THE
EMBO
JOURNAL

Rotational motion and rheotaxis of human sperm do not require functional CatSper channels and transmembrane Ca^{2+} signaling

Christian Schiffer, Steffen Rieger, Christoph Brenker, Samuel Young, Hussein Hamzeh, Dagmar Wachten, Frank Tüttelmann, Albrecht Röpke, U. Benjamin Kaupp, Tao Wang, Alice Wagner, Claudia Krallmann, Sabine Kliesch, Carsten Fallnich, Timo Strünker

Review timeline:

Submission date:	30th Apr 2019
Editorial Decision:	4th Jun 2019
Revision received:	31st Oct 2019
Editorial Decision:	21st Nov 2019
Revision received:	2nd Dec 2019
Accepted:	6th Dec 2019

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 4th Jun 2019

Thank you for the submission of your manuscript (EMBOJ-2019-102363) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and solidity of your work, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, major concerns are stated regarding the lack of sufficient insights into the Ca2+ dependence of the sperm rolling driving mechanisms (ref #1). Also, in support of your results, complementary experiments in the context of more physiological hormone stimulations (referee #1) and altered viscosity (referee #2, pt. b)) are requested. Also reviewer #2 points out that the claims made on a dispensable role of CatSper in mouse sperm motility are not sufficiently supported at this stage (referee #2, a), see also comment referee #3). Further, the reviewers raise a number of issues related to additional experiments needed, complementary methods annotation, terminology and data illustration that would need to be conclusively addressed

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. However we also concur with the referees that in light of the contradictory literature on this context, the current findings will need to achieve the level of robustness required for The EMBO Journal. Also, it will be essential to revisit how the current findings can be reconciled with the other existing studies.

REFEREE REPORTS:

Referee #1:

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In this interesting study the authors studied the role of CatSper channel and Ca2+ influx in sperm rotational motion and rheotaxis. The authors used human sperm from infertile patients that lack CATSPER2 and sperm from CatSper1-/- mice in Ca2+ and Ca2+-free solution to conclude that Ca2+ influx and change in cytoplasmic Ca2+ is not required for both sperm rotational motion and rheotaxis.

The results are solid and for the most part, do support the conclusions offered by the authors. However, there are two weakness in the study. The minor that needs some further clarification is the potential role of Ca2+ in rheotaxis. Figure 4I-L suggest that Ca2+ does have a role in rheotaxis as it is skewed when mutant sperm is placed in Ca2+-free solution. The question here is whether the same is seen in wild-type sperm and does this reflect disruption of the Ca2+ gradient along the flagellum or reduction in basal Ca2+ due to the fairly long incubation at very low external Ca2+. This is quite necessary with the finding of the present work is so different from previous findings by others.

A more significant shortcoming of the study is that the authors report what does not control sperm rotational motion and rheotaxis but do not provide even a clue what does, beside providing a vague statement in the discussion that sperm architecture is required. However, the effect of HCO3- on rotation frequency would suggest that cAMP may also modulate sperm rotational motion and rheotaxis. Moreover, to enhance the physiological significance of the findings that are opposite from finding in mine sperm, the authors should test whether CATSRER is required for sperm rotational motion and rheotaxis stimulated by hormones that regulate sperm function.

Minor:

A is missing in Figure 3.
In test when Fig. 4 is first mentioned, change (Fig. 4A, C, G, E) to (Fig. 4A, C, E, G)

Referee #2:

In their manuscript entitled "Rotational motion and rheotaxis of human sperm: the role of CatSper and transmembrane Ca2+ signaling" (EMBOJ-2019-102363), Schiffer et al. investigate rotation of both human and mouse sperm around their longitudinal axis (rolling), a motility pattern that promotes rheotaxis (the navigation of sperm in fluid flow). Specifically, the authors (re)examine the role of both the voltage- and alkaline-activated CatSper Ca2+ channel and elevations in the intracellular Ca2+ concentration in general in longitudinal rolling and rheotaxis.
Schiffer et al. show data that support the notion that rolling and rheotaxis persist in CatSper-deficient human sperm. The authors, furthermore, demonstrate that human sperm undergo rolling and rheotaxis when extracellular Ca2+ is <20 nM (and thus, no influx of Ca2+ occurs). Last, Schiffer and coworkers investigate rolling and rheotaxis in CatSper-deficient mouse sperm. Both motility patterns apparently persist and, thus, the authors conclude that "passive features of the flagellar beat enable sperm rolling and rheotaxis rather than Ca2+ signaling mediated by CatSper or other mechanisms controlling transmembrane Ca2+ flux".
The authors use whole-cell patch-clamp recordings from human sperm of healthy donors and compare results to data from patients that suffer from the deafness-infertility syndrome (DIS). In these patients, the CATSPER2 gene is deleted. Furthermore, immunochemistry and super-resolution microscopy are combined with dark- / bright-field microscopy, optical trapping of single sperm with laser tweezers in microfluidic chambers, and trajectory analysis of sperm in the absence and presence of fluid flow.
The findings presented by the authors are of interest to a wide range of individuals working in the fields of reproductive physiology as well as cell biology in general. The data are solid and the authors provide evidence for most of their claims. However, a few concerns - most of them minor - should be addressed before publication in The EMBO Journal (see below).

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major concerns:
a) My major concern is that the data presented on mouse sperm by far lacks the level of scientific scrutiny and detail that the authors have shown when examining human sperm. Not least because the mouse sperm data contradict previously published results and are therefore somewhat controversial (which, of course, is not a bad thing) mouse sperm rolling and rheotaxis should either be as thoroughly examined as in human sperm (my recommendation) or these results should be regarded as preliminary at this stage and, consequently, be left out.

b) A second point that would substantially strengthen the manuscript in my view is few additional experiments in media of higher viscosity. The authors discuss that "rheotaxis might be compromised at certain shear velocities and/or fluid viscosities." It would be highly informative to learn whether the apparent lack of any rolling and rheotaxis phenotype in CatSper-deficient human sperm persists under more natural (i.e., more viscous) conditions. I guess that the elegant approach that the authors have used could relatively easy be adapted to test in media of higher viscosity.

minor concerns:
1) Results, p.5, ll. 127-128: "These sperm lacked CatSper-mediated Ca2+ influx (Fig. 1A-B) and membrane currents (Fig. 1C), confirming..."
B and C describe currents.

2) Results, p.6, ll. 151-153: "...provided a measure of the rotation frequency (Fig. 2G). The rotation frequency of optically trapped control sperm from healthy donors was constant for several tens of seconds (Fig. 2G)."
The heterogeneity of rotation frequencies among the sperm population is striking. Is the skewed distribution (to larger values) indicative of a small subset of 'hyperactive' sperm? Please comment.

3) Results, p.6, ll. 170-171: "A stimulus buffer (stimulus stream) and sperm in control buffer (control stream) were separated by a barrier stream containing fluorescein in control buffer; the buffers were..."
These labels (i.e., "control / stimulus stream") do not correspond to the figure (Fig. 3A).

4) Abstract / Discussion: In their abstract, the authors conclude that their "results strongly support the concept that passive features of the flagellar beat enable sperm rolling and rheotaxis rather than Ca2+ signaling mediated by CatSper or other mechanisms controlling transmembrane Ca2+ flux."
The discussion, however, lacks a paragraph dedicated to such "passive features of the flagellar beat."
The authors should either elaborate (in the discussion) or rephrase the statement (in the abstract).

Referee #3:

This is a very comprehensive and detailed study by an experienced group. It tackles a fundamental aspect of sperm movement and function (rolling and rheotaxis). As specific inhibitors of CatSper are challenging to develop the use of a CatSper deletion patient provides a clear (and rare) experimental tool. A series of experiments examined the role of CatSper in rotational movement and rheotaxis of human spermatozoa. This work was complimented importantly by patients with a deletion in CatSper which showed that functional CatSper was not necessary. Moreover, in light of this, further experiments with CatSper KO mice (CatSper 1) showed a different result to what has previously been demonstrated although the authors provided no clear explanation for the discrepancies (L239).
.

Minor things to consider.
1. The details of the patients (CATSPER2-/-) are required such as semen analysis (? and HA), genetic details (ephys data is not proof of homozygous deletion, line 128, see Luo et al 2019 (PMID: 30629171) and deletions reported for DIF patients are different). Was the distribution equal in all 4 patients (Fig 1 D/E)?
2. I was surprised that CatSper subunits were still present in the patient (Fig 1). This is very different to the mouse. Can the authors speculate?

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3. Should the recent data on efcab9 be included?
4. In the Introduction section the authors should separate out experiments on animal and humans e.g. nobody knows what happens in humans - as the authors state more complex real models of the oviduct are required in humans.
5. It would be very helpful if the dim dark field technique (along with CASA) could be used for finding these men. Can the authors provide more details of how this may work on a routine basis?
6. Would it be helpful to have a cartoon/model of how the authors think the system now works without CatSper.
7. Did sperm from the CATSPER2-/- patient penetrate viscous media?
Whilst the mouse data is very interesting it may make a separate report once the discrepancies with other data are delineated. Just a thought

1st Revision - authors' response 31st Oct 2019

Please see next page.

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Response to all referees:

We thank the editor and the referees for their efforts to evaluate the manuscript, their overall positive assessment of the work, and the constructive criticism. We followed their suggestions, performed additional experiments, and revised the manuscript accordingly. The parts that have been changed are marked in red. A new figure (Figure 5) shows the results of additional experiments with mouse sperm. Moreover, Figure 1 shows now data on another CatSper-deficient patient. New data on sperm from healthy donors and CatSper-deficient human sperm are included in Figure 2, Figure 4, Figure 6 , and Figure EV1. We modified the Material and Methods section accordingly. Finally, to improve readability, we rephrased some sentences without changing the meaning. We did not mark such purely cosmetic and/or editorial changes.

Response to Referee #1:

In this interesting study the authors studied the role of CatSper channel and Ca2+ influx in sperm rotational motion and rheotaxis. The authors used human sperm from infertile patients that lack CATSPER2 and sperm from CatSper1-/- mice in Ca2+ and Ca2+-free solution to conclude that Ca2+ influx and change in cytoplasmic Ca2+ is not required for both sperm rotational motion and rheotaxis.

1. The results are solid and for the most part, do support the conclusions offered by the authors. However, there are two weakness in the study. The minor that needs some further clarification is the potential role of Ca2+ in rheotaxis. Figure 4I-L suggest that Ca2+ does have a role in rheotaxis as it is skewed when mutant sperm is placed in Ca2+-free solution. The question here is whether the same is seen in wild-type sperm and does this reflect disruption of the Ca2+ gradient along the flagellum or reduction in basal Ca2+ due to the fairly long incubation at very low external Ca2+. This is quite necessary with the finding of the present work is so different from previous findings by others.

This is the first study that investigates whether CatSper and/or Ca^{2+} influx is required for rolling and rheotaxis of *human* sperm. There, initially, was no conflict, because previous findings were obtained with *mouse* sperm (see, however, below).

Control sperm from healthy donors ("wild-type") become immotile in the absence of extracellular Ca^{2+} (Figure 3H), presumably, due to an influx of Na^{+} via CatSper and the ensuing depletion of ATP (Torres-Flores et al. Human Reproduction 2011). The rapid decay of motility in Ca^{2+}-free buffer prevents rheotaxis experiments with control sperm from donors. Instead, we used the CatSper-deficient sperm from DIS patients. The motility of CatSper-deficient human sperm is preserved in Ca^{2+}-free buffer, similar to CatSper-deficient mouse sperm (e.g., Jin et al. Biology of Reproduction 2007). In Figure 4I-L of the original manuscript, we showed the results of a single rheotaxis experiment with CatSper-deficient human sperm (n = 1). We repeated this experiment now several times. The spider-web plot in the new Figure 4L shows the mean binned angular frequencies of four experiments. The

enlarged data set indicates that, by and large, rheotaxis of CatSper-deficient human sperm is similar in the presence and absence of Ca^{2+}. We hope that these new results answer the referee's questions.

2. A more significant shortcoming of the study is that the authors report what does not control sperm rotational motion and rheotaxis but do not provide even a clue what does, beside providing a vague statement in the discussion that sperm architecture is required. However, the effect of HCO3- on rotation frequency would suggest that cAMP may also modulate sperm rotational motion and rheotaxis. Moreover, to enhance the physiological significance of the findings that are opposite from finding in mine sperm, the authors should test whether CATSRER is required for sperm rotational motion and rheotaxis stimulated by hormones that regulate sperm function.

We are not sure whether we understand the comments correctly. We show that CatSper is dispensable for rolling and rheotaxis in both human and mouse sperm. Thus, our findings on human and mouse sperm are similar rather than opposing. Moreover, we assume that "*hormones that regulate sperm function*" refers to CatSper agonists such as progesterone. Whether progesterone "*stimulates*" rheotaxis is not known. Following the referee´s suggestion, we examined rheotaxis of control and CatSper-deficient human sperm in the absence and presence of progesterone (100 nM). In the presence of the hormone, the fraction of control sperm and CatSper-deficient sperm undergoing rheotaxis (Figure 6C, D) was similar. Thus, under these experimental conditions and in terms of the parameters that we analyzed, progesterone-activation of CatSper does not affect rheotaxis. We caution, however, against rush interpretations. As outlined in the discussion, the potential action of progesterone on the rheotactic performance needs a detailed study on its own, following the demanding experimental and analytical approach introduced by Kantsler and colleagues (Kantsler et al. eLife 2014). This holds also true concerning a potential cAMP/bicarbonate-control of rheotaxis.

3. A is missing in Figure 3.

4. In test when Fig. 4 is first mentioned, change (Fig. 4A, C, G, E) to (Fig. 4A, C, E, G)

We apologize for these mistakes and changed the figure and the text accordingly.

Response to Referee #2:

In their manuscript entitled "Rotational motion and rheotaxis of human sperm: the role of CatSper and transmembrane Ca2+ signaling" (EMBOJ-2019-102363), Schiffer et al. investigate rotation of both human and mouse sperm around their longitudinal axis (rolling), a motility pattern that promotes rheotaxis (the navigation of sperm in fluid flow). Specifically, the authors (re)examine the role of both the voltage- and alkaline-activated CatSper Ca2+ channel and elevations in the intracellular Ca2+ concentration in general in longitudinal rolling and rheotaxis.
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The authors use whole-cell patch-clamp recordings from human sperm of healthy donors and compare results to data from patients that suffer from the deafness-infertility syndrome (DIS). In these patients, the CATSPER2 gene is deleted. Furthermore, immunochemistry and super-resolution microscopy are combined with dark- / bright-field microscopy, optical trapping of single sperm with laser tweezers in microfluidic chambers, and trajectory analysis of sperm in the absence and presence of fluid flow.
The findings presented by the authors are of interest to a wide range of individuals working in the fields of reproductive physiology as well as cell biology in general. The data are solid and the authors provide evidence for most of their claims. However, a few concerns - most of them minor - should be addressed before publication in The EMBO Journal (see below).

1. ***My major concern is that the data presented on mouse sperm by far lacks the level of scientific scrutiny and detail that the authors have shown when examining human sperm. Not least because the mouse sperm data contradict previously published results and are therefore somewhat controversial (which, of course, is not a bad thing) mouse sperm rolling and rheotaxis should either be as thoroughly examined as in human sperm (my recommendation) or these results should be regarded as preliminary at this stage and, consequently, be left out.***

We agree with the referee. We performed additional experiments to quantify rolling and rheotaxis in wild-type and CatSper-deficient mouse sperm. The new Figure 5 shows that rolling and rheotaxis were similar in wild-type and CatSper-deficient mouse sperm (see also Movies EV13-16). This new data set supports the conclusion that CatSper is also dispensable for rolling and rheotaxis of mouse sperm.

2. ***A second point that would substantially strengthen the manuscript in my view is few additional experiments in media of higher viscosity. The authors discuss that "rheotaxis might be compromised at certain shear velocities and/or fluid viscosities." It would be highly informative to learn whether the apparent lack of any rolling and rheotaxis phenotype in CatSper-deficient human sperm persists under more natural (i.e., more viscous) conditions. I guess that the elegant approach that the authors have used could relatively easy be adapted to test in media of higher viscosity.***

Following the referee´s suggestion, we studied rolling and rheotaxis of control and CatSper-deficient human sperm at more physiological viscosity (Menezo et al, European Journal of Obstetrics, Gynecology, and Reproductive Biology 1997; Miki and Clapham Current Biology 2013), using buffer containing 0.2% methyl cellulose. In addition, we studied rolling also in highly viscous buffer containing 1% methyl cellulose. Figure 2F and N of the revised manuscript show that the rolling frequency of both control and CatSper-deficient human sperm decreased with increasing viscosity. Furthermore, Figure 6A and B of the revised manuscript show that rheotaxis of wild-type and CatSper-deficient sperm was rather similar in 0.2% methyl cellulose. Thus, rolling and rheotaxis of CatSper-deficient human sperm persisted also in more viscous media. Future works need to study rheotaxis over a broader range of flow velocities and viscosities, which is, however, beyond the scope of the present manuscript (see also our response to referee #1).

3. Results, p.5, ll. 127-128: "These sperm lacked CatSper-mediated Ca2+ influx (Fig. 1A-B) and membrane currents (Fig. 1C), confirming..."
B and C describe currents.

We corrected Figure 1.

4. Results, p.6, ll. 151-153: "...provided a measure of the rotation frequency (Fig. 2G). The rotation frequency of optically trapped control sperm from healthy donors was constant for several tens of seconds (Fig. 2G). "The heterogeneity of rotation frequencies among the sperm population is striking. Is the skewed distribution (to larger values) indicative of a small subset of 'hyperactive' sperm? Please comment.

We are not sure whether we understand this question correctly. We assume that the question refers to the frequency histogram for trapped (Fig. 2J) versus freely moving sperm (Fig 2D, 25 mM bicarbonate). Figure 2D shows the distribution for sperm from one particular sample as a representative. There is, however, a considerable inter-individual variation (see the Figure R1 below).

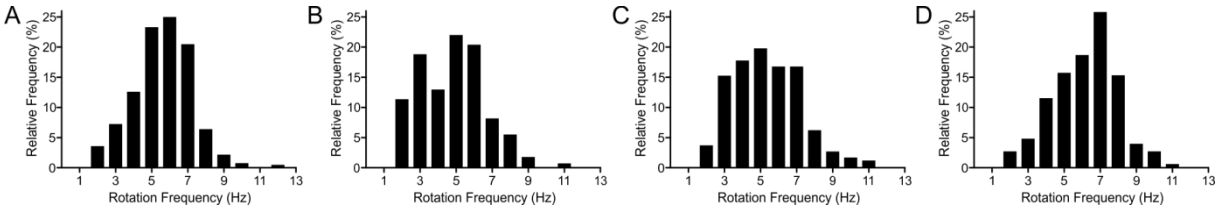

Figure R1: Representative rotation-frequency histograms of capacitated sperm from four different donors

The histogram in Figure 2J includes measurements of sperm from different samples. We suggest that the heterogeneity reflects the inter-individual variations rather than subsets of hyperactive sperm.

5. Results, p.6, ll. 170-171: "A stimulus buffer (stimulus stream) and sperm in control buffer (control stream) were separated by a barrier stream containing fluorescein in control buffer; the buffers were..."These labels (i.e., "control / stimulus stream") do not correspond to the figure (Fig. 3A).

We are sorry for this mistake, which we corrected in the new figure.

6. Abstract / Discussion: In their abstract, the authors conclude that their "results strongly support the concept that passive features of the flagellar beat enable sperm rolling and rheotaxis rather than Ca2+ signaling mediated by CatSper or other mechanisms controlling transmembrane Ca2+ flux." The discussion, however, lacks a paragraph dedicated to such "passive features of the flagellar beat." The authors should either elaborate (in the discussion) or rephrase the statement (in the abstract).

We agree and rephrased the abstract accordingly.

Response to Referee #3:

This is a very comprehensive and detailed study by an experienced group. It tackles a fundamental aspect of sperm movement and function (rolling and rheotaxis). As specific inhibitors of CatSper are challenging to develop the use of a CatSper deletion patient provides a clear (and rare) experimental tool. A series of experiments examined the role of CatSper in rotational movement and rheotaxis of human spermatozoa. This work was complimented importantly by patients with a deletion in CatSper which showed that functional CatSper was not necessary. Moreover, in light of this, further experiments with CatSper KO mice (CatSper 1) showed a different result to what has previously been demonstrated although the authors provided no clear explanation for the discrepancies (L239).

Minor things to consider.

1. The details of the patients (CATSPER2-/-) are required such as semen analysis (? and HA), genetic details (ephys data is not proof of homozygous deletion, line 128, see Luo et al 2019 (PMID: 30629171) and deletions reported for DIF patients are different).

In Figure EV1, we now show the results from array-Comparative Genomic Hybridization analysis (array-CGH) for the four patients that were included in the original manuscript and for an additional patient that we identified in the meantime. In all five patients, chromosome 15 features the signature deletion of DIS (Zhang et al. Journal of Medical Genetic 2007; Hildebrand et al. European Journal of Human Genetics 2010), the deletion is homozygous, and includes the *CATSPER2* gene. We are currently working out the exact break points of the chromosomal deletion for each individual patient. The patients underwent a complete andrological and hearing-impairment work up. According to the WHO manual for semen analysis, all five patients are normozoospermic, but their sperm fail to undergo hyperactivation. We refrain, however, from disclosing more detailed information on the patients' clinical phenotype in the present manuscript. We argue that Movies EV9 and EV10 are sufficient to demonstrate that we could purify large amounts of highly motile and

morphologically normal sperm form the patients' ejaculate. We are currently preparing a separate manuscript, providing the first comprehensive and complete characterization of the clinical phenotype (molecular genetics, andrology, and audiology) of the deafness-infertility syndrome as a whole.

2. Was the distribution equal in all 4 patients (Fig 1 D/E)?

We could study the distribution only in 2 patients. We assume, but do not know for certain, that this is similar among the five patients.

3. I was surprised that CatSper subunits were still present in the patient (Fig 1). This is very different to the mouse. Can the authors speculate?

The deletion of *Catsper1*, *2*, *3*, or *4* in mice abolishes the expression of functional CatSper channels. Moreover, it is unequivocal that in mice, the deletion of *Catsper1* disrupts the expression of the entire CatSper-channel complex. These results suggested that the expression of pore-forming CatSper subunits is interdependent. However, to the best of our knowledge, it is not known whether also mouse sperm deficient for *Catsper2*, *3*, or *4* lack the entire CatSper-channel complex. Thus, we don´t know whether the preserved expression of CatSper 2 and 3 in CatSper2-deficient human sperm is different to mouse sperm. This should be addressed in future studies, using the CatSper2$^{-/-}$ mice generated by the late David Garbers and colleagues.

4. Should the recent data on efcab9 be included?

Yes; we now refer to the recent study on Efcab9 by the Chung lab.

5. In the Introduction section the authors should separate out experiments on animal and humans e.g. nobody knows what happens in humans - as the authors state more complex real models of the oviduct are required in humans.

We rephrased the introduction accordingly.

6. It would be very helpful if the dim dark field technique (along with CASA) could be used for finding these men. Can the authors provide more details of how this may work on a routine basis?

We are not sure whether we understand this comment correctly. In fact, the dim dark field technique is not suited to identify CatSper-deficient men, because the rolling behavior is not affected in CatSper-deficient sperm. We suggest analyzing the rolling behavior as a surrogate for rheotaxis. We assume that sperm with impaired rolling fail to undergo rheotaxis, which might render men sub- or infertile. The rolling analysis can be combined with routine CASA, which is often performed by dark field microscopy. The combination of CASA with rolling analysis might require a slight modification of the dark-field set up of the particular

microscope and a small program written in the ImageJ macro language. We are prepared to share this technique/tool with the scientific community after its introduction.

7. Would it be helpful to have a cartoon/model of how the authors think the system now works without CatSper.

We refrained from providing a model, because it requires additional studies to develop new concepts as to how rolling and rheotaxis is actually enabled.

8. Did sperm from the CATSPER2-/- patient penetrate viscous media?

We did not perform a classical Kremer test. The CatSper-deficient sperm swam, however, progressively even in highly viscous medium (Figure 2N; Movie EV5).

9. Whilst the mouse data is very interesting it may make a separate report once the discrepancies with other data are delineated. Just a thought

We thank the referee for this suggestion. It is certainly advantageous to include both data set on mouse and human sperm in one and the same manuscript. Therefore, we performed additional experiments to obtain also a comprehensive data set on mouse sperm, bolstering our conclusions that rolling and rheotaxis in mouse and human sperm do not require CatSper (see response to referee #2).

2nd Editorial Decision 21st Nov 2019

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your amended study was sent back to two of the referees for re-evaluation, and we have received comments from both of them, which I enclose below.

As you will see the referee finds that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation as listed below, which need to be adjusted at re-submission.

REFEREE REPORTS:

Referee #1:

The authors mostly addressed all my concerns. They argue that testing effects of hormones and cAMP involve new set of studies that will take many months to complete. I will have to go with this because of the significance of the studies and wait for another work to clarify the mechanism of sperm rotational motion and rheotaxis. In view of this, I support publication of the manuscript in EMBO.

Referee #2:

In their substantially revised manuscript entitled "Rotational motion and rheotaxis of human sperm: the role of CatSper and transmembrane Ca2+ signaling" (EMBOJ-2019-102363R), Schiffer et al. have addressed all my previous concerns. Accordingly, the manuscript is now suitable for publication in The EMBO Journal.

2nd Revision - authors' response 2nd Dec 2019

The authors performed the requested editorial changes.

3rd Editorial Decision 6th Dec 2019

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Timo Strünker
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-102363

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We included only healthy donors that were normozoospermic according to the WHO laboratory manual for the examination and processing of human semen
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	We did not perfom animal studies. For experimemnts with sperm, blinding was not neccessary, because we used objective measures and analysis methods
5. For every figure, are statistical tests justified as appropriate?	We did not use statistical test. We provide raw data or mean +- s.d.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Because we did not use statistical tests, we did not assess whether the data are normally distributed.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	We provide the standard deviation.
Is the variance similar between the groups that are being statistically compared?	We did not compare groups statistically.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	1. commercial polyclonal rabbit anti-CatSper 4 (ACC-304, Alomone Labs, Israel); 2. custom polyclonal rabbit anti-CatSper 3; epitope directed against amino acids 384-402 (Peptide Speciality Laboratories GMBH, Heildelberg)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male C57BL/6N wild-type and Catsper1-/- mice; at least 25 days old. Catsper1-/- mice (Ren et al., 2001) were generously provided by David Clapham (Janelia Research Campus, USA). Mice were kept specific pathogen-free in ventilated cages . Maximally five mice were housed per cage.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mice were handled and sacrificed in accordance with the German Animal Welfare Act and the district veterinary office under approval by the LANUV (AZ.84-02.04.2012.A192).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confiim compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Institutional ethical committees of the Medical association Westfalen-Lippe and the Medical Faculty of the University of Münster; reference number 4INie
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Semen samples of human semen were obtained from healthy volunteers and DIS patients with their prior written consent
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We provide data in Expanded View Movies.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA