The Protein Interactions and Functions of Transient Receptor Potential Melastatin 7 (TRPM7) Ion Channel

by

Danny Chan

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Cell and System Biology University of Toronto

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An abstract of a thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Cell and System Biology

University of Toronto

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Ion channels are proteins that facilitate ion diffusion across cell membrane. Nevertheless, various groups of ion channels can act as surface receptors and play important roles in signal transduction. Transient Receptor Potential Melastatin 7 (TRPM7) ion channel has been implicated in diverse cellular functions including actomyosin cytoskeletal remodeling and anoxic neuronal death. However the mechanisms behind TRPM7’s physiological roles remain undetermined. TRPM7 possesses unusually long intracellular domains and a functional C-terminal alpha kinase domain that may contribute to regulation of channel activity and signal transduction. We therefore identified proteins that interact with TRPM7 C-terminus. Pull-down assays coupled with LC-MS/MS revealed that cytoskeletal proteins (actin and tubulin) and synaptic vesicle proteins (VAMP2 and SNAP25) associate with the TRPM7. In addition, we further found that TRPM7 does not directly bind microtubules or single dimeric tubulin subunits. Thus one or more microtubule binding proteins is involved in the association between TRPM7 and microtubules.
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Thesis

1. Introduction

1.1 Transient Receptor Potential (TRP) Channels

Ion channels are multimeric membrane protein complexes that facilitate diffusion of ions across the cell membrane. The basic function of ion channels is important for many cellular processes such as maintenance of membrane potential and the propagation of action potentials. Nevertheless, ion channels are more than simply pore proteins on the surface of cells; various groups of ion channels act as cell surface receptors and play important physiological roles in cellular signal transduction. Activation of these channels allows further amplification of the initial signal. Transient Receptor Potential (TRP) channels are a superfamily of ion channels that carry out diverse physiological functions; they act as important receptors and signal transducers in multi-cellular organisms including fruit flies, zebrafish, mice and humans (Venkatachalam and Montell, 2007). In terms of structure (Figure 1) TRP family channels possess a six transmembrane domain pore region and intracellular N- and C-terminal tails giving them a predicted topology similar to voltage gated potassium (K⁺) channels (Clapham et al., 2001; Yellen, 2002). However unlike voltage gated channels the majority of TRP channels are constitutively active, non-selective cation channels that display little sensitivity to membrane potential. TRP channels are however regulated by changes in the extra and intracellular environment and more recent evidence indicates they may be regulated by protein binding on intracellular domains (Clark et al., 2006).

TRP channels were first discovered in Drosophila, functioning as light sensing proteins that are required for phospholipase C (PLC)-dependent light response in the
photoreceptor cells of *Drosophila* eyes (Montell and Rubin, 1989). The mammalian TRP superfamily, now containing 28 identified members, is broadly divided into group 1 and 2 and further separated into six subfamilies. Group 1 TRP channels are segregated from Group 2 TRPs based on sequence and topological differences. The four subfamilies of Group 1 TRPs: TRPC, TRPV, TRPM and TRPA possess great sequence homology to the first identified member, *Drosophila TRP*, whereas the two subfamilies that comprise group 2 TRP channels, TRPP and TRPML share little sequence homology with *TRP* (Montell and Rubin, 1989). Unlike most ion channels, TRP channels are identified by their protein sequence homology rather than by selectivity or function. The first identified mammalian TRP, Transient Receptor Potential Canonical 1 (TRPC1) (Wes et al., 1995), together with all mammalian TRPC proteins appear to be analogous to the *Drosophila* TRPs (Clapham, 2003). Over the years, more TRP members have been identified. The identity of Transient Receptor Potential Vanilloid subfamily (TRPV1) was revealed by using expression cloning to isolate a functional cDNA encoding a vanilloid receptor that is activated by capsaicin (Caterina et al., 1997; Jordt and Julius, 2002), and the Transient Receptor Potential Melastatin (TRPM) subfamily proteins bear homology to Melastatin (TRPM1) which was first identified as a prognostic marker for metastasis of melanoma (Duncan et al., 1998; Ramsey et al., 2006). The first TRPA (Ankyrin) protein, human TRPA1, was identified as a gene product containing sequential ankyrin repeats that was down-regulated following oncogenic transformation of fibroblasts (Jaquemar et al., 1999). As the name implies, Transient Receptor Potential Polycystin (TRPP) channels, which belong to Group 2 TRPs, were named after the disease Polycystic Kidney Disease 2, and its first member (TRPP1, previously known as PKD2) was discovered as a mutated
protein causing Polycystic Kidney Disease 2 (Mochizuki et al., 1996). TRPML1, the founding member of the Transient Receptor Potential Mucolipin subfamily, was identified as a protein in which its mutated version is responsible for the lysosomal storage disorder mucolipidosis IV. Such disease is characterized as a severe neurodegeneration disease.

Due to their diverse physiological roles TRP channels have become targets of intensive research, especially the TRPC channels which were originally thought to mediate "store operated calcium entry", and TRPV channels which are readily activated and now appear to be the main mediators of temperature and perception by sensory neurons. The mammalian TRP channels are non-selectively permeable to cations such as $\text{Ca}^{2+}$ and $\text{Na}^{+}$ and are generally thought to be important sensory receptors and transducers that respond to changes in the intracellular state as well as extracellular stimuli including light, temperature, pH, alcohol, pressure and mechanical stresses (Venkatachalam and Montell, 2007). In addition, many of the TRP channels have been implicated as disease associated proteins. Genetic mutations in some TRP members can lead to consequences beyond the cellular level, affecting the whole organism (Table 1). Of particular interest are two members of the TRPM subfamily, TRPM2 and TRPM7, which are implicated in governing cell death following anoxic injury (Aarts and Tymianski, 2005a; Aarts and Tymianski, 2005b; Fonfria et al., 2004; Fonfria et al., 2005; Hara et al., 2002)}. However, the mechanism of cell death regulation is not well understood and further investigation is required in order to reveal how these channels contribute to cell viability.
Figure 1. TRP channels vs Voltage-gated Potassium (K⁺) channels

TRP channels and voltage-gated potassium channels have similar pore forming structure (between transmembrane segment 5 and segment 6) and both possess long intracellular domains.
Table 1. TRP Channel Subfamilies and Their Related Diseases. The 6 subfamilies of TRP channels, with the exception of TRPAs, are found to be disease associated due to genetic variations.

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<tr>
<td>TRPV (vanilloid)</td>
<td>TRPV5 - Renal Ca(^{2+}) disorder (Gkika et al., 2006; Renkema et al., 2005)</td>
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<td>TRPA (ankyrin)</td>
<td>-------------------------------</td>
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<tr>
<td>TRPM (melastatin)</td>
<td>TRPM6 - Hypomagnesemia with secondary hypocalcemia (Schlingmann et al., 2002; Walder et al., 2002)</td>
</tr>
<tr>
<td>TRPP (polycystin)</td>
<td>TRPP2 - Polycystic kidney disease (Kimberling et al., 1988; Peters and Sandkuijl, 1992; Wu and Somlo, 2000)</td>
</tr>
<tr>
<td>TRPML (mucolipin)</td>
<td>TRPML1 - Mucolipidosis type IV (Zeevi et al., 2007; Zeevi et al., 2009)</td>
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1.1.1 Structural features of TRP Channels

All TRP members contain six transmembrane segments with the pore loop positioned between the fifth and sixth transmembrane domains which together form the cation permeable pore (Figure 2) (Schindl and Romanin, 2007; Venkatachalam and Montell, 2007). Other than the pore forming region, TRP family channels also possess intracellular N- and C-terminal tails giving them a structure similar to voltage gated potassium (K+) channels (Clapham et al., 2001; Yellen, 2002). Group 2 TRP proteins are differentiated by an extra segment that forms a large extracellular loop separating the first and second transmembrane segments. Group 1 TRP channels, with the exception of the TRPM subfamily, have multiple intracellular, N-terminal ankyrin repeats (Gaudet, 2008). These repeats are protein-protein interaction motifs that can be found in a large variety of proteins involved in transcription, cell-cycle regulation and ion transport (Mosavi et al., 2004). In addition, TRPCs, TRPMs and the non-mammalian TRPN members contain a C-terminal TRP domain situated on the intracellular tail in close proximity to the sixth transmembrane domain but lacks a known function. The most conserved portions of the TRP domain are the two “TRP boxes” flanking the border of the TRP region (Venkatachalam and Montell, 2007). “TRP box 1” presents no sequence variation in TRPC channels but slight differences in TRPM and TRPN channels. “TRP box 2” is a proline-rich region that has higher sequence variation relative to “TRP box 1” among all three subfamilies.
Figure 2. Structures of TRP Channels

TRP Channels are divided into 2 groups based on sequence homology.

(Left) Subfamilies of Group 1 TRPs: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin) and TRPM (melastatin).

(Right) Subfamilies of Group 2 TRPs: TRPP (polycystin) and TRPML (mucolipin).

The following domains are indicated: ankyrin repeats (A); coiled-coil domain (CC); protein kinase domain (K); TRP domain (TRP). Pore loop (P), allowing influx of cations (+++), between the 5th and the 6th transmembrane segments are also shown in this figure.
1.1.2 Physiological Roles of TRP channels

The channels within the TRP superfamily have diverse functions in vertebrates. Some TRPC members are found to be important for modulating neurite extension (TRPC5) and both vasoconstriction and vasodilation (TRRC3, C4 and C6) (Clapham et al., 2001; Clapham, 2003). Interestingly, all mammalian TRPC proteins are activated by Phospholipase C (PLC) (Montell, 2005b), and several members of TRPC may be Ca^{2+} store-operated (Wes et al., 1995); they can be activated by depletion of Ca^{2+} from internal stores.

One of the most important documented physiological roles of TRP channels is in mediating sensation. TRP channels respond to a large range of external sensory modalities such as hearing, taste, olfaction, vision, thermal and mechatransduction (Clapham, 2003; Venkatachalam and Montell, 2007). Several mammalian TRP channels are thought to be crucial for chemosensation including TRPM5 and TRPV1. TRPM5 is required for the taste modality and the responses to sweet and bitter are greatly reduced in TRPM5 knockout mice (Montell, 2005b; Nelson et al., 2001; Talavera et al., 2005a); whereas TRPV1 is activated by capsaicin, the inflammatory vanilloid compound that gives spicy foods their hot sensation (Caterina et al., 1997). Furthermore, TRPV1 responds to many other chemicals, such as piperine (McNamara et al., 2005) from black pepper and allicin from garlic (MacPherson et al., 2005).

Mammalian thermal sensation to a large range of temperatures can be attributed to multiple TRP channels that are activated by temperature changes. Some TRP members, including TRPV1 and TRPV2, respond to uncomfortable heat levels (> 43°C) (Caterina et al., 1997) and very hot (> 52°C) (Caterina et al., 1999), respectively, while other TRP
channels such as TRPV3 (30 – 39°C) (Watanabe et al., 2002) and TRPV4 (25 – 34°C) (Smith et al., 2002; Xu et al., 2002) account for the perception of moderate temperatures. TRPM8 (23 – 28°C) and TRPA1 (< 18°C) are activated in the cool temperature range and in addition are activated by coolness evoking compounds, such as menthol and icilin (Clapham, 2003; McKemy et al., 2002; Peier et al., 2002). Some members of the TRP superfamily also display activation by mechanotransduction and TRPM3 and TRPV4 can act as osmosensors. TRPM3 currents are enhanced by hypotonic extracellular solutions and cell swelling (Grimm et al., 2003), whereas TRPV4 is activated in vitro by hypotonicity and TRPV4 knock-out mice exhibit defects in osmotic regulation (Liedtke et al., 2000; Liedtke et al., 2003). TRPP1/TRPP2 heteromeric channels have been shown to function as a mechanosensor in the cilia of renal epithelial cells (Woudenberg-Vrenken et al., 2009).

1.2 The TRPM Channels

The functionally diverse subfamily of TRPM channels is comprised of eight members and subdivided into 3 different groups based on sequence homology: TRPM1/3, TRPM4/5, and TRPM6/7. TRPM2 and TRPM8 are not placed in any subset due to low sequence similarities (Clapham, 2003; Fleig and Penner, 2004; Venkatachalam and Montell, 2007). TRPM2, TRPM6 and TRPM7 channels have a very unusual architecture and are termed chanzymes due to the fact that they are channels that possess functional enzymatic domains on their C - terminal ends. As mentioned in Section 1.1, the TRPM subfamily proteins was first identified as a prognostic marker for metastasis of melanoma (Duncan et al., 1998; Ramsey et al., 2006). Instead of the N-terminal ankyrin repeats that
other group 1 TRP members carry, the TRPM proteins contain a highly conserved coiled-coil (CC) region in the C-terminus, following the sixth transmembrane segment. Coiled-coil domains are protein interaction domains that allow multimerization of up to four individual proteins with similar CCs. The TRPM CC is thought to act as a general self-assembly domain for TRPM channels. Tsuruda et al. has demonstrated with gel filtration experiments that the isolated, recombinant coiled-coils from several TRPM subunits (TRPM2 – 3 and TRPM6 – 8) are capable of self-assembly (Tsuruda et al., 2006).

Although TRPM1 is the first identified member of TRPM subfamily, it is still enigmatic; its function or channel activity have not been well studied. In addition to the full length protein channel (TRPM1-L), TRPM1 has a splice variant (TRPM1-S) which interacts with the functional TRPM1-L and prevent its translocation to the plasma membrane therefore down-regulating the activity of TRPM1 (Xu et al., 2001). Studies in TRPM3 have demonstrated that its channel activity is up-modulated in response to hypotonic extracellular solution and cell swelling. Alternatively, TRPM3 can be activated by store depletion (Lee et al., 2003) or sphingolipids (Grimm et al., 2005). Alternative splicing can be a way to change the properties of channels such as conductance and ion permeability. TRPM3α1 and TRPMα2 are splice variants with altered ionic selectivity due to changes in the pore region (Oberwinkler et al., 2005). TRPM3α1 channels are monovalent cation selective, whereas TRPM3α2 channels are well permeated by Ca\(^{2+}\) and Mg\(^{2+}\) but inhibited by extracellular monovalent cations. Other TRPM3 isoforms have also been reported but their functions are unclear (Lee et al., 2003).

TRPM4 and TRPM5 appear to be unique among all TRP channels due to their monovalent-cation selectivity and voltage-modulated properties (Hofmann et al., 2003;
Launay et al., 2002; Liu and Liman, 2003; Nilius et al., 2003; Prawitt et al., 2003; Ullrich et al., 2005). Both of these channels exhibit prominent voltage-dependent inactivation at negative membrane potentials (Barr et al., 2001; Clapham, 2003). Similar to many other TRP channels, both TRPM4 and TRPM5 channel activities are enhanced by phosphatidylinositol-4,5-bisphosphate (PIP$_2$) and Ca$^{2+}$ (Liu and Liman, 2003; Nilius et al., 2006; Zhang et al., 2005). The TRPM5 channel also has dual functions in sensation as it is responsible for sensing temperature in the range of 15-35°C (Talavera et al., 2005b) and involved in sweet taste transduction (Talavera et al., 2008). The presence of quinine inhibits TRPM5 current resulting in the suppression of sweet taste and evoking bitterness.

TRPM8 is a thermally regulated channel that is activated by cool temperatures and coolness evoking compounds such as menthol (McKemy et al., 2002; Peier et al., 2002). TRPM8 activity is also regulated by PIP$_2$, as depletion of PIP$_2$ reduces activity enhanced by menthol or thermal cool (Liu and Qin, 2005).

TRPM2 forms a non-selective cation channel permeable to Ca$^{2+}$ that is activated by intracellular Ca$^{2+}$ and adenosine diphosphophate ribose (ADPR). TRPM2 possesses a C-terminal ADP pyrophosphatase domain (homologous to NUDT9 ADP-ribose hydrolyase domain) that is critical to the activation of TRPM2 (Perraud et al., 2001; Sano et al., 2001; Wehage et al., 2002). However it is the binding of ADPR to the TRPM2 ADP-ribosylase rather than the enzymatic activity, which is unconfirmed, that regulates TRPM2 conductance (Perraud et al., 2001; Sano et al., 2001; Wehage et al., 2002). Second messengers including nicotinamide adenine dinucleotide (NAD) (Sano et al., 2001), cyclic adenosine diphosphoribose (cADPR) (Kolisek et al., 2005) and arachidonic acid (Hara et al., 2002) also display positive modulation of TRPM2 function.
Furthermore, the TRPM2 channel can be activated by oxidative and nitrosative stresses such as hydrogen peroxide (H$_2$O$_2$) (Hara et al., 2002).

TRPM6 and TRPM7 are unique channels that possess both ion channel and protein kinase activities (Schlingmann et al., 2002). The C-terminal kinase domain bears little sequence homology to conventional protein kinases (e.g., PKA) but is instead homologous to the atypical alpha-kinase family including myosin heavy chain kinase A (MHCK A) from *Dictyostelium* (Cote et al., 1997; Futey et al., 1995) and mammalian elongation factor 2 kinase (eEF2 kinase). Alpha kinases are structurally homologous to other catalytically active protein kinases except for the inclusion of a zinc-finger domain (Liu and Qin, 2005; Ryazanova et al., 2004; Yamaguchi et al., 2001). Both TRPM6 and TRPM7 channels are permeable to divalent cations which include Mg$^{2+}$ and Ca$^{2+}$. In addition, they share important biophysical features with endogenous magnesium inhibited currents (MIC) to provide an effective mode of feedback regulation to maintain intracellular Mg$^{2+}$ level (Liu and Qin, 2005; Nadler et al., 2001; Schlingmann et al., 2007; Schmitz et al., 2003; Voets et al., 2004; Yamaguchi et al., 2001).

With the exception of TRPM7 several alternate isoforms arisen by RNA splicing have been identified for each of the TRPM family proteins (Murakami et al., 2003; Oancea et al., 2009; Uemura et al., 2005). Changes in coding sequences due to alternative splicing can have profound consequences on the regulation and activity of channels. Several TRPM channel isoforms with N-terminal splicing display dominant negative effect (TRPM2/4) (Murakami et al., 2003; Zhang et al., 2003) or become either non-functional (TRPM6) (Chubanov et al., 2004) or constitutively active (TRPM3) (Lee et al., 2003). The change of channel functions is possibly caused by removal of a potential
regulatory region which determines subunit interaction and channel formation (Vazquez and Valverde, 2006).

1.3 Characteristics and Domains of TRPM7

TRPM7 is a non-selective cation channel, which is permeable to mono- and divalent cations, specifically to Mg$^{2+}$ and Ca$^{2+}$, as well as trace metals such as Zn$^{2+}$, Mn$^{2+}$ and Ni$^{2+}$ (Monteilh-Zoller et al., 2003; Nadler et al., 2001; Penner and Fleig, 2007). TRPM7 is ubiquitously expressed but with highest mRNA levels detected in heart, kidney and brain tissues (Montell, 2005a; Nadler et al., 2001; Runnels et al., 2001; Takezawa et al., 2004). To date, the detailed physiological mechanism for TRPM7 activation is unclear, however TRPM7 appears to be sensitive to changes in [Mg$^{2+}$]$_i$ and [Ca$^{2+}$]$_e$ (Nadler et al., 2001) and may be activated by metabolic stress as hydrogen peroxide (H$_2$O$_2$) has been shown to markedly enhance inward TRPM7 current (Aarts et al., 2003). In addition, TRPM7 is reportedly regulated by Src-family kinases (Jiang et al., 2003), and PIP$_2$ levels have been shown by several groups to be important for TRPM7 channel activation. It remains controversial whether the receptor-mediated activation of PLC, leading to the hydrolysis of PIP$_2$, results in activation or deactivation of the TRPM7 channel (Langeslag et al., 2007; Runnels et al., 2002). Other research has also shown that TRPM7 currents are activated following a decrease in extracellular pH from physiological pH (7.4) to pH 4.0 (Jiang et al., 2005; Venkatachalam and Montell, 2007).
Figure 3. Predicted and confirmed protein interaction domains and consensus motifs of TRPM7

TRPM7 contains a N-terminal that is highly conserved among all TRPM subfamily members. The snapin binding sequence (87-326) is also located within this region. The transmembrane domain (TM) of TRPM7 forms the ion channel. The α-kinase domain, coiled-coil region and TRP box are located in the C-terminal domain of TRPM7. In addition, the C-terminal autophosphorylation region (P) that may be important for substrate binding is also shown.
1.3.1 The N-terminus of TRPM7

TRPM7 possesses long intracellular domains as do other members of the TRP superfamily (Figure 1), however the role of these intracellular domains in channel function and physiology is poorly defined. Work to date shows that the truncation of the TRPM N-terminal sequence (Murakami et al., 2003) or the expression of the N-terminus alone (Zhang et al., 2003) can produce a dominant negative effect that inhibits the activities of full-length, functional TRPM channels, indicating the presence of regulatory sites in this highly conserved N-terminal region. A recently published paper used confocal microscopy and immunoprecipitation to demonstrate that TRPM7 resides on the synaptic vesicle member of sympathetic neurons and forms molecular complexes with synaptic vesicle proteins including synapsin I, synaptotagmin I and Snapin, a synaptic vesicle protein whose role is important for vesicle docking. More importantly, using an in \textit{vitro} binding assay, recombinant snapin (AA. 43-68) was found to directly interact with a region on the recombinant TRPM7 N-terminus (AA 87-327) (Figure 3). Further studies using TRPM7 knockdown by siRNA and expression of a TRPM7 pore mutant revealed the interaction between TRPM7 and Snapin, as well as the channel activity, are critical for vesicle fusion events and neurotransmitter (acetylcholine secreting synaptic vesicles) release in sympathetic neurons (Brauchi et al., 2008; Krapivinsky et al., 2006).
1.3.2 The TRP Domain and Coiled-coil Domain of TRPM7

TRPM channels have diverse C-terminal domains but share a common C-terminal cytoplasmic TRP domain and a coiled-coil domain (Figure 3). As mentioned previously in Section 1.1.1, two TRP boxes located within the TRP domain possess great sequence homology among the members of TRPC, TRPM, and TRPN subfamilies (Venkatachalam and Montell, 2007). The coiled-coil domain is a widespread protein-protein interaction structural motif that is found in a variety of protein classes including motor proteins, fibrous proteins and membrane fusion proteins (Fujiwara and Minor, Jr., 2008; Lupas and Gruber, 2005; Woolfson, 2005). Using gel filtration, Tsuruda et al. demonstrated that recombinant proteins containing the putative coiled-coils from TRPM channels are capable to self-assemble into multimers. Furthermore, a TRPM8 coiled-coil deletion mutant was shown to be unresponsive to TRPM8 stimuli, indicating that TRPM8 coiled-coil is required for the proper channel formation and to carry out its function for thermal sensation and response to chemical stimuli such as menthol (Tsuruda et al., 2006). Similarly, another research using site mutagenesis by Erler et al. demonstrated that a single site mutation in the TRPM8 coiled-coil region can disrupt multimeric channel assembly which leads to non-functional channels, confirming that coiled-coil domain interactions are required for proper channel maturation (Erler et al., 2006). A recent study using X-ray crystallography also demonstrated that recombinant TRPM7 coiled-coils self-assemble into a four-stranded antiparallel architecture, forming functional homologous as well as heterologous interactions with TRPM6 subunits whose coiled-coil domain possesses great sequence similarity to TRPM7’s (Fujiwara and Minor, Jr., 2008).
1.3.3 The Atypical Alpha-kinase Domain of TRPM7

A sub-group of the TRPM protein family has been designated as “chanzymes” due to the unique feature of being a functional ion channel with an integrated enzymatic domain at the C-terminus. TRPM6 and TRPM7 contain an atypical functional kinase known as the alpha-kinase. The first member of the alpha-kinase family reported was the myosin heavy chain kinase A (MHCK A) from *Dictyostelium* (Cote et al., 1997; Futey et al., 1995), whereas the elongation factor 2 kinase (eEF-2 kinase) was the first mammalian alpha-kinase identified (Ryazanov et al., 1997). Three additional mammalian alpha-kinases have now been reported: the lymphocyte alpha-kinase (Hs LAK), muscle alpha-kinase (Hs MAK) and heart alpha-kinase (Hs HAK) (Ryazanov et al., 1997; Ryazanova et al., 2004). Despite the lack of detectable sequence homology to conventional protein kinases (CPKs) such as Protein Kinase A (PKA) (Yamaguchi et al., 2001), the majority of the structure, sequence motifs, and the position of amino acids important for enzymatic activity appear to be very similar between CPKs and alpha-kinases (Drennan and Ryazanov, 2004). One main structural difference between these two kinase families is that the C-terminal lobe of alpha-kinases includes a highly conserved zinc-finger that is not present in the structure of CPKs. The purpose of this zinc-finger domain remains unclear though it may play a secondary role unnecessary for kinase function that involves stabilization and positioning of substrates, similar to other zinc-fingers that bind nucleic acids, proteins or small ligands (Drennan and Ryazanov, 2004; Yamaguchi et al., 2001). In addition to structural differences, the region of phosphorylation in substrate targets of the two kinase families shows dissimilarities. As the name implies, alpha-kinase tends to phosphorylate amino acids located within alpha-helices (Ryazanov et al., 1999) while
CPKs phosphorylate amino acids within loops, turns or irregular structures (Drennan and Ryazanov, 2004; Pinna and Ruzzene, 1996).

Currently the functions and regulation of TRPM7 alpha-kinase domain are not well understood. According to one study the kinase activity is essential for channel function (Runnels et al., 2001). However, other groups of researchers including Schmitz et al. (2003) and Matsushita et al. (2005) have used mutation and deletion of C-terminal kinase domain to demonstrate that the TRPM7 channel is functionally independent of its alpha-kinase. Yet changes in kinase activity may alter the sensitivity of the channel to Mg\(^{2+}\) inhibition (Matsushita et al., 2005; Schmitz et al., 2003). In a study using truncation mutants and site mutations, Yamaguchi et al. showed that TRPM7 alpha-kinase dimer formation plays a critical role in regulating enzyme activity. The TRPM7 alpha-kinase assembles into a dimer through the exchange of an N-terminal segment near the kinase domain that extends from residue 1551 to residue 1577 (Yamaguchi et al., 2001). This N-terminal segment can be divided into 2 sections; an “activation sequence”, encompassing residues 1553-1562, that is important for kinase activity and a “dimerization sequence”, residing on amino acids 1563-1570, that is critical for both dimerization and channel activity of TRPM7 (Crawley and Cote, 2009). A few proteins have been reported to bind the TRPM7 kinase domain including phospholipase C (PLC), annexin I, myosin II heavy chain A-C and tropomodulin (Clark et al., 2006; Dorovkov et al., 2008; Dorovkov and Ryazanov, 2004; Nadler et al., 2001). These data may provide insight in TRPM7 kinase functions and the mechanisms to regulate kinase activity.

Phospholipids C (PLC) is a well studied enzyme that is particularly important for signal transduction. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to generate
diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Once released IP$_3$ diffuses through the cytosol to bind to IP$_3$ receptors on the endoplasmic reticulum (ER) membrane causing the release of Ca$^{2+}$ from intracellular stores. In 2001 Runnel et al. first identified TRPM7 with yeast two-hybrid screening by using the C2 domain of PLC$\beta$ as bait (Runnels et al., 2001). Later research by the same group found that PLC$\beta$ was co-immunoprecipitated from cell lysates containing both PLC$\beta$ and recombinant full-length TRPM7 proteins, but not from cell lysates containing PLC$\beta$ and recombinant TRPM7 proteins lacking the kinase domain. Results from these two studies together confirm association between TRPM7 alpha-kinase domain and the C2 domain of PLC$\beta$ (Runnels et al., 2002). Runnels et al. also suggested that PLC is a potential regulator of TRPM7 through direct interaction with the alpha-kinase domain even with no further experimental analysis and data to confirm or support this idea.

Recent research into TRPM7 alpha-kinase function has revealed that all three non-muscle isoforms of the myosin II heavy chain (IIA, IIB and IIC) are target substrates of TRPM7 kinase. Myosin II protein interacts with actin and form bipolar filaments through electrostatic interactions of the myosin heavy chain (MHC) tail (Hostetter et al., 2004). Interactions with actin and the formation of MHC filaments are important for actin stability (Clark et al., 2006). In vitro kinase assays were used to show that TRPM7 wild type C-terminal fusion protein but not the kinase dead version phosphorylates the helical portion of different MHCII proteins (Clark et al., 2006). In addition, binding and phosphorylation of MHCII by TRPM7 is Ca$^{2+}$ and kinase dependent; the addition of bradykinin, a PLC coupled receptor agonist, induces Ca$^{2+}$ influx and activates TRPM7 alpha-kinase, promoting its interaction with myosin II heavy chain (Clark et al., 2006;
Clark et al., 2008b). More importantly TRPM7 kinase autophosphorylation is critical for its subsequent substrate-level phosphorylation. Kinase-dead mutants or deletion of the autophosphorylation sites located upstream of the kinase domain (Figure 3) completely abolished the phosphorylation of myosin II heavy chain (Clark et al., 2008c; Clark et al., 2008a). Furthermore it has been demonstrated by using an artificial substrate that TRPM7 kinase activity remains unchanged between the fully autophosphorylated and incomplete autophosphorylated state. As an indication, the massive autophosphorylation of the Ser/Thr-rich domain controls substrate phosphorylation of TRPM7 alpha-kinase not by enhancing enzymatic activity but by securing the position of the substrate and providing access to the enzymatic domain (Clark et al., 2008c). The evidence of myosin II heavy chain as a substrate of TRPM7 kinase suggests a role in the regulation of the actomyosin cytoskeleton similar to the myosin II heavy chain kinase (MHCK A) present in the Dictyostelium. Phosphorylation of the myosin II heavy chain results in disassembly of myosin II bipolar filaments and potentially inhibits myosin II-dependent contractile events or allows cytoskeleton remodeling (Cote and Bukiejko, 1987; Luck-Vielmetter et al., 1990; Steimle et al., 2001). Similar examination of TRPM7 kinase substrates has uncovered TRPM7 phosphorylation sites in the N-terminal functional domain of tropomodulin. Tropomodulin is an actin binding protein that also interacts with tropomyosin. The actin-capping ability of tropomodulin allows it to cap the pointed end (for disassembly) of actin filament to prevent disassembly of beta-actin subunits. Association with tropomyosin and actin promotes inhibition of actin depolymerization, which stabilizes the structure of actin cytoskeleton (Kostyukova et al., 2005; Kostyukova et al., 2007; Kostyukova and Hitchcock-Degregori, 2004). Further analysis in
tropomodulin phosphorylation revealed that mutations mimicking N-terminal phosphorylation of tropomodulin caused a loss of the capping ability of tropomodulin with no effect on the ability of the N-terminal tropomodulin domains to bind tropomyosin. Once again, this indicates a potential role in the regulation of the dynamics of actin filaments by TRPM7 alpha-kinase domain (Dorovkov et al., 2008).

TRPM7 is also implicated in regulating cytoskeletal dynamics by its association with annexin I. Using an in vitro kinase assay Dorovkov et al. found that annexin I is a substrate of TRPM7 alpha-kinase which is phosphorylated at a conserved serine residue (Ser5), located within the N-terminal amphipathic-helix of annexin I (Dorovkov and Ryazanov, 2004). Similar to myosin II, annexin I phosphorylation is stimulated by increased [Ca^{2+}]. Considering both annexin I and TRPM7 have been implicated in regulating cell proliferation (Gerke et al., 2005; Hanano et al., 2004) and cell death (Aarts et al., 2003; Arur et al., 2003), it is highly possible that phosphorylation of annexin I by the TRPM7 kinase has a crucial role in cell survival/death regulation and signal transduction (Dorovkov and Ryazanov, 2004).
1.4 The Potential Physiological Roles of TRPM7

The physiological role of TRPM7 is not fully understood. However it has been implicated in widespread functions including Mg\textsuperscript{2+} homeostasis, regulation of cell survival and cell morphology.

1.4.1 TRPM7 and Mg\textsuperscript{2+} Homeostasis

Initially, the TRPM7 channel was identified in parallel by two separate research groups; one found TRPM7 as a PLC interacting protein using yeast two-hybrid screening and a bifunctional protein with kinase and ion channel activities (Runnels et al., 2001), while the other group was searching for the channel responsible for MIC (magnesium inhibited current) (Nadler et al., 2001). According to Nadler et al., the first electrophysiological analysis of TRPM7 over-expression in HEK-293 cells showed large currents regulated by millimolar levels of intracellular Mg-ATP and with the permeation properties of a divalent cation influx pathway (Nadler et al., 2001). TRPM7 and its closely related homologue TRPM6 have since been characterized as ion channels essential for cellular magnesium transport and homeostasis. Both channels are divalent cation permeable, preferably to Mg\textsuperscript{2+} and Ca\textsuperscript{2+} (Venkatachalam and Montell, 2007). TRPM7 is characterized as Mg\textsuperscript{2+} and Ca\textsuperscript{2+} selective and like many non-selective cation channels may flux a variety of mono and divalent cations. TRPM7 and TRPM6 are unique in that they have a relatively higher conductance for Mg\textsuperscript{2+} than Ca\textsuperscript{2+} (Monteilh-Zoller et al., 2003; Penner and Fleig, 2007). Original publications described TRPM7 as an outwardly rectifying channel with a conductance of 40 – 108pS (Nadler et al., 2001). However TRPM7 channel function varies significantly depending on cell type (Chubanov et al., 2004; Monteilh-Zoller et al., 2003). TRPM7 is considered to be a significant
regulator of Mg\(^{2+}\) in that RNA interference or deletion of TRPM7 results in a significant
decrease in intracellular magnesium concentrations ([Mg\(^{2+}\)]\(_{i}\)), growth arrest (smooth
muscle cells) and eventually cell death in rapidly dividing cell types such as DT-40 B
lymphocytes and gastric cancer cells (Hanano et al., 2004; Nadler et al., 2001).
Moreover, supplementation of extracellular Mg\(^{2+}\) was able to restore proliferation and
rescue TRPM7-deficient cells (Montell, 2003; Schmitz et al., 2003). Inhibition of
TRPM7 channel activity or cation conductance can be achieved by increasing [Mg\(^{2+}\)]\(_{i}\) and
intracellular Mg-ATP. Similarly reduction of the intracellular levels of these regulators
leads to activation of TRPM7-like currents with outward rectification due to divalent
influx at physiologically negative voltages and monovalent outward fluxes at positive
voltages (Penner and Fleig, 2007). A recent study focusing in TRPM7 expression
revealed a potential mechanism to regulate TRPM7 expression. Osteoblasts pre-exposed
to conditions of reduced extracellular Mg\(^{2+}\) and Ca\(^{2+}\) levels showed a significant increase
in TRPM7 channel expression, whereas the expression of TRPM6 remained stable,
suggesting compensatory mechanisms afforded by TRPM7 in order to maintain
intracellular ion homeostasis. In addition pre-incubation of cells in reduced extracellular
Mg\(^{2+}\) conditions led to up-regulation of TRPM7 channel activity, specifically to an
increase in Ca\(^{2+}\) and Mg\(^{2+}\) influx. Reduction of TRPM7 expression by siRNA prevented
corresponded Ca\(^{2+}\) and Mg\(^{2+}\) influx and inhibited cell proliferation (Abed and Moreau,
2007). Beginning from the initial research of TRPM7, the physiological role of this
channel in controlling cellular Mg\(^{2+}\) level has been extensively studied. Compiled
research shows that TRPM7 acts as Mg\(^{2+}\) sensor, maintains cellular [Mg\(^{2+}\)], and its
channel activity and expression are modulated by intracellular and extracellular Mg\(^{2+}\) availability, suggesting and confirming the vital role of TRPM7 in Mg\(^{2+}\) homeostasis.

### 1.4.2 The Critical Role of TRPM7 in Cell Survival/Death

Aside from its role in Mg\(^{2+}\) homeostasis, TRPM7 has also been implicated to be a critical player in cell growth, proliferation and anoxic cell death. As a regulator of cell survival/death, TRPM7 was suggested to be a potential target for the pharmacological treatment of gastric cancer (Kim et al., 2008). Research in human gastric cancer cells has shown that blockade of TRPM7 channels or suppression of TRPM7 expression by siRNA inhibited cancer cell growth and induced the death of these cancer cells.

TRPM7 may represent the only ion channel known that is essential for cell viability (Penner and Fleig, 2007), however the underlying mechanism of cell survival/death regulation by TRPM7 is unknown. Down-modulation of TRPM7 with siRNA silencing in retinoblastoma cells resulted in decelerated cell proliferation and arrest in cell progression (Hanano et al., 2004). Similar knock-out experiment with hairpin RNA in primary human lung mast cells reduced TRPM7 non-selective cationic current and induced cell death (Wykes et al., 2007). Surprisingly, the consequences of both TRPM7 knock-out experiments could not be rescued by extracellular Mg\(^{2+}\) supplementation, implying such effects from TRPM7 depletion were not Mg\(^{2+}\) dependent. Another study showed that melanophores from TRPM7 – mutant zebra fish were deleted during animal development from necrotic but not apoptotic cell death. In addition, melanin synthesis facilitated the death of these cells, indicating that melanophores may require TRPM7 to detoxify and prevent accumulation of cytotoxic
intermediates of melanin synthesis (McNeill et al., 2007). TRPM7 may play a role in metabolite detoxification in other cell types as well and it has been predicted that failure of TRPM7 expression may cause accumulation of cytotoxic intermediates that lead to eventual cell death.

Recent research directed into ischemic neuronal injury has provided new insight into the physiological role of TRPM7, and yet, increased the complexity of the potential roles that TRPM7 plays in cell survival and cell death. An in vitro stroke model, initially established by Choi and colleagues, uses oxygen-glucose deprivation (OGD) treatment to mimic ischemic conditions (Choi et al., 1987; Choi et al., 1988; Goldberg et al., 1987). Under OGD, primary murine cortical neurons treated with glutamate receptor (GluR) antagonists can survive up to 1 hour however the GluR antagonism becomes ineffective for prolonged OGD exposure (> 1.5 hours) (Aarts and Tymianski, 2005b; Choi et al., 1987). This observation led to the identification of a neuronal Ca\(^{2+}\) accumulation and a detectable cation current, I\(_{\text{OGD}}\), with the properties of a Ca\(^{2+}\) permeable and non-selective cation conductance. Surprisingly, inhibition of I\(_{\text{OGD}}\) by Gd\(^{3+}\), a general calcium channel blocker, successfully prevented neuronal death under prolonged OGD exposure. I\(_{\text{OGD}}\) was later confirmed to be mediated by TRPM7 and blocking I\(_{\text{OGD}}\) or suppressing TRPM7 expression blocked TRPM7-like currents, anoxic Ca\(^{2+}\) uptake, reactive oxygen species (ROS) production, and anoxic death of cortical neurons. TRPM7 suppression eliminated the need for glutamate receptor antagonists and permitted the survival of neurons destined to anoxic death (Aarts et al., 2003; Aarts and Tymianski, 2005b). This study demonstrated another key role of TRPM7 in regulating anoxic cell death but also led to
ambiguity as to whether TRPM7 channel function is required for cell viability or is responsible for cell death in certain diseases or cell injuries.

1.4.3 TRPM7/ Cytoskeletal Protein Interactions: Diverse Cytoskeletal Related Functions of TRPM7

Recent studies of TRPM7 function have shown that TRPM7 channel activity, kinase function or both are important for shear-stress cellular response, cell morphology regulation and vesicular transport. TRPM7 is expressed in vascular smooth muscle cells and its cell surface expression has been shown to increase following fluid flow across the membrane. In particular shear force induced by fluid flow causes localized insertion of TRPM7 into the plasma membrane. Furthermore, TRPM7 vesicular insertion and TRPM7 currents are correlated to the amount of force generated by fluid flow indicating a potential role in physiological or pathological response to vessel wall injury (Oancea et al., 2006b).

As previously described in Section 1.3.3., the protein kinase domain of TRPM7 phosphorlyates two actin interacting proteins: myosin II and tropomodulin (Clark et al., 2006; Clark et al., 2008b; Dorovkov et al., 2008). Interaction with these cytoskeletal accessory proteins suggests TRPM7 kinase-dependent activity might alter the stability of actomyosin filament and influence cytoskeletal remodeling. Phosphorylation of myosin II leads to disassembly of the myosin bipolar filament (Clark et al., 2006; Dulyaninova et al., 2005; Oancea et al., 2006a), and tropomodulin in its phosphorylated state results in actin filament destabilization due to the loss of actin-capping ability of tropomodulin (Dorovkov et al., 2008). Channel activity of TRPM7 is also thought to be important for
cell morphology. Studies in Flp-In T-Rex 293 cells and HEK-293 cells show that TRPM7 over-expression causes cell rounding associated with a loss of cell adhesion that depends upon the cation activity of the TRPM7 channel (Nadler et al., 2001; Su et al., 2006). In addition, down-modulation of TRPM7 with shRNA exhibits the opposite effect; cells become more spread and adhesive. Contrarily, another group of researchers, Clark et al. found that TRPM7 over-expressing neuroblastoma cells display enhanced spreading and cell-matrix adhesion (Clark et al., 2006). In spite of the controversy, it has been proposed that Ca^{2+} influx via the TRPM7 channel may be important for activation of the alpha-kinase and subsequent MHCII phosphorylation. Moreover, Ca^{2+} may act directly at the level of integrin activation, which leads to the remodeling of the actomyosin cytoskeleton to promote cell spreading (DeMali et al., 2003). Ca^{2+} is an important second messenger in actin remodeling including polymerization, severing of filaments and F-actin–membrane interactions (Forscher, 1989; Sun et al., 1999).

Synaptic vesicles mediate the release of neurotransmitters into the synaptic cleft in response to action potentials. The TRPM7 channel has recently been shown to play a critical role in cholinergic synaptic vesicle priming and release of transmitter from peripheral sympathetic neurons. Knockdown of TRPM7 protein using siRNA substantially decreased the frequency of the acetylcholine vesicle (ASVs) fusion events. Similarly, expression of a dominant negative TRPM7 (dnTRPM7) pore mutant dramatically decreased the frequency of the spontaneous fusion events (Brauchi et al., 2008). Instead of regulating fusion activity, the kinase domain of TRPM7 was shown to control the speed of vesicular transport. TRPM7 kinase-dead mutant expression increased vesicle mobility (Brauchi et al., 2008). Multiple experiments have demonstrated that
synaptic vesicles and juxtamembrane secretory vesicles are mobile and that inhibition of protein phosphorylation alters synaptic vesicle mobility (Gaffield et al., 2006; Guatimosim et al., 2002; Li and Murthy, 2001; Takahashi et al., 2003). It is possible that TRPM7 complexes with synaptic vesicle proteins and microtubule interacting proteins while the alpha-kinase domain phosphorylates these proteins, which allows the control of the motility of vesicles along microtubules.
1.5 Objectives and Goals of Thesis Project

TRPM7 is a relatively new cation channel protein that potentially dictates cell proliferation, survival and morphology and has become the subject of intense investigation by a small number of diverse research groups. Its functions and the underlying mechanisms behind its physiological roles are as yet not well understood. Moreover, the machinery involved in TRPM7 activation and functions remain undetermined. The TRPM7 channel is thought to function independently from its intrinsic kinase however both the channel and kinase activities are required for certain cellular functions such as synaptic vesicle release. Conflicting reports on many aspects of TRPM7 function and physiology have made the regulatory mechanism of TRPM7 harder to explain. In addition to the atypical alpha-kinase domain, TRPM7 possesses unusually long intracellular tails, and so far, the N-terminus contains no recognized consensus motif. Researches into other ion channels that also possess long intracellular domains have shown that protein-channel domain interaction is important for regulation of channel formation and functions, as well as downstream signal transduction. The voltage gated potassium channel, Kv7, is structurally similar to TRPM7, and the long intracellular C-terminal region of Kv7 is critical for channel tetramerization and trafficking (Kanki et al., 2004; Maljevic et al., 2003; Schmitt et al., 2000; Schwake et al., 2003). Moreover, calmodulin interaction with its C-terminal domain is also required for proper channel assembly and function; calmodulin binding enhances Kv7 channel activity (Wen and Levitan, 2002; Yus-Najera et al., 2002). N-methyl D-aspartate receptor (NMDAR) is a specific type of ionotropic glutamate receptor. Its co-activation by glutamate and glycine triggers Ca$^{2+}$ influx into the cells. The long intracellular C-terminal
domain of NR1 subunit of NMDAR provide competitive binding site for alpha-actinin and calmodulin. Binding of calmodulin reduces channel opening time, whereas channel closing time is decreased when alpha-actinin interacts with the C-terminus of NR1 subunit (Rycroft and Gibb, 2002; Rycroft and Gibb, 2004; Wyszynski et al., 1997). More importantly, binding of calmodulin and alpha-actinin provide modulation of NMDAR channel activity, indirectly regulate activation of second messenger (Ca$^{2+}$) signaling pathways. Similarly, I hypothesize that the intracellular domains of TRPM7 serve as key structural protein binding regions or TRPM7 regulatory sites so that protein interactions may alter channel function and kinase activity, which is important for TRPM7 regulatory functions in cell morphology and cell death. The main objective of this project is to identify proteins that interact with the intracellular domains of TRPM7 and more specifically the C-terminal domain.

TRPM7 protein interactions will be investigated by pull down with fusion proteins containing discrete TRPM7 domains and motifs such as the alpha-kinase. Purified TRPM7 fusion proteins will be incubated with mouse brain lysate, a tissue with relatively high endogenous TRPM7 expression, and interacting proteins identified using mass spectrophotometry. Identified interactions will be confirmed by co-immunoprecipitation and direct binding assays. Interacting proteins will also be compared to known interactions and physiological roles for TRPM7 as a mean to evaluate experimental validity and result consistency. Both confirmation of published TRPM7 interactions in an endogenous system (mouse brain) and the identification of novel interactions will be useful to aid postulating new models to explain the concealed mechanisms for potential physiological roles of TRPM7.
2. Materials and Methods:

2.1 Cloning

2.1.1 Conventional Cloning Methods

Conventional Sub-Cloning usually involves techniques of restriction digest, dephosphorylation, DNA blunting, DNA ligation, agarose gel electrophoresis, bacterial transformation, plasmid isolation and DNA sequencing. Restriction enzyme sites chosen on both mouse TRPM7 cDNA (Figure 4), FLAG-TRPM7 pcDNA4/TO template and GE Health Care pGEX-5X® system plasmids (vector) are listed in Table 2.

2.1.1.1 Restriction Digest

Restriction digest was performed with commercial 10X buffers and restriction enzymes, each in 1/10 of final volume. Digests were incubated at the optimal temperature indicated for maximal enzymatic activity for a duration of one to sixteen hours.

2.1.1.2 DNA Blunting

DNA blunting was used to remove 3’ overhang or fill in 5’ overhangs on cDNA restriction fragments that were required for blunt-end ligation. The blunting enzyme, T4 DNA polymerase of Quick Blunting Kit (New England Biolabs) was used to completely fill in 5’ overhangs of linearized DNA by addition of dNTPs to complementary strand for all TRPM7 insertion fragments listed in Table 1. Reactions were carried out in 1X Blunting Buffer (100mM Tris-HCl, 50mM NaCl, 10mM MgCl$_2$, 0.025% Triton X-100 and 5mM dithiothreitol) for one hour at 37 °C. Blunting enzyme was heat-inactivated at 70°C for 10 minutes.
<table>
<thead>
<tr>
<th>Clone #</th>
<th>Name of Fusion Construct</th>
<th>Restriction Enzyme sites for TRPM7 Bluescript</th>
<th>Blunting of TRPM7 Insert Required (Y/N)?</th>
<th>pGEX-5X-(#)</th>
<th>Restriction Enzyme sites for pGEX plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α kinase</td>
<td>EcoRI (5’) + BsaBI (3’)</td>
<td>Y</td>
<td>1</td>
<td>Smal</td>
</tr>
<tr>
<td>4</td>
<td>C-terminus-N</td>
<td>BsrGI (5’) + BsaBI (3’)</td>
<td>Y</td>
<td>2</td>
<td>Smal</td>
</tr>
<tr>
<td>5</td>
<td>C-terminus-C</td>
<td>BsrGI (5’) + NotI (3’)</td>
<td>Y (before NotI digestion)</td>
<td>2</td>
<td>Smal / NotI</td>
</tr>
<tr>
<td>6</td>
<td>Coiled-coil</td>
<td>BbvCI (5’) + AlwNI (3’)</td>
<td>Y</td>
<td>3</td>
<td>Smal</td>
</tr>
<tr>
<td>7</td>
<td>Long coiled-coil</td>
<td>BsrGI (5’) + Sall (3’)</td>
<td>Y (before Sall digestion)</td>
<td>2</td>
<td>Smal / Xhol</td>
</tr>
</tbody>
</table>
2.1.1.3 Dephosphorylation

Linearized pGEX-5X® vector was dephosphorylated to prevent re-circularization and re-ligation of empty vectors. Dephosphorylation was carried out using 5μg linearized vector, 1 μL (10U) calf intestinal alkaline phosphatase (New England Biolabs) in 1X NEBuffer 3 (100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂ and 1mM dithiothreitol) for 1 hour at 37 °C. The phosphatase was heat-inactivated at 70°C for 10 minutes.

2.1.1.4 DNA Ligation

The pGEX-5X plasmid system (GE Health Care) was used as vectors to carry insert DNA. Phosphoryl-ester bonds were formed with the help of DNA ligase (Fermentas Rapid DNA Ligation Kit) to attach insert DNA to the linearized plasmid DNA vector in 1X Ligation buffer. Ligation was performed with approximately 1 vector: 5 inserts ratio to achieve optimal ligation efficiency.

2.1.1.5 Bacterial Transformation

Chemically competent bacterial cells, DH5-α Escherichia coli (sub-cloning efficiency), 10-beta E.coli (high efficiency), and BL21 (DE3)pLysSE. coli were used for purification of plasmids, ligation product transformation, and protein expression, respectively. In general, 2 – 5μL (100-300ng) of DNA was mixed with 50 μL of thawed chemically competent bacterial cells and incubated on ice for 30 minutes. Bacteria were heat-shocked on 42°C water bath for 45 seconds and placed on ice for 2 minutes. Bacteria were grown in 950ul of LB medium [1% peptone, 0.5% Yeast Extract, 0.5% NaCl] at 37°C shaker (~200rpm) for 1 to 1.5 hours, allowing the recovery of bacteria and
expression of antibiotic resistance proteins. Only the transformation with 10-beta *Escherichia coli* required 10-fold dilution in the growing medium prior plating. Aliquots of the bacteria were plated onto Agar plates [1% tryptone, 1% agar, 0.5% NaCl] containing 100µg/ml ampicillin, and plates were incubated at 37°C overnight.

### 2.1.1.6 Agarose Gel Electrophoresis

DNA was separated by size on 1% agarose gels containing 0.5µg/ml ethidium bromide in 1X TAE (0.04M Tris – Acetate, 0.001M EDTA). DNA samples were mixed with 1/5 volume of Fermentas 6X loading dye and run at 80V against a DNA ladder (Fermentas, DNA Ladder Plus). DNA was visualized under UV light and sizes of DNA fragments were estimated by comparison to molecular weight of DNA ladder standards.

### 2.1.1.7 DNA Gel Extraction

Following agarose gel electrophoresis (see section 2.1.1.6), DNA bands were excised and purified from the agarose using the Qiagen- QIAquick Gel Extraction Kit (Qiagen). Agarose gel was dissolved in solution provided by manufacture, at 50°C for 10 minutes to release DNA embedded in agarose gel. DNA was then captured with binding columns and excess salt was removed with washing buffer. DNA was eluted off the column.

### 2.1.1.8 Plasmid DNA Isolation

For small scale plasmid isolation (Mini-prep) bacterial cells were grown in 5mL LB medium with appropriate antibiotics at 37°C, overnight in a shaking incubator.
Bacteria were pelleted from 3mL of cell suspension by centrifugation at 8000g for 5 minutes at room temperature. The bacterial pellet was then prepared according to manufacturer’s instructions for plasmid isolation using the QIAprep® Mini-prep Kit (Qiagen). Bacterial cells were first lysed with SDS and NaOH containing solution and chromosomal DNA was precipitated with acetic acid, whereas plasmid DNA remains in solution and captured with DNA binding columns provided. Plasmid DNA was further washed and eluted off the column.

2.1.2 Polymerase Chain Reaction (PCR) Cloning

PCR was used to amplify the desired TRPM7 insert DNA from the full-length FLAG-TRPM7 pcDNA4/TO template DNA. The forward primer: GAATTC\textsuperscript{4697}CACAGAGTATTCCCTCGTCTCT\textsuperscript{4719} and reverse primer: CTCGAGGGCCTCTAGTTCTATAACATCAGACGAA\textsuperscript{end} and forward primer: GAATTC\textsuperscript{4427}GCTGCTGGATATAGTGAATGTTGTAAGACT\textsuperscript{4456} and reverse primer: CTCGAGGGCCTCTAGTTCTATAACATCAGACG\textsuperscript{end} were used to amplify the segments of interest for Clone 2 and Clone 3 (Table 3 and Figure 4), respectively. PCR was performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs) with final concentrations of 1X Phusion HF Buffer, 200µM dNTPs, 1uM primer mix, 2ng/ul Template and Phusion DNA Polymerase 0.02U/µl. An initial denaturation step occurred at 98°C for 1 minute, with subsequent denaturation periods of 10 seconds at 98°C. PCR reactions were allowed to cycle with 68°C annealing temperature for 15 seconds and elongation at 72°C for 25 seconds.
Following PCR, electrophoresis was used to confirm PCR product sizes. Amplified DNA segments were ligated to PCR II-Blunt-TOPO vector (Invitrogen) as described in section 2.1.1.4 for 10 minutes. Ligation products were used to transform DH5-α *Escherichia coli* cells as mentioned previously in section 2.1.1.5, and plated on Agar/ Kanamycin (50μg/mL) plates. Five colonies for each clone were selected and grown in 5mL LB/ Kanamycin (50μg/ml) overnight at 37°C. Plasmid DNA was isolated from bacterial cells (section 2.1.1.7), restriction digested with 10U each of EcoRI and XhoI (section 2.1.1.1) and run on a 1% agarose gel to confirm the presence of the TRPM7 insert. Insert DNA was excised, purified with Qiagen- QIAquick Gel Extraction Kit (Qiagen) and ligated into the appropriate pGEX-5X vector according to Table 3, which was linearized with EcoRI / XhoI and gel purified. Ligation products were transformed into 10-beta *Escherichia coli* as described in 2.1.1.5. Six bacterial colonies were chosen for screening; restriction digest analysis and DNA sequencing were used to confirm construct identities.
Figure 4. cDNA regions selected to generate TRPM7-GST fusion proteins.

Recombinant DNA constructs encoding TRPM7-GST fusion proteins were generated by conventional cloning (Clone #1, 4-7) and PCR cloning (Clone #2-3) (refer to section 2.1.1 and 2.1.2 and Appendix 1 for details). The targeted regions for sub-cloning, including the α-kinase domain, the coiled-coil domain and the autophosphorylation region are all located on the C-terminus of TRPM7 cDNA.
<table>
<thead>
<tr>
<th>Clone #</th>
<th>Name of Fusion Construct</th>
<th>Restriction Enzyme Sites Added</th>
<th>pGEX-5X-(#)</th>
<th>Restriction Enzyme sites for pGEX plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>α kinase- C</td>
<td>Forward: EcoRI Reverse: XhoI</td>
<td>1</td>
<td>EcoRI / XhoI</td>
</tr>
<tr>
<td>3</td>
<td>Long α kinase- C</td>
<td>Forward: EcoRI Reverse: XhoI</td>
<td>2</td>
<td>EcoRI / XhoI</td>
</tr>
</tbody>
</table>
2.2 Glutathione S-transferase (GST) Fusion Protein Induction and Purification

BL21(DE3)pLysS *Escherichia coli* containing GST-TRPM7 fusion constructs were grown in 2X YT medium [1.6% peptone, 1% Yeast Extract, 0.5% NaCl] with 100ug/ml ampicillin at 37°C overnight. An aliquot of BL21 cells for each construct was transferred to 10X volume fresh 2X YT medium + 100ug/ml ampicillin and incubated at 37°C for approximately 2 hours until the O.D. \(_{600}\) was 0.6 – 0.8. GST fusion protein expression was induced with the addition of 2mM of IPTG for 1.5 hours. Bacteria were washed and re-suspended in ice-cold glutathione S-transferase (GST) Lysis Buffer- [50mM Tris, pH 7.5, 300mM NaCl, 1.5mM MgCl\(_2\), 0.2mM ethylenediaminetetraacetic acid (EDTA), 0.5mM DTT, 1% Triton X-100 and protease inhibitors] (Clark et al., 2006). Bacteria were lysed with ultra-sonication for 5 times of 10 second pulse. Insoluble materials were removed by centrifugation at 11000xg and lysates were filtered through 0.45um syringe filters. The supernatant was transferred to a fresh tube and incubated with Glutathione–Sepharose 4B beads (GE Health-care) for 2 hours at 4°C. The beads were washed 3 times with GST lysis buffer. GST fusion proteins immobilized on Glutathione–Sepharose beads were directly used for **Pull-Down Assay**.

2.3 Pull-Down Assay

GST fusion proteins, purified on Glutathione–Sepharose beads, were incubated with mouse brain lysate (refer to **Brain Homogenization**, section 2.7) for 2 hours at 4°C with constant vertical rotations. Beads were washed three times with 1X PBS [137mM NaCl, 2.7mM KCl, 4.3mM Na\(_2\)HPO\(_4\), 1.47mM KH\(_2\)PO\(_4\)]. Interacting proteins bound to
GST-TRPM7 fusion proteins were processed for mass spectrophotometry analysis by either MALDI-TOF or LC-MS/MS. Proteins processed for MALDI-TOF were removed from glutathione-sepharose beads by heating in 2X sample buffer [0.125M Tris, 2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue] (Laemmli et al., 1970). Proteins were separated by size using denaturing 1D SDS-PAGE and the gels were post-stained with Coomassie Brilliant Blue dye. TRPM7 interacting proteins, processed for LC-MS/MS, were eluted from glutathione–Sepharose beads with Elution Buffer [50mM Tris-HCl pH 8.0, 10mM reduced glutathione].

### 2.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples denatured in sample buffer were loaded into well and separated in 4% acrylamide stacking gel and 10% acrylamide separating gel at 120V for 1.5 hours using the standard Laemmlli buffer system (Laemmli et al., 1970).

### 2.5 Coomassie Gel Staining and Destaining

Following electrophoresis, polyacrylamide gels are stained with Coomassie Brilliant Blue R250 [0.25% in 50% methanol, 10% acetic acid] for 1 hour and destained with Destaining Buffer (4 parts methanol: 1 part acetic acid: 5 parts water) overnight with constant agitation.
2.6 Western Blot

Following SDS-PAGE, proteins were transferred to nitrocellulose membranes in 1X transfer buffer [25mM Tris, 192mM glycine, 20% methanol] at 100V for 1 hour. To visualize protein bands, nitrocellulose blots were stained with Ponceas S. Membranes were blocked with blocking buffer - 5% non-fat + 1X TBST [150mM NaCl, 10mM Tris, 0.1% Tween-20] at room temperature for an hour. Blots were then probed with Anti-GST HRP conjugated antibodies in 1:10,000 dilutions, in blocking buffer for 1 hour. Non-specific antibody binding was removed by a 15-min TBST wash and three 5-min TBST washes. HRP-labeled secondary antibody binding was detected using chemiluminescent substrate (Pierce) and exposure to film (Kodak).

2.7 Brain Homogenization

Each gram of stripped mouse brain (Pel-Freeze® Biologicals) was homogenized in approximately 3mL of homogenization solution A [0.32M Sucrose, 1mM NaHCO₃, 1mM MgCl₂, 0.5mM CaCl₂] using a motor-driven (Jacobs Multi Craft & SDS Adaptor) homogenizer with up and down strokes until no visible, intact brain tissue was observed (approx 5 strokes per gram of brain). Brain homogenate was centrifuged at 1400xg at 4°C. The supernatant was transferred to a new tube and saved on ice. The pellet was resuspended in a 10% volume for 10 min of homogenization solution A using 3 additional homogenization strokes, and centrifuged at 1400xg at 4°C for 10 min. The second supernatant was pooled with previous supernatant and centrifuged at 710xg at 4°C for 10 min. Supernatant was frozen in dry ice with methanol and stored in -80°C.
2.8 In-gel Tryptic Digest

Prior to MALDI-TOF Mass Spectrometry analysis, it is necessary to excise, destain and extract protein bands from SDS-PAGE gel. Bands of interest were excised from Coomassie stained gel by using a razor blade and destained with 100mM (NH₄)HCO₃ in 50% Acetonitrile for 30 minutes. This step was repeated until colourless gel pieces were achieved, followed by gel dehydration for 10 min with 100% Acetonitrile. Gel pieces were dried with speed-vacuum.

Dried gel pieces were re-hydrated with 10mM Dithiothreitol in 25mM (NH₄)HCO₃ at 56°C for 1 hour and residual liquid was removed. 50mM iodoacetamide with 25 mM (NH₄)HCO₃ solution was added to gel for alkylation, at room temperature for 45 minutes in the dark. Washing of gel was completed with 50mM (NH₄)HCO₃ for 10 minutes at room temperature. Gel pieces were rehydrated with 100% Acetonitrile at room temperature for 10 minutes. Alkylation and rehydration were repeated and gel was speed-vacuumed to complete dryness.

Rehydration solution, containing a final concentration of 12.5ng/µl trypsin and 50mM (NH₄)HCO₃ was added to merely cover gel pieces from section 2.8.1 and incubated for 10 minutes at 4 °C. Rehydration solution was removed and gel pieces were left overnight at 37°C in 50mM (NH₄)HCO₃ solution.

Supernatant was transferred to another tube after a spin of 14000rpm at 1 minute. 20mM (NH₄)HCO₃ was added and incubated for 10 minute at room temperature. Supernatant was moved to the same tube mention previously. Formic acid extraction was performed 3 times with 5% formic acid in 50% Acetonitrile for 20 minutes at room
temperature. Supernatant was subsequently transferred, pooled and dried with speed-vacuum after formic acid extraction.

2.9 Co-Immunoprecipitation

Approximately 500μl of brain homogenate (∼1.5μg of proteins) was incubated with 4μg of rabbit anti-TRPM7 antibody (Millipore) or normal rabbit IgG (Cell Signaling Technology®) for 2 hours at 4°C with constant vertical rotations. Incubation was repeated after addition of Protein-A Sepharose beads (Sigma-Aldrich) to brain/antibody mixture. Samples were centrifuged at 9000rpm for 1 minute to remove supernatant. Beads were washed with 1X PBS + 0.5% Trition X-100 for 3 times, with subsequent spinning at 9000rpm to remove washing buffer. Protein-A beads were boiled in same volume of 2X sample buffer at 70°C for 5 minutes.

2.10 Microtubule Binding Protein Assay

The commercial Microtubule Binding Protein Spin-down Assay Kit from Cytoskeleton Inc. allows the identification of proteins that will bind to microtubules (MTs) in vitro. This assay relies on the fact that MTs pellet at high speed centrifugation (100,000xg). Any proteins that associate with MTs will be also pelleted along with the MTs.

Purified GST-fusion proteins (see section 2.2) were subjected to this test. Instructions in the Cytoskeleton Inc. manual of this assay were followed. In general, tubulin subunits were allowed to self-assembly into microtubule in buffer provided at 35°C for 20 minutes. The test proteins (GST-fusion proteins) and microtubules were
mixed and incubated for 30 minutes. Samples were subjected for ultra-centrifugation (100,000xg) for 40 minutes. Supernatant and pellet fractions of samples were collected separately and screened on SDS-PAGE gel or Western blot.

2.11 *In vitro* Interaction Assay

GST-fusion protein was purified and attached to Glutathione–Sepharose beads as described in section 2.2. Commercially purified tubulin (Cytoskeleton Inc.) in unpolymerized and polymerized forms were added to GST-fusion protein and allowed for 2 hour incubation at 4°C with constant vertical rotations. Beads were washed three times with 1X PBS. 100ul of supernatant was collected and the rest discarded. Equal volume of 2X sample buffer was added to both supernatant and bead fractions, and then boiled at 100°C for 10 minutes. Samples were subjected to SDS-PAGE analysis.
3. Results

3.1 Generation of Glutathione S-Transferase (GST) Fusion Constructs

In order to study protein-protein interactions with the intracellular domains of TRPM7, seven GST-TRPM7 fusion constructs were generated according to regions of interest on protein map of TRPM7 shown in Figure 3. TRPM7 fusion proteins were mainly selected from regions of the C-terminus of TRPM7 due to the fact that several consensus domains have been previously identified and described. In contrast, the long N-terminal domain of TRPM7 has no recognized consensus motifs other than being highly homologous to the N-terminus of other TRPM family members. Regions of interest were sub-cloned into pGEX-5X® vectors as described in the Materials and Methods Section. Fusion constructs identities were verified with restriction digest analysis (Figure 4). DNA band patterns of the six restriction-digested constructs (#1-4, and 6-7) observed on 1% agarose gel are consistent with expected sizes (Table 4). Construct #5, C-terminus – C (refer to Figure 4) was omitted from further experiments due to its redundancy with sequences and motifs found in Constructs #2 and 3. The identities of the fusion constructs were further confirmed with DNA sequencing analysis.

Different GST fusion proteins were expressed in BL21 pLysS E.coli and analyzed on Western blot to examine proper expression of these recombinant proteins in bacteria. The fusion protein molecular weights (Figure 6), are compared to the estimated GST fusion protein sizes which are predicted based on the length of inserts and the size of GST tag (Table 5). The gel migration of each of the seven fusion proteins was consistent with the predicted molecular weights. This data provides secondary confirmation of
successful in-frame sub-cloning as well as proper expression of fusion proteins in bacterial cells.
Figure 5. Restriction Digest of GST Fusion Constructs

Six restriction-digested GST fusion constructs (#1-4, 6-7) (refer to Figure 4) were resolved on 1% agarose gel to examine the DNA fragment patterns. The DNA fragment band sizes after restriction digest (resolved on the gel) were compared to the expected fragment sizes (Table 4) for each DNA constructs.
<table>
<thead>
<tr>
<th>Clone #</th>
<th>Restriction Digest</th>
<th>Fragment Sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRV</td>
<td>2000, 3800</td>
</tr>
<tr>
<td>2</td>
<td>EcoRV</td>
<td>2000, 3860</td>
</tr>
<tr>
<td>3</td>
<td>EcoRV</td>
<td>2280, 3860</td>
</tr>
<tr>
<td>4</td>
<td>EcoRV/PstI</td>
<td>620, 1600, 2200, 2900</td>
</tr>
<tr>
<td>6</td>
<td>EcoRI</td>
<td>620, 5000</td>
</tr>
<tr>
<td>7</td>
<td>EcoRV/PstI</td>
<td>260, 780, 1200, 4000</td>
</tr>
</tbody>
</table>
Figure 6. Expression of GST-Fusion proteins in BL21 *Escherichia coli*

GST fusion proteins were over-expressed in BL21 pLysS *Escherichia coli* cells and later boiled in 2X sample buffer. GST- TRPM7 fusion proteins were detected with GST antibody and observed on Western blot to confirm correct protein expressions. Each lane is labeled as Clone # and compared to the predicted GST fusion protein sizes listed on Table 5.
Clone #

1  2  3  4  5  6  7

kD
150
100
75
50
Table 5 Predicted Molecular Weights* of TRPM7- GST Fusion Proteins

<table>
<thead>
<tr>
<th>Clone #</th>
<th>TRPM7 Construct Name</th>
<th>Estimated GST Fusion Protein Size (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-kinase</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>α-kinase – C</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>Long α-kinase - C</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>C terminus - N</td>
<td>111</td>
</tr>
<tr>
<td>5</td>
<td>C-terminus - C</td>
<td>116</td>
</tr>
<tr>
<td>6</td>
<td>Coiled-coil</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>Long coiled-coil</td>
<td>74</td>
</tr>
</tbody>
</table>

*GST fusion protein sizes were estimated based on the length of TRPM7 DNA insert (bp) and size of GST tag (26 kDa).
3.2 Pull-Down Assays and Identification of TRPM7 Interacting Proteins

TRPM7-GST fusion proteins or GST alone were overexpressed in BL21LysS E.coli. The bacteria were pelleted, lysed and the GST proteins were purified from the lysate using glutathione-sepharose beads. Purified fusion proteins were then used for pull-down experiments to capture and identify TRPM7 interacting proteins in mouse brain homogenate. Two types of mass spectrometry were used to identify the TRPM7 interacting proteins: MALDI-TOF MS and LC-MS/MS.

3.2.1 Matrix-assisted Laser Desorption/Ionisation - Time Of Flight Mass Spectrometry (MALDI-TOP MS) Analysis

Pull-down samples of Alpha-Kinase - C and Long Alpha-Kinase fusion proteins (refer to Figure 4) were first analyzed on 1-dimensional (1-D) SDS-PAGE gel. Selection of TRPM7 associating proteins on 1D-SDS PAGE gel was based on the comparison of protein band patterns of to the band patterns of 3 different controls: (i) plain bacterial lysate/ brain control, (ii) empty plasmid (GST tag)/ brain control and (iii) fusion protein control (no brain). Theoretically, any defined protein bands that were not observed in controls could be excised for MALDI-TOF MS analysis, as illustrated in Figure 7a. A total of eight protein bands (arrowed protein bands in Figure 7b) were selected from samples 2(B) and 3(B) through protein pattern comparison with the 3 controls: (i) the bacterial lysate/ brain control (not shown), (ii) the empty plasmid/ brain control (not shown), and (iii) 2(0) and 3(0) (without brain incubation controls), respectively. However, with the use of MALDI-TOP MS analysis for the purpose of protein identification, only three TRPM7 interacting proteins were identifiable (excluding the
two falsely selected GST tags (#2 ~20 and ~25kD)). Interestingly, among all three proteins, two are cytoskeletal proteins, beta-actin (#3 ~50kD) and beta-tubulin (#2 ~55kD), and one is cytoskeletal associating protein, nebulin-related anchoring protein (#3 ~20kD) (shown in Table 6). Beta-actin and beta tubulin are well known subunits that comprise the polymeric cytoskeleton proteins, F-actin and microtubule, relatively. F-actin provides structural support and it is crucial for functions including cell motility and cytokinesis. Microtubules also serve as structural component within cells and are involved in many cellular processes such as mitosis and vesicle transport. Nebulin-related anchoring protein (NRAP) is a cytoskeletal interacting proteins that possesses a C-terminal actin binding site. Its function is not well understood but it has been suggested to have an anchoring function, linking the terminal actin filaments of myofibrils to protein complexes located beneath the sarcolemma (Luo et al., 1997b; Luo et al., 1997a). As indicated in our result, TRPM7 association with cytoskeleton or cytoskeletal interaction proteins strongly suggests that TRPM7 has a role in cytoskeleton-related functions as proposed by other researches including modulation of cell morphology, regulation of synaptic vesicle transport and fluid induced stress response.
Figure 7. 1-D SDS-PAGE gel analysis of Pull-Down samples

a. Hypothetical 1-D SDS-PAGE gel image. Band pattern of pull-down sample is compared to band patterns of 3 controls: plain bacterial lysate/brain control, empty plasmid (GST tag)/brain control and fusion protein control (no brain). Distinct protein bands (circled) that do not appear on control lanes are excised.

b. The screening of protein band patterns in pull-down samples. Alpha-Kinase - C and Long Alpha-Kinase recombinant proteins were used as the baits to identify TRPM7 interactors from mouse brain lysate and protein samples were resolved on 1D SDS-PAGE gel and detected using Coomassie Brilliant Blue stain. Band patterns of Alpha-Kinase - C (2(B)) and Long Alpha-Kinase (3(B)) pull down samples were compared to band patterns of the 3 controls: (i) the bacterial lysate/brain control (not shown), (ii) the empty plasmid/brain control (not shown), and (iii) 2(0) and 3(0) (without brain incubation controls), respectively. Arrowed protein bands were excised and subjected to MALDI-TOF analysis to reveal their identities.
Table 6. TRPM7 Interacting Proteins Identified by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Bait</th>
<th>Estimated Interactor Size (kD)</th>
<th>Protein Identity</th>
<th>Number of Peaks/Recognized Peptide Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-Fusion Protein #2</td>
<td>60</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Beta-tubulin</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>GST</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>GST</td>
<td>40</td>
</tr>
<tr>
<td>GST-Fusion Protein #3</td>
<td>60</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Beta-actin</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Nebulin-related anchoring protein</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.2.2 Liquid Chromatography Mass Spectrometry (LC-MS/MS)

MALDI-TOF MS may only require 1-10µM of purified proteins (peptides) for protein identification but is limited in its dynamic ability to detect proteins such that contaminating proteins or proteins expressed at high concentrations (> 10µM) can overwhelm the detection system. In addition, the sensitivity of MALDI-TOF is significantly reduced when combined with the limited resolving power of 1D gel electrophoresis and visible proteins stains. 1D-SDS PAGE gel separates proteins based on size and what appear to be single bands can often contain multiple proteins. The detection of one or more proteins within the band may be lost or ‘hidden’ if one of the band constituents has a significantly higher concentration. Coomassie Brilliant Blue stain also limits protein detection in that its sensitivity is limited to visible bands that contain 50-100-ng of protein. In addition different proteins with similar gel migration may be excluded if similar bands appear in both control and test lanes. Accordingly we used a second approach to identify proteins interacting with the kinase domain of TRPM7. LC/MS-MS is a powerful proteomic approach for analysing complex mixtures of proteins. Although it is not as sensitive as MALDI-TOF, LC-MS/MS is not as easily saturated nor complicated by the limitations of gel electrophoresis. Pull-down samples can be directly subjected to tryptic digest and LC-MS/MS without the need of resolving protein bands on 1D-SDS PAGE gel. Thus mixtures of proteins in different samples were separated with the help of liquid chromatography. Identified proteins in each sample were listed. Proteins in pull-down samples with four different GST fusion proteins (Clone #1 - 4) were compared to proteins in control samples. Protein names that do not appear in control sample lists are possible TRPM7 interactors (Table 7.1 – 7.4).
Three cytoskeletal proteins, alpha/beta-tubulin and beta-actin are found to be attached to #2 - #4 GST-fusion protein/bead complexes (Table 7.2 – 7.4) but not associating with GST or glutathione beads alone even though GST may be a potential contaminant due to its abundance in cells. The recombinant alpha-kinase region of TRPM7 (clone #1) only interacts with microtubule components, alpha/beta-tubulin, but not beta-actin, which suggests the surrounding sequence of alpha-kinase domain are required for the binding to actin or actin associating proteins. In addition to cytoskeletal proteins, synaptosomal components required for synaptic vesicle release, vesicle associated membrane protein 2 (VAM2) and synaptosomal-associated protein 25 (SNAP25), were also identified as proteins associated with TRPM7 (Long Alpha-Kinase TRPM7 recombinant protein). The LC-MS/MS results are largely consistent with the suggested physiological roles of TRPM7 in cell morphology regulation (Clark et al., 2006; Dulyaninova et al., 2005; Oancea et al., 2006a) and synaptic vesicle release (Brauchi et al., 2008; Krapivinsky et al., 2006). A Na⁺/K⁺ ATPase subunit, alpha-3 subunit, is also detected as a TRPM7 interacting protein (Table 7.2 & 7.4). K⁺/Na⁺ ATPase is an abundant protein in neurons and critical for the reuptake of K⁺ following action potential firing. Moreover, protein-protein interactions of Na⁺/K⁺ ATPase is important for protein activation (Src family kinase) and mediating signal transduction (Xie and Cai, 2003). Thus the interaction of TRPM7 with Na⁺/K⁺ ATPase may be a potential mechanism for TRPM7 alpha-kinase regulation or activation.
### Table 7.1 LC-MS/MS Analysis on TRPM7 Associating Proteins in Pull-Down Assay using Alpha-Kinase Fusion Protein as The Bait

<table>
<thead>
<tr>
<th>Fusion Protein</th>
<th>Identified Interactors</th>
<th>Peptide Sequences Recognized</th>
<th>Confident Level</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clone #1 - α-Kinase</strong></td>
<td>tubulin, alpha 1C [Mus musculus], gi</td>
<td>148225011</td>
<td>ref</td>
</tr>
<tr>
<td></td>
<td>tubulin, beta 2 [Mus musculus], gi</td>
<td>157819845</td>
<td>ref</td>
</tr>
</tbody>
</table>

### Table 7.2 LC-MS/MS Analysis on TRPM7 Associating Proteins in Pull-Down Assay using Alpha-Kinase - C Fusion Protein as The Bait

<table>
<thead>
<tr>
<th>Fusion Protein</th>
<th>Identified Interactors</th>
<th>Peptide Sequences Recognized</th>
<th>Confident Level</th>
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</thead>
<tbody>
<tr>
<td><strong>Clone #2 - α-kinase - C</strong></td>
<td>tubulin, alpha 1C [Mus musculus], gi</td>
<td>148225011</td>
<td>ref</td>
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<tr>
<td></td>
<td>tubulin, beta 2 [Mus musculus], gi</td>
<td>157819845</td>
<td>ref</td>
</tr>
<tr>
<td></td>
<td>actin, beta [Mus musculus], gi</td>
<td>13592133</td>
<td>ref</td>
</tr>
<tr>
<td></td>
<td>vesicle associated membrane protein 2 [Mus musculus], gi</td>
<td>117616908</td>
<td>gb</td>
</tr>
<tr>
<td></td>
<td>synaptosomal-associated protein 25, isoform CRA_a [Mus musculus]</td>
<td>(K)AWGNNQDGVVASQPAR(V) 80 – 94%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATPase, Na+/K+ transporting, alpha 3 polypeptide, isoform CRA_a [Mus musculus], gi</td>
<td>148692349</td>
<td>(R)LNIPVSQVNPR(D) (R)QGAIVAVTDGVNDSPALK(K) (K)GVIISEGNETVEDIAAR(L) 50 – 79%</td>
</tr>
</tbody>
</table>
Table 7.3 LC-MS/MS Analysis on TRPM7 Associating Proteins in Pull-Down Assay using Long Alpha-Kinase Fusion Protein as The Bait

<table>
<thead>
<tr>
<th>Fusion Protein</th>
<th>Identified Interactors</th>
<th>Peptide Sequences Recognized</th>
<th>Confident Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone #3 - Long α-kinase - C</td>
<td>tubulin, alpha 1C [Mus musculus], gi</td>
<td>148225011</td>
<td>ref</td>
</tr>
<tr>
<td></td>
<td>tubulin, beta 2 [Mus musculus], gi</td>
<td>157819845</td>
<td>ref</td>
</tr>
<tr>
<td></td>
<td>actin, beta [Mus musculus], gi</td>
<td>13592133</td>
<td>ref</td>
</tr>
</tbody>
</table>

Table 7.4 LC-MS/MS Analysis on TRPM7 Associating Proteins in Pull-Down Assay using C - Terminus - N Fusion Protein as The Bait

<table>
<thead>
<tr>
<th>Fusion Protein</th>
<th>Identified Interactors</th>
<th>Peptide Sequences Recognized</th>
<th>Confident Level</th>
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3.3 Confirmation of TRPM7 Specific Interactions Using Co-Immunoprecipitation

Consistent results from both MALDI-TOF and LC-MS/MS analyses represent good validity of pull-down experiments; however it is insufficient to indicate specific associations between TRPM7 and cytoskeletal proteins. Due to its abundance in cells, cytoskeleton may be potential contaminants for pull-down assay and produce non-specific interaction. To ensure specific associations, TRPM7 antibody was used for co-immunoprecipitation (Co-IP), attempting for co-purification of beta-actin and beta-tubulin together with TRPM7. Due to the low expression of TRPM7 and different detection conditions (different TRPM7 antibody used), TRPM7 was detected in mouse brain lysate with beta-tubulin (Figure 8b), but not in the mouse brain lysate in the separated Western blot that also was probed for beta-actin (Figure 8a). Regardless of the detection of TRPM7 in straight brain lysate, both beta-actin (Figure 8a) and beta-tubulin (Figure 8b) were co-immunoprecipitated along with TRPM7 using TRPM7 antibody in both co-immunoprecipitation experiments. Neither TRPM7 nor the two associating proteins appear on the Normal Rabbit IgG control lane; it indicates that beta-actin and beta-tubulin directly or indirectly interact with TRPM7 but not the IgG proteins.
Figure 8. Co-Immunoprecipitation of TRPM7

Specific associations between TRPM7 and interactors were further confirmed using co-IP. A rabbit anti-TRPM7 antibody (Millipore) or control rabbit IgG were used to precipitate endogenous TRPM7 and its associated proteins from mouse brain lysate. Following incubation with brain lysate the antibodies were precipitated from solution using Protein-A Sepharose and heated in SDS sample buffer. Eluted proteins were separated by SDS-PAGE, blotted on nitrocellulose and probed with antibodies against beta-actin or beta-tubulin. Whole mouse brain lysate (Brain) was used as a positive control to confirm detection of TRPM7, beta-actin or beta-tubulin in the starting material. Western analysis confirmed that both beta-actin and beta-tubulin are co-immunoprecipitated with TRPM7 but not control antibody.
3.4 Examination of Tubulin Association with TRPM7

Both alpha-tubulin and beta-tubulin were identified through LC/MS-MS analysis (Table 7), and it is possible that TRPM7 interacts with microtubules rather than single subunits of tubulin heterodimers. To further investigate the association between TRPM7 and cytoskeletal proteins, we examined whether TRPM7 can bind directly to microtubule and if the binding involves individual microtubule subunits or polymerized microtubules. Alpha-tubulin and beta-tubulin form the dimeric subunit of microtubules (MTs). Two sets of experiments (the microtubule binding protein assay and the in vitro protein interaction assay) were done to test for direct interaction of TRPM7 and MTs.

3.4.1 The Microtubule Binding Protein Assay

The microtubule binding protein assay was used to examine if TRPM7 bound to MTs directly or indirectly. Purified tubulin can be polymerized into microtubules and pelleted by high speed centrifugation. Proteins that directly interact with MTs will also be pelleted. Microtubule associated proteins (MAPs), proteins that bind soluble tubulin subunits or MTs, were used as a positive control and bovine serum album (BSA) was used as a negative control for this assay. MAP interacted with MTs and was found in the pellet fraction, but remained in the supernatant when it was centrifuged without MTs (Figure 9a), whereas BSA stayed in the supernatant regardless of the presence of MTs. Possibly due to prolonged storage of purified GST proteins at low temperature, GST protein formed aggregation hence a small amount of GST was detected as a pellet while most of it was found in the supernatant fraction. Although majority of the tubulin proteins formed a pellet during ultra-centrifugation, TRPM7 fusion protein (Long Alpha Kinase),
containing partial TRPM7 C-terminus, still remained in the supernatant fraction on the Coomassie stained SDS-PAGE gel (Figure 9b) and Western blot (Figure 9c). This result indicates that TRPM7 does not directly interact with polymerized microtubules and may instead require an accessory or adaptor proteins for cytoskeletal associations.

3.4.2 In vitro Protein Interaction Assay

To further address whether TRPM7 can form direct interactions with polymerized or unpolymerized form of dimeric tubulin subunits, in vitro protein interaction assays was used. Recombinant TRPM7 proteins (Long Alpha-Kinase – Clone #3) were immobilized on glutathione resin and both unpolymerized and polymerized forms of tubulin were allowed to interact with the recombinant protein. Coomassie stained SDS-PAGE gel and western blot analysis showed that neither polymerized (Figure 10a and 10d) nor unpolymerized (Figure 10c and 10d) forms of tubulin bind TRPM7 recombinant proteins, therefore they remain in the supernatant fraction. This data confirms that TRPM7 does not form a direct protein-protein binding relationship with MTs or soluble tubulin subunits but requires one or more intermediate proteins to act as linkers between TRPM7 and the microtubule cytoskeleton.
Figure 9. Examination for Direction Interaction between TRPM7 and Microtubule using Microtubule Binding Protein Assay.

The Microtubule binding assay was used to test for direct interaction between TRPM7 and microtubules. Purified tubulins were self-assembled into microtubules, which were later incubated with purified GST fusion protein (Long Alpha Kinase). Following ultracentrifugation of samples, both pellet (P) and supernatant (S) fractions of were collected and run on SDS-PAGE gel.

(a) Controls of Microtubule Binding Protein Assay

4 sets of control samples were run on SDS-PAGE gel and proteins were detected with Coomassie Brilliant Blue stain:

i) Positive control: Microtubule & MAP: (Tub + MAP)
ii) Negative control: Microtubule & BSA: (Tub + BSA)
iii) MAP control: (MAP)
iv) BSA control: (BSA)

(b) & (c) Analysis of TRPM7-Microtubule interaction using Microtubule Binding Protein Assay

3 sets of test protein samples were run on SDS-PAGE gel and GST fusion proteins were detected with either (b) Coomassie Brilliant Blue stain or (c) GST antibody on Western Blot:

i) Long Alpha-Kinase recombinant protein (~5ug) & Microtubule: (#3 – Tub)
ii) Long Alpha-Kinase recombinant protein (~2ug) & Microtubule: (#3 (1/2) – Tub)
iii) GST & Microtubule: (GST – Tub)
Figure 10. Examination of Direction Interaction between TRPM7 and Microtubule using *in vitro* Protein Interaction Assay.

Purified tubulin subunits or polymerized tubulins (microtubule) were incubated with purified GST fusion protein (*Long Alpha-Kinase* – Clone #3) that was pre-attached to glutathione resin. Both supernatant (S) and pellet (P) samples were run on a SDS-PAGE gel and Western blot to test for direction interaction between TRPM7 and polymerized tubulin (microtubules) (a and b) or unpolymerized tubulin monomers (c and d) at the two indicated salt concentrations.

(a) & (b) Test of interactions between *Long Alpha-Kinase* recombinant protein and polymerized form of tubulin (microtubule)

2 sets of test protein samples were run on SDS-PAGE gel and proteins were detected with either (a) Coomassie Brilliant Blue stain or (b) GST antibody or tubuline antibody on Western Blot:

i) Alpha-Kinase recombinant protein & Microtule: (#3 – Tub)
ii) GST & Tubulin: (GST – Tub)

(c) & (d) Test of interactions between *Long Alpha-Kinase* recombinant protein and unpolymerized tubulin subunits

2 sets of test protein samples were run on SDS-PAGE gel and proteins were detected with either (a) Coomassie Brilliant Blue stain or (b) GST antibody or tubuline antibody on Western Blot:

i) Alpha-Kinase recombinant protein & Microtule: (#3 – Tub)
ii) GST & Tubulin: (GST – Tub)
10c

#3 - Fusion Protein (75kD)

Tublin (50kD)

GST (28kD)

10d

#3 - Fusion Protein

Tubulin

GST
4. Discussion

4.1 Identification of TRPM7 Associating Proteins

In this study MALDI-TOF MS analysis was used to identify proteins from mouse brain lysate that interact with intracellular C-terminal domains of TRPM7. Distinct fusion proteins, each containing the TRPM7 kinase domain, were shown to interact with the cytoskeletal protein subunits beta-actin and beta-tubulin as well as nebulin-related anchoring protein (refer to Figure 4 & Table 6). This result was confirmed in a separate analysis using LC-MS/MS which identified beta-actin and beta-tubulin as well as alpha-tubulin as TRPM7 kinase domain binding partners. The use of recombinant TRPM7-GST fusion proteins as bait for pull down experiments and mass spectrometry was validated by the reproducible identification of interacting proteins in separate analyses, the high number of peptide sequences identified for each protein target and the consistency of the findings reported here and that of published research. A study using TRPM7 immunoprecipitation by Clark et al. (2006) has shown that beta-actin was present in the TRPM7 precipitant, forming molecular complex with TRPM7 (Clark et al., 2006). Likewise, our co-immunoprecipitation experiment using a TRPM7 antibody coupled with immunoblot detection (Figure 8a and 8b) showed similar result that both beta-actin and beta-tubulin co-precipitated with TRPM7. Together these results confirmed the interaction between endogenous TRPM7 with beta-actin and beta-tubulin and also suggested a cytoskeletal-related function of TRPM7 dependent on the specific association between TRPM7 and the cytoskeleton.

Beta-actin and alpha/beta-tubulin subunits are the building blocks that compose actin filaments and microtubules, respectively. Association with these cytoskeletal
subunits indicates a potential role for TRPM7 in cytoskeletal related functions that involve interaction with actin filaments and microtubules. A body of evidence, together with our results, now suggests that TRPM7 contributes to cytoskeletal cellular physiology including stress-induced response, cell morphology regulation, and modulation of cell adhesion possibly through the regulation of actin disassembly and cytoskeleton rearrangement (Birukov et al., 2002; Chubanov et al., 2004; Clark et al., 2006; Clark et al., 2008b; Oancea et al., 2006a; Su et al., 2006). The association of the TRPM7 C-terminus with actin, as indicated by our result, is consistent with the suggested role of the alpha-kinase in regulating myosin II-dependent contractile responses and actomyosin remodeling (Clark et al., 2008a; Clark et al., 2008b). Regulation of actomyosin contractility is essential to modulate focal adhesion assembly (DeMali and Burridge, 2003; Geiger and Bershadsky, 2002; Linder and Aepfelbacher, 2003), and TRPM7 is implicated in directing the assembly of focal adhesion by regulating actomyosin rearrangement through myosin II heavy chain phosphorylation (Chubanov et al., 2004; Clark et al., 2006; Su et al., 2006). Alternatively, the association with actin may simply serve to facilitate TRPM7-myosin II interactions. TRPM7 is a membrane embedded channel and myosin II is a cytosolic protein that travels along actin filament and thus there may be very low probability for an independent TRPM7-myosin II interaction. However, TRPM7 association with cytoskeletal elements such as actin filaments may dramatically increase the likelihood of TRPM7-myosin II interaction.

Spatial reorganization of the cytoskeleton is a critical protective mechanism for endothelial cells against mechanical stress created by frequent fluid flow, providing structural support and integrity to cells to resist shear forces induced by increased fluid
flow (Galbraith et al., 1998; Malek and Izumo, 1996). In situ morphologic studies reveal the formation of a type of actin structure known as stress fibers. In non-muscle cells, stress fibers are bundles of actin microfilaments whose assembly is involved in actin-myosin interactions and cross-linking by actin associating proteins including α-actinin, myosin and tropomyosin (Lu et al., 2008a; Lu et al., 2008b). Under the conditions of increased fluid flow rate, it has been shown that actin stress fibers are formed in a unique orientation that is parallel to the direction of shear stress vector in endothelial cells, which contrasts with the random cytoskeletal arrangement of endothelial cells culture grown under static conditions (Nehls and Drenckhahn, 1991). More importantly, the onset (within 2 minutes of fluid exposure) of cytoskeletal remodeling under shear stress may involve the phosphorylation of myosin by myosin light chain kinase (MLCK) and Rho-associating kinase. The TRPM7 alpha-kinase may play a similar role in facilitating endothelial cell cytoskeletal arrangement by phosphorylating the myosin II heavy chain in later stage of shear stress induction. Such a role would explain the observations by Oancea et al. who observed the insertion and accumulation of TRPM7 channels in endothelial cells, at membrane regions exposed to shear stress force (Oancea et al., 2006a).

In addition to cytoskeletal proteins, two components of synaptic vesicle release were identified by LC-MS/MS analysis as proteins interacting with the c-terminal domain of TRPM7. Synaptosomal proteins including both the synaptic vesicle-specific proteins (VAMP2) and the non-vesicular components of the fusion machinery that reside on the membrane (SNAP25) were found to associate with TRPM7 fusion proteins containing the alpha-kinase domain (Table 7.2). This data is consistent with the work of
Krapivinsky et al. (2006) who found that TRPM7 resides on the membrane of cholinergic vesicles in peripheral sympathetic neurons and forms molecular complexes with synaptic vesicle proteins including synaptotagmin I, synapsin I and snapin (Krapivinsky et al., 2006). Prior to the release of synaptic vesicles, the components of fusion machinery, such as SNAP 25 and syntaxin, are required for docking and fusion of synaptic vesicles (Pevsner et al., 1994; Sollner et al., 1993). The identified association with SNAP25 in our data allows us to predict that TRPM7 is involved in the docking process of synaptic vesicles, also a function suggested by Krapivinsky and colleagues (Krapivinsky et al., 2006). It should be noted that Krapivinsky et al. has found that a discrete region of the TRPM7 N-terminus directly interacts with the synaptic vesicle protein snapin. However we cannot eliminate the possibility that other domains of TRPM7, such as the C-terminal kinase, may also bind synaptic vesicle proteins. The use of the isolated kinase from the TRPM7 C-terminus as bait for my pull-down experiments reveals that this region could also contribute to the interaction with synaptic vesicular proteins and the formation of molecular complexes within the synaptic vesicle membrane. This result is pertinent to the finding of Bracuchi and colleagues who has shown that the TRPM7 kinase domain provides control to vesicle motility (Brauchi et al., 2008). Potentially, the TRPM7 C-terminal domain interacts with synaptic vesicle proteins to function as a regulator of vesicular transport and to monitor vesicle mobility. Moreover, my identification of tubulin as a TRPM7-interacting protein suggests that TRPM7 may associate with microtubule proteins in order to regulate vesicular transport along microtubules. However, results from the microtubule binding protein assay (refer to Figure 9b - c) and in vitro protein interaction assay (refer to Figure 10a - d) indicate that the TRPM7 kinase
does not directly interact with tubulin or polymerized tubulin microtubules. Thus additional microtubule binding or accessory proteins must be involved in the association between TRPM7 and microtubules.

4.1.1 Limitation of LC-MS/MS Affects Detection of TRPM7 Associating Proteins

LC-MS/MS is a powerful tool suitable for the identification of novel protein interactions. Protein complexes that associate with the bait protein in pull-down experiments can be separated through high pressure liquid chromatography, subjected to tryptic digest in solution and identified using mass spectrometry. The results obtained from Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis are consistent with the physiological role of TRPM7 suggested by other researchers. However, other components involved in TRPM7 functions seem to be missing. For instance, only VAMP2 and SNAP25 were detected in TRPM7 protein complexes while other synaptic vesicular proteins such as synapsin and synaptotagmin were not identified. Thus the results obtained here may be limited by the sensitivity of LC-MS/MS and the masking effect exhibited by abundance proteins or polymeric interactions. The sensitivity of LC-MS/MS is based on the sample component ratio. The signal from a protein with high number can overwhelm the signal from a low number protein present in the same mixture, causing a masking effect on lower abundance proteins. Therefore proteins with higher expression or abundance are more likely to be detected with LC-MS/MS. Association with multimers or polymers may cause a greater masking effect. Cytoskeletal proteins are among the most abundant proteins in a cell and the formation of a protein complex between TRPM7, microtubules and F-actin is very likely to mask the signals
given by other TRPM7 associated proteins. More importantly, binding to one molecule of
cytoskeleton may result in the presence of large number of monomers, alpha/beta-tubulin
and beta-actin, in the sample mixtures. Thus a combination of association with high
abundance protein and polymers could hide the identity of other TRPM7 associated
proteins in LC-MS/MS analysis. The Na⁺ / K⁺ ATPase, as another identified TRPM7
associated protein, was not susceptible to the masking effect by cytoskeletal proteins due
to its abundance in neurons. On the other hand, synaptic vesicle proteins, whose
abundance is dependent on synaptic activities in neurons (Burre et al., 2006), may not be
able to escape the masking effect. The fact that only two synaptic vesicle proteins,
VAMP2 and SNAP25, are weakly detected in the pull-down sample mixtures by LC-
MS/MS may indicate other synaptic vesicle fusion machinery proteins, such as
synatotagmin and syntaxin, were overwhelmed by the abundance of cytoskeletal proteins,
hence undetectable. Therefore, presumably not all TRPM7 associating proteins are
identified in this experiment.
4.2 Potential Model of TRPM7 Functions in Cytoskeletal Remodeling and Shear Stress Response

As mentioned previously, TRPM7 channels localize at plasma membrane regions that are under local shear stress induced by fluid flow (Oancea et al., 2006a). Actin stress fibers also form in a parallel alignment to the stress stress vector, which helps endothelial cells to resist the shearing force created by blood flow and by collision with red and white blood cells (Nehls and Drenckhahn, 1991). Shear stress-induced changes in cell morphology and the formation of stress fibers in endothelial cells involve rearrangement of the actin filaments (Wagner et al., 2002). Modulation of actin cytoskeleton remodeling may involve covalent modification of Myosin II and tropomyosin/tropomodulin, which are important components required for structural integrity of actin and stress fiber formation (Nehls and Drenckhahn, 1991;Ngu et al., 2008). Phosphorylation of myosin II (Clark et al., 2006; Kendrick-Jones et al., 1983; Smith et al., 1983) and tropomodulin (Mudry et al., 2003;Wagner et al., 2002) have been suggested as a regulatory mechanism to alter their binding affinity to cytoskeleton, leading to disassembly of actin filament rearrangement. Thus phosphorylation of these actin associated proteins may be important for the formation of stress fiber in response to shear stress force.

Myosin II is a class of actin binding protein that moves along actin filaments. All myosin II proteins are composed of two heavy chains and two pairs of light chains. Moreover, myosin II molecules form bipolar filaments through electrostatic interactions of the myosin heavy chain (MHC) tail (Hostetter et al., 2004). The globular motor domain of each MHC catalyzes ATP hydrolysis and interacts with actin. Phosphorylation of MHC promotes dissociation of myosin II filaments and potentially leads to actomyosin remodeling. In addition to myosin II, tropomodulin and tropomyosin are also actin
binding proteins. Tropomodulin possesses both actin-capping domains and tropomyosin binding domain. The actin-capping ability of tropomodulin allows it to cap the pointed end (for disassembly) of actin filament to prevent dissociation of beta-actin subunits, and together with tropomoyosin association, tropomodulin inhibits depolymerization of actin and stabilizes the structure of the actin cytoskeleton (Kostyukova et al., 2005; Kostyukova et al., 2007; Kostyukova and Hitchcock-Degregori, 2004). Phosphorylation of tropomodulin within its N-terminal domain by TRPM7 kinase results in a loss of actin-capping ability while the C-terminal actin-capping and tropomoyosin binding ability are unaffected (Dorovkov et al., 2008). Conversely, another study examining tropomodulin functions demonstrated that phosphorylation of tropomodulin increased its association with cytoskeletal proteins (Wagner et al., 2002) and such interaction helps stabilize the cytoskeleton (Krieger et al., 2002; Mudry et al., 2003; Ono and Ono, 2002).

Regulation of actin dynamics is important for many biological processes including cytokinesis, cell migration, cell adhesion and mechanical stress response. As previously described, TRPM7 is suggested to regulate actin dynamics by phosphorylating the myosin II heavy chain and tropomodulin. It has been shown to be involved in shear stress response and alteration of cell morphology. These data all indicate that TRPM7 has cytoskeletal related function(s) and perhaps provide insight into a potential model for a fluid-induced shear stress response involving TRPM7. Presumably under normal conditions TRPM7 would be dispersed throughout the plasma membrane (Figure 11a). As the fluid flow rate increases TRPM7 channels are inserted and localize at the cell membrane region under induced shear stress force (Figure 11b). The localization of TRPM7 should permit its interaction with myosin II heavy chain and tropomodulin, as
well as substrate phosphorylation by the TRPM7 kinase domain. As a result myosin filaments would dissociate from each other while tropomodulin would also lose its ability to cap actin at the N-terminal domain, followed by actin depolymerization and cytoskeleton rearrangement (Figure 11c). Changes in tropomodulin binding affinity to actin may be only a temporal effect. In addition, phosphorylation at different regions of tropomodulin could have dual effect on actin structure; N-terminal phosphorylation of tropomodulin would facilitate actin reorganization, whereas phosphorylation at other sites of tropomodulin, possibly by other kinases may enhance tropomyosin binding and actin capping ability in the C-terminal domain in which would promote ultimate stabilization of actin stress fibers (Figure 11d). As a result, actin stress fibers can provide structural support to endothelial cells with alignment parallel to fluid flow (stress vector) to resist shear force and to prevent cell tearing.
Figure 11. Proposed Model of TRPM7 Function in Endothelial Shear Stress Response

(a) In resting state, TRPM7 channels are disturbed throughout the plasma membrane.

(b) TRPM7 channels are inserted and localize at the region of cell membrane under shear force caused by increase in fluid flow (arrow represents direction vector of shear force).

(c) TRPM7 C-terminal domain interacts with tropomodulin and myosin II heavy chain followed by substrate phosphorylation with TRPM7 alpha-kinase to allow disassembly and reorganization of actin filaments.

(d) Actin stress filaments are formed aligning parallel to shear force vector and stabilized by tropomodulin and myosin II filaments to resist shear stress induced by fluid flow.
11a

11b
4.2.1 Future Studies of TRPM7 Shear Stress Response

Several lines of research could be undertaken to address the proposed role of TRPM7 in modulating cytoskeletal dynamics in the shear stress response. First, a TRPM7 kinase-dead mutant and a channel mutant (with key point mutations that abolish ion conductance) can be used to examine the effect of TRPM7 kinase activation and channel activity on the formation of actin stress fibers under fluid induced shear stress condition. An endothelial cell line such as human microvascular endothelial cells (HMEC-1) could be transfected with wild-type or mutant TRPM7 constructs and subsequently exposed to fluid flow in a perfusion system. The dynamics of the actin cytoskeleton could then be observed using actin markers such as Texas Red-conjugated phalloidin (Birukov et al., 2002; Clark et al., 2008b) or with GFP-β-actin transfected cells for live images. The loss of actin stress fiber formation in cells expressing the kinase-dead mutant would confirm that TRPM7 kinase activity is required for actin cytoskeletal remodeling and actin stress fiber formation. With the use of Western analysis and phosphor-antibodies to examine phosphorylation changes of actin or accessory proteins in wild type or kinase-dead mutant expressing cells could help to capture proteins that are involved in the fluid shear stress response but not known as a TRPM7 kinase substrate. Next, mutation of TRPM7-specific phosphorylation sites on myosin II heavy chain and tropomodulin could be used to mimic either the dephosphorylated or phosphorylated state of the two substrates. Over expression of phosphorylation mutants could be used to confirm that TRPM7 directed phosphorylation is involved in regulating cytoskeleton dynamics and actin-binding proteins. The loss of stress fiber formation in endothelial cells transfected with negative phosphomutants would indicate that TRPM7 kinase activity is required for stress fiber
formation by phosphorylating of myosin II and tropomodulin as a protective mechanism to resist shear force.

4.3 Potential Model of TRPM7 Functions in Synaptic Vesicle Release

Synapses are highly specialized intercellular junctions that mediate the transmission of information between axons and target cells. Neurons communicate through synapses by means of neurotransmitter release. The exocytosis of synaptic vesicles allows neurotransmitter content to diffuse through synaptic cleft and bind appropriate receptors on postsynaptic membrane. The cytoskeleton is an important structural component of synapses. The formation and morphological changes of the synapse ultimately requires the dynamic actin cytoskeleton (Al-Alwan et al., 2003; Averbeck et al., 2004; Dillon and Goda, 2005). The cytoskeleton also provides functional supports necessary for neuronal activities, especially for neurotransmitter release. Synaptic vesicle transport along the axon to a synapse requires the cooperation of microtubules and kinesin. Furthermore, regulation of synaptic vesicle populations in the reserve pool involves the action of the actin-based cytoskeleton reversibly tethered to synapsin, a synaptic vesicle-associated phosphoprotein that resides on the membrane of synaptic vesicle (Benfenati et al., 1991; Hirokawa et al., 1989). TRPM7 has been functionally implicated in synaptic vesicle docking and release processes, as well as interaction with cytoskeleton associated proteins. However the underlying mechanism and machinery involved in synaptic vesicle release and whether the process involves TRPM7 and cytoskeletal accessory proteins remain unclear. Many questions have arisen from the limited experimental data available. Krapivinsky et al. have shown that TRPM7
resides on the cholinergic synaptic vesicle membrane of sympathetic neurons (peripheral neurons) (Krapivinsky et al., 2006), but whether TRPM7 is also present in the synaptic vesicle membrane of central cortical neurons is questionable. During synaptic vesicle docking the vesicular protein VAMP2 interacts with membrane-associated SNARE proteins, SNAP25 and syntaxins to form a stable complex that later associates with synaptotagmin (Pevsner et al., 1994; Sollner et al., 1993). Subsequently, proteins such as snapin also interact with SNAP25 to assist the docking event (Ilardi et al., 1999). Krapivinsky and his colleagues demonstrated using co-immunoprecipitation experiments that TRPM7 only forms molecular complexes with synaptic vesicle proteins including synapsin I, synaptotagmin I and snapin but not with other synaptic vesicle proteins (VAMP2) or membrane fusionary proteins such as SNAP25 and syntaxins. In addition, the N-terminal domain of TRPM7 was shown to directly interact with snapin (Krapivinsky et al., 2006) but no experimental data supports that snapin interacts other vesicle docking proteins such as synapsin I or synaptotagmin I. How can a TRPM7 antibody co-immunoprecipitate the two synaptic vesicle proteins, synapsin I and synaptotagmin I along with TRPM7? My results from pull-down assays and LC-MS/MS data provide a possible idea to explain the missing link. The C-terminal domain of TRPM7 may also associate with synaptic vesicle proteins, possibly with VAMP2, synapsin I or synaptotagmin I.

In addition to be functionally important for synaptic vesicle docking and fusion, TRPM7 alpha-kinase domain also regulates synaptic vesicle mobility by an unknown mechanism (Brauchi et al., 2008). Synaptic vesicles are transported away from the cell body and down the axon to the synaptic cleft. This process of axonal transport is now
known to occur on microtubules. The microtubules in axons are positioned and oriented with their plus (+) ends toward the axon terminal. Kinesin and dynein are motors responsible for transport on microtubules. Kinesin transports materials towards the (+) end and proceeds from the cell body to the synaptic junctions, whereas dynein accounts for transport in the opposite direction, moving substances toward cell body. Studies of vesicle transport indicate that phosphorylation of kinesin provides a possible mechanism to alter vesicle motility by reducing kinesin ability to bind vesicles and microtubules (Morfini et al., 2002; Okada et al., 1995; Sato-Yoshitake et al., 1992). Phosphorylated kinesin has been shown to have significantly reduced ability to bind synaptic vesicles. Moreover, synaptic vesicles pre-incubated with phosphorylated kinesin associated less frequently with microtubules than synaptic vesicles pre-incubated with unphosphorylated kinesin, which in turn may reduce the efficiency for synaptic vesicle transport hence a decrease in synaptic vesicle motility (Sato-Yoshitake et al., 1992). Similarly, TRPM7 alpha-kinase may regulate synaptic vesicle motility through the phosphorylation of kinesin or other microtubule associating proteins.

Synaptic vesicle docking and fusion are mediated by the assembly of a stable SNARE core complex of proteins including both synaptic vesicle membrane proteins including VAMP2, snapin (Ilardi et al., 1999) and synaptotagmin (Yoshida et al., 1992)) and the plasma membrane proteins syntaxin (Bennett et al., 1992) and SNAP-25 (Oyler et al., 1989)). A model to explain TRPM7 function in synaptic vesicle docking and fusion may involve the interaction of TRPM7 with these SNARE proteins (Figure 12). Prior to the vesicle docking event, TRPM7 could interact with snapin and other synaptic vesicle proteins, syntaxin I and synaptotagmin I. As synaptic vesicles approach to the presynaptic
membrane and the docking process starts, the synaptic vesicle proteins, VAMP2 and
synaptotagmin form the SNARE complex with the target membrane SNARE proteins,
SNAP25 and syntaxins (Pevsner et al., 1994; Sollner et al., 1993). Snapin may also
interact with SNAP25 to assist the docking event (Ilardi et al., 1999). Once a stable
SNARE complex is formed the fusion of the synaptic vesicle membrane into cell
membrane may start.
Figure 12. Proposed Model of TRPM7 Function in Synaptic Vesicle Release

Synaptic vesicle is transported along microtubule by kinesin. TRPM7 regulates synaptic vesicle motility by phosphorylating kinesin. During the docking process, SNARE proteins on synaptic vesicle (VAMP2, snapin and synaptotagmin) bind SNARE proteins on cell membrane (SNAP25 and syntaxin) to form a docking complex, following by fusion event of synaptic vesicle.
4.3.1 Future Studies of TRPM7 Associated Synaptic Vesicle Release

To investigate the proposed function of TRPM7 in mediating synaptic vesicle transport, a TRPM7 kinase-dead mutant or channel mutant with abolished ion conductivity could be used to examine the effect of TRPM7 kinase and channel activity on synaptic vesicle release. Both peripheral cholinergic neurons (superior cervical ganglia neuron cell line) and cortical neurons (HCN-1A cell line) would be transfected with TRPM7 kinase and channel mutant constructs. Recording of excitatory postsynaptic potential (EPSP), as a mean to evaluate synaptic vesicle release activity, in this comparison study can help to determine if TRPM7-mediated synaptic vesicle release is only limited to peripheral cholinergic neurons. Alternatively, the use of an in vitro binding or kinase assay and co-immunoprecipitation could check for direct binding of TRPM7 to kinesin or synaptic vesicle proteins. Western blot would also be useful in examining phosphorylation changes in wild type or kinase-dead mutant expressing cells in order to identify synaptic vesicle proteins or microtubule associating proteins as substrates for the TRPM7 kinase. Next, mass spectrometry can be used to pinpoint phosphorylation sites on positive substrates of TRPM7 kinase. Finally, mutations at phosphorylation sites mimicking TRPM7 substrate phosphorylation and dephosphorylation could determine the effect of TRPM7 kinase-dependent phosphorylation on synaptic vesicle mobility. Total Internal Reflection Fluorescence (TIRF) Microscopy can be applied for such a purpose to observe changes in synaptic vesicle mobility in cells co-transfected with the phosphomutants and VAMP2- pHluorin, a synaptic vesicle marker. Any altered activities (changes in vesicle mobility) observed in cells expressing phospho-mutants of vesicle proteins or microtubule associating proteins
would be informative to show that TRPM7 dependent phosphorylation is required to regulate synaptic vesicle motility.
5. Summary

This project was designed to gain insight into the functions and protein-protein interaction of the TRPM7 channel. C-terminal TRPM7-GST fusion proteins containing mainly the alpha-kinase domain were used as bait to capture endogenous TRPM7 associating proteins in mouse brain lysate. Initially, MALDI-TOF mass spectrometry was used to determine TRPM7 interactors in samples (from pull-down assay) resolved on 1D SDS-PAGE gel electrophoresis. In total, three TRPM7 associating proteins were identified using MALDI-TOF analysis: beta-tubulin, beta-actin and nebulin-related anchoring protein. Due to the resolving limit of 1D SDS-PAGE gel and detection limit of Coomassie Brilliant Blue stain, we used an alternative approach, LC-MS/MS, to identify TRPM7 associating proteins. According to the result from LC-MS/MS analysis, identified TRPM7 associated proteins can be divided into 3 different groups: cytoskeleton proteins, synaptic vesicle proteins and ATPase. The identification of cytoskeletal proteins and synaptic vesicle proteins is not surprising since it is consistent with the suggested functions of TRPM7 in cytoskeleton remodeling and synaptic vesicle release from literatures. Moreover, our result suggests that in addition to the N-terminal domain, the C-terminal region of TRPM7 may also associate with synaptic vesicle proteins to bring assistance to the docking and fusion events of synaptic vesicles.

Co-immunoprecipitation experiments were used to confirm specific association of TRPM7 with cytoskeletal protein, beta-actin and beta-tubulin. Both beta-actin and beta-tubulin were co-precipitated along with TRPM7 by TRPM7 antibody but not by normal rabbit IgG. As an indication, TRPM7 may associate with the two cytoskeletal proteins through direct interactions. However, further investigation with microtubule binding
proteins assay and *in vitro* protein interaction assay has disproved this possibility; both experimental results consistently indicate that TRPM7 does not directly interact with microtubules or soluble tubulin subunits. Therefore one or more microtubule binding or accessory proteins must be involved in the association between TRPM7 and microtubules. This study has contributed evidence to strengthen the small body of published literature that implicates TRPM7 in cytoskeleton remodeling and synaptic vesicle release. Based on the results presented here and the findings from other research groups we have postulated physiological models to explain the role that TRPM7 may play in regulating cytoskeletal dynamics and synaptic vesicle release.
6. Reference List


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Appendix 1

Cloning with TRPM7 cDNA

TRPM7 cDNA carried by pcDNA\textsuperscript{TM}4 TOPO plasmid from invitrogen was used. Multiple restrictions sites (as listed in Tables below) located on TRPM7 cDNA were targeted to excise the different C-terminal regions of interest (refer to Materials and Methods section, Figure 4) for the purpose to generate fusion constructs (#1, 4-7). Fusion constructs #2 and #3 were generated with PCR cloning instead of conventional cloning methods due to the difficulties to locate suitable restriction sites on TRPM7 cDNA map. Excised TRPM7 cDNA segments were inserted into pGEX-5X vectors (refer to Appendix 2).
Conventional Cloning: Fusion Constructs and Restriction Sites Used

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Name of Fusion Construct</th>
<th>Restriction Enzyme sites for TRPM7 Bluescript</th>
<th>Location on TRPM7 cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α kinase</td>
<td>EcoNI (5’) + BsaBI (3’)</td>
<td>4960 → 5761</td>
</tr>
<tr>
<td>4</td>
<td>C-terminus-N</td>
<td>BsrGI (5’) + BsaBI (3’)</td>
<td>3482 → 5761</td>
</tr>
<tr>
<td>5</td>
<td>C-terminus-C</td>
<td>BsrGI (5’) + NotI (3’)</td>
<td>3482 → 5590 (end)</td>
</tr>
<tr>
<td>6</td>
<td>Coiled-coil</td>
<td>BbvCI (5’) + AlwNI (3’)</td>
<td>3639 → 4275</td>
</tr>
<tr>
<td>7</td>
<td>Long coiled-coil</td>
<td>BsrGI (5’) + SalI (3’)</td>
<td>3482 → 4739</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction Cloning

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Name of Fusion Construct</th>
<th>Restriction Enzyme Sites Added</th>
<th>Location on TRPM7 cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>α kinase- C</td>
<td>Forward : EcoRI Reverse: Xhol</td>
<td>4697 → 5590 (end)</td>
</tr>
<tr>
<td>3</td>
<td>Long α kinase- C</td>
<td>Forward : EcoRI Reverse: Xhol</td>
<td>4456 → 5590 (end)</td>
</tr>
</tbody>
</table>
Appendix 2

pGEX-5X Features and Restriction Map

The pGEX-5X vector contains cDNA of Glutathione S-Transferase, ampicillin resistant gene (Amp), and LacIq gene. In addition, pGEX-5X vector has 3 different versions to account for 3 different open reading frames as shown in figure below.
pGEX-5X-1 (27-4584-01)
Factor Xα

Ile Glu Gly Arg+ Gly Ile Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
ATC GAA GGT CGT GGG ATC CCC GAA TTC CGG GGT C3A C3C GAG CGG GCG CAT CGT GAC TGA
BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-5X-2 (27-4585-01)
Factor Xα

Ile Glu Gly Arg+ Gly Ile Pro Gly Ile Pro Gly Ser Thr Arg Ala Ala Ala Ser
ATC GAA GGT CGT GGG ATC CCC GGA ATT CCC GGG TCG ACT C3A GGG GCC GGA TCG TGA
BamHI EcoRI SmaI SalI XhoI NotI Stop codons

pGEX-5X-3 (27-4586-01)
Factor Xα

Ile Glu Gly Arg+ Gly Ile Pro Arg Asn Ser Arg Val Asp Ser Ser Gly Arg Ile Val Thr Asp
ATC GAA GGT CGT GGG ATC CCC AAG AAT TCC CGG GTC GAC TCG AGC GCC CGC ATC GTG ACT GAC TGA
BamHI EcoRI SmaI SalI XhoI NotI Stop codons

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pGEX

~4300 bp

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Diagram of pGEX vector with restriction sites and transcription start sites.