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Dissecting the signaling pathways involved in the function of sperm flagellum

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Abstract

The mammalian flagellum is a specific type of motile cilium required for sperm motility and male fertility. Effective flagellar movement is dependent on axonemal function, which in turn relies on proper ion homeostasis within the flagellar compartment. This ion homeostasis is maintained by the concerted function of ion channels and transporters that initiate signal transduction pathways resulting in motility changes. Advances in electrophysiology and super-resolution microscopy have helped to identify and characterize new regulatory modalities of the mammalian flagellum. Here, we discuss what is currently known about the regulation of flagellar ion channels and transporters that maintain sodium, potassium, calcium, and proton homeostasis. Identification of new regulatory elements and their specific roles in sperm motility is imperative for improving diagnostics of male infertility.

Keywords

Sperm ion channels; CatSper; Hv1; Slo3; Slo1; Capacitation; Fertility; Motility; Progesterone; pH; Flagellum; EFCAB9

Introduction

The tail of mammalian sperm cells is represented by a single motile cilium known as the flagellum that generates its movement to propel the cell through the female reproductive tract and deliver paternal genetic material into an egg. Sperm cells rely on vigorous motility that is initiated once they are released from the seminal plasma coagulum. The motility initiation is partially driven by an increase in the intraflagellar pH caused by ion channels and transporters that move protons out of and bicarbonate into the cell. Intracellular alkalization is also an important part of a set of molecular changes known as ‘capacitation’ or final sperm maturation that sperm cells undergo in the female reproductive tract making them competent to reach and fertilize the egg [1,2]. Capacitation includes the alteration of membrane fluidity due to cholesterol removal, the change of intracellular pH, protein

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Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: PVL has a financial interest in YourChoice Therapeutics, Inc.

tyrosine phosphorylation, induction of 'hyperactivated' asymmetrical motility, and the acquisition of the ability to perform the acrosome reaction [3,4]. The former event, hyperactivation, is defined by a powerful asymmetrical flagellar bending mode, which allows sperm cell to penetrate the egg's protective vestments. The latter, acrosome reaction [5], is an exocytotic event that takes place in the sperm head during fertilization. Specifically, an acrosome is a vesicle located in the anterior segments of the sperm head that contains hyaluronidase and other hydrolytic enzymes required for sperm penetration through zona pellucida, a protective layer of the egg.

All these physiological processes are possible because of the finely orchestrated signaling pathways between cytoskeletal protein complexes and plasma membrane elements, such as ion channels and transporters [6]. Dysfunction of these proteins or their regulation can cause defects in ciliary motility, which can lead to infertility [7,8]. In this review, we summarize recent advances in the function of ion channels and transporters and their corresponding signaling pathways in the mammalian sperm flagellum.

Bicarbonate signaling/HCO_3^- transporters

The uterus and oviduct provide an alkaline environment of pH ~7.9 and a very high concentration of bicarbonate (~50 mM, as measured in rabbit female reproductive tract) [9]. High concentration of bicarbonate is crucial for capacitation. Specifically, HCO_3^- directly activates the atypical bicarbonate-dependent soluble adenylyl cyclase (sAC) [10] that generates cAMP required for activation of cAMP-dependent protein kinase A (PKA; Figure 1a and b). PKA signals downstream by activating tyrosine kinases [11] that phosphorylate various protein targets. Bicarbonate elevation inside sperm cells can be achieved in several ways: via CO_2 diffusion through the membrane, because of its conversion into bicarbonate by carbonic anhydrases [12], and via a subset of Cl^-/HCO_3^- exchangers, such as SLC26A3 and SLC26A6 [13]. These exchangers can directly shuttle bicarbonate between the extracellular and intracellular environments [13]. In mice and humans, the cystic fibrosis transmembrane conductance regulator channel (CFTR) works in association with these transporters, to provide a sustained regulation of Cl^- transport (Figure 1a and b). As reported in Refs. [14,•15], mutation or deficiency in SLC26A3 gene is associated with impaired sperm motility in humans and mice. Interestingly, a new member of this family, SLC22A14, has also been shown to be required for male fertility in mice [16]. Additionally, the bicarbonate can be imported inside the murine sperm via the Na^+/HCO_3^- transport mechanism [17]; however, the molecular identity of this cotransporter is yet to be revealed. Overall, the HCO_3^-/sAC/cAMP/PKA signaling pathway is essential for the process of capacitation and male fertility, as demonstrated by sAC-deficient mice [18,19] and mice lacking catalytic subunit Cβ2 of sperm-specific PKA [20].

Additional hallmark of capacitation is cholesterol removal. Cholesterol is in general an essential stabilizing component of the mammalian plasma membrane. Moreover, in sperm cells its removal from the flagellar membrane is a key part of the sperm maturation process inside the female reproductive tract which is enriched in albumin and bicarbonate [9,21]. It is known that albumin sequesters cholesterol from plasma membrane (Figure 1a and b) only

in the presence of bicarbonate; however, the molecular mechanism of such cholesterol sequestration is still poorly understood [22]. Cholesterol removal is associated with changes in sperm plasma membrane fluidity, the activation of a signal transduction pathway involving protein kinase A and tyrosine kinase and resulting in protein tyrosine phosphorylation and capacitation [23].

Na^+/H^+ exchangers

Sperm intraflagellar pH is regulated by a concerted action of ion channels and transporters. Na^+/H^+ (NHE) exchangers, such as SLC9B1 and SLC9B2, export protons from the cell in exchange for sodium and are required for sperm motility and male fertility in mice [24]. Another member of the same family, SLC9C1, also known as sperm-specific Na^+/H^+ exchanger (sNHE), has been proposed to play a role in pH regulation [25]. *SLC9C1*-deficient male mice are infertile, but their infertility is partially rescued by the addition of ammonium chloride, which increased the intracellular pH. Additionally, their infertility was completely rescued by the addition of cell-permeable cAMP [25]. As has been reported, *SLC9C1*-deficient male infertility results not from the absence of sNHE, but from inactive sAC that likely forms a complex with sNHE (Figure 1a) [26]. Interestingly, sea urchin sNHE has been shown to function as a genuine Na^+/H^+ exchanger gated by voltage and further modulated by cAMP [•27]. Further studies are needed to clarify sNHE's function in mammalian male fertility, particularly in humans.

H^+ channel

Proton efflux by ion channels is another efficient mechanism for intracellular pH regulation. The first proton voltage-gated ion channel, Hv1, was identified in 2006 [28,29]. Hv1 is expressed in human (and not mouse) spermatozoa [30], and a subset of these channels [•31] are positioned near the pH-dependent, Ca^{2+}-permeable sperm-specific cation channel of sperm (CatSper) [32]. Specifically, the Hv1 channel is distributed asymmetrically to one side of the flagellar midline and according to the model [•31], it can activate only a subset of CatSper channels, resulting in asymmetrical local Ca^{2+} influx and perhaps flagellar rotation. A well-recognized feature of the native and recombinant Hv1 channel is its voltage-gating dependency on transmembrane pH-gradient [28,29]. Additionally, Hv1 can be further upregulated by fatty acids [33] and the endogenous cannabinoid anandamide [30], which is synthesized by the cumulus oophorus [34] (Figure 1b). In addition to the full-length Hv1, human sperm cells contain its N-terminally cleaved isoform (Hv1Sper), which has slightly different voltage-gating properties [35]; however, the precise function of Hv1 in human sperm motility will be elucidated only after a male infertile patient associated with mutation in *Hvcn1* (the gene that encodes Hv1) is identified.

Ca^{2+} ion channel/CatSper

While ascending the female reproductive tract, the intracellular Ca^{2+} concentration of sperm raises through the activation of CatSper, which acts as the main flagellar calcium gate. CatSper is located in the principal piece of sperm flagellum [32] and is regulated by several endogenous stimuli, such as alkaline pH in rodents [32,36] and primates [30,37], as well as by progesterone (P4) and prostaglandins [37,38] produced by the cumulus oophorus in

primates (Figure 1a and b). In human spermatozoa, CatSper activation by P4 occurs through P4 binding to the membrane receptor α/β hydrolase domain-containing protein 2 (ABHD2) [39]. ABHD2 is a lipid hydrolase that upon P4 binding hydrolyzes the endocannabinoid 2-arachidonoylglycerol (2-AG), removing it from the plasma membrane (Figure 1b). The 2-AG inhibits CatSper, and its removal leads to CatSper activation, followed by Ca^{2+} influx [39].

One of the downstream effects of P4-induced Ca^{2+} influx is the powerful asymmetrical flagellar bending mode known as hyperactivation. The precise mechanism of how intraflagellar calcium evaluation triggers hyperactivation by affecting dynein motility is not yet known; however, it has been shown recently that at high Ca^{2+} concentrations, Ca^{2+} binding protein EFCAB1 directly suppresses the velocity of microtubule sliding by outer-arm dynein and thus induces asymmetric flagellar bending in *Ciona intestinalis* sperm [40]. EFCAB1-deficient mice, however, are fertile, and their sperm have normal hyperactivated motility, which indicates that at least for mice, EFCAB1 is not required for hyperactivation [41].

In addition to conducting Ca^{2+}, CatSper is also positively regulated by cytoplasmic Ca^{2+} [••42]. The CatSper channel is a complex of at least 10 different subunits—CatSper 1 through 4 are pore-forming and CatSper beta, gamma, delta, epsilon, and zeta [••43] are auxiliary subunits. In addition, a novel member of the CatSper complex has been recently revealed: EF-hand calcium-binding domain-containing protein 9 or EFCAB9 [••42] (Figure 1a), which is a calmodulin-like protein that binds Ca^{2+} and acts as a dual calcium and pH sensor for CatSper. EFCAB9 exists as a complex with CatSper-zeta, and male mice with either CatSper-zeta or EFCAB9 deficiencies, or lacking both subunits, are subfertile [••42,••43]. EFCAB9—CatSper-zeta interaction is pH-dependent, and their complex breaks down at alkaline pH, thus allowing more efficient CatSper opening [••42]. The dissociation between EFCAB9 and CatSper-zeta likely increases the open probability of the channel. Interestingly, EFCAB9 also serves as an important structural element for the murine CatSper complex, as its absence results in a different organization of the CatSper nanodomains along the flagellum. Future studies will be needed to confirm functional importance of EFCAB9 for human sperm. Advances in super-resolution imaging revealed that CatSper is arranged in quadrilateral parallel lines along the sperm tail [44] (Figure 2a) by forming four lines that span the entire length of sperm principal piece (Figure 2a—b). CatSper channels have also been suggested to form a zig-zag pattern by linking two channel complexes within each line of CatSper in the flagellum [45] (Figure 2c). Indeed, it was further revealed that each of those four lines exists as two-row structures of CatSper nanodomains, and both EFCAB9 and CatSper-zeta are essential for this two-row formation. *EFCAB9*-deficient mice have the same quadrilateral parallel CatSper lines along the flagellum but each of these lines is made only of a single row of CatSper complexes[••42]. It was further suggested that CatSper-zeta and EFCAB9 bind to each other within a single CatSper channel complex (Figure 2); however, it is also possible that CatSper-zeta from one CatSper complex interacts with EFCAB9 from another CatSper complex located within the same two-row structure (Figure 2c). Such an arrangement could allow a coordinated opening of the channels within the line and ensure efficient and fast signal propagation along the flagellum in a domino-like effect. Such a hypothesis would require rigorous genetic proof, as well as reconstitution of an entire

CatSper complex in a heterologous expression system—a task that has not yet been achieved by any research group.

Ca^{2+} pump/Na^+ – Ca^{2+} exchanger

In the human epididymis, sperm cells are kept quiescent by the acidic extracellular pH and low basal intracellular Ca^{2+} level kept around 100 nM, which is 15,000 times lower than the concentration measured in cauda epididymal plasma ~1.5 mM [46,47]. This gradient is maintained mainly by ATP2B4 in mouse, a Ca^{2+}-pump, which transports Ca^{2+} outside powered by ATP consumption [48]. *ATP2B4*-deficient male mice are infertile, because of severely impaired sperm motility [49]. When the $[Ca^{2+}]_i$ is elevated, Na^+/Ca^{2+} exchanger is also involved in calcium clearance [48]; however, Ca^{2+} clearance and the role of ATP2B4 in human male fertility need to be elucidated. After the spermatozoa enter the female reproductive tract and begin to capacitate, intracellular calcium elevation plays a significant role in the initiation of hyperactivated motility and is required for the onset of acrosome reaction. In addition, cytoplasmic Ca^{2+} promotes tyrosine phosphorylation via activation of sAC [50], which further upregulates PKA by cAMP generation. Mice sperms also rely on the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, and calcineurin-deficient mice are infertile [51].

TRP channels and Na^+–K^+ ATPase

In addition to the stimuli mentioned above, CatSper is also regulated by membrane depolarization [36,37]. Interestingly, murine CatSper is less voltage-dependent than human one since the slope factor of $k_{\text{mouse CatSper}}$ = 30—32, while $k_{\text{human CatSper}}$ = 17—21 [36,37]. Moreover, the half-maximal voltage activation $V_{1/2\ \text{mouse CatSper}}$ = +11 mV and $V_{1/2\ \text{human CatSper}}$ = +70 mV [36,37]. These numbers indicate that only a small fraction of human CatSpers will be opened at physiological-relevant membrane potentials. The progesterone shifts $V_{1/2}$ of human CatSper to +30 mV in capacitated sperm cells [37]; however, to produce larger current, CatSper still requires both intracellular alkalization and significant membrane depolarization [37]. Therefore, membrane depolarization must occur before CatSper is activated.

Once inside the female reproductive tract, mammalian spermatozoa are exposed to increased temperatures of 37 °C, as opposed to 34.7 °C in the epididymis [52]. Additionally, human sperms also express a temperature-activated non-selective cationic channel TRPV4 [••53] that contributes to membrane depolarization by allowing positively charged ions, such as sodium and calcium, to enter the flagellum. Therefore, TRPV4 is an excellent candidate for the sperm-depolarizing channel that ensures human CatSper activation. Besides TRPV4, other members of TRP channel family (such as TRPV1 and TRPM8) were reported to be expressed in mammalian sperm flagellum; however, their functional detection by direct electrophysiology recordings did not confirm their functional presence [••53,54]. Sperm membrane potential is also controlled by K^+ and Na^+ equilibrium maintained by the electrogenic Na^+/K^+-ATPase α4 (encoded by the *ATP1A4,* in mouse and human) [55,56]. Na^+/K^+-ATPase is crucial for male fertility, since *ATP1A4*-deficient mice [55] is infertile.

Therefore, Na^+/K^+-ATPase α4 is currently being considered a promising male contraceptive target [•57].

Sperm K^+ channels

Potassium current in murine sperm is carried by sperm-specific Slo3 protein (encoded by the *KCNU1* gene [58]), which is a strongly pH-sensitive channel activated by intracellular alkalization [59]. The Slo3 channel is important for male fertility, since mice lacking *KCNU1* are infertile [60,61]. Human sperm also possess a potassium-conducting channel, KSper [62]; however, the molecular identity of human KSper is more complicated. Calcium-sensitive Slo1 channel (encoded by the *KCNMA1* gene) was found to be expressed in human spermatozoa [63], in addition to Slo3 protein (Figure 1b) [64]. Surprisingly, human Slo3 channels are less pH-dependent, with currents showing activation already at pH = 6.7, a pH at which mouse Slo3 currents are not active [65]. Additionally, human KSper is regulated by intracellular calcium and inhibited by progesterone [63] and was suggested to be represented by Slo1. Calcium and progesterone sensitivities of human KSper were further confirmed by Brenker et al.; however, the group has suggested that the molecular identity of human KSper is Slo3 [64]. No human patient with either ablations or mutations in either KCNU1 or KCNMA1 genes was reported so far; however, according to Ref. [66], a male infertile patient deficient in sperm potassium current KSper, but with intact KCNU1 and KCNMA1 genes was reported. Future studies are needed to define the role of both *KCNU1* and *KCNMA1* genes in human sperm fertility. Specifically, the correlation between mutations in either of these genes with male infertility will be required to proof their importance for human sperm physiology.

Concluding remarks

Over the last few years, significant advances have been made in the identification of the macromolecular membrane complexes that regulate mammalian sperm motility, and a deeper understanding of their concerted action has been achieved. A combination of traditional methods, novel imaging techniques, and electrophysiology recording of ion channels from sperm cells has provided deeper insights into how these cells function; however, in order to ultimately unveil how a membrane transport system orchestrates different sperm motility patterns, computational modeling will be required. Specifically, it will be important to understand how the alteration of ion influx in response to changes in the flagellar environment modulates the beating patterns, such as basal and hyperactivated motility. This will require determination of the relative locations of the key flagellar ion transport systems and detailed characterization of their function under physiological conditions.

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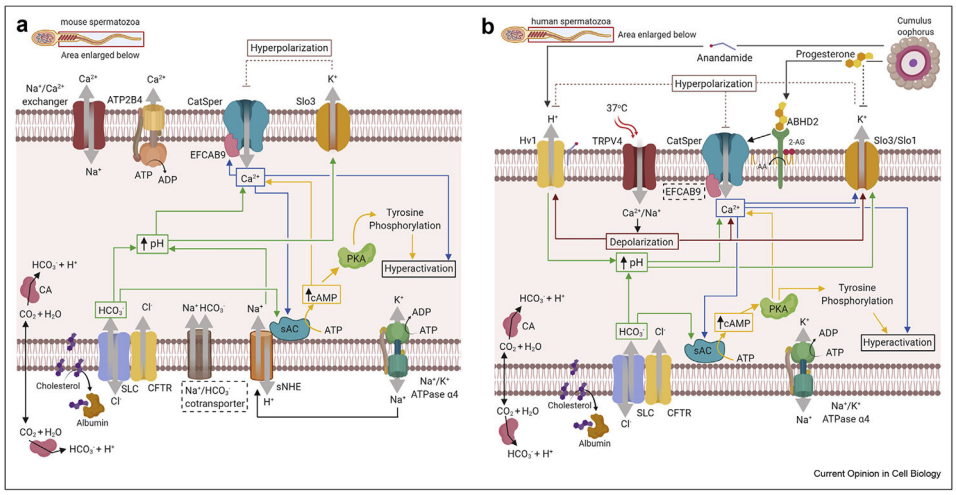

Figure 1.

(a) Signaling pathways in the murine sperm flagellum. pH-activated CatSper opens and carries Ca^{2+} into the cell. This channel is additionally regulated by intracellular Ca^{2+} via its EFCAB9 cytoplasmic subunit. Calcium clearance mechanisms of mouse sperm is provided mainly by ATPase (ATP2B4) and by Na^+/Ca^+ exchanger. Intracellular alkalization can be caused by the action of NHE exchangers. Sperm capacitation is triggered by an increase in intracellular HCO_3^-, which enters the cell in two different ways. First, CO_2 can diffuse through the membrane and then is converted by CA into HCO_3^-. Second, extracellular HCO_3^- can be carried into the cell by Cl^-/HCO_3^- SLC cotransporter interacting with Cl^--permeable CFTR. Na^+/K^+ gradient across the plasma membrane and, hence, membrane potential are maintained by the Na^+/K^+-ATPase α4. This gradient could be used to power the sNHE exchanger to promote influx of Na^+ and efflux of H^+ and to ensure intracellular alkalization. Potassium enters the cell through the Na^+/K^+-ATPase α4 and can leave through the Slo3 channel, which in mouse is activated by intracellular alkalization. Efflux of K^+ causes hyperpolarization, further inhibiting CatSper. HCO_3^- and intracellular Ca^{2+} can trigger activation of sAC, which produces cAMP, leading to activation of PKA and tyrosine kinases, resulting in broad tyrosine phosphorylation leading to sperm capacitation and hyperactivation. Capacitation is also associated with cholesterol removal from the plasma membrane by albumin. Solid lines represent activation, and dotted lines represent inhibition. Dotted rectangles indicate proteins with yet to be conformed functional role in male fertility.

(b) Signaling pathways in human sperm flagellum. P4 released from the cumulus oophorus binds to ABHD2, which cleaves 2-AG to AA and thus removes the inhibition imposed by 2-AG on the CatSper channel. CatSper opens and carries Ca^{2+} into the cell. Warm temperature (37 °C) in the female reproductive tract activates the TRPV4 channel, allowing cations (Na^+ and Ca^{2+}) to enter the cell and to cause membrane depolarization, which further promotes CatSper opening. Besides P4 and depolarization, CatSper also requires intracellular alkalization to produce maximal current. This can be achieved by proton efflux through Hv1, which is activated by anandamide and fatty acids released from the cumulus oophorus. As mentioned in Figure 1a, HCO_3^- enters the cell via CO_2 diffusion

and via conversion by CA into HCO_3^-. Additionally, it can be imported by SLC cotransporter interacting with Cl^--permeable CFTR. As in murine sperm, HCO_3^- activates sAC. Flagellar alkalinity further upregulates CatSper. Potassium enters the cell through the Na^+/K^+-ATPase α4 and leaves through the human KSper (Slo3/Slo1) channel, which is activated by depolarization and intracellular Ca^{2+}, and is inhibited by P4. Efflux of K^+ causes hyperpolarization, thus negatively regulating CatSper and Hv1. As in murine sperm, cAMP elevation triggered by HCO_3^- and intracellular Ca^{2+} leads to PKA and tyrosine kinase activation, resulting in broad tyrosine phosphorylation. The latter completes sperm capacitation and ensures hyperactivation. Additionally, cholesterol removal from the plasma membrane by albumin further facilitates capacitation. Solid lines represent activation, and dotted lines represent inhibition. Dotted rectangles indicate proteins with yet to be conformed functional role in human male fertility. **Abbreviations:** TRPV4, transient receptor potential cation channel subfamily member 4 vanilloid 4 channel; sAC, soluble adenylyl cyclase; PKA, protein kinase A; CatSper, cation channel of sperm; Hv1, proton voltage-gated ion channel; ABHD2, α/β hydrolase domain-containing protein 2; 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; KSper/Slo1/Slo3, potassium channel of sperm/Slowpoke homolog 1/3; SLC, solute carrier family anion exchanger; CFTR, cystic fibrosis transmembrane conductance regulator; sNHE/SLC9C1, sperm-specific Na^+/H^+ exchanger; EFCAB9, EF-hand calcium-binding domain-containing protein 9; ATP2B4, ATPase plasma membrane Ca^{2+} transporting 4; ATP, adenosine triphosphate; ADP; adenosine diphosphate; cAMP, cyclic adenosine monophosphate; P4, progesterone; CA, carbonic anhydrases. (a) and (b) figures are created with Biorender.com.

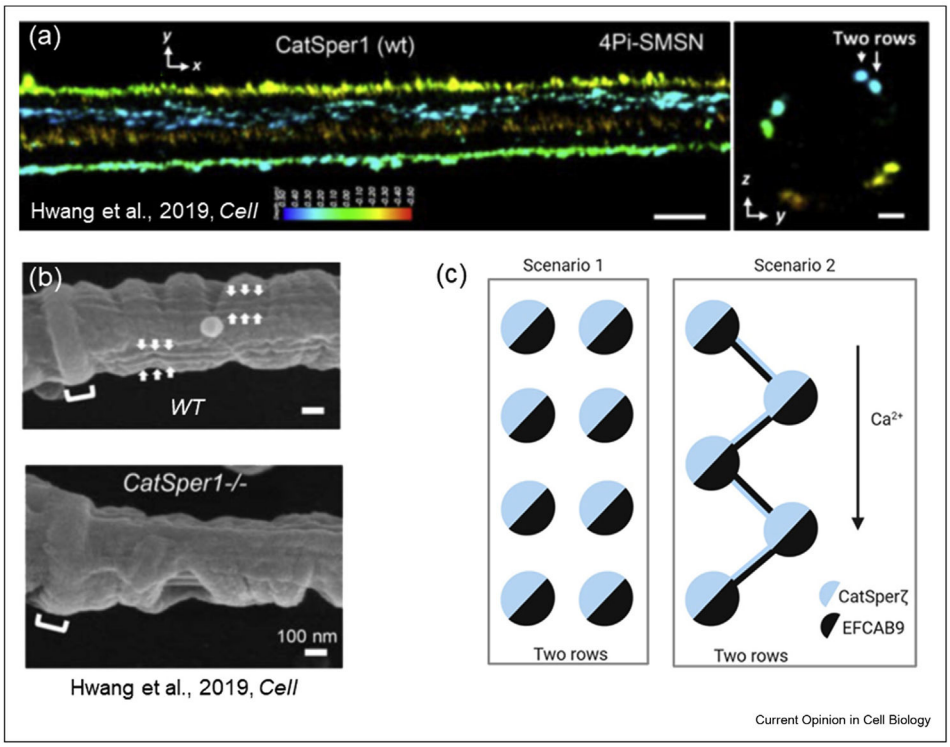

Figure 2. Two possible flagellar arrangements of CatSper nanodomains.

(a) Reproduced from Ref. [••42]: 4Pi single-molecule switching nanoscopy images of murine CatSper1 in wild type (WT) flagella. x–y projection colors encode the relative distance from the focal plane along the z axis. Scale bar, 500 nm. Right panel: y–z cross sections (100 nm thick). Two-row structures are indicated with arrows. Scale bar, 200 nm. **(b)** SEM images of the principal piece of flagella from WT (top panel) and CatSper1$^{-/-}$ (bottom panel) mice. As indicated by arrows, double-row lines are observed on both sides of WT flagellum within each longitudinal column. These structures are absent in CatSper1$^{-/-}$ flagellum. Scale bar, 100 nm. (a) and (b) figures are from Ref. [••42]. **(c)** Two possible scenarios of CatSper flagellar nanodomain architecture. As shown in (a), CatSper forms two parallel rows and as suggested by Hwang et al. [••42], CatSper-zeta associates with EFCAB9 within the same CatSper complex (left panel). According to an alternative scenario (right panel), CatSper-zeta and EFCAB9 link together two neighboring channels in a zig-zag manner, in which CatSper-zeta from one CatSper complex is associated with EFCAB9 from the neighboring CatSper complex. This hypothetical arrangement would not only structurally link CatSper complexes, but also link them functionally. This linkage could permit more efficient signal propagation along the flagellar length and could be responsible for either synchronization of CatSper opening or longitudinal propagation of Ca^{2+} waves.