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The involvement of Fyn kinase in resumption of the first meiotic division in mouse oocytes

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Key words: meiosis, oocyte, Fyn, GVBD, exit from metaphase, polar body

Abbreviations: AI, anaphase of the first meiotic division; APC, anaphase promoting complex; CA, constitutively active; CLSM, confocal laser-scanning microscope; cRNA, complementary RNA; DIC, differential interference contrast; DN, dominant negative; ECL, enhanced chemiluminescence; GV, germinal vesicle; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotrophin; LH, luteinizing hormone; MI, metaphase of the first meiotic division; MII, metaphase of the second meiotic division; MPF, maturation promoting factor; MTs, microtubules; PBI, polar body I; PMSG, pregnant mares' serum gonadotrophin; RMI, resumption of the first meiotic division; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SFKs, Src family kinases; TI, telophase of the first meiotic division; WB, western blot; WT, wild type; χ^2 , chi-square

The process of resumption of the first meiotic division (RMI) in mammalian oocytes includes germinal vesicle breakdown (GVBD), spindle formation during first metaphase (MI), segregation of homologous chromosomes, extrusion of the first polar body (PBI) and an arrest at metaphase of the second meiotic division (MII). Previous studies suggest a role for Fyn, a non-receptor Src family tyrosine kinase, in the exit from MII arrest. In the current study we characterized the involvement of Fyn in RMI. Western blot analysis demonstrated a significant, proteasome independent, degradation of Fyn during GVBD. Immunostaining of fixed oocytes and confocal imaging of live oocytes microinjected with Fyn complementary RNA (cRNA) demonstrated Fyn localization to the oocyte cortex and to the spindle poles. Fyn was recruited during telophase to the cortical area surrounding the midzone of the spindle and was then translocated to the contractile ring during extrusion of PBI. GVBD, exit from MI and PBI extrusion were inhibited in oocytes exposed to the chemical inhibitor SU6656 or microinjected with dominant negative Fyn cRNA. None of the microinjected oocytes showed misaligned or lagging chromosomes during chromosomes segregation and the spindle migration and anchoring were not affected. However, the extruded PBI was of large size. Altogether, a role for Fyn in regulating several key pathways during the first meiotic division in mammalian oocytes is suggested, particularly at the GV and metaphase checkpoints and in signaling the ingression of the cleavage furrow.

Introduction

Meiosis of mammalian oocytes starts during embryonic life and arrests around birth, at prophase of the first meiotic division, characterized by the presence of a germinal vesicle (GV). Meiotic arrest lasts at least until puberty, when selected oocytes resume their first meiotic division (RMI) and are ovulated after being exposed to the midcycle surge of luteinizing hormone (LH).¹ RMI is manifested by chromosomes condensation, GV breakdown (GVBD), formation of the spindle and its migration towards the oocyte cortex, segregation of homologous chromosomes, extrusion of the first polar body (PBI) and an arrest at metaphase of the second meiotic division (MII).²

Progression through meiotic and mitotic divisions is controlled by maturation promoting factor (MPF), a heterodimer that consists of the cyclin-dependent kinase I (p34cdc2) and a

regulatory subunit (cyclin-B).²⁻⁴ MPF activity is low in oocytes at the GV stage. The intra-oocyte drop in cAMP concentration during GVBD^{5,6} leads to an increase in MPF activity and thus to the formation of the spindle. MPF activity stays elevated during the first meiotic division (MI). Degradation of cyclin-B accompanies the metaphase-anaphase transition and the extrusion of PBI,⁷ followed by formation of the MII spindle and a second meiotic arrest.^{8,9} Release from MII arrest is triggered by an increase in intracellular Ca²⁺ concentration induced by the fertilizing spermatozoon. Inactivation of MPF during exit from both MI and MII stages is controlled by the anaphase promoting complex (APC), a multi-subunit E3 ligase that ubiquitinates cyclin-B, thereby targeting it for degradation by the 26S proteasome.^{3,8,10,11}

Src family kinases (SFKs) are nine non-receptor protein tyrosine kinases sharing a homologous structure.¹² Members of the SFKs are able to compensate for one another.^{13,14} Src, Yes, Fyn

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and Yrk (a non mammalian SFK) are found in a broad range of tissues, including oocytes,^{1,15-21} while Lck, Fgr, Lyn, Hck and Blk are restricted to cells of the haematopoietic lineages.²² Activated SFKs catalyze the transfer of γ -phosphates from ATP molecules to tyrosine residues of target proteins, thereby transmitting extracellular signals downstream to cellular components.²²

Fyn is a 59 kDa SFK involved in a vast variety of cell functions, including cell cycle,²² apoptosis,²³ adhesion,²² control of Sertoli cell structure,²⁴ B-cell activation²⁵ and growth factor-induced mitogenesis.²⁶⁻²⁸ Several studies suggest an involvement of Fyn in the regulation of microtubules (MTs) organization,^{16,17,21,29,30,32-34} while according to others Fyn also affects actin-dependent processes.^{24,30,35-42} Furthermore, Fyn^{-/-} female mice show decreased fertility.^{14,32}

In our previous studies we have determined the expression and localization of Fyn, Yes and Src in rat oocytes fixed at the MII stage. Fyn is localized to the oocytes cytoplasm, cortex and spindle, Yes to the cytoplasm and the cortex, whereas Src is found only at the cytoplasm.^{20,43,44} Fyn coimmunoprecipitates with β -tubulin and phosphorylates β -tubulin in vitro,⁴⁴ indicating an association with the oocyte MTs.

We have further demonstrated that the release from MII arrest in rat oocytes activated by SrCl₂, puromycin or ionomycin is inhibited in the presence of SFKs inhibitors, PP2 or SU6656.^{20,45,46} As we have recently shown, the same applies to mouse oocytes.⁴⁷ Moreover, inhibition of SFKs by SU6656 also inhibited the degradation of cyclin-B and the release from MII arrest in fertilized rat oocytes.⁴⁵ Altogether, these observations imply a role for SFKs in general and for Fyn in particular, in the signal leading to cyclin-B degradation downstream to the Ca²⁺ elevation in mammalian oocytes during the exit from MII arrest.

Although the importance of SFKs in regulating cell cycle of somatic cells and in activating mammalian oocytes is determined, the data regarding their role in RMI is still limited. Inhibition of SFKs by PP2 caused inhibition of GVBD and the exit from MI.²¹ Inhibiting SFKs with either siRNA knockdown or gene deletion also inhibited the exit from MI, but had no effect on the process of GVBD or extrusion of PBI.³² Though both studies indicate defects in chromosome alignment in some fixed oocytes, it is still unclear whether the defected alignment inhibits the exit from MI. The limited evidence implicating SFKs in RMI encouraged us to elucidate the role of SFKs, particularly Fyn, in RMI in oocytes, by several approaches including live cell confocal imaging, immunoblotting, immunostaining, microinjection of selective complementary RNAs (cRNAs) and the use of chemical inhibitors.

Results

Fyn degradation during RMI. Changes in the amount of the endogenous Fyn protein (59 kDa) within oocytes during RMI in vitro were recorded by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (WB) analysis. ERK1/2 (42 kDa) or p34cdc2 (34 kDa) served as reference for the amount of total oocytes proteins analyzed in all experimental groups. The amount of Fyn decreased considerably

three hours after milrinone was washed out (Fig. 1A). Milrinone (Primacor) is a phosphodiesterase III inhibitor that causes high levels of cAMP in the oocytes cytoplasm⁴⁸ and thus inhibits the intra-oocyte drop in cAMP concentration leading to GVBD.^{5,6} No other significant changes were observed during the next 8 hours of RMI in vitro (Fig. 1B), when the majority of the oocytes reached the MII stage. Fyn degradation was correlated with the process of GVBD (Fig. 1C). The phenomena of Fyn degradation during in vitro maturation was also observed in in vivo maturation (Fig. 1D). In order to examine whether the decrease in the amount of Fyn during RMI involves the proteasome pathway we used two proteasome inhibitors, MG132 (50 μ M) and Lactacystin (50 μ M).^{49,50} As indicated in the literature, the proteasome is essential in mouse oocytes for the exit from MI and for PBI extrusion, but not for the process of GVBD.⁵⁰ Hence, we assessed by light microscopy in our system, the influence of proteasome inhibition on the two processes of PBI and GVBD. Fourteen hours after removal of oocytes from milrinone, none of the proteasome-inhibitors treated oocytes extruded PBI (0/71 of the MG132-treated oocytes; 0/60 of the Lactacystin-treated oocytes), while 91.4% (64/70) of the control oocytes extruded PBI. Exposing milrinone-treated oocytes to the proteasome inhibitors at the indicated concentrations did not affect the rate of GVBD (96.7, 95.3 and 96.7% for control, MG132-treated and Lactacystin-treated oocytes, respectively; 150 oocytes in each treatment). Though our results confirm previous published studies regarding the role of the proteasome in meiosis, our current data indicated that inhibition of the proteasome did not affect the degradation of Fyn and the amount of Fyn in oocytes matured in vitro for three hours was similar in treatment and control groups (Fig. 1E).

Localization of Fyn during RMI. Localization of Fyn within in vitro matured oocytes was examined by microinjection of cRNA of Fyn-GFP or Fyn-Venus (Fig. 2) into live oocytes or by confocal laser-scanning microscope (CLSM) imaging of fixed oocytes immunostained with rabbit anti-Fyn antibody. Immunostaining showed homogenous localization of Fyn throughout the oocyte cytoplasm and concentration at the spindle and cortex areas (Fig. 3Bb). In order to track temporal changes of Fyn localization within in vitro matured oocytes, the localization of Fyn was examined by imaging live oocytes with spinning-disk confocal microscopy, ensuring minimal laser exposure and thus minimum damage to the oocytes. Two cRNA constructs with opposite conjugated-fluorophore orientations, wildtype Fyn-GFP (WT-Fyn-GFP; fluorophore at the carboxy-terminus of Fyn; Fig. 2) or wildtype Fyn-Venus (WT-Fyn-Venus; fluorophore at the amino-terminus of Fyn; Fig. 2), mixed with Histone-H2B-RFP cRNA, were microinjected into live GV oocytes. The carboxy-terminal orientation of the GFP-conjugate allowed the translated Fyn-GFP protein to be myristoylated and palmitoylated on the free amino-terminus, leading to an increased hydrophobicity of the protein and contributing to its membrane-association (Fig. 3Aa-e). The orientation of the Venus-conjugate at the amino-terminus (Fig. 2) obstructed the translated Fyn protein from being myristoylated and palmitoylated, but allowed its localization to the meiotic spindle (Fig. 3Aa'-e'). Concordantly,

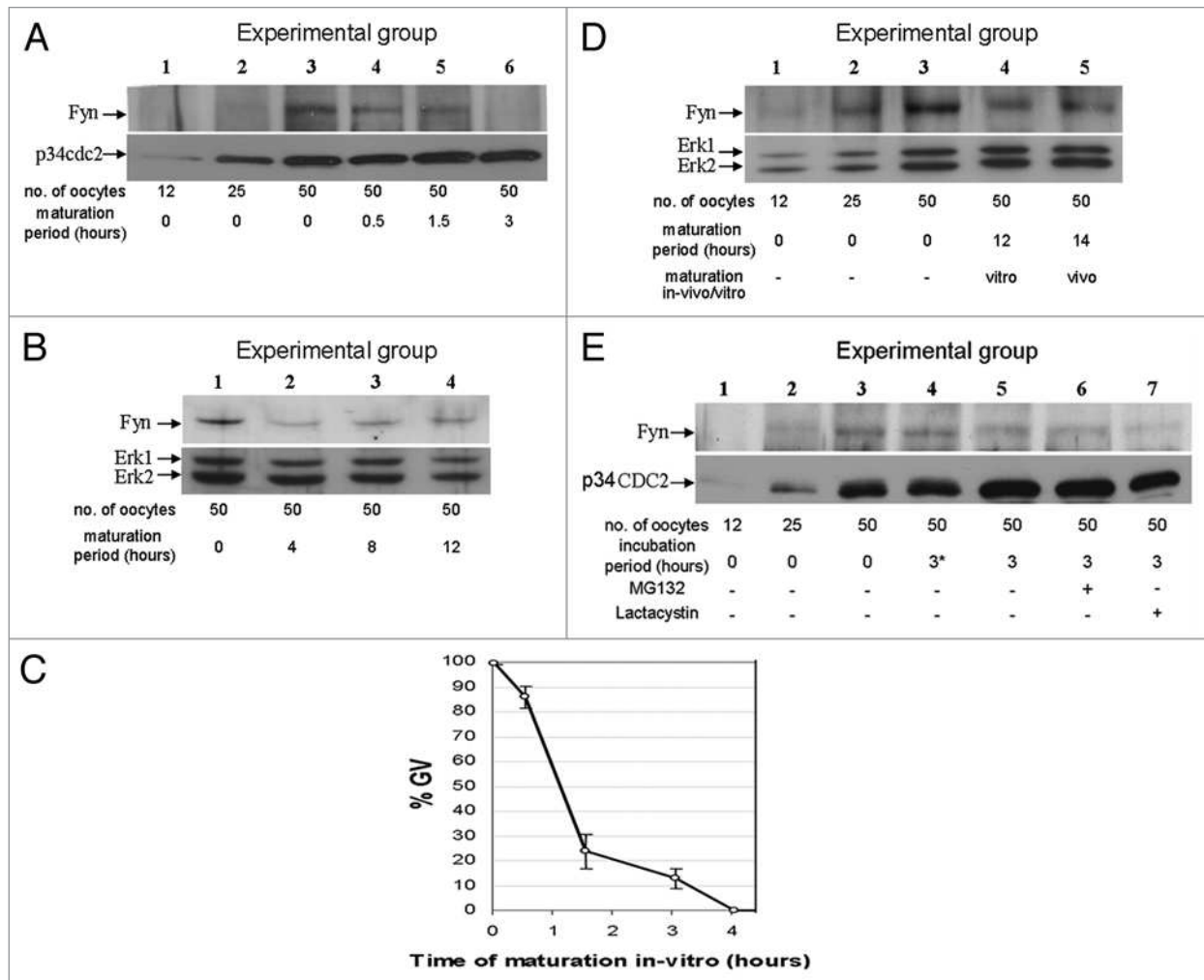


Figure 1. Degradation of Fyn during RMI. Oocytes were lysed and subjected to SDS-PAGE and WB for detection of Fyn protein (59 kDa). p34cdc2 (34 kDa) or ERK1/2 (42 kDa) served as indicators for the number of oocytes in all experimental groups. (A) Batches of 12, 25 and 50 (A1, A2 and A3, respectively) GV mouse oocytes and batches of 50 oocytes cultured for 0.5, 1.5 and 3 hours (A4, A5 and A6, respectively) after milrinone wash out. (B) Batches of 50 mouse oocytes, before (B1) or after 4, 8 and 12 hours of culture after release from milrinone (B2, B3 and B4, respectively). (C) The relative percent of GV oocytes in each lane, as a function of RMI in vitro culture period, are presented. (D) Batches of 12, 25 and 50 (D1, D2 and D3 respectively) GV mouse oocytes and 50 oocytes matured in vitro (C4; 12 hours after milrinone wash out) or matured in vivo (C5; 14 hours after hCG injection). Three independent experiments were performed. A representative experiment is presented. (E) Batches of 12, 25 and 50 (E1, E2 and E3 respectively) GV mouse oocytes collected immediately or oocytes after 3 hours culture without milrinone (E3) or after 3 hours culture without milrinone, but with 50 μ M MG132 (E4) or 50 μ M Lactacystin (E5).

the combined localization of the two injected exogenous Fyn conjugates, matched the signal detected in oocytes immunostained for endogenous Fyn (Fig. 3Bb), i.e., homogenous localization of Fyn throughout the cytoplasm and concentration at the cortex and spindle areas.

WT-Fyn-GFP was recruited to the cortical area designated for PBI extrusion, 15 minutes prior to PBI extrusion, whereas 15 minutes after PBI extrusion, the kinase resumed a uniform distribution pattern throughout the cortex (Fig. 3Aa-c; Suppl. movie 1). Fyn was also detected at the cleavage furrow area during PBI extrusion (Suppl. movie 1). The localization of WT-Fyn-GFP protein (Fig. 3Aa-e; Suppl. movie 1) during the whole RMI process was similar to that of constitutively active Fyn (CA-Fyn; Suppl. movie 2) and dominant negative Fyn

(DN-Fyn; Suppl. movie 3) proteins. Interestingly, the WT-Fyn-Venus protein (Fig. 3Aa'-e'; Suppl. movie 4) was homogeneously distributed throughout the spindle whereas the DN-Fyn-Venus protein was specifically localized at the poles of the meiotic spindle in mouse oocytes (Fig. 3Ba). Moreover, the expressed WT-Fyn-Venus protein showed higher concentration at the spindle area in MI and MII rat oocytes (Fig. 3Ca and b, respectively).

Experimentally expressed GFP-only and Venus-only, which served as negative-controls, were homogeneously localized throughout the oocyte cytoplasm (Fig. 3Da and b, respectively). The amount of WT-Fyn-GFP protein, translated six hours after microinjection of their cRNA into GV oocytes (85 kDa; Fig. 3E2), was measured by SDS-PAGE and WB analysis and was found to be 6.7 ± 0.8 times higher than the amount of the

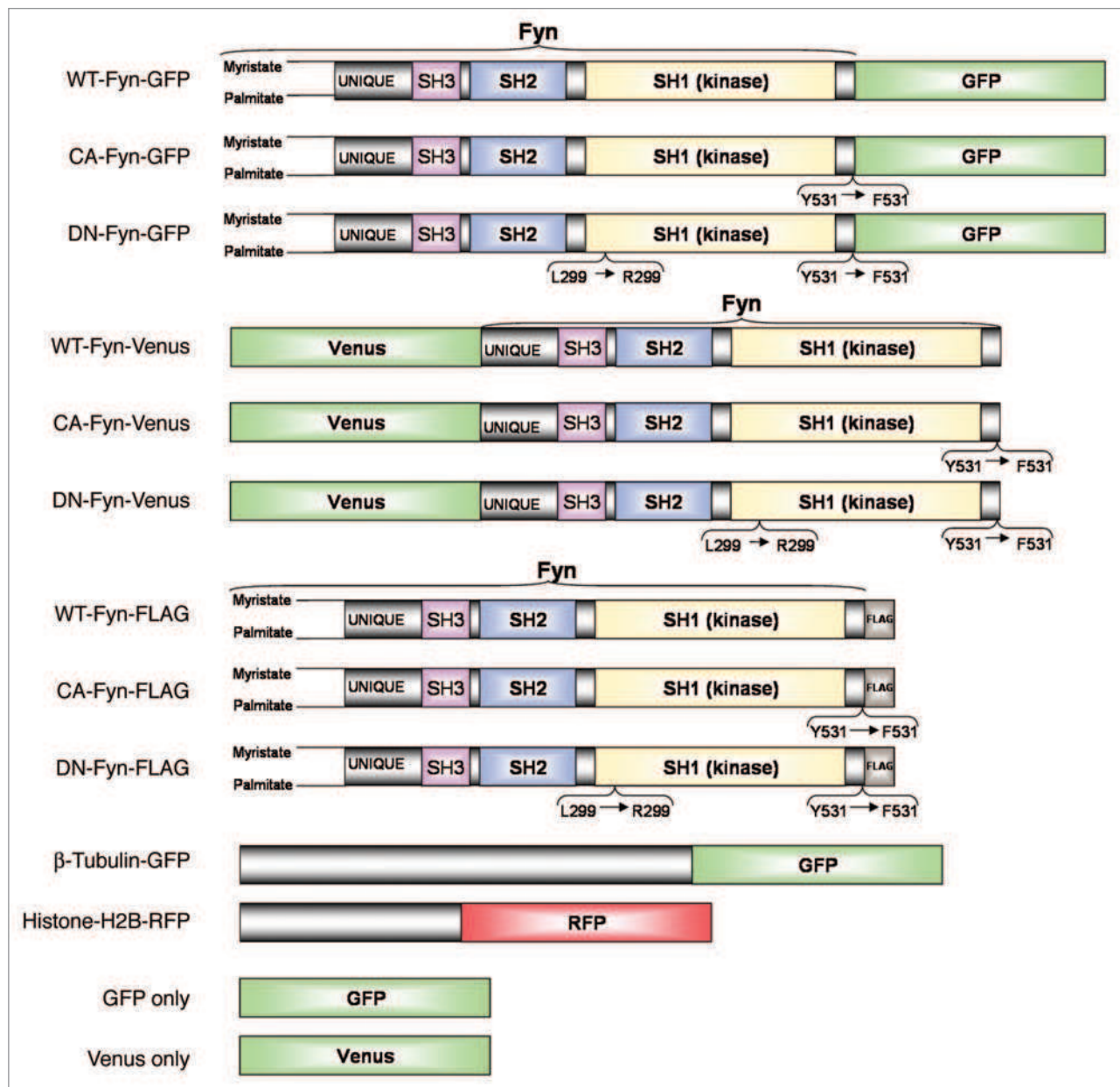


Figure 2. Proteins translated in oocytes microinjected with cRNA.

endogenous Fyn protein (59 kDa) in non-injected GV oocytes (Fig. 3E1).

The effect of Fyn inhibition on duration and rate of RMI. First, we examined the involvement of Fyn in the entire process of RMI of GV oocytes by assessing PBI extrusion, scored via the Differential Interference Contrast (DIC) channel. Inhibition of Fyn significantly inhibited the duration and rate of RMI (Fig. 4A). Only 25, 44.4 and 44.8% of the SU6656-exposed, DN-Fyn-FLAG (Fyn conjugated a tag of 8 amino acid FLAG had already been constructed in our lab; Fig. 2) and DN-Fyn-Venus cRNA microinjected oocytes extruded the PBI, respectively, compared to 84, 77.9, 72.7 and 78% of the control, WT-Fyn-FLAG, CA-Fyn-FLAG and WT-Fyn-Venus cRNA microinjected oocytes, respectively. Moreover, the average time

span elapsed from milrinone wash out to PBI-extrusion was longer in oocytes subjected to SU6656 (657 min), or microinjected with DN-Fyn-FLAG (655 min) or DN-Fyn-Venus cRNA (713 min) than in non-injected oocytes (611 min) or WT-Fyn-FLAG cRNA injected oocytes (604 min; $p < 0.05$; two-sample t-test for unequal sample sizes and unequal variances).

In the attempt to evaluate the involvement of Fyn in specific cell cycle events during RMI we followed developmental stages of the oocytes according to the next criteria: the disappearance of the GV as seen at the DIC channel was defined as GVBD stage; the appearance of condensed chromosomes at the 561 nm channel and of non-organized spindle at the 488 nm channel were defined as MI stage (assessed only in the FLAG-injected oocytes); the appearance of segregated chromosomes at the 561nm channel

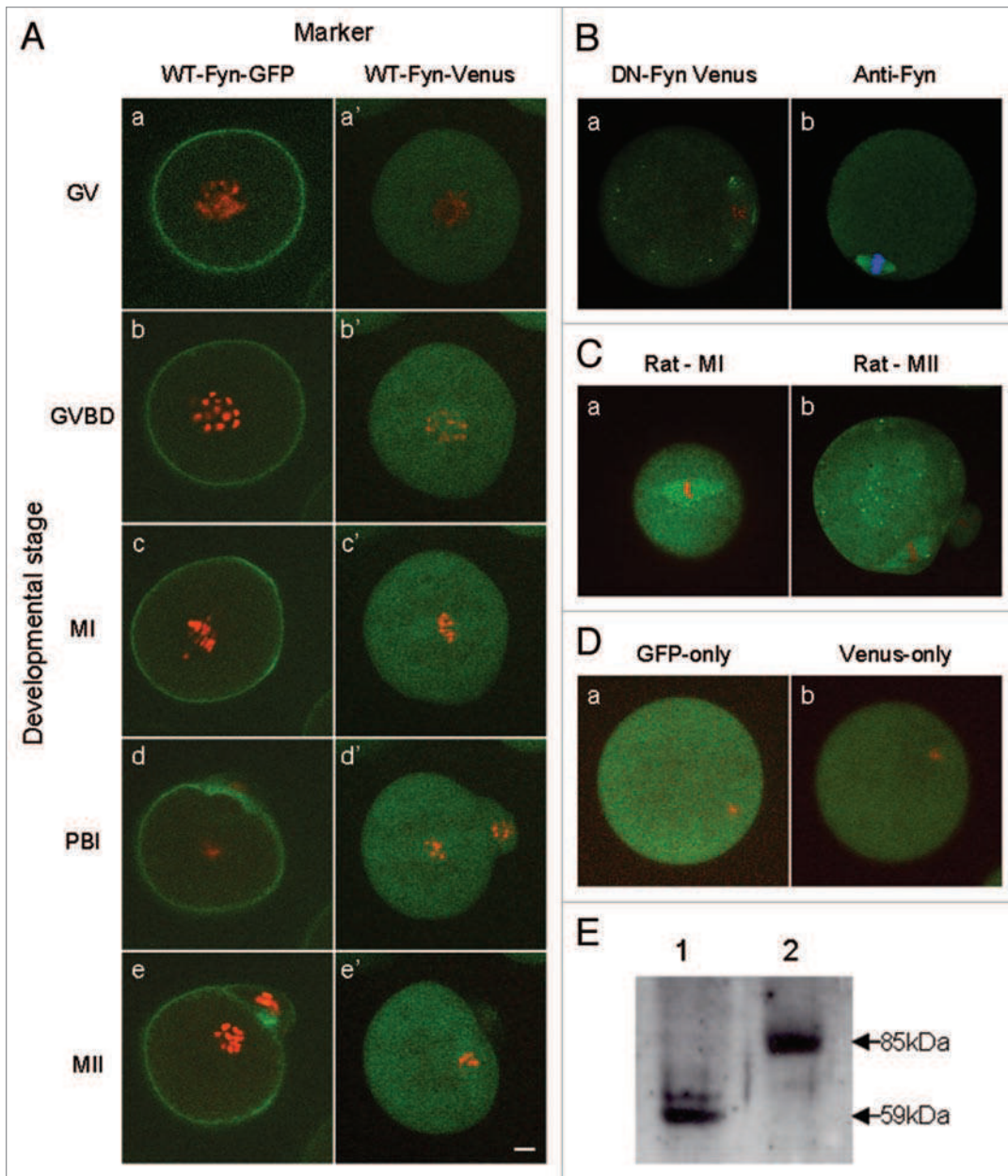


Figure 3. Localization of Fyn during RMI. (A) Spinning-disk confocal images of representative live mouse oocytes microinjected with Histone-H2B-RFP (red) for chromosomes detection, together with one of two cRNA constructs of WT-Fyn, conjugated to fluorophores with opposite orientations: GFP fluorophore at the carboxy-terminus of Fyn (Aa–e; green) or Venus fluorophore at the amino-terminus of Fyn (Aa'–e'; green). Injected oocytes were cultured for six hours in M2 medium in the presence of milrinone, to allow translation of the cRNA, and were then transferred into milrinone-devoid M2 culture medium. Four-dimensional (x, y, z, t) images in three channels (488 nm, 561 nm and DIC) were acquired using the Volocity 5 software at intervals of 15 or 30 minutes with an automatic movement of the objective in the z-axis ($\Delta z = 5 \mu\text{m}$) over a total depth of 45 μm . Scale bar, 10 μm . (B) Confocal images of representative MII mouse oocytes microinjected with (a) Histone-H2B-RFP (red) together with DN-Fyn-Venus (green) or stained (b) rabbit anti-Fyn antibody (1:100), followed by goat anti-rabbit AlexaFlour488-conjugated secondary antibody (1:400; green) mixed with DNA-specific fluorochrome (Hoechst 33342; blue). (C) Spinning-disk confocal images of representative live, in vitro matured rat MI (Ca) or MII (Cb) oocytes microinjected with WT-Fyn-Venus cRNA. (D) Spinning-disk confocal images of representative live mouse oocytes (control) microinjected with GFP-only (Da; green) or Venus-only (Db; green) mixed with Histone-H2B-RFP (red) cRNA at the MII stage after RMI in vitro. (E) SDS-PAGE and WB analysis indicating the amount of endogenous Fyn (59 kDa) in 50 non-injected GV oocytes (E1) and of exogenous Fyn-GFP (85 kDa; E2), six hours after microinjection of cRNA into 8 GV oocytes. A representative experiment is presented.

was defined as either anaphase or telophase of the first meiotic division (AI/TI; assessed only in the FLAG injected oocytes); the appearance of a spindle at the 488 nm channel after PBI extrusion was defined as MII stage (assessed only in the FLAG-injected oocytes).

Inhibition of Fyn significantly inhibited GVBD (Fig. 4B). Only 38.7%, 75% and 67.1% of the SU6656-treated, DN-Fyn-FLAG and DN-Fyn-Venus cRNA microinjected oocytes completed GVBD, respectively, compared to 90.4%, 91.7%, 95.3% and 90.4% of the control, WT-Fyn-FLAG, CA-Fyn-FLAG and WT-Fyn-Venus cRNA microinjected oocytes, respectively. Moreover, the average duration of the transition from GV to GVBD in SU6656-treated oocytes (127 min) and Fyn-FLAG cRNA injected oocytes (81 min) was statistically longer than that of non-injected control oocytes (59 min), WT-Fyn-FLAG cRNA injected oocytes (56 min), WT-Fyn-Venus (59 min) and CA-Fyn-FLAG (62 min; $p < 0.05$). However, we can not conclude that the average duration of the transition from GV to GVBD was affected by the microinjected DN-Fyn-Venus cRNA (68 min) because it was measured in only 74 oocytes.

Inhibition of Fyn inhibited the exit from MI. Only 62.6% of the DN-Fyn-FLAG injected oocytes went through the transition from MI to AI/TI, compared to 89.4% of the WT-Fyn-FLAG injected oocytes ($p < 0.05$; Fig. 4D). More important, none of the oocytes showed misalignment of the chromosomes during MI stage or lagging chromosomes during chromosomes segregation. Moreover, the dynamics of the timing of exit from MI was not affected (Fig. 4D), nor were the spindle migration and anchoring to the cortex.

Inhibition of Fyn prolonged the process of the PBI extrusion. Although the timing of the start of the ingression of the cleavage furrow was similar in all groups, the average time needed to complete the extrusion of the PBI in DN-Fyn-FLAG cRNA was longer than the WT-Fyn-FLAG cRNA microinjected oocytes (38 min, as compared to 20 min; $p < 0.05$; Fig. 4E).

Inhibition of Fyn had no significant effect ($p > 0.05$) on the transition from GVBD to MI (Fig. 4C) or from PBI to MII (Fig. 4F) and showed no effect on anchoring of the MII spindle to the cortex.

The effect of Fyn inhibition or activation on spindle length and PBI volume. Inhibition of SFKs by SU6656 results in reduction in size of the spindle structure in MII rat oocytes.^{44,47} The current study shows that the inhibition of SFKs in mouse oocytes causes a reduction of the spindle poles structure (Fig. 5Ab). However, we did not observe a significant effect on the spindle structure in MII oocytes fixed six hours after microinjection of DN-Fyn-FLAG (Fig. 5Ac).

In order to examine a potential effect of Fyn inhibition or activation on the length of the spindle during RMI, GV mouse oocytes were microinjected with cRNA of Histone-H2B-RFP and β -tubulin-GFP mixed with either WT-Fyn-FLAG, CA-Fyn-FLAG or DN-Fyn-FLAG. Spindle length was defined as the distance between its two poles. Spindles length (Fig. 5B) and shape were similar in all the experimental groups. The average length of MI-spindles was 26.3 ± 0.71 (mean \pm SE), 25.2 ± 1.05 and 25.7 ± 0.55 μm in WT-Fyn-FLAG, CA-Fyn-FLAG and DN-Fyn-FLAG

cRNA microinjected oocytes, respectively—all statistically similar ($p > 0.05$). The average length of MII-spindles were 24.2 ± 0.72 , 23.2 ± 0.9 and 23 ± 0.61 μm in WT-Fyn-FLAG, CA-Fyn-FLAG and DN-Fyn-FLAG cRNA microinjected oocytes, respectively—all statistically similar ($p > 0.05$).

In order to examine the effect of Fyn inhibition on the volume of the extruded PBI, groups of GV mouse oocytes were subjected to several treatments: non-injected oocytes incubated in the presence or absence (control) of 10 μM SU6656 (added seven hours after oocytes were removed from milrinone); oocytes microinjected with WT-Fyn-FLAG, DN-Fyn-FLAG, WT-Fyn-Venus or DN-Fyn-Venus cRNA. The average volume of PBI was significantly larger ($p < 0.05$) in SU6656-treated oocytes (9644.2 ± 854.5 μm^3) and in DN-Fyn-FLAG cRNA microinjected oocytes (11462.1 ± 1879.7 μm^3) than in the non-injected control oocytes (7058.3 ± 310.9 μm^3) and in the WT-Fyn-FLAG (7588.3 ± 585.4 μm^3), WT-Fyn-Venus (7760.5 ± 758.5 μm^3) or DN-Fyn-Venus cRNA microinjected oocytes (7916.2 ± 773.1 μm^3 ; Fig. 6).

Discussion

The involvement of Fyn in GVBD. One can assume that the meiotic stages in which Fyn is involved are those that exhibit changes in the expression of Fyn. Therefore, we examined, by SDS-PAGE and WB analysis, the changes in the amount of endogenous Fyn protein during RMI in vitro and in vivo. The amount of Fyn decreased considerably during RMI and correlated with GVBD. More important, when Fyn was inhibited by a chemical inhibitor, or by microinjection of DN-Fyn cRNA, the process of GVBD was inhibited. The effect of SU6656, that inhibits Fyn, Yes and Src, on GVBD was more dramatic, though it is possible that SU6656 inhibits other related kinases in the oocytes, as indicated in somatic cells.⁵¹ These results can imply that Fyn takes part in the signal to GVBD, however, because the developmental stages later than GVBD seemed to be normal in oocytes microinjected with CA-Fyn-FLAG, the requirement for Fyn degradation once the GVBD was already triggered, would therefore not seem to be absolute, but only a coincidence. Studies of the involvement of SFKs in GVBD in mouse oocytes show contradicting data: Zheng et al.²¹ showed that mouse oocytes matured in the presence of another SFK inhibitor, PP2, were unable to undergo GVBD, whereas McGinnis et al.³² showed that SKI606, a chemical inhibitor of SFKs, induced GVBD in mouse oocytes cultured under conditions that promote metaphase arrest. Differences in the affinity and selectivity among the various inhibitors, SKI606, PP2 and SU6656, may account for the contradictory data.

We ruled out the involvement of the proteasome in the down-regulation of Fyn since we have shown that the amount of Fyn in in vitro matured mouse oocytes was similar in the absence or presence of proteasome inhibitors. It seems therefore, that Fyn is downregulated or degraded via other pathways, such as the calpain, endosome or lysosome pathways as suggested for Fyn in other cell types.^{38,39,52,53}

The localization of Fyn at the meiotic spindle. Activated Src-family kinases are possibly associated with the meiotic spindle during meiosis.¹⁶ Differences in intracellular localization of

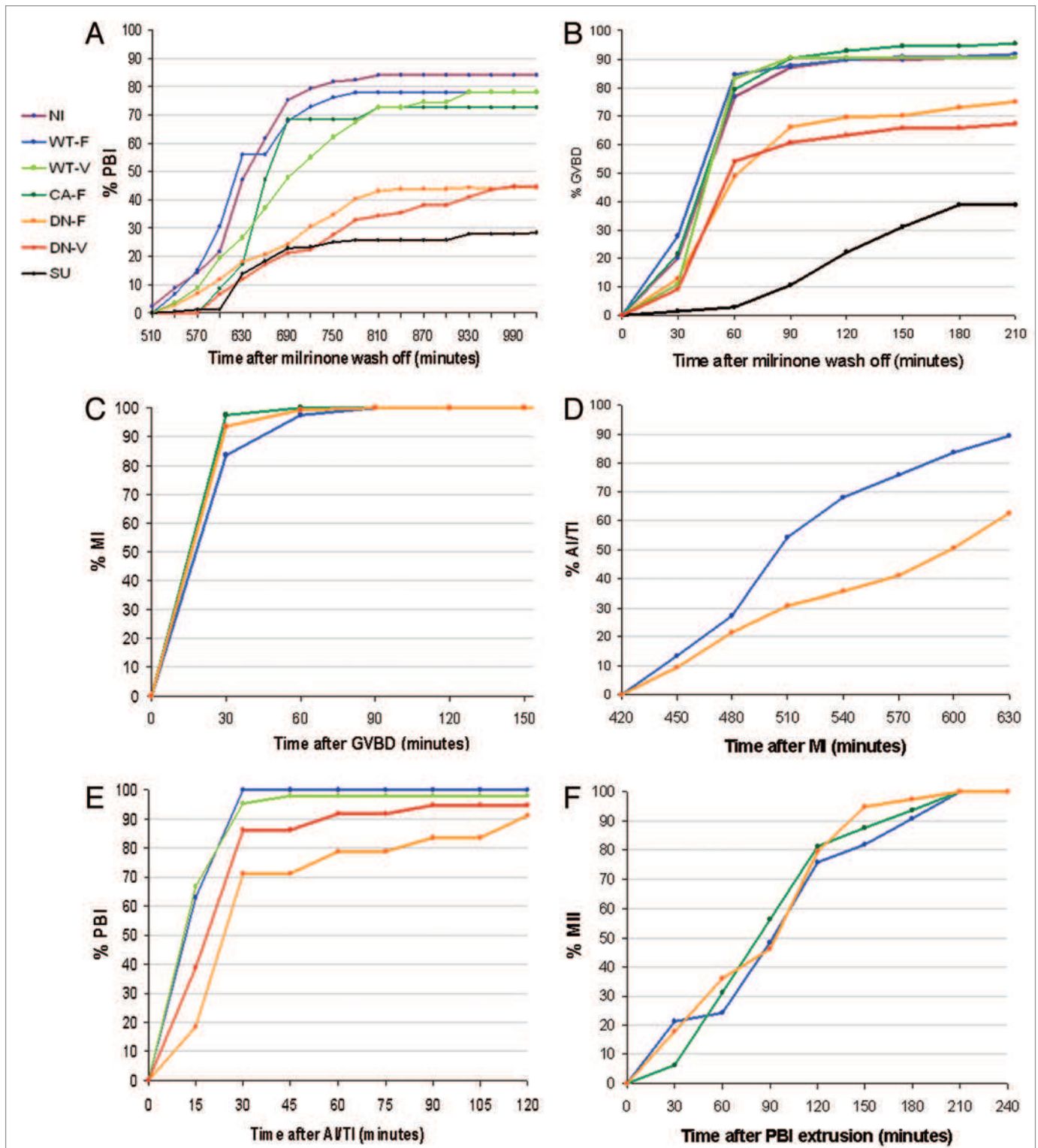


Figure 4. For figure legend, see page 1584.

the various SFKs could indicate their unique roles during meiosis. Immunostaining of rat MII oocytes showed that Fyn, Src and Yes are localized in the cytoplasm, while Yes and Fyn are concentrated at the cortex and Fyn concentrates at the meiotic spindle as well.^{43,44,47} Inhibition of SFKs by SU6656 results in

disruption and reduction in size of the MII spindle structure, reduction in spindle size, misalignment of the chromosomes and appearance of MTs throughout the cytoplasm in a dose-response manner.^{44,47} The exposure of MII rat oocytes to a tubulin-depolymerizing agent, nocodazole, caused disappearance of

Figure 4 (See previous page). The effect of inhibition or activation of Fyn on duration and rate of RMI. Groups of mouse oocytes at the GV stage were subjected to several treatments: non-injected oocytes incubated in the presence of 10 μ M SU6656 (SU; 132 oocytes; black) or its absence (NI, non-injected control; 125 oocytes; purple); oocytes microinjected with cRNA of Histone-H2B-RFP and β -tubulin-GFP mixed with WT-Fyn-FLAG (WT-F; 97 oocytes; blue), CA-Fyn-FLAG (CA-F; 121 oocytes; dark green) or DN-Fyn-FLAG (DN-F; 170 oocytes; orange); oocytes microinjected with cRNA of Histone-H2B-RFP mixed with WT-Fyn-Venus (WT-V; 83 oocytes; pale green) or DN-Fyn-Venus (DN-V; 74 oocytes; red). Oocytes were incubated in M2 medium in the presence of 1 μ M milrinone for six hours to allow translation of the cRNA. Milrinone was washed out and oocytes were imaged at intervals of 15 minutes by spinning-disk confocal microscopy. Non-injected oocytes were kept in the incubator and served as a control for the RMI in vitro process. Four-dimensional (x, y, z, t) image stacks in a three channels (488 nm, 561 nm and DIC) were processed and analyzed by the Volocity 5 software. Several parameters were scored: the transition from GV to PBI as observed through the DIC channel was classified as complete RMI (A); the disappearance of the GV as observed through the DIC channel was classified as GVBD stage (B); the appearance of condensed chromosomes as observed through the 561 nm channel and of non-organized spindle as observed through the 488 nm channel was classified as metaphase of the first meiotic division (MI; C); the appearance of segregated chromosomes as observed through the 561 nm channel was classified as either anaphase or telophase of the first meiotic division (AI and TI, respectively; D); the appearance of extruded polar body as observed through the DIC channel was classified as PBI stage (E); the appearance of a spindle as observed through the 488 nm channel after PBI extrusion was classified as metaphase of the second meiotic division (MII; F).

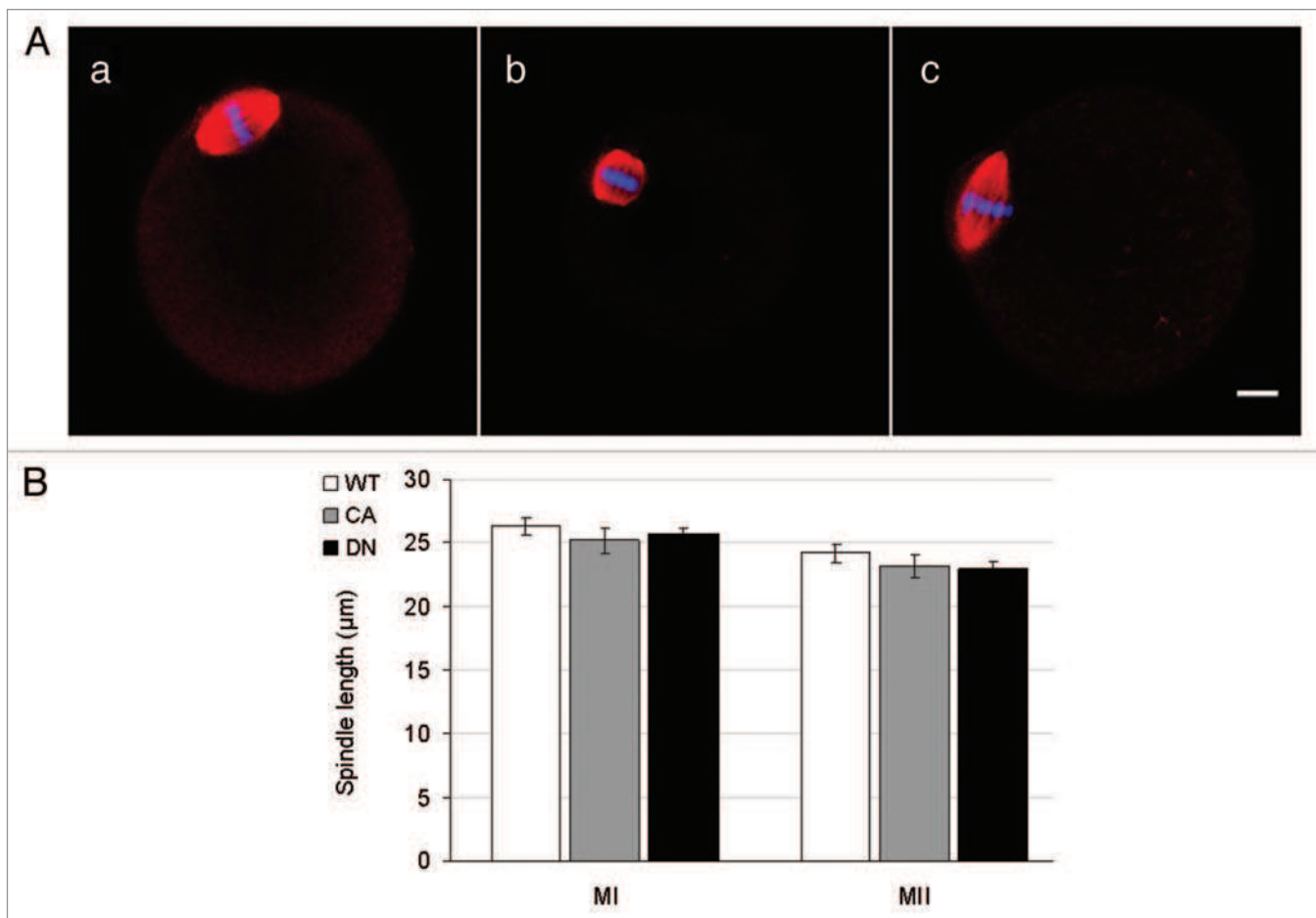


Figure 5. Effect of SFKs inhibition on spindle length. (A) CLSM images of representative fixed MII mouse oocyte after incubation in the absence (a) or presence (b) of 10 μ M SU6656 for 30 minutes or after microinjection of DN-Fyn-FLAG cRNA and incubation for six hours (c). Oocytes were fixed and permeabilized and then exposed to rabbit anti-Fyn antibody (1:100), followed by goat anti-rabbit AlexaFluor488-conjugated secondary antibody (1:400; green). Chromosomes were detected by a DNA-specific fluorochrome (Hoechst 33342; blue). Scale bar, 10 μ m. Three independent experiments were performed. (B) Mouse oocytes at the GV stage were microinjected with Histone-H2B-RFP and β -tubulin-GFP cRNA mixed with WT-Fyn-FLAG cRNA (WT; 30 oocytes), CA-Fyn-FLAG cRNA (CA; 16 oocytes) or DN-Fyn-FLAG cRNA (DN; 68 oocytes). Oocytes were incubated for six hours in M2 medium in the presence of 1 μ M milrinone to allow translation of the cRNA. Milrinone was washed out and oocytes were imaged by spinning-disk confocal microscopy. The distance between the MI and MII spindle-poles was measured in the 3D images acquired by the 488 nm channel using Volocity 5 software and was scored as spindle length. Each bar represents mean \pm SE.

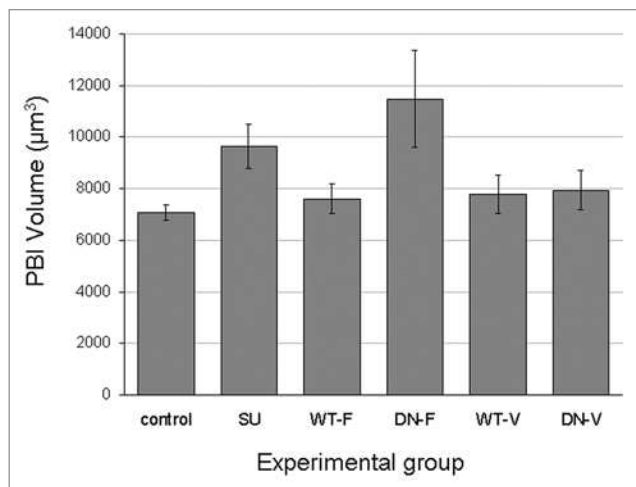


Figure 6. Effect of Fyn inhibition or activation on PBI volume. Mouse oocytes at the GV stage were subjected to several treatments: non-injected oocytes incubated in the presence of 10 µM SU6656 (SU; 48 oocytes); SU6656 was added seven hours after removal of milrinone from culture medium or its absence (NI—non-injected control; 42 oocytes); oocytes microinjected with cRNA of WT-Fyn-FLAG (WT-F; 49 oocytes), DN-Fyn-FLAG (DN-F; 52 oocytes), WT-Fyn-Venus (WT-V; 33 oocytes) or DN-Fyn-Venus (DN-V; 25 oocytes). Oocytes were imaged by spinning-disk confocal microscopy. The volume of PBI was measured in the DIC channel using Volocity 5 software [Volume of oblate spheroid = $4/3 \times \pi \times (\text{length})^2 \times \text{height}$]. Each bar represents mean \pm SE.

the spindle and abolished Fyn labeling at the spindle area. After washing the nocodazole off, the oocytes re-exhibited a fully recovered spindle formation accompanied by Fyn labeling.⁴⁴ The current study demonstrates Fyn localization at the area of meiotic MI and MII spindles of mouse oocytes matured in vitro and immunostained with anti-Fyn antibodies or microinjected with WT-Fyn-Venus while the localization of DN-Fyn-Venus was at the spindle poles. The differences in the spindle localization between the DN- and the WT-forms of Fyn-Venus could be explained by the allosteric open-conformation of the DN form that allows its concentration at the poles, compared to the closed conformation of the WT form that enables a homogenous localization at the spindle. This could imply the importance of the SH2 domain of Fyn for its association with key factors on the spindle poles during metaphase. Although meiotic spindle assembly in mammalian oocytes occurs in the absence of centrioles,⁵⁴ it can be established by the presence of γ -tubulin at the spindle poles.⁵⁵ Fyn co-localizes with both mitotic spindle and poles in T lymphocytes⁵⁶ and coimmunoprecipitates through its SH2 and SH3 domains with γ -tubulin in activated mast cells.³⁴ Pretreatment of mast cells with PP2 inhibits the formation of MTs and reduce the amount of tyrosine-phosphorylated-proteins in γ -tubulin complexes, suggesting that SFKs are involved in the initial stages of MTs formation.³⁴ other findings demonstrated an elevated level of phosphorylated tyrosine at the poles of MII spindles in mouse oocytes, which eventually disappeared as MII progressed to AII.¹⁶ It is possible that phosphorylated Fyn is one of the factors responsible for the high level of phosphorylated tyrosine at the spindle poles, either by accumulating

at the poles and/or by phosphorylating tyrosine residues of other proteins.

In contrast to cultured somatic cells, poleward flux in mouse eggs is critically dependent upon kinesin 5 and its inhibition leads to spindle shortening by depolymerization of the poles.⁵⁷ In the current study, a similar phenomenon of reduction of the spindle length caused by shortening of the spindle poles was observed when SFKs were inhibited with 10 µM SU6656. This could imply that SFKs take part in spindle elongation at the poles area. The existence of functional overlapping or compensatory relationship among the various SFKs, regarding their involvement in spindle stabilization can explain how no effect on spindle characterization was observed when Fyn alone was inhibited by DN-Fyn cRNA, but a significant effect was observed when all SFKs-Fyn, Yes and Src-were inhibited by SU6656.

The involvement of Fyn in the exit from metaphase. Our previous studies showed that the degradation of cyclin-B and the release from MII arrest in rat oocytes was inhibited in the presence of SFKs inhibitors.^{20,45,46} As we have recently shown, the same applies to mouse oocytes.⁴⁷ Moreover, Microinjection of CA-Fyn protein into rat oocytes caused release from MII arrest in a dose-dependent manner.⁴⁴ Microinjection of CA forms of either Fyn or Yes cRNA caused release from MII arrest. Microinjection of DN forms of either Fyn or Yes cRNA inhibited SrCl₂- or sperm-induced exit from MII arrest in rat oocytes.⁴⁵ In the present study, inhibition of Fyn by microinjecting DN-Fyn-FLAG cRNA into mouse oocytes resulted in inhibition of the exit from MI. Our results imply the involvement of Fyn not is being specific for the exit from MII, but rather to an exit from metaphase in general. However, it is also possible that overexpression of DN-Fyn can also inhibit other related SFKs in the oocytes. Furthermore, the exit from MI and MII arrest in fixed mouse oocytes was inhibited also in Fyn^{-/-} mice oocytes and in oocytes exposed to the SFKs inhibitors, SKI606 or PP2.^{16,21,32} A possible mechanism for a role of SFKs in somatic cell mitosis has been suggested based on studies of SFKs in cells arrested during mitosis after exposure to the tubulin depolymerizer, nocodazole, or in cells microinjected with SFKs inhibitory antibodies.^{22,57-60} The metaphase-stage elevated activity of Fyn, Yes and Src correlates with phosphorylation of Src by p34cdc2 kinase at several serine and threonine residues. However, an antibody that recognizes Src alone did not prevent cell division; only the cross-inhibitory peptide antibodies against Fyn, Yes and Src were effective. These studies, combined with our results, suggest a possible negative feedback mechanism during the cell cycle: accumulation of cyclin-B during metaphase leads to high activity of p34cdc2, which, in-turn, phosphorylates SFKs and induces their elevated activity. High activity of SFKs can participate in induction of the degradation of cyclin-B and the exit from metaphase arrest.

The involvement of Fyn in PBI extrusion. Various studies have demonstrated the involvement of Fyn in several actin functions, including cytokinesis in somatic cells.^{24,37-42} However, in some cases it was hard to distinguish between cell cycle and cytokinesis. Lou et al.⁶¹ demonstrated a disruption in the organization of microvilli, cortical granules and filamentous actin at the cortical area overlaying the meiotic spindle in MII mice

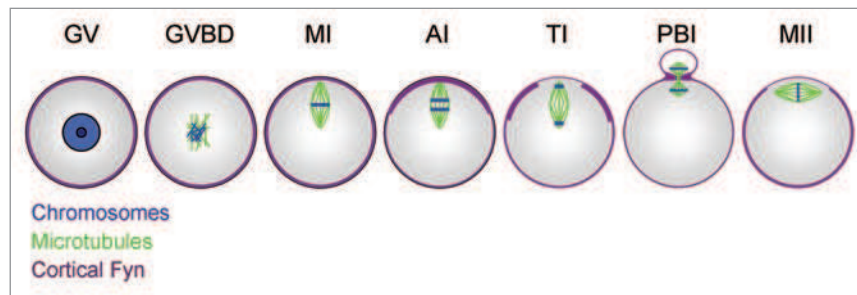


Figure 7. Changes in the localization of cortical Fyn during RMI. Cortical Fyn is recruited to the cortical differentiation area designated for PBI extrusion during AI stage. Fyn is concentrated to the cortical area around the midzone of the meiotic spindle during TI stage and at the cleavage furrow area during extrusion of PBI. After PBI extrusion, Fyn resumes a uniform distribution pattern throughout the cortex.

oocyte exposed to SKI606 or in Fyn-null oocytes. By using live image confocal microscopy of oocytes microinjected with cRNA of WT-Fyn-GFP, CA-Fyn-GFP or DN-Fyn-GFP, we were able to examine the exact process of cytokinesis and show that all forms of Fyn were recruited to the cortical differentiation area designated for PBI extrusion, already 15 minutes prior to extrusion. Fyn was concentrated at the cleavage furrow and cortical area surrounding the meiotic spindle midzone during TI and at the cleavage furrow area during PBI extrusion. Fyn resumed a uniform distribution pattern throughout the cortex 15 minutes after PBI extrusion. The summary of these observations is illustrated in **Figure 7**. Moreover, inhibition of Fyn by microinjection of DN-Fyn-FLAG cRNA did not change the starting time of the cleavage furrow ingression, but caused a significant increase in the total duration needed for the ingression and the completion of the extrusion of PBI, compared to WT-Fyn-FLAG cRNA. We also observed a considerable influence of Fyn inhibition on the volume of the extruded PBI. The average volume of PBI was larger in oocytes exposed to SU6656 and in DN-Fyn-FLAG microinjected oocytes than in the non-injected, control oocytes or in oocytes microinjected with WT-Fyn-FLAG, WT-Fyn-Venus or DN-Fyn-Venus cRNA. The fact that the protein translated from the microinjected DN-Fyn-FLAG cRNA and concentrated at the cortex of the oocytes,⁴⁵ caused a more significant delay in PBI extrusion and extrusion of a bigger PBI than proteins translated from microinjected WT-Fyn-Venus or DN-Fyn-Venus cRNA that localize at the cytoplasm, could suggest that cortical Fyn is the main contributor to the cleavage furrow ingression. It is possible that inhibition of Fyn by the cortical DN-Fyn-FLAG cRNA extends the duration of the cleavage furrow ingression; as a result the area restricted for formation of the cleavage furrow is more extended and a larger PBI is extruded. In conclusion, the recruitment of Fyn to the area designated for PBI extrusion, the delay in timing of the extrusion process and the extrusion of a larger PBI by DN-Fyn-FLAG injected oocytes, demonstrate the involvement of cortical Fyn in the regulation of the process of PBI extrusion, possibly by facilitating the organization and function of actin-filaments and/or related molecular machinery in the area of the cleavage furrow.

Altogether, we propose that during RMI in mammalian oocytes Fyn takes part in regulating several key signaling pathways leading to the exit from GV and MI and in the ingression of the cleavage furrow.

Materials and Methods

Animals. OF1 female mice (7–9 weeks-old; Charles River Laboratories International, Chatillon-sur-Chalaronne, France) and Wistar Han IGS female rats (3–4 weeks-old; Charles River Laboratories International) 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 superstimulated with 5 IU of pregnant mares' serum gonadotrophin (PMSG; Syncro-Part, Sanofi, France) and superovulated with 5 IU of human chorionic gonadotrophin (hCG; Sigma Chemical Co., St. Louis, MO) 48–54 hours later.

Oocytes collection and culture. Non-stimulated female mice and rats were sacrificed and their ovaries removed into prewarmed (37°C) M2 medium (M-7167; Sigma) supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma). GV oocytes arrested at prophase I were released from the follicles into M2 medium containing 1 μ M milrinone (Sigma). Milrinone (Primacor) is a phosphodiesterase III inhibitor that causes high levels of cAMP in the oocytes cytoplasm⁴⁸ and thus inhibits the intra-oocyte drop in cAMP concentration leading to GVBD.^{5,6} Oocytes were then cultured under mineral oil (Sigma) at 37°C. RMI was synchronized in all oocytes by transferring them into milrinone-free M2 medium.

Mature, ovulated MII mouse oocytes were removed from the oviductal ampullae into M2 medium 14 hours after hCG administration. Cumulus cells were removed by hyaluronidase (H-3631; Sigma).

Chemical inhibitors. A selective SFKs inhibitor (SU6656; Calbiochem, San Diego, CA) was added to the mouse oocytes culture medium.²⁰ SU6656 is known to inhibit Fyn, c-Src and c-Yes⁵¹ by diffusing into cells, attaching to the ATP binding pocket of SFKs and hence acting as a competitive inhibitor. We used SU6656 at a concentration of 10 μ M since this was the effective concentration that blocked activation of rat²⁰ and mouse⁴⁷ oocytes.

Mouse oocytes were also incubated in the presence of the proteasome catalytic activity inhibitors (50 μ M MG132 or 50 μ M Lactacystin; Calbiochem).

WB analysis. Batches of oocytes were collected as described by Talmor-Cohen et al.²⁰ Proteins were separated by SDS-PAGE.

Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham, Buckinghamshire, UK). WB analysis was performed with either rabbit anti-Fyn IgG (1:200; sc-16; Santa Cruz, Santa Cruz, CA), rabbit anti-p34cdc2 IgG (1:1,000; c-19; Santa Cruz) or rabbit anti-ERK1/2 (1:1,000; sc-94; Santa Cruz). Bound antibodies were recognized by goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000; 111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was performed by an enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA). Intensity of bands was quantified and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining and CLSM. Oocytes were fixed and permeabilized as described elsewhere⁴³ and were then incubated in the presence of rabbit anti-Fyn (1:100; sc-16; Santa Cruz) or mouse anti- β -tubulin (1:5,000; T8328; Sigma) primary antibodies, prior to incubation with AlexaFlour488-conjugated goat anti-rabbit (1:400; A11034; Invitrogen—Molecular Probes, Carlsbad, CA) or Cy-3-conjugated donkey anti-mouse (1:400; 715-165-151; Jackson ImmunoResearch Laboratories) secondary antibodies, respectively, mixed with Hoechst 33342 (1 μ g/ml; Sigma).

Stained oocytes were visualized and photographed with CLSM, Zeiss-LSM410 (Carl Zeiss MicroImaging, Oberkochen, Germany), equipped with a 25 mW krypton-argon laser (488 nm and 568 nm lines), a 10 mW helium-neon laser (633 nm line) and an UV laser (Coherent Laser Group, Santa Clara, CA). A 40X NA/1.2 planapochromat water-immersion lens (Axiovert 135 M; Zeiss) was used for all imaging.

Preparation and microinjection of cRNA. The full-length sequences of human cDNA encoding the WT, CA or DN forms of Fyn kinase (I.M.A.G.E. clone ID number 3613878, ResGen, Carlsbad, CA), inserted into pCMV-Tag 4A with an in-frame FLAG at the 3'-end (Stratagene, Cedar Creek, TX) had already been constructed in our lab (Fig. 2).⁴⁶ As indicated in the literature, 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.^{12,62-65} A point mutation at L299 in the ATP binding site of the kinase makes Fyn a kinase-dead mutant, capable of inhibiting endogenous Fyn.^{12,28,62,63,66,67} Furthermore, the activity of the different forms of Fyn had previously been assessed by an in vitro autophosphorylation activity of Fyn. The purified protein of the CA-Fyn-FLAG was highly autophosphorylated whereas the WT-Fyn-FLAG and DN-Fyn-FLAG had only a minimal autophosphorylation activity or none at all.⁴⁶ WT-Fyn, DN-Fyn and CA-Fyn were amplified and inserted into pRN-N1, with an in-frame GFP at the 3'-end. The 3'-end orientation of FLAG and GFP epitopes allowed eukaryotic post translational modifications, i.e., amino-terminal myristoylation and palmitoylation of Fyn within oocytes, thus enabling Fyn membrane targeting. Fyn conjugated to Venus at the 5'-end was also constructed in pSPE3 vectors in order to examine the cytoplasmic localization of Fyn. All plasmids were sequenced and analyzed. They were linearized using SfiI restriction enzyme

(New England Biolabs, Ipswich, MA) and cRNA transcripts were prepared in vitro using T3 mMESSAGE mMACHINE kit (Ambion, Austin, TX). The cRNA transcripts were then purified on RNeasy columns (Qiagen, Duesseldorf, Germany) and eluted by nuclease free water to a final concentration of 0.4–1 μ g/ml. Aliquots of 4 μ l were stored at -80°C.

The expression of Fyn protein translated in GV oocytes injected with Fyn cRNA, was measured by both WB analysis and Reticulocyte Lysate System.⁴⁵

Histone-H2B-RFP cRNA and β -tubulin-GFP cRNA⁶⁸⁻⁷⁰ were donated by Dr. Marie-Helene Verlhac, Pierre and Marie Curie University, Paris, and were used for imaging of chromosomes and MTs, respectively.

The in vitro synthesized cRNA of Fyn-GFP or Fyn-Venus, mixed with Histone-H2B-RFP, or of Fyn-FLAG mixed with Histone-H2B-RFP and β -tubulin-GFP were microinjected into the cytoplasm of oocytes at the GV stage at a volume of ~5% of the oocyte volume, using the Eppendorf micromanipulator system (Leitz, Heidelberg, Germany).

Live cell imaging. Oocytes injected with cRNA were cultured for 6 hours in M2 medium supplemented with milrinone to allow protein translation. For imaging, oocytes were put into oil covered, 10 μ l medium within a specially designed chamber, maintained at 37°C on a heated microscope stage. Signal was recorded via three channels (488 nm, 561 nm and DIC) at intervals of 15 or 30 minutes by a C910 CCD digital-camera (Hamamatsu, Hamamatsu City, Japan) attached to an advanced confocal inverted microscope (Axiovert 200M; Carl Zeiss MicroImaging) with a CSU10 Spinning Disk head (Yokogawa, Tokyo, Japan), operated by Volocity 5 software (Improvision, Coventry, England). Three-dimensional images were created by the movement of the objective along the z-axis ($\Delta z = 5 \mu$ m) over a total depth of 45 μ m. Four-dimensional (x, y, z, t) image stacks were processed and analyzed by the Volocity 5 software.

Fyn inhibition and activation. In order to characterize the involvement of Fyn in RMI, we employed several approaches for inhibiting or activating Fyn within oocytes. Mouse oocytes were subjected to several treatments: (1) GV oocytes incubated in the presence or absence (control) of 10 μ M SU6656. (2) GV Oocytes microinjected with Histone-H2B-RFP cRNA and β -tubulin-GFP cRNA mixed with either WT-Fyn-FLAG cRNA, CA-Fyn-FLAG cRNA or DN-Fyn-FLAG cRNA. As shown previously, Fyn-FLAG concentrates at the cortex and spindle area of oocytes.⁴⁵ (3) GV oocytes microinjected with Histone-H2B-RFP cRNA mixed with WT-Fyn-Venus cRNA or with DN-Fyn-Venus cRNA. This was performed to examine whether the opposite orientation of the tag in Fyn-Venus that localizes homogeneously to the oocyte's cytoplasm, acts different than Fyn-FLAG during oocyte RMI. Since Fyn-Venus localizes to the spindle, we did not co-inject it with β -tubulin-GFP.

Statistical analysis. Data were evaluated by Pearson's χ^2 (chi-square) test for independence or by independent, two-sample t-test for unequal sample sizes and unequal variances. $p < 0.05$ was considered significant.

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Note

Supplementary materials can be found at:

www.landesbioscience.com/supplement/LeviCC9-8-Sup.pdf
www.landesbioscience.com/supplement/LeviCC9-8-Sup1.avi
www.landesbioscience.com/supplement/LeviCC9-8-Sup2.avi
www.landesbioscience.com/supplement/LeviCC9-8-Sup3.avi
www.landesbioscience.com/supplement/LeviCC9-8-Sup4.avi

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