ANALYSES OF METACASPASE 1 AND A POTENTIAL APAF-1 ORTHOLOGUE DURING HEAT STRESS-INDUCED PROGRAMMED CELL DEATH IN CHLAMYDOMONAS REINHARDTII

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Master of Science

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by

Celso Santos Goncalves Catumbela

May, 2017

ANALYSES OF METACASPASE 1 AND A POTENTIAL APAF-1 ORTHOLOGUE DURING HEAT STRESS-INDUCED PROGRAMMED CELL DEATH IN CHLAMYDOMONAS REINHARDTII

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Celso Santos Goncalves Catumbela

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APPROVED:

Anne Gaillard, PhD
Thesis Director

Todd Primm, PhD
Committee Member

James Harper, PhD
Committee Member

John B. Pascarella, PhD
Dean, College of Science and Engineering Technology

DEDICATION

To my parents, Jose and Ana Luisa Catumbela, and my "Ma", Lynne Preston, whose exceptional love and support never ceases to inspire me.

"*Tula utima a manjangue tchossi chassalapo ame ndichitata*"

In memoriam

Aaron Alexander Preston

Jan. 14, 1991 – Sept. 6, 2013

ABSTRACT

Catumbela, Celso Santos Goncalves, *Analyses of Metacaspase 1 and a potential APAF-1 orthologue during heat stress-induced programmed cell death in Chlamydomonas reinhardtii*. Master of Science (Biology), May, 2017, Sam Houston State University, Huntsville, Texas.

Purpose

The purpose of this thesis was to expand on the knowledge of the PCD machinery of unicellular eukaryotes. To this end, the first half of this thesis employs the single cell green alga, *Chlamydomonas reinhardtii*, and examines the potential roles of metacaspase type I (MCA1) in modulating the events of programmed cell death (PCD). The second half of this thesis attempts to elucidate the function of a potential orthologue of APAF-1, a key apoptotic protein reported in multicellular eukaryotes, and hypothesized to be present in the unicellular *C. reinhardtii*.

Methodology

Using an *MCA1* knockout strain of *C. reinhardtii* (*mca1*), verified via PCR and DNA sequence analysis, the first half of this thesis utilizes the following techniques to reveal the potential roles of MCA1: fluorescence microscopy and DNA laddering, as well as colony formation and colorimetric assays. All experiments were conducted in triplicate. The data was analyzed via manual cell counts, and qualitative analyses were performed by a single analyst. Statistical software (SAS 9.4 and JMP 13) analyses were conducted by two independent personnel. For the second half of this thesis, proteomic analysis of *C. reinhardtii* was conducted through usage of whole-cell extraction, sucrose gradient centrifugation, SDS-PAGE, Coomassie blue staining, and Western blotting.

Findings

Genetic analysis of *mca1* revealed that the disruption of *MCA1* is linked to the rapid transmission of certain PCD events due to heat stress in *C. reinhardtii*. Results show that in response to heat stress, *MCA1* knockout is associated with significantly increased plasma membrane disruption in *C. reinhardtii*. Moreover, heat-stressed *mca1* cells consistently displayed increased DNA laddering, relative to WT. Furthermore, in response to heat stress, *mca1* populations displayed more rapid accumulation of ROS, as well as a significantly greater ROS response with prolonged heat stress exposure. Notably, *MCA1* knockout did not alter the rate of phosphatidylserine (PS) externalization, or cell proliferation upon the onset of heat stress. Together, our data suggest the potential that MCA1 acts as a negative regulator of certain heat stress-induced PCD phenotypes within *C. reinhardtii*.

In the second half of this thesis, proteomic analysis revealed that prior published results suggestive of APAF-1 presence in *C. reinhardtii* could be generated in the absence of antibodies against human APAF-1. Intriguingly, comparable results were obtained through sole usage of goat anti-rabbit IgG, or goat anti-mouse IgG (1:5000, both). Moreover, *in silico* analysis revealed the primary APAF-1 homologs in *C. reinhardtii* to lack key conserved domains (CARD and AAA16). Together, the biochemical and *in silico* data refuted the previous report of a potential APAF-1 orthologue in *C. reinhardtii*. This thesis provided novel data to aid in the understanding of the unicellular PCD machinery, as well as recommendations for future studies.

KEY WORDS: Chlamydomonas reinhardtii, Metacaspase, MCA1, PCD, APAF-1, Heat stress, Reverse genetics.

ACKNOWLEDGEMENTS

Without a doubt, the completion of this Master's thesis is due to the unrivaled efforts of Dr. Anne Gaillard, who guided me through the numerous challenges of scientific research, and yet provided me with an environment that nurtured independent learning. Our shared love of cats, wine, and Keanu Reeves was the foundation of our successful pairing.

I would also like to thank my thesis committee members, Drs. James M. Harper and Todd Primm, as well as Dr. Madhusudan Choudhary, whose tutelage throughout the years has helped me better understand and love biology as I do today. In addition to my graduate school professors, I am also grateful to Dr. Maria Todd, as well as Heather Petty, whose words and affection inspired me to pursue a research career.

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CHAPTER I

INTRODUCTION

A review of cellular death

Regulated cell death. Regulated or programmed cell death (PCD) refers to the array of genetically programmed physiological processes by which cells cease to exist. PCD is conserved among eukaryotes, and is present in both multicellular and unicellular organisms (Mcarthy, 2003, Moharikar *et al*., 2007, Shemarova, 2010). Within multicellular organisms, PCD plays a key role in regulation of development. Notably, PCD is also a prominent feature in mammalian-pathogen interactions, and has been linked with malignant, autoimmune, and neurodegenerative diseases. Numerous studies have shown that cells undergo PCD due to stressors that disrupt cell survival, as well as due to a pre-determined fate (Aballay & Ausubel, 2001, Alberts *et al*., 2002, Dekkers *et al*., 2013).

Mechanisms of PCD. Cells require an array of complex steps in order to remain alive, but as myriad studies have shown, cellular death involves a number of steps just as intricate (Alberts *et al.,* 2002, Shemarova, 2010, Mcilwain *et al.,* 2017).

PCD domains. The death-domain superfamily consists of protein domains that are key to apoptotic and inflammatory signaling cascades. Members of this superfamily include Caspase Recruitment Domains (CARDs), Pyrin Domains (PYDs), Death Domains (DDs), and Death Effector Domains (DEDs) (Alberts *et al.,* 2002, Shemarova, 2010). A number of PCD pathways have been well characterized in mammals (Fig. 1).

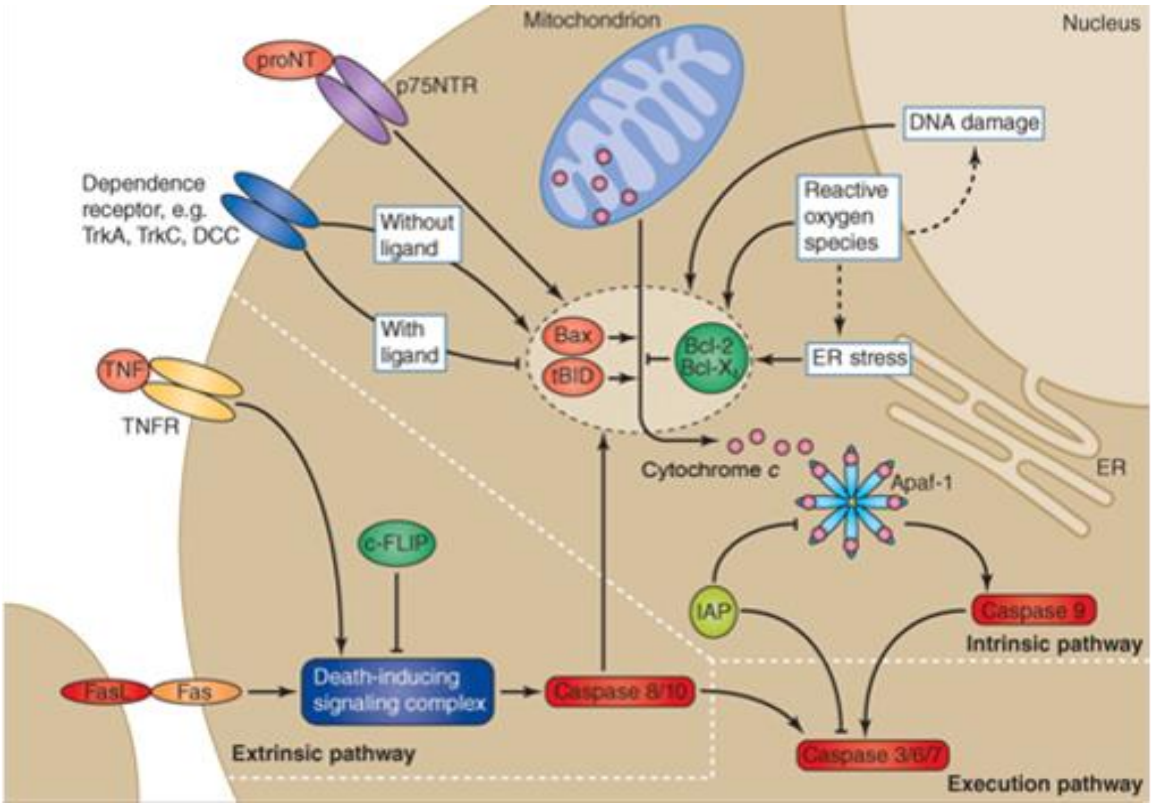

Figure 1. Schematic of the programmed cell death machinery as characterized in mammalian cells. This figure is derived from a delayed open access journal (6 months), and adapted with permission from: Dekkers, M. P., Nikoletopoulou, V., & Barde, Y. (2013). Cell biology in neuroscience: Death of developing neurons: New insights and implications for connectivity. *The Journal of Cell Biology*, 203(3), 385-393.

In mammalian cells, the binding of an extracellular ligand to a DD-containing tetrameric death receptor, such as members of the tumor necrosis factor (TNF) superfamily, initiates canonical PCD. Ligand binding induces homotypic interactions between the death receptor and intracellular proteins containing either Fas-associated death domain (FAD) or TNER-associated death domain (TNADD). Subsequently, the adaptor protein associated with the death receptor undergoes conformational changes so that its DED is capable of interacting with the initiator caspases-8 and-10 (Shemarova, 2010).

PCD proteases. Though capable of being initiated by a number of stimuli, PCD pathways are ultimately dependent on the action of proteases capable of inducing the downstream degradation characteristic of PCD. PCD is canonically regulated by cysteine protease (CP) family members. Originally, the CP family included only caspases; however, recent reports have revealed two additional members, metacaspases and paracaspases (Uren *et al.,* 2000). Further studies reported that though similar in function, these proteases display distinct domain architectures (Fig. 2) (Choi & Berges, 2013).

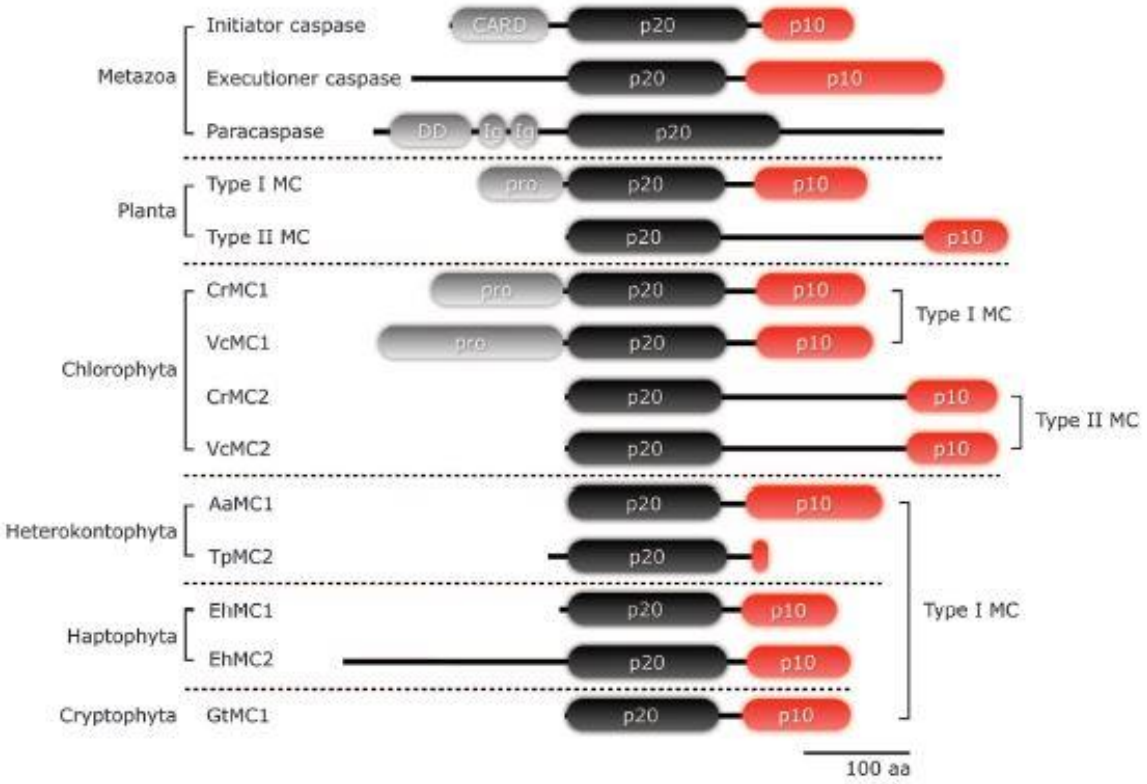

Figure 2. Distinct domain organization of caspases, paracaspases, and type I and II metacaspases. Shown are the domain architectures of initiator and executioner caspases in metazoans (caspase-9 and -6, respectively), a paracaspase in humans (PCASP1), and type I and II metacaspases in plant (AtMC1 and AtMC4 of *Arabidopsis thaliana*, respectively) and plant-like organisms. The catalytic domain of CP family members encodes a p20, p10, and/or a prodomain consisting of PCD domains (e.g., CARD, DD, Immunoglobulin-like). In order of appearance (top to bottom), the species abbreviations used are: Cr, *Chlamydomonas reinhardtii*, Vc, *Volvox carteri f. nagariensis*, Aa, *Aureococcus anophagefferens*, Tp, *Thalassiosira pseudonana*, Eh, *Emiliana huxleyi*, Gt, *Guillardia theta*. This figure is derived from an open access journal, and adapted with permission from: Choi, C. J., & Berges, J. A. (2013). New types of metacaspases in phytoplankton reveal diverse origins of cell death proteases. *Cell Death and Disease*, *4*(2). doi:10.1038/cddis.2013.21

Caspases. Caspases are proteases containing cysteine at their active sites, which they use to cleave aspartate residues of target proteins. Currently, fourteen caspases have been identified, whose physiological roles are as diverse as they are numerous. Recent evidence suggests that caspases can be subdivided into two categories in accordance with such physiological roles: 1) involved in apoptosis (caspase-2,-3,-6,-7,-8,-9, and -10), and 2) involved in the inflammatory response (caspase-1,-4,-5,-11,-12,-13, and -14) (Fink & Cookson, 2005, Lee *et al.,* 2011). Though similar in structure, the apoptotic caspases display differences in their temporal roles, and can be further categorized into two groups: 1) initiator caspases (e.g., caspase-2,-8,-9, and -10), which are responsible for initiating caspase-dependent death cascades, and 2) effector caspases (e.g., caspase-3,-6, and -7), which are responsible for the cleaving of cellular substrates that ultimately leads to PCD hallmarks (Fink & Cookson, 2005).

Activation of initiator caspases occurs via their long prodomain, which contains DEDs, and allows them to dimerize with DED-containing adaptor proteins and form the death-inducing signaling complex (DISC) (Acehan *et al.,* 2002, Fink & Cookson, 2005). Afterwards, DISC is capable of recruiting procaspases 8 and 10, whose aggregation leads to the autocatalytic cleavage of their prodomains, and results in their activation. These active initiator caspases are then capable of activating the effector caspases, which proceed to induce the degradation of nuclear or cytoplasmic targets, at which point PCD is irreversible. Interestingly, mammalian cells also possess caspase-9, which differs from other caspases due to not requiring cleavage in order to be active, but rather, it interacts with the apoptotic peptidase activating factor (APAF-1) adaptor protein. The interaction between APAF-1 and caspase-9 is promoted by the release of cytochrome *c* from the

mitochondria (described in more detail later), and occurs via the intrinsic PCD pathway (Moharikar *et al.,* 2007, Shemarova, 2010, Mcilwain *et al*, 2017).

Metacaspases. The conserved nature of PCD mechanisms in eukaryotes allows both animal and plant cells to undergo PCD in response to extracellular or intracellular stimuli. However, a notable difference lies in the proteases responsible for inducing PCD events. Unlike the caspases found in animal cells, the cysteine proteases in plant cells cleave at glycine and lysine residues of target proteins and are known as metacaspases (Merchant *et al.,* 2007, Moharikar *et al.,* 2007, Fagundes *et al.,* 2015, Kasuba *et al.,* 2015). Metacaspases are subdivided into types I (MCA1) and II (MCA2), in accordance with their structure (Fig. 2). Typically, the *N*-terminus of type I metacaspases contains a prodomain, as well as a zinc-finger motif. Type II metacaspases lack a prodomain, and instead have a linker region of 160-180 amino acids. Recent findings have indicated that both metacaspase types play a role in the PCD response of plants. Studies in rice (*Oryzea sativa*) have shown the upregulation of metacaspase genes (*OsMC*) during abiotic and biotic stress. Tomatoes (*Esculentum personicum*) inoculated with the fungal pathogen *Botrytis cinerea* show upregulation of the type II metacaspase, LeMCA1. Notably, unicellular organisms have been shown to possess metacaspases that are similar in structure and function to mammalian caspases (Fagundes *et al*., 2015). Identification of the yeast metacaspase, YCA-1, has prompted a search for metacaspases in other unicellular organisms (Shemarova, 2010). Of note, recent reports have also identified a novel group of metacaspases classified as type III (MCA3), which are present in Haptophyta (*Prymnesium parvum*), Cryptophyta (*Guillardia theta*), and Heterokontophyta (*Thalassiosira pseudonana*, *Phaeodactylum tricomutum*, and

Ectocarpus siliculosus). These metacaspases exhibit domain rearrangements unlike those of types I and II; notably, type III metacaspases display the p10 domain in their *N*-terminus, rather than the *C*-terminus (Fig. 3). The effect of these rearrangements is not yet clear, and their classification remains putative; however, such nonlinear rearrangements can allow for evolutionary innovation, and have been reported in other types of proteins (e.g., histone and homeobox proteins, DNA methyltransferases, ABC transporters) (Choi & Berger, 2013).

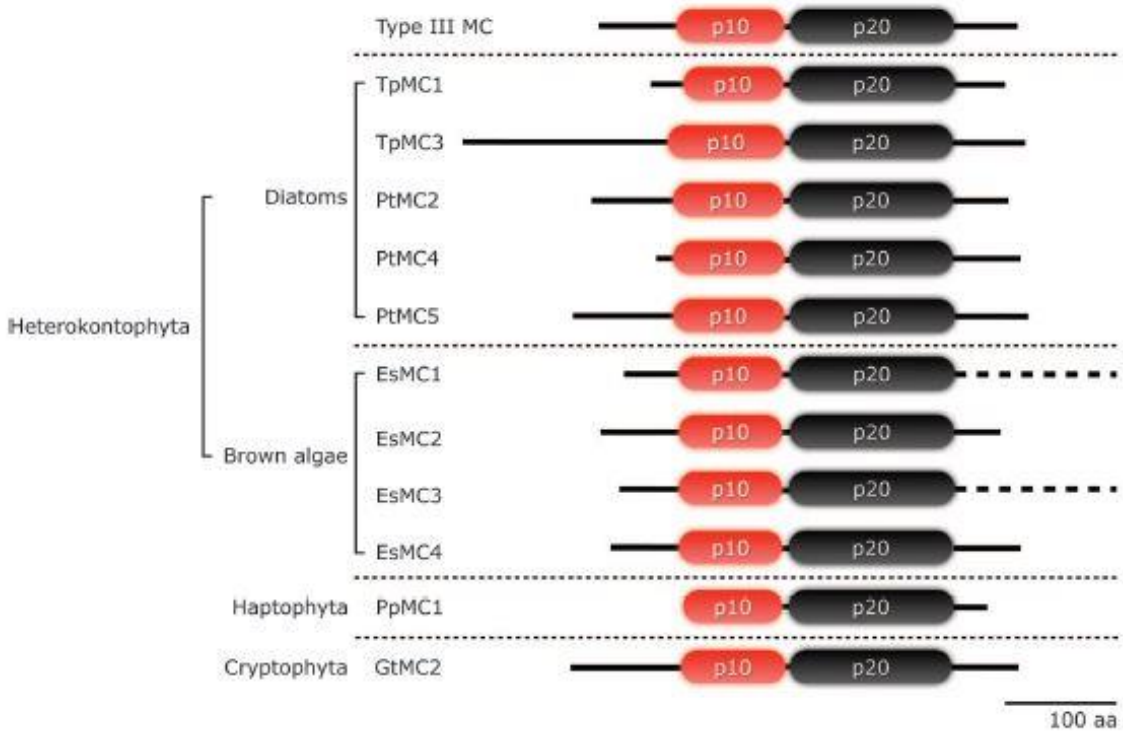

Figure 3. Type III metacaspases display a distinct rearrangement of the p10 domain. Shown are the type III metacaspases identified in Heterokontophyta (*Thalassiosira pseudonana*, *Phaeodactylum tricomutum*, and *Ectocarpus siliculosus*), Haptophyta (*Prymnesium parvum*), and Cryptophyta (*Guillardia theta*). The p10 domain of these metacaspases is located in the *N*-terminus, rather than the *C*-terminus. Due to the large size of EsMC1 and EsMC3, the total length of their *C*-terminus is not displayed. This figure is derived from an open access journal, and adapted with permission from: Choi, C. J., & Berges, J. A. (2013). New types of metacaspases in phytoplankton reveal diverse origins of cell death proteases. *Cell Death and Disease*, *4*(2). doi:10.1038/cddis.2013.21

In silico analyses of unicellular green algae such as *Chlamydomonas reinhardtii*, *Coccomyxa subellipsoidea*, and *Chlorella variabilis*, generated further support for the presence of metacaspases in lower eukaryotes (Moharikar *et al.*, 2007). Further studies revealed metacaspases to be present in animals, plants, algae, and even bacteria. Comparative phylogenomic analyses of the *C. reinhardtii* genome identified two sequences predicted to encode MCA1 and MCA2 (Merchant *et al*., 2007). Notably, Murik *et al.* (2013) reported that during H_2O_2-induced PCD in *C. reinhardtii*, the secondary induction of oxidative stress via dehydroascorbate exposure leads to greater upregulation of *MCA2* than *MCA1*. Furthermore, the usage of caspase inhibitors during the late stages of PCD has been reported to suppress PCD within *C. reinhardtii* exposed to mastoparan, a toxin (wasp venom) capable of stimulating various pathways (e.g., oxidative stress, phosphatidic acid, Ca^{2+}) (Yordanova *et al.*, 2013). Nonetheless, further studies are necessary to elucidate the role, type, and molecular partners of metacaspases within unicellular organisms.

Paracaspases. Relative to other CP family members, paracaspases display the least expansion in domain organization, and therefore remain the most similar to the ancestral CP (Staal *et al.*, 2016). Similar to metacaspases, paracaspases can be categorized into either types I or II, in accordance with their domain organization. While type I paracaspases consist of a DD, an immunoglobulin-like domain, and a caspase-like domain (Fig. 2), type II contains solely a caspase-like domain. Three paralogs of type I paracaspases (PCASP1, PCASP2, PCASP3) have been identified to date, and while mammals possess only PCASP1, a number of deuterostomes such as tunicates, lampreys, and lancelets have been shown to possess PCASP2 and PCASP3 (Hulpiao *et al.*, 2015,

Staal *et al.,* 2016). On the other hand, type II paracaspases have been identified in sponges, cnidarians, ctenophora, and trichoplax (Staal *et al.,* 2016, Uren *et al*., 2000).

Like other members of the CP family, paracaspases are employed in key cellular processes. In the single-celled haploid amoeba, *Dictyostelium discoideum*, paracaspases are responsible for regulation and localization of the contractile vacuolar system, thus being critical to maintenance of cell morphology (Saheb *et al.,* 2013). Within humans, PCASP1 is encoded by the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (*MALT1*) gene. MALT1 is a key component of the CARMA1-Bcl10-MALT1 (CBM) signaling complex, which is critical to T and B cell antigen receptor signaling. Further studies revealed PCASP1/MALT1 to be important for numerous signaling complexes involving Bcl10 and CARMA1-like proteins, which are characterized by a CARD and coiled-coil domain (CC), and belong to the CARD-CC protein family (Hulpiao *et al.*, 2015, Staal *et al.,* 2016).

Organelles involved in PCD. The regulatory roles of the CP family are made complex not only due to their spatial and temporal activity, but also due to their association with key cellular organelles (Bajt *et al.*, 2006, Kaczanowski *et al.,* 2011).

Mitochondria. The permeabilization of the outer mitochondrial membrane (OMM) is a critical step in various forms of PCD, and can occur via molecular alterations to inner mitochondrial membrane (IMM) components. Various death stimuli can trigger the opening of the transition pore in the IMM, which in turn allows for the accumulation of water and small molecules within the intermembrane space, and subsequent rupture of the OMM. Moreover, the interplay between anti-apoptotic Bcl2 family members (Bcl2, Bcl-X_L, Bcl-W, Mcl1, and A1), containing four *Bcl2*-homology (BH) domains, and pro-

apoptotic Bcl2 proteins (Bax, Bak, and Bok), in possession of BH 1-3 domains, are also key to modulation of the OMM. The heterodimerization of anti-apoptotic and pro-apoptotic Bcl2 proteins inhibits OMM permeabilization. The disruption of OMM potential can be promoted by BH3-only proteins (Bad, Bim, Bid, Noxa, Puma, Bik/Blk, Bmf, Hrk/Dp5, Beclin-1, and Mule), which can activate pro-apoptotic Bcl2 proteins, while inhibiting anti-apoptotic Bcl2 family members (Shamas-Din *et al.*, 2011). Independent of the selected mechanism of OMM permeabilization, the subsequent release of cytochrome *c* is integral to the apoptotic cascade via its role in the formation of the apoptosome (Fig. 1) (Haupt *et al.*, 2003, Broker *et al*., 2005, Moharikar *et al*., 2007, Amaral *et al.*, 2010).

APAF-1. Typically, cytochrome *c* acts as an electron acceptor, and its activity is regulated by two other mitochondrial proteins, OMI/HtrA2 and Smac/DIABLO. However, in multicellular eukaryotes, the transmission of apoptotic signals to the mitochondria leads to the release of cytochrome *c*. Upon its release into the cytosol, cytochrome *c* binds to APAF-1 and activates it (Fig. 4).

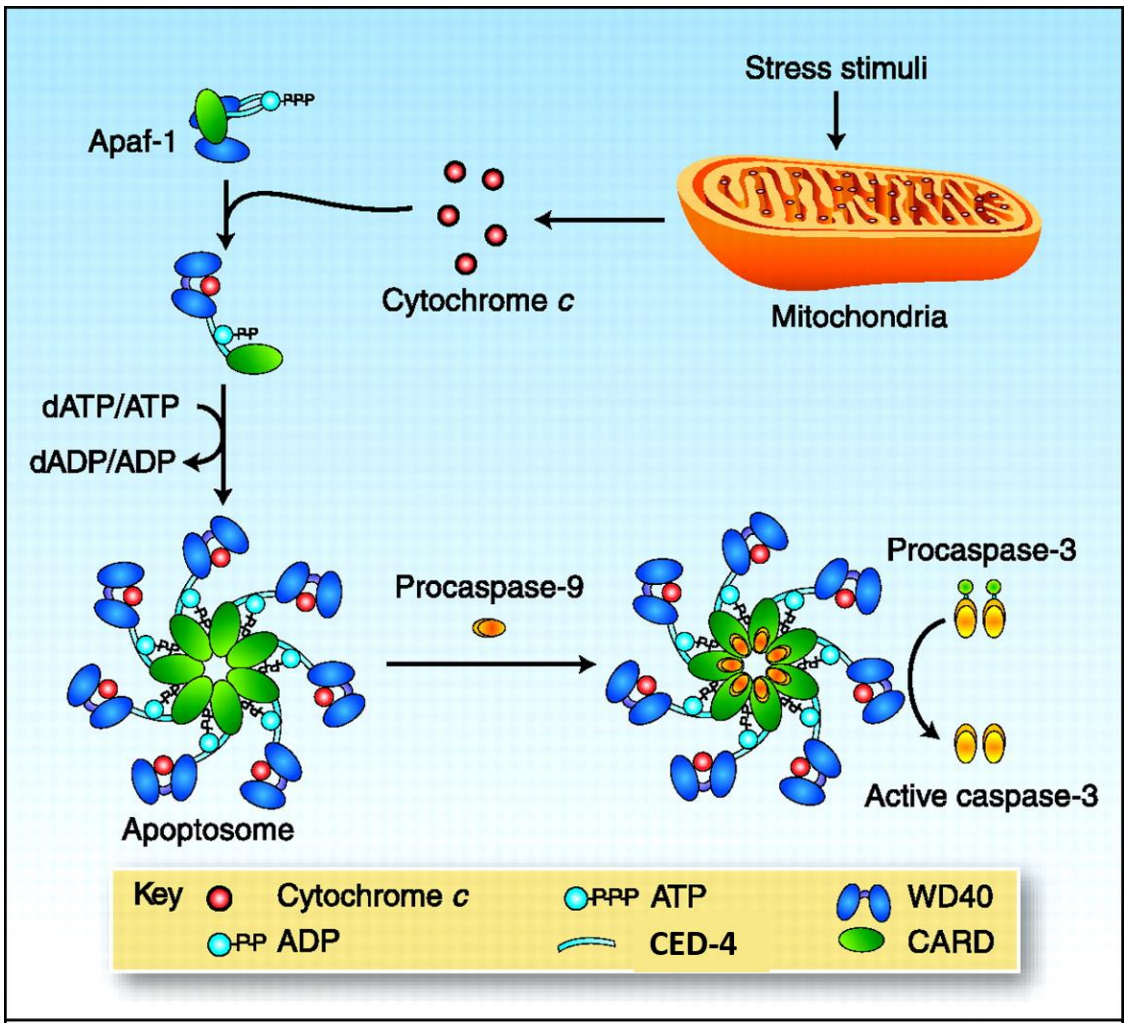

Figure 4. Schematic depiction of apoptosome complex formation. When present in the cytosol due to stress (e.g., toxic, heat, oxidative) cytochrome *c* interacts with APAF-1, and triggers apoptosome formation via a process dependent on hydrolysis of dATP. The apoptosome complex activates the initiator caspase, procaspase-9 via interaction between the CARD present in the NH_2-terminus region of APAF-1, and the CARD present in the prodomain of procaspase-9. Active caspase-9 is then capable of recruiting and activating effector caspases. This figure is adapted with permission from: Ledgerwood, E. C., & Morison, I. M. (2009). Targeting the Apoptosome for Cancer Therapy. Clinical Cancer Research, 15(2), 420-424. doi:10.1158/1078-0432.ccr-08-1172

APAF-1 is a 130 kDa protein comprised of a CARD at its *N*-terminus, a CED4 homology domain, and 12 or 13 WD40 repeats at its *C*-terminus. When bound to cytochrome *c*, APAF-1 is then capable of forming the apoptosome, a ring-like heptamer of APAF-1 molecules with CARD regions facing its center (Acehan *et al.,* 2002, Shemarova, 2010, Kasuba *et al.*, 2015). The apoptosome recruits procaspase-9, which unlike other initiator caspases, contains a CARD that allows it to interact with, and be activated by the apoptosome. APAF-1 orthologues have been identified in lower multicellular eukaryotes, such as *C. elegans*, *D. melanogaster*, and *Schmidtea mediterranea*, which can induce PCD in a cytochrome *c*-independent and/or dependent fashion (Alberts *et al.,* 2002, Moharikar *et al.,* 2007, Kaczanowski *et al*., 2011). Interestingly, Moharikar *et al*., (2007), identified a 130 kDa protein using antibodies for the human APAF-1 in UV-C treated *C. reinhardtii*, a single-celled green alga. Together, these findings suggest that an APAF-1-like protein might be present in unicellular eukaryotes, and raises the possibility that apoptosome formation may also occur during unicellular PCD. However, *in silico* analyses attempting to identify the potential APAF-1 orthologue in *C. reinhardtii* revealed the prime candidate to possess the expected WD-40 domains, while lacking the CARD and AAA16 domains (Moharikar *et al*., 2007). It is possible that the potential APAF-1 orthologue identified in *C. reinhardtii* and the *in silico*-derived sequence are not the same, raising the possibility that the organism might not possess a true orthologue.

Apoptosis inducing factor. Apoptosis inducing factor (AIF), a mitochondrial protein located in the IMM and participant in oxidoreductase functions, has also been shown to possess apoptotic activity when released into the cytosol. Following OMM

permeabilization, AIF translocates into the nucleus, and induces DNA degradation and peripheral chromatin condensation. Regulation of cytosolic AIF occurs via interaction with heat shock protein 70 (hsp70). Interestingly, AIF has been reported to be active during apoptosis in both a caspase-dependent and -independent manner. (Bajt *et al.*, 2006, Kaczanowski *et al.*, 2011).

Endonuclease G. Further support for the role of the mitochondria in PCD is noted in the activity of the mitochondrial enzyme, endonuclease G. Endonuclease G is a member of the ββα-*me finger* family of nucleases, and displays non-specific DNA/RNA nuclease activity (Toro-Londono *et al.*, 2011). Notably, endonuclease G is present in a wide-array of multicellular and unicellular organisms, and has been reported to induce DNA fragmentation in a caspase-independent manner. However, under certain physiologic conditions, endonuclease G has been noted to interact with caspase-activated exonucleases. Moreover, endonuclease G has also been hypothesized to interact with AIF, thus suggesting a potential role in caspase-dependent PCD (Bajt *et al*., 2006, Kaczanowski *et al.*, 2011).

p53. The role of the mitochondria in cell death is critically regulated by the transcription factor, p53, which can activate pro-apoptotic Bcl-2 proteins and activate the intrinsic pathway of apoptosis (Haupt *et al*., 2003, Amaral *et al*., 2010). A wide-array of studies have shown p53 to be critical to a wide array of cellular processes, such as the canonical manifestation of PCD known as apoptosis (described in more detail later), DNA repair, cell-cycle arrest, cellular stress responses, and senescence (Vazquez *et al*., 2008, Amaral *et al*., 2010). Through induction of apoptosis, p53 can modulate cell number in multicellular organisms, as well as unicellular populations, via the removal of

damaged, infected, or excess cells. The activity of p53 is regulated by Mouse double minute 2 homolog (Mdm2), a protein that promotes the proteasomal-degradation of p53. However, under stress conditions, p53-stabilizing signals allow it to escape degradation, after which p53 can promote the upregulation of *APAF-1*, and pro-apoptotic Bcl-2 members such as *Puma*, *Bax*, and *Noxa* (Haupt *et al*., 2003, Amaral *et al*., 2010). *Puma* expression has been reported to mediate the translocation of cytosolic Bax to the OMM. Once present in the OMM, Bax homodimers facilitate the release of cytochrome *c*, thus promoting apoptosome formation. Noxa is a pro-apoptotic Bcl-2 protein and its upregulation is likely to aid in shifting the mitochondrial balance of Bcl-2 family members towards pro-apoptosis (Haupt *et al*., 2003).

Lysosomes. Recent studies suggest that the amount of lysosomal proteins present in the cytoplasm, in conjunction with the degree of lysosome permeabilization, is indicative of the PCD pathway (Li *et al*., 2000). Lysosomes can be indirectly involved in apoptosis by promoting the release of mitochondrial proteins and ROS via the disruption of OMM permeability. Of note, ROS has been reported to induce permeabilization of the lysosome, and promoting the release of lysosomal proteases into the cytosol. Interestingly, the aspartic lysosomal protease, cathepsin D, has been shown to induce PCD in T lymphocytes through activation of Bax. Potentially, the interaction between these two organelles promotes an ROS-cathepsin D feed-forward loop, involving an initial rise in ROS due to death stimuli, capable of inducing the continuous release of ROS directly and/or indirectly through mitochondrial and/or lysosomal leakage, respectively. However, lysosomes may also directly influence PCD via the release of cysteine lysosomal proteases, such as cathepsin B, which has been shown to play a role in

bile salt-induced apoptosis following translocation to the nucleus. Notably, lysosomal proteases have also been reported to induce caspase activity through direct cleavage of executioner caspases (Vancompernolle *et al*., 1998, Broker *et al.*, 2005).

Endoplasmic reticulum. Under stress conditions the ER can initiate PCD via cytoplasmic Ca^{2+} influx, and/or congestion of misfolded proteins (Narvaez & Welsh, 2001, Shiraishi *et al.*, 2006). The effector caspase 12 zymogen, which is located on the cytosolic face of the ER, is activated in response to ER damage, and consequentially triggers effector caspases. Elevation of cytoplasmic Ca^{2+} levels by the ER also induces the activation of calcium-activated neutral proteases, also known as calpains, which can act downstream of caspase-dependent pathways. Similar to lysosomes, the ER is also capable of influencing PCD via interaction with the mitochondria. Studies seeking to elucidate the 3D structure of organelle membranes have estimated that 5-20% of the total surface area of mitochondria is in close contact with the ER. Key to the aforementioned studies, was the biochemical isolation and identification of the ER's mitochondria-associated membranes (MAMs). MAMs are separated by a mere 10-25 nm, and their association is facilitated by Mitofusin 2 (localized in the OMM and ER membrane), as well as the Mmm1/Mdm10,12,34 complex (Mmm1 is localized in the ER membrane, Mdm 12 present in the cytosol, and Mdm 10 and 34 localized in the OMM). Interestingly, MAMs contain an array of enzymes responsible for regulating the lipidome. Reports have indicated that formation of MAMs is required for an array of key cellular processes, such as regulation of the morphology and functions of mitochondria, Ca^{2+} transport from the ER to mitochondria, formation of autophagosomes, phosphatidylserine import from the ER to mitochondria, and cell survival (Grimm *et al.*, 2012, Vance *et al.*, 2014). Further

studies on the role of the ER, and MAMs in particular, are key to understanding the complex role of organelles, and their interactions, during cellular death.

The cross-talk between organelles and the myriad proteases associated with PCD are key to a number of cellular processes. These interactions also vary in the type and number of molecules/organelles involved, and in turn, have allowed for the characterization of numerous forms of PCD (Alberts *et al.*, 2002, Shemarova, 2010).

Types of PCD. The process of PCD varies across life domains, is capable of being initiated by a number of stimuli, and possesses distinct hallmarks. The myriad forms of PCD can be regulated by CP family members (caspase-dependent PCD), or alternatively, by non-CP family members (caspase-independent PCD) (Fig. 5).

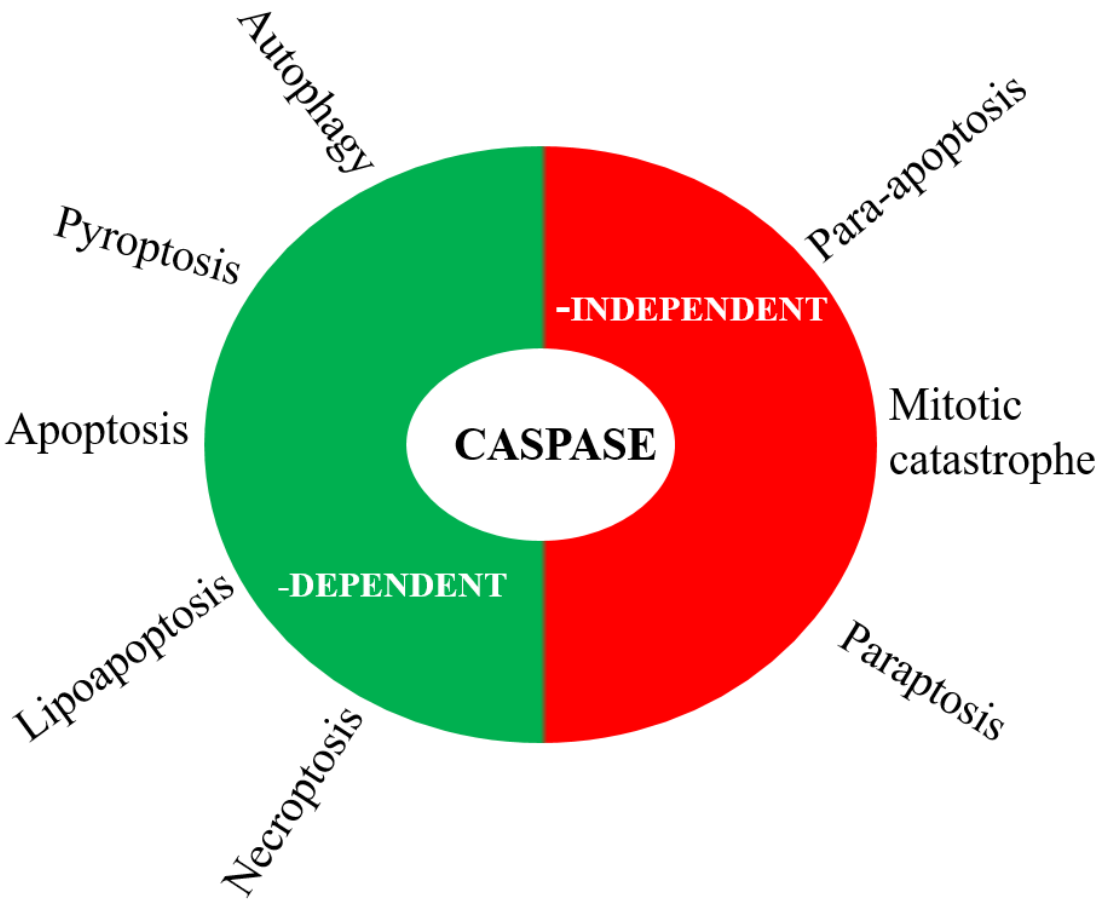

Figure 5. Schematic representation of caspase-dependence and -independence of programmed cell death types. Shown are certain forms of PCD that are either regulated by caspases (green), or not dependent on caspase activity (red). The respective location of PCD types on the wheel is not indicative of their degree of dependence/independence on caspases.

Caspase-dependent PCD. As the name suggests, these pathways require the activity of caspases in order for the downstream death signaling cascade to take place.

Apoptosis. This type of PCD can be characterized by a number of events, including: nuclear condensation, cytoplasm shrinkage, membrane blebbing, induction of proteases and endonucleases, and release of cytochrome c due to disruption of mitochondrial membrane potential. Apoptosis can be triggered by cellular damage via noxious agents or disease, as noted in its response to radiation-induced DNA damage, after which the cell may die via a *p53*-dependent pathway (Elmore, 2007, Kasuba *et al.*, 2015). Once induced, apoptosis utilizes a wide array of initiator (caspase-2,-8,-9,-10,-11,-12) and effector (caspase-3,-6,-7) CP proteases (Wang *et al.,* 2001, Lee *et al.,* 2008, Leblanc *et al.,* 2014, Mcilwain *et al*., 2017**)**. Moreover, apoptosis has also been reported to be triggered by the absence of survival factors, such as growth factors or hormones. The wide variety of pathological and physiological stimuli is a testament to the key role of apoptosis in the development and defense of living organisms (Elmore, 2007, Kasuba *et al.,* 2015).

Lipoapoptosis. This PCD form has been notably linked with obesity and aging, and is due to a disturbance in the lipid balance of cells (Unger & Orci, 2002, Kasuba *et al.,* 2015). Unger & Orci (2002) reported that when unoxidized long-chain fatty acids (LCFA) are present within nonadipose tissue, such as due to metabolic disturbances, they are then capable of entering the toxic de novo ceramide pathway and initiating lipoapoptosis, thus resulting in cell death. The caspase-dependence of lipoapoptosis is made evident by the activity of caspase-2. In *Xenopus* extracts, the down-regulation of caspase-2 is reported to impair cell death via LCFA accumulation. Conversely, disruption

of LCFA accumulation, via metabolic treatment, is reported to inhibit caspase-2 activation, thus providing further support for the caspase-dependence of lipoapoptosis (Johnson *et al*., 2013).

Necroptosis. Necroptosis is a recently discovered type of PCD that is key to homeostatic maintenance in metazoans. This deceptively-named PCD form consists of hallmarks similar to those of necrosis, however, this form is mediated by members of the receptor-interacting serine/threonine-protein kinase (RIPK) and mixed lineage kinase domain-like protein (MLKL) families (Cai *et al.*, 2013, Kasuba *et al.*, 2015, Pasparakis & Vandenabeele, 2015). Due to the observed cell swelling and subsequent membrane rupture, necroptosis is believed to act in a pro-inflammatory manner. Interestingly, caspase-8 has been reported to suppress necroptosis, while promoting apoptosis. This is evidenced in the interaction of caspase-8 with cellular FLICE (FADD-like IL-1 – converting enzyme)-inhibitory protein (c-FLIP), a key anti-apoptotic protein. Studies have shown that caspase-8/c-FLIP heterodimers are key to suppression of necroptosis. Additionally, in the absence of c-FLIP, caspase-8 fails to inhibit necroptotic death (Oberst, 2015).

Pyroptosis. Also known as caspase-1-dependent cell death, this mode of PCD is infamously associated with microbial infections, but can also be triggered by various other stimuli, such as stroke, heart attack, and cancer. When active, caspase-1 induces conversion of inactive interleukin-1β (IL-1β) and IL-18 into mature inflammatory cytokines, which ultimately lead to rapid disruption of plasma membrane integrity, and consequential release of pro-inflammatory intracellular content. Specifically, caspase-1 induces a net increase in osmotic pressure by producing plasma membrane pores that

dissipate cellular ionic gradients. In turn, the cell is subjected to water influx that ultimately leads to swelling, and subsequent osmotic lysis and release of intracellular content. The ubiquitous presence of mechanisms against pyroptosis amongst various pathogens, which allows them to persevere, and ultimately induce diseases, is a testament to the critical role of pyroptosis as a defense mechanism (Fink & Cookson, 2005, Suzuki *et al*., 2007, Bergsbaken *et al*., 2009).

Autophagy. Though referred to as Type II cell death, the classification of autophagy as a true form of cell death has been a topic of debate. Autophagy is a cell survival mechanism that employs the lysosome for removal of unwanted cellular components within cells. Autophagy can be stimulated by high temperatures, as well as conditions of hypoxia and starvation, and plays a role in cellular remodeling in response to stress, damage, or differentiation induced by cytokines (Tsujimoto & Shimizu, 2005, Kasuba *et al.,* 2015). Recent studies on mice have indicated that heterogeneous disruption of the autophagy gene, *Beclin 1*, leads to increased tumorigenesis (Broker *et al.,* 2005). Studies in organisms such as *Drosophila melanogaster* and *S. cerevisiae* helped reveal that autophagy is regulated by phosphoinositide-3 kinase (PI3K) types I and III. PI3K type I is mediated by growth factors, and PI3K type III is regulated by the autophagy related 12-homolog (Atg12) and Atg8 pathways (Sinha *et al.,* 2005). Autophagy can be categorized by the manner in which cellular components are translocated to the lysosome; these being, macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. During macro-autophagy, the cell utilizes the lysosome's autophagic vacuoles, known as autophagosomes, which are double membrane vesicles that can fuse with lysosomes in order to degrade the stored cellular components. In micro-autophagy, the lysosome itself

is capable of taking in cellular components via invagination of the lysosomal membrane. Chaperone-mediated autophagy involves the chaperone-mediated translocation of cellular components across the lysosomal membrane, and is dependent on the lysosomal-associated membrane protein 2A (LAMP-2A) receptor's ability to recognize the chaperone protein complex carrying the targeted cellular components (Glick *et al*., 2010). Studies have shown that caspases are responsible for a high degree of cross-talk between apoptosis and autophagy. Studies on myeloma cells have reported that Bcl-2-associated transcription factor 1 (BCLAF-1), a protein capable of inducing autophagy, is cleaved and inactivated by caspase-10, thus inhibiting autophagic death. Moreover, caspase-9 is suggested to regulate autophagy via interaction with atg7, which promotes atg7-mediated formation of autophagosomal LC3-II. Furthermore, caspase-3 has been shown to cleave atg4D at the DEVD^{63}K motif, and consequentially promoting atg4D-coordinated autophagy (Lamy *et al*., 2013).

Caspase-independent PCD. The absence of caspase activity in certain forms of PCD introduces another layer of PCD regulation (Broker *et al*., 2005).

Para-apoptosis. This is a non-canonical form of apoptosis, which is a morphologically distinct type of PCD, in which swelling of the mitochondria and endoplasmic reticulum leads to cytoplasmic vacuolization, as well as condensation of the chromatin (Broker *et al.*, 2005, Jimenez *et al.*, 2009, Kasuba *et al.*, 2015). This pathway is mediated by the activity of mitogen activated protein kinases (Broker *et al.*, 2005). Interestingly, studies on mice helped reveal a variant form of para-apoptosis, known as paraptosis (Jimenez *et al.*, 2009, Kasuba *et al.*, 2015).

Paraptosis. This PCD type is capable of being triggered by the insulin-like growth factor I receptor (IGF-1), as well as TAJ/TROY, a member of the TNF family of receptors (Broker *et al*., 2005). Interestingly, this variant form of para-apoptosis has also been shown to be activated by a catalytic mutant of Caspase-9 that is APAF-1-independent. Though morphologically similar, paraptosis has been noted to be resistant to caspase inhibitors, and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL)-negative (Jimenez *et al.*, 2009, Kasuba *et al.*, 2015).

Mitotic catastrophe. This type of PCD is triggered by DNA damage and microtubule destabilizing agents. Consequentially, mitotic failure occurs in response to cell cycle checkpoint defects, and subsequent development of aneuploid cells. Mitotic catastrophe can occur in a p53-independent manner, typically around metaphase, or in a p53-dependent manner, after failed mitosis. Mitotic catastrophe is resistant to caspase inhibitors and Bcl2 overexpression; however, studies have also noted that this form of PCD is typically followed by caspase activation and permeabilization of the mitochondrial membrane (Broker *et al*., 2005).

The importance of PCD is evidenced in its conservation across all life domains. The multiple forms of PCD, as well as their distinct layers of regulation, shed light onto the complexity of cellular death. Interestingly, myriad studies on unregulated cell death, as well as the various types of PCD, have led to the hypothesis that cell death might be more aptly described as a spectrum, rather than a dichotomy.

Role of PCD. Cells capable of undergoing a genetically controlled death provide a significant boost to their population's fitness; however, if unregulated, PCD can also be detrimental to the population (Kaczanowski *et al*., 2015).

Role in multicellular organisms. Within multicellular organisms, PCD has evolved to play different roles at varying stages of development (Kaczanowski *et al.,* 2015). In vertebrates, PCD is key to regulation of organ and tissue development. During early development of the vertebrate nervous system, PCD allows for the elimination of any neuron whose axons fail to innervate their target. It is hypothesized that the initial excess of neurons allows the organism to ensure that all targets are properly innervated. The ability of PCD to facilitate the removal of cells with a temporary function provides insight into its key role in multicellular organisms (Aballay & Ausubel, 2001). Interestingly, the aforementioned role in neuronal development is also present in *Drosophila melanogaster*. Normal adult eye differentiation in *D. melanogaster* is dependent on the elimination of superfluous interommatidial cells that surround the photoreceptor clusters (Denton *et al*., 2013). The PCD-mediated loss of redundant cells is also critical for rat eye development. Sinha *et al*. (2005) identified a mutation to the *Nuc1* gene in rats, that when present in a single locus, hinders the PCD-mediated loss of nuclei from lens fiber cells, and leads to a novel eye phenotype.

The key role of PCD is not limited to neuronal developmental events. During the metamorphosis of a tadpole into a frog, the cells that constitute the tadpole tail undergo PCD, thus making the tail absent in the advanced stage of the organism (Alberts *et al.,* 2002, Fagundes *et al*., 2015). Similarly, the PCD-mediated loss of interdigital cells is also key to the proper sculpting of paws in mice (Alberts *et al.,* 2002). Even in multicellular organisms with few cells, such as *Caenorhabditis elegans*, PCD remains just as critical (Dekkers *et al*., 2013). Aballay and Ausubel (2001) noted that when infected with

Salmonella typhimurium, excess germ cells present in the *C. elegans* gonad will undergo PCD, thus removing cells that could become detrimental to the developing organism.

Role in unicellular organisms. The role of PCD within unicellular organisms has been the subject of much debate over the years. Studies on unicellular organisms such as *Saccharomyces cerevisiae*, *C. reinhardtii*, the protozoans *Leishmania* and *Plasmodium*, and the dinoflagellate *Amphidinium carterae*, have shown each of the respective PCD hallmarks to be similar to those of multicellular organisms (Kaczanowski *et al.*, 2011, Kasuba *et al.*, 2015). These hallmarks include a disrupted plasma membrane, increased vacuolization, translocation of mitochondrial proteins, degradation of internal cellular components, and cell shrinkage (Jimenez *et al.*, 2009, Kaczanowski *et al.*, 2011, Kasuba *et al.*, 2015).

Studies in unicellular organisms suggest that PCD programs precede the existence of multicellular species. A contemporary theory is that PCD provides unicellular populations with the capacity to optimize their numbers (cell death/survival) in response to environmental conditions (nutrients, stressors, etc.,), thus aiding in the proliferation of their genome. Additionally, PCD might also confer unicellular organisms with an added layer of security for the population, through removal of cells incapable of promoting and maintaining genome clonality. Interestingly, studies using the unicellular chlorophyte, *Dunaliella viridis*, have shown the presence of a myriad of types of non-apoptotic cell-death programs. These programs were dubbed as such due to utilizing the PCD molecular machinery (e.g., PCD proteases, endonucleases, etc.), yet displaying a characteristic set of PCD phenotypes, such as cytoplasmic swelling and chromatin clusters, that are not observed during apoptosis (Jimenez *et al.*, 2009, Kasuba *et al.*, 2015). Jimenez *et al.*

(2009), noted that in response to different intensities of a stimulus, *D. viridis* was capable of undergoing varying types of non-apoptotic PCD. More intriguing, such findings raise the possibility that cell death/cell survival is closer to a continuum rather than a dichotomy, in which apoptosis and necrosis represent varying extremes. An understanding of the numerous types of PCD is therefore key to elucidating its crucial function within eukaryotic species.

Unregulated cell death. In stark contrast to the controlled death of a cell, hallmarks of unregulated cell death, or necrosis (hereafter referred as such), include random clumping and degradation of nuclear DNA, presence of autophagosomes, swelling of the cell and cellular organelles, and rupture of the plasma membrane. Necrosis has been linked to a number of human malignancies, such as Alzheimer's disease, Parkinson's disease, epilepsy, amyotrophic lateral sclerosis, and Huntington's disease. Similar to PCD, necrosis can be initiated by various stimuli, such as toxin exposure, hypoglycemia, prolonged hypoxia, extreme temperature changes, nutrient deprivation, and ischemia (Rieckher & Tavernarakis, 2009).

Mechanisms of necrosis. Necrotic cell death mechanisms vary widely, and are noted to be triggered by hyperactive mutations to ion channel receptors. Studies on *C. elegans* have provided further insight into the mechanisms associated with necrosis. Syntichaki and Tavernarakis (2004) identified two degenerin family members, *deg-1* and *mec-4*, whose hyperactivity leads to initiation of necrosis in distinct *C. elegans* neurons. Notably, *mec-4* gain-of-function mutations induced necrosis in touch receptor neurons, whereas *deg-1* gain-of-function mutations initiated necrosis in interneurons of the posterior touch sensory circuit. Studies in *Drosophila* have also noted that

downregulation of excitatory amino acid transporters (EAAT) induces necrosis-like hallmarks in neuropils, or regions of unmyelinated nerve fibers (Rieckher & Tavernarakis, 2009). The role of ion channel receptors in necrosis is further evidenced in reports that downregulation of the calcium binding chaperones, calreticulin and calnexin, inhibits necrosis in *mec-4* gain-of-function mutants. Similarly, inhibition of Ca^{2+} release from the ER, through mutations of ryanodine receptors (RyR) such as *unc-68* and *itr-1*, has also been shown to inhibit necrosis. However, the activity of cellular agents of necrosis is also dependent on interaction between distinct organelles (Syntichaki & Tavernarkis, 2004, Rieckher & Tavernarakis, 2009).

Organelles associated with necrosis. Similar to PCD, the organelles associated with necrosis also display a high degree of interaction.

Mitochondria. The pro-apoptosis Bcl-2 proteins, Bax and Bak, are key to the initiation of PCD. Interestingly, studies suggest that Bax and Bak may be just as integral to the activation of necrosis. The protein cyclosporin A (CsA) belongs to a family of metabolites tasked with inhibiting the mitochondrial transition pore from opening, and consequentially stopping molecules from accumulating in the intermembrane region. The action of CsA is reported to inhibit necrotic cell death via cell injuries such as oxidative stress, pH-dependent ischemia, or Ca^{2+} ionophore toxicity (Lemasters *et al.*, 1999). Recent findings suggest that the absence of Bax and Bak is also capable of providing cells with greater resistance to necrosis by inhibiting the mitochondrial transition pore from opening. Moreover, the size of infarctions in triple-knockout mice lacking Bax, Bak, and cyclophilin D, a critical regulator of necrosis, is reported to be comparable to those of double-knockout mice lacking Bax and Bak. The aforementioned effects are

reversed upon introduction of wild-type Bax in either mutant cell types, thus suggesting that pro-apoptosis Bcl-2 proteins may play a role at both ends of the cell death spectrum (Whelan *et al*., 2012).

Endoplasmic reticulum. The role of the ER in necrosis stems from defects to its inositol-1,4,5-triphosphate and/or RyR receptors, which are followed by an increase in cytosolic Ca^{2+} levels. High Ca^{2+} levels have been shown to induce the localization of the cytosolic protease, calpain, to the lysosome, which leads to lysosomal membrane degradation and subsequent release of lysosomal proteases. In turn, the proteolytic activity of lysosomal proteases in the cytoplasm induces the rupture of the plasma membrane and breakdown of the cell (Broker *et al.,* 2005). Moreover, given the key role of MAMs during PCD, and in a number of cellular processes, they could potentially be a factor in the array of irregular activities observed during necrosis (Grimm *et al.,* 2012, Vance *et al.,* 2014).

Lysosomes. Studies have also shown that defects in lysosomal function, as seen in *cup-5* mutants, lead to enlarged lysosomes capable of aggregating around the swollen nucleus, as well as lysosomal rupture (Artal-Sanz *et al.,* 2006). Further support for a lysosomal role in necrosis is derived from *C. elegans* studies, which revealed that the alkalization of lysosomes leads to the inhibition of necrotic cell death (Broker *et al*., 2005). Modulation of lysosomal pH allows extracellular pathogens to avoid removal by macrophages, thus promoting resistance against host defense mechanisms. The fungal pathogens *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*, as well as the bacterial pathogens *Mycobacterium tuberculosis*, *Salmonella enterica*, and

Listeria monocytogenes, have been reported to disrupt autophagosomal acidification in order to persist in host cells (Danhoff *et al*., 2016).

The critical role and conservation of cell death mechanisms in living organisms has been reported in a wide array of studies, particularly in mammals. The discovery of novel molecules and organelles involved in cellular death, has also stimulated an increase in the diversity of studied organisms (Kasuba *et al.,* 2015). Nonetheless, the empirical literature is plagued by a number of gaps that inhibit us from further revealing this critical process.

Gaps in the literature.

The high Ca^{2+} levels responsible for the opening of the mitochondrial transition pore, as well as the numerous molecules that affect the permeability of distinct organelles, are major role players in both PCD and necrosis. A number findings have also identified a wide array of PCD types that have led to the speculation that cell death cannot be characterized as a mere dichotomy, but rather, that it is best viewed as a wide spectrum consisting of regulated and unregulated forms of cell death. The multiple gaps in our understanding of cell death are due to myriad factors, including the complexity of its mechanisms, the intricate cross-talk between organelles, and the array of unidentified role players of cell death. Moreover, the relative lack of diversity in the model organisms used in cell death studies have led to a greater understanding of PCD in multicellular eukaryotes, such as mammals, compared to unicellular eukaryotes, such as *C. reinhardtii* (Moharikar *et al.,* 2007, Amaral *et al.,* 2010).

Proposed study

Knowledge of PCD is largely derived from studies in multicellular organisms. As characterized in mammals, caspases are key to the regulation of PCD. The lack of caspases in a vast array of organisms, as well as the conservation of PCD phenotypes across all life domains, prompted the search for caspase orthologues. The recent discovery of metacaspases and paracaspases helped fill an extensive gap in the literature; nonetheless, these novel CPs remain poorly understood (Tsiatsiani *et al.*, 2011). The current study takes a reverse genetic approach to study MCA1 in *C. reinhardtii*, to determine PCD phenotypes that are potentially modulated by MCA1, and further elucidate the role of metacaspases.

Within eukaryotes, APAF-1 is responsible for formation of the apoptosome complex, and the subsequent recruitment of caspases that allow it to play a regulatory role during PCD. Though *C. reinhardtii* has been suggested to possess an APAF-1 gene product, its function remains elusive (Moharikar *et al.*, 2007). If indeed present, *C. reinhardtii* would be the sole unicellular organism to possess a verified APAF-1 orthologue, thus suggesting a key role in this species, which may further elucidate the evolutionary origin of eukaryotic PCD. Moreover, the current study seeks to identify the function of APAF-1 within *C. reinhardtii*. Within mammals, the apoptosome interacts with the prodomain of caspase 9, and to date, APAF-1 has not yet been shown to interact with caspase orthologues; therefore, this study seeks to determine if an interaction between the APAF-1 and MCA1 prodomains occurs. Of note, at the onset of this thesis an MCA2 mutant *C. reinhardtii* was not available for acquisition.

Why C. reinhardtii? In response to various forms of stress (e.g., heat, oxidative, toxic), unicellular organisms, such as alveolates of the class Dinophyceae (*Peridinium gatunense*, *Alexandrium tamarense*, *Amphidinium carterae*) and green algae of the class Chlorophyceae (*C. reinhardtii*, *Chlorella saccharophila*, *Dunaliela tertiolecta*) have been reported to display PCD phenotypes (e.g., ROS accumulation, increased vacuolization, TUNEL positivity, DNA laddering) akin to multicellular organisms (Kasuba *et al.*, 2015). *C. reinhardtii* is a fitting model for cell death studies due to a number of factors: 1) different types of stress can be homogenously applied to all cells in a culture; 2) their heat-stress response is well documented, and temperatures of ~42 ^{o}C have been reported to induce PCD; 3) they possess only two types of putative metacaspases (MCA1 and MCA2); and 4) relative to higher eukaryotes, they possess fewer gene families, thus being a simpler model for understanding eukaryotic PCD mechanisms (Schroda *et al.*, 2015).

Hypotheses & research design. The following hypotheses are made.

H1$_0$. *MCA1* knockout does not affect heat stress-induced PCD phenotypes in *C. reinhardtii*.

H1$_1$. *MCA1* knockout does affect heat stress-induced PCD phenotypes in *C. reinhardtii*.

Research design for H1. To test this hypothesis both WT (CC-4533) and *MCA1* knockout (*mca1*) cells were exposed to heat-stress at 42 ^{o}C (for PCD induction), or control conditions. The cells were then subjected to cell death assays and analyzed for the following PCD phenotypes: externalization of phosphatidylserine (PS), DNA laddering, plasma membrane disruption, accumulation of ROS, and compromised cell proliferation.

$H2_0$. Heat stress-induced PCD in *C. reinhardtii* will have no effect on formation of an apoptosome complex.

$H2_1$. Heat stress-induced PCD in *C. reinhardtii* will have an effect on formation of an apoptosome complex.

$H3_0$. MCA1 is not required for formation of an apoptosome complex in *C. reinhardtii*.

$H3_1$. MCA1 is required for formation of an apoptosome complex in *C. reinhardtii*.

Research design for Hypotheses 2 and 3. These hypotheses were tested by first conducting whole-cell extraction of heat-stressed, or control (WT) and *mca1* cells. The lysates from heat-stressed cells, and respective controls, were then subjected to sucrose gradient centrifugation, SDS-PAGE, and Coomassie blue staining, followed by Western blotting with an antibody against human APAF-1.

Impact of study

The observation that PCD occurs in both multicellular and unicellular organisms (Alberts *et al.,* 2002, Shemarova *et al.,* 2010, Kasuba *et al.,* 2015) has forced us to re-think our understanding of altruistic cell death. Adding to the mystery, some of the molecular components associated with PCD are also well conserved in both structure and function (Aballay & Ausubel, 2001, Shemarova, 2010, Murik *et al.,* 2013). Though reported in the *C. reinhardtii* genome, the role of metacaspases in this organism has been seldom analyzed, which inhibits us from further elucidating the mechanisms regulating unicellular PCD. Through an analysis of a *C. reinhardtii MCA1* knockout strain (*mca1*), this study sought to reveal the potential role of MCA1 in certain PCD phenotypes akin to

those of multicellular organisms (ROS accumulation, plasma membrane disruption, DNA laddering, PS externalization, and compromised cell proliferation). As will be discussed in the first half of this thesis (Chapter II), our data indicates that *MCA1* knockout is associated with significantly increased rates of plasma membrane disruption and DNA laddering within heat-stressed *C. reinhardtii* populations. Moreover, heat-stressed *mca1* populations were also linked to significantly earlier displays of immediate ROS accumulation, and a significantly greater ROS response with prolonged heat stress exposure. Conversely, disruption of *MCA1* was not associated with a significant effect on the rate of PS externalization or cell proliferation of *C. reinhardtii*.

As indicated in the second half of this thesis (Chapter III), proteomic analysis revealed that prior published results suggestive of APAF-1 presence in *C. reinhardtii* could be generated in the absence of antibodies against human APAF-1. Specifically, we recreated comparable results through sole usage of either goat anti-rabbit IgG, or goat anti-mouse IgG (1:5000, both). *In silico* analysis also revealed the prime APAF-1 candidates in *C. reinhardtii* to lack key conserved domains (CARD and AAA16). Together, our data refute the prior published report of a potential APAF-1 orthologue in *C. reinhardtii*. This dissertation provides key novel data to the empirical literature, as well as recommendations for future studies.

CHAPTER II

ANALYSIS OF METACASPASE 1 DURING HEAT STRESS-INDUCED PROGRAMMED CELL DEATH IN CHLAMYDOMONAS REINHARDTII

This dissertation follows the style and format of the *Journal of Cell Biology (JCB)*

Abstract

Programmed cell death (PCD) refers to the array of genetically-controlled physiological processes by which a cell ceases to exist. PCD is conserved across living organisms, and canonically, is regulated by members of the cysteine protease (CP) family. Initially, the CP family consisted only of caspases; however, the recent discovery of metacaspases and paracaspases has significantly impacted our understanding of the evolution of PCD. Metacaspases are subdivided into types I (MCA1) and II (MCA2), and are noted to be present in organisms whose PCD machinery is poorly understood, such as the unicellular green alga, *Chlamydomonas reinhardtii*. This study sought to expand our understanding of metacaspases via analysis of an *MCA1* knockout strain of *C. reinhardtii* (*mca1*). Results show that in response to heat stress, *mca1* populations are associated with significantly increased plasma membrane disruption. Moreover, heat-stressed *mca1* cells consistently displayed increased DNA laddering, relative to WT. Furthermore, in response to heat stress, *mca1* populations exhibited more rapid accumulation of ROS, as well as a significantly greater ROS response with prolonged heat stress exposure. Notably, *MCA1* knockout did not alter the rate of phosphatidylserine (PS) externalization, or affect cell proliferation. Together, our data suggest the potential that MCA1 modulates the early cellular response to heat stress-induced PCD within *C. reinhardtii*, and may function as a negative regulator of certain PCD events.

KEY WORDS: Chlamydomonas reinhardtii, Metacaspase, MCA1, PCD, Heat stress, Reverse genetics, ROS, DNA laddering, PS externalization, Plasma membrane disruption.

Background

The three life domains, Eukarya, Bacteria, and Archaea, inhabit a wide array of environments. Consequentially, all living organisms are exposed to numerous agents (molecular, environmental, etc.,) capable of compromising their survivability (Alberts *et al*., 2002). Each domain has developed an intricate array of mechanisms to mediate interactions with various stimuli. Programmed cell death (PCD) is one such mechanism, and refers to the array of genetically-controlled physiological processes by which cells cease to exist (Aballay & Ausubel, 2001, Alberts *et al*., 2002, Shemarova, 2010)

PCD is conserved across all three life domains, and its direct role in mediating cell-host interactions is key to an organism's fitness. PCD can be triggered by various stimuli, including the presence of toxic agents, an absence of growth factors, and disturbance to homeostasis (Unger & Orci, 2002, Kaczanowski *et al*., 2011, Kasuba *et al*., 2015). Organisms undergo PCD in a variety of forms (e.g., apoptosis, pyroptosis, lipoapoptosis) that are modulated by cysteine protease (CP) family members (Unger & Orci, 2002, Fink & Cookson, 2005, Leblanc *et al.,* 2014, Mcilwain *et al*., 2017). However, other forms of PCD (e.g., para-apoptosis, mitotic catastrophe) have also been reported to occur independently of such proteases (Broker *et al*., 2005, Jimenez *et al*., 2009, Kasuba *et al*., 2015).

In mammals, PCD is regulated by the CP family members known as caspases. These contain a cysteine residue at their active sites that are used to cleave at aspartate residues of target proteins, and consequently, promote the induction of PCD phenotypes. Upon stimulation of PCD, initiator caspases, which possess a death effector domain (DED)-containing prodomain, are capable of dimerizing with DED-containing adaptor

proteins, thus forming the death-inducing signaling complex (DISC). Formation of the DISC promotes the aggregation and activation of the caspases responsible for cleaving specific targets, and ultimately inducing PCD-associated events (Alberts *et al.*, 2002, Fink & Cookson, 2005). PCD is crucial to the normal development of vertebrates and invertebrates. For example, in vertebrates, PCD mediates the removal of excess neurons that fail to innervate their target within mammals, and eliminates the tail during the metamorphosis of a tadpole into a frog (Alberts *et al*., 2002, Fagundes *et al*., 2015). Within invertebrates, such as *Caenorhabditis elegans*, PCD modulates the removal of gonadal cells infected by extracellular pathogens, such as *Salmonella typhimurium* (Aballay & Ausubel, 2001). A number of studies on mammalian-pathogen interactions have also linked PCD irregularities with autoimmune, neurodegenerative, and malignant diseases (Mcarthy, 2003, Moharikar *et al.*, 2007, Dekkers *et al.*, 2013, Shemarova, 2010).

Our understanding of PCD is largely derived from studies of multicellular eukaryotes, and consequently, its mechanism and role in unicellular organisms is poorly understood. Uren *et al.*, (2000) identified two novel CP family members, metacaspases and paracaspases. These were revealed to be present across numerous genomes, including those of organisms lacking caspases. The domain organization of paracaspases is the least evolved amongst CP family members. Notably, paracaspases are involved in key cellular processes, such as regulation of antigen receptor signaling within humans, and cell morphology within the single-celled haploid amoeba, *Dictyostelium discoideum*, (Hulpiao *et al*., 2015, Staal *et al*., 2016). Intriguingly, metacaspases have been identified in numerous organisms that lack caspases, suggesting a key role in the evolution of PCD (Merchant *et al.*, 2007, Moharikar *et al*., 2007, Fagundes *et al*., 2015).

Metacaspases are cysteine proteases that cleave their targets at glycine and lysine residues. Metacaspases are subdivided into types I (MCA1) and II (MCA2), in accordance with their structure. The *N*-terminus of type I metacaspases contains a proline-rich prodomain, and a zinc-finger motif, while type II metacaspases have a linker region of 160-180 amino acids, but lack a prodomain (Fagundes *et al.,* 2015, Kasuba *et al.,* 2015). Of note, recent reports have also revealed a putative category of metacaspases, type III (MCA3), which display a distinct rearrangement of the p10 domain in the *N*-terminus, instead of the *C*-terminus, the relevance of which remains unknown (Choi & Berges, 2013). *In silico* analyses have noted that the genome of certain organisms, plants in particular, can encode various metacaspases of either type (Fig. 6), the consequence of which is not yet fully understood (Tsiatsiani *et al*., 2011).

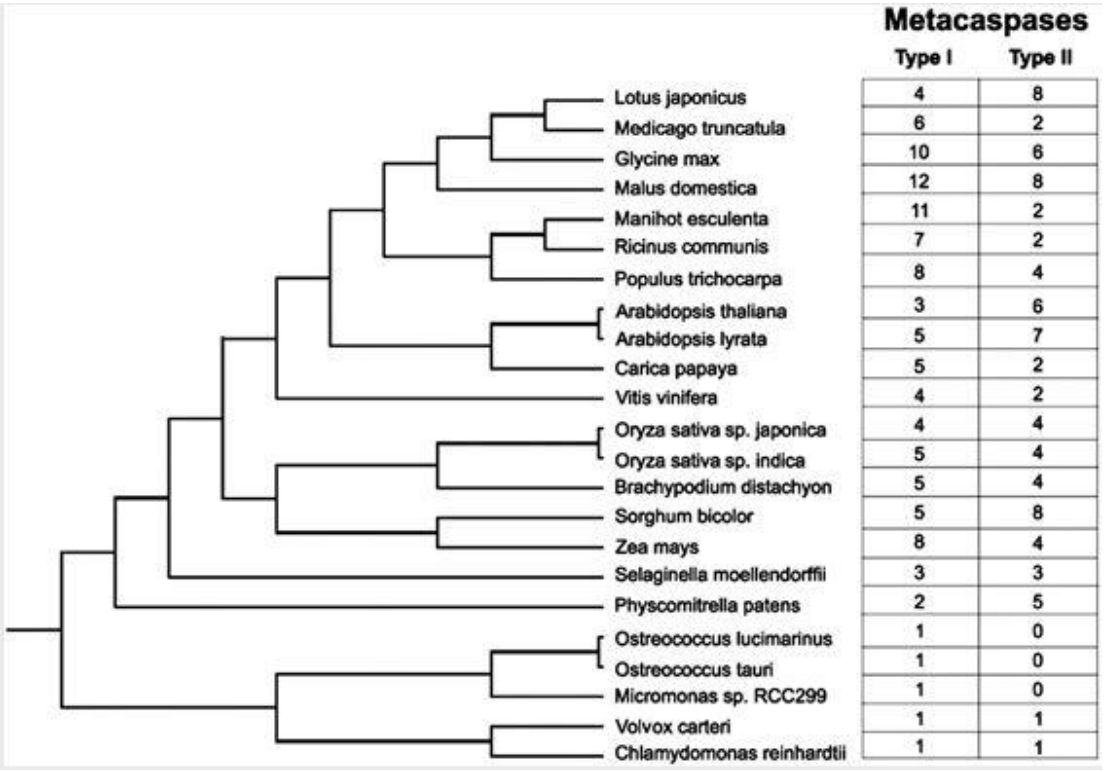

Figure 6. Distribution of metacaspase types-I and –II across plant and plant-like genomes. This figure is adapted with permission from: Tsiatsiani, L., Breusegem, F. V., Gallois, P., Zavialov, A., Lam, E., & Bozhkov, P. V. (2011). Metacaspases. *Cell Death and Differentiation, 18*(8), 1279-1288. doi:10.1038/cdd.2011.66

Studies have shown that tomatoes (*Esculentum personicum*) subjected to *Botrytis cinerea*, a fungal pathogen, undergo upregulation of the type II metacaspase, LeMCA1. The metacaspase genes present in rice, *Oryzea sativa*, have been reported to be upregulated during biotic and abiotic stress (Fagundes *et al.*, 2015). Of note, the unicellular green alga, *Chlamydomonas reinhardtii* genome encodes two metacaspases, MCA1 and MCA2 (Fig. 6) (Merchant *et al.*, 2007, Moharikar *et al.*, 2007, Tsiatsiani *et al.*, 2011); however, their role in PCD is unclear. For example, a study by Merchant *et al*. (2007) reported that during H_2O_2-induced PCD in *C. reinhardtii*, the addition of dehydroascorbate leads to a greater upregulation of *MCA2* than *MCA1*. A finding that could be due to either redundant, or antagonistic roles during PCD within *C. reinhardtii*. Furthermore, reports have indicated that PCD induced via mastoparan, a toxin (wasp venom) capable of stimulating various pathways (e.g., oxidative stress, phosphatidic acid, Ca^{2+}), can be suppressed in *C. reinhardtii* via caspase inhibitors during the late stages of PCD (Yordanova *et al.*, 2013). The current study seeks to examine the role of metacaspases during heat stress-induced PCD within *C. reinhardtii*. Specifically, this study investigated the effect of *MCA1* disruption on heat stress-induced PCD using a knockout strain. The *MCA1* knockout strain of *C. reinhardtii* (*mca1*) was acquired from the *Chlamydomonas* Resource Center (CRC), and generated via electroporation of a calcium and integrin-binding 1 (CIB1) cassette into the CC-4533 (WT) strain (Zhang *et al.*, 2014).

C. reinhardtii is reported to undergo PCD events akin to multicellular organisms (Kasuba *et al.*, 2015); thus, we expected that *MCA1* knockout within *C. reinhardtii* would delay the onset of PCD phenotypes known to occur in higher eukaryotes: DNA laddering,

plasma membrane disruption, phosphatidylserine (PS) externalization, the accumulation of reactive oxygen species (ROS), and compromised cell proliferation. Interestingly, our data indicated that *MCA1* knockout mutant populations are associated with an increased rate of plasma membrane disruption in response to heat stress. However, both the parent strain, CC-4533 (WT), and the *mca1* strain displayed a similar rate of PS externalization, as well as comparable cell proliferation. Additionally, the onset of ROS accumulation in response to heat stress occurred earlier in *mca1* cells (10 min), relative to the WT (20 min). Interestingly, we also noted that prolonged heat stress led to a second burst of ROS accumulation in both populations (~3 hrs.), that was significantly greater in *mca1* populations (45.6% of total cells), relative to WT (21% of total cells). Qualitatively, we also noted that *MCA1* disruption appears to lead to an increased rate of DNA laddering. Further analyses, both genetic and functional, are necessary to fully elucidate the role of *MCA1* in *C. reinhardtii*. Nonetheless, our data refute the null hypothesis that *MCA1* knockout does not affect heat stress-induced PCD phenotypes in *C. reinhardtii*, and instead suggest a potential role for MCA1 in modulation of PCD.

Results

Confirmation of *MCA1* knockout. The *MCA1* knockout mutant was generated via electroporation of the CIB1 cassette (~2,243 bps) into two separate regions of the *MCA1* locus (coding sequence and intron) and a chromosome 8 intron of the WT strain (Li *et al.*, 2016). The disruption of the target locus of the *mca1* strain was verified in accordance with Li *et al.*, 2016. Following the extraction of DNA from WT and *mca1* cells, PCR was performed using primers specific for *MCA1* (*MCA1* forward primer (G1): 5’-AGACTTGGTGGGGAACAGTG-3’, *MCA1* reverse primer (G2): 5’-

CAGTACCTCCAGACTTCCGC-3’). The lack of CIB1 insertions in WT strains was predicted to allow the amplification of *MCA1* (predicted amplicon size: ~1,692 bps); however, in the mutant strain, the presence of disruptive CIB1 cassettes was expected to inhibit the amplification of *MCA1* within the allotted elongation time (Fig. 7A). The resulting amplicons were subjected to gel electrophoresis, and the results indicated that *MCA1* was not amplified in the *mca1* strain (Fig. 7B).

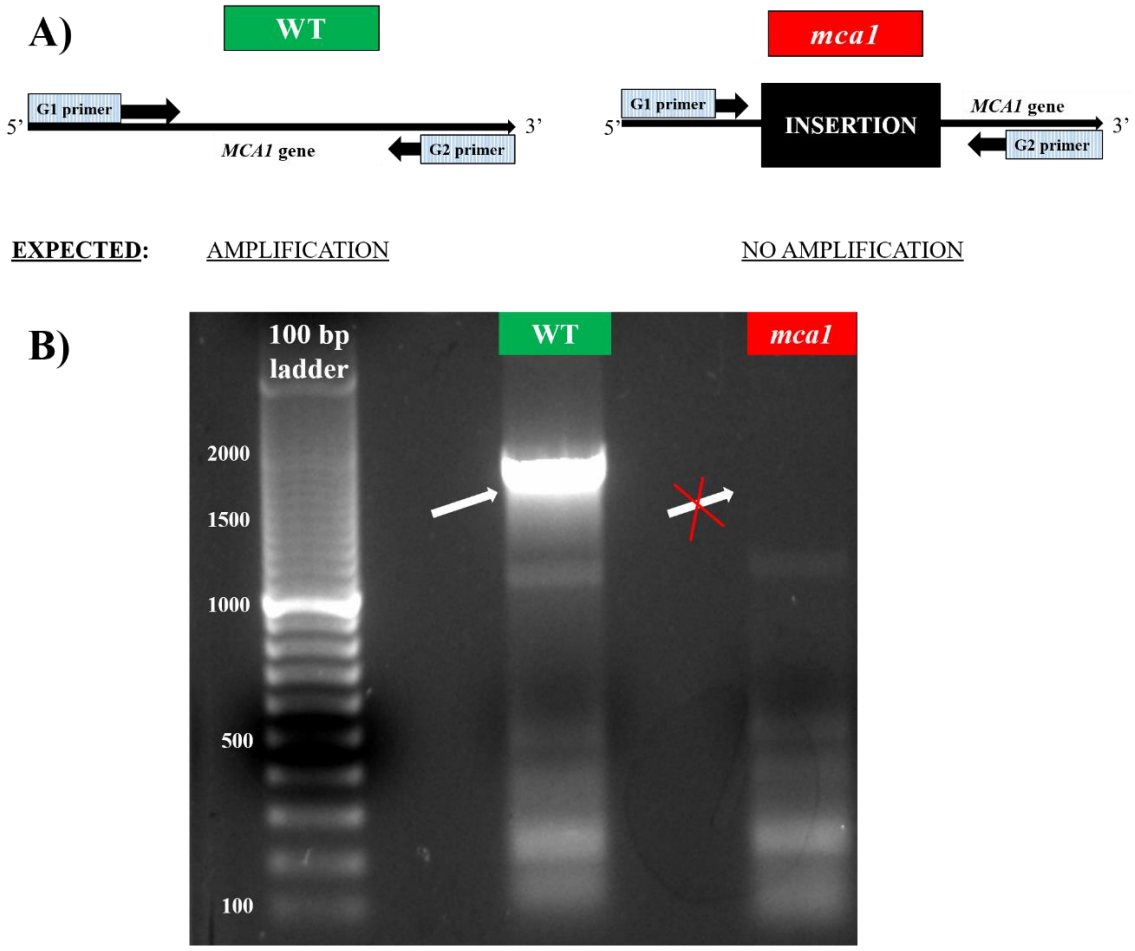

Figure 7. Agarose gel electrophoresis revealing a disrupted *MCA1* locus in the *mca1* strain of *C. reinhardtii*. Image of agarose gel electrophoresis results using primers for *MCA1* (expected size: ~1692 bps) and either WT, or *mca1*-derived DNA template. Normal arrow indicates the presence of an expected PCR amplicon, and crossed arrow indicates the absence of an amplicon.

Afterwards, the DNA lysate from the *mca1* strain was subjected to PCR using two separate sets of primers to confirm the presence of the CIB1 insertion within the *MCA1* locus of *mca1*: the first set (*mca1* A) consisted of a forward primer for *MCA1* (G1) and a reverse primer for the 5' CIB1 insertion junction (C1); the second set (*mca1* B) included a reverse primer for *MCA1* (G2) and a forward primer for the 3' CIB1 insertion junction (C2). The presence of a CIB1 insertion in the *MCA1* sequence of the mutant strain was predicted to allow for amplification using either primer set, but with variations in amplicon size (Fig. 8A). As expected, gel electrophoresis of the resulting amplicons indicated the presence of a CIB1 insertion in the target locus (Fig. 8B). To further validate *MCA1* disruption, the WT and *mca1* genomic DNA were sent for sequence analysis using the four separate primers. The resulting analysis indicated that the *MCA1* locus was indeed disrupted in the *mca1* strain (Table 1), and that the CIB1 insertion was present in the first exon of *MCA1* (Fig. 9).

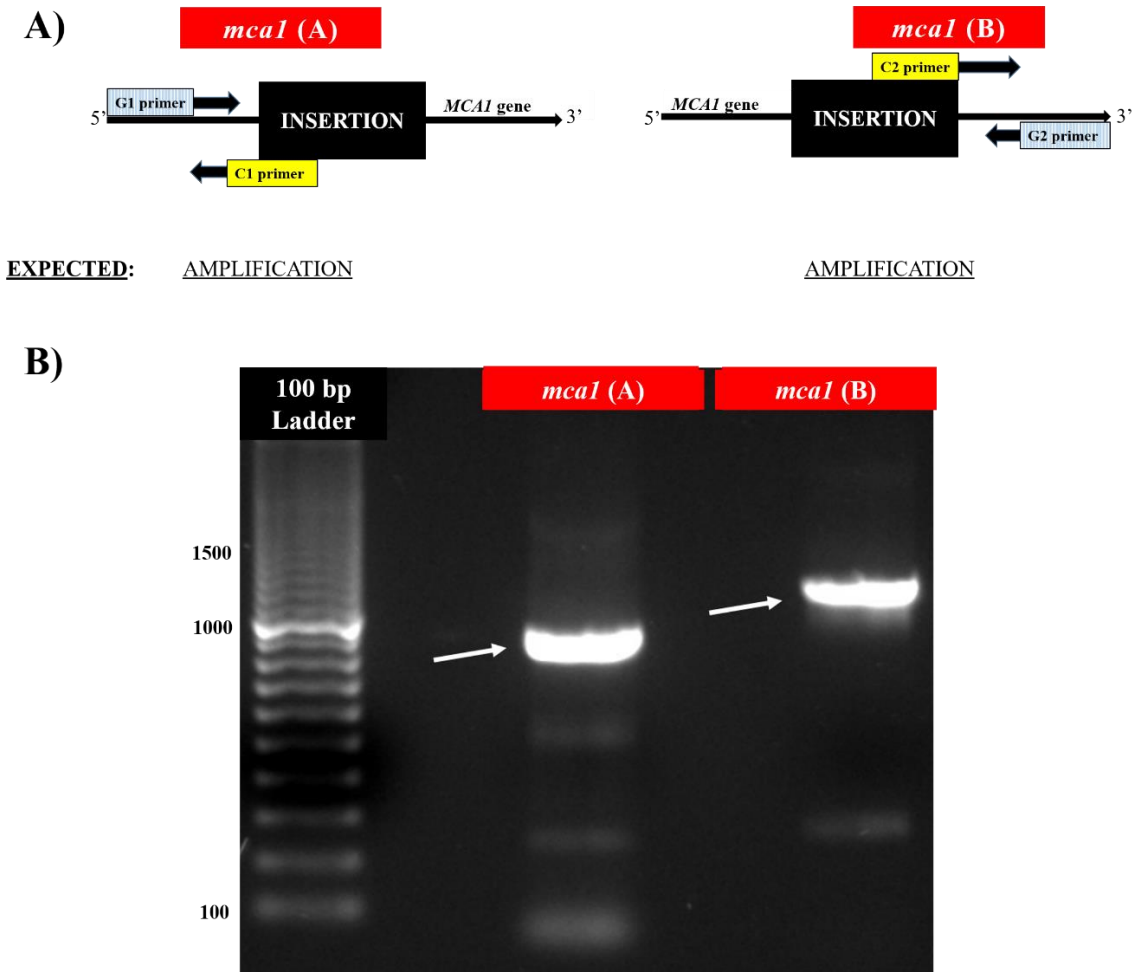

Figure 8. Presence of a CIB1 insertion in the target locus of the *mca1* strain of *C. reinhardtii*, as revealed by agarose gel electrophoresis of PCR amplicons. Agarose gel image depicting the presence of amplicons derived from PCR (arrows) using designated primer sets (*mca1* A or *mca1* B). The *mca1* A set consisted of a forward primer for *MCA1* (G1) and a reverse primer for the 5' CIB1 insertion junction (C1); the *mca1* B set included a reverse primer for *MCA1* (G2) and a forward primer for the 3' CIB1 insertion junction (C2).

Table 1

BLASTn results indicating the homology between the analyzed *mca1* strain sequences and the *MCA1* locus (NW_001843738.1) of *C. reinhardtii*.

Subject sequences	**Max score**	**Total score**	**E value**	**Identity to *MCA1***
MCA1 forward primer (**G1**)-derived sequence	1238	1471	0.0	99%
MCA1 reverse primer (**G2**)-derived sequence	1213	1278	0.0	89%
CIB1 5' region reverse primer (**C1**)-derived sequence	1243	1270	0.0	89%
CIB1 3' region forward primer (**C2**)-derived sequence	1011	1171	0.0	92%

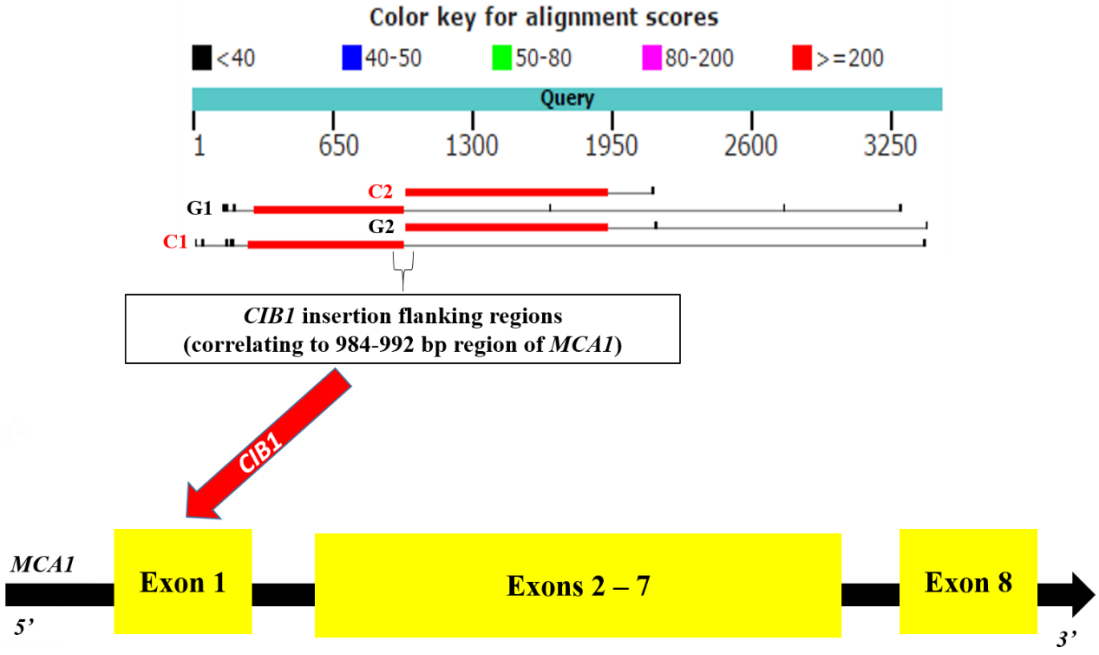

Figure 9. Schematic depiction of a CIB1 insertion within the target locus (first exon) of the *mca1* strain, as revealed via BLASTn. The *MCA1* forward (**G1**)-, *MCA1* reverse (**G2**)-, *CIB1* 5' region reverse primer (**C1**)-, and *CIB1* 3' region forward primer (**C2**)-derived sequences were subjected to homology searches against *MCA1* of *C. reinhardtii* via BLASTn. The *MCA1* FASTA sequence (query) is derived from the NCBI database (accession: NW_001843738.1). The analyzed amplicon sequences were used as the subject sequences.

***mca1* populations display increased plasma membrane disruption.** Disruption of the plasma membrane is a hallmark for various forms of PCD, as well as necrosis (Alberts *et al*., 2002, Elmore, 2007). WT and *mca1* strains were exposed to heat stress, and at various time points, samples were collected and subjected to an Evans Blue assay in order to assess membrane disruption. Viable cells can remove Evans Blue dye present in the cytosol (e.g., via efflux pumps); however, progressive disruption of the plasma membrane impairs the removal of Evans Blue dye from the cytosol. Though indirectly, Evans Blue staining allows for the visualization, and subsequent quantification, of cells whose plasma membrane has been disrupted (Baker & Mock, 1994, Osterloh *et al.,* 2002, Radu & Chernoff, 2013). Due to plasma membrane rupture typically occurring at the later stages of PCD (Elmore, 2007), Evans Blue staining was first examined at a wide-range of time-points. This revealed no significant difference in the rate of plasma membrane disruption between WT and *mca1* populations subjected to heat stress (Fig. 10A). However, both strain populations displayed a substantial increase in the rate of plasma membrane disruption between 15 and 45 min post-heat stress (WT: 20.5% to 94.3% of total cells, *mca1*: 17.8% to 92.5% of total cells, respectively). In the absence of heat stress there was negligible (> 1%) plasma membrane disruption (Fig. 10B).

Because of the marked increase in staining between 15 and 45 min after initiating heat stress, a second experiment was conducted to examine the response in more detail for the first40 min after heat stress. Heat-stressed *mca1* populations exhibited a significant increase in the amount of cells undergoing plasma membrane disruption, relative to WT (Fig. 11A). Negligible amounts of staining were observed for cells of either strain tested at room temperature (25 ^{o}C) (Fig. 11B**)**.

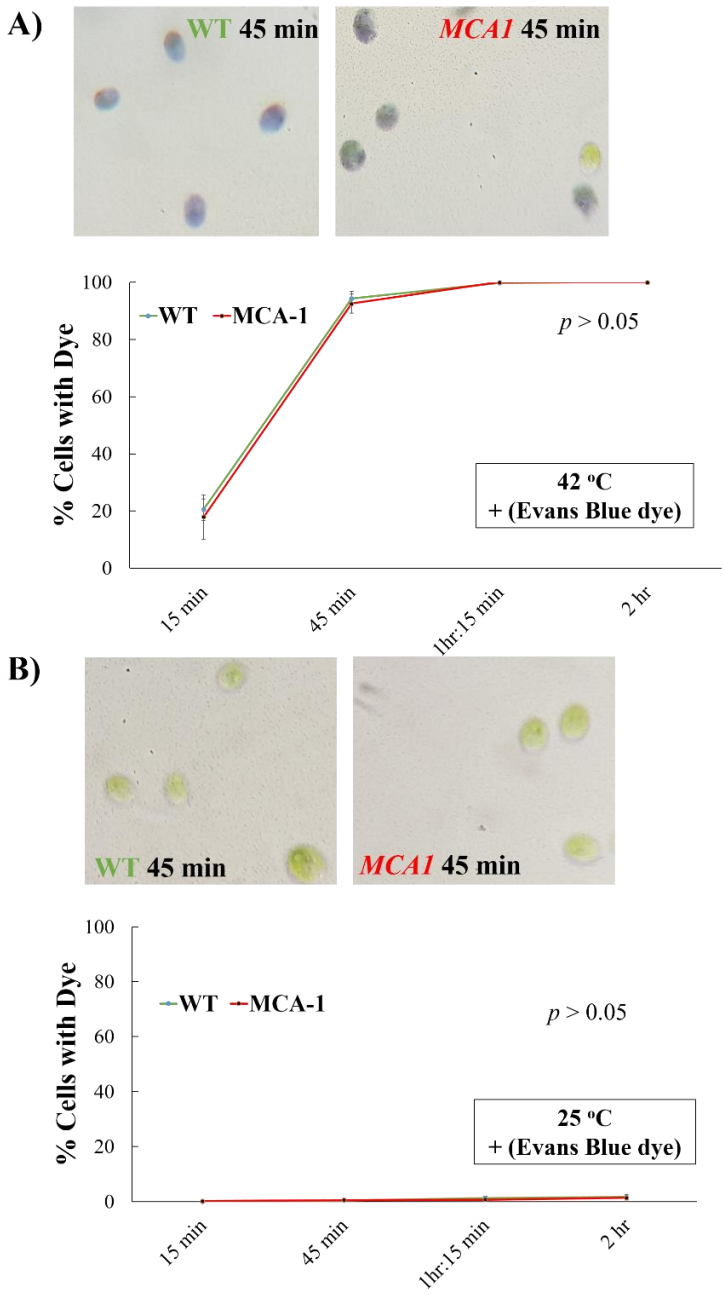

Figure 10. Mean (±SD) of *C. reinhardtii* cells displaying plasma membrane disruption in presence or absence of 42 ºC heat stress over a 2-hour timespan, as revealed by Evans Blue assays. Graphs show the percentage of WT or *mca1* populations displaying Evans Blue dye presence in the cell cytosol. At indicated time-points, results were determined via manual counts of cells in a random field of view (2-8 cells) until 200 cells were counted. Results derive from three independent assays. A Two-Way Repeated Measures ANOVA (*p* value of main effect displayed) with post hoc analysis using Tukey's multiple comparison test was conducted via statistical software (SAS 9.4 and JMP 13). Statistical significance was defined as $p < 0.05$. Individual graphs show the *p* value of the main effect (temperature x strain x observation).

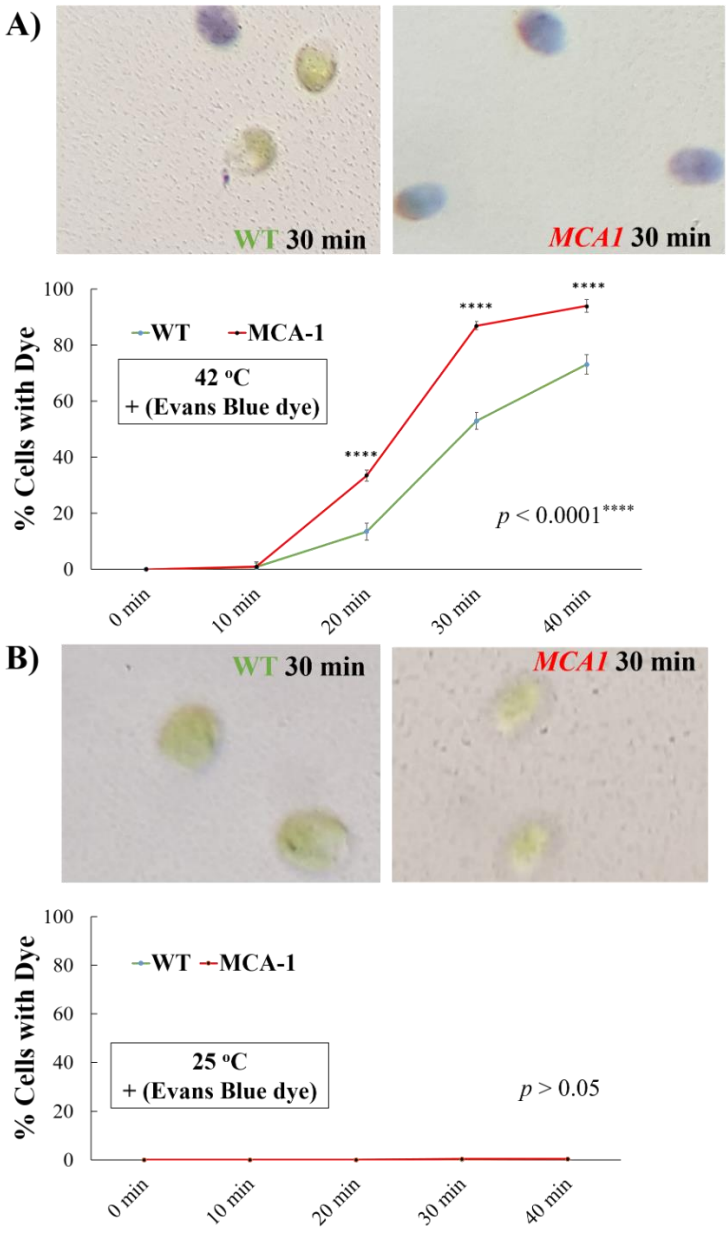

Figure 11. Mean (±SD) of *C. reinhardtii* cells exhibiting plasma membrane disruption in presence or absence of 42 °C heat stress over a 40-minute timespan, as revealed by Evans Blue assays. Graphs show the percentage of WT or *mca1* populations displaying Evans Blue dye presence in the cytosol. At indicated time-points, results were determined via manual counts of cells in a random field of view (2-8 cells) until 200 cells were counted. Results derive from three independent assays. A Two-Way Repeated Measures ANOVA with post hoc analysis using Tukey's multiple comparison test was conducted via statistical software (SAS 9.4 and JMP 13). Statistical significance was defined as $p < 0.05$. Individual graphs display either the p value of the main effect (temperature x strain x observation), or the post hoc analysis p value (****).

Since efflux pump disruption can also occur while the plasma membrane remains intact, plasma membrane disruption was further tested via a fluorescence microscopy assay using a membrane-impermeable SYTOX Green fluorophore. SYTOX Green is a DNA-intercalating dye whose fluorescence is significantly enhanced (>1000 fold) upon DNA binding (Thakur *et al.*, 2015). The results indicated that fluorescence was significantly greater in *mca1* cells exposed to heat stress (Fig. 12). Intriguingly, heat-stressed *mca1* cells also exhibited a substantial decrease in fluorescence post-50 minutes, suggesting the possibility of either plasma membrane repair, or inhibition of fluorophore binding. Both temperature and negative (no dye) controls displayed negligible (< 3%), or no fluorescence, respectively (Fig. 12).

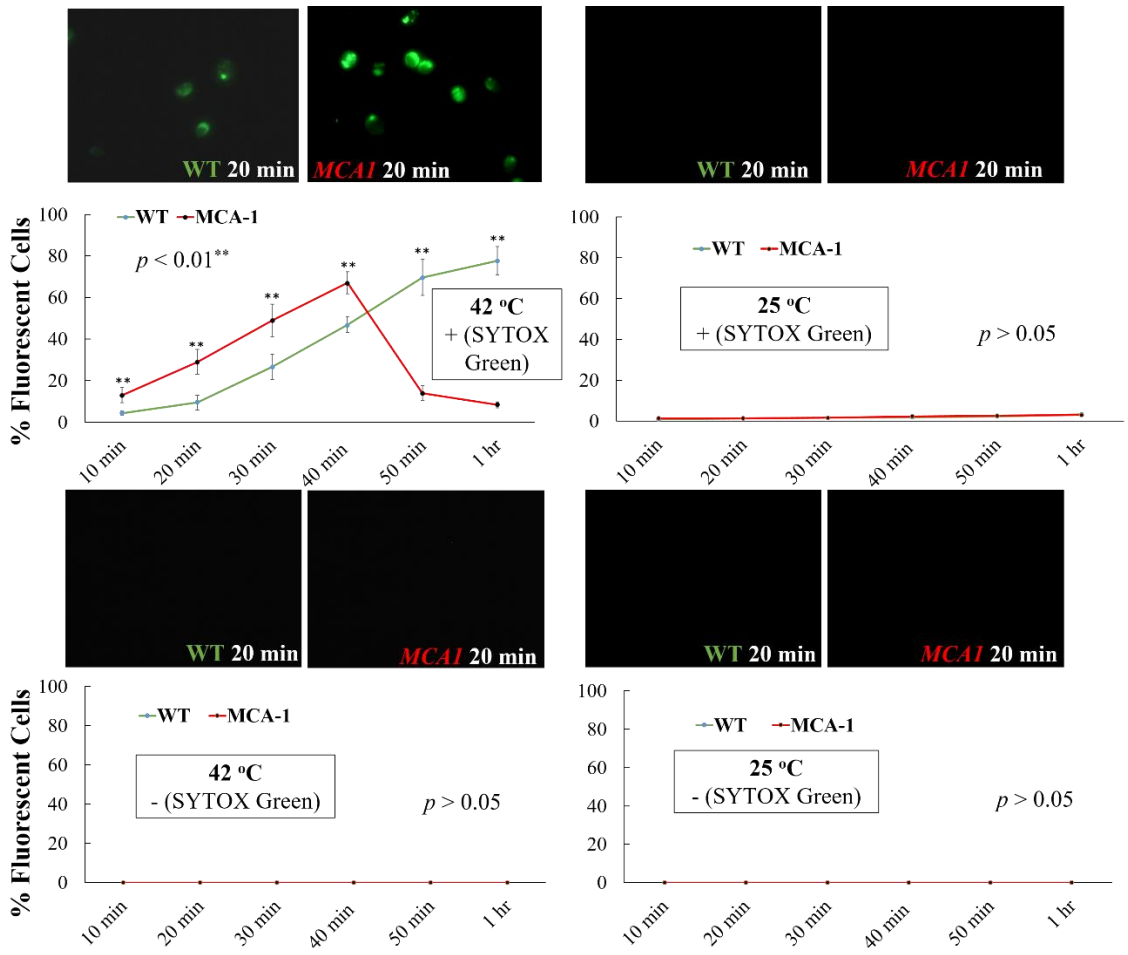

Figure 12. Mean (±SD) of *C. reinhardtii* cells exhibiting plasma membrane integrity disruption in the presence or absence of 42 °C heat stress, as revealed by SYTOX Green-induced fluorescence. Graphs show the percent of fluorescent cells in WT or *mca1* populations exposed to either SYTOX Green dye, or no dye. At indicated time points, results were determined via manual counts of cells in a random field of view (2-8 cells) until 200 cells were counted. Results derive from three independent assays. A Two-Way Repeated Measures ANOVA with post hoc analysis using Tukey's multiple comparison test was conducted via statistical software (SAS 9.4 and JMP 13). Statistical significance was defined as $p < 0.05$. Individual graphs display either the p value of the main effect (temperature x strain x observation), or the post hoc analysis p value (**).

WT and *mca1* populations display a similar rate of PS externalization.

Regulation of phospholipid bilayer asymmetry is one of the mechanisms by which cells maintain plasma membrane integrity. PS is a lipid typically restricted to the intracellular side of the plasma membrane; however, PCD activation promotes its movement to the extracellular side of the plasma membrane (Marino & Kroemer, 2013). Using Annexin V Alexa Fluor 488 conjugate staining, both WT and *mca1* populations displayed a similar degree of PS externalization in response to heat stress (Fig. 13). Temperature and dye controls indicated the absence of PS externalization-induced fluorescence in either strain (Fig. 13).

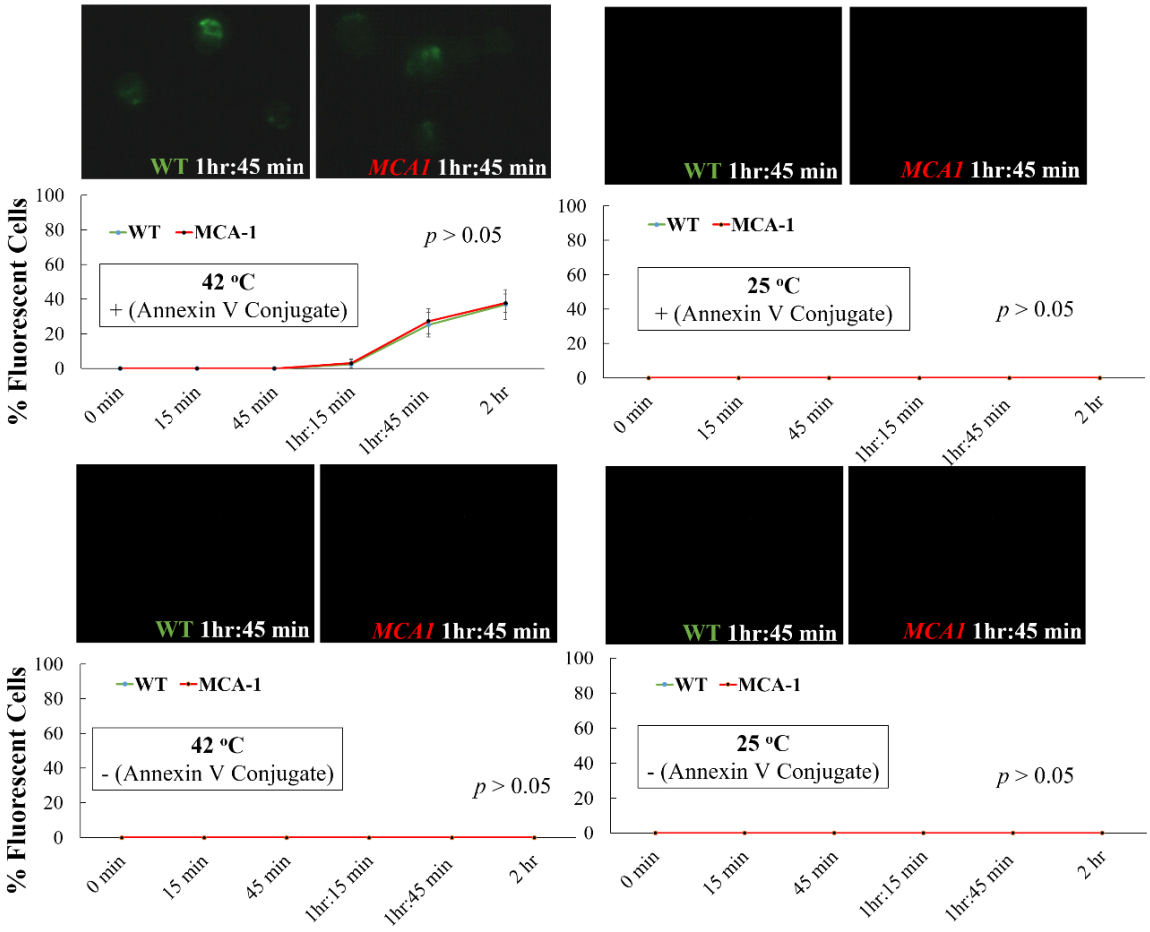

Figure 13. Mean (±SD) of *C. reinhardtii* cells displaying PS externalization in the presence or absence of 42 ºC heat stress. Graphs show the percent of fluorescent cells in WT or *mca1* populations incubated with either Annexin V Alexa Fluor 488 conjugate or no dye. At indicated time points, results were determined via manual counts of cells in a random field of view (2-8 cells) until 200 cells were counted. Results derive from three independent assays. A Two-Way Repeated Measures ANOVA with post hoc analysis using Tukey's multiple comparison test was conducted via statistical software (SAS 9.4 and JMP 13). Statistical significance was defined as $p < 0.05$. Individual graphs display the *p* value of the main effect (temperature x strain x observation).

MCA1 gene knockout alters ROS accumulation. The onset of PCD promotes the disruption of organelle membrane integrity, and consequentially, ROS is released in conjuction with various proteases to promote the transmission of death signals (Vancompernolle *et al.*, 1998, Broker *et al.*, 2005). Using a CM-H_2DFCDA assay, *MCA1* knockout was associated with an irregular accumulation of ROS that was dependent on the length of heat stress exposure. Specifically, there was a marked increase of ROS in *mca1* within 10 min; similar levels were not seen in WT until 20 min later. In each case, ROS level declined rapidly and remained low until a second burst of ROS was seen at approximately 3 hrs of heat stress; and, although it occurred in both strains, it was significantly greater in the *mca1* cells (45.6% of total cells in population), relative to the WT (21% of total cells in population) (Fig. 14). Both the temperature and dye controls indicated negligible rates of ROS accumulation (< 1% and 0%, respectively) (Fig. 14).

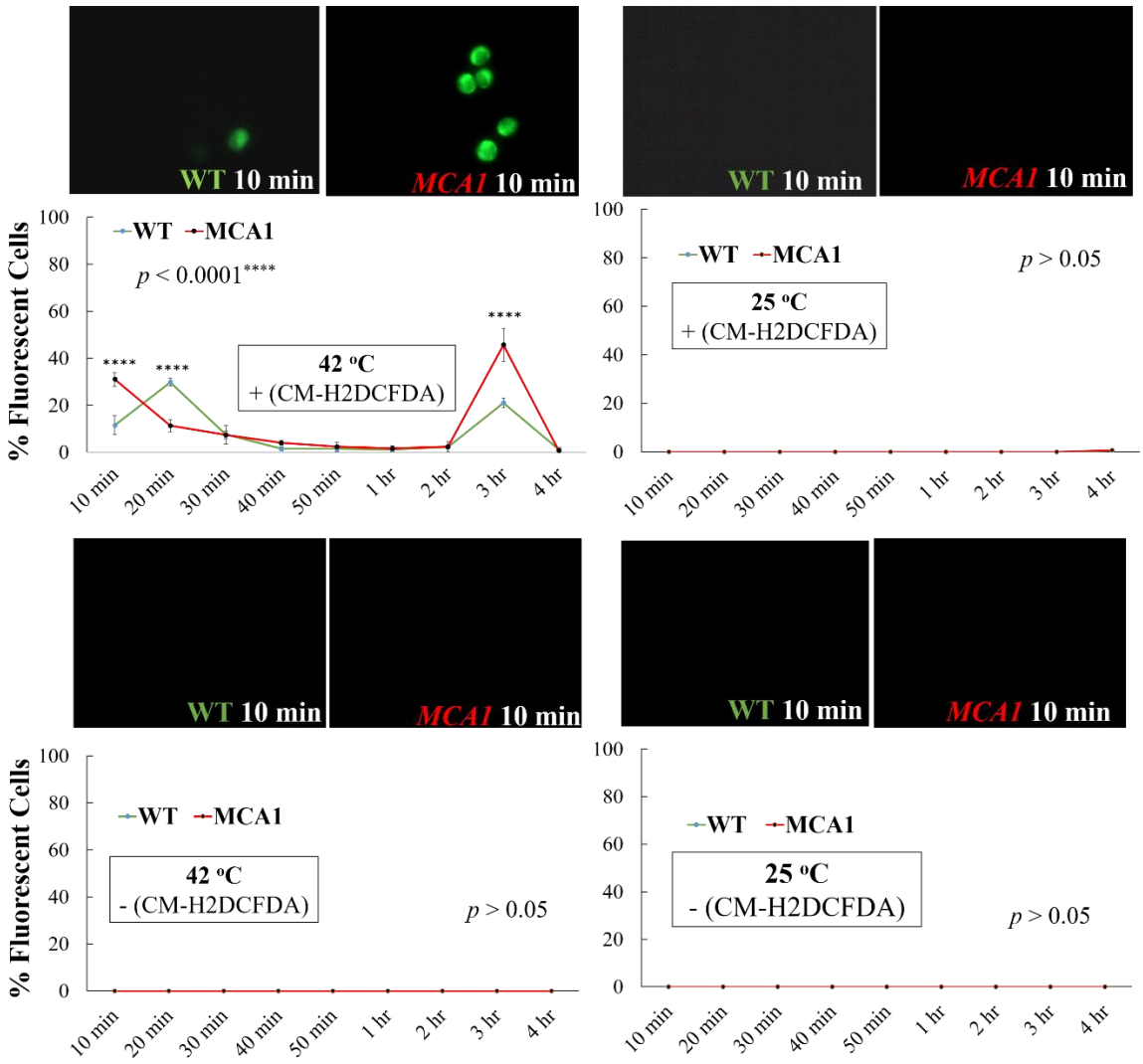

Figure 14. Mean (±SD) of *C. reinhardtii* cells displaying ROS accumulation in the presence or absence of 42 °C heat stress. Graphs show the percent of fluorescent cells in WT or *mca1* populations exposed to either CM-H_2DCFDA or no dye. At indicated time points, results were determined via manual counts of cells in a random field of view (2-8 cells) until 200 cells were counted. Results derive from three independent assays. A Two-Way Repeated Measures ANOVA with post hoc analysis using Tukey's multiple comparison test was conducted via statistical software (SAS 9.4 and JMP 13). Statistical significance was defined as $p < 0.05$. Individual graphs display either the p value of the main effect (temperature x strain x observation), or the post hoc analysis p value (****).

Heat stress promotes increased DNA laddering in *mca1* cells. The temporal laddering of DNA via apoptotic factors, such as endonuclease G, is critical for both caspase-dependent, and -independent PCD (Bajt *et al.*, 2006, Toro-Londono *et al.*, 2011, Kaczanowski *et al*., 2011). *MCA1* knockout cells exposed to heat stress showed a higher rate of DNA laddering relative to WT after 60 min (Fig. 15).

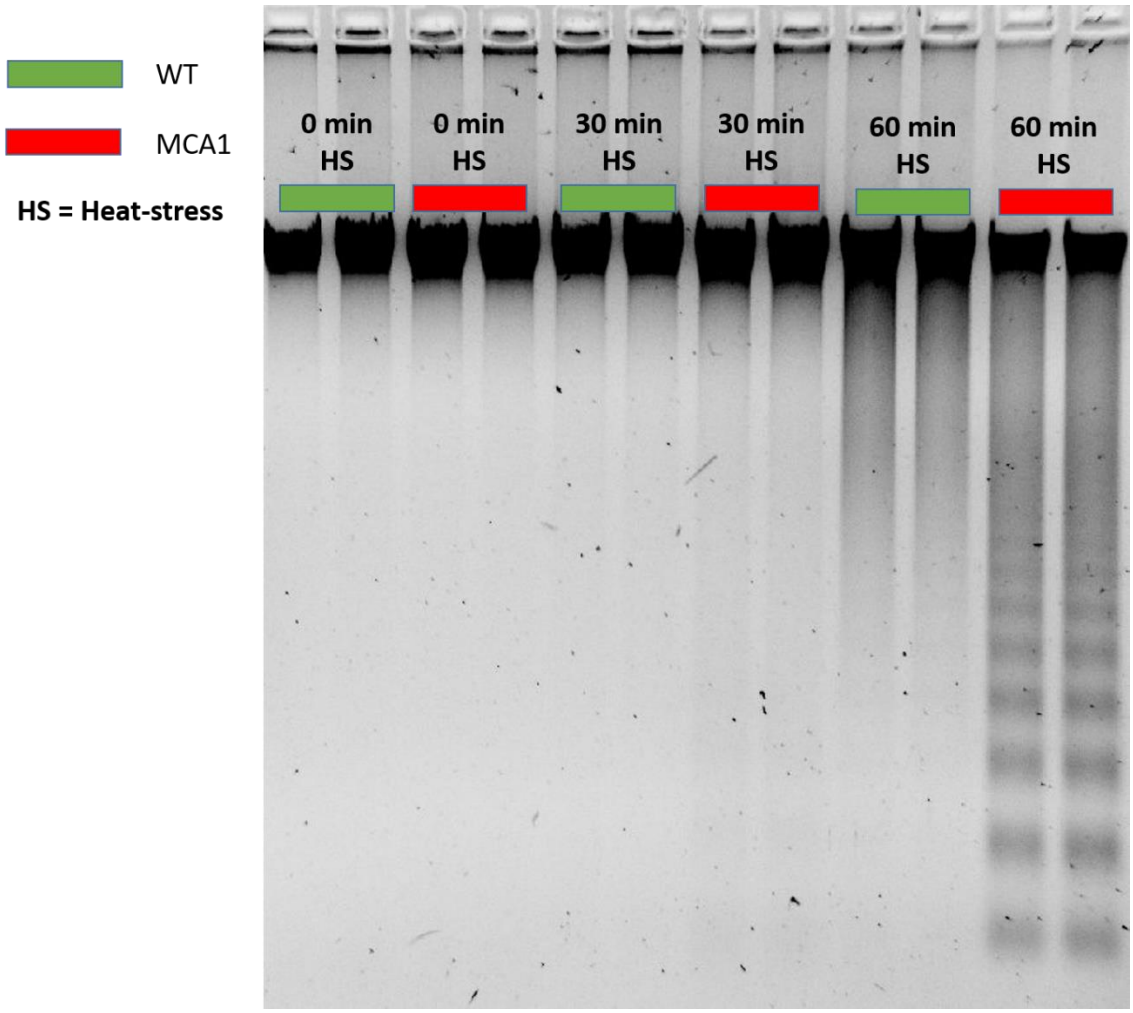

Figure 15. DNA laddering rates of WT and *mca1 C. reinhardtii* in response to heat stress. At indicated time points, DNA was extracted from cells exposed to 42 ºC heat stress, and subjected to agarose gel electrophoresis (285 µg per lane). Image is inverted and representative of results derived from triplicate experiments.

Cell proliferation of WT and *mca1* cells is similarly compromised upon the onset of heat stress. The plasma membrane serves as the first site of interaction between an organism and its habitat, and consequently, the integrity of the plasma membrane is directly linked with the organism's ability to proliferate in their environment (Alberts *et al.*, 2002). Due to the previous findings suggesting that *MCA1* knockout promoted the rapid onset of PCD phenotypes in *C. reinhardtii*, e.g., increased disruption of the plasma membrane, a colony formation assay was performed in order to determine if the cell's ability to enter the cell cycle was similarly compromised upon the onset of heat stress. To this end, both strains were exposed to 42 ^{o}C, and at various time-points, $5x10^4$ cells were plated onto TAP-media plates. Samples were then allowed to incubate at 25 ^{o}C until colony growth was most evident (6 days). Due to feasibility, the data was analyzed only qualitatively, and results appeared to suggest that heat stress similarly compromised WT and *mca1* cells (Fig. 14). As expected, cells of both strains grew most readily in absence of heat stress; however, shortly following heat stress exposure (30 min) the cell proliferation of both strains was similarly compromised (Fig. 16).

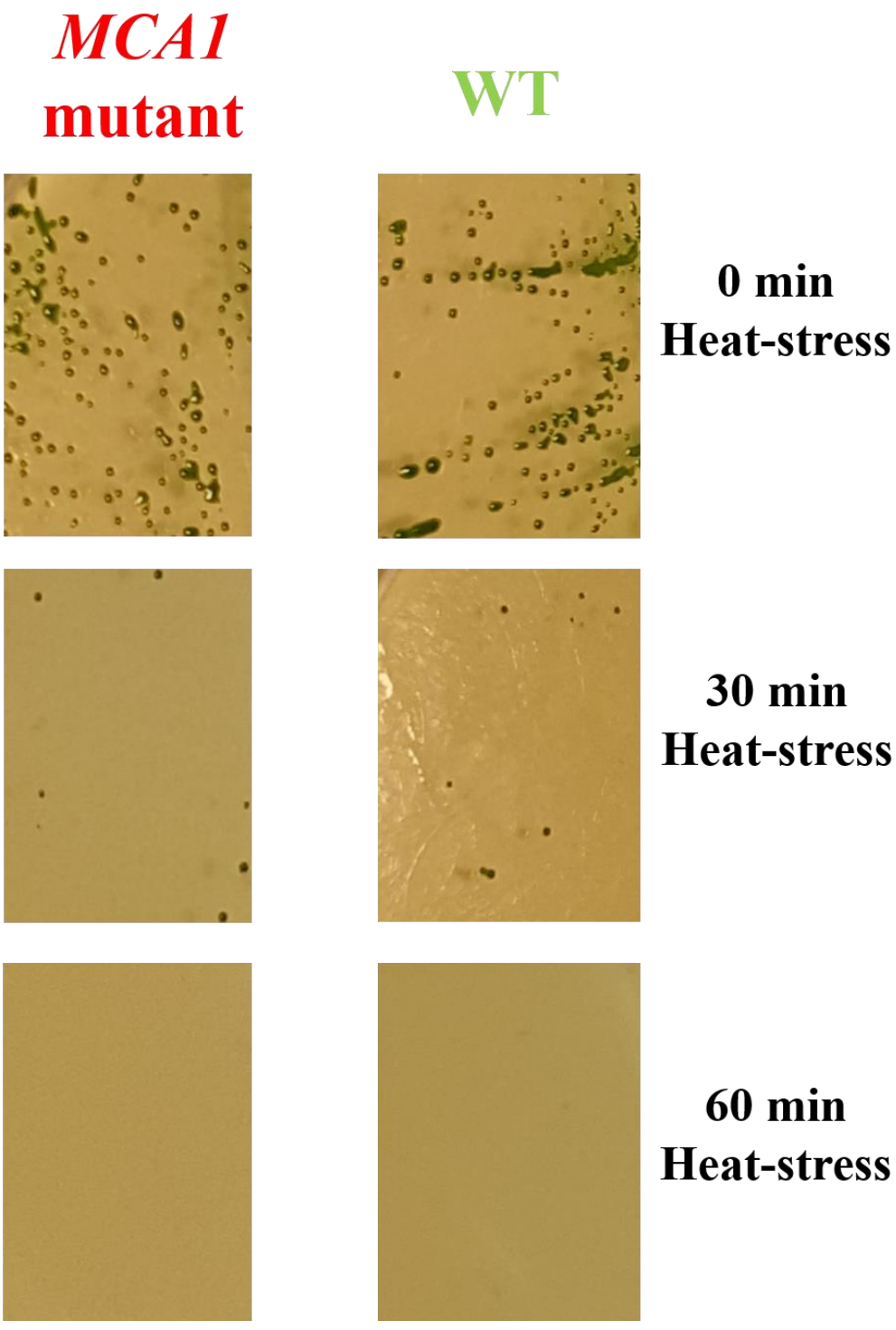

Figure 16. Representative images of the effect of 42 ºC heat stress on the colony formation capacity of WT and *mca1* populations. At the indicated time-points, $5.0x10^4$ of cells were spread onto TAP media plates, and allowed to incubate at 25 ºC until colony formation was visible (6 days). Results were qualitatively analyzed and derive from triplicate experiments.

Discussion

We sought to determine if MCA1 regulates PCD in *C. reinhardtii*. The critical role of CP family members in regulation of PCD (Fink & Cookson, 2005, Shemarova, 2010) led us to initially hypothesize that *MCA1* knockout would lead to the delayed onset of PCD phenotypes. Intriguingly, our findings suggest that instead, disruption of *MCA1* promotes a more rapid propagation of certain PCD events. In response to heat stress, *MCA1* mutants exhibit more rapid plasma membrane disruption. Our data do not indicate the mechanisms behind this effect. However, the type-I metacaspase, Yca1p has been reported to modulate plasma membrane integrity during the hyperosmotic stress response of *Saccharomyces cerevisiae* (Silva *et al.,* 2005). Additionally, another type-I metacaspase, Atmc2, has been shown to inhibit cell death during the hypersensitive response of *Arabidopsis thaliana* (Coll *et al.,* 2010). Similar to the former report, MCA1 in *C. reinhardtii* could also regulate the plasma membrane, and as supported by the latter study, the type-I metacaspase of *C. reinhardtii* could similarly inhibit PCD.

Though not yet reported for metacaspases, caspases have been shown to activate both Xkr8 (in mammalian cells) and CED-8 (in *C. elegans*), proteins involved with promoting PS externalization, otherwise known as scramblases (Lisa-Santamaria *et al.,* 2009, Marino & Kroemer, 2013). However, we found no significant difference in the rate of PS externalization between heat-stressed WT and *mca1* cells; although, it is possible that a second metacaspase, MCA2, instead plays this role in *C. reinhardtii*. The role of MCA2 within *C. reinhardtii* is poorly understood; however, MCA2 expression is noted to be upregulated following the addition of dehydroascorbate (biochemical marker of oxidative stress) to *C. reinhardtii* cells undergoing H_2O_2-induced PCD (Murik *et al*.,

2013), suggesting that MCA2 could also act as a modulator of PCD within *C. reinhardtii*. On the other hand, PS externalization has also been reported to be critical to non-PCD cellular processes, such as regulation of cell morphology (Rysavy *et al.*, 2015). Therefore, it is possible that *C. reinhardtii* possesses metacaspase-independent mechanisms that affect the localization of PS. An analysis of the effects of *MCA2* knockout in this species is needed.

Despite these apparent differences in plasma membrane disruption in response to heat stress, we found that the cell proliferation of both WT and *mca1* cells was similarly compromised after 30-60 minutes of heat stress; nonetheless this is consistent with the plasma membrane disruption that occurred in the early onset of heat stress. However, unlike our Evans Blue and SYTOX Green findings, we did not see differences in the rate at which cell proliferation was inhibited in either strain; notably, this analysis was only qualitative (visual) and not quantitative (CFU counts) due to feasibility. Our SYTOX Green data also indicated that heat stress exposure post-50 minutes leads to a significant decrease in the SYTOX Green-induced fluorescence of *mca1* cells. Since heat stress is noted to similarly compromise the cell proliferation of both strains, it is unlikely that the post-50 minute decrease we observed is due to the plasma membranes being repaired; however, it is possible that the discrepancy between Evans Blue and SYTOX Green staining data is due to the inhibition of SYTOX Green-DNA interaction. We found that *mca1* cells consistently displayed a substantial increase in DNA laddering after 60 minutes of heat stress. Therefore, it is possible that the increased rate of DNA fragmentation in heat-stressed *mca1* cells is capable of promoting a change in DNA such that it poorly binds to SYTOX Green, i.e., increased accumulation of single-stranded

DNA, and consequentially, leads to a decrease in SYTOX Green-induced fluorescence of *mca1* cells at later stages of heat stress. Notably, SYTOX Green, a non-membrane permeable DNA-intercalating dye, is capable of binding single-, or double-stranded DNA rapidly ($1.5 \times 10^6\ M^{-1}\ s^{-1}$), and with high affinity ($k_d = 40$ nm) (Thakur *et al.*, 2015). As a result of DNA binding, SYTOX Green fluorescence is significantly enhanced (>1000-fold); however, this fluorescence emission is noted to be at its lowest enhancement possible when bound to single-stranded DNA (Roth *et al*., 1997, Thakur *et al*., 2015). Our DNA laddering assay of choice was not specific for single-stranded DNA; therefore, different types of DNA laddering assays are necessary to elucidate the cause of our Evans Blue and SYTOX Green discrepancies. Additionally, our lack of DNA laddering observations specific for time points between 30 and 60 minutes also inhibits us from further clarifying this discrepancy.

Similarly elusive is the mechanism behind our observed increase of DNA laddering in heat-stressed *mca1* cells. Caspases, as well as mitochondrial proteins such as apoptosis inducing factor (AIF) and endonuclease G, have been reported to play a key role in regulated DNA fragmentation. Our results suggest that similarly to caspases, MCA1 might also play a role in regulating DNA fragmentation; however, metacaspases have not yet been linked with DNA laddering. Additionally, AIF and endonuclease G have not only been shown to act in CP family-dependent and –independent manners, but have also been suggested to interact with one another; thereby, adding another layer of complexity to the mechanisms modulating DNA degradation and further complicating the analysis of our results (Bajt *et al.*, 2006, Toro-Londono *et al.*, 2011, Kaczanowski *et al.*, 2011).

We found that ROS accumulation occurred earlier in the heat-stressed *MCA1* knockout strain, and in the event of prolonged stress, ROS accumulation was significantly more abundant in *mca1* populations. The lack of similar observations in the controls for either strain suggests the potential that MCA1 plays a role in modulating ROS accumulation in response to heat stress. Interestingly, such a role has not only been reported for effector caspases (e.g., caspase-3,-7, and -9), but also in the *Candida albicans* type I metacaspase (*Ca*MCA1), and the *Litchi chinensis* type II metacaspase (*Le*MCA2) (Brentnall *et al*., 2013, Jung & Kim, 2014, Wang *et al*., 2016). In numerous organisms, ROS is key to the propagation of various forms of PCD, and its accumulation is critical to activation of mitochondrial-induced death cascades. In *S. cerevisiae*, H_2O_2-induced apoptosis is inhibited by the absence of Yca1p (type-I MCA). ROS accumulation has also been reported to promote lysosomal leakage, and induce the release of lysosomal proteases (i.e., cathepsin D) capable of stimulating the breakdown of the outer mitochondrial membrane (OMM). Consequentially, OMM leakage allows for the release of additional ROS, thus initiating a positive-feed-forward loop that ultimately leads to increased ROS accumulation (Broker *et al.*, 2005, Kaczanowski *et al.*, 2011). Hypothetically, the earlier display of ROS accumulation in our heat-stressed *mca1* cells could generate a similar feed-forward-loop, and in turn, account for our observed rapid transmission of select PCD phenotypes. However, our analysis is complicated by reports of metacaspase-independent mechanisms capable of promoting similar PCD phenotypes, and their potential interaction with metacaspase-dependent agents in response to death stimuli. Intriguingly, we noted that both *C. reinhardtii* strains displayed ROS accumulation in two distinct bursts, in response to either the immediate onset, or

prolonged exposure to heat-stress. Interestingly, heat-stressed *mca1* populations displayed an initial burst of ROS accumulation in half the time as heat-stressed WT cells, and notably, the late and secondary burst of ROS accumulation was twice as abundant in heat-stressed *mca1* populations, relative to heat-stressed WT. Though we lack the data for earlier time-points (pre-10 min), the noted lack of ROS accumulation in the 25 ^{o}C controls for either strain suggests that in absence of heat stress (0 min), ROS levels would likely be basal. Just as likely, we may have also missed an earlier rise of ROS levels in heat-stressed WT cells (1-9 min). Importantly, we must once again note that the mechanisms influencing ROS accumulation within unicellular organisms are poorly understood. Future analyses will benefit from gauging earlier time-points, and whether ROS feed-forward loops occur in *C. reinhardtii*.

Our study serves as a foundation for understanding the potential role(s) of MCA1 during heat stress-induced PCD in *C. reinhardtii*. We conducted analyses of various PCD phenotypes, and in turn, have noted a number of cellular events linked to the disruption of *MCA1*. Notably, we refute our null hypothesis that *MCA1* knockout does not affect heat stress-induced PCD phenotypes in *C. reinhardtii*, and find that *MCA1* disruption is linked with a significantly more rapid transmission of certain PCD events in response to heat stress. Similar to caspases, metacaspases are largely linked with the positive regulation of PCD; however, our findings suggest that MCA1 could act as a negative regulator of PCD. Type-I metacaspases have been reported to both positively and negatively regulate PCD. In turn, the potential exists that the type I metacaspase of *C. reinhardtii*, MCA1 could also inhibit PCD; and thus, account for our findings.

Importantly, we verified that the *mca1* strain encodes an insertion within the first exon (8 total) of *MCA1*. The presence of a CIB1 cassette (~2 kbp) within the coding sequence of *MCA1* suggests that the gene product is either not synthesized, or non-functional within *mca1* cells. Though unlikely, it is also possible that a constitutively active MCA1 isoform is generated, and notably, would more easily explain our findings. Of note, since the *mca1* strain is reported to contain a CIB1 insertion in an intron of *MCA1*, and within an intron of a gene coding for an NYN-domain family member (chromosome 8), we cannot rule out the possibility that such genomic alterations might also influence our results. Unfortunately, our strain source (CRC) does not further specify the location of these insertions; however, we must note that the presence of these insertions within a single intron on chromosome 8, and a single intron on chromosome 12, makes them less likely to alter gene function(s). The lack of complementation experiments using the WT MCA1 gene, data on the role(s) of MCA2, the pattern(s) of MCA1 expression, and the poor understanding of metacaspases in general, inhibits us from further elucidating the role of MCA1 within *C. reinhardtii*. Nonetheless, the current study identifies distinct PCD phenotypes in need of further analyses.

Materials & Methods

Cell strains and culture conditions. The *Chlamydomonas* CC-4533 (cw15 cell-wall-deficient) and *mca1* strains were obtained from the *Chlamydomonas* Resource Center (CRC), University of Minnesota. CC-4533 strain is the parental strain for the mutant generated via electroporation. The *mca1* strain has three reported CIB1 cassette insertions conferring resistance to paromomycin (antibiotic): chromosome 12 coding sequence (Cre12.g517541 base 4050836) and intron (Cre12.g517541 base 4050973), and

chromosome 8 intron (Cre08.g361350 base 886975) (Li *et al.*, 2016). In accordance with Whitman, 1986, all cells were grown in liquid Tris-Acetate-Phosphate (TAP) medium, with aeration and under a 14-h/10-h light cycle.

DNA extraction and quantification. Sample DNA was extracted in accordance to Durand *et al.* (2014) using the DNeasy Plant Mini Kit (Qiagen), with some modifications. Cells were diluted to $1x10^7$ cell density in 50 mL of TAP media. Harvested cells were centrifuged at 4000 RPM for 4 minutes, and after removal of supernatant, subjected to modified kit lysis buffer (added 5mg of Proteinase K, and 20 µL of 10% SDS). Remaining steps were conducted in accordance to the Mini-Protocol for Purification of Total DNA from Plant Tissue (DNeasy Plant Handbook). DNA was quantified via Take3 Multi-Volume Plate (BioTek).

PCR amplification and verification of *MCA1* disruption. PCR was conducted in accordance to standard protocol (denaturation for 30 secs. at 95 ^oC, annealing for 45 secs. at 58 ^oC, elongation for 2 mins. at 72 ^oC for 40 cycles), and using the CRC-recommended primer sequences (*MCA1* locus forward primer (G1): 5'-AGACTTGGTGGGGAACAGTG-3', *MCA1* locus reverse primer (G2): 5'-CAGTACCTCCAGACTTCCGC-3', -CIB1 cassette 5' region reverse primer (C1): 5'-GCACCAATCATGTCAAGCCT-3', CIB1 cassette 3' region forward primer (C2): 5'-GACGTTACAGCACACCCTTG-3'). Following, PCR amplicons were subjected to gel electrophoresis, and sequence analysis (Lone Star Labs) using all four primers. These procedures were for the purpose of verifying the *C. reinhardtii MCA1* knockout strain.

Bioinformatics. The analyzed sequences were subjected to homology searches in the *C. reinhardtii* genome via BLASTn. The *MCA1* FASTA sequence was derived from

the NCBI database (accession: NW_001843738.1). The analyzed amplicon sequences were used as the subject sequences.

Gel electrophoresis. DNA was separated by 1% agarose-Tris-acetate-EDTA (agarose-TAE) gel electrophoresis, and ran for 30 minutes at 100 V. For all samples, DNA gels were visualized using a Chemi Doc XRS+ molecular imager (BioRad).

Induction of PCD. Previous reports indicate that *C. reinhardtii* PCD is induced at ~42 ºC (Schroda *et al.*, 2015). To induce PCD in cultured cells, samples were incubated in a hot water bath at 42 ºC, under constant light, for the indicated time. Designated assays also utilized temperature (25 ºC) and negative (no dye) control samples.

Cell Counts. For standardization, 10 µL of samples were placed under 40-100x objectives. All cell counts were conducted by one analyst, and for each trial, determined by random choice of field of view (2-8 cells) until counting a total of 200 cells.

Evans Blue assay. Cells were diluted to a density of $5.0x10^6$ cells in 10 mL of TAP media. At the indicated time-points, 500 µL of sample was incubated at a 1:1 ratio with Evans Blue dye for 15 minutes. Samples were then washed three times with TAP media, and the number of cells were counted.

Fluorescence microscopy. For all samples, cells were diluted to optimized cell density in 1 mL of TAP media, and incubated in accordance to the manufacturer instructions (Life Technologies) for the following fluorophores: CM-H_2DCFDA (ROS accumulation, 5.0 $x10^6$ cell density); SYTOX Green (cell membrane integrity and DNA staining, 8.0 $x10^6$ cell density); Annexin V Alexa Fluor 488 conjugate (externalization of PS, 5.0 $x10^6$ cell density). At selected time points, fluorescence emission was visualized

and data were analyzed via Motic Images Plus 3.0 software, using a Nikon Eclipse E-400 microscope equipped with a Nikon Y-FL Epi-fluorescence attachment at excitation wavelength range of 450-515 nm. Photographs were taken using a Moticam 2000 camera.

DNA Laddering Assay. Following DNA extraction at the indicated time-points, 285 µg of sample DNA was loaded into a 1% TAE gel, and separated for 30 minutes at 100 V.

Colony Growth Assay. Samples were quantitated to 5.0 $x10^6$ cell density in 1 mL, and at designated time-points, 100 µL of diluted sample (5.0 $x10^4$) was spread onto a TAP-solid plate. Colony formation was assessed after six days of growth due to the optimal visualization of colonies.

Data Analysis. All experiments were conducted in triplicate, and all quantitative data are represented as means $\pm$ SD. The DNA laddering and viability assay results were analyzed qualitatively. Statistical analysis was performed by two independent personnel via 2-Way Repeated Measures ANOVA (SAS 9.4 and JMP 13 software) with post hoc analysis using Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

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CHAPTER III

DETERMINING THE PRESENCE AND ROLE OF A PUTATIVE APAF-1 ORTHOLOGUE IN CHLAMYDOMONAS REINHARDTII

This dissertation follows the style and format of the *Journal of Cell Biology (JCB).*

Abstract

Apoptosis, the canonical manifestation of programmed cell death (PCD) in metazoans, is characterized by irregular cellular events, such as cytoplasm shrinkage and release of cytochrome *c*. The apoptotic machinery has been extensively studied in multicellular organisms, and is noted to be conserved. For example, APAF-1 is a protein critical for intrinsic apoptosis within multicellular organisms. More recently, a study identified a putative APAF-1 orthologue in the unicellular green alga, *C. reinhardtii*. This study sought to confirm this earlier report, as well as determine whether an APAF-1/cytochrome *c*/MCA1 complex forms during heat stress-induced PCD within *C. reinhardtii*. Interestingly, we obtained results that were comparable to those of the prior published report using solely secondary antibodies. That is, even in the absence of primary antibodies against human APAF-1, as used in the prior published report, APAF-1-sized bands (~130 kDa) are identified via sole incubation with goat anti-rabbit and goat anti-mouse IgG (1:5000, both). The data suggest that the antibody against human APAF-1 lacks the specificity required to accurately determine if APAF-1 is present in *C. reinhardtii*. Moreover, *in silico* analysis indicated the prime APAF-1 candidates in *C. reinhardtii* to lack key conserved domains (CARD and AAA16). Thus, the identification of APAF-1 in unicellular organisms remains solely reported *in silico*.

Key words: Chlamydomonas reinhardtii, APAF-1, Western blot, unicellular, green alga, PCD.

Background

Within animal cells, programmed cell death (PCD) is typically manifested in the form of apoptosis. Apoptosis can be triggered by an array of stimuli, such as radiation or growth factor withdrawal, after which the cell undergoes a series of genetically programmed physiological processes (Elmore, 2007, Leblanc *et al*., 2014, Kasuba *et al.*, 2015). The PCD phenotypes associated with apoptosis include induction of proteases and endonucleases, cytoplasm shrinkage, membrane blebbing, the release of cytochrome *c*, phosphatidylserine (PS) externalization, and nuclear condensation (Elmore, 2007, Kasuba *et al*., 2015).

Our understanding of PCD is largely derived from studies of multicellular eukaryotes, particularly mammals. In mammalian cells, the PCD form known as apoptosis occurs via either extrinsic or an intrinsic (mitochondrial) pathway. The extrinsic apoptotic pathway is activated by the binding of an extracellular ligand to a death domain (DD)-containing tetrameric death receptor, such as members of the tumor necrosis factor (TNF) superfamily. Ligand binding induces homotypic interactions between the death receptor and intracellular proteins containing either Fas-associated death domain (FAD) or TNER-associated death domain (TNADD). Subsequently, the adaptor protein associated with the death receptor undergoes conformational changes so that its death effector domain (DED) is capable of interacting with caspases (Shemarova, 2010). Caspases are proteases containing cysteine at their active sites, which they use to cleave specific proteins at their aspartate residues, and belong to the cysteine protease (CP) family. The temporal and spatial activity of caspases is critical to the proper breakdown of a cell (Fink & Cookson, 2005, Lee *et al.*, 2011).

The intrinsic apoptotic pathway is coordinated by mitochondria, and can be triggered by specific stimuli (e.g., cytotoxic stress, DNA damage, and cytokine deprivation), as well as indirectly via the extrinsic pathway. The transmission of apoptotic signals to mitochondria leads to the breakdown of the outer mitochondrial membrane (OMM), and subsequent release of cytochrome *c*, an electron acceptor. Upon its release into the cytosol, cytochrome *c* binds to APAF-1 and activates it. APAF-1 is a 130 kDa protein comprised of a caspase recruitment domain (CARD) at its *N*-terminus, a CED4 homology domain, and 12 or 13 WD40 repeats at its *C*-terminus. When bound to cytochrome *c*, APAF-1 is then capable of forming the apoptosome, a ring-like heptamer of APAF-1 molecules with CARD regions facing its center (Acehan *et al.,* 2002, Shemarova, 2010, Kasuba *et al.*, 2015). The apoptosome recruits procaspase-9, which unlike other initiator caspases, contains a CARD that allows it to interact with, and be activated by, the apoptosome (Fig. 17).

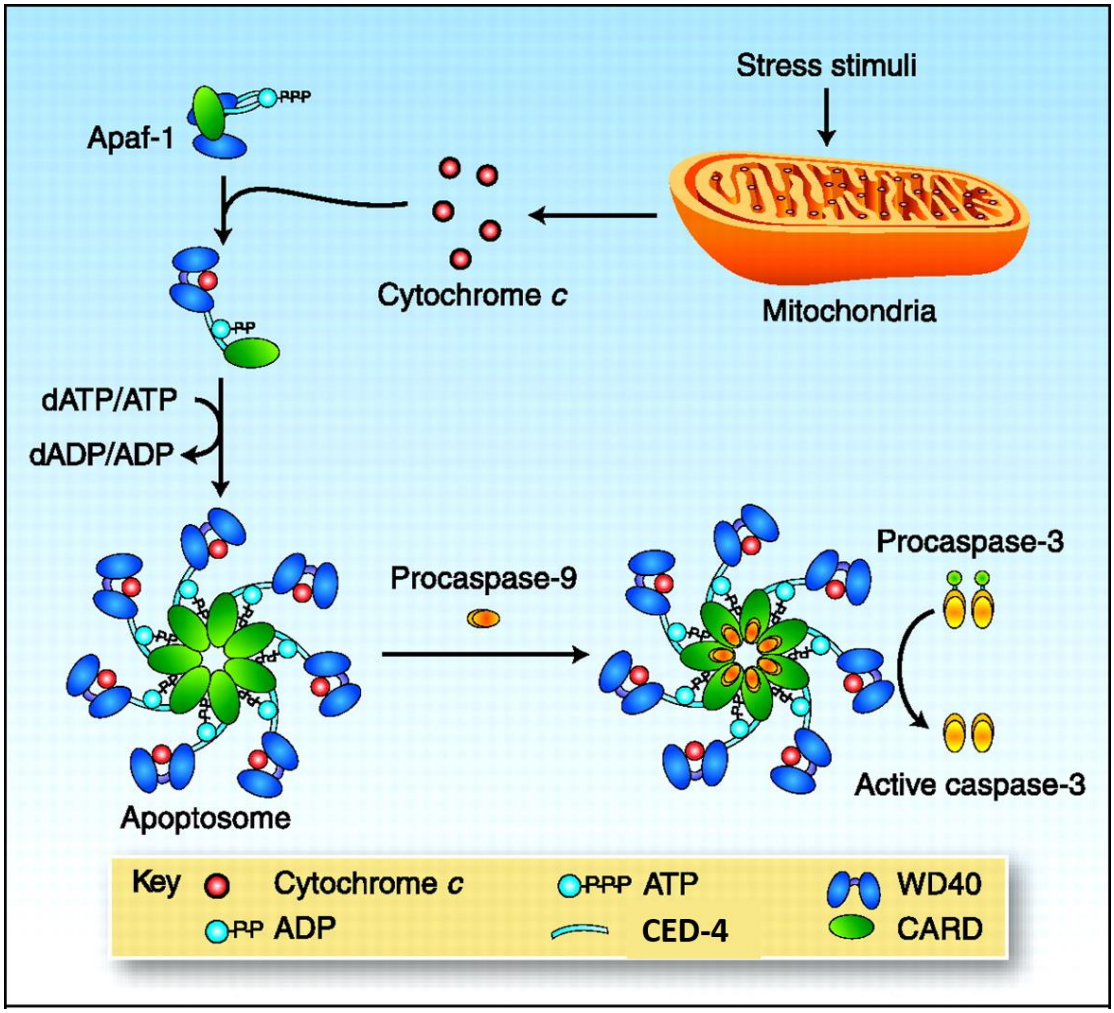

Figure 17. Schematic depiction of apoptosome formation. When present in the cytosol due to stress (e.g., toxic, heat, oxidative) cytochrome *c* interacts with APAF-1, and triggers apoptosome formation via a processes dependent on hydrolysis of dATP. The apoptosome complex activates the initiator caspase, procaspase-9 via the CARD domain present in both the NH_2-terminus region of APAF-1, and the prodomain of procaspase-9. Active caspase-9 is then capable of recruiting and activating effector caspases. This figure is adapted with permission from: Ledgerwood, E. C., & Morison, I. M. (2009). Targeting the Apoptosome for Cancer Therapy. Clinical Cancer Research, 15(2), 420-424. doi:10.1158/1078-0432.ccr-08-1172

APAF-1 orthologues have been identified in lower multicellular eukaryotes such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Schmidtea mediterranea* that can induce PCD in a cytochrome *c*-independent and/or dependent fashion (Alberts *et al.*, 2002, Moharikar *et al.,* 2007, Kaczanowski *et al*., 2011). Interestingly, APAF-1 has been biochemically identified solely in multicellular eukaryotes. However, the high degree of conservation of cytochrome *c* across living organisms, as well as the increase in homology searches identifying putative APAF-1 sequences in unicellular eukaryotes, such as the parasite, *Leishmania*, and the green alga, *Chlamydomonas reinhardtii* (Gannavaram & Debrabant, 2012, Kletzin *et al.,* 2015), suggest that the apoptosome could potentially modulate PCD in unicellular eukaryotes. Notably, Moharikar *et al*. (2007), identified a 130 kDa protein using antibodies against human APAF-1 (polyclonal, rabbit) in UV-C treated *C. reinhardtii*, a single-celled green alga. This finding suggests that an APAF-1-like protein might be present in lower unicellular eukaryotes, and raises the possibility that an apoptosome complex might form during UV-C-induced PCD. However, *in silico* analyses attempting to identify an APAF-1 orthologue in *C. reinhardtii* revealed the primary homolog to possess the expected WD-40 domains, while lacking the CARD and AAA16 domains (Moharikar *et al*., 2007). Though it is possible that the *in silico*-derived sequence and the antibody-bound protein are not the same, the *in silico* finding raises the possibility that the organism might not possess a true APAF-1 orthologue.

The current study sought to confirm the presence, and the potential role of the putative APAF-1 orthologue in *C. reinhardtii*. We hypothesized that heat stress-induced PCD would be capable of promoting apoptosome formation. Moreover, since the

prodomain of caspase-9 is key to its interaction with the apoptosome (Bratton & Salvesen, 2010), we hypothesized that the type I metacaspase, MCA1, which also contains a prodomain, would be capable of interacting with the potential APAF-1 orthologue/cytochrome *c* complex. Notably, the first half of this thesis indicated that MCA1 could potentially act as a negative regulator of PCD (Chapter II); nonetheless, we sought to determine whether the apoptosome complex forms, and potentially interacts with MCA1 in *C. reinhardtii*. To this end, heat-stressed and control (25 ^{o}C) cells underwent whole cell protein extraction, followed by sucrose gradient sedimentation (10-30% density), and the subsequent collection of fractions. Interestingly, rabbit polyclonal antibodies against human APAF-1 (at 1:100 and 1:1000) failed to display target specificity, and produced results comparable to those derived from usage of solely goat anti-rabbit IgG, or goat anti-mouse IgG (1:5000).

In silico analysis indicated that the primary APAF-1 homologs in *C. reinhardtii* lack key PCD domains (CARD and AAA16). Together, the data suggest that the identification of an APAF-1-sized band in *C. reinhardtii*, as conducted by Moharikar *et al.*, (2007), appears to be artefactual. Therefore, the findings of this study refute the prior published report, and posit that APAF-1 thus far remains identified solely in multicellular eukaryotes.

Results

Primary antibody against human APAF-1 displays poor specificity in *C. reinhardtii* cells. *APAF-1* has been identified biochemically solely in multicellular eukaryotes, including humans, and encodes a 130 kDa adaptor protein (Acehan *et al.*, 2002, Shemarova, 2010, Kasuba *et al.*, 2015). Using UV-C treated *C. reinhardtii* cells,

Moharikar *et al.*, (2007) identified a similarly sized protein using antibodies for human APAF-1. To determine the potential role of this hypothesized APAF-1 orthologue, a Western blot analysis of protein lysate derived from WT and *mca1* cells exposed to either 1-hour of heat stress (42 ^{o}C), or room temperature (control) was performed. Western blot analysis of control *mca1* cells using both rabbit polyclonal primary (1:1000) and goat anti-rabbit/-mouse secondary antibodies (1:5000) revealed a considerable amount of non-specific binding (Fig. 18). Blotting lysates from WT cells generated comparable results (not shown). Similar attempts using lower concentrations of primary and secondary antibodies generated poor results (not shown). To determine the cause of the non-specificity seen in the Western blot analyses, *mca1*-derived lysate was subjected to SDS-PAGE, followed by transfer onto a nitrocellulose membrane. Afterwards, the membrane was cut in half, and while one membrane half was probed with primary (1:100 or 1:1000) and secondary antibodies (1:5000), the other membrane half was probed with secondary antibodies only (1:5000). In both membrane halves, similarly-sized protein bands were identified, which included an approximately 130 kDa band (Fig. 19).

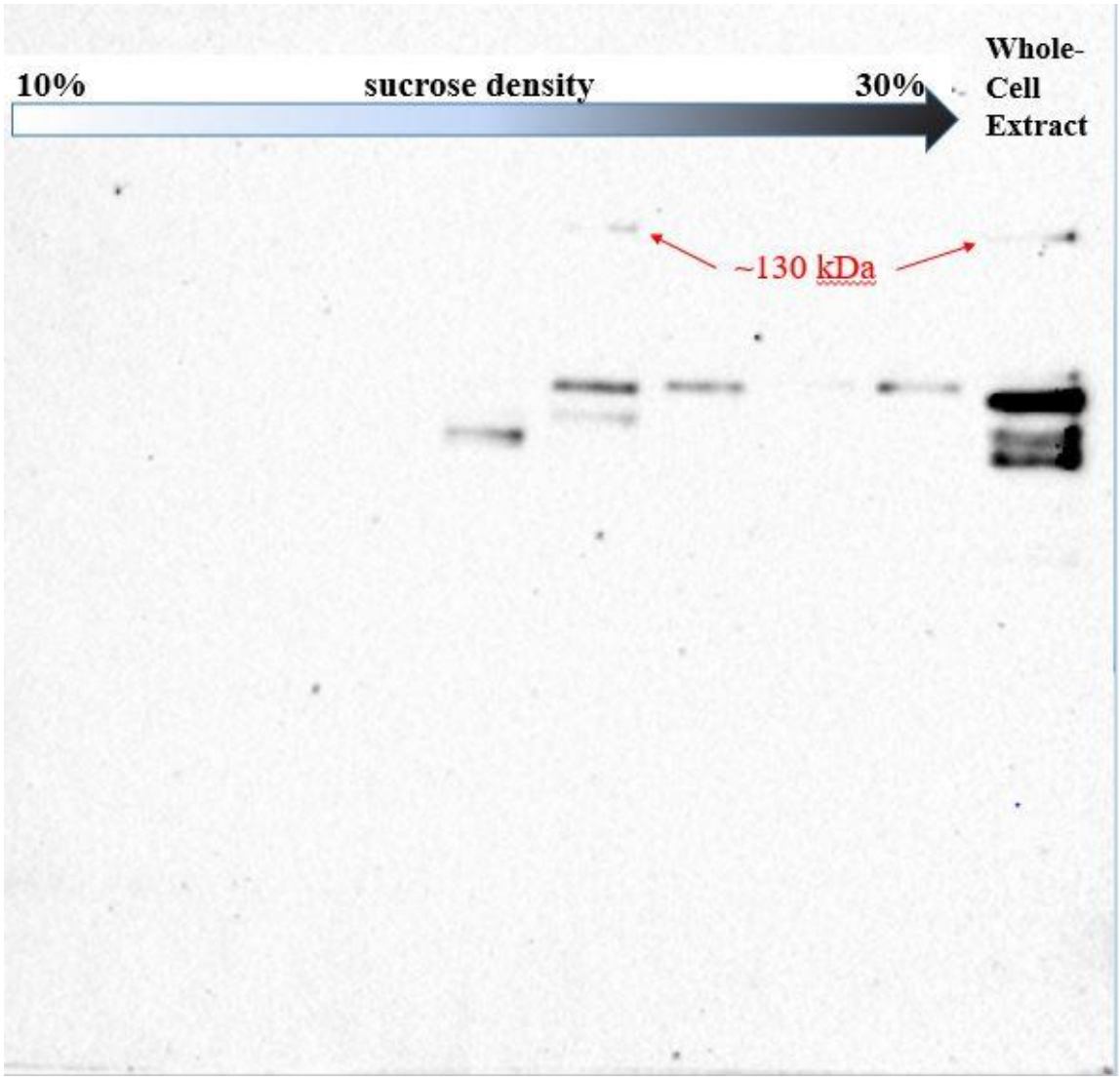

Figure 18. Non-specific binding generated by antibodies against human APAF-1 in *mca1 C. reinhardtii*, as revealed by Western blot analysis. Maximum volume (45µL) of protein lysate derived from sucrose gradient fractions or whole-cell extraction of control *mca1* cells were subjected to SDS-PAGE, followed by transfer onto a nitrocellulose membrane. Following, membrane was probed overnight at 4 ^{o}C using rabbit polyclonal antibodies for human APAF-1 (1:100) and rabbit secondary antibodies (1:3000) for 1-hr.

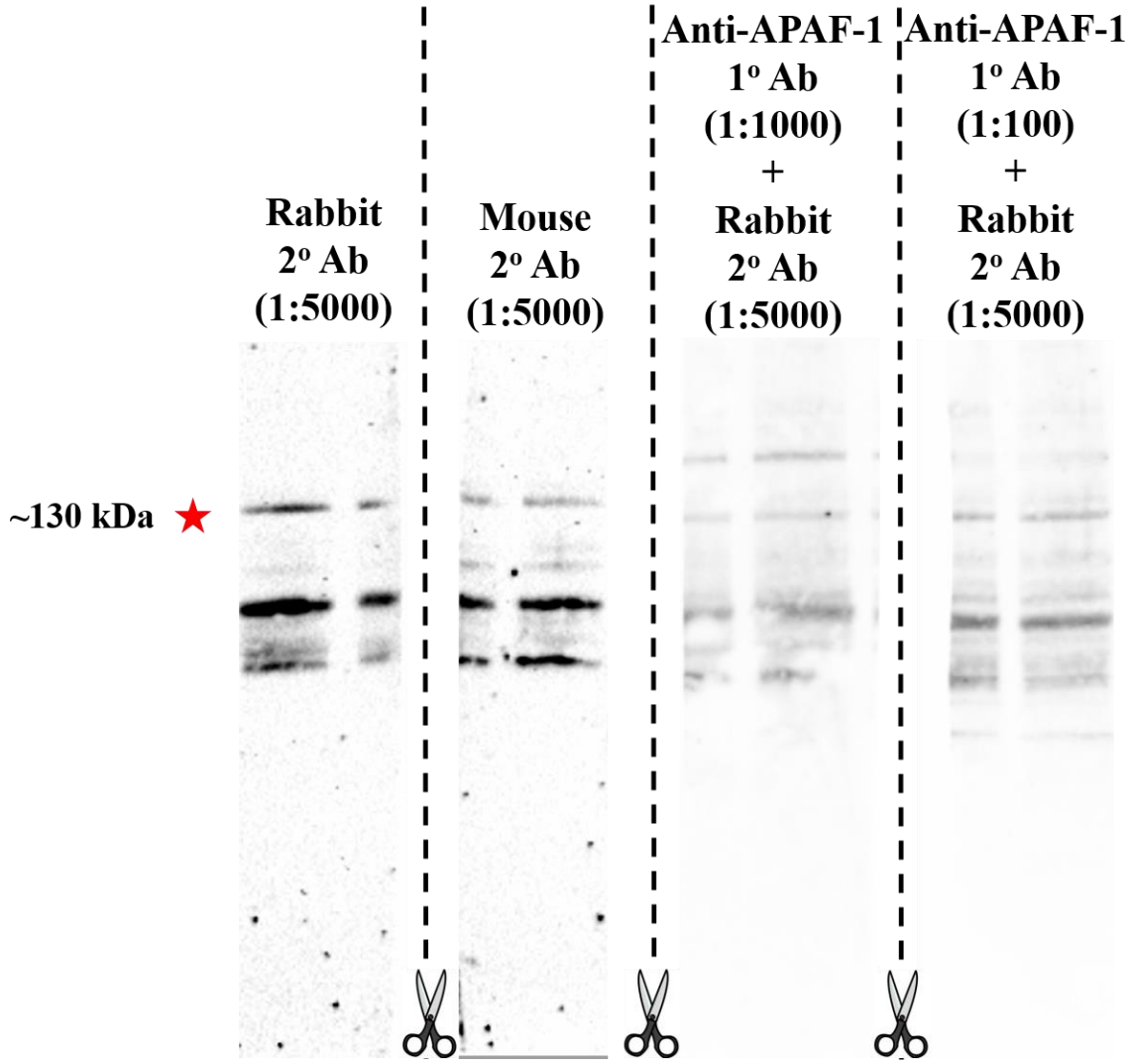

Figure 19. Western blot analysis revealing APAF-1-sized bands in the presence and absence of primary antibodies against human APAF-1. Maximum volume (45µL) of protein lysate derived from whole-cell extraction of control *mca1* cells was subjected to SDS-PAGE, followed by transfer onto a nitrocellulose membrane. The membrane was cut into separate sections and probed with either primary and secondary antibodies, or solely using secondary antibodies. Incubation with antibody occurred either overnight (primary) or for 1-hr (secondary), at indicated dilutions.

Primary APAF-1 homolog in *C. reinhardtii* lacks key domains. In mammals, the CARD-domain present in the *N*-terminus of APAF-1 allows it to interact with the CARD-containing prodomain of caspase-9 (Acehan *et al.*, 2002, Shemarova, 2010, Kasuba *et al.*, 2015). *In silico* analysis to determine the presence of human APAF-1-like genes in *C. reinhardtii* indicated that the primary homolog (E value: 3e-30) has few identical amino acids (30%), and possesses only the WD-40 repeat domains, while lacking the conserved CARD and AAA16 domains (Table 2).

Table 2

BLASTP results of prime *C. reinhardtii* sequences encoding a potential human APAF-1 orthologue.

Significant alignments	Total score	E value	Identity	Conserved domains present (CARD/AAA16 /WD40 repeats)	Accession (NCBI)	References
Candidate APAF-1 protein	640	3e-30	30%	WD40 repeats	XP_00169 6109	Merchant *et al.*, 2007
Receptor of activated protein kinase C1	216	2e-29	26%	WD40 repeats	XP_00169 8065	Merchant *et al.*, 2007
Flagellar associated protein	386	5e-29	23%	WD40 repeats	XP_00169 0131	Merchant *et al.*, 2007
Transcriptional repression protein	366	2e-26	34%	WD40 repeats	XP_00169 6066	Merchant *et al.*, 2007
Centriole proteome protein	358	3e-25	29%	WD40 repeats	XP_00169 9653	Merchant *et al.*, 2007

Heat-stress promotes substantial protein loss in *C. reinhardtii* cells. To determine if MCA1 and the potential APAF-1 orthologue in *C. reinhardtii* interact, a whole-cell extraction of WT and *mca1* cells was performed. The lysates of both strains underwent sucrose gradient centrifugation (10-30% density), followed by Coomassie blue staining to determine the efficiency of protein separation. The results indicated that the chosen sucrose gradient allowed for efficient protein separation in control samples of either strains (Fig. 20). Coomassie staining also indicated the substantial loss of proteins in heat-stressed samples of both strains (Fig. 21). Western blots using primary antibodies against human APAF-1 (1:100 and 1:1000), and/or secondary antibodies (1:5000, goat anti-rabbit), failed to display any protein binding when visualized under the molecular imager (not shown).

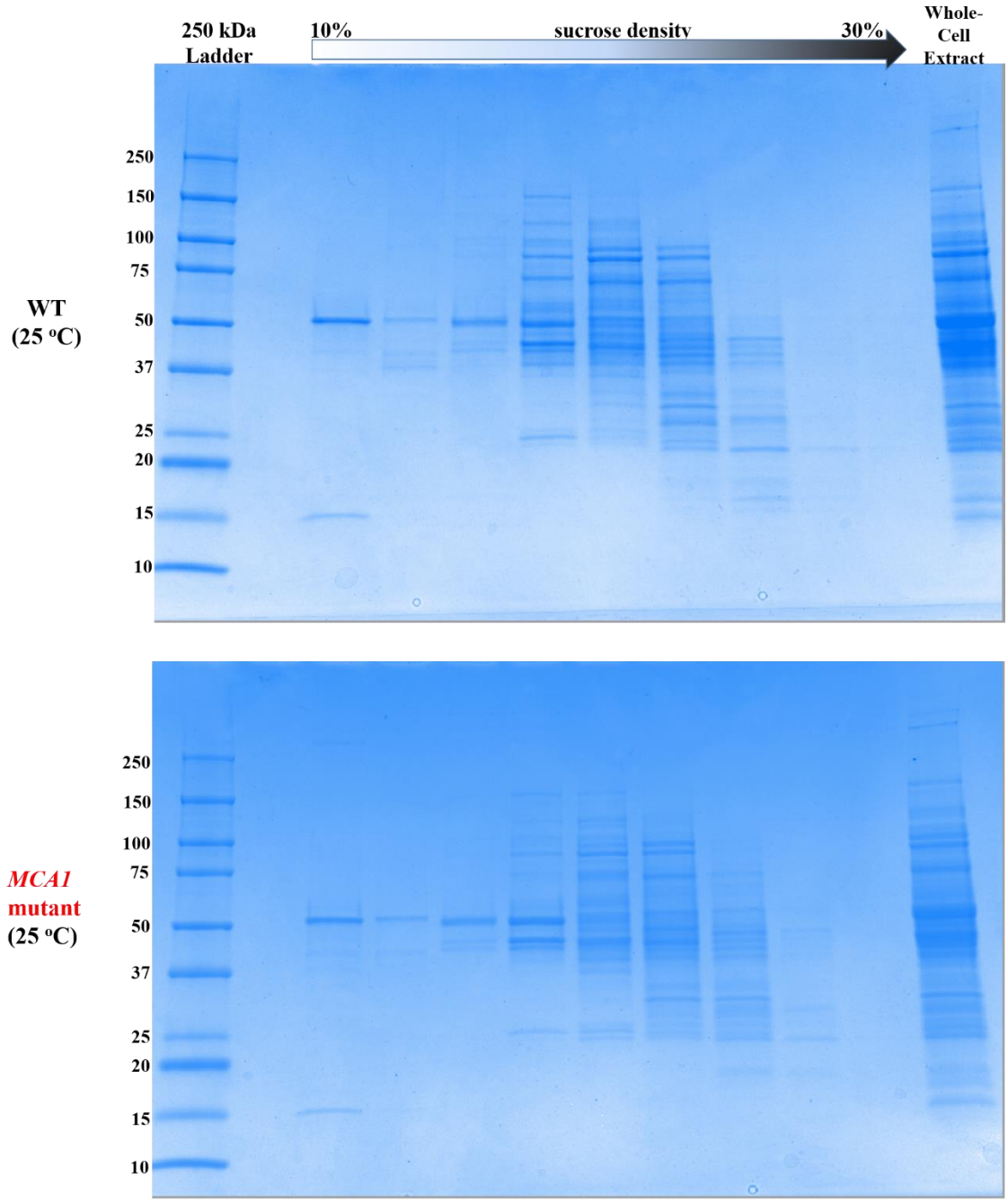

Figure 20. Coomassie blue stain results depicting efficient protein extraction and separation in both strains. Maximum protein volume (45 µL) from sucrose gradient (10-30%) fractions (1-9), and whole cell extract from both strains was subjected to SDS-PAGE, followed by overnight incubation with Coomassie blue, and visualization.

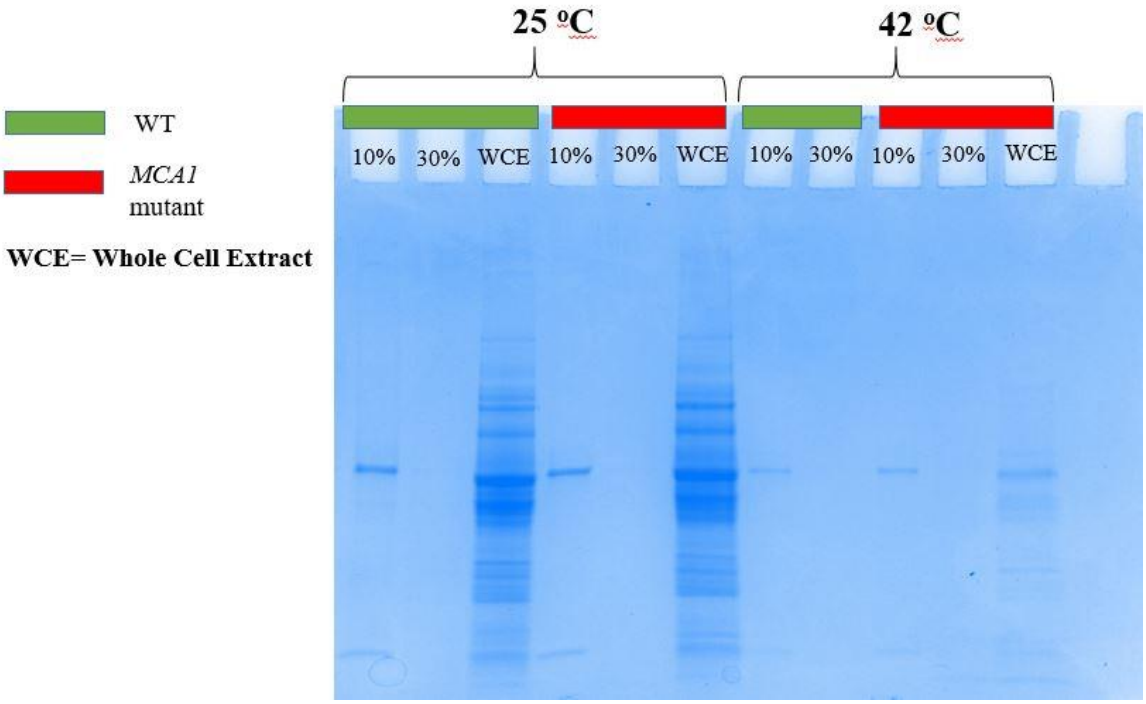

Figure 21. Representative image of Coomassie blue stain results revealing substantial loss of proteins in heat-stressed samples of both strains. The maximum volume (45 µL) of protein lysate derived from whole-cell extraction (WCE), and sucrose fractions (10% and 30%) from both strains was subjected to SDS-PAGE (WCE lysate from heat-stressed WT samples not included due to experimental error). Afterwards, gels were incubated overnight with Coomassie blue dye, followed by visualization.

Discussion

APAF-1, a protein critical for the intrinsic apoptotic pathway, has been solely identified biochemically in multicellular eukaryotes. However, PCD displays conservation across the three life domains, and the recent reports of putative *APAF-1* sequences in the genomes of unicellular eukaryotes, such as *Leishmania* and *C. reinhardtii*, are of particular interest to studies seeking to elucidate this process (Moharikar *et al.*, 2007, Gannavaram & Debrabant, 2012, Kletzin *et al.*, 2015). Our goal was to determine the presence and role of a potential APAF-1 orthologue in *C. reinhardtii*. We hypothesized that heat stress-induced PCD would lead to apoptosome formation. Further, in the event of apoptosome formation, we hypothesized that the presence of a prodomain in MCA1 would allow for interaction with APAF-1 during apoptosome formation.

While testing our hypotheses, the data indicated that the prior published report of a potential APAF-1 orthologue in *C. reinhardtii* was likely artefactual. Specifically, we noted that Western blot analysis using solely secondary antibodies generated results comparable to those derived from usage of primary antibodies against human APAF-1. In multicellular eukaryotes, the CARD domain is critical for the interaction between APAF-1 and procaspase-9, and is key to the activity of an array of PCD modulators (Shemarova, 2010, Kaczanowski *et al.*, 2011). Our *in silico* analysis further disagreed with the potential presence of an APAF-1 orthologue in *C. reinhardtii*, as evidenced by the low amino acid identity (23-34%) and lack of key conserved domains (CARD and AAA16) in the primary APAF-1 homologs. Interestingly, previous *in silico* attempts to identify an APAF-1 orthologue in the *C. reinhardtii* genome revealed a similarly low amino acid

identity (40%) and presence of solely WD-40 domains (Moharikar *et al*., 2007). Importantly, the prodomain of metacaspases has not yet been reported to contain a CARD (Choi & Berges, 2013); thus, if present in *C. reinhardtii*, an APAF-1 orthologue may not require such a domain to interact with metacaspases. Further, Moharikar *et al.* (2007) provided a cropped image of the Western blot analysis; therefore, we cannot verify whether the primary antibodies against human APAF-1 used in the earlier report also displayed a similar lack of specificity, i.e., non-specific bands. Together, our biochemical and *in silico* results suggest that an APAF-1 orthologue has yet to be biochemically identified within *C. reinhardtii*.

Notably, heat stress exposure inhibited the efficient extraction of proteins. Though PCD can be induced through various means, we opted to use heat stress due to our familiarity with the method. In previous experiments, we observed that heat stress induced the early transmission of PCD phenotypes in both WT and *mca1*cells, though notably more so in the latter (Chapter II). In turn, this could account for the substantial protein loss we observed, and be further aided by the cell-wall-deficiency (*cw15* mutation) inherent to both of our *C. reinhardtii* strains.

In all, our data is inconclusive and neither supports, nor rejects our null hypotheses, these being that heat stress will not induce apoptosome formation and that MCA1 is not required for formation of an apoptosome complex. Specifically, this inconclusiveness is due to the lack absence of available APAF-1 antibodies for *C. reinhardtii*. Due to the aforementioned finding, as well as time constraints, we chose not to attempt the optimization of heat stress exposure lengths necessary for efficient protein extraction. Consequently, we cannot determine whether our observation of substantial

protein loss post one hour of heat stress would hold at other time-points. Nonetheless, we recommend that future studies seeking to extract proteins from heat-stressed *C. reinhardtii* cells, particularly the CC-4533 strain, optimize their length of exposure to time frames shorter than one hour.

Reproducibility and verification are integral to the scientific method. While attempting to further analyze the findings of Moharikar *et al.* (2007) we found our results to be different. Similar to Moharikar *et al.* (2007), we identified an APAF-1-sized band; however, our attempts to validate our findings led to the discovery that the rabbit polyclonal primary antibodies against human APAF-1, as used in the prior published report, lacked the specificity necessary to indicate the presence of APAF-1 in *C. reinhardtii*. Thus, the results of this thesis were key to the empirical PCD literature, as evidenced by our assertion that the findings of Moharikar *et al.* (2007) were artefactual. Due to being a key regulator of multicellular PCD, the identification of APAF-1 in unicellular organisms would allow for a significant stride in our understanding of PCD evolution. However, our findings indicate that an APAF-1 orthologue in *C. reinhardtii* has yet to be identified biochemically, and remains solely reported in multicellular eukaryotes.

Materials & Methods

Cell strain and culture conditions. The *Chlamydomonas* CC-4533 (cw15 cell-wall-deficient) and *mca1* strains were obtained from the *Chlamydomonas* Resource Center (CRC), University of Minnesota. The CC-4533 strain is selected for control purposes due to being the parental strain of the mutant. The *mca1* strain has one verified CIB1 insertion: chromosome 12 coding sequence (Cre12.g517541 base 4050836), and

two unverified reported CIB1 insertions (chromosome 12 intron, Cre12.g517541 base 4050973; and chromosome 8 intron, Cre08.g361350 base 886975) (Li *et al.*, 2016). In accordance with Whitman, 1986, cells were grown in liquid Tris-Acetate-Phosphate (TAP) Medium, with aeration and under a 14-h/10-h light cycle.

PCD induction. Heat stress-induced PCD was conducted via incubation of samples in a water bath at 42 ^{o}C, for designated time-points. For controls, samples remained at room temperature (25 ^{o}C), for respective periods of time.

Lysate preparation. Sample proteins were prepared via whole-cell extraction. Four pea-sized loopfuls of either WT or *mca1* cells were inoculated into 300 mL of Tris-Acetate-Phosphate (TAP) medium, and after three days of growth, transferred into a flask containing 700 mL of TAP medium. After an additional three days of growth, samples were transferred to 50 mL conical tubes (20 total), and 10 tubes were subjected to heat-stress for 1 hr., while the remaining tubes served as controls. Designated sample halves were subjected to an initial spin at 4000 RPM for 4 min, and after removal of supernatant, were washed by resuspension to their original volume in 10 mM Hepes. After the wash (1x), the supernatant was poured off and cells were resuspended to 1 mL of ice cold HMDEK buffer containing Halt Protease Inhibitor Single-Use Cocktail (Lot # QJ228425) (1:100). The cells were then vortexed at setting 6.5 for 1 min using a 15 mL conical tube containing 1 mL of acid-washed glass beads. The cell suspension was transferred to a thick-walled centrifuge tube, and centrifuged at 23,000 RPM for 2 hours at 4 ^{o}C. Proteins were incubated with 5X sample buffer (1:4) at 100 ^{o}C for 5 min. Sample proteins were then aliquoted into microcentrifuge tubes, and stored at -20 ^{o}C.

Sucrose gradient centrifugation. To determine if APAF-1 was inactive in the cytosol (monomer form), or active in complex formation, we sought to determine if the putative APAF-1 protein was present in low, and/or high sucrose density fractions. To this end, 750 µL of designated protein samples were carefully pipetted into a thick-walled ultracentrifuge tube containing 4 mL of sucrose in HMDEK buffer (10-30% sucrose density). The tubes were then centrifuged at 36,000 RPM for 16 hrs. at 4 ^{o}C. Following, the centrifuged protein content of each sample was separated into 9 fractions, and after incubation with 5x Laemmli buffer at 100 ^{o}C for 5 min, was stored at -20 ^{o}C.

SDS-PAGE and Coomassie blue. To verify for the efficient separation of proteins, 35 µL of sample protein fractions were loaded onto a 4-20% Criterion TGX precast gel (BioRad). Gels were ran at 70 V for an initial 20 min, and then at 140 V for 1 hr. Afterwards, gels were stained overnight with Coomassie blue, and visualized under a Chemi Doc XRS+ molecular imager (BioRad).

Western blotting. Due to solely determining the presence of APAF-1, the sample protein content was not quantified. After electrophoresis of maximum sample load, proteins were transferred to a nitrocellulose membrane at 80 V for 1 hr, and verified via Ponceau S stain, in accordance to standard protocol. Indicated membranes were then probed with rabbit polyclonal human anti-APAF-1 (1:100-1:3000; Sigma Aldrich; Lot #: 061M1463, Product #: A8469) as a primary antibody, HRP-linked anti-IgG as a secondary antibody (rabbit: 1:3000, mouse: 1:3000; Bio-Rad; Catalog #: 172-1019, Catalog #: 172-1011, respectively), and developed using a chemiluminescence kit (Clarity Western ECL Substrate, Bio-Rad). The resulting membranes were visualized under a Chemi Doc XRS+ molecular imager (Bio-Rad).

Bioinformatics. Protein homology searches were conducted through BLASTP, using the non-redundant protein database for a relatively liberal search in the *C. reinhardtii* proteome. The reference sequence, human APAF-1 isoform B (accession: NP_001151.1), was derived from the NCBI database.

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CHAPTER IV

SUMMARY

The goal of this Master's thesis was to further expand our understanding of the PCD machinery in unicellular eukaryotes. Specifically, this thesis focused on elucidating the role(s) of MCA1, a novel member of the CP protease family (Chapter II), and APAF-1, an apoptotic protein key to PCD in multicellular organisms (Chapter III). The disruption of *MCA1* was noted to be associated with increased plasma membrane disruption in response to heat stress. On the other hand, *MCA1* knockout was not linked to an irregular rate of PS externalization, or alter cell proliferation of either strain upon the onset of heat stress. Additionally, within heat-stressed *C. reinhardtii* populations, the disruption of *MCA1* was linked with an early display of ROS accumulation, and a significantly greater ROS response with prolonged heat stress exposure. Furthermore, the rate of DNA laddering also appeared to be increased in heat-stressed *mca1* cells, relative to WT. Interestingly, Western blot and *in silico* analyses of WT and *mca1* cells refuted the prior published report suggesting the biochemical identification of APAF-1 within the unicellular green alga, *C. reinhardtii*. Moreover, our data also suggest the potential limitations of heat stress during the proteomic analysis of *C. reinhardtii*.

Contributions to the literature

Due to their novel discovery, as well as being largely present in organisms whose PCD machinery is seldom studied, metacaspases are poorly understood. *C. reinhardtii* has been used as a tool for understanding a wide array of cellular mechanisms; however, similar to other metacaspase-containing organisms, its PCD machinery is in desperate need of understanding. This thesis attempts to counter this trend via reverse genetic

analysis of *MCA1*, and in turn, identifies PCD events potentially modulated by MCA1 in *C. reinhardtii*. Notably, these findings fall short of indicating whether MCA1 is directly or indirectly associated with our analyzed PCD phenotypes. Nonetheless, this Master's thesis provides a foundation for future studies attempting to further elucidate the role of MCA1 in *C. reinhardtii*.

APAF-1 is a key apoptotic protein reported in multicellular eukaryotes, and its potential identification in unicellular organisms has major implications to our understanding of PCD mechanisms and evolution. The scientific method relies on the ability of scientists to verify, and even refute such critical findings. Thus, the current thesis benefits the scientific community by negating a prior published report of APAF-1 presence in the unicellular green alga, *C. reinhardtii*.

Recommendations for future studies

Future studies would benefit from utilizing *mca2*, and complemented strains to ascertain whether metacaspase-dependent, or –independent pathways are activated as a result of heat stress. This thesis relied on fluorescence microscopy to determine the induction of PCD events, however, future attempts are advised to use more detailed analytic methods, e.g., flow-cytometry, spectrophotometry, etc., which would allow for better elucidation of PCD mechanisms. Furthermore, different means of PCD-induction (e.g., H_2O_2-, UV-C-exposure, etc.,) will also aid in determining irregularities that are distinct to particular types of stress. Different forms of inducing PCD might also be more easily optimized for techniques such as protein extraction. PCD is extensively under-studied in unicellular organisms, and as exemplified by this thesis, multiple layers of verification are recommended to assess the PCD machinery in *C. reinhardtii*.

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VITA

Celso Santos Goncalves Catumbela

EDUCATION

Master of Science candidate in Biological Sciences at Sam Houston State University, August, 2014 – May, 2017

Thesis title: "Analyses of Metacaspase 1 and a Potential APAF 1 Orthologue During Heat-Stress Induced Programmed Cell Death in *Chlamydomonas reinhardtii*."

Bachelor of Science (May, 2013) in Biology, Southwestern University, Georgetown, TX.
Minor (May, 2013) in Sociology, Southwestern University, Georgetown, TX.

ACADEMIC EMPLOYMENT

Graduate Research Assistant, Department of Science and Engineering Technology, Sam Houston State University, August, 2014 – Present. Responsibilities include: providing research assistance in laboratory projects and laboratory maintenance.

PRESENTATIONS AT PROFESSIONAL MEETINGS

March 2016
Graduate Oral Presentation at Spring Meeting of American Society for Microbiology Conference "Genetic analysis of Metacaspase 1 during heat stress-induced PCD in *Chlamydomonas reinhardtii*"

November 2016
Graduate Poster Presentation at American Society for Microbiology Conference "Effects of *Metacaspase-I* Knockout during Heat-Stress Induced Programmed Cell Death in *Chlamydomonas reinhardtii*". ***2nd Place: Samuel Kaplan Poster Award***

April 2016
Culture Presentation at Sam Houston State University Annual International Week **"**Angola, no I did not just make it up, we do exist... for now".

October 2015

Graduate Student Speaker at Southwest Regional Meeting of Society of Developmental Biology "Functional analysis of novel conserved mixed-isoform B56δ/γ within the canonical Wnt signaling pathway in *Xenopus laevis*".

July 2015

Graduate Student Poster Presentation at Annual Meeting of Society of Developmental Biology "Functional analysis of novel conserved mixed-isoform B56δ/γ within the canonical Wnt signaling pathway in *Xenopus laevis*".

March 2015

Graduate Student Paper Competition at Annual Meeting of Texas Academy of Science "Functional analysis of novel conserved mixed-isoform B56δ/γ within the canonical Wnt signaling pathway in *Xenopus laevis*".