Zygote **25** (December), pp. 686–696. © Cambridge University Press 2017
doi:10.1017/S0967199417000533 First Published Online 16 October 2017

The potential roles of c-Jun N-terminal kinase (JNK) during the maturation and aging of oocytes produced by a marine protostome worm

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Date submitted: 14.03.2017. Date revised: 29.07.2017. Date accepted: 02.08.2017

Summary

Previous investigations have indicated that c-Jun N-terminal kinase (JNK) regulates the maturation and aging of oocytes produced by deuterostome animals. In order to assess the roles of this kinase in a protostome, oocytes of the marine nemertean worm *Cerebratulus* were stimulated to mature and subsequently aged before being probed with phospho-specific antibodies against active forms of JNK and maturation-promoting factor (MPF). Based on blots of maturing oocytes, a 40-kD putative JNK is normally activated during germinal vesicle breakdown (GVBD), which begins at 30 min post-stimulation with seawater, whereas treating immature oocytes with JNK inhibitors downregulates both the 40-kD JNK signal and GVBD, collectively suggesting a 40-kD JNK may facilitate oocyte maturation. Along with this JNK activity, mature oocytes also exhibit high levels of MPF at 2 h post-stimulation. However, by ~6–8 h post-GVBD, mature oocytes lose the 40-kD JNK signal, and at ~20–30 h of aging, an ~48-kD phospho-JNK band arises as oocytes deactivate MPF and begin to lyse during a necroptotic-like mode of death. Accordingly, JNK inhibitors reduce the aging-related 48-kD JNK phosphorylation while maintaining MPF activity and retarding oocyte degradation. Such findings suggest that a 48-kD JNK may help deactivate MPF and trigger death. Possible mechanisms by which JNK activation either together with, or independently of, protein neosynthesis might stimulate oocyte degradation are discussed.

Keywords: *Cerebratulus*, GVBD, MPF, nemertean, SP600125

Introduction

During oogenesis in animals, oocytes in the prophase-I stage of meiosis grow larger while also developing a prominent nucleus, called the 'germinal vesicle' (GV). After reaching full size, such GV-containing immature oocytes are usually incapable of being fertilized and instead must first undergo a maturation process that involves nuclear disassembly [= 'germinal vesicle breakdown' (GVBD)] and further meiotic progression before becoming fertilizable oocytes or eggs (Stricker, 1999; Deguchi *et al.*, 2015). In starfish and nemertean worms, immature oocytes are able to remain intact for more than 48 h in the absence of maturation-promoting stimuli, whereas non-inseminated mature oocytes begin an aging process that ultimately results in their death within about a day (Yuce & Sadler, 2001; Stricker *et al.*, 2016). Similarly, by ~6–12 h post-ovulation, an aging-induced loss of viability can prevent mature oocytes of many mammals from being properly fertilized (Fissore *et al.*, 2002; Miao *et al.*, 2009). Collectively, such findings indicate that after being spawned or ovulated, uninseminated mature oocytes undergo an accelerated aging process that restricts their window of opportunity for normal development.

The factors potentially mediating oocyte aging and death have been intensively analyzed, both to understand basic properties of cell cycle progression and to optimize assisted reproductive technologies,

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in which the use of aged oocytes can substantially reduce the likelihood of achieving successful outcomes (Miao *et al.*, 2009; Tiwari *et al.*, 2015). In particular, some research on oocyte aging has focused on the roles played by cell-cycle-related kinases, such as the Cdc2 kinase of maturation-promoting factor (MPF) and several kinds of mitogen-activated protein kinases (MAPKs). These studies have documented that the activity of MPF normally declines during oocyte aging and that treatments that maintain MPF activity help to keep oocytes intact, collectively indicating that elevated MPF activity serves to prevent oocyte degradation during aging (Kikuchi *et al.*, 1995, 2000; Wu *et al.*, 1997; Fissore *et al.*, 2002; Ono *et al.*, 2011). Conversely, the activation of two common types of MAPKs – extracellular signal regulated kinases (ERKs) and p38 MAPKs – can accelerate the onset of apoptotic death in aged oocytes (Sasaki & Chiba, 2004; Sadler *et al.*, 2004; Ebeling *et al.*, 2010).

Along with ERK and p38 MAPKs, somatic cells express three types of c-Jun N-terminal kinase types of MAPKs (JNK1, JNK2, JNK3) that are differentially spliced to yield 46-kD and 54-kD isoforms (Davis, 2000). In *Xenopus* oocytes, a 40-kD version of the somatic cell 46-kD JNK protein and a 49-kD equivalent of 54-kD JNK have been identified (Messaoud *et al.*, 2015) and, based on several lines of evidence, JNK activation is thought to promote GVBD in this species (Bagowski *et al.*, 2001). Similarly, JNK is activated in extensively aged starfish oocytes (Sadler *et al.*, 2004) and treatment with a JNK inhibitor can reduce fragmentation during aging of pig oocytes (Petrova *et al.*, 2009; Sedmikova *et al.*, 2013), collectively indicating that, in addition to perhaps stimulating GVBD, JNK may promote cellular demise during oocyte aging.

However, such studies of JNK MAPKs have focused on oocytes produced by deuterostome lineages of animals (e.g. chordates and echinoderms), despite the fact that the bulk of diversity within the kingdom Animalia occurs across phyla and species of protostome animals (e.g. worms, molluscs, and arthropods). Thus, to expand previous analyses, this investigation examined uninseminated oocytes of the marine protostome *Cerebratulus* sp., a 'ribbon worm' that belongs to the phylum Nemertea (Stricker, 1987; Stricker *et al.*, 2013).

Previously, it has been shown that, as in other animals, maturing oocytes of the nemertean *Cerebratulus* activate MPF during the process of GVBD (Stricker *et al.*, 2013). Accordingly, if MPF levels are kept low, *Cerebratulus* oocytes can be maintained in an immature state, and such GV-containing specimens remain intact for several days without exhibiting marked signs of cellular degradation (Stricker *et al.*, 2016). Conversely, cohort oocytes that had undergone a GVBD-related increase in MPF during maturation subsequently begin to deactivate MPF and lyse after only 1 day of aging, indicating that MPF deactivation in mature oocytes is correlated with an accelerated onset of death (Stricker *et al.*, 2016). Given such findings coupled with previous reports that oocyte degradation is potentially mediated by JNK and MPF in deuterostomes, phospho-specific antibodies are used here in conjunction with pharmacological modulators to analyze these kinase activities in maturing and aged oocytes of the protostome worm *Cerebratulus*.

Materials and Methods

Animals

Adult male and female *Cerebratulus* sp. worms were collected at False Bay on San Juan Island, WA, USA. Instead of attempting to stimulate spawning as typically occurs in the field during summer months (Stricker, 1987), oocytes were obtained by puncturing gravid females with forceps to release prophase-arrested oocytes through the body wall. Such isolated oocytes were initially incubated in calcium-free seawater (CaFSW) to reduce spontaneous maturation before being transferred to filtered seawater (SW) with or without inhibitors and maintained at 11–15°C.

Stock solutions

For stock solutions that were prepared at 1000× the working concentration, JNK inhibitors [AS601245 (Enzo Life Sciences, Farmingdale, NY, USA) and SP600125 (LC Labs, Woburn, MA, USA)] and the JNK agonist anisomycin (Cayman Chemical, Ann Arbor, MI, USA) were mixed in dimethylsulfoxide (DMSO), whereas the protein synthesis inhibitor cycloheximide (AG Scientific, San Diego, CA, USA) was dissolved in distilled water. In various tests, <0.4% DMSO and water aliquots added to SW yielded similar results as SW treatments without a vehicle (Stricker, data not shown). Hence, controls reported here were simply treated with SW alone.

GVBD and degradation percentages

To calculate GVBD and degradation percentages, oocytes from 2–10 females were incubated in monolayers within 1–2 ml of test solutions that had been added to 24-well-plate chambers. At various time points, ~100–200 specimens were examined for either maturation or morphological signs of death using a ×10 magnification objective on an inverted microscope. While assessing degradation totals, any immature specimens within the incubation chamber

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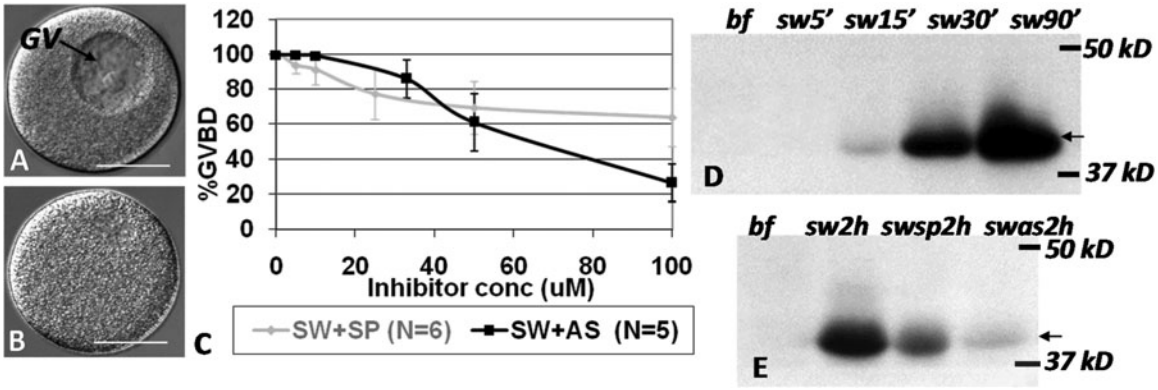

Figure 1 (*A*) Immature prophase-arrested oocyte of *Cerebratulus* exhibiting a conspicuous germinal vesicle (GV) prior to stimulation of maturation. (*B*) A mature oocyte after stimulation by seawater to trigger GVBD. (*C*) Reduction in levels of seawater-induced GVBD caused by two JNK inhibitors SP600125 (SP) and AS601245 (AS). (*D*) Immunoblot probed with a phospho-JNK antibody to detect activated JNK, showing the apparent activation of a 40-kD JNK isoform during GVBD, which begins by 30 min post-stimulation with seawater (sw). (*E*) Phospho-JNK immunoblot, showing a marked reduction in the 40-kD putative JNK signal caused by treatment with JNK inhibitors (sp, as). bf = before GVBD is stimulated by incubating prophase-arrested oocytes in seawater. Scale bars = 50 μm.

were excluded from the tally. Only mature specimens lacking a GV were monitored for cell death, which in turn was judged as having begun when marked flattening and/or lysis was visible.

Immunoblotting

Immunoblots of liquid-nitrogen-frozen oocytes were carried out as described in detail previously (Stricker, 2011; Escalona & Stricker, 2014). For immunoblotting, several hundred oocytes were rapidly removed from the culture dish and frozen over the course of each 20 h to 40 h-long experiment. In so doing, the individual pellets generated at the various time points collectively comprised a gradient ranging from non-aged specimens at the beginning of the time-lapse run to a mixture of aged and dying oocytes at the end. No attempt was made to sort different oocyte types (e.g. GV-containing, mature/intact, mature/degrading, or dead) before each freezing event. From such frozen pellets, 25 μg of total protein was loaded per lane and subsequently incubated with phospho-specific antibodies (#9251 phospho-JNK; #9111 phospho-Cdc2 Y15; #9114 phospho-Cdc2 T161) from Cell Signaling Technology (Danvers, MA, USA). To maximize productivity, blots were cut into horizontal strips of differing MWs (Escalona & Stricker, 2014) before being probed with multiple antibodies without the inclusion of housekeeping proteins for loading controls. Such controls were omitted, because phospho-JNK blots were also treated with a phospho-Cdc2 antibody that generates an oppositely trending signal to the JNK bands, thereby providing a convenient way of detecting loading artefacts, which were then eliminated from further analysis. Moreover, in order to avoid the influence of an occasionally misloaded lane, band intensities were routinely quantified from several independently conducted runs. All immunoblotting and live-cell analyses were conducted using oocytes from at least two females, and statistical significance was assessed via Mann–Whitney *U*-test or Student's *t*-test for $N <$ or > 20, respectively (Smythe & Stricker, 2005).

Results

Maturing nemertean oocytes activated a putative 40-kD JNK before arresting at metaphase I

After removal from the ovary, prophase-arrested oocytes of *Cerebratulus* sp. that had been kept in calcium-free seawater to prevent spontaneous maturation exhibited a prominent GV and lacked surrounding follicle cells (Fig. 1*A*). Following transfer to calcium-containing seawater (SW) to trigger maturation, 90–100% of the tested oocytes began GVBD by ~20–30 min, and such maturing specimens arrested at metaphase I within 1–1.5 h after initiating GVBD (Fig. 1*B*).

To assess the potential roles of JNK activation during maturation, oocytes were treated with SW solutions of two commonly used JNK inhibitors SP600125 (SP) and AS601245 (AS). Based on previous analyses of vertebrate oocytes (Petrova *et al.*, 2009; Du Pasquier *et al.*, 2011), such pharmacological blockers were used at doses ranging from 1 to 100 μM (Fig. 1*C*). However, even with vigorous mixing prior to addition, both inhibitors precipitated to varying degrees in seawater, thereby delivering lower-than-calculated concentrations in solution and perhaps contributing to some of

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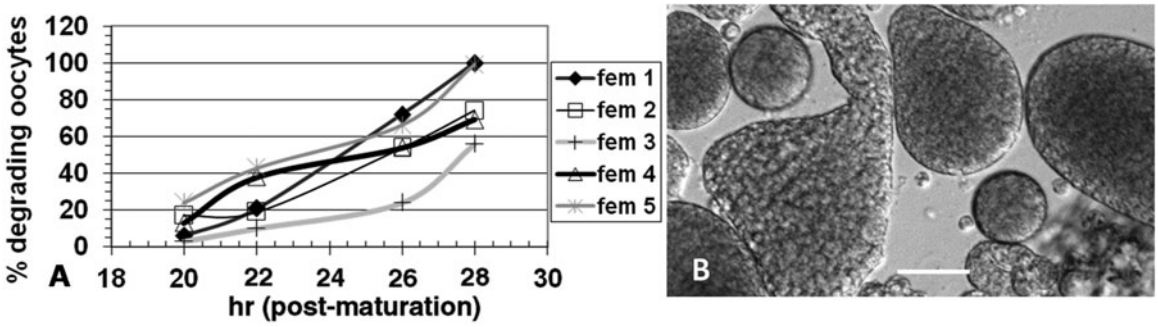

Figure 2 (*A*) Interspecimen variation in the onset of aging-induced degradation for oocytes produced by five female (fem) worms. (*B*) Typical necroptotic-like morphology of degrading oocytes at ~30 h of aging, showing flattening and subsequent lysis without displaying classic apoptotic features of cell death. Scale bar = 50 μm.

the batch-to-batch variation evident in results reported here and elsewhere (Yue & Lopez, 2016). Nevertheless, each JNK inhibitor caused a moderate blockage of GVBD while also reducing MPF activation (Stricker, data not shown). At the highest drug concentrations tested, such effects resulted in a significantly reduced ($P < 0.05$) level of oocyte maturation compared with controls in SW alone (Fig. 1*C*).

Similarly, in maturing oocytes that were frozen at several times following SW stimulation and subsequently immunoblotted with a phospho-JNK antibody against active JNK isoforms, a single putative phospho-JNK band at ~40 kD MW consistently appeared directly before GVBD and continued to be evident in mature, metaphase I-arrested specimens examined at 2 h post-stimulation (Fig. 1*D*). Accordingly, in oocytes treated with SW solutions of a JNK inhibitor concentration that was verified as allowing >80% GVBD (25 μM SP or 30 μM AS), the intensity of the phospho-JNK signal in mature experimentals was diminished compared with that exhibited by SW controls (Fig. 1*E*).

After maturation, aged oocytes degraded via a necroptotic-like death that began about a day post-GVBD

As noted previously (Stricker *et al.*, 2016), uninseminated mature oocytes began to degrade approximately 1 day after undergoing GVBD. At the onset of degradation, oocytes tended to expand, flatten, and gradually become lighter in colour before eventually lysing without forming noticeable cytoplasmic blebs (Fig. 2*A*). Collectively, such morphological characteristics suggested a more necroptotic type of cell death (Stricker *et al.*, 2016), rather than the typical apoptotic demise described for other animal oocytes, in which aging specimens typically shrink, exhibit a denser cytoplasm, and generate marked blebs before dying (Sadler *et al.*, 2004; Tiwari *et al.*, 2015).

The precise kinetics of oocyte death varied among *Cerebratulus* females (Fig. 2*B*). Thus, before quantifying degradation levels, control oocytes were periodically monitored until a particular time point for each female was reached, in which time approximately 70–90% of the oocyte batch had started to degrade. At these time points, which collectively averaged about 30 h of aging across all females tested, pervasive degradation had begun throughout the culture dish, but accurate oocyte counts could still be conducted, because full cellular decay was not yet completed.

During aging, the 40-kD phospho-JNK signal was rapidly lost, and a 48-kD phospho-JNK band appeared just before oocyte degradation

In immunoblotting analyses of uninseminated mature oocytes probed with the phospho-JNK antibody against activated JNK isoforms, the ~40-kD band rapidly diminished during aging. Thus, by 6–8 h, less than half of the peak intensity remained, and by 12–14 h, the band was essentially no longer visible (Fig. 3*A*, *B*). After loss of the 40-kD signal, a new faint band began to appear at ~48 kD from 14 to 24 h of aging (Fig. 3*A*, *B*). Subsequently, in specimens that continued to be aged for another day, a strong signal at ~48 kD consistently arose around the time when about 50% of the oocytes within each batch began to degrade, which in turn ranged from about 20 to 30 h post-GVBD in the various females examined (Fig. 3*A*, *B*). Thus, in all batches, the two putative phospho-JNK signals showed contrasting expression patterns, as the lower-MW band was exhibited by mature specimens that had been frozen at 2 h post-GVBD but not by markedly aged specimens, whereas the higher-MW signal was consistently visible in blots of degrading oocytes but not in blots of freshly matured specimens (Fig. 3*C*).

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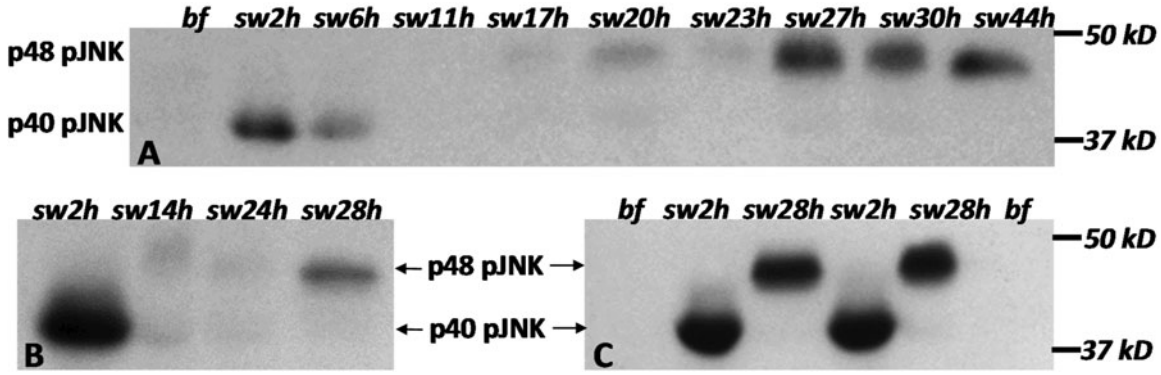

Figure 3 (*A–C*) Phospho-JNK immunoblots of immature, mature, and aging oocytes, showing an increase in the 40-kD phosphorylation during seawater (sw)-induced maturation. In moderately aged oocytes, the 40-kD band is lost prior to the appearance of a 48-kD band around the time that most oocytes begin to degrade (~24–30 h); bf = before GVBD is stimulated by incubating prophase-arrested oocytes in seawater.

JNK inhibitors retarded oocyte degradation while diminishing the 48-kD phospho-JNK signal

To assess the roles of the JNK activity during oocyte aging, prophase-arrested oocytes were incubated in SW solutions of varying concentrations of JNK inhibitors and allowed to age for ~30 h while periodically counting degradation only in post-GVBD specimens within the culture dish. Based on such analyses, both JNK inhibitors reduced the amount of oocyte degradation exhibited by mature oocytes in a dose-dependent fashion (Fig. 4*A*, *B*). However, such reductions were only temporary, as, unlike immature prophase-arrested oocytes that typically continued to remain intact for several days (Stricker *et al.* 2016 and Stricker, data not shown), all mature oocytes that had been aged for ~30 h in either control or experimental dishes eventually degraded when aged further overnight.

For subsequent analyses of JNK inhibitor effects, a 25-μM or 33-μM concentration was adopted for SP or AS, respectively, in order to ensure that the drugs did not markedly confound degradation counts simply by altering maturation levels. In addition, after checking oocytes for the successful completion of GVBD at 2 h post-stimulation, batches with >20% prophase-arrested oocytes were discarded, and in the remaining batches with >80% GVBD, immature specimens were easily recognized and excluded from the counts (cf. Fig. 1*A*, *B*). Based on such drug dosages and counting criteria, both inhibitors significantly ($P < 0.05$) retarded oocyte degradation in mature specimens that were counted at ~30 h of aging (Fig. 4*C–E*). Furthermore, such effects were particularly pronounced, when the inhibitor was included with seawater at the very onset of the experiment ('sw + inhibitor'), rather than being added at 2 h post-seawater-stimulation to oocytes that had already completed maturation ('sw then inhibitor') (Fig. 4*C*), suggesting the possible need to block JNK activation during oocyte maturation in order to obtain optimal degradation reductions. In any case, immunoblots confirmed that each JNK inhibitor significantly reduced ($P < 0.05$) the intensity of the late-arising 48-kD phospho-JNK band exhibited by extensively aged controls (Fig. 4*F*, *G*).

JNK inhibitors helped maintain maturation-promoting factor (MPF) activity in aging oocytes

As an age-associated decline in MPF activity is known to mediate the aging and degradation of oocytes in many species, including *Cerebratulus* (Stricker *et al.*, 2016), active MPF levels were tracked using phospho-specific antibodies to the Cdc2 kinase subunit of MPF during oocyte aging in the presence or absence of a JNK inhibitor. As verified previously via correlative phosphorylation assays of an MPF target (Stricker & Smythe, 2003), increasing levels of the stimulatory phosphorylation at T161 of Cdc2 along with decreased phosphorylation at the inhibitory Y15 site of Cdc2 indicated a rise in MPF activity during maturation. Such MPF activation normally began during GVBD and eventually reached peak levels in freshly matured specimens (Fig. 5*A*, *B*). Subsequently, as reported elsewhere (Stricker *et al.*, 2016), MPF activity continued to remain elevated until just before oocyte degradation, when the phospho-T161 signal decreased and the inhibitory Y15 site on Cdc2 was re-phosphorylated (Fig. 5*A*, *B*). In females whose aging oocytes underwent relatively early degradation, the onset of MPF deactivation also tended to be accelerated, whereas MPF deactivation was generally delayed in batches obtained from females that exhibited slower kinetics of oocyte degradation (Stricker *et al.* 2016 and Stricker, data not shown). Accordingly, both types

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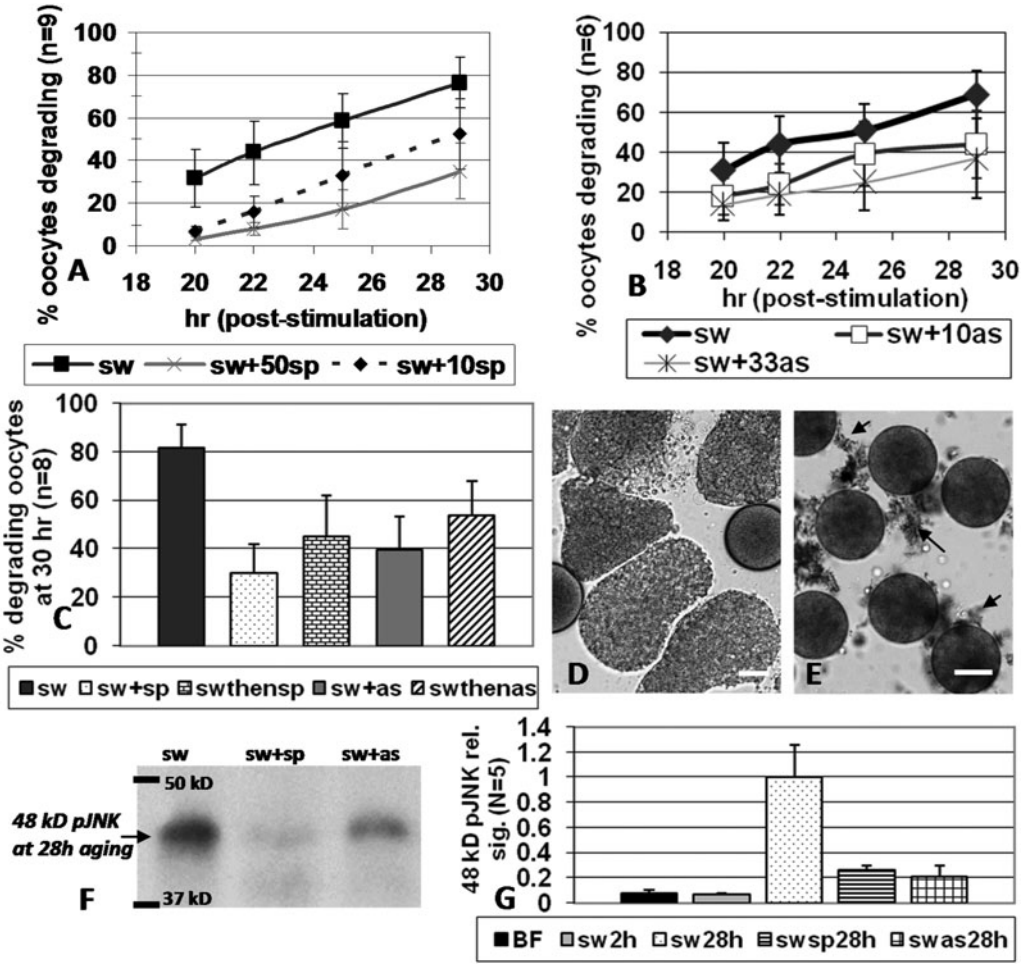

Figure 4 (*A*, *B*) Dose–response curves of varying JNK inhibitor concentrations (0–50 μM), showing the ability of SP600125 (sp) and AS601245 (as) to retard aging-induced oocyte degradation in seawater (sw). (*C*) Reductions in oocyte degradation levels at 30 h of aging are maximized when prophase-arrested oocytes are initially treated with a seawater (sw) solution of a JNK inhibitor (sw + sp, sw + as) [sp = 25 μM; as = 33 μM], rather than allowed to mature before adding the inhibitor (sw then sp, sw then as), suggesting JNK activation during maturation may facilitate subsequent aging-induced degradation. (*D*, *E*) Examples of several flattened, degrading control oocytes in sw alone at 44 h of aging (*D*) vs. cohorts treated with a sw solution of 25 μM SP600125 at 44 h; arrows mark precipitated inhibitor around intact oocytes. (*F*, *G*) A 25-μM or 33-μM seawater (sw) solution of the JNK inhibitor SP600125 (sp) or AS601245 (as), respectively, significantly reduces the intensity of the 48-kD phospho-JNK signal at 28 h of aging, compared with sw controls; bf = before GVBD is stimulated by incubating prophase-arrested oocytes in seawater. Scale bars = 50 μm.

of JNK inhibitors served to keep active MPF levels elevated longer, as evidenced by a more prolonged phosphorylation of the stimulatory T161 site than exhibited by aged SW controls lacking a JNK inhibitor (Fig. 5*C*, *D*).

The JNK agonist anisomycin retarded oocyte degradation, perhaps by blocking protein synthesis

As the bacterial metabolite anisomycin has been used to activate JNKs in various cells (Cano *et al.*, 1994; Fosbrink *et al.*, 2010), immature oocytes that were treated with SW solutions containing 1 to 50 μM anisomycin matured to metaphase I arrest (data not shown) before being further aged for approximately 1 day. Contrary to what might be expected, mature oocytes continually incubated in 25 μM of the putative JNK agonist actually displayed a temporary delay, rather than an acceleration, in degradation onset compared with cohort controls in SW alone (Fig. 6*A*, *B*, *D*). However, given that anisomycin could have also served as a protein synthesis inhibitor at concentrations used in this study, other batches of mature oocytes were treated with 100 μg/ml of cycloheximide, an alternative blocker of protein neosynthesis that in some cells does not activate JNK (Oksvold *et al.*, 2012). As with anisomycin incubations, cycloheximide caused a similar retardation in oocyte

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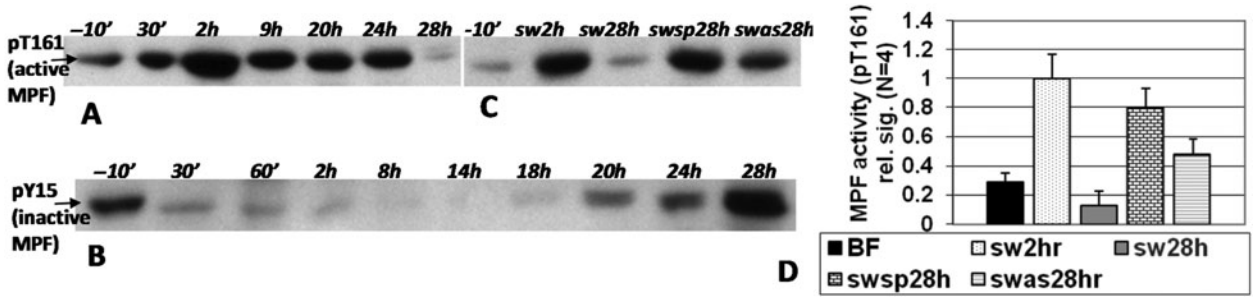

Figure 5 (*A*, *B*) Normal patterns of the stimulatory phospho-T161 Cdc2 signal (*A*) and the inhibitory phospho-Y15 Cdc2. (*B*) signal of maturation-promoting factor (MPF) during oocyte aging; both phosphorylations indicate a marked drop in MPF activity around 20–28 h of aging, the time point when most uninseminated oocytes begin to degrade (cf. Fig. 2*A*). (*C*, *D*) Oocytes treated with a 25-μM or 33-μM solution of the JNK inhibitor SP600125 (sp) or AS601245 (as), respectively, maintain MPF activity longer during aging, compared with controls in seawater (sw) alone. Such results are consistent with the finding that MPF deactivation is temporally linked to oocyte degradation; BF = before GVBD is stimulated by incubating prophase-arrested oocytes in seawater.

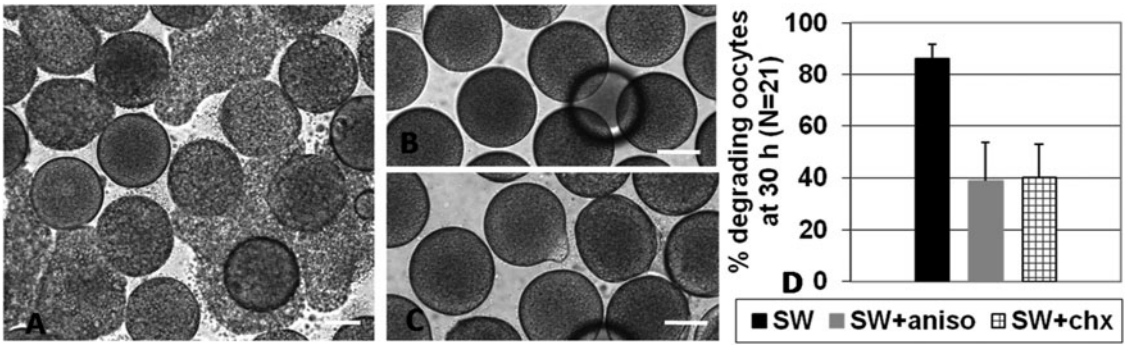

Figure 6 (*A–D*) Protein synthesis inhibitors [25 μM anisomycin (aniso) or 100 μg/ml cycloheximide (chx)] retard oocyte degradation levels at 30 h of aging, compared with seawater (sw) controls lacking an inhibitor. (*A*) SW controls with numerous flattened and lysing oocytes at 36 h; (*B*, *C*) Intact cohort oocytes at 36 h after treatment with anisomycin (*B*) or cycloheximide (*C*). (*D*) Summary graph of data.

degradation (Fig. 6*A*, *C*, *D*). Moreover, incubation in a 500-nM dose of another protein synthesis inhibitor, rapamycin, reduced degradation levels by ~25% compared with seawater controls (Stricker, data not shown), collectively suggesting that at least some of the anisomycin-induced delay in oocyte degradation was due to an inhibition of protein synthesis.

Discussion

The 40-kD putative JNK as a potential regulator of GVBD during *Cerebratulus* oocyte maturation

Control oocytes of *Cerebratulus* that are triggered to mature by seawater activate a putative 40-kD isoform of JNK during GVBD. Moreover, treatment with two kinds of JNK inhibitors reduced not only GVBD levels but also the amount of 40-kD phosphorylation detected by a phospho-JNK antibody against activated JNKs. Such findings coincide with results obtained from several studies of *Xenopus* oocytes that show an apparent increase in JNK activation during GVBD and an associated stimulatory role for active JNK during progesterone-induced meiotic resumption (Amar *et al.*, 1997; Bagowski & Ferrell, 2001; Bagowski *et al.*, 2001; Du Pasquier *et al.*, 2011).

However, a recent investigation of *Xenopus* GVBD has identified a progesterone-induced post-translational modification of a 42-kD ERK MAPK that causes the ERK isotype to cross-react with a phospho-JNK antibody designed to detect JNK activation (Yue & Lopez, 2016). Thus, depending on the size and formulation of the gels used to separate proteins, such a cross-reactive band could overlap with, and be misinterpreted as, a 40-kD JNK signal in blots identifying JNK as a positive regulator of GVBD. Similarly, data suggesting JNK activity is required for GVBD in *Xenopus* could be confounded not only by an off-target effect of the SP600125 JNK inhibitor used to block GVBD but also by a non-JNK-mediated phosphorylation of c-Jun in assays of JNK activity (Yue & Lopez, 2016).

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Accordingly, by employing more specific molecular probes to tease apart the contributions of JNK vs. ERK signaling during *Xenopus* oocyte maturation, it has been shown that even though pharmacological inhibitors of JNK or ERK downregulate GVBD, the use of constitutively active MEKK1 to activate both ERK and JNK causes GVBD, whereas activated MKK7, which stimulates JNK without activating ERK, fails to promote maturation (Yue & Lopez, 2016). Similarly, microinjections of constitutively active JNK do not induce GVBD (Mood *et al.*, 2004). Thus, along with identifying a 42-kD ERK isotype that cross-reacts in phospho-JNK blots, such data call into question previous conclusions that JNK plays a key role in regulating the maturation of *Xenopus* oocytes (Yue & Lopez, 2016).

Given the conflicting findings reported for *Xenopus* oocyte maturation, results presented here suggesting that GVBD in *Cerebratulus* oocytes may depend on the activation of a 40-kD JNK isoform need to be interpreted with caution, especially as the overall patterns of ERK activation during GVBD and oocyte aging in *Cerebratulus* are similar to those reported here for the 40-kD putative JNK band (Stricker & Smythe, 2006; Stricker *et al.*, 2016). However, it should be noted that ERK activation is not required for GVBD in *Cerebratulus* oocytes (Smythe & Stricker, 2005; Stricker *et al.*, 2010). Thus, if the JNK inhibitors used in this study block GVBD by an off-target effect, the non-JNK-mediated inhibition of maturation is not simply be due to ERK inactivation. In any case, non-specific side effects of pharmacological inhibitors in general can certainly influence experimental results. Thus, the potential role of JNK signaling during GVBD in *Cerebratulus* should be further evaluated, once genomic data and more specific molecular probes become readily available for this worm.

However, regardless of whether the ~40-kD phosphorylation associated with oocyte maturation in *Cerebratulus* is mediated by JNK or ERK activation or a combination of both, it is clear that the process of maturation sets a cellular clock ticking toward a more rapid degradation onset, given that immature specimens can remain intact for several days, whereas uninseminated mature oocytes typically flatten and lyse after only about 1 day of aging (Stricker *et al.*, 2016). Similarly, oocytes of the nemertean *Cerebratulus lacteus* can be kept immature in calcium-free seawater for 1 day before being matured in normal seawater and subsequently fertilized to generate batches with >50% normal embryos, whereas 1-day-old mature specimens rarely develop normally after insemination (Stricker & Smythe, 2000), collectively indicating that mature oocytes undergo an accelerated loss of viability. Thus, identifying signaling pathways unique to post-GVBD oocytes that might account for this more rapid decline in developmental capabilities should prove useful in attempts to manipulate the process of oocyte aging.

Activation of a 48-kD JNK during oocyte aging as a trigger of cellular degradation

Compared with the putative 40-kD JNK isoform that is activated during GVBD, the ~48-kD band recognized by the active JNK antibody represents a distinct signal based on several lines of evidence. For example, the putative 40-kD phospho-JNK band remains evident for only a few hours of aging, whereas signs of a weak band at the 48-kD MW begin to be detected after loss of the 40-kD signal. The fact that these weak bands occur at 48 kD rather than at some intermediary MWs argues against the idea that the 48-kD band simply represents a gradual conformational shift in the initial 40-kD protein, as has been noted in previous analyses of AMP-activated kinase signaling in these oocytes, in which a marked gradient of band mobilities is readily evident in time-lapse immunoblots (Stricker *et al.*, 2010). In any case, after ~10–12 h of exhibiting weak signals, a strong phosphorylation arises at ~48 kD in tight temporal correlation with the onset of obvious cellular degradation, and the fact that the 40- and 48-kD bands consistently display non-overlapping and opposing expression trends over time argues against either one of these phosphorylations patterns being solely due to gel loading artefacts.

Accordingly, the fact that the 40-kD signal decreases early in the aging process whereas the 48-kD phosphorylation increases at the end of aging may also explain previous findings related to JNK signaling in aged pig oocytes (Petrova *et al.*, 2009). In that study, treatments with JNK inhibitors reduced the levels of oocyte fragmentation, indicating that an aging-related activation of JNK may promote degradation; yet, the overall amount of phosphorylated JNK detected by a phospho-JNK antibody against activated JNK actually declined, rather than increased, during aging (Petrova *et al.*, 2009; Fig. 2). Given that such analyses reported total phosphorylated JNK levels without discriminating lower- vs. higher-MW isoforms of activated JNK, it is possible that, depending on the relative abundances of activated JNK isoforms, an opposing trend in the two phospho-JNK signals such as reported here could have yielded a drop in total JNK phosphorylation, even though the activation of an individual JNK isoform actually serves to trigger oocyte degradation.

Regardless of whether or not similar trends in JNK isoform phosphorylations occur in other oocytes, several lines of evidence indicate that the 48-kD band detected in phospho-JNK blots of *Cerebratulus* oocytes tracks the activation of a bone fide JNK isoform. First, unlike the 40-kD signal that might be confused

https://doi.org/10.1017/S0967199417000533 Published online by Cambridge University Press

with a cross-reacting ERK signal, the 48-kD band has not been demonstrated to overlap with any non-JNK MAPK family members possessing a similar electrophoretic mobility, and similarly none of the selective manipulations utilizing targeted molecular probes suggests that the 49-kD phospho-JNK band represents anything other than an active JNK signal in *Xenopus* oocytes (Yue & Lopez, 2016). Second, although the SP600125 JNK inhibitor can have off-target effects beyond just inhibiting JNK activity (Bain *et al.*, 2007), oocytes examined in this study were routinely subjected to another JNK inhibitor, AS601245, as an alternative to SP600125 treatment. Accordingly, based on *in vitro* assays of these two JNK inhibitors tested on a panel of over 70 kinases, both inhibitors relatively infrequently display equal off-target effects on the same non-JNK substrate (Bain *et al.*, 2007). Thus, the fact that each drug with its fairly distinct inhibition profile not only reduces the 48-kD phospho-JNK signal but also retards oocyte degradation supports the view that the observed effects of these modulators on aged oocytes are due to JNK inhibition. Finally, JNK activation generally plays a key role triggering the apoptotic demise of various types of differentiated and pluripotent somatic cells (Wada & Penninger, 2004; Almuedo-Castillo *et al.*, 2014). Similarly, in *Xenopus* oocytes, osmostress activates a 49-kD JNK prior to apoptosis (Messaoud *et al.*, 2015), and the phosphorylation-induced activation of JNK exhibiting either a lower, or an undefined, MW also occurs during aging of oocytes produced by *Xenopus* (Du Pasquier *et al.*, 2011) and a starfish (Sadler *et al.*, 2004), respectively, collectively providing a widespread precedent for JNK activation triggering cellular demise.

Possible pathways by which 48-kD JNK signaling could accelerate oocyte degradation

When assessing potential mechanisms by which JNK might regulate cell death, it is important to re-iterate that with respect to MPF activity in *Cerebratulus* oocytes, JNK inhibitors have two opposing effects, depending upon the maturation state and age of the oocytes that are treated. In maturing oocytes that exhibit only the activated 40 kD isoform of JNK, inhibitors of JNK activity serve to block phosphorylation of T161 on Cdc2 and thereby reduce the amount of MPF activation needed to achieve control levels of GVBD. Conversely, in aging post-GVBD oocytes that exhibit both high MPF activity and a switchover to the 48-kD, rather than 40-kD, JNK, such drugs prevent T161 dephosphorylation, thereby maintaining active MPF and causing a concomitant delay in degradation.

Accordingly, based on previous studies of somatic cells (Gutierrez *et al.*, 2010), the activation of a 48-kD JNK in aging *Cerebratulus* oocytes could accelerate the degradation process by downregulating the Cdc25 phosphatase that normally removes the inhibitory Y15 phosphorylation on Cdc2 during MPF activation. Thus, JNK-mediated Cdc25 inhibition could contribute to the aged-related deactivation of MPF that promotes oocyte death (Kikuchi et al, 1995, 2000). Consistent with a model of JNK activation deactivating MPF via Cdc25 inhibition are the coupled findings that MPF activity normally drops just before aging *Cerebratulus* oocytes degrade (Stricker *et al.*, 2016) and that the 48-kD phospho-JNK signal elevates as MPF's inhibitoryY15 site is re-phosphorylated in degrading oocytes (Fig. 5*B*). However, in addition to simply deactivating Cdc25, 48-kD JNK activation in degrading oocytes presumably also downregulates by an unknown mechanism the phospho-T161 site on Cdc2, given that JNK inhibitors promote a more prolonged T161 phosphorylation compared with that exhibited by aged controls (Fig. 5*A*, *B*, *D*).

As an alternative, or a supplement, to such a post-translational mode of triggering death, JNK-mediated alterations in protein synthesis could also modulate oocyte aging, given that protein profiles are known to change in aged vs. fresh oocytes (Jiang *et al.*, 2011; Zhang *et al.*, 2013; McGinnis *et al.*, 2014; Trapphoff *et al.*, 2015). Moreover, as noted here, as well as in other oocytes (Yuce & Sadler, 2001; Tang *et al.* 2013), protein synthesis inhibitors retard the onset of degradation during aging. Currently, there is no direct evidence linking JNK signaling to the apparent need for protein neosynthesis during oocyte degradation. However, in somatic cells, activated JNK is known to affect gene expression by phosphorylating c-Jun and various other transcription factors (Bogoyevitch & Kobe, 2006). Thus, the 48-kD phospho-JNK signal demonstrated here, along with the inhibitory effects of protein synthesis blockers on oocyte degradation, could indicate that a modulation of transcription factor activity by JNK contributes to the synthesis of new protein(s) that promote oocyte death during aging.

Further analyses, such as utilizing RNAi methods to alter JNK activation and its potential downstream targets, are needed to define more fully how JNK might trigger oocyte degradation. In addition, the fact that JNK inhibitors can substantially reduce the 48-kD phospho-JNK signal but still not completely maintain MPF activity or retard degradation onset (cf. Figs 4*A–C*, *G*, and 5*C*) may indicate that other non-JNK molecules, such as ERK (Stricker *et al.*, 2016) or p38 (unpubl. obs.) MAPK isoforms, could also play key roles either independently or in conjunction with JNK to regulate oocyte aging and death. Nevertheless, findings presented here support the view that, as is the

https://doi.org/10.1017/S0967199417000533 Published online by Cambridge University Press

case with deuterostome oocytes undergoing stress- or aging-induced apoptosis, JNK activation contributes to the necroptotic-like demise of aged oocytes produced by this marine protostome worm.

Acknowledgements

The use of facilities at the Friday Harbor Laboratories of the University of Washington is gratefully acknowledged.

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