Fyn kinase is involved in cleavage furrow ingression during meiosis and mitosis

Mattan Levi, Bernard Maro and Ruth Shalgi

Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Ramat-Aviv, Tel-Aviv 69978, Israel

Correspondence should be addressed to R Shalgi; Email: shalgir@post.tau.ac.il

Abstract

Fertilization of mammalian oocytes triggers their exit from the second meiotic division metaphase arrest. The extrusion of the second polar body (PBII) that marks the completion of meiosis is followed by the first mitotic cleavage of the zygote. Several lines of evidence in somatic cells imply the involvement of Fyn, an Src family kinase (SFK), in cell cycle control and actin functions. In this study, we demonstrate, using live cell confocal imaging and microinjection of Fyn cRNAs, the recruitment of Fyn to the oocyte's cortical area overlying the chromosomes and its colocalization with filamentous actin (F-actin) during exit from the meiotic metaphase. Fyn concentrated asymmetrically at the cortical site designated for ingression of the PBII cleavage furrow, where F-actin had already been accumulated, and then redispersed throughout the entire cortex only to be recruited again to the cleavage furrow during the first mitotic division. Although microinjection of dominant negative Fyn did not affect initiation of the cleavage furrow, it prolonged the average duration of ingression, decreased the rates of PB extrusion and of the first cleavage, and led to the formation of bigger PBs and longer spindles. Extrusion of the PBII was blocked in oocytes exposed to SU6656, an SFK inhibitor. Our results demonstrate, for the first time, a continuous colocalization of Fyn and F-actin during meiosis and imply a role for the SFKs, in general, and for Fyn, in particular, in regulating pathways that involve actin cytoskeleton, during ingression of the meiotic and mitotic cleavage furrows.


Introduction

Meiosis of mammalian oocytes starts during embryonic life and arrests around birth, at prophase of the first meiotic division (MI), characterized by the presence of a germinal vesicle (GV; Mehlmann 2005). Meiotic arrest persists at least until puberty, when selected oocytes resume their MI in a process referred to as oocyte maturation. Maturation includes formation of a centered spindle, migration of the spindle along its long axis toward the nearest part of the oocyte cortex, and formation of an area devoid of microvilli and enriched with filamentous actin (F-actin) at the cortical area overlying the spindle (Maro & Verlhac 2002). When the spindle reaches the cortex, the metaphase–anaphase transition is triggered, the first polar body (PBI) is formed in this actin-rich cortical domain, and the oocyte is arrested at metaphase of the second meiotic division (MII). The spindle of MII oocytes lies in parallel to the oocyte surface, under a cortical domain enriched with microfilaments and devoid of microvilli. The cleavage furrow site of the second polar body (PBII) extrusion is restricted to this region. At fertilization, the spermatozoon fuses with the MII oocyte and triggers it to resume its MII. The fertilized oocyte undergoes a series of events that are collectively called oocyte activation. During anaphase and telophase of the second meiotic division (AII and TII respectively), two cortical bulges form above the two sets of segregated chromosomes. One of these bulges enlarges while the other retracts, causing the spindle to rotate. Ingression of the cleavage furrow takes place during spindle rotation, causing the extrusion of the PBII that indicates the completion of meiosis. The extrusion of both PBs is a unique case of cytokinesis as well as the most extreme example of size difference between the two daughter cells (Maro & Verlhac 2002). The formation of pronuclei (PN) marks the beginning of embryonic development, followed by the first mitotic division that results in cleavage of the one-cell zygote into two blastomeres (Dard et al. 2008).

Src family kinases (SFKs) are nine homologous, non-receptor protein tyrosine kinases, capable of compensating one for another (Stein et al. 1994, Roche et al. 1995). Src, Yes, and Fyn are found in a broad range of mammalian cells, including oocytes (Sato et al. 1996, Talmor-Cohen et al. 2004, Mehlmann & Jaffe 2005, Meng et al. 2006, McGinnis et al. 2007, Zheng et al. 2007).
Several lines of evidence indicate the importance of Fyn in the control of cell cycle (Thomas & Brugge 1997). Several lines of evidence indicate the importance of Fyn for various actin functions (Thomas et al. 1995, Yasunaga et al. 1996, Ng et al. 2005, Samayawardhena et al. 2007) and its requirement for peripheral membrane targeting of F-actin (Sandilands et al. 2007). Pro-B cells from Fyn−/− mice, cultured at defined serum-free conditions, arrest at telophase during cytokinesis (Yasunaga et al. 1996). During anaphase, Fyn is localized at the cleavage furrow of hematopoietic pro-B cells (Yasunaga et al. 1996) and hybridoma T cells (Campbell et al. 1998). It is suggested that Fyn specifically stimulates ROCK-mediated stress-fiber formation in fibroblasts (Xu et al. 2007), and is implied that it plays a unique role upstream to phosphatidylinositol 3-kinase and Rac GTPases, in promoting the reorganization of F-actin in mast cells during spreading and chemotaxis (Samayawardhena et al. 2007).

We have previously demonstrated that rat oocytes activation, triggered by SrCl2, puromycin, or ionomycin, is inhibited in the presence of SFK inhibitors, PP2 and SU6656 (Talmor-Cohen et al. 2004, Tomashov-Matar et al. 2007, 2008) and have recently shown that the same applies to mouse oocytes (Levi & Shalgi 2009, Levi et al. 2010). We were able to demonstrate the localization of Fyn to the cytoplasm, cortex, and MII spindle of immunostained rat oocytes (Talmor-Cohen et al. 2004).

Although Fyn−/− mice are viable, females produce only two to three litters after which reproduction fails (McGinnis et al. 2009). Furthermore, Fyn−/− Src+/− female mice show decreased fertility (Stein et al. 1994), however, the molecular mechanism responsible for this phenotype is still unknown. In this study, we have used a parthenogenic model. Unlike fertilization, which produces a developmentally heterogeneous population of embryos, parthenogenesis yields a synchronous population, thereby facilitating the analysis of the meiotic processes. Our results obtained after the microinjection of Fyn cRNAs into the mouse oocytes and their examination by live cell confocal imaging imply a possible involvement of Fyn in F-actin functions during the meiotic and mitotic processes of cleavage furrow ingestion.

**Results**

In this study, oocyte activation was triggered parthenogenically in MII mouse oocytes microinjected with Fyn cRNAs. PBII extrusion was imaged by spinning-disk confocal microscopy, using the Volocity 5 software (Improvision, Coventry, UK). The MII spindle was cortical in all oocytes, while the GV was centrally located in more than 80% of the oocytes. Our results demonstrate that microinjection of the cortically oriented dominant negative (DN)-Fyn-FLAG cRNA, rather than the cytoplasmically oriented DN-Fyn-Venus cRNA, caused a significant decrease in the percent of oocytes extruding PBII (Fig. 1A; P<0.05) and undergoing the first mitotic cleavage (Fig. 1B; P<0.05). Only 60.8% of the DN-Fyn-FLAG cRNA-microinjected oocytes extruded PBII, compared with 98.8, 95.8, and 100% of the wild-type (WT)-Fyn-FLAG, WT-Fyn-Venus, and DN-Fyn-Venus cRNA-microinjected oocytes respectively (Fig. 1A). Oocytes injected with DN-Fyn-FLAG exhibited high rate of PBII extrusion, similar to noninjected positive control oocytes (96.6% of 118 oocytes). A more dramatic effect on PBII extrusion (37.9% of 108 oocytes extruded PBII) was observed in noninjected, negative control oocytes, exposed to 10 μM SU6656, a specific inhibitor of the SFKs. Microinjection of DN-Fyn-FLAG cDNA did not affect PN formation (100% in all groups; Supplementary Movies 1 and 2, see section on supplementary data given at the end of this article) even in oocytes that did not extrude PBII. Furthermore, only 27.3% of the DN-Fyn-FLAG cRNA-microinjected oocytes cleaved, compared with 100,

Figure 1 The effect of Fyn inhibition on the occurrence and duration of PBII extrusion (A) and on the first mitotic cleavage (B). Mouse oocytes at the MII stage were microinjected with Histone-H2B-TRIM27 cRNA mixed with WT-Fyn-FLAG (WT-F; 119 oocytes; blue), DN-Fyn-FLAG (DN-F; 147 oocytes; orange), WT-Fyn-Venus (WT-V; 26 oocytes; green), or DN-Fyn-Venus (DN-V; 56 oocytes; red) cRNAs. Oocytes were cultured in M2 medium for 6 h, allowing for cRNA translation, and were then parthenogenically activated for 6 min by 8% ETOH, washed, and imaged every 15 min by spinning-disk confocal microscopy. Four-dimensional (x,y,z,t) image stacks were acquired at three channels (488 and 561 nm and DIC), processed, and analyzed by the Volocity 5 software. Telophase was indicated when two sets of distant segregated chromosomes appeared (561 nm channel) and was classified as telophase of the second meiotic division (TII; A) or as telophase of the first mitotic division (T; B) if it occurred after pronuclei (PN) formation. Oocytes were classified as being at the PBII stage once the extruded PBII was identified (DIC channel).
100, and 87.5% of the WT-Fyn-FLAG, WT-Fyn-Venus, and DN-Fyn-Venus cRNA-microinjected oocytes respectively (Fig. 1B). These observations encouraged us to further examine the effect of Fyn inhibition on other parameters during the process of PBII extrusion. Parameters such as timing of initiation and duration of the cleavage furrow ingression, spindle length, and the volume of the extruded PBII were examined by live cell imaging of Fyn cRNA-microinjected oocytes.

Although the cleavage furrow ingression was initiated in all groups at approximately at the same interval after TII (Figs 2 and 3A, white bars), the average duration of the process of PBII extrusion (Fig. 3A, gray bars) was significantly longer \((P<0.05)\) in DN-Fyn-FLAG cRNA-microinjected oocytes \((61.5 \pm 7.2 \text{ min})\) than in WT-Fyn-FLAG, WT-Fyn-Venus, or DN-Fyn-Venus cRNA-microinjected oocytes \((26.9 \pm 1.2, 25.8 \pm 2.5, \text{ and } 26 \pm 2.3 \text{ min respectively})\). Furthermore, the average volume of PBII extruded by DN-Fyn-FLAG cRNA-microinjected oocytes \((14485 \pm 2588 \mu \text{m}^3)\) was considerably larger \((P<0.05; \text{ Fig. } 3B)\) than that extruded by noninjected control oocytes or by WT-Fyn-FLAG, WT-Fyn-Venus, or DN-Fyn-Venus cRNA-microinjected oocytes \((4826 \pm 270, 5460 \pm 716, 5460 \pm 716, \text{ and } 6188 \pm 299 \mu \text{m}^3 \text{ respectively})\). Unlike the effects of the cortical DN-Fyn-FLAG, the cytoplasmic DN-Fyn-Venus affected neither the duration of ingression nor the volume of the extruded PBII, the rate of its extrusion, or the process of the first cleavage. Interestingly, though microinjection of DN-Fyn-FLAG cRNA affected neither the length of the MII spindle (Fig. 4) nor its anchoring to the cortex (Fig. 2B; Supplementary Movies 1 and 2), the TII spindle was affected and appeared longer than the one in WT-Fyn-FLAG cRNA-microinjected oocytes (Fig. 4). The longer the TII stage lasted, the more elongated the spindle became (Supplementary Movies 1 and 2).

Our observations of PBII inhibition led us to examine the localization of Fyn in live mouse oocytes during meiosis, using confocal microscopy of oocytes microinjected with Fyn cRNAs. Fyn and F-actin colocalize and are homogeneously distributed throughout the cortex of GV oocytes (Fig. 5Aa). F-actin accumulated in the area designated for the formation of the cleavage furrow (Fig. 5Ab), while Fyn was homogeneously distributed throughout the cortex (Fig. 5Ab') before being recruited to the cortical bulges overlying the two sets of chromosomes during AII and TII. The spindle started rotating once one of these bulges enlarged and the other retracted (Fig. 2Ac). When the spindle was fully rotated, cleavage took place and PBII was extruded (Fig. 2Ad).

During the extrusion of PBII, Fyn and F-actin concentrated asymmetrically at the cortical area closest to

![Figure 2](https://www.reproduction-online.org)

**Figure 2** Effect of Fyn inhibition on the extrusion of PBII. Spinning-disk confocal images of representative live MII oocytes microinjected with Histone-H2B-TRIM27 (red) and β-tubulin-GFP (green) mixed with WT-Fyn-FLAG (A; 1 out of 119 imaged oocytes) or with DN-Fyn-FLAG (B; 1 out of 147 imaged oocytes) cRNAs. Oocytes were cultured in M2 medium for 6 h, allowing for cRNA translation, and were then parthenogenetically activated for 6 min by 8% EtOH, washed, and imaged by the spinning-disk confocal microscope. Four-dimensional \((x,y,z,t)\) image stacks were acquired at the 488 and 561 nm channels (Aa–e; Ba–e) and the DIC channel (Aa'–e'; Ba'–e'), using the Velocity 5 software at 10 min intervals with an automatic movement of the objective at the z-axis \((\Delta z=5 \mu \text{m})\) over a total depth of 45 μm. Scale bar (lower left) = 10 μm.
Unlike the effects of the cortical DN-Fyn-FLAG, the cytoplasmic DN-Fyn-Venus affected neither the duration of ingestion nor the volume of the extruded PBII, the rate of its extrusion, or the process of the first cleavage; suggesting that the cortical portion of Fyn is the main contributor to the ingestion of the cleavage furrow during cytokinesis in oocytes. Furthermore, our previous data indicate that the rate of extrusion of PBII and its size were also affected by the inhibition of cortical Fyn (Levi et al. 2010). Interestingly, the TII spindle in DN-Fyn-FLAG cRNA-microinjected oocytes was longer than in control oocytes. These differences may result from different manifestations in different oocytes of the effect of inhibition of Fyn by the microinjected DN-Fyn-FLAG cRNA: in some oocytes, the cleavage furrow progression during PBII extrusion was prevented, while in others the duration of the whole process was prolonged. The longer the TII stage lasted, the more elongated the spindle became, and as a result the area restricted for the formation of the cleavage furrow expanded. Once the spatial restrictions for cleavage furrow formation became milder, a larger PBII was extruded.

Further support for a role for SFKs in cytoskeleton organization comes from the analysis of Dsrc64 null flies (Thomas & Brugge 1997). The generation of a mature oocyte involves cytoskeleton-coordinated changes. Female flies, homozygous for a null mutation in Dsrc64, have a partial infertility defect that may be linked to oocyte development. The cytoplasm of the oocyte and its surrounding nurse cells is connected by cytoskeleton structures called ring canals that allow transport of nutrients from the nurse cells into the oocyte. Dsrc64 null females have smaller oocytes than WT, the spindle midzone (Fig. 5Ac′ and Ac″) and at the cleavage furrow during ingestion. Finally, once PBII was extruded, Fyn resumed its homogenous cortical distribution pattern (Fig. 5Ba′) before translocating again to the cortical cleavage furrow area above the spindle midzone during the first mitotic cleavage (Fig. 5Bb″).

Discussion

Our results demonstrated that inhibition of Fyn by microinjecting oocytes with the cortically oriented DN-Fyn-FLAG cRNA, rather than with the cytoplasmically oriented DN-Fyn-Venus cRNA, inhibited both PBII extrusion and the first mitotic cleavage. Furthermore, the average duration of the process of PBII extrusion was significantly longer and the average volume of the extruded PBII was larger in DN-Fyn-FLAG cRNA-microinjected oocytes than in control oocytes.

Figure 3 Effect of Fyn inhibition on the duration of cleavage furrow inversion and PBII volume. (A) Mouse oocytes at the MII stage were microinjected with WT-Fyn-FLAG (WT-F; 81 oocytes), DN-Fyn-FLAG (DN-F; 53 oocytes), WT-Fyn-Venus (WT-V; 60 oocytes), or DN-Fyn-Venus (DN-V; 75 oocytes) cRNAs. Oocytes were cultured in M2 medium for 6 h, parthenogenically activated, and imaged by spinning-disk confocal microscopy. TII stage was indicated when two sets of distant segregated chromosomes appeared through the 561 nm channel. The interval between the TII stage and the onset of the cleavage furrow inversion (DIC channel; white bars; TII to ingression), and the duration of the whole inversion process (DIC channel; gray bars; ingression) were assessed using the Volocity 5 software. Each bar represents mean ± S.E.M. (B) Mouse oocytes at the MII stage were microinjected with WT-Fyn-FLAG (WT-F; 58 oocytes), DN-Fyn-FLAG (DN-F; 31 oocytes), WT-Fyn-Venus (WT-V; 19 oocytes), or DN-Fyn-Venus (DN-V; 35 oocytes) cRNAs. Noninjected oocytes served as control (27 oocytes). Oocytes were cultured in M2 medium for 6 h, parthenogenically activated, and imaged by spinning-disk confocal microscopy. The volume of PBII was measured via the DIC channel using the Volocity 5 software (Volume of an oblate spheroid = 4/3 × π × (length)² × height). Each bar represents mean ± S.E.M.

Figure 4 Effect of Fyn inhibition on spindle length. Mouse oocytes at the MII stage were microinjected with cRNA of Histone-H2B-TRIM27 and β-tubulin-GFP mixed with WT-Fyn-FLAG (WT-F; 27 oocytes) or DN-Fyn-FLAG (DN-F; 35 oocytes) cRNAs. Oocytes were cultured in M2 medium for 6 h, allowing the translation of the cRNA, activated parthenogenically and imaged by spinning-disk confocal microscopy. Spindle length was determined as the distance between the spindle poles of both MII (white bars) and TII (gray bars) oocytes. It was measured at the three-dimensional images acquired via the 488 nm channel, using the Volocity 5 software. Each bar represents mean ± S.E.M.
caused probably by an incomplete transfer from the nurse cells’ cytoplasm into the oocyte. Ring canals of Dsrc64 null females are smaller and occasionally become detached. In addition, although WT ring canals stain normally with phosphotyrosine antibodies, ring canals from Dsrc64 null females are devoid of phosphotyrosine epitopes. Ring canals may be similar to cell cleavages, which is consistent with McGinnis & Albertini’s recent paper (2010) in which the concentration of phosphotyrosine at the cortex and spindle poles of Fyn\(^{+/+}\) mice oocytes was reduced.

Ng et al. (2005) have shown that cytokinesis is inhibited in sea urchin oocytes exposed to SU6656 or microinjected with SH2 domains of Src or Fyn. They also demonstrated that phosphorylation is required for progression of the cleavage furrow and that isolated membranes containing SFKs become phosphorylated on their tyrosine residues during cytokinesis. Moreover, the morphology of F-actin organization at the cortical area overlying the spindle seems to be disrupted in MII mice oocytes exposed to SKI606, an SFK inhibitor, or in Fyn\(^{−/−}\) mouse oocytes (Luo et al. 2009). Proteins, phosphorylated on their tyrosine residues, are concentrated at the cortex of mice oocytes in the region adjacent to the maternal chromatin and the forming PBII (McGinnis et al. 2007). Phosphorylation of protein tyrosines increased during the MI to MII progression. The level of phosphorylated proteins was significantly higher in the oocyte cortex (McGinnis & Albertini 2010).

Our observations, along with that of Maro & Verlhac (2002), demonstrate the chain of events during oocyte maturation. During oocyte maturation, the spindle migrates along its long axis toward the nearest cortical region. Simultaneously, an area devoid of microvilli and enriched with actin microfilaments begins to form at the cortex overlying the spindle. When the spindle is situated perpendicular to the cortex, the metaphase–anaphase transition is triggered and the PBI forms in this actin-rich cortical domain. In MII oocytes, the spindle lies parallel to the surface, which is the region restricted for ingression of the cleavage furrow (Maro & Verlhac 2002). Our results demonstrated that F-actin accumulates in this area designated for formation of the cleavage furrow, whereas Fyn is homogenously distributed throughout the cortex before being recruited to the cortical bulges overlying the two sets of chromosomes.

**Figure 5** Localization of Fyn and actin during meiosis. (A) GV and MII mouse oocytes were microinjected with WT-Fyn-GFP (Fyn-GFP; green) and UtrCH-TRIM27 (UTR-TRIM27; red) cRNAs and were cultured in M2 medium for 6 h, allowing for cRNA translation. Oocyte activation was triggered parthenogenically in MII oocytes. Oocytes at the GV, MII, and PBII stages were imaged by Zeiss-510 confocal laser-scanning microscope via the DIC (a–c), 488 nm (a’–c’; Fyn), and 543 nm (a”–c”; actin) channels. Colocalization images (a’’’–c’’’) were created by the LSM-510 software from the analysis of the 488/543 nm-crossed histograms. At least 30 oocytes at each stage were imaged. Representative oocytes are shown. In all cases, Fyn colocalized with the cleavage furrow. (B) MII mouse oocytes were microinjected with WT-Fyn-GFP (Fyn-GFP; green) and triggered parthenogenetically as described earlier. Oocytes at the pronuclei (PN) and two-cell stages were imaged by Zeiss-510 confocal laser-scanning microscope via the DIC (a–b) and 488 nm (a’–b’) channels.
during All and TII. One of these bulges expands while the other shrinks, resulting in spindle rotation. When the spindle was fully rotated, cleavage took place and the PBII was extruded. Unlike the symmetric localization of Fyn around the spindle midzone during the extrusion of PBI (Levi et al. 2010) and PBII, Fyn concentrated asymmetrically at the cortical area closest to the spindle midzone, the area designated for ingestion of the cleavage furrow, where F-actin have also been accumulated. Fyn resumed its homogenous cortical distribution pattern before translocating again to the area above the spindle midzone designated for ingestion of the cortical cleavage furrow during the first mitotic cleavage. In general, Fyn is localized to the oocyte cortex during most of the meiotic division. However, during cytokinesis, when actin translocates to the cleavage furrow, Fyn is recruited to the same place. Our results demonstrate, for the first time, a continuous colocalization of Fyn and F-actin during meiosis.

Prolonged physiological arrests and asymmetrical cell divisions are unique characteristics of the two meiotic divisions in mammalian oocytes. The accuracy required for orchestrating the precise execution of these processes is crucial for production of embryos with the appropriate ploidy. Our results imply, for the first time, a mechanism in which Fyn is recruited by the cytoskeletal factors to the area of cleavage furrow during ingestion only after the accumulation of F-actin. Thereafter, cortical Fyn is involved in regulation of cytokinesis, possibly by facilitating the organization and function of related molecular machineries that involve F-actin. We postulate that Fyn phosphorylates tyrosine residues and thus affects the activity of factors involved in the cleavage furrow ingression. Though only limited data exist regarding the factors that take part in cleavage furrow ingression during PBII extrusion in mammalian oocytes, future studies should concentrate on examining several key factors such as myosin II, Aurora-B, and Anillin that are known to localize and function in this region during cytokinesis (Eggert et al. 2006).

Materials and Methods

Animals

OF1 female mice (7–9-weeks old; Charles River Laboratories International, Chatillon-sur-Chalaronne, France) and ICR female mice (7–9-weeks old; Harlan Laboratories, Jerusalem, Israel) were housed in air-conditioned, light-controlled animal facilities. Animal care was in accordance with institutional guidelines and was approved by the local authorities. Some female mice were superovulated with 5 IU pregnant mare’s serum gonadotrophin (Syncro-Part, Sanofi, France) and super-ovulated 48–54 h later with 5 IU human chorionic gonadotrophin (hCG; Sigma Chemical Co.).

Collection and culture of oocytes

GV oocytes

Nonstimulated female mice were killed and their ovaries were removed into prewarmed (37 °C) M2 medium (M-7167; Sigma) supplemented with 4 mg/ml BSA (Sigma). GV oocytes were released from the follicles into M2 medium containing 1 μM milrinone (Sigma), and cultured in 20 μl drops of M2 medium under mineral oil (Sigma) at 37 °C. Milrinone, a phosphodiesterase III inhibitor, causes high level of intra-oocyte cAMP that keeps the oocytes at the GV state (Tsafiri et al. 1996).

Ovulated MII oocytes

Female mice were killed 14 h after hCG administration and their oviductal ampullae were removed into prewarmed (37 °C) M2 medium supplemented with 4 mg/ml BSA. MII oocytes were removed from the oviductal ampullae, their cumulus cells were dispersed by hyaluronidase (H-3631; Sigma), and the oocytes were cultured in 20 μl drops of M2 medium under mineral oil at 37 °C.

Preparation and microinjection of cRNA

The full-length sequences of human cDNAs encoding the WT or DN forms of Fyn kinase (I.M.A.G.E. clone ID number 3613878, ResGen, Carlsbad, CA, USA), inserted into pCMV-Tag 4A with an in-frame FLAG (eight amino acids tag) at the 3′-end (Stratagene, Cedar Creek, TX, USA) had already been constructed in our laboratory (Tomashov-Matar et al. 2007). A point mutation at Y531 in the regulation site of Fyn opens the conformation of Fyn kinase and activates it by exposing the SH1 and SH2 domains to the cytoplasm. A point mutation at L299 in the ATP-binding site of the kinase makes Fyn a kinase-dead mutant, capable of inhibiting endogenous Fyn. Furthermore, the activity of the different forms of Fyn had previously been assessed by an in vitro autophosphorylation activity assay of Fyn. The purified protein of the constitutively active (CA)-Fyn-FLAG was highly autophosphorylated, whereas the WT-Fyn-FLAG and DN-Fyn-FLAG proteins had only a minimal autophosphorylation activity, or none at all (Tomashov-Matar et al. 2007). The 3′-end orientation of the FLAG epitope allowed eukaryotic posttranslational modifications, i.e. amino-terminal myristoylation and palmitoylation of Fyn within oocytes, thus targeting Fyn protein toward the membrane (Tomashov-Matar et al. 2007). WT-Fyn and DN-Fyn cDNAs were amplified and inserted into pSPE3 vectors, with an in-frame Venus fluorophore at the 5′-end. This orientation prevents the posttranslational modifications and targets Fyn protein toward the cytoplasm (Levi et al. 2010). All plasmids were sequenced and analyzed and they were linearized using SfiI restriction enzyme (New England Biolabs, Ipswich, MA, USA). In vitro cRNA transcripts, prepared using the T3 mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA), were purified on RNeasy columns (Qiagen) and eluted by nuclease-free water to a final concentration of 0.4–1 μg/ml. Aliquots of 4 μl were stored at −80 °C. The expression of the Fyn protein translated in oocytes microinjected with Fyn cRNA...
was measured by both western blot analysis and reticulocyte lysate system (Tomashov-Matar et al. 2007). Histone-H2B-TRIM27 cDNA and β-tubulin-GFP cDNA (Brunet et al. 1998, Ledan et al. 2001, Tsurumi et al. 2004) were donated by Dr Marie-Helene Verlhac, Pierre and Marie Curie University, Paris, France and were used for imaging of chromosomes and microtubules respectively.

A conjugate of calponin homology domain of utrophin, an actin-binding protein, with TRIM27 fluorescent protein (UtrCH-TRIM27) was used for imaging of F-actin. UtrCH-TRIM27 cDNA was donated by Prof. William M Bement, Department of Zoology, University of Wisconsin, Madison, WI, USA. UtrCH-TRIM27 protein reports faithfully the distribution of F-actin in fixed and live cells without significantly altering the dynamics of actin-based processes (Burkel et al. 2007).

In vitro synthesized cRNAs (Supplementary Figure 1, see section on supplementary data given at the end of this article) were microinjected into the cytoplasm of oocytes at either the GV or MII stages at a volume of ~5% of the oocyte volume, using the Eppendorf micromanipulator system (Leitz, Heidelberg, Germany). Microinjected oocytes and embryos were cultured for 6 h in M2 medium under mineral oil at 37 °C to allow protein translation.

**Parthenogenesis and live cell imaging**

At the end of the protein translation period, several microinjected MII oocytes were exposed to 8% EtOH for triggering parthenogenic activation (Verlhac et al. 1994). Unlike fertilization that produces a developmentally heterogeneous population of embryos, parthenogenesis yields a synchronous population, thereby facilitating the evaluation of the initial changes that take place within the oocytes shortly after the onset of oocyte activation.

For imaging, oocytes were transferred to 10 μl droplets of M2 medium under oil in a specially designed chamber, maintained at 37 °C on a heated microscope stage. Signal was recorded by either Zeiss-510 confocal laser-scanning microscope (Carl Zeiss Microlmaging, Oberkochen, Germany) via three channels, 488 and 543 nm and differential interference contrast (DIC), or by a C910 CCD digital camera (Hamamatsu, Hamamatsu City, Japan) attached to a digital camera (Hamamatsu, Hamamatsu City, Japan) via three channels, 488 and 561 nm and DIC channels. Three-dimensional (x,y,z) images were created at 10 min intervals by the movement of the objective along the z-axis (Δz = 5 μm) over a total depth of 45 μm. Four-dimensional (x,y,z,t) image stacks were processed and analyzed by the Volocity 5 software.

**Statistical analysis**

Data were evaluated by Pearson’s χ² test for independence or by independent, two-sample t-test for unequal sample sizes and unequal variances. P<0.05 was considered significant. A one-way ANOVA statistical analysis showed similar results.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0312.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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