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ADP-ribosyl cyclases regulate early development of the sea urchin

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Abstract

ADP-ribosyl cyclases are multifunctional enzymes involved in the metabolism of nucleotide derivatives necessary for Ca^{2+} signalling such as cADPR and NAADP. Although Ca^{2+} signalling is a critical regulator of early development, little is known of the role of ADP-ribosyl cyclases during embryogenesis. Here we analyze the expression, activity and function of ADP-ribosyl cyclases in the embryo of the sea urchin – a key organism for study of both Ca^{2+} signalling and embryonic development. ADP-ribosyl cyclase isoforms (SpARC1-4) showed unique changes in expression during early development. These changes were associated with an increase in the ratio of cADPR:NAADP production. Over-expression of SpARC4 (a preferential cyclase) disrupted gastrulation. Our data highlight the importance of ADP-ribosyl cyclases during embryogenesis.

Keywords

ADP-ribosyl cyclase; cyclic ADP-ribose; NAADP; early development; gastrulation

Introduction

Changes in the concentration of cytosolic Ca^{2+} ions constitute a widespread and evolutionary conserved signalling pathway (Berridge *et al.*, 2000). These signals regulate diverse cellular processes ranging from cell division to cell death (Berridge *et al.*, 2000). It is perhaps not surprising then that Ca^{2+} plays a central role during early development (Slusarski and Pelegri, 2007; Whitaker, 2006). For example Ca^{2+} is necessary and sufficient for egg activation at fertilization (Whitaker, 2006) and is involved in later processes such as axis determination (Kume *et al.*, 1997), convergent extension (Wallingford *et al.*, 2001) and neural induction (Moreau *et al.*, 2008). Ca^{2+} signals regulate a multitude of events including those during embryogenesis.

A major mechanism for generating Ca^{2+} signals is through the production of Ca^{2+} mobilizing messengers that release Ca^{2+} from intracellular Ca^{2+} stores (Berridge *et al.*,

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2000). Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are two such messengers that target the ER and acidic Ca^{2+} stores (such as lysosomes), respectively (Lee, 2012; Patel and Docampo, 2010). Interestingly, despite their different mechanisms of action, both are synthesized by the same family of enzymes, the ADP-ribosyl cyclases via cyclisation and base-exchange reactions (Lee, 2000; Malavasi *et al.*, 2008). This family includes CD38 a well characterized cell surface antigen (Howard *et al.*, 1993). Indeed, all identified ADP-ribosyl cyclases appear to have exo-cytoplasmic catalytic sites suggesting compartmentalized messenger synthesis (De Flora *et al.*, 2004) although their topology might not necessarily be fixed (Zhao *et al.*, 2012). In addition to synthesis, ADP-ribosyl cyclases can also metabolize cADPR (and NAD) to ADP-ribose to regulate Ca^{2+} influx (Perraud *et al.*, 2001) and NAADP to ADP-ribose phosphate (Graeff *et al.*, 2006) which together with dephosphorylation (Berridge *et al.*, 2002; Schmid *et al.*, 2012) may serve to terminate NAADP-mediated Ca^{2+} signals. ADP-ribosyl cyclases are important multifunctional Ca^{2+} signalling enzymes.

ADP-ribosyl cyclase activity was first inferred in sea urchin egg homogenates over three decades ago (Clapper *et al.*, 1987). As a basal deuterostome, the sea urchin holds an important position in animal evolution and has been extensively used by both Ca^{2+} signalers and developmental biologists. But only relatively recently have molecular correlates of ADP-ribosyl cyclase activity been identified in this model organism. Four isoforms have been described in *Strongylocentrotus purpuratus* (SpARC1-4) (Churamani *et al.*, 2007; Ramakrishnan *et al.*, 2010). SpARC1, 2 and 3 were independently cloned and referred to as SpARC β , α and γ , respectively (Davis *et al.*, 2008). SpARC1/ β is unusual in that it is intracellular although its precise subcellular location (cortical granules in eggs v the ER) is unclear (Churamani *et al.*, 2007; Davis *et al.*, 2008). SpARCs show differences in catalytic activity with respect to their ability to metabolize the NAD surrogate NGD and the relative rates of cADPR versus NAADP production (Churamani *et al.*, 2008; Ramakrishnan *et al.*, 2010). SpARC2 for example preferentially produces NAADP over cADPR whereas SpARC4 preferentially produces cADPR over NAADP when assayed in parallel. These differences are likely attributable to a single residue within the active site because point mutation can convert the preference of SpARC4 to that of SpARC2 (Ramakrishnan *et al.*, 2010). An ADP-ribosyl cyclase has recently been cloned from the sea star (Ramos *et al.*, 2014) but its catalytic properties were not characterized and its relationship to other ADP-ribosyl cyclases is not clear at present. Our molecular understanding of these enzymes is advancing,

ADP-ribosyl cyclases play established roles in adult tissues which range from immune responses (Partida-Sanchez *et al.*, 2001) to social behaviour (Jin *et al.*, 2007). We know little however about the part played by ADP-ribosyl cyclases during development. We showed that CD38 is critical for development of *Xenopus laevis* identifying a specific role for ADP-ribosyl cyclase in elongation of the anterior-posterior axis and differentiation of skeletal muscle (Churamani *et al.*, 2012). Like mammals, *Xenopus* possess two ADP-ribosyl cyclases (CD38 and Bst1/CD157). Here we begin to define the role of ADP-ribosyl cyclases in development of the sea urchin, which possess an expanded ADP-ribosyl cyclase gene portfolio (Churamani *et al.*, 2007; Ramakrishnan *et al.*, 2010).

Materials and Methods

Embryology

Strongylocentrotus purpuratus eggs were fertilized and embryos grown as 0.5% (v/v) suspensions in filtered sea water (FSW) at 16°C. The embryos were then collected at various time points (egg, 2, 3, 10, 21, 27.5, 33.5, 46, 58 and 63h post-fertilization) according to standard protocols (Ettensohn *et al.*, 2004). For RT PCR and activity measurements, the suspensions were gently centrifuged, the FSW aspirated and the pellets (~ 250 µL packed volume) flash frozen and stored at -80°C prior to use.

Semi-quantitative RT-PCR

Total RNA was isolated from the frozen egg/embryo samples using the RNeasy kit (Qiagen), according to manufacturer's instructions. The samples were treated with on-column DNase to eliminate genomic DNA contamination. cDNA was synthesised from 2 µg of RNA from each embryo sample with oligo-dT primers using the ImProm-II kit (Promega) according to manufacturer's instructions. Water was added in place of reverse transcriptase in control reactions. PCR was performed using Platinum Taq Polymerase (Invitrogen) and the primers listed in Table S1. Cycle numbers for SpARC1-4 (35, 27, 33, 35, respectively) and ubiquitin (27) were determined empirically such that the amplification was in the exponential phase. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide (5 µg/ml) and visualized on an UV transilluminator. Images were captured using Image Quant software (GE Healthcare).

ADP-ribosyl cyclase activity measurement

Frozen egg/embryo samples were thawed, homogenized in a buffer comprising 20 mM HEPES (pH 7.2) supplemented with complete™ EDTA free protease inhibitors and sonicated (5 x 5s) to form a 25% v/v homogenate. Homogenates were diluted 10-fold in to a reaction mix containing a final concentration of either 20 mM HEPES (pH 7.2) and 1 mM NAD (for cyclase activity measurements) or 50 mM nicotinic acid (pH 4.8) and 1 mM NADP (for base-exchange activity measurements). The reactions were allowed to proceed for up to 3h. To terminate the reactions, samples were diluted 10-fold with water and heated at 60°C for 5 min. Samples were then centrifuged for 3 min at 21000 x g to remove any particulate matter. The products in the supernatants were resolved on an AG MP1 anion exchange HPLC column using a concave-up gradient of trifluoroacetic acid as described in (Churamani *et al.*, 2007). Protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce) and bovine serum albumin as the standard according to manufacturer's instructions.

Generation of SpARC4 mCherry

A construct encoding SpARC4 tagged with mCherry at its N-terminus was generated by replacing the sequence of the *myc* tag of pCS2+ encoding N-terminally *myc*-tagged SpARC4 *myc* described in (Ramakrishnan *et al.*, 2010) with that of mCherry. To achieve this, the coding sequence of mCherry was amplified by PCR using the primers listed in Table S1 and cloned into the ClaI and EcoRI sites of pCS2+ SpARC4 *myc*. Capped mRNA

for SpARC4-mCherry was generated from the SP6 promoter of pCS2+ using the mMessage mMachine kit (Ambion).

Microinjection

Eggs were arranged in rows on dishes coated with protamine sulfate (1% w/v) in FSW supplemented with 4 mM para amino benzoic acid (PABA). The eggs were fertilized and immediately microinjected with SpARC4 mRNA (2 mg/ml) according to standard protocols (Ettensohn *et al.*, 2004). Following microinjection, the embryos were gently washed ten times with FSW to remove PABA and incubated in a humidified chamber at 12°C for approximately 15 h before inspection.

Confocal microscopy

After hatching, the free-floating embryos were gently trapped on a clean glass slide using a cover slip and clay feet. Microscopy was performed at 12°C using a Zeiss LSM 700 confocal microscope. Both bright field and DIC mode were used to capture images of whole embryos. Fluorescence images of embryos expressing SpARC4-mCherry were captured upon excitation at 555nm.

Results and Discussion

ADP-ribosyl cyclase expression is developmentally regulated

In previous studies, transcripts for SpARC2 (Churamani *et al.*, 2008) and SpARCa, β and γ (Davis *et al.*, 2008) were detected in sea urchin eggs. To extend these studies, we systematically analyzed the expression of all known SpARC isoforms including SpARC4 during early development by semi-quantitative PCR. Amplicons of the expected size for SpARC1 (972 bp), SpARC2 (638 bp) and SpARC3 (619 bp) were detectable in the egg (Figure 1) in accord with earlier reports (Churamani *et al.*, 2008; Davis *et al.*, 2008). SpARC4 transcripts (expected amplicon size: 298 bp) were also detectable. The results indicate that all SpARCs are maternally expressed.

SpARC expression showed marked changes during subsequent development (up to 58h). Transcript levels for SpARC1 were low in egg and early embryos but increased from 27.5 h post-fertilization corresponding to the gastrula transition (Figure 1A). Conversely, SpARC2 levels were relatively high in egg and early embryos but decreased during the gastrula transition (Figure 1C). For SpARC3, the transcript levels in the egg were low, decreasing further to almost negligible levels by 10 h (Figure 1C). But at 21 h (corresponding to late blastula stage), SpARC3 expression increased (Figure 1C). Finally, for SpARC4, the expression was low in the egg and early embryos but increased significantly from 27.5 h post-fertilization, again corresponding to the gastrula transition (Figure 1D).

Taken together, these results suggest that expression of each SpARC isoform is uniquely regulated during sea urchin embryogenesis. The presence of SpARC1 transcripts in embryos potentially reconciles previous discrepancies regarding the subcellular location of SpARC1/ β . Davis *et al.* concluded that SpARC β is located within cortical granules in eggs (Davis *et al.*, 2008) but these structures undergo exocytosis at fertilisation and thus are absent in

somatic cells. This isoform must therefore reside elsewhere during later development possibly in the ER as reported for SpARC1 by Churamani et al. (Churamani *et al.*, 2007).

ADP-ribosyl cyclase activity is developmentally regulated

Previous studies have identified ADP-ribosyl cyclase activities in the sea urchin egg (Graeff *et al.*, 1998; Wilson and Galione, 1998). Little however is known regarding ADP-ribosyl cyclase activities in developing sea urchin embryos. We therefore measured endogenous cyclisation activity by HPLC using NAD as a substrate. cADPR production was observed at all the developmental time points tested (Figure 2A). Cyclisation activity was 2 ± 0.6 nmol/mg/h in the egg. Activity decreased during subsequent development reaching a minimum by ~ 20 h. Activity then recovered to that measured in the egg by ~ 50 h (advanced prism stages) and further increased at ~ 70 h (pluteus stage). At this point the production of cADPR was approximately two-fold higher than in eggs (Figure 2A).

Base-exchange activity was also measured in parallel. For these experiments, homogenates were incubated with NADP and nicotinic acid at acidic pH to favour NAADP production. Like cADPR, NAADP was produced at all the developmental time points analysed (Figure 2B). Base-exchange activity in the egg (5.3 ± 0.6 nmol/mg/h) was higher than cyclisation activity. Activity then decreased reaching a minimum at the blastula stage (27 h) and remaining relatively constant through gastrula (33 h), prism (46 h), and the advanced prism (53 h) stages. NAADP production rebounded at the pluteus stage (68 h) but remained lower than that in eggs (Figure 2B).

cADPR and NAADP production are therefore differentially regulated during embryogenesis. This is highlighted in Figure 2C where the ratio of cyclisation and base-exchange is calculated at each developmental time point. The analysis shows that in eggs and early embryos, NAADP production is favoured (ratio < 1) whereas later in development NAADP and cADPR production are more comparable (ratio ~ 1).

ADP-ribosyl cyclases regulate gastrulation

We have previously shown that recombinant SpARC isoforms have unique catalytic properties (Churamani *et al.*, 2007; Churamani *et al.*, 2008; Ramakrishnan *et al.*, 2010). Notably, SpARC4 preferentially produces cADPR over NAADP (Ramakrishnan *et al.*, 2010) whereas SpARC1 and SpARC2 show the opposite preference (Churamani *et al.*, 2007; Churamani *et al.*, 2008). The increase during early development of both the endogenous cyclisation:base exchange activity ratio (Figure 2C) and expression of SpARC4 (Figure 1D) prompted us to probe the role of SpARC4 in embryogenesis. To achieve this, development of embryos over-expressing SpARC4 was compared with control embryos from the same batch.

The control embryos developed as expected. Ciliated blastula stage embryos with intact fertilization envelopes were observed 15 h post-fertilization (Figure 3A). The embryos hatched between 20-21 h. The free-swimming embryos initiated gastrulation after 46 h (Figure 3B). The archenteron was fully developed and a pair of tri-radiate spicules were seen at the end of 3 d. By 5 d, the pluteus stage of the elongated larva with arms and tri-partitioned stomach was visible (Figure 3C).

Confocal microscopy of embryos injected with mRNA encoding SpARC4 tagged with mCherry confirmed expression of the construct at 14 h post-fertilization. Expression persisted for at least 6 days of development. SpARC4-mCherry expressing embryos developed similarly to the control embryos up to the ciliated blastula stage (Figure 3A). Importantly, most SpARC4-mCherry expressing embryos failed to gastrulate (Figure 3B). Some embryos underwent delayed but abnormal gastrulation, such that the archenteron was curved and fused to the embryo wall at an abnormal angle with evidence of abnormal spicule formation (Figure S1). These effects are likely specific to SpARCs, and unrelated to overexpression, since many proteins can be expressed in the embryo without deleterious consequences (for example see (Gokirmak *et al.*, 2012)). Interestingly, a recent study showed that knockdown of sea star ADP-ribosyl cyclase caused lethality at the gastrula stage consistent with a conserved role for ADP-ribosyl cyclases in echinoderm development (Ramos *et al.*, 2014). Thus, both overexpression and knockdown of ADP-ribosyl cyclases disrupts gastrulation pointing to a precise level of enzyme activity for sustaining echinoderm development.

Conclusion

In summary, we provide an analysis of ADP-ribosyl cyclase expression, activity and function during sea urchin development. Our results identify a potential role for ADP-ribosyl cyclases during gastrulation. Ca^{2+} signalling during this critical period has been previously described in vertebrate embryos (Webb and Miller, 2006). Sea urchins are unique in their expanded ADP-ribosyl cyclase complement even within echinoderms given that sea stars appear to possess only a single ADP-ribosyl cyclase (Ramos *et al.*, 2014). Both cADPR and NAADP contribute to Ca^{2+} signals at fertilization of sea urchin eggs (Galione *et al.*, 1993; Lee *et al.*, 1993) which contrasts to mammalian eggs which are driven primarily by IP_3 generated by a sperm-specific phospholipase C (Saunders *et al.*, 2003). We suggest that whilst the role of Ca^{2+} in regulating developmental events is likely conserved in deuterostomes, the underlying mechanisms for generating Ca^{2+} signals may differ. Further analysis of ADP-ribosyl cyclases during sea urchin development is thus warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Berridge G, Cramer R, Galione A, Patel S. Metabolism of the novel Ca^{2+} -mobilizing messenger nicotinic acid-adenine dinucleotide phosphate via a 2'-specific Ca^{2+} -dependent phosphatase. *Biochem J.* 2002; 365:295–301. [PubMed: 11936953]
2. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol.* 2000; 1:11–21. [PubMed: 11413485]

3. Churamani D, Boulware MJ, Geach TJ, Martin AC, Moy GW, Su YH, Vacquier VD, Marchant JS, Dale L, Patel S. Molecular characterization of a novel intracellular ADP-ribosyl cyclase. *PLoS ONE*. 2007; 2:e797. [PubMed: 17726527]
4. Churamani D, Boulware MJ, Ramakrishnan L, Geach TJ, Martin AC, Vacquier VD, Marchant JS, Dale L, Patel S. Molecular characterization of a novel cell surface ADP-ribosyl cyclase from the sea urchin. *Cell Signal*. 2008; 20:2347–2355. [PubMed: 18824228]
5. Churamani D, Geach TJ, Ramakrishnan L, Prideaux N, Patel S, Dale L. The signaling protein CD38 is essential for early embryonic development. *J Biol Chem*. 2012; 287:6974–6978. [PubMed: 22223651]
6. Clapper DL, Walseth TF, Dargie PJ, Lee HC. Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. *J Biol Chem*. 1987; 262:9561–9568. [PubMed: 3496336]
7. Davis LC, Morgan AJ, Ruas M, Wong JL, Graeff RM, Poustka AJ, Lee HC, Wessel GM, Parrington J, Galione A. Ca^{2+} signaling occurs via second messenger release from intraorganelle synthesis sites. *Curr Biol*. 2008; 18:1612–1618. [PubMed: 18951023]
8. De Flora A, Zocchi E, Guida L, Franco L, Bruzzone S. Autocrine and paracrine calcium signaling by the CD38/NAD⁺/cyclic ADP-ribose system. *Ann N Y Acad Sci*. 2004; 1028:176–191. [PubMed: 15650244]
9. Ettensohn CA, Wessel GM, Wray GA. The invertebrate deuterostomes: an introduction to their phylogeny, reproduction, development, and genomics. *Methods Cell Biol*. 2004; 74:1–13. [PubMed: 15575600]
10. Galione A, McDougall A, Busa WB, Willmott N, Gillot I, Whitaker M. Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs. *Science*. 1993; 261:348–352. [PubMed: 8392748]
11. Gokirmak T, Campanale JP, Shipp LE, Moy GW, Tao H, Hamdoun A. Localization and substrate selectivity of sea urchin multidrug (MDR) efflux transporters. *J Biol Chem*. 2012; 287:43876–43883. [PubMed: 23124201]
12. Graeff R, Liu Q, Kriksunov IA, Hao Q, Lee HC. Acidic residues at the active sites of CD38 and ADP-ribosyl cyclase determine nicotinic acid adenine dinucleotide phosphate (NAADP) synthesis and hydrolysis activities. *J Biol Chem*. 2006; 281:28951–28957. [PubMed: 16861223]
13. Graeff RM, Franco L, De Flora A, Lee HC. Cyclic GMP-dependent and -independent effects on the synthesis of the calcium messengers cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate. *J Biol Chem*. 1998; 273:118–125. [PubMed: 9417055]
14. Howard M, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RME, Walseth TF, Lee HC. Formation and hydrolysis of cyclic ADP-Ribose catalysed by lymphocyte antigen CD38. *Science*. 1993; 262:1056–1059. [PubMed: 8235624]
15. Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnyder NA, Yamada K, Noda M, Seike T, Fujita K, et al. CD38 is critical for social behaviour by regulating oxytocin secretion. *Nature*. 2007; 446:41–45. [PubMed: 17287729]
16. Kume S, Muto A, Inoue T, Suga K, Okano H, Mikoshiba K. Role of inositol 1,4,5-trisphosphate receptor in ventral signaling in *Xenopus* embryos. *Science*. 1997; 278:1940–1943. [PubMed: 9395395]
17. Lee HC. Enzymatic functions and structures of CD38 and homologs. *Chem Immunol*. 2000; 75:39–59. [PubMed: 10851778]
18. Lee HC. Cyclic ADP-ribose and Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) as Messengers for Calcium Mobilization. *J Biol Chem*. 2012; 287:31633–31640. [PubMed: 22822066]
19. Lee HC, Aarhus R, Walseth TF. Calcium mobilization by dual receptors during fertilization of sea urchin eggs. *Science*. 1993; 261:352–355. [PubMed: 8392749]
20. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, Vaisitti T, Aydin S. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol Rev*. 2008; 88:841–886. [PubMed: 18626062]

21. Moreau M, Neant I, Webb SE, Miller AL, Leclerc C. Calcium signalling during neural induction in *Xenopus laevis* embryos. *Philos Trans R Soc Lond B Biol Sci*. 2008; 363:1371–1375. [PubMed: 18198153]
22. Partida-Sanchez S, Cockayne DA, Monard S, Jacobson EL, Oppenheimer N, Garvy B, Kusser K, Goodrich S, Howard M, Harmsen A, Randall TD, et al. Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. *Nat Med*. 2001; 7:1209–1216. [PubMed: 11689885]
23. Patel S, Docampo R. Acidic calcium stores open for business: expanding the potential for intracellular Ca^{2+} signaling. *Trends Cell Biol*. 2010; 20:277–286. [PubMed: 20303271]
24. Perraud AL, Fleig A, Dunn CA, Bagley LA, Launay P, Schmitz C, Stokes AJ, Zhu Q, Bessman MJ, Penner R, Kinet JP, et al. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature*. 2001; 411:595–599. [PubMed: 11385575]
25. Ramakrishnan L, Muller-Steffner H, Bosc C, Vacquier VD, Schuber F, Moutin MJ, Dale L, Patel S. A single residue in a novel ADP-ribosyl cyclase controls production of the calcium mobilizing messengers, cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate. *J Biol Chem*. 2010; 285:19900–19909. [PubMed: 20385553]
26. Ramos I, Reich A, Wessel GM. Two-pore channels function in calcium regulation in sea star oocytes and embryos. *Dev*. 2014; 141:4598–4609.
27. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC zeta: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Dev*. 2003; 129:3533–3544.
28. Schmid F, Fliegert R, Westphal T, Bauche A, Guse AH. Nicotinic acid adenine dinucleotide phosphate (NAADP) degradation by alkaline phosphatase. *J Biol Chem*. 2012; 287:32525–32534. [PubMed: 22851169]
29. Slusarski DC, Pelegri F. Calcium signaling in vertebrate embryonic patterning and morphogenesis. *Dev Biol*. 2007; 307:1–13. [PubMed: 17531967]
30. Wallingford JB, Ewald AJ, Harland RM, Fraser SE. Calcium signaling during convergent extension in *Xenopus*. *Curr Biol*. 2001; 11:652–661. [PubMed: 11369228]
31. Webb SE, Miller AL. Ca^{2+} signaling and early embryonic patterning during the blastula and gastrula periods of zebrafish and *Xenopus* development. *Biochim Biophys Acta*. 2006; 1763:1192–1208. [PubMed: 16962186]
32. Whitaker M. Calcium at fertilization and in early development. *Physiol Rev*. 2006; 86:25–88. [PubMed: 16371595]
33. Wilson HL, Galione A. Differential regulation of nicotinic acid-adenine dinucleotide phosphate and cADP-ribose production by cAMP and cGMP. *Biochem J*. 1998; 331:837–843. [PubMed: 9560312]
34. Zhao YJ, Lam CM, Lee HC. The membrane-bound enzyme CD38 exists in two opposing orientations. *Sci Signal*. 2012; 5:ra67. [PubMed: 22969159]

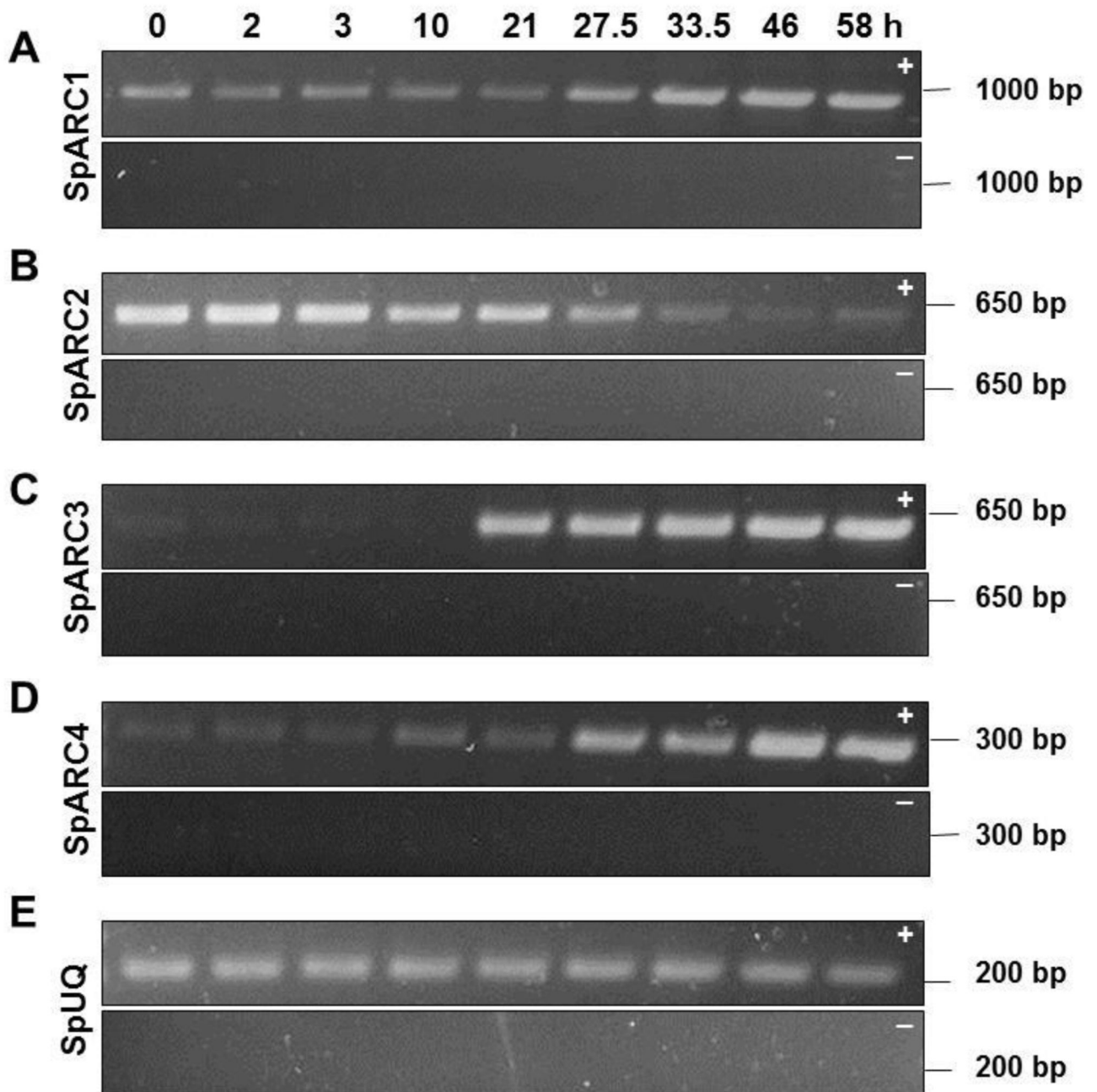


Figure 1. ADP-ribosyl cyclase expression is developmentally regulated.

Semi-quantitative RT-PCR analysis for SpARC1 (A), SpARC2 (B), SpARC3 (C), SpARC4 (D) and ubiquitin (SpUQ, E). Analyses was performed using cDNA synthesised from egg and embryos at the indicated time post-fertilization either in the presence (top lanes; +) or absence (bottom lanes; -) of reverse transcriptase. Size of the molecular weight markers are indicated to the right. Results are representative of 3 independent fertilizations. Embryos were developed at 16°C.

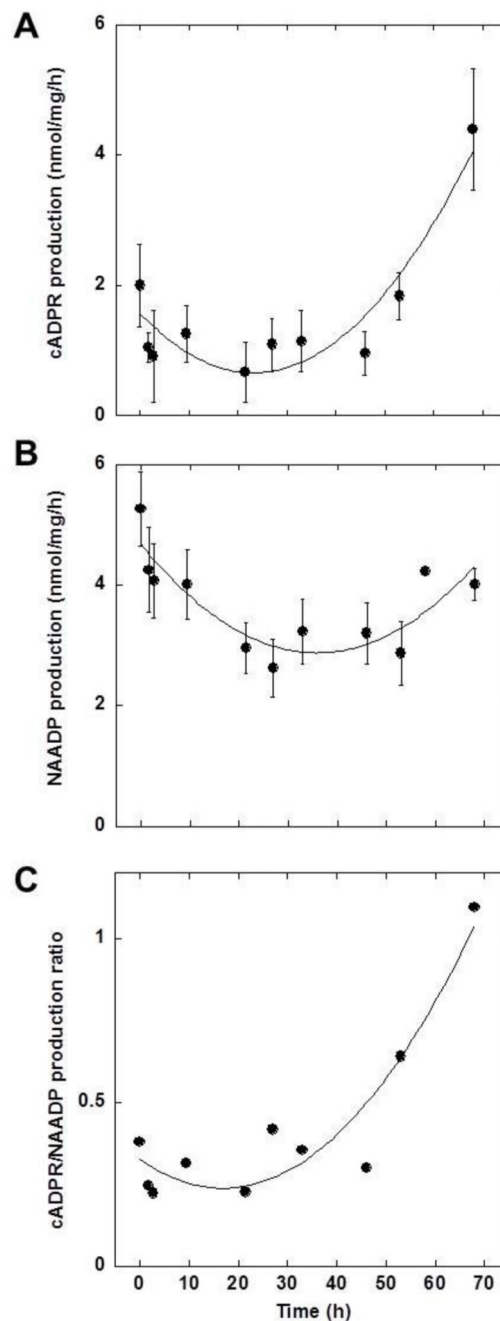


Figure 2. ADP-ribosyl cyclase activity is developmentally regulated.

HPLC analyses of cADPR (A) and NAADP (B) production by egg and embryo homogenates prepared at the indicated time post-fertilization. (C) Ratio of cADPR:NAADP production. Data are presented as mean $\pm$ S.E.M of 3 independent fertilizations. The embryos were developed at 16°C.

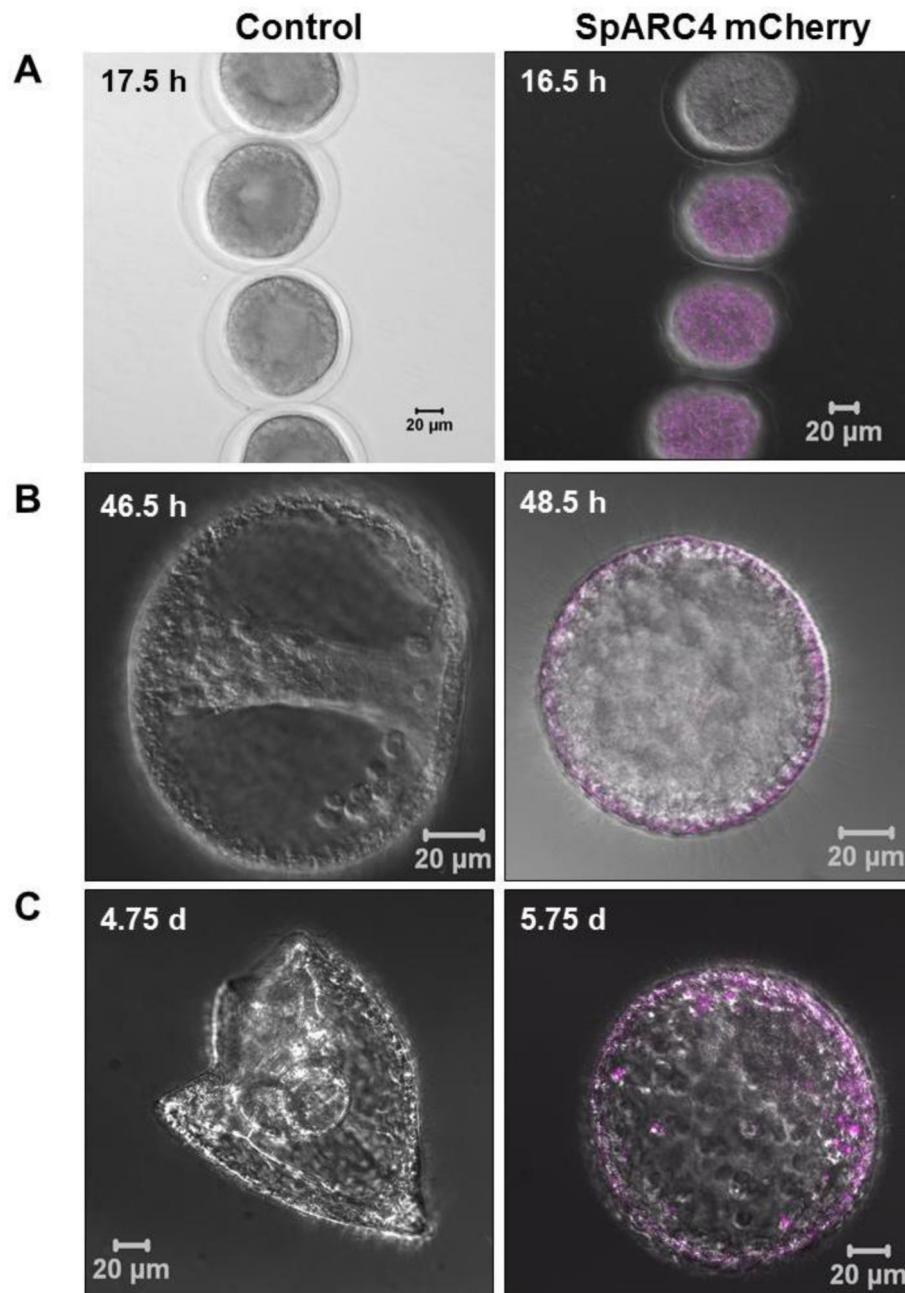


Figure 3. Over-expression of SpARC4 disrupts gastrulation.

Morphology of control embryos (left) or embryos injected with SpARC4-mCherry mRNA (right) at the indicated time post-fertilisation corresponding to blastula (A), gastrula (B) and prism/pluteus (C) stages. Images on the right are overlaid with confocal images of mRFP. Results are representative of 3 independent fertilizations. Embryos were developed at 12°C.