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<b>14. ABSTRACT</b> Our research focuses on the apical apoptotic enzyme caspase 2 (C2), which has been proposed as a critical activating protease for breast cancer chemotherapeutic agents such as doxorubicin and etoposide. Our lab has previously shown that C2 is held inactive by nutrient flux through the pentose phosphate pathway via phosphorylation at serine 135, which is mediated by calmodulin-dependent protein kinase II (CaMKII). We now report recent exciting advancements in the understanding of caspase-2 metabolic regulation using <i>Xenopus laevis</i> egg extract: as nutrient levels wane and NADPH/CaMKII activity drops, the acidic phosphoserine-binding protein 14-3-3 $\zeta$ is released from caspase-2, leaving caspase-2 vulnerable to PP1-mediated dephosphorylation and subsequent activation. Importantly, this mechanism appears to be conserved in mammalian oocytes, further supporting that, in fact, caspase-2 is likely to be metabolically regulated even in mammals. This work has been accepted for publication in the June 16, 2009, edition of <i>Developmental Cell</i> . In extending these findings to breast cancer, we have produced phospho-specific antibodies to potential mouse caspase-2 phosphorylation sites, and are currently examining the metabolic regulation of these phosphorylation sites in somatic cells.					
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## INTRODUCTION

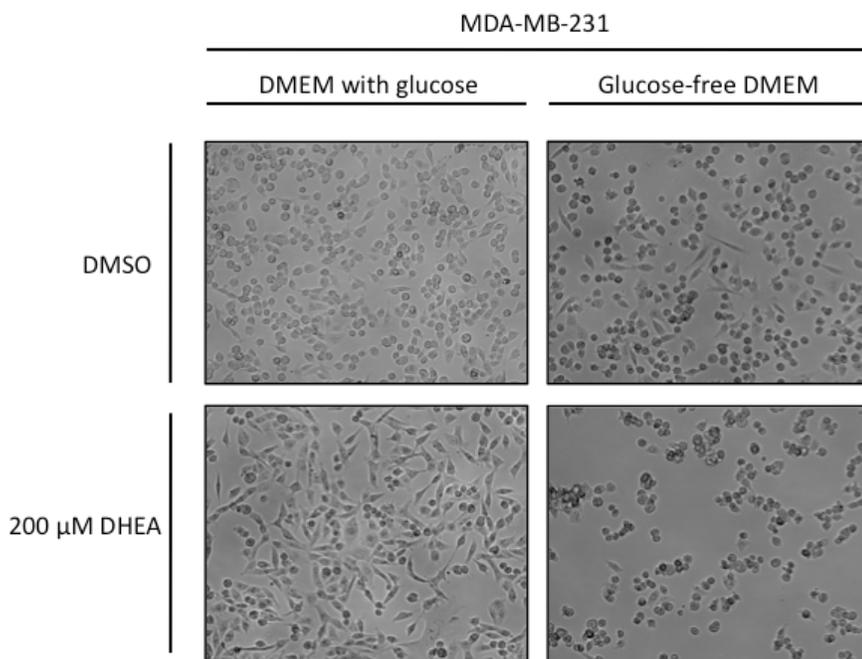
A major obstacle impeding the use of cytotoxic agents in breast cancer treatment is the cancer cell's ability to evade cell death pathways. Therapies which induce apoptosis in otherwise resistant cancer cells have the potential to treat a variety of cancers, including breast cancer. Our research focuses on the apical apoptotic enzyme caspase 2 (C2), which has been proposed as a critical activating protease for breast cancer chemotherapeutic agents such as doxorubicin and etoposide, as well as other genotoxic agents. Our lab has previously shown that C2 is held inactive by nutrient flux through the pentose phosphate pathway (PPP). C2 inactivation in response to nutrient abundance is a consequence of C2 phosphorylation by CaMKII, which prevents downstream engagement of the executioner pathway. CaMKII is itself activated by the PPP byproduct NADPH. The mechanism of CaMKII activation and subsequent C2 inhibition by NADPH is still unknown, as is the mechanism of C2 activation in response to declines in NADPH levels. Additionally, extensive studies have shown that cancer cells commonly have aberrant metabolic pathways, leading us to ask the question of whether metabolism is involved in evasion of cell death pathways in breast cancer. In this vein, we hypothesize that breast cancer cells may be taking advantage of alterations in their metabolic status and increased flux through the PPP in order to suppress C2 and evade cell death by cytotoxic agents. By elucidating the role of metabolism and C2 phosphorylation mechanisms in breast cancer cells, we will better understand the importance of metabolic pathways and cell death evasion in tumor chemoresistance.

## BODY

Our lab has previously shown that C2 is held inactive by nutrient flux through the pentose phosphate pathway via phosphorylation at serine 135, which is mediated by calmodulin-dependent protein kinase II (CaMKII). We now report recent exciting advancements in the understanding of caspase-2 metabolic regulation using *Xenopus laevis* egg extract. We now show that caspase-2 phosphorylated at S135 binds 14-3-3 $\zeta$ , thus preventing caspase-2 dephosphorylation. Moreover, we determined that S135 dephosphorylation is catalyzed by protein phosphatase-1, which directly binds caspase-2. Although caspase-2 dephosphorylation is responsive to metabolism, neither PP1 activity nor binding is metabolically regulated. Rather, release of 14-3-3 $\zeta$  from caspase-2 is controlled by metabolism and allows for caspase-2 dephosphorylation. Accordingly, a caspase-2 mutant unable to bind 14-3-3 $\zeta$  is highly susceptible to dephosphorylation. Although this mechanism was initially established in *Xenopus*, we now demonstrate similar control of murine caspase-2 by phosphorylation and 14-3-3 binding in mouse eggs. The fact that this mechanism is conserved in mammalian oocytes supports that, in fact, caspase-2 is likely to be metabolically regulated even in the mammalian system. This extensive biochemical work, on which I am a co-first author, has been accepted for publication in the June 16, 2009, edition of *Developmental Cell* and is appended to this report. This work is crucial to our understanding of the role of caspase-2 and metabolism in breast cancer because it extends our biochemical understanding of caspase-2 and its interactions with other proteins. Importantly, a paper was recently published which reports that 14-3-3 $\zeta$  (the isoform implicated in caspase-2 phospho-S135 binding and suppression) overexpression in breast cancer defines high risk of disease and promotes cancer cell survival (Neal, CL). This is extremely interesting given our current findings in that it supports conservation of the mechanism of caspase-2 activation in breast cancer cells.

In extending our findings of caspase-2 to breast cancer, we have produced phospho-specific antibodies to potential mouse caspase-2 phosphorylation sites, and are currently examining the metabolic regulation of these phosphorylation sites in somatic cells. We are also optimizing caspase-2 overexpression experiments with various metabolic treatments (DHEA to inhibit glucose-6-phosphate dehydrogenase, thus inhibiting NADPH production, and D-methylmalate to stimulate the malic enzyme, thus promoting NADPH production through a non-pentose phosphate pathway mechanism) and are in the process of evaluating C2 phosphorylation status using our C2-directed phospho-antibodies. We have further extended these metabolic experiments to breast cancer cells, and specifically to the breast cancer cell line MDA-MB-231. As shown in Figure 1, when these cells were incubated in glucose-rich media (regular DMEM) and treated with the G6PDH inhibitor DHEA the cancer cells are able to survive. Conversely, when the breast cancer cells are incubated in glucose-free media (DMEM without glucose) and treated with DHEA, they die rapidly. We are in the process of demonstrating that this cancer cell death is due to metabolically regulated caspase-2 dephosphorylation and activation. In theory, breast cancer cells incubated in glucose-rich media may be able to derive sufficient NADPH from nutrients in the media such that

the cells are not as susceptible to DHEA treatment. In contrast, breast cancer cells incubated in glucose-free media are more vulnerable to decreases in NADPH levels: as NADPH levels drop in the cell, 14-3-3 is signaled to release from phosphorylated caspase-2, thus leaving the metabolically-regulated phosphorylation site vulnerable to dephosphorylation by the constitutively-bound phosphatase. At the same time, drops in NADPH levels also cause a decrease in the caspase-2-directed kinase activity, and the end result of the two effects is caspase-2 dephosphorylation, oligomerization, and activation.



**Figure 1. Pentose phosphate pathway inhibition promotes death of breast cancer cells.** MDA-MB-231 cells were cultured in either regular DMEM or glucose-free DMEM for 12 hrs. Cells were then treated with 200  $\mu$ M DHEA for 6 hrs and brightfield pictures were obtained.

In addition to the publication appended to this report and the ongoing tissue culture work described, we have also been working on elucidating the mechanism of CaMKII activation by NADPH. Below I have listed the current status of my progress based on the approved Statement of Work from my grant:

## **Statement of Work**

### **A. Training plan tasks**

1. Attend weekly Signal Transduction Colloquia sponsored by the Department of Pharmacology and Cancer Biology, interact with seminar speakers  
*Status: currently attending all appropriate seminars*

2. Attend weekly Science of Oncology Symposia sponsored by the Duke Comprehensive Cancer Center  
*Status: attending all Science of Oncology seminars*
3. Attend oncology clinic at the Duke Breast Program  
*Status: have attended breast oncology clinic one time*
4. Develop skills to effectively ask scientific questions  
*Status: ongoing*
5. Practice thoughtful, creative, and effective experimental design  
*Status: ongoing*
6. Analyze the implications for public health and human disease in all of my scientific work  
*Status: ongoing*
7. Develop effective scientific writing techniques by preparing manuscripts  
*Status: prepared the manuscript for publication in Developmental Cell and was In charge of all review experiments, rebuttal letter writing, and manuscript revisions*
8. Maintain a strong fund of knowledge of the published literature in breast cancer and apoptosis  
*Status: ongoing*

## **B. Research tasks and Methods**

1. Determine the mechanism of CaMKII activation by NADPH
  - a. CaM/CaMKII binding assays in the presence and absence of G6P/NADPH in *Xenopus* egg extract
    - i. Silver staining
    - ii. 2D gel electrophoresis
    - iii. Mass spectrometry to identify novel binding partners

*Status: I have pursued the silver staining experiments extensively with varying wash conditions and have concluded that there is too much background binding to see any differential bands. 2D gel electrophoresis and mass spectrometry approaches are currently being considered.*

- b. Recombinant glutathione-bound CaMKII in the presence and absence of G6P/NADPH in *Xenopus* egg extract
  - i. Silver staining
  - ii. 2D gel electrophoresis
  - iii. Mass spectrometry to identify novel binding partners

*Status: We are working on obtaining the construct to make GST-CaMKII or are interested in cloning CaMKII into the pGEX vector ourselves.*

- c. NADPH affinity binding column for depletion of *Xenopus* egg extract of NADPH-interacting factors

- i. Evaluate depleted fractions for C2 phosphorylation activity in the presence of exogenous NADPH
- ii. Elution of NADPH binding partners and add-back of eluate to depleted extract to test for recovery of C2 phosphorylation activity

*Status: We have not yet attempted this approach; our belief is that perhaps CaMKII activation is directly due to something further downstream of NADPH,*

*such as fatty acids. Thus, we are not yet convinced that direct binding of NADPH is playing a role in CaMKII activation, but will pursue this further if needed.*

- d. Evaluate CaMKII activity in presence and absence of G6P/NADPH
  - i. Immunoblotting with phospho-T286 CaMKII antibody
  - ii. *In vitro* kinase assays to look at CaMKII phosphorylation at novel sites, mapping of novel sites

*Status: Our preliminary data suggests that in the presence of G6P, phosphorylation of CaMKII at its autophosphorylation site (T286) is increased.*

*Since we have seen increased phosphorylation at this canonical site, we have not pursued mapping of an alternative site.*

2. Analysis of C2 phosphorylation status in normal and breast cancer cell lines
  - a. Use a phospho-C2 antibody to analyze various breast cancer cell lines
    - i. Production of phospho-C2 antibody
    - ii. Immunoblotting a spectrum of breast cancer cell lines with phospho-C2 antibody

*Status: Serum for phospho-antigens was received; three different antigens were used: phospho-MC2 S157, S164, and S157/164. I have affinity-purified the phospho-S157 and S164 antibodies and have validated their specificity. I am currently using the phospho-antibodies to determine the metabolically-regulated caspase-2 phosphorylation site in somatic cells. I am optimizing these experiments to determine the appropriate treatment concentrations and timecourses and expect to determine the metabolically-regulated phosphorylation site very soon.*

- iii. Extend analysis to primary tumor samples and correlate C2 phosphorylation status with disease progression

*Status: As soon as the caspase-2 phosphorylation site in somatic cells is validated as described above, I will move to immunoblotting a panel of breast cancer primary and tissue culture cells to determine basal C2 phosphorylation status.*

- b. Introduction of C2 phospho-mutants to chemoresistant cells
    - i. Treat with chemotherapeutics such as doxorubicin, in the presence and absence of DHEA and/or malate
    - ii. Determine chemosensitivity using a cleaved caspase 3 antibody

*Status: Will pursue as soon as the appropriate phosphorylation site is validated and cloning for the corresponding phospho-mutant is completed.*

3. Analysis of CaMKII activity in normal and breast cancer cell lines
  - a. Evaluate cell lines using an autophosphorylated-CaMKII antibody (phospho-T286)
  - b. Evaluate activity of cell lysates against a radioactive CaMKII substrate
  - c. Extend analysis to primary tumor samples and correlate CaMKII activity with disease progression

*Status: I have preliminarily screened some breast cancer cell lysates by immunoblotting with the pT286 CaMKII antibody, and do see some appreciable differences in CaMKII activity. I would like to repeat these data and then choose*

*a panel that represents a wide range of CaMKII activity; I will then compare the phosphorylation status of C2 between these cell lines and would expect to see a direct correlation between CaMKII activity and C2 phosphorylation.*

4. Metabolomic profiling of breast cancer cell lines

- a. Use mass spectrometry to measure intermediary metabolites
- b. Use statistical analysis to create metabolic “signatures” for each cell line
  - i. Create a signature to differentiate between normal and cancerous breast epithelial cells
  - ii. Correlate metabolomic data with analysis of CaMKII activity and C2 phosphorylation status

*Status: Once breast cancer cell lines with differing CaMKII activity and C2 phosphorylation status have been identified, we will use these cell lines to pursue metabolic profiling to create “signatures” for each selected cell line.*

## KEY RESEARCH ACCOMPLISHMENTS

- Caspase-2 is dephosphorylated at S135 in *Xenopus laevis* egg extract prior to caspase-2 processing and downstream effector caspase activation
- Caspase-2 dephosphorylation is inhibited in the presence of G6P to stimulate the pentose phosphate pathway
- Inhibition of PP1 by okadaic acid addition prevents caspase-2 dephosphorylation, caspase-2 processing, cytochrome c release, and downstream caspase activation
- PP1 depletion using GST-Inhibitor-2 prevents caspase-2 dephosphorylation
- PP1 directly binds caspase-2 in the absence of a targeting subunit
- PP1 binds caspase-2 at V106/H108
- PP1 binding and activity are not regulated by metabolism
- Caspase-2 phosphorylated at S135 selectively binds 14-3-3 $\zeta$  but not other 14-3-3 isoforms
- 14-3-3 release from caspase-2 occurs immediately prior to maximal caspase-2 dephosphorylation and downstream caspase activation
- 14-3-3 release is inhibited in the presence of G6P, and 14-3-3 binding is the point of metabolic control in caspase-2 activation
- The mechanism of caspase-2 activation is conserved in mouse caspase-2 in oocytes
- **Remaining work as described above are preliminary and still in progress**

## REPORTABLE OUTCOMES

### **Manuscript:**

Metabolic control of oocyte apoptosis mediated by 14-3-3 $\zeta$ -regulated dephosphorylation of caspase-2

Leta K. Nutt\*, Marisa R. Buchakjian\*, Eugene Gan, Rashid Darbandi, Sook-Young Yoon, Judy Q. Wu, Yuko J. Miyamoto<sup>2</sup> Jennifer A. Gibbon, Josh L. Andersen, Christopher D. Freel, Wanli Tang, Changli He, Manabu Kurokawa, Yongjun Wang, Seth S. Margolis, Rafael A. Fissore, and Sally Kornbluth

Scheduled for publication in *Developmental Cell* in June, 2009

## CONCLUSIONS

Our lab has previously shown that C2 is held inactive by nutrient flux through the pentose phosphate pathway via phosphorylation at serine 135, which is mediated by calmodulin-dependent protein kinase II (CaMKII). We now report recent exciting advancements in the understanding of caspase-2 metabolic regulation using *Xenopus laevis* egg extract. We now show that caspase-2 phosphorylated at S135 binds 14-3-3 $\zeta$ , thus preventing caspase-2 dephosphorylation. Moreover, we determined that S135 dephosphorylation is catalyzed by protein phosphatase-1, which directly binds caspase-2. Although caspase-2 dephosphorylation is responsive to metabolism, neither PP1 activity nor binding is metabolically regulated. Rather, release of 14-3-3 $\zeta$  from caspase-2 is controlled by metabolism and allows for caspase-2 dephosphorylation. Accordingly, a caspase-2 mutant unable to bind 14-3-3 $\zeta$  is highly susceptible to dephosphorylation. Although this mechanism was initially established in *Xenopus*, we now demonstrate similar control of murine caspase-2 by phosphorylation and 14-3-3 binding in mouse eggs. The fact that this mechanism is conserved in mammalian oocytes supports that, in fact, caspase-2 is likely to be metabolically regulated even in the mammalian system. This work is crucial to our understanding of the role of caspase-2 and metabolism in breast cancer because it extends our biochemical understanding of caspase-2 and its interactions with other proteins.

In extending our findings of caspase-2 to breast cancer, we have produced phospho-specific antibodies to potential mouse caspase-2 phosphorylation sites, and are currently examining the metabolic regulation of these phosphorylation sites in somatic cells.

### D. Outcomes of the proposed research

Completion of the proposed work will result in the publication of at least two manuscripts: one which describes a molecular connection between metabolism and cell death signaling by detailing the mechanism of CaMKII activation by NADPH, and another which describes how metabolism affects C2 phosphorylation status and CaMKII activity in breast cancer cells, and how metabolism may be manipulated to increase chemosensitivity in these cells. Dissemination of this work will contribute to the understanding of metabolism and chemoresistance in breast cancer.

## **Metabolic control of oocyte apoptosis mediated by 14-3-3 $\zeta$ -regulated dephosphorylation of caspase-2**

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Keywords: caspase-2, glucose-6-phosphate, protein phosphatase-1, 14-3-3, oocyte

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Running Title: 14-3-3 $\zeta$  regulates caspase-2 activation

## SUMMARY

*Xenopus* oocyte death is partly controlled by the apoptotic initiator, caspase-2. We reported previously that oocyte nutrient depletion activates caspase-2 upstream of mitochondrial cytochrome *c* release. Conversely, nutrient-replete oocytes inhibit caspase-2 via S135 phosphorylation catalyzed by calcium/calmodulin-dependent protein kinase II. We now show that caspase-2 phosphorylated at S135 binds 14-3-3 $\zeta$ , thus preventing caspase-2 dephosphorylation. Moreover, we determined that S135 dephosphorylation is catalyzed by protein phosphatase-1, which directly binds caspase-2. Although caspase-2 dephosphorylation is responsive to metabolism, neither PP1 activity nor binding is metabolically regulated. Rather, release of 14-3-3 $\zeta$  from caspase-2 is controlled by metabolism and allows for caspase-2 dephosphorylation. Accordingly, a caspase-2 mutant unable to bind 14-3-3 $\zeta$  is highly susceptible to dephosphorylation. Although this mechanism was initially established in *Xenopus*, we now demonstrate similar control of murine caspase-2 by phosphorylation and 14-3-3 binding in mouse eggs. These findings provide an unexpected evolutionary link between 14-3-3 and metabolism in oocyte death.

## INTRODUCTION

Apoptosis is a form of programmed cell death characterized by activation of cysteine proteases known as caspases, which dismantle the cell in an orderly, energy-dependent manner. Caspases are divided into two classes, initiator or effector, based on their position in the apoptotic hierarchy. Initiator caspases (e.g. caspases-2, -8, and -9) occupy the apical position in cell death pathways and are activated by dimerization, which involves recruitment of adaptor proteins to the caspase prodomain via homotypic interactions between their respective caspase recruitment domains (CARDs) (Danial and Korsmeyer, 2004). Once active, initiator caspases cleave and activate effector caspases to promote apoptosis.

In response to certain apoptotic cues, caspase-2 (C2) activation occurs on a platform consisting of the adaptor protein RAIDD, which binds directly to the C2 prodomain, and the death-domain-containing protein PIDD, which oligomerizes RAIDD (Baliga et al., 2004; Duan and Dixit, 1997; Read et al., 2002; Tinel and Tschopp, 2004). PIDD expression is p53-inducible and has been linked to genotoxic stress (Hanoux et al., 2007; Panaretakis et al., 2005; Robertson et al., 2002). Whether and how RAIDD or PIDD might be regulated post-translationally to control apoptosis is unknown.

The nutrient status of a cell may be important in the decision to initiate apoptosis. Decreased glucose uptake and glycolytic rate occur upon growth factor withdrawal in lymphocytes, and this precedes commitment to caspase activation (Rathmell et al., 2003). Artificial maintenance of glycolysis confers apoptotic resistance under these conditions (Zhao et al., 2007). Furthermore, AKT kinase exerts an anti-apoptotic effect in many cell types and requires glucose uptake in order to do so (Majewski et al., 2004; Pastorino et al., 2002; Rathmell et al., 2003; Zhao et al., 2007). When hexokinase is bound to mitochondria it can suppress the pro-apoptotic release of mitochondrial cytochrome *c* (cyt *c*) (Majewski et al., 2004; Pastorino et al., 2002). It has also been demonstrated that the pro-apoptotic Bcl-2 family member, BAD, forms a complex with and regulates the activity of glucokinase at the mitochondria (Danial et al., 2003).

We reported previously that nutrient depletion in *Xenopus* oocytes promotes apoptosis through activation of C2 (Nutt et al., 2005). This is particularly interesting in light of work by Yuan et al. demonstrating that the primary phenotype of C2 knockout mice is excess oocytes in females, suggesting that oocytes might be particularly susceptible to C2-mediated death (Bergeron et al., 1998). In exploring links between metabolism and C2, we discovered that maintenance of NADPH levels by flux through the pentose phosphate pathway (PPP) induces a suppressive phosphorylation of *Xenopus* C2 on S135. A non-phosphorylatable mutant of C2 (S135A) induced apoptosis even in the presence of high levels of NADPH.

In analyzing regulation of C2, we found that NADPH promotes activation of calcium/calmodulin-dependent protein kinase II (CaMKII), which catalyzes S135 phosphorylation. Thus, for oocytes to undergo apoptosis upon nutrient depletion (decreasing PPP flux), a phosphatase must be required to dephosphorylate C2. Although a large number of kinases have been implicated in apoptotic regulation, potential links between phosphatases and cell death are poorly understood. For protein phosphatases-1 and -2A, specificity is typically conferred by a targeting subunit which either directs the catalytic subunit to its substrates or alters its subcellular localization (Cohen, 2002; Shenolikar, 1994). Recently, however, it has been shown that the PP1

catalytic subunit may also bind directly to substrates via substrate motifs similar to those found on targeting subunits (Margolis et al., 2003; Vietri et al., 2006). In these cases, targeting subunits may be dispensable for dephosphorylation. Regulation of PP1 activity and specificity may furthermore occur through PP1 binding to inhibitory proteins (I1, I2), and substrate dephosphorylation may also be modulated by additional post-translational modifications and/or binding partners. We recently reported this mechanism for the cell cycle phosphatase Cdc25 (Margolis et al., 2006). In this example, 14-3-3 protein binds to Cdc25, masking PP1 access to phospho-S287. Cdc25 remains phosphorylated and suppressed until 14-3-3 dissociates, thus leaving S287 vulnerable to PP1 (Margolis et al., 2006; Margolis et al., 2003).

Although NADPH is required for apoptotic suppression in the oocyte, it is not known if C2 activation might also be a locus of metabolic control. We now demonstrate that PP1-mediated C2 dephosphorylation is required for C2 activation, and dephosphorylation is indirectly regulated by PPP flux. In searching for novel C2 prodomain interactors, we discovered that 14-3-3 $\zeta$  binds the C2 prodomain. Moreover, S135 dephosphorylation, which is required for C2 activation, depends upon removal of 14-3-3 $\zeta$ . Importantly, 14-3-3 $\zeta$  removal is under tight metabolic control; stimulation of the PPP results in 14-3-3 $\zeta$  binding, and nutrient depletion promotes 14-3-3 $\zeta$  release. Finally, we have also uncovered evidence of a parallel regulatory C2 activation pathway in mammalian oocytes, in that perturbation of murine C2 phosphorylation or 14-3-3 binding appears to control the viability of mouse eggs. These data provide evidence that the metabolic regulation of C2 activation depends upon an evolutionarily conserved association with 14-3-3 and this association mediates the phosphorylation status and activity of C2 during apoptosis in the oocyte.

## RESULTS

### Metabolic suppression of caspase-2 dephosphorylation

Our previous work indicated that nutrient abundance inhibited C2 through CaMKII-mediated S135 phosphorylation. These findings, coupled with observations placing C2 upstream of mitochondrial cyt *c* release, implied that C2 should be dephosphorylated prior to caspase-3 (C3) activation. To evaluate this we monitored the phosphorylation status of endogenous C2 in egg extracts using an antibody directed against phosphorylated S135 (Fig. 1A). Endogenous C2 was immunoprecipitated using either a *Xenopus* C2 antibody directed against the C-terminal 20 amino acids of the protein or the corresponding preimmune serum, and immunoprecipitates were analyzed for C2 pS135. This experiment was performed in the presence of the C2 inhibitor VDAD-CHO to ensure that loss of C2 pS135 was not due to C2 processing. As shown in Figure 1B, endogenous C2 was dephosphorylated at S135 over time, and this preceded C3 activation.

These observations raised the interesting possibility that an S135-directed phosphatase might be regulated by metabolism, thus activating C2 upon nutrient depletion. To examine C2 dephosphorylation in isolation we prepared radiolabeled, S135-phosphorylated C2 prodomain (pro) fused to GST as a dephosphorylation substrate. Incubation of this construct in egg extract resulted in dephosphorylation of C2 S135 immediately preceding C3 activation, as would be expected if C2 dephosphorylation was required for its own activation and downstream caspase activity (Fig. 1C; the time course of C3 activation differs between extracts, but C2 dephosphorylation prior to C3 activation was consistent across multiple extracts). When we repeated this assay in the presence of G6P to increase PPP activity, C2 dephosphorylation and C3 activation were inhibited (Fig. 1D and Supplemental Data, Fig. 1). These data suggested that C2 dephosphorylation and activation were under metabolic control.

We also show that dephosphorylation of GST-C2 pro preceded C2 activation as demonstrated by processing of *in vitro* translated full-length C2 (Fig. 1E). These data suggested that both endogenous full-length and isolated pro-C2 were dephosphorylated at S135 over time, and this preceded C2 processing and C3 activation.

### PP1 interacts with caspase-2 prodomain

To identify the phosphatase responsible for C2 dephosphorylation, we titrated the phosphatase inhibitor okadaic acid (OA) into extracts and monitored C3 activation, mitochondrial cyt *c* release, GST-C2 pro dephosphorylation, and full-length C2 processing (Figs. 2A-C). OA follows a biphasic dose response curve in the extract, with nM/low  $\mu$ M doses inhibiting PP2A and higher ( $\sim 10 \mu$ M) concentrations inhibiting PP1 (Margolis et al., 2003). OA doses known to inhibit PP1 but not those inhibiting only PP2A abrogated C3 activation, cyt *c* release, and C2 dephosphorylation (Figs. 2A and B).  $10 \mu$ M OA also prevented processing of full-length C2 (Fig. 2C). These data (and the fact that  $>95\%$  of OA-inhibitable phosphatases in the extract is PP1 or PP2A) strongly suggested that PP1 might dephosphorylate C2 S135.

To confirm that PP1 was the S135-directed phosphatase we depleted extract of endogenous PP1 using GST-I2 as an affinity resin and monitored samples for GST-C2

pro dephosphorylation. As shown in Figure 2D, PP1 depletion markedly inhibited C2 dephosphorylation. Also consistent with a role for PP1, GST-C2 pro was able to bind PP1 in extracts but not PP2A (Fig. 2E), and we also did not observe binding to the OA-inhibitable phosphatase PP5 (data not shown). Additionally, endogenous C2 co-immunoprecipitated with endogenous PP1 (Fig. 2G).

PP1 is commonly directed to its substrates by a targeting subunit (Cohen, 2002). We reasoned that a targeting subunit involved in C2 dephosphorylation would be a component of the PP1-C2 complex. Thus, GST-C2 pro was incubated in extract and bound proteins were analyzed via far western assay with digoxigenin-labeled PP1. Interestingly, we did not see binding of PP1 to any protein in the affinity precipitate other than C2 pro itself (data not shown). These data were reminiscent of previous work from our lab demonstrating that PP1 directly binds Cdc25 in the absence of a targeting subunit (Margolis et al., 2003). Similar results have also been reported for the retinoblastoma protein (Vietri et al., 2006). Therefore, we hypothesized that PP1 might mediate S135 dephosphorylation through direct C2 binding. To test this we examined the interaction between GST-C2 pro and recombinant PP1 catalytic domain. As shown in Figure 2F (right two lanes), GST-C2 pro bound directly to recombinant PP1 *in vitro*, consistent with the idea that PP1 and C2 interact in the absence of a targeting subunit.

### **PP1 binding is required for caspase-2 activation**

Only a handful of PP1 substrates have been shown to bind PP1 directly (Margolis et al., 2003; Vietri et al., 2006). Analyses of PP1 binding motifs on direct substrates and targeting subunits have identified VxF as a PP1 docking site (Egloff et al., 1997; Wakula et al., 2003). We scanned C2 pro for VxF motifs and failed to identify a candidate, however, we mutated several valines and downstream residues in the hope that these residues might cooperate to confer PP1 docking. As shown in Figures 3A and B, mutation of V18/L20 and V106/H108 demonstrated that V106A/H108A disrupted PP1 binding both in extract and in buffer. This double mutant was phosphorylated by CaMKII to the same extent as WT (Fig 3C). To determine if PP1 binding was essential for C2 dephosphorylation, we assayed dephosphorylation of these mutants over time. We found that V106A/H108A was markedly refractory to dephosphorylation, while V18A/L20A was rapidly dephosphorylated (Fig. 3D). Moreover, V18A/L20A demonstrated metabolic regulation similar to WT C2 pro (Figure 3D). Consistent with these observations, microinjection of flag-tagged, full-length C2 V106A/H108A mRNA into *Xenopus* oocytes did not effectively induce death, whereas an equivalent amount of WT protein readily killed the oocytes (Figs. 3E and F).

Although C2 dephosphorylation was under metabolic control and mediated by PP1, this did not necessarily indicate that this interaction was controlled by metabolism. Therefore, we wished to determine whether PP1 bound C2 constitutively or whether binding was induced just prior to caspase activation. As shown in Figure 3G, GST-C2 pro bound PP1 constitutively, and binding was not reduced by G6P (also see Fig. 2F, first 4 lanes). More importantly, when His-tagged PP1 was incubated in control- or G6P-treated extracts, retrieved, and then incubated with CaMKII-phosphorylated GST-C2 pro *in vitro*, we saw that C2 was dephosphorylated to a similar degree regardless of G6P treatment (Fig. 3H, first four lanes). Thus, although C2 dephosphorylation was

metabolically regulated, regulation was not at the level of phosphatase activity or binding.

### **14-3-3z binds caspase-2**

Since C2 dephosphorylation was regulated by metabolism but the C2/PP1 interaction and PP1 activity were not, we hypothesized that an additional factor(s) must be involved. To identify C2 interactors we used C2 pro as bait in a yeast two-hybrid screen and identified the phosphoserine binding protein 14-3-3z. In agreement with this finding, we determined that GST-C2 pro bound endogenous 14-3-3 in G6P-treated extract (Fig. 4A). In a converse experiment, it was determined that binding of endogenous C2 to GST-14-3-3 $\zeta$  but not  $\epsilon$  was enhanced in the presence of G6P (Fig. 4B), however, there did appear to be some constitutive background binding to 14-3-3 $\epsilon$ . Given this observation, we confirmed that 14-3-3 $\zeta$  preferentially bound CaMKII-phosphorylated C2 by phosphorylating GST-C2 pro at S135 *in vitro* and incubating it with different 14-3-3 isoforms. We show in Figure 4C that GST-C2 pro pS135 bound 14-3-3 $\zeta$  but not a 14-3-3 $\zeta$  mutant unable to bind target proteins (14-3-3 $\zeta$  K49E) or 14-3-3 $\epsilon$  (Zhang et al., 1997). These results suggested that C2 selectively binds 14-3-3 $\zeta$  when nutrients are abundant.

These observations raised the possibility that binding of 14-3-3 $\zeta$  might control C2 activation. In this regard, it was interesting that release of pre-bound endogenous 14-3-3 $\zeta$  from GST-C2 pro in extract was observed to occur before C3 activation (Fig. 4D). Furthermore, addition of excess His-14-3-3 $\zeta$  blocked full-length C2 processing, whereas equal amounts of 14-3-3 $\zeta$  K49E or 14-3-3 $\epsilon$  were unable to do so (Fig. 4E). Finally, addition of excess 14-3-3 $\zeta$  to extract also prevented C3 activation (Fig. 4F). These data suggested that 14-3-3 $\zeta$  binding might, indeed, control C2 activation.

### **14-3-3 $\zeta$ binding regulates caspase-2 dephosphorylation**

As described above, our lab reported that cell cycle-regulated activation of Cdc25 depends upon 14-3-3 release (Margolis et al., 2006; Margolis et al., 2003). We speculated that C2 dephosphorylation might also be regulated at the level of 14-3-3 release, particularly because PP1 did not appear to be under metabolic control. To address this we first examined the timing of 14-3-3 release from GST-C2 pro compared to dephosphorylation. As shown in Figure 5A, 14-3-3 $\zeta$  released from C2 pro prior to maximal C2 dephosphorylation. Next, we examined GST-C2 pro dephosphorylation in the presence of excess His-14-3-3 $\zeta$  WT or K49E. We saw that prephosphorylated GST-C2 pro was dephosphorylated in control extract or extract with 14-3-3 $\zeta$  K49E, but dephosphorylation was significantly decreased in the presence of excess 14-3-3 $\zeta$  WT (Fig. 5B). This experiment suggested that the inhibitory effect of 14-3-3 $\zeta$  on C2 is directly related to the interaction between the two.

Mutation of GST-C2 pro S135 to Ala abrogated 14-3-3 $\zeta$  binding through loss of the phosphoserine docking site, and binding was also lost when C2 pro D137 was mutated to Ala, thus disrupting the downstream sequence required for efficient interaction (Fig. 5C). It is important to note that the C2/14-3-3 $\zeta$  binding motif is non-canonical in nature: classical 14-3-3 binding sequences are defined as RSXpSXP (mode I) and RXY/FXpSXP (mode II), although the literature suggests considerable binding site

degeneracy (Rittinger et al., 1999; Yaffe et al., 1997; Yang et al., 2006). Although canonical motifs are thought to confer high-affinity binding, 14-3-3 interactions with atypical motifs may result in binding subject to novel regulation, which appears to be the case for metabolic regulation of C2 (Yaffe et al., 1997).

If 14-3-3 $\zeta$  binding controlled C2 dephosphorylation, we would expect C2 D137A to be dephosphorylated more rapidly than WT. We first confirmed that CaMKII was able to phosphorylate GST-C2 pro WT and D137A to the same extent *in vitro* (Fig. 5D). Next, GST-C2 pro WT or D137A was prephosphorylated with CaMKII and incubated in extract, and GST-C2 pro phosphorylation status was assessed. As shown in Figure 5E, C2 D137A was dephosphorylated more rapidly than WT, consistent with a role for 14-3-3 $\zeta$  in inhibition of C2 dephosphorylation. We also depleted extract of PP1 using GST-I2, demonstrating that PP1 is required for C2 D137A dephosphorylation (Fig. 5F). We were able to reinforce these conclusions *in vitro* using recombinant components; addition of excess His-14-3-3 $\zeta$  was sufficient to block dephosphorylation of GST-C2 pro by His-PP1 in buffer (Fig. 3H, last two lanes). In agreement with the idea that 14-3-3 $\zeta$  binding controls C2 dephosphorylation (rather than vice versa), addition of sufficient OA to inhibit PP1 did not interfere with 14-3-3 $\zeta$  release, suggesting that these are separable events (Fig. 5G, upper panel).

### **14-3-3 $\zeta$ release is metabolically regulated**

As described above, G6P blocks C2 dephosphorylation and activation, yet PP1 was not metabolically regulated. Since S135 dephosphorylation was more rapid in the absence of 14-3-3 $\zeta$ , this suggested that metabolic suppression of C2 might result from a block in 14-3-3 $\zeta$  removal. As shown in Figure 5H, 14-3-3 $\zeta$  release from GST-C2 pro was suppressed in the presence of G6P even with CaMKII inhibition, demonstrating that CaMKII activity and 14-3-3 $\zeta$  release are completely separable. Finally, if 14-3-3 $\zeta$  removal is the point of metabolic control, then C2 D137A should not be susceptible to metabolic suppression of C2 pS135. This was, in fact, the case as dephosphorylation of GST-C2 pro D137A rapidly proceeded regardless of G6P treatment. Moreover, D137 dephosphorylation occurred despite inhibition of cyt *c* release by Bcl-xL to prevent downstream apoptotic events (Fig. 6A; note that G6P and Bcl-xL were used at sufficient concentrations to suppress endogenous apoptosis). We also show in Figure 6B that *in vitro* translated full-length C2 D137A was readily processed in the presence of G6P whereas C2 WT was not, demonstrating that 14-3-3 $\zeta$  binding was critical for C2 suppression by metabolism.

Furthermore, GST-C2 pro D137A prephosphorylated at S135 was readily susceptible to recombinant PP1 *in vitro*, demonstrating that this mutation did not interfere with PP1 binding or activity (Fig. 6C). Consistent with these observations, microinjection of full-length C2 D137A mRNA into *Xenopus* oocytes induced death more robustly than injection of WT (Fig. 6D). Therefore, activation of C2 upon nutrient depletion relies upon removal of 14-3-3 $\zeta$ ; when this point of control cannot be exerted (D137A mutant), C2 activation is uncoupled from the nutrient status of the cell.

### **Metabolic regulation of caspase-2 is conserved in the mammalian oocyte**

Although our data indicated that C2 phosphorylation and 14-3-3 binding were crucial for inhibition of *Xenopus* oocyte death, we wished to determine whether this might also be important for mouse oocyte survival, particularly given the presence of excess oocytes in C2 knockout mice (Bergeron et al., 1998). We first identified a metabolically-regulated phosphorylation site on mouse C2 (mC2). Since it is impossible to obtain sufficient mouse oocytes for biochemical experiments, we used *Xenopus* egg extract as a tool for identifying mC2 phosphorylation sites. Incubation of GST-mC2 pro WT or candidate phospho-mutants in extract with G6P identified S164 as a potential site (Fig. 7A). Moreover, an *in vitro* CaMKII assay also suggested that GST-mC2 pro S164 might be a CaMKII target (Fig. 7B). Furthermore, GST-mC2 pro S164A was shown to be deficient in 14-3-3 binding, and a 14-3-3 binding mutant analogous to *Xenopus* C2 D137A (D166A) was validated (Fig. 7C; it is important to note that like *Xenopus* C2, the mC2 14-3-3-binding sequence is also non-canonical). We tested full-length mC2 constructs by microinjection into *Xenopus* oocytes and observed that mC2 S164A behaved much like *Xenopus* C2 S135A in inducing robust oocyte death (Figs. 7D and E).

In *Xenopus* oocytes, NADPH generation by the PPP promotes CaMKII activity and phosphorylation of C2 S135. Accordingly, we previously reported that full-length C2 WT mRNA injected into oocytes was rapidly suppressed via PPP flux (Nutt et al., 2005). In contrast, concomitant microinjection of a CaMKII inhibitor, negating the contribution of NADPH, enhanced the potency of C2. To determine if similar regulation was conserved in mammals, we examined mC2 in the context of mouse egg apoptosis. In fresh postovulatory mouse eggs where we would expect abundant NADPH and CaMKII activity, overexpression of full-length mC2 WT induced death more slowly than mC2 S164A (homologous to *Xenopus* S135, Fig. 7F). Also similar to *Xenopus* C2, the mC2 14-3-3 binding mutant was more potent than WT (Fig. 7G).

A decrease in G6P dehydrogenase activity has been demonstrated in aged mouse oocytes (de Schepper et al., 1987). Therefore, we speculated that age-related decreases in PPP flux might sensitize mouse eggs to C2-dependent death. As predicted, postovulatory aged eggs were markedly more susceptible than fresh eggs to death induced by WT C2 (Figs. 7H and I; note that mC2 S164A and D166A were also more potent in aged oocytes as expected, suggesting that additional cell death-inhibitory factors may be inactivated, or stimulatory factors activated during aging). These data demonstrated that our observations in *Xenopus* oocytes were conserved in the murine egg, suggesting that decreases in metabolism may contribute to oocyte death during aging.

## DISCUSSION

The role of C2 in oocyte death was established by experiments demonstrating that C2 knockout mice are characterized by excess oocytes (Bergeron et al., 1998). Subsequently, we have reported that C2 is inhibited when oocyte nutrients are sufficient to stimulate PPP-mediated NADPH production, which promotes CaMKII-mediated phosphorylation and inhibition of C2. In this report, we demonstrate that C2 activation is also under tight metabolic control. Removal of the suppressive C2 S135 phosphorylation is catalyzed by PP1, but this step is not metabolically regulated. Rather, the point of metabolic control is at the level of 14-3-3 $\zeta$ , whose removal from C2 is required for S135 dephosphorylation. These observations suggest that C2 is dormant in nutrient-replete oocytes until stockpiles are exhausted, thus engaging a 14-3-3-release pathway to activate C2 and trigger oocyte death.

We have now demonstrated that PP1 is the C2 S135-directed phosphatase. The interaction between C2 and PP1 is both direct and constitutive, and we have mapped the PP1 binding site as C2 V106/H108. Mutation of this site prevents PP1 binding and C2 dephosphorylation. Thus, C2 joins a small list of proteins regulated by PP1 in the absence of a targeting subunit.

Since PP1 binding and activity toward C2 are independent of metabolism, we deduced that an additional factor(s) must dictate C2 phosphorylation under these conditions. We now show that 14-3-3 $\zeta$  binding to C2 is under metabolic control, and a C2 mutant unable to bind 14-3-3 $\zeta$  is dephosphorylated regardless of nutrient status. Therefore, factors controlling 14-3-3 $\zeta$  removal from C2 are critical for metabolic control of oocyte apoptosis.

The mechanism underlying 14-3-3 $\zeta$  release from C2 is not yet clear. 14-3-3 binding regulates the activity of many pro-apoptotic proteins, and in these cases its release depends upon post-translational modification (e.g. JNK phosphorylates 14-3-3 promoting its release from BAD and BAX) (Datta et al., 2000; Nomura et al., 2003; Sunayama et al., 2005; Tsuruta et al., 2004; Zha et al., 1996). We have not observed phosphorylation of 14-3-3 $\zeta$  in extract prior to its release from C2. GST-C2 pro phosphorylated at S135 and incubated in nutrient-deplete extract (treated with DHEA to inhibit PPP) is deficient in *in vitro* binding of recombinant 14-3-3 $\zeta$ , but similarly incubated GST-14-3-3 $\zeta$  protein retains its ability to bind *in vitro* translated, phosphorylated C2 (LKN and SK, unpublished). It may be that any post-translational modification necessary for 14-3-3 $\zeta$  release is targeted to C2 itself. We also do not observe C2 pro phosphorylation outside of S135, suggesting that distinct post-translational C2 modifications or prodomain binding factors may play a role in 14-3-3 $\zeta$  release.

In the *Xenopus* oocyte, overexpression of full-length C2 S135A was a potent inducer of apoptosis when compared to WT. Young, healthy oocytes contain substantial internal nutrients, and as such we hypothesized that C2 was suppressed by S135 phosphorylation as it was translated (Nutt et al., 2005). Extending these studies to the mouse egg, we have now identified a homologous site on mC2 that appears to be important in the metabolic regulation of mouse egg apoptosis. It is currently not clear if somatic mC2 is also regulated through S164 phosphorylation or if this pathway is exclusive to oocytes.

In fresh mouse eggs expected to have robust PPP and CaMKII activity, overexpression of full-length mC2 WT induced death more weakly than either the phospho- or 14-3-3-binding mutants. It has been suggested that activity of the rate-limiting step in the PPP (G6P dehydrogenase) markedly decreases with age in mouse oocytes (de Schepper et al., 1987). Along those lines, we demonstrated that postovulatory aged mouse eggs were substantially more sensitive to injection of mC2 than fresh eggs, most likely due to a decrease in NADPH-dependent CaMKII activity. Given these data, it is attractive to speculate that a loss of PPP operation *in vivo* may underlie oocyte depletion in females as they age: over time, oocytes lose G6P dehydrogenase activity, thus promoting a drop in NADPH and concomitant loss of CaMKII activity. This would, in essence, remove the metabolic impediment to 14-3-3 release, thus priming C2 for PP1-mediated dephosphorylation and activation and leaving the oocyte readily susceptible to apoptosis induced by a variety of stressors (e.g. oxidative damage, waning hormonal stimulation). Taken together, our observations also raise the interesting possibility that pathologic alterations in C2-directed PP1 activity or changes in 14-3-3z levels could contribute to changes in female fertility.

## **EXPERIMENTAL PROCEDURES**

### ***Xenopus* extract and oocytes**

Mature female frogs were induced to lay eggs as described previously (Smythe and Newport, 1991). Jelly coats were removed with 2% cysteine (pH 8.0) and eggs were washed x3 with modified Ringer's solution (MMR: 1 M NaCl, 20 mM KCl, 10 mM MgSO<sub>4</sub>, 25 mM CaCl<sub>2</sub>, 5 mM Hepes, pH 7.8, 0.8 mM EDTA) and x3 in egg lysis buffer (ELB: 250 mM sucrose, 2.5 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, 50 mM KCl, 10 mM Hepes, pH 7.7). Eggs were centrifuged at 400 g, and cytochalasin B (5 µg/mL; Calbiochem), aprotinin/leupeptin (5 µg/mL), and cyclohexamide (50 µg/mL) were added. Lysis was performed by centrifugation at 10,000 g, 12 min. Crude cytosolic egg extracts were supplemented with 2 mM ATP and 20 mM phosphocreatine.

Stage VI oocytes were prepared from frogs primed with pregnant mare serum gonadotropin 3–5 days before excision of ovaries. Ovaries were digested with 2.8 U liberase (Roche) in OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5) for 1.5 hr at room temperature (RT). Oocytes were washed with OR-2 buffer and stored in OR-2 + 1% fetal bovine serum, 0.2% gentamicin overnight at 18°C. Healthy stage VI oocytes were selected for microinjection.

### **Recombinant protein cloning and expression**

pGEXKG N-terminal GST-*Xenopus* C2 pro, mC2 pro, 14-3-3ζ and ε, I2, and His-PP1 were expressed in BL21 *E. coli* as described previously (Gasco et al., 2002).

*Xenopus* C2 was cloned as previously described into pGEXKG and pSP64T (Nutt et al., 2005). The Quikchange kit (Stratagene) was used to generate *Xenopus* C2 mutants from the WT template using the primers detailed in Supplemental Data.

To produce radiolabeled *in vitro* translated protein, pSP64T C2 cDNA was added at 20 ng/µL to rabbit TNT reticulocyte lysate (Promega) containing 0.4 µCi/µL <sup>35</sup>S-Translabel, 1x (minus-Cys, minus-Met) amino acid mix, and additional components in accordance with the manufacturer's protocol.

### **Antibodies**

Anti-PP1 (polyclonal, 1:1000) and PP2A (monoclonal, 1:2000) were purchased from Upstate; anti-cyt c (polyclonal, 1:500) from Pharmingen; anti-14-3-3 (polyclonal, 1:1000) from AbCam, anti-actin (monoclonal, 1:4000) from Sigma-Aldrich, anti-flag (polyclonal, 1:1000) from Cell Signaling, anti-CaMKII pT286 (polyclonal, 1:1000) from AbCam, and anti-CaMKII (monoclonal, 1:1000) from BD Transduction Laboratories. *Xenopus* polyclonal antibodies were generated as described in Supplemental Data.

### **Caspase Assay**

3 µL egg extract was incubated in 90 µL DEVDase buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) with the colorimetric peptide substrate Ac-DEVD-pNA (200 mM final concentration; BIOMOL Research Labs, Inc.). Reactions were incubated at 37 °C for 30 min. Absorbance of the colorimetric product was measured at 405 nm using a BioRad microplate reader.

### **Caspase-2 dephosphorylation assay**

30  $\mu\text{g}$  GST-C2 pro was phosphorylated at S135 as follows: glutathione sepharose-bound proteins were incubated with recombinant CaM and CaMKII in CaMK buffer (1 M HEPES, pH 7.5, 1 M DTT, 1 M  $\text{MgCl}_2$ , 10% Tween, 0.2 M ATP, 1 M  $\text{CaCl}_2$ ) in the presence of 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP. Radiolabeled GST-C2 pro was then incubated in extract at RT. Samples were washed with buffer containing salt and detergent, eluted with SDS sample buffer, resolved on SDS-PAGE, and analyzed by autoradiography.

### **Cytochrome c release assay**

15  $\mu\text{L}$  crude extract was diluted in 15  $\mu\text{L}$  ELB and filtered through a 0.1  $\mu\text{m}$  ultrafree-MC filter (Millipore). The filtrate was mixed with SDS sample buffer, resolved by 17.5% SDS-PAGE, and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was probed with an anti-cyt c antibody.

### **Inhibitor-2 affinity depletion of PP1**

*Xenopus* extract was depleted of PP1 by three one-hour incubations at 4°C with GST or GST-I2 bound to glutathione sepharose. Depletion was confirmed by PP1 immunoblotting; anti-actin was used to assure equal loading.

### **Recombinant protein affinity binding assay**

Recombinant GST-C2 pro (20  $\mu\text{g}$ ) or GST-14-3-3 ( $\zeta$  and  $\epsilon$ , 20  $\mu\text{g}$ ) were bound to glutathione sepharose and incubated in egg extract. Sepharose-bound proteins were washed with buffer + 300 mM NaCl and 0.1% Triton-X, resolved by SDS-PAGE, and analyzed by immunoblotting.

### **14-3-3 release assay**

20  $\mu\text{g}$  GST-C2 pro bound to glutathione sepharose was incubated in extract + G6P to promote binding of endogenous 14-3-3 $\zeta$ . Sepharose-bound proteins were retrieved and washed with low-salt buffer. GST-C2 pro with endogenous 14-3-3 $\zeta$  was then added to fresh extract and samples of sepharose-bound proteins were retrieved over time, washed with buffer + 300 mM NaCl and 0.1% Triton-X, eluted with SDS sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting with a pan-14-3-3 antibody.

### **Oocyte injection assays**

pSP64T *Xenopus* full-length C2 cDNA templates were linearized with *Xba*I, and mRNA was generated using the Stratagene mRNA capping kit. Translation levels were tested with TNT reticulocyte lysate *in vitro* translation as described above or affinity precipitation from translationally-competent egg extract where specified.

Mouse C2 was cloned into pSP64T and mRNA was produced as previously described (Kurokawa et al., 2007). In brief, the cDNAs were linearized with *Xba*I and transcribed *in vitro* using the mMessage/mMachine capping kit (Ambion, Austin, TX). Mouse oocytes were collected and microinjected with mC2 mRNA as previously described (Fissore et al., 2002; Kurokawa et al., 2007).

### **Additional reagents**

D-Glucose 6-phosphate sodium salt (Sigma-Aldrich) was used at 10 mM. Okadaic acid (Sigma-Aldrich) was used at 10  $\mu$ M unless otherwise indicated. Glutathione Sepharose was purchased from Amersham Biosciences, Dynabeads Protein A from Invitrogen, Calmodulin and CaMKII from CalBiochem, and VDVAD-CHO from BioMol.

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## **ACKNOWLEDGEMENTS**

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## FIGURE LEGENDS

### Figure 1. Caspase-2 S135 dephosphorylation is sensitive to metabolism

(A) Glutathione sepharose-bound GST-C2 pro WT or S135A was phosphorylated *in vitro*  $\pm$  recombinant CaMKII. Samples were resolved by SDS-PAGE and immunoblotted using an antibody specific to *Xenopus* C2 pS135.

(B) Anti-C2 and its corresponding preimmune serum were titrated to determine equal IgG loading (upper panel). Extracts treated with 100  $\mu$ M VDVAD-CHO were immunoprecipitated at the indicated time points for one hour using preimmune- or C2-IgG bound to Dynabeads Protein A. Samples were resolved by SDS-PAGE and immunoblotted for C2 pS135 (second panel). In parallel, extracts were analyzed for processing of radiolabeled *in vitro*-translated full-length C2 or C3 activity (lower panels). White arrow = full-length C2; PI = preimmune; IP = immunoprecipitate.  $n = 3$

(C) Left panel: Extracts were analyzed for C3 activity over time using the caspase substrate Ac-DEVD-pNA. Right panel: In parallel, GST-C2 pro was prephosphorylated with CaMKII and [ $\gamma$ - $^{32}$ P]ATP; sepharose-bound proteins were incubated in extract over time and samples were resolved by SDS-PAGE and detected by autoradiography. Data are demonstrated quantitatively in the left panel.  $n=5$

(D) Glutathione sepharose-bound GST-C2 pro was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP as described in (A) and sepharose-bound proteins were incubated in extract  $\pm$  G6P. Samples were taken over time and detected by autoradiography. In parallel, extracts  $\pm$  G6P were analyzed for C3 activity using Ac-DEVD-pNA (Supplemental Data).  $n = 3$

(E) Top panel: GST-C2 pro pS135 was analyzed over time as described in (A). Middle panel: In parallel, processing of full-length C2 was examined by autoradiography. Quantitation is provided (bottom panel). White arrow = full-length C2.  $n = 3$

### Figure 2. Caspase-2 dephosphorylation is mediated by PP1

(A) Left panel: Control- or OA-treated extracts were analyzed for C3 activity with the substrate Ac-DEVD-pNA. Right panel: Cyt *c* release was measured in control- or OA-treated samples. Cytosol was analyzed by SDS-PAGE and immunoblotted with an anti-cyt *c* antibody and anti-actin. OA = okadaic acid.  $n = 3$

(B) GST-C2 pro bound to glutathione sepharose was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP. Sepharose-bound proteins were incubated in extracts  $\pm$  OA, and samples were resolved by SDS-PAGE and detected by autoradiography.  $n = 3$

(C) Extracts were treated with OA and processing of radiolabeled full-length C2 was examined by autoradiography. White arrow = full-length C2.  $n = 3$

(D) Glutathione sepharose-bound GST-C2 pro was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP. In parallel, extracts were depleted of endogenous PP1 with GST- or GST-Inhibitor-2 (I2) (left panel). Prephosphorylated GST-C2 pro was incubated in depleted extracts and samples were retrieved over time and detected by autoradiography (right panel).  $n = 4$

(E) Glutathione sepharose-bound GST or GST-C2 pro was incubated in extract and affinity-purified proteins were resolved by SDS-PAGE and immunoblotted for PP1. The membrane was co-probed for PP2A. In = input; NS = non-specific.

(F) (First four lanes): Glutathione sepharose-bound GST or GST-C2 pro was incubated in extract  $\pm$  G6P and affinity-purified proteins were resolved by SDS-PAGE and

immunoblotted for PP1. (Right two lanes): Glutathione sepharose-bound GST or GST-C2 pro was incubated in buffer with recombinant PP1 catalytic domain. Sepharose-bound proteins were resolved by SDS-PAGE and immunoblotted for PP1.

(G) Anti-C2 and its corresponding preimmune serum were titrated to determine equal IgG loading (left panel). Extracts were immunoprecipitated using preimmune- or C2-IgG bound to Dynabeads Protein A. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted for PP1; rabbit IgG heavy chain is also shown. In = input; PI = preimmune; IP = immunoprecipitate.

### **Figure 3. PP1 binding is required for caspase-2 activation**

(A) Glutathione sepharose-bound GST-C2 pro WT, V18A/L20A, or V106A/H108A was incubated in extract, resolved by SDS-PAGE, and immunoblotted for PP1. NS = non-specific.

(B) Glutathione sepharose-bound GST-C2 pro WT, V18A/L20A, or V106A/H108A was incubated in buffer with recombinant PP1 catalytic domain. Sepharose-bound proteins were resolved by SDS-PAGE and immunoblotted for PP1. NS = non-specific.

(C) Glutathione sepharose-bound GST, GST-C2 pro WT, or V106A/H108A was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP. Samples were resolved by SDS-PAGE and examined by autoradiography. Black arrow = GST-C2 pro; white arrow = GST.

(D) Glutathione sepharose-bound GST-C2 pro V106A/H108A or V18A/L20A was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP and incubated in extract  $\pm$  G6P. Samples were resolved by SDS-PAGE and detected by autoradiography. n = 3

(E) Flag-tagged full-length C2 WT or V106A/H108A mRNA was incubated in translationally-competent extract + 100  $\mu$ M VDAD-CHO. Translated flag-tagged C2 was retrieved with anti-flag agarose and samples were analyzed by immunoblotting with anti-flag.

(F) Percent survival of *Xenopus* oocytes microinjected with  $\beta$ -globin, full-length C2 WT, or V106A/H108A mRNA. Equivalent levels of C2 mRNA expression were determined as described in (E). n = 3

(G) Glutathione sepharose-bound GST-C2 pro was incubated in extract  $\pm$  G6P and samples were retrieved over time and immunoblotted for PP1 and GST. n = 3

(H) Glutathione sepharose-bound GST-C2 pro was prephosphorylated with [ $\gamma$ - $^{32}$ P]ATP and incubated in buffer with His-PP1 pre-incubated in extract  $\pm$  G6P or excess His-14-3-3 $\zeta$  (27  $\mu$ M). Samples were analyzed by SDS-PAGE and autoradiography. Black arrow = GST-C2 pro; white arrow = His-PP1. n = 4

### **Figure 4. Caspase-2 binds 14-3-3 $\zeta$**

(A) Glutathione sepharose-bound GST or GST-C2 pro was incubated in extract + G6P. Affinity-purified proteins were resolved by SDS-PAGE and immunoblotted with a pan-14-3-3 antibody. Black arrow = GST-C2 pro; white arrow = GST.

(B) Glutathione sepharose-bound GST, GST-14-3-3 $\epsilon$ , or GST-14-3-3 $\zeta$  was incubated in extract  $\pm$  G6P, and sepharose-bound proteins were resolved by SDS-PAGE and immunoblotted for *Xenopus* C2.

(C) Glutathione sepharose-bound GST-C2 pro was phosphorylated at S135 and incubated with either control conditions, His-14-3-3 $\zeta$  WT, His-14-3-3 $\zeta$  K49E, or His-14-3-

3ε in buffer. Sepharose-bound proteins were analyzed by SDS-PAGE and immunoblotted with a pan-14-3-3 antibody. In = input.

(D) Upper panel: Extracts were analyzed for C3 activity using the caspase substrate Ac-DEVD-pNA. Lower panel: In parallel, glutathione sepharose-bound GST-C2 pro was incubated in extract + G6P to promote binding of endogenous 14-3-3ζ, and this construct was then incubated in fresh extract. Samples were retrieved and analyzed for 14-3-3 binding. n = 4

(E) Extract was incubated with radiolabeled full-length *in vitro*-translated C2 in the presence of either control conditions, His-14-3-3ζ WT, His-14-3-3ζ K49E, or His-14-3-3ε. Samples were analyzed for C2 processing by autoradiography. White arrow = full-length C2. n = 3

(F) Extracts ± His-14-3-3ζWT were analyzed for C3 activity using the caspase substrate Ac-DEVD-pNA. n = 3

### **Figure 5. 14-3-3ζ binding prevents caspase-2 dephosphorylation**

(A) Upper panel: Glutathione sepharose-bound GST-C2 pro was pre-bound to endogenous 14-3-3ζ and then incubated in fresh extract; samples were analyzed for 14-3-3 binding. The membrane was co-probed with a GST antibody. In parallel, glutathione sepharose-bound GST-C2 pro was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and incubated in extract. Samples were analyzed by autoradiography (data not shown). The results of these experiments are depicted quantitatively (lower panel). n = 3

(B) Upper panel: Glutathione sepharose-bound GST-C2 pro was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and incubated in extracts with control conditions, His-14-3-3ζWT, or His-14-3-3ζK49E. Samples were analyzed by autoradiography. Quantitation of GST-C2 pro phosphorylation is provided relative to total protein (lower panel). n = 4

(C) Glutathione sepharose-bound GST, GST-C2 pro WT, S135A, or D137A was incubated in extract + G6P and affinity-purified proteins were resolved by SDS-PAGE and immunoblotted for 14-3-3.

(D) Glutathione sepharose-bound GST, GST-C2 pro WT, or D137A was phosphorylated with CaMKII and [ $\gamma$ -<sup>32</sup>P]ATP in buffer. Sepharose-bound proteins were analyzed by autoradiography. Black arrow = GST-C2 pro; white arrow = GST.

(E) Glutathione sepharose-bound GST-C2 pro WT or D137A was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and incubated in extracts. Samples were retrieved over time and analyzed by autoradiography. n = 3

(F) Glutathione sepharose-bound GST-C2 pro D137A was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP. In parallel, extracts were depleted of endogenous PP1 with GST- or GST-I2 (experiment 2D was performed in parallel; extent of PP1 depletion is demonstrated in Fig. 2D). Prephosphorylated GST-C2 pro D137A was incubated in depleted extracts and samples were resolved by SDS-PAGE and detected by autoradiography. n = 2

(G) Glutathione sepharose-bound GST-C2 pro was incubated in extract + G6P to promote binding of endogenous 14-3-3ζ. Sepharose-bound GST-C2 pre-bound to endogenous 14-3-3ζ was incubated in fresh extract in the presence of either OA or G6P. Sepharose-bound proteins were analyzed for 14-3-3 binding by immunoblotting. n = 3

(H) Upper panel: Glutathione sepharose-bound GST-C2 pro was pre-bound to endogenous 14-3-3 $\zeta$  and incubated in extract  $\pm$  G6P and EGTA (5 mM). Samples were analyzed for 14-3-3 binding by immunoblotting. Lower left panel: In parallel, GST-C2 pro was incubated in extracts with [ $\gamma$ -<sup>32</sup>P]ATP  $\pm$  G6P and EGTA; samples were analyzed by autoradiography. Lower right panel: Samples of G6P and EGTA-treated extracts were analyzed for CaMKII activation by immunoblotting for CaMKII pT286. n = 3

**Figure 6. The interaction between caspase-2 and 14-3-3 $\zeta$  is metabolically regulated**

(A) Upper panel: Glutathione sepharose-bound GST-C2 pro D137A was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and incubated in extracts  $\pm$  G6P or Bcl-xL. Samples of sepharose-bound proteins were analyzed by autoradiography. Lower panel: In parallel, extracts treated with the same conditions were analyzed for C3 activity using the caspase substrate Ac-DEVD-pNA. n = 3

(B) Radiolabeled *in vitro* translated full-length C2 WT or D137 was incubated in extracts  $\pm$  G6P. Samples were retrieved over time and analyzed by SDS-PAGE and autoradiography.

(C) Glutathione sepharose-bound GST-C2 pro D137A was prephosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and incubated in buffer with His-PP1 that had been pre-incubated in extract. Samples of sepharose-bound proteins were analyzed by autoradiography. n = 2

(D) Percent survival of *Xenopus* oocytes microinjected with full-length C2 WT or D137A mRNA. n = 3

**Figure 7. Conservation of caspase-2 metabolic regulation in the mouse egg**

(A) Glutathione sepharose-bound GST-mC2 pro WT, S24A, S157A, T158A, or S164A was incubated in *Xenopus* extracts  $\pm$  G6P and [ $\gamma$ -<sup>32</sup>P]ATP. Sepharose-bound proteins were analyzed by autoradiography.

(B) Glutathione sepharose-bound GST-mC2 pro WT, S157A, T158A, or S164A was phosphorylated *in vitro* with CaMKII and [ $\gamma$ -<sup>32</sup>P]ATP. Sepharose-bound proteins were analyzed by autoradiography.

(C) Glutathione sepharose-bound GST-mC2 pro WT, S164A, or D166A was incubated in *Xenopus* extract + G6P. Sepharose-bound proteins were analyzed by SDS-PAGE and immunoblotted for 14-3-3.

(D) Percent survival of *Xenopus* oocytes microinjected with mC2 WT, S164A, or  $\beta$ -globin mRNA. n = 3

(E) *Xenopus* oocytes microinjected with mC2 WT or S164A mRNA as described in (D) shown as representative micrographs. n = 3

(F) Percent death of fresh mouse eggs microinjected with mC2 WT or S164A mRNA, 19 hrs post-injection. n = 3

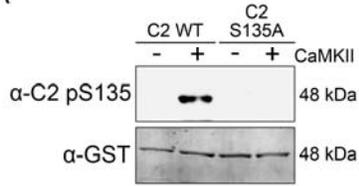
(G) Percent death of fresh mouse eggs microinjected with mC2 WT or D166A mRNA (24 hrs post-injection; this is a separate experiment from (E) and the time course of death varied somewhat between batches of eggs). n = 3

(H) Percent death of aged mouse eggs microinjected with mC2 WT or S164A mRNA, 8 hrs post-injection. n = 3

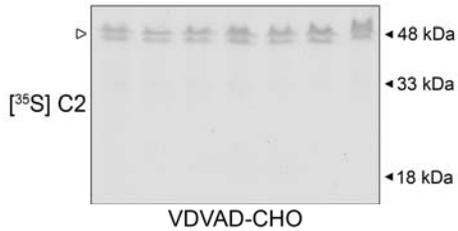
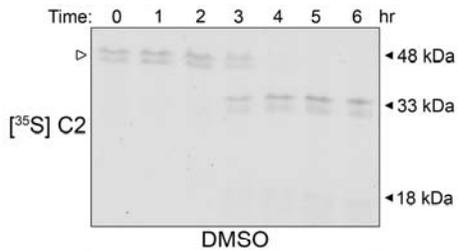
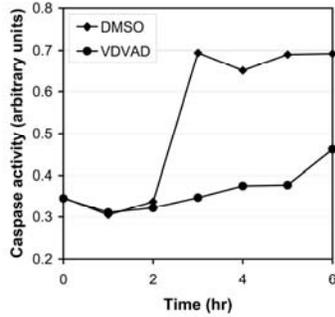
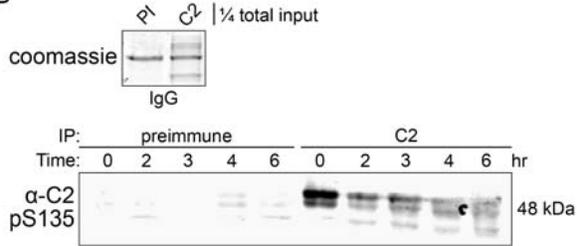
(I) Percent death of aged mouse eggs microinjected with mC2 WT or D166A mRNA, 6 hrs post-injection (note that death of the aged eggs in G and H was consistently accelerated relative to the fresh postovulatory eggs). n = 3

**Figure 1**

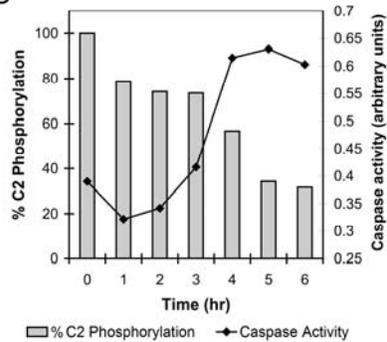
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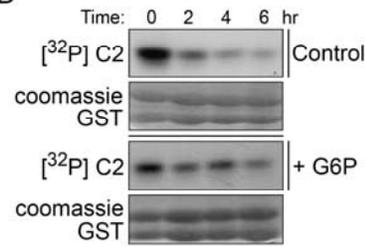
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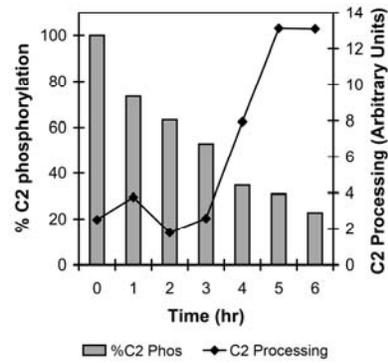
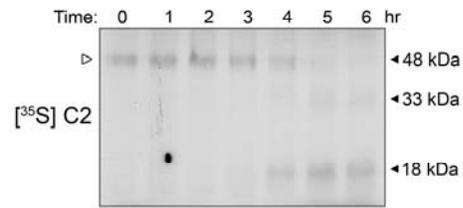
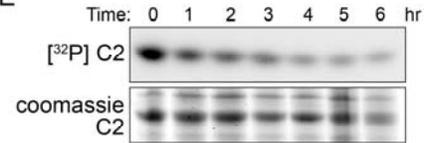
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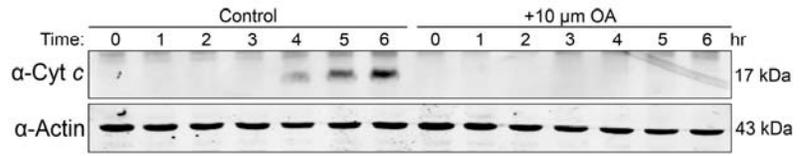
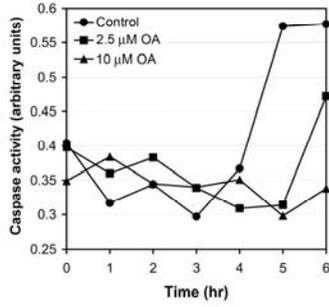


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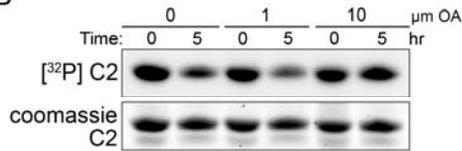


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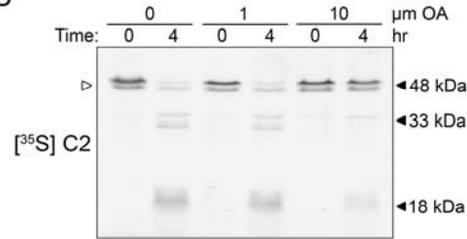
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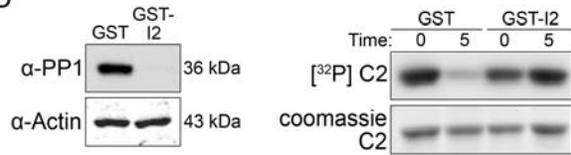
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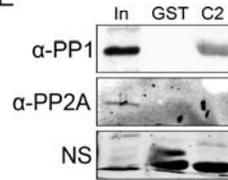
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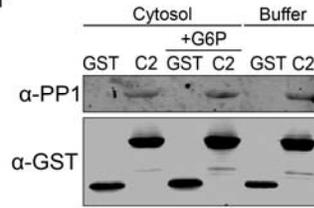
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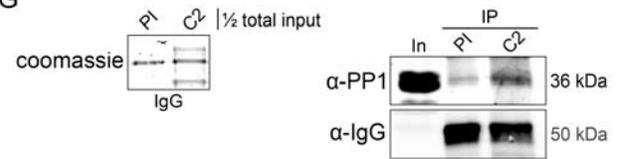
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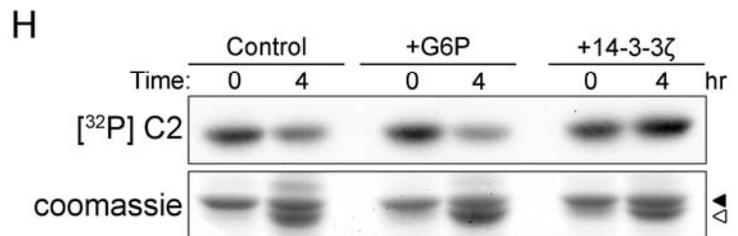
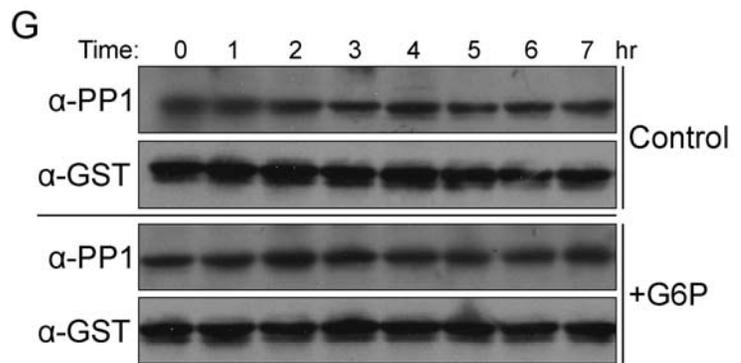
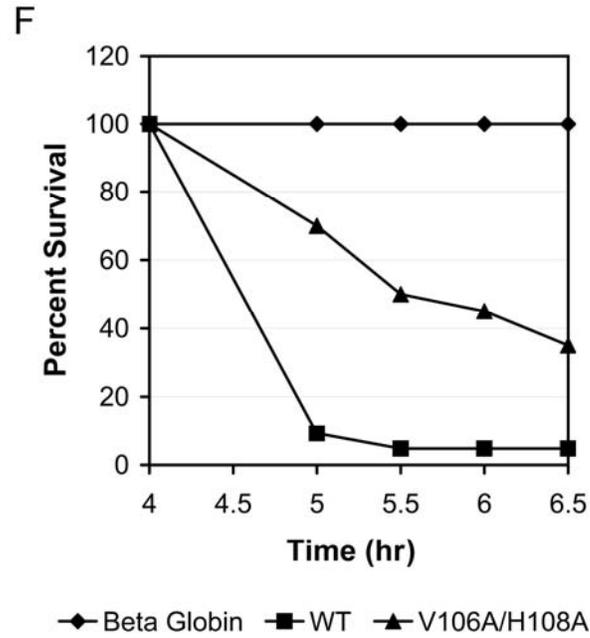
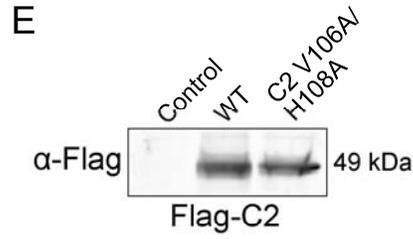
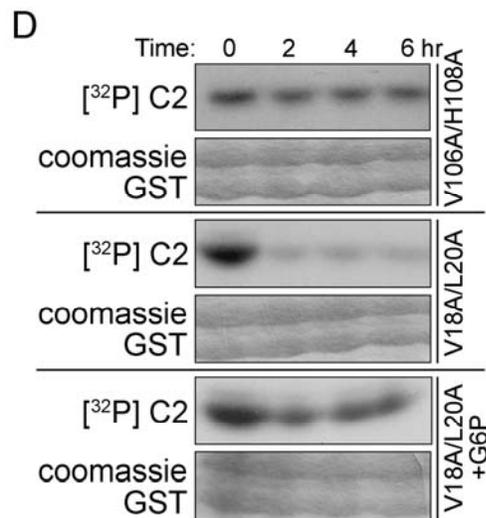
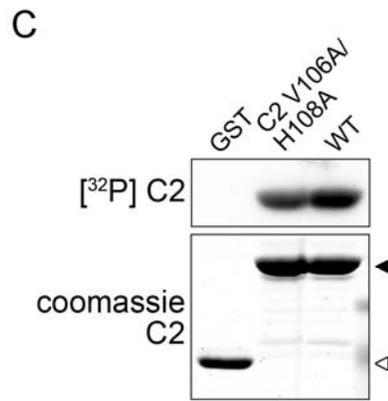
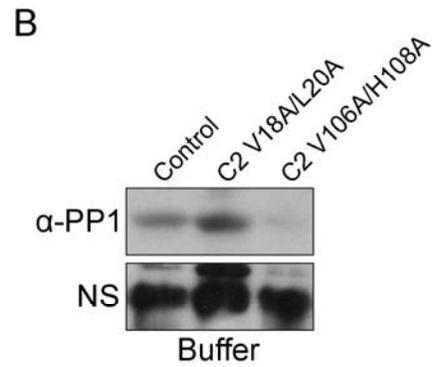
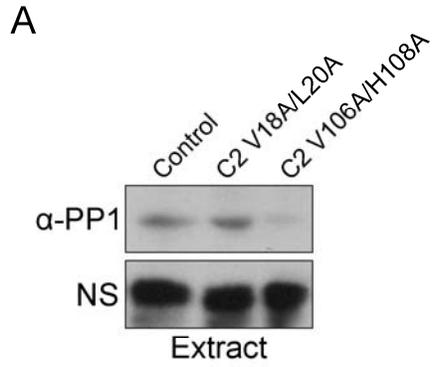


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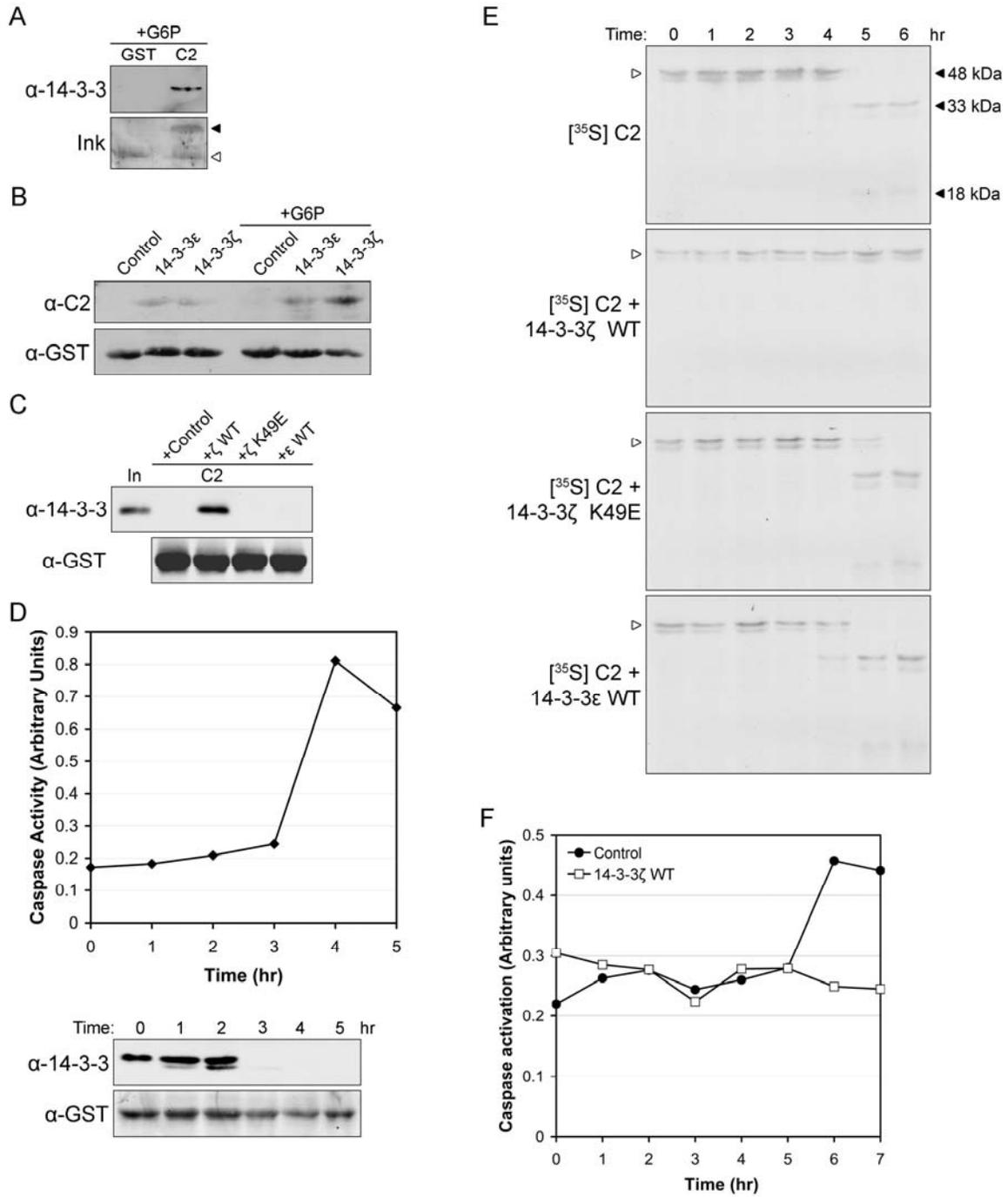


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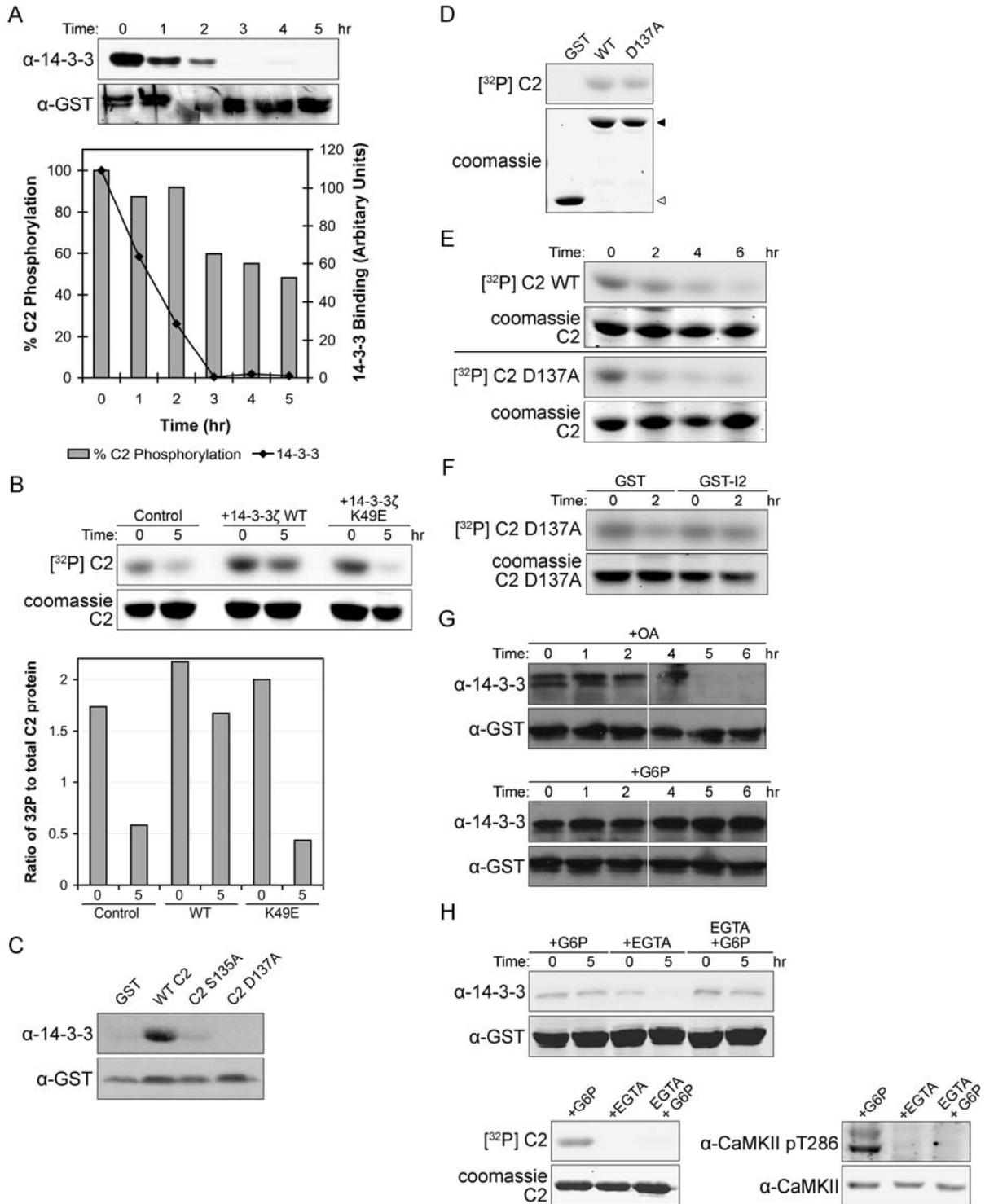




**Figure 4**

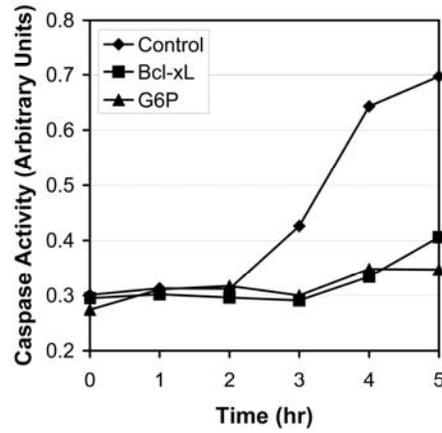
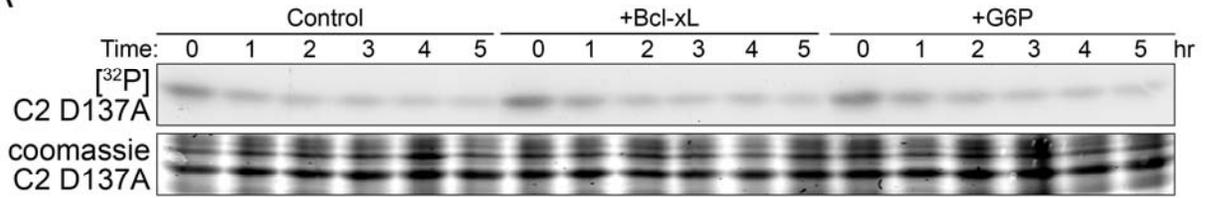


**Figure 5**

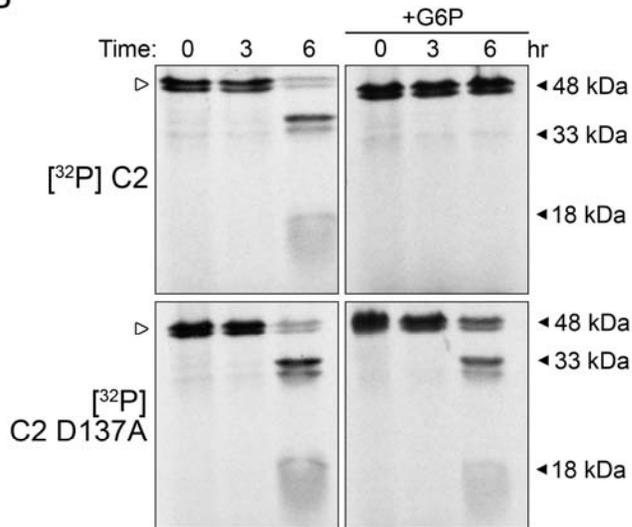


**Figure 6**

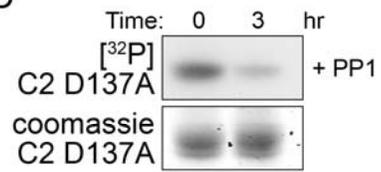
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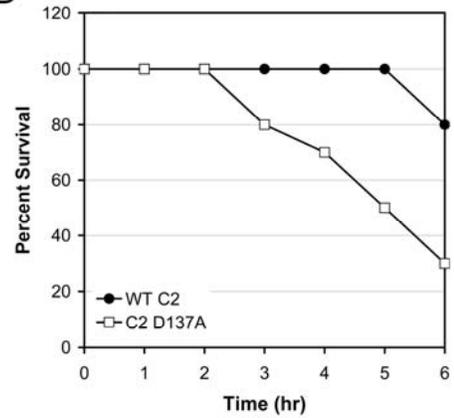
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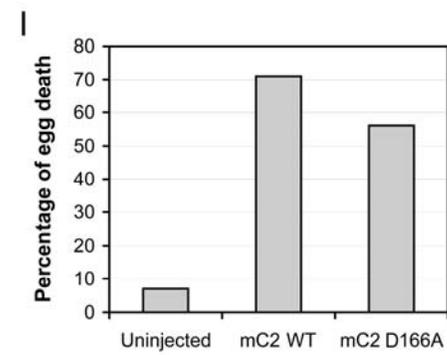
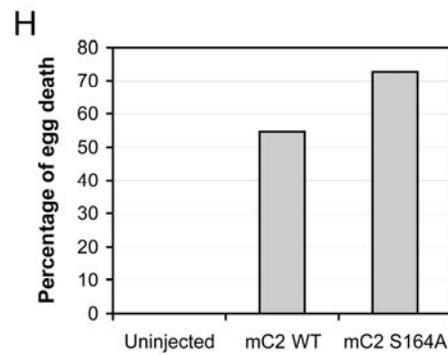
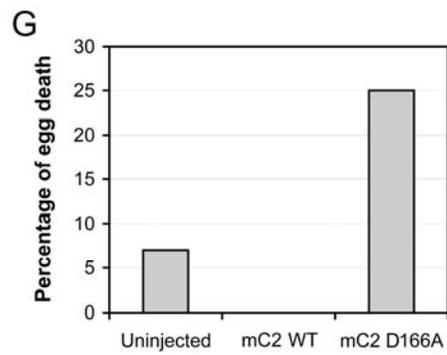
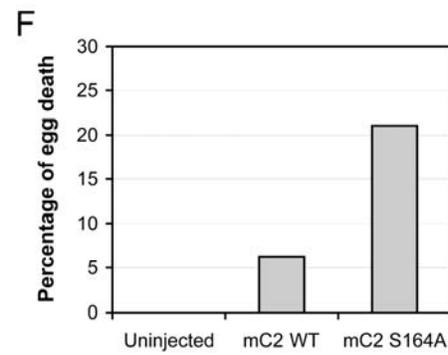
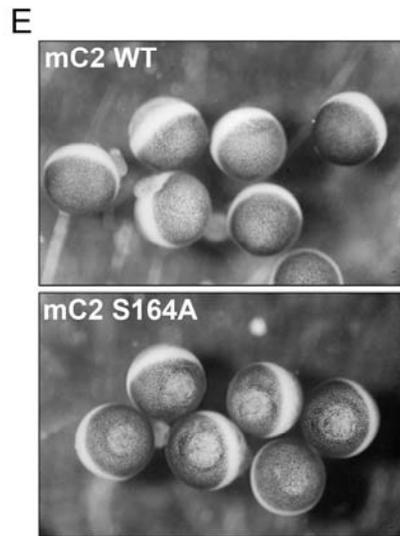
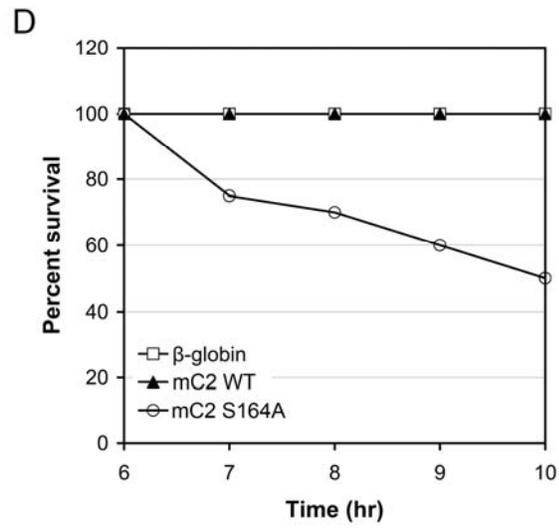
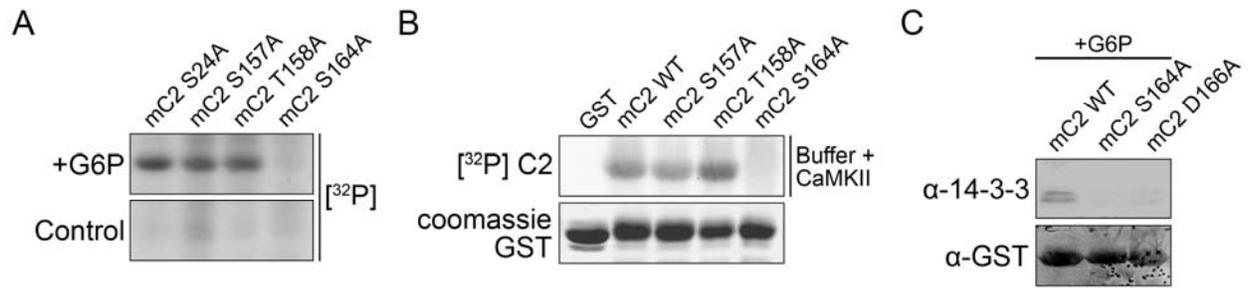


**C**



**D**





Supplemental Data

