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Roles of the cumulus-oocyte transzonal network and the Fragile X protein family in oocyte competence

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In brief: RNA granules travel through the cumulus cell network of transzonal projections which is associated with oocyte developmental competence and RNA packaging involves RNA binding proteins of the Fragile X protein family**.**

Abstract

The determinants of oocyte developmental competence have puzzled scientists for decades. It is known that follicular conditions can nurture the production of a high-quality oocyte, but the underlying mechanisms remain unknown. Somatic cumulus cells most proximal to the oocyte are known to have cellular extensions that reach across the zona pellucida and contact with the oocyte plasma membrane. Herein, it was found that transzonal projections (TZPs) network quality is associated with developmental competence. Knowing that ribonucleo-particles are abundant within TZPs, the distribution of RNA binding proteins were studied. The Fragile X-Related Proteins (FMRP, FXR1P, and FXR2P) and two partnering protein families, namely cytoplasmic FMRP interacting protein (CYFIP) and nuclear FMRP interacting protein (NUFIP), exhibited distinctive patterns consistent with roles in regulating mRNA packaging, transport and translation. Expression of GFP-FMRP fusion protein in cumulus cells showed active granule formation and their transport and transfer through filipodia connecting with neighboring cells. Near the projections' ends was found the cytoskeletal anchoring protein Filamin A and active protein synthesis sites. This study

highlights key proteins involved in delivering mRNA to the oocyte. Thus, cumulus cells appear to indeed support the development of high-quality oocytes via the transzonal network.

Introduction

Oocyte quality can be defined simply as the competency to resume meiosis, be fertilized, and undergo development to a stage beyond the activation of the embryonic genome, at which point the blastomeres acquire some ability to adjust independently to the surrounding environment. This definition focuses on the time interval spanning the embryonic program, which begins with gamete preparation during oogenesis, concurrent with folliculogenesis. Bovine oocytes acquire the potential to resume meiosis when they reach full size in antral follicles at about 3 mm in diameter (Fair *et al.*, 1995). *In vitro* culture has shown clearly that most embryo lethality occurs before embryonic genome activation (Plourde *et al.*, 2012; Dieci *et al.*, 2016) and that developmental competence is affected by conditions experienced by the oocyte during the later stages of folliculogenesis (Nivet *et al.*, 2012).

Folliculogenesis is a highly regulated process in which the different cell types comprising the ovarian follicle are interdependent and work as a syncytium. Our main interest is in the close relationship between the gamete and its surrounding somatic cells, maintained throughout folliculogenesis even after the glycoprotein shell or zona pellucida is secreted (Anderson and Albertini, 1976; Gilula *et al.*, 1978). Cumulus cells maintain physical contact with the oocyte by extending cellular processes through this shell. We have shown that ribonucleoprotein complexes of considerable size transit through these transzonal channels (Macaulay *et al.*, 2014; Macaulay *et al.*, 2016). However, the mechanisms that control mRNA transit remain unknown. Using a pulldown assay for *de novo* synthesized transcripts found inside the channels, several

genes potentially associated with mRNA shuttling were identified at the top of this list and Fragile X-Related Proteins (FXRPs) were found (Macaulay *et al.*, 2014). This group of proteins is composed of Fragile X Multi-Role Protein (also known as Fragile X Mental Retardation Protein) (FMRP), Fragile X Related Protein 1 (FXR1P) and Fragile X Related Protein 2 (FXR2P). Therefore, we hypothesize that an extensive network of transzonal channels is a hallmark of a competent oocyte and that the proteins involved in carrying mRNA include FXRPs.

The role of FXRPs in the ovary has not been fully explored, even though the correlation with female reproductive lifespan was shown long ago (Schwartz *et al.*, 1994; Vianna-Morgante *et al.*, 1996; Murray *et al.*, 1998; Sullivan *et al.*, 2005). In human, *FMR1* (coding for FMRP) acquired a destabilizing sequence in the form of repeating CGG trinucleotides inserted within the location corresponding to the 5' UTR of the mRNA. This sequence is subject to expansion during DNA replication.

Perturbations in *FMR1* leading to lower FMRP expression is associated with a decrease in female reproductive lifespan, causing about 20% of women to develop Fragile X-Associated Primary Ovarian Insufficiency (FXPOI) (Murray *et al.*, 1998; Sherman, 2000; Wheeler *et al.*, 2014). The most important symptom of the FXPOI syndrome is reaching menopause before the age of 40 due to early depletion of oocyte stocks (De Caro *et al.*, 2008; Gleicher *et al.*, 2014; Jiao *et al.*, 2018). The premutation is known to result in lower levels of FMRP that impacts oocyte competence through a yet unknown mechanism.

FMRP is known to interact *in vitro* with other members of the FXRPs family, which include the FXR1 and FXR2 proteins (FXR1P and FXR2P) (Zhang *et al.*, 1995). In neurons, these proteins form complexes that can bind mRNA to form granules,

which are involved in mRNA transport and the control of translation (Davidovic *et al.*, 2007; Graber *et al.*, 2013; El Fatimy *et al.*, 2016). FMRP also interacts with the cytoplasmic FMR1-interacting protein (CYFIP) and the nuclear FMRP interacting protein (NUFIP) families (Bardoni *et al.*, 1999; Schenck *et al.*, 2001). Proteins of the CYFIP family are responsible for cytoskeleton remodeling and are involved with FMRP in the extension of actin-based cytoplasmic projections such as dendrites (De Rubeis *et al.*, 2013; Pathania *et al.*, 2014; Hsiao *et al.*, 2016). By contrast, those of the NUFIP family are either nuclear and bind only to the specific nuclear FMRP isoform 12 or cytoplasmic RNA-binding proteins that localize with ribosomes and FMRP (Bardoni *et al.*, 1999; Bardoni *et al.*, 2003).

This study aimed to assess whether an association exists between the transzonal network, members of the FXRPs family and its interacting partners, and oocyte developmental competence. Therefore, localization of FMRP, FXR1P, FXR2P, CYFIP1, CYFIP2 and NUFIP1 within the transzonal projections (TZPs) was performed to observe their possible involvement in mRNA transport from cumulus cells to the oocyte. We found that the target proteins were abundant within TZPs under the form of granules consistent with mRNA packaging, transport, and translation. Also, active protein synthesis sites were found at the edge of the oocyte plasma membrane near and overlapping the tips of TZPs.

Material and Methods

Ethics statement

This project was evaluated and approved by the Animal Care Council of Laval University. The research project does not involve the use of animals dedicated for the purposes of this project. Cattle and pig ovaries were collected at different slaughterhouses during their normal operation. For Figure 1, ovaries from Holstein

dairy cows were recovered at the abattoir INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy. For other figures, cow ovaries were collected at Abattoir Bolduc, Buckland, QC, Canada. Pig ovaries were collected at the Olymel SEC slaughterhouse, Vallée-Jonction, QC, Canada. All animals and slaughterhouses facilities are subjected to routine veterinary inspection and operate in accordance with the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications (Italy) and under the legislation and supervision of the Canadian Food Inspection Agency (Canada). Tissue collection did not alter the normal flow of events at the slaughterhouses including animal demise and post-mortem processing. This study did not require handling animals on university premises.

Ovary collection

Ovaries from dairy cows aged four to eight years and from prepubertal gilts were collected in local slaughterhouses. Ovaries were placed immediately in a warm saline solution (0.9% NaCl) containing of an antimycotic antibiotic (Sigma-Aldrich, Oakville, ON, Canada) and were maintained at 37ºC (bovine) or 34ºC (porcine) during transport.

Oocyte collection

Ovaries were rinsed twice in the saline solution. Cumulus-oocyte complexes (COC) were collected by aspirating visible follicles between 3 and 6 mm in diameter and were placed in a 50 ml Falcon flask. COC selection based on morphology was carried out in phosphate-buffered saline solution with polyvinyl alcohol (PBS-PVA). Only oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were included in the study (n = 20 oocytes per group). Bovine oocytes from high-efficiency or low-efficiency ovaries were categorized based on the number of antral follicles in the 3–6 mm size range visible in the ovarian cortex, as described

previously (Modina *et al.*, 2007; Modina *et al.*, 2014). This categorization was used as a proxy variable for bovine oocyte developmental competence.

Immunofluorescence

Complete description of the antibodies used is this study can be found in Supplemental Table S1. Immunolocalization was performed as described previously (Macaulay *et al.*, 2014; Macaulay *et al.*, 2016). Selected COCs were denuded partially by repeatedly drawing them into a pipette. The resulting oocytes were fixed for 10 min in 4% paraformaldehyde in PBS-PVA and permeabilized for 20 min in 1% Triton X-100. Non-specific sites were blocked using 5% bovine serum albumin for 1 h. Incubation with primary antibody was for at least 16 h at 4°C. Oocytes were then washed 3 times for 5 min each at room temperature and incubated for 1 h with a 1:1000 dilution of secondary antibody: Alexa-Fluor 488 goat anti-rabbit, Alexa-Fluor 555 goat anti-rabbit (ThermoFisher Scientific, Watham, MA, USA) or Alexa-Fluor 488 goat anti-chicken (Biotium, San Francisco, CA, USA). After three washings for 5 min each, the oocytes were incubated with 1.5% actin stain 670 Phalloidin (Cytoskeleton, Denver) for actin filament detection and Hoechst dye 33342 (Invitrogen, product no. H21492) diluted 1:1000 for DNA staining. Controls with nonimmune primary antibody and secondary antibody were run in parallel.

TZP network quality assessment

Using the actin staining, TZPs are highlighted and imaged. Ten images spaced 1 µm apart at the equator of the oocyte are taken to make a maximum intensity projection (MIP). TZPs are handdrawn, and the intensity values are the average of the value of the pixels that has been identified (drawn) as part of a TZP. We use a 16-bit detector and measurements can take a value between 0 and 65,535. An in-house developed

script is used to output an intensity value, a length and a straightness value for each TZP. The script is available here:

https://github.com/alexandrebastien/ImageJ-Script-Collection/blob/master/ULaval_Misc1.0.0/scripts/Plugins/ULaval/TZPs_Analyzer.ijm

Candidate protein detection

Primary antibodies against FMRP and FXR1 have been described previously (Mazroui *et al.*, 2002; El Fatimy *et al.*, 2012). Anti-FXR2 (Atlas antibodies, Stockholm, Sweden), anti-CYFIP1 (Thermo-Fisher Scientific) anti-NUFIP1 (Thermo-Fisher Scientific) were used at 1 µg/ml, antiCYFIP2 (Thermo-Fisher Scientific) at 2 µg/ml, and anti-Filamin A (Sigma-Aldrich) at 1% (vol.).

Cell transfection

Intact COCs were matured *in vitro* for 22 h using standard maturation medium as previously described (Plourde *et al.*, 2012). Cumulus cells were stripped from matured COCs by gently pipetting and plated in six-well plates at a concentration of 1.5-2 million cells per mL in DMEM (Life Technologies, Burlington, ON, Canada) supplemented with sodium bicarbonate (MP Biomedial, Santa Anna, CA, USA), bovine albumin (Sigma), Fugizone (Life Technologies). Cells were left in culture for 3-5 days prior to two transfections one day apart. Plasmid delivery was carried out with the TransIT X2 system from Mirus (Madison, WI, USA). The construct was done in the pcDNA 3.1 (+) plasmid (Life Technologies) containing eGFP and human *FMR1* cDNA producing a functional fusion protein (Davidovic *et al.*, 2007). Time-lapse microscopy was conducted 12 h post-transfection.

Live-cell and time-lapse imaging

RNA granules were tracked *in vivo* using Syto RNASelect green-fluorescent cell stain solution (Thermo-Fisher Scientific) prewarmed to 37°C. Selected COCs and partially

denuded oocytes were incubated for 30 min in 5 µM of Syto RNASelect green-fluorescent cell stain solution (Thermo-Fisher Scientific). They were then rinsed twice for 5 min in culture medium, placed immediately in maturation medium in 280 µm diameter wells in a custom-made dish. Video recording was done using a Zeiss Live-Cell LSM700 confocal microscope in a humidified atmosphere at 37°C with 5% CO_2. Images of transzonal projections were taken at 40X while images of transfected cumulus cell culture were taken at 10X and 20X. All images were captured using ZEN software at several intervals ranging from 1.5 to 50 seconds.

Active translation

Active translation was detected using a commercial kit (Click-iT Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit, Thermo-Fisher Scientific). Selected COCs were labeled by incubation with 100 µl of Click-iT OPP reagent at 20 mM for 30 min at 37°C. Complexes were then washed once in PBS and fixed with 4% paraformaldehyde for 15 min. Fixed COCs were permeabilized for 15 min in 0.5% Triton X-100 at room temperature, washed twice in 100 µl of PBS and mixed with 100 µl of Click-iT Plus OPP reaction cocktail (prepared according to the manufacturer's protocol with 1X Click-iT reaction buffer in deionized water and 1X Click-iT reaction buffer additive) for 30 min at 37°C away from light. COCs were then removed from the reaction mixture and washed once in 100 µl of Click-iT reaction rinse buffer.

Data analysis

Immunofluorescence images were analyzed using ImageJ software to determine oocyte fluorescence intensity and corresponding TZPs total length and to localize foci of the protein of interest. Transzonal characteristic means were compared using Student's t-test while protein foci means were compared using one-way ANOVA with

the Tukey post hoc test for Fragile X-Related Proteins family or Student's t-test for CYFIP family members. Statistical significance was set at $p < 0.05$. JASP software was used for statistical analysis. Each group contained n = 15 oocytes.

Results

Using visual follicle count, oocytes were collected from High quality ovaries (more than 10 follicles of 2 to 5 mm in diameter) and Low-quality ovaries (less than 10 follicles) as published previously (Modina *et al.*, 2007; Tessaro *et al.*, 2011; Modina *et al.*, 2014). This allowed for the production of two groups of oocytes with contrasting developmental potentials. It has been shown that high-quality oocytes, as defined in these studies, reach the blastocyst stage *in vitro* 32.2 ± 1.9% of the time versus 8.2 ± 1.2% of the time for the corresponding low-quality oocytes (Modina *et al.*, 2007). Based on fluorescent intensity, oocytes recovered from high-efficiency ovaries had a significantly better developed transzonal network ($p < 0.001$) than those from low-efficiency ovaries (Figure 1A). The TZPs tended to be longer overall ($p = 0.087$) in oocytes recovered from high-efficiency ovaries (Figure 1B).

Our interest being in the transport of mRNA cargos within TZPs, labeling of total RNA combined with live-cell time-lapse image tracking showed the presence of granules sent from the cumulus cell body into the TZPs (Figure 2). Granular movement appeared to be mostly towards the oocyte with little reflux. We also noted that the granules appeared to be larger as they approached the entrance to the zona pellucida (Figure 2). In the zona pellucida, Fragile X-Related Proteins family only showed minor differences in distribution (Figures 3). In oocytes, FMRP, FXR1P and FXR2P overlapped with the TZP actin cytoskeleton (Figure 3). However, the general distribution patterns of the FXRPs differed (Figure 3). FMRP foci were found in majority in the first half of the zona pellucida closer to the cumulus cells (Figure 3).

The majority of FXR2P foci were also found in the first half of the zona but more on the outskirts which coincides with the beginning of the TZPs (Figure 3), while FXR1P foci were found throughout the zona pellucida and along the entire length of the projections (Figure 3). This suggests distinctive roles for the FXRPs in RNA packaging, transport and control. FXRPs and partner proteins abundance and distribution within cumulus-oocyte complexes might be a conserved trait in view of the similar results obtained for porcine FMRP (Supplemental Figure 1) and FXR2P (Supplemental Figure 1). A few foci were detectable throughout the projections with FXR1P again being uniformly distributed (Supplemental Figure 1).

The cytoplasmic FMRP interacting proteins CYFIP1 and CYFIP2 have a very different distribution in bovine oocytes even though both occur in granular form in the cytoplasm and in the surrounding cumulus cells (Figure 4). Both are known to interact with FXRPs. CYFIP1 foci spanned the entire TZP length like strings of pearls and produced larger foci in the first half of the zona (Figure 4). CYFIP2 foci were present in the cytoplasm of cumulus cells but in a distinctly granular form inside projections (Figure 4). They were partial to the outer half of the zona pellucida much like FMRP and FXR2P.

The distributions of porcine CYFIP1 and CYFIP2 foci were also similar to the bovine case. CYFIP1 (Supplemental Figure 2) had the same string-of-pearls-like arrangement and appeared more abundant than CYFIP2, with foci close to the oolemma.

The nuclear-interacting FMR proteins, NUFIP1 and NUFIP2, were also associated with newly synthesized mRNA transcripts in cumulus cells and found previously in TZPs (Macaulay *et al.*, 2014). This was not expected since these partner proteins are better known for their localization in the nuclear compartment. As

anticipated, NUFIP1 was very abundant in all bovine cumulus cell nuclei but surprisingly, extra-nuclear foci were found throughout TZPs including in the bulging ends (Figure 5). The NUFIP1 signal was more diffuse in the oocyte cytoplasm. In porcine, the distribution of NUFIP1 was also very similar to that in bovine COCs with foci abundant throughout the projections and in the end bulb (Supplemental Figure 3).

To show that FMRP can actively generate granules that can be carried through cellular extensions, cumulus cells were cultured and transfected with a plasmid containing a GFP-FMRP sequence generating a functional fusion protein (Davidovic *et al.*, 2007). Transfection efficiency was about 25%. Typically, cumulus cells extend filipodia to neighboring cells connecting each other. Cells expressing the construct contained abundant GFP-FMRP granules that moved along the cellular extensions similarly to the RNA granules detected in transzonal projections. Transfer of GFP-FMRP granules was observed where delivery was done from a transfected cell to another that was not expressing the construct (Figure 6). The figure shows a large fluorescent granule disappearing within the cell's cytoplasm and two additional granules that were tracked to move toward and to be delivered to the receiving cell (Figure 6 side panels).

Since FXRPs and their interacting proteins are known to be involved in binding, packaging, transporting and controlling the translation of mRNA in neurons (Khandjian *et al.*, 2004; Stefani *et al.*, 2004; Darnell *et al.*, 2011; El Fatimy *et al.*, 2016), they are also known as being involved in the development of the neuronal network. In the present study, we localized Filamin A (FLNA), a candidate protein found in neurons and proven to interact with FMRP (Bolduc *et al.*, 2010). FLNA is a known anchoring molecule that interacts with the actin filament and cell surface membrane glycoproteins such as integrins (Li *et al.*, 1999). Consistent with its known

roles, FLNA was detected mainly at the base of TZPs where the cumulus cell and oocyte membranes are juxtaposed (Figure 7).

Cumulus cell mRNA sent through the transzonal network in complexes with RNA-binding FXRPs and related partners must be released and translated locally for projection development and maintenance purposes or transferred to the oocyte via synapse-like vesicular secretion as shown previously (Macaulay *et al.*, 2014; Macaulay *et al.*, 2016). Using a fluorescent amino acid analog, no active translation was detected along the projection length, confirming the repressed state of the mRNA during transport. However, numerous hubs of active translation were found near and overlapping the projection end bud (Figure 8). Results in this study as whole shed light on, at least, part of the mechanism by which cumulus cell mRNA is packed then transported through TZPs to where they are released for translation.

Discussion

The factors that control oocyte quality are multifactorial and complex. In this study, we sought to better understand the role of transzonal network quality. We also show an mRNA transport system in which the FXRPs and partners participate. In the first perspective, we hypothesized that a poorly developed transzonal network could have a negative impact on oocyte quality. We tested this hypothesis using a model of developmental competence where ovaries with signs of reduced antral follicle count produce low developmental competence oocytes (Modina *et al.*, 2007; Tessaro *et al.*, 2011). The present results indicate that these low-quality oocytes harbor an underdeveloped transzonal network. This agrees with other reports using different models of oocyte developmental competence where lower number of TZPs are found in COCs from smaller size follicles in mouse (El-Hayek and Clarke, 2015) and COCs of gilts under heat stress harbor less TZPs (Yin *et al.*, 2020). This suggests that conditions during folliculogenesis can modulate the quality of the transzonal network,

which in turn could reduce the overall support from cumulus cells essential for the acquisition of developmental competence.

Cumulus cells are known to nurture the oocyte through the network of TZPs by providing cyclic nucleotides which control meiotic resumption (Gilchrist *et al.*, 2016) as well as energetic substrates which palliate to an inefficient glycolytic pathway in the oocyte (Sutton-McDowall *et al.*, 2010; Richani *et al.*, 2021). Recently, we have demonstrated that the projections also actively deliver mRNA to the oocyte (Macaulay *et al.*, 2014; Macaulay *et al.,* 2016). This mRNA transfer appears to be important for developmental competence. Indeed, in bovine COCs, TZPs are not constantly loaded with RNA. The timeframe of RNA accumulation in these channels, while the COCs are still enclosed in follicles, coincides with acquisition of developmental competence. In the case of COCs recovered from slaughterhouse ovaries, the timing of aspiration post mortem was found to be a determinant of developmental competence: waiting for 4 hours before COCs collection increases blastocyst formation to 30.5 ± 1.9% versus 14.7 ± 0.5% after 2 hours (Blondin *et al.*, 1997). Under these conditions, RNA accumulation in transzonal projections was also found to peak at 4 hours before COCs retrieval (Macaulay *et al.*, 2016). In this model of developmental competence, the quality of the TZP network cannot be a factor since neither oocyte morphology nor the number of TZPs differs. Taken together, this supports the notion that a low-quality network and reduced network activity can both negatively impact oocyte developmental competence.

Based on this evidence suggesting that oocyte competence requires delivery of mRNA from cumulus cells, we next sought to investigate how this transfer occurs. As we previously reported (Macaulay *et al.*, 2014; Macaulay *et al.*, 2016), and again shown here, mRNAs are shuttled in granules across TZPs. FXRPs were selected as

356 candidates from an initial survey of newly synthesized transcripts found in bovine
357 cumulus cells and TZPs (Macaulay *et al.*, 2014; Macaulay *et al.*, 2016).

358 Also, the involvement of one member of the FXRPs, namely FMRP, has been
359 demonstrated in women fertility where a reduced expression significantly increases
360 the risk of experiencing menopause before the age of 40 (Schwartz *et al.*, 1994;
361 Vianna-Morgante *et al.*, 1996; Murray *et al.*, 1998; Sullivan *et al.*, 2005). This
362 phenotype has been described as a normal pool of primordial follicles followed by
363 growth-impaired follicles and, finally, an extensive presence of atretic follicles
364 (Hoffman *et al.*, 2012; Lu *et al.*, 2012). The molecular mechanism through which the
365 RNA binding protein impacts oogenesis and follicular demise has not yet been
366 described. However, knock-out mouse models with *Fmr1* or *Fxr2* inactivated
367 individually have not been associated with specific reproductive phenotypes (The
368 Dutch-Belgian Fragile X Consortium, 1994; Bontekoe *et al.*, 2002) while *Fxr1-KO* is
369 non-viable (Mientjes *et al.*, 2004).

370 Our results indicate that all three FXRPs are present in TZPs in an evolutionary
371 conserved manner. Moreover, the *in vitro* transfection assay reported here confirms
372 FMRP can form granules that can be carried and transferred to neighboring cumulus
373 cells. In both bovine and porcine COCs, there is a great presence of FXR1P granules
374 along the TZPs while FMRP was mostly found on the outskirt of the zona with fewer
375 granules along the TZPs. FXR2P was mostly restricted to the outskirt of the zona
376 corresponding to the origin of the projections. Since all three FXR proteins contribute
377 to the formation of messenger ribonucleoprotein complexes for silencing and transport
378 of mRNA (Graber *et al.*, 2013; El Fatimy *et al.*, 2016), FMRP and FXR2P are likely

involved in silencing of mRNA as it enters the projections whereas FXR1P is more involved in the active shuttling.

We also detected CYFIP1 and CYFIP2, which are not mRNA-binding but rather actinremodeling proteins able to interact with FMRP (Schenck *et al.*, 2001; Pathania *et al.*, 2014). CYFIP1-containing granules appeared to be abundant throughout the transzonal network whereas CYFIP2 occurred mostly in the outer two thirds of the zona. Previous studies have shown that the absence of CYFIP1 in the brain is associated with decreased actin-based cytoplasmic protrusions and increased translation of certain other proteins (Napoli *et al.*, 2008; Bozdagi *et al.*, 2012; Abekhoukh *et al.*, 2017). These protein partnerships seem necessary for the structuration of the cytoskeleton involved in cellular extensions.

Also found with FXRPs is NUFIP1, an mRNA-binding protein that associates with FMRP (Bardoni *et al.*, 2003). NUFIP1 is a nucleocytoplasmic mRNA-shuttling protein from the nucleus where it is found adjacent to active transcription sites and in the cytoplasm in association with ribosomes (Bardoni *et al.*, 2003). In most cell types, NUFIP1 is mainly nuclear (Bardoni *et al.*, 2003; ◘abart *et al.*, 2004). In the present study, while NUFIP1 was not found in oocytes, it was found unexpectedly abundant in TZPs, suggesting the presence of ribosomes and mRNP complexes supporting its role in mRNA transport from cumulus cells to the oocyte.

The roles of FXRPs and partner proteins in packaging and transporting mRNA and regulating translation imply remote protein synthesis, which could be necessary to establish and maintain the transzonal network and transfer mRNA and/or proteins to

401 the oocyte. One candidate for the network building and maintenance function is a
402 protein that we had previously noted, namely filamin A (FLNA), a cross-linking
403 protein known to anchor actin filaments to membrane glycoproteins (Li *et al.*, 1999).
404 Interaction between FLNA and FMRP has been reported in *Drosophila*. In FMR1-
405 knock-out animals, FLNA is downregulated, which may cause the characteristic thin
406 and elongated appearance of dendritic spines (Bolduc *et al.*, 2010). Since FLNA is a
407 major constituent of intercellular connectivity, its inactivation has major life-impairing
408 consequences, including decreased female fertility (Li *et al.*, 1999; Feng *et al.*, 2006).
409 Murine studies have shown FLNA enrichment of the cortex from the germinal vesicle
410 to the MII stages and that knockdown decreases cytoplasmic actin mesh and cortical
411 actin content (Wang *et al.*, 2017). In the present study, FLNA was predominantly
412 found around the tip of TZPs, suggesting that it plays a role in maintaining contact
413 between cumulus cells and the oocyte. In addition, detection of active translation sites
414 has confirmed the presence of distal protein synthesis localized in the same area near
415 the ends of TZPs. Standard fluorescent confocal microscopy does not have sufficient
416 resolution to precisely position the signal to determine if it is exclusively within the
417 tip of TZPs or adjacent to it in the oocyte cortex. Both are not mutually exclusive.
418 Nonetheless, our data clearly show active translation sites closely localized to the
419 projections' end supporting the translation of transported mRNAs.

420 In conclusion, we propose that the development and functionality of the
421 transzonal network is an essential determinant of oocyte competence. Our observations
422 constitute evidence that in addition to small molecules, mRNA is transported from
423 cumulus cells to the oocyte through the network and that FXRPs and NUFIP1 are
424 likely involved. The abundance and distribution of these proteins individually support
425 distinctive roles in mRNA packaging and transport. Translation sites were detected at
426 the distal end of TZPs. Although the results do not confirm that amongst the mRNAs

being translated are some originating from the cumulus cells, we speculate that cumulus cells provide proteins to the oocyte in a controlled manner. The proposed transport mechanism appears to operate in porcine as well as bovine cumulus-oocyte complexes. Conditions that decrease FMRP expression would result in decreased CYFIP1 activity and decreased FLNA expression, which would lead to less connectivity between cumulus cells and the oocyte and hence an overall reduction of physiological support for the oocyte.

Declaration of interest

The authors have no conflict of interest to disclose.

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Author contribution statement

E.K.N., M.T.L., M.M., A.B. performed the experiments. E.K.N. and IG drafted the manuscript. E.W.K. provided the custom antibodies and GFP-FMRP expression vectors, A.M.L. and V.L. provided the high and low quality ovaries. ADM generated the time-lapse data of Fig 6. C.R. designed and supervised the study. E.W.K., A.M.L, V.L., R.V. and C.R. performed critical revision of the manuscript. All authors have given approval for publication of the present version of this manuscript.

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635

636 **Figure legends**

637 **Figure 1. Correlation between transzonal network development and oocyte**
638 **quality**. Bovine ovaries were classified as high-efficiency or low-efficiency based on
639 ovarian morphology (Modina *et al.*, 2007). (A) Ovaries with a morphology associated
640 with COCs leading to better embryonic rates exhibit a greater brightness in their TZPs
641 (AU, Arbitrary Unit ($p < 0.001$). (B) TZPs tended to be longer overall in oocytes from
642 high efficiency ovaries ($p = 0.087$).

643 **Figure 2. Progress of an RNA granule through the zona pellucida (ZP) from a**
644 **bovine cumulus cell towards the oocyte.** Live-cell imaging shows the movement of
645 an RNA granule (purple arrow) for an average 12 sec period for each picture. Total
646 RNA was stained with Syto RNASelect and is represented by the cyan blue color, Oo,
647 oocyte; ZP, zona pellucida, CC, cumulus cells. Scale bar = 20 μm.

648 **Figure 3. Different localization of the Fragile X-Related Proteins in transzonal**
649 **projections (TZPs) of bovine cumulus oocyte-complexes (COCs)**.
650 Immunofluorescence confocal microscopy images showing the actin filament of the
651 TZP (red) and the presence of A) FMRP, B) FXR1P and C) FXR2P within TZPs
652 (green). Actin filaments of TZPs were stained with Acti-stain 670 phalloidin (red);
653 primary antibodies against FMRP, FXR1P and FXR2P were subsequently incubated
654 with secondary antibodies conjugated with Alexa Fluor 488 (green). Scale bars = 20
655 μm.

Figure 4. Distribution of the cytoplasmic interacting family of FMR proteins 1 and 2 (CYFIP1 and CYFIP2) in bovine cumulus-oocyte complexes (COCs). Immunofluorescence confocal microscopy images showing the actin filament of the TZP (red) and the localization of A) CYFPIP1 and B) CYFIP2 in TZPs (green). TZPs were stained with Acti-stain 670 phalloidin (red). Primary antibodies against CYFIP1, and CYFIP2 were subsequently incubated with secondary antibodies conjugated with Alexa Fluor 488 (green). Scale bars = 20 μm.

Figure 5. **Distribution of nuclear interacting FMR protein 1 (NUFIP1) in bovine cumulusoocyte complexes (COCs).** Immunofluorescence confocal microscopy images showing the actin filament of the TZP (red) and the localization of NUFIP1 (green) within the TZPs in bovine COCs.

TZPs were stained with acti-stain 670 phalloidin (red). DNA was stained with Hoechst 33432 dye (blue). Scale bars = 20 μm.

Figure 6. GFP-FMRP expression in bovine cumulus cells. Cultured cumulus cells were transfected with a construct generating a GFP-FMRP fusion protein. Mobile green fluorescent granules were detected in the cells' filipodia. Side panels represent a time-lapse sequence in seconds showing the transfer of a large granule to the cytoplasm of the neighboring non-transfected cell (white arrow head) as well as the tracking of two other granules. In the yellow rings, coalescing of GFP-puncta into a larger granule is observed. The same phenomenon is observed in the red rings, and the fused puncta migrates to the neighbor cell situated in the top of the images. Scale bar= 20 □m.

Figure 7. Detection of filamin A (FLNA) at the junction of oocyte membrane and cumulus cells in a bovine oocyte. Immunofluorescence confocal microscopy showing localization of FLNA in transzonal projection (TZP) actin. TZPs were visualized using Acti-stain 670 Phalloidin (red); Primary antibodies against FLNA were subsequently incubated with secondary antibodies conjugated with Alexa Fluor 488 (blue). Scale bar = 20 μm.

Figure 8. *De novo* translation of mRNA in a bovine oocyte.FXR1P (green) was stained with Alexa Fluor 555; *de novo* translation sites (magenta) were stained with Alexa Fluor 488; Scale bar = 20 μm.

Supplemental Figure 1. Different localization of the Fragile X-Related Proteins in transzonal projections (TZPs) of porcine cumulus oocyte-complexes (COCs). Immunofluorescence confocal microscopy images showing the actin filament of the TZP (red) and the presence of FMRP, FXR1P and FXR2P within TZPs (green). Actin filaments of TZPs were stained with Actistain 670 phalloidin (red); primary antibodies against FMRP, FXR1P and FXR2P were subsequently incubated with secondary antibodies conjugated with Alexa Fluor 488 (green). Arrows show some of the granules. Scale bars = 20 μm.

Supplemental Figure 2. Distribution of the cytoplasmic interacting family of FMR proteins 1 and 2 (CYFIP1 and CYFIP2) in porcine cumulus-oocyte complexes (COCs). Immunofluorescence confocal microscopy images showing the actin filament of the TZP (red) and the localization of A) CYFPIP1 and B) CYFIP2 in TZPs (green). TZPs were stained with Actistain 670 phalloidin (red). DNA was stained with Hoechst 33432 dye (blue). Primary antibodies against CYFIP1, and CYFIP2 were subsequently

703 incubated with secondary antibodies conjugated with Alexa Fluor 488 (green). Arrows
704 show some of the granules. Scale bars = 20 μm.

705 **Supplemental Figure 3. Distribution of nuclear interacting FMR protein 1**
706 **(NUFIP1) in porcine cumulus-oocyte complexes (COCs).** Immunofluorescence
707 confocal microscopy images showing the actin filament of the TZP (red) and the
708 localization of NUFIP1 (green) within the TZPs in bovine COCs. TZPs were stained
709 with acti-stain 670 phalloidin (red). Arrows show some of the granules. Scale bars =
710 20 μm.

711

712 **FIGURE 1**

713

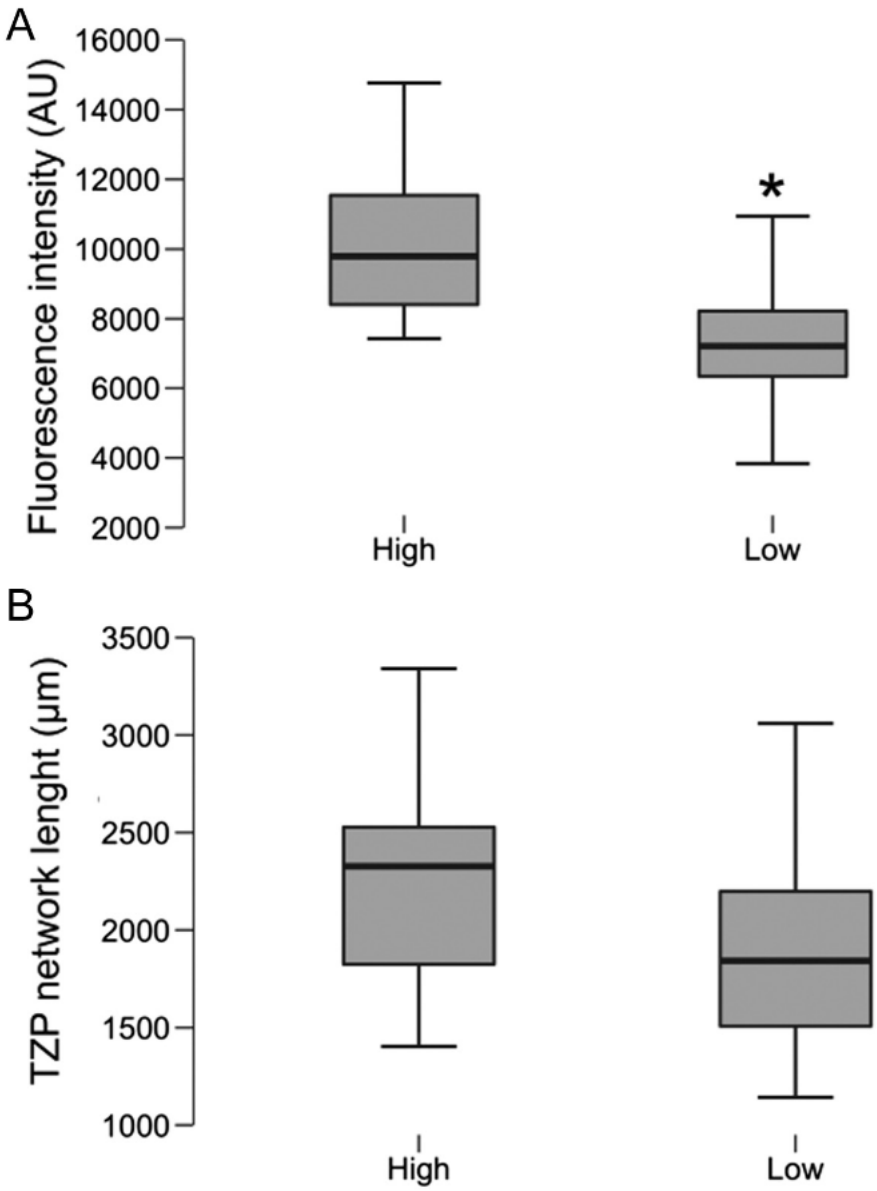

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FIGURE 2

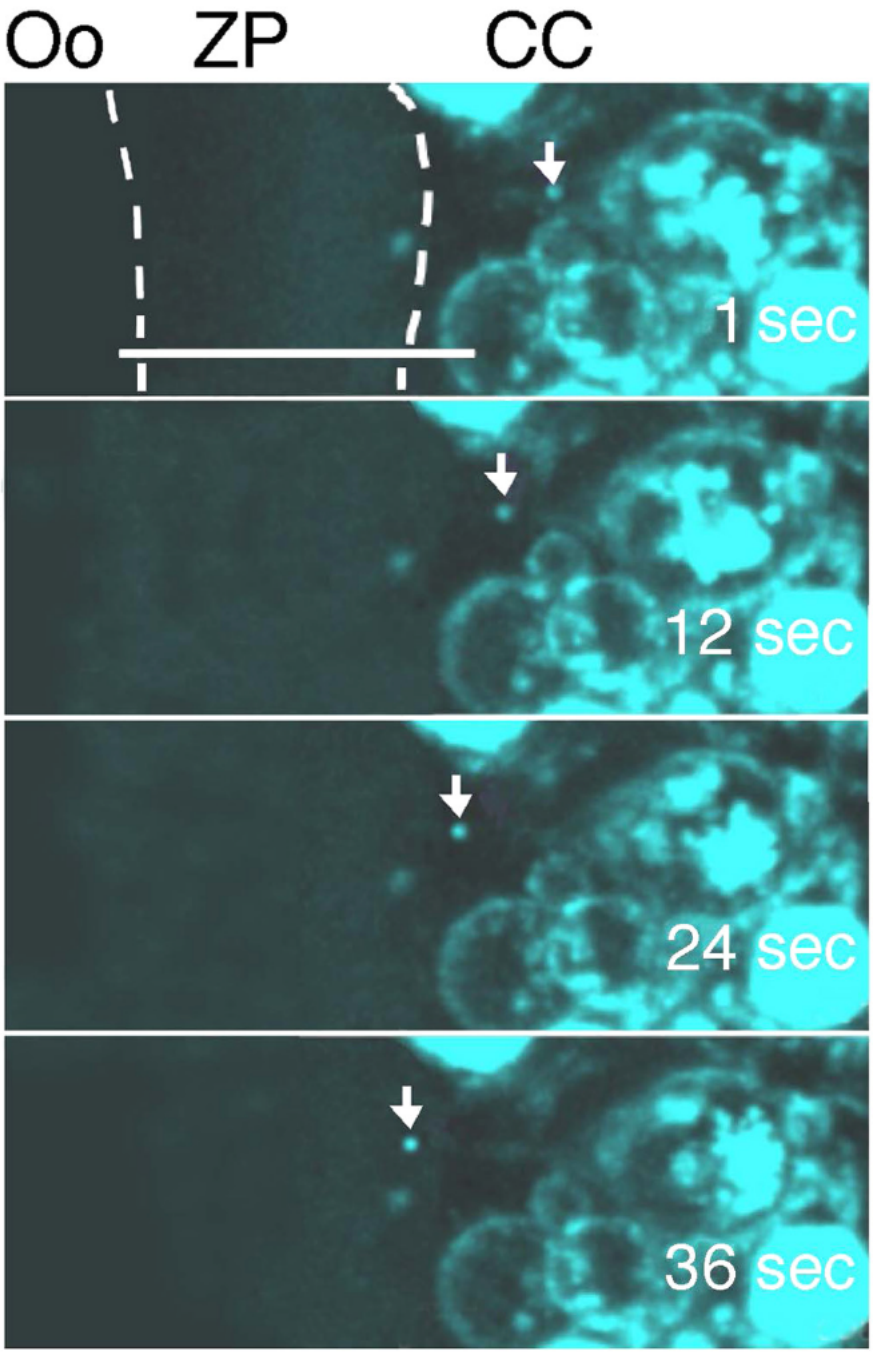


FIGURE 3

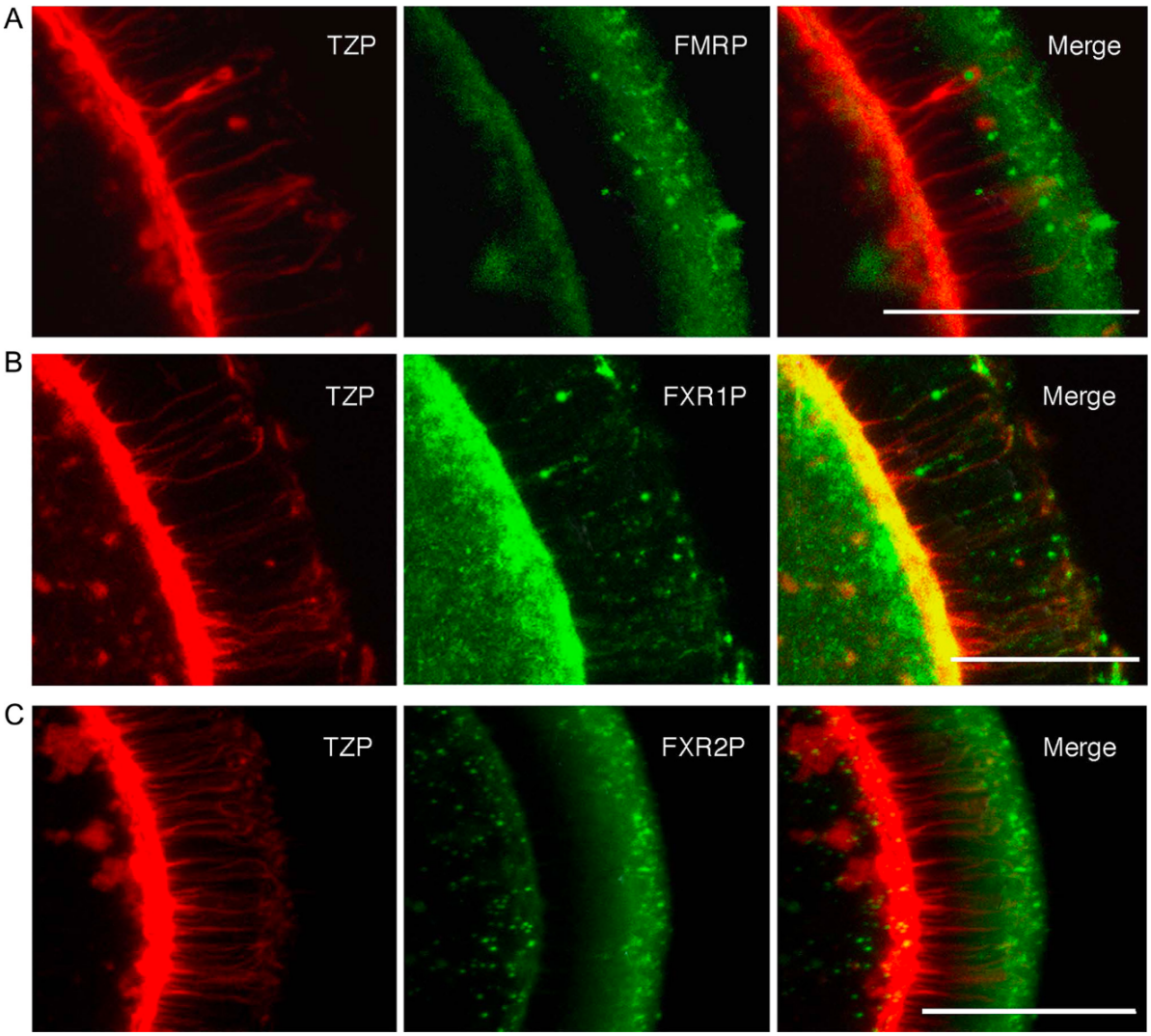


FIGURE 4

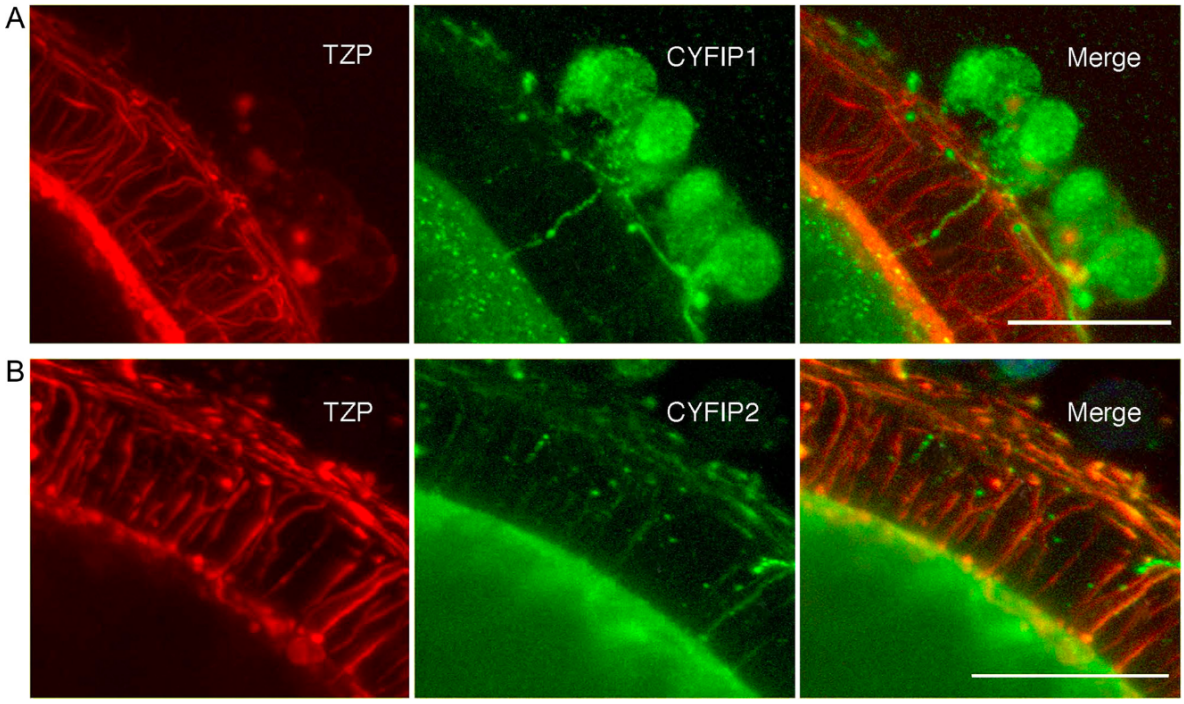
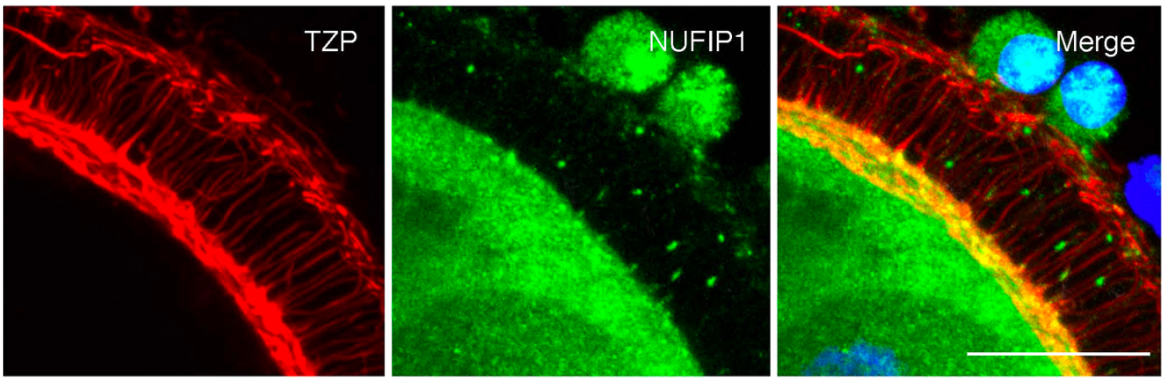
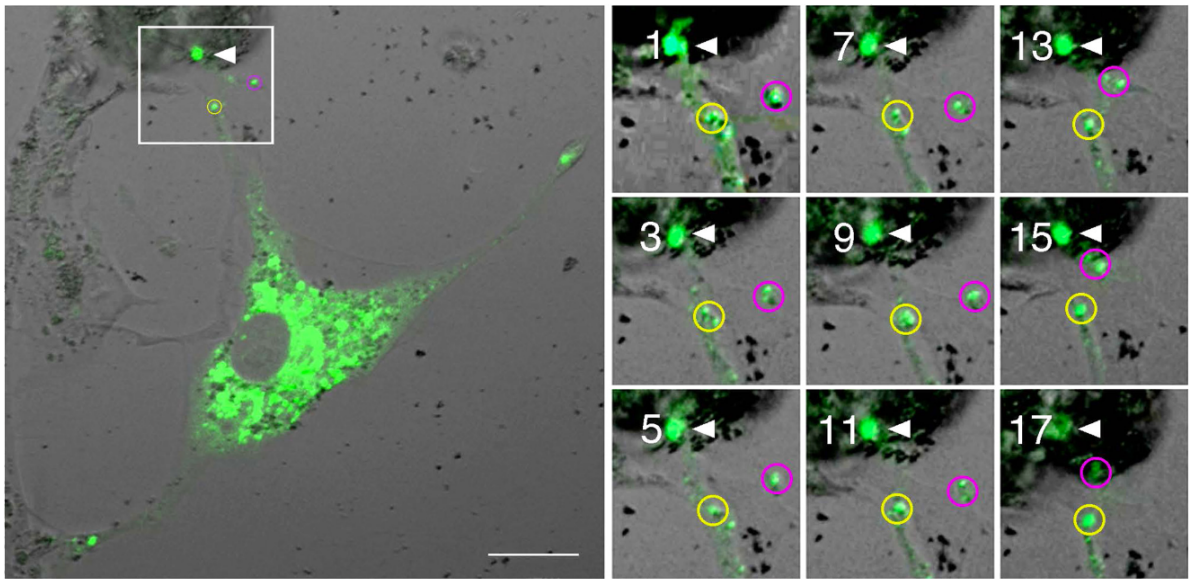


FIGURE 5



735 **FIGURE 6**



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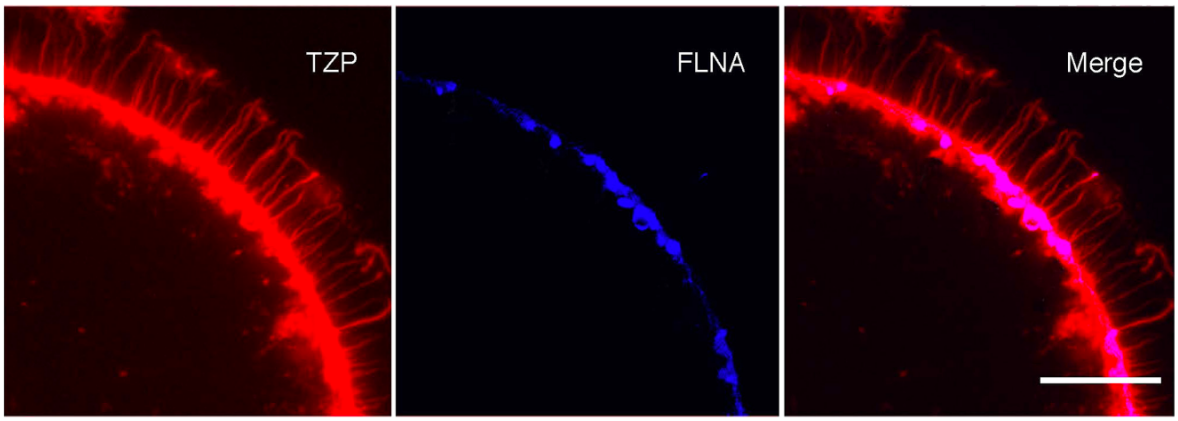
738 **FIGURE 7**



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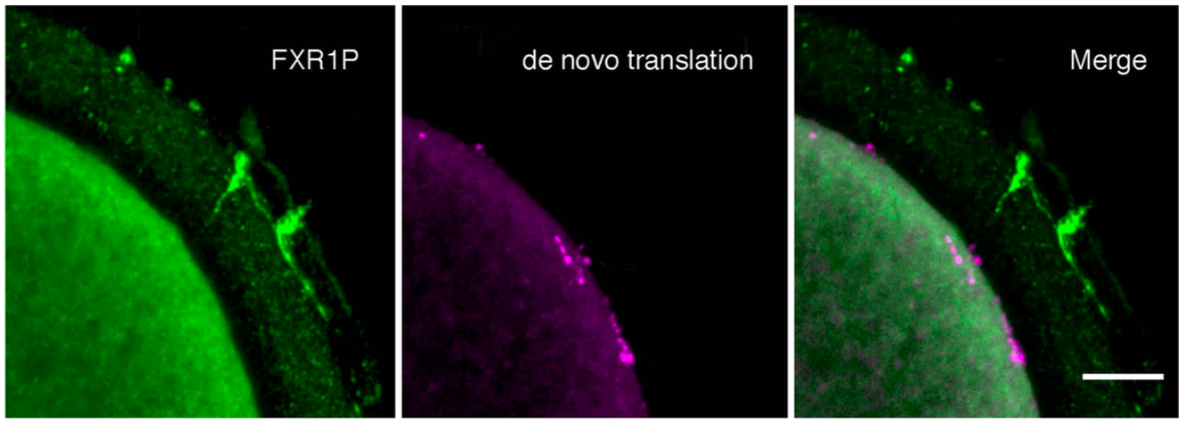
741 **FIGURE 8**



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745 **Supplemental Table S1.** List of the antibodies used in this study

Antibody Name	**Supplier, Catalogue Number**	**Dilution used in Immunofluorescence**	**RRID**
FMRP	Custom	1:100	
FXR1	Custom	1:1000	
Anti-FXR2, fragile X mental retardation, autosomal homolog 2	Atlas antibodies, HPA022997	1 µg/ml	AB_1849208
CYFIP1 Polyclonal Antibody	Thermo Fisher Scientific, PA5-31984	1 µg/ml	AB_2549457
CYFIP2 Polyclonal Antibody	Thermo Fisher Scientific, PA5-67174	2 µg/ml	AB_2664376
NUFIP1 Polyclonal Antibody	Thermo Fisher Scientific, PA5-56308	1 µg/ml	AB_2644885
Anti-Filamin A Antibody, clone TI10	Millipore, MAB1680	1:100	AB_94323
Goat Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	Thermo Fisher Scientific, A-11008	1:1000	AB_10563748
Rabbit anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific, A-21431	1:1000	AB_2535852
Goat Anti-Chicken IgY(H+L) Antibody, CF488A Conjugated	Biotium ,20020-1,	1:1000	AB_10854234

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