C2cd6-encoded CatSper τ Targets Sperm Calcium Channel to Ca²⁺ Signaling Domains in the Flagellar Membrane

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Highlights

- CatSper τ encoded by *C2cd6* is a C2 membrane-associating domain containing protein
- CatSper τ loss-of-function impairs sperm hyperactivation and male fertility
- CatSper τ adopts ciliary trafficking machineries for flagellar targeting via C2 domain
- CatSper τ targets the CatSper channel into nanodomains of developing sperm flagella

SUMMARY

In mammalian sperm cells, regulation of spatiotemporal Ca²⁺ signaling relies on the quadrilinear Ca²⁺ signaling nanodomains in the flagellar membrane. The sperm-specific, multi-subunit CatSper Ca²⁺ channel, which is crucial for sperm hyperactivated motility and male fertility, organizes the nanodomains. Here, we report CatSper τ , the *C2cd6*-encoded membrane-associating C2 domain protein, can independently migrate to the flagella and serve as a major targeting component of the CatSper channel complex. CatSper τ loss-of-function in mice demonstrates that it is essential for sperm hyperactivated motility and male fertility. CatSper τ targets the CatSper channel into the quadrilinear nanodomains in the flagella of developing spermatids, whereas it is dispensable for functional channel assembly. CatSper τ interacts with ciliary trafficking machinery in a C2-dependent manner. These findings provide insights into the CatSper channel trafficking to the Ca²⁺ signaling nanodomains and the shared molecular mechanisms of ciliary and flagellar membrane targeting.

Keywords: Domain organization, Flagellar membrane targeting, Sperm motility, Ca²⁺ channel, CatSper, Ca²⁺ signaling, Male fertility

Introduction

Compartmentalization of the plasma membrane into distinct domains is an important mechanism to control signaling. Cilia and flagella are microtubule-based projections generated from basal bodies and ensheathed by the specialized membrane of distinctive lipid compositions (Garcia III et al., 2018; Walters et al., 2020). A conserved intraflagellar transporter (IFT) system assembles the axoneme - the core structure common to both cilia and flagella - whereas ciliary and flagellar membranes contain concentrated ion channels and membrane receptors for signal transduction (Pablo et al., 2017; Trötschel et al., 2020; Wang et al., 2021). Over the past decades, our increased knowledge of ciliary targeting of membrane proteins has established that vesicular transportation and IFTs enable the cargoes to pass transition zone and membrane diffusion barrier (Nachury and Mick, 2019; Nachury et al., 2010). Cilia function as sensory antenna are crucial for diverse biological processes including phototransduction, olfaction, and Hedgehog signaling.

As specialized cilia, flagella of mammalian sperm have one particular task. The flagellum provides propulsion for the sperm cells to reach and fertilize the eggs in the female reproductive tract. For this arduous journey, flagellar membrane proteins also play essential roles in signal transduction to regulate sperm motility, highlighting their importance in male fertility (Wang et al., 2021). However, how flagellar membrane assembly is coordinated with other early spermiogenesis process remains largely unknown. Just like cilium biogenesis, IFT systems are predicted to deliver flagellar membrane proteins. Yet, the absence of IFT components compromises axonemal growth and results in very short or no flagella (Avidor-Reiss and Leroux, 2015), making it difficult to directly test this idea. It remains unclear whether flagellar membrane targeting uses the same ciliary membrane targeting machinery or distinct motors, and how it is achieved.

In mammalian sperm cells, spatiotemporal regulation of Ca^{2+} signaling relies on the quadrilinear organization of the Ca^{2+} signaling nanodomains in the flagellar membrane (Chung et al., 2014; Hwang et al., 2019), where the sperm-specific, multi-subunit CatSper Ca^{2+} channel complexes assemble into zigzag rows in each quadrant (Zhao et al., 2021). Previous studies have found that only spermatozoa with four intact CatSper nanodomains can develop hyperactivated motility, undergo acrosome reaction, and successfully arrive at the fertilizing site (Chung et al., 2017; Chung et al., 2014; Ded et al., 2020). Thus, flagellar targeting of the CatSper channel and its localization into the quadrilinear nanodomains is crucial for sperm motility and male fertility. However, its complexity in composition (Hwang et al., 2019; Lin et al., 2021; Zhao et al., 2021), the inseparable loss-of-function phenotypes of each transmembrane (TM) subunit in mice (Chung et al., 2011; Hwang et al., 2019; Qi et al., 2007), and the lack of heterologous systems for functional reconstitution have limited our understanding of the mechanism of the CatSper assembly and delivery to flagella.

Here, we report CatSper τ (tau, *CatSper Targeting subunit*) containing C2 membrane-associating domain encoded by *C2CD6* is critical for CatSper flagellar targeting and trafficking into the quadrilinear nanodomains. CatSper τ loss-of-function studies in mice reveal that CatSper τ targets the preassembled CatSper complexes to elongating flagella, where CatSper τ links the channel-carrying vesicles and motor proteins. C2 domain truncation of CatSper τ is sufficient to compromise the spatiotemporal trafficking of the CatSper channel into the nanodomains in developing spermatids and during sperm maturation. We demonstrate that mutant sperm still form the CatSper channel, albeit significantly decreased, and conduct calcium, but they fail to

hyperactivate, rendering the male infertile. These findings provide new insight into the molecular mechanisms of flagellar targeting of membrane proteins in general and organizing the CatSper Ca^{2+} signaling nanodomains in mammalian spermatozoa.

Results

CatSper τ is a New CatSper Component with the Membrane-Associating C2 Domain

The unique quadrilinear CatSper nanodomains within flagellar membrane suggest that the CatSper channel complex might require a membrane-targeting molecule specialized for this task. C2CD6 (previously known as ALS2CR11) was identified as one of the proteins significantly reduced in *CatSper1*-null spermatozoa that lack the entire CatSper channel complex (Hwang et al., 2019; Zhao et al., 2021). The testis-specific *C2CD6* gene encodes two isoforms: both long (>200 kDa) and short (~70 kDa) forms contain the conserved membrane-associating C2 domain in both mouse and humans (Figures 1A and 1B). Thus, we hypothesize that C2CD6 is a new CatSper component that mediates CatSper trafficking to the flagellar membrane and/or spatial partitioning to the nanodomains.

We first examined whether C2CD6 expression is CatSper-dependent (Figures 1C-1F). Immunoblot analysis of sperm from a mouse cauda epididymis showed that protein levels of both long and short forms of C2CD6 are severely reduced in *CatSper1*- and *CatSperd*-null sperm that lack the entire CatSper channel, compared with those in WT sperm (Figures 1C and 1D, right). C2CD6 is localized in the principal piece in both mouse and human sperm (Figures 1D and 1E). 3D structured illumination microscopy (SIM) further revealed a quadrilateral arrangement of C2CD6 (Figure 1F), a hallmark of the CatSper Ca^{2+} signaling domain (Chung et al., 2017). In addition, the decreased C2CD6 proteins distribute discontinuously (Figures 1C and 1D, middle) in *Efcab9*-null sperm that contain overall only ~20% of the protein levels of CatSper subunits and exhibit a fragmented pattern of the linear CatSper nanodomains (Hwang et al., 2019). All these results suggest that C2CD6 is associated with the CatSper channel complex in cauda sperm. C2CD6 is a newly identified *bona fide* CatSper component – we herein name it CatSper τ . Intriguingly, in *CatSper1*- or *CatSperd*-null sperm, CatSper τ is not completely absent, but still detected despite having even lower the protein levels compared to those of *Efcab9*-null sperm (Figures 1C and 1D). This minor yet obvious presence of CatSper τ in the absence of the channel complex distinguishes it from all the other previously reported CatSper subunits.

CatSper τ Loss-of-Function Causes Male Infertility with Defective Sperm Hyperactivation

This expression pattern of CatSper τ further supports our hypothesis that CatSper τ regulates flagellar localization of the CatSper channel. To test this idea, we generated *CatSper τ* mouse models by CRISPR/Cas9 genome editing (Figures 2 and S1). By injecting two pairs of guide RNAs - one targeting the first and 13th exons and the other for the first and second exons - we obtained a knockout line that deletes 43.2 kb encoding almost the entire genomic region of CatSper τ^S and two mutant *CatSper τ* lines that delete 134 bp (*134del*) or 128 bp (*128del*) of the protein coding region (Figures S1B-S1E). The mutant alleles were predicted to express mRNAs that encode frame-shifted proteins with early termination (Figure S1F). Our initial characterization

found that homozygous *CatSper^{128del}* and *CatSper^{134del}* mice showed the identical phenotypes. *CatSper^{134del}* line was used as *CatSper*-mutant mice throughout this study unless indicated. We examined the protein levels of CatSper τ in mutant and knockout models in testes from WT, homozygous *CatSper*-mutant (*CatSper^{ΔΔ}*) and knockout (*CatSper^{-/-}*) males (Figures 2A and S1G). CatSper τ , mainly enriched in the microsome fraction, is absent from *CatSper^{-/-}* testes (Figure S1G) whereas a few proteins with different molecular weights are distinguished from *CatSper^{ΔΔ}* testes by CatSper τ antibodies (Figures 2A and S1G). To characterize the proteins expressed specifically in *CatSper^{ΔΔ}* testes, we examined *CatSper* mRNA levels in *CatSper^{ΔΔ}* testes (Figures S1H-S1L). In *CatSper^{ΔΔ}* testis, ~40% of the *CatSper* transcript is expressed compared with that of WT testes (Figure S1I). RT-PCR and Sanger sequencing demonstrated that *CatSper^{ΔΔ}* testes express truncated *CatSper* mRNAs with 134 bp deletion or 186 bp deletion lacking exon 3 additionally (Figures S1J and S1K). These mRNAs are expected to generate mutant CatSper τ proteins with partial deletions in C2 domain at its N-terminus (Figure S1L). However, the mutant CatSper τ proteins were not detected sperm from *CatSper^{ΔΔ}* (i.e., *CatSper^{ΔΔ}* sperm; Figures 2B, 2C, and S2A, S2B), demonstrating that the C2-domain truncated CatSper τ is expressed in testes but fails to traffic to the *CatSper^{ΔΔ}* sperm flagella.

Both homozygous *CatSper*-mutant and knockout mice have no gross abnormality in survival, appearance, or behavior. *CatSper^{ΔΔ}* and *CatSper^{-/-}* females have normal reproductive ability and give birth with no difference in litter size. However, both *CatSper^{ΔΔ}* and *CatSper^{-/-}* males are infertile (Figures 2D, 2E, and S2C), despite normal testis histology, sperm morphology, and sperm counts in cauda epididymis (Figures 2C and S2A, S2D, and S2E). Although the *CatSper^{ΔΔ}* males express mutant CatSper τ containing truncated C2 domain in testis, they show identical physiological characteristics to those of *CatSper^{-/-}* males. The shared phenotypes between *CatSper^{ΔΔ}* and *CatSper^{-/-}* mice highlight that the C2 domain is essential for CatSper τ function. We noted that *CatSper^{ΔΔ}* mice are not only suitable for CatSper τ loss-of-function in mature cauda sperm but also ideal to investigate the critical N-terminal subdomain of C2 in the native context during male germ cell development. Therefore, we mainly used the *CatSper*-mutant mice in this study.

To understand how CatSper τ loss-of-function causes male infertility, we analyzed sperm motility and their flagellar movement in *CatSper^{ΔΔ}* males (Figures 2F, 2G, and S2F-S2H). Computer-assisted semen analysis (CASA) revealed that swimming velocities (i.e., VCL and VAP) and lateral head displacement were significantly lower in *CatSper^{ΔΔ}* sperm than those in sperm from *CatSper^{+Δ}* males (i.e., *CatSper^{+Δ}* sperm) after incubating them under capacitation condition (Figure S2F). *CatSper^{ΔΔ}* sperm also failed to increase the maximum angle of midpiece curvature (α -angle) and to reduce tail beating speed (Figures 2F, 2G, and S2G, S2H; Video 1), other known features of hyperactivation (Qi et al., 2007). All the results demonstrate that CatSper τ loss-of-function impairs hyperactivated motility and causes male infertility.

CatSper τ -Deficiency Reduces the CatSper Protein Levels and Disorganizes the Nanodomains

Under capacitating conditions, a complete lack of or insufficient Ca^{2+} entry prevents sperm from developing hyperactivated motility, controlling PKA activity, and developing the subsequent protein tyrosine phosphorylation (pTyr) (Chung et al., 2017; Chung et al., 2014; Navarrete et al., 2015). *CatSper*^{Δ/Δ} sperm prematurely potentiate capacitation-associated PKA activity (Figure S2I) and pTyr (Figures 2H and 2I) compared to *CatSper*^{+/Δ} sperm, suggesting a compromised Ca^{2+} influx. Thus, we hypothesized that *CatSper*_τ deficiency dysregulates the level of CatSper channel and/or its function in sperm. We examined CatSper TM (CatSper1-4, β and δ) and non-TM (CatSperζ and EFCAB9) subunit levels and found that they are only ~10% of mature *CatSper*^{Δ/Δ} sperm from cauda epididymis compared to those of WT sperm (Figures 3A and 3B). Interestingly, we found that the CatSper channel, probed by α-CatSper1, is localized at the distal region of the principal piece in the *CatSper*^{Δ/Δ} sperm (Figures 3C and S3A, S3B). 3D SIM imaging further suggested that the CatSper channel lacking *CatSper*_τ does not exhibit the typical quadrilateral nor continuous linear distribution of the nanodomains (Figure 3D). Based on these results, we propose that *CatSper*_τ might play a crucial role in linear and quadrilateral distribution of the channel complexes in the flagellar membrane during tail formation and/or epididymal maturation.

To test this idea, we first examined the protein levels of CatSper subunits and the nanodomain organization of WT and *CatSper*^{Δ/Δ} sperm from the caput and corpus epididymis (Figures 3E and S3C-S3G). Corpus epididymal sperm from *CatSper*^{Δ/Δ} males express around 10 - 25% of CatSper subunits compared to those in WT (Figures S3C and S3D). WT sperm from the caput epididymis displayed four CatSper nanodomains (Figure 3E, top), indicating the linear Ca^{2+} signaling domains are organized during spermiogenesis in testis. In *CatSper*^{Δ/Δ} caput sperm, however, we noted a few striking differences. First, the CatSper channel presented only 2 or 3 discontinuous linear nanodomains (Figure 3E, bottom), suggesting its role in organizing and maintaining the nanodomains. Second, regardless of the low protein levels, the mutant CatSper channel is still detected in the proximal principal piece, as in the WT channel of cauda sperm, suggesting changes in CatSper channel distribution in *CatSper*^{Δ/Δ} sperm during epididymal maturation. Indeed, the CatSper channels showed transitions in their localization from the proximal to distal region of the flagella in *CatSper*^{Δ/Δ} sperm and diminution of the protein levels (Figures S3D, S3F and S3G).

CatSper_τ Is Dispensable for Functional CatSper Channel Assembly

Delineation of the *CatSper*_τ function in flagellar trafficking is complicated by the significantly low protein levels of the CatSper channel in *CatSper*^{Δ/Δ} sperm flagella (Figures 3 and S3). To better understand why the protein levels are reduced, we first tested whether the functional CatSper channel is assembled in epididymal *CatSper*^{Δ/Δ} sperm.

Testis co-immunoprecipitation (coIP) showed that *CatSper*_τ is in complex with CatSper1 and CatSperδ in WT testis (Figure 4A). However, we found that all examined CatSper subunits could form immunocomplexes with CatSper1 and CatSperδ in both *CatSper*^{Δ/Δ} (Figures 4B and S4A) and *CatSper*^{-/-} (Figures 4C and S4B) testes. These results suggest *CatSper*_τ is dispensable in forming the CatSper channel complex in testes (Figures 4B, 4C, and S4A, S4B).

To directly test whether the functional CatSper channel is assembled without *CatSper*_τ, we measured CatSper current (*I*_{CatSper}) by electrophysiological recording (Figures 4D-4G). The inward

current from *CatSper*^{ΔΔ} sperm is only ~6% of the current from WT sperm but still significantly larger than that of *CatSper*^Δ-null sperm (Figures 4D-4F), which lack the entire CatSper channel (Chung et al., 2011). Furthermore, adding 10 mM NH₄Cl increased *I*_{CatSper} in *CatSper*^{ΔΔ} sperm (Figure 4G), consistent with alkalization activation of CatSper (Kirichok et al., 2006). Thus, *CatSper*^{ΔΔ} sperm assembled the functional CatSper channel with the previously known key characteristics despite the ~10% protein levels of CatSper subunits (Figures 3 and S3).

Intriguingly, Ca²⁺ conductance in *CatSper*^{ΔΔ} sperm was not enough to develop hyperactivated motility in *CatSper*^{ΔΔ} sperm (Figures 2 and S2). In agreement, we found that the reduced motility of *CatSper*^{ΔΔ} sperm either by extended incubation or intracellular Ca²⁺ chelation can be only partially rescued (Figures 4H and 4I), suggesting limited CatSper-mediated Ca²⁺ entry during recovery or capacitation. In particular, *CatSper*^{ΔΔ} sperm fail to fully recover their curvilinear velocity (VCL) compared to the initial velocity after inducing capacitation (Figures S4C and S4D). This is in stark contrast to *Efcab9*^{-/-} sperm, which present ~30% proteins levels of CatSper subunits and ~60% Ca²⁺ conductance, were able to recover nearly the full extent of VCL and ALH (Chung et al., 2017; Hwang et al., 2019).

CatSper_τ Is Essential for CatSper Channel Targeting to Flagella in Developing Spermatids

Given that the CatSper channel lacking CatSper_τ is functional (Figures 4 and S4), we next tested whether the CatSper_τ loss-of-function compromises targeting of the assembled CatSper channel complex to the flagella in developing germ cells. Flagellated mouse spermatids undergo dramatic morphological changes during development (Clermont et al., 1993). Notably, the C2-truncated mutant CatSper_τ did not affect the CatSper subunits to form complexes as early as in round spermatids (Figures 5A and 5B) isolated by STA-PUT (Miller and Phillips, 1969). As thin flagellum - without the outer dense fiber and the fibrous sheath - begins to protrude at this stage (Figure 5C), we hypothesized that the CatSper_τ regulates the transport of the CatSper channel from the cell body to the developing tail.

To test this hypothesis, we compared the intracellular localization of the CatSper_τ and the CatSper channel, probed by α-CatSper1 or δ, in developing WT and *CatSper*^{ΔΔ} spermatids according to the developmental steps, well classified by the morphological characteristics (Figures 5C-5E and S5). We observed that the CatSper_τ as well as the CatSper channel complex are localized at the prospective principal piece of WT flagella as early as steps 6-8 spermatids (Figures 5D and S5A). In *Catsper*^{ΔΔ} spermatids, however, confocal imaging detected that the C2-domain truncated mutant CatSper_τ, as well as CatSper1 and δ, is mostly enriched in the cell body until step 12 (Figure 5E and S5B). At steps 13-14 spermatids, a weak mutant CatSper_τ signal was detected but the positive mutant signal eventually disappeared in later steps of mutant spermatids (Figure 5E) and epididymal sperm (Figures 2C and 3C), suggesting removal of the mutant proteins. By contrast, weak and punctate CatSper1 or δ signals observed in *CatSper*^{ΔΔ} spermatids from the steps 13-14 remain in the spermatids until at later steps (Figures 5E and S5B) and in the epididymal sperm (Figures 2C and 3C). All these results illustrate that C2 domain truncation compromises the flagellar targeting of CatSper_τ and assembled CatSper channel in developing spermatids.

Small amount of CatSper τ remains in the cauda epididymal *CatSper1*-null sperm that lack the entire CatSper channel complex (Figure 1), suggesting CatSper τ could be an upstream molecule among the CatSper components for the CatSper targeting in developing spermatids. Thus, we tested whether CatSper τ can traffic to the flagella without associating with the assembled CatSper channel. In *CatSperd*- or *CatSper1*-null testis, CatSper τ was not found in the immunocomplex of the CatSper pore (CatSper1) or auxiliary TM (CatSper δ) subunits, respectively (Figure 5F). This result indicates that CatSper τ normally associates with the fully assembled channel complex; the small amount of CatSper τ detected in *CatSper1*-null sperm (Figure 1D) is presumably targeted to the flagella without associating with the channel complex. To further clarify that CatSper τ traffics to sperm flagella in absence of the CatSper channel, we observed the intracellular localization of CatSper τ in developing *CatSper1*-null and *CatSperd*-null spermatids. Contrary to the first appearance of the CatSper channels in *CatSper τ ^{ΔΔ}* spermatids at steps 13-14 (Figure 5E), CatSper τ was observed in the flagella of *CatSper1*-null and *CatSperd*-null spermatids as early as the step 6 (Figures 5G and S5D) just like in WT spermatids (Figure 5D). The CatSper τ signal, however, becomes gradually weaker in the spermatids lacking CatSper channel complex at later stages (steps 13-16). These results demonstrate that CatSper τ serves as an upstream CatSper component in the CatSper flagellar targeting, which requires association with the channel complex to remain in the flagella after trafficking.

CatSper τ Targets the Channel Complex to the Quadrilinear Nanodomains in the Flagella

CatSper τ loss-of-function dysregulates the flagellar targeting of the CatSper channel in developing spermatids (Figures 5 and S5), which results in impaired quadrilinear distribution of the CatSper channels in epididymal sperm flagella (Figures 3 and S3). Thus, we hypothesized that CatSper τ promotes quadrilinear distribution of the assembled CatSper channel in developing spermatids. To test this idea, we examined the nanodomain structures in developing spermatids by using 3D SIM imaging. CatSper τ and the assembled CatSper channel complex are all arranged quadrilinearly along the principal piece of WT spermatids at steps 13-16 (Figures 6A and S6A). In *CatSper τ ^{ΔΔ}* spermatids, however, the weak signals of the mutant CatSper τ and CatSper channel are resolved as discontinuous nanodomains (Figure 6B). Intriguingly, we were able to further resolve the CatSper τ distribution in *CatSper1*- and *CatSperd*-null spermatids at steps 13-14 (Figures 6C and S6B) when the positive CatSper τ signal becomes weaker (Figures 5G and S5D). Despite discontinuity in the signals, CatSper τ clearly showed the typical quadrilateral distribution along the principal piece in *CatSper1*-null and *CatSperd*-null spermatids, supporting the idea that CatSper τ presumably contributes to the four-nanodomain formation.

It is of note that condensed *CatSper τ ^{ΔΔ}* spermatids (Figures 5 and S5) and mature epididymal sperm (Figures 3 and S3) retain the small amount of the CatSper channel in the flagella, suggesting the possible involvement of another CatSper components in the CatSper channel targeting to sperm flagella, i.e. CatSper δ (Chung et al., 2011). Intriguingly, CatSper δ forms the complex that contains the auxiliary TM subunits alone but not the pore-forming subunits in the absence of CatSper1 (Chung et al., 2011; see also Figure 6D). Thus, we tested whether the auxiliary TM subunits are also involved in flagellar trafficking in developing spermatids. Confocal imaging revealed that the auxiliary TM subunits, probed by α -CatSper δ , show positive signal transiently at the prospective principal piece of *CatSper1*-null spermatids at steps 10-14 (Figure

6E). Given that CatSper δ did not immunocomplex with other pore subunits nor CatSper τ in *CatSper1*-null testis (Figures 5F and 6D), this result indicates that a complex comprised of the auxiliary TM subunits can traffic to flagella alone in developing spermatids just like CatSper τ . Closer observation by 3D SIM imaging, however, unraveled its bilateral distribution along the flagella of *CatSper1*-null spermatids at step 13 (Figure 6F). This result indicates that the CatSper δ -complex containing TM auxiliary subunits can traffic to the flagella but is not sufficient to distribute to the four linear nanodomains. By contrast, CatSper1 was not associated with other pore subunits in the absence of CatSper δ (Figure S6C) and fail to traffic to the flagella of developing spermatids from *CatSperd*-null males (Figure S6D). Thus, CatSper pore-forming subunits are presumably not able to traffic to flagella without other components. All these results from the developing spermatids and epididymal sperm from WT, *CatSper1*^{-/-}, *CatSperd*^{-/-}, and *CatSper* ^{Δ/Δ} males support that CatSper τ is a key component not only for flagellar targeting of the assembled CatSper channel but for their quadrilateral compartmentalization (Figure S6E).

CatSper τ Mediates the CatSper Channel Localization via Cytoplasmic Vesicles and Motor Proteins

CatSper τ traffics to elongating flagella independent of CatSper channel complex in developing spermatids (Figures 6 and S6). To understand the molecular mechanisms of CatSper τ in flagellar targeting and to determine the effect of the C2 truncation in the subcellular localization, we expressed recombinant WT and C2-domain truncated mutant CatSper τ (Δ 103-164; see also Figure S1) in the heterologous systems (Figure 7A). We found the lentiviral transduced WT CatSper τ was enriched as puncta at the ciliary base in ciliated hTERT-RPE1 (Figures 7B and S6F) and 293T (Figure S6G). Intriguingly, mutant CatSper τ was simply diffused throughout the cytoplasm and barely formed puncta. WT CatSper τ puncta were also observed from non-ciliated 293T (Figures S7A-C) and hTERT-RPE1 (Figure 7C) cells near the centrosome. This specific localization pattern of WT CatSper τ in the heterologous systems indicates CatSper τ can be recruited close to the centrosome originated basal body prior to the flagellar targeting. Furthermore, transiently expressed recombinant CatSper1 and CatSper ζ were observed near the WT CatSper τ puncta (Figures 7D and S7D), suggesting that CatSper τ brought those CatSper subunits to the basal body. These results indicate that CatSper τ , when associated with the assembled CatSper channel complex, can modulate transportation of the channel to the basal body.

Our results highlight that CatSper τ has pivotal roles in intracellular transport and trafficking of the CatSper channel to the flagellar and nanodomains via the membrane-associating C2 domain (Figures 3, 5, 6, and S3, S5, S6). We hypothesized that CatSper τ mediates the interaction between the membrane vesicles and the motor proteins for translocalization of the cargoes. i.e., the CatSper channel. Thus, we identified CatSper τ interactome in testes by using immunoprecipitation (IP)-based mass spectrometry and performed functional annotation (Figures 7E, 7F, and S7E-S7I; Tables S1 and S2). A total 811 proteins were identified from the IP with α -CatSper τ -359 (WT, N=704; *CatSper* ^{Δ/Δ} , N=706) and IP with normal IgG (WT, N=193) (Figure S7E; Table S1). 117 proteins with fold changes above the fold change of CatSper τ in WT over *CatSper* ^{Δ/Δ} testis (WT/*CatSper* ^{Δ/Δ} = 2.74) were considered to significantly associate with CatSper τ (Figures 7E and S7F). It is especially noted that we identified the membrane vesicle-

associated proteins (ARF5, COPB1, COPB2, RAB11B, and RAB5B), motor proteins (MYO6, MYO7A, and DYNC112), and ciliary/flagellar proteins (TRAF3IP1, DYNC2LI1, and IFT140) from the CatSper τ interactome, suggesting CatSper τ manages transportation of cargoes in membrane vesicles. The pathway analysis of the CatSper τ interactome by Ingenuity Pathway Analysis further revealed that protein targeting and vesicle movement were enriched in CatSper τ interactome (Figure S7G). Functional annotation of gene ontologies showed that the biological process terms, such as protein targeting, vesicle-mediated transport, and intraciliary transport, were significantly enriched (Figures 7F and S7H; Table S2). All these results further support the predicted CatSper τ functions to modulate cargo transportation. In addition, the enriched molecular function and cellular component ontologies represent that the CatSper τ interactome proteins have binding ability to protein-containing complex or motor activity, and localize at ciliary basal body, the transition zone, or vesicle membrane (Figures S7H and S7I). We propose that CatSper τ is a molecular linker to connect CatSper-carrying vesicles and the motor proteins, thereby enabling the intracellular transportation, flagellar targeting, and quadrilinear arrangement of the CatSper channel complexes in developing spermatids (Figure 7G).

Discussion

CatSper τ Targets CatSper Channel to the Flagella by Adopting Ciliary Trafficking Machinery

Cilia and flagella are the specialized cellular projection that extend from the cell body via the shared structural core, the axoneme. Both cellular compartments are equipped with membrane receptors and ion channels, and function as signal receivers in eukaryotic systems (Nachury and Mick, 2019; Wachten et al., 2017). Our comparative mass spectrometry analysis of CatSper τ immunocomplex from WT and the C2-truncated CatSper τ testes identified proteins mainly involved in vesicle transportation and ciliary trafficking (Figure 7), suggesting CatSper τ adopts conventional ciliary trafficking machinery. Analogous to ARF4 and ARF-like ARL6 that recognize and package the cargoes for ciliary targeting (Deretic et al., 2005; Jin et al., 2010), CatSper τ interactome contains ARF5, RAB11B, and a RAB11 effector RAB11FIP1, which are likely to sort and package the CatSper complexes into carrier vesicles. Cytoplasmic dynein or myosin motors normally deliver cargo-carrying vesicles to the ciliary base directly from the Golgi or via pericentriolar endosomes (Morthorst et al., 2018). We also found cytoplasmic myosin (MYO6 and MYO7A) and dynein (DYNC112) motors in the CatSper τ interactome. We propose that these cytoplasmic motors further deliver CatSper-carrying vesicles to the flagellar base to dock and fuse cargoes to the periflagellar membrane. As previously shown for several GPCRs (i.e., SSTR3, MCHR1, and GPR161) which rely on IFT-A and TULP3 molecules to enter the cilia (Mukhopadhyay et al., 2010; Mukhopadhyay et al., 2013), we found an IFT-A component (IFT140) in the CatSper τ associated proteins, presumably to take on the CatSper complex from the periflagellar region to the flagellar membrane.

In developing spermatids, CatSper τ showed polarized distribution along the flagellum (Figure 5). Furthermore, CatSper τ was enriched at the pericentriolar region when expressed in heterologous systems (Figure 7). These localization patterns support that CatSper τ can mediate between CatSper cargoes and the early endosome to transport the channel into the flagellum. Together with CatSper τ -dependent localization of CatSper1, these results suggest that CatSper τ

contributes to polarized transportation of cargo. CatSper τ , however, likely coordinates CatSper flagellar targeting together with additional players in the native system as small fractions of CatSper channels still traffic to the flagellum in *CatSper τ* -mutant spermatids (Figure 5).

CatSper τ is a Major CatSper Component in Organizing the Ca²⁺ Signaling Nanodomains

A functional CatSper channel can be assembled without either CatSper ζ -EFCAB9 binary complex or CatSper τ , demonstrating that the contribution of these non-TM subunits to the channel assembly is more likely to be marginal (Chung et al., 2017; Hwang et al., 2019; see also Figure 4). For example, although discontinuous, four CatSper nanodomains still remain in *Efcab9*-null sperm that express CatSper channel containing CatSper τ , indicating that CatSper ζ -EFCAB9 is dispensable for the channel trafficking to each quadrant in mature spermatozoa (Chung et al., 2017; Hwang et al., 2019). Compared to the absence of EFCAB9-CatSper ζ , we note that CatSper τ loss-of-function reduces the protein levels of the CatSper complex more significantly and disarranges the channel localizations more severely in mature spermatozoa (Figures 1 and 3; see also Chung et al., 2017; Hwang et al., 2019). CatSper τ can localize in a quadrilinear fashion along the flagellum even without the assembled CatSper channel (Figure 6). The C2-truncated mutant prevents the CatSper channel from forming the four linear domains in the spermatids (Figure 6). Furthermore, CatSper τ is able to regulate CatSper ζ intracellular localization in heterologous systems (Figure 7). Therefore, among the CatSper components reported so far, CatSper τ is the major component that targets the channel complex to flagella and organizes the quadrilinear nanodomains.

Genetic abrogation of the CatSper pore-forming or the auxiliary TM subunits results in entire loss of the CatSper channel complex in mature spermatozoa (Wang et al., 2021). This “all-in or all-out” expression pattern indicates that the pore-forming and auxiliary TM subunits are required to form a functional CatSper channel unit, also demonstrated by a recent atomic structure of the CatSper channel complex isolated from mouse sperm (Lin et al., 2021). The same study also reported CatSper τ (C2CD6) as one of the high confidence proteins from the mass spectrometry analysis of the purified CatSper complexes from mouse testes and epididymis. Yet, the resolution of the structures for the intracellular domains was low and did not resolve the intracellular components of the CatSper channel other than EFCAB9-CatSper ζ (Lin et al., 2021). Whether the unassigned, intracellular electron densities from the existing structure include CatSper τ remains to be elucidated. Our recent studies also suggest an E3 ubiquitin-protein ligase, TRIM69, as another new CatSper component; its protein level and intraflagellar distribution in epididymal sperm is CatSper-dependent (Hwang et al., 2019; Zhao et al., 2021). Of note, our CatSper τ interactome from testes also includes TRIM69 (Table S1). Human CatSper τ is predicted to have ubiquitinated lysine (Akimov et al., 2018). Therefore, TRIM69 might transiently associate with CatSper τ , i.e., for post-translational modification, and contribute to the CatSper channel targeting and/or nanodomain organization.

Membrane-Associating C2 Domain is Essential for CatSper τ Function

Diverse C2 domain containing proteins function in membrane trafficking, fusion, and signal transduction through its membrane-association and/or Ca²⁺ sensing ability (Brunger et al., 2018;

Corbalan-Garcia and Gomez-Fernandez, 2014). Multiple ciliopathy genes also encode C2-domain containing proteins, highlighting the functional importance of C2 domain in modulating ciliary cargo transportation (Zhang and Aravind, 2012). For example, mutation of *MKS6* encoding CC2D2A causes Meckel syndrome. CC2D2A is localized at the transition zone, and its mutation prevents ciliary trafficking of the membrane proteins in mammalian cells (Garcia-Gonzalo et al., 2011). Another C2 protein, C2CD3, localizes at the centrosome and recruits ciliary proteins to dock vesicles to the mother centriole (Ye et al., 2014). C2CD3-interacting CEP120 contributes to form centriole appendages (Tsai et al., 2019) and its C2 domain mutation causes ciliopathy (Joseph et al., 2018).

C2 domain is composed of eight β -strands that form a conserved barrel structure (Nalefski and Falke, 1996; Nishizuka, 1988). The loops between β -strands form the membrane-associating region, which interacts with membranal phosphate via their positive charged or Ca^{2+} -bound acidic residues (Corbalan-Garcia and Gomez-Fernandez, 2014). Our *CatSper τ* -mutant mice clearly demonstrate that the C2 domain truncation, which is predicted to lose the first loop, is sufficient alone to impair the polarized localization of CatSper τ and its interaction with the vesicular components, resulting in the failure of the CatSper complex trafficking to the flagella (Figures 5 and 7). As most ciliary C2 domain proteins stay at the transition zone, it is intriguing that CatSper τ traffics not only to the flagella but also localizes into the four CatSper nanodomains. It is possible that the CatSper τ C2 domain also plays a role in Ca^{2+} sensing and/or domain stabilization in mature sperm flagella, which remains to be further investigated.

The CatSper Levels Determine Sperm Capability to Maintain Ca^{2+} Homeostasis and Motility

Ca^{2+} is crucial for endurance and dynamic regulation of sperm motility. Ca^{2+} overload (Sanchez-Cardenas et al., 2018; Tateno et al., 2013) as well as depletion (Hwang et al., 2019; Marquez et al., 2007) renders sperm less motile. Under physiological conditions, Ca^{2+} homeostasis in mature spermatozoa would be mainly achieved via the balance between CatSper-mediated Ca^{2+} influx and PMCA4-mediated Ca^{2+} extrusion. $[\text{Ca}^{2+}]_i$ in *Pmca4*-null sperm is maintained abnormally high (Schuh et al., 2004). CatSper-deficient sperm gradually lose their motility under both non-capacitating and capacitating conditions (Hwang et al., 2019; Qi et al., 2007). In the current study, we find that there are critical levels of the CatSper proteins and I_{CatSper} which can replenish $[\text{Ca}^{2+}]_i$ to recover the diminished motility when $[\text{Ca}^{2+}]_i$ is depleted. Sperm lacking EFCAB9-CatSper ζ contain ~30% of CatSper proteins and conduct around half of I_{CatSper} compared to WT sperm, presumably due to the loss of gate inhibition (Chung et al., 2017; Hwang et al., 2019). These levels were sufficient to recover the motility of the mutant sperm when the available channels are stimulated. By contrast, *CatSper Δ/Δ* sperm retain ~10% of CatSper proteins but conduct only ~6% of I_{CatSper} compared to WT sperm. Apparently, this level of CatSper activity was insufficient to fully recover sperm motility or swimming speeds, even under the capacitating condition that activates the channel (Figure 4). Human sperm from subfertile patients show significantly reduced Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ after inducing CatSper activation (Kelly et al., 2018). Therefore, spermatozoa with more than this critical threshold of functional CatSper channels and the nanodomain structure are likely to manage the spatiotemporal Ca^{2+} changes during their long travel to fertilize eggs in the female reproductive tract (Chung et al., 2014; Ded et al., 2020). Therefore, the levels of CatSper proteins and/or I_{CatSper} can serve as a prognosis to determine clinical approaches, i.e., IVF vs. ICSI.

In summary, we have provided fundamental insights into the flagellar targeting mechanisms of the CatSper channel and its quadrilinear nanodomain organization (Figure 7G). We have identified CatSper τ as a new CatSper component and demonstrated that the C2-domain containing CatSper τ is a key regulator for CatSper flagellar targeting by mediating the channel complex and ciliary trafficking machineries. CatSper τ is in complex with the assembled CatSper channel and traffics the channel complex to developing spermatid flagella in a C2-dependent manner. CatSper τ interacts with vesicle and motor proteins, which are expected to transport CatSper-carrying vesicles to flagella in developing spermatids. We further show that CatSper τ is required for the quadrilinear arrangement of the targeted CatSper channel, which is crucial for sperm hyperactivation and male fertility. This study highlights the pivotal roles of CatSper τ in flagellar targeting and quadrilinear arrangement of the CatSper channel complex.

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Author contributions

J.-J.C. conceived and supervised the project. J.-J.C. and J.Y.H. designed, performed, and analyzed experiments. J.-J.C. and J.Y.H. generated *CatSper τ* mutant mice. Y.L. and M.I. created *CatSper τ* knockout mice. J.Y.H. performed molecular and cell biology experiments including expression construct generation, virus particle production, immunoprecipitation, immunocytochemistry, confocal and SIM imaging, sperm motility analysis, IP mass spectrometry and proteomics data analysis. H.W. did electrophysiological recordings, and H.W. and J.Y.H. analyzed the electrophysiology data. Y.L. performed IVF and histology experiments. M.I. provided crucial reagents and materials. J.Y.H. assembled figures. J.Y.H. and J.-J.C. wrote the manuscript with the input from the co-authors.

Declaration of interests

The authors declare no competing interests.

Figures and Figure legends

Figure 1. CatSper τ is a new CatSper-associated C2 domain containing protein encoded by C2cd6. (A) Diagrams of human and mouse C2CD6 (CatSper τ) isoforms. C2 domain in human CatSper τ (amino acid (aa) position 66 – 226) and a counterpart region in mouse CatSper τ (aa position 111 – 270) are represented in orange and red boxes, respectively. Arrowheads indicate epitope regions of two CatSper τ antibodies (α - τ -359 and α - τ -482) used in this study. (B) Predicted structures of human (left) and mouse (right) CatSper τ C2 domains. N- and C-termini are colored in blue and red, respectively. (C-D) Association of CatSper τ with the CatSper channel in mouse sperm from cauda epididymis. (C) Immunoblotting of CatSper τ in *Efcab9*-null (*Efcab9*^{-/-}) and *CatSperd*-null (*d*^{-/-}) sperm. Arrowheads indicate long (CatSper τ^L) and short (CatSper τ^S) isoforms. Acetylated tubulin (AcTub) is probed as a loading control. (D) Confocal images of immunostained CatSper τ in WT (left), *Efcab9*-null (middle), and *CatSper1*-null (right) epididymal sperm. DIC Images (top) and the corresponding fluorescence (middle) are shown. Each inset area in the fluorescence images is magnified (bottom). (E) A confocal image of immunostained CatSper τ in human sperm from ejaculate. Shown is a merged view of fluorescence and corresponding DIC images. Hoechst was used for DNA counterstaining. H, head; MP, mid-piece; PP, principal piece (D and E). (F) Quadrilinear arrangement of CatSper τ in WT mouse cauda sperm visualized by 3D structural illumination microscope (SIM) imaging. z-axis information is color-coded in x-y projection (left) and y-z projection (cross-section) is shown (right). Arrowheads indicate annulus, the junction between the midpiece and the principal piece (D-F). See also Figure S1.

Figure 2. Genetic disruption of *CatSper* impairs male fertility and sperm hyperactivation.

(A-C) Validation of *CatSper*-mutant mice generated in this study. *CatSper* τ protein expression was examined from the testis (A) and the cauda sperm (B and C) of homozygous *CatSper*-mutant males by immunoblotting (A and B) and immunostaining (C). Arrows and asterisks indicate normal and mutant *CatSper* τ , respectively. Na⁺/K⁺ ATPase and acetylated tubulin (AcTub) were probed as loading controls (A and B). WT testes (A) and sperm from heterozygous *CatSper*-mutant males (+/Δ) were used for positive control (B and C). (D) Percent pregnancy rates of fertile females mated with *CatSper*-mutant (+/Δ and Δ/Δ) and knockout (+/- and -/-) males. Pregnancy rates of females mated with *CatSper*-134del (+/Δ, N=5; Δ/Δ, N=4) and 128del (+/Δ, N=3; Δ/Δ,

N=3) are combined. (E) *In vitro* fertilization rates of *CatSper*^{+/-} (N=4, 97.5 ± 1.3%) and *CatSper*^{-/-} (N=4, 0.0 ± 0.0%) sperm with cumulus oocyte complexes (COCs). Circles indicate *in vitro* fertilization rates of individual males. ****p*<0.001. (F) Flagella waveforms of *CatSper*^{+/ Δ} (left) and *CatSper* ^{Δ / Δ} (right) sperm from cauda epididymis. Tail movements of the sperm tethered to imaging chamber were recorded at 200 fps speed before (0 min, top) and after (90 min, bottom) inducing capacitation *in vitro*. Overlays of flagellar waveforms for two beat cycles are color-coded in time. (G) Maximum angles of the primary curvature in the midpiece (α -angle) of cauda sperm. Heterozygous (+/ Δ , gray bars) and homozygous (Δ / Δ , red bars) *CatSper*-mutant sperm before (0min; +/ Δ , 45.76 ± 2.96°; Δ / Δ , 23.28 ± 1.16°) and after (90 min; +/ Δ , 92.71 ± 3.02°; Δ / Δ , 26.13 ± 2.51°) inducing capacitation (Cap). Tail parallel to head is set to 0°. Circles indicate the measured α -angle of the individual sperm cells (N=45) from 3 males in each group. **p*<0.05 and ****p*<0.001. (H-I) Excessive development of protein tyrosine phosphorylation (pTyr) in *CatSper* ^{Δ / Δ} cauda sperm during capacitation. (H) Immunoblotting of protein tyrosine phosphorylation in *CatSper*-mutant sperm cells before and after capacitation (0 and 90 min, respectively). (I) Confocal images of pTyr-immunostained *CatSper*-mutant sperm cells. Shown are images of *CatSper*^{+/ Δ} (top) and *CatSper* ^{Δ / Δ} (bottom) sperm before (left, 0 min) and after (right, 90 min) inducing capacitation. Data represented as mean ± SEM (E and G). Hoechst to counterstain DNA (C and I). See also [Figures S1](#) and [S2](#) and [Video S1](#).

Figure 3. CatSper τ loss-of-function diminishes CatSper subunits and disorganizes the CatSper nanodomain in epididymal sperm. (A-B) Reduced protein levels of CatSper subunits in *CatSper*^{Δ/Δ} sperm from cauda epididymis. (A) Immunoblotting of CatSper subunits in *CatSper*^{Δ/Δ} cauda sperm. Arrowheads indicate the bands of the target subunits (Aux-TM: auxiliary transmembrane, Pore: pore-forming, and Non-TM: non-transmembrane). *CatSper*^{d^{-/-}} is a negative control for the absence of the whole channel. Acetylated tubulin (AcTub) is a loading control. (B) Relative protein levels of the CatSper subunits in *CatSper*^{Δ/Δ} cauda sperm. Around 10% of each subunit is detected in *CatSper*^{Δ/Δ} sperm compared to WT sperm; CatSper1 (8.4 ± 2.1%), 2 (16.6 ± 4.5%), 3 (8.8 ± 1.7%), 4 (3.0 ± 1.2%), β (10.0 ± 1.2%), δ (9.3 ± 2.8%), ζ (10.3 ± 1.7%) and EFCAB9 (EF9, 12.0 ± 1.4%). Circles indicate relative levels of CatSper subunits in *CatSper*^{Δ/Δ} sperm from individual males. Protein levels were quantified by measuring the band density from the independent western blots. Data represented as mean ± SEM. N=3. (C) Diminished CatSper signals detected at the distal flagella in *CatSper*^{Δ/Δ} sperm. Shown are confocal images of immunostained CatSper τ (top) and CatSper1 (bottom) in WT (left) and *CatSper*^{Δ/Δ} (middle and right) cauda sperm and the corresponding DIC images (right). Sperm heads were counterstained with Hoechst. A magnified inset is shown (bottom middle). (D-E) 3D SIM images of CatSper1 in cauda (D) and caput (E) epididymal sperm from WT (top) and *CatSper*^{Δ/Δ} (bottom) males. Colors in x-y projections encode z-depth distance from the focal plan (D and E, left). y-z cross sections are shown at right (E). Arrowheads indicate annulus (C, D, and E). See also Figure S3.

Figure 4. Functional CatSper channel is assembled in the absence of CatSper τ . (A) CatSper τ in complex with CatSper channel in testis. (B-C) CatSper subunits in complex without CatSper τ in testes. Pore (Catsper1 and 2) and auxiliary transmembrane subunits (CatSper β and δ) were detected from CatSper δ - (B) and CatSper1- (C) immunocomplexes from solubilized testis microsome. (D-E) Representative traces of CatSper current ($I_{CatSper}$) from WT, CatSper Δ/Δ , and CatSper $\delta^{-/-}$ corpus sperm. $I_{CatSper}$ was elicited by voltage ramp (-100 to +100 mV) from 0 mV holding potential (D) or by step protocol (-120 to +120 mV) in 20 mV increments (E). The cartoons represent pH and monovalent ion composition in pipet and bath solutions (D and E). (F) Inward $I_{CatSper}$ measured from WT (gray, N=9), CatSper Δ/Δ (red, N=9), and CatSper $\delta^{-/-}$ (blue, N=8) corpus sperm at -100 mV. (G) Increase of inward $I_{CatSper}$ by intracellular alkalization. The currents before (solid) and after (hatched) adding 10 mM NH₄Cl to bath solution were recorded at -100 mV from WT (gray), CatSper Δ/Δ (red), and CatSper $\delta^{-/-}$ (blue) corpus sperm (N=5, each). Bars and circles indicate the average $I_{CatSper}$ and the currents from individual sperm, respectively (F and G). Insets show magnified $I_{CatSper}$ traces in CatSper Δ/Δ and CatSper $\delta^{-/-}$ sperm (F and G). (H-I) Effect of intracellular Ca²⁺ (Ca²⁺_i) changes on motility of WT, CatSper Δ/Δ , and CatSper $\delta^{-/-}$ cauda sperm. (H) Time-course changes on total sperm motility. Motility was compared from vehicle (*left*) or BAPTA-AM (*right*) treated WT (black), CatSper Δ/Δ (red), and CatSper $\delta^{-/-}$ (blue) sperm. Different letters indicate significant differences between the genotypes at each time point. (I) Relative motility changes after Ca²⁺_i chelation and recovery. Motility at 90 min time points from Ca²⁺_i chelation (grey) and recovery (red) were normalized with the motility before loading BAPTA-AM (M2 in H) in each genotype. Relative motility after Ca²⁺_i chelation and recovery within each genotype was compared. N=3. ns, non-significant, * p <0.05, ** p <0.01, and *** p <0.001 (F, G, and I). Data represented as mean $\pm$ SEM (F, G, H, and I). See also [Figure S4](#).

Figure 5. Loss of CatSper τ function delays CatSper channel targeting to the flagella during sperm development. (A-B) Formation of the CatSper channel complex in WT and $CatSper^{t^{\Delta/\Delta}}$ round spermatids. (A) Separation of germ cell populations by STA-PUT. Fractions enriched in somatic and diploid germ cells (*left*), round spermatids (*middle*), and condensed spermatids (*right*) are shown. (B) Detection of CatSper pore (CatSper1 and 2) and auxiliary transmembrane

subunits (Aux-TM; CatSper β , ϵ , and δ) from the CatSper-immunocomplexes in both WT and *CatSper* ^{Δ/Δ} round spermatids (RS). WT and *CatSperd*-null (*d*^{-/-}) testes lysates were used for positive and negative control, respectively. (C) Depiction of flagella development in elongating spermatids. Mouse spermatids from developmental steps from 6 to 16 were classified into four groups by morphological characteristics: steps 6-8 (spherical cell body and protruding axoneme), steps 10-12 (hook-shaped sperm head and elongating axoneme), steps 13-14 (condensed sperm head and flagella with developed principal piece), and steps 15-16 (compartmentalized mitochondrial sheath at midpiece). (D-E) Confocal images of immunostained CatSper τ (*top*) and CatSper1 (*bottom*) in WT (D) and *CatSper* ^{Δ/Δ} (E) spermatids. Magnified insets show the proximal principal piece of *CatSper* ^{Δ/Δ} spermatids (E, scale bar = 2 μ m). (F-G) CatSper τ trafficking to the prospective principal piece of the flagella in elongating spermatids in the absence of the CatSper complex. (F) Interaction of CatSper τ with CatSper1 or δ in WT, but not in *CatSperd*-null (*d*^{-/-}) or *CatSper1*-null (*1*^{-/-}) testis. (G) Confocal images of CatSper τ in *CatSperd*-null spermatids. CatSper τ transiently localizes to the flagella of *CatSperd*-null spermatids. Arrows and arrowheads indicate flagella in step 6-8 spermatids and annulus, respectively (D, E, and G). Hoechst to counterstain DNA (D, E, and G). See also [Figure S5](#).

Figure 6. CatSper τ is a major CatSper component to regulate the channel targeting to the nanodomains in elongating spermatids. (A-B) 3D SIM images of CatSper τ (top), CatSper1 (middle), and CatSper δ (bottom) in WT (A) and CatSper $\tau^{\Delta/\Delta}$ (B) spermatids at steps 13-14. (C) 3D SIM imaging of CatSper τ in CatSper δ -null spermatid at step 13. An inset representing the y-z cross section image depicts quadrilateral arrangement. z-axis information was color-coded. α -CatSper τ -482 was used for A, B, and C. (D-F) Minimal contribution of the auxiliary TM subunits to flagellar trafficking in the absence of the tetrameric channel. (D) CatSper Aux-TM subunits (CatSper β , δ , and ϵ) co-immunoprecipitated in CatSper1-null (1-/-) testis but not with CatSper τ . See also Figure 5F. WT and CatSper δ -null testes were used for positive and negative control, respectively. (E) Confocal images of immunostained CatSper δ in CatSper1-null spermatids. Corresponding DIC images of immunostained spermatids are shown at bottom. A magnified inset (scale bar = 2 μ m) shows faint CatSper δ signal in flagellum. Hoechst to counterstain DNA. (F) A 3D SIM image of CatSper δ in a step 13 CatSper1-null spermatid. y-z cross section shown at inset depicts bilateral localization. Information for z-axis was color-coded. Arrows and arrowheads indicate flagella (E) and annulus (A, B, C, E, and F), respectively. See also Figure S6.

Figure 7. CatSper τ modulates the intracellular localization and flagellar targeting of the CatSper channel by interacting with cytoplasmic vesicles via C2 domain. (A) Recombinant short forms of WT (CatSper τ S:WT-EGFP) and C2-truncated mutant CatSper τ (CatSper τ S:Mut-EGFP) proteins. EGFP-tagged proteins were expressed by lentiviral transduction in heterologous system. (B-C) Confocal images of ciliated (B) and non-ciliated (C) hTERT-RPE1 cells stably expressing EGFP-tagged WT (left) and mutant (right) CatSper τ . ARL13B (B) and CENTRIN1 (C) were immunostained to label cilia and centrosome, respectively. Enlarged insets are shown (bottom).

(D) Confocal images of immunostained CatSper1 (*top*) and CatSper ζ (*bottom*) in 293T cells. FLAG-tagged CatSper1 (CS1-FLAG) and CatSper ζ (CS ζ -FLAG) were transiently expressed in 293T cells stably expressing WT (*middle*) and mutant (*right*) CatSper τ . Non-transduced 293T cells were used for negative control (*left*). Magnified insets are shown (*top right*). Hoechst to counterstain DNA (B, C, and D). (E) Quantitative analysis of CatSper τ -interactome from WT and CatSper $\tau^{\Delta/\Delta}$ testis. Each identified protein represented as a dot was mapped according to its spectra-intensity in WT (y-axis) and CatSper $\tau^{\Delta/\Delta}$ (x-axis). The fold change of CatSper τ (red dot) in WT over CatSper $\tau^{\Delta/\Delta}$ testis was 2.74. The proteins with the fold changes above this value were considered to interact with CatSper τ significantly (blue dots, N=118) in testes. (F) Functional annotation of CatSper τ -interacting proteins. Enriched biological process gene ontologies are represented as bubbles and their functional categorization was visualized by a semantic similarity-based scatter plot. Bubble colors and sizes indicate false discovery rate (FDR) and proportion of total proteins annotated with the gene ontologies, respectively. (G) Proposed model for CatSper τ -mediated flagellar targeting of the CatSper channel. CatSper τ complexes with the vesicles carrying the channel complex, localizes them close to basal body and, and transports them to the elongating flagella of the developing spermatids in a quadrilateral formation. See also [Figures S6 and S7](#), and [Tables S1 and S2](#).

STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-mCatSper1	Laboratory of David E. Clapham (Ren et al., 2001)	Cat#CatSper1; RRID:AB_2314097
Rabbit polyclonal anti-mCatSper2	Laboratory of David L. Garbers (Quill et al., 2001)	N/A
Rabbit polyclonal anti-mCatSper3	Laboratory of David E. Clapham (Qi et al., 2007)	N/A
Rabbit polyclonal anti-mCatSper4	Laboratory of David E. Clapham (Qi et al., 2007)	N/A
Rabbit polyclonal anti-mCatSper β	Laboratory of David E. Clapham (Chung et al., 2011)	N/A
Rabbit polyclonal anti-mCatSper δ	Laboratory of David E. Clapham (Chung et al., 2011)	N/A
Rabbit polyclonal anti-mCatSper ϵ	Laboratory of Jean-Ju Chung (Chung et al., 2017)	N/A
Rabbit polyclonal anti-mCatSper ζ	Laboratory of Jean-Ju Chung (Chung et al., 2017)	N/A
Rabbit polyclonal anti-mEFCAB9	Laboratory of Jean-Ju Chung (Hwang et al., 2019)	N/A
Rabbit polyclonal anti-mCatSper τ (α -CS τ -359)	This study	N/A
Rabbit polyclonal anti-mCatSper τ (α -CS τ -482)	This study	N/A
Rabbit polyclonal anti-ARL13B	ProteinTech	Cat# 17711-1-AP RRID:AB_2060867
Rabbit monoclonal anti-phospho-PKA substrate (clone 100G7E)	CST	Cat# 9624 RRID:AB_331817
Rabbit polyclonal anti-ARL13B	ProteinTech	Cat# 17711-1-AP RRID:AB_2060867
Rabbit monoclonal anti-DYKDDDDK (clone D6W5B)	CST	Cat# 86861 RRID:AB_2800094
Mouse monoclonal anti-acetylated tubulin (clone 6-11B-1)	Sigma-Aldrich	Cat# 7451; RRID:AB_609894
Mouse monoclonal anti-Na ⁺ /K ⁺ ATPase (clone H-3)	SantaCruz	Cat# sc-48345 RRID:AB_626712
Mouse monoclonal anti-phosphotyrosine (clone 4G10)	EMD Millipore	Cat# 05-321; RRID:AB_309678
Mouse monoclonal anti-Centrin1 (clone 20H5)	EMD Millipore	Cat# 04-1624; RRID:AB_10563501
Goat anti-mouse IgG-HRP	Jackson ImmunoResearch	Cat# 115-035-003; RRID:AB_10015289

Goat anti-rabbit IgG-HRP	Jackson ImmunoResearch	Cat# 111-035-144; RRID:AB_2307391
VeriBlot	abcam	Cat# ab131366 RRID: N/A
Normal rabbit IgG	R&D Systems	Cat# AB-105-C RRID:AB_354266
Bacterial and Virus Strains		
NEB® 10-beta Electrocompetent E. coli (High Efficiency)	NEB	Cat# C3019H
Chemicals, Peptides, and Recombinant Proteins		
EmbryoMax M2 Medium (1X), Liquid, with phenol red	EMD Millipore	Cat# MR-015-D
EmbryoMax Human Tubal Fluid (HTF) (1X), liquid, for Mouse IVF	EMD Millipore	Cat# MR-070-D
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat# 12100-046
HAM's F12	Gibco	Cat# 21700-075
FBS	Thermofisher	Cat# 10437-028
Collagenase Type IV	Worthington Biochemical Corp	Cat# LS004188
Poly-D-Lysine	Sigma Aldrich	Cat# P0899
BAPTA-AM (CAS 126150-97-8)	Calbiochem	Cat# 196419
Pluronic™ F-127	Invitrogen	Cat# P3000MP
Dimethyl Sulfoxide, DMSO	AmericanBio	Cat# AB03091
Critical Commercial Assays		
Deposited Data		
Raw images of immunoblot	This study	http://dx.doi.org/10.17632/mw3wpmjpyt.1
Experimental Models: Cell Lines		
Human Embryonic Kidney (HEK) 293T	ATTC	Cat# CRL-3216; RRIC:CVCL_0063
hTERT-RPE1	ATCC	Cat# CRL-4000; RRID:CVCL_4388
Human Embryonic Kidney (HEK) 293T expressing CatSper ^{WT} -EGFP	This study	N/A
Human Embryonic Kidney (HEK) 293T expressing CatSper ^{S:Mut} -EGFP	This study	N/A
hTERT-RPE1 expressing CatSper ^{WT} -EGFP	This study	N/A
hTERT-RPE1 expressing CatSper ^{S:Mut} -EGFP	This study	N/A
Experimental Models: Organisms/Strains		
Mouse: B6.129S4-Catsper1 ^{tm1Clph/J}	Laboratory of David E. Clapham (Ren et al., 2001)	RRID:IMSR_JAX:018311
Mouse: B6.129S4-Catsperd ^{tm1.1Clph/J}	Laboratory of David E. Clapham (Chung et al., 2011)	RRID:IMSR_JAX:021451

Mouse: Efcab9 ^{tm1CHNG}	Laboratory of David E. Clapham (Hwang et al., 2019)	N/A
Mouse: CatSper ^{tm1CHNG}	This study	N/A
Mouse: B6D2-C2cd6<em1Osb>	This study	BRC No.: #11219; CARD ID: #3023
Mouse: C57BL/6NCrl	Charles River Laboratories	Crl:029
Mouse: ICR/Slc	Japan SLC	N/A
Mouse: B6D2F1/Slc	Japan SLC	N/A
Recombinant DNA		
Mammalian expression vector for mouse CatSper ^{S:WT} -HA (<i>phCMV3-mCatSper^{S:WT}</i>)	This study	N/A
Mammalian expression vector for mouse CatSper ^{S:Mut} -HA (<i>phCMV3-mCatSper^{S:Mut}</i>)	This study	N/A
Mammalian expression vector for mouse CatSper1-FLAG (<i>phCMV3-mCatSper1-Flag</i>)	Laboratory of Jean-Ju Chung (Hwang et al., 2019)	N/A
Mammalian expression vector for mouse CatSper ζ -V5 (<i>phCMV3-mCatSperζ-V5</i>)	Laboratory of Jean-Ju Chung (Hwang et al., 2019)	N/A
Mammalian expression vector for mouse CatSper δ (<i>pcDNA3-mCatSperδ</i>)	Laboratory of David E. Clapham (Chung et al., 2011)	N/A
Mammalian expression vector for mouse CatSper ζ -FLAG (<i>phCMV3-mCatSperζ-Flag</i>)	This study	N/A
Mammalian expression vector for mouse CatSper δ -FLAG (<i>phCMV3-mCatSperδ-Flag</i>)	This study	N/A
Lentiviral expression constructs for mouse CatSper ^{S:WT} -EGFP (<i>pLenti-CMV-CatSper^{S:WT}-EGFP-Hygro</i>)	This study	N/A
Lentiviral expression constructs for mouse CatSper ^{S:Mut} -EGFP (<i>pLenti-CMV-CatSper^{S:Mut}-EGFP-Hygro</i>)	This study	N/A
ORF clone of mouse <i>CatSper</i> ORF	Open Biology	Cat# MMM1013-211693533
<i>pLenti CMV GFP Hygro</i> (656-4)	Addgene	Cat# 17446
<i>psPAX2</i>	Addgene	Cat# 12260
<i>pMD2.G</i>	Addgene	Cat# 12259
Software and Algorithms		
FIJI	Schindelin et al., 2012	http://fiji.sc
Zen 2012 SP2	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html
SCA evolution	Microptic	https://www.micropticsl.com
R	R Foundation	https://www.r-project.org/

Ingenuity Pathway Analysis	QIAGEN	https://digitalinsights.qiagen.com/product-s-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/
REVIGO	Supek et al., 2011	http://revigo.irb.hr
Other		
Protein A/G PLUS-Agarose	SantaCruz	Cat# sc-2003
SureBeads™ Protein A Magnetic Beads	BioRad	Cat# 1614011
CellVision 4 Chamber 20 micron	CellVision	Cat# CV 1020-4CH

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for the resources and reagents should be directed to the Lead Contact, Jean-Ju Chung (jean-ju.chung@yale.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

CatSper1, *CatSperd*, and *Efcab9*-null mice (Chung et al., 2011; Hwang et al., 2019; Ren et al., 2001) are maintained on a C57BL/6 background. WT C57BL/6 mice were purchased from Charles River Laboratory. WT B6D2F1 and ICR mice were purchased from Japan SLC. Mice were cared according to the guidelines approved by Institutional Animal Care and Use Committee (IACUC) for Yale University (#20079) and by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (#Biken-AP-R03-01-0).

Generation of *CatSper*-mutant and knockout mice by CRISPR/Cas9 and Genotyping

CatSper-mutant and knockout mice were generated on C57BL/6 and B6D2F1 background, respectively, using CRISPR/Cas9 method. For *CatSper*-mutant mice, two guide RNAs (gRNAs), 5'-GCCACTCACGGACAAGAACA-3' and 5'-TCTCCAAATGGTATCAAGTT-3', in px330 were injected into pronuclei of the fertilized eggs obtained from super-ovulated females after mating with males. 2-cell embryos were transplanted into pseudo-pregnant females and founders' tails were biopsied to extract genomic DNA (gDNA). To examine the large truncation at target region by CRISPR/Cas9 editing, PCR was performed with F1 (5'-AAGACAGCTCCTGAGACGTG-3') and R1 (5'-GGGGGTTGGAGTATGGAGGA-3') primers. The PCR products were Sanger sequenced; founder females, which carry mutant allele with 128 or 134 bp deletion at coding region (128del and 134del, respectively) were mated with WT C57BL/6 males to test germline transmission of the mutant allele. Two mutant mice lines were maintained, and genotyping was performed with F1-R1 primer pair for mutant allele and F2 (5'-ATCCACTGCCACTGCCTAGA-

3')-R1 primer pair for WT allele. For *CatSper*-knockout mice, two CRISPR RNAs (crRNAs), 5'-AGCGCTCCACCTACGCCTAC-3' and 5'-TCAGAGGTCTTCGGTAAATG-3', were annealed to SygRNA® Cas9 Synthetic trans-activating CRISPR RNA (tracrRNA; Sigma Aldrich), and then mixed with TrueCut™ Cas9 Protein v2 (ThermoFisher) to form crRNA:tracrRNA:Cas9 ribonucleoprotein (RNP) complexes. The RNP complexes were introduced into the fertilized eggs obtained from super-ovulated WT B6D2F1 females by electroporation using an NEPA21 electroporator (Nepagene). Likewise, the treated eggs that developed into the 2-cell stage were transplanted into pseudo-pregnant ICR females, and gDNA was obtained from the founder animals by toe clipping. PCR was carried out using F1' (5'-TGCACGTGGTCCAGGAAA-3') and R1' (5'-TTGCTGGGGGAGACTCACTTA-3') primers or F2' (5'-TGGTGCAGTATGGTGATAAGG-3') and R1' primers to detect the knockout or WT allele, respectively. The PCR products from the knockout allele were subjected to Sanger sequencing to verify the detailed deletion size.

Cell Lines

Mammalian cell lines

HEK293T cells (ATCC; derived from female embryonic kidney) were cultured in DMEM (GIBCO) containing 10% FBS (ThermoFisher) and 1x Pen/Strep (GIBCO) at 37 °C, 5% CO₂ condition. hTERT-RPE1 (ATCC; derived from female retina pigmented epithelium) were cultured in 1:1 mixture of DMEM and Ham F12 (DMEM/F12, GIBCO) supplemented with 10% FBS (ThermoFisher), 1x Pen/Strep (GIBCO), and 10 µg/ml Hygromycin B (Invitrogen) at 37 °C, 5% CO₂ condition. HEK293T cells and hTERT-RPE1 cells stably expressing CatSper^τ^{S:WT} and CatSper^τ^{S:Mut} were cultured in the mediums for HEK293T cells and hTERT-RPE1 cells containing 50 µg/ml and 20 µg/ml Hygromycin B (Invitrogen), respectively.

Bacterial strains

NEB® 10-β and NEB® Stable (NEB) bacterial strains were used for molecular cloning.

Sperm Preparation

Mouse sperm preparation and In Vitro Capacitation

Epididymal spermatozoa were collected from cauda, corpus, and cauda epididymis of adult male mice by swim-out methods in M2 medium (EMD Millipore) or HEPES-buffered saline (HS; Chung et al., 2017), or direct release into Toyoda-Yokoyama-Hosi (TYH) medium. Collected cauda sperm were incubated in human tubular fluid (HTF; EMD Millipore) at 2.0 – 3.0 x 10⁶ cells/ml concentration to induce capacitation for 90 minutes at 37 °C, 5% CO₂ condition. For *in vitro* fertilization (IVF), sperm were capacitated in TYH medium at a concentration of 2.0 x 10⁶ cells/ml for 120 minutes at 37 °C, 5% CO₂ condition to induce capacitation.

Human sperm preparation

Frozen human sperm vials from healthy donors were purchased from Fairfax Cryobank. The vials were thawed and mixed with warm HS. After washing with HS two times, human sperm were placed on top of 20% Percoll (Sigma Aldrich) in HS and incubated at 37 °C for 30 minutes. Immotile sperm in top layer were discarded and motile sperm were collected by centrifugation at 2,000 x g, followed by resuspension with HS.

Testicular Germ Cell Preparation

Testicular cells preparation

Testes from adult male mice were collected and tunica albuginea, a capsule layer, was removed to harvest seminiferous tubules. Collected seminiferous tubules were washed with ice-cold PBS two times and chopped to dissociate germ cells. Dissociated testicular cells and chopped seminiferous tubules in PBS were filtered with cell strainer with 40 µm mesh size (Fisher Scientific). Filtered testicular cells were used for immunostaining.

STA-PUT germ cell separation

STA-PUT velocity sedimentation was applied to separate spermatogenic cell types (Bryant et al., 2013). Seminiferous tubules were collected from six to eight testes and washed with ice-cold PBS two times and DMEM (GIBCO) one time. Pulled seminiferous tubules were dissociated by incubation in 1 mg/ml collagenase Type IV (Worthington Biochemical Corp) at 37 °C for 10 minutes. Dissociated tubules were washed two times with DMEM by centrifugation at 500 x g for 5 minutes and incubated in EDTA-free 0.25% trypsin (GIBCO) with 15 unit/ml DNase 1 (NEB) at 37 °C for 10 minutes to dissociate testicular cells. Dissociated testicular cells in trypsin were mixed with DMEM (GIBCO) containing 10% FBS (Thermofisher) and 4 unit/ml DNase 1 (NEB), followed by filtering with 100 µm-pore cell strainer (Fisher Scientific). The filtrates were centrifuged and washed with DMEM (GIBCO) containing 10% FBS (Thermofisher) and 0.5% BSA (Sigma Aldrich) at 500 x g for 5 minutes. Washed germ cells were filtered with cell strainer with 40 µm mesh pore. 2.0 – 3.0 x 10⁸ cells were loaded on top of 2 – 4% of BSA (Sigma Aldrich) gradient in DMEM (GIBCO) and sedimented for 2 hours to separate different types of testicular cells. Medium was fractionated and morphology of testicular cells in each fraction was examined using Axio observer Z1 microscope (Carl Zeiss) to enrich round spermatids.

METHOD DETAILS

Antibodies and Reagents

Rabbit polyclonal antibodies to recognize mouse CatSper1 (Ren et al., 2001), 2 (Quill et al., 2001), 3, 4 (Qi et al., 2007) β, δ (Chung et al., 2011), ε, ζ (Chung et al., 2017), and EFCAB9 (Hwang et

al., 2019) were described previously. Polyclonal CatSper τ antibodies were generated by immunizing rabbits with KLH carrier-conjugated peptides corresponding to 359th – 377th (EKLREKPRERLERMKEEYK, α - τ -359; Open Biosystems) or 482nd – 500th (QIVEENEMPHLPKTSEPED, α - τ -482; Sigma Aldrich) residues of CatSper τ . Antisera from the immunized rabbits were affinity-purified using the peptides crosslinked to AminoLinkTM Coupling Resin (Pierce). All other commercial antibodies and reagents used in this study are listed in the key resource table.

RNA Extraction, cDNA Synthesis, and RT-PCR

Total RNA was extracted from adult WT and homozygous *CatSper1-134del* mutant male testes using RNeasy Mini kit (QIAGEN). 500 ng of the extracted RNA was used for cDNA synthesis using iScriptTM cDNA Synthesis kit (Bio-Rad) according to manufacturer's instruction. cDNAs were subjected to end-point PCR using OneTaq[®] 2X Master Mix (NEB) or quantitative PCR (qPCR) using iTaq Universal SYBR Green Supermix (Bio-Rad). Primers, F3 (5'-CGGTTAGTGGCAGATAGGGC-3'), R3, (5'-TTGCTCTGCAGAGAACCTGG-3'), F4 (5'-TTTCCCACCCAGTCATCTAA-3'), R4 (5'-CTTCATCTCGCCAAACCTAA-3'), F5 (5'-CCTTGGCCAACAGCGTTTAA-3'), R5 (5'-TCTTTCCAACCTTTCCCGGG-3'), F6 (5'-AGCATTGGGGTTCCTGAAGC-3'), and R6 (5'-TCAACTCTCAGGAGCCCAAAC-3'), were used for end-point PCR and qF1 (5'-CAAGGGTAAAGGCACAGGAA-3'), qR1 (5'-TTTATGTGAATCGCCAGACAG-3'), qF2 (5'-GACAAAAAGGGATAATAAGGGAAG-3'), and qR2 (5'-TGAAATAGCTTCATATTTTCTGTGATG-3') were used for qPCR. *TBP* was used as a reference gene to normalize transcript levels by ddCt method.

Molecular Cloning and Lentivirus Production

Transient expression constructs

Mammalian expression constructs for CatSper1 (*phCMV3-CatSper1-Flag*) was described in previous study (Hwang et al., 2019). Mouse *CatSperd* or z ORFs (Chung et al., 2011; Hwang et al., 2019) were subcloned into *phCMV3* to express C-terminal FLAG-tagged CatSper subunits (*phCMV3-CatSperd* or *z-Flag*) using NEBuilder[®] HiFi DNA Assembly Kit (NEB). A stop codon was placed at the upstream of HA-encoding sequences of *phCMV3* vector for FLAG-tagged CatSper subunit cloning.

Lentiviral Transfer constructs

An ORF clone of mouse *CatSper1* (NM_175200) Open Biosystems, clone 40090362) was subcloned into *phCMV3* (*phCMV3-CatSper1^{S:WT}*) encoding short form of CatSper τ (CatSper τ ^{S:WT}) tagged with HA at C-terminus. A CDS of the mouse mutant CatSper τ (CatSper τ ^{S:Mut}) with C2 domain truncation (Δ 103-163) was cloned into *phCMV3* vector by assembling two CDS fragments amplified from *phCMV3-CatSper1^{S:WT}* (*phCMV3-CatSper1^{S:Mut}*) using NEBuilder[®] HiFi DNA

Assembly Kit (NEB). ORF sequences of CatSper $\tau^{S:WT}$ (*phCMV3-CatSper $\tau^{S:WT}$*) and CatSper $\tau^{S:Mut}$ (*phCMV3-CatSper $\tau^{S:Mut}$*) were amplified using Q5[®] Hot Start High-Fidelity 2X Master Mix (NEB) and subcloned into *pLenti-CMV-GFP-Hygro* (656-4) (gifted from Eric Campeau & Paul Kaufman; Addgene plasmid # 17446) using NEBuilder[®] HiFi DNA Assembly Kit (NEB) (*pLenti-CMV-CatSper $\tau^{S:WT}$ -EGFP-Hygro* and *pLenti-CMV-CatSper $\tau^{S:Mut}$ -EGFP-Hygro*, respectively).

Lentivirus Production

Cloned lentiviral expression vectors (*pLenti-CMV-CatSper $\tau^{S:WT}$ -EGFP-Hygro* and *pLenti-CMV-CatSper $\tau^{S:Mut}$ -EGFP-Hygro*), and lentiviral packaging (*psPAX2*, Addgene plasmid # 12260) and envelop (*pMD2.G*, Addgene plasmid # 12259) plasmids (gift from Didier Trono) were transfected into cultured HEK293T cells using polyethylenimine (PEI). Culture medium was harvested after 1-3 days transfection. Collected medium with virus particles were centrifuged at 1,000 x g for 15 minutes at 4 °C and the supernatant were mixed with 4X lentivirus concentration solution - 40% (w/v) polyethylene glycol 8000 (PEG 8000, Fisher Scientific), 1.2 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH7.2 - followed by incubation at 4 °C for overnight. Precipitated virus particles were pelleted by centrifugation at 1,600 x g for 1 hour at 4 °C and resuspended with DMEM (GIBCO). Concentrated virus particles were frozen and stored at - 80 °C until use.

Recombinant Protein Expression in Mammalian Cells

Transient protein expression

Mammalian expression vectors encoding FLAG-tagged mouse CatSper proteins (1, δ , and ζ) were transiently expressed in HEK293T cells. Cultured HEK293T cells were transfected with plasmid to express recombinant proteins using PEI. After 36 – 48 hours from the transfection, cells were used for immunostaining.

Stable protein expression

HEK293T and hTERT-RPE1 cells were transduced with the lentiviral particles to express EGFP-tagged mouse WT and mutant CatSper τ (CatSper $\tau^{S:WT}$ -EGFP and CatSper $\tau^{S:Mut}$ -EGFP, respectively). Briefly, HEK293T and hTERT-RPE1 cells were placed in DMEM containing 10% FBS, 10 μ g/ml polybrene (EMD Millipore), and lentiviral particles, and subjected to cytopspin centrifugation at 1,000 x g for 1 hour at room temperature (RT). The cells were incubated at 37°C, 5% CO₂ for one day and the medium was changed to DDM (HEK293T) or DMEM/F12 (hTERT-RPE1) containing 10% FBS and 1X Pen/Strep. The lentivirus-transduced HEK293T and hTERT-RPE1 cells to express mouse CatSper $\tau^{S:WT}$ -EGFP and CatSper $\tau^{S:Mut}$ -EGFP were passaged one time and cultured in the FBS- and Pen/Strep-containing media supplemented with and 50 μ g/ml (HEK293T cells) or 20 μ g/ml (hTERT-RPE1) hygromycin B (Invitrogen). Stable protein expression was checked by epifluorescence microscope (ZOE Fluorescent Cell Imager, BioRad).

Protein Solubilization, Extraction, and Immunoblotting

Testis microsome

Mouse testes were homogenized in 0.32M sucrose and the homogenates were centrifuged at 4 °C, 1,000 x g for 10 minutes to remove cell debris and nucleus. Supernatant were collected and centrifuged at 100,000 rpm for 60 minutes at 4°C to separate cytosolic (cyto) and microsome (mic) fractions in supernatant and pellet, respectively. Microsome proteins were solubilized in PBS containing 1% Triton X-100 and cOmplete Mini, EDTA-free Protease Inhibitor Cocktail (Roche) for 2 hours at 4°C with gentle rocking. The microsome lysates were centrifuged at 18,000 x g for 1 hour at 4 °C to obtain soluble (supernatant) and insoluble (pellet) fractions. The insoluble microsome fractions were further lysed with 2X LDS sampling buffer by vortexing for 10 minutes at RT followed by centrifugation at 18,000 x g for 30 minutes at 4 °C. Cytosolic and solubilized microsome proteins mixed to 1X LDS buffer and insoluble microsome proteins in 2X LDS buffer were volume-equivalented and denatured by boiling at 75°C with 50 μM at dithiothreitol (DTT) for SDS-PAGE and immunoblotting to examine the protein partitioning. Primary antibodies used for the immunoblotting were: rabbit polyclonal anti-mouse CatSper τ (α -CS τ -359 and α -CS τ -482, 1 μg/ml each) and mouse monoclonal anti-Na⁺/K⁺ ATPase (1:500; SantaCruz) and anti-acetylated tubulin (1:2,000; clone 6-11B-1, Sigma Aldrich). Goat anti-mouse or rabbit IgG conjugated with HRP were used for secondary antibodies (1:10,000; Jackson ImmunoResearch). Solubilized microsome fractions were also used for coIP.

Testicular germ cells

Round spermatids enriched by STA-PUT were lysed with 1% Triton X-100 in PBS supplemented with EDTA-free protease inhibitor cocktail (Roche) by gentle rocking at 4°C for 2 hours. The lysates were centrifuged at 18,000 x g for 30 minutes at 4°C and solubilized proteins in supernatant were collected. Solubilized proteins were subjected to coIP.

Epididymal sperm cells

Whole sperm proteins were extracted as previously described (Hwang et al., 2019). Collected epididymal sperm from corpus and cauda were washed with PBS and lysed with 2X LDS by vortexing for 10 minutes at RT. The lysates were centrifuged at 4°C, 18,000 x g for 10 minutes. The supernatants were mixed to 50 μM DTT and denatured by boiling at 75°C for 10 minutes. Denatured sperm proteins were subjected to SDS-PAGE and immunoblotted. The used primary antibodies were: Rabbit polyclonal anti-mouse CatSper1, CatSper2, CatSper3, CatSper4, CatSper β , CatSper δ , CatSper τ , and EFCAB9 at 1 μg/ml, mouse CatSper ζ (2.7 μg/ml), and rabbit monoclonal anti-phospho-PKA substrate (clone 100G7E, CST, 1:1,000) and mouse monoclonal anti-phosphotyrosine (1 μg/ml; clone 4G10, Sigma Aldrich) and acetylated tubulin (1:20,000; clone 6-11B-1, Sigma Aldrich). HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were used for secondary antibodies (1:10,000; Jackson ImmunoResearch).

Co-immunoprecipitation

Solubilized testis microsome and round spermatids were subjected to coIP. Solubilized proteins in lysis buffer were mixed with SureBeads™ Protein A Magnetic Beads (BioRad) conjugated with either 1 µg of rabbit polyclonal anti-CatSper1, CatSperδ, or CatSperτ (α-CSτ-482), or 0.5 µl of rabbit monoclonal anti-DYKDDDDK (clone D6W5B, CST). After incubation for overnight at 4°C, the resins were washed with lysis buffer and eluted immunocomplexes were eluted with 2X LDS buffer supplemented with 50 µM DTT by boiling at 75°C for 10 minutes. The elutes were subjected to SDS-PAGE and immunoblotting. Primary antibodies for immunoblotting were: rabbit polyclonal anti-mouse CatSper1, CatSper2, CatSper3, CatSperβ, CatSperδ, CatSperε, and CatSperτ at 1 µg/ml, and rabbit monoclonal anti-DYKDDDDK (1:2,000; clone D6W5B, CST). For secondary antibodies, VeriBlot (1:200-1:500; Abcam) and HRP-conjugated goat anti-mouse or rabbit IgG (1:10,000; Jackson ImmunoResearch) were used.

IP Mass Spectrometry and Proteomics Analyses

Sample preparation

Testis microsome of WT and *CatSper*^{Δ/Δ} males were prepared and solubilized as described above. Lysates were centrifuged at 18,000 x g for 1 hour at 4°C and supernatants were mixed with Protein A/G PLUS-Agarose (SantaCruz) and incubated for 1 hour at RT to remove mouse immunoglobulin. Flow through were collected followed by incubation with anti-CatSperτ (α-CSτ-359; WT and *CatSper*^{Δ/Δ}) or normal rabbit IgG (R&D SYSTEMS; WT) crosslinked to Protein A/G PLUS-Agarose (SantaCruz) using 20 mM dimethyl pimelimidate (Sigma Aldrich) at 4°C for overnight. The resins were washed with 1% Triton X-100 in PBS and eluted two times by incubation with 0.1M glycine, pH2.3 at RT for 5 minutes. Elutes from five repeats were pulled and incubated with 4-volumes of acetone at – 20°C for overnight to precipitate proteins. The mixture was centrifuged at 18,000 x g for 1 hour at 4°C followed elution with 8M urea, pH7.4. The elutes in 1X LDS with 50 mM DTT were boiled at 75°C for 5 minutes and subjected to SDS-PAGE. The gels were run shortly and stained using Imperial™ Protein Stain (ThermoFisher). Stained bands were cut subjected to LC-MS/MS

Protein LC-MS/MS

The gel pieces were washed and dehydrated with acetonitrile for 10 minutes. The gel pieces were dried and subjected to trypsin-digestion for overnight at 37°C. Digested peptides were reverse-phase eluted (Peng and Gygi, 2001) and subjected to mass spectrometric analysis using an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (ThermoFisher). Peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of each peptide, which were matched with protein database using Sequest (ThermoFisher) to identify peptide sequences. Data was filtered to between a one and two percent peptide false discovery rate. Identified CatSperτ-interactomes were subjected to pathway analysis and functional annotation by using Ingenuity Pathway Analysis (QIAGEN) and STRING Version 11 (<https://string-db.org>; Szklarczyk et al., 2019).

Enriched gene ontologies were functionally categorized by using REVIGO (<http://revigo.irb.hr>; Supek et al., 2011) and visualized by using R software.

Immunocytochemistry

Collected epididymal sperm, testicular cells, and cultured cells were subjected to immunocytochemistry. Epididymal sperm were washed with PBS and attached to glass coverslip by centrifugation at 700 x g for 5 minutes. The attached sperm cells were fixed with 4% PFA in PBS for 10 minutes at RT and washed with PBS three times. Dissociated testicular cells in PBS were fixed with PFA in 4% concentration for 5 minutes followed by centrifugation at 250 x g for 3 minutes to attach to coverslips coated with poly-D-lysine (Sigma Aldrich). Testicular cells were air-dried shortly. Cultured cells on glass coverslip coated with poly-D-lysine were washed with PBS and fixed with 4% PFA in PBS for 10 minutes at RT. The fixed epididymal sperm, testicular cells, and cultured cells on glass coverslips were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at RT and blocked with 10% normal goat serum in PBS for 1 hour at RT. Blocked samples were incubated with primary antibodies at 4°C for overnight. Primary antibodies used for the immunocytochemistry were: rabbit polyclonal anti-CatSper1 (10 µg/ml), CatSperδ (10 µg/ml), CatSperτ (α-CSτ-359, 20 µg/ml; α-CSτ-482, 10 µg/ml), and ARL13B (1:200, Proteintech), rabbit monoclonal anti-DYKDDDDK (1:200; clone D6W5B, CST), and mouse monoclonal anti-pTyr (1:100, clone 4G10, Sigma Aldrich), anti-acetylated tubulin (1:200, clone 6-11B-1, Sigma Aldrich), and anti-CENTRIN1 (1:100, clone 20H5, Sigma Aldrich). Immunostained samples were washed with 0.1% Triton X-100 in PBS one time and PBS two times followed by incubation with goat anti-rabbit or mouse IgG conjugated with Alexa 488 or Alexa 568 (1:1,000, Invitrogen) in blocking solution for 1 hour. After incubation with secondary antibodies, coverslips were mounted with Vectashield (Vector Laboratory) and imaged with Zeiss LSM710 Elyra P1 using Plan-Apochromat 63X/1.40 and alpha Plan-APO 100X/1.46 oil objective lens (Carl Zeiss). Hoechst (Invitrogen) were used for counter staining.

Structured Illumination Microscopy

Immunostained mouse epididymal sperm and elongated spermatids (step 13-16) in dissociated testicular cells were prepared as described in *Immunocytochemistry*. 3D structural illumination microscopy (SIM) imaging was performed with Zeiss LSM710 Elyra P1 using alpha Plan-APO 100X/1.46 oil objective lens (Carl Zeiss). Z-stack images was acquired with 100 or 200 nm intervals and each section was taken using images were taken using 5 grid rotations with a 51 nm SIM grating period and a laser at 561 nm wavelength. Raw images were processed and rendered using Zen 2012 SP2 software (Carl Zeiss).

Mating Test and *In Vitro* Fertilization

Adult female mice with normal fertility were housed with heterozygous or homozygous *CatSpert* mutant or knockout males over two months, and the pregnancies and litter sizes were recorded.

For IVF, Cauda epididymal spermatozoa from WT or knockout males were released into the TYH medium drops at a concentration of 2.0×10^5 cells/ml. After 2 hours of incubation at 37°C, 5% CO₂ condition, the capacitated spermatozoa were transferred to new TYH medium drops containing cumulus-oocyte complexes (COCs) obtained from super-ovulated B6D2F1 females at a final sperm concentration of 2.0×10^5 cells/ml. After 6 hours of incubation, the COCs were treated with hyaluronidase and washed in clean TYH drops repeatedly to denude the cumulus cells. To determine the fertilization success, abnormal or parthenogenic eggs were excluded and the ones bearing two pronuclei were counted as fertilized eggs.

Histology Analysis

Testes from WT and *CatSpert*-knockout males were fixed in Bouin's fluid (Polysciences), dehydrated in ethanol, embedded in paraffin wax, and sectioned at a thickness of 5 µm on an HM325 microtome (Microm). The paraffin sections were adhered to microscope slides, rehydrated with graded concentrations of ethanol, stained with 1% periodic acid (Nacalai) and Schiff's reagent (Wako), and counter-stained with Mayer's hematoxylin solution (Wako). After dehydration with graded concentrations of ethanol and xylene, the microscope slides were mounted with Entellan® new (Sigma Aldrich) mounting medium and observed under an Olympus BX-53 microscope.

Sperm Motility Analysis

Flagellar Waveform Analysis

Sperm flagellar movement were analyzed as previous studies (Hwang et al., 2019; Hwang et al., 2021). In brief, non-capacitated or capacitated cauda epididymal sperm (2×10^5 cells/ml) were placed into fibronectin-coated imaging chamber for Delta-T culture dish controller (Bioptech) filled with 37°C HEPES-buffered HTF medium (Chung et al., 2017). Flagellar movements of the sperm cells of which heads were tethered on the plate were recorded for 2 seconds with 200 frame per seconds (fps) speed using pco.edge sCMOS camera equipped in Axio observer Z1 microscope (Carl Zeiss). Original image stacks were rendered to movies. Beating frequency and α -angle of sperm tail were measured and overlaid-images of sperm flagellar waveform were generated by using FIJI software (Schindelin et al., 2012).

Computer-Assisted Sperm Analysis

Non-capacitated and capacitated sperm cells (3.0×10^6 cells/ml) were loaded in 20 µm-depth of slide chamber (CellVision) and sperm motility was examined on 37°C warm-stage. Motile sperm were imaged with Nikon E200 microscope under 10x phase contrast objective (CFI Plan Achro

10X/0.25 Ph1 BM, Nikon) and recorded at 50 fps speed using CMOS video camera (Basler acA1300-200µm, Basler AG). The recorded movies were analyzed by Sperm Class Analyzer software (Microptic). Over 200 total sperm were imaged for each group.

Ca²⁺ handling Assay

Intracellular Ca²⁺-dependent sperm motility changes (Ca²⁺ handling assay) were examined as performed previous study (Hwang et al., 2019). Briefly, epididymal sperm from WT, *CatSperd*-null and *CatSper*^{ΔΔ} sperm in M2 medium (3.0 x 10⁶ cells) were loaded with 5 µM BAPTA-AM (bis-(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester, Calbiochem) or vehicle (0.05% DMSO and 0.01% F-127) and incubated for 90 minutes at 37 °C. After incubation, sperm cells were washed with HS medium by centrifugation two times at 700 x g for 2 minutes to remove BAPTA-AM and eluted with HTF medium (EMD Millipore). Eluted sperm in HTF was incubated at 37°C, 5% CO₂ condition for 90 minutes to restore intracellular Ca²⁺ level via activated CatSper channel by inducing capacitation. Motilities of over 200 sperm cells were imaged and analyzed at each time point as described in *Computer-Assisted Sperm Analysis*.

Electrophysiology

Corpus epididymal sperm were washed and resuspended in HS medium followed by attached on the 35mm culture dish. Gigaohm seals were formed at the cytoplasmic droplet of sperm (Kirichok et al., 2006). Cells were broken in by applying voltage pulses (450-600 mV, 5 ms) and simultaneous suction. Whole-cell CatSper currents were recorded from WT, *CatSperd*^{-/-}, and *CatSper*^{ΔΔ} sperm in divalent-free bath solution (DVF, 150 mM NaCl, 20 mM HEPES, and 5 EDTA, pH 7.4). Intrapipette solution for CatSper current recording consists of 135 mM CsMes, 10 mM HEPES, 10 mM EGTA, and 5 mM CsCl adjusted to pH 7.2 with CsOH. To induce intracellular alkalinization, 10 mM NH₄Cl was added to bath solution by perfusion system. Data were sampled at 10 Hz and filtered at 1 kHz. The current data were analyzed with Clampfit (Axon, Gilze, Netherlands), and figures were plotted with Grapher 8 software (Golden Software, Inc., Golden, Colorado).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were carried out using Student's t-test. Differences were considered significant at *p<0.05, **p<0.01, and ***p<0.001.

DATA AND SOFTWARE AVAILABILITY

The raw images of immunoblotting in this study are deposited in Mendeley Data at <http://dx.doi.org/10.17632/mw3wpnjpyt.1>. All software used in this study is listed in the [Key Resource Table](#).

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1013 **SUPPLEMENTAL INFORMATION**

1014 Supplemental Information includes seven figures, two tables, and a movie can be found with
1015 this article online at xxx.

1016 **Supplementary Figures and Legends**

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Figure S1. Generation of *CatSper* deletion mutant and knockout mice, related to Figures 1 and 2 (A) Genome structure of mouse *C2CD6* gene encoding *CatSper* τ . Represented are two variants (NM_026600 and NM_175200) that encode the long (*CatSper* τ^L , NP_080876) and short (*CatSper* τ^S , NP_780409) isoforms, respectively. Marked in red (coding region) and gray (UTR) boxes are the exons in scale. (B-C) Generation of *CatSper* deletion mutant (Δ/Δ) and knockout (-/-) mice using CRISPR/Cas9 genome editing. (B) Mutant alleles with 134 bp (*CatSper*-134del) and 128 bp (*CatSper*-128del) deletion in the *CatSper* τ coding region. The deletions are expected to cause out-of-frame mutations, resulting in early termination of protein translation from the start codon. (C) Knockout allele with 43 kb deletion at genomic region encoding *CatSper* τ . (D-E) Genomic DNA PCR for genotyping of *CatSper*-mutant (D) and knockout (E) mice with the primer pairs as marked in panels B and C. (F) Alignment of the predicted amino acid sequences translated from WT and the two mutant alleles in B. Amino acids in red indicate non-native sequences from the frameshift before termination. (G) Detection of truncated *CatSper* τ protein in *CatSper*-134del $^{\Delta/\Delta}$ testes. Testis homogenates from WT, *CatSper*-134del $^{\Delta/\Delta}$, and *CatSper* knockout mice were separated into cytoplasm (Cyt) and microsome (Mic) fractions and subjected to immunoblotting. (H-J) Detection of truncated *CatSper* mRNA in *CatSper*-134del $^{\Delta/\Delta}$ testes. (H) Exon composition of the *CatSper* transcript (NM_175200). Expected PCR product sizes for each primer pair are marked. A red box indicates the deleted coding region in *CatSper*-134del $^{\Delta/\Delta}$ mouse genome. (I) Real-time RT-PCR and (J) end-point RT-PCR analyses of *CatSper* mRNA expression in *CatSper*-134del $^{\Delta/\Delta}$ testes. (K) Diagrams of the truncated *CatSper* transcripts. Sanger sequencing of the F4-R4 PCR product reveals 134-bp (*middle*) or 186-bp (*bottom*) deleted *CatSper* transcripts in *CatSper*-134del $^{\Delta/\Delta}$ testes. (K) Sequence alignment of the WT and predicted mutant *CatSper* τ sequences translated from the open reading frames of the truncated *CatSper* transcripts. Red characters are the non-native amino acid sequences by deletion and frameshift. The sequence corresponding to C2 domain is yellow-boxed.

Figure S2. Characterization of *CatSper* mutant and knockout mice, related to Figure 2 (A-B) Absence of the truncated *CatSper* τ in *CatSper*^{-128del} ^{Δ/Δ} sperm by immunostaining (A) and immunoblotting (B). As *CatSper*^{-134del} ^{Δ/Δ} and *CatSper*^{-128del} ^{Δ/Δ} show identical phenotypes

from initial characterization, *CatSpert-134del*^{Δ/Δ} mice were used as *CatSpert*^{Δ/Δ} throughout this study unless indicated. (C) Litter sizes from WT, heterozygous, and homozygous females mated with heterozygous and homozygous *CatSpert*-mutant (+/Δ, 8.1 ± 0.3; Δ/Δ, 0.0 ± 0.0) and knockout (+/-, 10.0 ± 0.4; -/-, 0.0 ± 0.0) males. Females are all fertile as shown. Litters from the females mated with *CatSpert-134del* (circles; +/Δ, N=6, litter number=12; Δ/Δ, N=4, litter number=0) and *128del* (triangles; +/Δ, N=3, litter number=7; Δ/Δ, N=3, litter number=0) males are shown together. (D) Sperm counts from the epididymis of heterozygous (2.13 ± 0.17 cells) and homozygous (2.11 ± 0.09 cells) *CatSpert*-mutant males. Sperm numbers from *CatSpert-134del* (circles; +/Δ, N=5; Δ/Δ, N=5) and *128del* (triangles; +/Δ, N=3; Δ/Δ, N=3) are represented together. (E) Histology of *CatSpert* knockout testis. PAS-stained *CatSpert*^{+/-} (left) and *CatSpert*^{-/-} (right) testes sections are shown. (F) Computer Assisted Sperm Analysis (CASA) to measure sperm motility parameters from *CatSpert*^{+/-} (gray bars) and *CatSpert*^{Δ/Δ} (red bars) males. The motility parameters were measured from sperm cells before (0 min) and after (90 min) inducing capacitation. VCL, curvilinear velocity; ALH, amplitude of lateral head; VAP, average path velocity; VSL, straight line velocity; BCF, beat cross frequency. **p*<0.05 and ***p*<0.01. N=3. (G) Flagella waveforms of sperm from *CatSpert-128del*^{+/-} (top) and *CatSpert-128del*^{Δ/Δ} (bottom) males. Sperm tail movements were recorded before (0 min, left) and after (90 min, right) capacitation. (H) Tail beating frequency of *CatSpert*^{+/-} (gray bars; 0 min, 11.26 ± 0.38 Hz; 90 min, 6.11 ± 0.72 Hz) and *CatSpert*^{Δ/Δ} (red bars; 0 min, 20.97 ± 2.28 Hz; 90 min, 26.13 ± 4.96 Hz) sperm. Circles indicate the tail beating frequency of individual sperm cells. N=45 from three animals for each group. ****p*<0.001. (I) Immunoblotting of phosphorylated PKA substrates from *CatSpert*-mutant sperm cells. The extent of phosphorylation was compared between *CatSpert*^{+/-} and *CatSpert*^{Δ/Δ} sperm incubated under capacitating conditions (Cap) at the indicated times. Data is represented as mean ± SEM (C, D, F, and H).

Figure S4. Marginal effects to CatSper channel complex assembly by CatSper ϵ malfunction, related to Figure 4 (A-B) Co-immunoprecipitation of CatSper complex with α -CatSper1 (A) and α -CatSper δ (B) in solubilized microsomes of $CatSper^{\Delta/\Delta}$ and $CatSper^{\delta-/-}$ testis. WT and $CatSper1$ - (A) or $CatSper\delta$ -null (B) testes were used for positive and negative control, respectively. (C-D) Curvilinear velocity (VCL) changes by handling intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in WT, $CatSper^{\Delta/\Delta}$, and $CatSper^{\delta-/-}$ spermatozoa. (C) VCL values of WT (black), $CatSper^{\Delta/\Delta}$ (red), and $CatSper^{\delta-/-}$ (blue) sperm were measured during pre-incubation with vehicle (DMSO, left) or intracellular Ca^{2+} chelator (BAPTA-AM, right) in M2 and incubation in HTF medium. Different letters indicate significant differences in pairwise comparison between genotypes at each time point. (D) Relative VCL changes by $[Ca^{2+}]_i$ chelation (gray) and recovery (red). VCL values at the 90 minutes points from $[Ca^{2+}]_i$ chelation and recovery were normalized by the average VCL values before incubation with BAPTA-AM (before 0 min, M2) in each genotype. Statistical analyses were conducted by comparing relative VCL values after $[Ca^{2+}]_i$ chelation and recovery within each genotype. ** $p < 0.01$. Data is represented as mean $\pm$ SEM (C and D). N=3.

Figure S5. Targeting of CatSper proteins to the flagella and nanodomains in developing spermatids, related to Figure 5 (A-B) Confocal images of immunostained CatSperδ in WT (A) and *CatSper*^{Δ/Δ} (B) spermatids. Fluorescence (*top*) and corresponding DIC (*bottom*) images and shown. (C) Confocal images of testicular spermatids from *CatSper1*^{-/-} (*left*) and *CatSperδ*^{-/-} (*right*) males stained with α-CatSper1 (magenta) and α-CatSperδ (yellow), respectively. CatSperδ signal detected from the cytoplasm (steps 6-14), near annulus (steps 13-14), and mid-pieces (steps 15-

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1117 16) of *CatSperd*^{-/-} spermatids are considered non-specific. Corresponding DIC images are shown
 1118 (*bottom*). (D) Confocal images of CatSper τ -stained spermatids from *CatSper1*^{-/-} males.
 1119 Corresponding DIC images of the immunostained *CatSper1*^{-/-} spermatids are shown (*bottom*).
 1120 Insets are magnified (scale bar = 2 μ m). Arrows and arrowheads indicate the elongating flagella
 1121 of steps 6-8 spermatids and the annulus, respectively (A, B, C, and D). Hoechst were used for
 1122 counter staining (A, B, C, and D).

Figure S6. Capability of CatSper τ to traffic to the flagellum and organize quadrilinear nanodomains in developing spermatids, related to Figure 6 and 7 (A) 3D SIM images of CatSper subunits in WT spermatids at developmental steps 15-16. Shown are the images of immunostained CatSper τ (*top*), CatSper1 (*middle*), and CatSper δ (*bottom*) color-coded in z-depth from focal plane. (B) 3D SIM images of CatSper τ in *CatSper1*^{-/-} spermatids at steps 13-14. x-y projection is color-coded to represent z-depth from focal plane (*left*). y-z cross section is shown (*right*). (C) Co-immunoprecipitation of CatSper1 in *CatSperd*-null testis. None of the examined CatSper pore subunits (CatSper2 and 3) are detected from the CatSper1-immunocomplex in *CatSperd*^{-/-} testis (*d*^{-/-}). WT and *CatSper1*-null (*1*^{-/-}) testes were used for positive and negative control, respectively. (D) Confocal images of CatSper1 in developing *CatSperd*-null spermatids. CatSper1 is not detected in the elongating tails of *CatSperd*-null spermatids at all steps examined. Corresponding DIC images are shown (*right*). (E) Summary of the flagellar targeting and quadrilateral distribution of the CatSper proteins in male germ cells. Lines and colors in each cell depict arrangement and distribution of the CatSper proteins, respectively, in germ cells from each genotype. (F-G) Intracellular localization of the recombinant WT and the C2-domain truncated CatSper τ in ciliated hTERT-RPE (F) and 293T (G) cells. EGFP-tagged short forms of WT CatSper τ (CS τ ^{S:WT}-EGFP, *left*) and the predicted C2-truncated mutant CatSper τ (CS τ ^{S:Mut}-EGFP, *right*; Figure S1) were stably expressed in hTERT-RPE (F) and 293T (G) cells using lentiviral system. Immunostained acetylated tubulin (AcTub, F) and ARL13B (G) represent cilia (magenta). Enlarged insets are shown (*bottom*). An arrow (D) and arrowheads (A, B, and D) represent flagella in step 6-8 spermatids and annulus, respectively. Hoechst were used for counter staining (D, F, and G).

Figure S7. CatSper τ is in complex with molecular motors and proteins in cytoplasmic vesicles, related to Figure 7 (A-C) Intracellular localization of recombinant CatSper τ in 293T cells. Epifluorescence (A) and confocal (B and C) images of short forms of WT (CatSper τ -S:WT-EGFP; A, top; B and C, left) and mutant (CatSper τ -S:Mut-EGFP; A, bottom; B and C, right) CatSper τ expressed in 293T cells by lentiviral transduction (see also Figure 7A). EGFP is tagged at C-termini of the recombinant proteins. Immunostained acetylated tubulin (B, AcTub) and CENTRIN1 (C) show centrosomal localization of WT CatSper τ . Scale bars in magnified insets are 10 μ m (A)

or 2 μ m (B and C). (D) Confocal images of immunostained CatSper δ in 293T cells expressing CatSper τ . FLAG-tagged CatSper δ (CatSper δ -FLAG) were transiently expressed in 293T cells that stably express WT (CatSper τ ^{S:WT}-EGFP) or mutant (CatSper τ ^{S:Mut}-EGFP) CatSper τ . 293T cells that are not transduced were used as negative control. Intracellular localization of CatSper δ is not changed by neither WT nor mutant CatSper τ in 293T cells. Magnified insets are shown (A, B, C, and D). Hoechst was used for counter staining (B, C, and D). (E-F) Number and proportion of the identified proteins from CatSper τ co-immunoprecipitation (coIP) mass spectrometry. (E) Venn diagram of total 811 proteins identified from three conditions of coIP mass spectrometry: coIP using α -CatSper τ -359 in WT (red, n=704) and CatSper τ ^{Δ/Δ} (black, n=706) testis, and coIP using rabbit normal IgG in WT testis (gray, N=193). (F) 768 proteins enriched more than 2-fold in the CatSper τ immunocomplex (white) compared to that from normal IgG (black) from WT testis (see also [Figure 7E](#)). Among them, 118 proteins including CatSper τ (blue) that showed more than 2.7-fold in WT over CatSper τ ^{Δ/Δ} were considered to CatSper τ -interactome, which were subjected to pathway analysis and functional annotation. (G-I) Enriched pathways (G) and gene ontologies (H and I) of the CatSper τ -interactome in testis. Canonical pathways (dark gray) and diseases or functions (light gray) from Ingenuity Pathway Analysis are shown (G). Representative biological process (blue), molecular function (red), and cellular component (purple) gene ontologies from functional annotation are listed (H). X-axis indicates negative logarithm of p value (G) or false discovery rate (FDR, H). (I) Molecular function (*top*) and cellular component (*bottom*) gene ontologies were functionally categorized. Enriched ontologies were represented as bubbles in scatter plot. Sizes and colors of the bubbles indicate percentage of proteins annotated with the ontologies and FDR, respectively.

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- 1177 **Video S1. Flagellar movement of tethered *CatSper*^{+/Δ} and *CatSper*^{Δ/Δ} sperm, related to**
 1178 **Figure 2** Uncapacitated (*top*, 0 min) and capacitated (*bottom*, 90 min) epididymal sperm from
 1179 *CatSper*^{+/Δ} (*left*) and *CatSper*^{Δ/Δ} (*right*) mice were tethered to imaging chamber and flagellar
 1180 movement was recorded at 37 °C. Videos are played at 100 fps (1/2 speed).
- 1181 **Table S1. Identified proteins from *CatSper* interactome in testis by immunoprecipitation**
 1182 **mass spectrometry, Related to Figure 7.**
- 1183 **Table S2. Lists of the enriched pathways and gene ontologies of *CatSper* interacting**
 1184 **proteins, Related to Figure 7.**

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