

RESEARCH ARTICLE

Binding of sperm protein Izumo1 and its egg receptor Juno drives Cd9 accumulation in the intercellular contact area prior to fusion during mammalian fertilization

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ABSTRACT

Little is known about the molecular mechanisms that induce gamete fusion during mammalian fertilization. After initial contact, adhesion between gametes only leads to fusion in the presence of three membrane proteins that are necessary, but insufficient, for fusion: Izumo1 on sperm, its receptor Juno on egg and Cd9 on egg. What happens during this adhesion phase is a crucial issue. Here, we demonstrate that the intercellular adhesion that Izumo1 creates with Juno is conserved in mouse and human eggs. We show that, along with Izumo1, egg Cd9 concomitantly accumulates in the adhesion area. Without egg Cd9, the recruitment kinetics of Izumo1 are accelerated. Our results suggest that this process is conserved across species, as the adhesion partners, Izumo1 and its receptor, are interchangeable between mouse and human. Our findings suggest that Cd9 is a partner of Juno, and these discoveries allow us to propose a new model of the molecular mechanisms leading to gamete fusion, in which the adhesion-induced membrane organization assembles all key players of the fusion machinery.

KEY WORDS: CD9, Izumo1, Juno, Fertilization, Membrane dynamics, Membrane organization

INTRODUCTION

Among the various fusion mechanisms occurring in living organisms, gamete fusion is by far the least understood, although it is the key biological process conditional for all somatic development and thus for eukaryotic life. Several molecular machineries of intracellular trafficking and of virus-cell fusion have been identified, in contrast with gamete fusion. Only three of the membrane molecular actors involved in mouse fertilization, Izumo1 on the sperm surface (Inoue et al., 2005), its newly identified oocyte-based receptor Juno (Bianchi et al., 2014) and the tetraspanin Cd9 on the egg surface (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Inoue et al., 2005; Rubinstein et al.,

2006), have so far been shown to be essential. These three molecules are also present on human egg and sperm cells. Izumo1 is a testis immunoglobulin superfamily type 1 (IgSF) protein, expressed at the plasma membrane of acrosome-reacted sperm (Satouh et al., 2012), and is highly conserved in mammals (Grayson and Civetta, 2012). Female mice deleted for the *Izumo1* gene have normal fertility, but males are completely sterile despite normal mating behavior and normal sperm production (Inoue et al., 2005). Similar features are observed for Cd9 on egg cells. *Cd9*^{-/-} female are healthy but severely subfertile because of defective sperm-egg interaction. Unfertilized eggs extracted *in vivo* after mating between wild-type (WT) females and *Izumo1*^{-/-} males or between WT males and *Cd9*^{-/-} females showed sperm that had crossed the zona pellucida (ZP) but were accumulated in the perivitelline space, unable to penetrate within the oolemma (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Inoue et al., 2005; Rubinstein et al., 2006). These results confirmed *in vitro* data showing the inhibitory effect of monoclonal antibodies raised against Izumo1 or Cd9 on sperm-egg fusion (Okabe et al., 1988; Chen et al., 1999). Intracytoplasmic injection of *Izumo1*^{-/-} sperm into WT oocytes or intracytoplasmic injection of WT sperm into *Cd9*^{-/-} oocytes leads to normal egg activation and term development after the transfer into the oviduct of pseudo-pregnant female mice (Miyado et al., 2000; Inoue et al., 2005). These results provide evidence that Izumo1 and Cd9 are crucial factors to enable fertilization. Juno, the recently identified receptor of Izumo1, is a membrane-tethered folate receptor also known as Folr4 (Bianchi et al., 2014). Female mice lacking Juno are infertile and Juno-deficient eggs do not fuse with normal sperm. Rapid shedding of Juno from the oolemma after fertilization suggests the involvement of this protein in the membrane blocking against polyspermy (Bianchi et al., 2014).

In vitro fertilization assays performed between hamster eggs and human sperm have shown the inhibitory effect of a monoclonal antibody raised against human IZUMO1 on fertilization, thus also suggesting the involvement of IZUMO1 in the human fertilization process (Inoue et al., 2005).

As *Izumo1*-deleted sperm adhere to the mouse eggs but do not fuse, and WT sperm adhere to *Cd9*-deleted eggs but do not fuse either, it was suggested that Izumo1 and Cd9 are involved in the gamete fusion step but not in the initial adhesion step (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Inoue et al., 2005; Rubinstein et al., 2006). This idea was recently reconsidered for both Cd9 and Izumo1. In-depth analysis of sperm-egg interaction revealed different types of adhesion for Cd9 (Jegou et al., 2008), one of which involved Cd9 and was required for fusion (Jegou et al., 2011). Izumo1 was found to generate cell-egg adhesion, and a site in the N-terminal region of Izumo1 was found to be important for its

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binding to the egg membrane (Inoue et al., 2013). Bianchi et al. showed that the binding ability of Izumo1 and Juno was conserved in several mammalian species, including humans (Bianchi et al., 2014).

This paper addresses the questions of (i) the interplay between Izumo1, Juno and Cd9 in the fertilization process, and (ii) the conservation of their roles in humans. This was achieved through the quantitative and dynamic characterization of the membrane organization resulting from the adhesion that Izumo1 and Juno can generate between the egg plasma membrane of WT, *Cd9*^{-/-} eggs or eggs expressing Cd9-EGFP proteins, and of three mammalian cell lines overexpressing Izumo1. Combining a biophysical approach based on single-cell micromanipulation with time-lapse confocal observations, we could characterize the temporal evolution of the intercellular adhesion specifically due to the binding of Izumo1 and Juno, as well as Izumo1 and Cd9 reorganization during this adhesion. We demonstrate that Izumo1 and Cd9 concomitantly accumulate in the contact area. The recruitment of Izumo1 persists in the absence of egg Cd9, but at a higher velocity. These results show the influence of Cd9 on Izumo1 membrane kinetics and suggest that Cd9 is a partner of the Izumo1 receptor Juno. Heterologous adhesion and fusion assays indicate that the membrane mechanisms in which Izumo1, Cd9 and Juno are involved in the gamete interaction process are conserved across species.

RESULTS

Robust Izumo1-dependent cell-egg adhesion is reached after a few minutes of contact

We selected mammalian cell lines (K562, MDA and U2OS) that did not spontaneously adhere to the egg plasma membrane (supplementary material Fig. S1). These cells were transfected with mouse *Izumo1*. We characterized both their adhesion to the egg membrane and the molecular organization in the contact area through dual-pipette assays combined with time-lapse confocal imaging. All Izumo1-expressing cells from all three cell lines were shown to develop an adhesion with the eggs within 1 min (supplementary material Fig. S1C). After 5 min of contact, a stepwise-increasing aspiration was applied to a K562-Iz holding pipette while maintaining a small traction between a K562-Iz cell and an egg in an attempt to

detach them, and an increasing deformation of cell and egg was observed while the contact area seemed to remain intact (Fig. 1A,B). This observation proves that the forces linking the membranes together are strong enough to resist tractions, to induce extreme cell deformations and even to detach a fragment of K562-Iz cell, as shown by a fluorescent patch corresponding to the cell-egg adhesion area that remained stuck on the egg plasma membrane. Despite this strong adhesion, neither membrane fusion (i.e. fusion of both inner and outer leaflets of the cell and egg membranes) nor membrane hemi-fusion (i.e. fusion of the outer leaflets of the cell and egg membranes) was observed between Izumo1-expressing cells and eggs (supplementary material Fig. S2). To exclude the possibility that the transfection process itself could generate the robust adhesion observed, control experiments were performed with K562-Cd9-EGFP cells, which all yielded negative results (supplementary material Fig. S1C). These observations demonstrate that the adhesion does neither depend on the cell type nor on the transfection procedure. To confirm the direct involvement of Izumo1 and its receptor Juno in this robust adhesion, the same dual-pipette tests were performed in the presence of anti-Izumo1 or anti-Juno antibodies. Whereas the non-inhibitory mb125 anti-Izumo1 antibody did not affect cell-egg adhesion, the presence of the inhibitory mb34 anti-Izumo1 antibody did inhibit cell-egg adhesion (supplementary material Fig. S1C). Indeed, in the presence of 2 μ g/ml anti-Juno monoclonal antibody, K562-Iz cells and eggs were found to detach easily, and no adhesion patch was observed upon separation of the cell and the egg, even after more than 10 min of contact (Fig. 1C; supplementary material Movie 1). These results prove that Izumo1 and its egg receptor Juno are directly involved in the observed strong cell-egg adhesion. They emphasize the high robustness of the Izumo1-induced cell-egg adhesion that takes place within the first minutes of contact with its egg receptor Juno.

Egg Cd9 and Izumo1 are concomitantly recruited in the adhesion area and the recruitment kinetics of Izumo1 accelerates in the absence of Cd9

Co-incubation of the K562-Iz cells with zona-free eggs confirmed that Izumo1 binds to the egg membrane and that this binding is microvillar-area-specific (Fig. 2; supplementary material Movie 2). One striking feature of the incubation experiments or dual-pipette

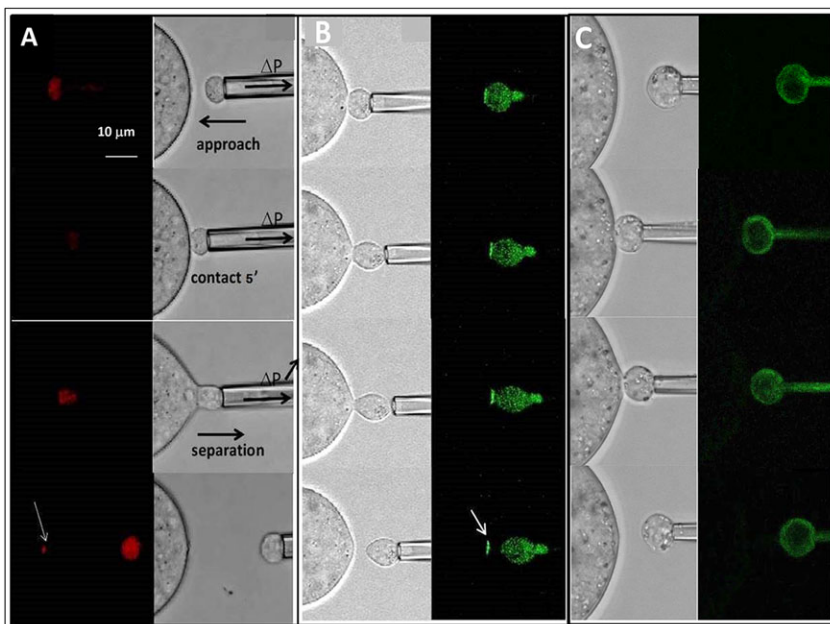


Fig. 1. Adhesion assays between oocytes and (A) K562-RFP-Iz and (B) K562-Iz-EGFP. After 5 min of contact time, the K562-Iz cell and the egg are pulled apart in an attempt to separate them, thus showing the deformation of the cell and of the egg. The aspiration in the K562-Iz pipette is increased in steps until cell separation occurs (aspiration in the K562 cell pipette increases from top to bottom). The bonds are strong enough to resist to traction that can induce high cell deformation and even detach a piece of K562-Iz cell (white arrows). (C) Adhesion assays between oocytes and K562-Iz-EGFP cells in presence of 2 μ g/ml anti-Juno monoclonal antibody. After 5 min of contact, the cells show little deformation upon traction and detach easily.

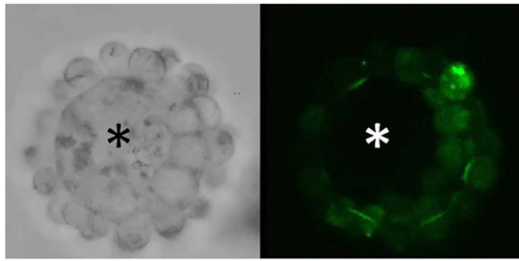


Fig. 2. Bright-field and confocal images of a zona-free oocyte with a high number of K562-Iz-EGFP cells attached to it. K562-Iz-EGFP are absent from the egg microvillar area (indicated by asterisks).

assays is the strong fluorescence enhancement that was systematically observed in each K562-, MDA- or U2OS-Iz-EGFP-egg contact areas (Fig. 1B; supplementary material Movie 3; supplementary material Fig. S2A and Fig. S3). Such fluorescence enhancement was also systematically observed when bringing into contact K562-RFP-Juno and K562-Iz-EGFP cells (supplementary material Movie 4). The Izumo1 molecules that contribute to the fluorescence enhancement of the contact areas were those trapped in the contact area and were therefore involved in the intercellular binding. By combining dual-pipette assays with time-lapse confocal observation, the kinetics of Izumo1 recruitment in the contact area were established in the presence (WT eggs) and absence (*Cd9* KO eggs) of *Cd9*. A good overview of the temporal and spatial evolution of Izumo1 in the contact area was achieved by following the distribution of fluorescence along the intersection line between the equatorial cell and egg planes as a function of time (Fig. 3A,B). Fig. 3A and B and supplementary material Movie 3 show a typical fluorescence

evolution as a function of time. The first signs of Izumo1 enrichment were observed within the first minute after the K562-Iz-EGFP cell and the egg were brought into contact. Intense isolated spots were nucleating in the contact area and the fluorescence intensity was increasing around these spots until it became uniform along the entire line. Fig. 3C compares the evolution of the fluorescence excess due to Izumo1 recruitment in the contact area between WT and *Cd9* KO eggs. For both systems, an increase in the fluorescence excess over contact time was observed, followed by a plateau reached after a few minutes. The first phase can be well described by a linear function with a positive slope associated with the velocity of Izumo1 enrichment. It is equal to 7 ± 1 fluorescence units/min in the presence of WT eggs (average \pm s.e.m. for five WT egg/K562-Iz-EGFP pairs) and to 20 ± 1 fluorescence units/min in the presence of *Cd9* KO eggs (three *Cd9* KO egg/K562-Iz-EGFP pairs) ($P=0.037$, Mann-Whitney *U* test). This dependence of Izumo1 enrichment velocity on *Cd9* is a striking result.

In order to further explore this question, the same experiment was performed with eggs expressing *Cd9*-EGFP and K562 cells expressing non-fluorescent Izumo1 (K562-RFP-Iz cells). As illustrated in Fig. 3D, the evolution of *Cd9* in the egg-cell contact area as a function of time showed striking similarities with Izumo1 enrichment (Fig. 3A; supplementary material Movie 5). A linear phase was obtained during the first minutes of contact, followed by a plateau. To ensure that this *CD9* enrichment was associated to egg-K562-Iz adhesion and was not the result of a simple mechanical contact, K562 cells without Izumo1 were brought into contact with *Cd9*-EGFP eggs and the evolution of *Cd9* in the contact area was recorded as a function of time. The two cells were maintained in close contact but did not develop any adhesion, due to the absence of Izumo1. As shown in Fig. 3D' and in supplementary material

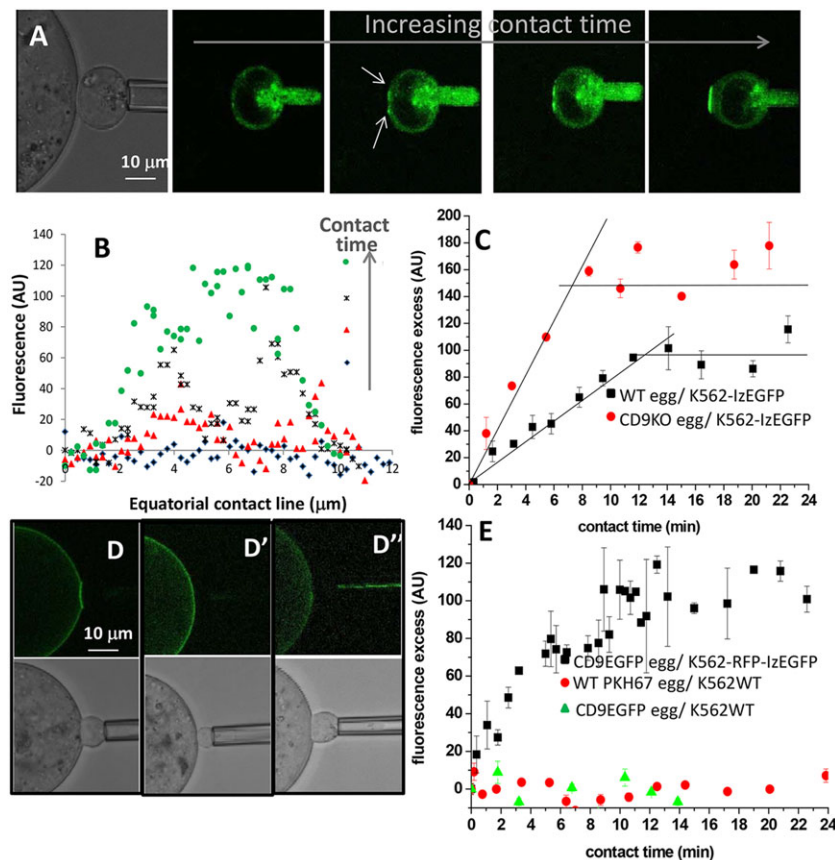


Fig. 3. Enrichment kinetics of Izumo1 in the cell-egg contact area. (A) Combination of dual-pipette assays with time-lapse confocal observation in the equatorial plane of cell and egg. A few tens of seconds after the K562-Iz-EGFP cell and the egg have been brought into contact, the first signs of Izumo1 enrichment in the contact area are observed. Intense isolated spots are nucleating in the contact area (arrows). The fluorescence intensity is increasing around these spots until it becomes uniform. (B) Associated temporal evolution of the fluorescence distribution along the intersection line between the equatorial cell and egg planes. (C) Representative curves of the temporal evolution of the fluorescence excess due to Izumo1 in the equatorial contact line for WT or *Cd9* KO eggs (five pairs were probed for WT eggs and three pairs for *Cd9* KO eggs) [fluorescence excess = $d_c(t) - d_c(0)$, with $d_c(t)$ equal to the mean fluorescence at contact time t and $d_c(0)$ equal to the mean fluorescence at contact time 0]. (D) Confocal images of K562-RFP-Iz cell/*Cd9*-EGFP egg pair, showing a *Cd9*-EGFP recruitment in the contact area, (D') K562-WT cell/*Cd9*-EGFP egg pair showing no *Cd9*-EGFP recruitment in the contact area and (D'') K562-RFP-Iz cell/PKH67-labeled WT egg pair showing no PKH67 recruitment in the contact area. (E) Representative associated temporal evolution curves of egg *Cd9*-EGFP for a K562-RFP-Iz cell/*Cd9*-EGFP egg pair (five pairs probed), of egg *Cd9*-EGFP for a K562-WT cell/*Cd9*-EGFP egg pair (six pairs probed) and of PKH67 for a K562-RFP-Iz cell/PKH67-labeled WT egg pair (five pairs probed).

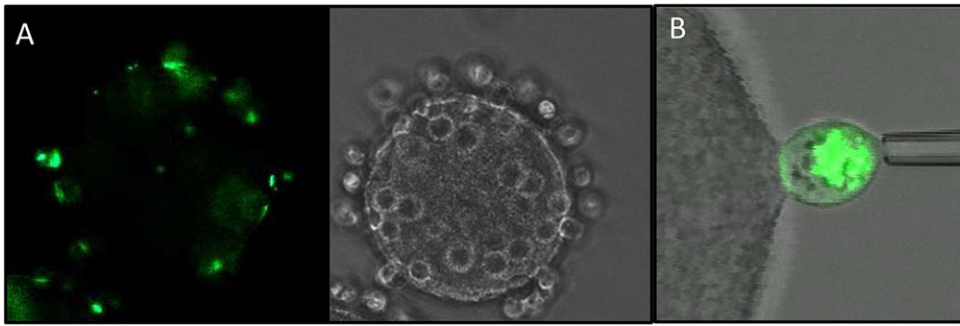


Fig. 4. Binding assays on human eggs. (A) Zona-free mature human oocytes were individually incubated with K562-Iz-EGFP cells for 45 min at 37 C with gentle agitation, washed and observed under an epifluorescence microscope. (B) Dual-pipette assays between a zona-free mature human oocyte and a K562-Iz-EGFP cell showing a robust adhesion and Izumo1 recruitment (five human egg/K562-Iz-EGFP pairs).

Movie 6, no enhancement of Cd9 was observed in the contact area, thus excluding the possibility that a non-adhesive contact could give rise to Cd9 recruitment. We also tested the possibility that the increase of Cd9 density in the contact area could stem from a putative local densification of the egg membrane (membrane micro-folding) triggered by the adhesion. Thus, WT eggs labeled with PKH 67, a green-fluorescent membrane linker, were brought into contact with K562-RFP-Iz cells. Despite the expected adhesion, no excess of fluorescence was observed in the contact area, excluding the densification hypothesis (Fig. 3D''; supplementary material Movie 7). These results prove that the fluorescence enhancement obtained in the contact area of a Cd9-EGFP egg and a K562-RFP-Iz cell was due to an enrichment of Cd9 correlated to the induced enrichment of Izumo1. Cd9 and Izumo1 recruitment were concomitant.

Molecules and mechanisms are conserved between mouse and human

The capacity of mouse Izumo1 to induce cell adhesion with human eggs was tested (i) by co-incubating K562-Iz-EGFP cells with human oocytes for 45 min (Fig. 4A), and (ii) by dual-pipette assays at single-cell level (Fig. 4B). For both types of experiments, K562-WT cells were used as control. Whereas no adhesion was observed in the absence of Izumo1, K562-Iz-EGFP remained attached to the egg membrane and an accumulation of Izumo1 in the contact area was observed. When preincubated with mb34 anti-Izumo1 antibody, K562-Iz-EGFP cells were unable to adhere to the egg anymore, thus confirming the direct involvement of Izumo1 in this adhesion.

In parallel, *in vitro* fertilization assays (IVF) were performed. Human zona-free eggs were inseminated with mouse sperm for 1 h, 4 h and overnight (19 h) in the presence or absence of mb34 anti-Izumo1 antibody (Table 1). Without mb34, fertilization rate and fertilization index were found to increase over co-incubation time. Following an overnight incubation, a large number (>15) of decondensed sperm nuclei were found inside the egg cytoplasm in the absence of mb34 antibody (Fig. 5A; supplementary material

Movie 8). On the other hand, no more than three decondensed sperm nuclei were observed when blocking Izumo1 with mb34 antibody (Fig. 5B; supplementary material Movie 9), thus showing the inhibition of the fusion process.

DISCUSSION

Three gamete membrane proteins have been shown to be essential for fertilization: the sperm protein Izumo1, its egg receptor Juno and the egg protein Cd9. It is essential to further understand the role of each protein and describe their interaction.

Our study mainly documents the interaction of these key proteins by showing their concomitant enrichment in the contact zone. Here, we demonstrate that the cell adhesion generated by Izumo1 and its receptor Juno takes place only a few seconds after the onset of contact and becomes very robust within a few minutes, which makes it compatible with the physiological gamete pre-fusion step. The consequence of this adhesion is not only an increased concentration of Izumo1 and therefore of any putative cis-membrane partner in the contact area, but also an increase of the egg tetraspanin Cd9, as evidenced by the concomitant enrichment of Izumo1 and Cd9 that we observed. Assuming that the observations made with three different cell lines are similar to those with sperm cells, the adhesion generated by Izumo1 during the adhesion phase of fertilization can provide the tight membrane apposition required in any fusion process. Furthermore, it is also able to concentrate in the fusion area the three essential molecular actors egg Cd9 and Juno and sperm Izumo1, as well as any of their respective, although yet unknown, cis-membrane partners.

As far as the roles of Cd9, Izumo1 and Juno are concerned, several roles can be proposed, such as adhesion protein, fusion protein or organizing the contact area for other partners that need to be identified and might be involved in adhesion or fusion.

Interestingly, the only sperm cells that seem to be able to fuse are acrosome-reacted sperm with Izumo1 gathered in the equatorial segment (Satouh et al., 2012), whereas egg Cd9 as well as sperm Izumo1 are able to interact in a cis way (Ellerman et al., 2009; Inoue et al., 2010a): the role of Izumo1 should therefore be to generate direct robust cell adhesion that would trigger a molecular organization suitable for fusion in the contact area of sperm and egg.

A direct role of Izumo1 in sperm-egg fusion is less probable, although a segment of its N-terminal region was recently found to have a helical structure and form ellipsoidal dimers that are reminiscent features of known fusion-related proteins, such as class I viral envelope proteins (Singh et al., 1999), SNAREs (Fasshauer et al., 1997; Rice et al., 1997), mitofusin (Koshiba et al., 2004; Detmer and Chan, 2007) and EEA1 (Christoforidis et al., 1999). Although it may be tempting to attribute to Izumo1 adhesion and fusion activities that are similar to those of SNARE proteins, i.e. first pulling the membranes together and then nucleating a fusion pore,

Table 1. Human zona-free eggs inseminated with mouse sperm in the presence or absence of mb34 anti-Izumo1 antibody

Insemination time (h)	Mb34 antibody present	Number of human eggs	Fertilization rate (%)	Fertilization index
1	No	8	50	1
4	No	4	75	1.5
	Yes	5	0	
19	No	4	75	>15
	Yes	2	50	1

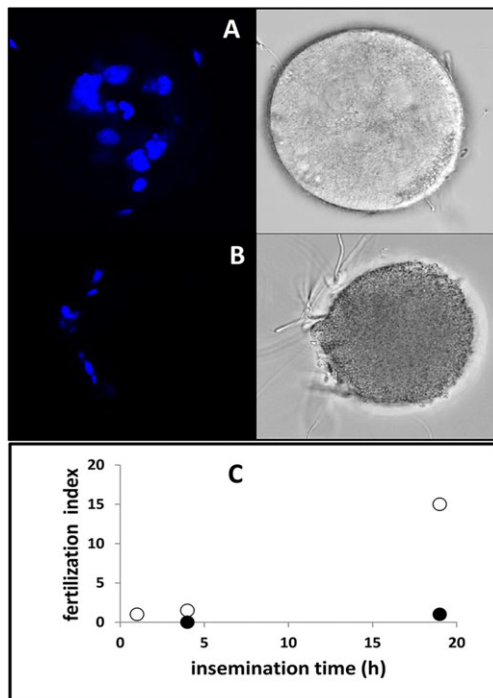


Fig. 5. Heterologous human egg-mouse sperm IVF. Human eggs were inseminated overnight with mouse sperm (A) in the absence (A) or presence (B) of mb34 anti-Izumo1 antibody. A large number (>15) of decondensed sperm nuclei were found in the egg cytoplasm in the absence of mb34 anti-Izumo1 antibody, whereas sperm bound to the egg membrane but did not penetrate the egg in the presence of mb34. (C) Fertilization index of human eggs as a function of insemination time in the absence (open circles) and in the presence (closed circles) of inhibitory mb34 anti-Izumo1 antibody.

there is no indication that Izumo1 does have these properties, as Izumo1 alone was neither able to induce the fusion nor the hemi-fusion of K562 and egg membranes. By co-incubating Izumo1-expressing HEK293 cells and Juno-expressing HEK293 cells without observing any fusion, Bianchi et al. reached the same conclusion (Bianchi et al., 2014). Other factors involved in the sperm are therefore absent in K562-Iz cells. One possibility could be that one of the Izumo1 cis-partners has fusogenic properties, even though ACE3, the only sperm protein thus far found to associate with Izumo1, does not appear to be involved in fertilization (Inoue et al., 2010a,b).

We have shown that Cd9 is enriched at the contact area between the egg and Izumo1-expressing cells. However, in its absence, these cells still adhere to the egg, showing that Cd9 is not essential for Izumo1-Juno bond formation. Nevertheless, we observed that Cd9 has an impact on the enrichment kinetics of Izumo1 at the contact zone between eggs and K562 cells. Cd9, like other tetraspanins, associates with several other surface proteins, which it might functionally regulate through membrane compartmentalization (Charrin et al., 2009). In addition, Cd9 and other tetraspanins dynamically accumulate, together with some of the molecules they associate with, at cell-cell contact areas. For example, endothelial Cd9 and Cd151 are recruited together with ICAM1 and VCAM1 to adhesion platforms formed upon interaction of endothelial cells with leucocytes (Barreiro et al., 2008). Furthermore, Cd81 is recruited to the immune synapse and regulates its maturation, especially the recruitment of Cd3, with which it interacts. On the egg membrane surface, Cd9 is known to organize other membrane proteins (Ziyyat et al., 2006). Regarding the dynamics of the proteins in the contact

zone, it has been shown that the tetraspanin Cd51 modifies the diffusion of the adhesion protein integrin $\alpha 6$ (Yang et al., 2012), consistent with the influence of Cd9 on Izumo1 recruitment kinetics.

A scenario that would reconcile these features and our observations suggests that on Cd9-positive eggs, Cd9 cis-associates with Juno (directly or indirectly) and thus forms part of a molecular complex with slower dynamics. Because these complexes diffuse more slowly at the egg membrane than isolated Juno molecules, the bond enrichment velocity would be higher in the absence of Cd9. Because Juno associates with Cd9, each Juno molecule involved in an intercellular bond would be related to an associated Cd9 and Izumo1 enrichment.

However, other scenarios are also possible. For instance, influence of Cd9 deletion on other gene expression, leading to an overexpression of Juno on the egg membrane, would indeed result in a higher probability to encounter Izumo1, consistent with the faster bond formation kinetics observed. Alternatively, the alteration of *Cd9* KO egg microvilli (Runge et al., 2007) might result in a smaller effective egg membrane surface in *Cd9* KO eggs than in WT eggs, which would lead to a higher enrichment velocity. Finally, but perhaps less likely, when present at the egg membrane, Cd9 might lead to an increase of the 'effective membrane viscosity' that would slow down the diffusion of Juno at the egg membrane and consequently reduce the kinetics of bond formation. However, when considered individually, none of these three hypotheses account for the concomitant Izumo1 and egg Cd9 enrichment. If not connected to Juno, even indirectly, one would have to imagine an independent process to account for this concomitance, such as a signaling process that would trap Cd9 in the area of contact as long as new bonds are formed between Izumo1 and its receptor.

Owing to Izumo1- and Juno-induced cell adhesion, Cd9 and Izumo1 accumulate in the adhesion area in a short time span compatible with the duration of the perfusion stage of physiological gamete adhesion.

It has long been known that human sperm are able to fertilize hamster eggs (Yanagimachi et al., 1976), and hamster zona-free oocytes have been used to assess the fertilizing ability of human sperm (Inoue et al., 2005), but to our knowledge the capacity for mouse sperm to adhere and fuse with human eggs has not been documented yet. In this study, we demonstrate that mouse sperm are able to adhere to and fuse with human eggs. The contribution of the mouse protein Izumo1 to the adhesion to human egg has been characterized through dual-pipette assays. Similar to mouse eggs, mouse Izumo1 protein was shown to generate robust adhesion with human oocytes, accompanied by a significant recruitment of Izumo1 in the contact area. In the presence of mb34 anti-Izumo1 antibody, this Izumo1-induced adhesion was inhibited, as was fusion of mouse sperm and human egg, as shown by the dramatic collapse of the fertilization index. These heterologous adhesion and fusion observations revealed the conserved nature of the sperm Izumo1/egg partner interactions between mouse and human. Again, it is tempting to propose a correlation between both phenomena (i.e. absence of Izumo1-induced adhesion and membrane fusion). This would suggest that fusion failure might originate from the absence of Izumo1- and Juno-induced adhesion and from the membrane reorganization resulting from this adhesion. Moreover, this process is conserved across species, as we show that the adhesion partners Izumo1 and its receptor Juno are interchangeable between mouse and human. The fact that mouse Izumo1 allows heterologous fusion with hamster eggs and that anti-human Izumo1 antibody drastically inhibits adhesion and fusion between human sperm and hamster eggs (Inoue et al., 2005) also supports this interchangeability among mammalian species.

In conclusion, we have established that within a time span of a few minutes, Izumo1 and its egg receptor Juno are not only able to generate a robust cell adhesion but also a local membrane organization, resulting in an accumulation of Cd9 and Izumo1, the essential proteins of fertilization. Because of the striking association of Izumo1 and Cd9 recruitment and of the slower Izumo1 dynamics induced by Cd9, we propose that the egg tetraspanin Cd9 is a partner of the Izumo1 receptor Juno. This time span of a few minutes is fully compatible with the prefusion contact time of the gametes in the physiological fertilization process. The observed accumulation of Izumo1 in the contact area during this period is also in agreement with the high Izumo1 density observed in the contact area of the fertilizing sperm and egg. Our results suggest a new scenario of the molecular mechanisms leading to gamete fusion: by inducing adhesion with Juno, Izumo1, the key actor in the sperm, accumulates at the fusion site and triggers the recruitment of Cd9, the key actor in the egg, both thereby conveying their own cis partners to build the gamete fusion machinery.

MATERIALS AND METHODS

Gametes

Human oocytes were donated by patients undergoing an assisted reproductive technology (ART) program for *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) in the assisted reproductive laboratory of Cochin's Hospital (Paris, France) after giving informed consent. Gametes were used according to the French Public Health code from 2013/10/28, part I, book II.

All procedures involving animal experimentation were conducted according to research animal protocols approved by the Ecole Normale Supérieure (ENS) Institutional Animal Care and Use Committee (B75-05-20).

The *Cd9*^{-/-} mouse line (Le Naour et al., 2000), the mouse line expressing Cd9-EGFP (a gift from K. Miyado, National Center for Child Health and Development, Tokyo, Japan) (Miyado et al., 2008) and WT mice from Charles River Laboratories were maintained at the ENS Paris animal facility. All mice lines were on the same C57BL/6 background.

Mouse sperm preparation

Sperm cells from 8- to 10-week-old male mice were expelled from the cauda epididymis and vas deferens into Ferticult IVF/3% BSA medium (FertiPro) under mineral oil. Sperm were then incubated at 37°C, 5% CO₂ in air for 2 h to induce capacitation.

Mouse egg preparation

Six- to eight-week-old WT, *Cd9*^{-/-} and CD9-EGFP female mice were superovulated by intraperitoneal injections, first of 5 IU pregnant mare serum gonadotropin (PMSG), followed by 5 IU human chorionic gonadotropin (hCG), 48 h apart. Cumulus-intact eggs were collected after 14 h into a Ferticult medium drop by tearing the oviductal ampulla from sacrificed mice. Eggs were separated from their cumuli by a brief incubation at 37°C in the presence of hyaluronidase IV-S (Sigma-Aldrich) (15 mg/ml). Mature eggs were selected on the basis of the presence of the first polar body. The ZP was subsequently removed by rapid treatment (<30 s) of the eggs with acidic Tyrode's solution (Sigma-Aldrich). Eggs were then incubated for 3 h at 37°C, 5% CO₂ in air to recover from the treatment.

Human egg preparation

In vitro-matured oocytes were obtained by incubation of metaphase I oocytes in micro-droplets of IVF culture medium under mineral oil, 5% CO₂, 5% O₂ and 37°C for 24 h. The ZP was chemically removed as described above or mechanically removed using a pair of micro-dissection scissors.

Transfection of K562, MDA and U2OS cells with mouse Izumo1, mouse Juno and human CD9

Mouse *Izumo1* cDNA (NM_00101813.1) encoding for Izumo1 (UniProt Q9D9J7) and the mutant *Izumo1*-C-terminal-EGFP were respectively

inserted (*Nhe1* and *Xho1* restriction sites) into the mammalian cloning vectors pCAGGS-RFP (a gift from X. Morin, ENS, France) and pEGFP-N1 (Clontech). The CD9-EGFP-coding region of human *Cd9* (NM_001769) was amplified by PCR. The PCR product was subcloned into pEGFP-N1 (Clontech). Using jetPEI (Polyplus) transfection reagent, K562 cells were transiently transfected with one of the two constructs encoding Izumo1, with the construct encoding human CD9-EGFP and with both the pCAGGS-RFP vector and the plasmid encoding the full mouse protein Juno (kindly provided by G. J. Wright, Wellcome Trust Sanger Institute, Hinxton, UK; see Bianchi et al., 2014). U2OS and MDA were transiently transfected with the Iz-EGFP construct. After 48 h, around 5% of the transfected cells were fluorescent for each group: red due to soluble RFP in the cell cytoplasm or green due to the presence of transmembrane Iz-EGFP or CD9-EGFP molecules. To test for the presence of Izumo1 or Juno on the cell membrane, the cells were incubated with primary non-inhibitory monoclonal anti-Izumo1 Mab125 (a gift from M. Okabe, Research Institute for Microbial Diseases, Osaka University, Japan; see Inoue et al., 2013) or anti-Juno/Folr4 monoclonal antibody, clone TH6 (BioLegend) in PBS for 1 h, washed and exposed for 30 min to goat-anti-rat Alexa 488 (Invitrogen) for RFP constructs and goat-anti-rat rhodamine (Invitrogen) for EGFP constructs. As expected, the same 5% red- or green-fluorescent cells did express Izumo1 or Juno at their membrane, whereas the remaining 95% non-fluorescent cells did not. This means that 100% of the red- or green-fluorescent cells did express Izumo1 or Juno, respectively. For dual-pipette experiments, cells expressing Izumo1 or Juno could therefore be selected on the basis of their fluorescence. In the following sections, the transfected cells will be referred as U2OS-Iz-EGFP, MDA-Iz-EGFP, K562-Iz-EGFP, K562-CD9-EGFP, K562-RFP-Iz and K562-RFP-Juno. Note that K562-RFP-Iz and K562-RFP-Juno cells are the only cells in which Izumo1 and Juno are not directly fluorescent, as these cells simultaneously express Izumo1 or Juno (not fluorescent) and soluble cytoplasmic RFP (fluorescent).

Zona-free binding assays of K562-Iz cells

WT zona-free eggs were individually incubated with ~2000 K562-RFP-Iz or K562-Iz-EGFP cells in a 20 µl drop of M2 (M7167, Sigma-Aldrich) 3% BSA medium for 45 min at 37°C with gentle agitation, washed and transferred into a 50 µl drop of M2 under mineral oil for confocal observation (Leica SP5II). Attached cells were counted all over the egg surface by performing 5 µm-thick vertical stacks. For human cell experiments, zona-free mature oocytes were individually incubated with ~8000 K562-Iz-EGFP or K562-WT cells for control in a 40 µl drop of 3% BSA-supplemented M2 medium under mineral oil for 45 min at 37°C.

Fusion assay

ZP-free mature human eggs were inseminated in the presence or absence of mb34 anti-Izumo1 antibodies (Inoue et al., 2013) with ~4000 capacitated motile mouse sperm in 20 µl Ferticult medium (FertiPro) for 1 h, 4 h or 19 h (overnight). Overnight insemination is the standard incubation time condition for human eggs (Tournaye et al., 1997; Ziyat et al., 2005; Barraud-Lange et al., 2008). For analysis, oocytes were washed and loaded with the DNA-specific fluorochrome Hoechst 33342 (Sigma-Aldrich) at 5 µg/ml for 5 min. Fusion was considered to have occurred when sperm nuclei were stained with Hoechst 33342 and decondensed.

Adhesion tests through dual-pipette assays

Dual-pipette assays (Chu et al., 2004) were used to test the adhesion between oocytes and the different cell lines used. On the stage of a Leica SP5II microscope, an egg and a cell were assembled onto the tips of two facing pipettes by gentle aspiration, brought into contact and maintained in that position for a controlled and adjustable time span. The aspiration in each pipette was accurately controlled and tuned.

Selection of cell lines that did not spontaneously adhere to the egg membrane

Those cell lines that did not spontaneously adhere to the egg were selected according to the following procedure: an egg and a cell were collected

and assembled onto the tips of two facing pipettes by low pipette suction (4 nN and 0.5 nN, respectively). The cell was brought into contact with the egg using a micromanipulator (supplementary material Fig. S1A). After 5 min of contact, the pipette bearing the cell was moved away. Depending on whether the cell was adhering to the egg or not, the cell remained attached either to the egg or to its pipette (supplementary material Fig. S1A', A''). The adhesion rate, shown in supplementary material Fig. S1B, was obtained for each cell line tested by submitting a high number of cell-egg pairs to this procedure. Only K562 and MDA cells were found to be 100% free of spontaneous adhesion to the egg membrane. Ten percent of U2OS cells showed a spontaneous adhesion to the egg, whereas between 50% and 100% of the COS7, HEK 293 and CHO cells were also adhering. These latter three cell lines, which spontaneously and strongly adhered to eggs, were considered unsuitable to study the action of one specific molecule. The three cell lines showing no or little spontaneous adhesion were transiently transfected to generate (i) K562 cells expressing Izumo1 (K562-RFP-Iz) at their membrane and RFP in the cytoplasm, (ii) K562, MDA and U2OS cells expressing Izumo1 with EGFP fused to the C-terminal part of Izumo1 (Iz-EGFP), thus allowing the direct visualization of the protein and (iii) K562 cells expressing CD9-EGFP at their membrane as control. Successfully transfected cells were then submitted to the same dual-pipette assays as described above. All Izumo1-expressing cells (100%) developed an adhesion with the eggs independent of the cell line, whereas CD9-EGFP-expressing K562 cells were still non-adherent to the eggs (supplementary material Fig. S1C).

Quantification of Izumo1-EGFP and CD9-EGFP recruitment

To quantify the enrichment kinetics of Izumo1 on K562 cells or of Cd9-EGFP on eggs, we combined dual-pipette assays and time-lapse confocal imaging by using the following procedure: the distribution of the fluorescence intensity over the contact line (Fig. 2A,B) between the equatorial plane of the cell and the egg was measured at contact time zero. Average fluorescence intensity was calculated from this distribution corresponding to the mean fluorescence intensity at contact time zero $d_c(0)$. The same step was carried out for several contact time points, providing a mean fluorescence intensity $d_c(t)$ for each time point. The fluorescence excess corresponding to $d_c(t) - d_c(0)$ was reported as a function of the cell-egg contact time. The temporal evolution of this fluorescence excess reflects the enrichment kinetics of the fluorescent molecule of interest and was compared between experiments by normalizing the intensity according to confocal parameters.

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.G., M.C. and F.P. developed the experimental approach; M.C., C.G., V.B.-L., C.B., A.Z., B.R., K.H. and A.N. performed experiments; C.G., M.C., F.P., N.R., E.P., P.S., C.B., E.R. and J.P.W. performed data analysis; C.G., M.C., N.R., E.P. and A.Z. prepared the manuscript.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.111534/-/DC1>

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DEV111534 – Supplementary material legends

Figure S1. Dual pipette tests probing the adhesion rate between ZP-free eggs and several cell lines transfected or non-transfected with Izumo1 or Cd9. (A) One cell is brought into contact with an egg and maintained in that position for 1 or 5 minutes. The aspirations in the pipettes are weak. (A') When pulling back the right pipette, the cells separate, meaning that the cell and the egg do not adhere. (A'') When pulling back the right pipette, the cells remain stuck, meaning that the cell and the egg do adhere. (B) Adhesion rate obtained for six different cell lines. (C) Adhesion rate obtained after the transfection with Izumo1 or Cd9 in presence or absence of mb125 or mb 34 anti-Izumo1 antibodies.

Figure S2. (A) Zona-free WT eggs were co-incubated for 1 h with K562-IzEGFP cells and rinsed. No Izumo1 is observed on the egg membrane except in the cell/egg contact areas, where fluorescence is strongly enhanced. The arrows indicate the Izumo1 enrichment of the contact areas. (B) Zona-Free WT eggs were co-incubated for 1 h with K562-RFP-Iz cells and rinsed. No RFP present in the K562-RFP-Iz, cytoplasm diffuses in the egg cytoplasm, meaning that no fusion has occurred. (C) K562-Iz cells were brought into contact with ZP-free eggs preloaded with Hoechst 33342. After 1 h of contact no Hoechst was observed inside the K562-Iz cells. (D) K562-RFP-Iz cells were brought into contact with ZP-free eggs stained with PKH67. After 1 h of contact, no PKH67 was observed on the cell membrane, meaning that neither fusion nor hemi-fusion had occurred.

Figure S3. Adhesion assays between WT-oocytes and (A) MDA-IzEGFP and (B) U2OS-IzEGFP. Izumo1 enrichment is observed in the contact area between MDA-IzEGFP and egg cells and U2OS-IzEGFP and egg cells. Pictures were taken after 20 min of contact.

Video 1. Inhibition of adhesion and of Izumo1 recruitment between WT eggs and K562- IzEGFP cells in presence of Juno antibody. Compare with Figure 1c.

Video 2. Stacks of a WT with a high number of K562-IzEGFP stuck to the microvillar area of the egg. Compare with Figure 2.

Video 3. Temporal evolution of fluorescence excess due to Izumo1 recruitment in the contact area of a WT egg and a K562-IzEGFP cell. Compare with Figure 1b and 3a.

Video 4. Izumo1 recruitment in the contact area of a K562-RFP-Juno cell and a K562-IzEGFP cell (five pairs probed).

Video 5. Time evolution of fluorescence excess due to egg Cd9 recruitment in the contact area of a Cd9EGFP egg and a K562-RFP-Iz cell. Compare with Figure 3d1.

Video 6. No adhesion and no fluorescence excess takes place in the contact area of Cd9EGFP eggs and K562-WT cells. Compare with Figure 3d2.

Video 7. Adhesion but no fluorescence excess takes place in the contact area of PKH67-labelled WT eggs and K562-RFP-Iz cell cells. Compare with Figure 3d3.

Video 8. Stacks of a human zona-free egg inseminated over night with mouse sperm. A large number of decondensed sperm nuclei are observed in the egg cytoplasm. Compare with Figure 5a.

Video 9. Stacks of two human zona-free eggs inseminated over night with mouse sperm and mb34 anti-IZUMO1 antibody. No more than three decondensed sperm DNA are observed in the egg cytoplasm. Compare with Figure 5b.

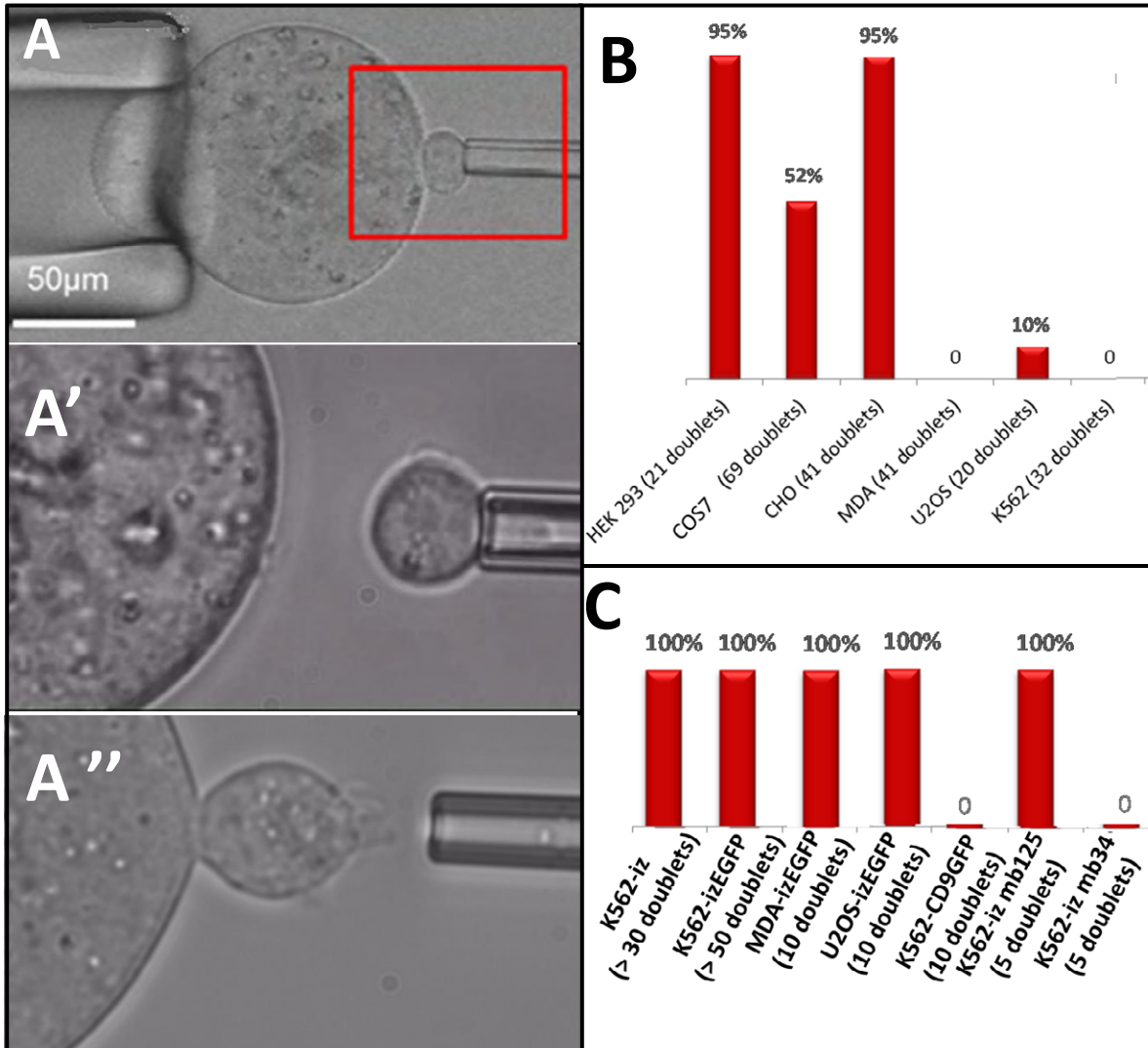


Figure S1

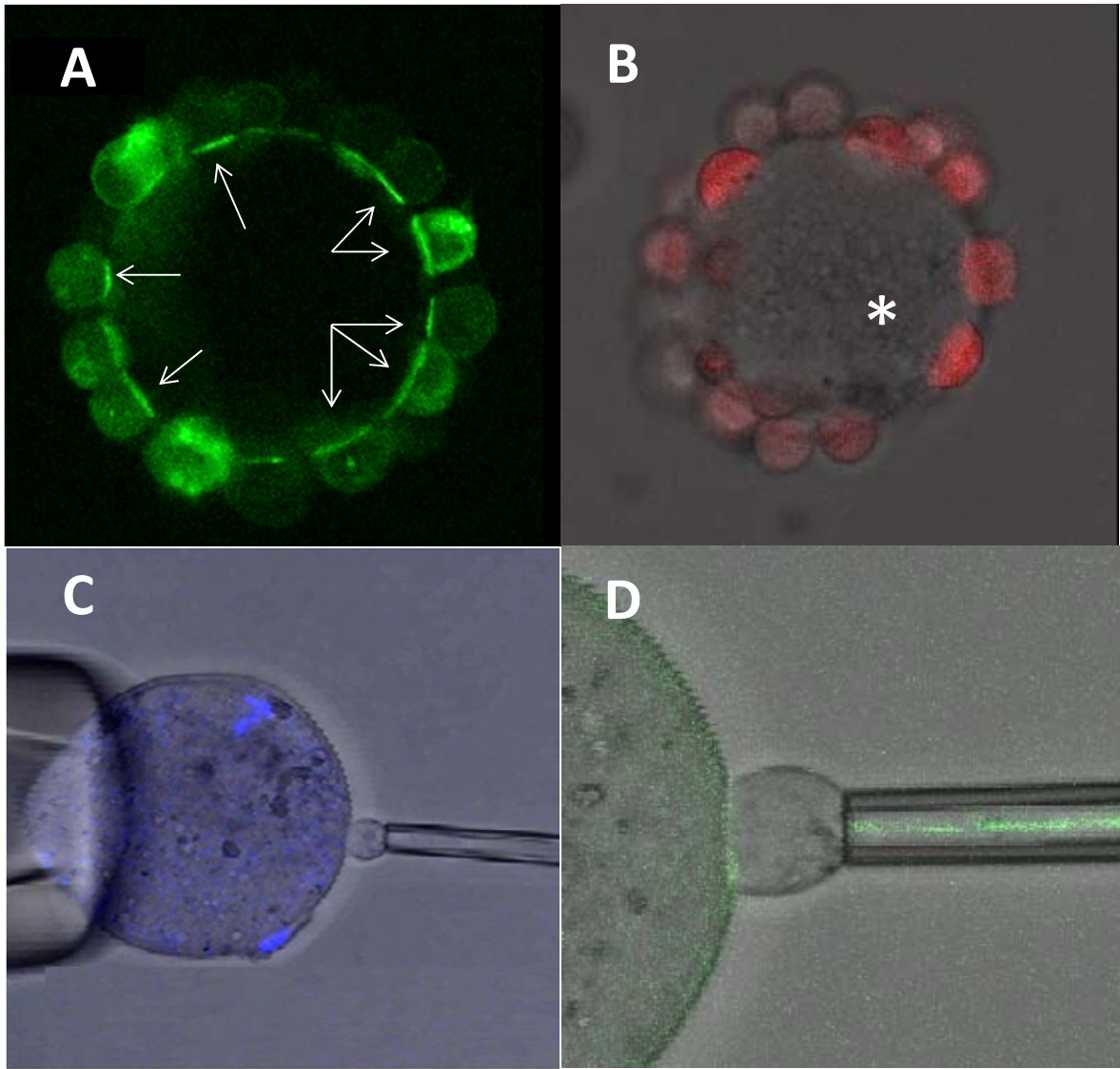


Figure S2

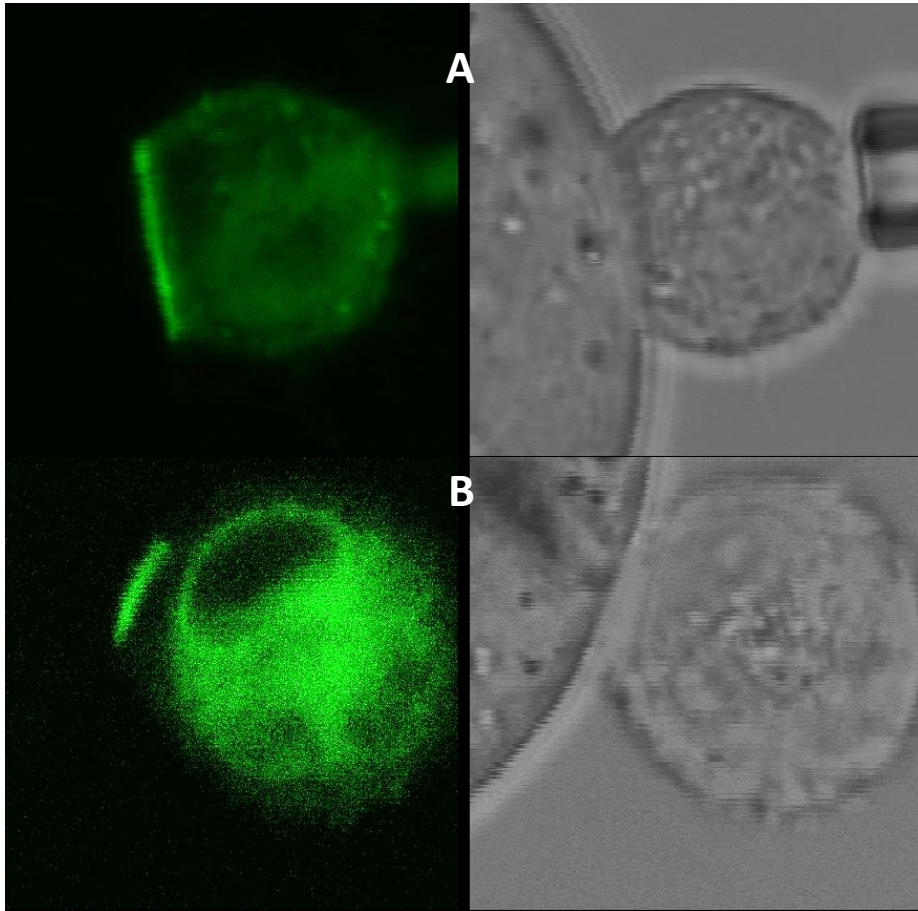
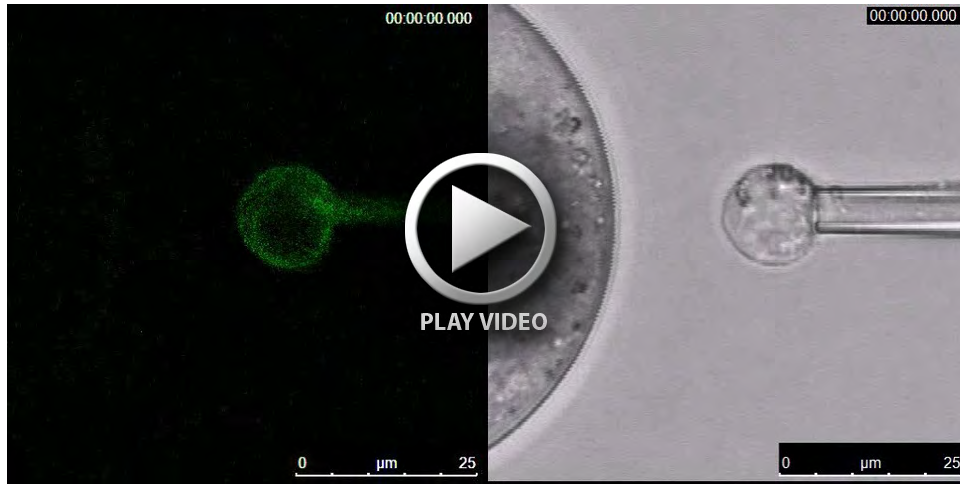
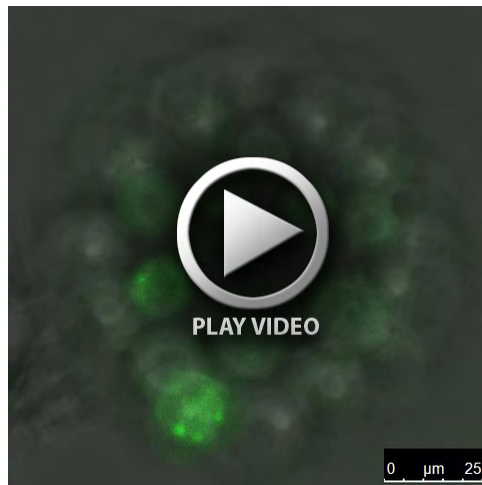


Figure S3



Movie 1.



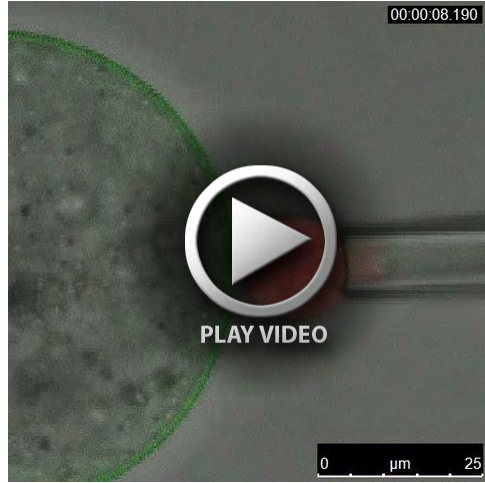
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Movie 3.



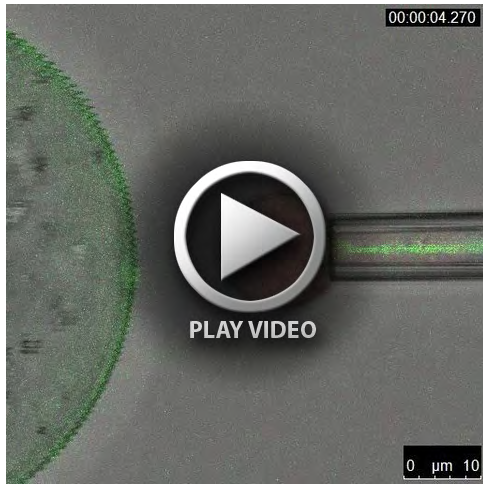
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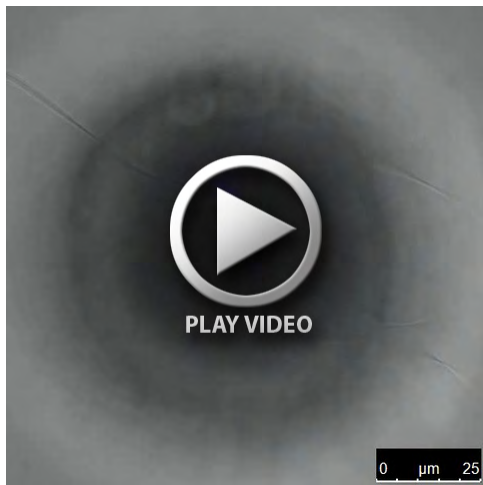
Movie 5.



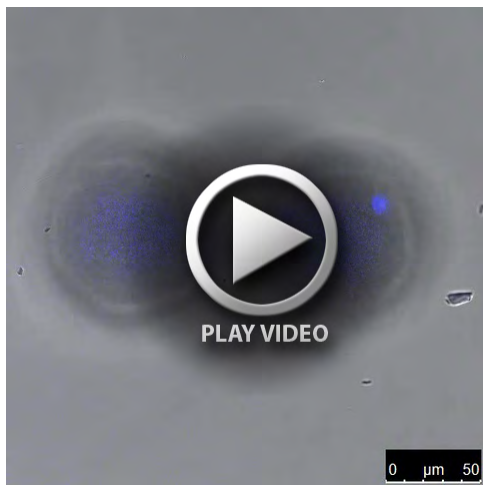
Movie 6.



Movie 7.



Movie 8.



Movie 9.