to 9); FSH, 37 U/L (ND to 13.5); LH, 42 U/L (2.4 to 13)	NA	NA	3 mo: T, 0.96 ng/mL (0.75 to 4.0); FSH < 0.4 U/L (ND to 13.5); AMH AMH 1,305 pmol/l (100–3,300)
ZNRF3 mutation	Splice site exon8:c.2767+5G > A	c.1661G > A p.Ser554Asn	c.2302A > G p.Arg768Gly	c.2302A > G p.Arg768Gly	c.1861T > G p.Arg621Ser
Allele Freq (ExAC) and Population	Novel	Novel	0.00487 South Asian	0.00487 South Asian	0.00003258 European
Diagnosis	Suspected 46,XY gonadal dysgenesis	46,XY Complete gonadal dysgenesis	46,XY DSD	46,XY DSD	46,XY DSD

Normal range refers to the range of basal levels in control subjects matched according to age and chromosomal sex with the case subjects. AMH, anti-Müllerian hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; NA, not available; ND, not detectable; T, testosterone; US, ultrasound.

B6.Y^{AKR} mice lacking just one copy of *Znrf3* suggests that heterozygosity for a disruptive *ZNRF3* mutation may be sufficient to cause 46,XY DSD, including complete gonadal dysgenesis, in the relevant human genetic background. Taken together, our data establish a testis-determining function for ZNRF3. ZNRF3 inhibits canonical WNT signaling in XY gonads during sex determination, permitting the establishment of robust *Sox9* expression. Our data reveal a molecular conflict between ZNRF3 and RSPO1 that governs mammalian sex determination.

Methods

Mouse Strains and Ethical Approval. Mice used were bred with licensed approval from the UK Home Office (PPL 30/2877 and 70/8988). Mice were housed in individually ventilated cages in a specific pathogen-free environment. Mice harboring *Znrf3* and *Rnf43* floxed alleles have been previously described (16). *Sf1*-eGFP reporter mice (33) were a kind gift from J. Bowles and P. Koopman (University of Queensland, Brisbane, QLD, Australia). All lines were maintained on C57BL/6J (B6) for at least six generations.

Generation of Embryos and Whole-Mount in Situ Hybridization. Noon on the day of the copulatory plug was counted as 0.5 dpc. Embryos collected at 11.5 dpc were staged accurately based on the number of tail somites. Whole-mount in situ hybridization (WMISH) analysis of embryonic tissues and probes for *Sox9*, *Wnt4*, and *Stra8* have been previously described (34). The *Lef1* probe (13) was a kind gift from Marie-Christine Chaboissier, Université Côte d'Azur, CNRS, INSERM, iBV, Nice, France. At least three independent biological samples from a given group were analyzed with a particular marker.

qRT-PCR. Total RNA was extracted using RNeasy plus micro kit (Qiagen), and reverse transcription (RT) was carried out with 250 ng of total RNA using the High capacity cDNA RT kit (Applied Biosystems). The qRT-PCR was performed with Fast SYBR Green Master Mix (Life Technologies) on a 7500 Fast Real-Time PCR system (Applied Biosystems). RNA expression levels were normalized to those of *Hrpt1* (endogenous control) using the ΔΔCt method. At least three samples for each genotype were analyzed. See *SI Appendix* for primer sequences.

Antibodies and Immunohistochemistry. Antibodies used in this study were FOXL2 (1:100; a kind gift from Dagmar Wilhelm, The University of Melbourne, Parkville, Australia), AMH (1:100; Santa Cruz sc28912), and GATA4 (1:50; Santa Cruz 25310). At least two independent biological replicates for each sex and genotype were analyzed in immunohistochemistry experiments.

Cell Proliferation Assays. Dams were injected with 9 mg/kg 5-ethynyl-2′-deoxyuridine (C10339; Thermofisher) and killed 1 h later. Gonads/mesonephroi were dissected from embryos and fixed in 4% paraformaldehyde for 4 h at 4 °C or 2 h at room temperature. Gonads underwent Click-iT chemistry according to manufacturer's instructions. GATA4 immunostaining was then performed before imaging using a Zeiss 710 multiphoton microscope.

TOPFlash Assays. The wild-type ZNRF3 construct (ZNRF3-HA) has been previously described (15). Point mutations were generated using site-directed mutagenesis

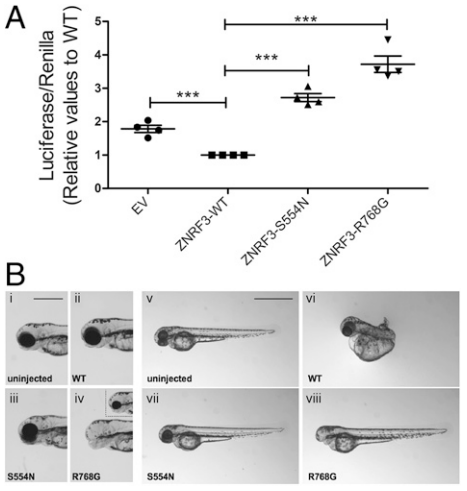

Fig. 4. Human *ZNRF3* variants disrupt ZNRF3 activity. (*A*) Effects of overexpressing wild-type and variant ZNRF3 on WNT/β-catenin signaling was tested by SuperTOPFlash luciferase reporter assay in KGN (ovarian granulosa cell tumor) cells for 48 h. The reporter constructs were transfected into KGN cells with either the empty vector (EV), wild-type (WT), or variant ZNRF3 expression vectors. The values are expressed relative to ZNRF3-WT. Each data point represents the mean of the value of each experiment ± SEM (n = four independent experiments; each experimental measurement was performed in quadruplicate). Pairwise comparison between the effect of ZNRF3-WT and all of the other conditions was performed using a two-tailed Student t test. ***$P \leq 0.0001$. (*B*) Lateral views of 58-h postfertilization uninjected control (*i* and *v*) or injected (12.5 pg, *ii–iv*; 50 pg, *vi–viii*) zebrafish embryos. Embryos injected with 12.5 pg of ZNRF3-WT or ZNRF3-S554N show no defects, but ZNRF3-R768G–injected embryos display reduction of the forebrain and either loss (*iv*) or reduction (*Inset* in *iv*) of the eyes. At the 50-pg dose, ZNRF3-WT embryos displayed dorsalization (*vi*), while ZNRF3-S554N–injected embryos showed no axis patterning defects (*vii*). At 50 pg, ZNRF3-R768G–injected embryos exhibited posteriorization of anterior structures (*viii*).

(QuikChange II XL Site-Directed Mutagenesis Kit; Agilent) according to manufacturer's instructions. T-cell factor/lymphoid enhancer factor (TCF/LEF) binding-site/mutated binding-site (TOPFlash/FOPFlash) reporter plasmids (M50 Super 8x TOPFlash and M51 Super 8x FOPFlash mutant; Addgene) were used for the detection of β-catenin–driven WNT transcriptional activity. See *SI Appendix* for further details.

Zebrafish Embryo Assays. To determine the effect of human variants on ZNRF3 function, we used an overexpression assay in zebrafish that assesses the impact of low (12.5 pg) and high (50 pg) doses of injected *ZNRF3* messenger RNA on early embryogenesis. Plasmids containing ZNRF3-HA were mutagenized using the QuikChange II Site-Directed Mutagenesis kit (Agilent) to incorporate the S554N and R768G point mutations. See *SI Appendix* for details.

Subjects, Samples, and Exome Sequencing. All patients with 46,XY DSD met the revised criteria of the Pediatric Endocrine Society European Society for Pediatric Endocrinology. This study was approved by the local French ethical committee (2014/18NICB; registration number IRB00003835), and consent to genetic testing was obtained from adult probands or from the parents when the patient was under 18 y. Exon enrichment was performed with Agilent SureSelect Human All Exon V4. Paired-end sequencing was performed on the Illumina HiSeq2000 platform with TruSeq v3 chemistry. See *SI Appendix* for details.

Single-Cell RNAseq. The Fluidigm C1 Autoprep system was used for cell capture and processing. Methodological details can be found in Stévant et al. (35) and *SI Appendix*.

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