The birth of quail chicks after intracytoplasmic sperm injection

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Short title: Quail chicks after ICSI

Authors: Shusei Mizushima[1], Gen Hiyama[1], Kogiku Shiba[2], Kazuo Inaba[2], Hideo Dohra[3], Tamao Ono[4], Kiyoshi Shimada[5] and Tomohiro Sasanami[1,*]

Affiliations: [1]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

[2]Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, Shizuoka 415-0025, Japan

[3]Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

[4]Department of Food Production Science, Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan

[5]WCU Major in Biomodulation, Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea

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*Corresponding to: Tomohiro Sasanami, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

Tel: +81-54-238-4526, Email: atsasan@ipc.shizuoka.ac.jp

SUMMARY

Intracytoplasmic sperm injection (ICSI) has been used successfully to produce offspring in several mammalian species including humans. However, ICSI has not been successful in birds because of the size of the egg and difficulty in mimicking the physiological polyspermy that takes place during normal fertilization. Microsurgical injection of 20 or more spermatozoa into an egg is detrimental to its survival. Here, we report that injection of a single spermatozoon with a small volume of sperm extract (SE) or its components led to the development and birth of healthy quail chicks. SE contains three factors—phospholipase Cζ (PLCZ), aconitate hydratase (AH), and citrate synthase (CS)—all of which are essential for full egg activation and subsequent embryonic development. PLCZ induces an immediate, transient Ca^{2}+ rise required for the resumption of meiosis. AH and CS are required for long-lasting, spiral-like Ca^{2}+ oscillations within the activated egg, which are essential for cell cycle progression in early embryos. We also found that coinjection of cRNAs encoding PLCZ, AH, and CS supported full development of ICSI-generated zygotes without the use of SE. These findings will help assist our understanding of the mechanism of avian fertilization and embryo development, as well as assisting in manipulation of the avian genome and the production of transgenic and cloned birds.

INTRODUCTION

Fertilization is an indispensable step for zygotic formation in sexual reproduction. In most animals, a single fertilizing spermatozoon evokes a temporal rise in intracellular Ca^{2+} ($[Ca^{2+}]i$) in an egg upon gamete fusion, and this $[Ca^{2+}]i$ plays essential roles in egg activation (Stricker, 1999; Runft et al., 2002). In birds, many (20–60) spermatozoa enter each egg before activating it (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). This polyspermic fertilization is one of the most obvious characteristics in some oviparous animals such as birds and reptiles with large eggs. In mammals, phospholipase Cζ (PLCZ) has been identified as the sperm-borne egg-activating factor as it induces a series of $[Ca^{2+}]i$ oscillations in the egg (Saunders et al., 2002). Interestingly, microinjection of PLCZ collected from chicken (Coward et al., 2005) or medaka (Coward et al., 2011) spermatozoa can also induce inositol trisphosphate (IP_3)-dependent Ca^{2+} oscillations in the mouse egg. In the newt, citrate synthase (CS) has been identified as another sperm-borne egg-activating factor (Harada et al., 2007).

Intracytoplasmic sperm injection (ICSI) has been very useful for studying the mechanisms of egg activation in mammals and urodeles (Yanagimachi, 2005; Morozumi et al., 2006; Iwao, 2012). Furthermore, this technology has contributed to the production of live offspring, and has also been applied clinically to humans. In contrast, ICSI has not yet been successful in producing offspring in birds because the natural polyspermic fertilization is difficult to mimic (Hrabia et al., 2003; Mizushima et al., 2009; Mizushima et al., 2010). All quail embryos produced by the injection of a single spermatozoon died before embryonic stage 6 (Mizushima et al., 2008) (nomenclature used in Hamburger and Hamilton [H&H], 1951). As mentioned above, multiple spermatozoa enter one egg before activation is complete during normal fertilization in birds (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). Therefore, we reasoned that a single spermatozoon does not contain sufficient egg-activating material to induce full activation of an egg. To test this hypothesis, we studied whether avian sperm extract (SE) was able

to enhance the development of ICSI-generated quail zygotes. We also tried to identify the chemical nature of SE components that would activate the quail egg and support subsequent embryonic development of the zygotes.

RESULTS

We first observed spatiotemporal changes in the $[Ca^{2}+]i$ rise in Fluo-8H AM-loaded quail eggs. Microinjection of 50 fmol IP_3 into an unfertilized egg evoked an immediate increase in $[Ca^{2}+]i$; the $Ca^{2}+$ signal propagated over the germinal disk and then peaked about 5 minutes after the injection (Fig. 1A,B and Supplementary Material Movie 1). Thereafter, $[Ca^{2}+]i$ decreased gradually and returned to the basal level within 30 minutes after injection. Microinjection of 2 ng SE per egg, equivalent to 200 spermatozoa per egg, evoked multiple, long-lasting spiral-like $Ca^{2}+$ waves that followed an initial transient $Ca^{2}+$ rise resembling the $[Ca^{2}+]i$ increase in IP_3-injected eggs (Fig. 1C,D and Supplementary Material Movie 2). These repetitive spiral-like $Ca^{2}+$ waves each originated from the injection site and continued for at least 1 hour. The $Ca^{2}+$ waves did not have a simple propagation pattern, but had irregular and complicated waveforms (Supplementary Material Movie 2). The mean fluorescence intensity of the entire germinal disk area oscillated with a mean interspike interval of around 1 minute (Fig. 1D). Although the injection of 50 fmol IP_3 into the egg caused very small oscillations within approximately 20 minutes, these oscillations were essentially different from spiral-like $Ca^{2}+$ oscillations due to significant differences in the mean amplitude of these oscillations (Supplementary Material Fig. S1). When fluorescence intensities were captured from different areas within the germinal disk, reciprocal repeating oscillations were observed (inset in Fig. 1D). These results indicated that SE contains a novel egg-activating factor that induced the spiral-like $Ca^{2}+$ oscillations, differing from those seen with the PLCZ/IP_3-induced $[Ca^{2}+]i$ increases. We repeated the SE injection experiment for six times (n=6) and found that the fundamental patterns in each egg were similar to each other.

To identify the active components that evoked the spiral-like Ca^{2}+ oscillations, gel filtration chromatography was used to fractionate SE (Fig. 2A). Only materials in fraction 3 could induce the spiral-like Ca^{2}+ oscillations (Fig. 2B,C). The spiral-like Ca^{2}+ oscillation-inducing activity in fraction 3 was adsorbed onto *Lens culinaris* agglutinin (LCA)-coated agarose beads (Fig. 2D). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) was then used to compare the components before and after LCA absorption (Fig. 2E). We found that three bands (70, 45, and 30 kDa) bound to the LCA–agarose beads. Liquid chromatography tandem mass spectrometry (LC-MS/MS), *de novo* protein sequencing, and protein identification software (PEAKS) (Ma et al., 2003) were used to identify the proteins. The 70 and 45 kDa proteins were identified as aconitate hydratase (AH) and CS, respectively (Supplementary Material Table S1). The 30 kDa protein was found to be a mixture of superoxide dismutase (SD), malate dehydrogenase (MD), and AH (Supplementary Material Table S1).

Neither porcine AH nor porcine CS induced any significant Ca^{2}+ release when microinjected individually into quail eggs (data not shown). However, simultaneous injection of these factors induced long-lasting repetitive Ca^{2}+ waves similar to those induced by injection of SE (Fig. 2F). Nevertheless, dual microinjections of AH and CS did not generate an immediate (*i.e.*, within 5 minutes) elevation of Ca^{2}+ (Fig. 2F). Immunodepletion of SE with anti-AH or anti-CS antibodies, but not with normal rabbit IgG, abolished the spiral-like oscillations (Fig. 3A-C), suggesting that both AH and CS were necessary for this phenomenon. To confirm that together AH and CS could induce spiral-like Ca^{2}+ oscillations in quail eggs, we synthesized cRNAs encoding quail AH and CS and microinjected these into unfertilized quail eggs. Coinjection of quail AH and CS cRNAs, like the coinjection of porcine AH and CS, induced spiral-like Ca^{2}+ oscillations in unfertilized quail eggs (Fig. 3D). Notably, onset of the cRNA-induced spiral-like Ca^{2}+ oscillations was delayed by about 15 minutes relative to onset of the protein-induced oscillations (*i.e.*, SE or porcine AH plus CS microinjection). This delay probably

reflected the time required for cRNA-to-protein translation. On the other hand, injection of PLCZ cRNA induced an immediate $[Ca^{2}+]i$ rise (Fig. 3E). Furthermore, double microinjections of PLCZ and CS or PLCZ and AH cRNAs induced a transient rise in $[Ca^{2}+]i$ without inducing spiral-like $Ca^{2}+$ oscillations (Fig. 3F,G). Importantly, when PLCZ cRNA was injected with the AH and CS cRNAs, both the transient $Ca^{2}+$ rise and the subsequent spiral-like $Ca^{2}+$ oscillations occurred (Fig. 3H). We employed heparin (Yue et al., 1995) and 2-aminoethoxydiphenyl borate (2-APB), a selective inhibitor for IP_3 receptor (IP_3-R) (Martin-Romero et al., 2008) to investigate the molecular events that leading to the induction of spiral-like $Ca^{2}+$ oscillations. An injection of 1 ng heparin with a mixture of PLCZ, AH, and CS cRNAs diminished the transient rise in $[Ca^{2}+]i$ without disturbing the spiral-like $Ca^{2}+$ oscillations (Supplementary Material Fig. S2B). The pre-incubation of eggs with 100 μM 2-APB before the injection had no effect on the induction of spiral-like $Ca^{2}+$ oscillations (Supplementary Material Fig. S2C). These results indicated that IP_3-R did not participate in signal transduction for the induction of spiral-like $Ca^{2}+$ oscillations in quail eggs.

On the other hand, when a microinjection of 25 fmol cyclic ADP ribose (cADPR), which has been shown to activate ryanodine receptors in sea urchins (Whitaker and Swann, 1993) and bovine eggs (Yue et al., 1995), was performed, irregular patterns of $Ca^{2}+$ waves were observed that were distinctly different from the IP_3 or PLCZ-generated transient rise in $Ca^{2}+$ (Supplementary Material Fig. S2D). Although the mean amplitude of these $Ca^{2}+$ waves did not differ from those of the CS plus AH-induced $Ca^{2}+$ oscillations, the mean interval of the oscillations in cADPR-injected eggs was significantly longer than those of CS- and AH-injected eggs (Supplementary Material Fig. S3). Furthermore, the removal of extracellular $Ca^{2}+$ by adding 20 μM BAPTA to the culture medium did not affect either the amplitudes or duration of spiral-like $Ca^{2}+$ oscillations, indicating that extracellular $Ca^{2}+$ was not required for this event (Supplementary Material Fig. S2H).

When quail SE proteins on Western blots were probed with anti-AH antibody, 70

156 kDa protein was evident; this AH in SE was approximately 10 kDa smaller than the AH
157 in unfertilized egg, liver, or kidney extracts (Supplementary Material Fig. S4A). Several
158 immunoreactive bands of approximately 45 kDa were also detected; however the nature
159 of these bands remains unknown (Supplementary Material Fig. S4A). These 45 kDa
160 proteins were not involved in the process of egg activation because they were not detected
161 in fraction 3 obtained by gel filtration chromatography (data not shown). Anti-CS
162 antibody detected a 45 kDa band in quail SE on Western blots, but a slightly lighter
163 molecule (44 kDa) was detected in the egg, liver, and kidney extracts (Supplementary
164 Material Fig. S4B). Ejaculated sperm were used to clone cDNAs encoding quail AH or
165 CS; notably, a sperm-specific AH cDNA lacked 105 bp that encoded 35 amino acids in
166 the N terminus, and a sperm-specific CS cDNA contained a 3-bp insert encoding 314th
167 arginine (Supplementary Material Fig. S5). Although such structure is not supposed to be
168 required for the egg activation because AH and CS derived from porcine heart induced
169 spiral-like Ca^{2}+ oscillation, these results suggest the existence of a specific form of AH
170 and CS in the quail sperm.
171 To investigate the relationship between quail egg activation and subsequent
172 embryonic development, we observed the effects of three factors (PLCZ, AH, and CS) on
173 the development of ICSI-generated zygotes. ICSI-treated eggs coinjected with 60 pg
174 PLCZ cRNA or 50 fmol IP_3 initiated the first cleavage at around 4.5 hours (data not
175 shown). Notably, this first cleavage was delayed by 1.5 hours relative to the
176 developmental time course that follows *in vivo* fertilization (data not shown). Moreover,
177 the development of these ICSI-derived zygotes was further delayed after 24 hours in
178 culture (Table 1; nomenclature used in Eyal-Giladi and Kochav, 1976). In contrast, when
179 ICSI-treated eggs were coinjected with 2 ng SE, 9 out of 15 embryos (60%) underwent
180 the first cleavage normally (data not shown) and developed to the IX and X stages after
181 24 hours in culture (Table 1). Furthermore, when ICSI-treated eggs were coinjected with
182 a mixture containing all three factors (cRNAs each encoding PLCZ, AH, or CS), 8 out of
183 17 embryos (47%) underwent the first cleavage normally (data not shown) and developed

184 to stages equivalent to those of eggs fertilized *in vivo* (Table 1). On the other hand, no
185 embryo developed normally if ICSI was made without any of these factors (Table 1).
186 Therefore, these results indicated that all of the three factors were essential for the normal
187 development of ICSI-derived embryos. The co-injection of PLCZ cRNA together with
188 cADPR also improved embryonic development (stage IX) after 24 hours in culture more
189 than either treatment with PLCZ cRNA or cADPR alone (Table 1).
190 Microinjection of 50 fmol IP_3 into ICSI-treated quail eggs improved the rate of
191 blastoderm development (86% in Table 1), but development of each such embryo was
192 arrested at H&H stage 6 (Table 2 and Fig. 4A). Likewise, the development of embryos
193 generated by dual injections of PLCZ and AH cRNAs, PLCZ and CS cRNAs, or PLCZ
194 cRNA and 25 fmol cADPR into ICSI eggs died at H&H stages 5, 6 or 8, respectively
195 (Table 2). Ultimately, we produced a live chick by coinjecting 2 ng SE and a single
196 spermatozoon into an unfertilized quail egg (Table 2 and Fig. 4B). The final hatchability
197 of the embryos transferred to the surrogate shell culture was 8%. A total of 5/12 (42%) of
198 the embryos developed past H&H stage 6, and one of these developed to just before
199 hatching (H&H stage 43). The resulting chick was female; after sexual maturation, she
200 produced healthy offspring by natural mating (Fig. 4C). It should be noted that the onset
201 of her sexual maturation (the onset of egg laying) occurred at 7 weeks and 3 days of age,
202 which was within the normal range of female Japanese quail (6-8 weeks of ages, Stein
203 and Bacon, 1976). Another healthy female offspring also hatched following coinjection
204 of a single spermatozoon and a mixture of the three PLCZ, AH, and CS cRNAs (Table 2
205 and Fig. 4D). The final hatchability of the embryos in this treatment group was 10%, with
206 4/10 (40%) of the embryos developing past H&H stage 6, and one developing to just
207 before hatching (H&H stage 43). Unfortunately, the hatching quail chick died before
208 sexual maturation due to an unknown reason. Because these 3 factors were found to be
209 solely essential for egg activation and the full-term development of ICSI-derived zygotes,
210 we concluded that SD and MD, identified by LC-MS/MS analysis, was not required for
211 these events and, thus, did not analyze them, further.

DISCUSSION

Role of Ca^{2}+ waves in quail development

To our knowledge, this is the first description of the pattern of Ca^{2}+ oscillation during *in vitro* fertilization in birds. In mammals including mice (Saunders et al., 2002), humans (Cox et al., 2002), pigs (Yoneda et al., 2006), and cattle (Yoon and Fissore, 2007), PLCZ has been isolated as a sperm-borne egg-activating factor. Thus, microinjection of its cRNA (Saunders et al., 2002) or recombinant protein (Kouchi et al., 2004) elicited long-lasting Ca^{2}+ oscillations similar to that observed in mouse eggs fertilized *in vitro*. In addition, the PLCZ-generated Ca^{2}+ oscillations serve as a sufficient trigger for the resumption of meiosis, pronuclear formation, and subsequent normal blastocyst development (Saunders et al., 2002; Cox et al., 2002; Yoneda et al., 2006). Here, we showed that quail eggs required two different kinds of Ca^{2}+ waves to enable full-term development following ICSI. Namely, *(i)* PLCZ induced a transient Ca^{2}+ rise, and *(ii)* AH and CS together induced long-lasting, spiral-like Ca^{2}+ oscillations. The difference between mammals and quail does not seem to arise because of the weakness of the egg activation activity of avian PLCZ because the Ca^{2}+ oscillation-inducing activity of chicken PLCZ for mouse eggs is equivalent to that of its mammalian counterpart (Coward et al., 2005). We suggest that egg activation mechanisms in the quail egg are distinctly different from those occurring in mammalian eggs, and this was supported by the following findings; *(i)* neither PLCZ nor IP_3 had the ability to induce spiral-like Ca^{2}+ oscillations, *(ii)* spiral-like Ca^{2}+ oscillations occurred irrespective of the presence or absence of a PLCZ-induced transient rise in Ca^{2}+, and *(iii)* heparin or 2-APB, an antagonist for IP_3-R, did not interfere with spiral-like Ca^{2}+ oscillations. Although the underlying mechanisms have not yet been elucidated in detail, the induction of spiral-like Ca^{2}+ oscillations may be mediated, at least in part, via ryanodine receptors rather than via IP_3-R because cADPR, an activator for ryanodine receptors could potently induce irregular patterns of Ca^{2}+ waves in eggs (Fig. S2D). Other unidentified receptors

240 responsible for the signal transduction inducing spiral Ca^{2}+ oscillations may exist
241 because the pattern of Ca^{2}+ waves (*i.e.* mean interval, Fig. S3B) as well as embryonic
242 development (Table 1,2) induced by PLCZ and cADPR differed from those induced by
243 PLCZ, AH and CS.
244 In mice, a single transient rise in [Ca^{2}+]*i* caused by artificial stimuli such as
245 electrical pulses or exposure to ethanol could induce partial egg activation (Tatone et al.,
246 1999; Ducibella et al., 2002; Jones, 2005). The eggs underwent second polar body
247 extrusion, but the chromatin rearrested on a monopolar third spindle. However, repeated
248 stimulations were capable of leading the eggs to full activation (Ducibella et al., 2002;
249 Jones, 2005). This was because the inactivation of cytostatic factor, the cytosolic protein
250 responsible for meiotic arrest at metaphase II, is achieved by repetitive Ca^{2}+ spikes, but
251 not by a single transient Ca^{2}+ rise (Ducibella et al., 2002; Jones, 2005). In addition, 24
252 electrical pulses induced an increase in cortical granule exocytosis (Ducibella et al.,
253 2002), which triggers the zona reaction preventing polyspermy (Jones, 2007). Although
254 the exact mechanism for the induction of Ca^{2}+ oscillations remains to be uncovered, the
255 theory that egg-derived PLCβ or PLCγ might enhance IP_3 generation via a positive
256 feedback of transient Ca^{2}+ rise after the introduction of PLCZ has been proposed for
257 mammalian somatic cells and ascidian eggs (Dupont and Dumollard, 2004; Igarashi et al.,
258 2007; Swann and Yu, 2008). Thus, the Ca^{2}+ oscillation in mammalian eggs is supposed to
259 be required for both the completion of meiosis as well as blocking polyspermic
260 fertilization.
261 What is the role of AH- and CS-derived spiral-like Ca^{2}+ oscillations in quail eggs?
262 Unlike mammals, treatment of the ICSI-activated quail egg by introducing PLCZ cRNA
263 or IP_3 did not lead to full-term development, whereas eggs that were microinjected with a
264 mixture of PLCZ, AH, and CS cRNAs reached the hatching stage. In addition, double
265 injections of CS and AH, but not a single injection of PLCZ induced spiral-like Ca^{2}+
266 oscillations (Fig. 3D,E), whereas these oscillations alone did not stimulate the first
267 cleavage of the eggs (Table 1). These results indicate that spiral-like Ca^{2}+ oscillations and

PLCZ/IP3-generated Ca^{2}+ signaling might contribute independently to different cellular events during fertilization. Thus, the PLCZ-induced transient Ca^{2}+ rise was indispensable for the resumption of meiosis, and the AH- and CS-induced long-lasting, spiral-like Ca^{2}+ oscillations worked as the major driving force for cell cycle progression in early embryos. Because there is no block to polyspermy before membrane fusion in birds (Wong and Wessel, 2006), the role of Ca^{2}+ oscillations in the process of the blockage of polyspermy appears to have been lost in quail eggs. How the spiral-like Ca^{2}+ oscillations are induced by CS and AH and how they enhance development of the early embryo must be answered by future studies. As described above, ryanodine receptors may be responsible for the induction of spiral-like Ca^{2}+ oscillations, however, the signal derived from ryanodine receptors alone appears to be insufficient to support full-term development of the bird *in vitro*.

Comparison of ICSI and normal polyspermic fertilization

Here, SE containing 2 ng of proteins—equivalent to approximately 200 spermatozoa—was found to be required for the full-term development of ICSI-generated quail zygotes. Previous reports demonstrated that chicken SE equivalent to a single or half of a spermatozoon induced pronuclear formation in mouse eggs (Dong et al., 2000), whereas a single quail or chicken spermatozoon could not activate the quail egg (Takagi et al., 2007; Mizushima, 2012). These results indicated that many spermatozoa providing sufficient amounts of PLCZ, AH, and CS proteins are necessary to ensure successful egg activation in the quail. Unlike mammals, polyspermy is normal in fertilized avian eggs. Here, many (20–60) spermatozoa penetrate the perivitelline membrane—homologous to the mammalian zona pellucida—and enter the egg's germinal disc (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). Wishart and Staines (1999) demonstrated that counts of fewer than 20 sperm-generated holes in the perivitelline membrane over the germinal disc were associated with reduced fertility in both chickens and turkeys. These reports indicated that at least 20 spermatozoa seem to be necessary to activate the avian egg. Why a much larger amount of SE factors is required for full-term development

following ICSI might be accounted for by the difference between *in vitro* insemination and our ICSI system. In the polyspermic newt egg, *in vitro* insemination showed that a few spermatozoa enter successively at different points, and small wave-like increases in [Ca^{2}+]*i* occur sequentially at each sperm entry site (Harada et al., 2011; Iwao, 2012). The Ca^{2}+ wave induced by one spermatozoon propagated over only one-eighth to a quarter of the egg surface, which suggests that many spermatozoa must enter to induce a Ca^{2}+ increase through the entire egg (Harada et al., 2011). On the other hand, complete activation of newt eggs by a single microinjection of newt SE needed a protein content equivalent to 330 spermatozoa (Harada et al., 2011). These results are consistent with our observations (Fig. 1C,D and Table 1). Although we did not assess the yield of PLCZ, AH, and CS proteins in the present study, successive entry of multiple spermatozoa into the avian germinal disc seems to be essential for full egg activation in birds. In our current ICSI system, the hatchability was low (8-10%). Some of the ICSI-assisted embryos might have been rescued by improving the surrogate shell culture system (system III in the present study), such as optimum oxygen supply. In fact, the previous finding demonstrated that the hatchability of intact in vivo fertilized eggs obtained from the anterior part of magnum was 19-25 % after the surrogate shell culture was performed and approximately 30-50% embryos died within 2 days of surrogate shell culture (Ono et al., 1994; Ono et al., 1996). This result implies that one of the reasons for the low hatchability in our current ICSI system may be due to a defect of the surrogate shell system. However, we anticipate that this lower rate may have also been explained by an inability of our current ICSI system to reproduce polyspermic fertilization. Further studies are needed to explore the mechanism of avian polyspermic fertilization. Unfortunately, no *in vitro* insemination systems are currently available because avian eggs are too large to handle in culture systems.

Conclusion

The ICSI technique has been well developed in mammals and has been successfully used to produce healthy offspring in humans, mice, hamsters, rats, rabbit, cattle, sheep, horses,

cats, pigs, and monkeys (Yanagimachi, 2005), but no chicks have been generated so far. To our knowledge, this is the first demonstration of full-term (zygote-to-adult) development of a bird following ICSI. Importantly, the resulting two offspring were female and they were not the result of parthenogenesis because the ZW sex-determining system in birds does not allow for parthenogenetic production of female chicks (Harada and Buss, 1981). The successful production of healthy chicks after ICSI has enormous implications for industrial, agricultural, and conservation applications including avian transgenesis, cloning technology, and in protecting endangered bird species. Furthermore, the discovery that sperm-borne AH and CS function as egg-activating factors responsible for embryonic development, and the unique pattern of Ca^{2}+ oscillations during egg activation in birds provides new insights into the molecular mechanisms of egg activation in vertebrates. Our results will also advance our understanding of the detailed molecular mechanisms underlying polyspermic fertilization in birds.

MATERIALS AND METHODS

Animals

Male and female Japanese quail, *Coturnix japonica*, 8–20 weeks of age (Motoki Corporation), were maintained individually under a photoperiod of 14L:10D (lights went on at 05:00) with *ad libitum* access to water and a commercial diet (Motoki Corporation). In domestic birds including quail, the ovulation of female birds is known to occur approximately 30 min after egg laying, with fertilization taking place within 15 minutes of ovulation (Woodard and Mather, 1964). In order to anticipate the time of fertilization *in vivo*, the egg-laying times of individual birds were recorded every day. All experimental procedures for the care and use of animals were approved by the Animal Care and Use Committee of Shizuoka University (Approval number 24-12).

ICSI and *ex vivo* culture

Ejaculated semen was collected from individual male birds immediately before copulation (Kuroki and Mori, 1997). To prepare SE, spermatozoa were washed

352 repeatedly in phosphate-buffered saline (PBS) and collected by centrifugation at 800 x *g*
353 for 3 minutes; fully washed spermatozoa were suspended in PBS. Spermatozoa were
354 disrupted by homogenization and sonication; clarified supernatant was collected via
355 centrifugation at 20,400 x *g* for 10 minutes and then stored as SE. Bicinchoninic acid
356 (BCA) protein assay kits (Pierce) were used to measure protein concentrations in the SE.
357 Unfertilized eggs were recovered from the anterior magnum within 1 hour after
358 oviposition (Mizushima et al., 2008). Each egg was microinjected with a single
359 ejaculated spermatozoon together with either 50 fmol IP_3 or 2 ng SE. The entire injected
360 volume was approximately 1 nl. All procedures used for ICSI were performed as
361 described by Hrabia et al. (2003) and Mizushima et al. (2008). Briefly, under a Hoffman
362 modulation contrast microscope (IX70, Olympus), IP_3 or SE solution was first drawn into
363 an injection micropipette, followed by a single ejaculated spermatozoon in the same
364 micropipette. The ovum was placed into DMEM in a plastic dish (35 x 18 mm; multidish
365 six wells, Nunclon) and both were then injected into the central area of the germinal disc
366 of the egg (approximately 30-50 μm in depth) using a micromanipulator connected to the
367 injector (IM-9B; Narishige) with silicon tubing filled with silicon oil under a
368 stereomicroscope (SZ11; Olympus). Rough estimate of the injection speed was
369 approximately 6 nl/min. Because the germinal disk of quail eggs is opaque, the
370 completion of the injection was visually confirmed by observing a swelling of the
371 injection site under a stereomicroscope. This manipulation was performed with the aid of
372 an image-processor system (Image Σ–III, Nippon Avionics). Regarding the pipettes for
373 ICSI, borosilicate glass capillary tubing (1-mm outer diameter, 0.75-mm inner diameter;
374 Sutter) was drawn with a pipette puller (P-97/IVF; Sutter), and the tip of the pipette was
375 cut with a microforge (MF-900; Narishige) such that the inner diameter at the tip was
376 approximately 5-7 μm. Each egg was cultured in Dulbecco's modified Eagle's medium in
377 a plastic cup at 41.5 °C in an atmosphere containing 5% CO_2 (Ono et al., 1994).
378 Individual embryos were then transferred to a large surrogate Japanese quail eggshell.
379 The shells were filled with thin chicken egg albumen and sealed tight with cling film. The

shell was secured by a pair of plastic rings and elastic bands. Embryos were then cultured for 63 hours at 37.5 °C and 70% relative humidity, with rocking at a 90° angle at 30-minutes intervals. Finally, individual embryos were transferred to a small surrogate chicken eggshell, a generous gift from the Avian Bioscience Research Center (ABRC), of Nagoya University. These were sealed with cling film using thin chicken egg albumen as a glue, and cultured at 37 °C with rocking at a 30° angle until hatching (Ono et al., 1994). For *in vivo* fertilized eggs, a zygote obtained from the anterior magnum approximately 1 hour after the expected time of fertilization was cultured as same procedure as that used for ICSI-derived zygotes.

Measurement of intracellular Ca^{2}+ concentration in quail egg

The Ca^{2}+-sensitive indicator dye, Fluo-8H AM (AAT Bioquest, Inc.) was used to measure all changes in [Ca^{2}+]*i*. Dye-loaded unfertilized eggs were injected with 50 fmol IP_3 (Sigma-Aldrich), 2 ng SE, 100 pg porcine AH (Wako Pure Chemical Industries), 100 pg porcine CS (Sigma-Aldrich), 25 fmol cADPR (Sigma-Aldrich), 60 pg quail PLCZ cRNA, 100 pg quail AH cRNA, 100 pg quail CS cRNA, or a defined combination thereof using a micromanipulator connected to the injector as described above. In cases of coinjection, the final concentration of each component of a mixture was equivalent the concentration of that component in the respective single-injection experiments. Rabbit anti-chicken AH polyclonal antibody (20 μg/ml; GeneTex), rabbit anti-chicken CS polyclonal antibody (20 μg/ml; GeneTex), or normal rabbit IgG (20 μg/ml; Sigma-Aldrich) was mixed with 2 mg/ml SE; each mixture was incubated overnight to neutralize the respective antigen in the SE. To examine the effects of heparin on [Ca^{2}+]*i* in the eggs, eggs were pre-injected with 1 ng heparin before the microinjection of each test substance. To evaluate the effects of 2-APB on [Ca^{2}+]*i* in the eggs, the microinjection and subsequent culture was performed in medium supplemented with 100 μM 2-APB. To remove extracellular Ca^{2}+ from the medium, 20 μM BAPTA was included in Ca^{2}+-deficient DMEM (GIBCO) and the microinjection and subsequent culture were performed in this medium. Fluorescent images of each injected egg were taken with a

digital CCD camera (ImagEM, C9100-13; Hamamatsu Photonics) connected to fluorescent stereomicroscope (M165 FC; Leica). The AQUACOSMOS (Hamamatsu Photonics) imaging software was used to measure background fluorescence from outside of the germinal disc and to then calculate the average fluorescence intensity of the germinal disc region (approximately 7 mm^2). The F0 value was set as a fluorescence intensity at the time of injection and time-course measurements in the same area were continued for at least 60 min (F value). F/F0 values were plotted as $[Ca^2+]i$ in the eggs. When the F/F0 value was more than 0.05 after 20 min of the injection, we interpreted this to indicate that spiral Ca^2+ oscillations had been induced. To analyze the spiral-like Ca^2+ waves, the fluorescence intensities at two regions (approximately 150 μm^2) within the germinal disk were quantitated as described above.

Cloning of AH and CS cDNAs

SE was subjected to separation on a Superdex 200 pg column (GE Healthcare); 10-ml fractions were collected; in all, eight fractions were prepared. Fraction 3 was treated with LCA–agarose beads overnight at 4 °C and the supernatant was collected via centrifugation at 20,400 *g* for 10 minutes. Fraction 3 or fraction 3 treated with LCA–agarose beads were resolved by SDS–PAGE (Laemmli, 1970) and subjected to Coomassie Brilliant Blue staining. For *de novo* protein-sequencing analysis, sequencing-grade trypsin was used as suggested by the manufacturer (Promega Corp.) to prepare and digest the proteins within the gel. The peptides recovered from the gel were analyzed by LC-MS/MS (NanoFrontier eLD; Hitachi High-Technologies Corp.) according to the manufacturer's instructions. A *de novo* sequencing software package, PEAKS, was used to identify proteins from the MS/MS data (Ma et al., 2003). We used primers designed from *de novo* sequence analysis and cDNA templates prepared from ejaculated quail spermatozoa and quail liver to amplify AH and CS sequences. The full-length sequences encoding quail AH and CS were obtained from ejaculated spermatozoa and liver using 5′ and 3′ rapid amplification of cDNA ends kits (Invitrogen) according to the manufacturer's instructions. The polymerase chain reaction products of

436 the quail AH or CS were cloned into the pGEM-T Easy vector (Promega Corporation)
437 and digestion with *Spe* I was used to linearize each recombinant plasmid. An mMessage
438 mMachine kit (Ambion) was used according to the manufacturer's instructions to
439 synthesize each plasmid. Microinjection of cRNA was performed as described above.
440 **Immunoblotting**
441 An ejaculated sperm and an unfertilized egg were collected as described above.
442 Ejaculated sperm, germinal disc of unfertilized egg, liver and kidney were homogenized,
443 sonicated and the supernatant was collected via centrifugation at 20,400 x *g* for 10
444 minutes. Protein concentration was measured by BCA protein assay kit (Pierce). Each
445 extract (10 μg protein per lane) was resolved by SDS-PAGE (Laemmli, 1970) on 12%
446 polyacrylamide gel and then transferred onto PVDF membrane (Millipore). Following
447 transfer and blocking for 30 minutes with 5% skimmed milk, the membrane was
448 incubated for 1 hour with rabbit anti-chicken AH polyclonal antibody (GeneTex) or
449 rabbit anti-chicken CS polyclonal antibody (GeneTex) and was subsequently incubated
450 for 30 minutes with goat anti-rabbit secondary antibodies conjugated with horseradish
451 peroxidase (Millipore)
452
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Author Contributions

SM and TS conceived and designed the study and wrote the manuscript. HD performed the LC-MS/MS and analyzed all LC-MS/MS data. KoS and KI assisted with the Ca^{2}+ imaging. SM, GH, and TS performed other experiments and analyzed the data. KiS performed the pilot study on ICSI with a Hoffman modulation contrast microscope. TO performed the pilot study on image enhancing of ova and *ex vivo* embryo culture. All authors approved the final manuscript. The authors declare no conflicts of interest.

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FIGURE LEGENDS

Fig. 1. Changes in $[Ca^{2}+]i$ in quail eggs showing fluorescent images of eggs injected with only 50 fmol IP_3 (A) or 2 ng SE (C). See also Supplementary Material Movies 1 and 2. Numbers at the top left indicate the time (min) after injection. The solid line circle shows the outline of the germinal disc. The small circle with a shaded line labeled 1 indicates the microinjection site. Small circles with shaded lines labeled 2 or 3 in panel C indicate the areas used for the $[Ca^{2}+]i$ time course measurements in panel D. Scale bar = 1 mm. (B and D) Time course measurements of the average $Ca^{2}+$ levels in the germinal disk (solid circles) in panels A and C are shown. The inset in D shows the time course measurements of $Ca^{2}+$ levels in the areas encircled by the shaded line in panel C (areas 2 and 3). A and B are representative results in 8 repeated experiments, and C and D in 6 repeated experiments.

Fig. 2. Identification of avian-specific egg-activating factors responsible for spiral-like $Ca^{2}+$ oscillations. (A) SE was subjected to separation on a Superdex 200 pg column and eight 10-ml fractions were collected. (B, C, and D) Changes in $[Ca^{2}+]i$ in quail eggs injected with fraction 2 (B), fraction 3 (C), or LCA–agarose-treated fraction 3 (D). (E) SDS–PAGE analysis of fraction 3, or fraction 3 treated with LCA–agarose. Proteins in the gel were visualized with Coomassie Brilliant Blue. (F) Changes of $[Ca^{2}+]i$ in quail egg injected with a mixture containing porcine AH (100 pg) and porcine CS (100 pg). A and E are results from a single experiment. B, C and D are typical examples of measurements of $[Ca^{2}+]i$ from 2 repeated experiments. F shows representative results from 8 repeated experiments.

Fig. 3. Changes in $[Ca^{2}+]i$ in quail egg after microinjection of egg-activating factors. Eggs were microinjected with anti-AH antibody-treated SE (A), anti-CS antibody-treated SE (B), normal rabbit IgG-treated SE (C), a mixture of AH and CS cRNAs (D), PLCZ cRNA alone (E), a mixture of PLCZ and AH cRNAs (F), a mixture of PLCZ and CS cRNAs (G) or a mixture of PLCZ, and cRNAs for AH and CS (H). It should be noted that

634 UV irradiation was not performed during the first 5 min after injection of cRNA aliquots
635 to avoid decomposition of the injected cRNA. The results representative of A and B are
636 from 2 repeated experiments, C from 3, D from 4, E from 6 , F from 4, G from 4 and H
637 from 13 repeated experiments, respectively.
638 **Fig. 4.** Development of a quail embryo derived from ICSI. (A) Quail embryo at H&H
639 stage 6 resulting from ICSI and coinjection of 50 fmol IP_3. Scale bar = 1 mm. (B)
640 Hatchling quail (named Megumi) resulting from ICSI and coinjection of 2 ng SE. Note
641 that Megumi hatched from a surrogate chicken eggshell. (C) Megumi and her offspring
642 produced by natural mating. (D) Another female quail was produced by ICSI with
643 coinjection of a mixture containing PLCZ, and cRNAs for CS and AH.

644

Table 1. Blastoderm development produced by ICSI at 24 hours of culture

Injecting sample	No. of eggs		No. of embryos						
			Developed to the stage* of						
	Injected	Developed (%)	IV	V	VI	VII	VIII	IX	X
In vivo fertilized egg	6	6 (100)							6
Sperm alone	26	5 (19)	2		2	1			
Sperm+60 pg PLCZ cRNA	13	6 (46)	2	1	1	2			
Sperm+50 fmol IP_3	29	25 (86)		3	6	9	7		
Sperm+2 ng SE	19	15 (79)	1	2			3	4	5
Sperm+100 pg AH and 100 pg CS cRNAs	3	0 (0)							
Sperm+60 pg PLCZ cRNA and 100 pg AH cRNAs	9	5 (56)		2	1	2			
Sperm+60 pg PLCZ cRNA and 100 pg CS cRNAs	8	5 (63)	1	1		3			
Sperm+60 pg PLCZ, 100 pg AH and 100 pg CS cRNAs	24	17 (71)	3	3	1		2	1	7
Sperm+25 fmol cADP ribose	5	1 (20)	1						
Sperm+60 pg PLCZ cRNA and 25 fmol cADP ribose	11	5 (46)			2	1	1	1	

*Developmental stages were determined according to Eyal-Giladi and Kochav (1976)

Mizushima et al. Table 1.

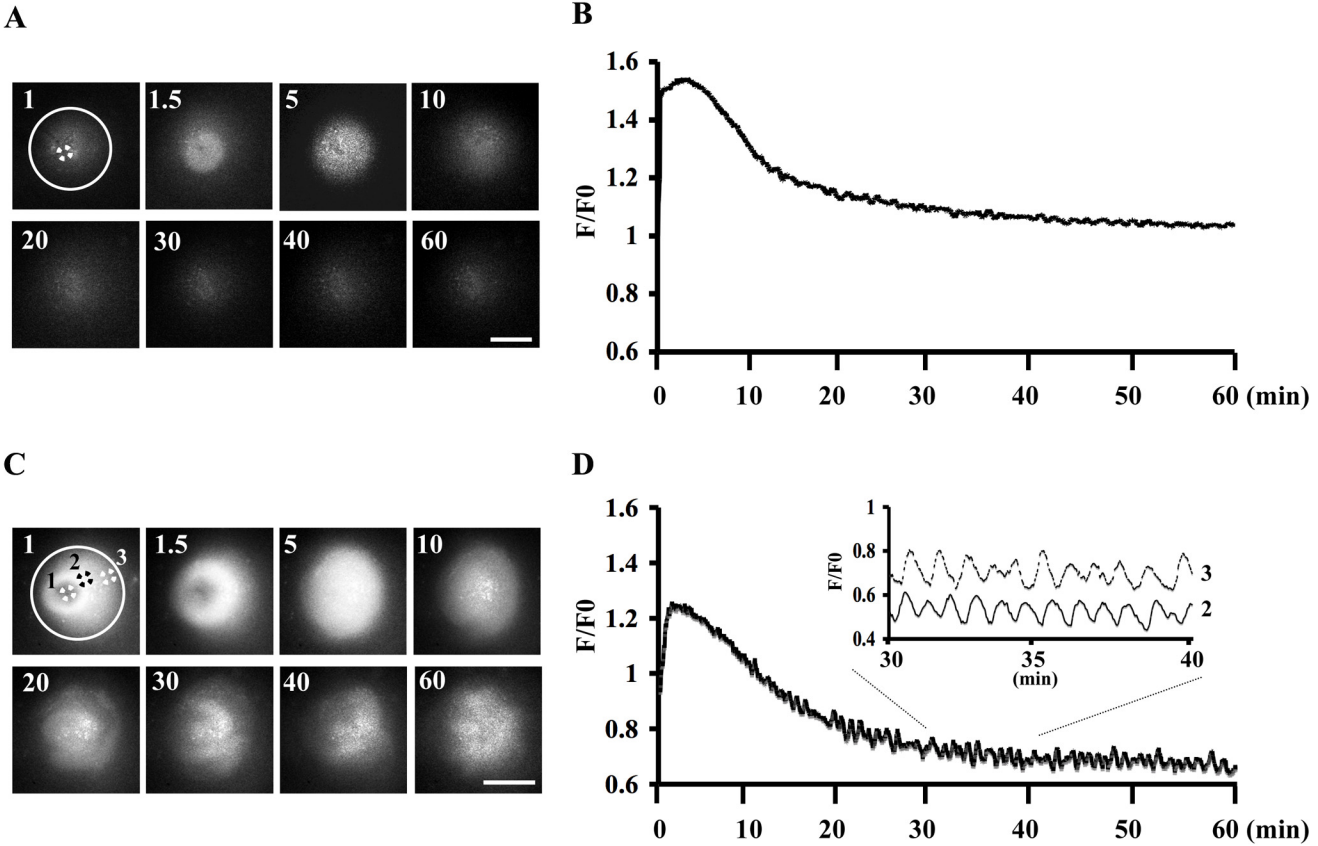
Table 2. Viability and hatchability of quail embryos produced by ICSI

Injecting sample	No. of embryos													
	Transferred to surrogate shell culture (%)	Developed to the stage of												
		VII	X	XIII	3	4	5	6	8	16	25	30	43	Hatch (%)
Sperm alone	1 (20)	1												
Sperm+60 pg PLCZ cRNA	2 (33)			1				1						
Sperm+50 fmol IP_3	16 (64)		2	3	2	1	3	5						
Sperm+2 ng SE	12 (80)		3		2	2				1	1	1	1	1 (8)
Sperm+100 pg AH and 100 pg CS cRNAs	0 (0)													
Sperm+60 pg PLCZ and 100 pg AH cRNAs	2 (40)				1		1							
Sperm+60 pg PLCZ and 100 pg CS cRNAs	3 (60)		1		1			1						
Sperm+60 pg PLCZ, 100 pg AH and 100 pg CS cRNAs	10 (59)		3	1		1		1			2		1	1 (10)
Sperm+25 fmol cADP ribose	0 (0)													
Sperm+60 pg PLCZ cRNA and 25 fmol cADP ribose	3 (60)				1		1		1					

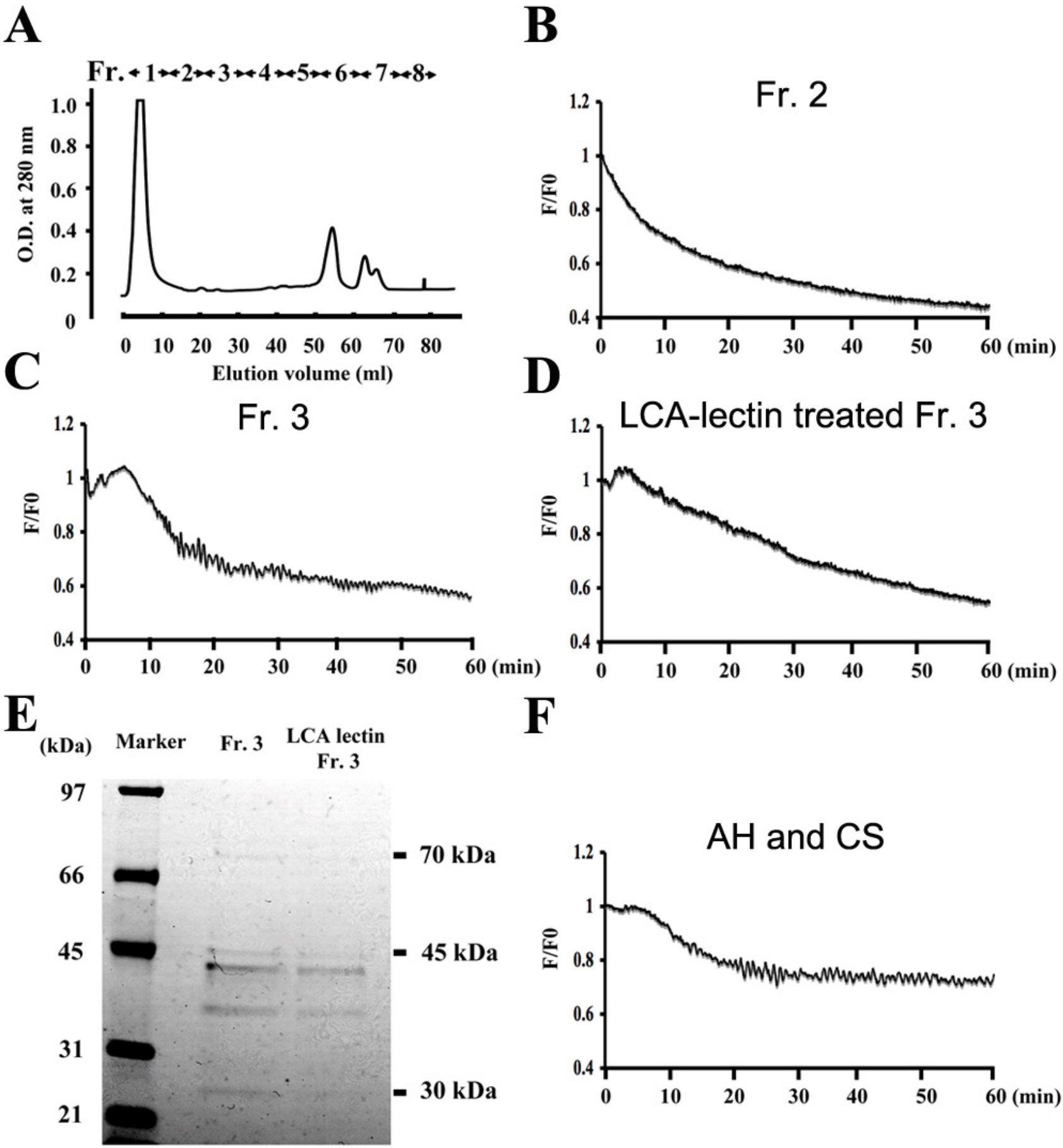
Developmental stages (Roman numerals) were determined according to Eyal-Giladi and Kochav (1976)

Developmental stages (Greek numerals) were determined according to Hamburger and Hamilton (1951)

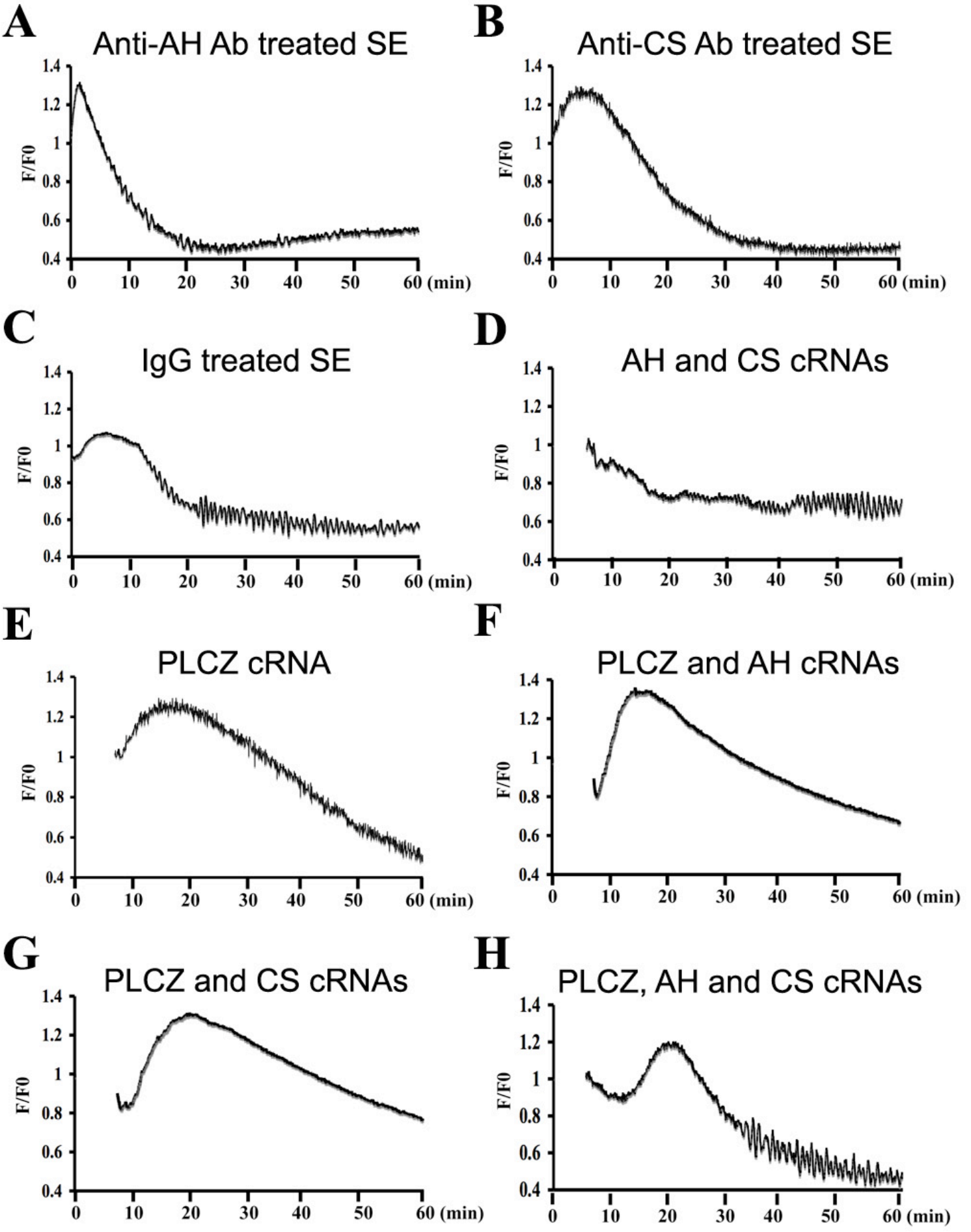
Mizushima et al. Table 2



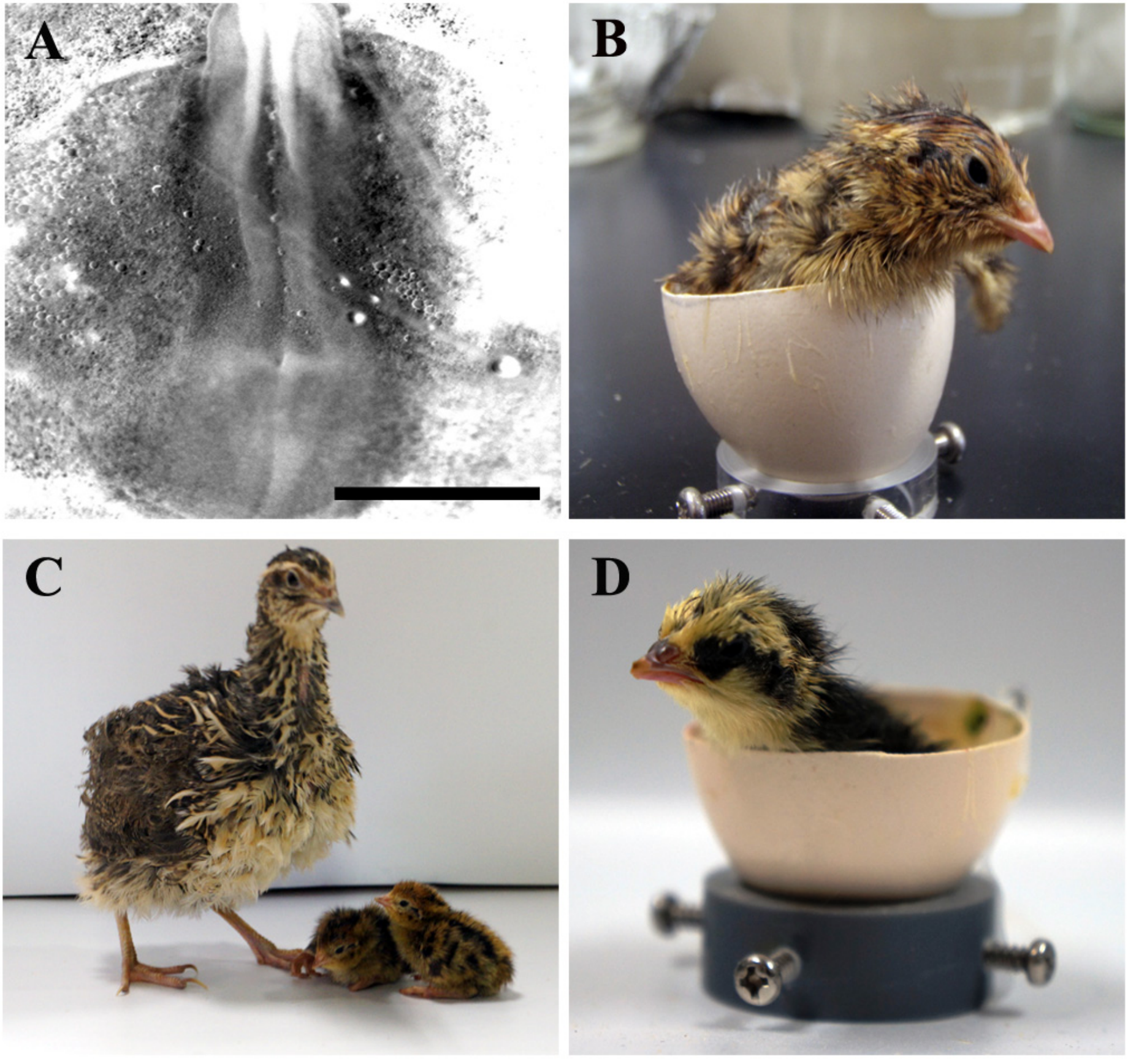
Mizushima et al. Figure 1



Mizushima et al. Figure 2



Mizushima et al. Figure 3



Mizushima et al. Figure 4

Supplementary Material

Table S1. LC-MS/MS identification of proteins in fraction 3.

Fig. S1. Mean amplitude of Ca^2+ oscillations in eggs 20-30 min after the microinjection of 50 fmol IP_3 or 2 ng SE. The mean amplitude of Ca^2+ oscillations differed significantly between IP_3- and SE-treated eggs (Student's unpaired *t* test; $p<0.005$). Values are expressed as the means ± SD of 4 (IP_3) or 6 (SE) repeated experiments.

Fig. S2. Effects of various chemicals on $[Ca^2+]i$ in quail eggs. (A-C) $[Ca^2+]i$ time-course measurements of eggs injected with PLCZ, AH, and CS cRNAs without further treatments (A), eggs injected with 1 ng heparin before a microinjection of the cRNAs (B), or eggs incubated in the presence of 100 μM 2-APB after the injection of the cRNAs (C). (D-G) $[Ca^2+]i$ time course-measurements of eggs microinjected with 25 fmol cADPR alone (D), AH cRNA and 25 fmol cADPR (E), CS cRNA and 25 fmol cADPR (F), or PLCZ cRNA and 25 fmol cADPR (G). (H) $[Ca^2+]i$ time-course measurements of eggs injected with PLCZ, AH, and CS cRNAs incubated in Ca^2+-deficient medium supplemented with 20 μM BAPTA. The results are representative of 4 repeated experiments (A-H).

Fig. S3. Measurements of the mean amplitude or interval of Ca^2+ oscillations in eggs. Eggs were microinjected with PLCZ, AH, and CS cRNAs, 25 fmol cADPR alone or PLCZ cRNA and 25 fmol cADPR. The mean amplitude (A) or interval (B) of Ca^2+ oscillations 30-45 min after the injection was calculated. Values are expressed as the mean ± SD of 3 independent experiments. An asterisk denotes a significant difference, $p<0.05$. Note that the mean interval of Ca^2+ oscillations produced by the cRNAs of 3 factors was significantly shorter than those of 25 fmol cADPR or PLCZ cRNA and 25 fmol cADPR treatment.

Fig. S4. Sperm-specific isoforms of quail AH and CS. Sperm-specific and somatic forms AH (A) and CS (B) were evident on Western blots probe with anti-AH or anti-CS antibodies. Tissues examined were SE, germinal disc of unfertilized egg (egg), liver (liv), and kidney (kid). Ten μg of protein was loaded into each lane.

Fig. S5. Characterization of quail sperm AH and CS. Amino acid sequences of AH (A) or of CS (B) derived from sperm (upper) or liver (lower) samples. Each amino acid difference between sperm-specific and somatic isoforms is indicated by an asterisk. The peptide sequences obtained by *de novo* sequencing analysis are shown in red.

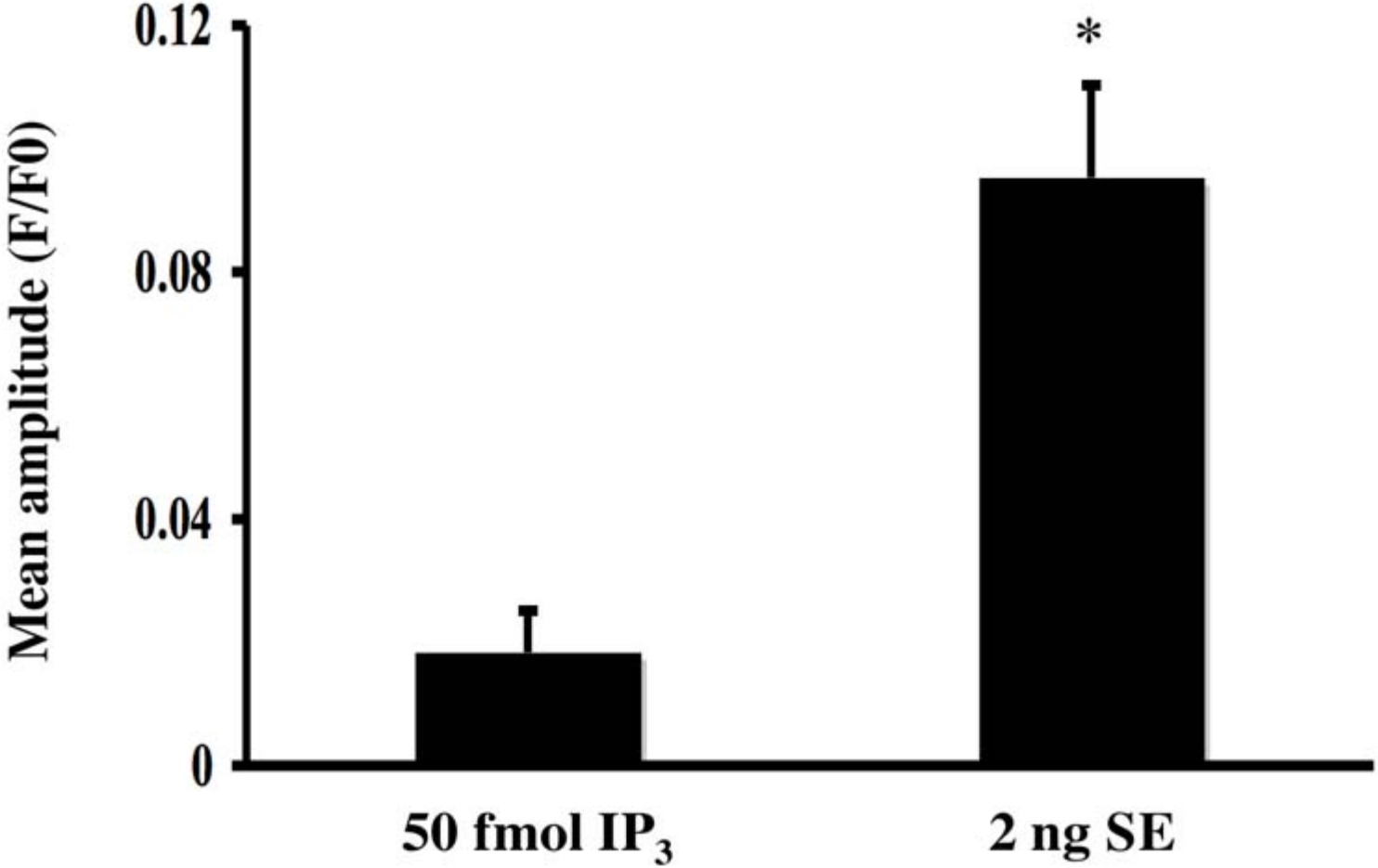
Movie 1. Changes in $[Ca^{2}+]i$ in Fluo-8H AM-loaded quail eggs after microinjection of 50 fmol IP_3. Images were captured and recorded every 0.5 seconds for 15 minutes. The images were converted into a single movie file, which is shown at a speed approximately 100 times faster than real time. Microinjection of IP_3 induced only a transient $Ca^{2}+$ rise.

Movie 2. Changes in $[Ca^{2}+]i$ in Fluo-8H AM-loaded quail eggs after microinjection of 2 ng SE. Images were captured and recorded every 2 seconds for 70 minutes. The images were converted into a single movie file shown at a speed approximately 100 times faster than real time. Note that the microinjection of SE induced a transient $Ca^{2}+$ rise and subsequent spiral-like $Ca^{2}+$ oscillations.

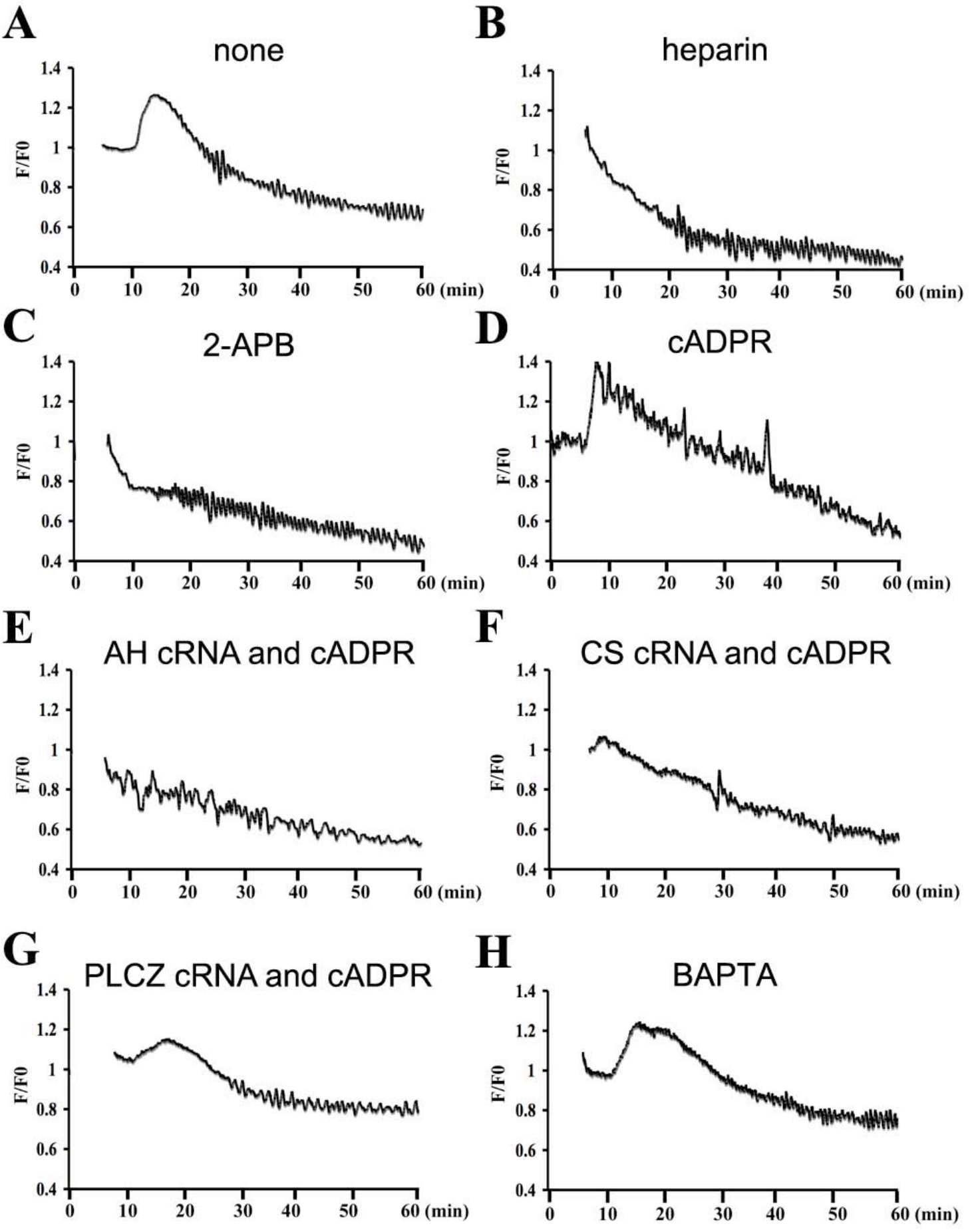
Table S1. LC-MS/MS identification of proteins in fraction 3

Band size (kDa)	Protein identified	Nominal mass (Da)	Accession No.
70	hypothetical protein RCJMB04_1a14 (aconitate hydratase)	85596	gi|53126140
70	aconitate hydratase, mitochondrial	85737	gi|45383738
70	heat shock protein 70kDa	69911	gi|118197127
70	stress-70 protein, mitochondrial precursor	73147	gi|57524986
45	PREDICTED: citrate synthase, mitochondrial-like	38664	gi|326935521
30	PREDICTED: superoxide dismutase[Mn], mitochondrial-like	26108	gi|326915675
30	PREDICTED: malate dehydrogenase, mitochondrial	36944	gi|50758110
30	aconitate hydratase, mitochondrial	85737	gi|45383738

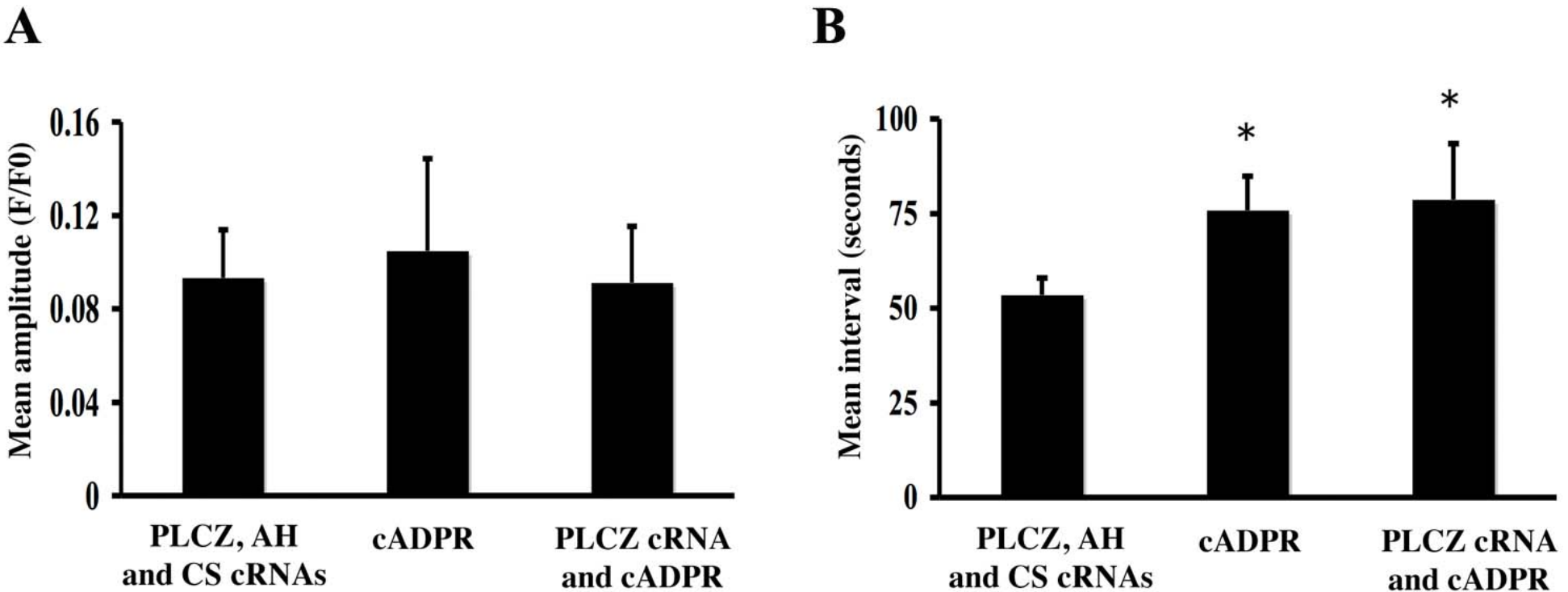
Mizushima et al. Table S1.



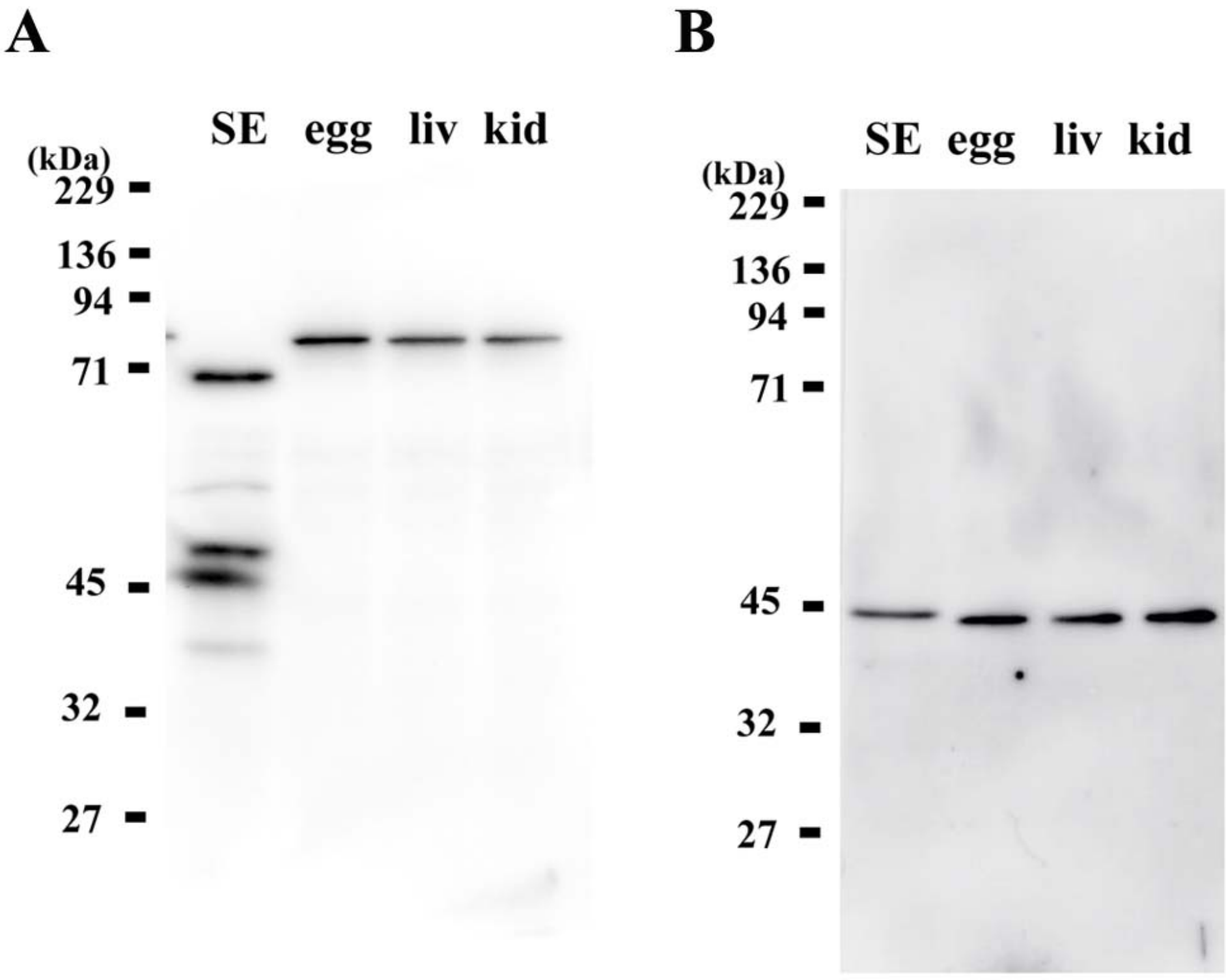
Mizushima et al. Figure S1



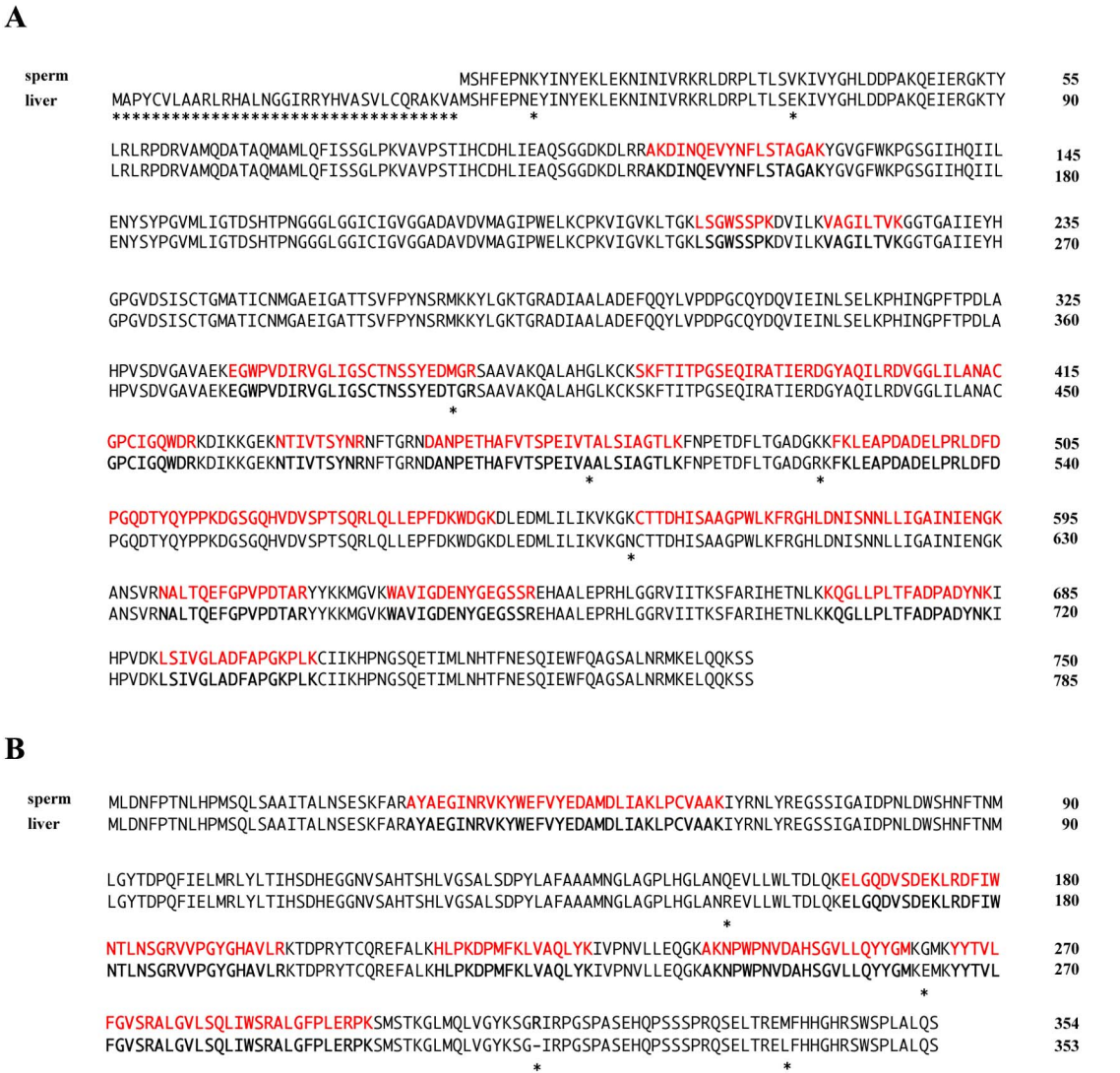
Mizushima et al. Figure S2



Mizushima et al. Figure S3



Mizushima et al. Figure S4



Mizushima et al. Figure S5