2008

CaMK-II Promotes Beta-Catenin-Dependent Transcription by Binding Flightless-I

Jamie McLeod
Virginia Commonwealth University

College of Humanities and Sciences
Virginia Commonwealth University

This is to certify that the thesis prepared by Jamie J.A. McLeod entitled CaMK-II Promotes β-Catenin-Dependent Transcription by Binding Flightless-I has been approved by her committee as satisfactory completion of the thesis requirement for the degree of Master of Science

Robert M. Tombes, Ph.D., Director of Thesis, Department of Biology

William B. Eggleston, Ph.D., Department of Biology and Forensic Science

Ghislaine Mayer, Ph.D., Department of Biology

Carmen Sato-Bigbee, Ph.D., Department of Biochemistry and Molecular Biology

Leonard A. Smock, Ph.D., Chair, Department of Biology

Fred M. Hawkridge, Ph.D., Interim Dean, College of Humanities and Sciences

Dr. F. Douglas Boudinot, Dean of the School of Graduate Studies

July 23, 2008

CAMK-II PROMOTES BETA-CATENIN-DEPENDENT TRANSCRIPTION BY BINDING FLIGHTLESS-I

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

JAMIE JOSEPHINE AVILA MCLEOD
B.S., Virginia Polytechnic Institute and State University, 2005

Director: ROBERT M. TOMBES, PH.D.
ASSOCIATE PROFESSOR, DEPARTMENT OF BIOLOGY

Virginia Commonwealth University
Richmond, Virginia
August, 2008

Acknowledgements

The writing of this thesis has been one of the most significant academic challenges I have ever had to face. Without the support, patience and guidance of the following people this work would not have been completed. It is to them that I owe my deepest gratitude.

- Dr. Robert M. Tombes who gave me the wonderful opportunity to work in his lab. His wisdom, knowledge and commitment to science inspire and motivate me to continue in this field.
- My friend and colleague, Charles Easley, who inspired me to continue my efforts no mater how discouraged I became. Chas has been an amazing friend and mentor whom without I may not have been able to come as far as I have.
- My committee members, Dr. William Eggleston, Ghislaine Mayer and Carmen Sato-Bigbee, whose suggestions and positive feedback have not only made me proud of my work but have encouraged me to continue these projects.
- Debbie and Jeff McLeod, my parents, who have always supported, encouraged and believed in me, in all my endeavors. They have so lovingly and unselfishly provided me with every opportunity possible to fulfill all my dreams and for that I am eternally grateful.

- My siblings, Ali, Jeff and John, who have been a constant support system to me during this entire process. They have been particularly helpful in reminding me to relax when the stress has become too high, while also showing me that overcoming the obstacles we are presented with in life is an adventure that we all must endure.
- My lab members, Sarah Rothschild, Ludmila Francescatto, Ali Myers, Collen Brosnahan, Bennett Childs and Jack Van Vleck whom have assisted me in this research project with interest and enthusiasm.

This thesis is dedicated to all my family.

Table of Contents

Page

List of Figures

Page

List of Abbreviations

α	Alpha
AMPA receptor	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Asp	Aspartic Acid
ATP	Adenosine Triphosphate
β	Beta
*Bam*HI	*Bacillus amyloliqufaciens* HI
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
C-terminus	Carboxy Terminus
Ca^{2+}	Calcium Ion
$CaC1_2$	Calcium Chloride
CaMK-II	Ca^{2+}/CaM Dependent Protein Kinase Type II
CaM	Calmodulin
Cdk	Cyclin-dependent kinase
D	Aspartic Acid
δ	Delta
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediamine Tetra-acetic Acid
EGTA	[ethylenebis(oxyethylenenitrilo)] Tetra-acetic Acid
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
Fli-I	Flightless-I
γ	Gamma
IgG	Immunoglobulin Gamma
IP	Immunoprecipitation
FBS	Fetal Bovine Serum
FLAG	DYKDDDDK Epitope Tag
g	Gram
GFP	Green Fluorescent Protein
GRABP	Gelsolin Related Actin Binding Protein
GSK	Glycogen Synthase Kinase
HB	Homogenization Buffer

HEPES	4-(2-Hydoxyethy) piperazine-1-ethanosulfonic acid
K	Lysine
kDa	Kilodalton
L2k	Lipofectamine 2000
Leu	Leucine
LRR	Leucine Rich Repeat
LRRFIP	Leaucine Rich Repeat Flightless-I Interacting Protein
Lys	Lysine
µ	mu/micro
m	Milli
M	Molar
M_r	Molecular Weight
MAPK	Mitogen-activated protein kinase
mEGFP	Monomeric Enhanced Green Fluorescent Protein
N-terminus	Amino terminus
NaCl	Sodium Chloride
NBT	Nitro Blue Tetrazolium
NIH/3T3	Mouse Embryonic Fibroblast Cell Line
NLK	Nemo-like kinase
NMDA	*N*-methyl *D*-aspartate
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Ser	Serine
Taq	*Thermus aquaticus*
TAK1	Transforming growth factor-activated kinase 1
TBS	Tris Buffered Saline
TBSTA	Tris Buffered Saline, 0.01% Tris-20 and Sodium Azide
Thr	Threonine
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Tyr	Tyrosine
Wnt	Wingless/Integration, Proto-oncogene, Ligand
Y	Tyrosine

Abstract

CaMK-II Promotes β-Catenin-Dependent Transcription by Binding Flightless-I

By Jamie J.A. McLeod, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Robert M. Tombes, Ph.D.
Associate Professor, Department of Biology

Transient intracellular elevations of Ca^{2+} are common signaling mechanisms used to allosterically regulate proteins. One potential target of Ca^{2+} is Ca^{2+}/calmodulin dependent protein kinase type II (CaMK-II). CaMK-II is a multi-functional protein kinase known to influence cellular pathways such as cell motility and cell cycle progression. Within the cell cycle, CaMK-II promotes the expression of the regulator protein cyclin D1, which is necessary for cell cycle progression. To further understand CaMK-II's role in cyclin D1 expression, the binding partners of cytosolic CaMK-II were studied using mass spectrometry. Several proteins were identified including β actin, β tubulins, tropomodulin-

3 and Flightless-I. Flightless-I was of the most interest because of its role as a transcriptional co-activator of β-catenin-dependent genes such as cyclin D1 and its ability to translocate out of the nucleus following serum stimulation or CaMK-II activation.

For this study, I sought to determine whether the interaction between CaMK-II and Flightless-I mediates transcription of cyclin D1. First, Flightless-I was linked to green fluorescent protein (GFP) and live cell imaging was performed. Under serum stimulation or constitutive CaMK-II expression, GFP-Flightless-I was cytosolic. Serum starvation or inhibition of CaMK-II expression resulted in the localization of GFP-Flightless-I to the nucleus. Next, a luciferase based reporter gene assay was used to evaluate the effect of Flightless-I and CaMK-II on β-catenin-dependent transcription of cyclin D1. Over-expression of Flightless-I or inhibition of CaMK-II both resulted in decreased β-catenin-dependent transcription; whereas suppression of Flightless-I by siRNA enhanced transcription. Taken together, these results suggest a novel mechanism whereby the interaction between CaMK-II and Flightless-I influences gene transcription necessary for cell cycle progression.

Introduction

Ca^{2+}/Calmodulin Dependent Protein Kinase Type II

Ca^{2+}/Calmodulin dependent protein kinase type II (CaMK-II) is a ubiquitously expressed multi-functional serine/threonine protein kinase. CaMK-II is expressed in a variety of cell types and is encoded by four genes (α, β, δ and γ), which give rise to over three dozen CaMK-II subtypes through alternative splicing (Tombes et al., 2003). Due to CaMK-II's ubiquitous nature, it has been shown to influence numerous signaling pathways. These pathways include neuron morphology and signaling, cell migration and cell cycle progression.

All four of the CaMK-II genes have a similar structure consisting of an amino-terminal catalytic and regulatory domain, a central variable domain and a carboxy-terminal association domain (Figure 1). The amino-terminus consists of 315-amino acids and contains ATP- and calmodulin-binding sites (Schulman et al., 1992). The next 30-100 amino acids comprise the variable domain where alternative splicing occurs leading to the multiple splice variants of CaMK-II (Tombes et al., 2003). Finally, the carboxy-terminal association domain allows for the twelve monomers of CaMK-II to oligomerize and form a dodecameric protein (Kolb et al., 1998; Lantsman and Tombes, 2005).

CaMK-II is unique from other members of the calmodulin-dependent kinases because of its ability to become independent of Ca^{2+}/CaM after initial activation. Before the binding of Ca^{2+}/CaM, CaMK-II is in an inactive state with the regulatory domain of the

enzyme folded over the catalytic domain causing autoinhibition (Hudmon and Schulman, 2002). When Ca^{2+}/CaM bind to CaMK-II the autoinhibitory arm is lifted, exposing the catalytic domain and allowing phosphorylation of the regulatory residue Thr^{287} to occur. Following phosphorylation of Thr^{287}, the enzyme becomes independent of Ca^{2+}/CaM and is able to phosphorylate target proteins. These phosphorylated proteins have been shown to regulate multiple cellular functions including, but not limited to, synaptic plasticity, synaptic vesicle mobilization, modulation of ion channels, smooth muscle contraction and gene regulation (Gaertner et al., 2004).

CaMK-II Function

CaMK-II is predominantly known for its role in regulating neuronal processes within the brain. These processes include regulating long term potentiation and synaptic plasticity; both are required in memory and learning. CaMK-II regulates these pathways through direct binding and/or phosphorylation of neuronal receptors and synaptic targets (Benfenati et al., 1992; Chen et al., 2001; Giese et al., 1998; He et al., 2000; Ito et al., 1991; Yoshimura et al., 2002). CaMK-II's roles in neuronal processes appear to be mediated by the interaction of CaMK-II with components of the actin cytoskeleton (Caran et al., 2001; Fink et al., 2003; Shen and Meyer, 1999; Shen et al., 1998)

The actin cytoskeleton is vital for maintaining cell morphology and signal transduction pathways necessary for cell motility. CaMK-II co-localizes with the actin cytoskeleton and influences cytoskeleton and focal adhesion dynamics. Focal adhesions are critical signaling and structural hubs found in migratory cells and are formed through

adaptor protein interactions (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Focal adhesion kinase (FAK) and paxillin are two such adaptor proteins essential for focal adhesion dynamics. CaMK-II activation has been shown to promote dephosphorylation of FAK and paxillin and results in focal adhesion destabilization and cell migration (Easley et al., 2008)

CaMK-II is also involved in cell cycle progression. Previous research has shown that upon CaMK-II inhibition, DNA synthesis is disrupted and cyclin D1 levels are decreased. Cyclin D1 is a cell cycle regulatory protein which activates the cyclin-dependent kinase, cdk4/6, to promote cell cycle progression out of the G1 phase of the cell cycle and into the S phase. When cyclin D1 levels are decreased through the inhibition of CaMK-II, the cell arrests in the G1 phase of the cell cycle (Morris et al., 1998). Furthermore, the signaling molecules $p44^{MAP\ kinase}$ (ERK1) and $p42^{MAP\ kinase}$ (ERK2) which also control G1 phase cell cycle progression, were unaffected. These results suggest that CaMK-II influences cyclin D1 levels to enable cell cycle progression; however the mechanism by which CaMK-II affects cyclin D1 has yet to be identified.

CaMK-II Binding Partners

CaMK-II mediates regulation within cellular pathways through phosphorylation and protein binding. Known binding proteins of CaMK-II include actin and tubulin (Easley et al., 2006; Shen et al., 1998), in addition to the newly discovered binding proteins tropomodulin-3, FLAP1, FLAP2 and Flightless-I (Seward et al., 2008). Each of these proteins functions correspond to some of the known regulator pathways of CaMK-II.

Actin

An actin localization domain within the variable region of CaMK-II is responsible for the association of certain CaMK-II's with stress fibers of the actin cytoskeleton (O'Leary et al., 2006). Upon actin binding, some CaMK-II isoforms have been shown to enhance dendritic arborization and synapse formation during brain development (Fink et al., 2003), while other isoforms act through the cytoskeleton to stabilize axons (Easley et al., 2006). It is believed that the association of CaMK-II with actin may provide more efficient substrate phosphorylation of the neuronal substrates of CaMK-II including AMPA receptors, NMDA receptors and MAP2 (Shen et al., 1998).

Tubulin

Tubulin is the main protein of microtubule filaments known to associate with the actin cytoskeleton and influence cellular processes involved in maintaining cell morphology as well as cell motility. The CaMK-II substrate, MAP-2, forms a bridge between tubulin and actin. Upon MAP-2 phosphorylation by CaMK-II, the interaction between tubulin and actin is severed. This loss of interaction has been suggested to cause dendritic morphogenesis critical to the formation of functional neuronal networks (Shen et al., 1998; Vallano and DeLorenzo, 1986; Vallano et al., 1986).

Tropomodulin-3

Tropomodulin-3 was recently discovered as a binding partner of CaMK-II

(Seward et al., 2008), however the interaction between Tropomodulin-3 and CaMK-II has not been further characterized. Tropomodulin-3 was identified to bind actin filaments and actin monomers, in addition to negatively regulating cell migration. Recent analysis revealed that, unlike previously characterized tropomodulins, tropomodulin-3 sequesters actin monomers with an affinity similar to its affinity for capping pointed ends of actin filaments (Fischer et al., 2006). These results show that the affects tropomodulin-3 has on actin dynamic regulation are different from the other members of the tropomodulin family, which only cap actin filaments (Fowler et al., 2003; Fujisawa et al., 2001; Krieger et al., 2001). The interaction of tropomodulin-3 with CaMK-II may promote this difference in function, while regulating actin dynamics important in cell motility.

Flightless-I

Flightless-I and the Flightless-I leucine rich repeat associated proteins 1 and 2 (FLAP1, FLAP2), are unique CaMK-II interacting proteins within the list of recently identified binding partners (Seward et al., 2008). Flightless-I is a gelsolin related actin binding protein (GRABP) that was originally identified in *D. melanogaster* mutants that had lost the ability to fly. The structure of Flightless-I consists of an amino-terminal leucine rich repeat domain, known to be important in protein-protein interactions, and a carboxy-terminal gelsolin-like domain which functions in actin binding and capping. Previous research has shown that Flightless-I, coupled with both association proteins, may be involved in cell migration as well as gene transcription (Archer et al., 2004; Cowin et al., 2007). With respect to cell motility, wild type Flightless-I enhances wound healing in

mice whereas overexpression of Flightless-I impairs the healing process (Cowin et al., 2007). Flightless-I has been shown to function as both a promoter and repressor of gene transcription. Flightless-I was shown to promote transcriptional co-activation of estrogen and thyroid receptors (Lee et al., 2004). However, Flightless-I also has been shown to inhibit the transcription of β-catenin-dependent promoters (Lee and Stallcup, 2006). There has been speculation that Flightless-I may serve as the link between signal transduction pathways and regulation of the cytoskeleton. However, the manner by which Flightless-I is able to translocate from the nucleus to the cytosol or how Flightless-I can both promote and inhibit transcription is not completely understood. The interaction between CaMK-II and Flightless-I may serve as the means for regulating Flightless-I function.

Rationale

Current CaMK-II research predominately focuses on CaMK-II's involvement in neuronal processes, specifically during development. However, CaM-II is known to be involved in multiple non-neuronal pathways. Of particular interest was CaMK-II's function in gene transcription necessary for cell cycle progression. Previous research has shown that inhibition of CaMK-II decreases cellular cyclin D1 levels, resulting in G1 phase cell cycle arrest (Morris et al., 1998). The study showed that this G1 phase cell cycle arrest was due to CaMK-II's effect on cyclin D1 and not through another G1 phase regulatory protein. The CaMK-II isoforms within the NIH/3T3 cells used in this study do not contain nuclear localization domains, thus the transcription of cyclin D1 could not be inhibited through direct CaMK-II contact within the nucleus. Lastly, known transcriptional regulators of cyclin D1 were evaluated to determine if these proteins were regulated through phosphorylation by CaMK-II, yet they are not.

To further these studies in hopes of discovering how CaMK-II regulates cyclin D1 levels, the binding proteins of CaMK-II were identified. Upon preliminary review of the proteins known functions, Flightless-I seemed to possess the most potential due to the presence of a nuclear localization domain as well as an ability to promote β-catenin-dependent transcription, such as cyclin D1.

The interaction between CaMK-II and Flightless-I was initially described in previous research. Immunoprecipation experiments using full length CaMK-II constructs in addition to truncated CaMK-II constructs found that full length CaMK-II is necessary for efficient Flightless-I binding (Seward et al., 2008). Kinase assays showed that Flightless-I is not a substrate of CaMK-II (Seward et al., 2008). Also, when performing immunoblots on cells transfected with wild-type versus constitutively active CaMK-II, researchers found that more Flightless-I bound constitutively active CaMK-II (Seward et al., 2008) suggesting an activity-dependent interaction. Finally, immunolocalization experiments in NIH/3T3 cells showed cytosolic Flightless-I localization during serum stimulation and upon serum starvation or CaMK-II inhibition Flightless-I localized to the nucleus (Seward et al., 2008). In this study we confirm Flightless-I localization using live cell imaging, as well as provide evidence that CaMK-II influences β-catenin-dependent transcription and thus cell cycle progression through an activity-dependent interaction with Flightless-I.

Materials and Methods

NIH 3T3 Cell Culture

The Swiss mouse fibroblast cell line, NIH/3T3, was maintained in DMEM (Gibco/Invitrogen, Carlsbad, CA) with 10% FBS, L-Glutamine, Penicillin, and Streptomycin at 37ºC in a 5% $C0_2$ incubator in plastic tissue culture dishes (Nunc, Rochester, NY). Serum free experiments were in DMEM with 0% FBS, L-Glutamine, Penicillin, and Streptomycin.

Cell Harvest

NIH/3T3 cell extracts were prepared by either scraping cells or detaching cells with 0.25% trypsin-EDTA, rinsing with PBS followed by centrifugation at 2000 x g for 5 minutes and then resuspending the cellular pellets in homogenization buffer. Homogenization buffer consisted of 20mM HEPES(pH7.4), 2.6mM EGTA, 20mM $MgCl_2$, 80mM β-glycerol phosphate, 0.1μM okadaic acid, 0.01mg/ml chymostatin, leupeptin, aprotinin, pepstatin, and soybean trypsin inhibitor. The cell samples were sonicated for two 5 second bursts on ice and centrifuged at 10,000 x g for 15 minutes at 4ºC.

Drug Treatment

KN-93, a reversible calmodulin antagonist specific for CaMK-II, was used at varying

concentrations and added directly to cell cultures.

Vectors

GFP was linked to the amino-terminus of human flightless-I cDNA (obtained from OPEN Biosystems, Birmingham ALA). The cyclin D1 promoter-luciferase vector encompasses the first ~1kb of the cyclin D1 promoter (Hilton et al., 2005). FLAG-tagged human β-catenin was provided by Dr. Barry Gumbiner, Univ. Virginia. The Super 8X TOPflash vector was obtained from Addgene, Cambridge MA (Veeman et al., 2003). All vectors were analyzed by restriction digestion and DNA sequencing, and by activity assays or immunoblots for determining the appropriate protein expression.

Transfection

Approximately 100% confluent cells in 100mm tissue culture dishes were sub-cultured into six-well cell culture dishes at approximately $5x10^5$ cells per well. A total of 3μg of plasmid DNA was transfected into each well of a six-well dish, with pBS-KS^+ used as a carrier when necessary. Lipofectamine 2000 (Invitrogen) was diluted in Opti-MEM with 3% FBS (Invitrogen, Carlsbad, CA) and the plasmid DNA was diluted in the cell culture medium described under cell culture. Cell culture medium was added immediately before transfection reagents were added. Cells were harvested 24-40 hours post-transfection.

Immunoblot

Cell lysate proteins were separated on 4-15% or 10% SDS-PAGE gels using the Mini-

Protean II gel electrophoresis system (Bio-Rad, Hercules CA). Proteins were transferred to 0.45μm nitrocellulose sheets for 1 hour at 100V and blocked with Tris Buffered Saline, 0.01% Tween, and Sodium Azide (TBSTA), containing 5% BSA and 2% pre-immune serum of the secondary antibody host for 30 minutes. Blots were then incubated for approximately 18hrs with primary antibodies at 1 μg/ml in 5% BSA/TBSTA. Blots were washed three times with TBSTA and incubated for 2 hours with alkaline phosphatase-conjugated secondary IgG at 2μg/ml in 5% BSA/TBSTA. The blots were washed three times with TBSTA with a final wash in 0.1M NaCl and 5mM $MgCl_2$ at pH 9.4 and developed with 0.25mg/mL of both nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Blots were digitally scanned and densitometry was performed using the Image J program (National Institutes of Health).

Imaging

Phase contrast and fluorescent images were taken on an Olympus IX-70 inverted microscope using a 12-bit camera (Olympus, Melville, NY). Fluorescent images were taken with two second exposures using a 595 nm dichroic long pass filter (Chroma, Rockingham, VT) illuminated by a mercury arc lamp. The images were processed using Olympus Microsuite, v.5.

Antibodies

The mouse anti-flightless-I antibody was a purified monoclonal, mouse IgG, at 1μg/mL (Covance, Berkeley, CA). Rabbit anti-GFP was purchased from Santa Cruz

Biotechnology, Santa Cruz CA. The goat anti-mouse antibody was purchased from Jackson Immunological Laboratories, West Grove, PA.

Luciferase assays

After 24-40hrs of transfection with a reporter vector and FLAG-β-catenin, cells were harvested (as described under cell harvest) and assayed in triplicate using the Enhanced Luciferase Kit (BD Biosciences). Luminescence readings were performed using a Wallac (Perkin Elmer) Victor^2 Multilabel 96-well counter. Luciferase values were normalized to total protein and are represented as fold-activation.

siRNA

siRNAs (up to 100 pico moles) were transfected with a total of 3.0 μg pBluescriptKS$^+$ or other DNA vectors into 5x10^5 NIH/3T3 cells using Lipofectamine 2000 as described under transfection. The flightless-I siRNA (Invitrogen) is listed 5' to 3'.
CCGCCGAGUGGUACAACAUUGACUU and
AAGUCAAUGUUGUACCACUCGGCGG.

Results

Constructing GFP-Flightless-I

To monitor the localization of Flightless-I in live cells, we constructed GFP-linked Flightless-I. A human Flightless-I clone was obtained from OPEN Biosystems, PCR-amplified and directionally inserted into the multiple cloning region of the mEGFP-C1 vector (BD Biosciences Clontech). The restriction enzymes *Sal*I and *Bam*HI were used to cut both the insert and the vector. Multiple tests were performed to confirm the ligation of Flightless-I into the mEGFP-C1 vector including a restriction enzyme digest, gene sequencing and Western blot.

First, a restriction enzyme digest was performed using the enzymes *Bam*HI and *Sal*I to remove the Flightless-I clone from the mEGFP-C1 vector. The Flightless-I clone is approximately 3.8kB in length, whereas the mEGFP-C1 vector is 4.7kB. DNA gel electrophoresis confirmed that the Flightless-I insert and mEGFP-C1 vector were present after the enzymatic digest as both a 3.8kB and 4.7kB band were observed on the gel (Figure 2A). Next, sequencing of the GFP-Flightless-I plasmid was performed to ensure the entire Flightless-I gene was successfully ligated into the vector without mutations and in the correct reading frame. Seven primers spanning the Flightless-I insert and regions within the GFP site of the vector were used. Each of the primers provided approximately 800bp of sequence and by aligning adjacent primer sequences the entire Flightless-I gene

was verified (Figure 2B). No mutations were found and the gene appeared in the correct reading frame. Finally, a Western blot was performed using NIH/3T3 cells overexpressing GFP-Flightless-I. The plasmid encoding GFP-Flightless-I was transfected into NIH/3T3 cells for 24 hours and the cell lysates were probed for Flightless-I using Western Blot analysis. Flightless-I migrates at approximately 145kDa and GFP-Flightless-I at approximately 170kDa. Western blotting indicates that both endogenous Flightless-I as well as GFP-Flightless-I were present as both a 145kDa and 170kDa band were detected (Figure 2C). These tests confirm that the Flightless-I gene was successfully ligated into the mEGFP-C1 vector.

Localization of GFP-Flightless-I in NIH/3T3 Cells

To determine if GFP-Flightless-I localizes in live cells as it does in fixed cells, GFP-Flightless-I was transfected into NIH/3T3 cells. In fixed cells, Flightless-I gradually localized to the cytosol under cell proliferating conditions and to the nucleus under growth inhibited conditions or during CaMK-II inhibition (Seward et al., 2008). Live cell imaging verified that cells overexpressing GFP-Flightless-I exhibit the same Flightless-I localization as cells expressing endogenous Flightless-I. Under growth stimulated conditions, GFP-Flightless-I localized to the cell cytosol (Figure 3A) and upon serum starvation GFP-Flightless-I gradually translocated to the nucleus (Figure 3B). The percentage of nuclear GFP-Flightless-I was averaged from at least ten cells per condition and ten regions of interest per cell to determine GFP-Flightless-I localization (Figure 3D).

These results are consistent with prior Flightless-I localization experiments and verify that GFP-linked Flightless-I retains intracellular Flightless-I location.

CaMK-II Activity Dependent Localization of GFP-Flightless-I

Previous research suggests that Flightless-I localization is dependent on CaMK-II activation (Seward et al., 2008). In order to further examine whether the activity state of CaMK-II drives Flightless-I localization, $T^{287}D$ CaMK-II and GFP-Flightless-I were co-transfected into NIH/3T3 cells. $T^{287}D$ CaMK-II is a constitutively active CaMK-II mutant with an Asp^{287} point mutant at Thr^{287}, the autophosphorylation site, which renders CaMK-II calcium independent. Twenty four hours post-transfection, serum-free medium was added to the cells. GFP-Flightless-I was imaged after 18 hours under serum-free conditions using epifluorescent microscopy. In cells expressing $T^{287}D$ CaMK-II, GFP-Flightless-I remained in the cytosol under serum starvation (Figure 3C). Localization was calculated by averaging total nuclear fluorescence from ten cells and within ten regions of interest per cell (Figure 3D). These results support prior localization experiments and confirm that CaMK-II activation directs Flightless-I localization.

β-Catenin Dependent Transcription

The second part of this study investigated the role of CaMK-II and Flightless-I on transcriptional regulation. To do this, the Super 8xTOPflash-luc (TOPflash) and cyclinD1-luc reporter vectors were obtained. TOPflash and cyclinD-luc are luciferase reporters of β-catenin-mediated transcriptional activation. Each vector contains a Tcf/Lef promoter

region upstream from the luciferase gene that allows for β-catenin binding. Upon binding, luciferase is expressed. The TOPflash vector contains eight Tcf/Lef binding sites; whereas cyclinD1-luc contains only one in addition to the first one thousand nucleotides of the cyclin D1 promoter.

Because the TOPflash reporter vector contains eight Tcf/Lef binding sites and cyclinD1-luc contains only one, an initial luciferase assay was performed to determine if the number of Tcf/Lef binding sites affects luciferase expression. NIH/3T3 cells were co-transfected with either cyclinD1-luc or TOPflash and FLAG-β-catenin. Forty hours post-transfection cells were harvested and luciferase-luminescence was measured using a luminometer. CyclinD1-luc has an approximate 2-fold activation in the presence of β-catenin; whereas TOPflash has an approximate 70-fold luciferase activation, due to the eight Tcf/lef binding sites of TOPflash (Figure 4A). These results confirm that both reporter vectors are β-catenin-dependent, with the TOPflash vector having significantly enhanced luciferase activation as compared to the cyclinD1-luc vector.

Flightless-I inhibits β-catenin-dependent Transcription

Flightless-I has been shown to inhibit Tcf/Lef transcription under serum starvation conditions (Lee and Stallcup, 2006). To determine whether Flightless-I overexpression impairs β-catenin-dependent transcription, Flightless-I was co-transfected into NIH/3T3 cells with either TOPflash or cyclinD1-luc and FLAG-β-catenin. Forty hours post-transfection a luciferase assay was performed. Increased Flightless-I significantly reduced luciferase-luminescence in the presence of either the cyclinD1-luc reporter (Figure 4B) or

the TOPflash reporter (Figure 4C). Furthermore, Flightless-I's effect on luciferase-luminescence was dose-dependent. Transfecting higher concentrations of Flightless-I further impaired β-catenin-dependent transcription (Figure 4B+C).

To investigate how Flightless-I regulates β-catenin-mediated transcription, we first evaluated whether Flightless-I promotes β-catenin-dependent degradation. A non-degradable $S^{45}A$ β-catenin plasmid was constructed by mutating Ser^{45} to Ala^{45}, the ubiquitination phosphophorylation site of glycogen synthase kinase-3 (GSK-3). Non-degradable $S^{45}A$ β-catenin was co-transfected into NIH/3T3 cells with the TOPflash reporter vector and increasing concentrations of Flightless-I. Although total luciferase-luminescence increased in the presence of $S^{45}A$ β-catenin, increasing concentrations of Flightless-I continued to have a dose-dependent inhibitory effect (Figure 4C). This result indicates that Flightless-I inhibition of Tcf/Lef gene transcription is independent of β-catenin degradation.

Effect of CaMK-II on β-catenin-dependent Transcription

Because Flightless-I inhibited β-catenin-dependent transcription through means other than direct β-catenin degradation, we next evaluated whether inhibition of CaMK-II would have a similar effect on β-catenin-dependent transcription. To address this question, NIH/3T3 cells were co-transfected with either cyclinD1-luc or TOPflash in addition to either FLAG-β-catenin or $S^{45}A$ β-catenin. Sixteen hours post-transfection, the CaMK-II inhibitor KN-93 was added for 24 hours. Luciferase assays showed decreased luminescence in the presence of KN-93. This inhibition occurred in a dose-dependent

manner using both cyclinD1-luc and TOPflash and either Flag-β-catenin or $S^{45}A$ β-catenin (Figure 5A+B). Thus, like Flightless-I expression, inhibition of CaMK-II decreases β-catenin-dependent transcription independent of β-catenin degradation.

In an attempt to enhance β-catenin-dependent transcription, we overexpressed either wild-type CaMK-II or $T^{287}D$ CaMK-II. Forty hours post-transfection, luciferase-luminescence was measured in each sample. The results show that whether in the presence of the cyclinD1-luc or TOPflash reporter vector and FLAG-β-catenin, luciferase-luminescence was inhibited during CaMK-II overexpression (Figure 6A+B). Based on these results, CaMK-II may be activating other transcription regulator proteins such as transforming growth factor activated kinase 1 (TAK1) which has been shown to stimulate nemo-like kinase (NLK) activity and results in decreased β-catenin-dependent transcription (Ishitani et al., 1999).

Flightless-I suppression promotes β-catenin Dependent Transcription

To confirm overexpression of Flightless-I caused inhibition of β-catenin-dependent transcription, Flightless-I suppression experiments were performed using small interfering RNA (siRNA). Flightless-I siRNA interferes with the expression of Flightless-I and results in a decrease of Flightless-I gene expression. NIH/3T3 cells were co-transfected for 40 hours with the CyclinD1-luc reporter vector and increasing concentrations of Flightless-I siRNA. Western immunoblot analysis of the cell lysates confirmed the inhibition of Flightless-I expression (Figure 7A) and a luciferase assay showed an increase in luciferase-luminescence upon increased concentrations of Flightless-I siRNA (Figure 7B).

To further substantiate this evidence and show that the effect siRNA Flightless-I was having on β-catenin-dependent transcription was a direct result of Flightless-I suppression, increasing amounts of GFP-Flightless-I were co-transfected into cells with siRNA. GFP-Flightless-I reversed the effects of siRNA Flightless-I, and once again β-catenin-dependent transcription was inhibited (Figure 7B).

Discussion

This study provides the first direct evidence linking CaMK-II and β-catenin-dependent transcription. Previous studies have shown that CaMK-II inhibition within NIH/3T3 cells promotes a decrease in cyclin D1 (Morris et al., 1998), a β-catenin-dependent gene. However, all CaMK-II variants in NIH/3T3 cells are strictly cytosolic, and the transcription factors necessary for cyclin D1 expression are not substrates of CaMK-II (Morris et al., 1998). Flightless-I, a known binding partner of CaMK-II, has previously been shown to be involved in cell motility and β-catenin dependent gene regulation (Archer et al., 2004; Cowin et al., 2007; Lee and Stallcup, 2006), which are two known CaMK-II functions (Easley et al., 2008; Morris et al., 1998). Our results, here, suggest a novel means by which CaMK-II influences β-catenin-dependent transcription by sequestering Flightless-I to the cytosol (Figure 8)

While CaMK-II activity appears to influence Flightless-I localization to the cytosol and thus promote β-catenin-dependent transcription, expression of $T^{287}D$ CaMK-II actually inhibited β-catenin-dependent transcription. This result seems contradictory to the data presented here, but in fact, this finding can be explained because CaMK-II is involved in other pathways that could impair β-catenin-dependent transcription. Previous research has shown that $T^{287}D$ CaMK-II promotes the loss of cell attachment due to rapid focal adhesions turn over, resulting in cell rounding which ultimately leads to cell death (Easley

et al., 2008). Also, not all of the phosphosphorylation substrates of CaMK-II have been identified so it is possible that CaMK-II may directly or indirectly promote the phosphorylation of proteins involved in β-catenin degradation such as GSK3-β or E3 ligase, resulting in decreased β-catenin-dependent transcription. Another possible explanation for a decrease in β-catenin-dependent transcription during overexpression of CaMK-II is that CaMK-II may be inhibiting β-catenin degradation resulting in an accumulation of β-catenin within the cytosol. Excess β-catenin has been shown to promote p53 activation which leads to repressed cyclin D1 transcription (Damalas et al., 1999; Rocha et al., 2003). Finally, CaMK-II may inhibit β-catenin-dependent transcription through the phosphorylation of Tak1 or NLK. These proteins have both been shown to down regulate transcriptional activation of β-catenin-dependent genes (Ishitani et al., 1999). Thus, expression of $T^{287}D$ CaMK-II is unable to promote β-catenin-dependent transcription due to the activation of other pathways which result in the inhibition of β-catenin-dependent transcription.

The canonical and non-canonical Wnt pathways are two separate pathways that may cross-talk due to the interaction between Flightless-I and CaMK-II. Activation of the canonical Wnt pathway has been shown to promote β-catenin-dependent transcription, while activation of the non-canonical Wnt pathway has been shown to regulate cell motility (Kohn and Moon, 2005; Moon, 2005) . Previous research has shown that the non-canonical Wnt pathway inhibits β-catenin-dependent transcription by promoting β-catenin degradation (Topol et al., 2003). Here we show that the activation of CaMK-II through the non-canonical Wnt pathway promotes β-catenin-dependent transcription by sequestering

Flightless-I to the cytosol (Figure 8). Thus, this research suggests a novel means for cross-talk to occur between the canonical and non-canonical Wnt pathways (Figure 9).

Recently, the differential expression of CaMK-II genes involved in zebrafish, *Danio rerio,* development were characterized and include *camk2a1,camk2b1, camk2g1, camk2g2, camk2d1, camk2d2*. Each gene was shown to be temporally expressed within many tissues; including the heart, brain, retina, gut, pectoral fins, and epidermal tissue during the first three days of development (Rothschild et al., 2007). My current research primarily focused on the interaction of one CaMK-II gene, δ CaMK-II, and Flightless-I. Both variants of the δ CaMK-II gene in zebrafish (*camk2d1 and camk2d2*) were shown to have low expression throughout the first three days of development in the hindbrain, forebrain, epidermal tissue and retinal epithelium (Rothschild et al., 2007). By mapping the expression of Flightless-I during early zebrafish development, we would hope to find similar Flightless-I localization patterns. The next step would be to analyze any effect the interactions between the two proteins are having on cyclin D1 levels, in a tissue specific manner throughout different stages of development. Using morpholino antisense oligonucleotides (MO) directed against the zebrafish δ CaMK-II genes, *camk2d1 and camk2d2,* the expression of δ CaMK-II would be inhibited. After designing cyclin D1 primers, RT-PCR could be performed on zebrafish containing the *camk2d1 and camk2d2* morpholinos to determine any changes in the levels of cyclin D1. This research could be furthered by not only assaying this interaction in δ CaMK-II zebrafish genes, but rather all of the known zebrafish CaMK-II genes.

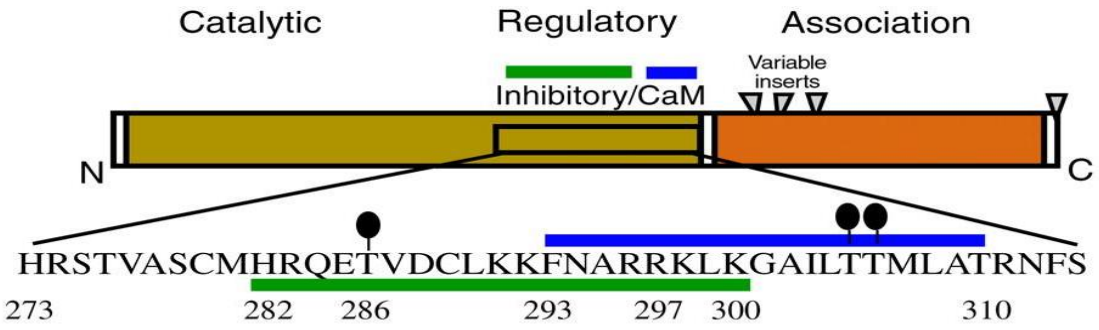

Figure 1. CaMK-II Structure. CaMK-II contains an amino-terminal catalytic domain, a central regulatory domain containing an inhibitory site as well as a Ca^{2+}/CaM binding site and a carboxy-terminal variable domain.

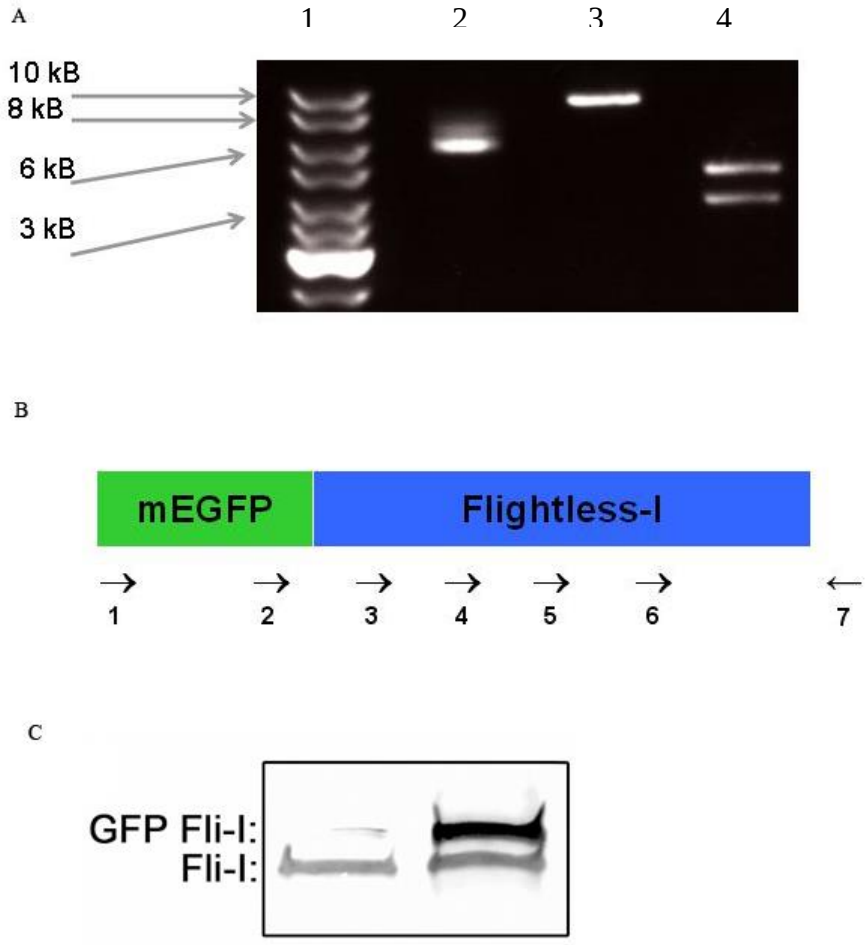

Figure 2. Constructing GFP-Flightless-I. GFP was ligated to the N-terminal side of human Flightless-I cDNA. **A.** DNA electrophoresis of a GFP-flightless-I restriction enzyme digest shows uncut GFP-Flightless-I in lane two, Sal-I linearized GFP-Flightless-I in lane three and Sal-I/BamHI double digested GFP-Flightless-I in lane four. **B.** A schematic of GFP-linked Flightless-I and the primers used to obtain sequence. **C.** 20μg of lysate from un-transfected (left lane) and GFP-Fli-I transfected cells (right lane) were hybridized with anti-Fli-I. Fli-I migrates at ~145kDa and GFP-Fli-I at ~170kDa.

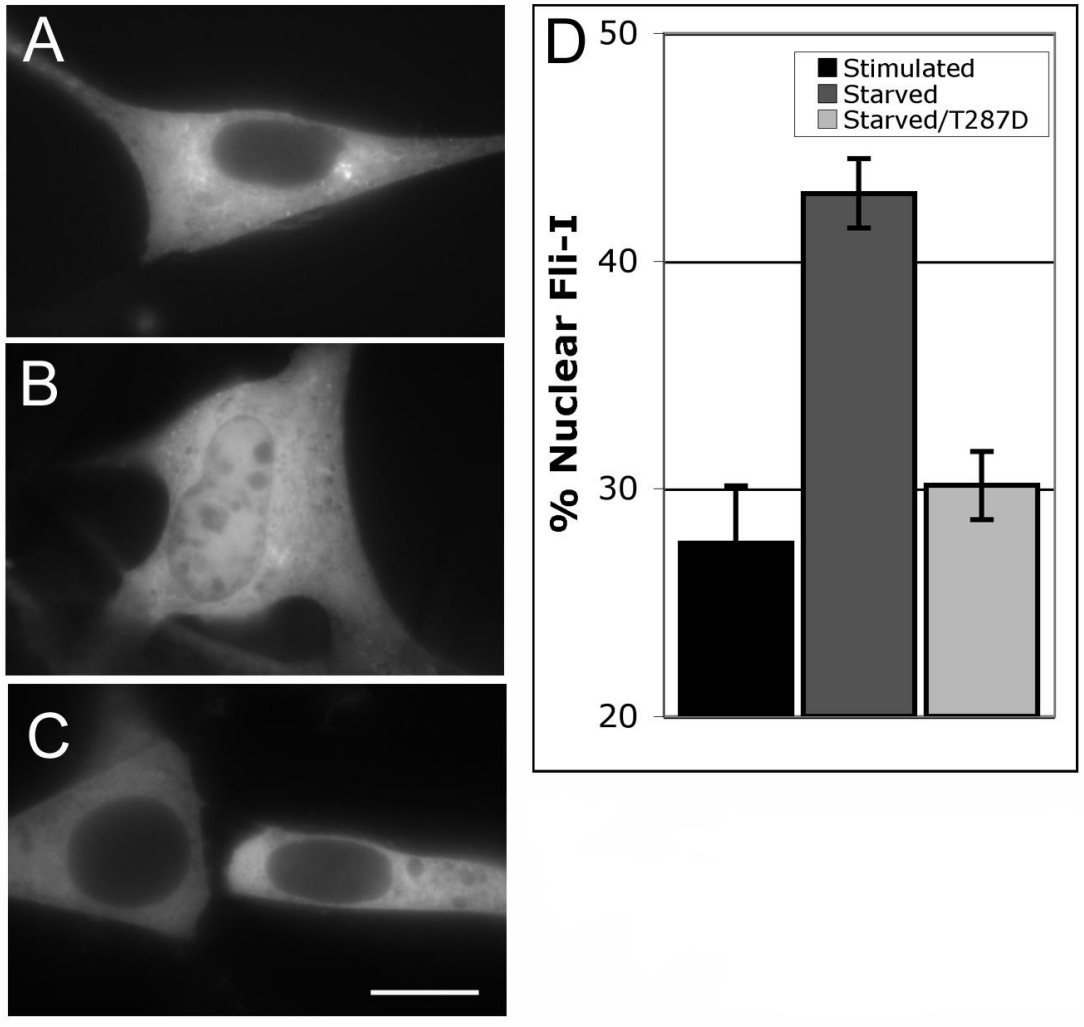

Figure 3. Flightless-I is retained in the cytosol by CaMK-II. GFP-Flightless-I was transfected alone **(A,B)** or with $T^{287}D$ CaMK-II **(C)** and then either maintained in **A:** complete medium or **B,C:** serum starved for 18 hours. Live conventional epifluorescent microscopy was used to image cells. Scale bar = 10μm. **D:** The percentage of total fluorescence that is nuclear was averaged from at least ten cells per condition and ten regions of interest per cell.

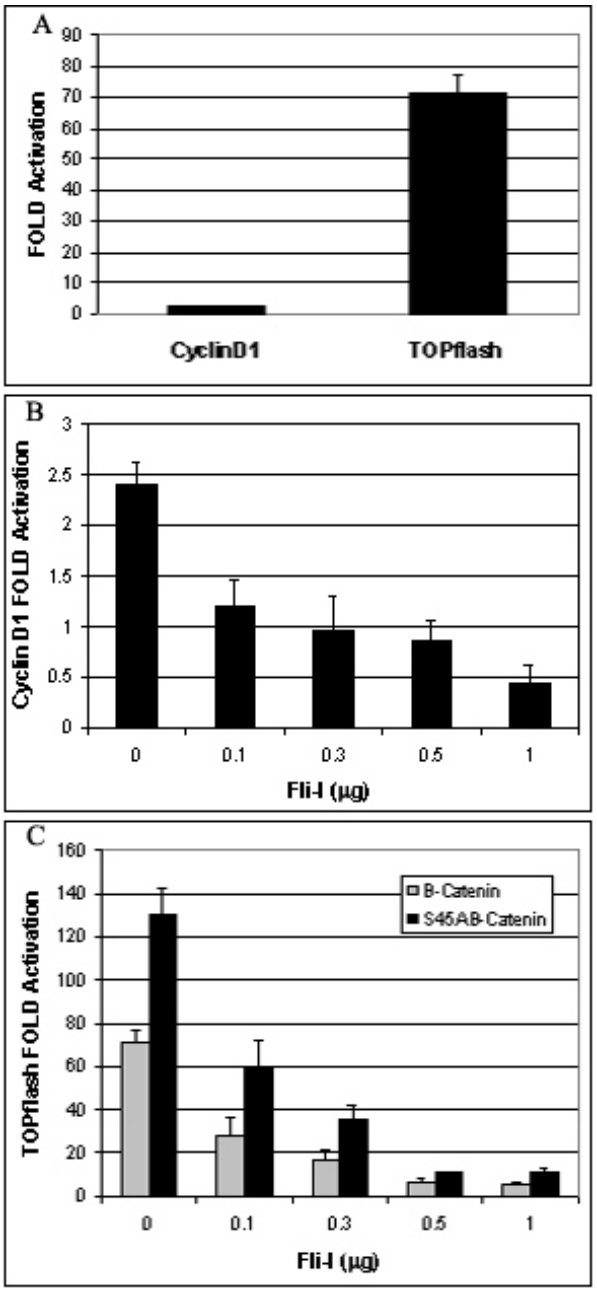

Figure 4. Flightless-I suppresses β-catenin-dependent transcription. Luciferase assays were performed 40 hours post-transfection of NIH/3T3 cells in complete medium. Fold-activation of luciferase was measured. **A:** Cells were co-transfected with 1µg FLAG-β-catenin and either 1µg CyclinD1-luc or 0.5µg TOPflash-luc. **B:** Indicated amounts (µg) of GFP-Fli-I were co-transfected with 1µg FLAG-β-catenin and 1µg CyclinD1-luc. **C:** Indicated amounts (µg) of GFP-Fli-I were co-transfected with 0.5µg TOPflash-luc and either 1µg of FLAG-β-catenin or 1µg of $S^{45}A$ FLAG-β-catenin.

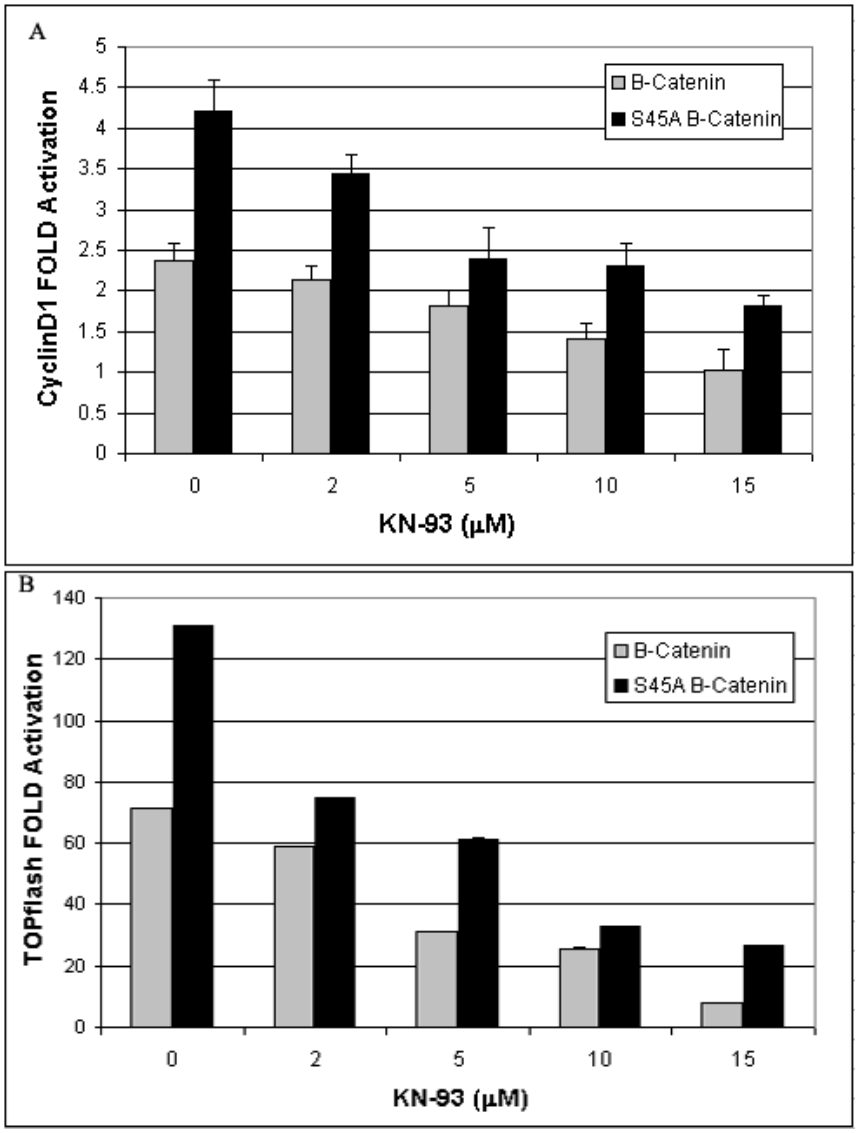

Figure 5. Inhibition of CaMK-II suppresses β-catenin-dependent transcription. Luciferase assays were performed 40 hours post-transfection of NIH/3T3 cells in complete medium. Fold-activation of luciferase was measured. Cells were co-transfected with either 1μg FLAG-β-catenin or 1μg of $S^{45}A$ FLAG-β-catenin in the presence of **A:** 1μg CyclinD1-luc or **B:** 0.5μg of TOPflash-luc. KN-93 was added in the amounts indicated (μM).

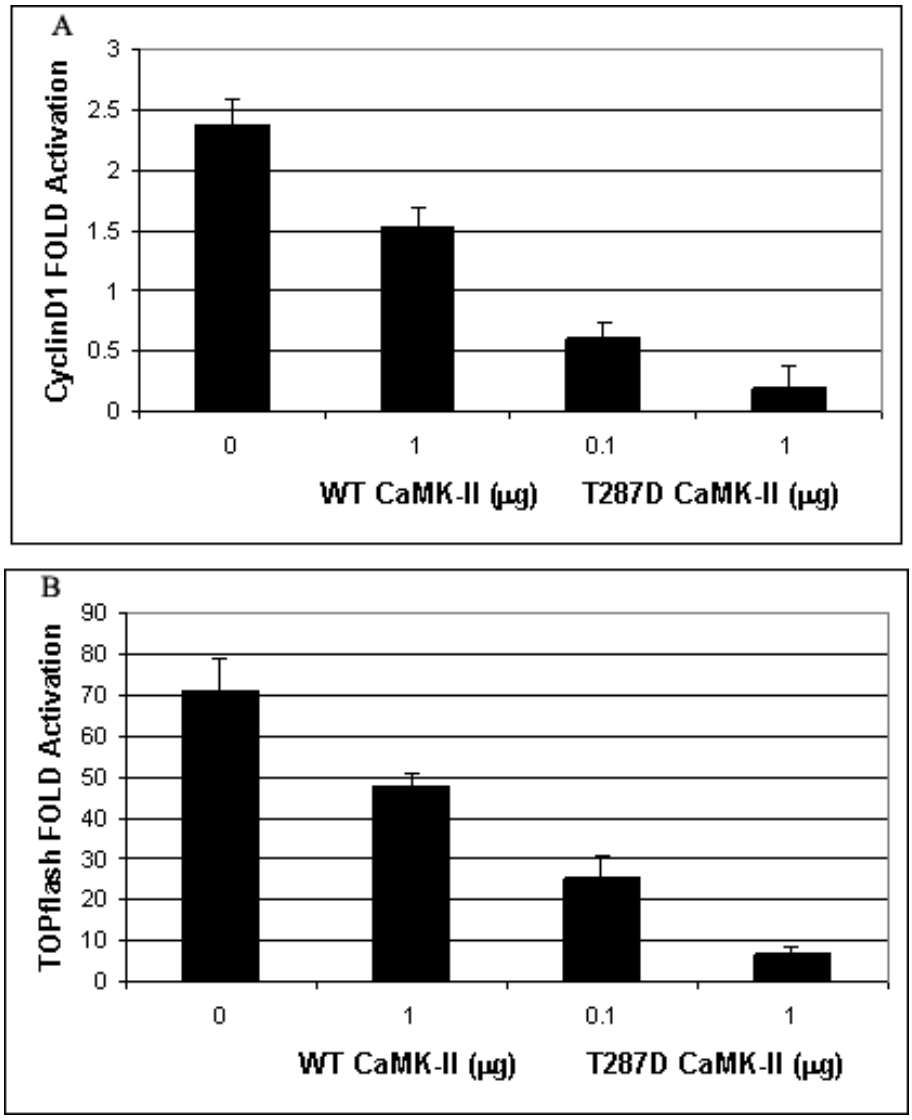

Figure 6. Over-expression of CaMK-II inhibits β-catenin-dependent transcription. Luciferase assays were performed 40 hours post-transfection of NIH/3T3 cells in complete medium. Fold-activation of luciferase was measured. Cells were co-transfected with 1µg FLAG-β-catenin in the presence of **A:** 1µg CyclinD1-luc or **B:** 0.5µg of TOPflash-luc. Wild-type δ CaMK-II or $T^{287}D$ CaMK-II were added in the amounts indicated (µg).

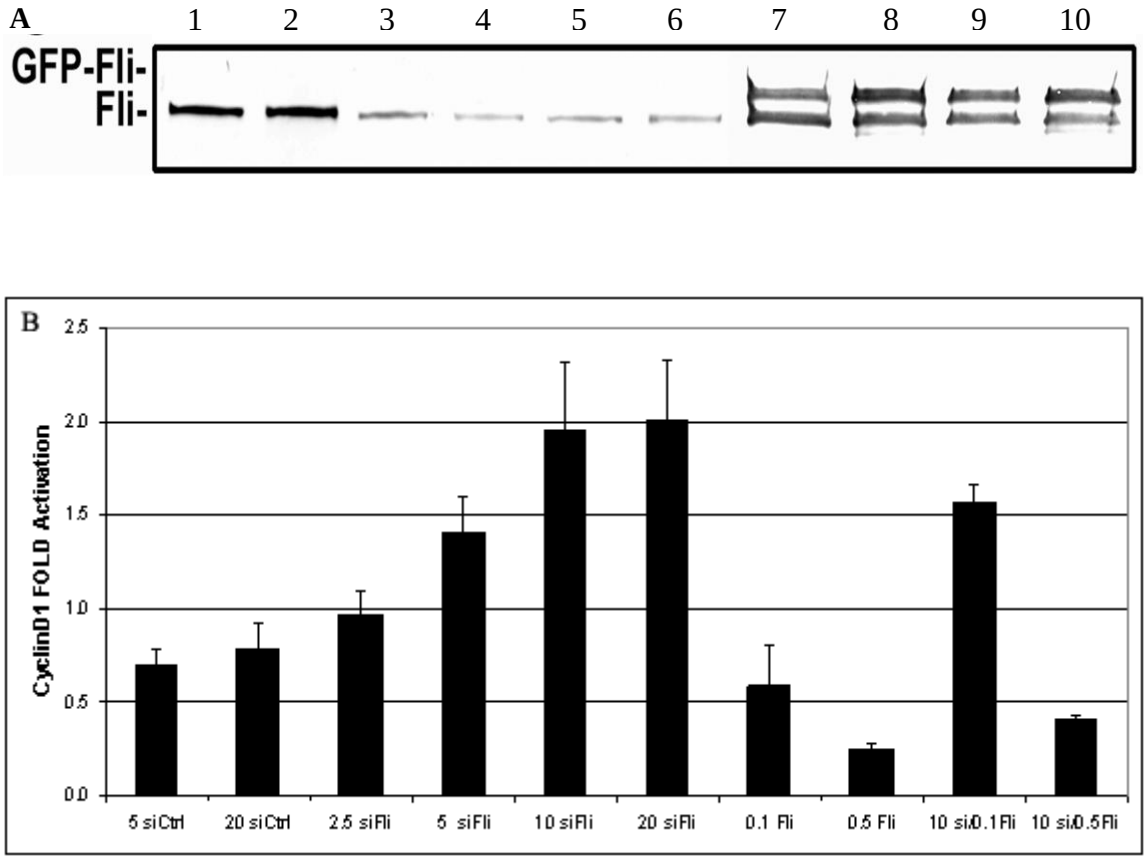

Figure 7. siRNA suppression of Flightless-I enhances cyclin D1 transcription. Luciferase assays were performed 40 hours post-transfection of NIH/3T3 cells in complete medium. Luciferase values are represented as fold-activation relative to cells transfected with cyclinD1-luc alone. **A:** Anti-Fli-I immunoblot of the relative levels of endogenous and transfected Fli-I corresponding to transfected cells used in lanes 1-10 of part B. **B:** Cells were co-transfected with 1.0μg of cyclinD1-luc and indicated amounts (pico moles) of either control or Flightless-I siRNA (lanes 1-6). Lanes 7-8 show cells co-transfected with 1.0μg of cyclinD1-luc and indicated amounts (μg) of GFP-Fli. Lanes 9-10 show cells co-transfected with 1.0μg of cyclinD1-luc and indicated amounts (μg) of GFP-Fli in the presence of 10picomoles of Flightless-I siRNA.

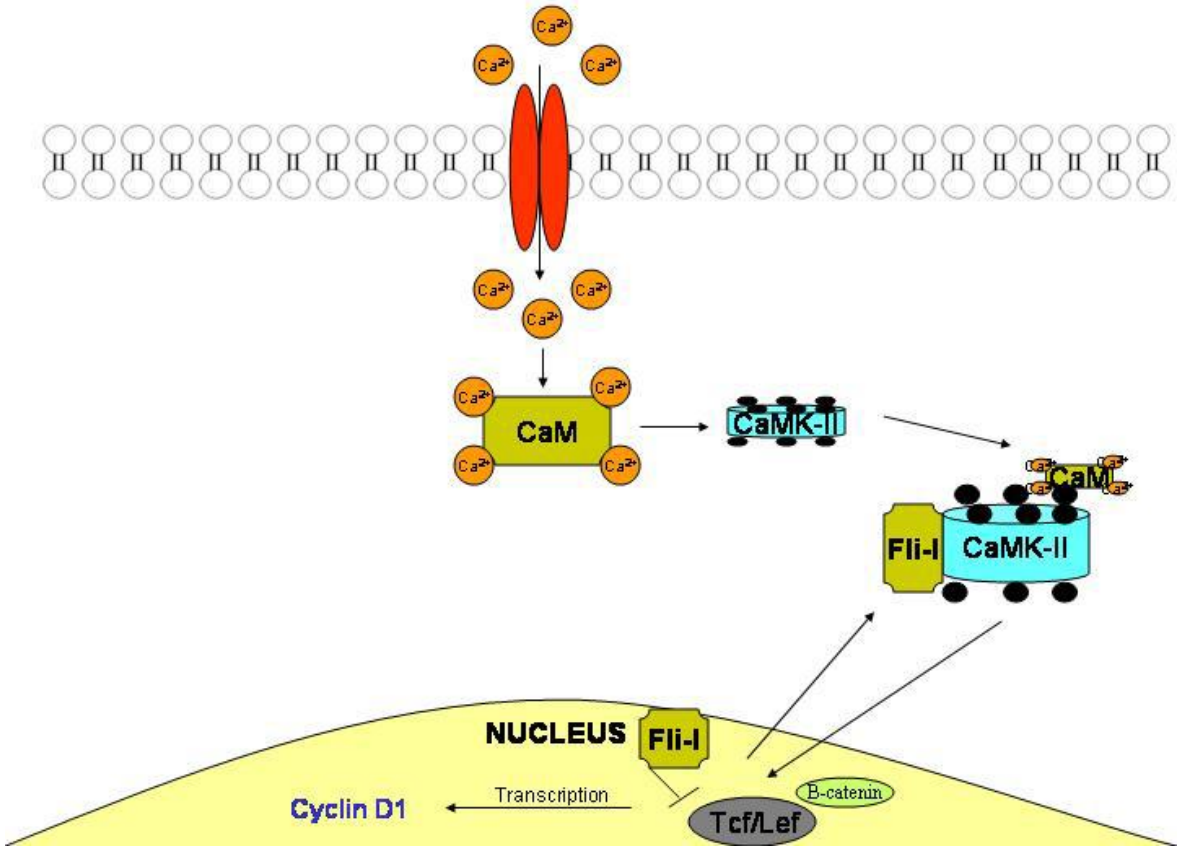

Figure 8. Model of CaMK-II and Flightless-I's Interaction. When intracellular Ca^{2+} levels rise, CaM binds Ca^{2+}. Ca^{2+}/CaM binds CaMK-II, activating the kinase. In its activated state, CaMK-II is capable of binding Flightless-I, which is otherwise sequestered to the nucleus. Within the nucleus Flightless-I inhibits Tcf/Lef dependent gene transcription.

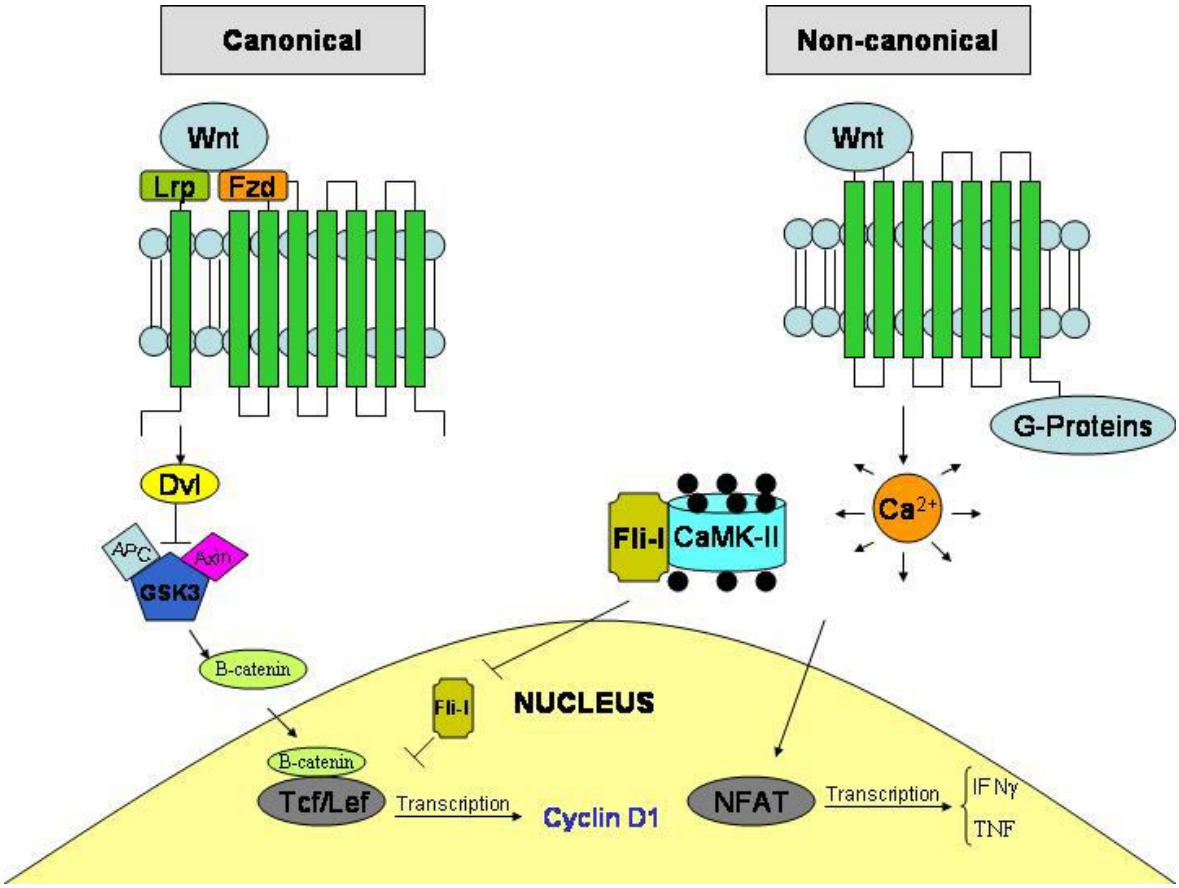

Figure 9. Model of CaMK-II's Role in Canonical and Non-canonical Wnt Pathways. Upon activation of the non-canonical Wnt pathway, intracellular Ca^{2+} levels rise and CaMK-II becomes activated. Activated CaMK-II sequesters Flightless-I to the cytosol thus promoting Tcf/Lef dependent gene transcription, the nuclear target of the canonical Wnt pathway.

Literature Cited

.

Archer, S.K., C.A. Behm, C. Claudianos, and H.D. Campbell. 2004. The flightless I protein and the gelsolin family in nuclear hormone receptor-mediated signalling. *Biochem Soc Trans*. 32:940-2.

Benfenati, F., F. Valtorta, J.L. Rubenstein, F.S. Gorelick, P. Greengard, and A.J. Czernik. 1992. Synaptic vesicle-associated Ca2+/calmodulin-dependent protein kinase II is a binding protein for synapsin I. *Nature*. 359:417-20.

Caran, N., L.D. Johnson, K.J. Jenkins, and R.M. Tombes. 2001. Cytosolic targeting domains of gamma and delta calmodulin-dependent protein kinase II. *J Biol Chem*. 276:42514-9.

Chen, H.X., N. Otmakhov, S. Strack, R.J. Colbran, and J.E. Lisman. 2001. Is persistent activity of calcium/calmodulin-dependent kinase required for the maintenance of LTP? *J Neurophysiol*. 85:1368-76.

Cowin, A.J., D.H. Adams, X.L. Strudwick, H. Chan, J.A. Hooper, G.R. Sander, T.E. Rayner, K.I. Matthaei, B.C. Powell, and H.D. Campbell. 2007. Flightless I deficiency enhances wound repair by increasing cell migration and proliferation. *J Pathol*. 211:572-81.

Damalas, A., A. Ben-Ze'ev, I. Simcha, M. Shtutman, J.F. Leal, J. Zhurinsky, B. Geiger, and M. Oren. 1999. Excess beta-catenin promotes accumulation of transcriptionally active p53. *Embo J*. 18:3054-63.

Easley, C.A., M.O. Faison, T.L. Kirsch, J.A. Lee, M.E. Seward, and R.M. Tombes. 2006. Laminin activates CaMK-II to stabilize nascent embryonic axons. *Brain Res*. 1092:59-68.

Easley, C.A.t., C.M. Brown, A.F. Horwitz, and R.M. Tombes. 2008. CaMK-II promotes focal adhesion turnover and cell motility by inducing tyrosine dephosphorylation of FAK and paxillin. *Cell Motil Cytoskeleton*. 65:662-74.

Fink, C.C., K.U. Bayer, J.W. Myers, J.E. Ferrell, Jr., H. Schulman, and T. Meyer. 2003. Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron*. 39:283-97.

Fischer, R.S., E.G. Yarmola, K.L. Weber, K.D. Speicher, D.W. Speicher, M.R. Bubb, and V.M. Fowler. 2006. Tropomodulin 3 binds to actin monomers. *J Biol Chem*. 281:36454-65.

Fowler, V.M., N.J. Greenfield, and J. Moyer. 2003. Tropomodulin contains two actin filament pointed end-capping domains. *J Biol Chem*. 278:40000-9.

Fujisawa, T., A. Kostyukova, and Y. Maeda. 2001. The shapes and sizes of two domains of tropomodulin, the P-end-capping protein of actin-tropomyosin. *FEBS Lett*. 498:67-71.

Gaertner, T.R., J.A. Putkey, and M.N. Waxham. 2004. RC3/Neurogranin and Ca2+/calmodulin-dependent protein kinase II produce opposing effects on the affinity of calmodulin for calcium. *J Biol Chem*. 279:39374-82.

Giese, A., B. Laube, S. Zapf, U. Mangold, and M. Westphal. 1998. Glioma cell adhesion and migration on human brain sections. *Anticancer Res*. 18:2435-47.

He, X., F. Yang, Z. Xie, and B. Lu. 2000. Intracellular Ca(2+) and Ca(2+)/calmodulin-dependent kinase II mediate acute potentiation of neurotransmitter release by neurotrophin-3. *J Cell Biol*. 149:783-92.

Hudmon, A., and H. Schulman. 2002. Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu Rev Biochem*. 71:473-510.

Huttenlocher, A., I.J. Frieden, and H. Emery. 1995. Neonatal onset multisystem inflammatory disease. *J Rheumatol*. 22:1171-3.

Ishitani, T., J. Ninomiya-Tsuji, S. Nagai, M. Nishita, M. Meneghini, N. Barker, M. Waterman, B. Bowerman, H. Clevers, H. Shibuya, and K. Matsumoto. 1999. The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature*. 399:798-802.

Ito, I., H. Hidaka, and H. Sugiyama. 1991. Effects of KN-62, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, on long-term potentiation in the rat hippocampus. *Neurosci Lett*. 121:119-21.

Kohn, A.D., and R.T. Moon. 2005. Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium*. 38:439-46.

Kolb, S.J., A. Hudmon, T.R. Ginsberg, and M.N. Waxham. 1998. Identification of domains essential for the assembly of calcium/calmodulin-dependent protein kinase II holoenzymes. *J Biol Chem*. 273:31555-64.

Krieger, I., A.S. Kostyukova, and Y. Maeda. 2001. Crystallization and preliminary characterization of crystals of the C-terminal half fragment of tropomodulin. *Acta Crystallogr D Biol Crystallogr*. 57:743-4.

Lantsman, K., and R.M. Tombes. 2005. CaMK-II oligomerization potential determined using CFP/YFP FRET. *Biochim Biophys Acta*. 1746:45-54.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-69.

Lee, Y.H., H.D. Campbell, and M.R. Stallcup. 2004. Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. *Mol Cell Biol*. 24:2103-17.

Lee, Y.H., and M.R. Stallcup. 2006. Interplay of Fli-I and FLAP1 for regulation of beta-catenin dependent transcription. *Nucleic Acids Res*. 34:5052-9.

Moon, R.T. 2005. Wnt/beta-catenin pathway. *Sci STKE*. 2005:cm1.

Morris, T.A., R.J. DeLorenzo, and R.M. Tombes. 1998. CaMK-II inhibition reduces cyclin D1 levels and enhances the association of p27kip1 with Cdk2 to cause G1 arrest in NIH 3T3 cells. *Exp Cell Res*. 240:218-27.

Ridley, A.J., M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, and A.R. Horwitz. 2003. Cell migration: integrating signals from front to back. *Science*. 302:1704-9.

Rocha, S., A.M. Martin, D.W. Meek, and N.D. Perkins. 2003. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. *Mol Cell Biol*. 23:4713-27.

Rothschild, S.C., J.A. Lister, and R.M. Tombes. 2007. Differential expression of CaMK-II genes during early zebrafish embryogenesis. *Dev Dyn*. 236:295-305.

Schulman, H., P.I. Hanson, and T. Meyer. 1992. Decoding calcium signals by multifunctional CaM kinase. *Cell Calcium*. 13:401-11.

Seward, M.E., C.A.t. Easley, J.J. McLeod, A.L. Myers, and R.M. Tombes. 2008. Flightless-I, a gelsolin family member and transcriptional regulator, preferentially binds directly to activated cytosolic CaMK-II. *FEBS Lett*.

Shen, K., and T. Meyer. 1999. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science*. 284:162-6.

Shen, K., M.N. Teruel, K. Subramanian, and T. Meyer. 1998. CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. *Neuron*. 21:593-606.

Tombes, R.M., M.O. Faison, and J.M. Turbeville. 2003. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene*. 322:17-31.

Topol, L., X. Jiang, H. Choi, L. Garrett-Beal, P.J. Carolan, and Y. Yang. 2003. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. *J Cell Biol*. 162:899-908.

Vallano, M.L., and R.J. DeLorenzo. 1986. Separation of microtubule-associated cAMP and calmodulin-dependent kinases that phosphorylate MAP-2. *Ann N Y Acad Sci*. 466:453-6.

Vallano, M.L., J.R. Goldenring, R.S. Lasher, and R.J. Delorenzo. 1986. Association of calcium/calmodulin-dependent kinase with cytoskeletal preparations: phosphorylation of tubulin, neurofilament, and microtubule-associated proteins. *Ann N Y Acad Sci*. 466:357-74.

Yoshimura, Y., T. Shinkawa, M. Taoka, K. Kobayashi, T. Isobe, and T. Yamauchi. 2002. Identification of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II in the postsynaptic density by protein sequencing and mass spectrometry. *Biochem Biophys Res Commun*. 290:948-54.

VITA

Jamie Josephine Avila McLeod was born August 31st, 1983 in Collingwood, Ontario to Debbie and Jeff McLeod. She received her Bachelor's of Science in biology from the College of Science at Virginia Polytechnic Institute and State University May 2005 in Blacksburg, Virginia.