The Ca\textsuperscript{2+}-activate Cl\textsuperscript{-} channel Ano1 controls microvilli length and membrane surface area in the oocyte

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Summary Statement: The chloride channel Ano1 regulates cell surface area by modulating the length of cellular extensions known as microvilli through interaction with ERM proteins and the cytoskeleton.
Abstract

Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (CaCC) play important physiological functions in epithelia and other tissues. In frog oocytes the CaCC Ano1 regulates resting membrane potential and the block to polyspermy. Here we show that Ano1 expression increases the oocyte surface, revealing a novel function for Ano1 in regulating cell morphology. Confocal imaging shows that Ano1 increases microvilli length, which requires ERM protein-dependent linkage to the cytoskeleton. A dominant negative form of the ERM protein moesin, precludes the Ano1-dependent increase in membrane area. Furthermore, both full-length and the truncated dominant-negative forms of moesin co-localize with Ano1 to the microvilli, and the two proteins co-immunoprecipitate. The Ano1-moesin interaction limits Ano1 lateral membrane mobility, and contributes to microvilli scaffolding therefore stabilizing larger membrane structures. Collectively these results reveal a novel role for Ano1 in shaping the plasma membrane during oogenesis, with broad implications on the regulation of microvilli in epithelia.
**Introduction**

Oogenesis encompasses the growth phase of vertebrate oocytes during which time they accumulate macromolecular complexes essential for supporting early embryogenesis (Machaca, 2009). This is followed by a maturation phase before oocytes acquire the competency to be fertilized. Oocyte maturation encompasses the reductionist meiotic division in addition to cytoplasmic remodeling that prepares the mature egg for fertilization and the egg-to-embryo transition. In the frog oocyte, maturation is initiated in response to steroid hormones that induce a commitment to progressing through meiosis until the process is arrested again at metaphase of meiosis II until fertilization (Machaca, 2009; Nader et al., 2013). An important component of oocyte maturation is the remodeling of the Ca\(^{2+}\) signaling machinery to support a specialized Ca\(^{2+}\) transient at fertilization (Machaca, 2009; Nader et al., 2013). The Ca\(^{2+}\) signal at fertilization possesses specialized spatial and temporal dynamics that encode cellular processes --such as the block to polyspermy and the completion of meiosis-- in a sequential fashion to mediate the egg-to-embryo transition. Ca\(^{2+}\) is in fact the universal signal for egg activation at fertilization in all sexually reproducing species investigated to date, although the fertilization-specific Ca\(^{2+}\) transient takes on different spatio-temporal dynamics in a species specific fashion (Stricker, 1999).

Frogs have evolved two complimentary, but mechanistically distinct, Ca\(^{2+}\)-dependent mechanisms to efficiently block polyspermy given their large egg size. The fast block to polyspermy is practically immediate after fertilization, and is due the stimulation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCC) leading to membrane depolarization, which precludes further sperm-egg fusion (Cross and Elinson, 1980; Jaffe et al., 1983). On a slower time scale Ca\(^{2+}\) induces the fusion of cortical granules, which release enzymes that modify the egg’s extracellular matrix and mediate the more sustained slow-block to polyspermy (Grey et al., 1974; Wolf, 1974).

CaCC were first described in the *Xenopus* oocyte as regulators of the resting membrane potential in addition to their role in the block to polyspermy (Duran and Hartzell, 2011; Machaca et al., 2001). CaCC represent the primary current in the oocyte and their biophysical properties have been well characterized (Hartzell et al., 2005; Kuruma and Hartzell, 2000). CaCCs are broadly distributed among different species from invertebrates to mammals; and across diverse tissues in vertebrates (Kidd and Thorn, 2000; Robertson and Martin, 1996). Functionally CaCCs regulate multiple physiological processes, including epithelial secretion,
vision, olfaction, vascular tone, and neuronal excitability (Hartzell et al., 2005; Machaca et al., 2001). However, our understanding of CaCCs was hampered for many years by controversy regarding their molecular identity. However, in 2008 the *Xenopus* oocyte CaCC was identified as TMEM16A using an expression cloning approach (Schroeder et al., 2008), and knockdown of endogenous *Xenopus* TMEM16A was shown to reduce native Ca$^{2+}$-activated Cl$^{-}$ current (Yang et al., 2008). Given that TMEM16A is an anion channel with 8 transmembrane domains Yang et al. renamed the protein Anoctamin 1 or Ano1 (for anion+octa) (Yang et al., 2008). Mammalian genomes encode 10 anoctamin homologs. Ano1 is widely expressed in various epithelia where it is important for Cl$^{-}$ secretion, as illustrated by secretion defects in Ano1 knockout mice (Ousingsawat et al., 2009). In addition to its role as a secretory channel in epithelia, Ano1 has also been shown to play important roles in gut smooth muscle, airway smooth muscle, nociception, the biogenesis of the primary cilium, and interestingly Ano1 expression has been shown to correlate with cancer providing a good biomarker for some cancers (Duran and Hartzell, 2011; Ruppersburg and Hartzell, 2014).

Here we describe a novel function of Ano1 that is independent from its channel activity. We show that Ano1 regulates the length and diameter of microvilli in *Xenopus* oocytes through its interaction with ERM proteins and the actin cytoskeleton. The number of microvilli in the oocyte has been estimated to be $\approx 40 \times 10^6$, and they expand the surface of the oocyte by 3-4 folds (Kado et al., 1981). The structure of a microvillus has been extensively described in the intestinal brush border (Brown and McKnight, 2010; Crawley et al., 2014). Microvilli are shaped around parallel bundles of actin fibers that stabilize the thin highly curved microvillus membrane against significant physical forces including surface tension, bending stiffness and membrane-cytoskeleton adhesion energy (Crawley et al., 2014; Nambiar et al., 2010). Single actin filaments are insufficient to overcome these forces generating the need for actin bundles that are cross-linked and stabilized by a family of actin-binding proteins, including villin, fimbrin and espin (Crawley et al., 2014). In turn actin bundles are physically linked to integral plasma membrane proteins and membrane lipids through two primary classes of proteins, the unconventional myosins and ERM proteins. The ERM family (Ezrin, Radixin and Moesin) of proteins share 70-80% identity among the different members, and are highly conserved throughout the animal kingdom (Arpin et al., 2011). The ERM proteins bind membrane lipids such as PIP2 and also connect the cytoskeleton to a wide variety of transmembrane proteins such as ions channels (e.g. CFTR) (Guerra et al., 2005), G-protein coupled receptors (e.g. κ-opioid receptor and β2-adrenergic receptor) (Heydorn et al., 2004;
Li et al., 2002), as well as adhesion proteins (e.g. ICAM1, CD44) (Bretscher et al., 2002). The ERM proteins share a common structure: the N-terminal (N_{ter}) domain --termed FERM (4.1 protein and ERM) or N-ERMAD (ERM Associated Domain)-- is composed of three lobes that mediate ERM binding to the plasma membrane. The C-terminal or C-ERMAD domain contains the binding site for actin (see Fig. 3A). At rest the protein is folded and the C-ERMAD and FERM domains are in contact resulting in auto-inhibition of ERM crosslinking function. ERM proteins need to unfold to unmask the functional sites allowing binding to their specific partners, a process promoted by phosphorylation of a threonine residue in the C-ERMAD domain (Fehon et al., 2010). The interaction of the ERM protein with its target at the plasma membrane involves either direct binding to the cytoplasmic portion of membrane proteins, or requires linker proteins such as EBP50/NHERF (ERM-Binding Phosphoprotein 50/Na-H^+ Exchanger Regulatory Factor) or E3KARP (NHE type 3 Kinase Regulatory Protein) that contain a PDZ domain (Fehon et al., 2010).

Data presented herein reveal a novel function of Ano1 that is independent of channel activity in modulating the structure of microvilli. Ano1 supports microvilli elongation through its interaction with ERM in the oocyte. This results in an expansion of membrane surface area as a function of Ano1 expression. Hence, Ano1 plays a critical role in regulating membrane surface area in the oocyte by physically linking to the cytoskeleton through ERM proteins. Oocyte maturation is associated with the loss of microvilli due to endocytosis a process that is critical for the biogenesis of the first polarized epithelium leading to blastocoel formation.
Results

Ano1 overexpression increases membrane capacitance

The effect of expression of *Xenopus laevis* Ano1 (10ng RNA) on CaCC currents in stage VI oocytes was evaluated using a voltage jump from the resting membrane potential of -30 mV to +40 mV (Fig. 1A). There was a significant increase in the resting current recorded at +40 mV in cells expressing Ano1 (Fig. 1B), that was associated with a decrease in membrane resistance (Fig. 1C) and a small but significant depolarization (Fig. 1D). At the resting membrane potential (i.e. -35.8 ± 0.8 mV, n=42) the driving force for chloride ions is minimal, with the reversal potential for Cl⁻ (E\(_{Cl}\)) in our recording conditions being between -25 mV and -30 mV (Barish, 1983; Costa et al., 1989). Furthermore, the current through CaCC in the *Xenopus* oocyte displays a strong outward rectification at depolarizing potential and low Ca\(^{2+}\) concentration. Together this explains the moderate effect of Ano1 expression on the membrane potential.

To increase intracellular Ca\(^{2+}\) and stimulate Ano1, we mobilized intracellular Ca\(^{2+}\) stores using ionomycin, and recorded Cl⁻ currents at the peak of the Ca\(^{2+}\) release phase (Courjaret and Machaca, 2014). As illustrated in Figure 1A and 1E the current recorded at +40 mV was greatly increased in response to intracellular Ca\(^{2+}\) release in Ano1 expressing cells. This indicates that the over-expressed Ano1 increases the Ca\(^{2+}\)-activated chloride currents in the oocyte with similar properties to the endogenous CaCC, as previously reported in axolotl oocytes (Schroeder et al., 2008). Strikingly however, these expected changes in membrane conductance properties following Ano1 expression were associated with a significant increase in the membrane capacitance (from 230.6 ± 4.4 nF to 311.8 ± 9.2 nF, Fig. 1F). This increase in membrane surface area is specific to Ano1, since overexpression of another ion channel, Orai1, does not affect membrane capacitance (Fig. 1G).

Ano1 expression changes the structure of the microvilli independent of channel function

The theoretical surface area of a smooth oocyte of 1.2–1.3mm in diameter would result in a cell capacitance of 45-55 nF (with a conversion factor of 1 µF/cm\(^2\), (Hille, 1992)). This value is close to the measured capacitance of *Xenopus* eggs where the microvilli have been lost during oocyte maturation. When the stage VI oocyte matures, a significant portion of the membrane area in microvilli is internalized leading to a loss of microvilli and flattening of the
cell membrane (Kado et al., 1981). While the apparent diameter of the cell does not change during oocyte maturation, the cell capacitance drops from 224.0±4.5 nF (n=51) to 74.3±2.8 (n=67 nF) (see also (Kado et al., 1981; Machaca and Haun, 2000; Peres and Bernardini, 1985)). This indicates that the microvilli surface accounts for the majority of the cell capacitance.

We imaged oocyte microvilli and evaluated their dimensions under different conditions. We first measured the size and shape of microvilli on the animal pole of naïve cells using Wheat Germ Agglutinin to stain the plasma membrane of live oocytes (El Jouni et al., 2008). Cells were cooled to 4°C during the staining process to limit vesicular trafficking (Fig. 2A). This was then compared with membrane structure in cells expressing Cherry-tagged Ano1 by imaging the red fluorescence of mCherry. The first striking observation was the presence of very long microvilli and, on some cells, of large vesicular-shaped structures consisting of broad membrane extensions of shorter length, similar to the length of microvilli in control cells (Fig. 2A, arrowheads). These structure are likely fused microvilli. Both features were absent in naïve cells (Fig. 2A). We first quantified the maximum microvillus length in naïve, Ano1 expressing cells, and cells expressing the Ca^{2+} channel Orai1-GFP. The length of the 10 longer microvilli was measured in a single orthogonal section for each cell and averaged. There was a significant increase in the maximum microvillus length when Ano1-mCh was expressed but not with Orai1-GFP (Fig. 2B). Additionally, the maximum microvillus diameter was also increased in Ano1-mCh expressing, but not Orai1 expressing, cells (Fig. 2C). To obtain an estimate of the average increase in the length of the microvilli due to Ano1 expression, we analyzed confocal z-stacks by plotting the intensity of the fluorescent signal across orthogonal sections from the membrane intracellular boundary to the tip of the microvilli (Fig. 2D-F). The width of the curves at half-maximum intensity provides a good estimate for the average microvillus length, which was significantly increased in Ano1 expressing cells (Fig. 2F). In addition, we used a mesh reconstruction algorithm of the oocyte surface (Fig. 2G) that allows the quantification of the actual membrane surface. In naïve cells the estimated actual membrane surface was 2,880±260 µm^2 (n=17) for a 400 µm^2 section, indicating that the convolutions increase the membrane surface by ~7.2x at the animal pole of naïve cells. This value is larger than the theoretical value of a ~5 fold increase for the whole cell but this is probably due to the uneven distribution of the microvilli and their higher density at the animal pole, and that the measure of the surface is highly sensitive to the mesh parameters. In cells expressing Ano1 the increase factor reached ~10.3x (Fig. 2H). The mesh
analysis suggests that the increased length of microvilli in Ano1 expressing cells is associated with a decrease in their density. To obtain an estimate of microvilli density in control versus Ano1 expressing cells, we used the average microvilli length and maximal diameter measured as discussed above to calculate microvilli density for a theoretical oocyte of 1.25 mm in diameter (surface area of 4.9 mm$^2$, equivalent to 49 nF at 1µF/cm$^2$). The measured surface area of a control oocyte is ~23 mm$^2$ (230 nF), yielding a total microvilli surface area of 18.1 mm$^2$. In control cells the average microvillus length is 1.74 µm with a maximum diameter of 0.42 µm. If we model the microvillus as a cone this results in an individual microvillus surface area of 1.29 µm$^2$, and a total number of ~14x10$^6$ (low estimate) with a density of 2.9x10$^6$ villi/mm$^2$. Ano1 expressing cells have a surface area of 31.2 mm$^2$ (312 nF) with an average microvillus length of 2.87 µm and a maximum diameter of 0.91 µm. This gives a microvillus surface area of 4.8 µm$^2$ and a total of 5.5x10$^6$ microvilli with a density of 1.1x10$^6$ villi/mm$^2$. This represents a 2-3 fold reduction in microvilli density following Ano1 expression. These calculations are consistent with the imaging data (Fig. 2A), the mesh analysis (Fig. 2G) and the dome-like structures, which could be due to several microvilli fusing together (Fig. 2A, arrowheads).

To assess whether Ano1 Cl$^-$ channel function is required for its role in modulating microvilli length, we blocked channel function using the Ano1 inhibitor MONNA (Oh et al., 2013) during the expression of Ano1. MONNA (1 µM) effectively blocked the resting CaCC current in both naïve oocyte (Fig. 3A), and in oocytes expressing Ano1 (Fig. 3B). However, despite the fact that MONNA blocked the CaCC current it had no effect on the increased capacitance observed following Ano1 expression (Fig. 3C). This is also visible in the confocal images recorded on cells overexpressing Ano1 where no change in the microvilli density or structure is visible (Fig. 3D). Collectively these results support the conclusion that Ano1 increases the membrane capacitance by increasing the length and diameter of microvilli while decreasing their overall density and that this effect does not require the activation of the channel.

Expression of the ERM protein, moesin, modulates oocyte surface area

Perez-Cornejo et al. comprehensively defined the Ano1 interactome, and identified the ERM protein family as robust Ano1 binding partners, thus providing a molecular link between the plasma membrane and the cytoskeleton (Perez-Cornejo et al., 2012). They also found that the
interaction between Ano1 and moesin influences the presence of both proteins at the plasma membrane. As discussed in the introduction the microvillus membrane associates with the actin cytoskeleton allowing it to overcome the physical forces against its bending to fit the structure of the microvillus. The observation that Ano1 expression increases microvilli length suggests that Ano1 links the plasma membrane to the cytoskeleton through ERMs, thus supporting the increased microvillus length and area. To test whether ERM proteins provide the molecular link between Ano1 and the cytoskeleton within the microvilli, we expressed different human moesin constructs and tested the effect on membrane surface area. We chose moesin as the ERM protein since it is known to be expressed in *Xenopus* oocytes (Thorn et al., 1999). Human and *Xenopus* moesin share 88% overall identity, reaching 97% in the functional FERM domain and 100% in the F-actin binding site (Fig. 4A; Supp. Fig. 1). Cells were injected with cRNAs coding for three different proteins: full length moesin (FL-moesin), a truncated form containing the FERM domain (the first 381 residues, Nter-moesin), and a protein encoding the F-actin-binding domain (382-end; Cter-moesin) (Fig. 4A) (Amieva et al., 1999). Oocytes tolerated the expression of FL-moesin well, however, prolonged overexpression of Nter-moesin lead to depigmentation of the animal pole and cell death at longer time points (Fig. 4B). Cter-moesin was poorly expressed (Fig. 6). Microvilli were visualized using WGA staining and orthogonal reconstruction of confocal z-stacks. There was no obvious effect of the FL-moesin and Cter-moesin expression on the plasma membrane (Fig. 4B). For cells expressing the Nter-moesin membrane structure depended on the extent of the discoloration of the animal pole of the oocyte. In non-discolored cells, some microvilli were longer while patches of the membrane lacked microvilli (Supp. Fig. 2). When the cells were discolored the membrane became more chaotic, showing large round shaped structures stained by WGA and no visible microvilli (Supp. Fig. 2). Therefore, for the rest of the experiments with Nter-moesin, analysis was limited to cells with no discoloration on the animal pole to avoid non-specific toxicity effects. The cell capacitance was significantly increased following expression of FL-moesin (Fig. 4C). In contrast, expression of the Nter truncation resulted in a reduction of membrane capacitance (Fig. 4C). The increase in membrane area following FL-moesin expression is likely due to its crosslinking function between the cytoskeleton and plasma membrane. The decrease in membrane capacitance correlated with the levels of expression of Nter-moesin in a dose-dependent fashion (Fig. 4D).

To better control the expression levels of the different moesin truncations, we further normalized for the cRNA copy number injected per oocyte, based on the size of the different
constructs (5ng WT, 4 ng Nter-moesin and 3 ng Cter-moesin). The results were comparable to what was obtained with the injection of 10ng of cRNA: a slight increase in the capacitance for the expression of the FL-moesin and a reduction for the Nter-moesin (Supp. Fig. 3A). There was no significant change in the membrane potential or in the membrane resistance (Supp. Fig. 3B and 3C). Importantly, expression of the different moesin constructs did not significantly alter the levels of the CaCC current at rest or after mobilization of store Ca\(^{2+}\) stores with ionomycin (Supp. Fig. 3D and 3E). This indicates that expression of FL, Nter or Cter moesin does not significantly alter the membrane residence of Ano1, and that the observed decrease in membrane capacitance in Nter expressing cells is due to a disruption of the molecular link between the cytoskeleton and the plasma membrane. The absence of any phenotype with Cter expression is likely due to the poor expression of this construct as shown below.

**Moesin interacts with Ano1**

We next co-expressed Ano1-mCh with FL-moesin and its fragments to evaluate their co-localization and influence on Ano1 expression and function. We did not observe any synergistic effect of co-expression of Ano1 with FL-moesin or Cter with both resulting in the same level of enhancement of membrane capacitance as observed with expression of Ano1 alone (Fig. 5B). In contrast, co-expression of Nter-moesin with Ano1 reverts the enhancement of membrane surface area observed following Ano1 expression (Fig. 5A and 5B). We interpret this result to indicate that Nter saturates the new Ano1 ERM binding sites generated following Ano1 overexpression, and as such inhibits the microvilli elongation and the associated increase in membrane surface area. However, another potential explanation is that moesin is somewhat involved in the trafficking of Ano1 to the plasma membrane and with the co-expression of the dominant negative Nter, trafficking is inhibited leading to a block of the increase in surface area. However this is not the case as revealed by measurements of the amplitude (Fig. 5C) and density (Fig. 5D) of the CaCC current (measured at the peak of the ionomycin-induced Ca\(^{2+}\) release event). Co-expression of Nter-moesin did not reduce the amplitude (Fig. 5C) or density (Fig. 5D) of the current, showing that overexpressed Ano1 reaches the plasma membrane to the same extent whether expressed alone of co-expressed with Nter.
We further investigated the interaction between Ano1 and moesin in vivo using co-localization and immunoprecipitation. The different moesin constructs used above were tagged with GFP and co-expressed with Ano1-mCh to evaluate their respective subcellular localization. When the N
ter-moesin-GFP was expressed the discoloration of the cell and reduction of the microvilli could be observed only after significantly longer periods of expression (3 to 5 days as opposed to 24/48h in the case of untagged N
ter-moesin), although the fluorescence was clearly observed before. We therefore used cells with clear expression of N
ter-moesin but without any discoloration for our analysis. In the case of the C
ter-moesin-GFP, the fluorescence of the GFP could be detected on some cells but was very faint or absent in most of them (Fig. 6A). The co-localization of the Ano1 and moesin was then quantified using orthogonal projections from z-stacks (Fig. 6 A). Two values were used as a reference: for maximum co-localization we measured the values obtained from an experiment where Ano1-mCh expressing cells were stained with WGA. For minimum co-localization (exclusion) we used the Pearson’s coefficient obtained from two proteins that are not located in the same cellular compartment: the Ca\textsuperscript{2+} sensor STIM1 (located in the endoplasmic reticulum) and the plasma membrane Ca\textsuperscript{2+} channel Orai1 (Fig. 6B, S/O) (Courjaret and Machaca, 2014). There was a high degree of co-localization between Ano1 and either FL-moesin or N
ter-moesin (Fig. 6B). For the C
ter-moesin the co-localization analysis was limited only to selected cells that expressed a significant GFP signal and showed reduced co-localization (Fig. 6B), however this could be due to the poor expression of C
ter-moesin in the oocyte. Nonetheless, these results indicate that moesin is located at the plasma membrane and is bound through its N-terminal domain, to its target. Next, to confirm that one of the targets of moesin in the plasma membrane is Ano1, we tested the ability of both proteins to co-immunoprecipitate. The experiments were performed on cells expressing the GFP tagged moesin constructs. As illustrated in Figure 6C, immunoprecipitation of the FL-moesin-GFP or N
ter-moesin-GFP pulls down Ano1 revealing a single immunoreactive band close to the theoretical value of 113 kDa for Ano1. These results show that Ano1 interacts with moesin in vivo through its N
ter domain leading to co-localization of both proteins to microvilli.

To further support the finding that moesin interacts with Ano1, we next performed FRAP experiments in order to measure Ano1 diffusion in the plane of the membrane (see methods). We used cells expressing Ano1-mCh with or without untagged moesin proteins and bleached a selected membrane area while monitoring the rate of fluorescence recovery over time (Fig. 7A and 7B). Proteins responsible for the recovery of fluorescence after photobleaching can
originate from the cytoplasm (fast diffusion) and from the plasma membrane (slower) (Goehring et al., 2010). In our case, the bleaching is performed using a line scan over the z-axis; consequently the intracellular pool of proteins under the membrane is bleached as well. The existence of a substantive intracellular Ano1 pool ready to fuse with the plasma membrane is also unlikely since, even under conditions of Ano1 overexpression, no detectable vesicular Ano1-positive fluorescence is visible (Fig. 6A). Consequently most of the fluorescence recovery after photobleaching is likely to occur from lateral diffusion in the plane of the plasma membrane. There was a significant increase in the rate of recovery in cells expressing the Nter-moesin but no major change for the FL or the Cter fragment (Fig. 7B and 7C). This indicates that the linkage of Ano1 to the cytoskeleton through ERM reduces its mobility in the membrane and is disrupted when the Nter-moesin competes for the Ano1 binding site.

To confirm the role of ERM-Ano1 interactions in mediating the increase in membrane surface area following Ano1 expression, we sought to express different fragments of Ano1 that would act as binding sites for ERM. Given that the Ano1 binding site for ERM proteins is not known, we expressed the large cytoplasmic N-terminal fragment of Ano1 (aa 1-322) and a longer fragment that ends just before the presumed 3rd transmembrane domain of Ano1 (Supp. Fig. 4). Expression of the 1-322 fragment of Ano1 effectively reduced membrane capacitance (Supp. Fig. 4C), without affecting CaCC current (Supp. Fig. 4B). This supports the role of ERM-Ano1 interactions in modulating membrane surface area.
Discussion

To increase the exchange capacity of a cell while limiting volume variations (i.e. increase the surface/volume ratio) epithelial cells, oocytes, lymphocytes, hepatocytes and placental cells grow thin extensions termed microvilli. They extend the membrane surface to facilitate exchanges (absorption and secretion), and depending on the cell type, can also serve mechanical functions, including physical support and mechanotransduction. Densely packed microvilli form a brush border extending ~10-fold the surface area of epithelial cells such as the ones lining the small intestine and the proximal tubule of the kidney (Crawley et al., 2014; Helander and Fandriks, 2014). *Xenopus* oocytes are covered with microvilli that vary in shape and density during the growth and development of the gamete. This is particularly obvious following oocyte maturation in preparation for fertilization. During that process the number of microvilli is drastically reduced at a fast pace (2000/sec) inducing a large drop in the membrane capacitance in a few hours, without any change in the apparent diameter of the oocyte (Kado et al., 1981; Machaca and Haun, 2000). This rapid internalization of membranes forming the microvilli generates an intracellular vesicular pool that is essential for the rapid cellularization during early embryogenesis (Muller and Hausen, 1995). Furthermore, this membrane internalization forms the initial step in the biogenesis of the first polarized epithelium in the embryo that supports vectorial transport of Na$^+$ and water into the blastocoel (Muller, 2001). This is because endocytosis during oocyte maturation is associated with a selective internalization on membrane proteins resulting in two populations of membranes the egg plasma membrane, which represents the future apical membrane of the epithelium; and the internalized vesicular pool, which represents the future basolateral portion, with distinct protein content. For example, Orai1 and PMCA are internalized during oocyte maturation but not Ano1 (El Jouni et al., 2008; Yu et al., 2009; Yu et al., 2010).

Here we show that Ano1 regulates microvilli length and as such membrane surface area independently of its channel function. Ano1 expression increases the oocyte surface area and microvilli length and diameter (Fig. 1 and 2). The Ano1-dependent effect on microvilli is mediated through ERM proteins, as co-expression of the dominant-negative $N_{ter}$ domain of moesin with Ano1 abolishes the Ano1-dependent increase in microvilli length and surface area (Fig. 5). Furthermore, full length moesin and the $N_{ter}$ domain co-localize and co-immunoprecipitate with Ano1 (Fig. 6). In addition, expression co-expression of Ano1 with $N_{ter}$-moesin increases Ano1 lateral mobility in the plane of the membrane supporting the conclusion that moesin links Ano1 to the cytoskeleton, and as such anchors it and limits its
lateral diffusion (Fig. 7). Collectively, our results support a model where Ano1 traffics to the microvillus where it binds ERM proteins and cross-links to the cytoskeleton. This stabilizes the microvillus membrane revealing an important role of Ano1 in microvilli biogenesis in the oocyte.

Not surprisingly expression of the Nter-moesin alone decreases membrane surface area (Fig. 4). This is likely partly due to inhibition of Ano1-ERM interaction, but also to the inhibition of other interactions between ERM and plasma membrane proteins and lipids. This is highlighted by the discoloration observed in the oocyte at high levels or after prolonged expression of Nter-moesin (Fig. 4). Interestingly however, co-expression of Ano1 with full length moesin does not show a synergistic effect over Ano1 expression alone. This could be due a physical limit on the length of the microvilli independent of the amount of available scaffolding proteins. The effect of Ano1 on microvilli can be readily dissociated from its channel function. Blocking the activation of Ano1 using MONNA does not affect the ability of overexpressed Ano1 to increase microvilli length and membrane capacitance. Conversely, when the Ano1-ERM link is perturbed, either by expressing the dominant-negative Nter-moesin mutant (Fig. 5) or the Ano1 N-terminal cytosolic fragment (Supp. Fig. 4), the global chloride current is not affected yet the increased capacitance due to Ano1 expression is reversed. This shows that Ano1 regulates microvilli length through a physical/scaffolding interaction and not through its Cl⁻ channel ion conducting property.

The role of plasma membrane channels in shaping the morphology of microvilli is largely unknown with the exception of channelrhodopsin-2 that has been shown to enlarge microvilli (Zimmermann et al., 2008). Expression of other plasma membrane channels such as CFTR or the sodium channel ENaC increases the cell capacitance, but this process is attributed to the increased fusion of transport vesicles at the plasma membrane (Awayda, 2000; Weber et al., 1999). Several integral membrane proteins are involved in shaping the microvillus structure through interactions with ERM proteins such as CD43, CD44, ICAM-2 (Bretscher et al., 2002; Yamane et al., 2011; Yonemura et al., 1999) or CD9 in the oocyte (Runge et al., 2007). The microvillus is a spatially defined compartment with some proteins, including ions channels, localizing to microvilli (or even in microvillus sub-regions), through binding to intermediate scaffolding proteins such as ERMs (Lange, 2011). The binding of membrane proteins to the cytoskeleton would therefore have the dual role of stabilizing the microvilli by reinforcing the scaffolding link between the plasma membrane and the actin backbone, and of precisely distributing channels to specific functional locations.
What is the function of the anchoring of Ano1 to the cytoskeleton? The distribution of Ano1 is not homogenous in the oocyte and it is known that CaCC localize preferentially to the animal pole of the oocyte at the expense of the vegetal pole (Machaca and Hartzell, 1998). This may in fact explain the increased microvilli density at the animal compared to the vegetal pole of the oocyte (Bluemink et al., 1983). We recently showed that Ca$^{2+}$ influx through store-operated Ca$^{2+}$ entry (SOCE) specifically activates distally located Ano1 through Ca$^{2+}$ transfer within the ER lumen and release through IP$_3$ receptors (Courjaret and Machaca, 2014). We refer to this as ‘mid-range Ca$^{2+}$ signaling’ as it spatially bridges elementary and global Ca$^{2+}$ signaling. SOCE “clusters” do not co-localize with Ano1. Consequently the clusters are unlikely to make it to the microvilli and constitute a specific compartment in terms of Cl$^-$ and Ca$^{2+}$ signaling. The anchoring of Ano1 to the cytoskeleton would therefore allow a very precise localization of the channel in the vicinity or away from specific Ca$^{2+}$ release sites.

Another important parameter is the function of the microvilli. In the oocyte the microvilli contact the macrovilli arising from the follicle cells and protruding through the vitelline envelope (Browne and Werner, 1984; Dumont and Brummett, 1978). At the contact point, gap junctions allow the passage of numerous molecules, particularly IP$_3$ and Ca$^{2+}$ produced by the stimulation of PLC in follicular cells, a complex coupling that varies depending on the subtypes of follicular cells involved and/or gap junctions (Arellano et al., 2012; Sandberg et al., 1992; Supplisson et al., 1991). Precise positioning of Ano1 within the microvilli by anchoring it to the cytoskeleton is likely to enhance the efficiency of a signal transferred from the follicular cells to the oocyte through gap junctions, avoiding cytoplasmic buffers.

In summary, we have identified a novel function of Ano1 that regulates microvilli length, diameter and density within the oocyte through linkage to ERM and the actin cytoskeleton. This in turn modulates the surface area of the cell. Given the wide distribution of Ano1 in different epithelia, which are often enriched in microvilli, and the conservation of the role of ERMs and the actin cytoskeleton, these findings are likely to translate to most epithelia.
Materials and Methods

Expression in Xenopus oocytes - Stage VI oocytes were harvested from wild type and lab bred *Xenopus laevis* (Xenopus Express, France and Nasco, U.S.A) using previously described procedures (Machaca and Haun, 2000). Oocytes were kept in 0.5x L15 (Sigma) and used no earlier than 24 hours after surgery.

Molecular biology - Human Moesin (Accession number: NP_002435, a gift from Criss Hartzell) was sub-cloned from pcDNA3.1 via Xba1 and HindIII restriction sites into the pSGEM vector using the following set of primers: 5’-ACTGCTCTAGATGCCAAAACGATC-3’; 5’-TCGATAAGCTTTTACATAGACTCAAATTCG-3’. Human Moesin N-terminus (1-381) was sub-cloned from pcDNA3.1 via Xba1 and HindIII restriction sites into the pSGEM vector using the following set of primers (5’-ACTGCTCTAGATGCCAAAACGATC-3’; 5’-TCGATAAGCTTTTACATAGACTCAAATTCG-3’). Human Moesin C-terminus (382-577) was sub-cloned from pcDNA3.1 via Xba1 and HindIII restriction sites into a psGEM vector using the primers (5’-ACTGCTCTAGATGCCAAAACGATC-3’; 5’-TCGATAAGCTTTTACATAGACTCAAATTCG-3’). To create Moesin-GFP fusion constructs, human moesin, moesin (1-381), moesin (382-577) were sub-cloned from pcDNA3.1 using SacII and Xba1 in-frame into pSGEM-GFP. The primers used are: For pSGEM-Moesin-GFP: 5’-ACTGCCCGCGGATGCCAAAACGATC-3’; 5’-TCGATTCTAGACATAGACTCAAATTCG-3’. For pSGEM-Moesin (1-381)-GFP: 5’-ACTGCCCGCGGATGCCAAAACGATC-3’; 5’-TCGATTCTAGACATAGACTCAAATTCG-3’. For pSGEM Moesin (382-577): 5’-ACTGCCCGCGGATGCCAAAACGATC-3’; 5’-TCGATTCTAGACATAGACTCAAATTCG-3’.

Electrophysiology - The ionic currents were recorded using standard two electrode voltage-clamp recording technique. Recording electrodes were filled with 3M KCl and coupled to a Geneclamp 500B controlled with pClamp 10.5 (Axon instruments). CaCC currents were recorded using depolarizing pulses to +40 mV and a previously described “triple jump” protocol (Courjaret and Machaca, 2014; Machaca and Hartzell, 1999). Membrane capacitance was monitored using the built in routine from pClamp to measure membrane parameters. Cells were continuously superfused with saline during voltage-clamp and
imaging experiments using a peristaltic pump. The standard extracellular saline contained (in mM) 96 NaCl, 2.5 KCl, 1.8 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, pH 7.4.

**Imaging** - Confocal imaging of live oocytes was performed using a LSM710 (Zeiss, Germany) fitted with a Plan Apo 63x/1.4 oil immersion objective or a TCS SP5 (Leica, Germany) fitted with a 63x/1.4-0.6 oil immersion objective. Confocal z-stacks were taken in 0.5 µm sections using a 1 Airy unit pinhole aperture. The images were recorded using Zen black 2012 (Zeiss) or LAS AF 2.4.1 (Leica) software. Micrographs of oocytes were taken using a Zeiss Lumar V12 Stereomicroscope controlled using Zen blue 2012 (Zeiss). Images were analyzed with ImageJ 1.46q (Schneider et al., 2012). Cell surface calculation was performed using the “iso-surface” function of the Bone J plugin (Doube et al., 2010). A 400 µm$^2$ image plane was selected and a mesh created to fit the 3d reconstruction of the surface. A resampling factor of 2 was used and a detection threshold for surface change of 20 pixels. The results are expressed as fold increase of the theoretical surface area (measured surface/400).

**Wheat Germ Agglutinin staining** - For WGA staining, the cells were cooled down to 4°C for 15 min then stained for 30 min at 4°C in Ringer solution containing 5µg.ml$^{-1}$ of Ax488-WGA conjugate (ThermoFischer Scientific). The cells were then washed in Ringer and kept on ice prior to imaging to minimize internalization of the membrane marker.

**Fluorescence Recovery After Photobleaching (FRAP)** - For FRAP experiments, orthogonal images across the membrane were obtained directly using X-Z scans: a line scan is performed at different Z positions and the Z-positioning is achieved using a “Super-Z” galvanometric stage (Leica) that allows for fast monitoring of the fluorescence across the plasma membrane. To bleach a defined region of the membrane, the zoom factor of the scanner was increased 2 times and the laser power (561 nm) set at 100% for 3 scans. Scanning speed was set a 400 Hz and image frequency to 0.1 Hz for both bleaching and recording.

**Immunoprecipitation and Western blot** - Oocytes were lysed in lysis buffer containing Triton-X100 (10mM Hepes, 150 mM NaCl, 1mM EGTA, 0.1 mM MgCl2, 0.5% triton-x100, pH 7.4). The immunoprecipitation was performed using Magnetic Associated Cell Sorting (MACS) using microbeads according to the manufacturer’s instructions (Miltenyi Biotec, Germany). The lysates were incubated with micro beads conjugated to an anti-GFP monoclonal antibody for 30 minutes on ice. The lysate-beads complexes were then sorted in the magnetic field of a µMACS. After the washing steps, the proteins were eluted with the
denaturing elution buffer provided in the kit. Western blotting was performed as previously described (Courjaret et al., 2013) and repeated three times on different samples. The Anti-Anol1 antibody was used at a dilution of 1/1000 (SAB2102460, QC13356 Sigma).

Statistics - Values are given as means ± SEM. Statistical analysis was performed when required using either Student paired and unpaired two-tailed t-test or ANOVA and corrected for multiple comparison. P is indicated as follows: * (P<0.05), ** (P<0.01), *** (P<0.001) and ns (not significant). Statistics were obtained using Prism 6.05 (Graphpad Software, La Jolla, USA).

Ethical approval- Animals were handled according to Weill Cornell Medical College IACUC approved procedures.
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Author Contributions

RC designed and performed experiments, analyzed data and wrote the paper. RH performed and analyzed experiments. SH, AI, MD and SD performed experiments. KM designed experiments, analyzed data and wrote the paper.
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**Figure 1: Overexpression of Ano1 in Xenopus oocytes increases membrane capacitance.**

Various physiological parameters were recorded on uninjected cells (naïve) and cells expressing Ano1-mCh (Ano1). **A**: Chloride currents recorded at rest (left traces) and during store depletion induced by ionomycin (Ion, 10 µM, right traces) following a voltage jump from -30 mV to +40 mV. **B**: Amplitude of the resting chloride currents ($I_{Cl1}$) recorded at +40 mV. **C**: Membrane resistance. **D**: Membrane potential. **E**: Bar chart summarizing the amplitude of the current ($I_{Cl1}$) following mobilization of store Ca$^{2+}$ with ionomycin in naïve and Ano1 expressing cells. **F**: Increase in the membrane capacitance of oocyte expressing Ano1. **G**: Overexpression of the Ca$^{2+}$ channel Orai1 does not increase cell capacitance. Statistics were performed using Student “t” test. The number of cells tested is indicated over the bars or in the plotting area when identical.
Figure 2: Microvilli structure following overexpression of Ano1 in Xenopus oocytes.

A: Confocal imaging was performed across the plasma membrane of naïve cells stained with Wheat Germ Agglutinin (WGA) or expressing Ano1-mCh (Ano1). Two confocal planes are illustrated; one at the very top of the microvilli (Tip) and one close to the base. The lower panel presents orthogonal projections across the confocal z-stack. There is a pronounced increase in microvilli length as well as the appearance of dome-like structures (white arrows) presumably resulting from microvilli fusion.  

B: The maximum length of 10 microvilli was averaged on each cell. A third data set obtained from cells expressing the Orai1-GFP protein was used as an additional control. Ano1 expression specifically extended the maximum length of the microvilli.  

C: The maximum diameter of the microvilli identified at the “base” plane was measured in the same cells as in B and reveals an increase in Ano1 expressing cells.  

D: Average microvillus length was evaluated using the fluorescence intensity across a z-stack, the base of the plasma membrane is used as a vertical reference.  

E: The intensity measured in D is plotted as a function of the z-axis illustrating microvilli extension.  

F: The thickness was then evaluated as the width of the curve in (E) at half-maximal intensity (dotted line).  

G: The oocyte surface at the animal pole was reconstructed using a mesh technique (see methods).  

H: Histogram summarizing the increase in surface induced by the membrane convolutions. Statistics were performed using either Student “t” test or ANOVA.
**Figure 3: The increase in membrane area does not require Ano1 Cl⁻ channel function**

Naïve cells and cells expressing Ano1 where incubated with the Ano1 inhibitor MONNA (1 µM) for the duration of the experiment. **A-B:** the resting chloride current was significantly reduced by MONNA in both naïve and Ano1 expressing cells. **C:** MONNA incubation had no effect on the membrane capacitance of naïve cells nor did it influence the increase in Cm recorded following Ano1 expression. **D:** Confocal imaging of the Ano1-cherry protein across the plasma membrane reveals no change in the microvilli structure in cells incubated in MONNA.
Figure 4: Expression of full length and truncated moesin in Xenopus oocytes.

A: Cartoon representing the structure of the ERM protein moesin. The FERM domain contains the binding site to plasma membrane proteins while the C-terminal end (C-ERMAD) binds actin. The arrow marked “381” indicates the position of the separation between the N_{ter}- and C_{ter}-moesin fragments used in this study. B: Photographs of oocytes expressing the different moesin proteins (top panels). Prolonged expression of N_{ter}-moesin induces a discoloration of the animal pole. The lower panels are orthogonal section obtained from confocal z-stacks of representative cells stained with WGA. C: Capacitance of oocytes injected with the different moesin constructs. The number of cells used is indicated in parentheses above the bars. Statistics were performed using ANOVA. D: Dose dependence of the membrane capacitance (Cm) upon injection of different amounts of cRNA coding for the N_{ter}-moesin.
Figure 5: Co-expression of moesin and Ano1

Oocytes were injected with Ano1-mCh alone or with either FL-moesin, Nter-moesin or Cter-moesin. **A:** Confocal images across the microvilli and orthogonal reconstruction of z-stacks of cells expressing Ano1-mCh with or without Nter-moesin. **B:** Capacitance of oocytes expressing Ano1-mCh and the three different forms of moesin, only the Nter fragment influences the capacitance. **C:** Maximum Cl⁻ current recorded on cells expressing Ano1-mCh and moesin constructs following store Ca²⁺ mobilization with ionomycin. **D:** Cl⁻ current densities obtained on cells expressing Ano1 and the moesin constructs. Statistics were performed using ANOVA.
Figure 6: Co-localization and co-immunoprecipitation of Ano1 and moesin

A: Orthogonal sections through z-stacks of confocal images from oocytes co-expressing Ano1-mCh with GFP-tagged FL-moesin (WT), Nter-moesin or Cter-moesin as indicated above the section and in the cartoon representation of the constructs. The overlay image (merge)
shows that both FL-moesin and N\textsubscript{ter}-moesin co-localize while the C\textsubscript{ter}-moesin expression levels are low and don’t co-localize with Ano1. \textbf{B:} The co-localization level was assessed using Pearson’s coefficient on orthogonal sections. The level of co-localization for STIM1 (ER) and Orai1 (plasma membrane) (S/O) was used as a reference “minimal” value, and the co-localization of WGA with Ano1-mCh as a “maximal” value (WGA, see text for details). FL- and N\textsubscript{ter}-moesin, but not C\textsubscript{ter}-moesin, show strong co-localization with Ano1. \textbf{C:} GFP-tagged FL- and N\textsubscript{ter}-moesin co-immunoprecipitate with endogenous Ano1. Lysates from oocytes expressing the different moesin constructs as indicated were Western blotted and probed with either anti-Ano1 or anti-GFP antibodies before (Input) and after pull-down using an anti-GFP antibody (IP Anti-GFP). Statistics are performed using ANOVA.
Figure 7: Fluorescence Recovery After Photobleaching

FRAP experiments were performed using a line scan over the z-axis to obtain fast orthogonal planes (see Methods). **A:** A defined area (dotted rectangle) was bleached using increased laser power over a zoomed area, and the recovery monitored for several minutes in cells expressing Ano1-mCh alone or in combination with N<sub>ter</sub>-moesin. **B:** Timeline of recovery from bleaching when the different N<sub>ter</sub>, C<sub>ter</sub> and FL-moesin constructs are co-expressed. **C:** Bar chart summarizing the half-time of recovery of the fluorescence when Ano1-mCh is co-expressed with various untagged moesin proteins. Statistics are performed using ANOVA.
**Supplemental Fig. 1:** Alignment of the sequences of moesin from *Xenopus laevis* and *Homo sapiens*. The alignment was performed using CLC software (V7.5.1, Qiagen), the key portions of the protein are highlighted.
Supplemental Fig. 2: Effects of the expression of moesin and its truncated variants on the plasma membrane structure. The membrane aspect is visualized after staining with WGA on cells injected with FL-moesin, N	extsubscript{ter} moesin or C	extsubscript{ter} moesin. Confocal planes have been taken at both ends of the microvilli. The lower panel represents orthogonal sections reconstructed from z-stacks.
Supplemental Fig. 3: Effects of the expression of moesin and its variants on the physiology of the oocyte. Different amounts of cRNA coding for the WT (5 ng), N\textsubscript{ter} (4ng) and C\textsubscript{ter} (3ng) have been injected in oocytes. The membrane capacitance (A), resting potential (B) and resistance (C) have been measured after 48h to 72h of expression. The chloride currents were also recorded at a membrane potential of +40 mV at rest (D) and during store depletion by ionomycin (E).
Supplemental Fig. 4: Effect of the expression of two N-terminal fragments of Ano1 on the membrane capacitance and chloride currents. A: Two stop codons were inserted in the pSGEM-xAno1 construct at position 322 and 510 to generate two N-terminal fragments: the first encoding amino acids 1-322 of Ano1 containing the cytoplasmic domain; and the second fragment 1-510, ending just before the 3rd transmembrane domain. The following primers were used for 1-322: 5’GTCAGGAAGTATTTTGGAGAGTAGATAGGACTGTACTTTGCC3’ and 5’GGCAAAGTACAGTCCTATCTACTCTAAAATACTTCCTGAC3’. For the 1-510 construct: 5’GAGAGACAGGTTTCCAGCTTAGCTCTATCTCCTCCAAAATACTTCCTGAC3’ and 5’GATGCCAACACATTTGTAGCTAGCTGGAAAACCTGTCTCTC3’. Both constructs were sequence verified and mRNA produced from NheI linearized vectors by in vitro transcription using the mMessage mMachine T7 kit (Ambion). B: Injection of RNA (20ng) coding for either fragment had no effect on the chloride current recorded after ionomycin application or at rest (not illustrated). C: The membrane capacitance was significantly reduced when the cytoplasmic 1-322 domain was expressed.