



HAL
open science

Inositol 1,4,5-Trisphosphate (IP₃) Responsiveness Is Regulated in a Meiotic Cell Cycle Dependent Manner Implications for Fertilization Induced Calcium Signaling

Mark Levasseur, Alex Mcdougall

► **To cite this version:**

Mark Levasseur, Alex Mcdougall. Inositol 1,4,5-Trisphosphate (IP₃) Responsiveness Is Regulated in a Meiotic Cell Cycle Dependent Manner Implications for Fertilization Induced Calcium Signaling. *Cell Cycle*, 2003, 2 (6), pp.610-3. <hal-03025805>

HAL Id: hal-03025805

<https://hal.science/hal-03025805v1>

Submitted on 16 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Report

Inositol 1,4,5-Trisphosphate (IP₃) Responsiveness Is Regulated in a Meiotic Cell Cycle Dependent Manner

Implications for Fertilization Induced Calcium Signaling

Mark Levasseur

Alex McDougall

School of Cell and Molecular Biosciences; University of Newcastle, The Medical School; Newcastle upon Tyne, UK

Correspondence to: Dr Mark Levasseur; Senior Research Associate; School of Cell and Molecular Biosciences; University of Newcastle; The Medical School; Framlington Place; Newcastle upon Tyne NE2 4HH UK; Tel.: 0191.222.3481; Email: m.d.levasseur@ncl.ac.uk and

Dr. Alex McDougall; Biologie du Developpment; Observatoire Oceanologique; Universite Pierre et Marie Curie; 06230 Villefranche-sur-Mer; France; Tel.: +33 493 76 37 72; Email: dougall@obs-vlfr.fr

Received 06/24/03; Accepted 08/04/03

Previously published online as a Cell Cycle E-Publication at:

<http://www.landesbioscience.com/journals/cc/tocnew26.php?volume=2&issue=6>

KEY WORDS

Meiotic cell cycle, calcium, IP₃, CDK1

ABSTRACT

Fertilization of mammalian eggs is known to trigger a series of transient rises in cytosolic calcium, known as calcium oscillations, the initiation and duration of which are crucial for meiotic exit and subsequent entry into embryogenesis. It is not known how these calcium oscillations are terminated when the zygote exits meiosis; it is thought that responsiveness to inositol 1,4,5-trisphosphate (IP₃) is involved, since the oscillations are known to be mediated by an IP₃ dependent mechanism. Here we report that IP₃ responsiveness is maintained throughout meiotic maturation and falls very rapidly after meiotic exit. We also show that inhibition of the major cell cycle kinase, CDK1, has no effect on responsiveness, but that prolonging CDK1 activity prevents the decline in responsiveness normally seen at meiotic exit. We conclude that CDK1 plays a role, but is not the only factor involved in controlling IP₃ responsiveness during the meiotic cell cycle.

INTRODUCTION

The fundamental process of fertilization serves not only to ensure exchange of maternal and paternal genetic material, but also to activate eggs from meiotic or mitotic cell cycle arrest, and in so doing initiate embryogenesis. A universal consequence of this sperm-egg fusion is a large rise in cytosolic calcium.^{1,2} In mammals, and the primitive marine chordate the ascidian, our chosen animal model, this initial rise in calcium is followed by a series of transient rises, known as calcium oscillations, and these constitute an essential trigger for meiotic exit and subsequent entry into embryogenesis.²⁻⁶ It has recently been suggested that the initial rise in calcium is initiated by delivery of a sperm specific phospholipase, PLC ζ .⁷ However, it is not known how the calcium oscillations are switched off when the zygote exits meiosis. It is thought that responsiveness to IP₃ is involved, since the oscillations are known to be generated by an IP₃ dependent calcium release from the endoplasmic reticulum (ER),⁸ and because IP₃ responsiveness is known to decline after meiotic exit,⁹⁻¹² although the timing and rate of this decline have not yet been established. We were interested in determining the link between the meiotic cell cycle and IP₃ responsiveness.

Here we have precisely measured IP₃ responsiveness in the ascidian oocyte and show that it is maintained throughout meiotic maturation, and falls very rapidly ($t_{1/2}=2.9 \text{ min} \pm 1.2$) following second polar body (pb2) extrusion, a morphological marker of meiotic exit. Meiotic cell cycle progression is principally controlled by the activity of cyclin B-dependent kinase (CDK1), the principle component of MPF.¹³⁻¹⁵ We show that inhibition of CDK1 has no effect on IP₃ responsiveness in the unfertilised ascidian oocyte, but that when CDK1 activity was maintained beyond the time of pb2 extrusion, IP₃ responsiveness was also maintained. These results imply that the fall in CDK1 activity is necessary but not sufficient for the fall in responsiveness to IP₃, and that another cell cycle activity is also involved.

MATERIALS AND METHODS

Biological Material. Specimens of the ascidian *Asciella aspersa* were collected locally from the Northumberland coast and kept in a tank of sea water at 10°C. Oocytes were harvested by first removing the cellulose tunic and then piercing the first gonoduct to release the oocytes which were immediately transferred to a small Petri dish containing 0.1% trypsin in filtered sea water (0.2 μM pore size filter, Millipore UK). After 1 hour of trypsin digestion, the outer chorions were then easily removed by shearing several times through the base of a glass pipette. Dechorionated oocytes were then kept in filtered sea water in dishes coated previously with a mixture of gelatine and

formaldehyde (1% each and rinsed prior to use) to prevent the now exposed membrane of the oocytes sticking to the plastic. All plastic and glassware was treated similarly if coming into contact with the oocytes.

Plasmid Fusion Constructs. Δ90 cyclin B was constructed by a PCR based approach from the previously described Δ90 cyclin B::GFP.¹⁸ mRNA for Δ90 cyclin B was then transcribed using a T3 mMESSAGE mMACHINE (Ambion) according to the kit protocol, and resulting mRNA dissolved in nuclease free water to a concentration of approximately 1 μg/μl and stored at -20°C prior to use.

p21::GFP fusion protein was constructed again using a PCR based strategy where the gene encoding p21 was amplified from pCEP/WAF, a kind gift from Dr R Patel, University of Leicester. The PCR product was then cloned into a derivative of the GST expression vector pGEX 6P-1, which had been engineered to contain the sequence coding for EGFP, resulting in a fusion protein consisting of an N-terminal GST tag, followed by p21 and EGFP at the C-terminus. Following bacterial expression, the protein was purified by GST-affinity chromatography according to the kit protocol (Amersham Pharmacia Biotech), buffer exchange into PBS and finally concentration on Microcon YM 30 spin concentrators (Millipore UK) to a final concentration of approximately 10 mg/ml. Aliquots were then stored at -70°C.

Microinjection and Fluorescence Imaging. The procedure for injecting oocytes was exactly as previously described.⁹ Intracellular calcium levels were measured by microinjecting Calcium Green-1 dextran (Molecular Probes, Oregon) to an approximate final concentration of 50 μM. The specimen was imaged using an epifluorescent microscope. Excitation light was from a Xenon lamp (Opti Quip, 150W) and was passed through 350 nm, 365 nm, 380 nm and 488 nm band pass filters housed in a filter wheel (Sutter Lambda 10-2, Sutter Instruments) to illuminate the specimen. A triple pass dichroic/emission filter set (Chroma set 61000, suitable for DAPI, FITC and TRITC) was used to direct light to, and send light from the specimen onto a cooled (-10°C) charge-coupled device (CCD) camera (MicroMax, Princeton Instruments). Images were processed using MetaFluor software (Universal Imaging)

Measuring IP₃ Responsiveness. Oocytes were coinjected with Calcium Green-1 and caged IP₃ (Calbiochem) to approximate final concentrations of 50 and 10 μM respectively. IP₃ responsiveness was quantified by measuring increase in Calcium Green-1 fluorescence as a result of release of caged IP₃ by time controlled flashes of 365 nm u.v. light.

Histone H1 and MBP Kinase Assays. These were used to measure CDK1 and MAP Kinase activities respectively and were performed exactly as previously described.⁹

RESULTS

Fertilization in the ascidian oocyte, which is arrested at metaphase of meiosis I, results in the generation of two series of calcium oscillations, punctuated by a brief period (the so-called gap phase) where calcium returns to basal levels and the first polar body is extruded. The oscillations then resume and finally stop just prior to extrusion of the second polar body (Fig. 1), an indication that meiosis is now complete. It is known that in the ascidian, these oscillations are generated by an IP₃-dependent mechanism,¹⁶ as is the case in mammalian eggs.⁸ We have previously shown in the ascidian that responsiveness to IP₃ is maintained throughout this period of oscillatory behaviour, even during the gap phase, and has fallen following extrusion of the second polar body,⁹ and see Figure 1. Such a decline has also been observed in mammalian oocytes, where IP₃ responsiveness has diminished by the time of pronuclear formation.¹⁰⁻¹² Here we have precisely measured the rate of loss of responsiveness to IP₃ and determined the exact point in the meiotic cell cycle at which this decline commences. Next we manipulated the major cell cycle kinase active during this period, CDK1, to determine if there is a causal relationship between CDK1 activity and IP₃ responsiveness. We did this by inhibiting CDK1 with p21 and by maintaining CDK1 activity with Δ90 cyclin B1 and measuring response to IP₃.

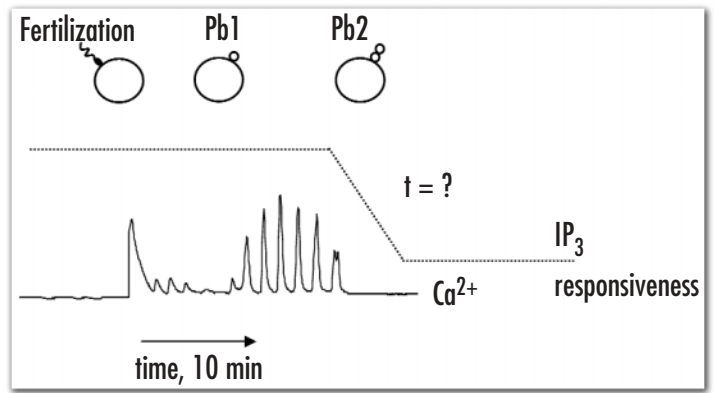


Figure 1. Pattern of calcium oscillations and IP₃ responsiveness following fertilisation in the ascidian oocyte. Intracellular free calcium was measured by injection of the ratiometric calcium indicator Fura-2 dextran. The trace (solid line) clearly shows the two phases of calcium oscillations separated by a brief gap following fertilisation in the ascidian oocyte. IP₃ responsiveness (as previously measured by injection of IP₃⁹) is indicated by the dotted line. Egg morphology indicates time of fertilisation, 1st and 2nd polar body extrusion.

The Decline in IP₃ Responsiveness Starts After Extrusion of the 2nd Polar Body and Falls Very Rapidly. Oocytes were co-injected with the calcium indicator dye Calcium Green-1 and the photo-releasable form of IP₃, caged IP₃. IP₃ responsiveness was then measured as a consequence of calcium release by recording increases in Calcium Green-1 fluorescence following sequential periods of u.v. light exposure which result in the uncaging of discrete doses of IP₃ (Fig. 2). The figure clearly shows that responsiveness remains the same during the first four 25 ms flashes, at which point the 2nd polar body was extruded. Further flashes of the same duration show an immediate, rapid and continuing decline to the point at which the tenth flash, administered approximately 7 minutes after 2nd polar body extrusion, elicits no response at all. In all, our results give a $t_{1/2}$ value for this decline of 2.9 minutes (± 1.2 min. $n = 25$ from 6 different animals). That only a

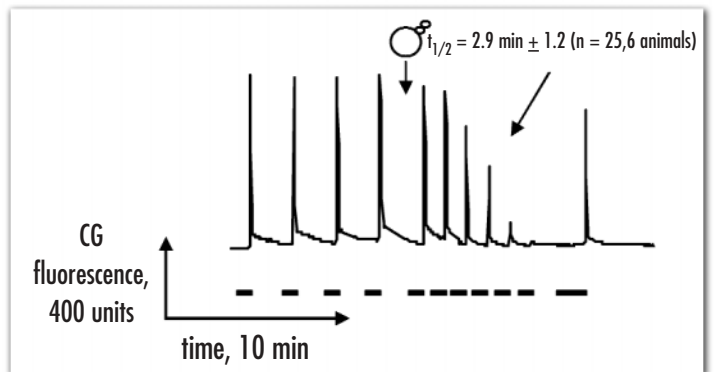


Figure 2. IP₃ responsiveness declines rapidly after 2nd polar body extrusion. Oocytes were co-injected with the calcium indicator dye Calcium Green-1 and caged IP₃ to give final concentrations of approximately 50 and 10 μM respectively. Sequential 25 millisecond flashes of 365nm u.v. light were administered resulting in extrusion of the 2nd polar body (as indicated by the egg morphology). IP₃ induced calcium release is indicated by the size of the Calcium Green-1 fluorescent signal, which declines rapidly after 2nd polar body extrusion ($t_{1/2}$ as shown). A final flash of 125 ms was found necessary to elicit a response, indicating that there has been a five-fold decline in responsiveness. Flash periods are indicated by the solid bars, increased length of which indicates the longer final flash time (not to scale).

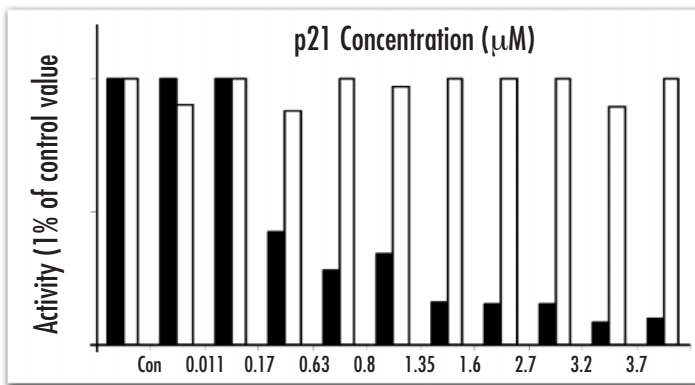


Figure 3. p21 is a specific inhibitor of CDK1 activity at low micromolar concentrations. Oocytes were injected with varying amounts p21::GFP protein (see materials and methods). Final concentration of p21 in each oocyte was estimated by comparing the resulting green fluorescent signal against a previously prepared calibration curve constructed by injecting known amounts of recombinant EGFP into oocytes and measuring the fluorescence produced. Samples of 3 oocytes with similar amounts of p21 were collected to represent a large range of concentrations, and these were then subjected to dual HH1 (a major substrate for CDK1 kinase)/MBP (a major MAP kinase substrate) assays. Values on the y-axis represent the resulting HH1 and MBP activities as a percentage of that found in control oocytes, HH1 activity is depicted by the solid bars, MBP by the white bars. Con is the control.

further flash of 125 ms, five times the duration previously used, was sufficient to elicit a response confirms our previous finding that there is a five-fold decline in responsiveness to IP₃ following meiotic exit.⁹ We are also confident that we are observing a genuine decline in responsiveness and not simply a depletion of available caged IP₃ in that the decline always correlates with pb2 extrusion, even though the flash protocol used to elicit pb2 differed from one experiment to another. Additionally it was noted (data not shown) that longer flashes, after responsiveness had declined, would always elicit a response, which would not be seen if the supply of caged IP₃ were exhausted. We assume that a sufficiently large supply of IP₃ remains caged at the centre and far side of the egg (where the u.v. flash penetrates progressively less efficiently) throughout the duration of each experiment.

From these data we conclude that the responsiveness to IP₃ falls rapidly following pb2 extrusion, and so were interested to determine whether this decline in responsiveness could be affected by preventing meiotic exit.

Inhibition of CDK1 Activity Has No Effect on IP₃ Responsiveness in the Unfertilised Oocyte. In order to investigate whether IP₃ responsiveness is related to the activity of the major cell cycle kinase, CDK1, we sought to inhibit CDK1 activity in the unfertilised oocyte. This we did using the known molecular CDK inhibitor p21.¹⁷ Bacterially expressed GST-tagged GFP::p21 fusion protein was made by affinity purification, and used to construct a dose response graph (Fig. 3). This confirmed that p21 is an effective inhibitor of CDK1 activity when present at a concentration of 1.6 µM or greater, and moreover that this is a specific inhibition, in that even at high concentrations, MAP kinase activity remains unaltered. A supra-optimal concentration of p21 was used in the following experiment.

The effect of CDK1 inhibition on IP₃ responsiveness was tested as above, by comparing the increase in Calcium Green-1 fluorescence obtained when control and p21 injected oocytes were subjected to identical duration u.v. flashes in the same field of view. Results (Table 1) showed that there was no significant difference in IP₃ responsiveness between control oocytes and oocytes injected with p21 ($p = 0.264$ using a standard two-tailed t-test, $n = 11$ from 6 animals)

Maintaining CDK1 Activity Prolongs IP₃ Responsiveness. In order to determine whether responsiveness to IP₃ could be maintained by CDK1 activity, we injected oocytes with $\Delta 90$ cyclin B, a non-destructible truncated form of cyclin B1, known to keep CDK1 active in ascidian oocytes.¹⁸

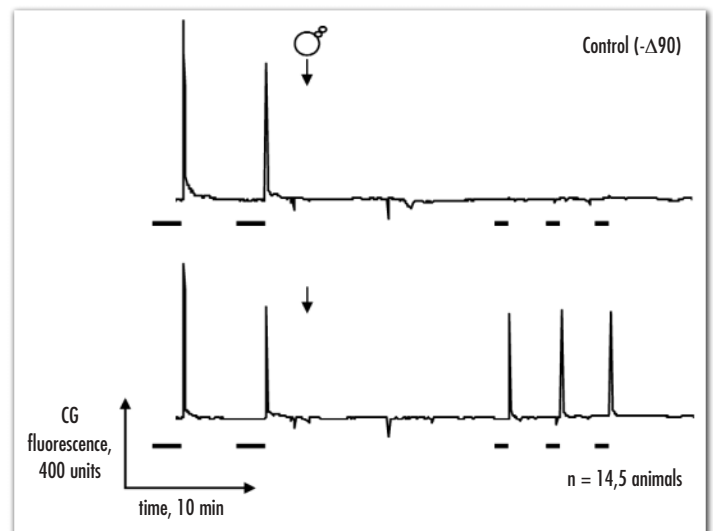


Figure 4. IP₃ responsiveness is maintained when CDK1 activity remains elevated. Pairs of oocytes, one control and one expressing $\Delta 90$ cyclin B to maintain elevated CDK1 activity¹⁵ were injected with as close to identical amounts of Calcium Green-1/caged IP₃ as possible in order that response to flashing in each is directly comparable. In the same field of view, 10ms duration 365nm u.v. flashes were administered and 2nd polar body was duly extruded in the control oocyte (as indicated by the egg morphology). That this does not happen in the $\Delta 90$ oocyte is confirmation that CDK1 activity is elevated. Later, further flashes of only 2ms produce no response in the control oocyte, but are sufficient to elicit calcium releases in the $\Delta 90$ oocyte, which is clear evidence of maintained IP₃ responsiveness. Flash periods are indicated by the solid bars, with the shorter bars indicating the later reduced flash times (not to scale).

We used a non-GFP tagged version of $\Delta 90$ to avoid the GFP fluorescence contributing to the Calcium Green-1 fluorescence measurements.

Pairs of oocytes, one control and one expressing previously injected $\Delta 90$ cyclin B mRNA, were coinjected with identical amounts of Calcium Green-1/caged IP₃, and then oocytes with the same CG signal were paired, since CG fluorescence relates directly to the amount of caged IP₃ present. This is important in that it then allows us to treat flash responsiveness as directly comparable between pairs of oocytes. The paired oocytes were then exposed to 10 ms flashes of u.v. light, two of which were sufficient to lead to pb2 extrusion in the control oocyte (Fig. 4). That the $\Delta 90$ oocyte fails to extrude a second polar body is evidence that we have successfully maintained CDK1 activity as shown previously.¹⁸ We then proceeded to administer further short, 2ms flashes to the oocytes after the time we know IP₃ responsiveness to have declined in the control oocyte. It can clearly be seen that the $\Delta 90$ oocyte gives a response after each flash where there is none in the control. This is clear evidence that IP₃ responsiveness can be maintained in the presence of sustained CDK1 activity ($n = 14$ from 5 different animals).

Table 1 **EFFECT OF CDK1 INHIBITION ON IP₃ RESPONSIVENESS IN UNFERTILIZED OOCYTES**

Sample	IP ₃ Responsiveness
control	213.6 + 22.5 ($n = 8$ from 6 animals)
p21	241.2 + 21.3 ($n = 11$ from same 6 animals)

Results shown mean IP₃ responsiveness, measured as rise in Calcium Green-1 fluorescence above baseline after u.v. flashing, \pm S.E.M.

DISCUSSION

The results we report here show that on the one hand inhibition of CDK1 activity in the unfertilised, metaphase arrested oocyte has no effect on IP₃ responsiveness, but on the other that maintaining CDK1 activity beyond the point in the meiotic cell cycle at which it normally declines results in prolonged responsiveness to IP₃. These results imply that CDK1 activity can play a role in regulating IP₃ responsiveness, but that there is another regulatory pathway involved, as has also recently been suggested in nemertean oocytes.¹⁹ It is possible that CDK1 is acting at the level of regulating the structure of the endoplasmic reticulum or ER,^{19,20} the membranes of which contain not only the IP₃ receptors, but also the calcium pumps for returning calcium to the lumen of the ER.²¹ That CDK1 activity is not solely responsible for regulating IP₃ responsiveness is also implied when we look at the situation in a normal fertilised oocyte. As described above, there is a period when the calcium oscillations pause and the CDK1 activity is low (Fig. 1), but that this is not due to a drop in IP₃ responsiveness, which we have already shown stays steady during this period.⁹ This is consistent with the idea that responsiveness to IP₃ does not only follow CDK1 activity, although it could be argued that the drop in CDK1 activity during the gap phase is so brief (only approximately 5 minutes) that a decline in responsiveness could not be detected before CDK1 activity rises again and responsiveness is restored. The extremely rapid decline in IP₃ responsiveness that we report here is highly unlikely to be due to proteolytic degradation of IP₃ receptors which has been reported to occur over a period lasting several hours in mouse eggs.²²⁻²⁴ We have also shown here that IP₃ responsiveness finally declines only after pb2 extrusion, when CDK1 activity is known to have already declined to basal levels and the calcium oscillations have already stopped. These observations seem to imply that another cell cycle pathway maintains IP₃ responsiveness until meiotic exit, and also that the calcium oscillations cease for a reason other than decline in IP₃ responsiveness.

Acknowledgements

We would like to thank Dr. R. Patel for the gift of the pCEP/WADF plasmid construct. This work was supported by a Wellcome Trust grant to AMD.

References

- Cuthbertson KSR, Whittingham DG, Cobbold PH. Free calcium increases in exponential phases during mouse oocyte activation. *Nature* 1981; 294:754-7.
- Stricker SA. Comparative biology of calcium signalling during fertilization and egg activation in animals. *Dev Biol* 1999; 211:157-76.
- Kline D, Kline JT. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol* 1992; 147:80-9.
- Swann K, Ozil JP. Dynamics of the calcium signal that triggers mammalian egg activation. *Int Rev Cytol* 1994; 152:183-222.
- Speksnijder JE, Corson W, Sardet C, Jaffe LF. Free Ca²⁺ pulses follow fertilisation in the ascidian egg. *Dev Biol* 1989; 135:182-90.
- McDougall A, Sardet C. Function and characteristics of repetitive calcium waves associated with meiosis. *Curr Biol* 1995; 5:318-28.
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC ζ : a sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development. *Development* 2002; 129:3533-44.
- Miyazaki S, Shirakawa H, Nakada K, Honda Y. Essential role of inositol 1,4,5-trisphosphate receptor/Ca²⁺ release channel in Ca²⁺ waves and Ca²⁺ oscillations at fertilization of mammalian eggs. *Dev Biol* 1993; 158:62-78.
- McDougall A, Levasseur M. Sperm triggered calcium oscillations during meiosis in ascidian oocytes first pause, restart then stop: correlations with cell cycle kinase activity. *Development* 1998; 125:4451-9.
- Fissore RA, Robl JM. Mechanisms of calcium oscillations in fertilised eggs. *Dev Biol* 1994; 166:634-42.
- Jones KT, Carroll J, Merriman JA, Whittingham DG, Kono T. Repetitive sperm-induced Ca²⁺ transients in mouse oocytes are cell cycle dependent. *Development* 1995; 121:3259-66.
- Machaty Z, Funahashi H, Day BN, Prather RS. Developmental changes in the intracellular Ca²⁺ release mechanism in porcine oocytes. *Biol. Reprod.* 1997; 56:921-30.
- Masui Y, Markert CL. Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. *J Exp Zool* 1971; 177:129-45.
- Newport JW, Kirschner M. Regulation of the cell cycle during early *Xenopus* development. *J Cell Biol.* 1984; 98:1247-55.
- Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. *Nature* 1989; 339:275-80.
- Yoshida M, Sensui N, Inoue T, Morisawa M, Mikoshiba K. Role of two series of Ca²⁺ oscillations in activation of ascidian eggs. *Dev. Biol.* 1998; 203:122-33.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993 366:701-4.
- Levasseur M, McDougall A. Sperm-induced calcium oscillations at fertilisation in ascidians are controlled by cyclin B1-dependent kinase activity. *Development* 2000; 127:631-41.
- Stricker SA, Smythe TL. Endoplasmic reticulum reorganizations and Ca²⁺ signalling in maturing and fertilized oocytes of marine protostome worms: the roles of MAPKs and MPF. *Development* 2003; 130:2867-79.
- FitzHarris G, Marangos P, Carroll J. Cell cycle – dependent regulation of structure of endoplasmic reticulum and inositol 1,4,5-trisphosphate – induced Ca²⁺ release in mouse oocytes and embryos. *Mol Biol Cell* 2003; 14:288-301.
- Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000; 1:11-21.
- Parrington J, Brind S, De Smedt H, Gangeswaran R, Lai FA, Wojcikiewicz R, Carroll J. Expression of inositol 1,4,5-trisphosphate receptors in mouse oocytes and early embryos: the type 1 isoform is upregulated in oocytes and downregulated after fertilization. *Dev Biol* 1998; 203:451-61.
- Jellerette T, He CL, Wu H, Parys JB, Fissore RA. Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilisation or parthenogenetic activation. *Dev Biol* 2000 223:238-50.
- Brind S, Swann K, Carroll J. Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm and adenophostin A but not to increase in intracellular Ca²⁺ or egg activation. *Dev Biol* 2000; 223:251-65.