REGULATION OF THE MEF-2 AND THE SMAD FAMILY OF FACTORS IN THE FREEZE TOLERANT WOOD FROG, *Rana sylvatica*

BY

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ABSTRACT

The wood frog, *Rana sylvatica* is a native North America capable of withstanding full body freezing when ambient temperatures drop below 0°C. During the freeze exposure, approximately 65-70% of the extracellular fluid gets converted into ice. Anoxia, ischaemia, osmotic and oxidative stress are some of the consequences which result from a freezing cycle. The dynamic nature of cells allows them to adapt to a wide array of stress conditions at different organizational levels. Transcription factors are key regulators of gene expression responsible for adaptation. In the present study, the MEF2 and SMAD family of transcription factors are demonstrated to have importance in the wood frog during freezing. The proteins were initially associated with developmental controls, however recent studies have found them to be involved in stress responses. Western blots were used to assess the expression and phosphorylation levels of MEF2A, MEF2C, SMAD1, SMAD2, SMAD3, and SMAD4 during torpor. It was generally found that MEF2A, MEF2C, and SMAD3 were post-translationally (phosphorylated) at Thr312, thr300, ser425 sites, respectively during 24h and 8h thawing. RT-PCR analysis of MEF2 and SMAD target genes (*calreticulin, glucose transporter-4, creatine kinase (brain and muscle)* and *serpine1, myostatin, tsc22d3*, respectively) revealed a modest up-regulation during 24h freezing in wood frog brain, heart, skeletal muscle, liver and kidney in selected transcripts. These results show that the two families of transcription factors are transcriptionally active during freezing, which comes as no surprise given the signals which regulate these proteins as well as the functions of the genes they activate.

ACKNOWLEDGEMENTS

Firstly, I would like to take this opportunity to formally thank my supervisor for the last 3 years, Dr. Ken Storey who not only has taught me how to "kick ass and take names" in everything but especially in the field of Science, although I am certain that he will not take credit for my fishing abilities. I am also forever grateful to Jan Storey who has shown nothing but love and support throughout my time in the Storey lab. It is evident that behind every great man, there is a great woman.

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Last, but most certainly not least "La Familia." This a long list but I'll keep it short and sweet: my father, mother, Abuelita, Orlando, Juan, Moises & Megan; my adorable nephews Alejandrito & Xaviercito who everyday remind how beautiful life is and why we must all fight a better future. As well as all my family, and people all over the world who have, and continue to suffer hardship – this could easily be them writing this text if it were not for the opportunity.

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List of Abbreviations

aa	Amino acid
AP1	Activator protein 1
APS	Ammonium persulfate
ATF	Activated transcription factor
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Calr	Calreticulin
CaMK	Ca2+/calmodulin-dependent protein kinase
CaN	Calcineurin
cDNA	Complementary deoxyribonucleic acid
ChREBP	Carbohydrate-responsive element-binding protein
CKB	Creatine kinase brain isoform
CKM	Creatine kinase muscle isoform
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enzymatic chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FoxO	Forkhead box O subclass
GDF-8	Growth differentiator factor-8, also known as myostatin
GILZ	Glucocorticoid-induced leucine zipper
GLUT	Glucose transporter
HAT	Histone acetylase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase

I-SMAD	Inhibitor SMAD
kb	Kilobase
kDa	Kilo Daltons
MADS	MCM1, Agamous, Deficiens and Serum response factor
MAPK	Mitogen-activated protein kinase
MEF-2	Myocyte enhancer factor 2
MH1/MH2	Mad homology domain 1 and 2
miRNA	micro RNA
mRNA	Messenger RNA
MW	Molecular weight
NF-κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf-2	Nuclear respiratory factor 2
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
R-SMAD	Receptor-regulated SMAD
RT	Reverse transcriptase
SBE	Smad binding element
SDS	Sodium dodecyl sulfate (lauryl sulfate)
Ser	Serine
Serpin	serine protease inhibitor
SMAD	Mothers against decapentaplegic homolog
SUMO	Small ubiquitin-like modifier protein
TBST	Tris buffered saline with tween-20
STAT	Signal transduction and transcription

TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGF-β	Transforming growth factor β
Thr	Threonine
T_M	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TSC22D3	TGFβ-stimulated clone 22 family domain 3
UPR	unfolded protein response
UV	Ultraviolet

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CHAPTER 1

GENERAL INTRODUCTION

Animals in the wild are continually faced with an ever-changing environment. This environment brings a variety of challenges such as temperature changes, water and nutrient availability, oxygen fluctuations, UV radiation, and shelter alterations amongst others. Nature has equipped living creatures with the ability to survive and flourish in pretty much all the corners of our Earth. However, this ability comes with a price, that explains the distribution or life among the planet. This "price" is mostly evident in those organisms which can withstand the extremes conditions. The Canadian winter is considered an environmental struggle for animals which demands a series of biochemical adaptations from its inhabitants.

1.1 Adaptations to Extreme Environments

Animals that reside in the polar regions of the planet have developed multiple methods of surviving the winter. Warm-blooded animals have three main mechanisms: they either remain active, migrate to warmer regions, or hibernate. Cold-blooded organisms hibernate or deal with freezing by two means: freeze-avoidance or freeze-tolerance. Freeze-avoidance is mostly used by intertidal and inshore marine fish and invertebrates and by terrestrial arthropods and other invertebrates and is based mainly on greatly increasing the concentrations of antifreeze metabolites and proteins in their body fluids that allow them to suppress their freezing and/or supercooling points below the ambient environmental temperature (Storey & Storey, 2008). The alternative, freeze-tolerance, is the phenomenon where the organism allows ice crystal formation in extracellular spaces while maintaining a liquid state within individual cells. Freeze tolerance is used by many insects, some intertidal marine molluscs, and a few terrestrially-hibernating amphibians and reptiles (Storey & Storey, 2004b).

For the vast majority of animals, freezing is damaging and/or lethal. The reasons for this are the multiple stresses which arise during tissue freezing. The challenges that face organisms during freezing include (i) physical damage to delicate tissues (especially blood vessels) caused by ice crystal formation, (ii) extreme cellular dehydration due to water outflow from cells into accumulating extracellular ice masses, (iii) ischemia due to plasma freezing that cuts off the supply of oxygen and nutrients to tissues and halts the removal of wastes and the intertissue transmission of blood-borne signals, (iv) subzero temperatures, and (v) the halting of all muscle-based physiological processes (e.g. movement, breathing, heart beat, etc). The problems are not limited just to freezing; thawing also requires controls including (iv) mechanisms to deal with reactive oxygen species (ROS) formation due to the sudden reperfusion of oxygenated blood, (v) re-uptake of extracellular water so as to not damage cells, and (vi) a need to coordinate the reactivated of vital signs after thawing Thus, it is evident why the freeze-tolerant phenotype is rare.

1.2 Freeze-Tolerant Model Organism

The freeze-tolerant wood frog, *Rana sylvatica* (also known as *Lithobates sylvaticus*) and is the primary model for studies on vertebrate freeze-tolerance. The geographic range of the wood frog extends from Alaska through Canada to the Great Lakes and up to Labrador (Fig. 1.1) (Behler & King, 1979). This distribution is basically across the northern forests, ending at the tree line, and subjects the frogs, that winter on the forest floor, to some of the harshest winter conditions the North has to offer. This suggests that the wood frog has highly developed adaptations to endure freezing in contrast to other species whose exposures are not as severe. Our laboratory has been

working extensively to discover the key behavioural, physiological and biochemical processes which aid vertebrate freeze-tolerance.

1.3 Physiology and Biochemistry of the Freeze-Tolerant Wood Frog

The wood frog has an impressive array of defenses to combat the damage that could be caused by freezing. One of the key mechanisms that aid survival is the control of ice formation. The frogs do this by limiting their ability to supercool and instead actively instigate ice nucleation at high subzero temperatures so that ice formation occurs at a slow rate that provides lots of time for the animals to make metabolic adjustments. Freezing can occur whenever the body temperature of the frog is below the freezing point of its body fluids (-0.5°C). Frogs can supercool somewhat but typically start freezing before their body temperature reaches -3°C. Contact with environmental ice or the formation of ice crystals on the skin surface typically triggers the nucleation of body fluids and sets off the propagation of ice through the body interior (Storey & Storey, 2004b). Ice nucleating bacteria found in the gut and on the skin surface are frequently involved in triggering ice formation. Wood frog plasma also contains what appear to be ice nucleating proteins that may play a role in mediating ice growth through the fluids spaces of the frog. Once freezing begins, vital signs such as muscle movement, heartbeat, breathing, and brain activity slowly diminish and stop.

Cellular dehydration is a fatal consequence of freezing which must be dealt with to ensure survival. While frozen, wood frogs face water loss through two means: (i) the whole body loses water due to evaporation across the skin, and ii) cells dehydrate as a consequence of water loss into extracellular ice masses. Defense against evaporative

water loss is mainly overcome by selecting a relatively humid covered hibernaculum under the leaf litter of the forest floor. Wood frogs can endure the conversion of about 65% of total body into ice (Lee *et al.*, 1992) and to limit further cellular dehydration, the frogs synthesize high levels of cryoprotectant, chiefly the blood sugar glucose (Storey & Storey, 1986). High glucose inside cells limits the amount of water that can be lost from them by the colligative resistance of high concentrations of dissolved molecules in the cytoplasm (Fig. 1.2) (Storey & Storey, 2004; Storey & Storey, 1986). When freezing initiates, glucose is produced from glycogen stores found in the liver, and is delivered to all the organs through the circulation. Blood glucose levels can reach as high as 300 mM when frozen in contrast to 5 mM when unfrozen (Storey & Storey, 1986). Glucose however is not the only player involved; urea has recently been discovered to play a role as an osmoprotectant and cryoprotectant during freezing in the wood frog (Constanzo & Lee, 2005).

In order to survive long term freezing exposures, wood frogs also undergo metabolic suppression to conserve fuel and energy reserves. Part of energy conservation comes from the cessation of body physiological activities. However, many intracellular enzymes, pathways, and functions are also strongly suppressed. A study on wood frog metabolic enzymes showed a freeze-induced reduction in the activities of 75% of the enzymes investigated (Cowan and Storey, 2001). Nevertheless, despite a strong overall reduction in the rates of transcription and translation during freezing, there is also evidence that there is differential gene expression during freezing (Storey *et al.*, 1997; Storey & Storey, 2004). These include several novel genes that have been discovered (*li16*, *fr10* and *fr47*) but whose function in freeze tolerance is not yet known (McNally *et*

al., 2002; McNally *et al.*, 2003; Cai & Storey, 1997a; Cai & Storey, 1997b). The molecular mechanisms and signal transduction pathways that mediate freeze-responsive gene expression and/or freeze-induced metabolic rate depression are in the process of being elucidated. The target transcription factors which are activated are also largely unknown. This is where my research is guided, at studying selected transcription factors in response to freeze exposure in the wood frog.

1.4 Selected Transcription Factors and Objective

My thesis is built on findings from previous preliminary work using commercially available scanning tools where whole transcriptomes were analyzed using array technology. This included results from cDNA array (gene chip) screening (University of Toronto MicroArray centre, http://www.uhnres.utoronto.ca/facilities/microarray.htm), and transcription factor screening using the Panomics TranSignal TF Protein array (http://panomics.com/) and the Marligen multiplex transcription factor assay (http://www.marligen.com/multiplex-testing-services.html). Gene chips were purchased from the University of Toronto and screening involved comparison of tissues from control and 4 h frozen *R. sylvatica*, according to the U of T protocol. Samples were also run on the Panomics and Marligen transcription factors assays that measured freeze-responsive changes in over 200 transcriptional regulators. The Panomics and Marligen identified a number of highly up-regulated transcription factors in the frozen wood frog. The MEF2 and SMAD3/4 transcription factors were included in this list. The 19K gene chip also indicated that several MEF2 and SMAD regulated genes were up-regulated in response to freezing in the wood frog.

The use of data from gene chips and arrays cannot be considered quantitative or definitive because these tools are designed for specific genomes of model organisms (e.g. human, rat, mouse, *Xenopus*, *Drosophila*, *Caenorhabditis elegans*, Zebrafish). Hence, heterologous screening with wood frog tissue extracts can lead to both false positive and false negative results in some cases although the lab's experience to date has been highly positive when other technologies have been applied to confirm gene chip results. Therefore, the insights gained from gene screening led to the design of the current project and led to the exploration of the responses of two transcription factor pathways and the downstream gene that they regulate to uncover the novel contributions that they make to freezing survival.

The present thesis explores the roles of MEF2 (Chapter 3) and SMAD (Chapter 4) transcription factors, that have previously been linked primarily with the regulation of developmental mechanisms (Potthoff & Olson, 2007; Black & Olson, 1998; Ross & Hill, 2007; Massagué *et al.*, 2005). My studies show that they also have stress activated responses. The research described in the following chapters verifies the leads generated from cDNA arrays and transcription factor array screening, explores two specific transcription factor pathways, and provides a much clearer vision of how transcription is regulated in the wood frog during freeze exposure.

Figure 1.1: Distribution of the wood frog, *Rana sylvatica* in North America. The geographic range of the wood frog extends from Alaska through Canada to the Great Lakes and up to Labrador

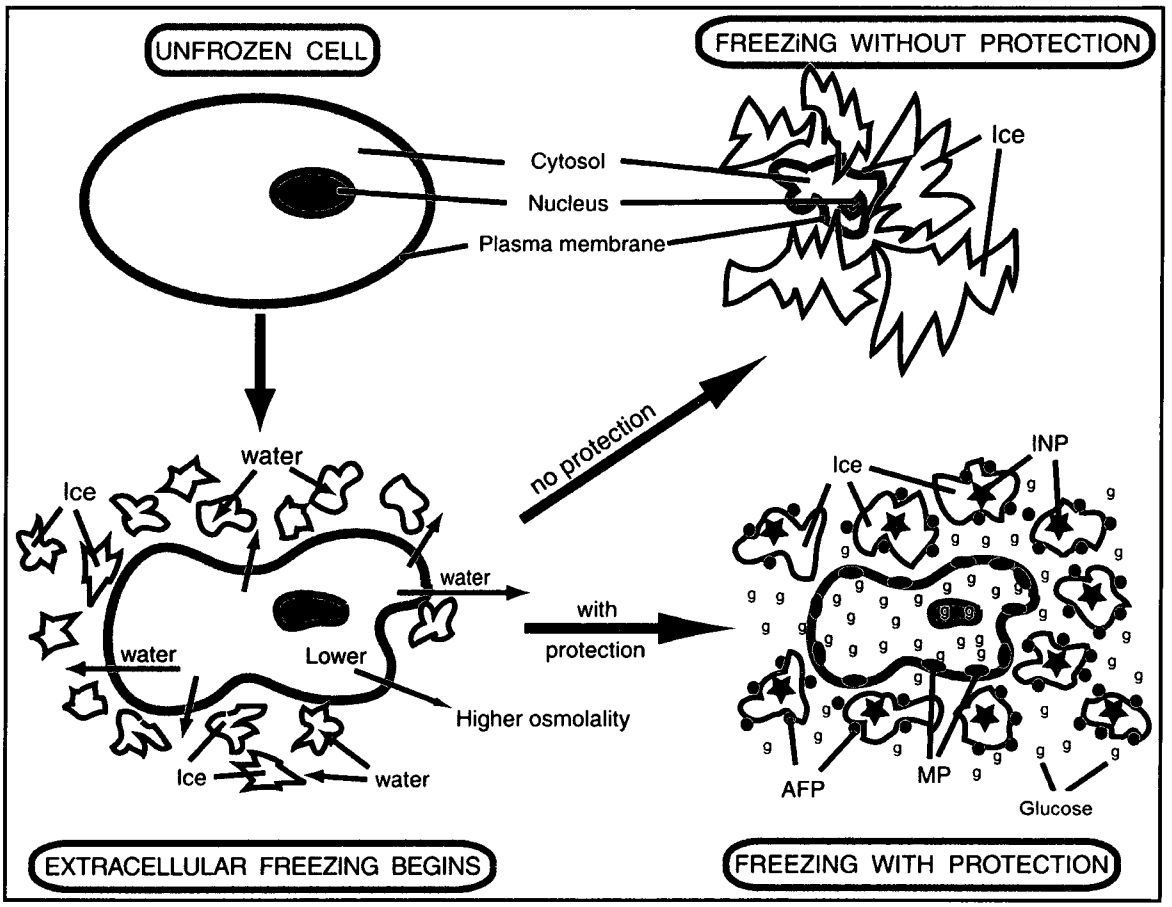
Copied from the US Environmental Protection Agency EcoRisk profile of the wood frog:

http://www.epa.gov/region01/ge/thesite/restofriver/reports/final_era/B%20-%20Focus%20Species%20Profiles/EcoRiskProfile_wood_frog.pdf

Figure 1.1

Figure 1.2: Cellular responses to freezing. Extracellular ice formation leads to severe dehydration and osmotic stress, which is fatal for unprotected cells. Freeze-tolerant cells have a variety of survival tactics including increasing their colligative properties with the use of small molecular weight molecules, glucose (g) in *R. sylvatica*, and ice-nucleating proteins (INP) which promote controlled ice formation.

Figure 1.2


UNFROZEN CELL
FREEZiNG WITHOUT PROTECTION
Cytosol
Nucleus
Plasma membrane
Ice
no protection
water
Ice
water
water
Lower
Higher osmolality
Ice
water
with
protection
Ice
INP
AFP
MP
Glucose
EXTRACELLULAR FREEZING BEGINS
FREEZING WITH PROTECTION

CHAPTER 2

EXPERIMENTAL PROTOCOLS

2.1 Animal Experiments

Male wood frogs, *Rana sylvatica* (5-7 g body mass), were collected from Ottawa area breeding ponds during the spring of 2007. The frogs were washed in a tetracycline bath and held in plastic boxes containing damp S*phagnum* moss at 5°C for 1 to 2 weeks prior to use. Frogs were then separated randomly into control and experimental groups. Control frogs were sampled directly from the 5°C acclimated condition. For freeze exposure experiments, wood frogs were transferred in plastic containers that were lined with dampened paper towels, closed, and placed in an incubator at a temperature of -4°C. Frogs cooled and when body temperature fell below about -0.5°C, ice formation was seeded by contact with ice formed on the paper. All frogs were nucleated within 45 minutes as shown from initial trials using wood frogs with thermistors taped onto their abdomens (Storey and Storey, 1985). Following the initial 45 minutes, incubator temperature was raised to -2.5°C and the length of freezing was timed from this point. Some frogs were sampled after 4 h or 24 h freezing exposure. Other frogs were frozen for 24 h and then transferred back to 5°C and sampled after 8 h of thawing. All frogs were sacrificed by pithing and each was dissected within 30 to 90 seconds with the help of 4-6 people. Tissues were flash frozen in liquid nitrogen and stored at -80°C until use. Tissues sampled were brain, heart, liver, kidney, and hind leg thigh skeletal muscle.

Protocols for the care, experimentation and euthanasia of the animals were approved by the Carleton University Animal Care Committee in accordance with the guidelines put forth by the Canadian Council on Animal Care.

2.2 Western Blotting

Total Protein Isolation

Soluble protein extracts were prepared from tissue samples of control, and frozen (4h and 24h). For cell free extracts, approximately 500 mg of frozen tissue was weighed and ground up using a mortar and pestle under liquid nitrogen, transferred to a 12 ml falcon tube and then quickly homogenized using a Polytron homogenizer in 1 ml of homogenization buffer (20 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 0 mM β-glycerophosphate and 1%v/v Triton X-100); 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) was added immediately prior to homogenizing. Samples were centrifuged for 15 min at 10,000 x g at 4°C and supernatants were transferred to clean tubes. Soluble protein concentrations were measured by the Coomassie blue dye-binding method and then protein concentrations were normalized to a set value by adding small amounts of homogenization buffer, and samples were then prepared for SDS-PAGE as explained below. This was done for four independent samples of each experimental treatment.

Cytoplasmic and Nuclear Protein Extractions

Nuclear extracts were made using a modified version of the method described by Dignam *et al.* (1983). Aliquots of approximately 500 mg frozen tissue were weighed, lightly ground using a mortar and pestle under liquid nitrogen, and transferred to a 12 ml falcon tube. A 500 µl aliquot of homogenization buffer was added [10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 10 mM DTT and 10 µl of protease inhibitor cocktail (Bioshop)] and tissues were then independently disrupted using a Dounce homogenizer.

Samples were centrifuged for 10 min at 8000 x g and 4°C. The supernatant was collected in a clean tube giving the Cytoplasmic extract. The pelleted nuclei were washed using homogenization buffer, resuspended in 150 µl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10% v/v glycerol, 10 mM DTT, and 1.5 µl of protease inhibitor cocktail) and then placed on a rocker at 4°C for 1 h at a slow speed. Samples were then centrifuged at 10,000 x g for 10 min and the supernatant (nuclear extract) was transferred to clean 1.5 ml tubes. Due to tissue availability, this procedure was done only for skeletal muscle and liver tissue with four independent samples from control and experimental frogs. Protein concentrations for both cytoplasmic and nuclear extracts were measured using the Bio-Rad assay. Protein concentrations were then normalized, and prepared for SDS-PAGE as explained below. Since the extracts were prepared to study the migration of transcription factors into the nucleus, it was crucial that the efficiency of the extraction be determined. This was accomplished by running samples on a SDS-PAGE gel, followed by Western blotting using a rabbit anti-histone H3 (Cell Signalling) at a dilution of 1:1000, followed by anti-rabbit secondary antibody (Bioshop Canada) for 1.5 h at 1:8000 dilution. Bands for histone H3 were found only in the nuclear extracts and not in cytoplasmic extracts, thus confirming the success of the isolation (data not shown).

Protein Concentration and Sample Preparation

The Coomassie blue dye binding method was used to determine protein concentration using the Bio-Rad prepared reagent with bovine serum albumin as a standard (BioRad, Hercules, CA). The reagent was diluted 5-fold and typically protein extracts were diluted 40-fold in distilled deionized water before assay. The assay

typically consisted of 10 µl of diluted protein and 190 µl of diluted Bio-Rad reagent in a microplate wells with each sample done in triplicate. Color development occurred over 10 min and then samples were read at 595 nm using a microplate reader. Protein concentration was then calculated based on a standard curve.

Sample concentrations were then normalized in order to minimize the variance in sample loading; this was done by adding calculated small amounts of the appropriate buffer. Samples were then mixed 1:1 v/v with 2X SDS-PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.4% w/v bromophenol blue) with 10% v/v fresh β-mercaptoethanol added. Samples were then boiled for 5 min and stored at -20°C. The final concentration in samples for standard Western blotting was typically 5µg/µl.

SDS Polyacrylamide Gel Electrophoresis and Immunoblotting

SDS resolving gels (8-12% acrylamide, 400 mM Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.04% v/v TEMED) were prepared with 5% upper stacking gels (5% acrylamide, 130 mM Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED). For each gel, an equal amount of soluble protein (ranging from 10-30 µg depending on the protein to be detected) was loaded into each well and then proteins were separated electrophoretically in SDS-PAGE running buffer (25 mM Tris-base, 190 mM glycine, 0.1%w/v SDS) at 180 V for 45 min. In order to estimate the size of the proteins, an aliquot of Kaleidoscope prestained molecular mass ladder (Fermentas) was loaded in one well. After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore) by wet transfer with pre-chilled transfer solution (25 mM

Tris pH 8.5, 192 mM glycine, and 20%v/v methanol) at 4°C for 1.5 h at 160 mA. The blots were then blocked with 2.5-5%w/v non-fat dried milk dissolved in Tris buffered saline containing Tween-20 (TBST: 20 mM Tris base, 140 mM NaCl, 0.05% v/v Tween-20), followed by an overnight incubation at 4°C with primary antibody diluted in TBST and small amount of sodium azide added; the blocking and antibody dilutions differed for each antibody (see as detailed in each chapter). Membranes were then washed multiple times with TBST and distilled/deionized water, and incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Bioshop Canada) for 1.5 h at a 1:8000 v/v dilution in TBST. Membranes were again washed with TBST and signal was detected using enzymatic chemiluminescence (ECL); 1 ml of a 1:1 v/v substrate solutions (hydrogen peroxide and luminol reagent) were first thoroughly mixed and then added to the membrane to initiate exposure. Chemiluminesence was detected using the Chemi-Genius Bioimaging system (Syngene, MD, USA) and band densities were analyzed using the associated Gene Tools software. Membranes were then stained for 10 min with Coomassie blue (0.25% w:v Coomassie Brilliant Blue R in 50% v:v methanol, 7.5% v:v acetic acid) and then destained for ~15 min with distaining mix (60% v/v methanol, 20% v/v acetic acid in ddH_2O.

2.3 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

General Precautions

All plastic including bottles, tubes and tips were autoclaved prior to use and gloves were worn at all times during operations involving nucleic acid manipulation. For RNA preparation and manipulation, tips and tubes were also treated with 0.1% v/v

diethylpyrocarbonate (DEPC) in double distilled water in order to inactivate RNAses that may have been present. Treated water and tips/tubes were then autoclaved to eliminate any lingering DEPC. This DEPC-ddH_2O was used for any RNA/DNA work.

Total RNA Isolation

All materials and solutions were treated with 0.1% v/v diethylpyrocarbonate (DEPC) and autoclaved prior to use. Total RNA was isolated from tissues using Trizol® reagent (Invitrogen), following manufacturer's instructions. Briefly, about 100 mg of tissue was homogenized in 1.5 mL Trizol using a Polytron homogenizer followed by addition of 300 µL of chloroform. The samples were then centrifuged at 10,000 x g for 15 minutes at 4 °C. The upper aqueous phase which contained total RNA was removed to a fresh tube containing 2-propanol (750 µL) allowing RNA to precipitate by incubation at room temperature for 10 min. The samples were centrifuged at 12,000 x g for 15 min at 4 °C and the RNA pellet was washed with 1 mL of 70% ethanol, followed by a second centrifugation at 12,000 x g for 5 min. The supernatant was removed and samples were allowed to air dry (about 10 min). The pellet was resuspended in about 30 µL of DEPC treated water. Four individual tissue samples were extracted from each experimental group. RNA concentration was determined by measuring the absorbances at 260/280 nm and quality was assessed by separating 2 µg of total RNA from each sample on a 1% agarose gel with ethidium bromide (EtBr) staining; the appearance of two sharp bands corresponding to the 28S and 18S ribosomal RNA (rRNA) indicated good quality RNA. Once the RNA concentrations and purities were assessed, normalization of RNA concentrations was done typically by diluting the samples to a final concentration of 1 µg/ µl or 0.5 µg/ µl.

First Strand cDNA synthesis and PCR amplification

Complementary DNA (cDNA) was synthesized from the total RNA samples. Approximately 15 µg of total RNA was diluted with DEPC treated water to a final volume of 10 µL. To each sample was added 1 µL of 200 ng/µL oligo-dT (5'-TTTTTTTTTTTTTTTTTTTTTTV-3'; V= A, G, or C) (Sigma Genosys) which forms a hybrid with the poly A tail of mRNA; this was incubated at 65°C in a water bath for 5 min. The samples were then immediately placed on ice and 4 µL 5X first strand buffer, 2 µL 10 mM DTT, 1 µL 10 mM dNTPs, and 1 µL reverse transcriptase enzyme Superscript II (all reagents from Invitrogen) were added; final volume was 19 µL. The mixture was then placed in a water bath at 42°C for 45 min and then transferred to 4°C. The resulting cDNA samples were serially diluted (10^{-1} and 10^{-2}), and all samples were kept at -80°C until used for PCR amplifications.

The Polymerase Chain Reaction (PCR) was used to amplify selected genes from the cDNA samples. A 25 µL PCR reaction typically consisted of 15 µL of DEPC-treated H_2O, 5 µL of cDNA sample dilution, 0.5 µL 10X PCR buffer (Homemade), 1.75 µL 50 mM $MgCl_2$ (Invitrogen), 1.25 µL of gene specific primer dilution (0.5 µM forward and 0.5 µM reverse primers, designed and ordered from Sigma Genosys – see below), 0.5 µL 10 mM dNTPs, and 1 µL of *Taq* Polymerase (Invitrogen). The PCR protocol started with an initial step of 2 min at 94°C, followed by 37 cycles of 94°C for 45 sec, optimal annealing temperature for 45 sec, and 72°C for 45 sec; the final step was 72°C for 4 min. This protocol was subject to optimization depending on the primers used and the size of the PCR product.

PCR products were electrophoretically separated on a 1-1.5%w/v agarose gel and stained with 0.01% v/v EtBr. The gel preparation consisted of adding 1.5 g of agarose (BioShop) to 150 mL of 1X TAE buffer made by adding 6 mL of 50X TAE buffer (242 g Tris base, 57.1 mL concentrated acetic acid, 100 mL of 0.5 M EDTA in 1 L water, adjusted to pH 8.5) to 294 mL of DEPC-treated water. The solution was then heated in a microwave and allowed to cool to room temperature. While the solution was fairly warm, 3 μL of EtBr (0.3 mg/300 mL) was added prior to pouring the solution onto a gel casting apparatus, where it was allowed to solidify. A 9 μL aliquot of PCR product was mixed with 1 μL of DNA loading buffer (0.25%w/v xylene cyanol FF, 30%v/v glycerol in ddH_2O) and electrophoresis was conducted using 1X TAE buffer. Quantification of the band intensity on the gel was performed using a ChemiGenius Bio Imaging System and the associated GeneTools software (SynGene, MD, USA). Band intensities were quantified and then normalized against the corresponding intensity of the *α-tubulin* band amplified as a control from the same cDNA sample. Gene specific primers were designed as described below and the optimal PCR conditions are stated in the appropriate chapters.

Primer Design for unknown mRNA sequences

Primer design was accomplished by first finding the mRNA sequence of the target gene from *Xenopus laevis* (African clawed frog) or *Silurana (Xenopus) tropicalis* (tropical clawed frog) from the NCBI database (www.ncbi.nlm.nih.gov). This sequence was then blasted using the nucleotide blast tool [Basic Local Alignment Search Tool (BLAST), http://blast.ncbi.nlm.nih.gov/Blast.cgi]. Sequences from different organisms were obtained and loaded using the bioinformatics software DNAMAN (Lynnon BioSoft) and the sequences were aligned. The selected sequences typically consisted of

anuran, avian, mammalian and fish sequences. The original *X. laevis/tropicalis* sequence was then loaded into Primer Designer (Scientific and Educational Software) software where primers were selected according to predicted physical properties along with homology analysis based on the DNAMAN alignment. Appropriate wobble base pairs were inserted accordingly and a final step of filtering was done based on physical properties and heterozygous dimerization as determined by IDT OligoAnalyzer (http://www.idtdna.com/analyzer/applications/oligoanalyzer). The designed primers were then ordered from Sigma-Genosys, diluted to PCR concentrations (5 μM), tested using RT-PCR, and the PCR products were run on agarose gel electrophoresis. The amplifications which gave expected PCR product sizes were physically cut out of gel and sent for sequencing to confirm that the amplified PCR product was indeed the target gene transcript.

2.4 Normalization and Statistical Analysis

Quantification of the band intensity of both Western blots and RT-PCR gels were performed using a ChemiGenius Bio Imaging System and the associated GeneTools software (SynGene, MD, USA). For Western blots, band intensities from chemiluminesce were quantified and normalized against the Coomassie brilliant blue stained band in the same lane to correct any minor variations in sample loading; the Coomassie stained band chosen was constant in intensity across all sample and was well separated from the area of the membrane containing the immunoreactive protein. For RT-PCR, band intensities were quantified and then normalized against the corresponding intensity of the *α-tubulin* band amplified as a control from the same cDNA sample.

All values were reported as mean ± S.E.M. Independent sample sizes were 3-5 for protein Western blotting and 4-8 for RT-PCR experiments. For each data set, the control group was normalized to 1. The means were analyzed by unpaired Student's t-test or one-way ANOVA, with Student-Newman-Keuls post hoc testing. Values of $P<0.05$ were accepted as being significantly different, symbolized as ★ on graphs.

CHAPTER 3

REGULATION OF THE MYOCYTE ENHANCING FACTOR-2 (MEF2) TRANSCRIPTION FACTORS IN THE WOOD FROG UNDER FREEZING EXPOSURE

3.1 INTRODUCTION

In order to endure the harsh winter months, many animals in the wild display a combination of adaptations that include behavioural, physiological, and metabolic adjustments. Freeze tolerance is one of these adaptations of winter survival, and is the subject of the present study using the freeze-tolerant wood frog, *Rana sylvatica*, as a model organism. Some of the metabolic adaptations displayed by wood frogs are achieved by strict transcriptional regulation of gene expression, which regulates the population of the transcriptome. The transcriptional machinery is composed of many proteins with distinct as well as overlapping functions. Key players in transcription are, as their names suggest, transcription factors. The roles of these proteins in response to freezing have begun to be elucidated and the present study focuses on the Myocyte Enhancer Factor 2 (MEF2) family of transcription factors.

MEF2 Family of Transcription factors

The MEF2 transcription factors are evolutionarily conserved proteins that belong to the MADS-box family. The MEF2 proteins consists of four members in vertebrates: MEF2A, MEF2B, MEF2C, and MEF2D (Black & Olson, 1997). These members are nearly identical in their N-termini which contain the two DNA-binding domains, MADS and MEF2. The MADS and MEF2 domains are the highly conserved portions of the proteins with >90% and >70% identity amongst vertebrates, respectively. The MADS domain (MCM1, Agamous, Deficiens and Serum response factor) of the transcription factor binds to A/T rich DNA with a $CTA(A/T)_4TAG/A$ sequence upon dimerization. The adjacent 29 amino acid extension known as the MEF2 domain is essential for DNA-binding affinity and co-factor interactions (Potthoff & Olson, 2007). The remaining C-

terminal region which is approximately 420 amino acid long is known as the transactivation domain and is where the nuclear localization sequence (NLS) as well as the regulatory sites are located. The transactivation domain contains many sites that are subject to post-translational modifications such as by phosphorylation, acetylation, or SUMOylation (Ma *et al.*, 2005; Grégoire *et al.*, 2006). Initially, these proteins were thought to be muscle-specific but later studies revealed that they are expressed in a wide range of cell types (Dodou *et al.*, 1995; Ornatsky *et al*, 1996).

Regulation of MEF2 Transcription factors

A large amount of data shows that MEF2 molecules are regulated by a variety of signals (see Figure 3.1). Two branches of the mitogen-activated protein kinase (MAPK) family are known to phosphorylate MEF2; these are the p38 and ERK5 MAPKs (Zhao *et al.*, 1999; Kato *et al.*, 2000). Upon phosphorylation, MEF2 undergoes transcriptional activation and recruits the machinery to transcribe its designated genes, which depend on tissue type as well as co-factors present (Potthoff & Olson, 2007). Calcium signalling is also a potent regulator of MEF2 proteins. In response to Ca^{2+} signals, MEF2 can be regulated through a variety of divergent and convergent signals typically through calmodulin. The reason for this is that calmodulin activates both calmodulin-dependent protein kinase (CaMK) and Calcineurin (CaN), which are proteins that have been shown to regulate MEF2 transcription factors directly, and in-directly through affiliated co-factors (McKinsey *et al.*, 2002).

Functions of MEF2 Transcription factors

MEF2 proteins are known to respond to a variety of signals, both extra- and intra-cellular. Initial studies revealed MEF2 transcription factors as key regulators of muscle differentiation and remodelling (Black & Olson, 1997). Further studies found that they are key regulators in apoptotic as well as anti-apoptotic processes in both lymphocytes and neurons (Potthoff & Olson, 2007). To date, MEF2 proteins have implications in a variety of cellular mechanisms ranging from vascular integrity, bone development, to tissue energetics and metabolism (Chang *et al.*, 2006; Arnold *et al.*, 2007; McGee *et al.*, 2006). Recently, implications in response physiologic and cellular stress have been investigated. MEF2 proteins also have important roles in cardiac hypertrophy, particularly via Ca^{2+} dependent signalling (Czubert & Olson, 2004), as well as other less documented stresses including oxidative and osmotic stresses (Al-Khalili *et al.*, 2004).

The present study focuses on the regulation of MEF2 transcription factors in response to freeze exposure in the wood frog, *R. sylvatica*. Preliminary studies in our lab have revealed that there is reason to suspect that MEF2 is transcriptionally active in the wood frog during freezing. The current findings provide evidence of that indeed the MEF2A and MEF2C transcription factors are regulated in response to freezing, and the signals are delivered efficiently to the nucleus, where they activate transcription of selected target genes. Selected downstream target genes were also studied including *glut-4*, which codes for the glucose transporter 4 (the insulin-dependent isoform), *calreticulin*, a Ca^{2+}/ER-chaperone, and *ckb* and *ckm*, which code for the brain and muscle isozymes of creatine kinase, respectively. All of these are known to be transcriptionally regulated by

MEF2 in other systems (Lui *et al*, 1994; Lynch *et al*, 2005; Ferrari *et al*, 1997; Shen *et al*, 2002).

3.2. MATERIALS AND METHODS

Animals and tissue collection

Male wood frogs were treated, sacrificed, and tissue collected as previously described in Chapter 2.

Protein Isolation and Western Blotting

Total protein as well as cytoplasmic and nuclear protein extracts were isolated and treated as previously described in Chapter 2. Samples were run on SDS-PAGE and transferred onto a PVDF membrane. Table 3.1 below is a summary of the probing conditions used for detecting MEF2 proteins.

Table 3.1. Summary of MEF-2 antibody experimental conditions used in western blots.

Primary Antibody	Size	Blocking Condition	Probing Conditions (dilution)	2° Antibody (dilution)	Source of Primary Antibody
Rabbit anti-MEF2A	~ 50 kDa	2.5% w/v milk for 10 min	Overnight Incubation (1: 1000)	Anti-Rabbit IgG-HRP (1:8000)	GenScript Corp.
Rabbit anti-phospho-MEF2A^{Thr312}	~ 50 kDa	2.5% w/v milk for 10 min	Overnight Incubation (1: 1000)	Anti-Rabbit IgG-HRP (1:8000)	GenScript Corp.
Goat anti-MEF2C	~ 43 kDa	2.5% w/v milk for 10 min	Overnight Incubation (1: 1000)	Anti-Goat IgG-HRP (1:8000)	SantaCruz Biotechnology
Rabbit anti-phospho-MEF2C^{Thr300}	~ 43 kDa	2.5% w/v milk for 10 min	Overnight Incubation (1: 1000)	Anti-Rabbit IgG-HRP (1:8000)	SantaCruz Biotechnology

RNA Isolation and RT-PCR

Total RNA was isolated and used to synthesize cDNA as previously described in Chapter 2. Primers were designed, tested, and PCR products were sent for sequencing in order to confirm that the target genes were amplified. Relative RT-PCR was then conducted in order to determine expression levels of selected genes. The PCR primers for each wood frog gene, the cDNA fragment size amplified, and the optimal melting temperature (T_M) for each were as shown below in Table3.2.

Table 3.2. Optimal conditions of RT-PCR of MEF2 related genes

Gene	Primers	T_M	Size
α-tubulin	Forward 5'-AAGGAAGATGCTGCCAATAA-3' Reverse: 5'-GGTCACATTTCACCATCTG -3'	53°C	600 bp
mef2c	forward: 5'-GGTTTCCATTCCGGTGAGCA-3'; reverse: 5'-TCCTTTGATTCACAGAGGGC-3'	65°C	360 bp
mef2a	forward: 5'-AGGCTCTGACAGAAGGAACA-3' reverse: 5'-CACAGAGATGTCCGATGAAC-3'	65°C	360 bp
Ckm	forward: 5'–GGACTGGCCGYAGCATYAAG-3' reverse: 5'-AGTGTCMACACCACCTGTGC-3'	65°C	600 bp
Ckb	forward: 5'-GACAGRCAYGGYGGCTACAA-3' reverse: 5'-GTCTTRTTGTCATTGTGCCA-3'	65°C	370 bp
calreticulin	forward: 5'-GAYAACAGCAAGGTKGARTC-3' reverse: 5'–TTCTTGCGWTCCTCYTCATC-3'	60°C	525 bp
glut-4	forward: 5'-GASATGAAGGAGGAGAAGAG-3' reverse: 5'-GAAGTTGGMGGTCCAGTTGG-3'	60°C	500 bp

3.3. RESULTS

Protein Expression levels of MEF2 transcription factors

The effects of freezing on MEF2 protein expression and regulation was assessed in five tissues of the wood frog, brain, heart, muscle, liver and kidney. This was achieved by performing western blots using soluble protein extracts and detecting targets using protein specific antibodies. The results show a uniform pattern with slight variance depending upon tissue. The anti-human MEF2 antibodies cross-reacted with frog MEF2 proteins in all tissues studied in the wood frog. Both antibodies raised against total MEF2A protein and those raised against a peptide containing the phospho-threonine 312 residue on MEF2A detected a single band at ~50 kDa which is the expected molecular mass for MEF2A. Similarly, antibodies detecting both total MEF2C phospho-MEF2C Thr300 detected a band at ~43 kDa, again the expected size of MEF2C.

Effects of freeze exposure on MEF2A

Tissue specific changes were discovered with respect to both expression levels and phosphorylation levels of MEF2A. Brain samples from wood frog showed a 1.4-fold increase in MEF2A protein levels along with a 1.7-fold increase in phosphorylation at Thr312 during 24 h of freeze exposure (Figure 3.2A). Levels returned to control values after thawing. In heart, freezing had no effect on MEF2A protein levels but a 1.4-fold increase in phosphorylated MEF2A content was observed after 8 h of thawing recovery (Figure 3.2B). A 2-fold increase in phospho-MEF2A content was seen in skeletal muscle during freezing and was sustained during thawing; total MEF2A protein also rose by a 1.3-fold during thawing (Figure 3.3A). Liver showed elevated phospho-MEF2A during the 24 h freeze, but after thawing levels returned to control values (Figure 3.3B). MEF2A

protein levels in kidney showed a 1.4 fold increase during freezing that returned to control levels during recovery (Figure 3.4). The amount of phospho-MEF2A Thr312 also rose by 2.1-fold during freezing and remained high (1.55-fold over controls) during thawing.

Effects of freezing on MEF2C

Similarly to MEF2A, MEF2C was regulated differently in terms of expression levels and phosphorylation state. In brain there was no change in expression of MEF2C during freezing cycle; however a 1.8-fold increase in phosphorylated MEF2C Thr300 was seen after 8 h thawing (Figure 3.2A). In the heart, no changes in total MEF2C protein were found whereas phospho-MEF2C rose by 1.4-fold during freezing (Figure 3.2B). Skeletal muscle showed a strong 2-fold increase in phospho-MEF2C during freezing that fell again after thawing along with a 1.5-fold increase in total MEF2C protein during thawing (Figure 3.3A). In wood frog liver there was 1.8-fold increase in MEF2C levels during freezing that remained high (1.6-fold over controls) during thawing. Phosphorylated MEF2C in the liver also increased during freezing by 1.6-fold and then rose much higher to 3.6-fold over control values during thawing (Figure 3.3B). In the kidney there were no changes in MEF2C total protein over freeze/thaw but phospho-MEF2C rose by 3-fold during freeze exposure and remained elevated (1.8-fold) after thawing (Figure 3.4).

Transcriptional regulation of MEF2 Transcripts

Protein levels of MEF2A and MEF2C increased significantly in selected tissues in response to freeze/thaw (Figure 3.2-3.4). To determine if this was due to freeze-

responsive up-regulation of the *mef2* genes, transcript levels of *mef2a* and *mef2c* were next measured in ground squirrel tissues. Gene specific primers were designed and their efficacy was assessed using RT-PCR. Those PCR products which produced the correct expected sizes were sent for sequencing to confirm that the target gene was amplified. Once the sequences confirmed target gene amplification, relative RT-PCR was used to assess differential expression of *mef2* transcripts in brain, heart, skeletal muscle, liver, and kidney during 24 h freeze exposure.

Expression of *mef2a* was found in all tissues studied in the wood frog. Transcript levels of *mef2a* were unchanged in brain, skeletal muscle and kidney after 24 h freezing. However, both heart and liver showed significant increases in *mef2a* transcript levels; levels increased by 1.9- and 1.4-fold, respectively (Figure 3.5A).

Transcripts of *mef2c* were found in all tissues. Transcript levels remained constant over freeze/thaw in brain, muscle and kidney. However, heart showed a 64% decrease in *mef2c* transcript levels in 24 h frozen frogs whereas in wood frog liver, mRNA levels increased by 2.4 fold (Figure 3.5B).

Nuclear insights to MEF2 Transcription factors

Since gene up-regulation occurs in the nucleus, the relative amount of a transcription factor that is localized in the nucleus is a strong indicator of the transcriptional state of the genes under its control. Hence, the relative amounts of MEF2A and C were assessed in nuclear fractions from wood frog muscle and liver under three experimental conditions: control, 4 h freezing and 24 h freezing.

Figure 3.7 shows results for skeletal muscle. The total amount of MEF2A in the nucleus remained relatively constant over the freezing time course (Figure 3.6A). However, phospho-MEF2A Thr312 content rose strongly by 2.1-fold after 4 h freezing and remained high at 1.5-fold over control levels after 24 h freezing. The pattern of response by MEF2C was much the same. Total MEF2C protein content in the nucleus did not change during freezing but the relative amount of phospho-MEF2C Thr300 rose sharply to 1.7- and 2.1-fold higher than control values after short and long term freezing, respectively (Figure 3.6A).

MEF2A responses in liver nuclear extracts were similar results to those seen in skeletal muscle. Protein levels of MEF2A showed a slight decrease during 4 h of freezing (a 40% decrease) but they returned to control levels during 24 h freeze. However, phospho-MEF2A Thr312 nuclear content rose strongly during freezing, by 2-fold after 4 h of freezing and 1.7-fold after 24 h being frozen (Figure 3.6B). Nuclear MEF2C protein levels were constant for the first 4 h of freeze exposure and rose by 1.4-fold after 24 h. Again, the amount of phospho-MEF2C Thr300 rose over time during freezing, being 1.3- and 1.5-fold higher than controls at 4 and 24 h frozen, respectively (Figure 3.6B).

Transcriptional regulation of downstream genes of MEF2 Tfs

The strong increases in the relative amounts of phosphorylated active MEF2 transcription factors in the nucleus during freezing suggests freeze responsive up-regulation of target genes under MEF2 control. Hence, selected known gene targets of MEF2 were next assessed by quantifying mRNA transcript levels of four downstream genes that code for glucose transporter 4, calreticulin, and the muscle and brain isozymes of creatine kinase.

The *glucose transporter-4* (*glut-4*) mRNA was found to have a tissue selective expression profile. *Glut-4* presence was detected in heart, skeletal muscle, and kidney of wood frogs (Figure 3.7A). In heart, transcript levels rose 2.5-fold after 24 h freezing whereas in skeletal muscle the increase was 1.7-fold. Transcript levels in kidney did not change.

Transcripts of *calreticulin* were found in all wood frog tissues tested suggesting that it is ubiquitously expressed. Relative RT-PCR analysis of mRNA levels in control versus 24 h frozen frogs showed that *calreticulin* was up-regulated in all tissues with the exception of kidney (Figure 3.7B). The muscle tissues studied, heart and muscle, showed a 4.8- and 2.3-fold increases in transcript levels, respectively. *Calreticulin* mRNA levels also rose by 40% in brain and 60% in liver of frozen frogs.

Creatine kinase has two main cytosolic isozymes that were originally named for the homozygous dimers found in muscle (MM) and brain (BB). These are coded by the genes *ckm* and *ckb*, respectively. In terms of expression profiles, *ckm* transcripts were not detected in brain and kidney of wood frogs and liver did not express either *ckb* or *ckm.* Transcripts of *ckb* were detected in four tissues (Figure 3.8A), and only heart and skeletal muscle showed changes in mRNA levels with 1.7- and 1.6-fold increases during 24 h frozen, respectively. The muscle isomer, *ckm*, was up-regulated in both muscle tissues. Cardiac and skeletal muscle extracts respectively showed a 2.8- and 1.8-fold increases in *ckm* transcript levels during 24 h frozen (Figure 3.7b).

Nucleotide and Amino Acid Sequence Analysis

The RT-PCR products were sent for sequencing and the nucleotide sequences obtained were analyzed. Sequences were confirmed as the desired targets by using a nucleotide Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were translated using DNAMAN software and all three open reading frames (ORFs) were analyzed by using a protein BLAST, identifying the desired reading frame. The polypeptide sequences were also inserted into Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) search to identify any domains which could be recognized.

Analysis of mef2 sequences

The designed *mef2a* primers gave a PCR product which was confirmed as the *mef2a* nucleotide sequence. The nucleotide sequence is shown in Figure 3.9. The nucleotide sequence and translated amino acid sequence is shown in Figure 3.9. The obtained nucleotide sequence produced a 140 amino acids and this was identified as a portion of MEF2A spanning amino acids 160-300 (as compared with the *Xenopus* sequence) and representing about 27% of the 516 amino acids of the protein. A protein domain search failed to recognize any conserved domains from the polypeptide sequence. A homology tree was also constructed using the obtained wood frog polypeptide sequence, and aligning it with the sequence from the African clawed frog, mouse, human, chicken, and zebrafish (Figure 3.9). The wood frog was found to have 76% shared identity with the African clawed frog, and 61% with the remaining phyla (mammals, aves and fish).

The partial *mef2c* sequence contained 512 bp and was translated to produce 170 amino acids (Figure 3.10). These corresponded to a region ranging from position 125 to 300 of *Xenopus* MEF2C, and therefore contained about 39% of the 432 amino acids of the typical protein. No conserved domains were identified upon searching. Threonine 167 (underlined and in bold) of the frog partial sequence was identified as corresponding to the Thr300 phosphorylation site of the full length protein. A homology tree was also produced from protein alignments of MEF2C using sequences from the Western clawed frog, mouse, human, chicken, common carp, and zebrafish (Figure 3.10). The tree demonstrates that MEF2C from the wood frog shares 93% identity with the clawed frog, mammals and aves, and 81% with bony fish.

Analysis of downstream gene sequences

A wood frog partial *glut-4* mRNA sequence was cloned; 475 bp were retrieved and encoded 158 amino acid residues (Figure 3.11). This segment covered the region between amino acids 275 and 430 of GLUT-4, representing about 31% of the 509 amino acids in the protein. Domain analysis of the sequence showed a sugar transporter domain. The *glut-4* polypeptide sequences from the African clawed frog, human, rat, mouse, pig, Coho salmon and Atlantic cod were aligned and used to construct the homology tree shown in Figure 3.11. The analysis shows a 91%, 71%, and 70% shared identity between wood frog and the African clawed frog, mammals, and fish respectively.

The partial cDNA clone of *calreticulin* produced a 477 bp fragment which was successfully sequenced and identified. The nucleic acid sequence was used to produce a polypeptide sequence of 158 amino acids (Figure 3.12). From polypeptide alignments,

this sequence was confirmed to be the region from position 210 to 367 of the 411 amino acid protein; therefore 38% of the wood frog calreticulin sequence was identified. A domain analysis showed that the amino acid sequence belonged to the calreticulin family. The calreticulin mRNA sequences from the wood frog was aligned with those from the Japanese wrinkled frog, Western and African clawed frog, chicken, human, and zebrafish (Figure 3.12). The homology tree produced showed that the Japanese wrinkled frog had highest % shared identity at 93%, followed by the clawed frog at 85%, chicken and human at 79%, and zebrafish at 71%.

The brain creatine kinase PCR product contained a 597 bp sequence that was confirmed as being a partial transcript of *ckb*. The translated amino acid sequence produced 198 amino acids, about 52% of the full 376 amino acids of the CKB protein (Figure 3.13). The location of the fragment was between amino acids 48 and 245 of CKB. Protein homology analysis between the wood frog, clawed frogs, aves, mammals, and zebrafish showed that the wood frog shares 88% homology with the clawed frogs and 86% with the remaining organisms (Figure 3.13).

The wood frog muscle partial sequence also encoded about half of the creatine kinase M protein (Figure 3.14). The 534 bp coded for 177 amino acids that corresponded to the region spanning amino acid positions 148 to 325 of the full protein. This accounted for 46% of the 381 amino acids of the typical vertebrate protein. The polypeptide sequences from clawed frogs, chicken, human and zebrafish were compared with that of the wood frog. The wood frog was found to have 90% shared identity with the clawed frog, followed by human and chicken at 88%, and lastly zebrafish with a shared homology of 86% (Figure 3.14). Domain analysis of both brain and muscle creatine

kinase amino acid sequences confirmed the presence of an ATP:guanido phosphotransferase, C-terminal catalytic domain.

3.4. DISCUSSION

The present study demonstrates that the MEF2 Family of transcription factors is subject to regulation during freeze exposure in the wood frog. Most studies of MEF2 transcription factors to date have focused on their roles in development and differentiation in mammals (Naya & Olsen, 1999; Potthoff & Olson, 2007). Hence, the present study which focuses on freeze-tolerance in anurans represents a completely novel function for MEF2s. The data show ubiquitous expression of MEF2A and MEF2C in wood frog organs, agreeing with mammalian studies that found that MEF2 was not limited to muscle-specific cells (Dodou *et al.*, 1995; Ornatsky *et al.*, 1996). The study demonstrates organ-specific expression and regulation of MEF2 in response to freeze/thaw in five frog organs, as well as differential responses to freezing by genes known to be under MEF2 control.

MEF2 is primary regulated post-translationally during freeze exposure in the wood frog

In general, MEF2A and MEF2C protein levels either remained stable or increased in response to freezing, thawing, or both. MEF2A levels were elevated in brain and kidney of 24 h frozen frogs (Figures 3.2A & 3.4) and also in thawed muscle (Figure 3.3A). MEF2C levels were elevated in response to freezing in liver and thawing in liver and muscle (Figures 3.3A & 3.3B). Analysis of *mef2a* and *mef2c* transcript levels revealed few freeze-responsive changes (Figures 3.5A & 3.5B). Heart and liver showed 2- and 1.4-fold increases in *mef2a* transcript levels during freezing and *mef2c* mRNA

levels rose by 2.4-fold in liver. This latter result for *mef2c* transcripts correlates with the rise in MEF2C protein in liver during freezing, providing evidence of freeze-responsive transcriptional control of MEF2C levels in this organ. However, in general, the data do not indicate widespread modification of MEF2 mRNA or protein levels in response to freeze/thaw and indicate instead that regulation is done by other means, most likely by post-translational modification.

Studies on other systems have shown that MEF2 transcription factors are subject to regulation via reversible phosphorylation (Black & Olsen, 1998) and the current data agree with this for frog organs. As can be seen from the generally phosphorylated states of MEF2 threonine residues, this appears to be a key method of regulating these proteins during freeze/thaw in the wood frog. For example, levels of MEF2A^{Thr312} increased by 70% in brain after 24 h frozen and similar changes were observed in muscle, liver and kidney (2-, 1.7, 2.14-fold increases, respectively). MEF2C ^{Thr300} phosphorylation state also increased in all tissues during freezing and/or thawing. Except for brain, all tissues showed at least a 1.4-fold increase in phospho-MEF2C^{Thr300} content in 24 h frozen frogs and MEF2C phosphorylation state was also high during thawing in brain, liver and kidney. MEF2 is known to be phosphorylated by p38 or ERK5/BMK1 (Zhao *et al.*, 1999; Kato *et al.*, 1997; Kato *et al.*, 2000) and, therefore, it is possible that these MAPKs also control the freeze-responsive phosphorylation of MEF2C in response to wood frog freeze/thaw.

The subcellular localization of MEF2 proteins is still not clear. Some studies have shown that MEF2 localizes only in the nucleus (Borghi *et al.*, 2001) whereas others report shuttling from cytoplasm to nucleus (Chen *et al.*, 2001). Lynch *et al.* (2005)

demonstrated that the localization is dependent on Ca^{2+} signalling, through calreticulin and/or calcineurin. The present study clearly identified a population of MEF2 proteins in the nucleus where they could regulate transcription. As with total tissue extracts, freezing had little effect on the total amounts of MEF2A and MEF2C in nuclear fractions but the amounts of phosphorylated $MEF2A^{Thr312}$ and $MEF2C^{Thr300}$ in the nucleus increased strongly after both short (4 h) and long (24 h) term freezing (Figures 3.7, 3.8). Nuclear levels typically rose by 1.5-2.0 fold in skeletal muscle and liver. In order to determine if the phosphorylated transcription factors are transcriptionally active in response to freezing, the transcriptional states of known MEF2 target genes were used to provide an insight.

Transcriptional regulation and roles of MEF2 target genes during freezing

The up-regulation of MEF2 target genes indicates that there is a probable need for enhanced levels of MEF2-regulated proteins to support freeze survival and/or thawing recovery by wood frog organs. Glucose, which is the main cryoprotectant of wood frogs, is synthesized by liver and then transported efficiently throughout the organism and taken up by other organs in order to provide intracellular protection during freezing. Hence, mechanisms that load glucose efficiently into cells are crucial to survival and this is the role of glucose transporters. A preliminary study reporting the results of a cDNA array screening of gene expression in heart of control versus frozen wood frogs (Storey, 2004a; Storey, 2004b) revealed a series of putative freeze-responsive genes. One of these was the glucose transporter-4 which was an up-regulated target during freezing. This finding was officially confirmed by this study. The *glut-4* mRNA transcript was found in heart, skeletal muscle and kidney but as expected was not expressed in liver and brain (Uldry &

Thorens, 2003). *Glut-4* mRNA was increased strongly during freezing by 2.5- and 1.7-fold increase in heart and skeletal muscle, respectively (Figure 3.13). Consequently, it is postulated that this would also be reflected in GLUT-4 protein levels, in order to allow rapid glucose uptake into these tissues during the early hours of freezing before blood circulation shuts down. Previous work on glucose transport in wood frogs demonstrates an increase in the number of glucose transporters as well as transport capacity (King *et al.*, 1993).

Calcium homeostasis must always be regulated due to the various functions that Ca^{2+} is involved in. Calreticulin is a Ca^{2+}-binding chaperone protein involved in many cellular functions such as Ca^{2+} transport, Ca^{2+} storage, protein folding, Ca^{2+}-dependent transcription, and in ER/UPR stress (Groenendyk *et al.*, 2004; Michalak *et al.*, 2002). Expression of the calreticulin gene was detected in all wood frog tissues studied and transcript levels showed strong freeze-responsive up-regulation in all tissues except in kidney. A 24 h freeze exposure caused a 1.5- 4.8-, 2.3- and 1.5- fold increases in *calreticulin* mRNA levels in brain, heart, skeletal muscle and liver, respectively (Figure 3.15). It appears that the up-regulation of *calreticulin* message was almost uniform throughout wood frog organs during freezing and this suggests that the protein was also probably elevated. This is not surprising considering the cellular functions of calreticulin, particularly its role in dealing with ER stress and the unfolded protein response, which has been shown to be activated in liver and skeletal muscle of wood frogs during freezing (Niles, 2007) as well as its recently discovered affiliation in wound healing (Nanney *et al.*, 2008; Johnson *et al.*, 2001).

Creatine kinase (CK) is a key enzyme of energy metabolism that is known to be transcriptionally regulated by MEF2. CK catalyzes a reversible reaction transferring a phosphate group from ATP to creatine, thereby producing phospho-creatine and ADP. The phosphocreatine pool is particularly important in muscle tissues where it provides an instantly available reserve that can be mobilized to buffer ATP levels as soon as contraction begins and before ATP production by glycolysis and/or the Krebs cycle can be ramped up to maximum. Cytosolic CK isozymes are hetero- or homodimers composed of two different CK subunits coded by two genes, *ckb* and *ckm*, under the regulation of MEF2 (Wallimann *et al.*, 1992; Ferrari *et al*, 1997; Shen *et al*, 2002). The mRNA levels of both *ckb* and *ckm* increased in both cardiac and skeletal muscles of wood frogs in response to freezing. Transcript levels of *ckb* rose 70% and 60% in heart and skeletal muscle, respectively; whereas *ckm* transcripts rose by 2.7- and 1.8 fold as compared with controls during the 24 h freeze exposure. These data indicate that both *ckb* and *ckm* were up-regulated in cardiac and skeletal muscle in response to freezing and suggest that CK protein levels may be similarly enhanced during freezing. Recent work in our laboratory found that the CK kinetic parameters were altered by reversible phosphorylation in skeletal muscle of frozen wood frogs (Dieni & Storey, 2009), but total CK protein levels were unchanged. Thus, it can be suggested that the up-regulation *ckb* and *ckm* seen here may be an anticipatory response in skeletal muscle that could support CK protein synthesis as soon as the frog thaws. Never-the-less it shows that creatine kinase is regulated at various steps of gene expression and demonstrates the importance of metabolic control in response to freezing.

Signalling to MEF2 transcription factors in response to freezing

The data in this chapter show that MEF2 proteins are activated in response to freezing in the wood frog. The primary regulatory candidates for MEF2 control are members of the MAPK pathways. Studies ranging through yeast, plants and mammals demonstrate that the p38 MAPK responds to osmotic stress (Sheikh-Hamad & Gustin, 2004) and hypothermia (Roberts *et al.*, 2002; Clanachan *et al.*, 2003). Indeed, there is research in our lab that provides support for a p38-mediated MEF2 activation. Greenway & Storey (2000) found that p38 MAPK undergoes increased phosphorylation, hence activation in liver, kidney, and heart within the first 12h of freezing and within 4 h of recovery from freezing in wood frogs. Interestingly, this response was found to be freeze-dependent as it was seen in response to two of the component stresses of freezing: anoxia and dehydration (Greenway & Storey, 2000). p38 MAPK activation in response to hyperosmotic and hypothermic stresses has also been observed in the heart of the marsh frog, *Rana ridibunda* (Aggeli *et al*, 2001; Aggeli *et al.*, 2002). Thus, it seems plausible that what is observed in wood frogs is a combination of these signals converging to regulate p38 MAPK which then relays signals to MEF2. Similarly to p38, ERK5 also responds to oxidative stress and hyperosmotic stress (Nishimoto & Nishida, 2006), but has not been reported to respond to hypothermia. The ERK5 MAPK pathway still remains relatively unexplored and therefore further research will be needed to determine whether this regulatory kinase affects MEF2 activation in the wood frog.

MEF2 activation may also be induced by direct and in-direct signals independent of MAPK pathways. Calcium-dependent signals have been found to have effects on MEF2 activity, and have proven to be a challenge to elucidate. This is because these

effects are signalled through both a Ca^{2+}-dependent protein kinase and a Ca^{2+}-dependent protein phosphatase (McKinsey *et al.*, 2002). Calcium signalling through calmodulin-dependent protein kinase (CaMK) can activate MEF2 by either directly phosphorylating MEF2 or indirectly by phosphorylating co-factors (Passier *et al.*, 2002; McKinsey *et al.*, 2001). Similarly, Ca^{2+} signals via calmodulin/calcineurin have also been shown to affect MEF2 transcriptional activity by dephosphorylating MEF2 and by dephosphorylating co-factors such as NFAT (Wu *et al.*, 2000; Blaeser *et al.*, 2000). The class II histone deacetylases HDAC4, 5 and 7 have been found to act as co-repressors of MEF2, which upon phosphorylation by CaMKIV, undergo nuclear export via 14-3-3 thus rendering MEF2 trancriptionally active (McKinsey *et al.*, 2001). Interestingly, unpublished data from our laboratory shows that HDAC 4 has a 0.4-fold downregulation in wood frog skeletal muscle during 24h freezing whereas liver shows a 2.3-fold increase ($P<0.05$). Nevertheless, the phosphorylation states of class II HDACs remain to be investigated in wood frog. Changes in calcium levels during freezing in wood frog tissues also remain to be explored, but if the freeze-tolerant phenotype shows similarities across phylogeny, then a role for Ca^{2+} in wood frog freeze tolerance, as observed in freeze-tolerant plants such as *Arabidopsis thaliana* (Yamazaki *et al.*, 2008), would not be unexpected. In fact, some aspects of Ca^{2+} metabolism are already known to change during freeze/thaw in wood frog muscle. Studies of sarcoplasmic reticulum (SR) Ca^{2+} binding and oxalate dependent Ca^{2+} update by the SR in muscle of frozen wood frogs showed a strong suppression to 48% and 8% of control values, respectively, and both of these parameters rebounded during recovery (Hemmings & Storey, 2001). These findings suggest that as the wood frog freezes, it does so with large pools of intracellular Ca^{2+} which is bound to

have signalling effects during freezing. In addition, as aforementioned, MEF2 transcriptional activity can be greatly influenced by co-factors, both activators and repressors. As the transcriptional machinery at work in the wood frog continues to be discovered, more insights regarding such co-factors will provide a clearer picture of the regulatory mechanisms during freezing.

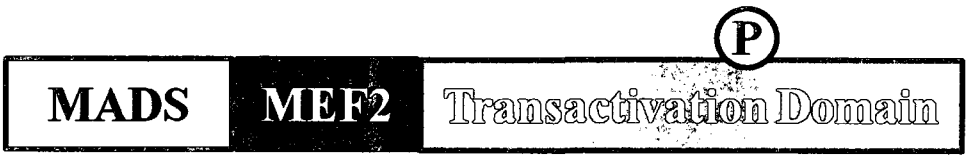
Conclusions

The research demonstrates that MEF2 undergoes phosphorylation in response to freezing and/or thawing in a tissue specific manner in the wood frog. MEF2 regulation was analyzed at the transcriptional, translational, as well as post-translational level and the studies reveal that in response to freezing, MEF2 proteins are regulated by phosphorylation. This signal was revealed to be transmitted to selected downstream genes in some tissues, especially in heart and skeletal muscle. The signals responsible for the transcriptional activation remain to be elucidated; however, there is evidence that supports p38 MAPK mediated activation. From the stress signals expected to be induced during freezing, parallel signal transduction pathways that regulate MEF2 remain suspects and future studies will shed more light on these signalling cascades.

Figure 3.1: Regulation of MEF2 Transcription Factors. The domain organization of MEF2 proteins (**A**).showing the three domains, MADS, MEF2 and transactivation domains (**TAD**) are displayed along with the typical phosphorylation site (**P**) on the TAD domain. The various ways that MEF2 proteins are regulated is shown in (**B**) displaying how p38 and ERK5 MAPK pathways along with Ca^{2+} signals and co-factors (activators and repressors) affect MEF2 transcriptional activity.

Figure 3.1

A


P
MADS
MEF2
Transactivation Domain

B

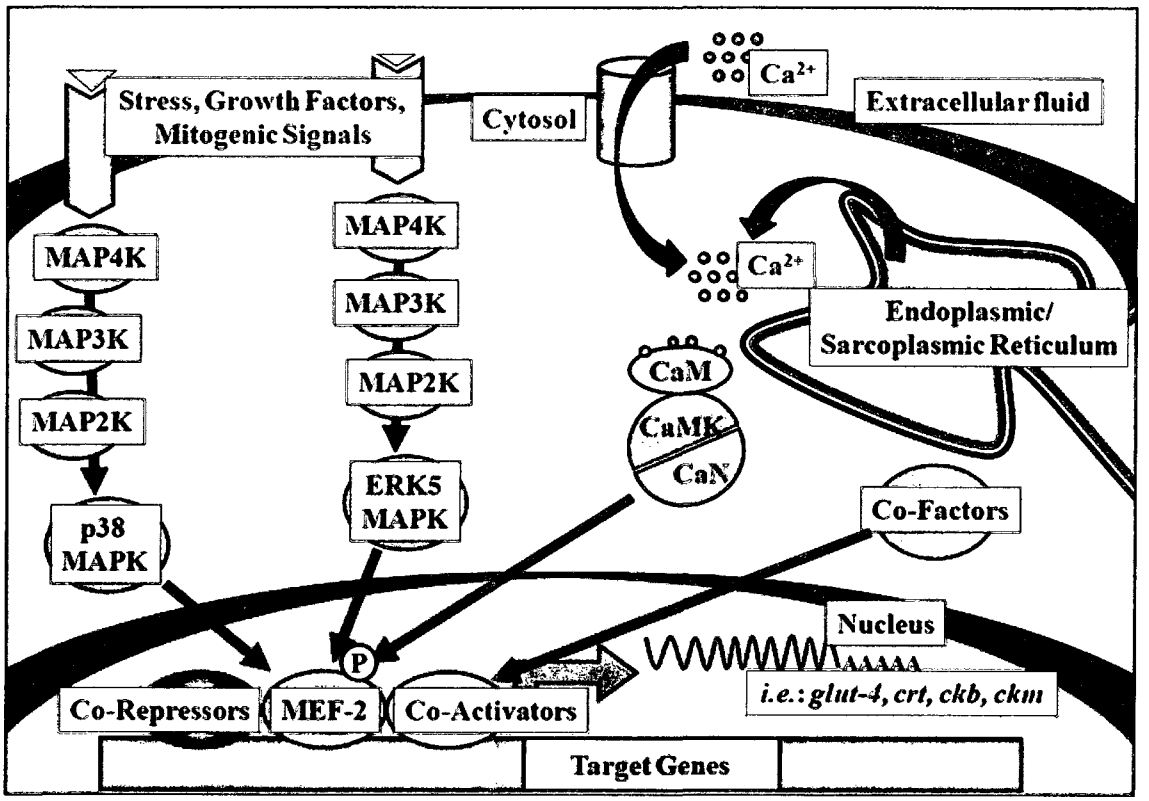

Stress, Growth Factors, Mitogenic Signals
Cytosol
Ca2+
Extracellular fluid
MAP4K
MAP3K
MAP2K
p38 MAPK
MAP4K
MAP3K
MAP2K
ERK5 MAPK
Ca2+
Endoplasmic/ Sarcoplasmic Reticulum
CaM
CaMK
CaN
Co-Factors
Nucleus
AAAAA
i.e.: glut-4, crt, ckb, ckm
P
Co-Repressors
MEF-2
Co-Activators
Target Genes

Figure 3.2: Western blot analysis showing effects of freezing on MEF2 proteins in wood frog brain and heart.

A) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the wood frog brain under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively. ★ - Significantly different from the corresponding control values using the Student's t-test, $P<0.05$.

B) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the wood frog heart under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 3.2

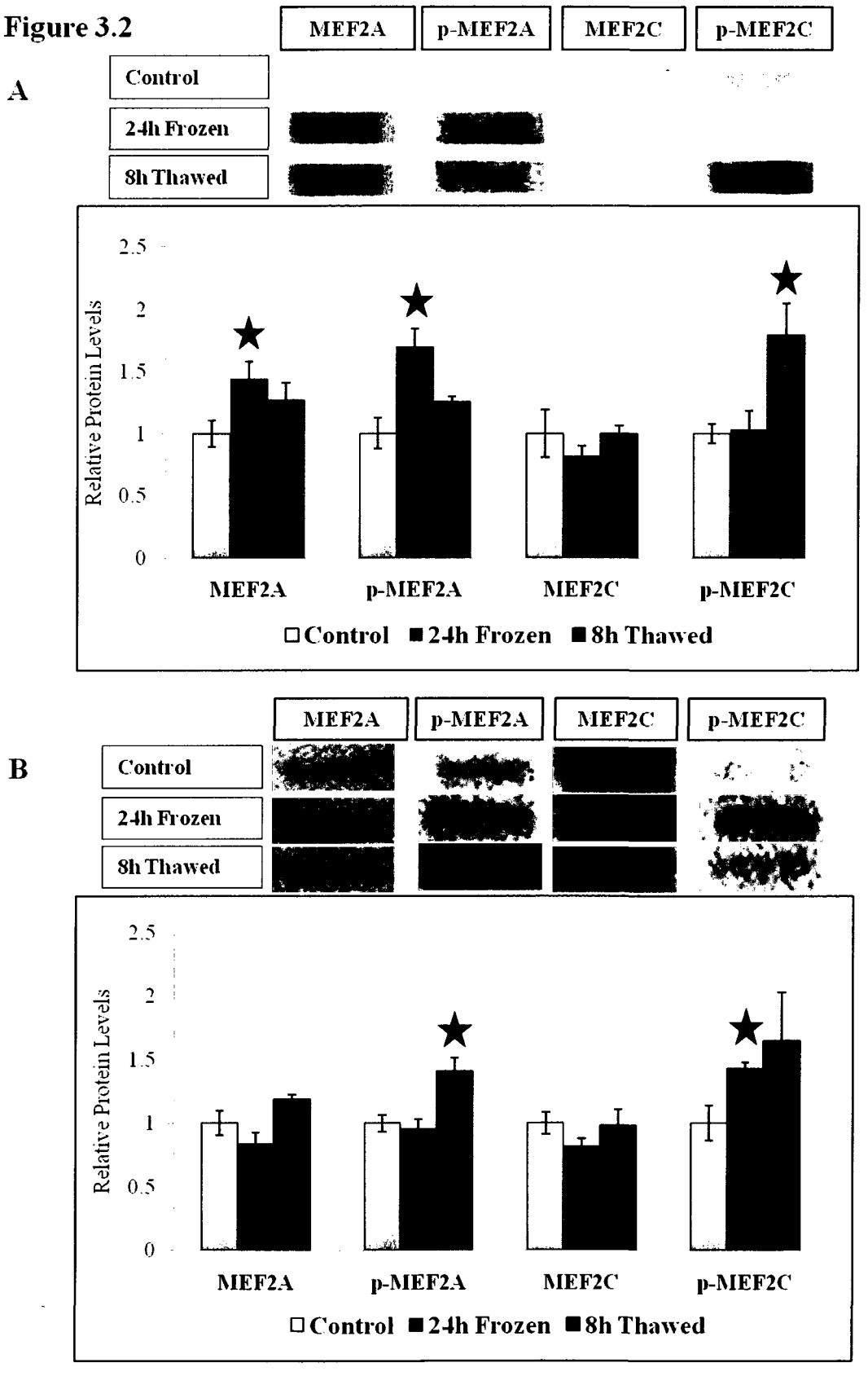
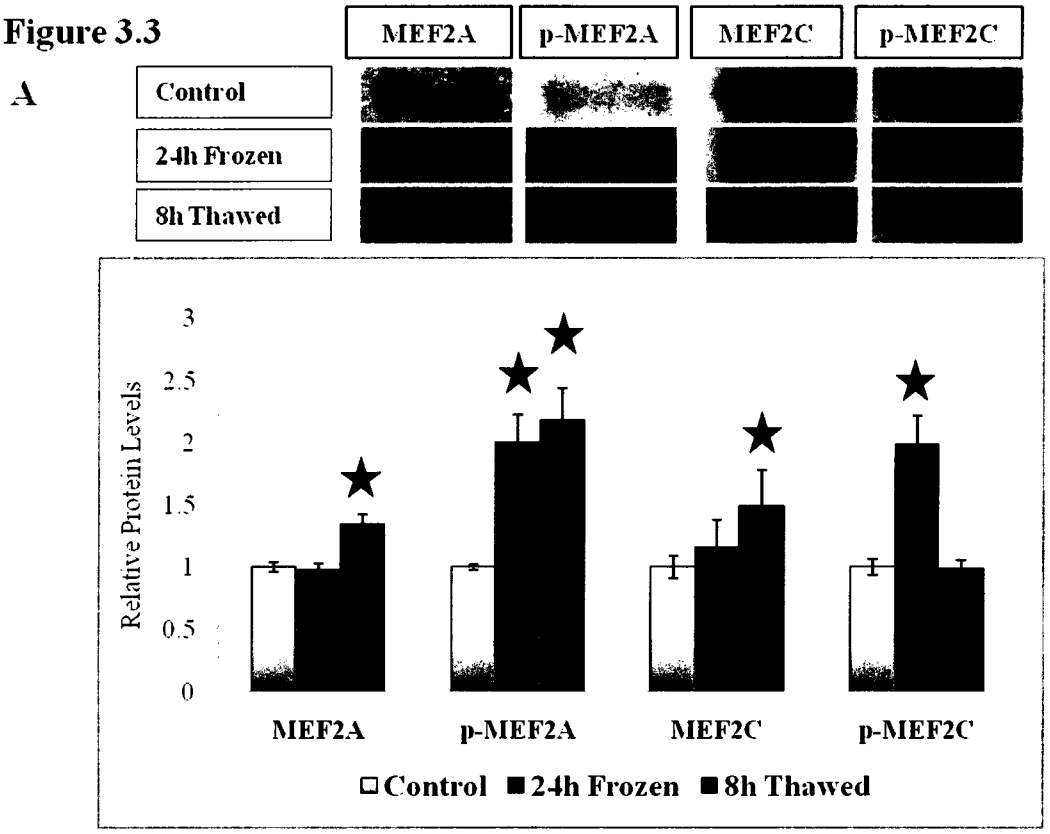

A
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Protein Levels
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
B
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Protein Levels
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed

Figure 3.3: Western blot analysis showing effects of freezing on MEF2 proteins in wood frog skeletal muscle and liver.

A) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the wood frog skeletal muscle under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

B) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the wood frog liver under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 3.3


A
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Protein Levels
3
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed

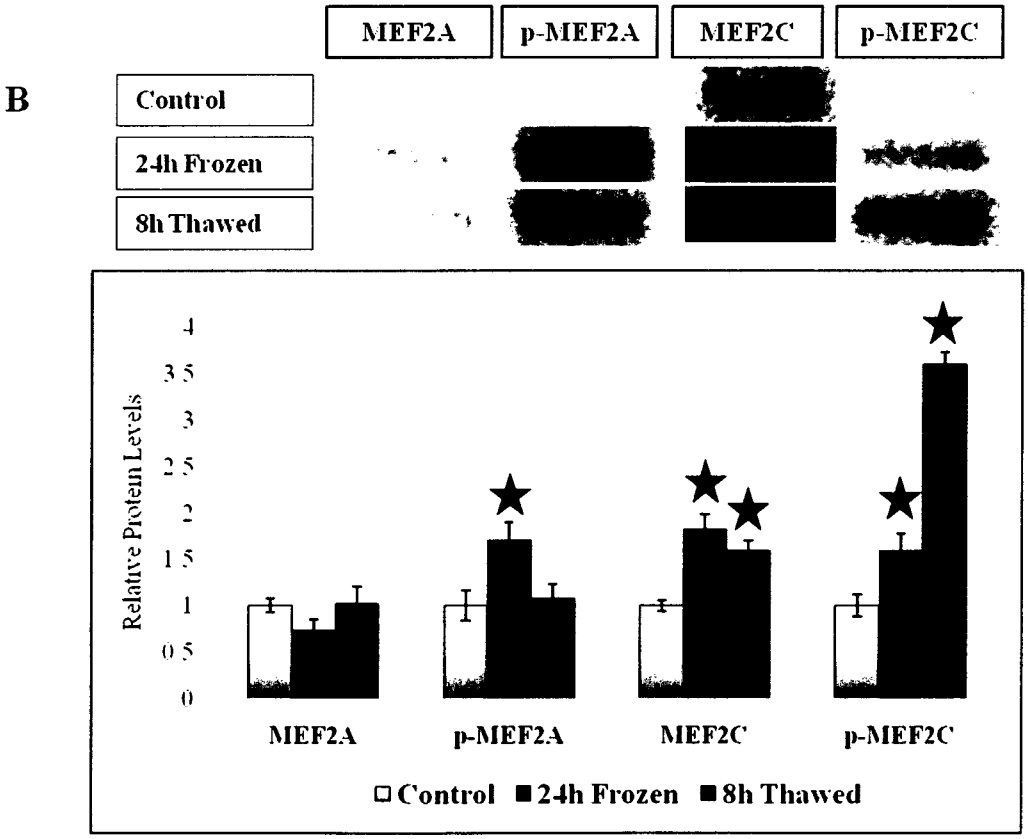

B
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Protein Levels
4
3.5
3
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed

Figure 3.4: Western blot analysis showing effects of freezing on MEF2 proteins in wood frog kidney.

Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the wood frog skeletal muscle under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 3.4

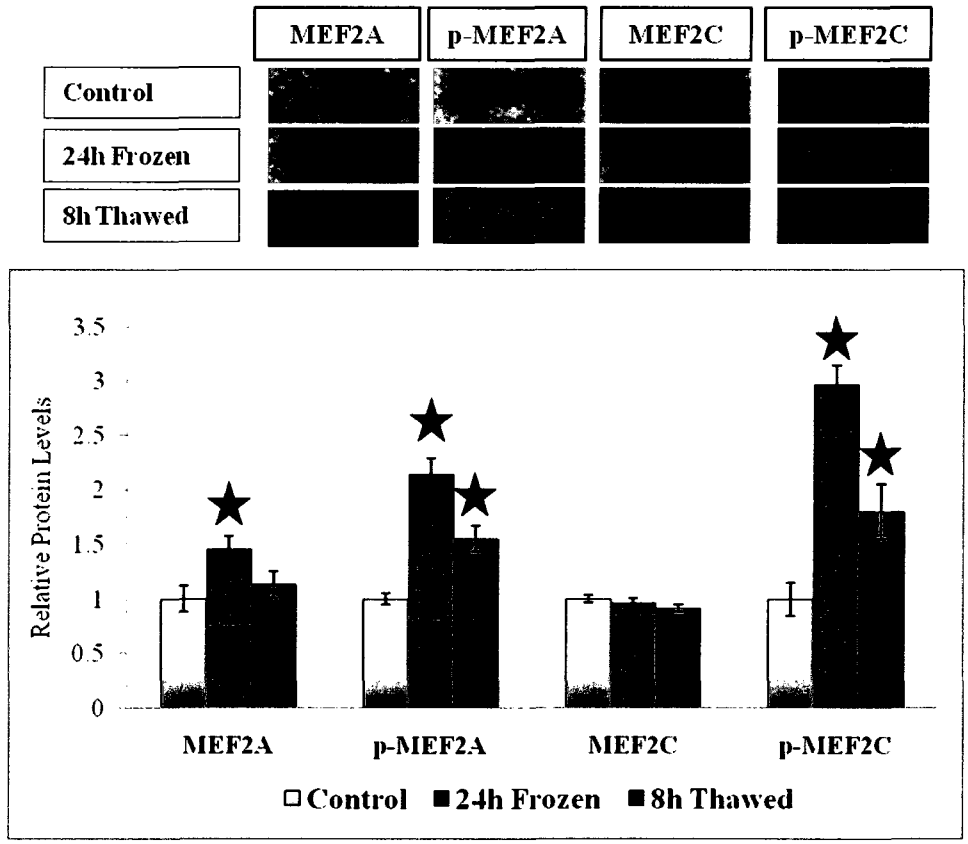

MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Protein Levels
3.5
3
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed

Figure 3.5: RT-PCR analysis showing the effects of freezing on *mef2a* and *mef2c* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

A) Expression of *mef2a* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *mef2a* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen. ★ - Significantly different from control values using Student's t-test, $P<0.05$.

B) Expression of *mef2c* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *mef2c* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 3.5

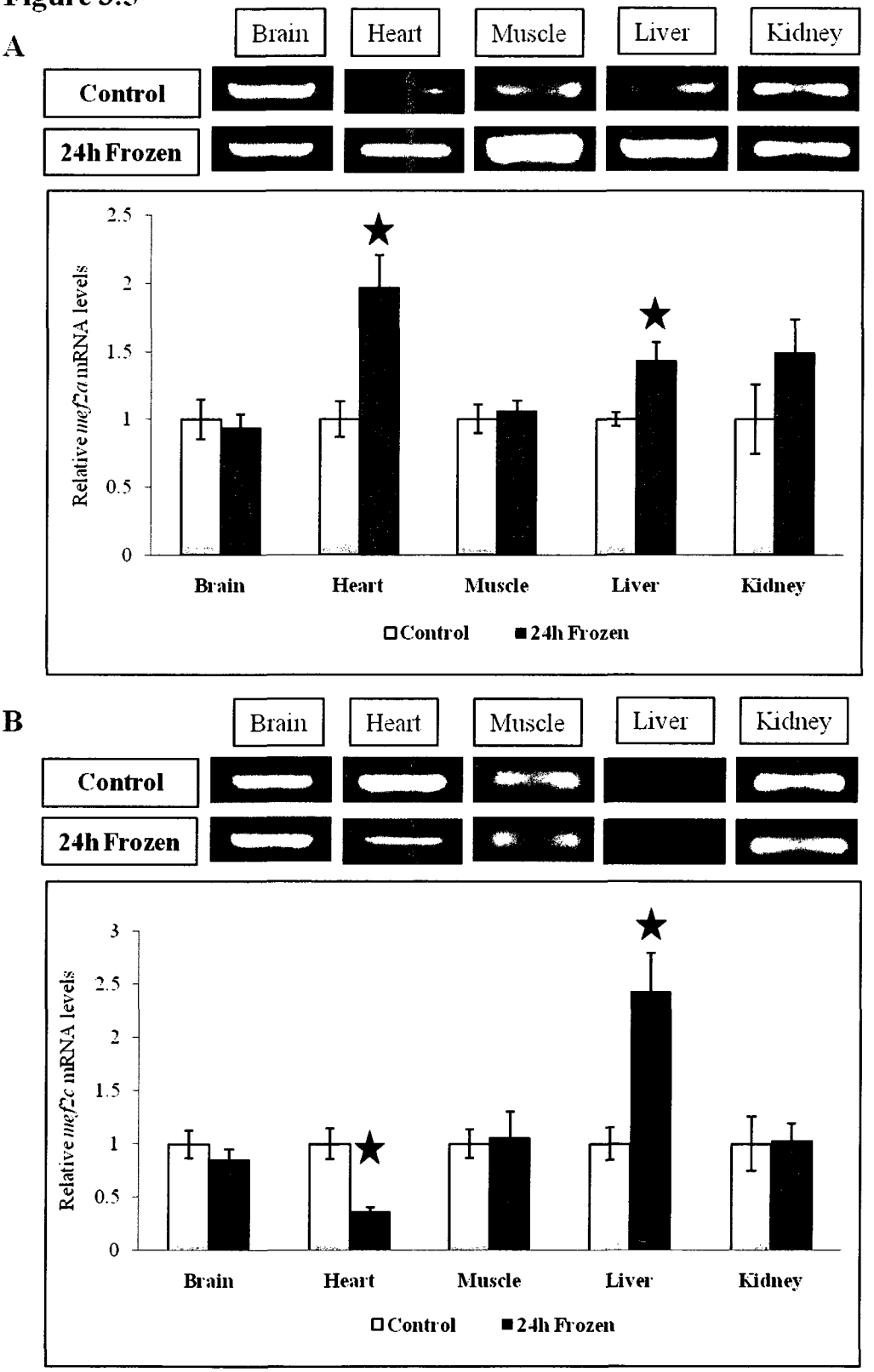

A
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative mef2a mRNA levels
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
B
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative mef2c mRNA levels
3
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 3.6: Western blot analysis showing effects of freezing on MEF2 proteins in nuclear extracts of wood frog skeletal muscle and liver.

A) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the nuclear extracts of control, 24h frozen and 8h thawed wood frog skeletal muscle. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

B) Expression and regulation of MEF2A and MEF2C and respective phosphorylated forms, $MEF2A^{thr312}$ and $MEF2C^{thr300}$ in the nuclear extracts in control, 24h frozen and 8h thawed wood frog liver. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 3.6

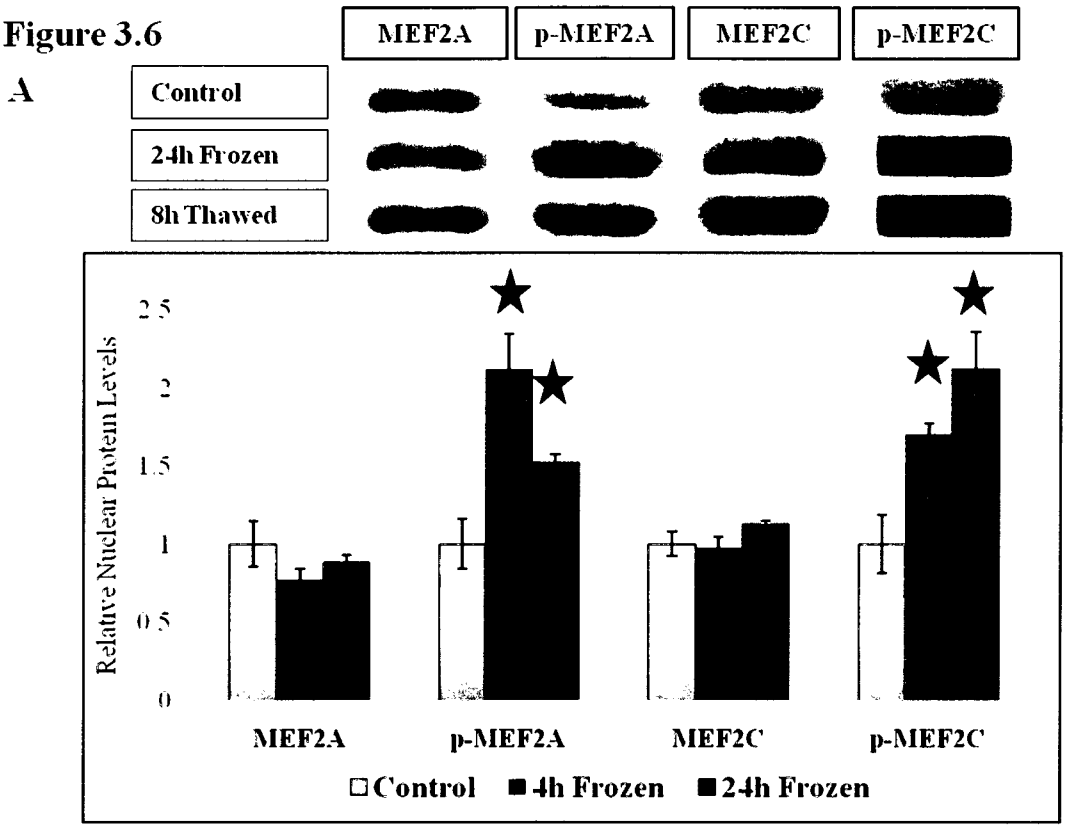

A
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Nuclear Protein Levels
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
4h Frozen
24h Frozen
B
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
24h Frozen
8h Thawed
Relative Nuclear Protein Levels
2.5
2
1.5
1
0.5
0
MEF2A
p-MEF2A
MEF2C
p-MEF2C
Control
4h Frozen
24h Frozen

Figure 3.7: RT-PCR analysis showing the effects of freezing on *glucose transporter-4* and *calreticulin* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

A) Expression of *glut-4* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *glut-4* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

B) Expression of *calreticulin* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *calreticulin* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 3.7

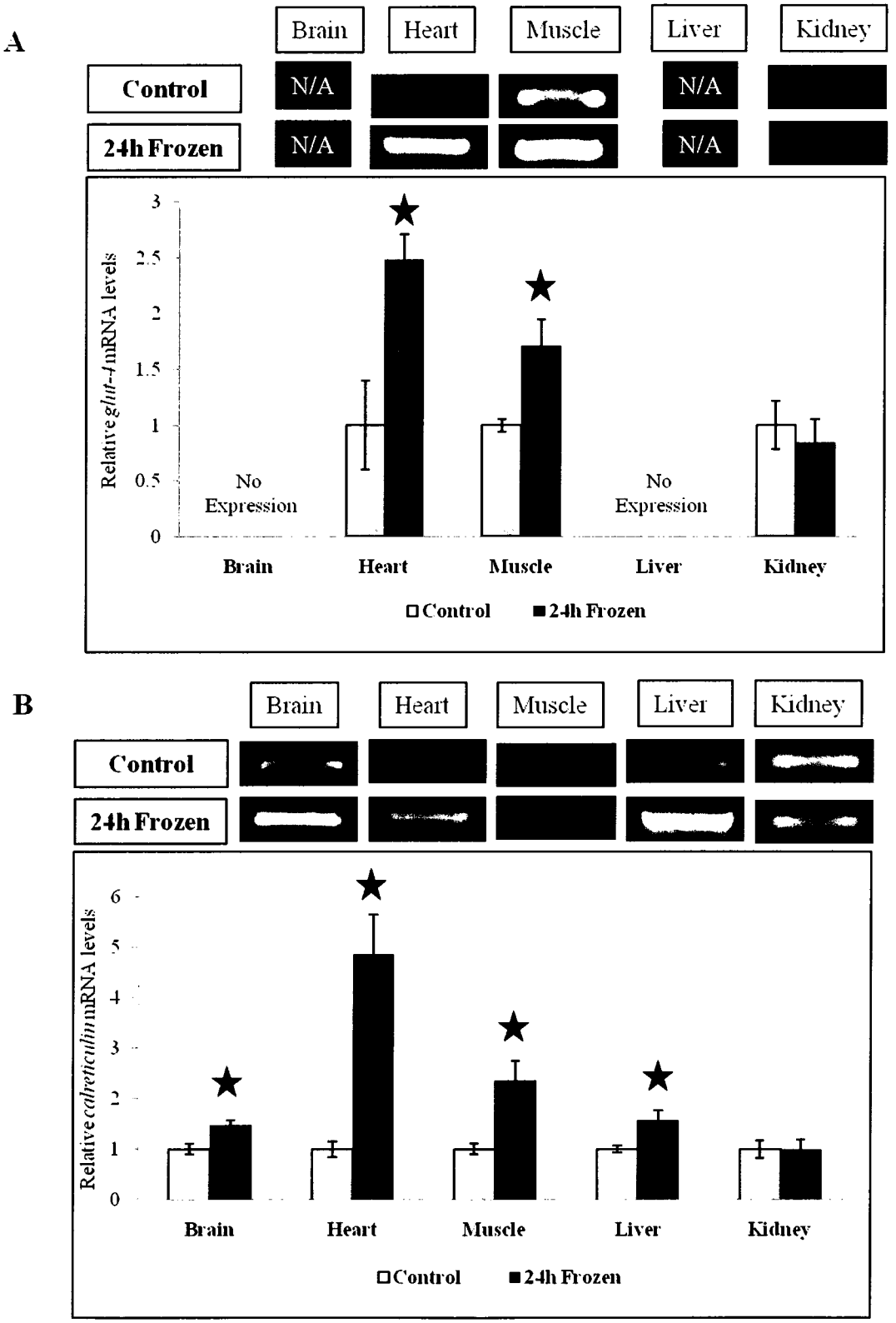

A
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
N/A
N/A
N/A
N/A
Relative glut-1 mRNA levels
3
2.5
2
1.5
1
0.5
0
No Expression
No Expression
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
B
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative calreticulin mRNA levels
6
5
4
3
2
1
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 3.8: RT-PCR analysis showing the effects of freezing on *brain creatine kinase* and *muscle creatine kinase* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

A) Expression of *ckb* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *ckb* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

B) Expression of *ckm* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *ckm* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 3.8

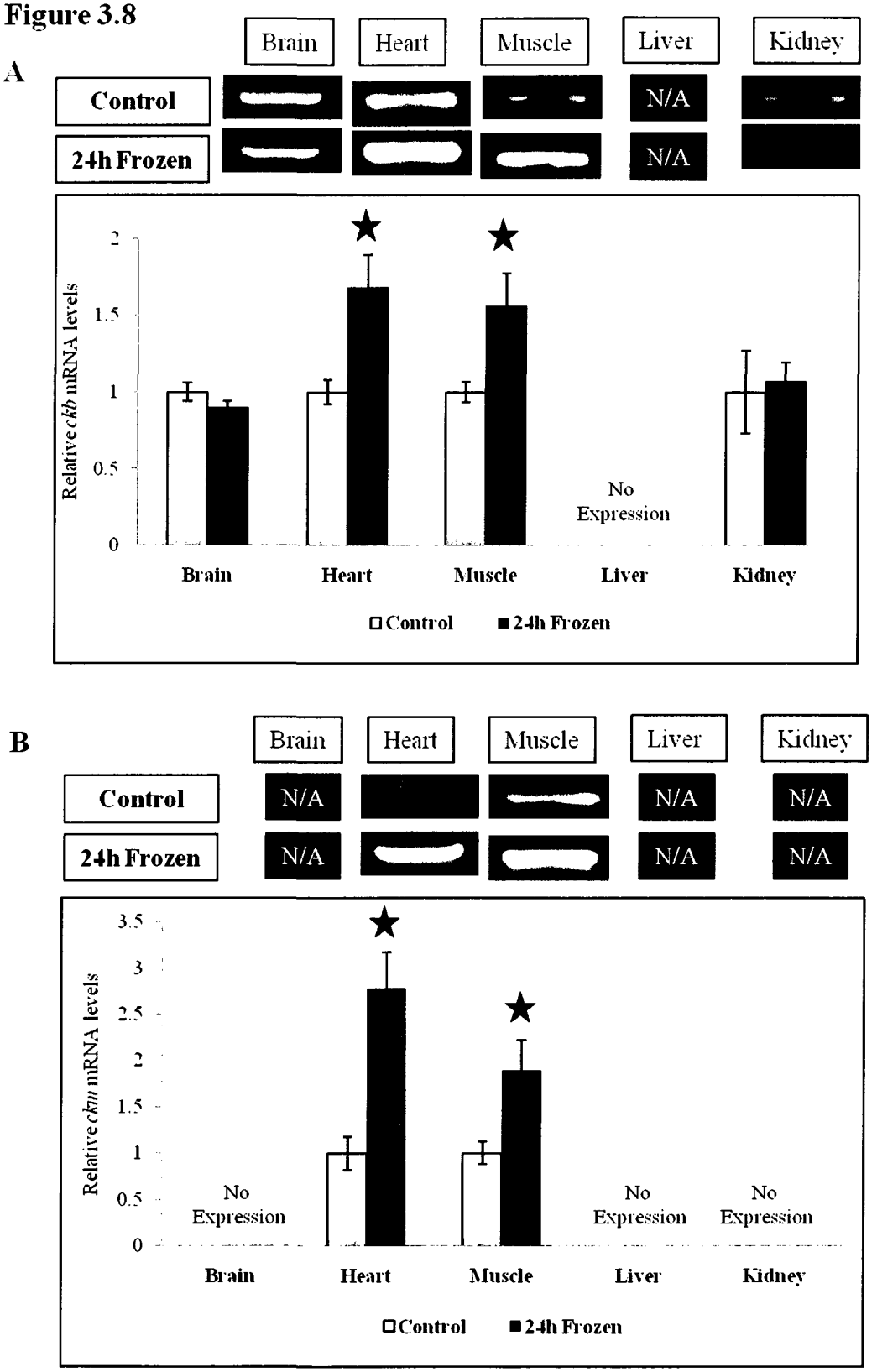

A
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
N/A
N/A
Relative ckb mRNA levels
2
1.5
1
0.5
0
No Expression
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
B
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
N/A
N/A
N/A
N/A
N/A
N/A
Relative ckm mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
No Expression
No Expression
No Expression
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 3.9: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial MEF2A sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for MEF2A gene product. The 144 amino acids were identified as the region covering amino acid position 130-270 of MEF2A, and comprises ~25% of the total size. No conserved domains were identified.

B) Homology tree produced from partial MEF2A amino acid alignments. The *R. sylvatica* sequence was compared with MEF2A polypeptide sequences from African clawed frog (*Xenopus laevis*, Genbank accession code: NP_001095216), mouse (*Mus musculus*, NP_001028885), human (*Homo sapiens*, AAH13437), chicken (*Gallus gallus*, NP_990195), and zebrafish (*Danio rerio*, CAK11495). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.9

```
1          GACAGAAGGAACACAGAGGCTGCGACAGCCCGGACCCCGACGGCTCGTACGTCCTGACCC
1             Q  K  E  H  R  G  C  D  S  P  D  P  D  G  S  Y  V  L  T
61         CCCACACCGAGGAAAAGTACAAAAAAATTAATGAGGAATTTGATAATATGATGCGCAATC
20         P  H  T  E  E  K  Y  K  K  I  N  E  E  F  D  N  M  M  R  N
121        ATAAAATTTCTCCTGGACTACAGCAGCCGAACTTCTCCATGTCTGTGACCATCCCAGTGT
40         H  K  I  S  P  G  L  Q  Q  P  N  F  S  M  S  V  T  I  P  V
181        CCAACCCATTGTCATACAGTTCTCCAGGGAATACCCTTGTCACCTCTTCACTGGCTGCCA
60         S  N  P  L  S  Y  S  S  P  G  N  T  L  V  T  S  S  L  A  A
241        GCACCTCATTAACAGACACAGGGATGATGTCTCCTCCTCAGACATCGCTGCACCGAAATG
80         S  T  S  L  T  D  T  G  M  M  S  P  P  Q  T  S  L  H  R  N
301        TGGCGTCTCCTGGGGTGTCGCAAAGACCATCAAGCACTGGAAATGCAGGTGTAATGTTAT
100        V  A  S  P  G  V  S  Q  R  P  S  S  T  G  N  A  G  V  M  L
361        GTTCATCGGACATCTCTGTGCCAAATGGAGCTGGTGCCAGTCCAGTCGGCAATGGGGTTT
120        C  S  S  D  I  S  V  P  N  G  A  G  A  S  P  V  G  N  G  V
421        GG
140        W
```

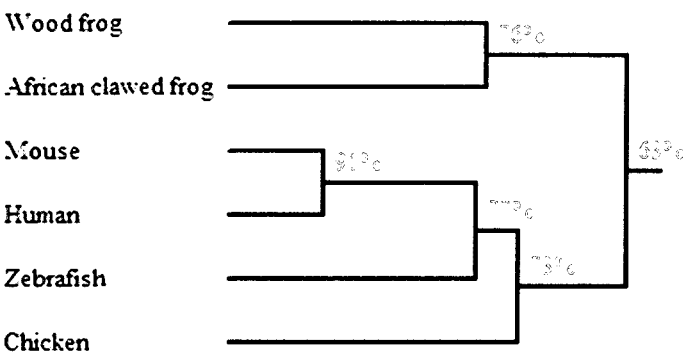


Figure 3.10: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial MEF2C sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for MEF2C gene product. The 170 polypeptide sequence was identified as the region covering positions 125 to 300 of MEF2C, meaning that 36% of the protein was identified. Underlined and in bold is the phosphorylation site at threonine 300. There were no conserved domains discovered from the sequence.

B) Homology tree produced from partial MEF2C amino acid alignments. The *R. sylvatica* sequence was compared with MEF2C polypeptide sequences from Western clawed frog (*Silurana tropicalis*, NP_001106387), mouse (*M. musculus*, NP_079558), human (*H. sapiens*, NP_002388), chicken (*G. gallus*, XP_001231662), zebrafish (*D. rerio*, AAC05226), and common carp (*Cyprinus carpio*, BAA33568). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.10

```
1          TTGATCTAATGATCAGCAGGCAAAGATTGTGTGCCGTTCCACCTCCCAATTTTGAGATGC
1             D  L  M  I  S  R  Q  R  L  C  A  V  P  P  P  N  F  E  M
61         CGGTTTCCATTCCGGTGAGCAATCATAACAGCTTGGCATACAGCAATCAAGGTTCACTGG
20         P  V  S  I  P  V  S  N  H  N  S  L  A  Y  S  N  Q  G  S  L
121        GTAATCATAATCTATTGCCATTGTCCCATCAGTCCTTGCAAAGAAACAGCATGTCTCCAG
40         G  N  H  N  L  L  P  L  S  H  Q  S  L  Q  R  N  S  M  S  P
181        GCTTGAATCATCGGCCACCCAGTGCAGGTAACACAGGTGGTCTGATGGGTGGGGACCTCA
60         G  L  N  H  R  P  P  S  A  G  N  T  G  G  L  M  G  G  D  L
241        CAAGCGGTGCAGGCACCAGTGCAGGAAATGGGTATGGCAATCATCGTAACTCACCAGGTC
80         T  S  G  A  G  T  S  A  G  N  G  Y  G  N  H  R  N  S  P  G
301        TGCTGGTCTCACCTGGTAACTTGAATAAAAATATGCAAACAAAATCACCACCGCCAATGA
100        L  L  V  S  P  G  N  L  N  K  N  M  Q  T  K  S  P  P  P  M
361        ACTTGGGGATGAACAATCGTAAACCTGACCTCAGAGTTCTTATTCCACCTGGCAGCAAGA
120        N  L  G  M  N  N  R  K  P  D  L  R  V  L  I  P  P  G  S  K
421        ATACAATGCCCTCTGTGAATCAAAGGATAAACAACTCACAATCTGCTCAGTCACTGGCTA
140        N  T  M  P  S  V  N  Q  R  I  N  N  S  Q  S  A  Q  S  L  A
481        CCCCAGTGGTTTCCGTAGCAACTCCAATTTCC
160        T  P  V  V  S  V  A  T  P  I  S
```

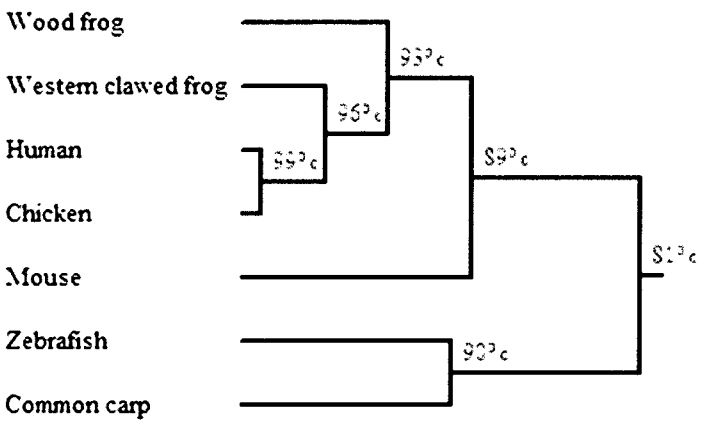


Figure 3.11: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial GLUT-4 sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for GLUT-4 gene product. The 158 polypeptide was identified as the region covering amino acid positions 275-430 of GLUT-4, and constitutes about 30% of the total size. Domain analysis of the sequence also showed a sugar transporter domain which spanned the entire sequence.

B) Homology tree produced from partial GLUT-4 amino acid alignments. The *R. sylvatica* sequence was compared with GLUT-4 polypeptide sequences from African clawed frog (*X. laevis*, ABL10365), mouse (*M. musculus*, BAB03251), human (*H. sapiens*, NP_001033), rat (Rattus norvegicus, NP_036883), Pig (*Sus scrofa*, NP_001121905), Coho salmon (*Oncorhynchus kisutch*, AAM22227), and Atlantic cod (*Gadus morhua*, AAZ15731). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.11

```
1          ATCCTGCAGATCTTCAGATCCAGACAATACCGGCAACCCATGATTGTGGCCATAATCCTC
1           I  L  Q  I  F  R  S  R  Q  Y  R  Q  P  M  I  V  A  I  I  L
61         CAACTCTCCCAACAACTTTCCGGAATCAATGCGATCTTCTATTATTCCACCGATATCTTC
21          Q  L  S  Q  Q  L  S  G  I  N  A  I  F  Y  Y  S  T  D  I  F
121        GCTAAAGCCGGAGTGGAGCAGCCGATCTACGCCACGATTGGGGCAGGAATCGTCAACACG
41          A  K  A  G  V  E  Q  P  I  Y  A  T  I  G  A  G  I  V  N  T
181        GCCTTCACGGTGGTGTCGCTGTTCCTTGTGGAGAGGGCAGGAAGACGGACCCTGCACCTG
61          A  F  T  V  V  S  L  F  L  V  E  R  A  G  R  R  T  L  H  L
241        GTGGGCCTGGCGGGAATGATCCTCTGTGCCTTATTAATGACGGTGGCCATGGTCCTACAG
81          V  G  L  A  G  M  I  L  C  A  L  L  M  T  V  A  M  V  L  Q
301        GAAACCATCCCTGCCATCAGCTCTCTCAGCATGGCCGCCATTTTTGGATTCGTGGCGTTC
101         E  T  I  P  A  I  S  S  L  S  M  A  A  I  F  G  F  V  A  F
361        TTCGAGGTGGGGCCTGGTCCTATTCCGTGGTTCATTGTGGCGGAACTCTTCAGTCAAGGT
121         F  E  V  G  P  G  P  I  P  W  F  I  V  A  E  L  F  S  Q  G
421        CCCCGGCCCGCAGCCATGGCCATCGCTGGATGTACCAACTGGACCTCCAACTTCA
141         P  R  P  A  A  M  A  I  A  G  C  T  N  W  T  S  N  F
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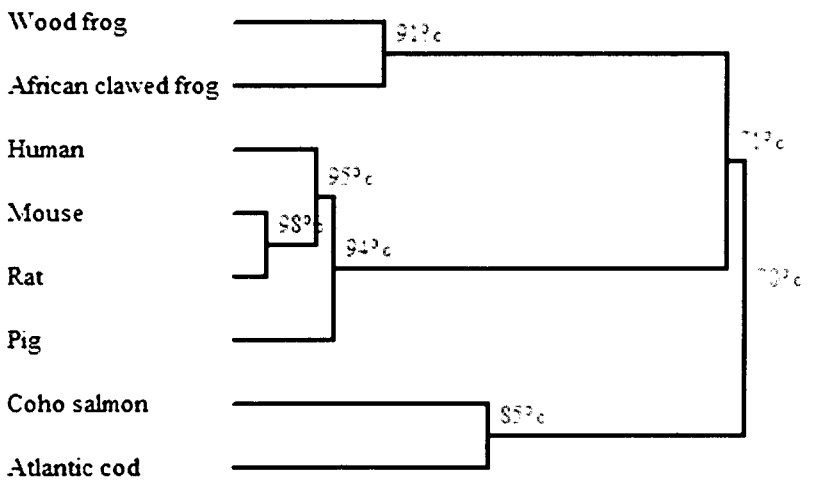

Figure 3.12: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial Calreticulin sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for calreticulin gene product. The 158 amino acids were identified as the region covering amino acid position 210-368 of calreticulin, which comprises ~38% of the total size. Domain analysis of the sequence also showed a calreticulin superfamily domain (shown underlined).

B) Homology tree produced from partial calreticulin amino acid alignments. The *R. sylvatica* sequence was compared with calreticulin polypeptide sequences from Japanese wrinkled frog (*Rana rugosa*, BAA11425), African clawed frog (*X. laevis*, NP_001080765), Western clawed frog (*S. tropicalis*, NP_001001253), human (*H. sapiens*, AAB51176), chicken (*G. gallus*, AAS49610), and zebrafish (*D. rerio*, NP_571122). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.12

```
1          TCAAGGACCCTGAGGCCAAGAAGCCCGATGAGTGGGATGAGCGTCCCAAAATTGATGATC
1             K  D  P  E  A  K  K  P  D  E  W  D  E  R  P  K  I  D  D
61         CCGAGGACAAGAAACCAGAGGACTGGGAAAAGCCAGAACATATCCCTGACCCAGATGCAG
20         P  E  D  K  K  P  E  D  W  E  K  P  E  H  I  P  D  P  D  A
121        TGAAACCAGAAGATTGGGATGAGGAGATGGATGGAGAGTGGGAGCCACCAGTCATCACAA
40         V  K  P  E  D  W  D  E  E  M  D  G  E  W  E  P  P  V  I  T
181        ATCCAGAGTACAAGGGAGAGTGGAAACCACGCCAGATTGACAACCCTGACTACAAAGGAA
60         N  P  E  Y  K  G  E  W  K  P  R  Q  I  D  N  P  D  Y  K  G
241        AGTGGGTGCACCCAGAGATCGACAACCCAGAATATACCCCAGATCCTACTCTGTACTCCT
80         K  W  V  H  P  E  I  D  N  P  E  Y  T  P  D  P  T  L  Y  S
301        ATGAAGACTTTGGAGCTTTGGGCCTTGATCTTTGGCAGGCGAAATCTGGCACCATCTTTG
100        Y  E  D  F  G  A  L  G  L  D  L  W  Q  A  K  S  G  T  I  F
361        ACAACTTCTTGATTACTGACGATGAGAAGTTTGCAAAGGAACAGGCCACTAACCCATGGG
120        D  N  F  L  I  T  D  D  E  K  F  A  K  E  Q  A  T  N  P  W
421        GAGTCACAAAGGAAGGAGAGAAAAAGATGAAGGAACTGCAGGATGAAGAGGATCGC
140        G  V  T  K  E  G  E  K  K  M  K  E  L  Q  D  E  E  D  R
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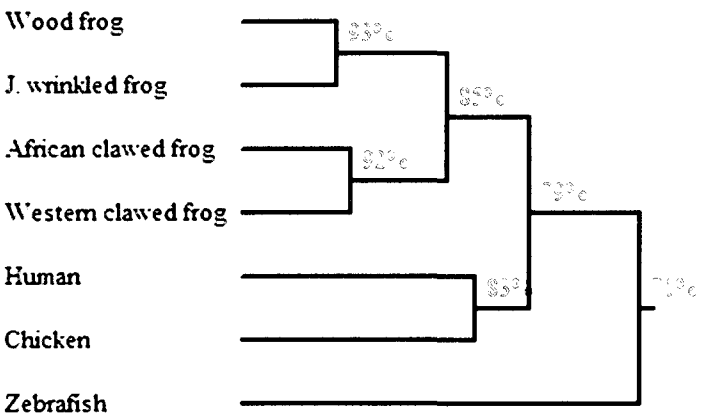

Figure 3.13: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial Brain Creatine Kinase (CKB) sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for CKB gene product. The 198 amino acid polypeptide was identified as the region covering amino acid position 48 to 245 of CKB, and constitutes ~52% of the total size. Domain analysis of the sequence revealed an ATP:guanido phosphotransferase, C-terminal catalytic domain (shown underlined).

B) Homology tree produced from partial CKB amino acid alignments. The *R. sylvatica* sequence was compared with CKB polypeptide sequences from African clawed frog (*X. laevis*, NP_001080363), Western clawed frog (*S. tropicalis*, NP_001037926), human (*H. sapiens*, NP_001814), mouse (*M. Musculus*, NP_067248) chicken (*G. gallus*, NP_990641), zebra finch (*Taeniopygia guttata*, ACH45596) and zebrafish (*D. rerio*, NP_001070631). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.13

```
1          TGGATTTACTGGTGGATGATGTCATTCAGACCGGAATTGACAACCCAGGCCACCCTTATA
1             D  L  L  V  D  D  V  I  Q  T  G  I  D  N  P  G  H  P  Y
61         TTCTGACCGTGGGAGCTGTTGCTGGTGATGAAGAGTGCTATGATGTGTTCGGTGATCTAT
20         I  L  T  V  G  A  V  A  G  D  E  E  C  Y  D  V  F  G  D  L
121        TTGACCCCATCATTGAAGACCGACATGGTGGCTACAAGCCAGGAGACCAGCACAAGACTG
40         F  D  P  I  I  E  D  R  H  G  G  Y  K  P  G  D  Q  H  K  T
181        ACCTGAAATCTGAACACTTGAAGGGTGGTGATGACCTGGACCCAAACTACGTCCTTAGTT
60         D  L  K  S  E  H  L  K  G  G  D  D  L  D  P  N  Y  V  L  S
241        CCCGTGTTAGAACTGGCAGAAGTATCCGTGGTTTTTGCCTCCCACCTCACTGCAGCCGTG
80         S  R  V  R  T  G  R  S  I  R  G  F  C  L  P  P  H  C  S  R
301        GGGAAAGACGAGGCATTGAAAAACTCTCCATTGAAGCTCTGGACAGCCTGGATGGAGATC
100        G  E  R  R  G  I  E  K  L  S  I  E  A  L  D  S  L  D  G  D
361        TGAAAGGAAAGTACTATGCTCTCAAGAGCATGACTGACGCAGAGCAGCAGCAACTCATCG
120        L  K  G  K  Y  Y  A  L  K  S  M  T  D  A  E  Q  Q  Q  L  I
421        ATGACCACTTCCTGTTTGACAAGCCAGTATCTCCCCTGCTTCTGGCCTCAGGGATGGCCC
140        D  D  H  F  L  F  D  K  P  V  S  P  L  L  L  A  S  G  M  A
481        GGGACTGGCCTGATGCCAGAGGAATTTGGCACAATGACAACAAGACTTTCCTTGTCTGGA
160        R  D  W  P  D  A  R  G  I  W  H  N  D  N  K  T  F  L  V  W
541        TCAATGAGGAGGATCATCTCCGTGTCATCTCCATGCAGAAGGGTGGCAACATGAAGG
180        I  N  E  E  D  H  L  R  V  I  S  M  Q  K  G  G  N  M  K
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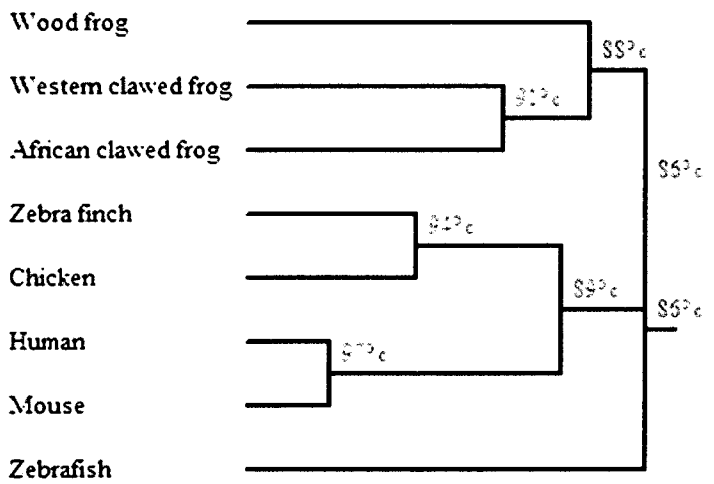

Figure 3.14: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial Muscle Creatine Kinase (CKM) sequence.

A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for CKM gene product. The 177 amino acid polypeptide was identified as the region covering amino acid position 148 to 325 of CKB, which comprised about 46% of the total size. Domain analysis of the sequence revealed an ATP:guanido phosphotransferase, C-terminal catalytic domain which spanned the entire sequence.

B) Homology tree produced from partial CKM amino acid alignments. The *R. sylvatica* sequence was compared with CKM polypeptide sequences from African clawed frog (*X. laevis*, NP_001080073), Western clawed frog (*S. tropicalis*, NP_989374), human (*H. sapiens*, NP_001815), chicken (*G. gallus*, NP_990838), and zebrafish (*D. rerio*, AAD54775). The percentage values correspond to the shared identity between the corresponding species.

Figure 3.14

```
1          TGAGCGCCGTGCCATTGAGAACTTGTCCATCAAAGCTCTCGACAGTCTGACTGGAGAGTT
1            E  R  R  A  I  E  N  L  S  I  K  A  L  D  S  L  T  G  E  F
61         CAAGGGAAAGTACTACCCCCTGAAGGACATGACAGACGCCGAGCAGCAGCAGCTGATTGA
21           K  G  K  Y  Y  P  L  K  D  M  T  D  A  E  Q  Q  Q  L  I  D
121        TGACCACTTCCTCTTTGACAAGCCTGTGTCTCCTCTTCTGCTGGCCGCCGGTATGGCCCG
41           D  H  F  L  F  D  K  P  V  S  P  L  L  L  A  A  G  M  A  R
181        TGACTGGCCCGATGCCCGTGGTATCTGGCACAACGACAACAAGTCCTTCCTTGTGTGGGT
61           D  W  P  D  A  R  G  I  W  H  N  D  N  K  S  F  L  V  W  V
241        AAATGAAGAGGACCACCTGAGAGTCATCTCCATGGAGAAGGGAGGCAACATGAAGACAGT
81           N  E  E  D  H  L  R  V  I  S  M  E  K  G  G  N  M  K  T  V
301        GTTCAAACGCTTCTGTGAGGGTCTTCAGAAGATTGAGGAGATCTTCAAGAGTGCAGGACA
101          F  K  R  F  C  E  G  L  Q  K  I  E  E  I  F  K  S  A  G  H
361        TCCCTTCAGCTGGAGCGAGCATCTTGGTTACATCCTTACTTGCCCATCCAACTTGGGCAC
121          P  F  S  W  S  E  H  L  G  Y  I  L  T  C  P  S  N  L  G  T
421        AGGTCTGAGAGGCGGAGTCCATGTCAAGCTTCCCAACCTCAGCAAACACCCCAAATTCGA
141          G  L  R  G  G  V  H  V  K  L  P  N  L  S  K  H  P  K  F  E
481        GGAGATCCTGACCAGACTGCGTCTGCAGAAGAGAGGCACAGGTGGTGTGGACAC
161          E  I  L  T  R  L  R  L  Q  K  R  G  T  G  G  V  D
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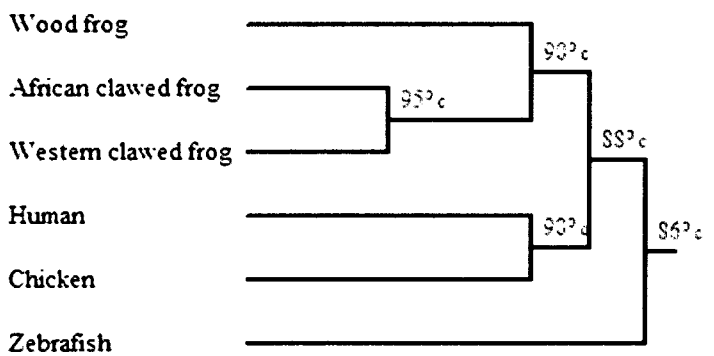


CHAPTER 4

THE REGULATION OF THE SMAD TRANSCRIPTION FACTORS IN THE WOOD FROG IN RESPONSE TO FREEZE EXPOSURE

4.1. INTRODUCTION

As previously emphasized in Chapters 1 and 3, the transcriptional machinery is tightly regulated during all stages of cell life. There are no exceptions and this is specially observed during the responses of cell to stress. With this in mind, it is expected that a cycle of freezing and thawing would disrupt normal cellular activity and thus activate the appropriate transcriptional machinery necessary to alter the expression of genes for optimal cell survival. The present chapter focuses on a second family of transcription factors, the SMAD proteins, and their role in natural freeze tolerance of the wood frog, *R. sylvatica*.

SMAD Family of Transcription Factors

The SMAD proteins are an evolutionarily conserved family of transcription factors, not only in homology, but also in function and regulation. The name originates from the sequence similarity of the invertebrate orthologs, Sma and Mad corresponding to the *Caenorhabditis elegans* and *Drosophila melanogaster* names, respectively (Liu *et al.*, 1996). In *Xenopus* and in mammals, there appears to be 8 family members: SMAD1 through 8. SMADS1, 2, 3, 5 and 8 are known as Receptor regulated SMADs (R-SMADS) meaning that they are substrates for TGF-β receptor-mediated signals. SMAD4 is known as a Co-SMAD, which serves as a common partner for all R-SMADs. The remaining SMAD proteins, SMAD6 and SMAD7 are inhibitory SMADs (I-SMADs) which act to dissipate SMAD activation signals. The SMAD-binding element found in SMAD-responsive gene promoters has been identified as 5'-GTCT-3'or its complement 5'-AGAC-3' (Massagué *et al.*, 2005).

SMAD proteins consist of two globular domains flanked by a linker region (Massagué *et al.*, 2005). The N-terminal region of the proteins contains the Mad Homology 1 (MH1) domain which is highly conserved in R-SMADs. This domain is responsible for DNA binding stabilized through a zinc atom (Chai *et al.*, 2003). The linker arm has signalling sites for post-translational modifications including phosphorylation and ubiquitination, as well as nuclear export signals. The MH2 domain is found at the C-terminal and is involved in protein-protein interaction and cytoplasmic retention signals (Massagué *et al.*, 2005). For this reason, the SMAD proteins are typically regulated at the linker arm, MH2 domain or the C-terminal tail.

Activation of SMAD Proteins

The SMAD transcription factors were initially found to be regulated by signals from the transforming growth factor β (TGF-β) superfamily of cytokines, therefore placing them downstream of the TGF-β receptors. The R-SMADS are primarily regulated by post-translational modifications, commonly in the form of phosphorylation. This phosphorylation takes place at serine residues in the C-terminal tail on a SSXS motif, in the range of amino acid residues 425-475 (Wrighton *et al.*, 2009). SMAD proteins routinely shuffle from cytoplasm to nucleus, but once receptor-mediated phosphorylation takes place, the activated R-SMADs translocate and accumulate in the nucleus. Upon phosphorylation, R-SMADs translocate into the nucleus where SMAD4, other co-factors as well as DNA retain their nuclear localization (Wrighton *et al.*, 2009). This activation causes the SMAD proteins to form hetero-complexes and exert their transcriptional influence. Once dephosphorylated, the reverse occurs and SMAD proteins are exported into the cytoplasm, rendering them transcriptionally inactive. Recently, SMADs have

also been discovered to be regulated by acetylation as well as SUMOylation (Inoue *et al.*, 2006; Simonsson *et al.*, 2006; Ohshima & Shimotohno, 2003). Thus, the complexity of regulating these proteins is being revealed.

Signalling to SMAD Transcriptional Factors

The SMAD proteins were initially identified as downstream substrates of TGF-β signals. TGF-β is a polypeptide growth factor involved in a variety of cellular functions including cell growth and differentiation, apoptosis, and immune responses, among others (Ross & Hill, 2007). Typically, TGF-β binds a TGF-β type II receptor on the cell surface thereby triggering the formation of a hetero-tetrameric complex (composed of TGF-β type I and type II receptors). Once activated, the cytoplasmic portion of the receptors, which have serine/threonine kinase activities, phosphorylate themselves and R-SMAD proteins (Massagué *et al.*, 2005). As previously explained, this phosphorylation induces the formation of R-SMAD complexes with SMAD4 as well as with other factors which then travel to the nucleus and induce transcription (see Figure 4.1). Recent studies on SMADs are beginning to reveal the cross-talk between signalling pathways and thus more methods of regulating these proteins.

Signals that regulate SMAD proteins have yet to be fully discovered. Nevertheless, we are beginning to see how complex this mechanism truly is. In addition to C-terminal receptor mediated phosphorylation, SMAD activity can also be regulated by post-translational modification sites on the linker arm, which can receive signals from multiple signalling cascades (Wrighton *et al.*, 2009). Another mode of regulation is the co-factors that SMAD proteins are affiliated with. SMAD proteins have been found to interact with

ATF3, AP1, FoxO, and MEF2, among others, thereby spreading their influence to regulating a vast array of genes (Kang *et al.*, 2003; Xia *et al.*, 2007; Allen & Utterman, 2007; Quinn *et al.*, 2001).

In this chapter, the role of SMAD proteins in the metabolic response to freezing by wood frog organs will be examined. Previous studies have given our lab insights which lead us to postulate a role of SMAD transcriptional activation in the wood frog during freezing. Here, SMAD regulation is investigated in the brain, heart, skeletal muscle, liver, and kidney of the wood frog during a freeze cycle. The transcriptional activity of SMAD proteins is also assessed by studying the transcript levels of downstream target genes. The results demonstrate that SMAD transcription factors do play a role in the transcriptional regulation of freeze-responsive genes.

4.2. MATERIALS AND METHODS

4.2.1 Animals and tissue collection

Male wood frogs were treated, sacrificed, and tissue collected as previously described in Chapter 2.

4.2.2 Protein Isolation and Western Blotting

Total protein as well as cytoplasmic and nuclear protein extracts were isolated and chemically treated as previously described in Chapter 2. Samples were run on SDS-PAGE and transferred onto a PVDF membrane. Table 4.1 below is a summary of the probing conditions used for detecting SMAD proteins.

Table 4.1. Summary of SMAD antibody experimental conditions used in western blots

Primary Antibody	Size	Blocking Condition	Dilution	Probing Conditions	Company
Rabbit anti-phospho-SMAD1/5 Ser463/465	~50 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	Cell Signalling
Rabbit anti-SMAD2	~50 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	Cell Signalling
Rabbit anti-phospho-SMAD2 Ser465/467	~50 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	Cell Signalling
Rabbit anti-SMAD3	~50 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	GenScript Corp.
Rabbit anti-phospho-SMAD3 Ser425	~50 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	GenScript Corp.
Rabbit anti-SMAD4	~60 kDa	5% w/v milk, 10 minutes	1: 1000	Overnight incubation	Cell Signalling

4.2.3 RNA Isolation and RT-PCR

Total RNA was isolated and used to synthesize cDNA as previously described in Chapter 2. Primers were designed, tested, and PCR products were sent for sequencing in order to confirm that the target gene had been amplified. Relative RT-PCR was then conducted to determine expression levels of selected genes. The following table summarizes the parameters used for the RT-PCR reactions.

Table 4.2 Optimal conditions for RT-PCR amplification of SMAD related genes

Gene	Primers	T_M	Size
α-tubulin	Forward: 5'-AAGGAAGATGCTGCCAATAA-3' Reverse: 5'-GGTCACATTTCACCATCTG-3'	53°C	600 bp
Smad2	Forward: 5'-TCTGAAATTTGGGGACTGAG-3' Reverse: 5'-CTGTGTCCATRCTTTGRTT-3'	60°C	480 bp
Smad4	Forward: 5'-CGCTTCTGCTTAGGTCAGCTGTC-3' Reverse: 5'-AGGGAAAGGTGTGCAGCTGGAGT-3'	60°C	150 bp
Serpine1	Forward: 5'-CATGTACCACTGGTCCGCCATCA-3' Reverse: 5'-CTGAGAACATGTCTTCAATGCCG-3'	60°C	150 bp
Myostatin	Forward: 5'-CGMCTGGAACARGCWCCTAA-3' Reverse: 5'-TGCCAARTWCCAGTGCCTGG-3'	60°C	400 bp
TSC22D3	Forward: 5'–TCGTKGCCATYGACAACAAG-3' Reverse: 5'–CTCTGGRCTKGCCAGGKTCT-3'	65°C	150 bp

4.3. RESULTS

The effects of freezing on the expression of SMAD proteins was investigated in wood frog brain, heart, skeletal muscle, liver, and kidney. This was achieved by using immunoblotting to evaluate protein levels in soluble protein extracts from control (5°C acclimated), frozen (24 hours at -2.5°C) and thawed (8 hours back at 5°C after 24 h frozen) frogs. The antibodies used were those that detected SMAD1, SMAD2, SMAD3, and SMAD4 as well as selected phosphorylated forms of SMADs 1-3. SMADs 1-3 were detected as single strong bands at a molecular weight of approximately 50 kDa whereas SMAD4 was found at ~60 kDa, all within the expected MW range of SMAD proteins in other vertebrates.

4.3.1 Effects of freezing on SMAD Expression

Tissue specific changes were observed for the SMAD proteins in response to freezing. There were not many significant changes observed in SMAD proteins in brain (Fig. 4.2A). SMAD4 protein content rose significantly by 1.6-fold in brain of 24 h frozen frogs but then decreased again when frogs were thawed for 8 h although levels were still significantly higher (1.2-fold) than control values. In addition, the phosphorylation state of SMAD3 changed significantly during freeze thaw, with the relative amount of pSMAD3 phosphorylated on serine 425 being 1.6- and 2-fold higher in brain of 24 h frozen and 8 h thawed frogs, respectively.

Muscle tissues showed greater changes in SMAD expression during freeze/thaw. Cardiac muscle showed significant decreases in SMAD2 protein content and in the relative phosphorylation states of phospho-SMAD1 and phospho-SMAD2. During freezing, the relative amount of phosphorylated SMAD1 Ser 465 was reduced to ~60% of the control value but levels rebounded when frogs thawed. SMAD2 protein levels and p-SMAD2 Ser465/467 content decreased to 54% and 35% their respective control values in heart from 24 h frozen frogs and after 8 h thawing these values remained low, 37% and 39% of control values. SMAD3 protein levels did not change during freeze/thaw but the content of phospho-SMAD3^{ser425} increased by 1.9- in frozen heart and remained high at 1.6-fold greater than control values after thawing. SMAD4 protein levels did not change with freeze/thaw in cardiac muscle (Fig 4.2B).

The hind leg skeletal muscle response was without a doubt the tissue with the most profound SMAD activation in response to freeze/thaw (Fig. 4.3A). The amount of phosphorylated SMAD1 Ser 465 increased by 1.4-fold during 24 h freezing and remained high after 8 h thawed (1.6-fold above controls). SMAD2 levels and amount of p-SMAD2 Ser465/467 both increased to 1.6- and a 1.8-fold above control values during freezing and were 1.6- and 2.4-fold higher than controls in thawed muscle, respectively. SMAD3 protein levels were unchanged during freezing but rose by 30% during thawing. The phosphorylation site of SMAD3 serine 425 increased by 34% and 45% in during freezing and thawing, respectively. Protein levels of SMAD4 were unchanged over the freezing cycle.

In liver, freeze/thaw had only a few effects on SMADs. SMAD2 protein levels were unchanged in liver of 24 h frozen frogs but levels decreased by 30% after thawing (Fig. 4.3B). SMAD4 protein levels decreased by 40% during freezing rebounded partially after 8 h thawed. Only SMAD3 showed a change in phosphorylation state with a 2.4-fold increase in phospho-SMAD3^{ser425} during freezing; levels remained high after thawing (2.1 fold above control). In kidney, p-SMAD1^{ser465} content rose by 2.2-fold during freezing but fully returned to control values after thawing (Fig. 4.4). By contrast, both SMAD2 protein and phosphorylated SMAD2$^{ser465/467}$ content were unaffected during freezing but increased strongly by 1.9- and 1.8-fold during thawing. SMAD3 levels decreased to 78% of the control value during freezing and so too did phospho-SMAD3^{se425} content that was 21% and 17% lower than control values during freezing and thawing, respectively. SMAD4 levels decreased by 43% during thawing.

RT-PCR was also performed to determine if the mRNA expression levels of *smad2* and *smad4* changed in response to freezing. Gene specific primers were designed (as described in Chapter 2) for *smad2* and *smad4* and were used in RT-PCR reactions with cDNA made from total RNA from control and 24 h frozen frogs. Tissue analysis found that with the exception of kidney, all studied tissues showed a relative increase in *smad2* transcript levels during freezing. Brain, heart, skeletal muscle, and liver showed 1.6-, 2.1-, 1.46-, and 1.48- fold increases in mRNA levels after 24 h freeze exposure (Fig 4.5A). *Smad4* transcript levels were stable in brain, and heart increased significantly in skeletal muscle and liver by 1.36- and 1.9-fold whereas kidney witnessed a 53% decrease in transcript levels in response to freezing (Fig 4.5B).

4.3.2 Cellular Localization of SMAD Transcription factors

The observed changes in SMAD protein and phosphoprotein contents suggest that SMAD-mediated gene expression is important to freeze/thaw adaptation but to upregulate gene expression transcription factors must be in the nucleus. Hence, nuclear levels of the R-SMADs were examined in nuclear fractions isolated from control and frozen (4 h and 24 h) skeletal muscle and liver. Fig 4.6A shows freezing had only a few effects on the nuclear content of SMADs in muscle. Phospho-SMAD2 content was prominently regulated with 1.52- and 1.77-fold increases in nuclear levels after 4 and 24 h of freezing, respectively. By contrast, the nuclear contents of p-SMAD1 and p-SMAD3 levels were reduced by 48% and 37% during short term freezing but returned to control levels in 24 h frozen frogs. In the liver p-SMAD1 and p-SMAD3 also decreased in the nucleus of 4 h frozen frogs by 30% and 50%, respectively, but contrary to the muscle results, liver p-SMAD2 also decreased by 40% during short term freezing as did total

SMAD2 protein (Fig 4.6B). The phosphorylation state of all three SMADs was elevated again in 24 h frozen frogs, p-SMAD1 and p-SMAD2 levels returning again to control values whereas p-SMAD3 content increased to 2.5-fold higher than control. Nuclear SMAD3 levels also rose by 1.35-fold 24 h frozen frogs.

4.3.3 Transcriptional regulation of downstream genes of SMAD Tfs

Relative increases in the amount of phosphorylated transcriptionally-active SMAD proteins during freezing suggested that some downstream SMAD target genes are probably upregulated in response to freezing. Therefore, relative RT-PCR analysis was used to assess transcript levels of selected target genes (*serpine1*, *myostatin*, *tsc22d3*) in tissues from control versus 24 h frozen frogs. The proteins encoded by these genes are SERPINE1 which is plasma serine protease inhibitor that inhibits plasminogen activation, myostatin which is a negative regulator of skeletal muscle mass, and TSC22 which is a transcription factor regulated by TGF-β signalling.

Serpine1 was found to be ubiquitously expressed in all five wood frog tissues tested. Furthermore, with the exception of kidney, *serpine1* transcripts were significantly up-regulated in frog tissues in response to freezing. Brain, heart, skeletal muscle, and liver showed 1.83-, 1.95-, 2.44-, and 2.46-fold increases in mRNA levels during freezing, respectively (Fig 4.7A).

The *myostatin* transcripts were only present in brain and skeletal muscle extracts. Heart, liver, and kidney did not have detectable levels of *myostatin* mRNA. Interestingly, the brain *myostatin* transcript levels were suppressed to 66% of their control values after

24 h of freezing whereas the skeletal muscle transcripts showed a 2.14-fold increase in transcripts after 24 h of being frozen (Fig 4.7B).

The mRNA levels of *tsc22d3* were also assessed in response to freezing. *Tsc22d3* was universally expressed in the wood frog tissues studied. The brain and kidney showed no significant changes in *tsc22d3* transcript levels during freezing but cardiac and skeletal muscle along with liver showed significantly higher mRNA levels with fold changes of 1.48-, 2.17-, and 1.81-fold, respectively (Fig 4.8).

4.3.4 Nucleotide and Amino Acid Sequence Analysis

As described in Chapters 2 and 3, the RT-PCR products for the genes of interest were sent for sequencing and the nucleotide sequences obtained were analyzed. Sequences were confirmed as encoding the desired products by using a nucleotide BLAST search as well as mRNA alignments using DNAMAN. The appropriate ORF was confirmed using protein BLAST and was also used for domain study.

Analysis of smad sequences

The primers designed for *smad2* gave a PCR product was confirmed as encoding a portion of the *smad2* sequence. The nucleotide sequence obtained contained 435 bp which translated into a 145 amino acid polypeptide which spanned the region covering amino acid residues 100 to 245 on the vertebrate protein. This segment of wood frog SMAD2 nucleotide sequence represented about 33% of the full length of the typical vertebrate 437 amino acid protein. Part of the obtained region corresponds to the MH1 domain of SMAD2 (Fig 4.9A). The obtained wood frog partial sequence was compared with equivalent portion of the SMAD2 sequences from Western clawed frog, human,

chicken, and zebrafish. The homology analysis showed that wood frog SMAD2 has 98% homology with terrestrial vertebrates, and 91% with zebrafish (Fig 4.9A).

The *smad4* PCR product was also confirmed as encoding a partial fragment of the *smad4* gene. The largest PCR product gave a partial nucleotide sequence of 268 bp and encoded a polypeptide sequence of 89 amino acids (Fig 4.9B). That represents about 18% of the full length *Xenopus* protein that is typically 482 amino acids long. This region was found to be situated in the MH2 domain of vertebrate SMAD4 encoding amino acid residues 360 to 449. The polypeptide sequence was also compared with SMAD4 sequences from the Western clawed frog, human, and zebrafish and was found to have a 97%, 94%, and 90% conserved homology, respectively (Fig 4.9B).

Analysis of downstream target sequences

The PCR product retrieved from the designed *serpine1* primers was confirmed as encoding the desired product, which was found to consist of 17% the *serpine1* sequence. The 209 nt amplicon coded a polypeptide sequence of 69 amino acids spanning the region from amino acids 263 to 332 on the *X. laevis* SERPINE1 protein which is 403 aa in size. SERPINE1 (Fig 4.10A). A conserved domain analysis found that the obtained sequence corresponds to a Serpin superfamily domain. The homology analysis of the polypeptide found that the wood frog sequence had 77%, 57%, 42%, and 34% similarities with the African clawed frog, human, zebrafish, and chicken sequences, respectively (Fig 4.10.A).

The *myostatin* PCR product gave a 369 nucleotide sequence which was determined to be the target gene. The mRNA sequence was used as a template for

translation and the correct ORF was shown to encode a 122 amino acid polypeptide (Fig 4.10B) that represented about 33% of the length of the typical vertebrate protein that ranges from 79 to 201 amino acids in the 4 comparison species examined. A domain analysis found that the obtained myostatin belonged to the TGF-β propeptide superfamily of proteins. The wood frog sequence was compared to the myostatin sequences from human, chicken, zebrafish, and the western clawed frog. The analysis revealed that the partial myostatin sequence wood frog shares 70% homology with terrestrial vertebrates and 64% with bony fish (Fig 4.10B). The polypeptide was also found to code the region from amino acid residue 77 to 199 as compared with the clawed frog sequence.

Gene specific primers designed for *tsc22d3* produced a 149 nucleotide PCR product, which through analysis was confirmed to be a partial amplicon of *tsc22d3*. The translated ORF for the nucleotide sequence gave a 49 amino acid sequence (Fig 4.11) which was confirmed to be TSC22D3. A domain analysis showed that the sequence belongs to the TSC22 superfamily. Furthermore, sequence analysis revealed that the obtained sequence shared greater homology with the TSC22D3 than with any of the other family members. The partial sequence for wood frogs corresponded to amino acids 38 and 87 of the 138 amino acid protein from *S. tropicalis*. A homology analysis found that the obtained TSC22D3 wood frog sequence shared 86% homology with human, 74% with zebrafish and chicken, and 69% with the Western clawed frog amino acid sequences (Fig 4.11).

4.4. DISCUSSION

The present study confirms that the SMAD family of transcription factors are subject to transcriptional regulation in response to freezing in the freeze-tolerant wood frog, *R. sylvatica*. To date, much of the TGF-β/SMAD research has been conducted on in the fields of development and differentiation (Massagué *et al.*, 2005). The roles of SMAD proteins in response to cellular stresses are only beginning to be elucidated. This work is novel in the sense that it the first to explore the responses and regulation of SMAD transcription factors in the phenomenon of natural freeze tolerance.

SMAD Tf regulation in response to freeze exposure

SMAD protein levels did not correlate well with *smad* transcript levels. In brain, cardiac and skeletal muscle, and liver, *smad2* transcript levels were up-regulated during freezing without a corresponding increase in SMAD protein levels. An explanation might be that this *smad2* up-regulation is anticipatory and leads to an accumulation of SMAD2 over the long term in the frozen state or, probably more likely, supports a rapid synthesis of SMAD2 as soon as thawing begins. This would implicate SMAD2 in a recovery role that helps to readjust cells for normal functions and/or reverse any freeze-induced damage when the animal thaws. This would explain the observed up-regulation of SMAD2 observed in skeletal muscle during 24 freezing and 8h thawing, and kidney, where the protein levels increase during recovery. *Smad4* transcript levels showed a similar story with only kidney showing a correlation between mRNA levels and protein levels. Thus, SMAD protein regulation in the wood frog has several layers of regulation, from transcriptional to translational, which appear to regulate independently of each other.

The analysis of SMAD protein levels showed an interesting variety of tissue-specific responses to the freeze/thaw cycle. The phosphorylation sites on SMAD1, SMAD2, and SMAD3 that were analyzed in phosphoprotein immunoblots are all found in the C-terminal regulatory region of the SMAD proteins which dictate activation when phosphorylated (Wrighton *et al.*, 2009; Souchelnytski *et al.*, 1997). Not surprisingly, each tissue seemed to have a unique pattern of response both for total SMAD protein levels and for the relative phosphorylation of the serine regulatory sites that were assessed. Protein levels of the three SMAD isoforms behaved differently in response to freeze/thaw and no consistent trend was observed. SMAD3 levels were only affected during thawing in two tissues, rising by 30% in skeletal muscle but decreasing by 22% in muscle. SMAD4 responded to freezing in two tissues (increasing in brain by 60% and decreasing in liver by 40%) and also decreased in thawed kidney by about 50%. SMAD2 levels changed in all tissues except brain and were strongly increased in muscle during freezing by 60% and in kidney during thawing by 90%. By contrast levels fell during freezing in heart and thawing in liver.

Changes in the phosphorylation state of SMAD proteins also showed tissue-specific responses to freeze/thaw. Levels of p-SMAD1 and p-SMAD2 showed the same pattern of change in all tissues: levels were unchanged in brain and liver, decreased in heart, and increased strongly in skeletal muscle and kidney. The content of p-SMAD1 and p-SMAD2 increased in response to freezing and remained high during thawing whereas in kidney p-SMAD1 rose by 2.2 fold during freezing but p-SMAD2 increased only during thawing. The phosphorylation state of SMAD3 was affected by freeze/thaw in all tissues and showed perhaps the strongest and most consistent response. The

phosphorylation levels of p-SMAD3 increased during freezing by 1.3- to 2-fold in brain, heart, muscle and liver and remained elevated during thawing. This suggests that activation of SMAD3 by phosphorylation during freezing may be triggering a common gene response in most tissues that could enhance survival.

Analysis of the nuclear extracts from muscle and liver revealed a peculiar twist to the SMAD story. In muscle, analysis of the R-SMADS revealed that nuclear protein levels remained relatively stable for the 24 hours of freezing. The only increase was found in phospho-SMAD2 which increased significantly in response to short and long term freezing, although total SMAD2 protein did not change. P-SMAD1 and p-SMAD3 both were found to be decreased at 4h freezing. In liver there were only small changes in total SMAD2 and SMAD3 but SMAD3 content rose during thawing as did the amount of p-SMAD3 which increased strongly during thawing by 2.5-fold. The observed changes correlate with the phosphorylation levels of p-SMAD3 detected in total tissue extracts. A peculiarity was in the nuclear levels of p-SMAD1 and p-SMAD3 which, according to their phospho-levels, were expected to up in nuclear muscle isolations.

Signals regulating SMAD Tfs in during freeze exposure

The signals responsible for SMAD regulation during freeze/thaw of wood frogs remain to be elucidated. Whether the signals are coming from TGF-β cytokines or elsewhere is currently not known. Ice nucleation in the wood frog typically initiates at 45 min incubation at -3°C, however there is a approximately 6h until blood completely freezes therefore there is plenty of time for hormones/cytokines to activate membrane receptors (Storey & Storey, 2004b). Furthermore, studies on human endothelial and

hepatic cells have shown that TGF-β/SMAD signals undergo activation in response to hypoxia (Akman *et al.*, 2001; Shi *et al.*, 2006; Zhang *et al.*, 2003). In addition, activation of TGF-β/SMAD, SMAD3 particularly, has also been observed in hyperglycaemia in mice (Isono *et al.*, 2002). The signalling cascades which activate these signals however remain to be discovered. In high glucose environments it appears that TGF-β has a link to the activation (Xia *et al.*, 2008) however in the case of hypoxia, SMAD signalling might be induced independent of TGF-β. Interestingly, in hypoxic stress TGF-β receptors are up-regulated but there is no evidence that TGF-β cytokines are responsible for the observed stimulation (Akman *et al.*, 2001). Ismael *et al.* (2008) found that TGF-β is capable of autocrine activity, thereby adding complexity to this pathway. Thus, at the moment, the signals that induce SMAD activation are unknown, but there are many venues available.

Transcriptional regulation of SMAD target genes and roles in response to freeze-survival

The transcriptional activity of SMAD transcription factors was assessed by studying SMAD responsive gene expression. The selected genes are SERPINE1, myostatin, and TSC22D3, all of which are downstream of TGF-β signalling through SMADs (Das *et al.*, 2008; Allen & Unterman, 2007; Shibanuma *et al.*, 1992). These findings provide evidence that although not universal, SMADs are transcriptionally active in response to freezing in the wood frog.

SERPINE1, as its alternate name plasminogen activator inhibitor-1 describes, is a serine protease inhibitor which inhibits plasminogen activation. When cleaved, plasminogen becomes plasmin, an enzyme responsible for degradation of blood clots

(Das et al., 2008). It does this by reversibly binding to the active site of the plasminogen activators (both tissue-type and urokinase-type PA) in a 1:1 stoichiometric manner (Lijnen, 2005). In response to freezing in the wood frog, we find that with the exception of kidney, *serpine1* mRNA levels were observed to increase by at least 1.8-fold in the organs studied (Fig 4.7A). These results suggest that there clearly is a need for higher amounts of *serpine1* transcripts and hence SERPINE1 protein during freezing. These data provide a link to the previously observed up-regulation of fibrinogen transcripts in frozen wood frogs (Cai & Storey, 1997a). It appears that blood clot formation during thawing is encouraged by both formation and preservation of blood clots at site of injury. Puncturing of the microvasculature by ice crystals is one of the main injuries that cause failure after transplant of organs that were previously frozen and is the main reason why organ banking via cryopreservation is still not an option for transplant medicine. Animals that display natural freeze tolerance must have found the solution to this problem. One solution is the up-regulation of fibrinogen when frogs freeze. This would increase the clotting capacity of the plasma and help the frog to deal rapidly with any freezing punctures to the vasculature that is detected during thawing (Cai & Storey, 1997a). Up-regulation of SERPINE1 would complement this system because it would inhibit the breakdown of clots that are resealing injured sites in the microvasculature until such time as the blood vessel walls are adequately repaired. Interestingly, our laboratory has also found that the up-regulation of a number of serpin types is a widespread response in various animal species that enter stress-induced hypometabolic including hibernation by thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*), anaerobiosis in red-eared slider turtles (*Trachemys scripta elegans*), and freezing in painted turtles (*Chrysemys*

picta marginata) (Storey & Storey, 2007). Thus, the fact that SMAD proteins regulate *serpine1* gene expression further supports the evidence that SMADs are transcriptionally active in wood frogs during freezing bouts.

The expression of *myostatin* was also investigated as a target gene of SMAD transcription factors in freeze-tolerant frogs. Myostatin, also known as growth differentiation factor 8 (GDF-8) is a member of the TGF-β ligand superfamily which negatively regulates skeletal muscle mass (McPherron *et al.*, 1997). In mouse embryos, myostatin is expressed during very early development in the myotome compartment of development somites (McPherron & Lee, 1997). In adults, however, myostatin has been reported in a variety of cell types predominantly in skeletal muscles but also in hearts, mammary glands, and in brain of brook trout (Sharma *et al.*, 1999; Ji *et al.*, 1998; Roberts & Goetz, 2001). In the present study, myostatin was expressed in skeletal muscle and brain of wood frogs. In rainbow trout, two myostatin isoforms were identified: myostatin I was ubiquitously expressed and myostatin II was found in skeletal muscle and brain (Recan et al., 2001). Although not experimentally confirmed, bioinformatic analysis of the *Silurana (Xenopus) tropicalis* genome shows that there might also be two myostatin isomers in the Western clawed frogs. If this is the case, taken together with the findings for rainbow trout, it appears that the wood frog *myostatin* transcript that was only found in muscle and brain might be an ortholog of the myostatin II isomer in trout. From the homology analysis of the sequenced *myostatin* gene, this cannot be concluded since the wood frog shares 73% homology with both *S. tropicalis* isomers. Analysis of transcript levels showed that there was a 33% decrease in *myostatin* levels in brain and a 2.15-fold increase in skeletal muscle during freezing. These results suggest that in brain, there is no

need to up-regulate myostatin during freezing. However, in skeletal muscle, there appears to be a need for *myostatin* transcription and probably translation as well. Up-regulation of myostatin during freezing would contribute to metabolic arrest by inhibiting growth responses in skeletal muscles while animals are in the frozen, hypometabolic state. This would help to focus limited energy supplies on other metabolic functions that are key to long term viability in the frozen state.

The effects of freeze exposure on *tsc22d3* transcripts were also studied in the wood frog. TSC22D3, or TGF-β-stimulated clone 22 domain family 3 alternatively known as GILZ (Glucocorticoid-induced leucine zipper), is a transcription factor regulated by TGF-β signalling (Shibanuma *et al.*, 1992). In mammals and in *S. tropicalis* the TSC22 family consists of four members: TSC22D1-4. In this study, *tsc22d3* transcripts were up-regulated in heart, skeletal muscle, and liver of 24 h frozen frogs by 1.5-, 2.2-, and 1.8-fold, respectively. This family of transcriptional regulators remains largely unstudied although preliminary work has found that TSC22 is induced by a number of signals including TGF-β, glucocorticoids, tumor necrosis factor α, interferon-γ, interleukin-1β, and lipopolysaccharide (Soundararajan *et al.*, 2007; Ohta et al., 1996). Work with a fish (black tilapia, *Oreochromis mossambicus*) has found that the osmotic stress transcription factor-1 (OSTF-1) which appears to be an ortholog of TSC22/GILZ, is induced gill cells in response to hyperosmotic stress (Fiol & Kültz, 2005; Fiol *et al.*, 2006). This was also found in mouse kidney cells in response to osmotic stress (Fiol *et al.*, 2007). Surprisingly, *tsc22d3* transcript levels were stable in wood frog kidney but levels were elevated in heart, muscle and liver of frozen frogs. It is possible that the *tsc22d3* response in these organs could be involved in dealing with the osmotic stress on cells that

is imposed by ice formation in extracellular compartment. The promoter of *tsc22* has been found to have a SMAD-binding element, however if SMAD activation leads to *tsc22d3* transcription remains to be discovered. Nevertheless, it appears that there is transcriptional regulation of *tsc22d3* in the wood frog during freeze exposure.

Conclusion

The data in this chapter provide the first revelation that SMADs are regulated in response to freezing in the wood frog. Although for the most part there was limited activation of R-SMADs, SMAD3 was strongly phosphorylated in brain, heart, skeletal muscle, and liver of frozen wood frogs. This SMAD3 activation was consequently observed to transcriptionally regulate SMAD target genes. Freeze responsive transcriptional activation was demonstrated by the up-regulation of *serpine1*, *myostatin*, and *tsc22d3* transcripts during the 24 h freeze exposure. Future studies will reveal if the increases in transcript levels of these genes correlates with elevated levels of their respective gene products. Furthermore, the signals that transduce SMAD3 activation remain to be discovered. Other studies involving hypoxia, hyperglycaemia or osmotic stress have given us reason to suspect that there is a link between these cellular stresses and the observed freeze-responsive transcriptional activation of SMAD target genes in the frozen wood frog.

Figure 4.1: Regulation of SMAD transcription factors. The domain organization of Receptor mediated-SMAD proteins (**A**).showing the three domains, MAD Homology 1 (MH1), the linker and the MAD Homology 2 domains along with the S-X-S motif which when phosphorylated (**P**), activates the R-SMAD proteins. Regulation of SMAD signals is demonstrated in (**B**). When a TGF-β ligand binds the receptors, the signal is transduced to the cytoplasmic serine/threonine kinase domain of the TGF- β type I and II receptors which phosphorylate R-SMAD proteins. The phospho-SMADs form hetero-complexes with other R-SMADs and the co-SMAD, SMAD4, and travel to the nucleus where they transcriptionally regulate target genes. Co-factors (both activators and repressors) can affect their transcriptional activity.

Figure 4.1

A


MH1
Linker
MH2
S-X-S
P
P

B

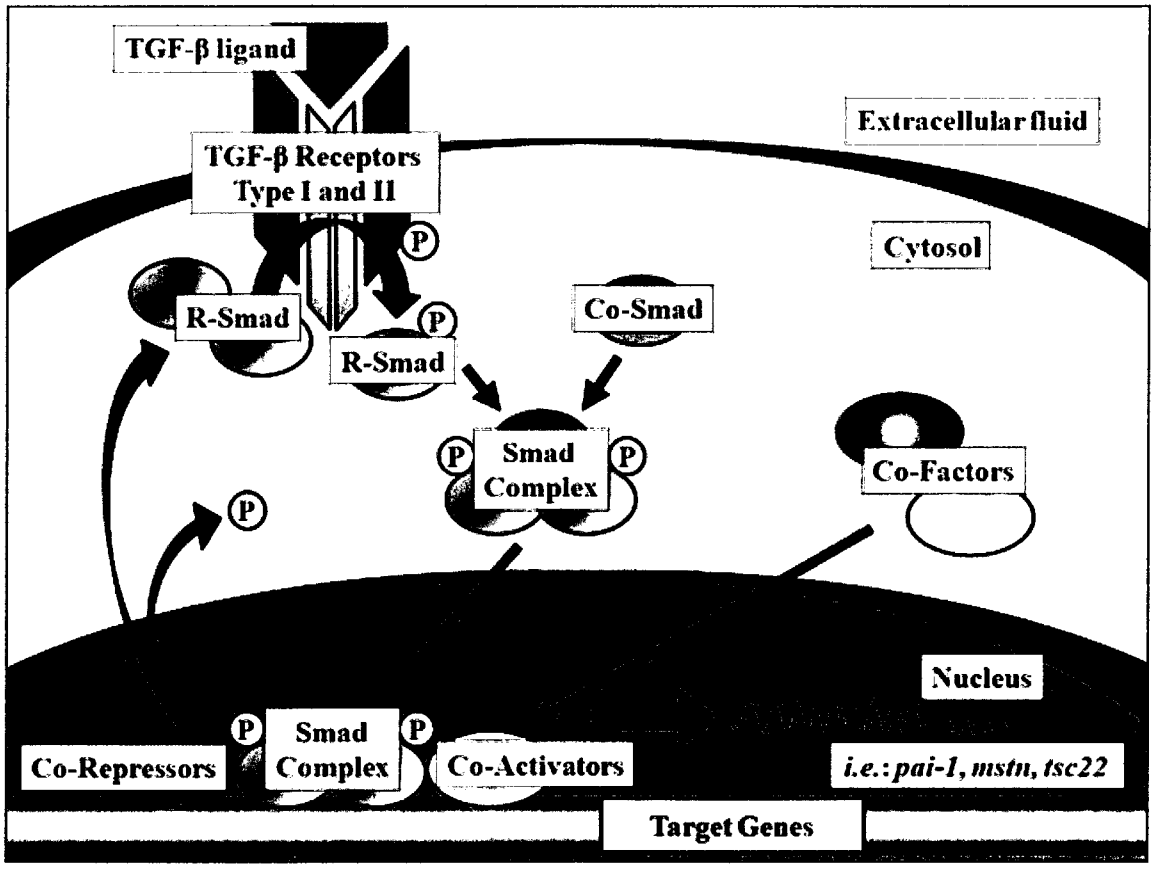

TGF-β ligand
TGF-β Receptors Type I and II
Extracellular fluid
Cytosol
R-Smad
R-Smad
Co-Smad
Smad Complex
Co-Factors
Nucleus
Co-Repressors
Smad Complex
Co-Activators
i.e.: pai-1, mstn, tsc22
Target Genes
P

Figure 4.2: Western blot analysis showing effects of freezing on SMAD proteins in wood frog brain and heart.

A) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog brain under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively. ★ - Significantly different from the corresponding control values using the Student's t-test, $P<0.05$.

B) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog heart under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 4.2

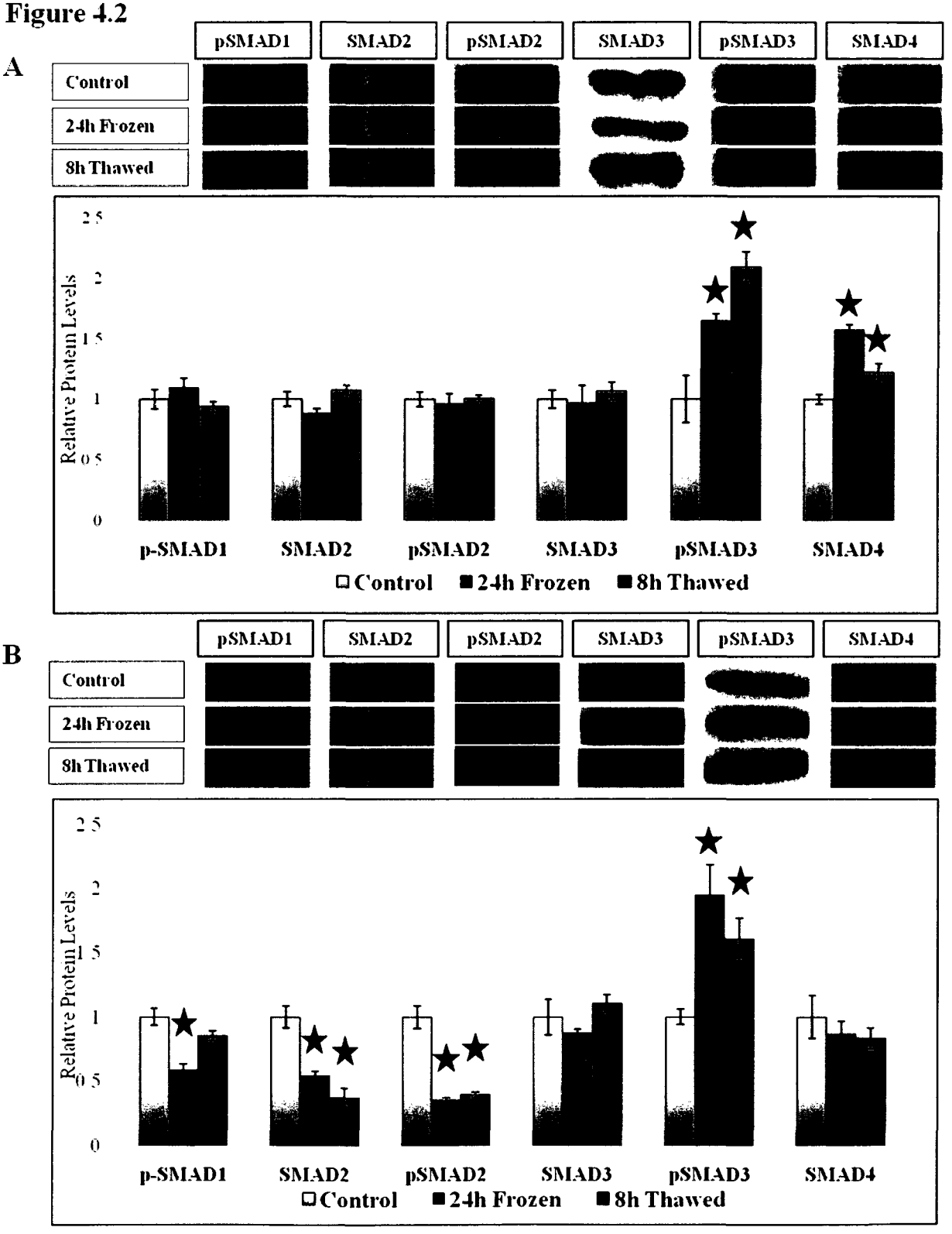

A
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
Relative Protein Levels
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
B
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
Relative Protein Levels
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed

Figure 4.3: Western blot analysis showing effects of freezing on SMAD proteins in wood frog skeletal muscle and liver.

A) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog skeletal muscle under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

B) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog liver under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 4.3

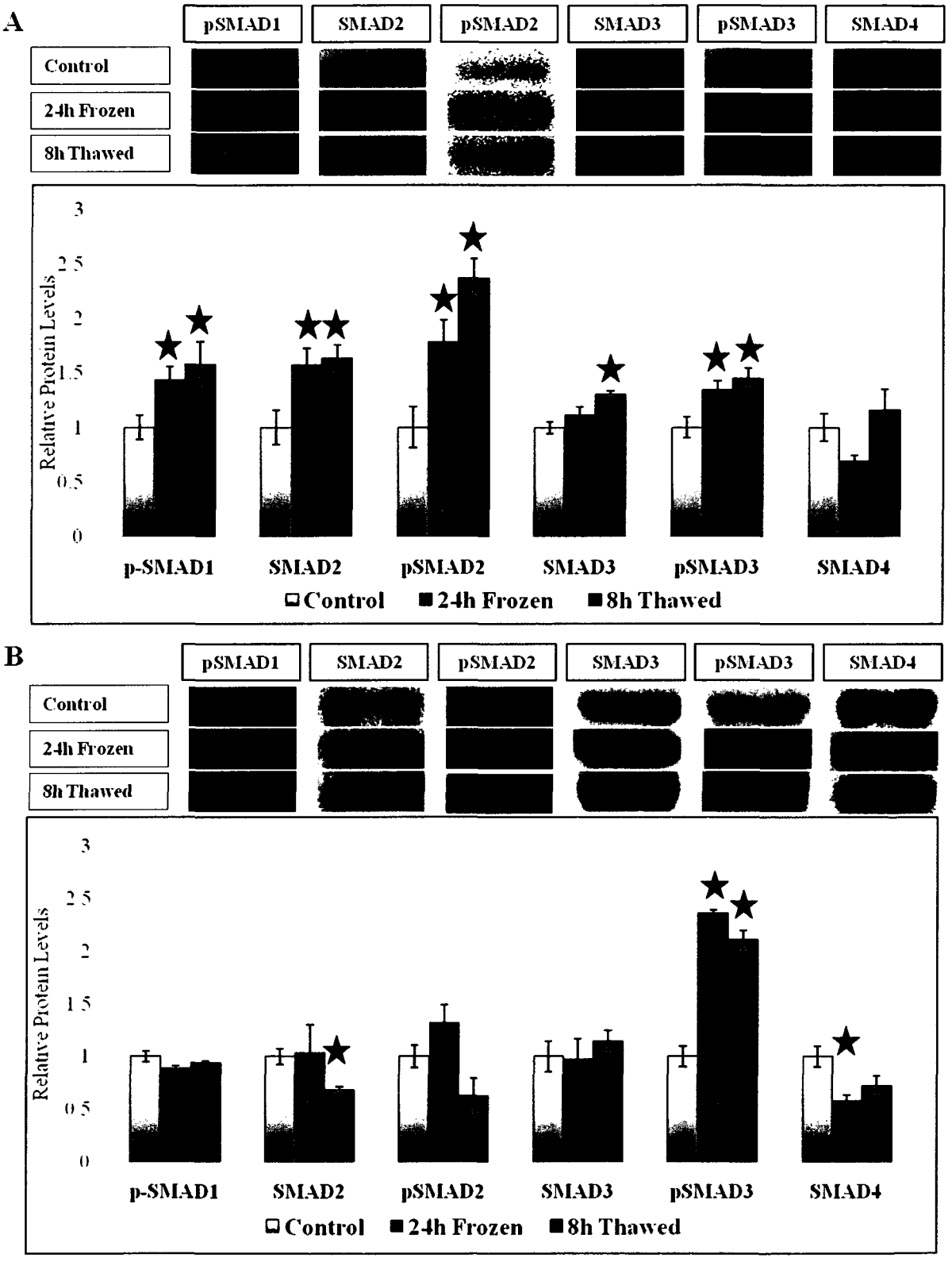

A
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
Relative Protein Levels
3
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
B
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
Relative Protein Levels
3
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed

Figure 4.4: Western blot analysis showing effects of freezing on SMAD proteins in wood frog kidney.

Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog skeletal muscle under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 4.4

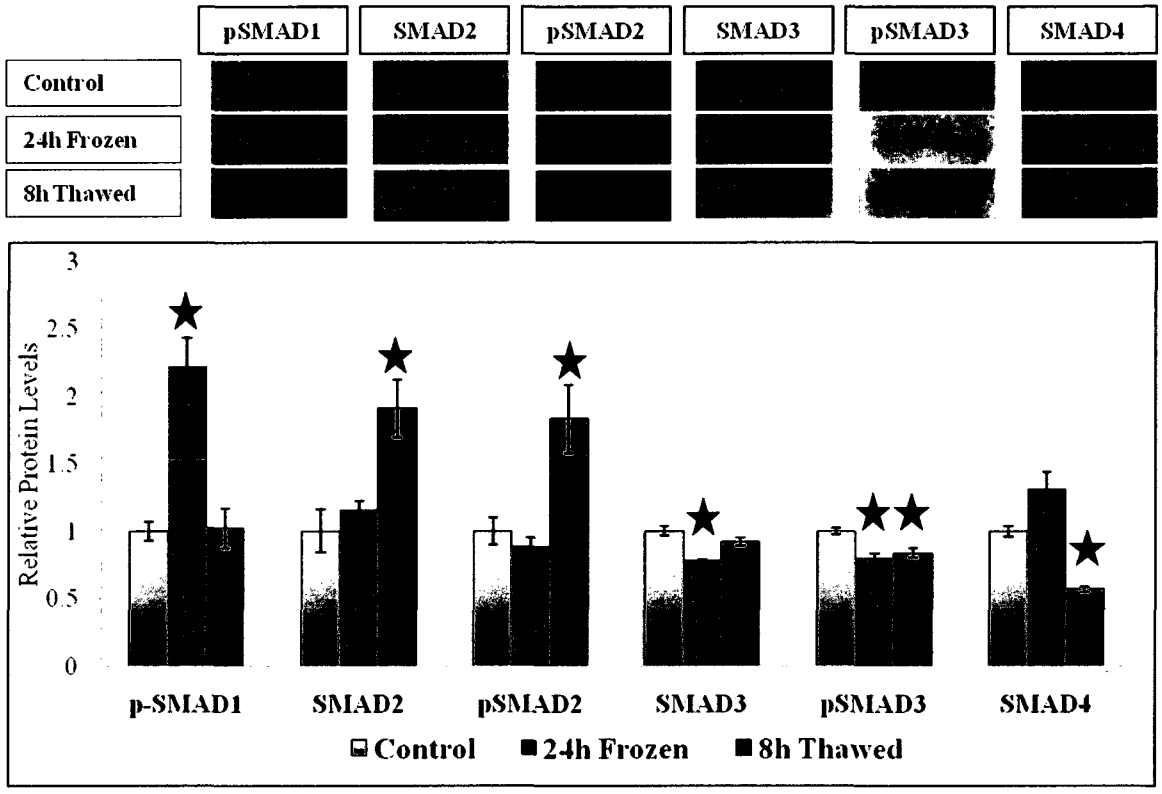

pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed
Relative Protein Levels
3
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
SMAD4
Control
24h Frozen
8h Thawed

Figure 4.5: RT-PCR analysis showing the effects of freezing on *smad2* and *smad4* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

C) Expression of *smad2* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *smad2* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen. ★ - Significantly different from control values using Student's t-test, $P<0.05$.

D) Expression of *smad4* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *smad4* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 4.5

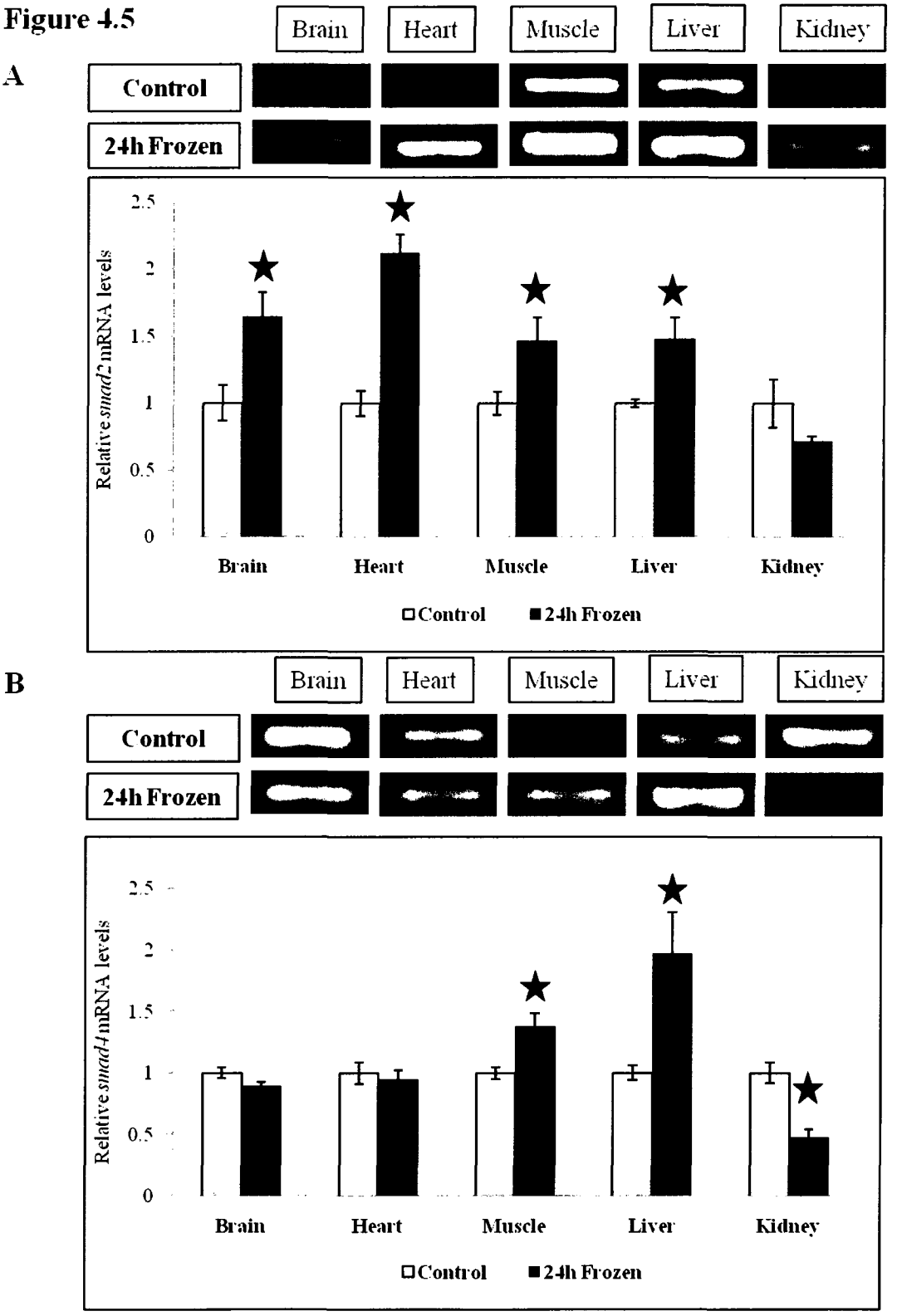

A
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative smad2 mRNA levels
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
B
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative smad4 mRNA levels
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 4.6: Western blot analysis showing effects of freezing on R-SMAD proteins in nuclear extracts of wood frog skeletal muscle and liver.

A) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog skeletal muscle under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

B) Expression and regulation of phospho-SMAD1$^{ser263/465}$, SMAD2, phospho-SMAD2$^{ser465/467}$, SMAD3, phospho-SMAD3^{ser425}, and SMAD4 in the wood frog liver under control, 24h freezing and 8h thawing conditions. Western blot and representative histograms showing normalized mean values (+ SEM, n=3-5 independent determinations) under the different experimental conditions. Light gray, black and dark gray bars represent control, 24 h frozen, and 8h thawed respectively.

Figure 4.6

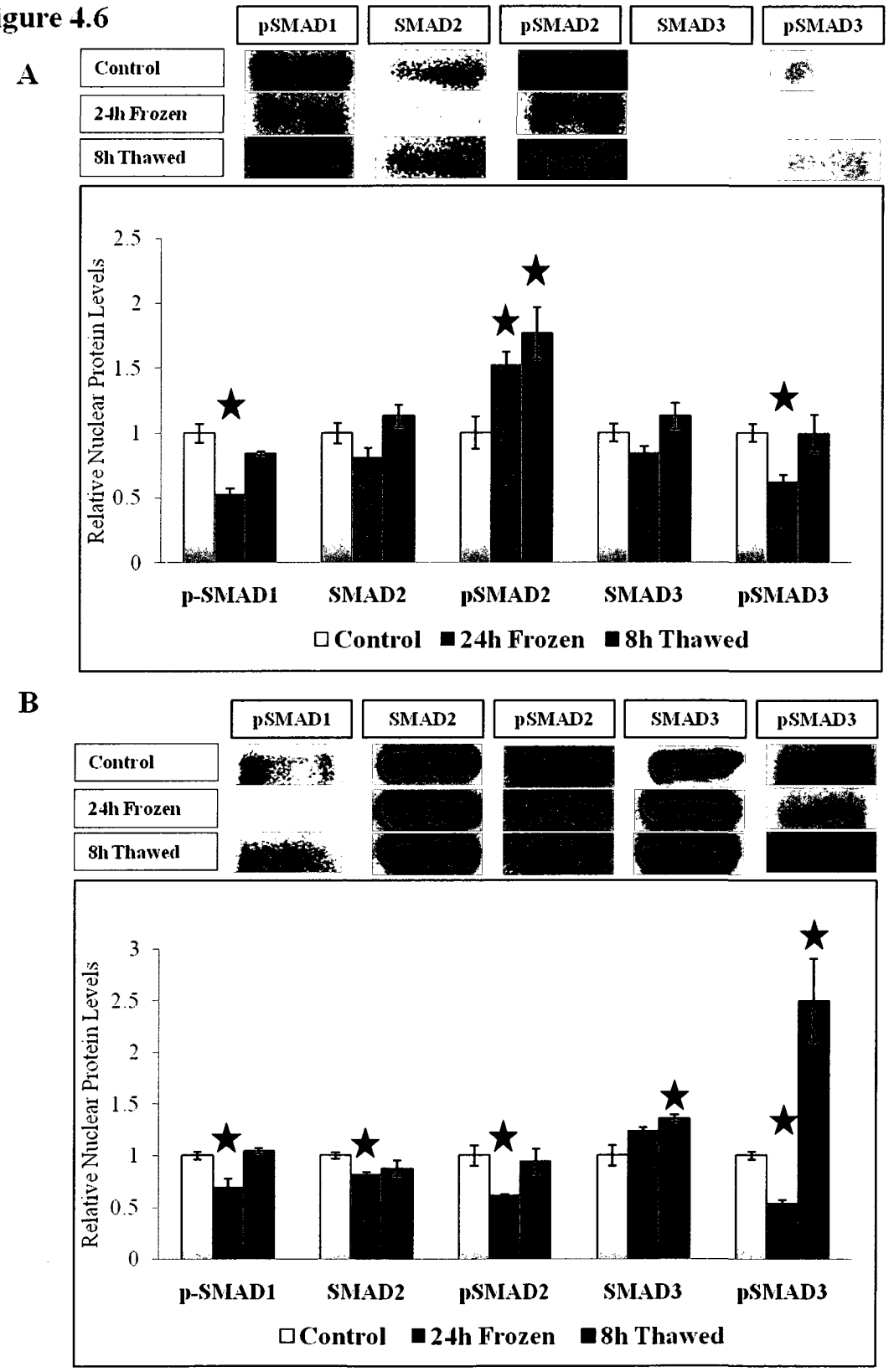

A
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
Control
24h Frozen
8h Thawed
Relative Nuclear Protein Levels
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
Control
24h Frozen
8h Thawed
B
pSMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
Control
24h Frozen
8h Thawed
Relative Nuclear Protein Levels
3
2.5
2
1.5
1
0.5
0
p-SMAD1
SMAD2
pSMAD2
SMAD3
pSMAD3
Control
24h Frozen
8h Thawed

Figure 4.7: RT-PCR analysis showing the effects of freezing on *serpine1* and *myostatin* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

A) Expression of *serpine1* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *serpine1* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen. ★ - Significantly different from the corresponding control values using the Student's t-test, $P<0.05$.

B) Expression of *myostatin* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *myostatin* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 4.7

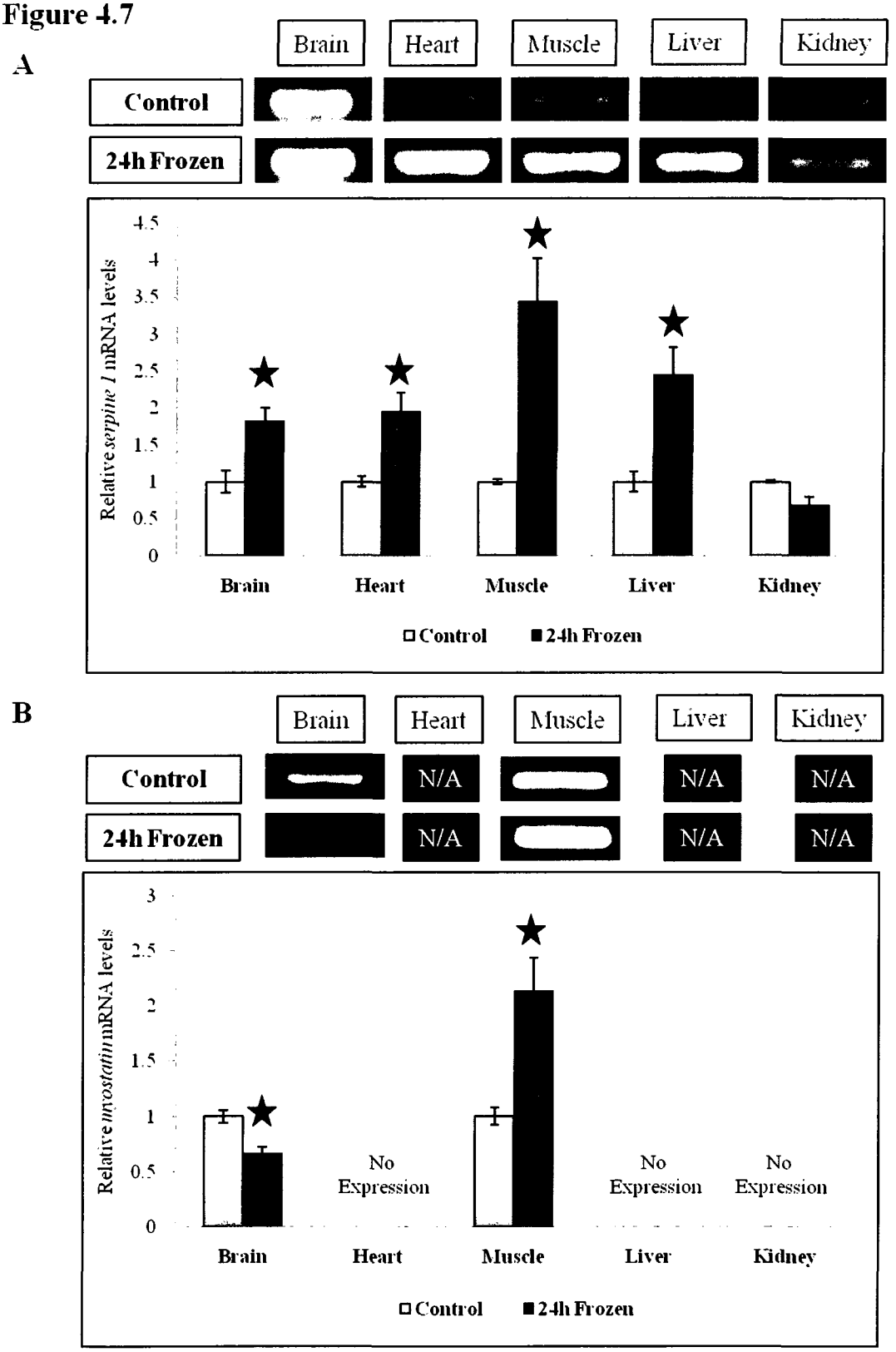

A
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative serpine 1 mRNA levels
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
B
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
N/A
N/A
N/A
N/A
N/A
N/A
Relative myostatin mRNA levels
3
2.5
2
1.5
1
0.5
0
No Expression
No Expression
No Expression
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 4.8: RT-PCR analysis showing the effects of freezing on *tsc22d3* mRNA levels in brain, heart, muscle, liver, and kidney of wood frog.

A) Expression of *tsc22d3* in response to freezing in the wood frog tissues. Representative RT-PCR bands of *tsc22d3* and corresponding histograms showing normalized mean values (± SEM, n=3-8 independent determinations) under the different experimental conditions. Light gray bars represent control and black represent 24 h frozen.

Figure 4.8

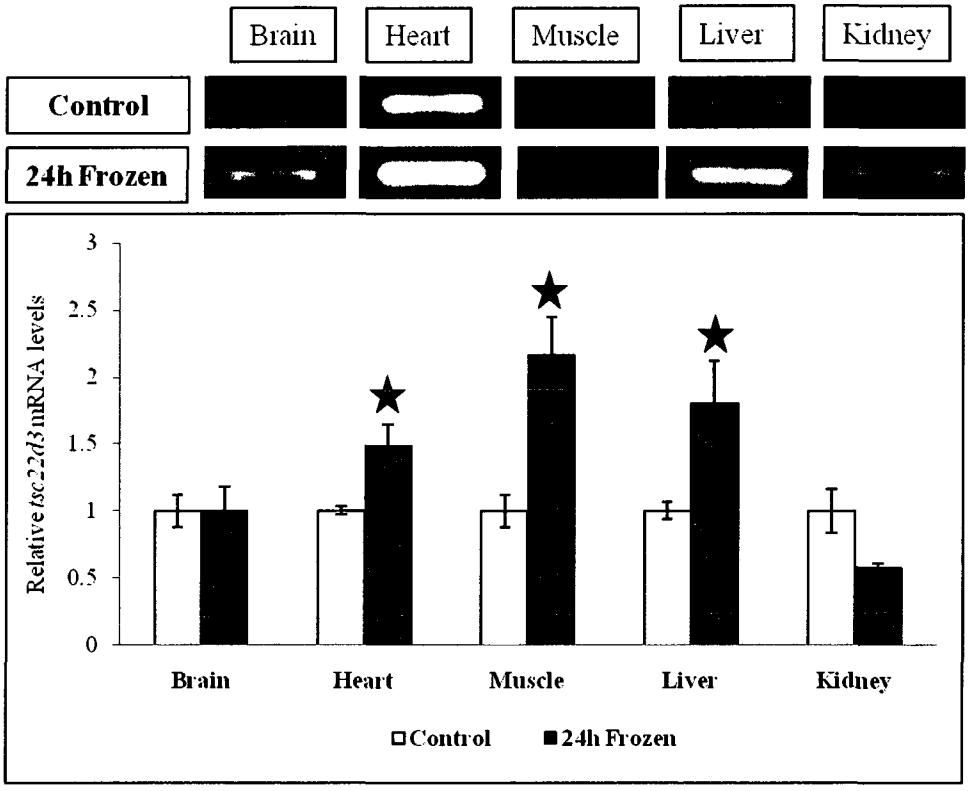

Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen
Relative tsc22d3 mRNA levels
3
2.5
2
1.5
1
0.5
0
Brain
Heart
Muscle
Liver
Kidney
Control
24h Frozen

Figure 4.9: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial SMAD2 and SMAD4 sequence.

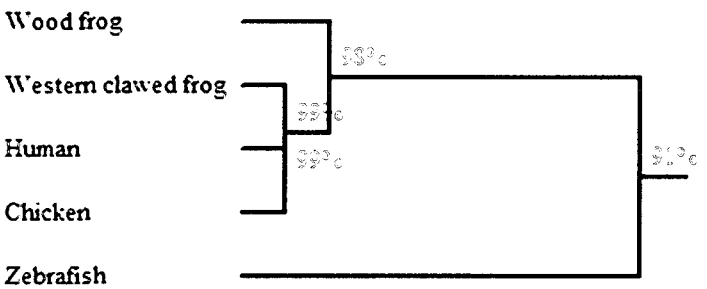
A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for SMAD2 gene product. The 145 amino acid polypeptide was identified as the region covering amino acid position 100 to 245 of SMAD2, which consist of 33% of the total size. An MH1 domain was identified (shown underlined). The *R. sylvatica* sequence was compared with *smad2* polypeptide sequences from African clawed frog (*X. laevis*, NP_001084329), Western clawed frog (*S. tropicalis*, NP_001011168), human (*H. sapiens*, NP_005892), chicken (*G. gallus*, NP_989892), and zebrafish (*D. rerio*, AAF06737). The percentage values correspond to the shared identity between the corresponding species.

B) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for SMAD4 gene product. The 89 amino acid polypeptide was identified as the region covering amino acid position 360 to 449 of SMAD4, which consist of 18% of the total size. The *R. sylvatica* sequence was compared with *smad4* polypeptide sequences from Western clawed frog (*S. tropicalis*, AAI35846), human (*H. sapiens*, NP_005350), and zebrafish (*D. rerio*, NP_001116172). The percentage values correspond to the shared identity between the corresponding species.

Figure 4.9

A

```
1          GCCCTTTACAGCTTCTCTGAACAAACCAGGTCTCTCGATGGCCGCCTTCAAGTGTCCCAC
1           A  L  Y  S  F  S  E  Q  T  R  S  L  D  G  R  L  Q  V  S  H
61         CGTAAAGGGTTACCGCACGTCATTTACTGCCGCCTCTGGCGCTGGCCAGACCTTCACAGT
21          R  K  G  L  P  H  V  I  Y  C  R  L  W  R  W  P  D  L  H  S
121        CACCACGAACTGAAGGCAATTGAAAATTGTGAATACGCATTTAATCTTAAGAAAGATGAA
41          H  H  E  L  K  A  I  E  N  C  E  Y  A  F  N  L  K  K  D  E
181        GTGTGCGTCAACCCTTATCACTATCAAAGAGTGGAAACTCCCGTCTTACCACCTGTATTA
61          V  C  V  N  P  Y  H  Y  Q  R  V  E  T  P  V  L  P  P  V  L
241        GTGCCGCGCCACACGGAGATCTTAACAGAACTTCCTCCTCTCGATGACTACCAACATTCC
81          V  P  R  H  T  E  I  L  T  E  L  P  P  L  D  D  Y  Q  H  S
301        ATTCCGGAAAACACTAATTTCCCAGCAGGCATCGAGCCTCAGAGCAACTATATACCAGAA
101         I  P  E  N  T  N  F  P  A  G  I  E  P  Q  S  N  Y  I  P  E
361        ACTCCACCTCCCGGGTACATTAGTGAAGATGGAGAAACTAGCGATCAGCAACTTAACCAA
121         T  P  P  P  G  Y  I  S  E  D  G  E  T  S  D  Q  Q  L  N  Q
421        AGCATGGACACAGAA
141         S  M  D  T  E
```



B

```
1          GGCGATCGCTTCTGCTTAGGTCAGCTGTCCAATGTACATACAACAGAAGCTATTGAGAGA
1           G  D  R  F  C  L  G  Q  L  S  N  V  H  T  T  E  A  I  E  R
61         GCAAGGTTACACATAGGGAAAGGTGTGCAGCTGGAGTGTAAAGGTGAAGGAGACGTGTGG
21          A  R  L  H  I  G  K  G  V  Q  L  E  C  K  G  E  G  D  V  W
121        GTGCGCTGTCTTAATGATCATGCCGTGTTTGTACAGAGCTACTACCTGGACAGAGAAGCT
41          V  R  C  L  N  D  H  A  V  F  V  Q  S  Y  Y  L  D  R  E  A
181        GGGCGTGCTCCCGGAGACGCCGTTCACAAAATTTATCCAAGTGCATATATTAAGGTATTT
61          G  R  A  P  G  D  A  V  H  K  I  Y  P  S  A  Y  I  K  V  F
241        GACCTGCGCCAGTGTCACCGACAAATAA
81          D  L  R  Q  C  H  R  Q  I
```

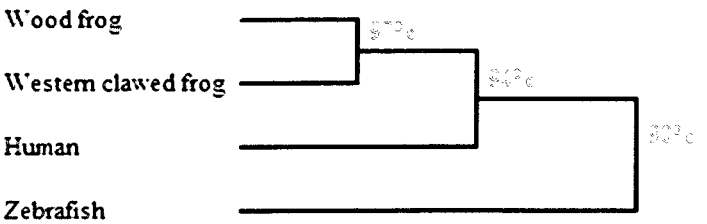


Figure 4.10: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial SERPINE1 and Myostatin sequence.

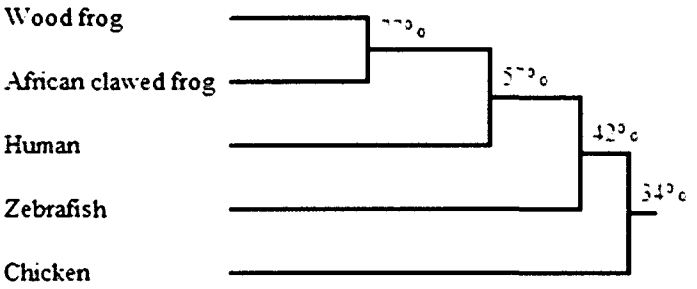
A) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for Serpine 1 gene product. The 69 amino acid polypeptide was identified as the region covering amino acid position 263 to 332 of Serpine 1, which consists of 17% of the total size. Homology tree produced from partial *serpine 1* amino acid alignments. The *R. sylvatica* sequence was compared with *serpine 1* polypeptide sequences from African clawed frog (*X. laevis*, NP_001090520), human (*H. sapiens*, NP_000593), chicken (*G. gallus*, NP_001077389), and zebrafish (*D. rerio*, NP_001108031). The percentage values correspond to the shared identity between the corresponding species.

B) Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for Myostatin gene product. The 122 amino acid polypeptide was identified as the region covering amino acid position 77 to 199 of myostatin, which consist of 33% of the total size. Homology tree produced from partial *myostatin* amino acid alignments. A TGF-β propeptide superfamily domain was found (shown underlined). The *R. sylvatica* sequence was compared with *myostatin* polypeptide sequences from Western clawed frog isomers 1 and 2 (*S. tropicalis*), human (*H. sapiens*, NP_005250), chicken (*G. gallus*, NP_001001461), and zebrafish (*D. rerio*, NP_571094). The percentage values correspond to the shared identity between the corresponding species.

Figure 4.10

A

```
1          TTATGAAGAACATGTACCACTGTCCGCCATCACGAACATCTTGACTCCAGAGCTCATCAA
1            Y  E  E  H  V  P  L  S  A  I  T  N  I  L  T  P  E  L  I  N
61         CCAATGGAAAGCCAATATGCAGAAATTAACCCGTCTCTTAGTTTTGCCTAAGTTTTCTCT
21           Q  W  K  A  N  M  Q  K  L  T  R  L  L  V  L  P  K  F  S  L
121        GGTCAGTGAGGTCAATCTGAAGACTCCGCTTGAGCGTCTCGGCATTGAAGACATGTTCTC
41           V  S  E  V  N  L  K  T  P  L  E  R  L  G  I  E  D  M  F  S
181        AGAGGAAAAAGCAGACTTCAGCCGCTTG
61           E  E  K  A  D  F  S  R  L
```



B

```
1          TGCCAAAGCTCCACCTCTGCAAGACATAATTGACCAGTATGATGTTCAAAGCGATGCAAG
1            A  K  A  P  P  L  Q  D  I  I  D  Q  Y  D  V  Q  S  D  A  S
61         CATTGAAGGGTCCTTAGAAGACGATGATTATCATGCCACTCCTGAAACATTCATTATAAT
21           I  E  G  S  L  E  D  D  D  Y  H  A  T  P  E  T  F  I  I  M
121        GCCAACACAAAATGATTTGGACTATGAAACATCCAAAACAAAAAAATGCTGTTATTTCAA
41           P  T  Q  N  D  L  D  Y  E  T  S  K  T  K  K  C  C  Y  F  K
181        ACTGAACTCTGAAATTCAGTCAACTAAAATATCCAAGGCGCAGTTATGGATACATCTAAA
61           L  N  S  E  I  Q  S  T  K  I  S  K  A  Q  L  W  I  H  L  K
241        GCCTGTCCAAATGCCTGCAACAGTTGTGGTGCAAATCCTGAGACTCATTAAACCCTTGAA
81           P  V  Q  M  P  A  T  V  V  V  Q  I  L  R  L  I  K  P  L  K
301        AGATGGTACAAGACACATTGGAATCCGAACATTAAAACTTGACATGAATCCAGGCACTGG
101          D  G  T  R  H  I  G  I  R  T  L  K  L  D  M  N  P  G  T  G
361        AACTTGGCA
121          T  W
```

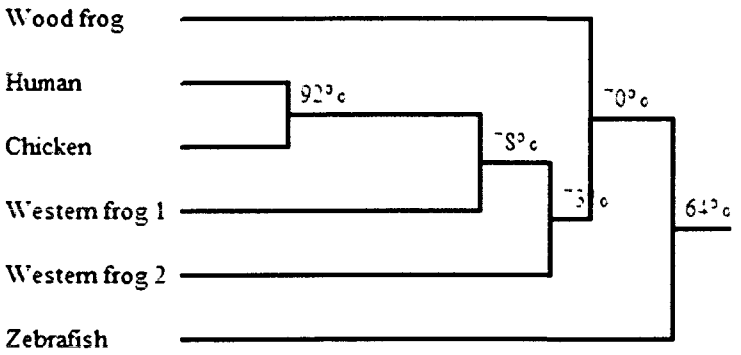
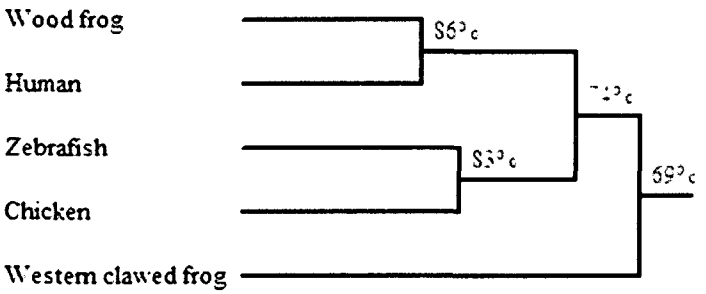


Figure 4.11: Nucleotide and deduced amino acid sequence for *R. sylvatica* partial TSC22D3 sequence.

Nucleotides and amino acids are numbered on the left. The putative amino acid sequence was deduced by blasting the open reading frames and confirming that the deduced amino acid sequence codes for TSC22D3 gene product. The 49 amino acid polypeptide was identified as the region covering amino acid position 38 to 87 of TSC22D3, which consist of 35% of the total size. Domain analysis revealed that the identified sequence belongs to the TSC22 superfamily of proteins (shown underlined). Homology tree produced from partial *tsc22d3* amino acid alignments. The *R. sylvatica* sequence was compared with *tsc22d3* polypeptide sequences from Western clawed frog (*S. tropicalis*, AAH90571), human (*H. sapiens*, CAI41544), chicken (*G. gallus*, NP_001070702), and zebrafish (*D. rerio*, AAH68985). The percentage values correspond to the shared identity between the corresponding species.

Figure 4.11

```
1          TCGTGGCCATCGACAACAAGATCGAGCAAGCCATGGATTTGGTTAAAAGCCACCTTATGT
1             V  A  I  D  N  K  I  E  Q  A  M  D  L  V  K  S  H  L  M
61         ATGCAGTTCGAGAAGAGGTGGAGGTGCTGAAGGAGCAGATCAAAGAGTTAGTGGAGAAAG
20         Y  A  V  R  E  E  V  E  V  L  K  E  Q  I  K  E  L  V  E  K
121        ACTCTCAGCTGAACCGATGGAACACGGGG
40         D  S  Q  L  N  R  W  N  T  G
```



CHAPTER 5

GENERAL DISCUSSION

The wood frog, *R. sylvatica,* is the main model model for research on vertebrate freeze tolerance. Our laboratory has become a leader in the field of natural cryobiology, answering many questions regarding the physiological and biochemical adaptations and regulatory mechanisms that are involved in conferring animal freezing survival. This fascinating phenomenon has allowed us to shed light on the complex regulatory mechanisms that contribute to the survival of a stress that is normally fatal for the vast majority of animal species. The uniqueness of the wood frog in handling the wide array of cellular stresses that are triggered by freezing has intrigued us into exploring the avenues possible. Initial studies were focused on the physiological characteristics of the animal during freezing. As the stepping stones were in place, studies moved to focus on the biochemical regulation ensuring freeze survival. Next, research moved to the gene level and studies began to investigate the molecular biology of the wood frog, ranging from signal transduction cascades to gene expression. Novel freeze-induced genes were discovered and characterized (McNally *et al.*, 2002, 2003; Cai & Storey, 1997b). The signals regulating gene expression as well as the proteins which dictate this expression have been the focus of the research presented in this thesis. More specifically, I have analyzed the regulation of two specific transcription factors and their role in gene expression during freeze exposure in the wood frog.

5.1 Transcriptional regulation during cellular stress

When cells are faced with stresses, they either deal with it using the available arsenal at their disposal, or they seek advice from the nucleus which provides them with new "lines of defence" that better equip cells for handling the task at hand. Transcriptional regulation in

response to perturbation of homeostasis differs in response to different challenges to ensure that the appropriate suites of genes are upregulated under temperature shock, hypoxia, ischemia, osmotic stress, shear stress, *etc.*. Indeed, all of these stresses are components of freezing and, thus it is not surprising that when wood frogs are faced with freezing, there are multiple transcriptional responses. Despite the global metabolic suppression that reduces overall energy expenditure in the frozen state, multiple genes are still upregulated to provide the changes in type and amount of specific proteins that are needed to deal with the consequences of freezing on cellular metabolism. Furthermore, upregulation of other genes contributes to metabolic rate depression in the frozen state as well as to enhancement of various protective mechanisms (e.g. chaperones, antioxidant defences) that are needed to sustain long term viability while frozen and/or to help cells make controlled transitions back to normal metabolism during thawing. All of these genes are under the control of specific transcription factors that each mediate the upregulation of a suite of genes to deal with each of the many consequences of freezing.

Our lab first investigated freeze responsive gene expression using cDNA libraries and found several known genes that were up-regulated during freezing as well as three novel wood frog genes. Among the first genes that were shown to be upregulated as a result of cDNA library screening were myosin light chain 2, fibrinogen α and γ, hsp7, phosphoglycerate kinase 1, acidic ribosomal phosphoprotein P0, and ribosomal protein L7 (McNally, 2002; Cai & Storey, 1997a; Wu & Storey, 2009; Wu & Storey, 2005; Wu *et al.*, 2008). The three novel genes discovered to be freeze-responsive were *fr10*, *fr47*, and *li16* (McNally *et al.*, 2002, 2003; Cai & Storey, 1997b). Since then, our laboratory has focused much of our research on discovering the signalling pathways involved in transmitting freezing signals into nuclear up-regulation of selected genes and mapping the target genes/proteins that are downstream of these signals.

Advancements in DNA microarray technology subsequently allowed us to evaluate huge numbers of genes and discover many putative freeze-responsive genes such as ferritin light chain, glutathione-S-transferase θ, hypoxia-inducible factor 1α, receptor for advanced glycosylation end products (RAGE), among others (Storey, 2004a,b). However, results from heterologous array screening require validation by other techniques, such as RT-PCR to quantify mRNA levels and immunoblotting to analyze protein levels. Nonetheless, data from DNA array screening implicated the importance of several transcription factors to freezing survival, and this, coupled with studies that specifically analyzed mitogen-activated protein kinase signalling cascades (p38, JNK) in wood frog tissues (Greenway & Storey, 2000) as well as data from specialized transcription factor arrays, indicated that quite a number of signal transduction pathways are involved in mediating cell/organ responses to freezing in freeze tolerant frogs. To date, some of these signal transduction pathways have been evaluated in wood frog organs. These include the several stress-activated transcription factors: STATs, Nrf-2, ATFs, FoxOs, ChREBP, ETS-1/2, NFκB, p53, and Rb/E2F (Du, 2005; Niles, 2007; Bouffard, 2007; and unpublished data). For my thesis, this investigation is further expanded by studying two families of transcription factors, the MEF-2s and the SMADs, and demonstrating whether they are subject to regulation during freezing in the wood frog.

5. 3 The MEF-2 Transcription Factors

The effects of freezing on the MEF2 family of transcription factors were also investigated. MEF2 proteins have been described as regulators of development, differentiation, as well as cell survival and death (Potthoff & Olson, 2007; Black & Olson, 1998). They are beginning to emerge as stress activated regulators of transcription involved in cardiac hypertrophy, oxidative stress, and osmotic stress (Passier *et al.*, 2002; Nishimoto & Nishida,

2006). In this thesis, I explored the roles of MEF2A and MEF2C in the wood frog response to natural freeze exposure.

The expression studies of MEF2 proteins revealed that they are regulated post-translationally during freezing. Protein levels of MEF2A and MEF2C in the observed tissues show little to no changes (Fig 3.2 to 3.4). Similarly, *mef2a* and *mef2c* transcript levels displayed changes only in heart and liver isolates (Fig 3.5). By far the impressive changes observed were in the phosphorylation state of these proteins during the freezing cycle. Brain, heart, muscle, liver and kidney had at least one of the MEF2 transcription factors that showed a >1.5-fold increase in phosphorylation state during freezing and thawing. This reveals a global need for MEF2 activation during freezing in the wood frog. Studies of the nuclear distribution of the MEF2 proteins in skeletal muscle and liver also showed a correlation with the observed responses of these proteins in total tissue extracts during short term and long term freezing. Post-translational regulation was evident in the increase of phospho-MEF2A^{Thr312} and phospho-MEF2C^{Thr300} during 4h and 24h freezing exposures in both tissues studied (Fig 3.6), thus demonstrating that the cellular signals are transmitted to the nucleus early during the freeze exposure.

Analysis of target genes revealed that freeze-responsive phosphorylation of MEF2s led to transcriptional activation. Transcript levels of *calreticulin, glucose transporter-4, brain creatine kinase,* and *muscle creatine kinase* were investigated using RT-PCR. Kidney mRNA species showed few changes whereas cardiac and skeletal muscle showed strong increases in *glut-4*, *calreticulin*, *ckb* and *ckm* (1.5 to 4-fold; Fig 3.7 and 3.8). Brain showed only a 1.5 fold increase in *calreticulin* transcript levels. The results are not surprising considering the function of the gene products. The glucose transporter 4 has a crucial role to play in freezing since glucose is the primary cryoprotectant used by the frog. Glucose is only synthesized in the liver during freezing,

and therefore, glucose uptake into cells of all other organs is crucial for freeze/dehydration protection. GLUT4 is the insulin-responsive glucose transporter that plays a main role in glucose uptake into muscle tissues. Screening of a cDNA microarray for freeze-responsive genes from wood frog heart also displayed an up-regulation in *glut-4* mRNA levels (Storey, 2004a,b); hence, my work validates this array result. Calreticulin is a Ca^{2+}-binding chaperone which has been described as serving a number of cellular functions ranging from cellular signalling, transcription, wound healing and ER/UPR stress responses (Groenendyk *et al.*, 2004; Michalak *et al.*, 2002; Nanney *et al.*, 2008; Johnson *et al.*, 2001). Recently, a study on wood frog skeletal muscle and liver during freezing revealed an activation of the ER/UPR stress response (Niles, 2007). In addition, calreticulin could have an important role to play during wound healing caused by any physical damage inflicted by extra-cellular ice crystals. Analysis of cDNA microarrays for freeze-responsive genes from wood frog brain and skeletal muscle also revealed a putative increase in the brain isozyme of creatine kinase (unpublished data). The reason for this appears to be anticipatory, since a work on creatine kinase revealed that protein levels remain stable and the enzyme is regulated by post-translational phosphorylation (Dieni & Storey, 2009). Nevertheless, it appears that creatine kinase is an important regulator of the bioenergetics of frozen tissues especially in skeletal muscle and heart.

5.2 The SMAD Transcription Factors

The SMAD proteins are primarily substrates for TGF-β signalling pathways. Their roles in gene regulation have been documented mostly in studies of development and differentiation but recently, the action of SMADS has also been linked with canncer, inflammation, and stress (Pagett *et al.*, 1998; Massagué *et al.*, 2005; Zhang *et al.*, 2003). SMAD proteins are separated into three classes, the receptor SMADs (SMAD1, 2, 3, 5, and 8), inhibitory SMADs (SMAD6

and 7), and the co-SMAD, SMAD4 (Massagué *et al.*, 2005). My thesis research now adds the Smad proteins to the growing number of transcription factors that are functional in response to natural freezing in the wood frog. Furthermore, this work shows that tissue specific responses are initiated in brain, heart, skeletal muscle, liver and kidney during a freezing cycle.

Most of the SMADs showed little to no regulation during freezing. This exception was the R-SMAD member, SMAD3. In brain, heart, skeletal muscle, and liver, SMAD3 phosphorylation at serine 425 increased significantly by 1.5-2.5 fold during 24 hours of freezing (Figs 4.2 to 4.4). This multi-organ response lead me to predict that SMAD3 had a significant role to play in freezing survival. The rest of the R-SMADs showed relatively few changes, although selective up-regulation in some cases. Interestingly, skeletal muscle showed the most changes during 24 h frozen and 8 h thawed whereas kidney showed the largest changes during thawing. Brain, heart and liver remained relatively stable with changes occurring only in p-SMAD3 during freezing and thawing. To determine whether this enhanced level of phosphorylation lead to the accumulation of R-SMADs into the nucleus, nuclear extracts were used to analyze the relative abundance of SMAD proteins over the first 24 hours of freezing (Fig 4.6). What was found is that there was little or no change during the first 24 hours of freezing in nuclear SMAD levels. In fact, it appears that during the initial 4 hours, there may even a decrease in levels. The phosphorylated R-SMADs demonstrated the same results in nuclear fractions as they did in total protein isolates. Nuclear muscle extracts demonstrated an increase in p-SMAD2 during 4h and 24h freezing where liver showed a 2.5-fold increase in p-SMAD3 during 24h freezing.

The assessments of the SMAD target genes correlated well with changes in the downstream target genes that were assessed. The studied genes: *serpine1*, *myostatin*, and *tsc22d3*, all showed evidence for transcriptional regulation. *Serpine1* showed the strongest

support for this with transcript levels in four organs rising 1.8-3.5 fold during 24h freezing (Fig 4.7A). The amplified myostatin partial mRNA was only found in brain and skeletal muscle but only showed an increase in muscle. In addition, *tsc22d3* transcripts were up-regulated in heart, skeletal muscle and liver by1.5-2.2 fold (Fig 4.7). The products of these genes, as previously discussed, could be linked with freeze survival processes in the wood frog. For instance, the up-regulation of *serpine1*, together with the up-regulation of *fibrinogen-α* and *fibrinogen-γ* (Cai & Storey, 1997) argues that enhancement of clotting capacity is important for dealing with any bleeding injuries that occur as a result of ice damage to the microvasculature. Elevated fibrinogen increases the ability to clot whereas SERPIN E1 helps to prevent clot breakdown; therefore, these are linked as part of the same protective machinery that can deal rapidly with any vascular damage caused by penetrating ice crystals. The observed up-regulation in myostatin transcripts, if translated into functional protein, would appear to be involved in inhibiting muscle growth which seems appropriate for an organism in torpor with a limited energy budget. An increase in myostatin has recently been described in the thirteen-lined ground squirrel during arousal from hibernation (Brooks *et al.*, 2009) which further supports the idea that myostatin is important to establishing or maintaining a hypometabolic state in skeletal muscle of overwintering organisms. The findings for *tsc22d3* transcripts pose a number of questions pertaining to the regulatory mechanisms and the role that this transcription factor plays in response to freezing. Interestingly, the cDNA microarray screens conducted by our lab have predicted similar results. Similar to the observed up-regulation of serpins in organisms facing hypometabolic conditions (thirteen-lined ground squirrel, red-eared slider, and the painted turtle; Storey & Storey, 2007), wood frog cDNA microarray studies on tissues from frozen frogs also showed increased transcript levels of several serpins in brain, heart, and muscle (serpinB,

serpinF, and serpinA, respectively). Interestingly, *serpine1* was not amongst these serpins. *Myostatin* also showed an up-regulation in array screening of wood frog extracts

5. 4 Summary

The present study concludes that MEF2 and SMAD transcription factors are subject to activation in the wood frog during freezing. In the case of MEF2, this activation was generally observed as an increase in phosphorylated MEF2A and MEF2C at sites threonine 312 and threonine 300, respectively. These changes occurred in multiple organs in response to both 24h freezing and 8h thawed. A study of the MEF2 downstream genes: *glut-4, calreticulin, ckb* and *ckm* also display evidence for transcriptional activation. Work on the SMAD proteins also revealed transcriptional activation. R-SMADs were investigated and like the MEF2 proteins, SMADs were found to be primarily regulated via phosphorylation. SMAD3 was the family member that showed the greatest activation, also identified as being relatively universal. *Serpine1*, *myostatin*, and *tsc22d3* are the SMAD target genes which were studied and found to be up-regulated in many tissues. This work provides evidence for transcriptional activation of MEF2 and SMAD proteins in the wood frog during freezing. This shows that in addition to the developmental controls which these two families of proteins regulate, they are also responsible for severe stress responses, thus adding to their phenotypic controls. This study is novel in that it shows these proteins are induced by natural vertebrate freezing, adding the MEF2 and SMAD proteins to the list of freeze-responsive proteins.

5.5 Future Studies

This study provides an insight into the molecular regulatory mechanisms in play during vertebrate freezing. These studies break new barriers as it shows that proteins typically involved

in development and differentiation are subject to activation in response to such an extreme cellular stress as freezing. Here, I show that SMADs and MEF2 transcription factors appear to have a role to play in response to freezing.

Signal transduction cascades which regulate these proteins remain to be investigated in the wood frog. This would involve an analysis of the upstream kinases regulating these transcription factors. The p38 MAPK pathway has been shown to be activated within the initial 12h of freezing in heart, liver and kidney of the wood frog (Greenway & Storey, 2000). In the marsh frog, *R. ribunda*, p38 MAPK was also demonstrated to be activated in response to hypothermia and hyperosmotic stress (Aggeli *et al*, 2001, 2002). ERK5 MAPK has been shown to be strong regulator of MEF2 proteins during oxidative and osmotic stress (Nishimoto & Nishida, 2006), and therefore might be a candidate for control of MEF2 activation during freezing in wood frogs. With respect to SMAD3 activation, studies on the phosphorylation states of TGF-β receptors would reveal whether TGF-β signals are responsible for the observed SMAD3 phosphorylation. If results show that this activation is TGF-β receptor-mediated, this would have interesting implications for the extracellular environment of frozen tissues, since phosphorylation was found to take place after animal was frozen. Furthermore, it will be instructive to determine whether MEF2 and SMAD3 responses in wood frogs are exclusive to freezing or are also observed in response to two of the component stresses of freezing: anoxia and/or dehydration. So far, the p38 MAPK activation was found only in response to freezing (Greenway & Storey, 2000).

Additional research should also be undertaken to determine the consequences of freeze-induced target gene upregulation on the levels of their protein products. There is not always a direct correlation between transcript and protein levels; indeed, this is illustrated even in this

thesis for the relationship between MEF2 and SMAD transcript versus protein levels. Hence, analysis of the protein levels of the target genes will be needed to confirm that freeze-induced gene up-regulation did in fact lead to elevated levels of the gene products. This is crucial since proteins are the real workhorses of the cells and thus gene product levels, as well as relative activities in the case of enzymes or transporters (where appropriate), should be investigated to conclude their importance during freezing.

MEF2 and SMAD proteins regulate a number of genes some of which were studied here. However, there are many more gene targets under their regulation, some of which might also be important during freezing. For instance, MEF2 transcriptionally regulates myosin light 2 (Chambers *et al.*, 1994; Evansa *et al.*, 1994) which has been shown to be up-regulated in hearts of frozen wood frogs (McNally, 2002). MEF2 also fine-tunes matrix metalloproteinase-10 transcripts, an enzyme linked to wound healing (Salmela *et al.*, 2004) and might be up-regulated in response to vertebrate freezing. TGF-β/SMAD signalling has also been found to regulate the cyclin dependent kinases p15 and p21 which are crucial for cell cycle arrest (Jörnvall *et al.*, 2001). Cell cycle regulation during freezing in the wood frog is currently being investigated and these questions will shortly be answered. In addition, a new paradigm to translational regulation is being revealed as microRNAs are investigated and MEF2 and SMAD transcription factors have already been discovered to regulate specific miRNAs (Liu et al., 2007; Hoover & Kubalak, 2008).

Co-factors are another dimension of to the regulatory mechanisms of MEF2 and SMAD proteins. Both families have been found to co-ordinate transcription with a number of proteins including themselves, thereby forming MEF2/SMAD complexes (Quinn *et al.*, 2001; Liu *et al.*, 2004). Both MEF2 and SMAD proteins are also known to bind to the acetylase and

transcriptional activator p300 (Ma *et al.*, 2005; Kassimatis *et al.*, 2006). HDACs also play a role as transcriptional repressors and regulators of epigenetic controls and indeed as previously discussed are strong regulators of the MEF2 machinery (McKinsey *et al.*, 2001). Co-immunoprecipitations of MEF2 and SMAD proteins would elucidate the protein complexes formed by these factors in response to freezing and provide more insights into the regulatory pathways involved in freeze-responsive gene expression as well as undoubtedly expand the list of known target proteins controlled by these transcription factors under freezing conditions. Therefore, as discussed in here, there is plenty of research remaining to be conducted and the present work constitutes some new stepping stones at the frontier of discovering the "breadth and depth" of transcriptional regulation in the phenomenon of natural freeze tolerance by the wood frog, *Rana sylvatica*.

APPENDIX I:

PROTEIN SEQUENCE ALIGNMENTS

MEF2A protein alignment

```
Wood_frog            ....VEALQKEHRGCDSPDPDGSYVLTPHTEEKYKKINEE     36
African_clawed_frog fkrdpalnk--n----------------------------    160
Mouse                .....tlrk-gln--e---a-dyfehs-ls-drfs-l--d    121
Human                .....alnk----------t-----------------    121
Zebrafish            .....klrn-g-nd-p-----dcfghs-lmddrfg-l---    121
Chicken              .....tlrk-gln--e---a-dyfehs-ls-drfs-l--d    121

Wood_frog            FDNMMRN.HKISPGLQQPNFSMSVTIPVSNPLS..YSSPG     73
African_clawed_frog ------s.-------p-qt-p----v-----ntlp-----    199
Mouse                s-fifkr.gp..---ppq-------v--ts-nals-tn--    158
Human                -------.---a---ppq-------v--ts-nals-tn--    160
Zebrafish            s-l-ykrcgp..ta-ppq----h-av--t--nams-.n--    158
Chicken              s-fifkr.gp..---paq-------v-----ntlt--n--    158

Wood_frog            NTLVTSSLAASTSLTDTGMMSPPQTSLHRNVASPGVSQRP    113
African_clawed_frog --m--a-----a----ar-l---p-t-----fpq-ypse-    239
Mouse                ss--sp-----st-a-ss-l---pat-----spgapqr.-    197
Human                ss--sp-----st---ss-l-----t-----spgapqr.-    199
Zebrafish            as-ssq--s-aa--s-ga-l----g-m--s-vp..pqr.-    195
Chicken              ss--sp-----s---s-t-l-----t-----spgapqr.-    197

Wood_frog            SSTGNAGVMLCSSDISVPNGAGASPVGNGVW.........    144
African_clawed_frog p-------------l-------t------fvnpraspshl    279
Mouse                p---s-sg--stt-lt------n------fvnsraspnli    237
Human                p------g--stt-lt------s------fvnsraspnli    239
Zebrafish            p---st.....................---fvnprgspgll    215
Chicken              p------gi-gtt-lt------t--------nsraspsll    237
```

MEF2C Protein Alignment

```
Wood frog           ....DLMISRQRLCAVPPPNFEMPVSIPVSNHNSLAYSNQ     36
African clawed frog nedi-------------------------p-------p    160
Human               nedi-------------------------s----v---p    160
Mouse               neef-n--kshkip-----sl----t----s----v---p    158
Chicken             nedi-----------------------------s--v---p    160
Zebrafish           nedi-----------i-qs-yd---------p---i-ghp    159
Common carp         nedi-----------i-qs-yd--i------p---i--hp    159

Wood frog           G.SLGNHNLLPLSHQSLQRNSMSPGLNHRPPSAGNTGGLM     75
African clawed frog av------------p--h-------vt-------------    200
Human               vs----p-----a-p----------vt-------------    200
Mouse               vst---p-----a-p----------vt-------------    198
Chicken             vs----p-----a-p----------vt-------------    200
Zebrafish           -a-m--p-----t-p----------vt---------....    195
Common carp         -a----p-m---a-p----------vt-------------    199

Wood frog           GGDLTSGAGTSAGNGYGNHRNSPGLLVSPGNLNKNMQTKS    115
African clawed frog -----t-------------------------------    240
Human               ------------------p-----------------a--    240
Mouse               ------------------p---------------i-a--    238
Chicken             -----t------------p-----------------a--    240
Zebrafish           ...........-------------------ms--l-a--    223
Common carp         -p--s--v----------------------sms----a--    239

Wood frog           PPPMNLGMNNRKPDLRVLIPPGSKNTMPSV........NQ    147
African clawed frog ------------------------------.......--    272
Human               ------------------------------sedvdlll--    280
Mouse               ------------------------------sedvdlll--    278
Chicken             ------------------------------sedvdlll--    280
Zebrafish           --s--msls.------------a------i........--    254
Common carp         ----tms-s--------------------i........--    271

Wood frog           RINNSQSAQSLATPVVSVATPIS.................    170
African clawed frog ----------------p----tlpgqgmggyptaisttyg    312
Human               ---------------------tlpgqgmggypsaisttyg    320
Mouse               ---------------------tlpgqgmggypsaisttyg    318
Chicken             ---------------------tlpgqgmggypsaisttyg    320
Zebrafish           --ss-------s---------tlpgevmggypsaintsyg    294
Common carp         -----------s---------tlpgqgmggypsalstsyg    311
```

Glucose Transporter -4 protein alignment

```
Wood frog           .........KDPEAKKPDEWDERPKIDDPEDKKPEDWEK     30
J. wrinkled frog    dwdflppkkv---------d------------------d-    239
African clawed frog dwdflppkki-----------------------------    233
Western clawed frog dwdflpakki---------d------------------d-    239
Chicken             dwdflppkki---------d----a-------s-----d-    240
Human               dwdflppkki---d-s--ed----a-----t-s-----d-    238
Zebrafish           dwdflppkki---------d--d-a-------t-----d-    238

Wood frog           PEHIPDPDAVKPEDWDEEMDGEWEPPVITNPEYKGEWKPR     70
J. wrinkled frog    ------------------------------------------    279
African clawed frog -----------------------------q-----------    273
Western clawed frog a----------------------------q--d--------    279
Chicken             ---------k------------------q--------r--    280
Human               ---------k------------------q-----------    278
Zebrafish           --n------k--d----d-------am-p----------k    278

Wood frog           QIDNPDYKGKWVHPEIDNPEYTPDPTLYSYEDFGALGLDL    110
J. wrinkled frog    -------------------------------a-----v---    319
African clawed frog -----------i------------d-----ds--v-----    313
Western clawed frog -----------i------------s---q-ds--v-----    319
Chicken             --------------------------n--a-ds--vi----    320
Human               ---------t-i---------s---si-a-dn--v-----    318
Zebrafish           -----s---t-----------sa-dai-kfdsi-v-----    318

Wood frog           WQAKSGTIFDNFLITDDEKFAKEQATNPWGVTKEGEKKMK    150
J. wrinkled frog    --v------------------e-h--kt------------    359
African clawed frog --v----------m-n---h-e-ygnet------a-----    353
Western clawed frog --v----------m-----y-e-ygnet------a-----    359
Chicken             --v-------------------e-fgnet--a---a-r---    360
Human               --v------------n--ay-e-fgnet-----aa--q--    358
Zebrafish           --v--------------vee-ekfg-dt--a--gp-----    358

Wood frog           ELQDEEDR.............................       158
J. wrinkled frog    -q----e-kkqeeeekkrkeqepaeeaeddddddddddee    399
African clawed frog -q------kkqeeeektrkeeepqeeededdddeeekeee    393
Western clawed frog -q------kkqeeeekkrkdeepqeeeeddddeeeekeee    399
Chicken             -q----q-qkqeeedkqrkeeegdedgdgdd.........    391
Human               dk----q-lkeeeedkkrkeeeeaedke.dd.........    388
Zebrafish           dq-e--e-kkreeeekskkddneedeedededepeeddht    398
```

Calreticulin protein alignment

```
Wood frog           ..........KDPEAKKPDEWDERPKIDDPEDKKPEDWEK     30
J. wrinkled frog    dwdflppkkv---------d------------------d-    239
African clawed frog dwdflppkki-----------------------------     233
Western clawed frog dwdflpakki---------d------------------d-    239
Chicken             dwdflppkki---------d----a-------s-----d-    240
Human               dwdflppkki---d-s--ed----a-----t-s-----d-    238
Zebrafish           dwdflppkki---------d--d-a-------t-----d-    238

Wood frog           PEHIPDPDAVKPEDWDEEMDGEWEPPVITNPEYKGEWKPR     70
J. wrinkled frog    ------------------------------------------    279
African clawed frog -----------------------------q-----------    273
Western clawed frog a----------------------------q--d--------    279
Chicken             ---------k------------------q--------r--    280
Human               ---------k------------------q-----------    278
Zebrafish           --n------k--d----d-------am-p----------k    278

Wood frog           QIDNPDYKGKWVHPEIDNPEYTPDPTLYSYEDFGALGLDL    110
J. wrinkled frog    -----------------------------a-----v---    319
African clawed frog -----------i------------d-----ds--v-----    313
Western clawed frog -----------i------------s---q-ds--v-----    319
Chicken             --------------------------n--a-ds--vi----    320
Human               ---------t-i---------s---si-a-dn--v-----    318
Zebrafish           -----s---t-----------sa-dai-kfdsi-v-----    318

Wood frog           WQAKSGTIFDNFLITDDEKFAKEQATNPWGVTKEGEKKMK    150
J. wrinkled frog    --v------------------e-h--kt------------    359
African clawed frog --v----------m-n---h-e-ygnet------a-----    353
Western clawed frog --v----------m-----y-e-ygnet------a-----    359
Chicken             --v------------------e-fgnet--a---a-r---    360
Human               --v------------n--ay-e-fgnet-----aa--q--    358
Zebrafish           --v--------------vee-ekfg-dt--a--gp-----    358

Wood frog           ELQDEEDR.............................       158
J. wrinkled frog    -q----e-kkqeeeekkrkeqepaeeaeddddddddddee    399
African clawed frog -q------kkqeeeektrkeeepqeeededdddeeekeee    393
Western clawed frog -q------kkqeeeekkrkdeepqeeeeddddeeeekeee    399
Chicken             -q----q-qkqeeedkqrkeeegdedgdgdd.........    391
Human               dk----q-lkeeeedkkrkeeeeaedke.dd.........    388
Zebrafish           dq-e--e-kkreeeekskkddneedeedededepeeddht    398
```

Brain Creatine Kinase protein alignment

```
Wood frog           .........DLLVDDVIQTGIDNPGHPYILTVGAVAGDEE     31
Western clawed frog klrdkqtpsgft--------v------f-m---c------     80
African clawed frog klrsrttssgftl-------v------f-m---c------     75
Zebra finch         klrdrvtpsgftl-------v------f-m---c------     75
Chicken             klrdrqtssgftl-------v------f-m---c------     80
Human               elrakstpsgftl-------v--------m---c------     80
Mouse               elrakctpsgftl--a----v--------m----------     80
Zebrafish           nlrdkqtpsgftl-------v------f-m---c------     80

Wood frog           CYDVFGDLFDPIIEDRHGGYKPGDQHKTDLKSEHLKGGDD     71
Western clawed frog s-e--ke---------------t------in--n------    120
African clawed frog s-e--k----------------t------in-an------    115
Zebra finch         s-e--ke----v----------s-------nadn-q----    115
Chicken             s-e--ke----v----------t-e-----nadn-q----    120
Human               s-e--k----------------s-e-----npdn-q----    120
Mouse               s----k--------e-----q-s-e-----npdn-q----    120
Zebrafish           t----ke-l--v----------t-k-----npdn------    120

Wood frog           LDPNYVLSSRVRTGRSIRGFCLPPHCSRGERRGIEKLSIE    111
Western clawed frog ---------------------s-----------a---m---    160
African clawed frog -------------------ys-----------a---m---    155
Zebra finch         ---------------------------------a-----v-    155
Chicken             ---------------------------------a-----v-    160
Human               ---------------------------------a----av-    160
Mouse               ---------------------------------a----av-    160
Zebrafish           -----------------------------------s--v-    160

Wood frog           ALDSLDGDLKGKYYALKSMTDAEQQQLIDDHFLFDKPVSP    151
Western clawed frog --a-------------nt---q------------------    200
African clawed frog --a-------------n---eq-----------------    195
Zebra finch         --g-------------rn---------------------    195
Chicken             --g--g----------rn---------------------    200
Human               --s------a-r--------e------------------    200
Mouse               --s------s-r--------e------------------    200
Zebrafish           --ga-------------d--ee-----------------    200

Wood frog           LLLASGMARDWPDARGIWHNDNKTFLVWINEEDHLRVISM    191
Western clawed frog ----------------------------------------    240
African clawed frog ----------------------------------------    235
Zebra finch         ----------------------------------------    235
Chicken             ----------------------------------------    240
Human               ----------------------------v-----------    240
Mouse               ----------------------------------------    240
Zebrafish           ----------------------------v-----------    240
Wood frog           QKGGNMK.................................    198

Western clawed frog -------dvfqrfctgltkieaifknkgsafmwnqhlgyv    280
African clawed frog -------evfnrfctgltkiesifknkgspfmwnqhlgyv    275
Zebra finch         -------evfnrfctgltkieslfkgknyefmwnphlgyi    275
Chicken             -------evftrfctgltqietlfksknyefmwnphlgyi    280
Human               -------evftrfctgltqietlfkskdyefmwnphlgyi    280
Mouse               -------evftrfctgltqietlfksknyefmwnphlgyi    280
Zebrafish           ------revfnrfctgltkieelfkekghefmwnehlgyv    280A
```

Muscle Creatine Kinase protein alignment

```
Wood frog           ..........................ERRAIENLSIK     11
African clawed frog ldpnyvlssrvrtgrsikgytlpphcsrg----v-k---q   160
Western clawed frog ldpnyvlssrvrtgrsikgyalpphcsrg------kh---   160
Human               ldpnyvlssrvrtgrsikgytlpphcsrg----v-k--ve   160
Chicken             ldpkyvlssrvrtgrsikgyslpphcsrg----v-k--ve   160
Zebrafish           ldpnyvlssrvrtgrsikgyalpphnsrg----v-k--ve   160

Wood frog           ALDSLTGEFKGKYYPLKDMTDAEQQQLIDDHFLFDKPVSP     51
African clawed frog --s--s-------------s--------------------   200
Western clawed frog --s--s-------------s--------------------   200
Human               --n--------------s--ek------------------   200
Chicken             --n--e-----r-----a--eq------------------   200
Zebrafish           --s--d-----------s------e---a-----------   200

Wood frog           LLLAAGMARDWPDARGIWHNDNKSFLVWVNEEDHLRVISM     91
African clawed frog -------g---------------t----------------   240
Western clawed frog ----s--g------------e--t----------------   240
Human               ----s------------------------------------   240
Chicken             ----s------------------t----------------   240
Zebrafish           --------------------e--t----------------   240

Wood frog           EKGGNMKTVFKRFCEGLQKIEEIFKSAGHPFSWSEHLGYI    131
African clawed frog q------e--r------t-------q-----m-n-----v   280
Western clawed frog q------e--r--------------q-----m-n-----v   280
Human               -------e--r---v----------k-----m-nq----v   280
Chicken             -------e--r---v--k-------k-----m-t------   280
Zebrafish           q------e------v---r------khn-g-m-n----fv   280

Wood frog           LTCPSNLGTGLRGGVHVKLPNLSKHPKFEEILTRLRLQKR    171
African clawed frog ----------------a----------------------   320
Western clawed frog ----------------a----------d---g-------   320
Human               --------------------ah------------------   320
Chicken             --------------------k--q--------h-------   320
Zebrafish           --------------------k--t-a--------------   320

Wood frog           GTGGVD.................................     177
African clawed frog ------taavggvfdisnadrlgfsevdqvqmvvdgvklm   360
Western clawed frog ------taavggvfdisnadrlgfsevdqvqmvvdgvklm   360
Human               ------taavgsvfdvsnadrlgsseveqvqlvvdgvklm   360
Chicken             ------taavgavfdisnadrlgfseveqvqmvvdgvklm   360
Zebrafish           ------tasvggvfdisnadrigsseveqvqcvvdgvklm   360
```

SMAD2 protein alignment

```
Wood frog           .....................ALYSFSEQTRSLDGRLQVSH     20
Western clawed frog tcseiwglstpntidqwdttg-------------------     119
Human               tcseiwglstpntidqwdttg-------------------     119
Chicken             tcseiwglstpntidqwdttg-------------------     119
Zebrafish           ncseiwglstpntieqwdtsg---ypd-------------     120

Wood frog           RKGLPHVIYCRLWRWPDLHSHHELKAIENCEYAFNLKKDE      60
Western clawed frog ----------------------------------------     159
Human               ----------------------------------------     159
Chicken             ----------------------------------------     159
Zebrafish           -------------------------r---t-----------     160

Wood frog           VCVNPYHYQRVETPVLPPVLVPRHTEILTELPPLDDYQHS     100
Western clawed frog ------------------------------------t--     199
Human               ------------------------------------t--     199
Chicken             ------------------------------------t--     199
Zebrafish           ------------------------------------tn-     200

Wood frog           IPENTNFPAGIEPQSNYIPETPPPGYISEDGETSDQQLNQ     140
Western clawed frog ----------------------------------------     239
Human               ----------------------------------------     239
Chicken             ----------------------------------------     239
Zebrafish           --------t----pn-----------------a----m--     240

Wood frog           SMDTE.................................       145
Western clawed frog ----gspaelspstlspvnhnldlqpvtysepafwcsiay     279
Human               ----gspaelspttlspvnhsldlqpvtysepafwcsiay     279
Chicken             ----gspaelspstlspvnhsldlqpvtysepafwcsiay     279
Zebrafish           ----gspaelspstlspvnhgmdlqpvtysepafwcsiay     280
```

SMAD4 protein alignment

```
Wood_frog           ...GDRFCLGQLSNVHTTEAIERARLHIGKGVQLECKGEG      37
Western_clawed_frog psg-------------r----------------------     392
Human               psg-------------r----------------------     395
Zebrafish           psg-------------r----------------------     390

Wood_frog           DVWVRCLNDHAVFVQSYYLDREAGRAPGDAVHKIYPSAYI      77
Western_clawed_frog -------s--------------------------------     432
Human               -------s--------------------------------     435
Zebrafish           -------s--------------------------------     430

Wood_frog           KVFDLRQCHRQI..........................        89
Western_clawed_frog -----------mqqqaataqaaaaaqaaavagnipgpgsv     472
Human               -----------mqqqaataqaaaaaqaaavagnipgpgsv     475
Zebrafish           -----------mqqqaataqaaaaaqaaavagnipgpgsv     470
```

Serpine 1 protein alignment

```
Wood frog           .....................YEEHVPLSAITNILTPE     17
African clawed frog gdfydvielpyegeelsmiiaap--kg----t------s-    281
Chicken             elwyniielpyhgemismlialpt-ntt-----iphistk    320
Human               ghyydilelpyhgdtlsmfiaap--ke-----l----saq    280
Zebrafish           gvdydviempyegesismllvtpf-kd-----lnke-sss    267

Wood frog           LINQWKANMQKLTRLLVLPKFSLVSEVNLKTPLERLGIED     57
African clawed frog --v----q-k-va----------l---d--k-------tn    321
Chicken             t-gs-mtt-vakrvqvi----ta-a-td--d--ka---t-    360
Human               --sh--g--tr-p----------et--d-rk---n--mt-    320
Zebrafish           r-h--rqe-r-iskq-si-r--mdt-id--st-s-m-lg-    307

Wood frog           MFSEEKADFSRL.............................     69
African clawed frog --t--m------ssekplyvseafqkikvevtergtrasa    361
Chicken             --d-s-sn-akitrteglhvshvlqktkievsedgtkasa    400
Human               --rqfq---ts-sdqeplhvaqalqkvkievnesgtvass    360
Zebrafish           i--qsr-----itteeplcvskvlqrvklevneegtkgss    347
```

Myostatin protein alignment

```
Wood frog      A......KAPPLQDIIDQYDVQSDASIEGSLEDDDYHATP      34
Western frog 1 -ikyllp-----eel-----l---d-s-----e------t     112
Western frog 2 -ikhllp-------l--k----k-e-sa-h--e----vsa     112
Human          virqllp-----rel-------r-d-sd-----------t     115
Chicken        vikqllp------el-------r-d-sd-----------t     115
Zebrafish      vvkqllp------qll-----lg-d-kd-av-e--e---t     116

Wood frog      ETFIIMPTQ.N.D..LDYETSKTKK.CCYFKLNSEIQSTK      69
Western frog 1 --i-t---e.s.-s.tg.-..-q-..-----fs-vv-yn-     144
Western frog 2 --v-----efgis..i-...m-e-pi--f--fs-kv-l--     147
Human          --i-t---e.s.-flmqvd.g-p-..--f--fs-k--yn-     150
Chicken        --i-t---e.s.-flvqm-.g-p-..--f--fs-k--yn-     150
Zebrafish      --imt-a-e.p.-pivqvd.r-p-..--f-sfspk--anr     151

Wood frog      ISKAQLWIHLKPVQMPATVVVQILRLIKP..LKDGTRHIG     107
Western frog 1 -a------y-----krt--f--tf--..-ks-n--a-st-     182
Western frog 2 --------------k-t------s-----..------yt-     185
Human          vv------y-r--et-t--f---------..m-----yt-     188
Chicken        vv------y-rq--k-t--f---------..m-----yt-     188
Zebrafish      -vr----v--r-aeeat--fl--s--m.-..v---g--r.     187

Wood frog      IRTLKLDMNPG.TGTW.........................     122
Western frog 1 ------e-g--.s---ksldvktalqnwlkqpastlgiei     221
Western frog 2 --s---e----.s---qsidvktvlqnwlrqpesnlgiei     224
Human          --s--------.--i-qsidvktvlqnwlkqpesnlgiei     227
Chicken        --s--------.--i-qsidvktvlqnwlkqpesnlgiei     227
Zebrafish      --s--i-v-a-v-s.-qsidvkqvltvwlkqpetnrgiei     226
```

TSC22D3 protein alignment

```
Wood_frog            ..............................VAIDNKI      7
Human                pcylinegicnrnidqtmlsillffhsasgasv-------    120
Zebrafish            svkldn...................sasgasv-------     54
Chicken              svkldn...................sasgasv-------     54
Western_clawed_frog  avtvds..................sssgasv-------     45

Wood_frog            EQAMDLVKSHLMYAVREEVEVLKEQIKELVEKDSQLNRWN     47
Human                --------n-----------i-----r-----n---e-e-    160
Zebrafish            --------n-----------i--------a--nn--e-e-     94
Chicken              --------n--------------------l--n---e-e-     94
Western_clawed_frog  ----------------------------i--n---eqe-     85

Wood_frog            TG...................................        49
Human                -llktlaspeqlekfqscls.............peep.ap    186
Zebrafish            sllknlaspeqlekfqsrlpsds.....slpldpqelgsp    129
Chicken              sllktlaspeqlekfqsrlp........sevlhpqeq.sp    125
Western_clawed_frog  nllktlaspeqlaqfqaqlqagsppsssstcsqppgstaa    125
```

Appendix II:

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