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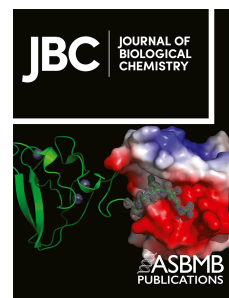
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p38 α and p38 β regulate osmostress-induced apoptosis

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ABSTRACT

Hyperosmotic shock induces cytochrome c release and caspase-3 activation in *Xenopus* oocytes. Different signaling pathways engaged by osmostress converge on the mitochondria to trigger cell death. The mitogen activated protein kinases (MAPKs) JNK1-1 and JNK1-2 are early activated by hyperosmotic shock and sustained activation of both isoforms accelerates the apoptotic program. Indeed, sustained activation of p38 accelerates osmostress-induced cell death, but the p38 isoforms involved are not well characterized. Here we study the expression and activation of *Xenopus* p38 isoforms in response to hyperosmotic stress. We find that p38 α , p38 β , and p38 γ are early activated by hyperosmotic shock and sustained activation of p38 α and p38 β accelerates osmostress-induced apoptosis. Moreover, microinjection of cytochrome c in the oocytes induces caspase-3 activation and p38 α and p38 β phosphorylation suggesting that caspases and kinases are interlinked in a positive feedback loop to promote cell death. In summary, we present a more complete view of the mechanisms involved in osmostress-induced apoptosis.

INTRODUCTION

Hyperosmotic shock has many damaging effects on cells by promoting water flux out of the cell, causing cell shrinkage and intracellular dehydration [1]. Cells respond to osmostress with compensatory molecular adaptations that allow them to restore homeostasis and cellular function [2]. However, when cells are no longer able to compensate for hyperosmotic stress and the amount of damage is too great, they trigger apoptosis [3-7].

We have reported that hyperosmotic shock induces cytochrome c release and caspase-3 activation in *Xenopus* oocytes [8]. Several mechanisms regulate osmostress-induced apoptosis in this cellular system. Hyperosmotic shock induces rapid calpain activation and high levels of Smac/DIABLO release from the mitochondria (early events) before significant amounts of cytochrome c are released to promote caspase-3 activation (late events) [9]. We also studied the role of Bcl-2 family members on osmostress-induced apoptosis. Bid is early proteolyzed in small amounts, by a yet not identified protease/caspase, contributing to cytochrome c release, and later on high amounts are proteolyzed by caspase-3 creating a positive feedback loop [10]. Hyperosmotic shock also activates very quickly the MAPKs (mitogen-activated protein kinases) p38 and JNK [8-11]. Simultaneous inhibition of both pathways, adding SB203580 plus SP600125, reduces osmostress-induced apoptosis [9]. Accordingly, sustained activation of p38 [9] or JNK [10] accelerates the release of cytochrome c and caspase-3 activation. We concluded from these studies that different pathways, early induced by osmostress, converge on the mitochondria to trigger apoptosis [9, 10, 12].

We also characterized the JNK isoforms activated by hyperosmotic shock and their role in apoptosis. JNK1-1 and JNK1-2 are early activated by osmostress and sustained activation of both isoforms accelerates the apoptotic program. Moreover, when caspase-3 is activated, JNK1-2 is proteolyzed at Asp385, increasing the release of cytochrome c and caspase-3 activity, thus creating another positive feedback loop [10]. Although p38 is involved in the apoptotic process, it is not clear which specific p38 isoforms are early activated by osmostress and how they regulate cell death. Interestingly, we reported that cytochrome c injection in *Xenopus* oocytes induces phosphorylation of p38 through caspase-3 activation, suggesting an additional positive feedback loop [11].

The p38 MAPK family is composed of 4 isoforms that have 60-70% homology between them. p38 α (MAPK14), p38 β (MAPK11), p38 γ (MAPK12) and p38 δ (MAPK13) are

encoded by different genes and have different expression patterns in tissues, with p38 α being the major component, expressed ubiquitously in most cell types, while the other isoforms are expressed specifically in different tissues. p38 β is preferentially expressed in the brain, p38 γ in skeletal muscle, and p38 δ in endocrine glands [13]. p38 MAPKs are activated by dual phosphorylation of tyrosine and threonine residues in a conserved Thr-Gly-Tyr motif, in the activation loop, by MKK3 and MKK6 [14-16]. In some circumstances, such as ultraviolet radiation, MKK4, an activator of JNK, may contribute to p38 activation [17]. Hyperosmotic shock has been described to activate MKK6 and MKK3 in mouse embryonic fibroblasts [18]. MKK6 activates p38 α , p38 β , and p38 γ in response to hyperosmotic shock, but does not activate so well p38 δ , while MKK3 activates the four isoforms, being the main activator of p38 δ [18]. Autophosphorylation may also contribute to the activation of p38 MAPKs [19, 20].

p38 activation can have a pro- or an anti-apoptotic function depending upon the stimuli and the cellular context [12, 21]. It seems clear that early transient activation of p38 promotes cell survival, whereas prolonged activation mediates cell growth arrest or apoptosis [12, 22–25]. In human erythrocytes, p38 activation favors hyperosmotic shock-induced apoptosis [26], although it is not clear which isoforms are relevant.

Little is known about the function of p38 in *Xenopus* oocytes. p38 α and p38 γ have been cloned and expressed in the oocytes and both isoforms are activated by a constitutively active MKK6 mutant (MKK6-DD) [27-29]. It has been described that progesterone activates p38 γ and regulates G2/M transition during meiotic progression [29]. However, nothing is known about the role of the different p38 isoforms in osmostress-induced apoptosis.

Here we study the expression and activation of p38 isoforms in *Xenopus* oocytes exposed to hyperosmotic stress. We find that p38 α , p38 β , and p38 γ are early activated by hyperosmotic shock and sustained activation of p38 α and p38 β accelerates osmostress-induced apoptosis. Moreover, cytochrome c injection in *Xenopus* oocytes induces caspase-3 activation and p38 α and p38 β phosphorylation indicating that caspases and kinases are linked in a positive feedback loop to promote cell death.

RESULTS

***Xenopus* p38 α and p38 γ are phosphorylated in response to hyperosmotic shock**

The expression of p38 isoforms was analyzed in *Xenopus laevis* oocytes by RT-PCR. PCR products were obtained for p38 α , p38 β , and p38 γ , but not for p38 δ (Figure S1), suggesting that p38 δ is not expressed in the oocytes.

Xenopus oocytes were microinjected with cRNAs corresponding to the wild type p38 α (Xp38 α), p38 γ (Xp38 γ), or their catalytically inactive mutants (-KR, -AF, -DA, see Experimental Procedures section). All Xp38 isoforms have a Myc epitope at the amino-terminal end allowing their detection by Western-blot. Oocytes injected with the different cRNAs expressed similar levels of the corresponding proteins (Figures 1A, S2A, S2D) and did not show any significant change in caspase-3 activity (Figure 1B) or cytochrome c release (Figures 1A, S3D) compared to water-injected oocytes. Oocytes expressing a constitutively active MKK6 (MKK6-DD), the kinase upstream of p38, showed a marked phosphorylation of p38 (Figures 1A, S3A) but not an increase in caspase-3 activity (Figure 1B). Treatment of oocytes with 300 mM sorbitol for 1 h and 3 h induced the phosphorylation of endogenous p38 at Thr181 and Tyr183, a large increase of phosphorylation in oocytes expressing Xp38 α or Xp38 α -KR, and a significant increase in those expressing Xp38 γ , Xp38 γ -KR or Xp38 γ -DA (Figures 1C, 1E, S2, S3). It is well known that the overall activity of p38 is correlated with the dual phosphorylation of Thr181 and Tyr183 [30]. As expected, the mutants Xp38 α -AF and Xp38 γ -AF, with residues Thr and Tyr mutated to Ala and Phe, were not phosphorylated in the oocytes treated with sorbitol (Figures 1C, 1E, S2, S3). There were no significant changes in caspase-3 activity and cytochrome c release between water-injected oocytes and those expressing Xp38 α , Xp38 γ isoforms, or their mutants (Figures 1D, 1F, S3E, S3F). As reported previously, expression of MKK6-DD accelerated caspase-3 activation and cytochrome c release in oocytes exposed to hyperosmotic shock (Figure 1D) [9].

***Xenopus* p38 β is phosphorylated in response to hyperosmotic shock**

Xenopus laevis p38 β (Xp38 β), *Xenopus tropicalis* p38 δ (XTP38 δ), or their respective mutants -AF and -KR, were expressed in *Xenopus* oocytes (Figures 2A, S4A, S4D). Treatment with 300 mM sorbitol induced phosphorylation of Xp38 β and Xp38 β -KR, but not Xp38 β -AF (Figures 2C, 2E, S4, S5). XTP38 δ was not phosphorylated in response to osmostress in some experiments (Figures 2C, 2D), but a slight phosphorylation of

XTp38 δ and XTp38 δ -KR was detected in others (Figures S4B, S4C, S4E, S4F). Expression of Xp38 β , XTp38 δ , or their mutants, did not alter the levels of caspase-3 activity induced by osmostress compared to water-injected oocytes (Figures 2B, 2D, 2F). As described before, oocytes expressing MKK6-DD presented increased caspase-3 activity compared to water-injected oocytes (Figure 2D).

Since XTp38 δ was not well phosphorylated in response to osmostress, we also studied the response of human p38 δ (Hp38 δ). As shown in Figure S6A, hyperosmotic shock induced phosphorylation of Hp38 δ , but to a lesser extent than Xp38 β . Hp38 δ -AF and Hp38 δ -KR mutants were not significantly phosphorylated by sorbitol treatment (Figure S6B). Expression of wild-type Hp38 δ or their mutants did not modify caspase-3 activity induced by hyperosmotic shock (Figure S6C-F).

In summary, the above results indicate that hyperosmotic shock induces phosphorylation of Xp38 α , Xp38 β , and Xp38 γ in *Xenopus* oocytes, and that expression of these isoforms, or their mutants, does not modify osmostress-induced apoptosis.

A constitutively active Xp38 α or Xp38 β accelerates osmostress-induced apoptosis

It is clear from the previous results that a constitutively active MKK6 accelerates hyperosmotic shock-induced apoptosis (Figures 1 and 2). To determine which p38 isoform/s is/are involved in this process, we expressed a constitutively active p38 α (Xp38 α -CA), a constitutively active p38 β (Xp38 β -DA/YL), or both (see Experimental Procedures section for details). These mutants contain a Myc sequence at the N-terminal end that allows us to verify their expression levels by Western blot.

Xp38-CA or Xp38-DA/YL were efficiently expressed in the oocytes, inducing a marked phosphorylation of p38, but not cytochrome c release or caspase-3 activation, like MKK6-DD (Figures 3A, 3B, S7, S8). These results indicate that sustained activation of Xp38 α , Xp38 β , or both, is not sufficient to induce apoptosis in untreated oocytes. However, treatment with 300 mM sorbitol for 1 h significantly increased caspase-3 activity in the oocytes expressing Xp38-CA, Xp38-DA/YL, or both, compared to water-injected oocytes (Figure 3D), and was correlated with massive release of cytochrome c (Figures 3C, S7B, S7E, S8E). As expected, MKK6-DD also increased caspase-3 activity and cytochrome release relative to water-injected oocytes (Figures 3C, 3D, S8E).

Very high levels of caspase-3 activity were reached 2 h after sorbitol treatment, with no significant differences observed between the conditions analyzed (Figure 3F).

These results suggest that sustained activation of Xp38 α and Xp38 β isoforms has a pro-apoptotic role in hyperosmotic shock-induced apoptosis.

Kinases and caspases are linked in a positive feedback loop: cytochrome c induces caspase-3 activation and phosphorylation of Xp38 α and Xp38 β

As we reported previously, injection of cytochrome c in *Xenopus* oocytes induces rapid caspase-3 activation (30 min) and p38 phosphorylation (1 h) [11]. This effect was blocked by a caspase-3 inhibitor, demonstrating that p38 phosphorylation was caspase-3 dependent [11]. These results suggested that p38 was engaged in a positive feedback loop. To clarify the p38 isoforms involved in this loop, each isoform was expressed in *Xenopus* oocytes, and subsequently injected with cytochrome c. Phospho-p38 levels were analyzed by Western blot one hour after cytochrome c injection. As shown in Figures 4A, 4B and S9, all the p38 isoforms were expressed at similar levels in the oocytes, but only Xp38 α and Xp38 β were phosphorylated 1 h after cytochrome c injection. As expected, caspase-3 activity was increased in the oocytes injected with cytochrome c (Figure 4C). We can rule out a nonspecific stress response due to the exogenous protein injection since we have previously shown that cytochrome c from yeast (*Saccharomyces cerevisiae*), which cannot trigger caspase-3 activation in *Xenopus* oocytes [31], does not induce p38 phosphorylation [11]. Importantly, injection of a specific caspase-3 inhibitor (Ac-DEVD-CHO) in the oocytes at 0.1 μ M (final concentration) decreased caspase-3 activity and phosphorylation of Xp38 α and Xp38 β induced by cytochrome c (Figures 5A, 5B). A higher concentration of the inhibitor (1 μ M) markedly decreased caspase-3 activity as well Xp38 α and Xp38 β phosphorylation (Figures 5C, 5D). In summary, our results suggest that sustained activation of Xp38 α and Xp38 β accelerates cytochrome c release (Figure 3), which in turn induces caspase-3 activation and phosphorylation of Xp38 α and Xp38 β (Figure 4), thus creating a positive feedback loop.

DISCUSSION

Xenopus oocytes have several advantages to study cell death mechanisms. Is a very good system to express wild type and mutant proteins. Since *Xenopus* oocytes at stage VI are transcriptionally inactive, all the effects observed are non-genomic. Cytochrome c release and protein kinase activation can be measured in single cells by Western blot [8, 11]. One of the most important advantages of *Xenopus* oocytes is that cytochrome c can be injected in the cells to investigate the signaling pathways activated downstream, independently of

the initial stimuli. This facilitates the study of positive feedback loops engaged during cell death. Hyperosmotic shock induces apoptosis in *Xenopus* oocytes through activation of different signaling pathways [9]. In previous work we described the basic properties of p38 in response to hyperosmotic shock [11]. Xp38 is quickly activated (5 min) with an ultrasensitive and sustained response [11]. When maximum p38 activation is attained, cytochrome c is released from the mitochondria to the cytosol, promoting caspase-3 activation. We also demonstrated that sustained activation of p38 accelerates cytochrome-c release and caspase-3 activation [9] and, interestingly, that cytochrome c release induces p38 phosphorylation in a caspase-3 dependent manner, suggesting a positive feedback loop [11]. However, it was not clear in the previous studies which p38 isoforms are involved in this process.

Here, we show that Xp38 α , Xp38 β , and Xp38 γ are detected by RT-PCR in *Xenopus laevis* oocytes (Figure S1). Ectopic expression of different isoforms in the oocytes allow to study their activation in response to osmostress. The three isoforms are activated by hyperosmotic shock (Figure 1 and 2). Xp38 δ was not detected by RT-PCR, and *Xenopus tropicalis* p38 δ (Xp38 δ) ectopically expressed in the oocytes was weakly phosphorylated in response to hyperosmotic shock. Likewise, human p38 δ (Hp38 δ) expressed in oocytes was phosphorylated in response to osmostress but to a lesser extent than Xp38 β . Based on these results, we consider that endogenous Xp38 δ is not expressed in oocytes. Even if expressed at low levels, it would hardly be activated by osmostress. MAPKs have a dual role in apoptosis, since they act as activators or inhibitors, depending on the cell type and the stimulus [12]. However, it seems clear that the kinetics and the intensity of the signaling pathway is going to be important for cell fate. Sustained activation of MAPKs usually favors caspase-3 activation, which in turn engages several positive feedback-loops promoting cell death [12, 32].

In this study we show that expression of a constitutively active Xp38 α or Xp38 β accelerates osmostress-induced apoptosis in *Xenopus* oocytes (Figure 3). Therefore, activation of both isoforms by hyperosmotic shock regulates the apoptotic program.

Xp38 γ is also activated by osmostress (Figure 1) but its role in apoptosis is uncertain. We could not obtain a constitutively active Xp38 γ , but it has been reported that sustained activation of this isoform induces meiotic progression in *Xenopus* oocytes [29]. Indeed, we observed that co-expression of MKK6-DD and Xp38 γ induced germinal vesicle break down (GVBD) in the oocytes, detected as the appearance of a white spot in the animal pole (data not shown). It has been reported that Xp38 γ induces meiotic progression

through activation of the phosphatase Cdc25C [29]. More studies are necessary to address the role of p38 γ in osmostress-induced apoptosis.

Importantly, we show that cytochrome c injection in the oocytes induces phosphorylation of Xp38 α and Xp38 β , but not Xp38 γ or XTp38 δ (Figure 4). Therefore, our results imply that p38 is activated by two different inputs at different times: First, hyperosmotic shock induces sustained activation of Xp38 α , Xp38 β (probably mediated by activation of MKK6) that accelerates the apoptotic program. Second, the confluence of different signaling pathways in the mitochondria promote cytochrome c release and caspase-3 activation, which in turn activates Xp38 α and Xp38 β creating a positive feedback loop. This loop, in combination with others engaged by caspase-3 [12], would make irreversible the apoptotic program.

How caspase-3 activation induces phosphorylation of p38 α and p38 β ? It has been reported that caspase-3 induces proteolysis and constitutive activation of MEKK1 [33, 34], which in turn activates JNK and p38 [35]. However, cytochrome c injection did not induce a rapid phosphorylation of JNK (data not shown), thus discarding proteolysis and constitutive activation of MEKK1. Another possibility is that caspase-3 activation would increase the levels of reactive oxygen species (ROS) through disruption of the functions of complex I and II of the electron transport chain [36], which in turn would activate p38 through activation of MINK and/or ASK1 [37, 38]. Sustained activation of p38 also induces important metabolic changes and enhances the respiration rate, increasing the production of mitochondrial ROS, which contributes to p38-induced apoptosis [39]. More studies are necessary to characterize the signaling pathway that induces Xp38 α and Xp38 β phosphorylation mediated by caspase-3 activation.

Sensing environmental changes in salinity and activation of p38 is an ancient function conserved in both unicellular organisms and animals [40, 41]. Although cells have developed mechanisms to adapt to osmotic changes and survive, when the stress is intense or persistent the cellular machinery initiates a death program.

In Figure 6, we present a model for osmostress-induced apoptosis, which summarizes our knowledge about this process. The apoptotic program is characterized by two different phases: In an **early phase**, hyperosmotic shock induces synchronic activation of different pathways: calpains, Bid cleavage (by an unknown protease), MAPKs phosphorylation (JNK1-1, JNK1-2, p38 α , p38 β , p38 γ) and release of Smac/DIABLO. We propose that this early phase is reversible, with crosstalk of anti-apoptotic and pro-apoptotic signals. In this phase, the oocytes could recover if the stress is removed or weakened. MAPKs

(JNK and p38 isoforms) are sensors of stress that could activate early anti-apoptotic substrates, but strong and/or sustained activation of MAPKs would activate late pro-apoptotic substrates. These substrates, not yet characterized, could include different Bcl-2 family members. We know that Bcl-X overexpression blocks osmostress-induced apoptosis [10]. In a **late phase**, the different signaling pathways converging on the mitochondria trigger cytochrome c release and caspase-3 activation. Caspase-3 induces JNK1-2 proteolysis at Asp385 and massive proteolysis of Bid at Asp52, which in turn induce more cytochrome c release and caspase-3 activation engaging two positive feedback loops [10]. Our previous results also indicate that caspase-3 increases Smac/DIABLO release [9]. Here we show that caspase-3 induces phosphorylation and activation of p38 α and p38 β . In addition, it has been reported that caspases can induce proteolysis of the calpain inhibitor calpastatin [42, 43], thus increasing calpain activation in a positive loop. Therefore, hyperosmotic shock induces different signaling pathways that converge on the mitochondria to engage an irreversible apoptotic program through caspase-3 dependent activation of multiple positive feedback loops.

In summary, we have now a panoramic view of the signaling pathways and feedback loops activated by osmostress that regulate apoptosis. However, much work remains to be done to understand this complex biological process. We know that sustained activation of several protein kinases (p38 α , p38 β , JNK1-1, JNK1-2) are pro-apoptotic, opening new avenues of research to identify their targets. A complete understanding of osmostress should also consider transient anti-apoptotic mechanisms engaged before cell death. Deciphering the mechanisms of osmostress-induced apoptosis will give insights in how apoptotic programs are regulated, and could be useful to understand some human illness caused by alterations in fluid osmolarity

EXPERIMENTAL PROCEDURES

Oocyte isolation and treatment

Oocytes were obtained from sexually mature *Xenopus laevis* females (purchased from Centre d'Élevage de Xenopes, Montpellier, or from Xenopus Express, Vernassal, France), anesthetized in 0.02 % benzocaine and portions of ovary were removed through a small incision on the abdomen. The incision was sutured, and the animal was returned to a separate tank until it had fully recovered from the anesthesia. It was then returned to a large tank in which all the frogs were kept for at least 4 weeks until the next surgery.

The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Autònoma de Barcelona (Permit Number: CEEAH 439) and all efforts were made to minimize animal suffering. The tissue was dissected into small pieces, if the ovaries were healthy enough to use, and oocytes were defolliculated for 2-3 h at room temperature with collagenase/dispase (0.8 mg/ml (Sigma), 0.48 mg/ml (Roche)) in Modified Barth's Saline (MBS: 5 mM HEPES, 88 mM NaCl, 1 mM KCl, 1 mM MgSO₄·7H₂O, 2.5 mM NaHCO₃, 0.7 mM CaCl₂, pH 7.8) with gentle agitation. The defolliculated oocytes were then washed thoroughly with MBS and transferred to Petri dishes. Stage VI oocytes were sorted manually and incubated overnight in MBS at 18°C. The next day, healthy survivors were selected and transferred to a Petri dish containing fresh MBS.

RT-PCR and DNA constructs

Total RNA was isolated from *Xenopus laevis* oocytes (stage VI) with the method described by McGrew [43] and kept at -70°C. First-strand cDNA was synthesized with RevertAid M-MuLV Reverse Transcriptase (Fermentas) in a 20 µL reaction using 250 ng of total RNA and poly (dT) primer. Reaction mixture was incubated for 1 h at 42°C and subsequently for 10 min at 70°C to terminate the reaction, chilled on ice and stored at -20°C. Specific primers were designed to amplify the four p38 isoforms (α , β , γ , δ). In addition, primers with appropriate restriction enzyme sites were designed for cloning the complete coding sequence of Xp38 β in the plasmid FTX5 (that contains a Myc tag sequence at the 5' end). *Xenopus laevis* p38 α (Xp38 α), p38 γ (Xp38 γ), human p38 δ (Hp38 δ), cloned in FTX5 vector, and human MKK6-DD (cloned in FTX4 vector, without Myc tag) were obtained from Eusebio Perdiguero (Centre de Regulació Genòmica, Barcelona) and Angel R. Nebreda (Institut de Recerca Biomèdica, Barcelona) and have been described previously [28, 29, 45]. MKK6-DD is a constitutively active kinase with the two phosphorylation sites in the activation loop Ser-207 and Thr-211 changed to Glu. *Xenopus tropicalis* p38 δ (Xtp38 δ) was obtained from an I.M.A.G.E cDNA clone (IRBHp990H0820D, Source BioScience), and specific primers were designed for subcloning the coding sequence in FTX5 vector. The primer sequences used are available upon request. PCR reactions were performed in a total volume of 50 µl, with 3 µl of RT product or 10 ng of cDNA plasmid (Xtp38 δ), 0.2 mM dNTP, 20 µM of each primer (Sigma) and 2.5 units of Pwo Super Yield DNA polymerase (Roche). PCR products were purified, digested with the appropriate restriction enzymes and cloned in pFTX5 vector.

Site directed mutagenesis and in vitro transcription

PCR was employed to mutate specific amino acids in the coding sequences. The mutagenesis was performed according to QuikChange Site-Directed Mutagenesis Kit (Stratagene) instruction manual. The primer sequences used for mutagenesis are available upon request.

As described before, Xp38 α was obtained from another laboratory. However, after sequencing this construct we detected two consecutive amino acids mutated (E99D and F100P) compared with the published sequence (GenBank, NCBI Reference Sequence: NM_001086831.1). Curiously, the two amino acids mutated (E99 and F100) are codified by an EcoRI restriction site sequence (GAATTC). We assumed that mutation of amino acids EF to DP in Xp38 α was a consequence of cloning or subcloning and therefore this construct was corrected by site directed mutagenesis to obtain the wild type E99 and F100 amino acids, as reported in GenBank. We also confirmed by RT-PCR and sequencing that endogenous Xp38 α mRNA codifies for EF amino acids at positions 99 and 100.

The mutants Xp38 α -AF (Thr181 and Tyr183 replaced by Ala and Phe, respectively) and Xp38 α -KR (Lys54 replaced by Arg) were also generated. These mutants have been reported as catalytically inactive and can act as dominant negative in some contexts [46-48]. Analogous mutants (-AF and -KR) were designed for Xp38 β , Xp38 γ and Hp38 δ . Xp38 γ -DA (Asp-171 replaced by Ala), a catalytically inactive mutant that inhibits progesterone-induced maturation was a gift of Eusebio Perdiguero [29]. Constitutively active p38 α (Xp38 α -CA) (Asp-177 and Phe-328 replaced by Ala and Ser respectively), and constitutively active p38 β (Xp38 β -DA/YL) (Asp-176 and Ala-176 replaced by Tyr and Leu, respectively) were generated in our laboratory. It has been reported that these mutants are constitutively active [49, 50].

All cloned genes and mutations generated were confirmed by DNA sequencing. In vitro transcriptions of capped RNAs (cRNAs) were obtained by using mMessage mMachine T7 Transcription Kit (Ambion).

Oocyte injection and hyperosmotic shock treatment

Xenopus oocytes (stage VI) were microinjected near their equator with 50 nL (5 ng) of the corresponding cRNAs or H₂O using a Nanoject II Automatic Nanoliter Injector (Drummond Scientific Company). Injected oocytes were incubated 18 h at 18°C and poor oocytes were eliminated the next day before treatment. Oocytes were exposed to

hyperosmotic shock by transferring them to a new dish containing MBS with 300 mM sorbitol, and pools of 20 oocytes were collected at different times and treated as described below. Previous studies have shown that 300 mM sorbitol induces cytochrome c release and caspase-3 activation between 1 h and 3 h, depending on the experiment [9]. In some experiments, oocytes were injected with cytochrome c (0.5 μ M final intracellular concentration) from horse heart (c-7752, Sigma) with or without the caspase-3 inhibitor Ac-DEVD-CHO (Molecular Probes) at 0.1 or 1 μ M final intracellular concentration and collected 1 h later. As a control, some oocytes were injected only with MBS, the solvent used for cytochrome c and Ac-DEVD-CHO.

Oocyte lysis and Western blot analysis

Pools of 20 oocytes were lysed by pipetting up and down in 200 μ L of ice-cold extraction buffer (0.25 M sucrose, 0.1 M NaCl, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2) containing 1 mM EDTA, 1 mM EGTA, protease inhibitors (10 μ g/ml leupeptin, 1 mM PMSF, 10 μ g/ml aprotinin) and phosphatase inhibitors (50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate). Samples were clarified by centrifugation at 14,500 rpm for 5 min and supernatants (cytosolic fraction) were collected and processed for immunoblotting or caspase assay as described below. The whole supernatants were denatured with Sample Buffer (50 mM Tris HCl, pH 6.8, SDS 2%, 100 mM dithiothreitol, 10% glycerol) and subjected to 10% or 15% SDS/PAGE and transferred to Immobilon-P membranes (Millipore). 16 μ L of cell extract was loaded in each well (equivalent to 32 μ g protein). In our experience, more errors are made trying to load similar amounts of protein measured with a colorimetric method. The uniformity of samples loading was verified by Ponceau (Sigma) staining of the blots. Membranes were blocked for 1 h with 5% dried skimmed milk in TBST (50 mM Tris, 150 mM NaCl, 100 mM KCl, pH 7.4, and 0.1% Tween 20) and then incubated with the following antibodies: anti-AMPK α (#2532, Cell Signaling), anti-pp38 (Thr180/Tyr182) (#9211, Cell Signaling), monoclonal anti-Myc (M4439, clone 9E10, Sigma), monoclonal anti- β -actin (A1978, Sigma), and anti-Cytochrome c (556432, BD Pharmingen). Antibody binding was detected with horseradish peroxidase-coupled secondary antibody and the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences). Blots were quantified with Image J and the ratio pp38/AMPK, pp38/Myc or cytochrome c (CC)/AMPK represented.

Assay for DEVDase activity

Caspase-3 activity was measured in terms of DEVDase activity assay in 96 cells opaque plates (OptiPlate, Ref. 6005270, PerkinElmer). 25 µl cytosolic fraction (corresponding to 2.5 oocytes) were diluted 1:1 with 25 µl lysis buffer and assayed with 50 µl 2× Reaction Buffer (20% glycerol, 40mM Hepes, 4mM DTT, pH 7.5) containing 200 µM of synthetic peptide Z-DEVD-AMC (Peptide Institute, Inc.). Fluorescence at 360 nm for excitation and at 460 nm for emission was measured after incubation of the samples for 60 min at 37°C. Caspase-3 activity was determined as the concentration of fluorescent AMC formation from Z-DEVD-AMC substrate and represented as arbitrary units (AU) of caspase-3 activity, giving value 1 to non-treated oocytes injected with H₂O.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed with GraphPad Prism 8.0.1 program. One-way ANOVA with a Dunnett Multiple Comparison Test was used in oocytes microinjected with different cRNAs comparing all columns versus water injected oocytes. Uncorrected Fisher's LSD test was used when several groups injected with cytochrome c were compared with their respective controls (non-injected). Values of $p < 0.05$ were considered statistically significant.

DATA AVAILABILITY

All the relevant data are contained within this article. Primer sequences used to generate the different constructs are available upon request to the corresponding author (JML).

SUPPORTING INFORMATION

The supporting information contains Figures S1-S9.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Figure 1. Xp38 α and Xp38 γ are activated by hyperosmotic shock. (A, C, E) Expression and phosphorylation of Xp38 α , Xp38 γ , and their mutants. Oocytes were injected with 50 nL of H₂O or cRNAs (5 ng in 50 nL) Xp38 α , Xp38 α -AF, Xp38 α -KR, Xp38 γ , Xp38 γ -DA, or MKK6-DD and 18 h later exposed to osmotic shock (300 mM sorbitol) for 1 h, 3 h, or non-treated. Expression of p38 isoforms was confirmed with Myc antibodies (A). MKK6-DD is a constitutively active mutant without Myc tag. pp38 and cytosolic cytochrome c (CC) levels were analyzed by Western blot and AMPK was used as a loading control. (B, D, F) Caspase-3 activity was measured in all the conditions assayed, giving value 1 to non-treated water-injected oocytes. Expression of Xp38 α , Xp38 γ , or their inactive mutants did not modify hyperosmotic shock-induced apoptosis. Results in panels B, D, F are the mean $\pm$ SD of three independent experiments. * $p < 0.05$ compared to water-injected oocytes (ANOVA and Dunnett's test). Western blots are representative of three independent experiments (see Figures S2 and S3 for additional experiments and blots quantification).

Figure 2. Xp38 β is activated by hyperosmotic shock. (A, C, E) Expression and phosphorylation of p38 isoforms and mutants. Oocytes were injected with 50 nL of H₂O or cRNAs (5 ng in 50 nL) Xp38 β , Xp38 β -AF, Xp38 β -KR, Xp38 δ , Xp38 δ -AF, Xp38 δ -KR, or MKK6-DD and 18 h later exposed to osmotic shock (300 mM sorbitol) for 1 h, 3 h, or non-treated. Expression of p38 isoforms was confirmed with Myc antibodies (A). pp38 and cytosolic cytochrome c (CC) levels were analyzed by Western blot and AMPK was used as a loading control. (B, D, F) Caspase-3 activity was measured in all the conditions assayed, giving value 1 to non-treated water-injected oocytes. Expression Xp38 β , Xp38 δ , or their inactive mutants did not modify osmostress-induced apoptosis. Data in B, D, and F are represented as mean $\pm$ SD of three or four independent experiments. * $p < 0.05$ compared to water-injected oocytes (ANOVA and Dunnett's test). Western blots are representative of three independent experiments (see Figures S4 and S5 for additional experiments and blots quantification).

Figure 3. Constitutively active Xp38 α (CA) or Xp38 β (DA/YL) expression accelerates hyperosmotic shock-induced apoptosis. (A, C, E) Oocytes were injected

with 50 nL of H₂O or cRNAs (5 ng in 50 nL) Xp38 α -CA, Xp38 β -DA/YL, a combination of both, or MKK6-DD and 18 h later exposed to osmotic shock (300 mM sorbitol) for 1 h, 2 h, or non-treated. Expression of p38 mutants was confirmed with Myc antibodies (A). pp38 and cytosolic cytochrome c (CC) levels were analyzed by Western blot and AMPK was used as a loading control. (B, D, F) Caspase-3 activity was measured in all the conditions assayed, giving value 1 to non-treated water-injected oocytes. Results represent the mean $\pm$ SD of seven independent experiments. * p <0.05, ** p <0.01 compared to water-injected oocytes (ANOVA and Dunnett's test). Western blots are representative of three independent experiments (see Figures S7 and S8 for additional experiments and blots quantification).

Figure 4. Cytochrome c injection in *Xenopus* oocytes induces caspase-3 activation and p38 α and p38 β phosphorylation. (A) Oocytes were injected with 50 nL of MBS or cRNAs (5 ng in 50 nL), Xp38 α , Xp38 β , Xp38 γ , or Xp38 δ and 18 h later injected with MBS (as a control) or horse cytochrome c (CC) (0.5 μ M final concentration) dissolved in MBS and pools of 20 oocytes were lysed 1 h later to analyze Myc, pp38, and AMPK by Western blot. A representative blot of two independent experiments is shown (see Figure S9 for additional experiment). (B) Blots were quantified with Image J and the ratio pp38/AMPK represented. Results are the mean $\pm$ SD of two independent experiments. *** p <0.001, **** p <0.0001 compared to oocytes of the same condition but non-injected with cytochrome c (ANOVA and Fisher's LSD test). (C) Caspase-3 activity was determined in all the extracts and represented as arbitrary units (AU), giving value 1 to oocytes injected with MBS. Results are the mean $\pm$ SD of two independent experiments. ** p <0.01, *** p <0.001 compared to oocytes of the same condition but non-injected with cytochrome c (ANOVA and Fisher's LSD test).

Figure 5. Caspase-3 inhibition reduces phosphorylation of p38 α and p38 β induced by cytochrome c injection. (A) Oocytes were injected with MBS, horse cytochrome c (CC) (0.5 μ M final intracellular concentration), or CC (0.5 μ M) plus caspase-3 inhibitor Ac-DEVD-CHO (0.1 μ M final intracellular concentration) and pools of 20 oocytes were lysed 1 h later to analyze Myc, pp38, and β -Actin by Western blot. Caspase-3 activity was determined as previously described (see values at the bottom of the blot). (B) pp38 and Myc levels were quantified from the blot with Image J and the ratio pp38/Myc represented in arbitrary units (AU), giving value 100% to oocytes expressing Xp38 α or

Xp38 β injected with cytochrome c. (C and D) Oocytes were treated and analyzed as described above, but Ac-DEVD-CHO was injected at 1 μ M final concentration. Note that caspase-3 activity was markedly reduced in C compared with B.

Figure 6. Model for osmostress-induced apoptosis. Like a heavy rain, hyperosmotic shock rapidly activates several signaling pathways that converge in the mitochondria to increase their permeability releasing cytochrome c to the cytosol. In *Xenopus* oocytes, hyperosmotic shock induces rapid calpain activation, Smac/DIABLO release from the mitochondria, cleavage of small amounts of Bid by an unknown protease, and activation of JNK1-1, JNK1-2, p38 α , p38 β , and p38 γ . Sustained activation of JNK1-1, JNK1-2, p38 α , and p38 β in combination with Smac/DIABLO, t-Bid, and calpains converge on the mitochondria to induce the release of cytochrome c into the cytosol, which in turn activates caspase-3. The activation of caspase-3 induces JNK1-2 and Bid proteolysis, as well as p38 α and p38 β phosphorylation. These events, in turn, promote additional cytochrome c release and caspase-3 activation engaging at least three positive feedback loops (L1-L3) to complete the cell death program.

Figure 1

Journal Pre-proof

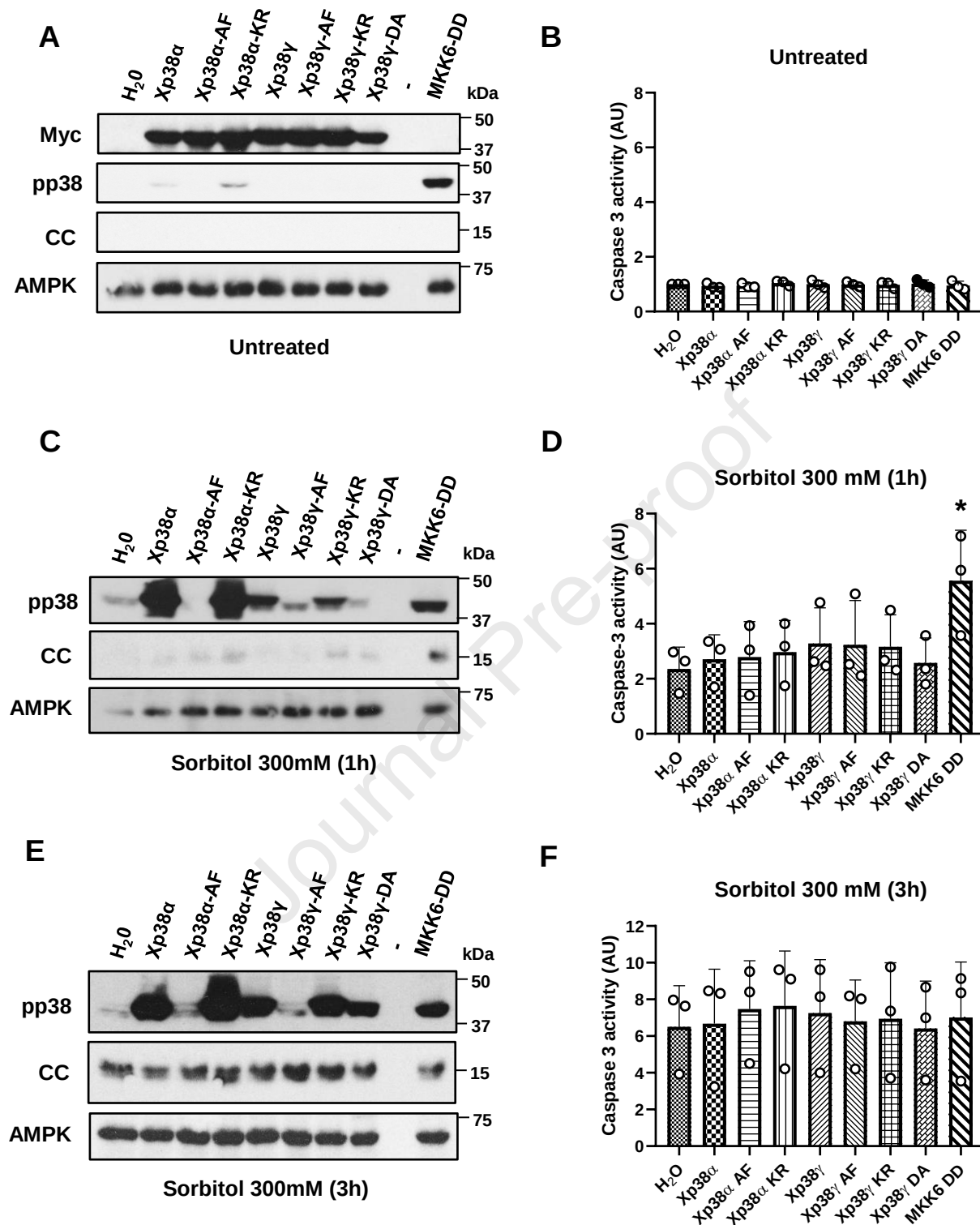
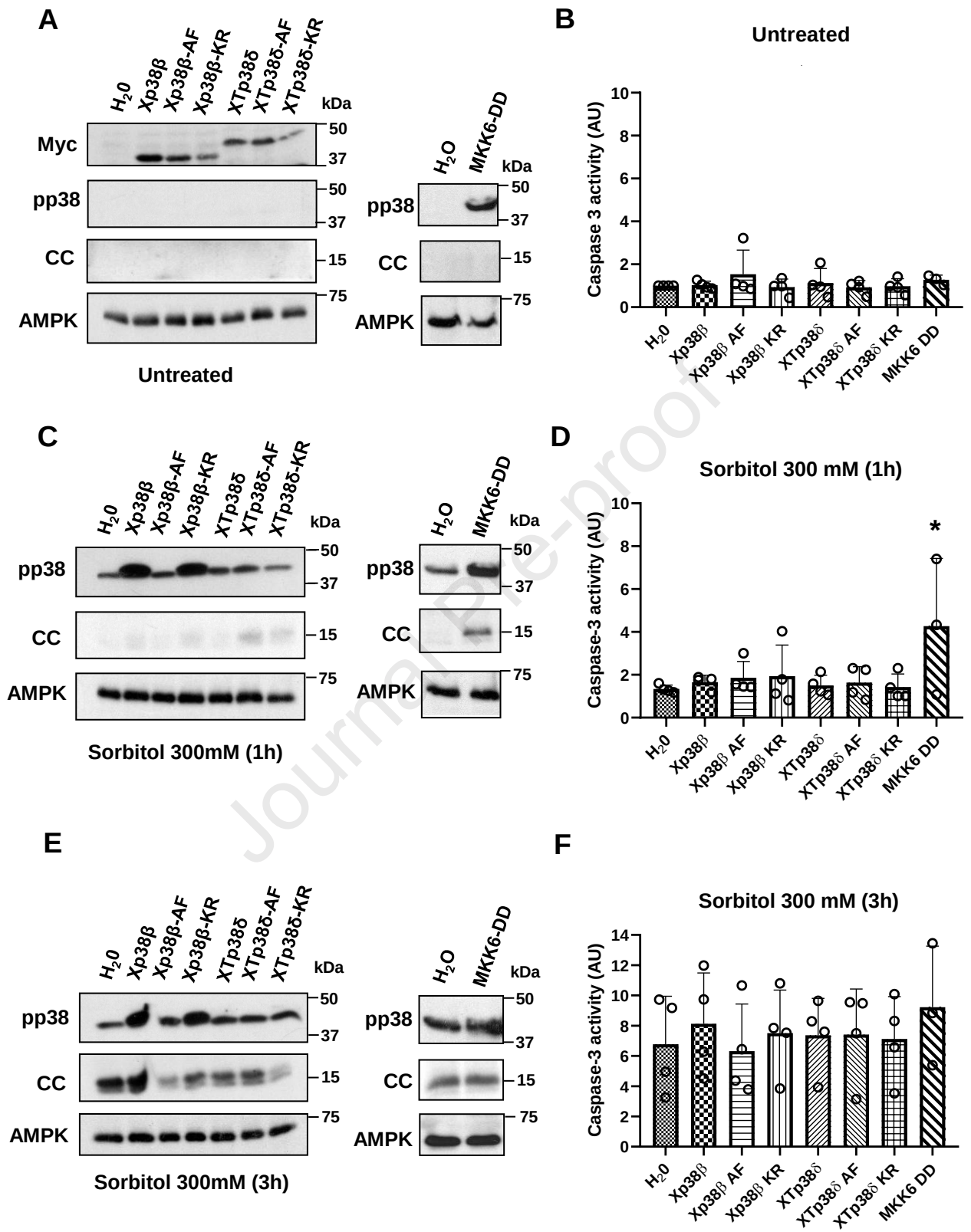
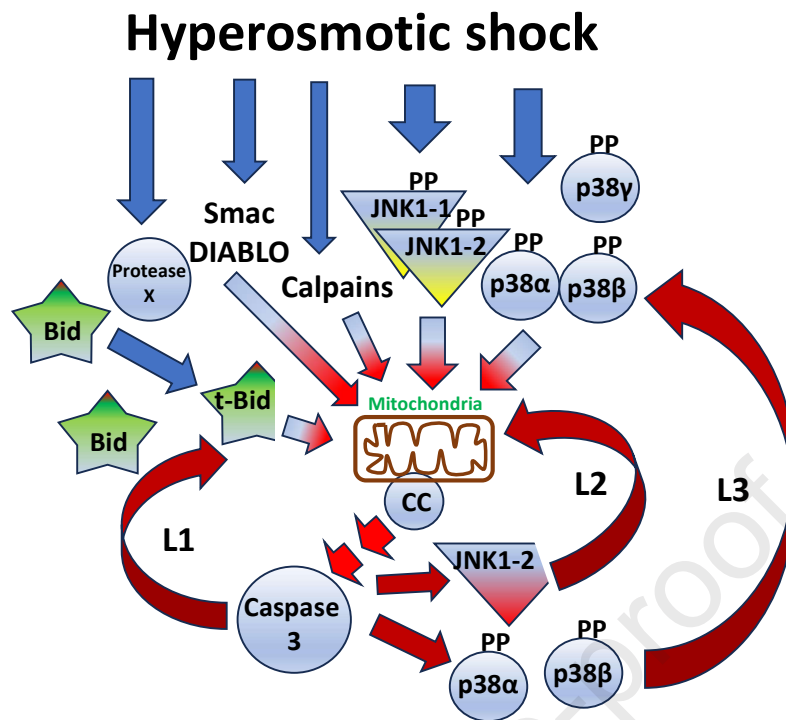
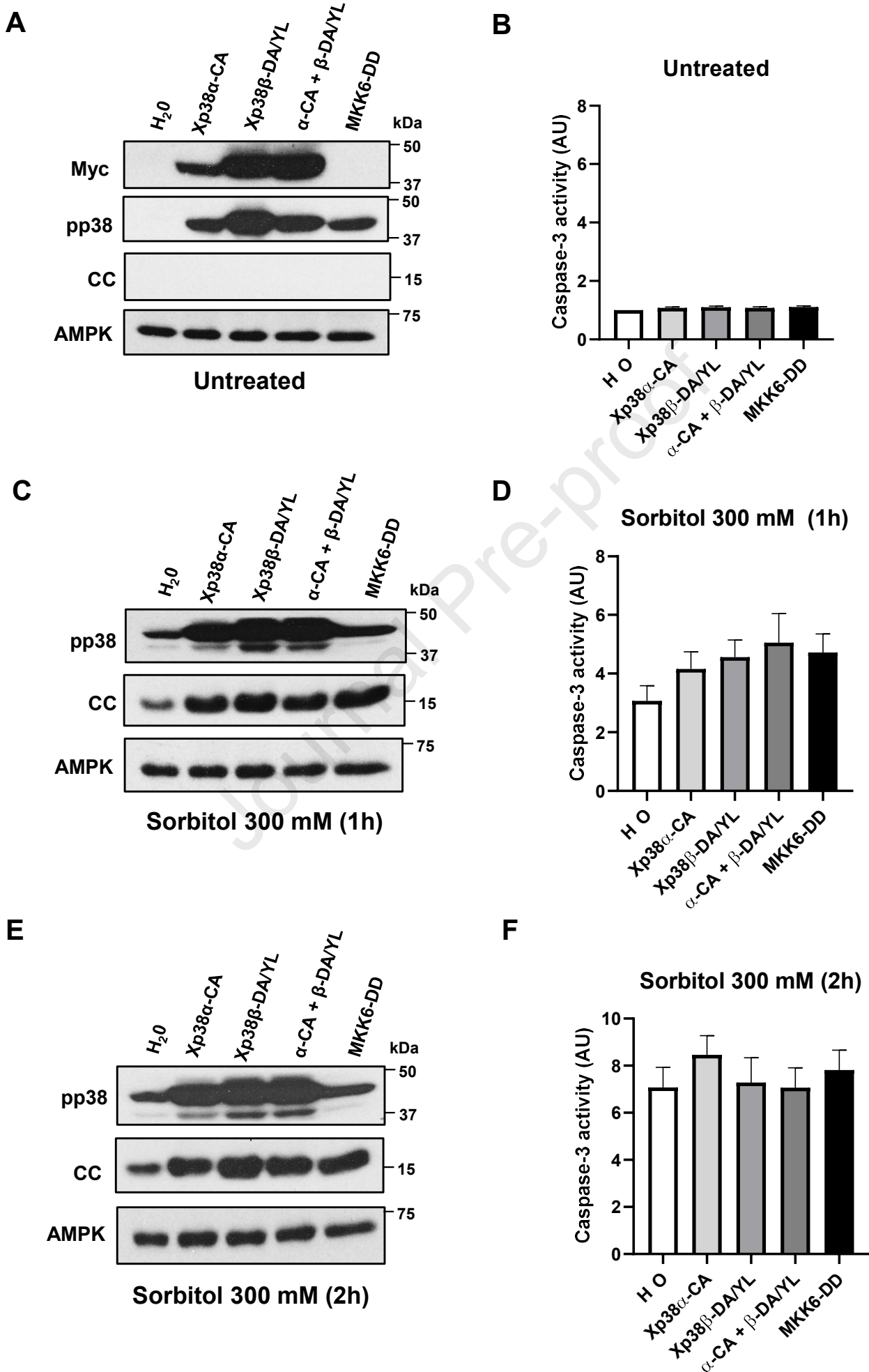


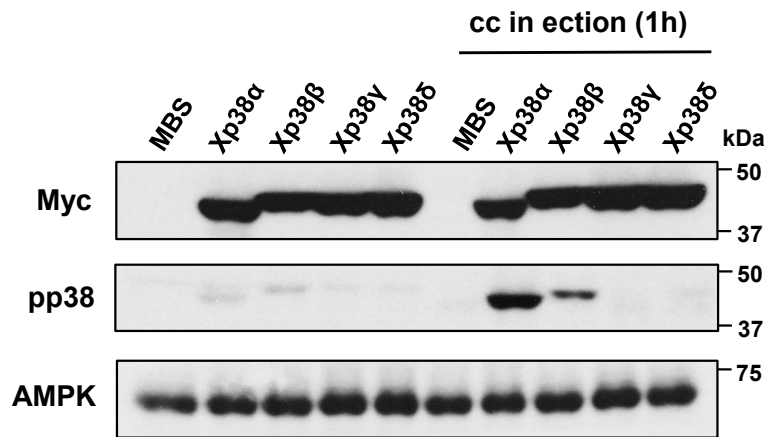
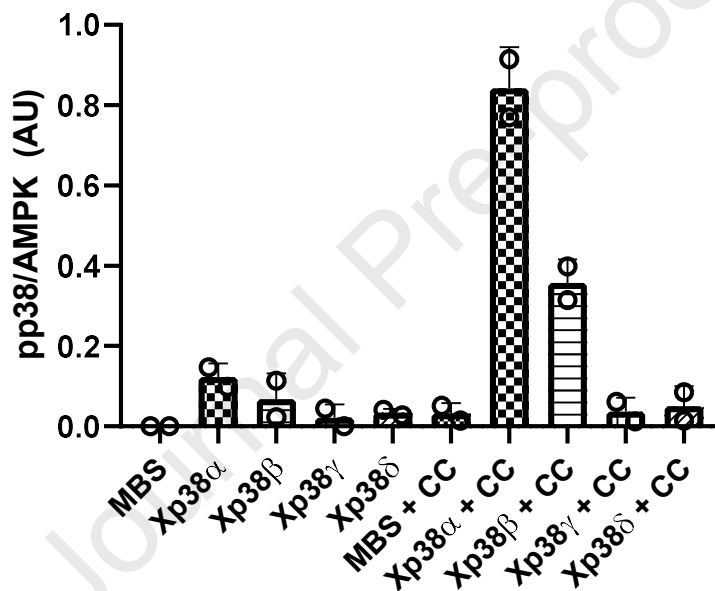
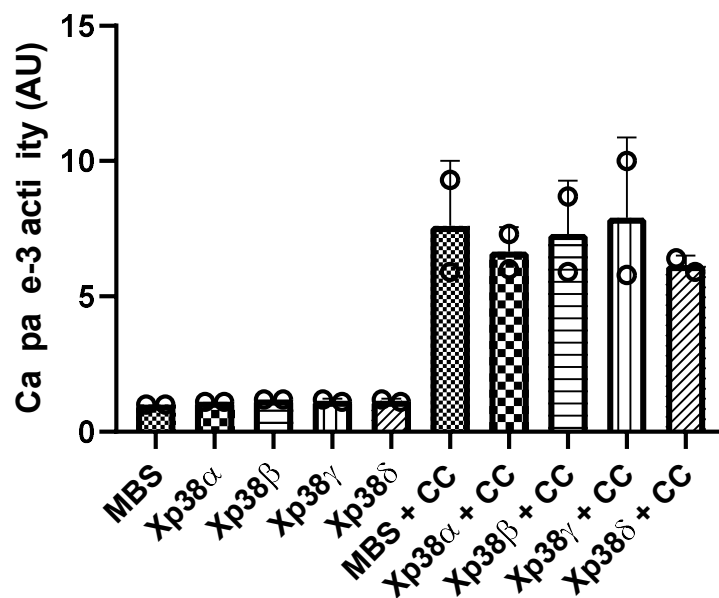
Figure 2

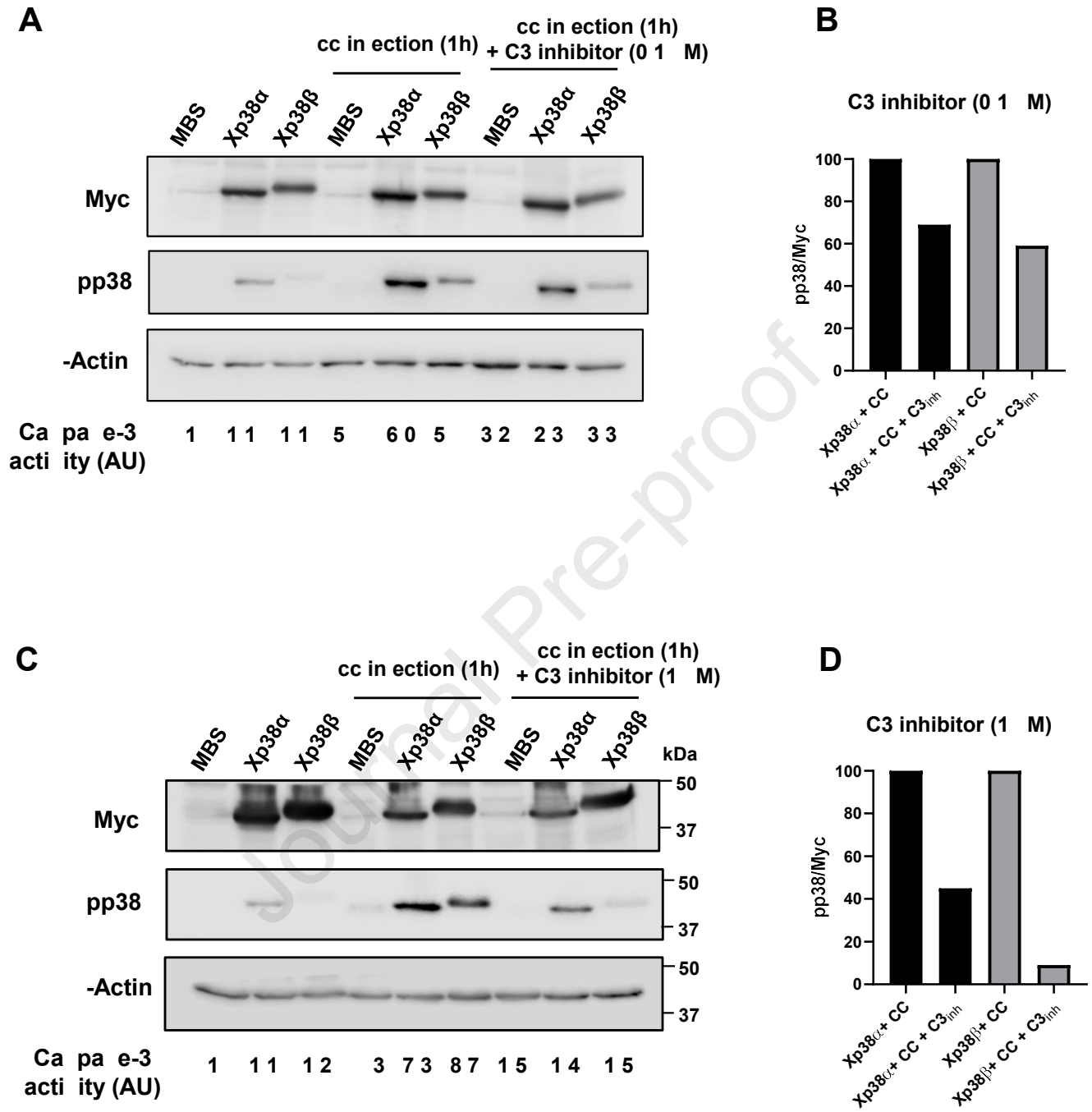
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CRediT author statement

Nabil Ben Messaoud: Methodology, Formal analysis, Investigation. **José M.**

López: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original Draft, Writing- Reviewing and Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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