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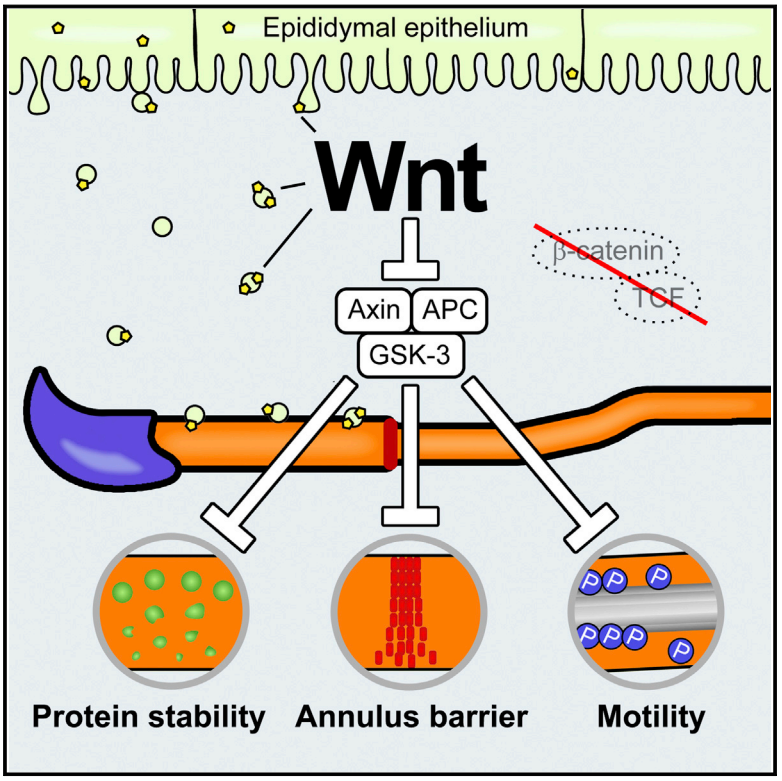
Post-transcriptional Wnt Signaling Governs Epididymal Sperm Maturation

Graphical Abstract



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In Brief

Although the impact of canonical Wnt signaling is predominantly thought to necessitate β-catenin-dependent transcription, a multifaceted set of Wnt functions in sperm development is implemented post-transcriptionally through mechanisms that maybe relevant to other tissues as well.

Highlights

- Mammalian spermatozoa are Wnt-responsive cells
- Wnt signaling controls sperm maturation independent of β-catenin
- Wnt ligands promote sperm proteome stability (Wnt/STOP) and sperm motility
- GSK3 is a septin 4 kinase that inhibits septin clustering

Koch et al., 2015, Cell *163*, 1225–1236
November 19, 2015
http://dx.doi.org/10.1016/j.cell.2015.10.029

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Post-transcriptional Wnt Signaling Governs Epididymal Sperm Maturation

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http://dx.doi.org/10.1016/j.cell.2015.10.029

SUMMARY

The canonical Wnt signaling pathway is of paramount importance in development and disease. An emergent question is whether the upstream cascade of the canonical Wnt pathway has physiologically relevant roles beyond β-catenin-mediated transcription, which is difficult to study due to the pervasive role of this protein. Here, we show that transcriptionally silent spermatozoa respond to Wnt signals released from the epididymis and that mice mutant for the Wnt regulator Cyclin Y-like 1 are male sterile due to immotile and malformed spermatozoa. Post-transcriptional Wnt signaling impacts spermatozoa through GSK3 by (1) reducing global protein polyubiquitination to maintain protein homeostasis; (2) inhibiting septin 4 phosphorylation to establish a membrane diffusion barrier in the sperm tail; and (3) inhibiting protein phosphatase 1 to initiate sperm motility. The results indicate that Wnt signaling orchestrates a rich post-transcriptional sperm maturation program and invite revisiting transcription-independent Wnt signaling in somatic cells as well.

INTRODUCTION

Canonical Wnt signaling is an evolutionarily conserved pathway, which plays a key role in numerous processes of development and disease (Anastas and Moon, 2013; Clevers and Nusse, 2012; Holland et al., 2013). At the heart of canonical Wnt signaling is the transcriptional regulator β-catenin, which in unstimulated cells is phosphorylated by glycogen synthase kinase 3 (GSK3) and then polyubiquitinated and thereby targeted for proteasomal degradation. Binding of Wnt proteins to the Frizzled (Fz) receptors and their low-density lipoprotein receptor related protein (LRP) 5 and 6 co-receptors triggers GSK3 inhibition to stabilize β-catenin, which enter the nucleus and regulate downstream target genes (Kikuchi et al., 2011; MacDonald et al., 2009).

The canonical Wnt signaling cascade is well characterized, and it is widely assumed that this pathway acts primarily through transcriptional response of β-catenin-dependent target genes. However, we have previously shown that Wnt signaling peaks in the G_2/M phase of the cell cycle in a variety of cells (Davidson et al., 2009). It appeared paradoxical that Wnt signaling should peak in G_2/M, when the hallmark of the pathway is transcriptional regulation, which in mitosis comes to a standstill. Importantly, De Robertis and colleagues showed that Wnt signaling stabilizes many other cellular proteins in addition to β-catenin (Taelman et al., 2010). Building on this discovery, we introduced Wnt-dependent stabilization of proteins (Wnt/STOP), which is independent of β-catenin, and peaks during mitosis to slow down protein degradation as cells prepare to divide (Acebron et al., 2014). This post-transcriptional branch of canonical Wnt signaling is required for proper chromosome segregation, endo-lysosomal biogenesis, as well as for cell growth and cell-cycle progression (Acebron et al., 2014; Huang et al., 2015; Ploper et al., 2015; Stolz et al., 2015). Wnt stabilizes proteins by inhibiting GSK3, a kinase, which creates phospho-degrons to target proteins for proteosomal degradation. GSK3 inhibition by Wnt signaling peaks in mitosis because the Wnt coreceptor LRP6 is activated by cyclin Y (Ccny) and its target kinase CDK14 (cyclin-dependent kinase 14) in a cell-cycle-dependent manner (Acebron et al., 2014; Davidson et al., 2009). In addition to Wnt/STOP, other post-transcriptional signals have been shown to branch off downstream of GSK3. For instance, Wnt-induced GSK3 regulation activates mTOR to increase protein translation and cell growth (Inoki et al., 2006) and modulates the activity of microtubule-associated proteins during axon growth (Salinas, 2007).

A main caveat of this post-transcriptional Wnt signaling model is the lack of genetic proof. This is because in vivo it is challenging to study Wnt responses under conditions where β-catenin transcriptional effects can be ruled out, e.g., in β-catenin mutant background, since such cells are often heavily perturbed due to the pervasive role of β-catenin in transcription and cell adhesion (Valenta et al., 2011). Given the paramount importance of Wnt signaling, it is essential to clarify whether the upstream cascade of the canonical pathway can act independent of β-catenin transcriptional response by obtaining genetic proof in vivo, which is the subject of this study.

One class of cells where confounding transcription-dependent effects of Wnt signaling can be ruled out is spermatozoa. These cells are akin to mitotic cells; i.e., they are arrested in a cell-cycle phase where the chromosomes are condensed and transcription has come to a halt (Braun, 1998). Coincidentally, mitosis is also

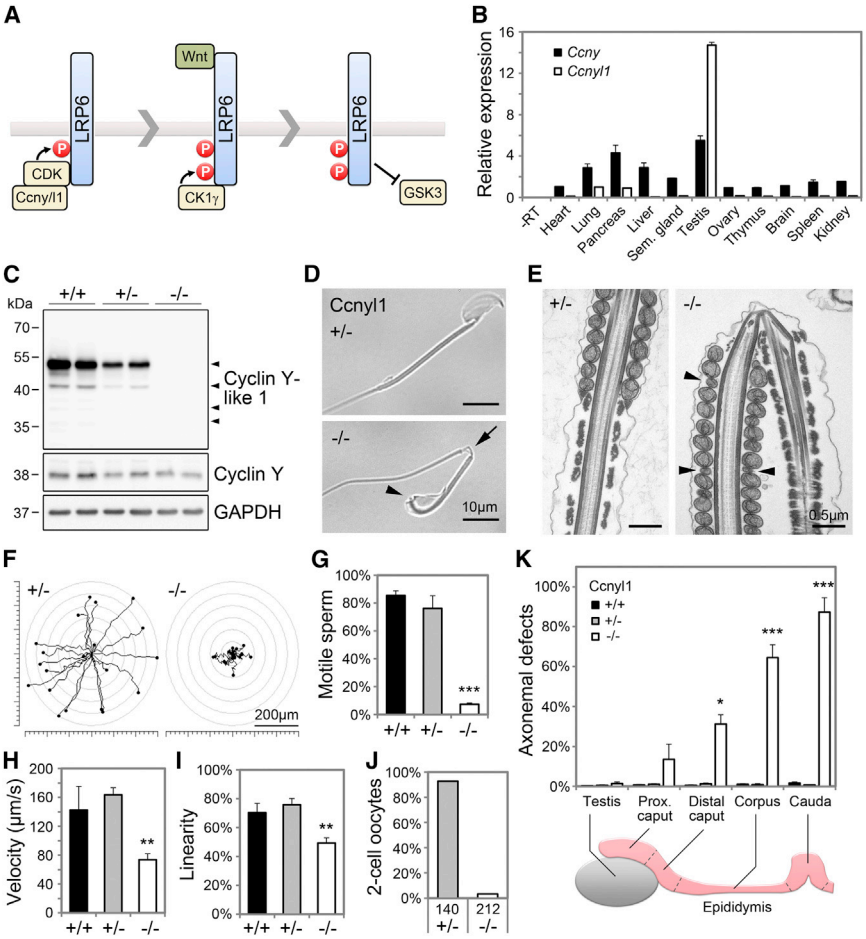

Figure 1. *Ccnyl1* Mutant Mice Show Sperm Maturation Defects

(A) Illustration of Cyclin Y (-like 1)-dependent LRP6 priming, required for Wnt-induced receptor activation.

(B) qPCR of cyclin Y (*Ccny*) and cyclin Y-like 1 (*Ccnyl1*) in wild-type mouse tissues, normalized to *HPRT*.

(C) Immunoblot of Ccnyl1 in testis lysates of 2 *Ccnyl1* mutant mice each.

(D) Bright field microscopy highlighting axonemal defects at the annulus (arrow) and connecting piece (arrowhead) of *Ccnyl1*$^{-/-}$ cauda sperm.

(E) Electron micrographs of the annulus region showing axonemal bending and heteromorphic mitochondria (arrowheads) in *Ccnyl1*$^{-/-}$ sperm.

(F) Representative track plots of cauda sperm from *Ccnyl1*$^{+/-}$ and *Ccnyl1*$^{-/-}$ mice. The graphs show 20 sperm each tracked for 2 s.

(G) Quantification of total motile cauda sperm. n ≥ 3 mice per genotype.

(H and I) Computer-assisted sperm motility analysis showing that the (H) curvilinear velocity and (I) linearity of the remaining motile cauda sperm were significantly decreased in *Ccnyl1*$^{-/-}$ mice.

(J) Quantification of B6/F1 oocytes in 2-cell stage after IVF with *Ccnyl1* mutant cauda sperm. Sperm were pooled from two mice per genotype. The total number of oocytes is indicated below.

(K) Quantification of sperm axonemal defects in different parts of the reproductive tract. *Ccnyl1*$^{-/-}$ sperm exhibit progressive structural defects during epididymal transit. n = 3 mice per genotype. See also Figure S1.

the cell-cycle phase where responsiveness to Wnt signaling is expected to peak (Davidson et al., 2009; Hadjihannas et al., 2012; Olmeda et al., 2003). Importantly, germ-cell-specific deletion of β-catenin has no effect on male fertility, with spermatocyte differentiation and sperm maturation proceeding normally (Rivas et al., 2014).

Despite their compacted structure, spermatozoa are not signaling inert. During a week-long maturation process mammalian spermatozoa respond to maturation signals as they transit the epididymis, a coiled tubule connected to the testis. Epididymal maturation of spermatozoa involves changes in protein composition and subcellular localization, necessary for motility and fertilization (Sipilä et al., 2009; Turner, 2008; Yeung and Cooper, 2002). Maturation is thus induced by factors secreted from the epididymis while spermatozoa are transcriptionally silent. The identity of the epididymal maturation factors has remained unresolved. Here, we show that spermatozoa respond to Wnt signals released from the epididymis. The Wnt regulator Cyclin Y-like 1 (Ccnyl1) is highly expressed in germ cells, and *Ccnyl1*$^{-/-}$ mice are male sterile due to immotile and malformed spermatozoa. Wnt signaling impacts spermatozoa through GSK3 by (1) reducing global protein poly-ubiquitination to maintain protein homeostasis; (2) inhibiting septin 4 phosphorylation to establish a membrane diffusion barrier in the sperm tail; (3) and inhibiting protein phosphatase 1 to initiate sperm motility. The results indicate that Wnt signaling orchestrates a post-transcriptional sperm maturation program.

RESULTS

Ccnyl1 Mutant Mice Show Sperm Maturation Defects

To investigate post-transcriptional Wnt signaling in mammalian physiology, we generated mice deficient for *cyclin Y-like 1* (*Ccnyl1*) (Figure 1A). In contrast to its ubiquitously expressed homolog *Ccny*, *Ccnyl1* RNA and protein are largely restricted to germ cells in the testis (Figures 1B, 1C, S1A, and S1B). As is the case for Ccny (Acebron et al., 2014; Davidson et al., 2009), Ccnyl1 protein also localizes to the plasma membrane in mammalian cell lines, where it associates with LRP6 and collaborates with Ccny in promoting Wnt signaling (Figures S1C–S1E; see also Davidson et al., 2009). *Ccnyl1*$^{-/-}$ mice appeared normal, but male *Ccnyl1*$^{-/-}$ mice were sterile due to severe sperm structural and motility defects. Sperm tails displayed hairpin bending at the annulus (Figure 1D) and in electron microscopy presented axonemal breakage at the junction between midpiece and principal piece (Figure 1E). Additionally, *Ccnyl1*$^{-/-}$ sperm exhibited heteromorphic mitochondria and a partial denudation of the distal midpiece

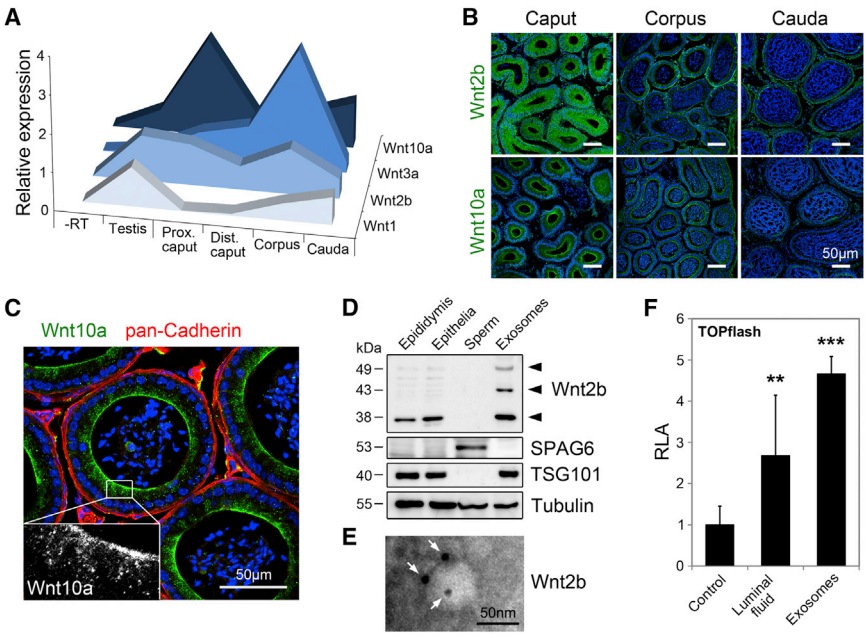

Figure 2. Exosomal Wnt Signaling in the Epididymis

(A) Real-time RT-PCR identified site-specific expression of indicated Wnt ligands in different parts of the male reproductive tract. The graph depicts the mean expression levels of six wild-type mice.

(B) Immunostaining of wild-type mouse epididymides showing a proximal-to-distal gradient of Wnt2b and Wnt10a protein.

(C) Immunolocalization of Wnt10a in caput epididymal sections of wild-type mice showing vesicle-like punctae that accumulate at the apical plasma membrane. The tubule margin is indicated by pan-Cadherin staining.

(D) Immunoblot of Wnt2b in exosome-enriched epididymal vesicle fractions of wild-type mice. Note the molecular weight shift of Wnt2b, characteristic for mature Wnt ligands.

(E) Immunogold labeling of Wnt2b in epididymal exosomes of wild-type mice. Arrows highlight the Wnt2b staining.

(F) Epididymal luminal fluid and exosomes were used for TOPflash reporter assays in HEK293T cells. Tissue from eight to ten wild-type mice was pooled for each experiment. The graph is representative of two experiments and shows relative luciferase activity (RLA) ±SD.

See also Figure S2.

(Figure 1E). Moreover, *Ccnyl1*$^{-/-}$ sperm showed a severe motility defect, with less than 10% of mutant sperm exhibiting progressive motility, while sperm movement was unaffected in heterozygous mice (Figures 1F and 1G). Residual motile mutant spermatozoa had reduced curvilinear velocity and linearity (Figures 1H and 1I). Consequently, *Ccnyl1*$^{-/-}$ sperm had a greatly reduced capacity to fertilize wild-type oocytes in vitro (Figure 1J). Thus, loss of *Ccnyl1* results in a complex sperm structure and motility phenotype, ultimately causing male infertility (see also Zi et al., 2015).

Dysfunctional spermatozoa can arise from defects in spermatogenesis and spermiogenesis in the testis, or aberrant sperm maturation during epididymal transit. Analysis of sperm from different regions of the male reproductive tract revealed that the hairpin phenotype appeared gradually (Figure 1K), suggesting a defect in sperm maturation. In agreement with this interpretation, total caudal sperm count, meiotic cell divisions, and germ cell differentiation in the testis were unaffected in *Ccnyl1*$^{-/-}$ mice (Figures S1F–1H). Moreover, testicular *Ccnyl1*$^{-/-}$ sperm were indistinguishable from controls and exhibited no axonemal damage (Figure S1I). Similarly, the epididymides of *Ccnyl1*$^{-/-}$ mice showed no structural abnormalities, and the distribution of major epididymal cell populations was unchanged compared to controls (Figures S1J and S1K). This indicates that the cellular malformations result from a sperm-intrinsic defect manifesting during epididymal transit.

Exosomal Wnt Signaling in the Epididymis

Consistent with a role for Wnt signaling in epididymal sperm maturation, qPCR showed expression of multiple Wnt ligands in the epididymis, including the canonical Wnt ligands *Wnt1*, *Wnt2b*, *Wnt3a*, and *Wnt10a* (Figure 2A) (Wang et al., 2013). Interestingly, these Wnts displayed compartmentalized expression patterns within the epididymis, and immunostaining of Wnt2b and Wnt10a in particular showed a proximal-to-distal protein gradient (Figures 2B and S2A). Active Wnt signaling in the proximal epididymis was confirmed by examining *BAT-gal* reporter mice, which express nuclear β-galactosidase under the control of β-catenin/TCF (Maretto et al., 2003) (Figure S2B). Reporter activity in these mice correlated with Wnt protein expression; i.e., it was high in the caput epididymis where peak Wnt2b and Wnt10a protein levels were seen but virtually absent in the cauda (Figures S2C and S2D). Interestingly, Wnt10a accumulated in vesicle-like punctae at the apical plasma membrane of epididymal principal cells (Figure 2C). This pattern is characteristic for vesicles released as epididymal exosomes, which are essential for sperm maturation (Sullivan et al., 2007). Since Wnt ligands can be transported on epithelial exosomes to facilitate long-range Wnt signaling (Gross et al., 2012; Luga et al., 2012), we asked whether epididymal exosomes can activate Wnt signaling. We therefore isolated extracellular vesicles from epididymal luminal fluid (Figures 2D and 2E). This exosome-enriched fraction harbored mature Wnt2b, which in immunoelectron microscopy localized to vesicles (Figures 2E and 2F). Importantly, this vesicle fraction robustly induced Wnt signaling in vitro (Figure 2F). In *Ccnyl1*$^{-/-}$ mice, epididymal Wnt activity was unaffected, and there was no difference in Wnt signaling in testis and epididymis (Figures S2E and S2F). We conclude that (1) the epididymis expresses multiple Wnt genes, (2) Wnt signaling decreases from caput to cauda, and (3) the epididymis releases active Wnt ligands on exosomes into the epithelial lumen.

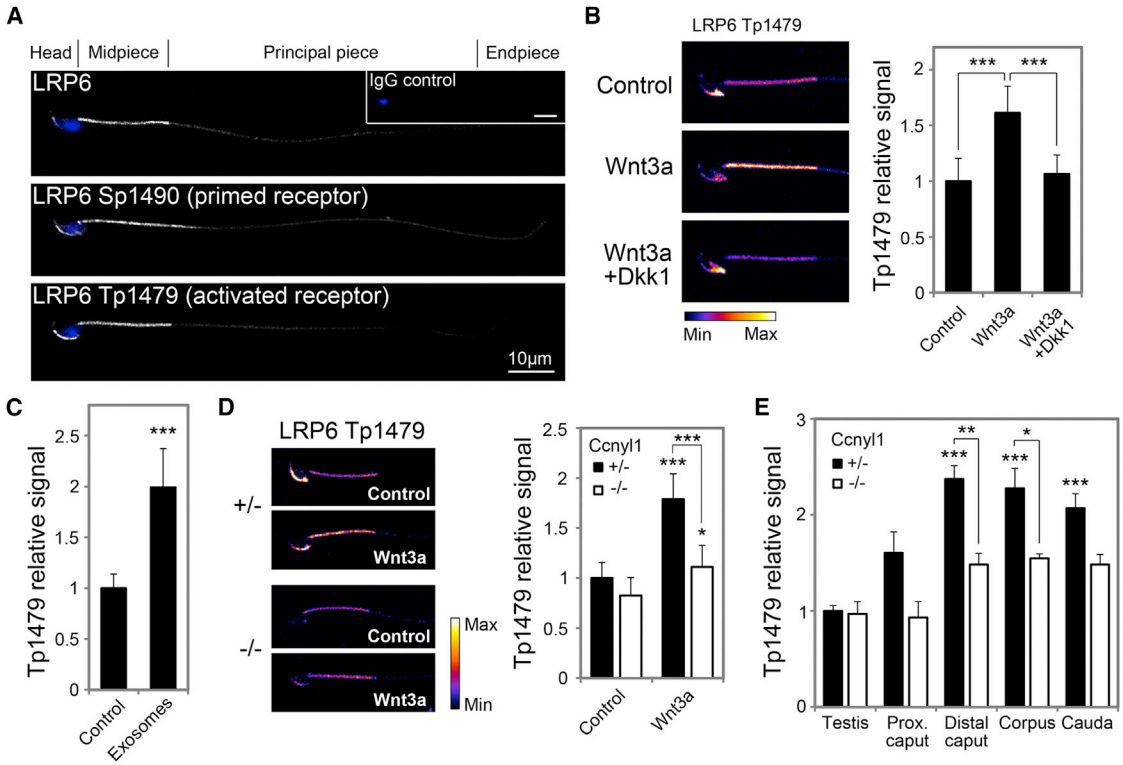

Figure 3. Mammalian Spermatozoa Are Wnt-Responsive Cells

(A) Immunostaining of LRP6 with the indicated antibodies in wild-type mouse cauda sperm showed localization along the tail, which was largely restricted to the midpiece, with sporadic staining in the acrosomal cap.

(B) Immunostaining of wild-type cauda sperm treated with Wnt3a CM for 1 hr showed increased LRP6 Tp1479 staining in the midpiece, which was blocked by co-administration of recombinant Dkk1. Exemplary heatmaps are shown on the left. The average signal intensity along the midpiece was measured and normalized to control treated sperm. n = 6 mice.

(C) Relative Tp1479 staining intensity in sperm treated with epididymal exosomes for 1 hr was increased compared to controls. n = 3 wild-type mice.

(D) *Ccnyl1*$^{-/-}$ sperm showed both lower basal LRP6 activity and reduced Wnt responsiveness. Heatmaps are shown on the left. n = 5 mice per genotype.

(E) Endogenous LRP6 activity monitored by Tp1479 staining in sperm isolated from indicated parts of the reproductive tract, normalized to total LRP6. *Ccnyl1*$^{-/-}$ mice exhibited reduced Tp1479 signal in all areas of the epididymis compared to controls. n = 5 mice per genotype.

See also Figure S3.

Mammalian Spermatozoa Are Wnt-Responsive Cells

Is it possible then that Wnt ligands signal to transcriptionally silent sperm? Active Wnt signaling can be monitored with LRP6 antibodies specific for the CK1γ phosphorylation site Tp1479 (“active LRP6”) (Davidson et al., 2005), adjacent to the cyclin Y/CDK14 priming phosphorylation site Sp1490 (“primed LRP6”) (Figure 1A). Both primed and active LRP6 proteins were detectable in mouse and bull spermatozoa, where they primarily localized to the midpiece, with additional staining in the head (Figures 3A and S3A). LRP6 activation was induced by exogenous Wnt3a treatment and was blocked by addition of the Wnt antagonist Dkk1 (Figures 3B and S3B). Additionally, epididymal exosomes activated LRP6 ex vivo (Figure 3C). Endogenous LRP6 phosphorylation was highest in sperm collected from the distal caput epididymis of wild-type mice (Figure S3C), consistent with peak Wnt signaling activity in this region, and decayed rapidly after removal of sperm from the epididymis (Figure S3D). Importantly, *Ccnyl1*$^{-/-}$ sperm had a blunted response to stimulation with exogenous Wnt3a, and they exhibited reduced LRP6 activation in all parts of the epididymis (Figures 3D and 3E). Taken together, the data (1) identify mammalian spermatozoa as Wnt-responsive cells and (2) suggest that deficiency in Ccnyl1-dependent Wnt signal transduction leads to malformed spermatozoa during their epididymal transit.

Ccnyl1 Mutant Spermatozoa Show Reduced Wnt/STOP Signaling

Since the results suggested that Wnts act as sperm maturation factors in the epididymis, we tested the involvement of Wnt/STOP signaling (Acebron et al., 2014). In this signaling mode, Ccny-dependent Wnt signaling protects part of the proteome from GSK3-dependent ubiquitination and proteasomal degradation, independent of β-catenin (Figure 4A). Consistent with this model, *Ccnyl1*$^{-/-}$ cauda sperm showed globally increased K48-linked protein ubiquitination (Figure 4B). In immunofluorescence analysis, bulk ubiquitinated proteins co-localized with proteasomes (20S subunit α5) in the sperm tail, in particular, in the distal midpiece of *Ccnyl1*$^{-/-}$ cells (Figure 4C). Moreover, protein levels of several Wnt/GSK3 targets, including

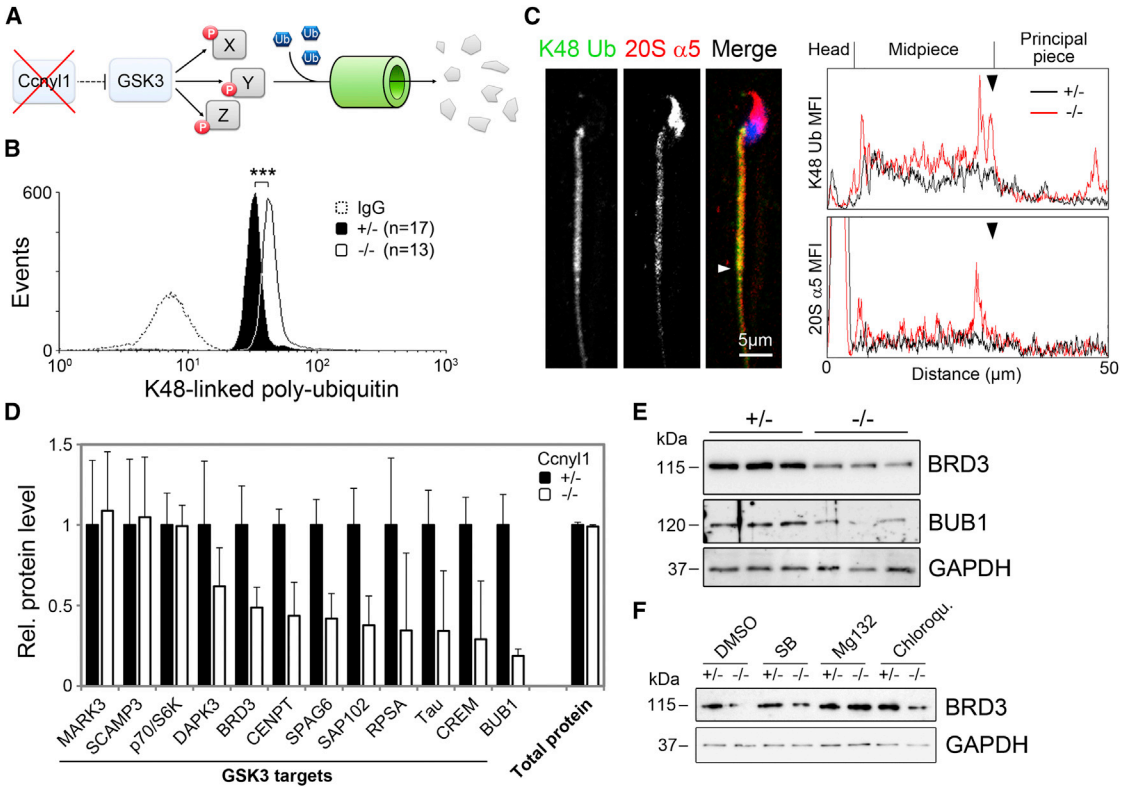

Figure 4. *Ccnyl1*$^{-/-}$ Spermatozoa Show Reduced Wnt/STOP Signaling

(A) Schematic depiction of GSK3-dependent destabilization of target proteins by phosphorylation, which leads to protein ubiquitination and proteasomal degradation.

(B) Fluorescence-activated cell sorting (FACS) profile of K48-linked poly-ubiquitin in cauda sperm of *Ccnyl1* mutant mice.

(C) Co-immunolocalization of K48-linked poly-ubiquitin and 20S proteasomal subunit α5 in *Ccnyl1*$^{+/-}$ cauda sperm showed overlapping staining in the distal midpiece. Representative profile plots of the mean fluorescence intensity (MFI) are shown on the right. Arrowheads indicate the position of the annulus.

(D) The protein level of GSK3 targets in cauda sperm samples were determined by immunoblot and normalized to GAPDH. Total protein levels were measured by bicinchoninic acid assay. n = 3–6 mice per genotype.

(E) Exemplary immunoblot of GSK3 target proteins BRD3 and BUB1 in sperm lysates of three *Ccnyl1* mutant mice each.

(F) BRD3 levels in *Ccnyl1* mutant cauda sperm after treatment with GSK3 inhibitor SB-216763 (20 μM), proteasomal inhibitor Mg132 (10 μM), or lysosomal inhibitor chloroquine (16 μM) for 6 hr at 37°C. Sperm were pooled from four mice per genotype.

See also Figure S4.

Bromodomain-containing protein (BRD) 3 and serine/threonine protein kinase BUB1 (Acebron et al., 2014; Taelman et al., 2010; Xu et al., 2009), were markedly decreased in *Ccnyl1*$^{-/-}$ sperm, whereas other specific proteins and bulk protein were unaffected (Figures 4D and 4E).

Importantly, treatment of sperm with inhibitors against GSK3, proteasome, or to a lesser extent lysosome increased BRD3 levels in *Ccnyl1*$^{-/-}$ cells (Figure 4F), suggesting that the sperm proteome is subject to GSK3-dependent degradation. We conclude that *Ccnyl1*$^{-/-}$ spermatozoa show aberrant GSK3/ubiquitination-dependent protein degradation. It has been proposed that sperm GSK3 activity is regulated by inhibitory serine phosphorylation (Somanath et al., 2004). However, in *Ccnyl1*$^{-/-}$ sperm, which exhibit enhanced GSK3 activity, GSK3 serine phosphorylation was unchanged in caput epididymal sperm, and even increased in caudal sperm (Figure S4), indicating that PKB/SGK3-dependent derepression of GSK3 does not account for increased GSK3 activity in *Ccnyl1*$^{-/-}$ mice.

Post-transcriptional Wnt Signaling Regulates Sperm Diffusion Barrier Function via Septin 4

Considering the large number of potential GSK3 target proteins that may be misregulated in *Ccnyl1*$^{-/-}$ sperm, their complex mutant phenotype may result from loss-of-function of multiple proteins. Yet, we sought to identify key regulators of sperm maturation that are controlled by GSK3. An intriguing candidate GSK3 target is septin 4, knockout mice of which share many features of *Ccnyl1* mutants, including male sterility, sperm tail hairpin bending, and dysmorphic mitochondria (Kissel et al., 2005; Matsuda et al., 2005). Septin 4 is a filament-forming GTPase, which during sperm maturation is required for the formation of a membrane diffusion barrier at the annulus, a cortical ring separating the midpiece and principal piece of the sperm tail (Figure 5A). Sequence inspection of septin 4 revealed three putative GSK3 phosphorylation sites in its N-terminal proline-rich domain (S68, S100, and S107), of which the latter two are highly conserved in vertebrates (Figure 5B). Of note, these sites are also

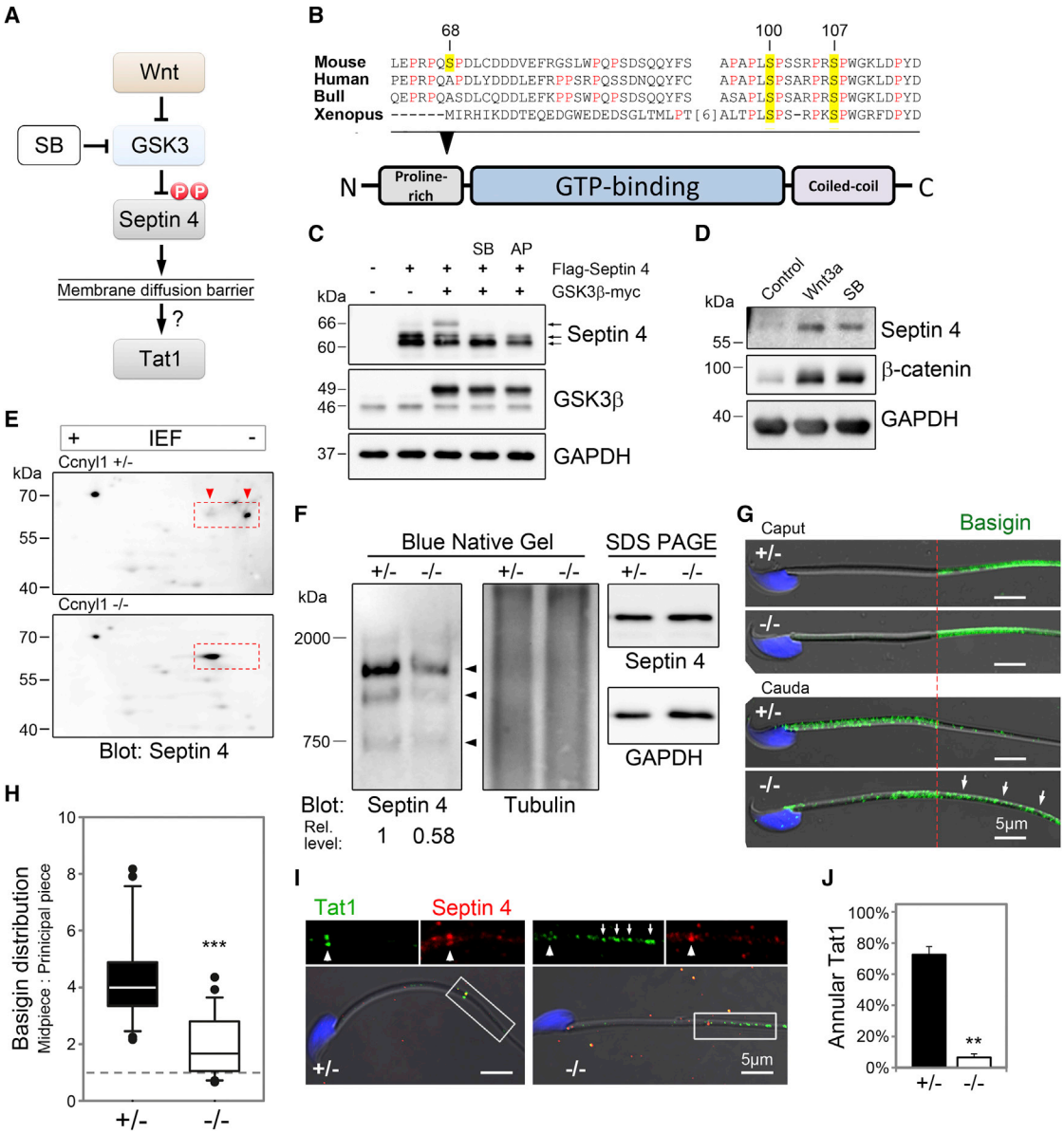

Figure 5. Wnt Signaling Regulates Sperm Diffusion Barrier Function via Septin 4

(A) Schematic presentation of the investigated signaling module.

(B) Partial sequence alignment of septin 4. Multiple putative phosphorylation sites (yellow) were identified in the N-terminal proline-rich domain. Proline residues are highlighted in red. Numbers indicate amino acid positions in the mouse protein.

(C) Immunoblot of mouse Flag-septin 4 co-expressed with GSK3β-myc in HEK293T cells with or without GSK3 inhibitor (SB) or alkaline phosphatase treatment (AP). GSK3 induced increased septin 4 phosphorylation. Arrows indicate major septin 4 species.

(D) Mouse spermatocyte-derived GC-2spd cells were treated with Wnt3a CM or SB for 72 hr. Both treatments increased endogenous septin 4 protein levels.

(E) Cauda sperm were analyzed by 2D gel electrophoresis. Arrowheads highlight a mobility shift of septin 4 in *Ccnyl1*$^{-/-}$ sperm.

(F) Protein extracts from cauda sperm were analyzed by blue native gel electrophoresis, which showed a loss of high-molecular-weight septin 4 complexes in *Ccnyl1*$^{-/-}$ cells (arrowheads). Membranes were reprobed for α-tubulin. An aliquot of each sample was subjected to reducing SDS electrophoresis to confirm equal loading. Data in (E) and (F) are representative of three independent experiments with samples pooled from three to five mice per genotype.

(G) Representative staining of basigin showing loss of diffusion barrier integrity specifically in cauda epididymis sperm of *Ccnyl1*$^{-/-}$ mice. The position of the annulus is indicated by the red line. Arrows highlight aberrant localization of basigin in the principal piece.

(H) Quantification of basigin distribution in *Ccnyl1* mutant cauda sperm. n = 3 mice per genotype.

(I) Co-localization of Tat1 and septin 4 in the sperm tail. Boxed areas are magnified above. Arrowheads indicate the position of the annulus. Arrows highlight mislocalized Tat1 in *Ccnyl1*$^{-/-}$ sperm.

(J) Quantification of Tat1 annular localization in *Ccnyl1* mutant cauda sperm. n = 3 mice per genotype.

See also Figure S5.

found in the pro-apoptotic septin 4 splice variant ARTS, which in sperm is implicated in protein degradation (Kissel et al., 2005). Co-expression of septin 4 with GSK3β in HEK293T cells induced a phosphorylation super-shift that was blocked by GSK3 inhibition (Figure 5C). Mutation of individual or all putative phosphorylation sites identified S100 as the major GSK3 target residue, while S107 most likely acts as a priming site that can also be phosphorylated by other kinases (Sitz et al., 2008) (Figure S5A). Septin 4 physically associated with endogenous GSK3 (Figure S5B); however, this interaction was inhibited when either one or all of the putative phosphorylation sites were replaced with alanine. In addition, in HeLa cells expression of GSK3β reduced septin 4 levels by protein destabilization, which was partially rescued by mutation of S100 and S107 (Figures S5C–S5F). Moreover, in mouse spermatocyte-derived GC-2spd cells, Wnt3a treatment or pharmacological GSK3 inhibition increased endogenous septin 4 levels (Figure 5D).

In sperm, Wnt signaling alters the polymerization properties of septin 4, rather than promoting its stabilization. In *Ccnyl1*$^{-/-}$ sperm, septin 4 levels were unchanged, but 2D gel electrophoresis showed a shift of septin 4 toward the positive pole, consistent with increased GSK3-dependent phosphorylation (Figure 5E). Moreover, septin 4 isolated from mutant sperm failed to form high-molecular-weight complexes, as revealed by blue native gel electrophoresis (Figure 5F). Septins form a membrane diffusion barrier at the annulus, and this barrier function restricts the localization of proteins, including basigin (Kwitny et al., 2010). In caput sperm, basigin is confined to the principal piece and undergoes relocation to the midpiece during sperm epididymal transit (Figure 5G). In *Sept4*$^{-/-}$ sperm basigin localizes over the whole tail length (Kwitny et al., 2010). *Ccnyl1*$^{-/-}$ sperm exhibited normal basigin localization in caput sperm but lost midpiece restriction during epididymal transit toward the cauda (Figures 5G and 5H), in line with an epididymal maturation defect. Moreover, the testis anion transporter 1 (Tat1/Slc26a8), which normally localizes to the sperm annulus and is essential for sperm terminal differentiation (Touré et al., 2007), was dispersed along the tail in the majority of *Ccnyl1*$^{-/-}$ sperm (Figures 5I and 5J).

It was suggested that during epididymal transit spermatozoa receive an unknown signal that triggers a post-translational modification of, e.g., septins, which opens a one-way gate to regulate protein diffusion in the sperm tail (Kwitny et al., 2010). Our results support this idea, whereby epididymal Wnt signaling inhibits GSK3, a septin 4 kinase, which negatively regulates the barrier function of septin filaments.

Wnt Signaling Unlocks Sperm Motility through GSK3

Although the morphological changes of *Ccnyl1*$^{-/-}$ sperm are consistent with septin 4 malfunction, this may not satisfactorily explain the reduced motility of overtly normal sperm (Figure 1H). It is known that the potential for motility already exists in immature sperm, and that serine/threonine protein phosphatase 1 (PP1) holds motility in check before epididymal passage (Vijayaraghavan et al., 1996). PP1 is a ubiquitous enzyme that dephosphorylates a wide range of protein substrates, and that is kept in an active state by GSK3-dependent phosphorylation of its inhibitory subunit PPP1R2 (Cohen, 1989; Vijayaraghavan et al., 1996). Together with our findings, this suggests a simple mechanism for inducing sperm motility during epididymal transit: Caput Wnt signals inhibit GSK3 and thereby inactivate PP1, thus removing the roadblock to sperm motility (Figure 6A). To test this model, we first confirmed phosphorylation of PPP1R2 T72 by GSK3, using two GSK3 inhibitors in HeLa cells (Figure S6A). PPP1R2 Tp72 was greatly increased in mitotic compared to non-mitotic HeLa cells and was reduced by Wnt3a treatment (Figures 6B and S6B). PPP1R2 phosphorylation was also greatly increased in *Ccnyl1*$^{-/-}$ sperm (Figure 6C), and this should lead to higher PP1 activity and hence reduced protein phosphorylation. Indeed, total phospho-serine in the tail of *Ccnyl1*$^{-/-}$ sperm was markedly reduced (Figure 6D). The phospho-serine differences, as well as changes in protein ubiquitination (Figure 4B), were only apparent in epididymal spermatozoa, but not in sperm progenitor cells or somatic cells in testis and epididymis, and they were not caused by differential gene expression (Figures S6C–S6F). Moreover, GSK3 inhibition was sufficient to increase pan-serine phosphorylation in caput sperm of *Ccnyl1*$^{-/-}$ mice (Figure S6G). Consistent with these findings, Wnt3a stimulation or pharmacological GSK3 inhibition both increased the velocity of submotile spermatozoa isolated from the distal caput epididymis of wild-type mice (Figure 6E). Likewise, in *Ccnyl1* heterozygous mice, Wnt3a, the GSK3 inhibitor BIO, and phosphatase inhibitor okadaic acid significantly increased the velocity of caput sperm to approximately the same extent (Figure 6F). In contrast, *Ccnyl1*$^{-/-}$ sperm completely failed to respond to Wnt3a. However, inhibition of either GSK3 or phosphatase, which act downstream of Ccnyl1 in the Wnt-PP1 cascade, partially rescued their motility.

Since motility defects are a major cause of male infertility, we also analyzed human sperm. We confirmed that (phospho)-LRP6 localizes to the midpiece of human testicular spermatozoa (Figure 6G). We then treated two independent disaggregated testicular biopsies with Wnt3a, or okadaic acid as a positive control. Wnt treatment increased the number of sperm with notable flagellar beating in both samples approximately twofold (Figure 6H). Low Wnt signaling in sperm could potentially be a cause for male infertility, and analysis of a published data set (Platts et al., 2007) showed that sperm from men with teratozoospermia, i.e., structural defects of the sperm, show remarkably reduced *Ccnyl1* RNA levels (Figure S6H).

Sperm Maturation Involves Epididymal Wnt Signaling and the Axin/APC Complex

We confirmed the requirement for epididymal Wnt signaling in sperm maturation by transgenic overexpression of the Wnt antagonist Dkk1 in the proximal epididymis. Mice harboring the proximal epididymis-specific *Lcn5-CreERT2* (Xie et al., 2013) were crossed with *Rosa26-Dkk1* mice, containing a floxed transcriptional stop signal (Wu et al., 2008). Tamoxifen injection in *Lcn5-CreERT2/Rosa26-Dkk1* mice induced moderate *Dkk1* expression in the caput epididymis (Figures S7A and S7B). *Dkk1* induction partially phenocopied the sperm phenotype of *Ccnyl1*$^{-/-}$ mice, including reduced LRP6 activation, increased PPP1R2 Tp72 levels, and decreased pan-serine phosphorylation (Figures 7A–7E). Importantly, sperm motility was significantly decreased following tamoxifen

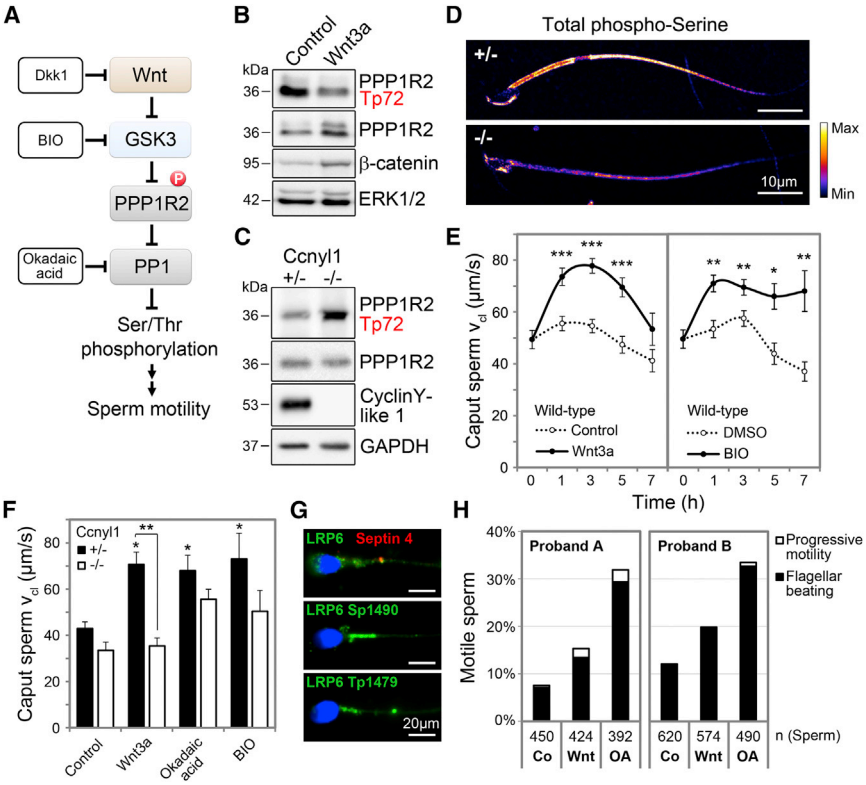

Figure 6. Wnt Signaling Unlocks Sperm Motility via GSK3

(A) Schematic representation of the investigated signaling module, and its inhibitors.

(B) Immunoblot of PPP1R2 phosphorylation in G_2/M-synchronized HeLa cells. Wnt3a reduced PPP1R2 inhibition.

(C) Representative immunoblot of PPP1R2 Tp72 in *Ccnyl1* mutant cauda sperm lysates pooled from three mice per genotype.

(D) Heatmap of serine phosphorylation in cauda sperm. Staining in the tail was decreased in *Ccnyl1*$^{-/-}$ cells.

(E) Wild-type sperm isolated from the distal caput epididymis were incubated with Wnt3a-conditioned media or 1 μM BIO, and curvilinear velocity was measured at the indicated times. n = 5 mice.

(F) Caput sperm were incubated with Wnt3a CM, okadaic acid, or BIO, and the velocity of motile sperm was measured after 3 hr. *Ccnyl1*$^{-/-}$ cells did not respond to Wnt3a but showed increased motility following phosphatase and GSK3 inhibition. n = 6 mice per genotype.

(G) LRP6 staining in human testicular sperm. Primed and active receptor localized to the midpiece, as in mouse and bull sperm. The annulus is indicated in the top panel by septin 4 staining.

(H) Quantification of sperm motility in testicular biopsies of two volunteers. Wnt treatment increased the number of motile sperm in both cases. OA, Okadaic acid.

See also Figure S6.

injection in *Lcn5-CreERT2/Rosa26-Dkk1* mice, but not in control animals (Figures 7F and S7C). These results corroborate that Wnt signals trigger the cascade that inhibits PP1 and activates motility as immature sperm pass through the epididymis.

To further corroborate the cell-autonomous Wnt signaling defect, we assessed the involvement of the GSK3/Axin/APC destruction complex in sperm maturation and Wnt/STOP. The tankyrase inhibitor XAV-939, which stabilizes Axin and thereby inhibits Wnt signaling (Huang et al., 2009), increased GSK3-dependent PPP1R2 phosphorylation in wild-type sperm (Figure 7G), decreased pan-serine phosphorylation in the sperm tail (Figure 7H), and blocked the Wnt-induced increase in caput sperm motility (Figure 7I). Moreover, XAV treatment increased overall protein ubiquitination in sperm, consistent with reduced Wnt/STOP (Figure S7D). Conversely, APC loss of function, which upregulates Wnt signaling, reduced PPP1R2 phosphorylation in MDA-MB-231 cells in vitro (Figure 7J). In addition, total serine phosphorylation and sperm motility were increased in APC^{min} sperm (Figures 7K and 7L), as was the stability of Wnt/STOP targets BRD3 and CREM (Figure S7E). Collectively, these observations outline a critical role for the GSK3/Axin/APC complex in sperm maturation and Wnt/STOP.

DISCUSSION

Wnts Act as an Epididymal Sperm Maturation Signal

The role of the epididymis in sperm maturation is well established, but the secreted signals inducing the maturation process are poorly understood. The first main conclusion of this study therefore is the discovery of Wnts as an epididymal sperm maturation signal. Our results support the following model: Wnt ligands produced by epithelial cells in the proximal epididymis are released into the lumen on signaling-competent exosomes, where they signal to transiting spermatozoa. Epididymal Wnt ligands activate LRP6, which is phospho-primed via Ccnyl1. Wnt signaling inhibits GSK3, to promote sperm maturation through at least three distinct mechanisms: (1) inhibiting protein poly-ubiquitination to maintain protein homeostasis (Wnt/STOP signaling); (2) promoting septin 4 polymerization, thereby maintaining the annular protein diffusion barrier; and (3) inhibiting PP1 to unlock sperm motility. In *Ccnyl1*$^{-/-}$ mutants, GSK3 fails to be inhibited, leading to sperm structural and motility defects and infertility.

The results indicate that Wnt signaling is particularly important in the caput epididymis, since LRP6 phosphorylation peaked in caput spermatozoa, and Wnt inhibition in the caput was sufficient to block sperm maturation. Moreover, Wnt reporter activity was most prominent in caput epididymis, which expresses high levels of β-catenin (Wang et al., 2013). Wnt ligands with high expression in the caput epididymis include *Wnt10a*, *Wnt2b*, and *Wnt1*, but *Wnt10a* and *Wnt2b* mutants apparently do not display male fertility deficits (Tsukiyama and Yamaguchi, 2012; Yang et al., 2015), while *Wnt1*-null mutants die perinatally (McMahon and Bradley, 1990). Multiple Wnts may

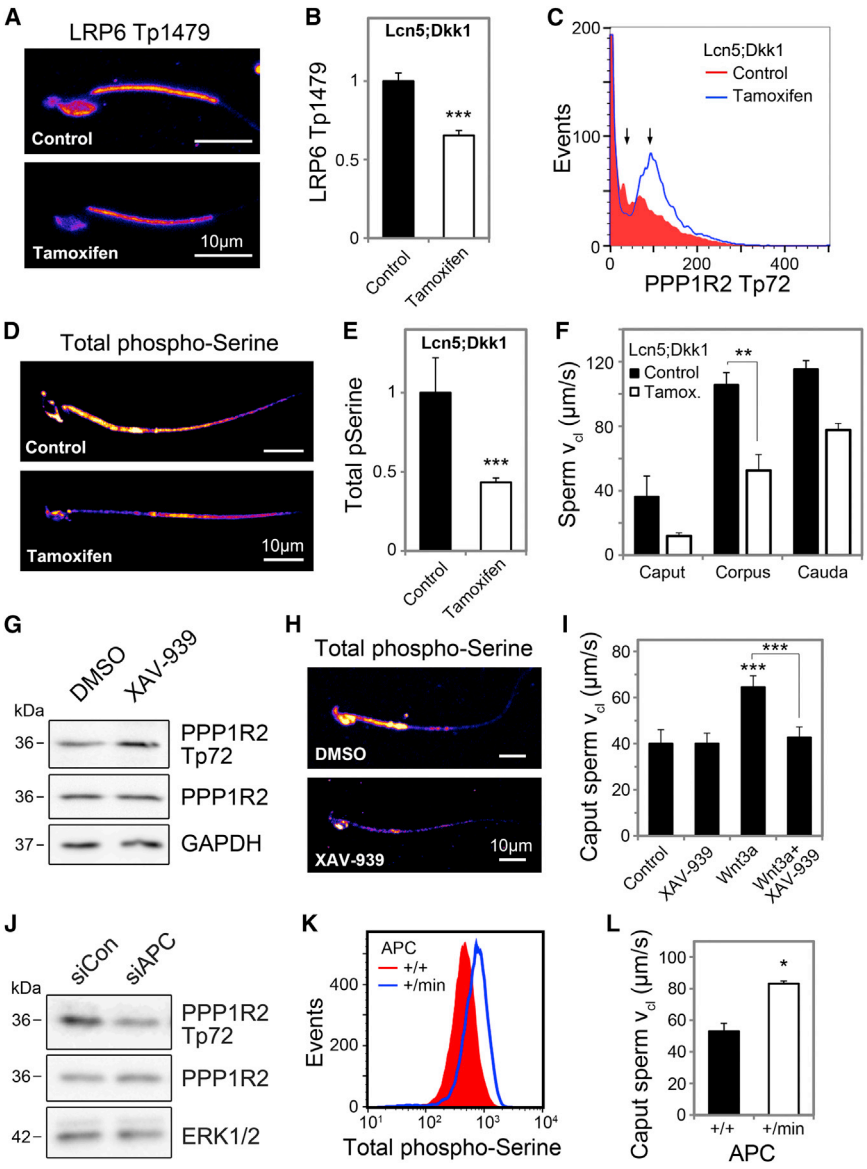

Figure 7. Sperm Maturation Involves Epididymal Wnt Signaling and the Axin/APC Complex

(A) Heatmaps and (B) quantification of LRP6 Tp1479 staining in *Lcn5-CreERT2/Rosa26-Dkk1* (Lcn5;Dkk1) cauda sperm following *Dkk1* induction. n = 3 mice per group.

(C) Flow cytometric analysis of PPP1R2 phosphorylation in Lcn5;Dkk1 cauda sperm. Samples were pooled from three mice per group.

(D and E) Heatmaps (D) and quantification (E) of total serine phosphorylation in Lcn5;Dkk1 sperm. n = 3 mice per group.

(F) Sperm motility analysis of Lcn5;Dkk1 mice. Motility was markedly decreased in all regions of the epididymis after *Dkk1* induction. n = 3 mice per group.

(G) Immunoblot of PPP1R2 phosphorylation in wild-term sperm treated with 5 μM tankyrase inhibitor XAV-939 for 1 hr.

(H) Heatmaps of total serine phosphorylation in wild-type sperm treated with XAV-939 for 1 hr. Samples in (G) and (H) were pooled from eight mice.

(I) Sperm motility analysis after treatment with Wnt3a-conditioned medium and XAV-939 for 1 hr. n = 4 wild-type mice.

(J) Immunoblot of PPP1R2 phosphorylation following APC depletion in MDA-MB-231 cells for 24 hr.

(K) FACS profile of total serine phosphorylation in APC^{min} cauda sperm. Samples were pooled from three mice per group.

(L) Motility analysis of APC^{min} sperm. n = 3 mice per group.

See also Figure S7.

therefore function redundantly and co-operatively in sperm maturation.

Previous studies with various Wnt pathway mutants have implicated Wnt signaling in adult testicular spermatogenesis, including germ cell apoptosis, differentiation, and proliferation (Boyer et al., 2012; Das et al., 2013; Li et al., 2005), but not in epididymal sperm maturation. The severe spermatogenic defects in previous genetic mouse models likely masked later defects in sperm maturation. Importantly, however, β-catenin is dispensable for all steps of spermatocyte development and sperm maturation (Rivas et al., 2014), consistent with our model of Wnts as post-transcriptional maturation signal. Interestingly, mutants of *Cdk16*, one of the five Ccny-dependent CDKs (CDK14 to 18), show sperm maturation deficits similar to Ccnyl1 mice, and the two proteins can interact in vivo (Mikolcevic et al., 2012; Zi et al., 2015), suggesting that a Ccnyl1/CDK16 complex mediates Wnt signaling during sperm maturation. *Cdk16* mutants additionally display terminal spermatogenesis defects, and this somewhat severer phenotype compared to $Ccnyl1^{-/-}$ sperm is probably due to partial redundancy of *Ccnyl1* with *Ccny*.

We note finally that the involvement of a druggable signaling pathway in sperm maturation may provide new opportunities for male infertility and contraception.

Post-transcriptional Wnt Signaling through a Canonical Upstream Cascade

It is commonly thought that the Wnt-Fzd-LRP-GSK3 signaling module acts predominantly by regulating β-catenin and its target genes. Various arguments have been raised against canonical Wnt signaling regulating proteins other than β-catenin (reviewed in, e.g., Metcalfe and Bienz, 2011; Wu and Pan, 2010). The main controversy is whether Wnt signaling regulates phosphorylation of kinase substrates beyond β-catenin and whether this is physiologically relevant. Studying this question genetically in vivo is challenging due to the pervasive role of Wnt signaling in transcriptional regulation. Here, we establish sperm maturation as the first genetic model to study Wnt signaling by the upstream

canonical pathway, where confounding transcriptional effects can be ruled out. Hence, the second main conclusion of this study is that post-testicular sperm are Wnt responsive, i.e., that Wnt signaling acts in transcriptionally silent sperm cells. Our data support the concept that the upstream canonical Wnt pathway can elicit a rich post-transcriptional response (Acebron et al., 2014; Kim et al., 2009; Taelman et al., 2010; Vinyoles et al., 2014).

We identified a number of Wnt/STOP target proteins in sperm and showed that stabilization of proteins is but one of the post-transcriptional functions of the Wnts that govern sperm maturation. Another role of Wnt signaling is to regulate protein function of septin 4 and the activity state of PP1 via PPP1R2. Septins are GTPases involved in cytokinesis, and hence our results suggest septin 4 to be a target of post-transcriptional Wnt signaling also in mitosis in somatic cells. In addition, the septin 4 variant ARTS is known to regulate critical homeostatic functions such as stem cell apoptosis and calcium signaling (Fuchs et al., 2013; Sharma et al., 2013) and may similarly be under Wnt control in somatic cells. PP1 regulates the β-catenin degradation complex (Kim et al., 2013; Luo et al., 2007), and hence in somatic cells Wnt signaling may inhibit PP1 possibly in a negative feedback loop.

Wnt signaling is thought to target a sub-pool of GSK3 in complex with the scaffold protein Axin, and only this pool of GSK3β participates in Wnt signaling and is insulated from other inhibitory signals, such as HGF and insulin (reviewed in Wu and Pan, 2010). GSK3 substrates other than β-catenin also require Axin for Wnt regulation (Acebron et al., 2014; Huang et al., 2015; Inoki et al., 2006; Kim et al., 2009, 2015; Stolz et al., 2015; Taelman et al., 2010; Vinyoles et al., 2014). This indicates that Axin mediates Wnt pathway insulation also in post-transcriptional Wnt signaling. Limiting amounts of Axin may achieve this by undergoing phosphorylation-dephosphorylation cycles, proposed to stabilize β-catenin (and presumably other GSK3 targets as well) across broad component stoichiometries (Kim et al., 2013). In agreement with this model, we found that both Axin and APC are involved in the regulation of sperm maturation and Wnt/STOP, which corroborates that the destruction complex is involved in regulation of proteins other than β-catenin.

In conclusion, we show that Wnt signaling elicits an unexpectedly rich response in transcriptionally silent spermatozoa. In light of this important lesson from germ cells, it appears fruitful to revisit also in somatic cells the degree to which Wnt signaling exerts its effects post-transcriptionally, notably during mitosis. There is evidence that Wnt pathway components upstream of mutated β-catenin or APC are also relevant in tumorigenesis (e.g., SFZD1 and DKK1; Vincan and Barker, 2008), and hence targeting of post-transcriptional Wnt signaling may offer new pharmaceutical avenues.

EXPERIMENTAL PROCEDURES

Mice

Sperm from mice carrying a flanked by loxP (floxed) allele of cyclin Y-like 1 (Ccnyl1$^{tm1a(EUCOMM)Wtsi}$/H) was obtained from the European Mouse Mutant Archive (EMMA) and used for in vitro fertilization of wild-type C57BL/6N oocytes. Heterozygous Ccnyl1-flox mice were bred with transgenic animals expressing Cre recombinase under the control of CMV promoter to achieve organism-wide gene knockout. β-catenin/TCF reporter mice (BAT-gal; Maretto et al., 2003) in a C57BL/6J genetic background were purchased from The Jackson Laboratory and bred with Ccnyl1-null mice. Mice with or without reporter element showed identical sperm phenotypes and were used interchangeably in most experiments. Lcn5-CreERT2 mice were generated as reported (Xie et al., 2013). APC^{min} and R26-Dkk1 mice harboring full-length mouse Dkk1 with a floxed transcriptional stop signal have been described (Moser et al., 1990; Wu et al., 2008). Wild-type (C57BL/6N) mice were obtained from Charles River Laboratories. All mouse experiments were approved by the State review board of Baden-Württemberg (protocol no. G159/13 to S.K. and C.N.) and performed according to federal and institutional guidelines.

Human Samples

Acquisition of human testicular biopsies was approved by the institutional review board at the University of Heidelberg Medical Center (protocol no. S-267/2014 to G.H.). Samples were obtained from volunteers undergoing scheduled surgery for testicular sperm extraction (TESE), following written, informed consent.

Data Analysis

Data were analyzed with an unpaired Student's t test in Excel 2007 (Microsoft) or Holm-Sidak post hoc test following one-way analysis of variance (ANOVA) in SigmaPlot 12 (Systat Software), assuming normal distribution. Data in Figure S6H were from data set GEO: GSE6969 (Platts et al., 2007) and analyzed by Mann-Whitney rank-sum test in Sigmaplot. Data are displayed as arithmetic mean ± SEM, unless indicated otherwise. Statistically significant results in all figures are indicated as *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.029.

AUTHOR CONTRIBUTIONS

S.K. and S.P.A. conceived, performed, and analyzed experiments. J.H. supervised animal husbandry and assisted with experiments. G.H. procured human samples. C.N. supervised all aspects of the project. S.K. and C.N. wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

Expert technical support by the DKFZ core facilities for flow cytometry, transgenics, light microscopy, electron microscopy, and the central animal laboratory is gratefully acknowledged. We thank X. Huang and Y. Zhang for Lcn5-CreERT2 mice, D. Baumann for Cela2a;Kras mice, N. Soshnikova for APC^{min} mice, and W. Becker and L. Leyns for reagents. This work was supported by the Deutsche Forschungsgemeinschaft (DFG), SFB 873.

Received: April 10, 2015
Revised: September 3, 2015
Accepted: September 23, 2015
Published: November 19, 2015

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