Metabolic Control of CaMKII-mediated Caspase-2 Suppression by B55β/PP2A

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Xiao-Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
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ABSTRACT

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Abstract

Apoptosis is a programmed form of cell death, essential for maintaining tissue homeostasis and eliminating dysfunctional cells. The process of apoptosis is executed by a family of cysteine proteases called caspases. High levels of metabolic activity confer resistance to apoptosis. Caspase-2, an apoptotic initiator, can be suppressed by high levels of nutrient flux through the pentose phosphate pathway (PPP). This metabolic suppression of caspase-2 is exerted via the inhibitory phosphorylation of S135 on the caspase-2 prodomain by activated Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII). However, it was unclear how CaMKII activity is regulated by nutrient flux.

After investigating how nutrient flux leads to activation of CaMKII, a recent study reported that coenzyme A (CoA) can directly bind to and activate CaMKII. However, by performing mass spectrometry (MS) analysis of CaMKII, and other biochemical assays, including gel filtration assays, immuno-precipitation assays, immuno-depletion assays, and in vitro kinase assays, in the Xenopus egg extract system, our studies show that the complete nutrient-driven CaMKII activation requires the additional release of a “brake” through the dephosphorylation of CaMKII at novel sites (T393/S395). Furthermore, this metabolically-stimulated dephosphorylation of CaMKII is mediated by the metabolic activation of protein phosphatase 2A (PP2A) in complex with the B55β targeting subunit. Importantly, our findings have been successfully replicated
in human 293T cells, including the metabolic activation of CaMKII, and also the suppression of this activation by B55β knockdown.

Our discovery represents a novel locus of CaMKII regulation and also provides a mechanism contributing to metabolic control of apoptosis. These findings may have implications for metabolic control of the many CaMKII-controlled and PP2A-regulated physiological processes, as both enzymes appear to be responsive to alterations in glucose metabolized via the PPP. Finally, our study reveals B55β as a potential target for cancer therapy, because of its importance in suppressing metabolic suppression of caspase-2 activation and apoptosis.
Dedication

To my parents, Xianyao Huang and Yuping Song, for their constant support for my research; and to my fiancée, Zixi Chen, for her companionship throughout this journey.
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List of Abbreviations

APAF1  apoptotic protease activating factor 1
BAD  Bcl-2-associated death promoter
Bak  Bcl-2 homologous antagonist killer
Bax  Bcl-2 associated X protein
Bcl-2  B-cell lymphoma 2
Bcl-XL  B-cell lymphoma-extra large
BH  Bcl-2 homology
Bid  BH-3 interacting-domain death agonist
bVAD-fmk  biotinylated valine-alanine-aspartate–fluoromethyl ketone
CAD  caspase-activated deoxyribonuclease
CaM  Calmodulin
CaMKII  Ca/calmodulin dependent protein kinase II
CARD  caspase recruitment domain
Cdc25  cell division cycle 25
Chk1  checkpoint kinase 1
CoA  coenzyme A
C subunit  Catalytic subunit
CREB  cAMP response element-binding protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroisoandrosterone</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>E1A</td>
<td>adenovirus early region 1A</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose-6-phosphate</td>
</tr>
<tr>
<td>G6PDH</td>
<td>G6P dehydrogenase</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1β converting enzyme</td>
</tr>
<tr>
<td>Ich-1</td>
<td>ICE and CED-3 homologue 1</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
</tr>
</tbody>
</table>
MnTBAP Mn(III)tetrakis (4-benzoic acid) porphyrin
MOMP mitochondrial outer membrane permeabilization
MS Mass spectrometry
NAC N-acetylcysteine
NADPH nicotinamide adenine dinucleotide phosphate
NLS nuclear localization signal
OA okadaic acid
PET positron emission tomography
PIDD p53-induced protein with death domain
PKA protein kinase A
PP1 protein phosphatase 1
PP2A protein phosphatase 2A
PPP pentose phosphate pathway
RAIDD RIP-associated Ich-1/Ced-3-homologue protein with a death domain
Ras rat sarcoma
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SiRNA small interfering RNA
SOD superoxide dismutase
tBid truncated Bid
TNF tumor necrosis factor
UPR        unfolded protein response
WT         wild type
z-VDVAD    benzyloxycarbonyl-Val-Asp-Val-Ala-Asp
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1. Introduction

1.1 Apoptosis: a form of programmed cell death

1.1.1 Classification of cell death

In multicellular organisms, the death of cells has been observed and reported for more than one and half centuries (reviewed in Majno and Joris, 1995). Research into this phenomenon has identified multiple circumstances for cell death. For instance, cell death occurring during embryonic development of animals is called developmental degeneration (Saunders, 1966). In this case, cell death is programmed to maintain the normal developmental process. A second circumstance of programmed cell death occurs in body tissues where cell death must be maintained in balance with cell division, which is crucial for homeostasis (reviewed in Broker et al., 2005). Both pathways are precisely regulated and the dysregulation of either one disrupts tissue homeostasis. This can causes diseases such as degenerative disorders (reviewed in Danial and Korsmeyer, 2004). In contrast with the concept of “programmed cell death”, upon receiving severe outside damage, tissues can also suffer from a more chaotic and passive form of cell death, called “necrosis”, characterized by rapid destruction of plasma membrane followed by the induction of inflammatory reaction (Hirsch et al., 1997).
While necrosis is an un-controlled form of cell death that cannot be stopped after the accidental initiation, programmed cell death is executed through precise signaling pathways which can be interfered with. Many researchers have characterized multiple forms of programmed cell death, including apoptosis and autophagy (reviewed in Bursch et al., 2000). More recently, in multiple circumstances, the regulated form of necrosis, called necroptosis, has been observed (Sun et al., 2012; Teng et al., 2005). Even with these multiple patterns of programmed cell death, the form most commonly referred to as the classical form is apoptosis (reviewed in Suzanne and Steller, 2013). Apoptosis remains the best-understood cell death pathway, known to play important roles in organismal conditions such as organogenesis, neurodegeneration, and tumorigenesis.

1.1.2 The history of apoptosis

The concept of “apoptosis” was first proposed in 1972 when it became recognized as a distinct process from necrosis, which until that time had been considered the general representation of cell death (Kerr et al., 1972). According to this manuscript, apoptosis was classified based on a series of characteristic morphological changes during the death process of dying cells, including the condensation of both cytoplasm and nucleus, the fragmentation of chromatin, the blebbing of plasma membrane, and finally destruction of cells and formation of a vacuole structure called
apoptotic bodies. Varying in sizes, apoptotic bodies contain cell organelles which are highly condensed but intact, and fragmented nuclear remnants sometimes, from original cells. Free apoptotic bodies are digested by phagocytes. Through the whole digestion process, no cellular content could be leaked to the outer micro-environment. In addition to categorizing apoptosis, this article also for the first time proposed the importance of apoptosis relative to cell division, as two sides of the coin necessary to maintain tissue homeostasis (Kerr et al., 1972). Although some controversy in the field of cell death was generated, this article largely stimulated scientists to further study cell death and finally enabled people to obtain a better understanding of apoptosis.

Various model organisms were used to investigate the mechanisms hiding beneath these characteristic morphological changes and controlling apoptosis. The apoptotic signaling pathway was first deciphered in *C. elegans*. This model was most amenable to early studies of apoptosis because the adult worms consistently contain 959 somatic cells; thus, mutations resulting in abnormal cell numbers in certain mutants could be readily detected (reviewed in Potts and Cameron, 2011). By performing genetic screens in *C. elegans*, two genes, *ced-3* and *ced-4*, were first identified to be essential for apoptosis (Ellis and Horvitz, 1986). The protein CED-3, encoded by *C. elegans* gene *ced-3*, was found to be a cysteine protease essential for the initiation of the apoptotic pathway. Importantly, CED-3 was also found to be similar to the human protein, interleukin-1β.
converting enzyme (ICE), which is now named caspase-1 (Yuan et al., 1993). After the identification of CED-3 as a *C. elegans* homolog of human caspase-1, more *C. elegans* apoptosis-related proteins were discovered to be homologs of human proteins. For example, the *C. elegans* protein CED-9 was discovered to be responsible for protecting cells from apoptosis, and was later found to be conserved with a human homolog protein, B-cell lymphoma 2 (Bcl-2) (Hengartner et al., 1992; Hengartner and Horvitz, 1994). Beyond *C. elegans* and humans, it is now well accepted that the general apoptotic pathways are largely conserved in different species, catalyzed by a family of cysteine proteases named caspases.

Besides caspase-1, more proteins were identified to be homologs of *C. elegans* protein CED-3. This group of proteins was nominated as “caspases” based on two of their biochemical properties: 1) to acknowledge them as cysteine dependent proteases, and 2) to recognize aspartic acid to cleave target proteins (Alnemri et al., 1996).

By cleaving various target proteins, the active caspases are responsible for causing those universal morphological changes characteristic of cells dying from apoptosis. For example, plasma membrane blebbing was shown to be caused by the caspase-mediated cleavage and activation of Rho-associated protein kinase 1 (ROCK1) (Coleman et al., 2001). Another example is the fragmentation of DNA in apoptotic cells, catalyzed by caspase-activated deoxyribonuclease (CAD). In normal situations, the
inhibitor of CAD (ICAD) interacts with CAD in the cytoplasm preventing CAD from translocating into nucleus and degrading DNA. However, when apoptosis is initiated, the active caspase-3 cleaves ICAD, resulting in its release from CAD and loss of its ability to suppress CAD’s activity (Enari et al., 1998).

1.2 Caspases: the initiators and executioners of apoptosis

1.2.1 Classification of caspases

As of today, 14 members of the caspase family have been identified, most of which play critical roles in the apoptotic process. Upon biosynthesis, all caspases are initially in their inactive precursor form called “procaspases”. The procaspases all contain similar domain structures: an N-terminal prodomain with diverse sizes, a large subunit in the middle, a small C-terminal subunit, and a linker region between the large middle and small C-terminal subunits. Aspartic acids exist at the end of the prodomain and in the linker region, and the activation of caspases requires cleavage at these sites. As a result of this cleavage, the prodomain and linker region are removed (Fig. 1-1) (reviewed in Nunez et al., 1998). Structural analysis of caspase-1 and caspase-3 revealed that the active form of caspase is a tetramer consisting of two heterodimers, formed by the association between the large and small subunits (Wilson et al., 1994; Walker et al., 1994; Rotonda et al., 1996).
Although all caspase family members show homology to each other, they can be further divided into three subfamilies, based on their different cellular functions and structures. The subfamily members functioning as the apical caspases in the whole apoptotic pathway are called initiator caspases, and their function is to cleave and activate downstream caspases. The members of this subfamily include caspase-2, 8, 9, and 10. The second subfamily contains all executioner caspases, including caspase-3, 6, and 7. These executioner caspases are able to cleave various downstream target proteins following activation by initiator caspases. Distinct from the first and second subfamilies whose members function in apoptotic pathway, the third subfamily members are inflammatory caspases, including caspase-1, 4, 5, 11, 12, 13, and 14 (reviewed in Fan et al., 2005). The structures of caspases differ among subfamilies. All initiator and inflammatory caspases contain in their procaspase forms, a long prodomain, consisting of domains responsible for protein-protein association, such as death effector domain (DED), or caspase recruitment domain (CARD). These domains enable them to interact with adaptor proteins, and play critical roles in the formation of the activation oligomer. However, executioner procaspases only possess a short prodomain, lacking the protein association domain, which means that the executioner procaspases cannot form activation oligomer, but can only be activated by the cleavage of initiator caspases (Fig. 1-1) (reviewed in Fuentes-Prior and Salvesen, 2004).
Initiator and executioner caspases differentiate in structures. The initiator caspases contain a large prodomain, which is responsible for protein-protein interaction. However, the executioner caspases do not. The activation process of caspases includes the cleavage and removal of prodoms and also linker regions between large subunits and small subunits.

**Figure 1-1: Structures of initiator and executioner caspases**
1.2.2 Extrinsic apoptotic pathway

In mammalian cells, the caspase cascade can be activated by two distinct mechanisms dependent on the origins of the stimuli: the extrinsic and the intrinsic pathways. As suggested by the nomenclature, the stimuli of the extrinsic pathway are extracellular, while the stimuli of the intrinsic pathway are intracellular.

The extrinsic pathway involves the activation of death receptors on the plasma membrane, such as tumor necrosis factor receptor (TNFR, alternatively named CD120) and Fas (alternatively named CD95), by certain death signals from outer microenvironment, such as tumor necrosis factor (TNF) and Fas ligand (FasL) respectively (reviewed in Smith et al., 1994). Death receptors are transmembrane proteins, containing a death domain (DD) on the cytoplasmic side, which is capable of recruiting adaptor proteins. In the case of Fas, one FasL (a homotrimer) is able to interact with three Fas proteins. As a result, the DD domains of Fas interact with each other and undergo conformational change that enables them to recruit adaptor proteins which also contain DD domain (Huang et al., 1996). Fas-associated death domain (FADD) is an adaptor protein containing both DD and DED domains, and it is recruited to the DD domain of activated Fas (Chinnaiyan et al., 1995). FADD then associates with procaspase-8 through DED domain, forming a complex named death inducing signaling complex (DISC) (Boldin et al., 1996; Muzio et al., 1996). On this platform, caspase-8 is activated, which
then activates the downstream executioner caspase-3 and further initiates the downstream apoptotic events (Fig. 1-2) (reviewed in Wang et al., 2005).
Upon receiving death signal from outer environment, initiator caspases are activated on the platform called DISC, and then activate downstream executioner caspases to induce apoptosis.

**Figure 1-2: Extrinsic apoptotic pathway**
1.2.3 Intrinsic apoptotic pathway

In contrast with the extrinsic apoptotic pathway, the intrinsic pathway is activated by intracellular death stimuli. These can be further divided into two categories: loss of apoptotic suppression or positive induction of apoptosis. Loss of apoptotic suppression includes withdrawal of growth factors or cytokines and nutrient deprivation. On the other hand, positive induction of apoptosis includes DNA damage triggered by radiation or chemicals, hypoxia, viral infection, oxidative stress, among others. Both categories typically activate apoptotic pathway in a mitochondria-dependent manner, featured by mitochondrial outer membrane permeabilization (MOMP) (reviewed in Elmore, 2007). A protein family, named the B-cell lymphoma 2 (Bcl-2) family, is responsible for regulating this characteristic MOMP event. The Bcl-2 family contains three subfamilies: pro-apoptotic Bcl-2 family, including Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak); the anti-apoptotic Bcl-2 family, including Bcl-2, B-cell lymphoma-extra large (Bcl-XL), and others; and the Bcl-2 homology domain 3 (BH-3) only family, including Bcl-2 like protein 11 (Bim), BH-3 interacting-domain death agonist (Bid), Bcl-2-associated death promoter (BAD), and others (reviewed in Cory and Adams, 2002). The pro-apoptotic members Bax and Bad are the main Bcl-2 family members that function on the mitochondria to activate the apoptotic pathway (Wei et al., 2001). The lack of either Bax or Bak alone in mice does not
produce a significant phenotype, however, the lack of both Bax and Bak in mice causes a
dramatic disruption of apoptosis, suggesting that they together are essential for
apoptosis induction (Lindsten et al., 2000). In the inactive form, Bax is a monomeric
cytosolic protein. In response to death stimuli, however, Bax is translocated to the
mitochondrial outer membrane and undergoes oligomerization (Hsu and Youle, 1998;
Antonsson et al., 2001). On the other hand, Bak is constantly located on mitochondria,
but it also undergoes conformational change and oligomerization in response to death
stimuli (Wei et al., 2000). Upon oligomerization, Bax and Bak are able to form pores on
the outer membrane of mitochondria, with sizes large enough to release apoptotic
proteins, such as cytochrome c, from the mitochondria to the cytosol. This cytochrome c
release is essential for the activation of the downstream apoptotic pathway (Bleicken et
al., 2013). Unsurprisingly, the activation of Bax and Bak is tightly regulated, both
negatively and positively. The anti-apoptotic Bcl-2 family members negatively regulate
Bax and Bak by interacting with them to block activation. For example, Bcl-XL was
shown to interact with Bak, forming a heterodimer which is unable to cause
mitochondrial outer membrane permeabilization (MOMP) (Sattler et al., 1997). In
addition, Bax was also shown to be suppressed by the hetero-dimerization with Bcl-2
(Oltvai et al., 1993; Korsmeyer et al., 1993). In contrast to the anti-apoptotic family
members that act as negative regulators of Bax and Bak, the BH-3 only subfamily,
positively regulates Bax and Bak. This subfamily is called BH-3 only, because its members only possess the third one of four Bcl-2 homology (BH) domains (BH-1, 2, 3, 4), while the other two subfamilies share 3 to 4 BH domains (reviewed in Huang and Strasser, 2000). BH-3 only proteins can promote the activation of Bax and Bak in two different ways, either by directly activating Bax and Bak, or by suppressing anti-apoptotic Bcl-2 proteins (reviewed in Hardwick and Soane, 2013). For example, after activation by cleavage, the truncated Bid (tBid) is able to oligomerize and activate Bak (Wei et al., 2000). On the other hand, Bim is able to interact with various anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-XL, Bcl-W, and Mcl-1, and Bad is able to interact with Bcl-2 and Bcl-XL (Chen et al., 2005). The three Bcl-2 subfamilies together form a triangle regulatory network to tightly regulate the MOMP event, which is an essential step in the intrinsic apoptotic pathway.

As mentioned above, MOMP directly causes the release of apoptotic proteins, such as cytochrome c, into the cytosol from the space between the outer and inner membrane of mitochondria. In the cytosol, cytochrome c leads to the formation of the caspase-9 activation platform, named the apoptosome. The apoptosome consists of cytochrome c, apoptotic protease activating factor 1 (APAF1), deoxyadenosine triphosphate (dATP), and procaspase-9 (Li et al., 1997). The procaspase-9 is processed and activated on this platform, after which the active caspase-9 is able to cleave and
activate executioner caspases, such as caspase-3 and caspase-7 (Fig. 1-3) (reviewed in Nicholson and Thornberry, 1997).
Upon receiving intracellular death signal, initiator caspases are activated, and then cleave and activate Bid. The truncated Bid (tBid) assist Bax and Bak to cause MOMP and to release cytochrome c into cytosol. In cytosol, cytochrome c interacts with pro-caspase-9 to form a platform called apoptosome, upon which the executioner caspases are activated.
1.3 Caspase-2: initiator of apoptosis

The extensive research on apoptotic pathways and caspases has shed light on the functions of many caspase family members. For example, caspase-8 has been demonstrated to initiate the extrinsic apoptotic pathway in response to extracellular death signals. Caspase-9 is activated upon the release of cytochrome c from mitochondria to cytosol and the formation of apoptosome in the intrinsic apoptotic pathway. Caspase-3, 6, and 7 are the executioner caspases activated by initiators. However, one of the first identified caspases and one of the most conserved caspases through different species, caspase-2, remained mysterious with unknown functions for a long time period (Kumar et al., 1992; Kumar et al., 1994; reviewed in Kumar, 2009). The initial study of caspase-2 functions was performed in caspase-2 knockout mice. However, caspase-2 deficiency in these mice did not generate a significant gross phenotype, except in their oocytes. Excessive numbers of oocytes were observed in the mice ovaries, and the oocytes were more resistant to apoptosis when treated with chemotherapeutics such as doxorubicin, in caspase-2 deficient mice. Subsequently, B lymphoblasts from caspase-2 deficient mice were shown to be more resistant to apoptosis induced by granzyme B than cells from wild type mice. However, in contrast, the cell death in caspase-2 deficient motor neurons was observed to be slightly up-regulated compared with wild type cells. Except for these differences, death of other cell
types showed no significant difference in caspase-2 deficient and wild type mice (Bergeron et al., 1998). Based on these observations, caspase-2 seems to be an essential positive apoptosis inducer in some circumstances, however, however, in most tissues, the role of caspase-2 seems not irreplaceable, and in rare situation, it can even act as a negative regulator of apoptosis. A reasonable guess is that caspase-2 share overlapping functions with other caspases, however, the observation of excessive oocyte numbers and higher resistance to apoptosis for B lymphoblasts in caspase-2 deficient mice suggests that caspase-2 could play special roles under certain stimuli. Although this research did not completely discover the functions of caspase-2, it provided clues for further study.

1.3.1 The relationship between caspase-2 structure and function

Structural studies of caspase-2 were also important for determining caspase-2 functions. One of the first studies of caspase-2 showed it to share homology to both ICE, which was later named caspase-1, and C. elegans ICE homologous protein CED-3. As a result of this study, caspase-2 was originally named ICE and CED-3 homologue 1 (Ich-1) (Wang et al., 1994). The N-terminal region of Ich-1, also called the prodomain region, contains a caspase recruitment domain (CARD) (Hofmann et al., 1997). The CARD domain has been shown to be responsible for protein-protein interaction, meaning that it facilitates caspase-2 interaction with itself and also with other proteins containing a
CARD domain, such as RIP-associated Ich-1/Ced-3-homologue protein with a death domain (RAIDD) (Chou et al., 1998). Compared with executioner caspases including caspase-3, 6, and 7, which only contain a short prodomain without the ability of protein-protein interaction, the long CARD-containing prodomain of caspase-2 suggested it be classified better into the subfamily of initiator caspases. The following research showed that the dimerization mediated by the CARD domain is able to drive the activation of caspase-2, which is a well-observed event for the activation of initiator caspases (Butt et al., 1998; Baliga et al., 2004). When a fusion protein containing the caspase-2 prodomain and the executioner caspase-3, which only contained a short prodomain lacking protein-protein interaction function, was generated, the fusion protein showed the ability to auto-process itself, allowing it to function as an initiator caspase (Colussi et al., 1998b). These studies together demonstrated that caspase-2, if not always, largely functioned as an initiator caspase. Similar to the function of DISC as the caspase-8 activation platform and the apoptosome as the caspase-9 activation platform, the existence of a platform for caspase-2 activation was also considered to be plausible. A protein complex was proposed to activate caspase-2 in cells, containing p53-induced protein with death domain (PIDD), RAIDD, and procaspase-2. This complex was named the PIDDosome (Fig. 1-4) (Tinel and Tschopp, 2004). However, the exact composition of the PIDDosome requires deeper investigation as the knockout of PIDD in mice did not appear to affect
the processing of procaspase-2 (Manzl et al., 2009; Kim et al., 2009b). It is possible that the function of PIDD could be replaced by other proteins, or PIDD might be only required in certain specific situations. On the other hand, other components of the PIDDosome might exist. This potential for additional proteins that serve as scaffolding factors for caspase-2 activation warrants further investigation.

While procaspase-2 shares many common features with other caspases, such as the long prodomain, the large subunit, and the small subunit, it also has the unique characteristic of a nuclear location signal (NLS) on the C-terminal of the prodomain (Colussi et al., 1998a; Baliga et al., 2003). Although caspase-2 was shown to mainly localize in the cytosol under normal conditions, it often translocates into the nucleus upon DNA damage in an importin-dependent manner (Zhivotovsky et al., 1999; Manzl et al., 2009). The function of caspase-2 in the nucleus requires further investigation, but based on the observation that it is present in the nucleus after DNA damage, it is highly possible that it plays a role in the DNA damage response.
A platform containing PIDD and RAIDD is proposed to interact with and activate caspase-2.
1.3.2 Caspase-2 as an initiator caspase

Despite some controversial studies showing the functions of caspase-2 in other or even opposite directions, more and more evidence has suggested that caspase-2 generally acts as an initiator caspase in the intrinsic apoptotic pathway, activated in response to various cellular stresses (reviewed in Krumschnabel et al., 2009). One of the most studied cellular stresses is DNA damage; caspase-2 has been reported to be important in apoptosis caused by DNA damage. Either caspase-2 inhibition by benzyloxy carbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone (z-VDVAD-fmk) or caspase-2 knockdown by antisense oligonucleotides interferes with the cytochrome c release that is caused by the DNA-damage-inducing drug etoposide. Caspase-2 knockdown/inhibition also protects against etoposide-induced apoptosis in Jurkat T-lymphocytes (Robertson et al., 2002). In addition, another RNA interference study showed that caspase-2 acts upstream of caspase-8 in inducing mitochondria-dependent apoptosis caused by etoposide treatment in T cell lines (Lin et al., 2004). In addition to T cell lines, caspase-2 has also been shown to mediate apoptosis induced by the DNA damage-inducing drugs including cisplatin and etoposide in multiple other cell lines. These include several cancer cell lines such as A549 human lung carcinoma cells and U2-OS human osteosarcoma cells (Cao et al., 2008). Such findings suggest that caspase-2
could potentially act as a tumor suppressor by inducing apoptosis in cancer cells. The mechanism of caspase-2 activation following DNA-damage was reported to be mediated through p53-induced expression of PIDD and the subsequent assembly of the caspase-2 activation platform the PIDDosome (Lin et al., 2000; Tinel and Tschopp, 2004).

Caspase-2 also mediates apoptosis induced by other types of cellular stresses, such as endoplasmic reticulum (ER) stress, heat shock, and oxidative stress. ER is the critical organelle to assist protein folding with the help of chaperones. However, when the amount of unfolded proteins is too much for the ER, the ER stress response, also called the unfolded protein response (UPR), is initiated. The purpose of the UPR is first to restore the balance between unfolded proteins and the capacity of the ER, but when this effort fails, apoptosis is activated to eliminate the dysfunctional cell (reviewed in Xu et al., 2005). Caspase-2 was found to be present on the ER in HeLa cells, detected by confocal microscopy. Following ER stress induced by brefeldin A or tunicamycin, caspase-2 was observed to be processed and activated, upstream of caspase-3 activation. Furthermore, caspase-2 knockdown by small interfering RNA (siRNA) suppressed apoptosis mediated by ER stress (Cheung et al., 2006).

Heat shock is another type of stress caused by the exposure of cells to high temperature. By performing a trapping assay using biotinylated valine-alanine-aspartate–fluoromethyl ketone (bVAD-fmk) as the bait to interact with caspases
activated by heat shock stress, caspase-2 was trapped by bVAD-fmk, suggesting the interaction between caspase-2 and bVAD-fmk (Tu et al., 2006). Considering that VAD-fmk is a pan-caspase inhibitor which interacts with active caspases at their catalytic sites, this study strongly indicated that caspase-2 is activated in response to heat shock stress (reviewed in Ekert et al., 1999). Furthermore, caspase-2/− mouse embryonic fibroblasts (MEF) cells were more resistant to heat shock induced apoptosis compared with control MEF cells, confirming that caspase-2 mediated apoptosis caused by heat shock stress (Tu et al., 2006).

Finally, oxidative stress is caused by the abnormal accumulation of reactive oxygen species (ROS), including peroxides and free radicals, which are able to cause severe cellular damage. This is a type of stress closely related to the development of cancers (reviewed in Halliwell, 2007). Similar to other cellular stresses, in oxidative stress, caspase-2 has been reported to mediate apoptosis in the muscle cells of mice (Braga et al., 2008). A common feature of caspase-2 mediated apoptosis in response to cellular stresses is that caspase-2 acts upstream of the mitochondria (Lassus et al., 2002). Later research showed that caspase-2 was able to cleave the BH-3 only Bcl-2 family protein Bid (Bonzon et al., 2006). The truncated form of Bid (tBid) was shown to oligomerize and activate the pro-apoptotic Bcl-2 family members Bax and Bak, which in turn caused mitochondria outer membrane permeabilization (MOMP), the release of
apoptotic proteins, such as cytochrome c, from mitochondria to the cytosol, and the activation of the downstream apoptotic pathway (Kim et al., 2009a; Wei et al., 2000; Guo et al., 2002).

1.3.3 Caspase-2 as a tumor suppressor

Considering the function of caspase-2 in initiating the intrinsic apoptotic pathway, the potential roles of caspase-2 in apoptosis-related diseases, such as cancers, are worth investigating. The first implication of caspase-2 as a tumor suppressor came from the gene mapping of human caspase-2 that located the gene CASP2 to the chromosome region 7q34–35, which was frequently altered in association with haematological oncogenesis (Kumar et al., 1995). The expression levels of caspase-2 in multiple types of tumor cells, compared with normal tissue cells, were also tested, and caspase-2 was shown to be significantly under-expressed in multiple types of tumors, including childhood acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), mantle cell lymphoma, gastric cancer, and metastatic brain tumors, suggesting that caspase-2 could be a suppressor for these types of cancers (Holleman et al., 2005; Hofmann et al., 2001; Yoo et al., 2004; Zohrabian et al., 2007; reviewed in Kumar, 2009). More direct evidence for a tumor suppressor function of caspase-2 comes from research using MEF cells from caspase-2−/− and caspase-2+/+ mice. It was shown that the caspase-2−/− MEF cells proliferated significantly faster in a tissue culture environment and also
formed tumors faster in nude mice, than caspase-2+/+ MEF cells, when MEF cells were equally transformed with oncogenes such as adenovirus early region 1A (E1A) and rat sarcoma (Ras). Furthermore, compared with caspase-2+/+ MEF cells, the caspase-2−/− MEF cells were more resistant to apoptosis when treated with either chemotherapeutics or radiation, suggesting that caspase-2 performed its tumor suppressor function by activating apoptotic pathway in response to cellular stresses (Ho et al., 2009).

1.4 Caspase-2 in the metabolic regulation of apoptosis

As mentioned earlier, caspase-2 knockout mice did not display a significant phenotype except the excessive number of oocytes in their ovaries and the higher resistance of oocytes to chemotherapy drug induced apoptosis, suggesting that caspase-2 played a unique and irreplaceable role in apoptosis of oocytes (Bergeron et al., 1998). However, how caspase-2 contributes to apoptosis in oocytes was still unclear.

In fact, cell death of oocytes has been reported for a long time. Most female germ cells die even before birth; less than two million oocytes survive the whole embryonic development process, compared with a stockpile of about seven million cells in the early embryonic stage (Baker, 1963). Even after birth, oocytes constantly undergo elimination, which eventually leads to female sterility, as the original oocyte stockpile is the only resource for females to be able to reproduce (reviewed in Morita and Tilly, 1999). Research trying to uncover the mechanism of oocyte death showed that the death of
mouse oocytes was significantly accelerated after treatment with the cancer chemotherapeutic drug, doxorubicin. The fact that this death was caused by apoptosis was confirmed when caspase inhibitor pretreatment of oocytes before doxorubicin treatment provided them with higher resistance to death. This study partially uncovered the mechanism of oocyte apoptosis by showing that the doxorubicin-induced apoptosis of oocytes were completely suppressed in Bax-deficient mice, but not in p53-deficient mice (Perez et al., 1997). Furthermore, the oocyte apoptosis could be suppressed by Bcl-2 overexpression, and enhanced in Bcl-2 knockout mice (Morita et al., 1999; Ratts et al., 1995). These findings position the control point mediating oocyte apoptosis upstream of mitochondrial outer membrane permeabilization (MOMP), but downstream of p53, which is consistent with the idea that caspase-2 plays an important role in oocyte apoptosis, because caspase-2 is an initiator caspase acting upstream of MOMP in response to cellular stresses.

Based on these observations, the mechanism of oocyte apoptosis was finally deciphered using a cell free system called Xenopus egg extract system prepared from the eggs laid by Xenopus laevis, a type of African frog (Nutt et al., 2005). The oocyte itself is not usable for biochemical analysis because of its small size. On the other hand, Xenopus egg extract system is perfect for the biochemical study of apoptotic pathway. Two decades ago, it was reported that the extracts presented many apoptotic events.
spontaneously, such as the release of cytochrome c from mitochondria, activation of caspases, and fragmentation of nucleus, if the extracts were incubated at room temperature for long enough time period (Newmeyer et al., 1994). Furthermore, these spontaneous apoptotic events could be suppressed by the addition of Bcl-2 protein, and the downstream events could be suppressed by treatment with caspase inhibitors (Kluck et al., 1997). These findings suggest that this system retained many properties of apoptotic pathway, and the apoptotic pathway in this system could be biochemically manipulated. Another advantage is the ease of using this system in which one can generate a large number of synchronized extracts, making this system an easy model system to operate.

1.4.1 Metabolic regulation of oocyte apoptosis through caspase-2

One unique feature of oocytes is that they contain a large amount of nutrient stocks, including glycogen and yolk proteins, to support them through the whole embryonic development process. Therefore, it is possible that these nutrient stocks suppress apoptosis to help oocytes survive, and apoptosis of the oocytes might happen because of the activation of apoptotic pathway induced by nutrient depletion.

To test this hypothesis, Nutt et al., a former researcher in Sally Kornbluth’s laboratory, prepared *Xenopus* egg extracts following the protocol generated by Newmeyer et al. and used this system to perform biochemical analyses of apoptotic
events (Newmeyer et al., 1994; Nutt et al., 2005). In the first step of this study, glucose-6-phosphate (G6P), the first downstream metabolite of glucose catabolism, was directly added into the extracts to test whether the nutrient abundance could have an impact on the spontaneous apoptosis in the extracts. Apoptosis was monitored by measuring caspase-3 activity, using a substrate of caspase-3 called DEVD-pNA. As the result, the activation of caspase-3 was totally suppressed by G6P treatment. Except for caspase-3 activation, G6P also inhibited other apoptotic events, including nuclear fragmentation and the release of cytochrome c from mitochondria. All these data suggested that apoptosis of the extracts were suppressed by metabolism (Nutt et al., 2005).

Secondly, because G6P could possibly undergo glycolysis or enter the pentose phosphate pathway (PPP), Nutt et al. investigated which metabolic pathway G6P went through to suppress apoptosis. Because the previous study had shown that G6P had been hardly metabolized through glycolysis in Xenopus oocytes, PPP was the preferred candidate (Dworkin and Dworkin-Rastl, 1989). Metabolism intermediates of glycolysis or PPP were added into the extracts, and apoptosis was monitored by reading caspase-3 activity. The results showed that only PPP intermediates, including 6-phosphogluconate and nicotinamide adenine dinucleotide phosphate (NADPH) could suppress apoptosis, while glycolysis intermediates, including glyceraldehyde-3-phosphate and pyruvate, failed (Fig. 1-5) (Nutt et al., 2005). The importance of the pentose phosphate pathway
(PPP) in suppressing apoptosis was further supported by the acceleration of spontaneous apoptosis when treating the extracts with dehydroisoandrostosterone (DHEA), an inhibitor of G6P dehydrogenase (G6PDH), which was able to stop G6P from entering the PPP (Schwartz and Pashko, 2004). The two main products of the PPP are NADPH and 5-carbon sugars. Because the NADPH treatment was able to suppress apoptosis, Nutt et al. proposed that NADPH was the key product of the PPP for the metabolic suppression of apoptosis (Fig. 1-5). Malate was used to test this hypothesis, because it can produce NADPH in an alternative way, catalyzed by malic enzyme. These studies found that apoptosis was suppressed when malate was added into the extracts, detected by both caspase-3 activation and cytochrome c release (Nutt et al., 2005).

The subsequent experiments of this study tried to uncover the locus of this metabolic suppression of apoptosis in the apoptotic pathway. As mentioned above, caspase-2 had been thought to be linked with oocyte apoptosis, positioning it as a highly potential candidate (Bergeron et al., 1998). As expected, radiolabelled recombinant procaspase-2 added into extracts underwent processing concordantly with the spontaneous apoptosis. And the processing could be suppressed by treating the extracts with G6P, NADPH, or malate, suggesting that the caspase-2 activation was under metabolic control. To examine whether the caspase-2 activation was related, or even critical, to apoptosis the extracts were treated with VDVAD-fmk, a peptide inhibitor of
caspase-2. The result showed suppression of both the processing of caspase-2 and the release of cytochrome c. In addition, Bcl-XL, an anti-apoptotic Bcl-2 family protein, could only suppress the release of cytochrome c, but not the processing of caspase-2, positioning caspase-2 upstream of Bcl-2 family proteins. Furthermore, zVAD-fmk, a pan-caspase inhibitor, failed to suppress either the caspase-2 processing or cytochrome c release, which was reasonable considering that caspase-2 had been reported by previous study to be not sensitive to zVAD inhibition compared with other caspases (Garcia-Calvo et al., 1998). This data positioned caspase-2 upstream of other caspases, because otherwise the inhibition of other caspases by zVAD treatment would suppress the processing of caspase-2, and importantly it showed the unique role of caspase-2 in suppressing cytochrome c release in the egg extracts. All these data together suggested that caspase-2 was suppressed by G6P through PPP, and this suppression of caspase-2 was the key step in the metabolic regulation of apoptosis in the extracts (Nutt et al., 2005).
Glucose-6-phosphate (G6P) undergoes PPP to generate NADPH, which is the key product for the metabolic suppression of apoptosis in *Xenopus* egg extracts.
1.4.2 Metabolically regulated caspase-2 phosphorylation mediated by Ca\(^{2+}\)/Calmodulin dependent protein kinase II (CaMKII)

Although the suppression of caspase-2 activation was shown to be critical in the metabolic suppression of apoptosis in the extracts, whether caspase-2 was directly suppressed by NADPH or not was still unclear. If the suppression was not due to a direct interaction, then caspase-2 was likely to be regulated by some upstream signal in between of NADPH production and caspase-2 activation. NADPH was known to be a reducing reagent in cells, therefore, it could act as a reducing factor directly on caspase-2 to suppress caspase-2 activation. Nutt et al. treated the extracts with other reducing reagents, including Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) which is known as a superoxide dismutase (SOD) mimetic, and N-acetylcysteine (NAC), but none of them could suppress the spontaneous apoptosis of the extracts (Nutt et al., 2005). This data suggested that NADPH did not suppress caspase-2 through this mechanism. Although the probability of other unknown direct mechanisms still existed, Nutt et al. speculated that caspase-2 was suppressed by NADPH probably through indirect mechanisms, mediated by some intermediate pathways.

As mentioned above, caspase-2 had been proposed to be processed and activated on a platform called PIDDosome, with the recruitment of caspase-2 to this platform achieved through interaction with RAIDD through the CARD domain (Duan and Dixit, 1997; Tinel and Tschopp, 2004). Nutt et al. tested whether RAIDD could assist with the
processing of procaspase-2, and observed that the addition of recombinant Glutathione S-transferase (GST)-tagged RAIDD could process procaspase-2 directly. However, this RAIDD-induced processing of procaspase-2 was suppressed by treatment of either G6P or NADPH. Furthermore, the interaction between GST-tagged RAIDD and the radiolabelled procaspase-2 added into the extracts was also significantly suppressed by the treatment of either G6P or NADPH. These data together suggested that G6P, metabolized through PPP, was able to impair caspase-2 recruitment to RAIDD and therefore suppress caspase-2 activation. Considering that the CARD domain was responsible for mediating this interaction, certain unknown changes (either post-translational modifications or the recruitment of unknown proteins) to the CARD domain on either procaspase-2 or RAIDD might occur in the presence of abundant nutrients (Nutt et al., 2005).

Nutt et al. first attempted to identify proteins associated with both procaspase-2 and RAIDD, but failed to detect any potential inhibitors of the CARD interaction. Therefore, Nutt et al. speculated that the CARD domain underwent certain post-translational modifications that could interfere with its function in protein-protein interaction. Phosphorylation was the first type of modifications examined. Using GST-tagged recombinant RAIDD or caspase-2 prodomain containing the CARD domain as the substrate, kinase assays were performed in the Xenopus egg extract system to detect
any potential phosphorylations. Although no phosphorylation of RAIDD was observed, Nutt et al. observed phosphorylation of the caspase-2 prodomain when the extracts were treated with G6P. Additionally, after immuno-precipitation using a caspase-2 specific antibody, the endogenous caspase-2 also showed phosphorylation stimulated by G6P. All these data suggested that G6P stimulates phosphorylation within the CARD domain of caspase-2 (Nutt et al., 2005).

To identify the kinase responsible for this stimulated phosphorylation on the caspase-2 CARD domain, Nutt et al. systematically screened potential kinases by using a panel of kinase inhibitors with varying specificities. The extracts were treated with G6P, and co-treated with kinase inhibitors against anti-apoptotic kinases including protein kinase A (PKA), protein kinase B (Akt), protein kinase C (PKC), and mitogen-activated protein kinase kinase (MAPKK or MEK). Kinase assays were performed subsequently to detect phosphorylation of the caspase-2 prodomain. None of these kinase inhibitors could inhibit the phosphorylation of the caspase-2 prodomain stimulated by G6P treatment, except a kinase inhibitor called UCN-01, which could inhibit a wide range of kinases. In the *Xenopus* egg extract system, it had been used to inhibit checkpoint kinase 1 (Chk1) and Ca²⁺/Calmodulin dependent protein kinase II (CaMKII), meaning that the kinase phosphorylating the caspase-2 prodomain would be either Chk1 or CaMKII (Graves et al., 2000; Hutchins et al., 2003). Chk1 was first tested; however, neither
treating the extracts with caffeine which was also capable of inhibiting Chk1, nor immuno-depletion of Chk1 in the extracts, could generate the same inhibitory effect as UCN-01. On the other hand, the phosphorylation of the caspase-2 prodomain stimulated by G6P treatment was inhibited by co-treatment with KN93, an inhibitor of CaMKII. Similar results were observed when the extracts were depleted with calmodulin, suggesting that the relevant kinase could interact with calmodulin. Additionally, Ca\(^{2+}\) could phosphorylate the caspase-2 prodomain by itself, and this stimulation of phosphorylation could be suppressed by co-treatment with ethylene glycol tetraacetic acid (EGTA). All these data consistently suggested that some member of CaMK family was responsible for phosphorylating the caspase-2 prodomain, stimulated by G6P (Nutt et al., 2005).

To test which member of CaMK family was the relevant one, Nutt et al. performed an in vitro kinase assay by incubating each CaMK family member (CaMKI, CaMKII, or CaMKIV) with GST-tagged caspase-2 prodomain, in the presence of Ca\(^{2+}\). CaMKII was the only member that could phosphorylate the prodomain. Furthermore, two specific inhibitors of CaMKII could inhibit this phosphorylation, confirming that CaMKII was the kinase responsible for the metabolically regulated phosphorylation of caspase-2 (Nutt et al., 2005).
Finally, Nutt et al. analyzed the amino acid sequence of caspase-2 prodomain for the characteristic sequence for CaMKII phosphorylation, and selected serine 73 (S73) and serine 135 (S135) (Xenopus numbering) as the candidate targeting sites. S73A and S135A mutants of caspase-2 prodomain were generated to test which site was phosphorylated by CaMKII. By performing kinase assays, Nutt et al. observed that the S135A mutant of the caspase-2 prodomain was no longer phosphorylated by the treatment of G6P, but the S73A mutant showed no suppressive effect. Besides this observed effect on phosphorylation of the caspase-2 prodomain, the S135A mutation, but not S73A mutation, also blocked other phenotypes caused by G6P treatment, including the suppression of procaspase-2 processing and the suppression of caspase-3 activation. Furthermore, the S135A mutant of procaspase-2 could directly induce apoptosis despite the presence of abundant nutrients. All the data together revealed that the spontaneous apoptosis in Xenopus egg extract system and also the apoptosis in Xenopus oocytes were suppressed by metabolism through the PPP. This metabolic suppression of apoptosis was mediated by an inhibitory phosphorylation on the S135 site of the caspase-2 prodomain, catalyzed a CaMKII (Fig. 1-6) (Nutt et al., 2005).
Figure 1-6: Metabolic suppression of caspase-2 through CaMKII

In the presence of abundant nutrients, such as G6P, CaMKII is activated and catalyze an inhibitory phosphorylation on prodomain of caspase-2. As a result, caspase-2 cannot interact with RAIDD and dimerize itself. The suppression of caspase-2 activation leads to the suppression of apoptosis.
1.4.3 Subsequent research regarding the metabolic regulation of caspase-2

Although the mechanism of oocyte apoptosis was largely deciphered, more questions remained unanswered. What are the phosphatases targeting S135 (Xenopus numbering) of the caspase-2 prodomain? Is dephosphorylation of S135 also under the regulation of metabolism? The stimulated phosphorylation of caspase-2 requires active CaMKII, but what are the mechanisms controlling the metabolic activation of CaMKII? Finally and importantly, are these mechanisms conserved through different species?

More detailed studies have been performed to answer these questions. By pre-phosphorylating the caspase-2 prodomain and testing the rate of its dephosphorylation in the egg extracts, Nutt et al. later demonstrated that the dephosphorylation of S135 was suppressed by the treatment of G6P. In addition, the phosphatase responsible for this dephosphorylation was identified to be protein phosphatase 1 (PP1), however, Nutt et al. did not observe the alteration of either the PP1 catalytic activity or the interaction between PP1 and caspase-2 stimulated by G6P treatment, suggesting that extra regulatory steps were existent (Nutt et al., 2009). The subsequent study identified 14-3-3ζ, which was able to recognize and interact with phosphorylated serine of target proteins, to interact with phosphorylated caspase-2. Additionally, 14-3-3ζ was found to be released from caspase-2 prior to the downstream activation of the caspase-3.
Furthermore, the processing of procaspase-2 was suppressed by the addition of recombinant histidine (His)-tagged 14-3-3ζ, suggesting that the interaction between 14-3-3ζ and caspase-2 was suppressive for the activation of caspase-2. 14-3-3 release had been reported to be important for the activation of Cdc25, therefore, similar mechanism could also regulate the dephosphorylation of S135 and the activation of caspase-2 (Margolis et al., 2006b; Margolis et al., 2003). This hypothesis was confirmed by the observation of 14-3-3ζ dissociation from caspase-2 happening prior to the caspase-2 S135 dephosphorylation, and further by successfully suppressing this dephosphorylation through the addition of recombinant 14-3-3ζ protein. Finally, Nutt et al. demonstrated that the 14-3-3ζ release from caspase-2 was also regulated by metabolism, by showing that the G6P treatment to the extracts was able to suppress 14-3-3ζ release (Fig. 1-7) (Nutt et al., 2009).

The mechanism of the metabolic control of 14-3-3ζ release from caspase-2 was also investigated (Andersen et al., 2011). 14-3-3ζ was identified by proteomics analysis to be a substrate of a member of Sirtuin deacetylases called Sirt1. Andersen et al. showed that G6P treatment of the extracts up-regulated the activity of Sirt1, which in turn deacetylated 14-3-3ζ stabilizing the interaction between caspase-2 and 14-3-3ζ. This interaction suppressed the dephosphorylation of S135 on the caspase-2 prodomain (Andersen et al., 2011).
Figure 1-7: Suppression of caspase-2 dephosphorylation by 14-3-3

In the top panel, the release of 14-3-3 from caspase-2 happens prior to the dephosphorylation of caspase-2 S135 by PP1, resulting in the activation of caspase-2. In the middle panel, the addition of excessive 14-3-3 prevents caspase-2 from being
dephosphorylated. In the bottom panel, G6P treatment suppresses the removal of 14-3-3 from caspase-2 and caspase-2 dephosphorylation.
To answer the question of whether the metabolic regulation of apoptosis through the inhibitory phosphorylation of caspase-2 could potentially be conserved through different species, Yang et al. observed the regulation of apoptosis by metabolism in *Drosophila*. Although the apoptotic pathways in *Drosophila* and *Xenopus Laevis* were not completely conserved, NADPH was shown to stimulate an inhibitory phosphorylation on Dronc, an initiator caspase in *Drosophila*, at the site of S130. In addition, the kinase responsible for this phosphorylation was also identified to be CaMKII (Yang et al., 2010). This study proved that the metabolic regulation of apoptosis through CaMKII could be conserved through species, and therefore could also potentially be conserved in some mammalian cell types.

The last question addressed is the identity of the mechanisms of CaMKII activation stimulated by nutrients. Recently, the Nutt laboratory partly answered this question by reporting that coenzyme A (CoA), generated in *Xenopus* egg extracts in the presence of abundant nutrients, binds to and activates CaMKII (McCoy et al., 2013b). This thesis shows that the nutrient-driven CaMKII activation additionally requires the release of a “brake”. Specifically, we identify two novel sites of CaMKII phosphorylation (T393/S395 on the *Xenopus* γ isoform L subunit; T371/S373 on the human homolog) located within the association domain, whose phosphorylation falls in the presence of high G6P levels. Dephosphorylation of these sites, catalyzed by protein phosphatase 2A
(PP2A), is necessary (albeit not sufficient) for metabolic activation of CaMKII, which is
the process of “brake” releasing. In addition, the nutrient-driven PP2A targeting to
CaMKII is driven by metabolically-regulated interaction of CaMKII with the PP2A
targeting subunit B55β. Furthermore, we show that this mechanism of CaMKII
regulation is largely conserved in mammalian cells. Together these findings provide
insight into the metabolic control of apoptosis and define a new mechanism for
controlling CaMKII, a protein critical for cell signaling in response to multiple stimuli.
2. Materials and methods

2.1 Preparation of Xenopus egg extracts and nutrient treatment

The S phase and ultra-fractionated S phase *Xenopus* egg extracts were prepared as previously described (Fig. 2-1) (Smythe and Newport, 1991). G6P was prepared as a 1M solution in water. Extracts were prepared at 4°C, treated with G6P at a final concentration of 20mM (water as control treatment), and incubated at room temperature.
The eggs laid by *Xenopus Laevis* are collected and packed after the removal of excess buffer. The S phase *Xenopus* egg extracts are the middle cytoplasmic fraction, generated by centrifugation.
2.2 Cell culture, nutrient treatment, and cell lysis

HEK 293T cells were maintained and grown in DMEM with 10% fetal bovine serum (FBS) medium at 37°C. Before nutrient treatment, cells were starved with glucose-free DMEM with 10% dialyzed FBS medium containing no D-glucose and sodium pyruvate at 37°C for 12 hours, and then treated with or without 25mM D-glucose (Sigma) for another 12 hours. In addition, 150µM dehydroepiandrosterone (DHEA) in dimethyl sulfoxide (DMSO) or DMSO was occasionally co-treated together with D-glucose to glucose-starved 293T cells. Cells were lysed in 50mM Tris pH 7.5, 150mM NaCl, 1mM dithiothreitol (DTT), and 1% NP-40, in the presence of 5µg/ml Aprotonin/Leupeptin (A/L), 100µM phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets from Roche, 20X) on ice for 0.5 hour. Cell lysates were collected after centrifugation with 12,000 rpm for 15 minutes.

2.3 SiRNA transfection

Lipofectamine RNAiMAX (Invitrogen) was used for siRNA transfection. PP2A-B55β siRNA was purchased from Santa Cruz Biotechnology to knock down B55β in 293T cells. Control siRNA was designed to target a non-mammalian protein, firefly luciferase (5’-CGUACGCGGAUACUUCGA-3’). 200,000 cells per well were plated on 6-well plates (Corning Life Sciences) one day before transfection. 50pM of B55β or
control siRNA, and 10µl RNAiMAX, were separately gently diluted in 200µl Opti-MEM I Reduced Serum Medium (Invitrogen). After 5 minutes, siRNA and RNAiMAX in Opti-MEM were gently mixed and added onto cells after the incubation at room temperature for 20 minutes. After the first round of transfection, the second round was repeated after 5 hours of incubation. Cells were then incubated for 48 more hours before any subsequent treatment.

**2.4 Plasmids and recombinant protein preparation**

**2.4.1 Gene cloning**

*Xenopus* CaMKIIγ was amplified by PCR using the primers 5’-

GGGGAATTCAATGGCCACTACCCAGACTTGCACC-3’ and 5’-

GGGCTCGAGTCACTGGAGAGGGGCTGCTGGTGC-3’. Purified PCR products were cloned into the EcoRI and XhoI sites of pENTR3C. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate point mutants in CaMKIIγ in pENTR3C. The T393A/S395A primers were 5’-

CAGATGGGATAAAAGGATCAGCAGGCTTGCAACACCACCACCTGAAG-3’ and its complement. The T393D/S395D primers were 5’-

CAGATGGGATAAAAGGATCAGCAGGATTGCAACACCACCACACTGAAG-3’ and its complement. The Gateway LR Clonase II Enzyme Mix (Invitrogen) was used to transfer CaMKIIγ wild type and mutants from pENTR3C into pDEST24. Bacmid DNAs
were produced by transforming pDEST24 plasmids into MAX Efficiency DH10Bac
Chemically Competent E. coli (Invitrogen) and transfected into SF9 cells using Cellfectin
II Reagent (Invitrogen). The viruses were amplified following the Baculovirus
Expression System with Gateway Technology manual (Invitrogen). After the final step
of amplification, supernatants containing viruses were stored in 4°C.

2.4.2 His-tagged protein production and purification

15ml of virus supernatants were mixed with 100 million SF9 cells, added to
100ml of total volume with Hyclone SFX-insect medium (Thermo Scientific), and
vortexed at 25°C for 36 hours. The SF9 cell pellets were collected by centrifugation and
stored at -80°C for later purification.

The SF9 cell pellets were re-suspended by 1X Xiaodong’s Buffer (20mM HEPES
pH 7.5, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM PMSF,
and 5μg/ml A/L), sonicated for 15 seconds with 30% amplitude for three times, and
centrifuged with 10,000 Xg for 1 hour. After centrifugation, supernatants were collected,
added into 3ml of nickel (Ni) beads (Qiagen), and rotated at 4°C for 2 hours. The beads
were washed with 300ml of lysis buffer with 500mM NaCl and 15mM imidazole,
followed by 20ml of lysis buffer without NaCl and imidazole. After the wash, the beads
were eluted by 1ml of lysis buffer with 250mM imidazole for six times. The elutions
were analyzed by western blot, and the ones with purified proteins were collected,
concentrated, and dialyzed three time into 1L 1X PBS with 1mM DTT at 4°C. Finally, the protein solutions were stored in -80°C.

2.4.3 GST-tagged protein production and purification

The GST-tagged Xenopus caspase-2 prodomain and B55β, and GST-only plasmids were transformed into BL21 competent cells and incubated at 37°C overnight. Colonies were inoculated into 50ml LB media at 37°C overnight, and then added into 1L LB media at 37°C. When the bacteria were amplified enough, 1ml of 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to bacteria to generate a final concentration of 1mM and incubated at 37°C for another three hours. The bacteria were collected by centrifugation.

2.5ml of buffer A (2.3M sucrose, 50mM Tris pH 7.5, 1mM EDTA) and 25μl of 100mM PMSF were added onto the bacteria pellets to re-suspend the pellets, followed by the addition of 10ml buffer B (50mM Tris 7.5, 10mM KCl, 1mM EDTA) containing 1μM DTT, 100μM PMSF, and 0.01g lysozyme. The mixtures were rotated at 4°C for 1 hour, then added with 263μl MgCl₂, 175μl 10% sodium deoxycholate, and 25μl deoxyribonuclease I (DNase I), sonicated for 15 seconds with 30% amplitude for three times, and finally centrifuged by 12,000 Xg for 0.5 hour at 4°C. The supernatants were collected, and rotated together with 2ml glutathione beads (GE healthcare) at 4°C for 2 hours. The beads were washed by 10ml buffer C (10mM HEPES pH 7.5, 1mM DTT)
containing 1mM DTT and 300mM NaCl for three times, and then washed by 10ml buffer
C twice. 1ml buffer C was left with the beads to generate a slurry of proteins bound to
the beads. 20µl of the slurries were used to analyze the concentration of recombinant
proteins by coomassie staining, compared with standard bovine serum albumin (BSA)
(New England Biolabs) with different amounts. The beads slurry was stored at 4°C.

2.5 Antibodies and related assays

2.5.1 Antibodies used for western blot

The following antibodies were used for western blot: anti-CaMKII Phospho T286
(Abcam), anti-CaMKII (BD Transduction Laboratories), anti-PP2A catalytic subunit
(Millipore), anti-PP5 (BD Transduction Laboratories), anti-PPP2R1A (Abcam), anti-
PPP2R2B (B55β) (Abcam), anti-pan B56 (Millipore), and anti-GST (Santa Cruz
Biotechnology). Proteins were measured by western blot either using LI-COR
Biosciences Odyssey software or enhanced chemiluminescence (ECL) method with
horseradish peroxidase (HRP) anti-mouse or rabbit immunoglobulin G (IgG) secondary
antibodies.

2.5.2 Co-immunoprecipitation

Two micrograms of anti-CaMKIIα (Sigma) or Mouse control IgG (Santa Cruz
Biotechnology) were incubated with 20µl of Dynabeads Protein G (Invitrogen) slurry
overnight at 4°C. 100µl of Xenopus egg extracts treated with or without G6P were
incubated with beads for 1 hr at 4⁰C. Beads were washed four times with wash buffer (described below), and eluted with 40µl 2X Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer diluted from 5X sample buffer (described below). Samples were resolved by SDS-PAGE for immunoblotting.

The wash buffer for co-immunoprecipitation: 300mM NaCl and 0.1% Triton X-100 (sigma) in 1x egg lysis buffer (ELB: 10mM HEPES pH 7.5, 50mM KCl, 2.5mM MgCl₂, 1mM DTT, and 0.25M sucrose, pH=7.7).

5X SDS-PAGE sample buffer: 150mM Tris pH 6.8, 30% glycerol, 10% SDS, 5% β-mercaptoethanol (BME), and 0.5% bromophenol blue.

2.5.3 Immuno-depletion

10µg PPP2R2B antibody (0.25µg/µl) or Rabbit control IgG (Santa Cruz Biotechnology) were incubated with 100µl of Dynabead Protein A (Invitrogen) overnight at 4⁰C. Beads were washed and divided into 3 equal parts. 90µl of Xenopus egg extracts were incubated with beads for 1 hour at 4⁰C and separated from beads by centrifugation of 12,000 rpm for 1 minute. This step was repeated for three times. 2µl extracts after depletion by either PPP2R2B antibody or Rabbit control IgG were resolved by 20µl 2X SDS-PAGE sample buffer, and analyzed by western blot for detecting the efficiency of immuno-depletion.
2.6 Pull-down assays

2.6.1 Calmodulin (CaM) sepharose pull-down assay

200µl Xenopus egg extracts were treated with G6P or water at room temperature for 0.5 hour. 40µl of CaM sepharose (Agilent technologies) was washed for twice with 1X ELB, then dipped into treated extracts, and incubated at 4°C for 1 hour. After incubation, beads were washed for four times with the same wash buffer as used in co-immunoprecipitation experiments, and eluted by 40µl 2X SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and analyzed by western blot.

2.6.2 GST-B55β pull-down assay

200µl Xenopus egg extracts were treated with G6P or water at room temperature for 0.5 hour. 40µl of GST-tagged Xenopus B55β or GST-only beads slurry containing 2µg proteins was washed for twice with 1X ELB, then dipped into treated extracts, and incubated at 4°C for 1 hour. After incubation, beads were washed for four times with the same wash buffer as used in co-immunoprecipitation experiments, and eluted by 40µl 2X SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and analyzed by western blot.

2.7 Phospho-antibody purification

The following peptides were synthesized to generate and purify phospho-antibody against T393 and S395 of Xenopus CaMKIIγ: [H]-VHNATDGIKGSTESCN-
[NH2] (non-phospho peptide), [H]-VHNATDGKGS-PThr-ESCN-[NH2] (phospho-T393 peptide), and [H]-VHNATDGKGS-PSer-CN-[NH2] (phospho-S395 peptide).

The non-phospho peptide and two phosphor peptides were conjugated with UltraLink Biosupport resin (Thermo Scientific). Sera were first run through non-phospho peptide column to eliminate antibodies which were able to recognize non-phospho CaMKIIγ. This process was repeated for 3 times to ensure the complete elimination. The eluted sera were then separately purified on two phospho-peptide columns to remove non-specific antibodies and to obtain highly purified antibodies respectively recognizing phospho-T393 and phospho-S395. This process was also repeated for 3 times to ensure complete purification.

1µl Xenopus CaMKIIγ wildtype and T393A/S395A mutant were separately added into 20µl 2X SDS-PAGE sample buffer, and analyzed by western blot to examine the specificity of two phospho-antibodies.

**2.8 Gel Filtration chromatography**

2ml of Xenopus egg extracts were treated with G6P or water, and co-treated with okadaic acid (OA) or DMSO. After incubation at room temperature for 4hrs, the extracts were centrifuged at 200,000 Xg and were fractionated by Superose 6 column (GE Healthcare Life Sciences). Each fraction collects approximately 400µl of elution. Before running samples, standard protein markers with molecular weights of 669kD, 443kD,
and 150kD were run through the column to determine the molecular weight pattern of
gel filtration fractions, analyzed by native-PAGE coomassie staining. For each sample,
nineteen fractions from number twenty first to thirty ninth were collected, resolved by
SDS-PAGE, and analyzed by western blot.

2.9 Mass spectrometry analysis

2.9.1 Sample preparation

*Xenopus* egg extracts were treated with G6P or water, and CaM sepharose was
added (Agilent Technologies). After incubation, the CaM sepharose beads were
collected by centrifugation and washed four times with 1x egg lysis buffer containing
500mM NaCl and 0.5% Triton X-100. Following buffer exchange into 50mM ammonium
bicarbonate, pH 8.0, samples were subjected to a micro Bradford assay (Pierce) and then
supplemented with 0.1% Rapigest SF surfactant (Waters Corporation).

Samples were reduced with 5mM DTT for 30 min at 70°C and free sulfhydryls
were alkylated with 10mM iodoacetamide for 45 min at room temperature. Proteolytic
digestion was accomplished by the addition of 500 ng sequencing grade trypsin
(Promega) with incubation at 37°C for 18 hours. Supernatants were collected following a
2 min centrifugation at 1,000 rpm, acidified to pH 2.5 with TFA and incubated at 60°C
for 1 hour to hydrolyze remaining Rapigest surfactant. Insoluble hydrolyzed surfactant
was cleared by centrifugation at 15,000 rpm for 5 min. Approximately 3µg of total
digested protein from each sample was reserved for direct unbiased Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) analysis to allow for protein loading normalization in the phosphor-peptide enriched dataset, while the remaining 90ug was brought to dryness using vacuum centrifugation and then re-suspended in 100ul 80% acetonitrile, 1% TFA, 50 mg/mL MassPrep Enhancer, pH 2.5 (Waters Corp). Peptides were subjected to phosphor-peptide enrichment using a 200ul TiO₂ Protea Tip (Protea Bio) and subsequently washed with 200uL 80% acetonitrile, 1% TFA, 50 mg/mL MassPrep Enhancer followed by 200uL 80% acetonitrile, 1% TFA. Peptides were eluted in 50uL 20% acetonitrile, 5% aqueous ammonia, pH 10.5 and then acidified to pH 2.5 with formic acid prior to drying using vacuum centrifugation. Phospho-peptide enriched samples were resuspended in 10uL 2% acetonitrile, 0.1% formic acid, 10mM citric acid prior to LC-MS/MS analysis.

**2.9.2 LC-MS/MS Analysis**

Each sample was subjected to chromatographic separation on a Waters NanoAquatry ultra performance liquid chromatography (UPLC) equipped with a 1.7μM BEH130 C18 75μM I.D. X 250mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 5 μL injection, peptides were trapped for 5 min on a 5μM Symmetry C18 180μM I.D. X 20 mm column at 20μl/min in 99.9% A. The analytical column was held at 5% B for 5 min
then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 300nl/min. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10μM tip orifice and coupled to a Thermo LTQ-Orbitrap XL mass spectrometer through an electrospray interface. For all samples, the instrument was set to acquire a precursor MS scan in the Orbitrap from m/z 400-2000 with r = 60,000 at m/z 400 and a target AGC setting of 1e6 ions. MS/MS spectra of the five most abundant precursor ions were acquired either in the Orbitrap with r = 7500 at m/z with a target AGC setting of 2e5 ions for non-enriched samples or in the ion-trap with a target AGC setting of 1e3 for enriched samples. Max fill times were set to 1000ms for full MS scans and either 500ms for Orbitrap MS/MS scans or 250ms for ion-trap MS/MS scans with minimum MS/MS triggering thresholds of 5000 counts. For all experiments, fragmentation occurred in the LTQ linear ion trap with a CID energy setting of 35% and a dynamic exclusion of 60s was employed for previously fragmented precursor ions.

2.9.3 Qualitative Identifications and Selected Ion Chromatogram Generation from Raw LC-MS/MS Data

Raw LC-MS/MS data files were processed in Mascot distiller (Matrix Science) and then submitted to independent Mascot searches (Matrix Science) against an Trembl database (v 40.14 Xenopus Laevis taxonomy, 12,530 forward entries) containing both forward and reverse entries of each protein. Search tolerances for LTQ-Orbitrap XL data
were 5 ppm for precursor ions and 0.02 Da for Orbitrap product ions or 0.8 Da for ion-trap product ions using full trypsin specificity. Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M) and phosphorylation (+79.9663 Da on S, T, and Y) were considered a variable modifications. All searched spectra were imported into Scaffold (v4.0, Proteome Software) and mascot ion scoring thresholds of 26 (p 0.05 Mascot identity score was 14.0) were set to achieve a false discovery rate of 0.0%. Probability of correct phosphorylation modification localization to a specific Ser, Thr or Tyr residue was performed by submitting each MS/MS spectrum to the AScore algorithm with AScores > 15 or >19 indicating a >90% or >99% probability of correct localization, respectively. Relative quantitation was performed in Skyline (v1.4.1, University of Washington) by applying the full MS precursor extracted ion chromatogram (EIC) function to integrate and measure peak areas (area under the curve, AUC) of each identified phospho-peptides. Reported AUC measurements for each phospho-peptide were the sum of the monoisotopic peak EIC as well as the second and third isotopomer EIC. To adjust for slight variations in starting CaMKII prior to TiO₂ enrichment, the average AUC of three non-phosphorylatable (i.e. did not contain a STY) peptides from the non-enriched LC-MS samples were used to generate a correction factor.
2.10 **Kinase assay**

40μl of glutathione Sepharose containing 4μg of recombinant GST tagged caspase-2 prodomain or GST-only proteins were incubated in *Xenopus* egg extracts together with 20μCi [γ-^{32}P]ATP, treated by 20mM G6P or water, at 30⁰C for 1hr. Samples were washed with the same wash buffer as in co-immunoprecipitation experiment, eluted with SDS-PAGE sample buffer, and resolved by SDS-PAGE for autoradiography.
3. Metabolic control of CaMKII activation through the B55β/PP2A

3.1 Introduction

CaMKII is an important Ca\textsuperscript{2+} signal mediator. Upon activation by the interaction with Ca\textsuperscript{2+}/CaM, CaMKII specifically phosphorylates the targeting serine/threonine residues on substrates and mediates multiple downstream signaling pathways. Highly expressed in brain tissues such as hippocampus, CaMKII is best studied in mediating the learning and memory process called long-term potentiation (LTP), which is a memory mechanism defined by synapse strengthening dependent on recent activities. Upon synaptic excitement, CaMKII activity is elevated by the increasing Ca\textsuperscript{2+} concentration in postsynaptic cells and mediates synaptic transmission (reviewed in Lisman et al., 2002). Kinase inhibitors were used to study the function of CaMKII in the process of synaptic transmission. Both the broad spectrum inhibitor H7 and the CaMKII-specific peptide inhibitor were found to reduce the potency of synaptic transmission (Waxham et al., 1993). The CaMKII\textalpha knockout mice showed deficiency of LTP, while presenting normal behaviors in most other aspects (Silva et al., 1992). Besides learning and memory, CaMKII has also been studied for its function in regulating the contractility of smooth muscles. By using the CaMKII inhibitor KN-93, CaMKII was
shown to be essential for maintaining the contractile force induced by histamine
treatment (Rokolya and Singer, 2000). CaMKII was thought to mediate smooth muscle
contractility through phosphorylating the light chain of myosin (Edelman et al., 1990).
Deficiencies within the CaMKII-mediated pathway results in heart dysfunction,
including cardiac arrhythmia and heart failure (reviewed in Hund and Mohler, 2014). In
addition, CaMKII could also regulate gene transcription by phosphorylating the
transcription factor, cAMP response element-binding protein (CREB), thereby
influencing the transcription of downstream genes (Sun et al., 1994). In addition to these
functions, CaMKII also functions in other aspects, such as regulating metabolism and
Ca\(^{2+}\) flux (reviewed in Braun and Schulman, 1995).

Considering the essential roles CaMKII plays in multiple signaling pathways, an
enormous number of studies have been performed to understand its structure and how
its activation is regulated. Four isoforms of CaMKII have been identified including
CaMKII α, β, γ, and δ. Although CaMKII is widely expressed in all types of tissues,
different tissues present different expression patterns for CaMKII isoforms, with at least
one isoform expressed (reviewed in Hudmon and Schulman, 2002). For example,
CaMKII is highly expressed in brain tissues, such as hippocampus and forebrain, but the
isoforms expressed are mainly α and followed by β (Erondu and Kennedy, 1985). In
contrast, the main CaMKII isoforms expressed in heart is δ, followed by γ, but not α and β (reviewed in Hund and Mohler, 2014).

The four isoforms of CaMKII share high homology to each other. Each isoform contains a catalytic domain near the N-terminus which is responsible for the kinase activity, an auto-regulatory domain in the middle region, and a C-terminal association domain (Kolodziej et al., 2000) (Fig. 3-1A). Although the catalytic domain and the auto-regulatory domain are highly conserved, alternative splicing regions exist in the association domain in some isoforms, generating differences in molecular weights and potentially functions among different isoforms (Hudmon and Schulman, 2002; reviewed in Griffith, 2004). For example, one variant of CaMKIIα isoform, called αB isoform was found to contain a specific nuclear localization signal (NLS) containing 11 amino acids in the association domain, which could translocate the CaMKII holoenzyme into the nucleus (Brocke et al., 1995; Srinivasan et al., 1994).

The CaMKII holoenzyme is a multi-subunit protein. Structural studies using CaMKII purified from rat brain showed that the holoenzyme was in the form of dodecamer (Bennett et al., 1983; Kuret and Schulman, 1984). The CaMKII holoenzyme was shown by electron microscopy to assemble into a wheel-like structure, with the C-terminal association domains interacting with each other and clustering in the center, and the N-terminal catalytic domain protruding outside (Fig. 3-1B) (Kanaseki et al.,
Considering that CaMKII has 4 different isoforms, the composition of the CaMKII holoenzyme was examined. CaMKII holoenzyme purified from rat brain presented a heteromultimeric composition, with primary α:β ratio of 3:1 and minor ratios of 6:1, 2:1, or 1:1 (Brocke et al., 1999; Vallano, 1989). Because of the higher expression level of CaMKIIα isoform, the excessive CaMKIIα was shown to form homomultimers (Brocke et al., 1999). Considering that different CaMKII isoforms have different molecular weights, the holoenzymes of CaMKII containing different compositions of isoforms vary in their total molecular weights.

The activity of CaMKII is regulated by a classical regulatory mechanism called auto-inhibition, existing in protein kinases and characterized by the existence of a specific structure within the kinase that acts as a pseudosubstrate and is able to interact with the kinases themselves to dock the catalytic active sites (reviewed in Kemp et al., 1994). For CaMKII, the auto-inhibition is catalyzed by the auto-regulatory domain located between the catalytic domain and the association domain in the linear CaMKII monomer structure. The auto-regulatory domain can be further divided into the N-terminal pseudosubstrate (auto-inhibitory) domain and the C-terminal CaM-binding domain, and two domains share some overlapping regions (reviewed in Hudmon and Schulman, 2002). In basal conditions, the pseudosubstrate sequence binds and inhibits the catalytic domain of CaMKII. This has been supported by inhibiting the kinase
activity of CaMKIIα from rat brain with a synthetic peptide containing its auto-
inhibitory domain (Colbran et al., 1989). However, Ca\textsuperscript{2+}/CaM binding to the CaM-
bounding domain disrupts the interaction between catalytic and auto-inhibitory domains,
and relieves CaMKII from inhibition, thereby activating the kinase and allowing access
to an autophosphorylation site conserved in all CaMKII isoforms (T286 for the α
isoform, and T287 for the β, γ, and δ isoforms) by conformational change (Colbran et al.,
1988). When two adjacent subunits in one CaMKII holoenzyme are both bound to
Ca\textsuperscript{2+}/CaM and activated, one subunit phosphorylates the adjacent one at T286 (for the α
isoform) located in the auto-regulatory domain (Hanson et al., 1994) (Fig. 3-1B). Once
autophosphorylated on T286, the Ca\textsuperscript{2+}/CaM off-rate drops over 1000-fold, largely
stabilizing and prolonging CaMKII activity. This phenomenon is called “CaM trapping”
(Meyer et al., 1992). Furthermore, the autophosphorylation of T286 significantly
suppresses the inhibitory potency of the auto-inhibitory domain, meaning that even if
Ca\textsuperscript{2+}/CaM dissociates from activated CaMKII subunits, those subunits with T286
autophosphorylation still retain autonomous (Ca\textsuperscript{2+}/CaM independent) kinase activity
(Colbran et al., 1989; Lai et al., 1987; Lou and Schulman, 1989; Miller et al., 1988; Thiel et
al., 1988). On the other hand, if T286 is dephosphorylated, CaMKII becomes dependent
on Ca\textsuperscript{2+}/CaM again (Lai et al., 1986). Therefore, based on all these properties, the T286
autophosphorylation can be utilized as an indicator of CaMKII activation. Besides T286,
more sites have been shown to undergo autophosphorylation, located in the CaM-binding domain. With T286 still autophosphorylated, the dissociation of Ca$^{2+}$/CaM from the CaM-binding domain induces further autophosphorylation at a series of sites including T305, T306, and S314 (Hanson and Schulman, 1992; Patton et al., 1990). The function of autophosphorylation on these sites is to prevent Ca$^{2+}$/CaM from interacting with the CaM-binding domain again, which is called “CaM capping” (reviewed in Hudmon and Schulman, 2002). Low basal level of T306 phosphorylation was also found, which played an inhibitory role by suppressing the interaction between Ca$^{2+}$/CaM and the CaM-binding domain of CaMKII (Ishida et al., 1994). Because we were focusing our studies on the mechanism of metabolic activation of CaMKII, we investigated the autophosphorylation specifically of T286 as the indicator of initial CaMKII activation.
Figure 3-1: The regulation of CaMKII activity

(A) Linear structure of CaMKII monomer. (B) The regulation of CaMKII activity by T286 autophosphorylation.
3.2 Results

3.2.1 Metabolic control of CaMKII activity

As reported previously, treatment of Xenopus egg extracts with G6P elevates the kinase activity of CaMKII (Nutt et al., 2005). To investigate the mechanisms of CaMKII activation, we still needed to confirm that the kinase activity of CaMKII was up-regulated by abundant nutrients. We first tested the autophosphorylation of T286 on CaMKII, an indicator of CaMKII activation, following the treatment of G6P in the extracts. By directly treating egg extracts with or without G6P and incubating at room temperature, we discovered that G6P increased phosphorylation of T286 on CaMKII, a known site of CaMKII auto-phosphorylation (Fig. 3-2A; note, the multiple phosphorylated bands are likely due to multiple isoforms and allelic variants in the pseudotetraploid Xenopus laevis). While the total CaMKII antibody used here recognized predominantly the CaMKIIα isoform, with increased exposure time, additional CaMKII bands became evident, corresponding to multiple bands of T286 phosphorylation. Compared with the significant increase of phosphorylation signal in the presence of G6P, all CaMKII bands were comparable for both presence and absence of G6P. This suggests that the observed increase in T286 phosphorylation following G6P treatment is likely generalizable to multiple CaMKII isoforms, at least to all isoforms recognized by the CaMKII antibody used. To test the speed of stimulating the autophosphorylation of
T286, the same experiment was performed with a shorter incubation. As a result, the T286 autophosphorylation was stimulated within 5 minutes of G6P treatment, suggesting that the stimulation of CaMKII T286 autophosphorylation is a rapid process as long as the extracts contain abundant nutrients.

However, although the autophosphorylation of T286 was considered an indicator of CaMKII activation, it was not equal to CaMKII activation. Therefore, we intended to observe the stimulation of CaMKII activity more directly. As reported previously, the *Xenopus* caspase-2 prodomain was shown to be a substrate of CaMKII (Nutt et al., 2005). We performed a kinase assay by adding GST-tagged recombinant *Xenopus* caspase-2 prodomain (or GST protein as the negative control) into egg extracts treated with G6P or water. We observed that the caspase-2 prodomain was more heavily phosphorylated in the presence of G6P, again consistent with the notion that the kinase activity of CaMKII is up-regulated by G6P (note that the GST protein was not phosphorylated even in the presence of G6P treatment, suggesting that G6P stimulated the phosphorylation of caspase-2 prodomain, not GST) (Fig. 3-2B). As also reported, CaMKII can physically bind the caspase-2 prodomain, which is stimulated by G6P treatment (McCoy et al., 2013a). The observation that the interaction between CaMKII and caspase-2 prodomain requires stimulation by G6P indicates that CaMKII potentially
undergoes certain changes in the presence of G6P, probably the elevation of its kinase activity.

Additionally, Nutt et al. reported that the human caspase-2 protein was also processed in *Xenopus* egg extracts and this processing could also be suppressed by G6P treatment. This suggests that the human caspase-2 could potentially be similarly regulated in certain human cells (Nutt et al., 2005). Furthermore, Nutt et al. also reported that human caspase-2 could be phosphorylated by the CaMKII protein, as determined by an in vitro kinase assay. These data suggest that the role of CaMKII on caspase-2 could be conserved between *Xenopus* and human cells (Nutt et al., 2005). Therefore, we tested whether the activation of CaMKII by abundant nutrients, which was an upstream pathway of caspase-2 phosphorylation, was also conserved in human cells. Interestingly, an up-regulation of T286 phosphorylation was also observed in human 293T cells after glucose starvation and re-supplementation, suggesting that the kinase activity of CaMKII was also stimulated by glucose abundance (Fig. 3-2C).

As also reported previously, in *Xenopus* egg extracts, the suppression of caspase-2 activation and the inhibitory phosphorylation of the caspase-2 prodomain depended on the activity of the pentose phosphate pathway (PPP), meaning that CaMKII was activated through PPP in *Xenopus* egg extracts (Nutt et al., 2005). Now that we know that CaMKII can also be activated by glucose in 293T cells, we tested whether this metabolic
activation of CaMKII depended on PPP in this mammalian setting. We co-treated cells with dehydroepiandrosterone (DHEA), an allosteric inhibitor of glucose-6-phosphate dehydrogenase (G6PDH), which catalyzed the rate-limiting step of PPP. The result showed that the glucose-induced T286 autophosphorylation of CaMKII in 293T cells was reduced by the co-treatment of DHEA, suggesting that the regulation of T286 autophosphorylation (or CaMKII activity) by glucose was also regulated through the PPP (Fig. 3-2D).
Figure 3-2: CaMKII activation is sensitive to metabolic status

(A) *Xenopus* egg extracts were treated with G6P or water for 0.5 hour (h) at room temperature, and analyzed for CaMKII T286 auto-phosphorylation using a phospho-T286 antibody (ab). Note that the middle and lower panels are from two different films as the CaMKII antibody recognized CaMKIIα (~50kDa) much more strongly than the other isoforms, so the detection of this isoform and the others required very different exposures. (B) GST tagged *Xenopus* Caspase-2 prodomain or GST bound to Glutathione Sepharose was incubated with *Xenopus* egg extracts supplemented with [γ-^{32}P] ATP and treated with G6P or water. Samples were resolved by SDS-PAGE and detected by autoradiography. CB: coomassie blue. (C) Glucose-starved 293T cells treated with or without glucose (25mM) were lysed and analyzed for CaMKII T286 auto-phosphorylation. (D) Glucose-starved 293T cells treated with or without glucose, and co-
treated with DHEA or DMSO were lysed and analyzed for CaMKII T286 auto-phosphorylation.
3.2.2 The phosphorylation status of CaMKII is altered by G6P treatment of egg extracts

Although autophosphorylation of T286 on CaMKII has been reported to be stabilized following G6P treatment, it may not be the only mechanism of regulating CaMKII as this autophosphorylation is downstream of the initial activation (McCoy et al., 2013a). Other mechanisms responsible for the initial stimulation of CaMKII activity are likely to exist. Therefore, we tried to determine whether other modifications of CaMKII, upstream of CaMKII activation, were modulated by metabolism. To examine the status of CaMKII in G6P-treated egg extracts, we resolved G6P-treated and untreated extracts by gel filtration and examined the profile of CaMKII fractionation. As shown in Fig. 3-3A, the apparent molecular weight of the CaMKII complex was increased following G6P treatment. It is possible that this G6P-induced shift in the CaMKII fractionation profile is caused by the association of additional proteins with CaMKII; even in the untreated extract, the molecular weight of CaMKII was still above 600K, meaning that CaMKII was already in its dodecameric (potentially active) form even without G6P treatment. It is worth noting that the shift might also be caused by the incorporation of other, higher molecular weight, CaMKII isoforms into the CaMKII holoenzyme.

To further investigate the mechanism of CaMKII activation, we looked for potential post-translational modifications of CaMKII induced by G6P treatment.
Endogenous CaMKII was precipitated from extracts treated with either G6P or buffer using Calmodulin (CaM) sepharose. These precipitates were then analyzed by Mass Spectrometry (MS) for post-translational modifications, such as acetylation and phosphorylation. The predominant CaMKII isoform identified by MS was CaMKIIγ L subunit. MS analysis failed to observe any significant difference of acetylation levels on CaMKII in the presence or absence of G6P treatment, it luckily identified several phosphorylation sites on CaMKII that were responsive to G6P addition. As expected, we observed an increase in T287 (T286 on CaMKIIα) phosphorylation in the sample treated with G6P, consistent with stimulation of T286 autophosphorylation in response to G6P treatment (Fig. 3-3C). More importantly, as shown in Fig. 3-3D and 3-3E, we identified two sites on CaMKIIγ L subunit, T393 and S395, whose phosphorylation was decreased in response to G6P treatment. These data suggested that regulated dephosphorylation of these two sites could influence the G6P-induced CaMKII activation in Xenopus egg extracts, meaning that certain phosphatase(s) might be activated by G6P treatment to dephosphorylate these sites and affect the downstream CaMKII activation. An interesting fact is that these two sites, T393 and S395, are located in the association domain of the CaMKIIγ L subunit. The association domain of CaMKII, as mentioned above, is responsible for protein-protein interaction, such as the oligomerization of the holoenzyme, and possibly the interaction with other proteins. Considering that G6P is
able to change the CaMKII holoenzyme molecular weight according to gel filtration, this change of molecular weight profile might be caused by the activation of those specific phosphatase(s). If this hypothesis is true, the upshift of the CaMKII profile in response to G6P treatment should be suppressed by co-treatment with phosphatase inhibitors.

To test this hypothesis, we performed the gel filtration experiment described in Fig. 3-3A, but co-treated with or without the phosphatase inhibitor, okadaic acid (OA). While G6P treatment alone still induced an upshift in the apparent molecular weight of CaMKII, as expected, the co-treatment with OA largely abrogated this upshift, suggesting that some OA-inhibitable phosphatase(s) might be required for the observed G6P-induced CaMKII molecular weight upshift (Fig. 3-3B). OA is a phosphatase inhibitor that mainly targets PP1 and PP2A, with a better efficacy for PP2A. The concentration of OA we used was not low enough to selectively inhibit PP2A, meaning that PP1 and PP2A might both be involved in suppressing the CaMKII molecular weight upshift stimulated by G6P. However, it was also reported that OA was not a specific inhibitor of only PP1 and PP2A, but could also inhibit other phosphatases including PP4, PP5, and PP6 (reviewed in Honkanen and Golden, 2002). Therefore, in addition to PP1 and PP2A, other phosphatases might still be involved in the upshift of CaMKII molecular weight. Combined with the mass spectrometric analysis, these data suggested
the possible involvement of some phosphatase(s), most possibly PP1 or PP2A, in G6P-induced CaMKII activation.
Figure 3-3: Modification of CaMKII by metabolism

(A) Xenopus egg extracts were treated with G6P or water for 4 h at room temperature and centrifuged at 200,000g. Cytosolic extracts were fractionated by gel filtration chromatography, and fractions were immunoblotted for CaMKII. (B) Xenopus egg extracts treated with G6P or water, and co-treated with okadaic acid (OA) or DMSO were fractionated and analyzed as in (A). (C-E) post-translational modifications of CaMKII were identified by MS analysis. (Upper): change of phosphorylation with or without G6P treatment. (Lower): tandem mass spectra used for identification and
localization of phosphorylation modifications on phosphopeptide containing corresponding site. (C) T287 phosphorylation levels with or without G6P treatment; (D) S395 phosphorylation levels with or without G6P treatment; (E) T393 phosphorylation levels with or without G6P treatment.
3.2.3 G6P treatment increases binding of PP2A to CaMKII

As mentioned above, some phosphatase(s) might be involved in the metabolic regulation of CaMKII activation, at least the metabolic stimulation of CaMKII molecular weight upshift. Considering that the change of apparent molecular weight of CaMKII is probably because of the incorporation of additional proteins to the CaMKII holoenzyme, the potential involved phosphatase(s) might interact with CaMKII in the presence of G6P, therefore contributing to the molecular weight upshift of CaMKII.

To determine whether some phosphatase(s) are indeed involved in regulating CaMKII activation, the interaction between CaMKII and potential phosphatases was examined. CaM sepharose was used to pull down endogenous CaMKII, and the proteins interacting with CaMKII, from Xenopus egg extracts treated with G6P or water. After incubating for one hour, the beads were washed intensively to reduce non-specific binding. Precipitates were immunoblotted for endogenous CaMKII, and more importantly, potential phosphatase(s), including PP1, PP2A catalytic (C) subunit, and PP5. The endogenous CaMKII pulled down by the same amount of CaM sepharose was similar with or without G6P treatment. Therefore, whether the interaction between the CaM complex (probably CaMKII) and phosphatase(s) was regulated by G6P treatment could easily be determined by directly comparing the intensity of phosphatase(s) bands in the presence and absence of G6P. Clearly, no interaction between the CaM complex
and PP5 was detected (Fig. 3-4A). Although lacking evidence of interaction does not definitively indicate that PP5 does not dephosphorylate CaMKII, this data at least suggests that PP5 does not contribute to the upshift of CaMKII molecular weight in response to G6P treatment. In contrast to the PP5 data, constitutive binding between PP1 and the CaM complex was observed with either G6P or water treatment (Fig. 3-4A). This observation was not surprising, because it was consistent with a previous report showing that PP1 was responsible for dephosphorylating CaMKII on the site of T286 in synaptic junctions (Shields et al., 1985; reviewed in Colbran, 2004). However, this association did not appear to be regulated metabolically, as the amount of PP1 pulled down by CaM sepharose with either G6P or water treatment was quite similar to each other, suggesting that PP1 was not the relevant phosphatase causing the upshift of CaMKII apparent molecular weight (Fig. 3-4A). Another reason that PP1 is unlikely to be the relevant phosphatase is that PP1 only dephosphorylates T286 on CaMKII, which playing an important role in stabilizing the kinase activity of CaMKII. This fact positions PP1 as a negative regulator of CaMKII activation. However, based on the gel filtration experiment and mass spectrometry analysis, the relevant phosphatase should be involved in CaMKII regulation in the presence of G6P, meaning that the relevant phosphatase should be a positive regulator of CaMKII. After excluding PP1 and PP5, only the PP2A C subunit exhibited increased interaction with sepharose following G6P.
treatment (Fig. 3-4A). This data suggests that PP2A is highly likely to be involved in the metabolic regulation of CaMKII activation, contributing to the dephosphorylation of sites identified by mass spectrometry analysis.

However, this data is not conclusive, because it only shows the interaction between the PP2A C subunit and the CaM complex, not directly between the PP2A C subunit and CaMKII. Therefore, it is still possible that the interaction is between the PP2A C subunit and CaM sepharose itself. Furthermore, although CaMKII is the most well-known protein that can potently bind CaM, as a key calcium (Ca\(^{2+}\)) sensor, CaM interacts with a large number of proteins besides CaMKII to mediate multiple Ca\(^{2+}\)-related signaling pathways. In fact, a large number of CaM-binding proteins have been systematically identified by scanning through the whole human proteome. Proteins identified included ribosomal proteins, proteasome proteins, and deubiquitinases. Thus, the PP2A C subunit might also interact with these proteins (Shen et al., 2005).

Considering these possibilities, the potential association between the PP2A C subunit and CaMKII requires further investigation.

To test whether the PP2A C subunit really interacts with CaMKII in response to G6P stimulation, other possibilities (such as interacting with CaM sepharose or other CaM-binding proteins) were examined. We first excluded the possibility that the PP2A C subunit interacts directly with CaM sepharose by depleting all CaM-binding proteins
including CaMKII. After incubating CaM sepharose with *Xenopus* egg extracts, most CaM-binding proteins were bound by CaM sepharose and largely depleted in the extracts. The efficacy of depletion was tested by analyzing the level of CaMKII and PP2A C subunit before and after depletion through immunoblotting. Results clearly demonstrated that the total PP2A C subunit level did not change despite depletion. This was not surprising because the PP2A C subunit is not a CaM-binding protein. In contrast, CaMKII was largely depleted; this demonstrates that the depletion itself was successful (Fig. 3-4B). After the depletion of CaM-binding proteins, CaM sepharose was incubated with either the depleted or un-depleted extracts treated with G6P or water in a pulldown assay to examine the interaction between the PP2A C subunit and CaM sepharose with or without depletion. The result showed that the G6P-stimulated interaction between the PP2A C subunit and the CaM complex was significantly suppressed by the depletion of CaM-binding proteins (Fig. 3-4C). This data suggests that the PP2A C subunit does not directly interact with CaM sepharose even in the presence of G6P treatment. However, another possibility is that the PP2A C subunit interacts with the CaM complex through other CaM-binding proteins. This possibility required further examination.

To clarify that CaMKII, and not another CaM binding protein, was interacting with the PP2A C subunit in response to G6P treatment, a specific anti-CaMKII antibody
was utilized to immunoprecipitate the endogenous CaMKII and PP2A C subunit. If the PP2A C subunit could be precipitated in the presence of G6P, it would suggest that CaMKII interacts directly with the PP2A C subunit, because other CaM-binding proteins cannot be precipitated by a specific anti-CaMKII antibody. The result showed that the endogenous PP2A C subunit could be co-immunoprecipitated with anti-CaMKII antibody at significant greater levels in the presence of G6P (Fig. 3-4D). Because the addition of G6P increased the general stickiness of the extracts, G6P treatment appeared to increase the background CaMKII immunoprecipitation in the presence of G6P, but the PP2A C subunit interaction was much more significantly elevated in the presence of G6P, compared with CaMKII. Together these data strongly suggest that CaMKII specifically interacts with PP2A, and this interaction is up-regulated by G6P-stimulated metabolism. Therefore, although not all phosphatases inhibited by OA were examined, PP2A is highly possible to be the relevant phosphatase that contributes to the metabolically-stimulated upshift of CaMKII apparent molecular weight and CaMKII activation.
Figure 3-4: CaMKII-PP2A interactions are regulated by G6P

(A) CaM sepharose was dipped into *Xenopus* egg extracts which had been treated with G6P or water for 0.5 h at room temperature, and incubated for 1 h at 4°C. Beads were retrieved by centrifugation and analyzed for the presence of candidate phosphatases by immunoblotting. (B) CaM sepharose was incubated with *Xenopus* egg extracts for 1 h at 4°C and then removed by centrifugation. This process was repeated 3 times. Depleted and un-depleted extracts were analyzed by CaMKII or PP2A immunoblotting. (C) CaM sepharose was incubated with either CaMKII-depleted or un-depleted extracts described in (B) treated with G6P or water. Beads were retrieved by centrifugation and analyzed by PP2A C subunit immunoblotting. (D) CaMKII antibody or control IgG coupled to protein G beads was dipped into *Xenopus* egg extracts treated with G6P or water, incubated for 1 h at 4°C, and retrieved for CaMKII or PP2A C immunoblotting.
3.2.4 B55β targets PP2A to regulate CaMKII activation

Functional PP2A holoenzyme is a multi-protein complex containing a catalytic (C) subunit with a molecular weight of about 36kD, a scaffolding (A) subunit with a molecular weight of about 65kD, and a regulatory (B) subunit with variable molecular weights. The C subunit is responsible for dephosphorylating targeting sites, the A subunit is responsible for the assembly of the holoenzyme by interacting with C and B subunits of PP2A, and most importantly the B subunit typically determines substrate specificity. The composition of the PP2A A and C subunits are quite simple, each of which contains only two isoforms. On the other hand, the composition of the B subunit family is much more complicated; this is to be expected as this family is responsible for mediating a wide range of cellular signaling pathways. Four different B subunit subfamilies have been identified: B (B55), B’ (B56), B’’ (PR72), and B’’’ (PR93). Additionally, each subfamily contains more than one member, all together forming a large B subunit family consisting of more than 16 members (reviewed in Shi, 2009). Although no homology was discovered between different B subunit subfamilies, all B subunit family members function via a similar mechanism. Upon recruitment to the PP2A holoenzyme by the A subunit, the B subunit leads the functional holoenzyme to specific substrates, by interacts with the substrates, upon receiving an upstream signal. The interaction between B subunits and their substrates has been investigated to some
degree. For example, Tau protein, whose hyperphosphorylation leads to polymerization in the brain and may result in Alzheimer’s disease, was identified to be dephosphorylated by PP2A (Goedert et al., 1995; reviewed in Goedert and Spillantini, 2006). This dephosphorylation by PP2A is mediated by the interaction between B55α subunit and Tau protein (Xu et al., 2008). Therefore, if PP2A is really positively involved in the metabolic regulation of CaMKII activation, a certain B subunit of PP2A must be present to specifically lead the PP2A holoenzyme to CaMKII, and the interaction between CaMKII and this B subunit should be observed in the presence of G6P treatment.

To identify the relevant B subunit family member that specifically mediates the metabolic regulation of CaMKII activity by PP2A, CaM sepharose was first used to precipitate endogenous CaMKII protein, and also the potential B subunits, in the extracts treated with G6P or water. The B55 and B56 subfamilies was preferentially speculated, because they have been implicated in the regulation of cell proliferation and death in several settings. For example, B56δ, a member of B56 subfamily, specifically leads the PP2A holoenzyme to dephosphorylate a site on cell division cycle 25 (Cdc25). This phosphorylation is important for Cdc25 activation and downstream entrance into mitosis (Margolis et al., 2006a). In addition, the B55 subfamily members showed high homology with another cell cycle related protein Cdc55, which was identified in
Saccharomyces cerevisiae, suggesting that B55 subfamily might also play roles in cell cycle regulation (Healy et al., 1991). More importantly, a recent study showed that the B55β/PP2A complex could dephosphorylate Cyclin E1, protecting it from being degraded and causing Cyclin E1 accumulation. The abnormally high activity of the B55β/PP2A was considered an important reason for the overexpression of Cyclin E1, which promoted cell proliferation, in many types of tumors (Tan et al., 2014). Therefore, this research identified a unique function of B55β/PP2A to promote cell proliferation, which was contrary to the previous opinion that PP2A normally acted as a tumor suppressor (reviewed in Janssens et al., 2005). This contradiction is reasonable considering that different B subunits may lead the PP2A holoenzyme to different substrates and to perform totally different functions.

Among all of the B subunits that we examined, we found B55β to up-regulate PP2A interaction with the CaM complex upon G6P treatment. However, by using a pan-B56 antibody to detect all B56 subfamily members, no B56 member showed G6P-stimulated interaction with the CaM complex, suggesting that the B56 subfamily was not involved in the metabolic regulation of CaMKII (Fig. 3-4A). In addition, as the scaffolding subunit, the A subunit should interact with both PP2A C and B subunits. Considering that both PP2A C and B subunits interact with CaM complex upon G6P treatment, we speculated that the PP2A A subunit should also interact with CaM.
complex. Therefore, the interaction between the A subunit and the CaM complex in response to G6P treatment was also examined. As expected, this interaction was indeed stimulated by G6P (Fig. 3-5A). In conclusion, all three components of the PP2A holoenzyme show stimulated interaction with CaM complex upon G6P treatment, suggesting that the B55β/PP2A interacted with CaM complex as a holoenzyme complex. However, because CaM sepharose was used in this experiment, the same problem exists as stated above—that the interaction between B55β/PP2A and the CaM complex might not be through CaMKII. Therefore, a more direct assay is necessary.

A more direct pulldown assay was performed to determine whether a direct interaction between B55β/PP2A and CaMKII occurs in extracts. The recombinant GST-tagged B55β protein was used to pull down endogenous CaMKII in extracts treated with G6P or water. G6P was found to stimulate the interaction between recombinant GST-tagged B55β protein and CaMKII (Fig. 3-5B). Importantly, besides the CaMKIIα isoform (shown slightly above 50kD), other isoforms of CaMKII were also observed to have G6P-stimulated interaction with B55β, when exposed for a longer period of time. Considering that the CaMKII isoform identified by previous MS analysis was CaMKIIγ, we were specifically interested in whether the interaction between the CaMKIIγ isoform and B55β was also stimulated by G6P in extracts. Therefore, the recombinant His-tagged CaMKIIγ L subunit and GST-tagged B55β proteins were added into *Xenopus* egg extracts.
treated with G6P or water, and the interaction between these two proteins was examined. Although G6P treatment increased background protein-protein interactions (GST protein alone could pull down some CaMKII protein), we were still able to observe that G6P could greatly stimulate the interaction between B55β and the recombinant CaMKIIγ protein (Fig. 3-5C). Taken together these data suggest that a multi-protein complex containing CaMKII, PP2A C subunit, A subunit, and B55β, forms in the presence of abundant nutrients (G6P), potentially contributing to the metabolic activation of CaMKII. Furthermore, these data also suggest that this increased protein complex formation stimulated by G6P seems to occur with multiple CaMKII isoforms, including CaMKIIα, and also the γ isoform which was identified in previous MS analysis.

Although it was clear that B55β was able to lead the PP2A holoenzyme to CaMKII in response to G6P treatment, we still needed to further confirm that this process positively contributed to the activation of CaMKII. An antibody specifically recognizing B55β was used to immuno-deplete the endogenous B55β protein in the extracts, and the efficiency of immuno-depletion was examined by immunoblotting. As shown in Fig. 3-5D, B55β was largely depleted from egg extracts without significant depletion of the PP2A C subunit, suggesting that the immuno-depletion was successful. To determine if B55β was required for the metabolic activation of CaMKII, the B55β-
depleted or un-depleted extracts were treated with G6P or water, and the T286 autophosphorylation of CaMKII was monitored. Compared with un-depleted extracts, the B55β-depleted extracts showed significantly diminished T286 autophosphorylation in response to G6P treatment (Fig. 3-5E).

In addition, by using the GST-tagged *Xenopus* caspase-2 prodomain as a substrate, we were able to monitor the ability of B55β-depleted and un-depleted extracts to phosphorylate the caspase-2 prodomain, treated with G6P or water. We observed that the un-depleted extracts could phosphorylate caspase-2 in the presence of G6P, however, this phosphorylation was largely diminished by B55β depletion (Fig. 3-5F). Since T286 autophosphorylation and caspase-2 phosphorylation were both indicators of CaMKII activation, both measures indicated diminished CaMKII activation in the B55β depleted extracts, suggesting that B55β is critical for the G6P-driven CaMKII activation in the *Xenopus* egg extract system. In addition, the fact that the immuno-depletion of B55β diminished multiple T286 auto-phosphorylation bands suggests that B55β is important for the activation of multiple CaMKII isoforms (Fig. 3-5E).

Moreover, when endogenous B55β was knocked down by B55β siRNA in 293T cells, the glucose-driven stimulation of CaMKII T286 auto-phosphorylation was suppressed, suggesting that the role of B55β in regulating CaMKII is evolutionarily conserved (Fig. 3-5G).
Figure 3-5: B55β regulates CaMKII activation

(A) CaM sepharose was incubated with *Xenopus* egg extracts treated with G6P or water and incubated for 1 h at 4°C. Beads were retrieved by centrifugation and analyzed by immunoblotting for PP2A regulatory subunits. (B) GST-B55β or GST bound to glutathione Sepharose was incubated for 1h at 4°C with *Xenopus* egg extracts treated with G6P or water. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. Note that the upper and middle panels are from two different films with different exposures as the CaMKII antibody recognized CaMKIIα much more strongly than the other isoforms. (C) *Xenopus* CaMKIIγ wild type (WT) protein expressed from baculoviral vectors in SF9 cells was added into *Xenopus* egg extracts for 0.5 h at room temperature, then the extracts were treated with G6P or water for 0.5 h at room temperature, and incubated with GST-B55β or GST bound to glutathione Sepharose for 1 h at 4°C. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. (D) B55β antibody or control rabbit IgG bound to Dynabead-linked Protein A was incubated with *Xenopus* egg extracts for 1 h at 4°C and removed with a magnet (repeated 3X). Extracts were analyzed by B55β and PP2A C
subunit immunoblotting. (E) The B55β-depleted or un-depleted extract was treated +/- G6P for 0.5 h, and analyzed by pT286 immunoblotting. (F) GST tagged Xenopus Caspase-2 prodomain or GST bound to Glutathione Sepharose was incubated with B55β-depleted or un-depleted Xenopus egg extracts supplemented with [γ-32P] ATP and treated with G6P or water. Samples were resolved by SDS-PAGE and detected by autoradiography. (G) B55β-specific or control siRNA-treated 293T cells were glucose starved for 12 h and then incubated with or without 25mM glucose. Lysates were analyzed by immunoblotting for pT286, CaMKII, and B55β.
3.2.5 PP2A dephosphorylates S395 on CaMKII in the presence of G6P

Based on our observation that B55β/PP2A was important for the metabolic regulation of CaMKII activation in both *Xenopus* egg extract system and mammalian cells, we were interested in identifying which sites on CaMKII were dephosphorylated by B55β/PP2A upon G6P treatment. Since the MS analysis showed that T393 and S395 of the CaMKIIγ L subunit were dephosphorylated in the presence of G6P, we postulated that these sites might be targeted by B55β/PP2A. To assess this, we generated phospho-antibodies recognizing each of these sites on the *Xenopus* CaMKIIγ isoform. The specificity of these two phospho-antibodies were tested by comparing the phosphorylation signal of wild type (WT) and T393A/S395A mutant CaMKIIγ proteins generated by transfecting baculovirus into SF9 cells. Unfortunately, the signal detected by phospho-T393 antibody was comparable between WT and the T393A/S395A mutant. Although we were unable to produce a high quality antibody specific for phosphorylated T393, we were successful with phospho-S395. As shown in Fig. 3-6A, WT CaMKIIγ protein exhibited basal phosphorylation of S395. However, the T393A/S395A mutation greatly diminished antibody recognition, attesting to its phoso-specificity. To confirm that the phosphorylation level of S395 was down-regulated by G6P treatment, we incubated *Xenopus* egg extracts with G6P or water, precipitated endogenous CaMKII with CaM sepharose, and immunoblotted with the
phospho-S395 antibody. As shown in Fig. 3-6B, phosphorylation of S395 was down-regulated in the presence of G6P (note that the γ isoform was shown in the loading control as the phospho-S395 antibody was designed based on a CaMKIIγ phosphopeptide sequence), confirming that the G6P-induced dephosphorylation of S395 identified by MS analysis was repeatable. Additionally, to test whether S395 of CaMKIIγ was dephosphorylated by B55β/PP2A, B55β immuno-depletion was performed. Phosphorylation of S395 was detected by phospho-S395 antibody in both B55β-depleted and un-depleted extracts treated with G6P or water. When B55β was depleted from Xenopus egg extracts, the G6P effect on S395 phosphorylation was inhibited, strongly suggesting that the dephosphorylation of S395 induced by G6P treatment was catalyzed through B55β/PP2A (Fig. 3-6C). Although the phospho-S395 antibody was unable to recognize the CaMKIIα isoform, since the B55β immuno-depletion could suppress the activation of all CaMKII isoforms stimulated by G6P and B55β could interact with multiple CaMKII isoforms including the CaMKIIγ isoform in the presence of G6P, it is quite possible that the S344 (the equivalent residue to S395 in the α isoform) is also dephosphorylated by B55β/PP2A in response to G6P treatment.

The phosphorylation level of S373 (the equivalent residue to Xenopus CaMKIIγ S395) of human CaMKIIγ was also examined in 293T cells treated with or without glucose for 12 hours after 12 hours of glucose pre-starvation. The phospho-S395
antibody did not detect any significant phosphorylation signal, regardless of glucose treatment, suggesting that the phoшpho-antibody failed to recognize human CaMKIIγ. However, considering that the requirement for B55β in the metabolic activation of CaMKII is conserved from *Xenopus* egg extracts to human cells, although the attempt by using the *Xenopus* phospho-S395 antibody failed, it is still highly possible that B55β/PP2A dephosphorylates human CaMKII at similar sites to promote the activation of human CaMKII in response to glucose treatment.

### 3.2.6 The phosphorylation status of T393 and S395 affects CaMKII activation

To determine whether the B55β-mediated dephosphorylation of S395 is critical for nutrient-driven activation of CaMKII, we first added CaMKIIγ WT, T393A/S395A mutant, and T393D/S395D mutant proteins (baculoviral produced) into *Xenopus* egg extracts, treated with G6P or water at room temperature, and monitored T287 (equivalent to T286 for CaMKIIα isoform) autophosphorylation by immunoblotting. As shown in Fig. 3-6D, after only 10 minutes of incubation with G6P, the T393A/S395A mutant exhibited higher stimulated phosphorylation of T287, compared with WT CaMKIIγ. Consistent with this data, the G6P-induced stimulation of CaMKII T287 autophosphorylation was significantly dampened by the T393D/S395D mutations (which may not perfectly mimic phosphorylation) (Fig. 3-6E). Considering the T287 (T286 for the α isoform) autophosphorylation of CaMKII is an indicator of CaMKII
activation, these data suggest that the dephosphorylation of S395 and possibly T393 stimulates the metabolic activation of CaMKII, while the phosphorylation of T393 and S395 suppresses it. In addition, to test this idea in a more direct way, we performed an in vitro kinase assay. We incubated CaMKIIγ T393A/S395A, or T393D/S395D with Calmodulin protein and the *Xenopus* GST-tagged caspase-2 prodomain, without the addition of egg extracts. We observed that the CaMKII T393A/S395A mutant was more able than the T393D/S395D mutant to phosphorylate the caspase-2 prodomain, consistent with the idea that the dephosphorylation of T393 and S395 is important for CaMKII activation (Fig. 3-6F). Finally, CaMKIIγ WT, T393A/S395A, or T393D/S395D were added into *Xenopus* egg extracts, treated with G6P or water, and pulled down using recombinant GST-tagged B55β protein to monitor whether the interaction between B55β and CaMKII was affected by phosphorylation status of T393 and S395. We found that, compared with the WT CaMKIIγ, the T393A/S395A mutant exhibited stronger interactions and the T393D/S395D mutant showed weaker interactions with the B55β protein compared with the WT CaMKIIγ (Figs. 3-6G and 3-6H). These data were contrary to our initial expectation, because we thought that if the only function of B55β/PP2A is to dephosphorylate T393 and S395, the T393D/S395D mutant needs to interact with B55β/PP2A more than WT and T393A/S395A mutant to dephosphorylate these two sites, while the T393A/S395A does not necessarily interact with B55β/PP2A
considering they were already dephosphorylated. However, these data suggest that the dephosphorylation of T393 and S395 might stabilize the binding of B55/β to CaMKII, meaning that B55/β/PP2A might perform additional functions in the metabolic regulation of CaMKII activation, other than only dephosphorylating T393 and S395. Collectively, these data suggest that the metabolic stimulation of T393 and S395 dephosphorylation through B55/β/PP2A could indeed positively contribute to CaMKII activation.
Figure 3-6: B55β-mediated T393/S395 dephosphorylation Contributes to CaMKII Activation

(A) Xenopus WT and T393A/S395A mutant CaMKIIγ expressed from baculoviral vectors in SF9 cells were analyzed by pS395 immunoblotting. (B) CaM Sepharose was incubated with Xenopus egg extracts treated with G6P or water, retrieved by centrifugation and analyzed by pS395 immunoblotting. (C) B55β-depleted or un-depleted Xenopus egg extracts with the addition of recombinant CaMKIIγ were treated with G6P or water. Recombinant CaMKIIγ was precipitated with CaM sepharose. Beads were retrieved by centrifugation and analyzed for pS395. (D) CaMKIIγ WT or T393A/S395A mutant was added into Xenopus egg extracts for 0.5 h at room temperature, then the extracts were treated with G6P or water and analyzed for pT287. (E) CaMKIIγ WT or T393D/S395D mutant was treated and processed as in (D). (F) GST tagged Xenopus Caspase-2 prodomain or GST bound to Glutathione Sepharose was incubated with Xenopus CaMKIIγ T393A/S395A or T393D/S395D mutants, Calmodulin, and [γ-32P] ATP at 30°C for 0.5 h. Samples were resolved by SDS-PAGE and detected by autoradiography. (G)
Xenopus CaMKIIγ WT or T393A/S395A mutant proteins were added into Xenopus egg extracts for 0.5 h at room temperature, then the extracts were treated with G6P or water for 0.5 h at room temperature, and incubated with GST-B55β or GST bound to glutathione Sepharose for 1 h at 4°C. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. (H) Xenopus CaMKIIγ WT or T393D/S395D mutant proteins were added into Xenopus egg extracts for 0.5 h at room temperature, then the extracts were treated with G6P or water for 0.5 h at room temperature, and incubated with GST-B55β or GST bound to glutathione Sepharose for 1 h at 4°C. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting.
3.2.7 Involvement of CoA in the B55β/PP2A regulation of CaMKII activation

Our data suggest that CaMKII needs to be released from a “brake” by B55β/PP2A to be activated. On the other hand, as mentioned above, the Nutt laboratory recently reported that Coenzyme A (CoA) was generated upon the addition of G6P into Xenopus egg extracts and was able to directly interact with and activate CaMKII (McCoy et al., 2013b). Although it is possible that the excessive amount of CoA used in the CaMKII activity assay directly overrides the “brake” and activates CaMKII, we were interested in whether CoA also plays a role in regulating the “brake” released by B55β/PP2A. First, Xenopus egg extract was treated by the direct addition of CoA or water, and incubated at room temperature. T286 autophosphorylation was tested by immunoblotting to indicate the kinase activity of endogenous CaMKII in extracts. A burst of T286 autophosphorylation was observed upon treatment with CoA throughout the region of molecular weights that all CaMKII isoforms covered, strongly indicating that CoA was able to stimulate the activity of multiple CaMKII isoforms, consistent with previous finding (Fig. 3-7A).

In addition, we examined whether CoA could also stimulate the interaction between CaMKII and the PP2A C subunit. CaM sepharose was dipped into the extracts that had been treated with CoA or water. The beads were washed and analyzed for potential binding of PP2A C subunit by immunoblotting. With similar endogenous
CaMKII protein bound to CaM sepharose, the PP2A C subunit showed significant CoA-stimulated binding (Fig. 3-7B). Although this experiment does not prove a direct interaction between CaMKII and the PP2A C subunit, it strongly suggests that CoA acts similarly to G6P in stimulating this interaction.

Furthermore, the interaction between the recombinant GST-tagged B55β protein and endogenous CaMKII protein in response to CoA treatment was also examined. Although the CoA treatment increased the background abundance of recombinant B55β protein, the interaction between B55β and CaMKII was up-regulated more significantly by CoA treatment, suggesting the ability of CoA to stimulate this interaction, consistent with our previous observation using G6P (Fig. 3-7C).

Finally, considering that G6P stimulated B55β/PP2A to dephosphorylate CaMKIIγ on S395 (and possibly T393), we tested whether the CoA treatment could also down-regulate the phosphorylation level of S395 on CaMKIIγ. *Xenopus* egg extracts were treated with CoA or water. Endogenous CaMKII was pulled down by CaM sepharose for immunoblotting with phospho-S395 antibody. Unsurprisingly, a decrease in S395 phosphorylation was observed when extracts were treated with CoA (Fig. 3-7D). Taken altogether, these data suggest that CoA plays at least a nominal role in regulating the release of CaMKII “brake” through B55β/PP2A.
Figure 3-7: Involvement of CoA in B55β/PP2A-mediated CaMKII regulation

(A) Xenopus egg extracts were treated with CoA or water for 0.5 hour (h) at room temperature, and analyzed for CaMKII T286 auto-phosphorylation using a phospho-T286 antibody (ab). (B) CaM sepharose was dipped into Xenopus egg extracts which had been treated with CoA or water for 0.5 h at room temperature, and incubated for 1 h at 4°C. Beads were retrieved by centrifugation, and analyzed for CaMKII or PP2A C subunit immunoblotting. (C) GST-B55β or GST bound to glutathione Sepharose was incubated for 1h at 4°C with Xenopus egg extracts treated with CoA or water. Beads were retrieved by centrifugation and analyzed for CaMKII or B55β immunoblotting. (D) CaM
Sepharose was incubated with *Xenopus* egg extracts treated with CoA or water, retrieved by centrifugation and analyzed for pS395 immunoblotting.
3.3 Discussion

3.3.1 The communication between CoA and B55β/PP2A-mediated CaMKII dephosphorylation

Multiple probabilities have been examined in studying the mechanisms of CaMKII activation by metabolism. Although this metabolically controlled CaMKII activation is still Ca²⁺ dependent because high concentration of EGTA could completely suppress this activation, Ca²⁺ flux was excluded from being the primary regulatory mechanism. Although calcium is a central regulator of CaMKII, previous work from our lab has shown that the centrifugal removal of Ca²⁺ stores from *Xenopus* egg extracts does not impede G6P-mediated activation of CaMKII shown by T286 autophosphorylation, suggesting that increased available Ca²⁺ does not underlie nutrient-dependent CaMKII activation.

Studies of CaMKII regulation have largely focused on T286 autophosphorylation after Ca²⁺/CaM binding. Indeed, G6P treatment of egg extracts impairs PP1-mediated dephosphorylation of T286 (McCoy et al., 2013a). However, this is unlikely to drive CaMKII activation as this phosphorylation is a result of activation. The Nutt laboratory reported that the G6P treatment of *Xenopus* egg extracts was able to up-regulate the level of CoA, which could directly activate CaMKII (McCoy et al., 2013b).

On the other hand, our data suggest that nutrient status is communicated to CaMKII, prompting activation, in part through the B55β/PP2A-mediated
dephosphorylation of CaMKII on S395 (γ isoform numbering), which releases the “brake” that restricts CaMKII activation. This suggests a novel locus for the control of CaMKII. Interestingly, based on data obtained by treating the egg extracts with CoA, this B55β/PP2A-mediated “brake” release of CaMKII also seems to communicate with the CaMKII activation mechanism mediated by CoA. However, available evidence is not conclusive of CoA directly inducing the dephosphorylation of CaMKII on S395 (γ isoform numbering) through B55β/PP2A. Considering that CoA has been reported to directly interact with CaMKII, it is possible that this interaction causes a conformational change on CaMKII, which could positively contribute to the interaction between CaMKII and B55β/PP2A. In addition, CoA might also target B55β to regulate CaMKII, either by directly binding B55β to change its conformation, or by indirectly modifying B55β through other intermediate signals. Therefore, it is worth further investigating how CoA is connected to the dephosphorylation of CaMKII on S395 (γ isoform numbering) as mediated by B55β/PP2A.

3.3.2 Effects of G6P-stimulated phosphorylation status change on CaMKII

In addition to T393 and S395 on Xenopus CaMKIIγ, the MS analysis also identified other sites showing changes in phosphorylation levels, including S311, S326, S333 and T421. However, differing from T393 and S395, all the other sites showed increased phosphorylation in response to G6P treatment. Although we have not yet
investigated these sites, they may also contribute to full CaMKII activation. The T393D/S395D mutant of CaMKIIγ is less potently activated by G6P than WT CaMKIIγ, indicated by T286 autophosphorylation immunoblotting. This finding is consistent with a requirement for phosphorylation of additional sites for full activation. In addition, considering that multiple sites have been identified as the autophosphorylation sites on CaMKII, it is possible that the sites showing increased phosphorylation when treated with G6P are also autophosphorylated after the activation of CaMKII. Even if these sites are not autophosphorylation sites, the kinase(s) responsible for phosphorylating these sites, and the question of whether the phosphorylation of these sites is under metabolic regulation, are worth pursuing.

More importantly, most of the sites identified by MS analysis to show altered phosphorylation status in response to G6P treatment are located in the association domain of CaMKII, which is known to be responsible for the self-association of the holoenzyme (Kolb et al., 1998; Shen and Meyer, 1998). However, our gel filtration results suggest that the oligomerization status of CaMKII itself is possibly not affected by these changes of phosphorylation status. Rather, G6P appears to shift the molecular weight of the full holoenzyme to an even higher range, suggesting the incorporation of additional factors into the CaMKII holoenzyme. Importantly, the molecular weight shift was largely abrogated by treatment with okadaic acid, consistent with the idea that
dephosphorylation of T393/S395 (at least S395) by B55β/PP2A might be required. In addition, although the functional consequence of other sites showing increased phosphorylation in response to G6P treatment is unknown, their phosphorylation might also contribute to the molecular change of the CaMKII protein complex. Therefore, it is attractive to speculate that the change of phosphorylation status on those sites might allow binding of additional regulatory factors to the CaMKII association domain. Interestingly, one variant of CaMKIIγ (CaMKIIγG-2) contains within its association domain a targeting sequence essential for ERK activation and contractility of smooth muscle cells—suggesting that the association domain can perform roles other than self-association (Marganski et al., 2005).

3.3.3 Regulation of T393/S395 phosphorylation status

Our data demonstrate a previously unknown role for the B55β targeting subunit of PP2A in regulating CaMKII. Often, the B subunit not only targets PP2A to the correct substrate(s), but also serves as a key locus of regulation. For example, Chk1-mediated phosphorylation B56 can inhibit PP2A-mediated dephosphorylation and activation of the Cdc25 mitotic regulator during DNA damage checkpoint signaling (Margolis et al., 2006a). Although it is possible that the CaMKII-B55β association is regulated at the level of CaMKII modification (such as a conformational change caused by CoA binding), we speculate that B55β may be differentially modified in a nutrient-dependent manner to
alter targeting of PP2A to CaMKII. As our data already showed, such a modification could be sensitive to CoA levels.

T393 and S395 are phosphorylated in the egg extracts before any treatment. Therefore, *Xenopus* egg extracts must contain a kinase(s) directed against these sites. As T393/S395 phosphorylation decreases prior to CaMKII activation, they are clearly not auto-phosphorylation sites. The relevant kinase is not currently known, but may provide an additional locus of metabolic regulation if its activity is high in the presence of abundant nutrients and low upon their depletion. Future experiments will be directed towards the identification and characterization of kinase(s) directed towards these sites.
4. Conclusions and perspectives

The work described in this thesis identifies a new locus of CaMKII regulation by metabolism—the dephosphorylation of T393/S395 (for CaMKIIγ isoform) by B55β/PP2A, and illuminates three important points for the future study.

First, our studies demonstrated specifically the metabolic control of spontaneous apoptosis in the *Xenopus* egg extract system, and suggested that this metabolic regulation could potentially be conserved in human cells, at least in certain types of cells. However, because our work in mammalian cells has been limited to 293T cells so far, more work must be performed to completely understand how conserved the metabolic regulatory mechanism of CaMKII is.

Second, importantly, CaMKII plays a role in multiple signaling pathways. Thus the same regulatory mechanism could also function to determine other aspects of cell fate.

Finally, considering: 1) that the B55β immuno-depletion could completely abolish the CaMKII activation that was stimulated by G6P treatment, and 2) that activated CaMKII suppresses caspase-2 activation and apoptosis, it is possible that B55β might serve as a chemotherapeutic target for certain types of cancers.
4.1 *Metabolic regulation of CaMKII in other CaMKII-mediated signaling pathways*

As mentioned above, CaMKII is ubiquitously expressed and has a diverse array of substrates. For example, CaMKIIγ regulates the contractility of smooth muscle cells. Multiple studies have reported that the altered metabolism caused by metabolic inhibition or hypoxia is able to affect contractile force generation and maintenance of smooth muscle cells (reviewed in Taggart and Wray, 1998). Although this effect has been associated with impaired Ca\(^{2+}\) flux, metabolically-controlled dephosphorylation of T393 and S395 might also contribute to the regulation of CaMKIIγ activation in smooth muscle cells.

In addition to its role in smooth muscle cell contractility, CaMKII is also well known to regulate the process of memory called long-term potentiation (LTP). Because the phospho-antibody of S395 only recognizes the CaMKIIγ isoform, we could not test the phosphorylation status of the corresponding site of S395 on the CaMKIIα isoform. However, based on data we have obtained, the importance of B55β for metabolic activation of CaMKII is universal for all CaMKII isoforms. This suggests that B55β likely also dephosphorylates the CaMKIIα isoform on sites corresponding to S395 of the CaMKIIγ isoform. It will be interesting to determine whether these sites play a similar role in regulating the highly abundant CaMKIIα isoform in neurons, which is important for LTP. Interestingly, it has been reported that LTP is regulated by metabolism,
especially the metabolism of a neurotransmitter called glutamate, whose biosynthesis is partly dependent on NADPH (Lynch et al., 1990). Considering that the regulation of CaMKII activation is also mediated through NADPH, it is likely that the metabolic regulation of CaMKII activation also plays a role in regulating LTP.

Similarly, B55β may control CaMKII in other physiological paradigms of regulation or metabolic control, warranting further investigation in other physiological settings.

**4.2 Potential targeting of B55β for cancer therapeutics**

Our data suggest that B55β/PP2A is required for both the metabolic activation of CaMKII and the downstream phosphorylation of the caspase-2 prodomain, which further suppresses caspase-2 activation. This leads to inhibition of spontaneous apoptosis in the *Xenopus* egg extract system. Based on the potential function of B55β/PP2A in suppressing caspase-2 and apoptosis in human cells, we speculate that B55β/PP2A could be a potential target for diseases caused by dysfunctional caspase-2 or apoptosis.

The ability of affected cells to “evade apoptosis” has been recognized as an important hallmark during the development of cancer in the famous review written by Dr. Robert Weinberg (reviewed in Hanahan and Weinberg, 2000). It has been realized for a long time that cancer cells acquire a resistance to apoptosis, even following
radiotherapy or chemotherapy. One of the most common changes seen in cancer cells is the loss-of-function mutation of tumor suppressor gene p53. This mutation results in a diminished ability of the cell to sense and respond appropriately to DNA damage by inducing apoptosis (Harris, 1996). Additionally, cancer cells have other strategies to avoid apoptosis, such as gain-of-function mutations in critical cell survival factors, or loss-of-function mutations for pro-apoptotic factors. For example, Bcl-2 family proteins are commonly reported to be altered in various types of cancer cells. In fact, the discovery of the anti-apoptotic member Bcl-2 was due to a gene translocation, which abnormally affected its expression level in lymphomas (Tsujimoto et al., 1985). Besides gene translocation, increased BCL-2 gene copy number was also observed in multiple types of cancers (Ikegaki et al., 1994; Monni et al., 1997). In addition to enhanced anti-apoptotic factors, abrogation of pro-apoptotic factors has been demonstrated in cancers: one of the best-studied pro-apoptotic members, Bax, shows mutations which impede its functions (Meijerink et al., 1998). Based on these findings, deficient apoptosis is an important element of tumorigenesis.

Importantly, caspase-2 has been reported to function as a tumor suppressor. As mentioned above, the caspase-2−/− mice form tumors faster than caspase-2+/− mice in the Eμ-Myc lymphoma model (Ho et al., 2009). Furthermore, the caspase-2−/− Mouse embryonic fibroblasts (MEF) cells obtained higher resistance to chemotherapy-induced
apoptosis, compared with caspase-2+/+ MEF cells, suggesting that the tumor suppressor function of caspase-2 is because of its function in initiating apoptosis (Ho et al., 2009). Additionally, caspase-2−/− mice develop Kras-dependent lung cancer faster than caspase-2+/+ mice, further supporting its role as a tumor suppressor (Terry et al., 2014). However, although the tumor suppressive function of caspase-2 is linked with its function as an initiator of apoptosis, the exact mechanism through which caspase-2 is abnormally regulated in cancer cells is unclear. Therefore, our discovery of the B55β/PP2A-CaMKII regulatory mechanism for caspase-2 might provide insight into mechanisms for suppressing tumorigenesis. More importantly, our studies also establish a link between apoptosis and metabolism, which could also play an important role during the development of cancers.

The importance of an altered metabolism pattern has been more and more recognized in recent years. Dr. Robert Weinberg updated his review “hallmarks of cancer” in 2011, and included the “reprogramming of energy metabolism” as a new hallmark of cancer development (reviewed in Hanahan and Weinberg, 2011). Actually, the abnormal metabolism pattern in cancers had been first documented more than half a century ago, by Dr. Otto Heinrich Warburg. He reported an abnormally rapid glucose uptake and consumption by glycolysis, resulting in high generation of lactate in tumors. This effect was called the “Warburg effect” (Warburg, 1956). Although inefficient, this
elevated process ensured the rapid generation of ATP, which is required for rapid
synthesis of biomolecules and cancer cell divisions. This elevation of glucose uptake has
been utilized in the diagnosis of cancers. By using the technique of positron emission
tomography (PET) scanning, the elevated uptake of radiolabelled 2-deoxy-2(18F)-fluoro-
D-glucose in tumor tissue can be visualized (reviewed in Gambhir, 2002). Multiple types
of cancers can be detected with this technology, including breast cancer, colorectal
cancer, prostate cancer, and so on (Franc and Hawkins, 2007; Lin, 2011; Shen et al., 2014).
Additionally, various drugs have been developed to target the enhanced glucose
metabolism in cancer cells (reviewed in Zhang and Yang, 2013). For example, as the
main entrance of glucose into human cells, glucose transporter 1 (GLUT1) has a high
potential as a target for cancer therapy. A GLUT1 inhibitor, phloretin, has shown
synergistic effect with chemotherapeutics in killing cancer cells (Cao et al., 2007). With
the development of cancer research, more drugs that are able to suppress glucose
metabolism with higher efficiency and specificity will be developed.

On the other hand, the pentose phosphate pathway (PPP), which is the
alternative glucose metabolism pathway, is also elevated in cancer cells. The elevated
PPP provides 5-carbon sugars, which are materials for the synthesis of biomolecules,
and NADPH, which is able to alleviate oxidative stress in cancer cells (reviewed in Patra
and Hay, 2014). Importantly, my research and other studies in our lab regarding the
metabolic regulation of caspase-2 reveal another potentially important role of elevated PPP in tumorigenesis, which is suppressing the apoptosis of cancer cells by inhibiting caspase-2 activation. Our lab has previously shown that the G6PDH inhibitor, DHEA, is able to induce apoptosis. This induction of apoptosis can be rescued by supplementation with malate, which produces NADPH through an alternative pathway catalyzed by malic enzyme (Yang et al., 2010). Because of cytotoxicity, DHEA cannot be used as a potential drug for cancer therapy, however, it supports the possibility of developing G6PDH inhibitors with low toxicity as anti-cancer drugs.

A problem with targeting either glycolysis or the PPP in cancer therapy is that these metabolic pathways are also required for normal cells, although at a lower rate than in cancer cells, which means that these drugs might cause severe adverse effects. Therefore, it is important to examine the factors downstream of glucose metabolism for their potential pharmacological value. Interestingly, our data revealed the requirement of B55β/PP2A in the PPP-stimulated CaMKII activation, caspase-2 inhibition, and the downstream apoptosis suppression, suggesting that the inhibition of B55β/PP2A or CaMKII could induce apoptosis in cancer cells. Considering the ubiquitous expression of CaMKII in all types of cells and its diversified functions in mediating Ca^{2+} signals, CaMKII itself might not be a feasible target for cancer therapy. On the other hand, although PP2A dephosphorylates a large variety of substrates, B55β specifically leads
the PP2A holoenzyme to CaMKII in the presence of abundant nutrients, meaning that B55β could potentially be a specific target for cancer therapy.

To examine the possibility of targeting B55β in treating cancers, the expression level of B55β and the phosphorylation status of CaMKIIγ S395 (or the corresponding site for other CaMKII isoforms) in various types of cancer cell lines must first be assessed. If B55β plays a role in suppressing cancer cell apoptosis, we should be able to observe the overexpression of B55β, and constant dephosphorylation of S395 on CaMKIIγ, at least in certain types of cancer cells. Additionally, if B55β is overexpressed in cancer cells, we speculate that it may undergo certain mutations that strengthen the interaction between B55β and CaMKII. This would enable B55β to constantly dephosphorylate S395 on CaMKIIγ and suppress caspase-2 mediated apoptosis, even with insufficient nutrient supply. Caspase-2 has been reported to be important for inducing apoptosis of ovarian cancer cells, therefore, it is possible that B55β is overexpressed in ovarian cancer cells to assist the metabolic activation of CaMKII to inhibit caspase-2 and apoptosis (Yang et al., 2014). In addition to ovarian cancer, B55β could also be a valuable target for other types of cancers where caspase-2 has been shown to function as a tumor suppressor, including lymphoma and lung cancer (Ho et al., 2009; Terry et al., 2014).

If the importance of B55β in suppressing apoptosis of cancer cells can be proven in certain cancer types, inhibitors of B55β which are able to suppress the interaction
between B55β and CaMKII are worth investigating. The efficacy of inhibitors to suppress CaMKII activation, and the ability of inhibitors to induce apoptosis of cancer cells should be assessed. Hopefully an inhibitor of B55β with strong ability of inducing apoptosis but with high specificity against CaMKII activation will be discovered in the future study.

4.3 Concluding remarks

The previous data from our lab revealed the metabolic suppression of spontaneous apoptosis in the *Xenopus* egg extract system through an inhibitory phosphorylation on the caspase-2 prodomain mediated by activated CaMKII. Mainly using the *Xenopus* egg extract system, our data partially answered the un-answered question of how CaMKII activation is regulated by metabolism, which is the release of a “brake” on CaMKII activation through the dephosphorylation of T393/S395 (γ isoform numbering) mediated by B55β/PP2A, stimulated by metabolism. Additionally, by repeating some experiments in human cells, we discovered that the regulation of CaMKII activation by B55β/PP2A could be conserved through different species.

Our finding not only supplements the whole signaling pathway controlling the metabolic suppression of apoptosis, but also reveals a novel regulatory locus of CaMKII regulation, which is the dephosphorylation of two novel sites, T393 and S395. In addition, because both T393 and S395 are located in the association domain of CaMKIIγ,
our study potentially presents a novel function of the association domain to regulate CaMKII activation.

Furthermore, because of the ubiquitous expression of CaMKII in all cell types and its multiple functions, our discovery of this novel mechanism of CaMKII regulation may be applied to other CaMKII-mediated signaling pathways, not just metabolic suppression of apoptosis. Finally, considering the importance of B55β/PP2A in suppressing caspase-2 activation and apoptosis, B55β could be a potential target of cancer therapy. By inhibiting the interaction between B55β and CaMKII, apoptosis in certain types of cancers might be induced; this suggests that B55β inhibitors might be useful as a combinatory treatment along with standard chemotherapeutics.
References


proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* 15, 1573-1581.


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