Functional Characterization of the Mammalian TRPV4 Channel:
Yeast Screen Reveals Gain-of-Function Mutations

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ABSTRACT

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Transient receptor potential (TRP) channels are a class of six-transmembrane (6-TM) cation-permeable channels that mediate flux of calcium and sodium into cells, leading to depolarization as well as activation of calcium-mediated second-messenger signaling pathways. The TRP channel family is large and diverse in terms of tissue expression, mechanism, and function; therefore, sub-classification is primarily through amino acid homology. A general role has emerged for TRP channels, though, in the processing of sensory stimuli at both the cellular and organismal level.

The goal of this study was to perform mutagenesis screens of mammalian TRP channels to reveal key structural determinants of channel activity (such as gating, permeation, and selectivity). We screened for gain-of-function alleles of TRP channels by their ability to rescue growth deficiency of a strain of the yeast Saccharomyces cerevisiae caused by lack of ion efflux. Channels were further characterized through electrophysiological analysis of their activity when heterologously expressed in Xenopus laevis oocytes.

Of the subset of mammalian TRP channels tested, only wild type TRPV4 rescued the ability of the yeast strain trk1Δ trk2Δ to grow on low potassium media. The TRPV4 channel is important in thermosensitive, osmosensitive, and mechanosensitive processes; recently, mutations of TRPV4 have been linked to human skeletal and neurodegenerative disorders. We obtained a loss-of-function variant of TRPV4 containing the substitutions K70E (N-terminal tail)
and M605T (intracellular linker between transmembrane helices S4 and S5) that failed to rescue low potassium growth of trk1Δ trk2Δ. Therefore, we screened for compensatory mutations that would restore the ability of the V4-K70E/M605T channel to rescue the yeast growth phenotype.

Five gain-of-function clones were isolated, containing a total of seven mutations: three substitutions in the N-terminal tail (R151W, P152S, L154F), one substitution in the pore-lining S5 transmembrane helix (M625I), one substitution in the C-terminal tail (H787Y), and two truncations of the C-terminal tail (N789Δ and Q790Δ). Each of these mutations was assayed, in both the variant V4-K70E/M605T and the wild type TRPV4 background, for effect on rescue of trk1Δ trk2Δ yeast low-potassium growth, as well as degree of salt sensitivity conferred on wild type yeast. We also performed two-electrode voltage-clamp (TEVC) recordings of the mutant channels expressed in Xenopus oocytes, obtaining preliminary data on the ability of the mutations to restore a calcium-activated sodium current to V4-K70E/M605T that was present in wild type TRPV4.

Given the known importance of the S5 helix in gating, the mutation M625I most likely has an effect on gating of the intracellular pore. This mutation showed strong rescue of low potassium growth and salt sensitivity in yeast, and preliminary data showed strong rescue of calcium-activated current in oocytes.

An autoinhibitory channel structure is formed by binding of the C-terminal calmodulin-binding domain to a portion of the N-terminus, which is disrupted by the binding of calcium-calmodulin to the C-terminal domain. The point mutations we isolated in the N- and C-termini lie just outside these respective regions, leading us to believe that the gain-of-function phenotype could be due to disruption of this autoinhibitory structure.
Although the C-terminal truncations were isolated with a gain-of-function phenotype in V4-K70E/M605T (rescue of low-potassium yeast growth), introduction of the truncations into wild type TRPV4 led to a loss-of-function phenotype: truncated channels no longer induced yeast salt sensitivity and exhibited no calcium-activated current in oocytes. This phenotype could be due to the loss of the calmodulin-binding domain, suggesting that the potentiation of channel activity by calcium involves mechanisms other than simply the disruption of the autoinhibitory domain. However, it is also possible that the phenotype is merely the result of reduced membrane expression: some studies indicate that truncation of the C-terminal tail leads to ER retention of the protein.

Taken together, the results of our three assays provide insight into the mechanisms of TRP channel function. Combined with what is already known about these channel regions, we are able to draw conclusions as to the potential contribution of these residues on channel activity and add to the body of evidence regarding mechanisms of function of the TRPV4 channel.
# TABLE OF CONTENTS

Table of Contents ........................................................................................................... i  
List of Figures ................................................................................................................... viii  
List of Tables .................................................................................................................... xi  
Acknowledgements .......................................................................................................... xii  
Dedication ........................................................................................................................ xiv  

Chapter 1: Introduction to TRP Channels ................................................................. 1  
1.1 General overview ........................................................................................................ 2  
1.1.1 TRP channels are cellular sensors ........................................................................ 2  
1.1.2 Historical significance: discovery of TRP channels ............................................. 4  
1.1.3 Examples of TRP channels found across species ................................................. 5  
1.1.3.1 *Saccharomyces cerevisiae* ............................................................................ 5  
1.1.3.2 *Drosophila melanogaster* ........................................................................... 5  
1.1.3.3 *C. elegans* ................................................................................................. 9  
1.1.3.4 Zebrafish .................................................................................................... 13  
1.1.3.5 *Xenopus* .................................................................................................. 14  
1.1.3.6 Mammals ................................................................................................... 16  
1.2 Mammalian TRP channels are classified by subfamily ...................................... 17  
1.2.1 TRPC ............................................................................................................ 17  
1.2.2 TRPV ........................................................................................................... 20  
1.2.3 TRPM ........................................................................................................... 22  
1.2.4 TRPA ........................................................................................................... 27
Chapter 2: Yeast Mutagenesis Screen ......................................................... 39

2.1 Introduction ..................................................................................... 40
  2.1.1 Gain-of-function screens of TRP channels ................................. 40
  2.1.2 Screening the ability of mammalian proteins to rescue the function of
deleted yeast proteins ................................................................. 41

2.2 Materials and Methods ................................................................. 44
  2.2.1 Yeast strains and media .............................................................. 44
  2.2.2 Plasmid construction ................................................................. 45
  2.2.3 Spot assays ............................................................................... 46
  2.2.4 Mutagenesis ............................................................................ 47
    2.2.4.1 TRPV4 screen ................................................................. 47
    2.2.4.2 TRPC6 screen ................................................................. 50
    2.2.4.3 TRPC5 screen ................................................................. 51

2.3 Results ......................................................................................... 53
  2.3.1 Which TRP channels rescue trk1Δ trk2Δ? ................................. 53
  2.3.2 A nonfunctional variant of TRPV4 ........................................... 53
  2.3.3 Screen for compensatory mutations allowing V4-K70E/M605T to rescue
      low potassium phenotype of trk1Δ trk2Δ .................................... 54
2.3.4 Salt sensitivity as an assay for constitutive channel activity and selectivity ................................................................. 55

2.3.5 Introducing the mutants into wild type TRPV4 ........................................... 57

2.3.5.1 Effect of mutants on ability of TRPV4 to rescue growth on low potassium ................................................................. 57

2.3.5.2 Effect of mutants on ability of TRPV4 to induce salt sensitivity ................................................................. 58

2.3.6 TRPC5 and TRPC6 screens yield no mutants that rescue trk1Δ trk2Δ ... 58

2.3.6.1 TRPC6 screen ................................................................. 58

2.3.6.2 TRPC5 screen ................................................................. 59

2.4 Discussion ........................................................................................................... 61

2.4.1 Heterologous expression of TRP channels in

Saccharomyces cerevisiae ................................................................. 61

2.4.2 Comparison of wild type TRPV4 and V4-K70E/M605T ......................... 64

2.4.3 Screen of V4-K70E/M605T for compensatory mutations ..................... 65

2.4.3.1 Rationale ........................................................................ 65

2.4.3.2 Screen yields mutants in three channel regions ......................... 66

2.4.3.3 Comparison of low potassium rescue versus salt sensitivity .... 67

2.4.4 Effect of mutations on wild type TRPV4 activity .............................. 70

2.4.4.1 Effect of mutations on ability of TRPV4 to rescue growth on low potassium ................................................................. 70

2.4.4.2 Effect of mutations on ability of TRPV4 to induce salt sensitivity ................................................................. 72
2.4.5 Summary of properties of TRPV4 mutants in yeast assay system ........ 73
2.4.6 A recent similar study confirms our findings ......................... 76
2.4.7 Screens of TRPC channels are unsuccessful despite multiple attempts .. 78

Figures and Tables for Chapter 2 ................................................. 80

Chapter 3: Electrophysiological Analysis of TRPV4 Mutants ................. 92
3.1 Introduction ........................................................................... 93

3.1.1 TRPV4 general properties .................................................. 93
3.1.2 TRPV4 is activated by a range of disparate stimuli .................... 93

3.1.2.1 Response to osmotic changes ........................................ 93
3.1.2.2 Activation by mechanical stimuli ................................... 94
3.1.2.3 Activation by 4α-PDD .................................................. 96
3.1.2.4 Activation by moderate heat ........................................ 97
3.1.2.5 Activation pathways of cell swelling, heat and 4α-PDD ........ 98
3.1.3 Calcium regulation ............................................................ 101
3.1.4 Electrophysiological analysis of TRPV4 mutants to uncover regulatory elements of the channel .................................................. 102

3.2 Materials and Methods .......................................................... 104

3.2.1 Cloning constructs to pGEMHE2 ...................................... 104
3.2.2 RNA synthesis ............................................................... 104
3.2.3 Oocyte preparation and injection of RNA ............................ 105
3.2.4 Electrophysiology ............................................................ 106
3.2.5 Electrophysiology Solutions .............................................. 107
3.3 Results .............................................................................................................. 108

3.3.1 TRPV4 exhibits basal activity but no response to
hypotonic stimulation ....................................................................................... 108

3.3.1.1 Wild type TRPV4 exhibits basal activity in standard solution
while V4-K70E/M605T does not; V4-ET-M625I/H787Y
rescues basal activity ....................................................................................... 108

3.3.1.2 No significant response to hypotonic shock seen ......................... 109

3.3.2 Response of TRPV4 to calcium in standard solution ....................... 109

3.3.2.1 Calcium application leads to increased sodium current in
wild type TRPV4 but not V4-K70E/M605T;
V4-ET-M625I/H787Y rescues phenotype .................................................. 110

3.3.2.2 Calcium must enter the cell to induce current increase ............ 110

3.3.2.3 Calcium-activated chloride current creates false magnitude
of response ....................................................................................................... 111

3.3.3 Effect of mutants from screen on calcium-activated sodium current in
V4-K70E/M605T and wild type TRPV4 ......................................................... 112

3.3.3.1 Loss of calcium-activated sodium current in V4-K70E/M605T
mainly caused by the K70E mutation ......................................................... 112

3.3.3.2 Double mutant M625I/H787Y rescues calcium-activated
sodium current in V4-K70E/M605T .......................................................... 113

3.3.3.3 N-terminal mutations only slightly rescue calcium-activated
sodium current in V4-K70E/M605T .......................................................... 114
3.3.3.4 C-terminal truncations do not rescue calcium-activated sodium current in V4-K70E/M605T, and eliminate it in wild type TRPV4; effect of other mutants not extensively tested in wild type TRPV4……... 115

3.3.4 Response of channels to 4α-PDD is weak or absent .................... 116

3.3.5 Ion selectivity ................................................................................. 116

3.4 Discussion .......................................................................................... 118

3.4.1 Use of two-electrode voltage clamp (TEVC) to assess function of TRPV4 mutants .............................................................. 118

3.4.2 Inability to recreate TRPV4 activity patterns of previous studies; novel calcium-activated current characterized ................................. 119

3.4.3 Channel mutants affect a calcium-activated sodium current; results recapitulate yeast growth assays .............................................. 121

3.4.4 Electrophysiological analysis of similar mutants from a recent study .... 124

Figures and Tables for Chapter 3 ................................................................ 127

Chapter 4: Conclusions ............................................................................ 141

4.1 Functional significance of TRPV4 .......................................................... 142

4.1.1 Thermosensation and Nociception .................................................. 142

4.1.2 Hearing .............................................................................................. 144

4.1.3 Vascular regulation ........................................................................... 144

4.1.4 Osmotic regulation ............................................................................ 146

4.1.5 Involvement in skeletal disorders and neurodegenerative diseases ...... 149

4.1.5.1 TRPV4 in human skeletal dysplasias ............................................ 149
4.1.5.2 TRPV4 in human neurodegenerative disorders .................. 152

4.2 Mutational analysis of TRPV4 ............................................. 154
  4.2.1 Experimental design: analyze loss-of-function allele of TRPV4 ...... 154
  4.2.2 V4-K70E/M605T loss-of-function phenotype due to the N-terminal
      substitution ................................................................. 156
  4.2.3 Analysis of gain-of-function mutants of V4-K70E/M605T .......... 157
    4.2.3.1 N-terminal mutants ............................................... 157
    4.2.3.2 Mutation in S5 pore-lining TM domain ......................... 159
    4.2.3.3 C-terminal mutations ............................................. 161
  4.2.4 Comparison of our mutants to a similar mutagenesis study .......... 163

4.3 Final thoughts and future directions ..................................... 167

Figures and Tables for Chapter 4 ............................................ 171

Chapter 5: Appendices ................................................................. 172

Appendix I: A single amino acid mutation attenuates rundown of voltage-gated
             calcium channels ...................................................... 173

Appendix II: Regulation of voltage-gated calcium channels by PKA and PIP2 .... 191

Appendix III: Expression of voltage-gated calcium channels in yeast .......... 201

References .................................................................................. 209
LIST OF FIGURES

Chapter 1: Introduction to TRP Channels

Figure 1.1.1 Predicted transmembrane topology, with location of select functional domains, of the TRP channel subfamilies ........................................ 34

Chapter 2: Yeast Mutagenesis Screen

Figure 2.3.1 TRPV4 rescues growth of $trk1^\Delta trk2^\Delta$ ............................................ 80
Figure 2.3.2 A variant of TRPV4 does not rescue $trk1^\Delta trk2^\Delta$ growth phenotype .... 81
Figure 2.3.3-1 Design of mutagenesis screen .......................................................... 82
Figure 2.3.3-2 Mutated clones of V4-K70E/M605T rescue growth of $trk1^\Delta trk2^\Delta$ on low potassium ................................................................. 83
Figure 2.3.3-3 Relative contribution to phenotype of residues from multiply-mutated clones .......................................................... 84
Figure 2.3.4 Not all of the mutants from the screen rescue the ability of V4-K70E/M605T to induce salt sensitivity in yeast ......................... 85
Figure 2.3.5.1 Effect of mutants on ability of wild type TRPV4 to rescue low $K^+$ growth of $trk1^\Delta trk2^\Delta$ .......................................................... 87
Figure 2.3.5.2 Effect of mutants on ability of wild type TRPV4 to induce salt sensitivity in BY4741 .......................................................... 88
Figure 2.4.6 Comparison of location of mutated residues isolated by our study and Loukin et al .......................................................... 90
Chapter 3: Electrophysiological Analysis of TRPV4 Mutants

Figure 3.1.1 Representative modes of TRPV4 activation ......................... 127
Figure 3.1.2 Regions of TRPV4 implicated in activation and calcium regulation .... 128
Figure 3.3.1.1 Basal activity seen in TRPV4 but not V4-K70E/M605T; restored in
V4-ET-M625I/M787Y .......................................................... 129
Figure 3.3.1.2 Response of channels to hypotonic stimulation ......................... 130
Figure 3.3.2.1 Calcium-activated sodium current ........................................ 131
Figure 3.3.2.2 Calcium is acting intracellularly ........................................... 132
Figure 3.3.2.3 Calcium-activated chloride current is contaminating the recordings .... 133
Figure 3.3.3 Location of mutations in TRPV4 transmembrane topology ............. 134
Figure 3.3.3.1 Mutation K70E completely abolishes calcium-activated current .... 135
Figure 3.3.3.2 Double mutant M625I/H787Y strongly rescues calcium-activated
sodium current in V4-K70E/M605T ............................................. 136
Figure 3.3.3.3 N-terminal mutations show little ability to rescue calcium-activated
current in V4-K70E/M605T ................................................................ 137
Figure 3.3.3.4 C-terminal truncations have no effect on calcium-activated current in
V4-K70E/M605T, but abolish current in wild type TRPV4 ....................... 138
Figure 3.3.5 Comparison of ion selectivity profile of channels .......................... 139

Chapter 4: Conclusions

Figure 4.1 Diagram of TRPV4 protein with location of gain-of-function mutations... 171
Chapter 5: Appendices

Appendix I:  
A single amino acid mutation attenuates rundown of voltage-gated calcium channels

Figure 1  
Amino acid sequences and transmembrane topology of the $\alpha_1$ subunit of voltage-gated $\text{Ca}^{2+}$ channels ................................................................. 189

Figure 2  
Mutations of I1520 in P/Q-type or homologous positions in N- and L-type channels affect their rundown ......................... 190

Figure 3  
Inhibition of WT and I1520 mutant P/Q $\text{Ca}^{2+}$ channels by MARCKS peptide ............................................................... 191

Figure 4  
Comparison of biophysical properties of WT and I1520H mutant P/Q $\text{Ca}^{2+}$ channels ......................................................... 192

Appendix II:  
Regulation of voltage-gated calcium channels by PKA and PIP$_2$

Figure 1  
PKA phosphorylation prevents PIP$_2$-induced inhibition of P/Q channels in inside-out patches ......................................................... 202

Figure 2  
Model of P/Q-type VGCC channel modulation by PIP$_2$ and PKA ...... 203

Figure 3  
In vitro phosphorylation assay of P/Q channel .................................. 204

Appendix III:  
Expression of voltage-gated calcium channels in yeast

Figure 1  
Mammalian VGCCs do not functionally rescue Cch1 ..................... 211

Figure 2  
Pulse-chase experiment does not pick up any detectable expression of L-type channel protein ......................................................... 212
LIST OF TABLES

Chapter 1: Introduction to TRP Channels

Table 1.1.3 Properties of representative TRP channels across selected species ........ 35

Table 1.2 Properties of mammalian TRP channels ............................................. 37

Chapter 2: Yeast Mutagenesis Screen

Table 2.3.4 Rescue of low potassium growth does not necessarily capitulate salt sensitivity ................................................................. 86

Table 2.4.5 Summary of yeast growth assays of V4-K70E/M605T and wild type TRPV4 ................................................................. 89

Table 2.4.7 Mutations isolated in TRPC5 screen, none of which exhibited rescue of trk1Δ trk2Δ ................................................................. 91

Chapter 3: Electrophysiological Analysis of TRPV4 Mutants

Table 3.4.3 Summary of effects of mutants across all assays ......................... 140
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Chapter 1:

Introduction to TRP Channels
1.1 GENERAL OVERVIEW

1.1.1 TRP channels are cellular sensors

Ion channels are a broad class of membrane-spanning proteins that mediate the passage of ions down their electrochemical gradient, resulting in changes to membrane voltage ($V_m$) that trigger neuronal action potential propagation and muscle contraction (Hille, 1992); in non-excitable cells, ion channels also play an integral role in cellular physiology since calcium is a key second messenger for a host of cellular activities including transcription, mitochondrial function, cell motility, and apoptosis (Berridge et al., 2003; Clapham, 2007). Different classes of ion channels are regulated in different ways: channels may open in response to changes in membrane potential (voltage-gated); by binding of a molecule to an extracellular receptor domain (ligand-gated) or binding of a second messenger to an intracellular site; or even sensory stimuli such as mechanical force (stretch- or shear-activated), light, and temperature.

The TRP channel superfamily comprises an eclectic group of proteins that share a predicted structure of six transmembrane helix domains with a pore-forming loop between the fifth and sixth helices proteins (Figure 1.1.1), which assemble into tetramers to form cation-permeable channels; they are classified mainly by amino acid homology due to fact that they have diverse properties of activation and selectivity as well as diverse functions. Further functionality is possible as the proteins can assemble as either homo- or hetero-tetramers (Clapham, 2003; Ramsey et al., 2006). Although TRP channels lack a series of arginine residues found in the fourth transmembrane domain of six-transmembrane voltage-gated cation channels (Montell, 2005), evidence of voltage-dependent gating has been observed (Nilius et al., 2005). Mechanisms of channel regulation will be further discussed later in this chapter.
On the basis of sequence homology, TRP channels are divided into seven subfamilies: TRPC for ‘canonical,’ as in closest to the first-cloned TRP channel from Drosophila; TRPV for ‘vanilloid,’ the compound found to activate the founding member of this group; TRPM for ‘melastatin,’ as the first member was identified in metastatic melanoma cells; TRPA for ‘ankyrin’ because it has 14 N-terminal ankyrin repeats compared to the 3-4 found in other TRPs; TRPP for ‘polycystin’ because mutation of these proteins leads to polycystic kidney disease; TRPML for ‘mucolipin’ as mutations lead to mucolipidosis type IV, a lysosomal storage disorder; and TRPN for ‘NOMPC,’ the Drosophila name of the first-characterized member of this subfamily – this is the one channel type not found in mammals (Montell et al., 2002; Clapham et al., 2003; Clapham, 2003; Ramsey et al., 2006).

What these proteins share, however, is a common role in processing sensory stimuli both at the cellular and multi-cellular level. TRP channels have been identified that are activated by temperature (both heat and cold), taste, touch, light, pain, osmolarity, pheromones, and more. TRP channels can be found throughout evolutionary history – from yeast, nematodes, and flies up to mice and humans. One can appreciate that the ability to process information about the external environment would be crucial to survival – temperature, to differentiate between permissive and hazardous habitats; taste, to avoid ingesting noxious or poisonous food sources; proprioception, to avoid cellular damage; pheromone sensing, to locate a suitable mate. It would appear that this role is conserved through evolution, with adaptations used by single-cell organisms building the basis for function of multi-cellular organisms. For example, for single-celled organisms such as yeast, ability to respond to changes in osmolarity would confer ability to survive a shifting aqueous environment; for humans, TRP channels now play a role in regulating osmotic balance in the kidney.
This chapter will provide an overview of TRP channel function: the characteristics of the main subfamilies of TRP channels, their diverse function across species, and their regulation.

1.1.2 **Historical significance: discovery of TRP channels**

The first example of this channel type was found through studies of *Drosophila* phototransduction: *trp* mutant flies exhibited a transient, rapidly decaying receptor potential during exposure to bright light (Cosens and Manning, 1969), in contrast to the sustained, gradually decaying potential in wild-type flies; hence the name, transient receptor potential. The *trp* gene was cloned (Montell et al., 1985) and found to encode a channel whose function was essential for light-activated calcium conductance (Hardie and Minke, 1992; Peretz et al., 1994), activated by some component of the light-activated phospholipase C (PLC) inositol cascade.

A second TRP channel, *trpl*, was isolated and determined to be responsible for the remaining ability of *trp* mutants to respond to light; *trpl* encodes a non-selective cation channel (Phillips et al., 1992; Niemeyer et al., 1996; Xu et al., 1997). A third homolog, *trpγ*, may also play a minor role in the visual response (Xu et al., 2000).

Many other types of TRP channels would eventually be isolated in *Drosophila* and other species, as we will explore in the next section.
1.1.3 Examples of TRP channels found across species

This section details the properties of the TRP channel types found in a range of organisms; see Table 1.1.3 for a summary. The mammalian channel types will be described in more detail in the next section.

1.1.3.1 *Saccharomyces cerevisiae*

In the yeast *Saccharomyces cerevisiae*, the TRP channel homolog Yvc1p (Palmer et al., 2001), also known as TRPY1, is expressed in the vacuolar membrane where it mediates the release of vacuolar calcium in response to hyperosmolarity (Denis and Cyert, 2002). Yeast have a protective response to hypertonic shock that includes cell shrinking, up-regulation of stress-response genes, and accumulation of intracellular glycerol; these responses are activated by signal transduction mechanisms, some of which involve Ca\(^{2+}\)-dependent signaling (Denis and Cyert, 2002).

Yvc1p was shown to be a mechanosensitive channel (Zhou et al., 2003), suggesting a model whereby water leaving the cell causes the vacuolar membrane to shrink, creating a stretch force that directly activates the channel. Yvc1p is also activated by cytoplasmic calcium (Palmer et al., 2001), which could act as a positive-feedback mechanism (Ca\(^{2+}\)-induced Ca\(^{2+}\) release or CICR).

1.1.3.2 *Drosophila melanogaster*

At least 15 genes encoding TRP channels have been isolated in *Drosophila melanogaster*, including the *trp* and *trpl* genes discussed earlier in this chapter. Just as *trp* and
trpl were found to mediate the animals’ response to light, many of the other TRP channels have been found to participate in a variety of sensory processes.

A group of TRP channels in the fly function as temperature sensors, mediating responses including thermosensation, thermotaxis, and cellular physiological functions. The first thermo-TRP identified in flies was painless, a gene isolated in a screen for larvae that did not roll away from noxious heat (39-41°C) (Tracey et al., 2003) – the ideal environment for the animal is in the 18-24°C range. The Painless protein was expressed in peripheral neurons that extend dendrites beneath the larval epidermis in a manner similar to vertebrate pain receptors, and was required for thermal as well as mechanical nociception. Expression of Painless was confirmed to be required for thermotaxis from noxious heat in adult flies, as well (Xu et al., 2006); it was characterized as a heat-activated, calcium-permeable channel of the TRPA subfamily (Sokabe et al., 2008).

A second TRPA-type channel gene, pyrexia (pyx), was isolated in a screen for altered temperature preference. When expressed in Xenopus oocytes, the Pyrexia channel was found to open around 40°C, and to be highly permeable to potassium. Pyrexia does not seem to mediate thermotaxis; flies lacking the channel are more susceptible to paralysis when exposed to noxious heat, suggesting that the channel is involved in protecting the fly from high-temperature stress or enabling the animal to be tolerant to higher temperatures. This theory is further strengthened by the observation that pyrexia is expressed throughout neurites (as opposed to channels such as painless that are expressed only at the tips of dendrites) – it has been suggested that this protects neurons from inappropriate firing when in a high-temperature environment (Lee et al., 2005b).

The dTRPA1 channel also senses warm temperatures. The gene was found by sequence analysis to be an orthologue of the mouse channel mANKTM1. Interestingly, whereas the mouse
channel is activated by cold temperatures, this *Drosophila* ion channel is activated by warm temperatures when expressed in *Xenopus* oocytes – specifically, in the range of 24-29°C (Viswanath et al., 2003). This temperature is warmer than the flies’ preferred range, but not as noxious as the range in which the Pyrexia or Painless channels are activated. Further work would confirm that this channel is necessary for thermotaxis: knockdown of the channel by RNAi or genetic knockout of the gene demonstrated that the channel is required for thermotaxis in the range of 18-24°C (Rosenzweig et al., 2005; Kwon et al., 2008).

Even TRP and TRPL play a role in *Drosophila* temperature sensing – a function completely separate from their role in photoreceptor neurons. These two channels are required for avoidance of cool temperatures (below 18°C) (Rosenzweig et al., 2008). In a recent screen, the Zuker lab isolated three genes also necessary for cold avoidance: *brivido-1*, -2, and -3. All three genes encode channels homologous to the TRPP subfamily; they were able to show that *brv-1* is expressed in neurons in the antennae that are selectively activated in cold temperatures (Gallio et al., 2011).

TRP and TRPL are not the only receptors to do double-duty in the fly. The Painless channel mediates avoidance of isothiocyanate (ITC), the pungent ingredient of wasabi; the channel is expressed in gustatory receptor neurons but not olfactory receptor neurons, indicating that the method of action is through taste rather than smell (Al-Anzi et al., 2006). Painless has even been shown to play a role in sexual receptivity of female flies: *pain* mutant females copulated with wild-type males significantly earlier than wild-type females, and the effect seemed to be mediated by cholinergic and GABAergic neurons since targeted RNAi knockdown of *pain* in these neurons phenocopied the effect seen in *pain* mutant females (Sakai et al., 2009).
Painless and pyrexia are also involved in gravitaxis. *Drosophila* spatial orientation is mediated by Johnston’s organ, a mechanosensory structure in the antenna – *painless* and *pyrexia* are expressed in this area (*painless* in the neurons, *pyrexia* in the associated cap cells) and are required for negative geotaxis (Sun et al., 2009), which is the fly’s inherent reaction to move upward against gravity (Hirsch and Erlenmeyer-Kimling, 1961).

In addition to proprioception, Johnston’s organ is responsible for hearing in the fly; TRP channels mediate this process as well: the TRPN-type channel NompC and the TRPV-type channels Nanchung (Nan) and Inactive (Iav) form heteromers that are required for transduction of auditory stimuli (Göpfert et al., 2006; Sun et al., 2009). Johnston’s organ appears to contain differential cell types that are involved in geotaxis versus hearing: *nompC* is expressed in a distinct subpopulation of neurons than *painless* while *nan* and *iav* are expressed throughout all neurons in the organ and are involved in both geotaxis and hearing (Sun et al., 2009), further supporting the hypothesis that Nan and Iav are not the primary mechanosensors but rather act downstream to enhance the excitatory signal (Göpfert et al., 2006).

The mechanosensitive properties of NompC are also utilized in locomotion in the fly: the channel is required for adult locomotion as well as larval crawling. NompC is expressed in dendrites of the sensory neurons that are activated during the peristaltic muscle contractions of larval locomotion, and controls the pace of crawling. Adult mutant flies have uncoordinated movements of the leg and wing and also have a reduced walking speed. The function of NompC in locomotion is dependent on its ankyrin repeats, which mediate association with microtubules (Cheng et al., 2010).
New roles continue to be discovered in *Drosophila* for this diverse group of channels, and the fly continues to be an important tool in the characterization of TRP channel function and regulation (Minke, 2010).

1.1.3.3 *C. elegans*

Through a combination of forward genetic screens and analysis of the complete genome sequence, a total of 17 TRP channels have been identified in the nematode *C. elegans*. As in the fly, many of the channels are involved in the processing of sensory modalities. In addition, worm TRP channels mediate processes such as gonadogenesis, fertilization, drug dependence, endocytosis and apoptosis, and more.

The first TRP channel to be cloned in *C. elegans* was *osm-9*, a TRPV-like channel isolated through searches for mutant animals with defects in avoidance of high osmolarity, olfactory adaptation, and mechanosensation (Colbert and Bargmann, 1995; Colbert et al., 1997). The OSM-9 channel is expressed in sensory neurons that mediate these processes: these include AWA and AWC, which mediate attractive chemotaxis; and ASH, which mediates repellant chemotaxis as well as avoidance of high osmotic stress and touch (Bargmann, 2006). Four homologous channel transcripts were identified and dubbed *osm-9/capsaicin receptor related genes (ocr-1-4)*. They are always expressed in cells expressing *osm-9*, though *osm-9* can be found in cells lacking *ocr* expression – the interaction of *osm-9* and *ocr-2* was demonstrated to drive their respective subcellular localization (Tobin et al., 2002). Mutation of *ocr-2* resulted in defects in chemotaxis, mechanosensation, and osmosensation of similar severity to *osm-9* while *ocr-1* showed no such sensory signaling deficits (Tobin et al., 2002); it was subsequently discovered that OCR-1 forms heteromeric channels with OCR-2 and OCR-4 that regulate the
proper timing of egg laying by promoting release of the neurotransmitter tyramine which inhibits egg laying (Jose et al., 2007). OSM-9 and OCR-2 also regulate the serotonergic neuron ADF: they are required for the proper expression of the key enzyme in serotonin biosynthesis, tph-1 (tryptophan hydroxylase) (Zhang et al., 2004).

OSM-9 and OCR-2 play another interesting role in worm behavior: regulation of the social feeding response. In social feeding, worms aggregate at a food source as opposed to remaining solitary; this is thought to be a protective response against a stressful environment. Mutation of osm-9 or ocr-2, or ablation of the ASH and ADL nociceptive neurons in which they are expressed, disrupts the aggregation response, presumably because they mediate detection of noxious chemicals (de Bono et al., 2002). Oxygen also plays a role in this response (worms prefer a lower oxygen level as it indicates presence of bacteria, their food source), and both channel proteins promote avoidance of high O₂, also through the action of ASH and ADL (Rogers et al., 2006).

Of the two TRPA channels in the worm, TRPA-1 and TRPA-2, only TRPA-1 has been characterized; it has been found to play a role in mechanosensitive behaviors including foraging and nose-touch response (Kindt et al., 2007) although it has not been determined if the channel is itself a mechano-transducer or if it plays an indirect role. The TRPN-type channel, TRP-4, is required for activation of the stretch-sensitive proprioceptive neuron DVA that regulates the body bends generated by the worm in locomotion (Li et al., 2006). TRP-4 was the first TRP channel conclusively shown to be directly gated by mechanical force (Kang et al., 2010).

Pkd-2 is the homolog of mammalian PKD2/TRPP2; it is expressed in male-specific neurons and is required for male mating behavior. During the complex and well-characterized mating sequence, male worms with mutated pkd-2 fail to locate the vulva of their mate (Barr and
Sternberg, 1999). As the process of vulval locating involves chemosensation and mechanosensation, it is thought that PKD-2 may mediate such processes in the male-specific sensory neurons. The PKD-2 channel has been demonstrated to be an intracellular Ca\(^{2+}\) release channel whose function is required for excitable cells that utilize calcium signaling for rapid responses to stimuli (Koulen et al., 2005).

TRP-1, TRP-2, and TRP-3 are the three *C. elegans* TRPC-type channels, with the highest sequence homology to the canonical Drosophila TRP. TRP-1 and TRP2 are a crucial modulator of the worms’ neurological response to nicotine. Feng et al. discovered through locomotor assays that the nematode exhibits a response to nicotine matching those of mammals, including hallmarks such as acute response, tolerance, withdrawal, and sensitization. As would be expected, this response is dependent on the family of nicotinic acetylcholine receptors (nAchR); however, mutation of trp-1 or trp-2 also disrupted nicotine-dependent behavior. Both of these TRP channels are expressed along with the nAchR encoded by acr-15 in the AVA command interneuron that is essential for the nicotine response; mutation of trp-1 or trp-2 led to greatly reduced nicotine-induced calcium response in AVA indicating that the channels functionally regulate nAchR activity. Interestingly, expression of human TRPC3 can rescue the nicotine phenotype, suggesting an evolutionarily conserved role for the channel (Feng et al., 2006).

Expression of trp-3 is enriched in sperm, and mutation leads to sterility. The sperm in trp-3 mutant males and hermaphrodites have normal morphology and motility, but are defective in the fertilization step when an oocyte is encountered. Translocation of TRP-3 to the plasma membrane during sperm activation is coincident with an increase in store-operated calcium entry, suggesting the channel is required for calcium influx during sperm-oocyte interactions required for fertilization (Xu and Sternberg, 2003). An evolutionary role for TRPC channels in
fertilization is suggested by the fact that many mammalian TRPCs are expressed in human sperm (Castellano et al., 2003) and mouse TRPC2 is required for the acrosome reaction in sperm (Jungnickel et al., 2001).

The *C. elegans* TRPM channel GON-2 is expressed in the gonad where it is required for normal gonadogenesis (Sun and Lambie, 1997). *Gon-2* is also expressed in the intestine, where it is involved in electrolyte homeostasis together with another TRPM channel, *gtl-1*. GON-2 carries a Mg$^{2+}$-regulated outwardly rectifying current while GTL-1 regulates the proper Mg$^{2+}$ responsiveness of this current; both channels are required for the proper absorption of trace metals such as Ni$^{2+}$ and Mg$^{2+}$ in the intestine (Teramoto et al., 2005). TRPM channels have also been implicated in magnesium homeostasis in mammals (Schlingmann et al., 2002; Monteilh-Zoller et al., 2003).

*Gon-2* and *gtl-1* are additionally required for proper defecation rhythms in the worm: the posterior body wall muscle contractions (pBoc) that induce defecation are maintained through inositol-1,4,5-trisphosphate (IP$_3$)-dependent Ca$^{2+}$ oscillations in the intestine epithelium and evidence suggests that GON-2 and GTL-1 carry this calcium current (Teramoto et al., 2005; Kwan et al., 2008; Xing et al., 2008).

Finally, the TRPML-type channel CUP-5 is required for normal endocytosis and apoptosis. Mutation of *cup-5* results in defective lysosome biogenesis and function, resulting in accumulation of large vacuoles containing endocytosis products that have not been properly degraded (Fares and Greenwald, 2001; Treusch et al., 2004; Campbell and Fares, 2010). In humans, mutations of the homolog TRPML1 cause similar defects leading to type IV mucolipidosis (Bassi et al., 2000).
1.1.3.4 Zebrafish

Another well-studied model organism, zebrafish (*Danio rerio*), utilizes TRP channels in sensory modalities, as well as calcium-mediated processes in kidney and muscle, in ways that are very similar to the functions we have already described in nematode and fly. Numerous zebrafish TRP channel homologs have been identified by sequence but functional analysis has not yet progressed past expression pattern profiling.

Zebrafish TRPC channels are involved in endothelial cell physiology. TRPC1 regulates angiogenesis – the channel mediates calcium influx that is necessary for VEGF-induced endothelial-cell permeability (Yu et al., 2010); although the function of zebrafish TRPC6 has not been studied, its expression in endothelial cells and gastrointestinal smooth muscle cells suggests a role in vaso-contractility similar to its mammalian counterpart (Möller et al., 2008). Meanwhile, TRPC2 is expressed in a subset of olfactory sensory neurons suggesting a role in olfactory coding and processing (Sato et al., 2005).

In mammals, mutation of the TRPP2 channel leads to autosomal-dominant polycystic kidney disease; in zebrafish, mutation of the TRPP2 channel also causes polycystic kidneys (Sun et al., 2004; Bisgrove et al., 2005).

The action of the zebrafish TRPV1 protein has not yet been established, but can be predicted from its sequence. Mammalian TRPV1 is gated by heat, protons, vanilloids, and cannabinoids (THC); in chicken and nematode the channel is gated by heat and protons but not THC – the protein lacks the amino acid residues critical for ligand binding. Sequencing of zebrafish TRPV1 revealed that it also lacks these residues, suggesting it will be found to be activated by heat and acidity but not ligands – a modality that appears to have evolved only in mammals (McPartland et al., 2007).
As mentioned earlier, the *Drosophila* TRPN channel (NompC) seems to be mechanosensitive and is essential for transduction of auditory stimuli. The zebrafish channel TRPN1 also functions in sensory hair cell mechanotransduction: knockdown of the gene results in deafness, with no detectable stimulus-evoked microphonic potentials. It has not been established if the channel is directly sensing mechanical stimuli (Sidi et al., 2003).

The zebrafish TRPA1 channel mediates the response to noxious chemicals, as it does in mammals (although it does not play a role in hearing transductions or thermosensation as do mammalian TRPA channels). Both orthologues (trpa1a and trpa1b), when transfected into HEK293 cells, resulted in calcium elevation in response to noxious chemicals (mustard oil and acreolin) (Prober et al., 2008); null mutants of the genes in zebrafish resulted in loss of noxious chemical avoidance (Moens et al., 2008).

### 1.1.3.5 Xenopus

Although most channel research involving the frog *Xenopus laevis* centers around the use of oocytes for heterologous expression of membrane channels, some research has been done on the frogs’ endogenous channels and TRP channel homologs have been identified.

The *Xenopus* TRPC1 channel has been proposed to be mechanosensitive: knockdown of the *trpc1* gene reduced the response of oocytes to hypotonic swelling, whereas expression of human TRPC1 elevated the response (Zhang and Hamill, 2000; Maroto et al., 2005). However, as we will discuss in Chapter 3, response to osmotic change involves chemical as well as mechanical pathways, so it is possible the channel is activated by the downstream effector of a signaling cascade as opposed to direct stretch activation.
TRPC1 also plays a role in axon path finding during frog embryogenesis. In cultured spinal neurons, application of the growth factors netrin-1 or BDNF induces an elevation of intracellular calcium that is blocked by knockdown of the \textit{trpc1} gene or pharmacological block of the channel; blocking the channel also inhibits growth-cone turning behavior (Wang and Poo, 2005). This was confirmed in vivo, when it was demonstrated that spinal commissural neurons fail to cross the midline in embryos lacking TRPC1 expression (Shim et al., 2005).

Expression of the \textit{Xenopus} TRPN1 channel is localized to epithelial cell cilia, including those of inner-ear hair cells, reminiscent of the \textit{Drosophila} homolog NompC that transduces auditory stimuli. However, it is unlikely that the frog channel is directly part of a mechanotranducing complex: it is expressed in the kinocilium, which is non-signal transducing, but not in the stereocilia, which is the part of the hair cell that triggers firing through mechanical deflection (Shin et al., 2005).

A TRPV channel (TRPV3) has been cloned in the related species \textit{Xenopus tropicalis}. Unlike mammalian TRPV3 which is activated by heat, the frog channel is activated by cold temperatures when expressed heterologously in \textit{Xenopus laevis} oocytes – the threshold for activation was 16ºC, which is just below the frogs’ known preferred temperature range (Saito et al., 2011). We previously outlined another instance of opposite temperature sensitivities between orthologs – the TRPA1 channel is activated by heat in flies, but by cold in mice. It has been hypothesized that the temperature sensitivity of thermoTRPs is dynamic, changing through evolution to adhere to the environmental range appropriate for the survival of the species.
1.1.3.6 Mammals

As alluded to in the previous sections, TRP channels have evolved to serve a variety of functions. Many of these can be grouped into the category of ‘sensation,’ triggering processes at the cellular as well as organismal level including thermosensation, chemosensation, mechanosensation, nociception, and osmosensation to name a few. TRP channel activity is therefore crucial for many processes of life, and events leading to dysfunction of these channels can have catastrophic consequences. In the next section we will explore the close to 30 known mammalian TRP channels in more detail in the context of the characteristics that make up the six subfamilies of mammalian TRP channels (as mentioned earlier, the TRPN family does not appear in mammals). A wealth of information has been uncovered about their myriad functions, as well as the diseases and disorders that have been traced to their mutation.
1.2 MAMMALIAN TRP CHANNELS ARE CLASSIFIED BY SUBFAMILY

In this section we provide an overview of the properties of the mammalian TRP channel subfamily members; Table 1.2 summarizes this information. Refer back to Figure 1.1.1 for the location of the functional hallmarks of each channel subtype.

1.2.1 TRPC

The TRPC family is comprised of the ‘canonical’ channels – that is, those found to have the highest homology to Drosophila trp (30-40% identity). Seven mammalian TRPC channels have been isolated, which are further subdivided by sequence and function into three groups – TRPC1/4/5, TRPC3/6/7, and TRPC2 (Clapham, 2003) – although it has been suggested that TRPC1 is dissimilar enough from TRPC4 and TRPC5 that it should comprise its own fourth group (Montell, 2005). TRPC channels assemble both as homomeric and heteromeric forms, leading to a greater functional repertoire.

Most studies support TRPC channels as being cation nonselective and receptor-operated, activated by a pathway involving phospholipase C (PLC) activation and hydrolysis of PIP$_2$ to DAG and IP$_3$, though there is not a clear picture of how these products actually activate the channel. Furthermore, some evidence exists of the channels being store-operated, meaning they are activated as a result of depletion of intracellular stores of calcium (Montell, 2005). TRPC channels are highly expressed in neurons, where they play a crucial role in mediating the influx of calcium. Calcium is not only important as a charge carrier in processes of membrane depolarization and neurotransmitter release – it is also acts as a second messenger, mediating processes of growth, survival, and differentiation. Therefore, the action of TRPC channels can
have great impact on neuronal physiology and have long-reaching functional effects such as modulation of synaptic plasticity (Bollimuntha et al., 2011).

TRPC1 was the first mammalian TRP channel to be identified and cloned (Wes et al., 1995). It is expressed almost ubiquitously through mammalian tissue types. Multiple studies have implicated it as a store-operated channel (Beech, 2005) although it may also be receptor-activated as evidenced by its response to diacylglycerol (Lintschinger et al., 2000); it may also be activated by membrane stretch (Maroto et al., 2005). Functionally, it has been implicated in a wide range of activities including vasoconstriction, secretion (such as in salivary gland cells), endothelial permeability, cell volume regulation, cell proliferation (including smooth muscle cells and embryonic stem cells), lipid raft integrity, and neurotransmission (glutamatergic) (Beech, 2005). The TRPC1-mediated neuronal store-operated calcium (SOC) pathway has been proposed as a therapeutic target for treatment of Huntington’s disease, as SOC activity is enhanced in cells expressing the mutant Huntingtin (Htt) protein (Wu et al., 2011).

TRPC4 and TRPC5 are highly homologous nonselective cation channels that are activated by receptors including the GPCR Gq/11 and receptor tyrosine kinases in a manner that is dependent on concentration of intracellular calcium, do not appear to be store-operated, and produce an unusual doubly-rectifying I-V relation (Okada et al., 1998; Schaefer et al., 2000; Strübing et al., 2001). Mouse knockout studies reveal that TRPC4 is required in agonist-induced vasoregulation and lung microvascular permeability (Freichel et al., 2001; Tiruppathi et al., 2002); some neuronal functions that have been suggested include neurotransmitter release (Munsch et al., 2003) and axonal regeneration after injury (Wu et al., 2008). TRPC5 regulates hippocampal growth cone morphology and neurite extension (Greka et al., 2003).
Both TRPC4 and TRPC5 assemble with TRPC1 to form heteromeric channels with distinct properties. Though TRPC1/TRPC4 or TRPC1/TRPC5 heteromeric channels retain the characteristic of $G_{q/11}$ receptor-activation seen in TRPC4 and TRPC5, they exhibit distinct biophysical properties including a much simpler I-V relation (one that is smoothly outwardly rectifying and gently negatively sloping at negative potentials) and in the case of TRPC1/TRPC5 a much smaller single-channel conductance (Strübing et al., 2001). More recent work in glomerular mesangial cells implicates the TRPC1/TRPC4 channel as mediating store-operated calcium entry through a mechanism by which they are activated by the ER calcium sensor STIM1 (stromal interaction molecule 1) (Sours-Brothers et al., 2009).

The second TRPC subgroup members, TRPC3, TRPC6, and TRPC7, share close to 80% protein sequence identity, are highly expressed in smooth and cardiac muscle, and encode non-selective cation channels exhibiting double-rectifying currents activated by Gq/11 receptors or directly by diacylglycerol (DAG) (Zitt et al., 1997; Hofmann et al., 1999; Okada et al., 1999; Inoue et al., 2001). The expression of these channels in smooth and cardiac muscle suggests functional roles in vascular tone and cardiac function – for example, TRPC6 is an essential component of the vascular alpha1-adrenoreceptor-activated cation channel involved in cardiac contractility (Inoue et al., 2001; Mohl et al., 2011); the vasoconstrictor endothelin-1 activates TRPC3 and TRPC7 (Peppiatt-Wildman et al., 2007). Roles in neuronal development and function have also been discovered: neuronal TRPC3 plays a role in BDNF signaling (Li et al., 1999) and neurotransmission (Singh et al., 2004; Hartmann et al., 2011); TRPC3 and TRPC6 together are involved in growth-cone guidance (Li et al., 2005).

The third subgroup contains a single member, TRPC2; in humans it is a pseudogene that encodes a nonfunctional truncated protein, but in rodents it appears to play an important role as a
pheromone sensor (Vannier et al., 1999). In rats the channel is localized to the microvilli of neurons in the vomeronasal organ (VNO) (Liman et al., 1999), and knockout of the gene in mice abolishes the ability of males to discriminate between males and females (Stowers et al., 2002; Leypold et al., 2002).

### 1.2.2 TRPV

The TRPV (vanilloid) family is so-named because its founding member, TRPV1, is activated by the vanilloid compound capsaicin (the active compound found in hot peppers) (Caterina et al., 1997). There are six mammalian TRPV channels, grouped functionally into TRPV1-TRPV4 (the thermo-TRPs, activated by heat) and TRPV5-TRPV6 (found mainly in epithelia of the kidney and intestine).

The TRPV1 cation channel is weakly calcium-selective and outwardly rectifying; it is activated not only by capsaicin but also noxious heat (>43°C), decreased pH (H⁺ ions), resiniferatoxin, endocannabinoid lipids (anandamide), and phosphorylation by PKC (Caterina et al., 1997; Premkumar and Ahern, 2000; Jordt and Julius, 2002; Ramsey et al., 2006; Studer and McNaughton, 2010). The channel is inhibited by the direct binding of PIP₂ (phosphatidylinositol-4,5-bisphosphate) to the C-terminal tail (Vlachová et al., 2003); relief of this inhibition by PLC-mediated PIP₂ hydrolysis is catalyzed by bradykinin and nerve growth factor, both of which are up-regulated in response to inflammation, which provides a molecular basis for the connection of TRPV1 activity to thermal hyperalgesia (Chuang et al., 2001; Prescott and Julius, 2003). Knockout of the mouse TRPV1 channel leads to impaired nociception, inflammatory response, and thermoregulation (Caterina et al., 2000). TRPV1 activity is also important in bladder
function (Birder et al., 2002), gastrointestinal inflammation and function (Geppetti and Trevisani, 2004), satiety (Ahern, 2003), and hearing (Zheng et al., 2003).

TRPV2 shares 50% identity with TRPV1 and is also a weakly calcium-selective cation channel, but has a higher noxious-heat activation threshold of 52°C (Caterina et al., 1999). It is also activated by cell swelling (Muraki et al., 2003). TRPV2 also appears to play a role in insulin secretion: translocation of the channel to the plasma membrane of pancreatic beta-cells occurs after stimulation by insulin-like growth factor-I (IGF-I), and knockdown of the channel leads to a reduction of glucose-induced insulin secretion (Kanzaki et al., 1999; Hisanaga et al., 2009).

TRPV3 also mediates a weakly calcium-selective and strongly outwardly rectifying cation current, but is activated by temperatures in the non-noxious range (>31°C); it is highly expressed in skin, tongue, and the nervous system (Peier et al., 2002a; Smith et al., 2002; Xu et al., 2002). The channel is activated by camphor, a natural compound that elicits a sensation of warmth in humans that is mediated by keratinocytes rather than sensory neurons; TRPV3 knockout mice showed impaired sensation of both moderate and noxious heat (Moqrich et al., 2005).

TRPV4, like TRPV3, is activated by moderate heat (>25°C) (Güler et al., 2002), is moderately calcium-selective and exhibits a gently outwardly rectifying I-V relation. In addition to heat it is activated by hypotonicity, not through direct mechanical force but rather by a metabolite of arachadonic acid, 5,6-epoxyeicosatrienoic acid (5,6-EET), that is produced during cell swelling (Strotmann et al., 2000; Liedtke et al., 2000; Watanabe et al., 2003a). It is also potently activated by the phorbol ester derivative 4αPDD (Watanabe et al., 2002a). Knockout of TRPV4 in mice leads to altered thermal sensitivity (Lee et al., 2005a), hearing impairment (Tabuchi et al., 2005), and defects in osmotic regulation that effect physiological processes
including in the kidney and nociceptive nerve fibers (Suzuki et al., 2003; Mizuno et al., 2003; Alessandri-Haber et al., 2003; Liedtke and Friedman, 2003).

TRPV5 and TRPV6 form a separate subgroup of TRPV channels: although they share 74% identity with each other, they are only 22-24% identical to the other TRPVs. They are also functionally quite distinct from the thermoTRPs: TRPV5 and TRPV6 are highly calcium selective ($P_{Ca}/P_{Na} > 100$), strongly inwardly rectifying, and constitutively active (Vennekens et al., 2000; Yue et al., 2001; Dekker et al., 2003). Both channels are highly expressed in the epithelia of the kidney and intestine where they may function as heteromers and are crucial for vitamin D-stimulated calcium update – mice in which TRPC5 has been deleted exhibit loss of renal calcium reabsorption and hypercalciuria (Hoenderop et al., 2003; 2005; van Abel et al., 2005).

1.2.3 TRPM

The TRPM subfamily is the most diverse, both in terms of sequence homology and function. Of the eight members, six fall into three main groups: TRPM1/3, TRPM4/5, and TRPM6/7; TRPM2 and TRPM8 stand alone due to their low sequence homology (Fleig and Penner, 2004).

The founding member, TRPM1, was originally named melastatin (MSLN) since it was isolated in a screen for genes down-regulated in mouse melanoma tumor-cell lines (Duncan et al., 1998). It is used as a prognostic marker for metastasis of localized melanoma, as its expression level is inversely correlated with aggressiveness of the melanoma, leading to suggestion that it could function as a tumor-suppressor gene (Hunter et al., 1998; Duncan et al., 2001) although it is still not clear how the channel might function in cell growth.
It was not until recently that evidence emerged showing TRPM1 does indeed function as an ion channel, producing small but identifiable currents in melanocytes and retina (Oancea et al., 2009). The TRPM1 channel mediates the depolarizing light response of ON-bipolar cells, which function to detect increases in light intensity, through a signal transduction cascade initiated by a metabotropic glutamate receptor (mGluR6) (Morgans et al., 2009; Koike et al., 2010). Mutation of TRPM1 is responsible for autosomal-recessive complete congenital stationary night blindness (Li et al., 2009; Audo et al., 2009; van Genderen et al., 2009).

The TRPM3 channel transcript is alternatively spliced into multiple functional channel variants; variants have also been found that differ only in the pore region (Grimm et al., 2003; Oberwinkler et al., 2005). For example, mouse TRPM3α1 is monovalent-selective while TRPM3α2 is divalent-selective, although both are constitutively active and display outwardly rectifying currents that are blocked by intracellular Mg$^{2+}$ (Oberwinkler et al., 2005). Most functional data to date concerns the monovalent-selective α2 variant. It has been shown to mediate calcium entry in response to hypotonicity (Grimm et al., 2003). TRPM3 activation by the neurosteroid pregnenolone sulfate (PS) has been linked to vascular smooth muscle contraction and calcium-induced insulin release from pancreatic islet cells (Wagner et al., 2008; Naylor et al., 2010), as well as PS-induced nociception. TRPM3 knockout mice lack nocifensive behavior upon injection of PS, and additionally have defects in avoiding noxious heat (Vriens et al., 2011).

TRPM4 and TRPM5 are unique among TRP channels as they are the only members to be monovalent-selective. Although impermeable to calcium, their activity is strongly activated by calcium and therefore by GPCRs coupled to PLC-dependent release of intracellular calcium stores; it is thought they are responsible for observed calcium-activated nonselective channel
activities (Launay et al., 2002; Hofmann et al., 2003; Prawitt et al., 2003). They exhibit a linear single-channel I-V relationship with a reversal potential of 0 mV, but they also exhibit voltage-dependence: the open probability decreases at negative potentials and increases at positive potentials, resulting in outward rectification of the steady-state I-V relationship (Launay et al., 2002; Hofmann et al., 2003; Nilius et al., 2003). TRPM4 is ubiquitously expressed and thought to play a general role in modulation of calcium driving force in both excitable and non-excitable cells – evidence of this has been seen in T cells, where knockdown of TRPM4 disrupts receptor-mediated calcium oscillations that are necessary for proper cytokine production (Launay et al., 2004). TRPM5, on the other hand, is primarily expressed in taste receptors and is responsible for transduction of sweet, bitter, and umami (amino acid) tastes through a mechanism whereby it is coupled to GPCRs that activate PLC (Pérez et al., 2002; Zhang et al., 2003). TRPM5 is also expressed in pancreatic beta cells, where it appears to act through a similar mechanism to mediate insulin release triggered by acetylcholine activation of muscarinic receptors leading to PLC activation (Prawitt et al., 2000; Gilon and Henquin, 2001).

TRPM6 and TRPM7 are quite unusual ion channels: they also possess a functional protein kinase domain in their C-terminal tails that, despite a lack of sequence homology to eukaryotic protein kinases such as PKA, shows structural homology to other catalytically active protein kinases with the notable difference that it contains a zinc-finger domain (Runnels et al., 2001; Yamaguchi et al., 2001; Ryazanova et al., 2004). Auto-phosphorylation has been demonstrated for both channels; rather than having a role in ion channel function, it appears to increase the rate of substrate phosphorylation by mediating kinase-substrate interaction (Matsushita et al., 2005; Clark et al., 2008). A well-described substrate for TRPM7 phosphorylation is annexin-1, a calcium-dependent membrane binding protein that functions in
membrane trafficking and reorganization (Dorovkov and Ryazanov, 2004). TRPM7 phosphorylation prevents annexin-1 from forming an N-terminal alpha helix that is essential for its interaction with both membranes and other proteins (Dorovkov et al., 2011).

As channels, TRPM6 and TRPM7 display outwardly rectifying current but pass only very small inward currents that are permeable to calcium and magnesium and are inhibited by $[\text{Mg}^{2+}]$; TRPM7 is inhibited by PLC-mediated PIP$_2$ hydrolysis and is ubiquitously expressed while TRPM6 is found primarily in kidney and intestine (Nadler et al., 2001; Runnels et al., 2002; Voets et al., 2004a). The high magnesium permeability of these channels suggests a role in Mg$_{2+}$ homeostasis. The widely expressed TRPM7 is required for cell viability: sustained channel activation during anoxia leads to cell death as demonstrated in cortical neurons (Aarts et al., 2003), and extracellular magnesium application is sufficient to rescue viability of TRPM7-deficient cells (Nadler et al., 2001; Schmitz et al., 2003). The kidney- and intestine-specific TRPM6 appears to be crucial for magnesium homeostasis in these organs: human TRPM6 mutations lead to autosomal recessive hypomagnesemia with secondary hypocalcemia, a disorder characterized by defects in renal and intestinal reabsorption of magnesium (Schlingmann et al., 2002; Walder et al., 2002).

TRPM2 is expressed in many tissues but primarily the brain, and forms a channel that is cation nonselective and voltage-independent as evidenced by its nearly linear I-V relation with a reversal potential of 0 mV. TRPM2 is activated by ADP-ribose (ADPR), a breakdown product of nicotinamide adenine dinucleotide (NAD), which binds to a carboxy-terminal nudix hydrolase domain that is highly homologous to the ADP pyrophosphatase NUDT9 but appears to have only residual enzymatic activity and mainly functions as a gating element (Perraud et al., 2001; Sano et al., 2001). TRPM2 may function as a cellular redox sensor to mediate stress-induced
apoptosis: the channel is responsive to TNF-alpha as well as reactive oxygen species including H$_2$O$_2$ – these may activate the channel directly, or indirectly through stimulation of ADPR production or other signaling cascades (Hara et al., 2002; Wehage et al., 2002; Perraud et al., 2005); evidence of this role has been shown in immune cells, during the oxidative burst of neutrophils (Heiner et al., 2003), as well as in dorsal root ganglion neurons (Naziroğlu et al., 2011). TRPM2 is also involved in regulation of insulin secretion in pancreatic beta cells (Inamura et al., 2003; Uchida and Tominaga, 2011).

TRPM8 is a thermo-TRP, but one that is activated by noxious cold temperatures (< 25°C) as well as ‘cooling’ compounds including menthol (the chemical found in mint herbs) and icilin; it is expressed in sensory neurons including those that project to the skin and tongue and mediate cold sensation as evidenced by defects in cold sensitivity in knockout mice (McKemy et al., 2002; Peier et al., 2002b; Nealen et al., 2003; Abe et al., 2005; Dhaka et al., 2007; Colburn et al., 2007; Bautista et al., 2007; Dhaka et al., 2008). It is also expressed in the prostate (where it was first identified), where it is suspected to act as an androgen-responsive channel; as it is up-regulated in prostate cancer is it used as a diagnostic marker (Tsavaler et al., 2001). TRPM8 channels are calcium permeable and display an I-V relation characterized by strong outward rectification and a reversal potential of 0 mV similar to TRPM4 and TRPM5 (Fleig and Penner, 2004); they are activated by PIP$_2$ (Rohács et al., 2005) and intracellular pH (Andersson et al., 2004). Temperature acts on TRPM8 by shifting the voltage dependence of the channel: lowered temperature shifts the voltage dependence from very positive potentials to the physiological range; menthol and icilin produce the same effect (Voets et al., 2004b).
1.2.4 TRPA

The mammalian TRPA ‘subfamily’ consists solely of the TRPA1 channel, also known as ANKTM1, a homolog of *Drosophila painless*. TRPA1 is a calcium-permeant, nonselective channel that is activated by noxious cold (temperatures <15ºC) (Story et al., 2003) as well as active components of mustard oil (allyl isothionate) and garlic (allicin) (Jordt et al., 2004; Macpherson et al., 2005); it is also regulated by PLC-coupled receptors (Bandell et al., 2004). Although the channel senses noxious cold in mammals, the *Drosophila* orthologue is the opposite: it is activated by noxious heat, as is the *Drosophila* Painless channel (Viswanath et al., 2003). TRPA1 is highly expressed in a subset of dorsal root ganglion (DRG) neurons that also contain the heat-activated TRPV1, while the other cold-sensitive channel TRPM8 is found in a separate population of DRG neurons (Story et al., 2003; Babes et al., 2004). The fact that the channel is also expressed in trigeminal ganglia neurons and is activated by pungent compounds such as mustard oil and the inflammatory peptide bradykinin suggests that the channel functions primarily as a transducer of pain rather than temperature (Jordt et al., 2004; Bandell et al., 2004); in fact, TRPA1 (but not other thermo-sensitive TRPs) is potently activated by nonsteroidal anti-inflammatory drugs (Hu et al., 2010). It has also recently been demonstrated as the downstream effector of histamine-mediated itch (Wilson et al., 2011).

TRPA1 is structurally unique in that it contains about 14 ankyrin repeats in the N-terminus—TRPV and TRPC channels only have three to four. One model that has been proposed is that these repeats could function as a mechanical spring, linking TRPA1 gating directly to cytoskeletal proteins; related to this function, there is evidence that TRPA1 could be the channel responsible for hearing transduction (Corey et al., 2004; Sotomayor et al., 2005; Nagata et al., 2005; Zhou et al., 2005).
1.2.5 TRPP

The first members of the TRPP family were identified as the causative mutations in patients with autosomal dominant polycystic kidney disease (ADPKD), a severe and prevalent disorder (affecting 1 in 400 to 1 in 1,000 people worldwide) characterized by bilateral cysts in renal epithelial cells that result in fatal renal failure in about half of patients by the age of 40-60 (Gabow et al., 1990; Hofherr and Köttgen, 2011). Mutations of TRPP1 (PKD-1) account for about 85% of cases, while mutations of TRPP2 (PKD-2) account for about 15% (Reeders et al., 1985; Kimberling et al., 1993; Mochizuki et al., 1996).

The TRPP2 protein sequence fit the predicted structure of a channel protein, with six transmembrane segments (6-TM) and a pore loop (Mochizuki et al., 1996), and indeed was shown to form a calcium-permeable non-selective cation channel (Koulen et al., 2002; Cantiello, 2004). Surprisingly, the PKD1 sequence predicts an 11-TM structure (with the 6 C-terminal TM domains bearing the most resemblance to a TRP channel) that contains domains thought to be involved in cell-cell or cell-matrix interactions (Anon, 1995; Hughes et al., 1995); no evidence has been found that it can form a channel on its own. The TRPP proteins therefore fall into two groups: those that encode 6-TM channels – TRPP2, TRPP3 (PKD2L1), and TRPP5 (PKD2L2) – and those with an 11-TM structure – PKD1, polycystin-REJ, PKD1L1, PKD1L2, and PKD1L3 (Hofherr and Köttgen, 2011).

Although TRPP2 does not appear to form a channel on its own, it was found to associate with PKD1 through C-terminal coiled coil domains (Qian et al., 1997; Tsiokas et al., 1997) and co-assembly of these proteins results in a functional channel (Hanaoka et al., 2000). Both channels are expressed in the primary cilia of renal epithelial cells, and knockout mice develop malformed cilia that lack characteristic calcium influx in response to fluid flow, suggesting that
the channel complex is important in mechanotransduction (Wu et al., 1998; Pazour et al., 2002; Nauli et al., 2003). PKD1 may regulate channel activity of TRPP2 directly via conformational coupling (Delmas et al., 2004), as well as indirectly through activation of signal transduction cascades including PI3-kinase activation (Boca et al., 2006). PKD1 also appears to translocate TRPP2 to the plasma membrane (Hanaoka et al., 2000) – TRPP2 channel accumulates in the endoplasmic reticulum due to an ER retention motif in the C-terminus, and forms an intracellular channel (Cai et al., 1999; Koulen et al., 2002).

The PKD1/TRPP2 channel complex also has functions that are outside of the kidney but seem to share a common mechanism: they are expressed in ovarian primary cilia and may function in follicle maturation (Teilmann et al., 2005), they are required in nodal monocilia to sense nodal flow during development of embryonic left-right asymmetry (Nonaka et al., 2002), and they are required for proper function of vascular smooth muscle cells (Qian et al., 2003); TRPP2 in the endoplasmic reticulum plays a role in apoptosis (Wegierski et al., 2009).

TRPP3 is a homologue of TRPP2 that lacks the ER retention motif, making it more amenable to electrophysiological study in heterologous systems such as *Xenopus* oocytes; it forms a constitutively active, calcium permeable, non-selective cation channel with a large conductance (Nomura et al., 1998; Chen et al., 1999). TRPP3 interacts with PKD1L3, and together they appear to function as the receptor for sour taste (LopezJimenez et al., 2006; Ishimaru et al., 2006; Huang et al., 2006). The second homologue of TRPP2 is TRPP5, but very little headway has occurred regarding its function.

PKDREJ is the human homologue of the sea urchin receptor for egg jelly (suREJ3); its localization to the sperm acrosomal membrane suggests a conserved function in sperm function and fertilization and knockout mice exhibit lowered reproductive success (Hughes et al., 1999;
PKDREJ is highly homologous with PKD1 with the notable exception that it contains a putative ion channel pore region between the last two transmembrane segments although no data has yet confirmed any ability to conduct ions (Butscheid et al., 2006).

There is even less known to date about the remaining PKD1 homologues, PKD1L1 and PKD1L2. PKD1L1 is similar to PKD1 in that it lacks an ion transport motif (Qian and Noben-Trauth, 2005); knockout of the gene in mouse results in situs inversus (mirror image reversal of inner organ placement) suggesting a role in the embryonic node during formation of left-right asymmetry (Vogel et al., 2010), a process in which PKD1/TRPP2 are also involved. PKD1L2 contains an ion transport motif (Yuasa et al., 2004); the channel has been implicated in neuromuscular disease as it is up-regulated in the ostes mouse model, a mutant strain characterized by chronic neuromuscular impairments including NMJ degeneration and myopathy (Mackenzie et al., 2009).

1.2.6 TRPML

The first mammalian TRPML channel, mucolipin-1 (TRPML1), was identified as being mutated in patients with mucolipidosis type IV (MLIV), a developmental neurodegenerative lysosomal storage disorder marked by mental and motor retardation as well as ophthalmologic abnormalities (Bargal et al., 2000; Sun et al., 2000; Bassi et al., 2000). The channel is localized to late endosomal-lysosomal vacuoles, which in affected patients appear to fill with membranes rather than being recycled; calcium is known to be required for lysosome fusion and biogenesis (Luzio et al., 2003). The TRPML1 knockout mouse phenocopies many hallmarks of the human disorder and causes lethality by the age of 8 months (Venugopal et al., 2007; Micsenyi et al., 2009).
Two other mucolipin family members were identified: TRPML2 and TRPML3 (Di Palma et al., 2002). The TRPML2 channel is inwardly rectifying, calcium-permeable, cation nonselective, and localizes to plasma membrane and the membranes of the endocytic pathway; however, little functional data exists to point to a clear physiological function (Flores and García-Añoveros, 2011).

TRPML3 is expressed in cytoplasmic compartments of cochlear hair cells and the plasma membrane of stereocilia, and is the gene mutated in the varitin-waddler (Va) mouse strain, which is characterized by early-onset hearing loss, vestibular defects, and pigmentation defects (Di Palma et al., 2002). The channel is mostly inactive at resting membrane potentials, exhibiting only small inwardly rectifying currents that are calcium permeable, while the mutant isoform present in the Va mouse is constitutively active with much larger currents at resting potential which leads to cation overload and cell death (Grimm et al., 2007; Nagata et al., 2008). TRPML3 is inactivated by low pH and is proposed to regulate the acidification of early endosomes: the channel is active at the pH range of 6-6.5 found in the early endosomes; as it conducts calcium out of the endosome the entry of H⁺ acidifies the vesicle until TRPML3 closes and the vesicle differentiates into a late endosome (Kim et al., 2008; Martina et al., 2009; Noben-Trauth, 2011).
1.3 SPECIFIC AIMS

In this chapter we have barely scratched the surface of the wealth of data that has been collected for this fascinating family of ion channels. Much insight has been gained concerning channel activation and physiological function of many TRP channel subtypes; knockout mouse models have been particularly instructive in linking TRP channels to disease models. Still, much remains unknown about the exact mechanisms of gating, permeation, and selectivity of TRP channels. Mutagenesis screens as well as targeted point mutations have been useful in uncovering key residues in these processes. Structure-function studies will benefit from the marked advances in biophysical techniques: although a full-length X-ray crystal structure has not yet been obtained for a TRP channel, structural data does exist through low-resolution electron cryomicroscopy for some full-length TRP channels (TRPM2, TRPC3, TRPV1, and TRPV4) while high-resolution NMR and X-ray crystal diffraction has shed light on the structure of cytoplasmic domains which contain a wealth of regulatory elements (Li et al., 2011). These structural data can then drive targeted mutagenesis and more directed functional assays.

The goal of the following study was to perform mutagenesis of mammalian TRP channels to uncover key residues important for channel activation. The next chapter (Chapter 2) details a gain-of-function mutagenesis screen of mammalian TRP channels, carried out in the yeast *Saccharomyces cerevisiae*. Mutated channels were screened for their ability to rescue a yeast growth phenotype caused by lack of ion efflux, thereby selecting for channels with increased or constitutive activity. In Chapter 3, the mutant channels obtained in the screen are subjected to electrophysiological analysis to probe how the mutations affect channel activity.
From this we can ascertain what functional contributions the mutated regions of the channel may have; in Chapter 4 we will analyze these findings in the context of the existing literature to date.
Figure 1.1.1  Predicted transmembrane topology, with location of select functional domains, of the TRP channel subfamilies. Transmembrane segments (orange rectangles and linker domains (grey lines) shown in plasma membrane positioned with extracellular side to top; N and C termini indicated. Following domains are indicated: ankyrin repeats (‘A,’ green), TRP domain (blue), coiled-coil domain (‘cc’, red), pore (‘P’ or ‘?’), kinase domain (pink), calmodulin (CaM)-binding domain (yellow). Adapted with permission from: Venkatachalam and Montell. TRP channels. Annu Rev Biochem 2007; 76:387-417. © 2007 Annual Reviews
Table 1.1.3 Properties of representative TRP channels across selected species.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Channel Name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>YEAST</td>
<td>TRPC</td>
<td>Yvc1p (TRPY1)</td>
</tr>
<tr>
<td>DROSOPHILA</td>
<td>TRPC</td>
<td>TRP</td>
</tr>
<tr>
<td></td>
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<td>TRPL</td>
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<tr>
<td></td>
<td></td>
<td>TRPγ</td>
</tr>
<tr>
<td></td>
<td>TRPP</td>
<td>Brivido-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brivido-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brivido-3</td>
</tr>
<tr>
<td></td>
<td>TRPA</td>
<td>Painless</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrexia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dTRPA1</td>
</tr>
<tr>
<td></td>
<td>TRPN</td>
<td>NompC</td>
</tr>
<tr>
<td></td>
<td>TRPV</td>
<td>Nanchung</td>
</tr>
<tr>
<td></td>
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<td>Inactive</td>
</tr>
<tr>
<td></td>
<td>TRPM</td>
<td>dTRPM</td>
</tr>
<tr>
<td></td>
<td>TRPML</td>
<td>dTRPML</td>
</tr>
<tr>
<td>C. ELEGANS</td>
<td>TRPC</td>
<td>TRP-1</td>
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<tr>
<td></td>
<td></td>
<td>TRP-2</td>
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<td></td>
<td>TRPP</td>
<td>PKD-2</td>
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<td>TRPA</td>
<td>TRPA-1</td>
</tr>
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<td>TRPN</td>
<td>TRP-4</td>
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<td>TRPV</td>
<td>OSM-9</td>
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<td>OCR-1</td>
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<td>TRPM</td>
<td>GON-2</td>
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<td></td>
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<td>GTL-1</td>
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<td></td>
<td>TRPML</td>
<td>CUP-5</td>
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Table 1.1.3 continued

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Channel Name</th>
<th>Functions</th>
</tr>
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<tbody>
<tr>
<td><strong>ZEBRAFISH</strong></td>
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<td></td>
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<tr>
<td>TRPC</td>
<td>TRPC1</td>
<td>Angiogenesis (endothelial cell permeability)</td>
</tr>
<tr>
<td></td>
<td>TRPC2</td>
<td>Olfaction? (expressed in olfactory neurons)</td>
</tr>
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<td></td>
<td>TRPC6</td>
<td>Vasoregulation? (expressed in endothelium, GI smooth muscle)</td>
</tr>
<tr>
<td>TRPP</td>
<td>TRPP2</td>
<td>Kidney regulation (mutation causes polycystic kidneys)</td>
</tr>
<tr>
<td>TRPA</td>
<td>TRPA1</td>
<td>Chemotaxis (noxious avoidance)</td>
</tr>
<tr>
<td>TRPN</td>
<td>TRPN1</td>
<td>Hearing (mechanosensation)</td>
</tr>
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<td>TRPV</td>
<td>TRPV1</td>
<td>Thermosensation – heat? (based on sequence analysis)</td>
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<td><strong>XENOPUS</strong></td>
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<tr>
<td>TRPC</td>
<td>TRPC1</td>
<td>Mechanosensation</td>
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<tr>
<td></td>
<td></td>
<td>Osmotic regulation</td>
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<td></td>
<td></td>
<td>Axon path-finding</td>
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<td>TRPN</td>
<td>TRPN1</td>
<td>Unknown (expressed in epithelial cells)</td>
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<td>TRPV</td>
<td>TRPV3</td>
<td>Thermosensation – noxious cold (&lt;16°C)</td>
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<td><strong>MAMMALS</strong></td>
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<tr>
<td>TRPC</td>
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<td>Vascular regulation</td>
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<td></td>
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<td>Endothelial permeability</td>
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<td></td>
<td></td>
<td>Mediate neurotransmitter release</td>
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<td></td>
<td></td>
<td>Pheromone sensor (rat TRPC2)</td>
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<tr>
<td>TRPP</td>
<td></td>
<td>Kidney regulation (mutation causes polycystic kidney disease)</td>
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<tr>
<td></td>
<td></td>
<td>Ovarian follicle maturation</td>
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<tr>
<td></td>
<td></td>
<td>Development of left-right asymmetry</td>
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<td></td>
<td></td>
<td>Taste (sour)</td>
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<td></td>
<td></td>
<td>Fertilization (sperm acrosomal activation)</td>
</tr>
<tr>
<td>TRPA</td>
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<td>Thermosensation – noxious cold</td>
</tr>
<tr>
<td>TRPV</td>
<td></td>
<td>Thermosensation – noxious heat</td>
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<tr>
<td></td>
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<td>Nociception</td>
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<td>Osmotic regulation</td>
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<tr>
<td>TRPM</td>
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<td>Osmotic regulation</td>
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<tr>
<td></td>
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<td>Smooth muscle contraction</td>
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<td>Cytokine production (receptor-mediated oscillations)</td>
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<td></td>
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<td>Taste (sweet, bitter, umami)</td>
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<td></td>
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<td>Thermosensation – noxious cold</td>
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<tr>
<td>TRPML</td>
<td></td>
<td>Lysosome biogenesis</td>
</tr>
</tbody>
</table>

Question marks indicate proposed function for channels that have not yet been fully characterized. References provided for channels not directly addressed in the text of this chapter. Mammalian TRP channel section is in no way meant to be comprehensive but rather is used to highlight evolutionarily conserved functions. nAChR – nicotinic acetylcholine receptor, GI – gastrointestinal.
Table 1.2 Properties of mammalian TRP channels.

<table>
<thead>
<tr>
<th>Name</th>
<th>Functions</th>
<th>Proposed Modes of Activation</th>
<th>Major Tissue Distribution</th>
<th>Selectivity $P_{Ca}/P_{Na}$</th>
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<td><strong>TRPC subfamily</strong></td>
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<tr>
<td>TRPC1</td>
<td>Vasoregulation, secretion, cell permeability, neurotransmission</td>
<td>Store-operated, receptor-operated, membrane stretch</td>
<td>Ubiquitous</td>
<td>Nonselective cation</td>
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<tr>
<td>TRPC4</td>
<td>Vasoregulation, cell permeability, neurotransmission, axonal regeneration</td>
<td>Receptor-operated</td>
<td>Brain, endothelia</td>
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<td>TRPC5</td>
<td>Growth cone morphology, neurite extension</td>
<td>Receptor-operated</td>
<td>Brain</td>
<td>9.5</td>
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<tr>
<td>TRPC3</td>
<td>Vasoconstriction, growth cone guidance, neurotransmission</td>
<td>Receptor-operated, DAG</td>
<td>Smooth muscle, cardiac muscle, brain</td>
<td>1.6</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Cardiac contractility, growth cone guidance</td>
<td>Receptor-operated, DAG</td>
<td>Smooth muscle, cardiac muscle, brain</td>
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<td>TRPC7</td>
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<td>TRPC2</td>
<td>Pheromone sensing (in rodents)</td>
<td>Store-operated?</td>
<td>VNO, testis</td>
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<td><strong>TRPV subfamily</strong></td>
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<tr>
<td>TRPV1</td>
<td>Inflammatory response, nociception, thermoregulation, bladder distention sensation</td>
<td>Vanilloids, noxious heat ($&gt;43^\circ C$), pH, anandamide, $\text{PIP}_2$ hydrolysis</td>
<td>CNS, bladder</td>
<td>9.6 (vanilloids), 3.8 (heat)</td>
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<td>Noxious heat ($&gt;52^\circ C$), hypotonicity</td>
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<td>TRPV3</td>
<td>Thermoregulation (keratinocyte-mediated)</td>
<td>Camphor, Moderate heat ($&gt;31^\circ C$)</td>
<td>CNS, skin, tongue</td>
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<td>Thermoregulation, hearing, osmoregulation (kidney and nociception)</td>
<td>Moderate heat ($&gt;25^\circ C$), hypotonicity, phorbol esters, 5,6-EET</td>
<td>CNS, kidney, lung, spleen, endothelia, liver, heart, keratinocytes</td>
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<td>TRPV5</td>
<td>Renal calcium reabsorption</td>
<td>Constitutive</td>
<td>Kidney and intestinal epithelia</td>
<td>&gt;100</td>
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<tr>
<td>TRPV6</td>
<td>Renal calcium reabsorption</td>
<td>Constitutive</td>
<td>Kidney and intestinal epithelia</td>
<td>&gt;100</td>
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<td></td>
</tr>
<tr>
<td>TRPM1</td>
<td>Vision (light intensity sensing)</td>
<td>Receptor-operated</td>
<td>Melanocytes, retina</td>
<td>Nonselective cation</td>
</tr>
<tr>
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<td>Smooth muscle contraction, nociception, insulin release</td>
<td>Hypotonicity, neurosteroids</td>
<td>Kidney (human), brain (mouse)</td>
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<td>TRPM4</td>
<td>Modulate calcium oscillations</td>
<td>Calcium</td>
<td>Ubiquitous</td>
<td>Monovalent cation selective</td>
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<td>Taste (sweet, bitter, umami), insulin release</td>
<td>Calcium, receptor-operated</td>
<td>Taste receptor cells, pancreas, intestine</td>
<td>Monovalent cation selective</td>
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<td>Magnesium homeostasis (renal and intestinal viability)</td>
<td>Inconclusive</td>
<td>Kidney, intestine</td>
<td>Divalent selective</td>
</tr>
<tr>
<td>TRPM7</td>
<td>Magnesium homeostasis (general cell viability)</td>
<td>$\text{PIP}_2$, phosphorylation</td>
<td>Ubiquitous</td>
<td>Divalent selective</td>
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<tr>
<td>TRPM2</td>
<td>Redox sensor (mediate apoptosis), insulin secretion</td>
<td>ADP-ribose, TNF-alpha, $\text{H}_2\text{O}_2$</td>
<td>Brain, immune cells, pancreas</td>
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<tr>
<td>TRPM8</td>
<td>Thermosensation</td>
<td>Noxious cold ($&lt;25^\circ C$), menthol, icilin, $\text{PIP}_2$, pH</td>
<td>Brain, sensory neurons (skin, tongue), prostate</td>
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Table 1.2 continued

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<tr>
<th>Name</th>
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<th>Major Tissue Distribution</th>
<th>Selectivity $P_{Ca}/P_{Na}$</th>
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<td>TRPA1</td>
<td>Thermal pain sensation, hearing</td>
<td>Noxious cold (&lt;15°C), mustard oil, garlic</td>
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<td>Nonselective cation</td>
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<td>pH</td>
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<td>TRPML3</td>
<td>Hearing</td>
<td>pH</td>
<td>Cochlear hair cells</td>
<td>Inconclusive</td>
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</table>

Within subfamilies, channels are not necessarily ordered numerically but rather by functional subdivision as detailed in text of chapter (dashed lines). Values of estimated selectivity taken from Montell 2005 Sci SKTE 272:re3. $P_{Ca}$; permeability to calcium; $P_{Na}$, permeability to sodium; PLC, phospholipase C; DAG, diacylglycerol; VNO, vomeronasal organ; CNS, central nervous system; 5,6-EET, 5,6-epoxyeicosatrienoic acid; ADP-ribose, adenosine diphosphate ribose; TNF-alpha, tumor necrosis factor alpha; $H_2O_2$, hydrogen peroxide.
Chapter 2:

Yeast Mutagenesis Screen
2.1 INTRODUCTION

2.1.1 Gain-of-function screens of TRP channels

High-throughput screens have been successfully utilized in a variety of TRP channel members, elucidating key residues in channel gating and function. The founding channel, *Drosophila* TRPC1, was discovered through a loss-of-function phenotype: the inability to sustain the light-induced receptor potential necessary for phototransduction in the retina (Montell et al., 1985). Much later, in 2000, a gain-of-function allele was discovered in a *Drosophila* screen: the receptor potential persisted long after the light stimulus was removed (Yoon et al., 2000) which led to blindness as well as rapid severe retinal degeneration. The cause was traced to the mutation F550I (Hong et al., 2002) located in the cytoplasmic end of transmembrane helix S5, the middle residue of a cluster of three phenylalanines that is at least partially conserved in many TRP channels. The mutation caused the channel to become constitutively active; this sustained calcium entry could cause cytotoxicity, explaining the retinal degeneration phenotype. High-throughput gain-of-function screens were developed as a more rapid way to obtain mutations that would elucidate channel function. Screens of yeast and mammalian TRP channels yielded mutations that resulted in constitutive channel activity; in both cases this included the residue analogous to the *Drosophila* TRPC F550, revealing evolutionarily conserved molecular mechanisms of channel gating through the TRP channel family.

The yeast TRP channel, TRPY1 (Yvc1p), is a mechanosensitive channel that resides in the vacuolar membrane and mediates the release of calcium from the vacuole into the cytoplasm in response to osmotic shock (Palmer et al., 2001; Denis and Cyert, 2002). Gain-of-function screens for mutants that caused an exaggerated response to a mild osmotic shock identified a
clone with the mutation F380L, which aligns exactly to the dTRPC1 mutation (F550) and is also in the middle of a three-phenylalanine cluster; single-channel patch-clamp of the mutant channel expressed in yeast vacuolar membranes confirmed that the channel has a higher open probability than wild-type channel (Zhou et al., 2007; Su et al., 2007).

Mammalian TRPV1 can be transformed into wild-type yeast with no effect on cell viability on standard medium. Addition of the channel activator capsaicin, however, leads to cell death, presumably due to cation overload. A gain-of-function screen was carried out to identify mutants that led to cell death even in the absence of capsaicin; this yielded the mutation M581T, which also aligns to the dTRPC1 residue F550 and resides between two phenylalanines. Electrophysiological analysis in *Xenopus* oocytes demonstrated that the mutant channel was constitutively active (Myers et al., 2008).

### 2.1.2 Screening the ability of mammalian proteins to rescue the function of deleted yeast proteins

As evidenced by the study described above and others, the yeast *Saccharomyces cerevisiae* is an ideal system in which to carry out large-scale mutagenesis screens of mammalian ion channels. In the case of Meyers et al., the channel was introduced into wild-type yeast and gain-of-function was assayed by cytotoxicity. However, one can also take the approach of deleting a yeast protein and testing the ability of a heterologous mammalian protein to rescue the resulting mutant phenotype.

The yeast *Saccharomyces* possesses multiple systems to maintain the necessary level of potassium in the cell for proper cellular function and membrane potential. Two of these, encoded by the genes *TRK1* and *TRK2*, are high-affinity and low-affinity potassium transporters,
respectively (Gaber et al., 1988; Ko and Gaber, 1991). While standard yeast media contains 7 mM KCl, wild-type cells can actually survive in media containing less than 0.1 mM KCl (Borst-Pauwels, 1981). In the *trk1Δ* strain, the requirement for external KCl increases to 3-5 mM KCl (Gaber et al., 1988); the double mutant *trk1Δ trk2Δ* requires 50-100 mM KCl in the media for proper growth (Ko et al., 1990). The ability of the double mutant strain to grow is dependent on the ability of the yeast to take up K\(^+\) though other non-specific mechanisms.

In 1995, Tang et al. showed that a mammalian channel protein (the guinea pig inwardly rectifying K\(^+\) channel gpIRK1) could substitute functionally for the yeast Trk1 and Trk2 proteins (Tang et al., 1995). The ability to use the rescue of a yeast mutation as functional readout for activity of the mammalian channel established a useful system for the study of channel function. For example, one study utilized this system to perform a high-throughput small molecule screen for K\(^+\) channel modulators, identifying a novel K\(^+\) channel inhibitor that was found to prevent Kv2.1-induced neuronal apoptosis (Zaks-Makhina et al., 2004).

An important technical consideration is that the yeast cell membrane is hyperpolarized, due to activity of a plasma membrane H\(^+\) ATPase (Peña et al., 1987; Serrano, 1991). Inwardly rectifying K\(^+\) channels open at hyperpolarizing voltages, allowing K\(^+\) influx at membrane potentials below the potassium equilibrium potential (E\(_K\)) (Hille, 1992), so they will be highly active in the yeast cell membrane. But what about mammalian channels with more complicated gating mechanisms?

GIRK channels are inwardly rectifying K\(^+\) channels that are gated by G proteins: direct binding of G\(_{\beta\gamma}\) results in an increase in channel open probability (Huang et al., 1997). The mammalian GIRK2 channel can be expressed in yeast but does not rescue growth of the *trk1Δ trk2Δ* strain on low potassium media. The Jan lab performed a screen for mutations of GIRK2
that would render the channel constitutively active and therefore able to rescue growth on low potassium media; subsequent electrophysiological analysis in *Xenopus* oocytes confirmed that the channels had basal currents 4- to 30-fold higher than wild-type GIRK2. This screen led to the identification of four residues in the transmembrane domains that play a crucial role in gating (Yi et al., 2001). These studies led us to believe that the yeast strain *trk1Δ trk2Δ* could be a useful background in which to screen for mutations in mammalian TRP channels that would give insight to channel function.

Since TRP channels are largely cation nonselective, we could take advantage of a commonly used yeast growth assay to study another permeant ion: sodium. Yeast can normally tolerate NaCl concentrations up to 2 M, due both to the low sodium permeability of the plasma membrane and the presence of proteins that actively extrude sodium (Haro et al., 1991; Rodriguez-Navarro et al., 1994). Expression of a channel that is constitutively active and permeable to sodium, however, would result in the influx of more sodium than the detoxification system could handle, leading to inhibition of growth on high-sodium media as the cells undergo programmed cell death (Nakamura and Gaber, 1998; Huh et al., 2002). The Jan lab employed this approach to characterize the selectivity of their GIRK2 mutants: GIRK2-S177W caused growth inhibition on high-salt media; it was consequently shown to be constitutively active and permeable to both Na\(^+\) and K\(^+\); GIRK2-S177T did not induce salt sensitivity as the mutation rendered the channel constitutively active without altering K\(^+\) selectivity (Bichet et al., 2004; Haass et al., 2007). Therefore, the assay of growth on high-sodium media could potentially be used in conjunction with the rescue of low potassium growth of *trk1Δ trk2Δ* to determine if mutations rendered a given TRP channel more or less active than wild type and whether or not the mutation alters the selectivity of the channel.
2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and media

Strains used in this study were BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; generously provided by the Miller lab) and SGY1528 (MATa ade2-1 can1-100 his3-11,15 leu2,112 trp1-1 ura3-1 trk1::HIS3 trk2::TRP1; gift from the lab of Stephen Kurtz). Cultures were grown at 30°C in either rich medium (YPD: 1% yeast extract, 2% peptone, 2% glucose; all from Fisher Scientific, Pittsburg, PA) or synthetic complete medium (SC: 0.67% yeast nitrogen base, 2% glucose, supplemented with 0.7 g/L of the appropriate amino acid dropout mix as required for auxotrophic growth); SC medium was supplemented with 100 mM KCl (Sigma-Aldrich, St. Louis, MO) to support growth of strain SGY1528. Standard yeast nitrogen base and amino acid dropout mixes from Sunrise Science Products (San Diego, CA); potassium-free yeast nitrogen base from Formedium (Norwich, UK). YPD and SC plates were made following the above recipe with the addition of 2% agar (Fisher Scientific, Pittsburg, PA).

Cells were transformed using the standard ‘quick’ transformation protocol: 250 µL of a culture in stationary phase was pelleted and re-suspended in a final concentration of 100 mM DTT, 160 mM lithium acetate, 32% PEG, and 50 µg salmon sperm carrier DNA along with approximately 300 ng of plasmid DNA; cells were heat-shocked at 45°C for 30 minutes, rinsed, and plated on the appropriate solid media. Individual colonies were re-streaked to fresh media after 2-3 days of growth at 30°C.
2.2.2 Plasmid construction

The following TRP channel cDNA clones were used in this study (GenBank accession numbers in parentheses), all obtained from Open Biosystems (Huntsville, AL): mouse TRPV3 (BC108984), human TRPV4 (BC117426), mouse TRPV5 (BC110555), mouse TRPV6 (BC016101), mouse TRPC5 (BC112972), and human TRPC6 (BC093658).

We actually obtained two versions of TRPV4 – the sequence of the first cDNA clone, LIFESEQ90218873, contained two residues that differed from the published sequence: K70E and M605T. We were not able to find any variant of TRPV4 in the literature with substitutions at these residues. We were later able to obtain true wild type TRPV4 clone (BC117426, verified NIH Mammalian Gene Collection). We characterized the properties of both clones, so throughout this work we will refer to the variant clone as V4-K70E/M605T (shortened to V4-ET when additional mutants are introduced, for ease of reading) to differentiate it from wild type TRPV4.

The coding sequence was amplified with primers corresponding to approximately 25 base pairs of the 5’ and 3’ ends of the coding region flanked on the outer end by a restriction enzymes recognition sequence which was used to clone the gene into yeast expression vectors (primers designed by hand by the author, synthesized by Invitrogen, Carlsbad, CA; Amplification with AccuPrime Pfx, Invitrogen, Carlsbad CA).

The yeast expression vectors used in this study were p426GPD (Mumberg et al., 1995) and p416MET25 (Mumberg et al., 1994), generously provided by the Miller lab and modified as described in the next paragraph. p426GPD is a high copy number plasmid due to a 2µ origin of replication (meaning a single cell may express 10-30 copies), driving gene expression off the constitutively active promoter of the yeast glyceraldehyde-3-phosphate dehydrogenase (GPD)
gene and utilizing the terminator sequence from yeast cytochrome-c oxidase (CYC1); it also contains the URA3 marker gene for auxotrophic selection. p416MET25 is a centromeric plasmid (1 copy present per cell) containing the promoter of the gene for O-acetyl homoserine sulphydrylase (MET25), commonly used in yeast studies because it is repressed when cells are grown in medium containing methionine; plasmid also contains the CYC1-terminator and the URA3 marker gene.

For both yeast expression vectors, the multiple cloning site (MCS) was excised and replaced with an oligo containing an MCS designed specifically for cloning of the mammalian TRP channel sequences. To allow visualization of the expressed protein in the yeast a GFP tag was cloned into the plasmid just downstream of the promoter (to tag the N-terminus of the protein); GFP sequence was obtained from the pMet25-GFP plasmid (LMB018 from the Miller lab). Modified plasmids were named p426-GPDgfp and p416-MET25gfp. Plasmid variants were also constructed with an HA tag in place of the GFP tag biochemical assays; HA sequence was obtained from the pMet25-HA plasmid (LMB019 from the Miller lab). Modified plasmids were named p426-GPD-HA and p416-MET25-HA.

The GIRK2-S177T channel, cloned into the pYES yeast expression vector, was generously provided by the laboratory of Lily Jan.

2.2.3 Spot assays

Spot assays (also known as drop tests) were used to measure differential growth of yeast strains on solid media. Saturated cultures (5 mL cultures grown overnight at 30°C) of strains to be tested were adjusted to equal ODs. Four-fold serial dilutions were prepared with sterile distilled water; aliquots of 5 μL were deposited on plates using a multi-channel pipette and
allowed to be absorbed into the media. Plates were incubated at 30°C and growth was scored visually 3-5 days later. Degree of rescue of given phenotype was scored visually in comparison to growth of positive control strain.

2.2.4 Mutagenesis

2.2.4.1 TRPV4 screen

Variant channel V4-K70E/M605T (V4-ET) in plasmid p416MET25-GFP was subjected to chemical mutagenesis by hydroxylamine and transformed into trk1Δ trk2Δ yeast; strains that rescued growth phenotype on low potassium media were isolated for study.

Hydroxylamine (HA) mutagenesis protocol was adapted from Rose and Fink (Rose and Fink, 1987) and Amberg et al. (Amberg and Burke, 2006). For each round of the screen, the HA solution was prepared fresh by dissolving 0.35 g of hydroxylamine hydrochloride (Sigma, St. Louis MO) in 5 mL of ice-cold 450 mM NaCl. For each reaction, 10 µg of plasmid DNA was added to 500 µL of the HA solution and incubated at either 70°C for 1 hour or 37°C for 24 hours. DNA was recovered using reagents from the QIAquick gel extraction kit (Qiagen, Valencia CA): 1 mL of buffer QX1 and 30 µL of QIAEX beads were added to the reaction and incubated at 10 minutes at room temperature, the beads were washed and plasmid was eluted with 25 µL resuspension buffer EB. Typical recovery of plasmid was 70-80%.

Mutagenized plasmid was transformed into trk1Δ trk2Δ yeast using standard full-scale transformation protocol: 25 mL exponentially growing culture (OD 0.5-0.8) was pelleted, washed with 10 mL of 1xLiAc/TE (100 mM LiAc, 10 mM Tris pH 7.5, 1 mM EDTA), and re-suspended in 1 mL of 1xLiAc/TE. Tubes were prepared with approximately 1 µg of
mutagenized plasmid DNA (or about 500 ng of GIRK2-S177T as a positive control), 100 µg of salmon sperm DNA, 100 µL of cells in 1x LiAc/TE, and 300 µL of 40%PEG4000 solution (50%PEG4000, 1 M LiAc, 100 mM Tris pH 7.5). Reactions were incubated at 30°C for 30 minutes, heat-shocked at 42°C for 15 minutes, rinsed with autoclaved distilled water, resuspended in 100 µL of water, and plated on low-potassium media (standard SC–ura media, containing 7.4 mM KH2PO4).

Plates were incubated at 30°C. Colonies were picked as they emerged and re-streaked on a fresh low potassium plate; plasmid was recovered from the yeast that grew on re-streak: miniprep of a saturated 5 mL yeast culture was performed using the EZNA Yeast Plasmid Kit (Omega Bio-Tek, Norcross GA), then transformed into E. coli and minipreped (Qiagen, Valencia CA) to increase yield. The coding sequence of the gene was sequenced (Genewiz, South Plainfield NJ) to locate the mutated residues.

A total of four rounds of mutagenesis were performed (a round consisting of HA treatment of 10 µg of plasmid and subsequent transformation into one 25 mL culture). Two of the HA treatments were at 70°C for an hour, the other two were at 37°C overnight. A total of 307 colonies grew; when re-streaked, only 8 of these grew. The preponderance of false positives in the first round is not entirely unexpected: first, low potassium media is not lethal to trk1Δ trk2Δ, it just causes the strain to grow extremely slowly so given enough time colonies will begin to emerge that contain un-mutated plasmid; second, the chemicals used to transform the yeast are somewhat toxic, so it is common for some colonies containing no plasmid to grow due to mutations induced in the yeast strain during the transformation.
Plasmid was recovered and sequenced from 7 of the 8 strains that grew on re-streak: 5 contained one or more mutations leading to an amino acid substitution, and the other 2 contained no mutations. It is worth noting that all 5 mutated strains emerged from the same round of mutagenesis, in which the DNA was exposed to HA for one hour at 70°C.

Mutated channels were cloned into fresh plasmid to eliminate any possible deleterious mutations, and transformed into fresh trk1Δ trk2Δ yeast as well as BY4741 yeast. Spot assays of the trk1Δ trk2Δ strains were performed to assess the extent of rescue of the low-potassium growth phenotype; spot assays of the BY4741 strains were performed on plates containing various levels of NaCl to test if the mutated channel caused increased salt sensitivity to the strain.

Two of the five channels contained more than one mutation. To independently test the contribution of each of the mutated residues to channel function the individual mutations were introduced into V4-K70E/M605T using site-directed mutagenesis through rolling circle amplification. For each mutation two complimentary oligonucleotides (to anneal to the target sequence on opposite strands of the plasmid) were synthesized containing the desired mutation flanked by 12-15 unmodified nucleotides on either side and phosphorylated on the 5’ end (Invitrogen, Carlsbad CA). Rolling circle amplification was performed using Accuprime Pfx (Invitrogen, Carlsbad CA) with an extension time of 2 minutes per kilobase. This was followed by incubation in the restriction enzyme DpnI (New England Biolabs, Ipswich MA) to digest the non-mutated parental DNA template (DpnI specifically digests the parental plasmid, which is methylated due to being isolated from E. coli DH5α). PCR product was transformed into E. coli; clones were isolated and sequenced to confirm the presence of the mutation, and coding
sequence of the gene was then sub-cloned into fresh plasmid in case mutations were introduced elsewhere in the plasmid.

In addition, site-directed mutagenesis was performed as described above to introduce the mutations from the screen into the wild type TRPV4 gene. Mutations isolated in pairs were cloned into wild type TRPV4 both singly and in the pairings in which they were isolated.

2.2.4.2 TRPC6 screen

TRPC6 gene in plasmid p416MET25-GFP was subjected to chemical mutagenesis by hydroxylamine and transformed into trk1Δ trk2Δ yeast, following protocol described above in which the plasmid DNA was exposed to HA for one hour at 70°C with the following modification in how yeast were transformed with the mutated plasmid: rather than aliquot the 1 mL of LiAc-washed cells into 10 reactions of 100 μL (9 reactions of mutated plasmid and one control), one large transformation was performed. First, 100 μL of cells were removed for the control reactions; this left 900 μL of cells to which were added a full round of HA-treated plasmid (approximately 8-9 μg after removal of the HA), 45 μL of salmon sperm DNA, and 2.7 mL of 40% PEG4000 solution. One of the weakly rescuing mutants found in the V4-K70E/M605T screen (V4-ET-L154F) was used as a positive control in addition to GIRK2-S177T.

A total of 11 rounds of mutagenesis were performed; 716 colonies grew and were re-streaked on the low-potassium media. However, not one of these re-streaks grew. Possible explanations for this inability to isolate mutations that would rescue the yeast phenotype will be explored in the discussion.
2.2.4.3 TRPC5 screen

Hydroxylamine mutagenesis was also performed on the TRPC5 gene in plasmid p416MET25-GFP as described for TRPC6, with HA incubation at 70°C for one hour. Rather than using QIAEX beads to recover plasmid, reaction was stopped by addition of 10 µl of 5 M NaCl and 25 µl of 2 mg/mL BSA; plasmid was then precipitated with ethanol and re-suspended in water. Negative control transformation (containing no DNA) was added to the positive controls GIRK2-S177T and V4-ET-L154F.

From the initial HA screen in which 13 reactions were transformed into trk1Δtrk2Δ a total of 1,136 colonies grew, but only six of these grew when re-streaked: of these, plasmid could not be recovered from two; another three were wild-type; one had a mutation leading to a conservative amino acid substitution [V389I] that, when cloned into fresh vector and transformed into fresh trk1Δ trk2Δ, produced no observable rescue of the phenotype.

It was suspected that the hydroxylamine might have lost efficacy (the chemical is highly hygroscopic), so another screen was performed with a new batch of HA consisting of 10 rounds of HA mutagenesis from which a total of 90 colonies were isolated and re-streaked. Thirty of these grew: twenty-five contained wild-type channel, and although the other five channels had mutations they did not show any rescue of the growth phenotype when they were transformed into fresh trk1Δ trk2Δ.

An error-prone PCR screen was also attempted: TRPC5 gene was amplified from the p416MET25-GFP construct with primers corresponding to the thirty base pairs of plasmid flanking the coding region. Standard Taq polymerase (Invitrogen, Carlsbad CA) was used, which is reported to have an error rate potentially as high as 1.1 x 10^-4 errors/bp (review of the different error rates reported in the literature can be found on the Invitrogen website). In some
rounds of PCR, 0.4 mM of MnCl$_2$ was added to increase the rate of mutation – MnCl$_2$ reduces template specificity of the polymerase (Beckman et al., 1985). The PCR product was co-transformed into $trk1\Delta\ trk2\Delta$ along with linearized p416MET25-GFP in a 5:1 ratio, which would be joined in the yeast through homologous recombination with the plasmid sequences present on the PCR product. In each round (cells from one 25 mL $trk1\Delta\ trk2\Delta$ culture), about 4,000 ng of PCR product and 800 ng of plasmid were co-transformed.

Six rounds of standard Taq PCR were transformed into $trk1\Delta\ trk2\Delta$ and 23 colonies were isolated. Of these, 5 grew when re-streaked: one was wild type; the other four contained mutations but none of these could rescue growth when transformed into fresh $trk1\Delta\ trk2\Delta$. Four rounds of MnCl$_2$-PCR were transformed; only 4 colonies were isolated, none of which grew when re-streaked.

In summary (excluding the first HA screening attempt with suspect reagents), between the HA and error-prone PCR methods, 20 rounds of potential mutants were transformed into $trk1\Delta\ trk2\Delta$ and 117 colonies were isolated, 35 of which grew when re-streaked; of those, 26 were found to contain wild-type channel and 9 contained mutated channels. However, none of the 9 mutants showed any rescue of low potassium growth when cloned into fresh plasmid and transformed into fresh $trk1\Delta\ trk2\Delta$. 

2.3 RESULTS

2.3.1 Which TRP channels rescue trk1Δ trk2Δ?

We tested the ability of a variety of channels (in yeast expression vector p416-MET25-GFP) to rescue the ability of trk1Δ trk2Δ to grown on low potassium media: TRPV3, TRPV4, TRPV5, TRPV6, and TRPC6. These channels were chosen due to properties that made them the best candidates to be functional in the heterologous yeast system. For example, TRPV5 and TRPV6 are constitutively active in mammalian systems, while TRPV4 is active at temperatures over 24°C (yeast are typically grown at 30°C).

Yeast transformed with each TRP channel were spotted on selective media containing a low concentration of potassium (7 mM K⁺), on which trk1Δ trk2Δ cannot usually grow. As a growth control the strains were also spotted on permissive media containing a high concentration of potassium (100 mM K⁺). Empty vector was used as a negative control, and the constitutively active GIRK2-S177T channel was used as a positive control. Of the TRP channels tested, the only channel that could rescue growth on low-potassium media was TRPV4 (Figure 2.3.1).

2.3.2 A nonfunctional variant of TRPV4

We obtained a variant of TRPV4 that could not rescue the low potassium growth phenotype in trk1Δ trk2Δ. The sequence of this channel contained two substitutions: K70E and M605T (Figure 2.3.2a). Residue 70 is located in the N-terminal tail, while residue 605 is located in the small intracellular loop linking transmembrane segments S4 and S5. We will refer to this channel as V4-K70E/M605T, or V4-ET when additional mutations are introduced (or for space requirements).
The two mutations were tested separately: V4-K70E completely abolished rescue of trk1Δ trk2Δ low potassium growth; V4-M605T rescued but to a much lesser degree than wild-type TRPV4 (Figure 2.3.2b).

2.3.3 Screen for compensatory mutations allowing V4-K70E/M605T to rescue low potassium phenotype of trk1Δ trk2Δ.

A mutagenesis screen was designed (Figure 2.3.3-1) to discover if compensatory mutations could be found which would allow the V4-K70E/M605T channel to rescue growth of the trk1Δ trk2Δ strain on low potassium medium, as wild type TRPV4 does. Five clones were isolated with mutations clustering in the N-terminus, S5 helix, and C-terminus (Figure 2.3.3-2). Three of the mutant channels, containing mutations in the S5 helix or C-terminus, demonstrated strong rescue (equal to or better than TRPV4); the two clones containing N-terminal mutations demonstrated weaker rescue (less than wild type TRPV4).

We will first describe the strong rescuers: V4-ET-M625I/H787Y, V4-ET-Q790Δ, and V4-ET-N789Δ. V4-ET-M625I/H787Y contains two point mutations: M625I in pore-lining transmembrane helix S5, and H787Y in the C-terminus. When tested separately, each of these mutations results in robust rescue of the phenotype, but neither rescues as strongly as the double mutant (Figure 2.3.3-3a).

V4-ET-Q790Δ is a C-terminal truncation. V4-ET-N789Δ is also a C-terminal truncation, but not a straightforward one. In this case it appears that a translocation event occurred such that the gene is normal from nucleotide 1 through 2361, but then a sequence corresponding to nucleotide 1782 picks up and continues through the stop codon at 2616. The second region is out of frame, however, so the net result is a full read through H787, followed by eight nonsense
residues (WAEDGDL) until a stop codon terminates the translocated region. To make sure that this eight-residue tail was not having an effect on the channel, a construct was made in which the channel was truncated after H787, and it behaves the same as the original clone (data not shown). Going forward we will refer to this clone as V4-ET-N789Δ – the first amino acid of the nonsense region is W, but residue 788 of TRPV4 actually is W, so this behaves like a truncation at N789.

The two clones that are weaker rescuers actually contain mutations in the same region of the N-terminus: one is a single mutation of L154F; the other has that same mutation in addition to R151W and P152S. The triple mutant V4-ET-R151W/P152S/L154F shows slightly stronger rescue than V4-ET-L154F; however, when the mutations are tested separately neither R151W nor P152S confers any rescue of low potassium growth (Figure 2.3.3-3b). It is worth noting that these N-terminal mutants were the result of a massive mutagenic event of cytosines in a very small region: R151W was the result of the nucleotide substitution c451t, P152S by c454t, L154F by c460t/c462t (in both clones); V4-ET-R151W/P152S/L154F also contained the silent mutations I153 and D156 caused by c459t and c468t respectively. This means that in total, six of the seven cytosines in the 18-nucleotide stretch from 451 to 468 were mutated to thymidine.

2.3.4 Salt sensitivity as an assay for constitutive channel activity and selectivity

We utilized a salt sensitivity assay in the wild type strain BY4741 (Figure 2.3.4) to further probe the functional differences between the wild type TRPV4, the variant V4-K70E/M605T, and the mutated V4-K70E/M605T channels from our screen that seemed to restore some functionality as measured by the low potassium growth phenotype.

All strains should grow normally on the 0 M NaCl control plates, whereas a strain expressing a channel that allows excess sodium influx will cause lethality on the 1.5 M NaCl
plate. The GIRK-S177T channel was used as a negative control: although it is constitutively active, it is potassium selective and therefore causes no lethality on high-sodium plates.

The wild-type BY4741 strain expressing TRPV4 is extremely salt sensitive (virtually no growth on 1.5 M NaCl) while one expressing V4-K70E/M605T is not (normal growth on 1.5 M NaCl). When tested separately, V4-K70E caused no measurable salt sensitivity while V4-M605T did induce robust salt sensitivity (though not equal in lethality to wild type TRPV4). These data are consistent with the observation in trk1Δ trk2Δ that V4-M605T alone rescues low-potassium growth (though not as strongly as wild type TRPV4) while V4-K70E does not.

The correlation between rescue of trk1Δ trk2Δ low-potassium growth and salt-sensitivity in BY4741 holds true for the weak rescuers isolated in the screen: N-terminal mutants V4-ET-L154F and V4-ET-R151W/P152S/L154F exhibit moderate salt sensitivity. While the triple mutant-containing V4-ET-R151W/P152S/L154F exhibits slightly higher salt sensitivity than the single mutant L154F of V4-ET-L154F, the other two residues when tested alone show no measurable salt sensitivity.

The correlation does not always hold true for the strong rescuers isolated in our screen. V4-ET-M625I/H787Y, the strongest rescuer of low-potassium growth of trk1Δtrk2Δ, does also cause severe salt sensitivity of BY4741. Testing the two mutations separately, M625I (in S5) caused salt sensitivity but H787Y (in the C-terminus) did not. This was in contrast to the low-potassium growth phenotype, whereby both mutants could rescue to some degree. The C-terminal truncations, V4-ET-Q790Δ and V4-ET-N789Δ, also show no measurable salt sensitivity.

Table 2.3.4 summarizes these results, comparing the ability of each channel to rescue low-potassium growth of trk1Δ trk2Δ to the level of salt sensitivity it confers to BY4741.
2.3.5 Introducing the mutants into wild type TRPV4

To further elucidate their role in channel function, the mutations isolated through the V4-K70E/M605T screen were introduced into wild type TRPV4. The resulting channels were transformed into the \textit{trk1}\textasciitilde trk2\textasciitilde and BY4741 strains, and the aforementioned yeast assays were performed.

2.3.5.1 Effect of mutants on ability of TRPV4 to rescue growth on low potassium

Overall, introduction of the mutations from the screen into the TRPV4 channel resulted in a channel that rescued growth of \textit{trk1}\textasciitilde trk2\textasciitilde on low potassium better than wild type TRPV4 did (Figure 2.3.5.1).

There were two exceptions to this observation. The first was TRPV4-P152S, which was originally isolated from the N-terminal triple-mutant V4-ET-R151W/P152S/L154F: when introduced into TRPV4 this mutation had no effect, exhibiting rescue to the same degree as wild-type TRPV4.

The second exception was the double mutant TRPV4-M625I/H787Y; in this case we could not successfully obtain transformants in \textit{trk1}\textasciitilde trk2\textasciitilde despite multiple attempts. We suspect that introduction of this double mutant into TRPV4 results in a channel with such a high level of basal activity that it caused lethality due to cation overload. Introduction into TRPV4 of either mutation alone results in a channel with stronger rescue of low potassium growth than wild type TRPV4, so introduction of both mutations together may have an additive effect that is simply too much for the cell to accommodate. Our electrophysiological data are consistent with this theory, and will be discussed in the next chapter.
2.3.5.2 Effect of mutants on ability of TRPV4 to induce salt sensitivity

The high degree of salt sensitivity that results by transforming wild type TRPV4 into BY4741 appears to be at the limit of our assay system. Therefore, we could not expect to determine from this assay if any of the mutations from our screen endowed the TRPV4 channel with a higher level of activity – we would only be able to determine if a mutation reduced salt sensitivity of TRPV4. This was found to be the case for the two C-terminal truncation mutants: no salt sensitivity was seen in Q790Δ, while N789Δ showed only slight salt sensitivity (Figure 2.3.5.2: a) representative spot assay, b) comparison of salt sensitivity of mutants in V4-K70E/M605T versus TRPV4).

As was the case in the previous section, the double mutant M625I/H787Y from V4-ET-M625I/H787Y could not be tested: multiple attempts to transform the construct into BY4741 were unsuccessful, consistent with the theory that this channel is lethal to BY4741, as it seemed to be to trk1Δ trk2Δ.

2.3.6 TRPC5 and TRPC6 screens yield no mutants that rescue trk1Δ trk2Δ

2.3.6.1 TRPC6 screen

The TRPC6 channel was subjected to mutagenesis by hydroxylamine following the same methods used in the TRPV4 screen. A total of 716 colonies were isolated on the test plates, but none of them actually contained a mutated channel: no colonies grew when re-streaked onto fresh selective media. Potential explanations for the failure of this screen will be considered in the discussion.
2.3.6.2 TRPC5 screen

The TRPC5 channel was subjected to mutagenesis by hydroxylamine following the same methods used in the TRPV4 screen. In the first pass, although over 1,000 colonies were isolated only six of them grew when re-streaked onto fresh selective media: plasmid could not be recovered from two of the strains, three contained wild-type channel, and one contained a conservative mutation (clone m3, see Table 2.4.7) that did not exhibit any detectable rescue of trk1Δ trk2Δ in a spot assay.

It was suspected that the low mutation rate could be due to a reduction in efficacy of the hydroxylamine reagent, which is extremely hygroscopic. Therefore, a new stock was ordered and the screen was repeated: 90 colonies were isolated, 30 of which grew when re-streaked onto fresh selective media. However, 25 of the 30 were wild type. The remaining 5 did contain mutations (Figure 2.4.7, clones m122, m123, m164, m171, m184) but none exhibited any rescue of trk1Δ trk2Δ in a spot assay.

In parallel to the hydroxylamine mutagenesis, an error-prone PCR approach was utilized: 23 colonies were isolated, 5 of which grew when re-streaked onto fresh selective media. One of these was wild type; the other 4 did contain mutations (Figure 2.3.6.2, clones m117, m118, m139, m140) but did not show any measurable rescue of trk1Δ trk2Δ in a spot assay. In an attempt to increase the likelihood of mutations, additional round of PCR were performed with the inclusion of MnCl2: only 4 colonies were isolated, and none of these grew when re-streaked onto fresh selective media.

To summarize the screen of TRPC5 (excluding the first pass of hydroxylamine mutagenesis that was likely performed with bad reagents): a total of 117 colonies were isolated; 35 colonies grew when re-streaked on fresh selective media, 26 of which were wild-type and 9 of
which contained mutations but did not show any measurable rescue of $trk1\Delta$ $trk2\Delta$ upon spot assay analysis. Potential explanations for the failure of this screen will be considered in the discussion.
2.4 DISCUSSION

2.4.1 Heterologous expression of TRP channels in *Saccharomyces cerevisiae*

The Julius lab demonstrated that the mammalian TRPV1 channel could be successfully expressed in yeast: although expression of the channel had no effect on the growth of wild-type yeast, addition of the channel activator capsaicin resulted in cell death presumably as a result of cation overload (Myers et al., 2008). As they point out, this provides support to other studies that indicate membrane voltage as having a minor role in thermo-TRP channel gating (Latorre et al., 2007; Matta and Ahern, 2007), as yeast are hyperpolarized compared to mammalian cells (Peña et al., 1987; Serrano and Rodríguez-Navarro, 2001).

We wanted to determine what other TRP channels could be functionally expressed in the yeast, by testing their ability to rescue growth of *trk1Δ trk2Δ* yeast on low potassium media – normally this strain can only grow on synthetic complete media that has been supplemented with a large amount of potassium (100 mM KCl, in addition to the 7 mM KH$_2$PO$_4$ present in standard SC media). This approach had the advantage of not having to add an exogenous channel activator (not all TRP channels have been shown to be ligand-gated), or rely on cell death as a measure of activity. The disadvantage would be that the TRP channel would have to be active at a high enough level to allow enough potassium in to overcome the *trk1Δ trk2Δ* deficiency – as mentioned in the Introduction, in the study of the GIRK channel it was only a mutated, constitutively-active channel that showed rescue; the wild-type channel did not (Yi et al., 2001). However, either outcome could be experimentally useful: if a TRP channel was functional and rescued *trk1Δ trk2Δ* we could explore how it is processed and trafficked to the membrane, or look for mutations that altered channel function; if a TRP channel did not rescue *trk1Δ trk2Δ* we
could screen for mutations that allowed it to rescue for example by making it constitutively active.

We chose to test TRPV3, TRPV4, TRPV5, TRPV6, TRPC5, and TRPC6: based on their previously described properties (Clapham, 2003; Ramsey et al., 2006), we felt there was likelihood that they would exhibit basal activity in the yeast.

TRPV1-V4 are known as are thermo-TRPs, as activation by heat has been demonstrated in heterologous systems (Ramsey et al., 2006). Yeast is typically grown at 30°C, so we chose the thermo-TRPs with activation temperatures in that range: TRPV3, which is activated above 30°C (Xu et al., 2002); and TRPV4, which is activated above 24°C (Güler et al., 2002). Both channels are Ca\(^{2+}\)-selective, but only weakly, so they would likely pass enough potassium to rescue trk1Δ trk2Δ growth. We did not test TRPV1, which had already been subjected to a similar screen by the Julius lab (Myers et al., 2008). TRPV2 was also not tested: since it does not exhibit any measurable basal activity when heterologously expressed in Xenopus oocytes (Myers et al., 2008) it was unlikely to do so in yeast; in addition, it is only activated at noxious temperatures (>53°C) (Caterina et al., 1999).

TRPV5 and TRPV6 were selected due to the fact that they were shown to be constitutively active and strongly inwardly rectifying (Vennekens et al., 2000; Dekker et al., 2003). They are also highly Ca\(^{2+}\)-selective – while this meant they were not likely candidates to rescue trk1Δ trk2Δ growth, they could be subjected to mutagenesis to identify residues that affect selectivity or trafficking. Analysis through SCAM was useful in identifying the location of the selectivity filter in TRPV5 (Dodier et al., 2007) and TRPV6 (Voets et al., 2004c); a random mutagenesis approach might identify other interesting properties.
TRPC5 and TRPC6 are members of the ‘canonical’ subfamily, those most analogous to the *Drosophila* TRP channel. TRPC channels are cation non-selective, double rectifying, and activated by G-protein coupled receptor (GPCR) stimulation. It is common to split the TRPC family functionally into two sub-groups: the first consisting of TRPC1, C4, and C5; the second consisting of TRPC3, C6, and C7. We chose to test TRPC5 and TRPC6 so we would have a representative channel from each sub-group.

Since the TRP channels were cloned into a yeast expression vector containing a GFP tag, we first examined their cellular localization pattern reasoning that visualization channel expression at the plasma membrane could be utilized later when studying the trafficking of the channels. Unfortunately, all channels examined showed very strong expression in the endoplasmic reticulum (ER) (data not shown). In yeast there are two easily identifiable populations of ER: perinuclear ER surrounding the nucleus, and cortical ER in the cell periphery. At first the strong ER expression of the channels led us to believe that the channels were getting stuck in the ER and not getting to the plasma membrane. Of course, the peripheral GFP fluorescence could include plasma membrane expression in addition to cortical ER. However, it would be very difficult to distinguish the two: even if a differential marker was available, the two regions are so close together that standard confocal microscopy would not be able to reliably separate them. We learned that the Jan lab had seen this expression pattern when heterologously expressing a potassium channel protein, but went on to see that the protein was able to rescue low-potassium growth in *trk1Δ trk2Δ* and so reasoned that enough channel was making its way to the plasma membrane (Helen Lai, personal communication; (Lai et al., 2005)). Therefore, we went ahead and tested the TRP channels in spot assays to determine if any could rescue low-potassium growth in *trk1Δ trk2Δ*. 
Of the TRP channels tested, the only channel that could rescue growth on low-potassium media was TRPV4 (Figure 2.3.1). TRPV4 is a non-selective cation channel expressed in a variety of tissues including kidney, lung, blood vessels; in addition to moderate heat, it is activated by hypo-osmolarity and phorbol ester derivatives (4αPDD) (Ramsey et al., 2006). Mutations in this gene have been linked to skeletal dysplasia disorders as well as neurodegenerative diseases (Verma et al., 2010; Wee et al., 2010); this will be discussed in more detail in Chapter 4. After we performed this experiment, another lab published a study asserting that TRPV4 does not respond to heat when expressed in yeast (Loukin et al., 2009). In their study, rat TRPV4 was expressed in a yeast strain lacking the gene for endogenous TRP channel (yvc1Δ); a rise in intracellular calcium was seen upon hypotonic shock, but not to heat (up to 50°C). Clearly, however, the TRPV4 channel exhibits enough basal activity in yeast grown at 30°C to allow adequate potassium entry to rescue growth of trk1Δ trk2Δ, even if this activity could not be picked up in their assay system.

2.4.2 Comparison of wild type TRPV4 and V4-K70E/M605T

We obtained a TRPV4 clone from OpenBiosystems (LIFESSEQ90218873) that contained two polymorphisms, K70E and M605T, and could not rescue growth of trk1Δ trk2Δ on low potassium media as wild type TRPV4 could. On first thought, one would assume that the mutation at residue 605 must be the one responsible for the loss of the V4-K70E/M605T channel’s ability to rescue the growth phenotype: it is close enough to the pore-forming units of the channel that it could have an effect, even indirect; meanwhile K70E is close to the end of the N-terminus, upstream of the ankyrin repeats that functionally define the region. Surprisingly, however, when the two mutations were tested separately K70E completely abolished rescue
while M605T showed only partial rescue (Figure 2.3.2b). This means that while M605T does have some effect on channel function, it is the K70E mutation that seems to be responsible for the complete failure of the channel to rescue the growth phenotype.

We saw this as a perfect opportunity to probe the workings of the TRPV4 channel. We knew the wild type channel could rescue, and the two polymorphisms in the variant channel clearly disrupted channel function. There were multiple reasons why this could happen – the mutations could disrupt proper protein folding, or disrupt the ability to interact with other proteins necessary for assembly and trafficking, meaning that a functional channel wasn’t even getting inserted into the membrane. On the other hand, it was possible that the channel was functional but the mutations altered the properties of the channel (open probability, ion selectivity, etc.) such that it was no longer able to allow enough potassium in to rescue \( trk1\Delta \) \( trk2\Delta \) growth. We wondered if we could find compensatory mutations that would allow V4-K70E/M605T to rescue \( trk1\Delta \) \( trk2\Delta \) as well as or even better than wild-type TRPV4.

2.4.3 Screen of V4-K70E/M605T for compensatory mutations

2.4.3.1 Rationale

We performed a gain of function (GOF) screen of the variant channel V4-K70E/M605T with the goal of identifying compensatory mutations that would restore the ability of V4-K70E/M605T to rescue low-potassium growth of \( trk1\Delta \) \( trk2\Delta \), hopefully back to the level of TRPV4 or maybe even better (Figure 2.3.3-1).

We mutagenized the channel DNA by exposing it to hydroxylamine (NH\(_2\)OH), a chemical mutagen that reacts with pyrimidines, preferentially cytosine (Freese et al., 1961).
Cytosine is converted to $N^4$-hydroxycytosine which pairs with adenine instead of guanine, resulting in C to T and G to A transitional mutations in the next round of replication (Singer and Fraenkel-Conrat, 1969; Humphreys et al., 1976). Hydroxylamine reacts with double-stranded DNA, and is easily removed from the reaction, making it ideal for plasmid mutagenesis (Rose and Fink, 1987; Amberg et al., 2005). Channels were screened for ability to rescue growth of $trk1\Delta\ trk2\Delta$ on low potassium media, and the degree of rescue was compared to that of wild type TRPV4.

2.4.3.2 Screen yields mutants in three channel regions

We isolated five mutated V4-K70E/M605T channels that could restore the ability of the channel to rescue the low-potassium growth of $trk1\Delta\ trk2\Delta$ (Figure 2.3.3-2). The degree of rescue varied – two channels only weakly rescued the phenotype whereas the other three strongly rescued the phenotype (of these, one exhibited even more robust rescue than the wild type TRPV4 channel). In the instances where multiple residues had been mutated, we separated the mutations to determine the degree to which each mutation contributed to the channel function (Figure 2.3.3-3).

The clone that exhibited the strongest rescue was V4-ET-M625I/H787Y, containing the substitutions M625I in the pore-lining transmembrane helix S5 and H787Y in the C-terminus. This resulted in a channel that exhibited even stronger growth on low potassium than wild type TRPV4. The two mutations were tested separately in V4-K70E/M605T: each mutation alone showed strong growth, but neither grew as well as the double mutant. The two C-terminal truncations, V4-ET-Q790Δ and V4-ET-N789Δ, also showed very strong rescue.
It is interesting to note that all three of the strong rescuers contain a mutation essentially on the same spot in the C-terminus: V4-ET-M625I/H787Y has a mutation at H787; V4-ET-Q790Δ and V4-ET-N789Δ are truncations immediately downstream. This would suggest a mutational hotspot; indeed, this region has been shown to be very important in channel regulation: the calmodulin-binding domain at residue 814 (Strotmann et al., 2003). Previous studies have identified that mutation of E797 leads to increased spontaneous TRPV4 activity (Watanabe et al., 2003b). In the related channel TRPV1, C-terminal truncations have been associated with constitutive channel activity (Myers et al., 2008). The other mutation in V4-ET-M625I/H787Y, M625I, is located in a region of transmembrane helix S5 that is mutated in patients with skeletal dysplasia: patients with Type 3 brachyolmia were found to have point mutations R616Q or V620I which resulted in a channel that displayed both higher basal current and a higher degree of activation by hypotonic stimulation or chemical agonist (Rock et al., 2008).

The remaining two clones exhibited rescued low potassium growth, but not to the same degree as wild type TRPV4: V4-ET-L154F and V4-ET-R151W/P152S/L154F in the N-terminus. At first it was assumed that L154F was solely responsible for the phenotype, as no measurable rescue was seen when R151W or P152S were tested separately. However, the triple mutant did rescue slightly better than L154F alone, suggesting a contribution of one or both of the other mutations.

2.4.3.3 Comparison of low potassium rescue versus salt sensitivity

If the mutations we have isolated are indeed resulting in increased channel activity this should be evident as an increase in salt sensitivity of yeast containing these channels: the extra
sodium influx would overwhelm the yeast’s natural detoxification systems and lead to programmed cell death (Nakamura and Gaber, 1998; Huh et al., 2002). Therefore, measuring the ability of mutant channels to grow on plates containing high levels of NaCl should serve as an assay of overactive channel activity. Of course, it is possible that the mutations could be affecting channel selectivity in addition to channel activity such that a channel could be rendered constitutively active but no longer Na$^+$ permeable, in which case the strain would still grow on high sodium plates. Such is the case with GIRK-S177T: although it is constitutively active, it is potassium selective and therefore has no effect on salt sensitivity; BY4741 yeast expressing this channel grow perfectly well on high NaCl and were therefore used in our assays as a negative control.

We examined the effect of TRPV4, V4-K70E/M605T, and our mutants on salt tolerance of the wild type yeast strain BY4741 (Figure 2.3.4). This strain can normally grow on plates containing an elevated level of sodium chloride (1.5 M versus 1.7 mM in standard SC media) but when the wild type TRPV4 is expressed the yeast die, presumably due to cation overload. The high degree of salt sensitivity conferred by the TRPV4 channel establishes that activity level of the channel is high enough for a lethal dose of sodium to enter the cell.

The V4-K70E/M605T channel, on the other hand, does not exhibit measurable salt sensitivity. There are many possible explanations for this effect: the channel could be nonfunctional, have a reduced open probability, or have altered selectivity such that the channel is open as often but does not allow sodium passage. When the two polymorphisms were tested separately it was observed that V4-K70E lacked the ability to cause salt sensitivity in the yeast while V4-M605T still exhibited salt sensitivity (albeit less severe than wild type TRPV4). These data are consistent with the observation that V4-M605T retains ability to rescue the low-
potassium growth phenotype while V4-K70E does not and strengthens the argument that the V4-K70E/M605T channel is not properly conducting ions due primarily to the mutation K70E.

By extension of this logic, one would hypothesize that the clones isolated in the screen that are weak rescuers of low potassium growth in \( trk1\Delta\ trk2\Delta \) would also show weak salt sensitivity in BY4741 whereas strong rescuers would show more severe salt sensitivity. Indeed, weaker rescuers V4-ET-L154F and V4-ET-R151W/P152S/L154F exhibit only moderate salt sensitivity. As with the low potassium growth rescue, the triple mutation of V4-ET-R151W/P152S/L154F shows slightly higher salt sensitivity than the single mutant V4-ET-L154F, but the other two residues when tested alone show no salt sensitivity.

The data are not so straightforward for the strong rescuers. V4-ET-M625I/H787Y, the strongest rescuer of low-potassium growth of \( trk1\Delta\ trk2\Delta \), causes severe salt sensitivity of BY4741. When tested separately, it was seen that salt sensitivity was solely conferred by V4-ET-M625I (mutation in S5); V4-ET-H787Y (C-terminal mutation) did not cause any salt sensitivity. This was surprising as V4-ET-H787Y can rescue the low potassium growth phenotype, albeit not to the same extent as the double mutant, so we would have expected at least slight salt sensitivity.

Most surprising to us was that the other two strong rescuers of low potassium growth, the C-terminal truncations V4-ET-Q790Δ and V4-ET-N789Δ, showed no salt sensitivity whatsoever. Coupled with the phenotype of V4-ET-H787Y, it becomes apparent that there is not a direct correlation between rescue of growth on low-potassium media and increased salt sensitivity.

Table 2.3.4 summarizes these results, comparing the ability of each channel to rescue low-potassium growth of \( trk1\Delta\ trk2\Delta \) to the level of salt sensitivity it confers to BY4741.
2.4.4 Effect of mutations on wild type TRPV4 activity

To further elucidate their role in channel function, the mutations isolated in the V4-K70E/M605T screen were introduced into wild type TRPV4. The resulting channels were transformed into the \( trk1\Delta trk2\Delta \) and BY4741 strains and the aforementioned yeast assays were performed.

Wild type TRPV4 shows a positive result in both of assays – that is, the channel rescues low-potassium growth in \( trk1\Delta trk2\Delta \) and causes increased salt sensitivity in BY4741. Meanwhile, V4-K70E/M605T gives a negative result in both cases – no low-potassium growth rescue in \( trk1\Delta trk2\Delta \), no salt sensitivity in BY4741. Our screen yielded mutations that endowed V4-K70E/M605T with the ability to rescue low-potassium growth of \( trk1\Delta trk2\Delta \); only a subset of these exhibited salt sensitivity in BY4741. How would these mutations affect the function of TRPV4? Would there be an additive effect, enabling the channel to rescue low potassium growth of \( trk1\Delta trk2\Delta \) to an even higher degree, or would they actually interfere with normal function of TRPV4?

2.4.4.1 Effect of mutants on ability of TRPV4 to rescue growth on low potassium

Overall, introduction of the mutations into wild type TRPV4 resulted in a channel that rescued growth of \( trk1\Delta trk2\Delta \) on low potassium either to the same degree as TRPV4 or better (Figure 2.3.5.1).

In the V4-K70E/M605T background the strongest rescue was seen in V4-ET-M625I/H787Y, which actually contained two mutations: the S5 mutant M625I and the C-terminal mutant H787Y. In the V4-K70E/M605T channel each of these mutations resulted in moderate rescue of the low-potassium growth phenotype when tested singly while the double
mutant rescued growth to a level that surpassed the level of rescue of TRPV4. When introduced into TRPV4, these mutations seem to further enhance the function of the channel: the single mutants TRPV4-M625I or TRPV4-H787Y lead to more robust growth on low potassium media than the strain expressing wild type TRPV4. One would predict that the double mutant in the TRPV4 background would lead to even higher growth levels, but we were not able to test this: despite multiple attempts to transform the construct into \(\text{trk1}^{\Delta}\text{trk2}^{\Delta}\), we never succeeded in isolating colonies containing the plasmid. We suspect that the introduction of both of these mutations into TRPV4 at once results in a channel with such a high level of basal activity that it causes lethality due to cation overload. Our electrophysiological data are consistent with this theory (discussed in the next chapter).

In the V4-K70E/M605T background, the two C-terminal truncations caused strong rescue of the growth phenotype: V4-ET-Q790\(\Delta\) resulted in rescue to the same level as TRPV4 while V4-ET-N789\(\Delta\) rescued better than TRPV4. When either of these truncations was introduced into TRPV4, the resulting channel could rescue the low-potassium growth phenotype better than wild type TRPV4.

In V4-K70E/M605T, the N-terminal mutations had resulted in only a weak rescue of low potassium growth phenotype, with the triple mutant rescuing slightly better than L154F alone while neither R151W nor P152S alone showed a phenotype. Although we hypothesized that these mutations would not affect wild type TRPV4, it was observed that TRPV4-R151W/P152S/L154F rescued to a higher degree than wild type TRPV4. Both TRPV4-L154F and TRPV4-R151W alone could also rescue to a higher degree; TRPV4-P152S was the only mutation that had no effect on its own. This confirmed that R151W has some effect on channel activity, despite having no discernable effect in V4-K70E/M605T.
2.4.4.2 Effect of mutants on ability of TRPV4 to induce salt sensitivity

Assaying the degree to which the mutations isolated in the screen affect the behavior of TRPV4 to induce salt sensitivity would not be as straightforward as the assay of low potassium growth (Figure 2.3.5.2). In trk1Δ trk2Δ, rescue of growth by wild type TRPV4 is strong but not absolute; therefore, if mutants had an additive effect this could be seen as more robust growth compared to wild type. Since the salt sensitivity phenotype of TRPV4 expressed in BY4741 is so severe, causing almost complete lethality, even if the mutations increased the activity of TRPV4 and led to increased salt sensitivity we would not be able to distinguish any further reduction in growth in this assay. What this assay could measure, however, was if any of the mutations actually decreased the ability of TRPV4 to induce salt sensitivity.

The salt sensitivity phenotype of V4-ET-M625I/H787Y appeared to be caused solely by one of the two mutated residues: when tested separately, the S5 mutation M625I exhibited severe salt sensitivity while the C-terminal mutation H787Y showed no sensitivity. The introduction of either of these mutations alone into TRPV4 does not lead to any apparent change in the level of salt sensitivity – all this tells us is that neither mutation impairs the ability of TRPV4 to induce salt sensitivity. Unfortunately the double mutant could not be tested in TRPV4 – as was the case for trk1Δ trk2Δ, multiple attempts to transform the construct into BY4741 were unsuccessful. The fact that the double mutant in TRPV4 is inviable, while the single mutant M625I is not, does suggest that the H787Y mutation must make some contribution to the phenotype that we can not discern from this assay.

The N-terminal mutations isolated in the V4-K70E/M605T screen exhibited slight salt sensitivity in BY4741: the triple mutant V4-ET-R151W/P152S/L154F showed a slightly stronger phenotype than the single mutant V4-ET-L154F; either R151W or P152 alone had no effect.
Introduction of these mutations into TRPV4 (each mutation separately or the triple mutant) does not lead to any apparent change in the level of salt sensitivity, so we can infer nothing new about these mutations other than the fact that they do not impair salt sensitivity of TRPV4.

Both of the C-terminal truncations from our screen, V4-ET-Q790Δ and V4-ET-N789Δ, failed to confer the ability to induce salt sensitivity on V4-K70E/M605T despite being the strongest rescuers of low potassium growth. We would have predicted that, like the mutant H787Y that also did not show salt sensitivity in V4-K70E/M605T, we would not see any change in the salt sensitivity assay in TRPV4. Surprisingly, however, both of the C-terminal truncations actually abolished the ability of TRPV4 to induce salt sensitivity in BY4741: no salt sensitivity at all was seen in TRPV4-Q790Δ, while only very slight salt sensitivity was retained in TRPV4-H787Δ.

2.4.5 Summary of properties of TRPV4 channel mutants in yeast assay system

In our mutagenesis screen we endeavored to identify mutations of the variant TRPV4 channel, V4-K70E/M605T, which would restore the ability of the channel to function in a yeast assay the way the true wild type TRPV4 channel did. We did not know if V4-K70E/M605T was simply a ‘dead’ channel – perhaps it was not trafficked to the plasma membrane, perhaps it could not properly fold and insert in the membrane; alternatively, perhaps it was a functional channel but simply had altered activity such that its conductance properties were different than the wild-type channel. The primary screening assay was the ability of the channels to rescue growth of \( trk1Δ\ trk2Δ \) on low potassium media, but we also examined the degree to which the channels induced cell death of the wild type yeast strain BY4741 on high-salt media (Table 2.4.5).
The two mutations in V4-K70E/M605T were K70E, close to the end of the N-terminus, and M605T in the intracellular loop between transmembrane helices S4 and S5. Although M605T did contribute somewhat to the inability of V4-K70E/M605T to rescue the yeast phenotype, we found that the inability of V4-K70E/M605T to function was primarily due to the K70E mutation.

We isolated mutations in three regions of the V4-K70E/M605T channel that allowed it to rescue the ability of the \( trk1\Delta trk2\Delta \) strain to grow on low-potassium media: the N-terminus (R151W/P152S/L154F), the pore-lining helix S5 (M625I), and the C-terminus (H787Y, N789\( \Delta \), and Q790\( \Delta \)). We went on to assay the ability of these mutants to induce salt sensitivity, as well as introduce the mutations into TRPV4 to determine how they would affect wild-type channel function (Table 2.4.5).

The N-terminal triple mutant V4-ET-R151W/P152S/L154F weakly rescued the ability of V4-K70E/M605T to rescue growth on low potassium, as well as induce weak salt sensitivity. L154F alone showed a similar level of low-potassium growth and salt sensitivity while R151W or P152S alone showed no effect; however, these two residues play some sort of additive role as the triple mutant rescued slightly better than L154F alone. When R151W, L154F, or the triple mutant were introduced into the wild-type TRPV4 they slightly improved the ability of TRPV4 to rescue low-potassium growth, so the increase of activity was not simply compensatory.

The S5 mutant M625I and the C-terminal mutant H787Y were isolated as a double-mutant in our mutagenesis screen of V4-K70E/M605T in the clone that showed the strongest rescue of low-potassium growth as well as strong salt sensitivity. When the mutants were tested separately they each showed strong rescue of low-potassium growth, although not as strong as the double mutant. In contrast, only M625I induced the strong salt sensitivity phenotype in V4-
K70E/M605T (H787Y did not appear to cause salt sensitivity). When each mutation was introduced singly into wild type TRPV4, the channel rescued low-potassium growth to a higher degree. The double mutant could not be tested, presumably because it increases activity of TRPV4 to such an extent as to be lethal to the yeast – one way to confirm this would be to use a yeast expression vector with an inducible promoter, so that we could control the timing and extent of channel expression.

We isolated two adjoining C-terminal truncation mutants, V4-ET-N789Δ and V4-ET-Q790Δ. Technically the former is the truncation H787Δ followed by a tail of eight nonsense residues, but the first nonsense residue is W which is what the next residue would have been (W788) so therefore we treat the channel as N789Δ. As we observed for the C-terminal point mutation H787Y in V4-K70E/M605T, both C-terminally truncated V4-K70E/M605T channels exhibited very strong rescue of low-potassium growth but did not cause any salt sensitivity. When introduced into wild type TRPV4, either truncation resulted in stronger rescue of low potassium growth. Surprisingly, however, both C-terminal truncations in TRPV4 completely abolished the salt sensitivity phenotype of the wild-type channel. This phenotype was not observed for the C-terminal point mutation, or for any other mutant isolated in our screen for that matter.

In the following chapter, we will report on our further exploration of the properties of these TRPV4 mutants through electrophysiological recordings in Xenopus oocytes.
2.4.6  A recent similar study confirms our findings

Subsequent to our findings, a very similar study was published in the Journal of Biological Chemistry (Loukin et al., 2010a). While it was disappointing to learn that another group had isolated TRPV4 gain-of-function (GOF) mutations that were quite similar to ours, their work displayed key differences – for example, the experimental design was distinct between the two studies, and we each isolated different mutated residues. Therefore, analysis of their study and comparison to ours can be particularly instructive in creating a fuller picture of TRPV4 function.

In their case, wild type rat TRPV4 channel (NM_023970) was screened for gain-of-function mutants as measured by their ability to block yeast proliferation, presumably through cation overload. The channel was subjected to mutagenesis by error-prone PCR and cloned into a vector under a galactose-inducible promoter (p416-GAL). Yeast transformed with the mutated plasmid were initially grown on repressive (dextrose-based) medium, and colonies were isolated that failed to grow when replica-plated to expressive (galactose-based) medium. Addition of the TRPV4 channel blocker ruthenium red (10 µM) could restore growth of these strains on galactose medium.

The authors isolated a total of six growth-inhibiting alleles, containing 7 mutations total, from two independent screens. These included a mutation in the N-terminus, a mutation in the intracellular loop between S2 and S3, two mutations in the S5 helix, mutations of a residue at the intracellular end of S6 that were isolated in three separate instances, and a mutation in the C-terminus that was isolated twice. Figure 2.4.6 shows the topographical location of their mutant alleles in comparison to ours.
The double-mutant allele R151Q/D456G only weakly inhibited growth; when they tested the N-terminal mutation R151Q alone they did not see any growth inhibition and concluded that the effect was solely due to the D456G mutation in the S2/S3 loop. The N-terminal residue 151 also came up in our screen, and had a very subtle phenotype: R151W alone did not enable V4-K70E/M605T to rescue low-potassium growth, but did contribute to the rescue phenotype of the C-terminal triple mutant R151W/P152S/L154F and did slightly increase the low-potassium growth phenotype of wild type TRPV4. The fact that this residue was isolated in both of our studies strengthens the case for this area playing a role in channel function. We did not isolate any mutations in the S2/S3 loop in our study.

They also isolated two mutations in the S5 transmembrane helix: L619P and L623P are approximately one helical turn apart; the phenylalanes would be predicted to cause kinks in the helix. Our mutant M625I was one of the strongest rescuers of low potassium growth in our screen. The location of all of these mutants in the pore-forming S5 helix suggests that they play a role in formation of the intracellular gate of the channel.

Finally, the authors isolated residues in the C-terminus, but they were not in the same area as the mutants isolated in our screen: M713V or M713I at the cytoplasmic end of S6, and W733R in the C-terminal tail. Our mutations were approximately 50 residues downstream in the C-terminal tail; it is likely that their enhancement of channel activity occurs through different pathways.

In the next chapter, we will discuss the electrophysiological analysis of the mutations isolated by these authors and how it relates to the analysis of the mutations we identified.
2.4.7 Screens of TRPC channels are unsuccessful despite multiple attempts

The mutagenesis screen performed on V4-K70E/M605T was relatively small-scale, yet produced a number of mutants that could recover the ability of the channel to rescue low-potassium growth of the trk1Δ trk2Δ yeast strain to a level comparable to wild type TRPV4. Therefore, we wanted to extend our experiments to other members of the TRP channel family. Neither of the two TRPC channels we tested, TRPC5 and TRPC6, could rescue the ability of trk1Δ trk2Δ to grow on low-potassium media; therefore, we performed mutagenesis screens with the goal of isolating mutants that would endow the channel with the ability to rescue the growth phenotype.

Unfortunately, neither screen produced a mutated channel that showed rescue of the low-potassium growth in trk1Δ trk2Δ. In the case of TRPC6, no mutated plasmids were recovered. In the case of TRPC5, mutated channels were isolated but did not show any measurable rescue in our assay system (Table 2.4.7). This was despite conducting the screens on a much larger scale, and introducing new mutagenesis techniques.

The TRPC6 channel was subjected to mutagenesis by hydroxylamine following the same methods used in the TRPV4 screen. Although the scale of the screen was larger, and a total of 716 colonies grew on the test plates, when these colonies were re-streaked onto fresh selective media none of them grew.

We next attempted a screen of the TRPC5 channel: initially, hydroxylamine mutagenesis was performed, but none of the mutant clones isolated actually exhibited measurable rescue of trk1Δtrk2Δ in a spot assay. A downside to the use of hydroxylamine is that it selectively mutates cytosines, resulting in C→T transitions, which limits the types of mutations you can hope to recover. Therefore, we switched to an error-prone PCR approach – theoretically, any residue
could be mutated with this approach. Again, a few mutant clones were isolated but did not show any measurable rescue of \textit{trk1Δ trk2Δ} in a spot assay.

Perhaps the TRPC channels are just unable to function in the yeast heterologous system. For example, they may require molecular partners for assembly and trafficking or activation that are not present in the yeast. Alternatively, it is possible that we did create gain-of-function channels but they were lethal to the yeast, therefore we could not isolate them. We did see similar phenomena in our TRPV4 screen – the double mutant M625I/H787Y was isolated in the screen of V4-K70E/M605T where it caused a very strong rescue of growth. When we tried to clone these two mutations into wild type TRPV4, we were never able to isolate transformants, presumably because the resulting increase of channel activity was far too large for the yeast to survive.
Figure 2.3.1 TRPV4 rescues growth of \textit{trk1}\textdagger \textit{trk2}\textdagger. Four-fold serial dilutions of \textit{trk1}\textdagger \textit{trk2}\textdagger transformed with indicated construct spotted on permissive (left panel, 100 mM K\textsuperscript{+}) or selective (right panel, 7 mM K\textsuperscript{+}) media. Degree of rescue scored visually in comparison to growth of positive control (GIRK2-S177T). Empty vector used as negative control.
Figure 2.3.2 A variant of TRPV4 does not rescue $trk1\Delta \ trk2\Delta$ growth phenotype.
a) Location in channel architecture of the two mutations found in variant channel (purple dots).
b) M605T slightly rescues low potassium growth, K70E does not. Four-fold serial dilutions spotted on permissive (100 mM K$^+$) or selective (7 mM K$^+$) media; degree of rescue scored visually in comparison to growth of GIRK2-S177T.
Figure 2.3.3-1 Design of mutagenesis screen. V4-K70E/M605T (“V4-ET”) plasmid DNA was subjected to hydroxylamine mutagenesis, transformed into trk1Δ trk2Δ, and plated on selective media (6-6.6 mM KCl) to isolate clones that gained ability to rescue growth on low potassium media. Plasmid DNA was isolated from these clones, and sequenced to determine if mutated channel (“V4-ET-X”) was present. Spot assays confirmed degree of rescue
Figure 2.3.3-2 Mutated clones of V4-K70E/M605T rescue growth of trk1Δ trk2Δ on low potassium. a) Four-fold serial dilutions of trk1Δ trk2Δ transformed with indicated construct (with mutations color-coded to match (b)) on permissive (100 mM K⁺) or selective (7 mM K⁺) media, degree of rescue scored visually in comparison to GIRK-S177T. b) Location of mutants in presumed transmembrane topography of V4-K70E/M605T. Filled purple circles indicate position of K70E and M605T. Colored circles indicate gain-of-function mutations: green for N-terminal, orange for S5 helix, and blue for C-terminal mutations.
Figure 2.3.3-3  Relative contribution to phenotype of residues from multiply-mutated clones.  
a) M625I (S5, orange) and H787Y (C-term, blue) both rescue, but not to the same degree as the double mutant.  
b) Of the three residues in the N-terminal triple mutant (green), only L154F can rescue the phenotype alone (though not to the same degree as the triple mutant).  
Four-fold serial dilutions of $trk1 \Delta \ trk2 \Delta$ transformed with indicated construct on permissive (100 mM K$^+$) or selective (7 mM K$^+$) media, degree of rescue scored visually in comparison to GIRK-S177T.  
Refer to topology in Figure 2.3.3-2(b) for location of mutations.
Figure 2.3.4  Not all of the mutants from the screen rescue the ability of V4-K70E/M605T to induce salt sensitivity in yeast. Four-fold serial dilutions of BY4741 transformed with indicated construct on permissive (0 mM Na\(^+\)) or selective (1.5 mM Na\(^+\)) media; degree of salt sensitivity (lethality) scored visually in comparison to wild type TRPV4. S5 (orange) and N-terminal (green) mutants rescue, but C-terminal (blue) do not. Refer to topology in Figure 2.3.3-2(b) for location of mutations.
Table 2.3.4 Rescue of low potassium growth does not necessarily capitate salt sensitivity.

<table>
<thead>
<tr>
<th>Channel</th>
<th>( trk1\Delta trk2\Delta: ) Growth on 7 mM K(^+)</th>
<th>BY4741: Sensitivity to 1.5 M Na(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>“V4-ET” V4-K70E/M605T</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V4-M605T</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>V4-K70E</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-M625I</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>V4-ET-H787Y</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-M625I/H787Y</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>V4-ET-Q790Δ</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-N789Δ</td>
<td>++++</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-R151W</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-P152S</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V4-ET-L154F</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V4-ET-R151W/P152S/L154</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

C-terminal point mutation and truncations (blue) rescue low potassium growth, but do not rescue salt sensitivity, of V4-ET (purple). Topography provided as reference for location of mutations in channel structure.
Figure 2.3.5.1  Effect of mutants on ability of wild type TRPV4 to rescue low K\(^+\) growth of *trk1Δ trk2Δ*. a) Four-fold serial dilutions of *trk1Δ trk2Δ* transformed with indicated construct on permissive (100 mM K\(^+\)) or selective (7 mM K\(^+\)) media; degree of rescue scored visually in comparison to GIRK-S177T. b) Table comparing effect of mutations in V4-K70E/M605T or wild type TRPV4 background. Refer to Table 2.3.4 for location of mutants in topology.
**Figure 2.3.5.2** Effect of mutants on ability of wild type TRPV4 to induce salt sensitivity in BY4741. 

**a)** Four-fold serial dilutions of BY4741 transformed with indicated construct on permissive (0 mM Na⁺) or selective (1.5 mM Na⁺) media, degree of rescue scored visually in comparison to wild type TRPV4.

**b)** Table comparing effect of mutations in V4-K70E/M605T or wild type TRPV4 background. Refer to Table 2.3.4 for location of mutants in topology. C-terminal truncations, but not point mutation, abolish salt sensitivity of wild type TRPV4.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Salt Sensitivity in V4-ET</th>
<th>Salt Sensitivity in TRPV4</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>++++</td>
</tr>
<tr>
<td>M625I</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>H787Y</td>
<td>—</td>
<td>++++</td>
</tr>
<tr>
<td>M625I/H787Y</td>
<td>++++</td>
<td>not viable</td>
</tr>
<tr>
<td>Q790Δ</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>N789Δ</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>R151W</td>
<td>—</td>
<td>+++</td>
</tr>
<tr>
<td>P152S</td>
<td>—</td>
<td>+++</td>
</tr>
<tr>
<td>L154F</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>R151W/P152S/L154F</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Table 2.4.2  Summary of yeast growth assays of V4-K70E/M605T and wild-type TRPV4.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>YEAST SPOT ASSAYS</th>
<th>Low potassium growth of trk1Δ trk2Δ</th>
<th>Sodium sensitivity of BY4741</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4 (wild type)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>V4-K70E/M605T (V4-ET)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mutants from screen: in V4-ET</td>
<td>in TRPV4</td>
<td>in V4-ET</td>
<td>in TRPV4</td>
</tr>
<tr>
<td>M625I</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>H787Y</td>
<td>++</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>M625I/H787Y</td>
<td>+++</td>
<td>not viable</td>
<td>+++</td>
</tr>
<tr>
<td>Q790Δ</td>
<td>+++</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>N789Δ</td>
<td>+++</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>R151W</td>
<td>—</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>P152S</td>
<td>—</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>L154F</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>R151W/P152S/L154F</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Topography provided as reference for location of mutations in channel structure.
Figure 2.4.6  Comparison of location of mutated residues isolated by our study and Loukin et al. Their mutants appear in white circles while mutants from our screen are in filled purple circles; open purple circles indicate position of the two pre-existing mutations in V4-K70E/M605T. Figure adapted from original publication in J. Biol. Chem: Loukin S et al. Forward genetic analysis reveals multiple gating mechanisms of TRPV4. JBC 2010; 285:19884-90. © the American Society for Biochemistry and Molecular Biology.
Table 2.4.7 Mutations isolated in TRPC5 screen, none of which exhibited rescue of *trk1Δ* *trk2Δ*

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Type of mutagenesis</th>
<th>Nucleotide(s)</th>
<th>Amino Acid(s)</th>
<th>Location in channel</th>
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</thead>
<tbody>
<tr>
<td>m3</td>
<td>hydroxylamine</td>
<td>g1165a</td>
<td>V389I</td>
<td>S2/S3 loop</td>
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<tr>
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<td>hydroxylamine</td>
<td>c140t</td>
<td>T47I</td>
<td>NT</td>
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<tr>
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<td>hydroxylamine</td>
<td>c2177t</td>
<td>A726V</td>
<td>CT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c2450t/c2451t</td>
<td>T817I</td>
<td>CT</td>
</tr>
<tr>
<td>m164</td>
<td>hydroxylamine</td>
<td>g2891a</td>
<td>G964D</td>
<td>CT</td>
</tr>
<tr>
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<td>hydroxylamine</td>
<td>c506t</td>
<td>P169L</td>
<td>NT</td>
</tr>
<tr>
<td>m184</td>
<td>hydroxylamine</td>
<td>g692a</td>
<td>S231N</td>
<td>NT</td>
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<td>D516G</td>
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<td></td>
<td></td>
<td>t2512c</td>
<td>F838L</td>
<td>CT</td>
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<td>t2801c</td>
<td>I934T</td>
<td>CT</td>
</tr>
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<td>V389A</td>
<td>S2</td>
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<td>epPCR</td>
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<td>E277V</td>
<td>NT</td>
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<tr>
<td></td>
<td></td>
<td>a2023c</td>
<td>N675H</td>
<td>CT</td>
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<tr>
<td></td>
<td></td>
<td>a2837g</td>
<td>E946G</td>
<td>CT</td>
</tr>
</tbody>
</table>
Chapter 3:

Electrophysiological Analysis of TRPV4 Mutants
3.1 INTRODUCTION

3.1.1 TRPV4 general properties

Much study has focused on the gating and regulation of the TRPV4 channel. In this chapter we probe the functional properties of the TRPV4 channel through electrophysiological analysis of the mutants isolated in the screen described in the previous chapter. First we will review what is currently known about the mechanisms of activation and regulation of TRPV4.

As outlined in Chapter 1, the TRPV4 channel is expressed in a wide variety of tissues; evidence suggests that this channel is a homotetramer and does not heteromultimerize with other TRPV isoforms. Channel activation results in a very weakly calcium-selective cation current characterized by a gently outwardly rectifying I-V relation (Figure 3.1.1) that can be blocked by ruthenium red (Voets et al., 2002; Ramsey et al., 2006; Plant and Strotmann, 2007).

3.1.2 TRPV4 is activated by a range of disparate stimuli

The TRPV4 channel is activated by hypotonic stimulation (cell swelling); moderate heat; and chemicals including arachadonic acid, endocannabinoids, and phorbol ester derivatives such as 4αPDD (Figure 3.1.1). Different channel regions have been implicated in the mechanism of action of these activators (see Figure 3.1.2 for particularly instructive mutations, discussed further in this section).

3.1.2.1 Response to osmotic changes

The first evidence of TRP channel osmosensitivity came from study of the OSM-9 protein in C. elegans: mutation of the osm-9 gene resulted in worms that no longer avoided areas
of high osmotic strength (Colbert et al., 1997). In mammals, TRPV4 was identified as the OSM-9 homologue – increase of extracellular osmolarity (hypertonicity) reduced TRPV4 activity in cultured cells while decrease of extracellular osmolarity (hypotonicity) increased channel activity (Strotmann et al., 2000; Liedtke et al., 2000) as measured by intracellular calcium increase.

The ability of TRPV4 to respond to cell swelling did not seem to be mechanical in nature, as activation could not be achieved by applying pressure to the cell membrane in cell-attached patch recordings (Strotmann et al., 2000). In fact, hypotonic stimulation was found to activate TRPV4 through a cell-signaling cascade involving arachidonic acid (Watanabe et al., 2003a). Cell swelling activates phospholipase A₂ (PLA₂), which catalyzes the release of arachidonic acid from membrane phospholipids and exposes it to hydrolysis by cytochrome P450 epoxygenase (EPG), resulting in formation of epoxyeicosatrienoic acids (EETs) that appeared to directly activate the TRPV4 channel, as application of EETs to cell-free inside-out patches induced single-channel activity (Watanabe et al., 2003a). What remains unclear is how PLA₂ is activated by cell swelling, or where EETs bind to the channel and how they trigger activation.

In direct contrast, a recent study has favored the model of TRPV4 being directly activated by mechanical stretch forces. Activation of TRPV4 in excised Xenopus oocyte membrane patches was observed upon application of pipette suction, with an increase in open probability as suction pressure was increased; this response was unaffected by inhibitors of PLA₂ or EPG (Loukin et al., 2010b).

3.1.2.2 Activation by mechanical stimuli

Loukin et al were not the first group to maintain that TRPV4 could be a direct mechanosensitive channel. A knockout mouse lacking the TrpV4 gene showed reduced
sensitivity to pressure applied to the tail as well as an almost two-fold increase in the threshold pressure required to activate the femoral nerve (Suzuki et al., 2003). In direct contrast to Strotmann’s observation (Strotmann et al., 2000), Suzuki et al. could demonstrate activation of TRPV4 by membrane stretch: in CHO cells, current was induced via inflation of cell volume by a factor of approximately 1.5 through application of positive pressure through a patch pipette (Suzuki et al., 2003). In another study expression of mammalian TRPV4 in ASH sensory neurons of *C. elegans* containing a defective *osm-9* gene was able to restore avoidance of mechanical stimulus (nose touch), high osmolarity, or noxious odors (Liedtke et al., 2003).

It is difficult to disentangle studies of osmotic response and mechanical response of the channel, as osmotic changes cause physical deformation to cells. For example, PLA$_2$ activation by hypotonicity could be a result of a mechanical process (such as unfolding of the plasma membrane leading to release of previously sequestered activating factors). If the TRPV4 channel was mechanically gated it would need to be in some way linked to an element of force transduction such as the cytoskeleton or extracellular matrix. Association of TRPV4 with actin was suggested by time- and space- correlated single photon counting imaging experiments (Ramadass et al., 2007) as well as laser scanning microscopy and FRET (Becker et al., 2009). A functional interaction was suggested with the finding that treatment of CHO cells exogenously expressing TRPV4 with the actin-destabilizing reagent latrunculin A disrupted membrane co-localization of TRPV4 and F-actin, and abolished response of the cells to hypotonic stress (Becker et al., 2009).

More compelling evidence has recently emerged of functional interaction between TRPV4 and cytoskeletal elements. Pull-down experiments demonstrated that the C-terminus of TRPV4 directly interacts with soluble or polymerized actin and tubulin in a complex that also
contains PKCε and CamKII (which are known to be involved in nociceptive signaling). TRPV4 stabilized microtubules in vitro even in the presence of depolymerizing agents. In vivo, TRPV4 and the actin and microtubule cytoskeleton exhibit bi-directional regulation: activation of TRPV4 results in microtubule disassembly (inducing morphological changes such as retraction of growth cones and restriction of neurite outgrowth), while stabilization of microtubules by Taxol significantly reduced Ca^{2+} influx activation. In this context, TRPV4 would serve as an integrator of mechanical (through associated cytoskeleton) and chemical (through associated kinases) stimuli in the cell (Goswami et al., 2010).

3.1.2.3 Activation by 4α-PDD

The strongest known exogenous activators of TRPV4 thus far are also the least physiologically understood – the 4α-phorbol ester derivatives.

Phorbol esters are a class of plant-derived compounds that promote tumor formation in mammals (Hecker, 1968). The response of mammalian cells to phorbol ester such as PMA (phorbol 12-myristate 13-acetate) was studied by multiple groups, and it was determined that the compounds bind and strongly activate PKC (protein kinase C), which is the gatekeeper to a multitude of cellular signaling cascades (Ashendel and Boutwell, 1981; Castagna et al., 1982; Nishizuka, 1984; Blumberg et al., 1984). Phorbol esters mimic the endogenous PKC activator diacylglycerol, produced by the degradation of PIP2 by phospholipase C (Nishizuka, 1984).

A class of 4α-phorbol ester derivatives was isolated, in which the hydroxyl group at C4 is in the α position instead of the β position. These 4α-phorbol ester derivatives do not activate PKC, and were long thought to be pharmacologically inactive compounds. However, Watanabe et al demonstrated that 4α-PDD (4α-phorbol 12,13-didecanoate) potently activates TRPV4
channels (EC$_{50}$ 0.2 µM) (Watanabe et al., 2002a), as does 4α-PMA (Strotmann et al., 2003). While the PKC-activating phorbol ester PMA (that is, 4β) does also stimulate TRPV4 activity, it is far less potent – the response is about 50 times less than that of 4α-PDD (Watanabe et al., 2002a). While an endogenous analog is still unknown, the robust activation of TRPV4 by 4α-PDD makes it an important tool in the study of the functional properties of the channel.

### 3.1.2.4 Activation by moderate heat

The ‘thermo-TRPs,’ TRPV1-TRPV4, are activated by heat, with each channel occupying a unique range of temperature sensitivity. While TRPV1 and TRPV2 are activated at noxious temperatures (>43°C and >53°C, respectively), TRPV4 is activated by moderate heat. When mammalian TRPV4 was heterologously expressed in *Xenopus* oocytes, the channel was activated by temperatures over 27°C (Güler et al., 2002). In HEK293 cells, one study saw an activation threshold temperature of 34°C (Güler et al., 2002) while another saw a threshold of 24°C (Watanabe et al., 2002b). The response of TRPV4 to temperature was dynamic, as evidenced by an increase in current as temperature was elevated up to 42°C at which point the channels became partially desensitized. The channel also became sensitized to heat, with repeated exposure resulting in a shift of the activation threshold to higher temperatures (Güler et al., 2002; Watanabe et al., 2002b). The importance of TRPV4 in temperature sensation was confirmed by the TRPV4$^{-/-}$ knockout mouse, which showed a preference for warmer floor temperatures and demonstrated a reduced sensitivity to moderately hot temperatures as measured by tail withdrawal from hot water (Lee et al., 2005a).
3.1.2.5 Activation pathways of cell swelling, heat, and 4α-PDD

The different activators of TRPV4 do not act in isolation: some act synergistically and others share common mechanisms of activation. Mutagenic analysis of TRPV4 has revealed certain residues and channel regions important in some modes of channel activation; Figure 3.1 highlights the mutations described below.

Even before it was determined that TRPV4 was gated by temperature, it was observed that ambient temperature had an effect on the response of the channel to osmolarity. One of the first observations upon cloning of the vertebrate TRPV4 channel was that response to hypotonicity was markedly increased at physiological temperatures (37°C for the rat channel, 40°C for the chicken channel) compared to room temperature (typically 22-24°C) (Liedtke et al., 2000). Indeed, temperature can modulate the response to all known TRPV4 activators: not only was there a significant increase in steady-state basal \([Ca^{2+}]_i\) levels at 37°C compared to room temperature (RT); but the response of the channel to osmotic stress, 4α-PDD, PMA, and shear stress was drastically elevated at 37°C (in the case of shear stress, there was no measurable response at RT) (Gao et al., 2003). Conversely, the response of the channel to temperature is affected by osmolarity. Exposure of TRPV4-transfected cells to a temperature of 45°C resulted in measurable calcium influx in isotonic (300 mOsm) solution; this influx was significantly reduced in hyperosmotic (410 mOsm) solution and significantly increased in hypo-osmotic (250 mOsm) solution (Güler et al., 2002).

A series of mutational analysis studies revealed more about the activation pathways of TRPV4. Deletion of the ankyrin repeat-containing N-terminal tail of TRPV4 abolished current activation by heat (Watanabe et al., 2002b) but not the ability of the channel to respond to hypotonicity (although it did slightly affect the time-course of activation) (Liedtke et al., 2000).
This was the first indication that heat and osmolarity act in distinct ways on the TRPV4 channel – this is consistent with the additive effect on channel activity of temperature and hypotonic stimulation. As discussed earlier, hypotonic stimulation of TRPV4 involves a pathway of PLA$_2$ activation and hydrolysis of arachidonic acid by cytochrome P450 epoxygenase. Vriens et al. demonstrated that inhibition of either of these enzymes rendered the TRPV4 channel unresponsive to hypotonic stimulation, but the channel could still be activated by heat or 4α-PDD (Vriens et al., 2004). A more recent study has revealed that PLA$_2$ inhibitors do not specifically inhibit HTS activation: given a long enough incubation period (oocytes were incubated with the PLA2 inhibitor bromophenacyl bromide for several hours), both HTS and 4α-PDD activation were inhibited (Loukin et al., 2010b). Response to heat was not tested in this study.

On the other hand, temperature and 4α-PDD do seem to share a common pathway of channel activation, distinct from that of osmolarity. The mutation of residue 556 in the N-terminal end of the third transmembrane helix (TM3) of TRPV4 from tyrosine to alanine (Y556A) severely impairs the ability of heat or 4α-PDD to activate the channel while having no effect on response to cell swelling (Vriens et al., 2004). Residue Y556 was targeted for mutagenesis because an earlier study of the TRPV1 channel found that a YS (tyrosine-serine) motif in the intracellular linker between TM2 and TM3 was involved in binding of the TRPV1 agonist capsaicin (Jordt and Julius, 2002). TRPV4 does not contain a YS motif in its TM2-TM3 linker but does in the very N-terminal end of TM3 (Y556, S557). The serine residue is not necessary for channel activation, but position 556 must contain an aromatic residue (tyrosine or phenylalanine) for the channel to be activated by 4α-PDD or heat (Vriens et al., 2004). Activation is severely impaired though not completely abolished – in the Y556A mutant, a five-
fold increase of 4α-PDD can still elicit a response (5 µM versus 1 µM for wild-type) (Vriens et al., 2007).

Residues in TM4 are also important for TRPV4 activation by 4α-PDD or heat, but not cell swelling. As in the study described above, these residues were targeted for study because of the homology to residues important for capsaicin binding of TRPV1. Mutation of leucine 584 (L584M) or tryptophan 586 (W586A), two hydrophobic residues located in the middle of TM4, severely decreased the sensitivity of the channel to 4α-PDD or heat but had no effect on activation by cell swelling. While mutation of the adjacent residue, methionine 587 (M587), had no measurable effect on response to 4α-PDD, the double mutant W586A, M587A displayed further reduced sensitivity to 4α-PDD than W586A alone suggesting that M587 does play a role in the interaction (Vriens et al., 2007).

Therefore, residues in both TM3 and TM4 mediate channel activation by 4α-PDD and heat, but not cell swelling. It is easy to imagine how this is the case for 4α-PDD, a chemical ligand that could fit in a binding site in the space between TM3 and TM4, created by the side chains of the residues identified in this study, in a manner similar to the binding of TRPV1 by capsaicin (Klausen et al., 2009). But how is thermal activation tied to these same residues? It has been hypothesized that thermal stimulation is transmitted by an endogenous lipid messenger that could bind the same site (Güler et al., 2002). In recordings from excised membrane patches TRPV4 can no longer be activated by heat, supporting the idea that a diffusible chemical messenger is involved (Watanabe et al., 2002b). TRPV1, in contrast, can still be activated by heat in excised patches (Tominaga et al., 1998).
3.1.3 **Calcium regulation**

Calcium is not only a permeant ion of the TRPV4 channel but also an important regulator of activity, playing a role in both channel activation and inactivation. Absence of extracellular calcium results in a marked reduction in spontaneous channel activity as well as much slower rates of channel activation by 4α-PDD or cell swelling (Strotmann et al., 2003); on the other hand, increase of either intracellular or extracellular calcium inhibits the channel in a voltage-dependent manner (Watanabe et al., 2002a; Voets et al., 2002), most likely as a feedback inhibition mechanism to prevent calcium overload. Figure 3.1 highlights mutations previously found to be important in calcium regulation, as we discuss further below.

The voltage-dependence of TRPV4 inhibition by calcium can be seen in the outward rectification of the I-V curve; \([\text{Ca}^{2+}]_e\) blocks the channel through binding of residue D682 in the pore (Voets et al., 2002). As \([\text{Ca}^{2+}]_e\) is increased the amplitude of the 4α-PDD elicited current decreases, and the current decays more rapidly. A residue in TM6 may play a role in this effect: introduction of mutation F707A leads to a slowing of the current decay even in the presence of extracellular calcium (Watanabe et al., 2003b). Increase of intracellular calcium has been shown to inhibit the channel with an IC$_{50}$ of about 400 nM, a physiologically relevant range (Watanabe et al., 2002a).

The mechanisms of calcium-dependent potentiation have been better characterized and involve a calmodulin (CaM)-binding domain on the C-terminal tail of the channel, which has shown to bind calmodulin in a calcium-dependent manner. The calmodulin-binding site is an alpha-helical region of about 20 residues, beginning at residue 814. Mutation of residues necessary for calmodulin binding (such as W222A, or truncation of the channel at K801 upstream of the domain) eliminated the ability of calcium to potentiate a current stimulated by
4α-PMA, and also resulted in a slowing of the inactivation rate by 4α-PMA or hypotonic shock (Strotmann et al., 2003).

Interestingly, this region of the channel has exactly the opposite effect on the TRPV6 channel in which it plays a role in slow channel inactivation; mutations similar to those that eliminate calcium potentiation of current in TRPV4 reduce inactivation in TRPV6 (Niemeyer et al., 2001). In addition, Ca^{2+}-CaM binding in TRPV6 is mediated by phosphorylation: PKC-mediated phosphorylation of a threonine residue in the CaM binding domain, while mutation of consensus sequences for protein-serine/threonine kinase phosphorylation in TRPV4 has no effect on channel activity (Strotmann et al., 2003).

Recent evidence has shed new light on the manner of Ca^{2+}-CaM activation of TRPV4. In low-calcium conditions, the CaM-binding site on the C-terminus forms a strong interaction with an area of the N-terminal tail (residues 117-136). Binding of Ca^{2+}-CaM to the C-terminus disrupts this interaction and facilitates current potentiation. Mutations that disrupt the interaction of the N- and C-terminal domains results in a channel that constitutively potentiates 4α-PMA-stimulated currents, supporting the hypothesis that the inter-channel interaction is an auto-inhibitory structure (Strotmann et al., 2010).

### 3.1.4 Electrophysiological analysis of TRPV4 mutants to uncover regulatory elements of the channel

In Chapter 2 we detailed a gain-of-function screen to identify mutations that would rescue the ability of the V4-K70E/M605T channel to rescue growth of trk1Δ trk2Δ yeast on low potassium media. This variant of mammalian TRPV4 contained two mutations, K70E at the beginning of the N-terminus and M605T on the intracellular linker between the S4 and S5
transmembrane domains, and has lost the ability to rescue low potassium growth or induce salt
sensitivity in the way that wild type TRPV4 can.

We identified mutations in three regions of the V4-K70E/M605T channel (N-terminus, pore-lining helix TM5, C-terminus) that gave different phenotypic profiles – mutants rescued low potassium growth to varying degrees, and induced salt sensitivity to different degrees in a way that did not always correlate with the low potassium rescue phenotype. To determine the mechanisms of action of these mutants, we heterologously expressed the channels in *Xenopus* oocytes and studied the electrophysiological properties of the channels in response to the channel activators described above, most of which can be easily applied to *Xenopus* oocytes. From this we would gain understanding of how the mutations were affecting channel activity at the mechanistic level – were they changing the open probability, conductance, or permeability of the channel?
3.2 MATERIALS AND METHODS

3.2.1 Cloning constructs to pGEMHE2

All TRPV4 wild type and mutant channels discussed in the previous chapter were cloned into the oocyte expression vector pGEMHE2_yu (generous gift of Yong Yu, Yang lab), a variant of the pGEMHE plasmid originally designed by the Hess lab at Harvard Medical School (Liman et al., 1992) from the backbone pGEM-3Z (Promega, Madison WI). The vector multiple cloning site (MCS) is flanked by 5’- and 3’ untranslated regions (UTRs) from the Xenopus β-globin gene to stabilize the RNA when it is injected into the oocytes leading to increased expression (Krieg and Melton, 1984), as well as a T7 promoter for in vitro RNA transcription. The genes were sub-cloned from the yeast vector to the oocyte vector: inserts were generated by 5’ digestion with SalI and 3’ digestion with XhoI, and ligated via T4 ligase to pGEMHE2 digested with SalI in the MCS using (all enzymes New England Biolabs, Ipswich MA). Correct orientation was confirmed by restriction digest analysis.

3.2.2 RNA synthesis

cRNA was synthesized through in vitro transcription using T7 polymerase (New England Biolabs). For each reaction 10-15 µg of vector was linearized just downstream of the 3’UTR with NheI (New England Biolabs), purified through phenol (pH 8.0)/chloroform extraction and precipitated with sodium acetate and ethanol. To this was added the following components for a final reaction volume of 50 µL in diethylpyrocarbonate-treated water (DEPC, Sigma): 10 mM G(PPP)G RNA Cap structure analog (New England Biolabs), 100 mM rNTP mix (containing equal proportion of rATP, rGTP, rCTP, rUTP; Roche), 100 mM dithiothreitol (DTT, Sigma),
50 mM MgCl₂ (Sigma), 1X T7 RNA polymerase transcription buffer (New England Biolabs), 100 U T7 RNA polymerase (New England Biolabs), and 15 U RNase inhibitor (Invitrogen). The reaction was incubated at 37°C for 2-2.5 hours. RNA was purified out through phenol (pH 5.2)/chloroform extraction and sodium acetate/ethanol precipitation and resuspended in DEPC-H₂O; concentration was determined by formaldehyde agarose gel electrophoresis.

### 3.2.3 Oocyte preparation and injection of RNA

Adult oocyte-positive female *Xenopus laevis* (Xenopus I, Xenopus Express, or Nasco) were anesthetized with tricaine methanesulfonate (Sigma) in accord with IACUC regulations. Ovarian lobes were removed and mature stage V-VI oocytes were isolated through defolliculation treatment with 2 mg/mL collagenase (Roche) under 200 rpm shaking in OR2 solution (84.2 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.6 with NaOH) for 1-1.5 hours or until about 80% of oocytes were free of follicular membrane. Oocytes were then rinsed and stored in ND96 solution (96 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, pH 7.6 with NaOH). For some studies of calcium-dependence of channel function, CaCl₂ was omitted from the ND96 solution.

cRNAs (typical concentration of 0.5 µg/µL) were injected into defolliculated oocytes using a Nanojet system (Drummond Scientific) which delivered approximately 50 nl per oocyte. Injected oocytes in ND96 solution were incubated at 18°C for 2-5 days before recordings.
3.2.4 Electrophysiology

Two-electrode voltage-clamp (TEVC) was utilized to measure channel function in the oocyte. Borosilicate electrodes (Warner Instruments, Hamden CT) with a resistance of 1-10 MΩ were filled with 3 M KCl and used to apply voltage clamp to the oocyte as it rested in a perfusion chamber at room temperature (approximately 22°C). Electrophysiological data were acquired with the Oocyte Clamp OC-725C (Warner Instruments, Hamden CT) head-stage and amplifier interfaced to a computer via a DigiData 1200 Series Interface (Axon Instruments) to convert the signal from analog to digital; data were recorded using Clampex 8.1 (Axon Instruments). Current was evoked every 2 s by a 70 ms test voltage stepped from -100 mV to +100 mV in 10 mV increments from a holding potential of -60 mV. This 42 s protocol was repeated over the course of minutes as different bath solutions were perfused, so that the I-V (current-voltage) relationship could be analyzed over time. Two variations of this protocol were used: a pre-pulse protocol in which a 30 mV pre-pulse was evoked for 70 ms before the voltage steps from -100 mV to +100 mV, and a positive potential protocol in which the voltage steps occurred only in the positive range (+20 mV to +100 mV).

Data were analyzed with Clampfit 8.1 (Axon Instruments): the peak current at each voltage was obtained for each run over the length of the experiment. These values were imported to Microsoft Excel and graphed. The time-course of channel activity was demonstrated by plotting the peak current at -100 mV and +100 mV over time. Data are represented as mean ± S.E.M (standard error of mean). Significance was determined by unpaired two-tail student t-test. In all figures, only statistically different (p < 0.05 or better) pair-wise comparisons within a given group are indicated.
3.2.5 Electrophysiology Solutions

Standard sodium bath solution consisted of 70 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 5 mM HEPES (pH = 7.4 with NaOH). Osmolarity of the solution was adjusted to 215 mOsm by addition of mannitol, measured with a VAPRO 5520 vapor pressure osmometer (Wescor an Elitech Group, Logan UT).

To test the effect of calcium, solutions were made with varying levels of CaCl₂: 0 mM, 0.1 mM, 1 mM, 2 mM.

To test the selectivity of the channel, bath solutions were modified to test one permeant ion at a time: in potassium bath solution KCl was increased to 70 mM and all NaCl was omitted (pH 7.4 with KOH), in N-methyl-D-glucamine (NMDG) bath solution all NaCl and KCl was omitted and replaced with 35 mM NMDG-Cl.

To prevent contamination by calcium-activated chloride currents chloride was replaced with sulphate, which is not only larger than chloride but also blocks the calcium-activated chloride channels (Hartzell et al., 2005). For the sodium bath solution, 70 mM NaCl was replaced by 35 mM Na₂SO₄ and CaCl₂ was replaced with the equivalent amount of CaSO₄ (KCl and MgCl₂ were not changed). Potassium bath solution contained 35 mM K₂SO₄ and 2 mM KCl (sodium omitted); NMDG bath solution contained 35 mM NMDG₂SO₄ (sodium and potassium omitted).

To test effect of osmolarity on the channel, all solutions could be made isotonic (215 mOsm as above), hypertonic (300 mOsm), or hypotonic (145 mOsM) by addition or omission of mannitol.
3.3 RESULTS

3.3.1 TRPV4 exhibits basal activity but no response to hypotonic stimulation

Electrophysiological properties of wild type and mutant variants of TRPV4 were studied using two-electrode voltage clamp (TEVC). The current-voltage (IV) relation was measured from -100 mV to +100 mV in 10 mV steps; this protocol was repeated over time as the oocyte was exposed to various solutions. Peak currents at -100 mV and +100 mV were plotted over time to yield a time-course of channel activity.

3.3.1.1 Wild type TRPV4 exhibits basal activity in standard solution while V4-K70E/M605T does not; V4-ET-M625I/H787Y rescues basal activity

Activity of wild type TRPV4, V4-K70E/M605T (V4-ET), and V4-ET-M625I/H787Y channels were measured in standard isotonic (215 mOsm) bath solution with sodium as the major permeant ion. V4-ET-M625I/H787Y was chosen for the initial characterization because it was the clone from the mutagenesis screen that produced the strongest rescue of yeast growth on low potassium media.

Wild type TRPV4 exhibits significant basal activity in standard bath solution, while V4-K70E/M605T produces barely any measurable current. The double mutant V4-ET-M625I/H787Y rescues this phenotype, exhibiting a level of basal activity even higher than that of wild type TRPV4 (Figure 3.3.1.1). It must be noted that the left side of this I-V relation is dampened due to the presence of ruthenium red. In preliminary experiments we observed that oocytes injected with the V4-ET-M625I/H787Y channel, and sometimes wild type TRPV4, quickly became sick with most dying within 1-2 days. Reasoning that the activity of theses
channels could be toxic to the cells, we began to incubate the oocytes in the known TRP channel blocker ruthenium red (3 µM) to block channel activity until the day of recording. Ruthenium red specifically blocks inward currents in TRP channels, so this prevented the cells from dying from cation overload. However, it was impossible to ever fully wash the ruthenium red out of the oocyte, so currents at negative potentials were dampened.

3.3.1.2 No significant response to hypotonic shock seen

TRPV4-expressing oocytes were subjected to hypotonic stimulation (HTS) through perfusion of a hypotonic bath solution (145 mOsm), which previous groups have demonstrated results in increased channel activity (Strotmann et al., 2000; Liedtke et al., 2000). However, a significant effect was not seen for wild type TRPV4, V4-K70E/M605T, or V4-ET-M625I/H787Y (Figure 3.3.1.2b). This could be due to the fact that we performed these experiments at room temperature: multiple groups have shown that the response to HTS is temperature-dependent, with a much more robust response seen at 37°C than room temperature (Liedtke et al., 2000; Gao et al., 2003). As before, these experiments were performed in the presence of ruthenium red, so the current at -100 mV is dampened.

3.3.2 Response of TRPV4 to calcium in standard solution

Next, we studied the response of the channel to calcium. Oocytes were perfused with standard isotonic solution (with NaCl as the major permeant ion) lacking calcium, then switched to standard isotonic solution containing calcium (CaCl₂, 0.1 mM or 1.0 mM). I-V relations were measured for upwards of ten minutes, until current reached steady state. For these experiments
the concentration of RNA injected was optimized to allow for oocyte survival without the addition of ruthenium red, so we could see the full I-V relation including the negative potentials.

### 3.3.2.1 Calcium application leads to increased sodium current in wild type TRPV4 but not V4-K70E/M605T; V4-ET-M625I/H787Y rescues phenotype

As seen in Figure 3.3.2.1, application of 1 mM CaCl₂ resulted in a large increase of both inward and outward current of wild type TRPV4, suggesting that calcium increases channel activation. V4-K70E/M605T did not exhibit significant calcium activation. The double mutant V4-K70E/M605T-IY rescued calcium activation of V4-K70E/M605T, exhibiting very large sodium current upon calcium application.

### 3.3.2.2 Calcium must enter the cell to induce current increase

Calcium must enter the cell to induce an increase in current, suggesting that calcium is acting on the intracellular side of the channel. This was tested in two ways. First, BAPTA, a calcium chelator, was injected into the oocytes before recording; after this treatment, no calcium-activated current was observed in wild type TRPV4, V4-K70E/M605T, or V4-ET-M625I/H787Y (Figure 3.3.2.2a). Unfortunately, it is very difficult to completely wash BAPTA out of the oocyte, so separate oocytes had to be used to test BAPTA versus BAPTA-free control. Therefore a second method was used to confirm these findings, utilizing a positive voltage protocol: from a holding potential of +20 mV, voltage was stepped in 10 mV increments from +20 mV to +100 mV (versus control protocol in which voltage is stepped from -100 mV to +100 mV, from a holding potential of -60 mV). At these positive voltages, the driving force does not favor calcium entering the cell. Application of calcium to the bath does not result in current increase during the
+20 - +100 mV protocol, but does when the protocol is switched back to the -100 mV to +100 mV range for the same oocyte (Figure 3.3.2.2.b).

3.3.2.3 Calcium-activated chloride current creates false magnitude of response

A problem with the standard bath solution is that the permeant ion is NaCl, so as sodium enters the cell through our channels, so does chloride. *Xenopus* oocytes contain a large number of calcium-activated chloride channels—when calcium enters the oocyte, these channels are activated and trigger a release of chloride from intracellular sources (endoplasmic reticulum). Therefore, in the previous experiments it is possible that what looks like increased sodium influx/efflux through the channel could actually be chloride.

To test this we used a channel that has been well characterized in our lab: the P/Q-type voltage-gated calcium channel (VGCC). It is a long-established observation that calcium entry through these channels activates chloride channels; therefore, experimental recordings are usually performed with barium (Ba\(^{2+}\)) as the permeant ion instead of Ca\(^{2+}\). A protocol was used in which a +30 mV pre-pulse was delivered to the cell, the voltage at which the inward driving force for Ca\(^{2+}\) through the VGCC is at its strongest. We then observed the I-V relation of the channel.

In Figure 3.3.2.3a, the left panel shows the typical I-V relation for a P/Q channel with 10 mM BaCl\(_2\) as the permeant ion. The right panel shows the P/Q channel in our standard NaCl bath solution with 10 mM CaCl\(_2\): this I-V relation looks quite similar to the recordings of TRPV4 (refer to part (b) of same figure), with a steep current increase at positive voltages. When the bath solution is switched to the mostly chloride-free solution with Na\(_2\)SO\(_4\) as the permeant ion, this current all but disappears. In the recordings of TRPV4-expressing oocytes this had been
thought to represent outward flow of calcium, but now it is apparent that it is inward flow of chloride.

Therefore, the bath solutions were modified to eliminate chloride as much as possible – chloride was replaced where possible by the larger anion sulphate (NaCl replaced by Na₂SO₄, etc; see Materials and Methods). In recordings done with Na₂SO₄ as the permeant ion, current increase is still seen upon calcium application, albeit on a smaller scale since the contaminating chloride current has been eliminated (Figure 3.3.2.3c). The results we obtained with NaCl held up: TRPV4 showed calcium-activated current increase while V4-K70E/M605T did not (Figure 3.3.2.3c). We could now proceed with electrophysiological analysis of the mutants obtained in the yeast screen.

3.3.3 Effect of mutants from screen on calcium-activated sodium current in V4-K70E/M605T and wild type TRPV4

Since the calcium-activated sodium current seemed to be a robust measure of channel activity, this was used as an assay to determine the effects of the various mutants isolated in the yeast screen on the TRPV4 channel. All experiments in this section were performed using the sulphate bath solutions (Na₂SO₄ instead of NaCl, etc.). Refer to Figure 3.3.3 for the location in the proposed transmembrane topology of the mutations of TRPV4 tested in this section.

3.3.3.1 Loss of calcium-activated sodium current in V4-K70E/M605T mainly caused by the K70E mutation

As shown in earlier figures, wild type TRPV4 exhibits a large calcium-activated sodium current that is eliminated in the V4-K70E/M605T channel containing mutations in the N-
terminus (K70E) and intracellular S4/S5 linker (M605T). The two mutations were tested separately to assess their relative contributions to the loss of the current (Figure 3.3.3.1). In the V4-K70E channel the calcium-activated sodium current was basically eliminated while V4-M605T still exhibited calcium-activated current. This is consistent with the finding in the yeast assay that mutation K70E is largely responsible for abolishing the ability of the channel to rescue growth on low potassium.

3.3.3.2 Double mutant M625I/H787Y rescues calcium-activated sodium current in V4-K70E/M605T

As expected from its ability to rescue yeast growth on low potassium, the double mutant channel M625I/H787Y strongly rescues the calcium-activated current in V4-K70E/M605T (Figure 3.3.3.2a).

In yeast, V4-ET-M625I/H787Y causes a rescue of yeast growth that is actually more robust than that of wild type TRPV4. We could not quantitate these TEVC recordings in a way that would directly compare comparable expression levels of TRPV4 versus V4-ET-M625I/H787Y, since the RNA of V4-ET-M625I/H787Y had to be diluted 20-fold compared to the other channel RNAs to retain viable oocytes. In addition, in general the variation in current is very large between batches due to such factors as the quality of oocytes, amount of RNA injected, and level of expression. However, the mere fact that V4-ET-M625I/H787Y causes such a strong lethality compared to wild type TRPV4 and produces a robust current even when expressed at much lower levels is a strong indication that the channel has a greatly increased activity compared to wild type TRPV4. Because the response of TRPV4 and V4-ET-M625I/H787Y was so strong, calcium-activated current was elicited through perfusion of 0.1
mM Ca\textsuperscript{2+} instead of 1 mM Ca\textsuperscript{2} to allow for longer recordings as the oocyte would not get sick as quickly.

The two mutations in V4-ET-M625I/H787Y were tested separately, and either V4-K70E or V4-M605T alone was able to rescue the calcium-activated sodium current (Figure 3.3.3.2b), although not to the level of the double mutant: the calcium concentration had to be increased from 0.1 mM back to 1 mM to see a measurable effect, and neither single mutant channel caused oocyte lethality at the level of the double mutant.

3.3.3.3 N-terminal mutations only slightly rescue calcium-activated sodium current in V4-K70E/M605T

Two clones isolated in the V4-K70E/M605T yeast mutagenesis screen had mutations in the same region of the N-terminus: the single mutant L154F and the triple mutant R151W/P152S/L154F. Both of these clones only very weakly rescued the yeast growth phenotype in V4-K70E/M605T.

Similarly, these mutations only weakly rescued the calcium-activated current in V4-K70E/M605T (Figure 3.3.3.3a). The single mutants V4-ET-R151W and V4-ET-P152S did not show any measurable rescue; the single mutant V4-ET-L154F and the triple mutant V4-ET-R151W/P152S/L154F showed a weak rescue but it was quite variable. The triple mutant more reliably showed a measurable calcium-activated current, although often it was small. In contrast, for the single mutant V4-ET-L154F we only isolated one oocyte that showed a robust calcium-activated current (about 10 µA); most of the oocytes tested had no measurable calcium-activated current. Figure 3.3.3.3b compares the triple mutant V4-ET- R151W/P152S/L154F to wild type TRPV4, to provide context for how weak the rescue of the phenotype is.
3.3.3.4 C-terminal truncations do not rescue calcium-activated sodium current in V4-K70E/M605T, and eliminate it in wild type TRPV4; effect of other mutants not extensively tested in wild type TRPV4

The two C-terminal truncations isolated in the mutagenesis screen, Q790Δ and W788Δ, had a phenotype unique to our other mutants. In V4-K70E/M605T the C-terminal truncations enabled the channel to very strongly rescue the low potassium growth phenotype, but did not cause any salt sensitivity; when introduced into wild type TRPV4, the channel could still rescue low potassium growth but the ability to induce severe salt sensitivity was completely abolished.

In oocytes, we first tested the effect of the C-terminal truncations on calcium-activated current in V4-K70E/M605T, and saw no rescue of current (Figure 3.3.3.4a). Strikingly, introduction of either C-terminal truncation into wild type TRPV4 completely abolished the calcium-activated current (Figure 3.3.3.4b), consistent with the finding that the C-terminal truncations abolished the ability of TRPV4 to induce salt sensitivity in yeast.

In the yeast assay, some mutants when introduced into TRPV4 seemed to increase activity of the channel, resulting in a stronger rescue of low potassium growth. Due to the variability of channel expression in oocytes as described earlier, we could not determine from these recordings if this was also the case for level of calcium-activated current. All we can conclude is that TRPV4 still exhibits robust calcium-activated current when the mutations M625I/H787Y, or the N-terminal mutations (R151W/P152S/L154F), are introduced into the wild type channel (data not shown) – the ability to cause a loss-of-function in wild type TRPV4 is unique to the C-terminal truncations.
3.3.4 Response of channels to 4α-PDD is weak or absent

We also attempted to stimulate channels by application of the phorbol ester derivative 4α-PDD at concentrations of 1 µM or 10 µM, but did not see any response (data not shown). This was surprising as previous studies had shown 4α-PDD to be a potent channel activator (Watanabe et al., 2002a) but it is quite possible that, as mentioned previously for hypotonic stimulation (HTS), temperature could play a role: Gao et al. demonstrated that the magnitude of either HTS or 4α-PDD was much higher at 37°C than room temperature (Gao et al., 2003). Unfortunately, we did not have the ability to control temperature of perfusate in our TEVC system; all experiments were performed at room temperature.

In a similar mutagenesis study performed by Loukin et al. that will be discussed further at the end of this chapter, TRPV4 activation by 4αPDD was only possible after oocytes were pre-exposed to agonist (3 µM) for about 20 minutes. (Loukin et al., 2010a).

3.3.5 Ion selectivity

Wild type TRPV4 showed basal current in standard bath solution (with sodium as the major permeant ion) while the polymorphic channel V4-K70E/M605T did not; the double mutant V4-ET-M625I/H787Y showed rescue of basal current to a level higher than that of wild-type TRPV4. To determine if these differences in channel activity were the result of differences in ion selectivity between the channels (as opposed to a change in gating properties), we compared the reversal potential of the channels in bath solutions containing as the major permeant ion sodium, potassium, or NMDG. N-methyl-D-glucamine is an organic monovalent cation of large size that is commonly used as a substitute for Na⁺ or K⁺ as it does not permeate most ion channels (Heinemann et al., 1992; Villarroel et al., 1995).
Figure 3.3.5a compares the I-V relations of wild type TRPV4 and V4-ET-M625I/H787Y in Na\(^+\), K\(^+\), or NMDG. There is no change in the reversal potential when switching between permeant ions, indicating that the channels are largely cation non-selective. The reversal potential is the same for wild type TRPV4 and V4-ET-M625I/H787Y. Figure 3.3.4b shows that the V4-K70E/M625T channel shows no basal activity regardless of the available cation. Thus, it is likely that the M625I/H787Y mutations cause a change in the activation/gating of the V4-K70E/M605T channel.
3.4 DISCUSSION

3.4.1 Use of two-electrode voltage clamp (TEVC) to assess function of TRPV4 mutants

In the yeast assay system, we could assess if the presence of the TRPV4 channel (wild-type or mutated) had a measurable effect on the growth of different yeast strains – if it could rescue the ability of the trk1Δ trk2Δ strain to grown without potassium supplementation, and if it could cause increased salt sensitivity in the wild-type BY4741 yeast strain. These measures of channel activity are indirect, and do not tell us anything about the nature of the change from a mechanistic standpoint – is the channel open more often, does the channel have increased conductance, has there been a change in the selectivity of the channel, etc. Therefore, we followed up on those findings by studying the properties of the channel through electrophysiological analysis.

Two-electrode voltage clamp (TEVC) of channels heterologously expressed in *Xenopus* oocytes has many technical advantages, as well as some drawbacks. TRPV4 channel activity has previously been confirmed in injected *Xenopus* oocytes (Güler et al., 2002); later we will discuss more recent studies of TRPV4 that also utilize TEVC (Loukin et al., 2010a; 2010b). Using TEVC, channel function can be screened fairly rapidly in a large number of oocytes. The current-voltage (I-V) relation of the channel can be measured, which provides information about reversal potential and overall channel activity. Channel activity can be monitored over time as various voltage protocols are applied, and various reagents perfused over the cell. Electrodes can remain in the oocyte for long periods of time with little to no damage to oocyte health, so that activity of the channel can be measured over long time-courses. Finally, in using mammalian cells one would have to contend with the activity of the endogenously expressed channel in the
cell line; the chance of the *Xenopus* oocyte containing a homolog to the mammalian channel being studied is far reduced.

There are some drawbacks of TEVC – for example, there is much variability in channel RNA expression level between batches of oocytes, making it difficult to establish exact levels of channel activity over large numbers of samples. Nonetheless, TEVC can be used to build to our knowledge of the activity of the TRPV4 mutants isolated in our screen.

### 3.4.2 Inability to recreate TRPV4 activity patterns of previous studies; novel calcium-activated current characterized

In previous studies of TRPV4 very little basal activity was seen when channels were heterologously expressed, but a gently outwardly rectifying I-V relation was seen in response to heat, hypotonic stimulation, or 4αPDD (Watanabe et al., 2002a; Voets et al., 2002; Vriens et al., 2004; Ramsey et al., 2006). In our preparation, however, we observed a large basal current for the wild-type TRPV4 (TRPV4). Only slight increase in current was seen when the cell was exposed to hypotonic solution, and there was no apparent activation by 4αPDD; this could be because channels expressed in oocytes were already maximally active at basal conditions, meaning application of activators would have little further effect.

It is possible that this discrepancy is due to differences in the experimental preparation: the majority of studies utilized recordings from mammalian cell lines transfected with TRPV, such as human embryonic kidney (HEK293) cells or Chinese hamster ovary (CHO) cells. One study used TEVC recordings from *Xenopus* oocytes (Güler et al., 2002); our standard bath solution was based on theirs, the composition of which (in mM) was 96 NaCl, 2 KCl, 1 MgCl₂, 0.1 CaCl₂, and 5 HEPES, adjusted to pH 7.4 with NaOH. Our only modification was to lower the
NaCl to 70 mM in order to allow for more addition of mannitol for studies of the effects of osmolarity. Guler et al., however, do not observe a notable basal current at room temperature. The studies performed in transfected mammalian cells were performed with bath solutions that were similar in composition, but with ion concentrations specific to the known properties of the cell type; for example, Watanabe et al. perfused HEK cells with a bath solution containing (in mM) 150 NaCl, 6 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH.

When we realized that the standard bath solution was leading to activation of calcium-activated chloride currents, the solutions were modified by replacing as much chloride as possible with sulphate (Na₂SO₄ instead of NaCl, etc). Even in these conditions, significant ‘basal’ sodium current was observed for wild type TRPV4; the reversal potential shifted to a slightly positive value. Hypotonic stimulation was not tested under these conditions but would be expected to be minimal since the channel is already basally active.

We discovered that the large sodium current observed in the standard bath solution seemed to be mainly due to the presence of calcium – when the 0.1 mM CaCl₂ was removed from the solution, the current was greatly reduced. As discussed in the introduction to this chapter, calcium is known to be important for channel activity (Strotmann et al., 2003). It is curious that previous studies would not have seen this large calcium-induced cation current, as all of their solutions contained CaCl₂ as ours did (including the study by Guler et al. that was performed in oocytes). Nevertheless, this calcium-activated sodium current afforded a reliable way to compare the activity of the TRPV4 channel mutants. Wild type TRPV4 exhibited a robust calcium-activated sodium current, while the polymorphic channel V4-K70E/M605T did not. We tested the ability of mutants isolated in Chapter 2 to rescue the calcium-activated current in V4-
K70E/M605T, and tested if they would have any effect on the wild type TRPV4 channel. Although we only have preliminary data at this point, they are in line with what was observed in the yeast assays and help us to formulate hypotheses about the functional significance of these mutations on channel activity.

Regarding the response of the channels to 4αPDD, it is surprising that no response was observed to this well-characterized potent channel agonist. We initially applied 1 µM of 4αPDD because this was the concentration used in previous studies; however, those studies were performed in HEK293 cells ((Watanabe et al., 2002a; Voets et al., 2002), and others). We increased the concentration to 10 µM and still saw no effect. A recent study was finally able to demonstrate activation of TRPV4 channels in *Xenopus* oocytes by 4αPDD; however, they found that this was only possible when the oocytes were pre-exposed to the 4αPDD (3 µM) for about 20 minutes (Loukin et al., 2010a). In addition, the activation they saw was slow and irreversible, unlike the action of 4αPDD on TRPV4 cells in mammalian culture where currents inactivate rapidly (Watanabe et al., 2003b).

### 3.4.3 Channel mutants affect a calcium-activated sodium current; results recapitulate yeast growth assays

The calcium-activated sodium current was used as an assay to compare activity of V4-K70E/M605T and mutants created in the screen of V4-K70E/M605T to the activity of wild type TRPV4, and assess how their relative activity profiles compared to what had been seen in the yeast assays. Table 3.4.3 summarizes the findings from all three assays.

Wild type TRPV4 exhibited a significant and reproducible calcium-activated current, which was lost in the polymorphic channel V4-K70E/M605T containing the substitutions K70E
(N-terminus) and M605T (S4/S5 linker); this mirrors our observations in yeast, where V4-K70E/M605T loses the ability to rescue low potassium growth or induce salt sensitivity. These polymorphisms were tested separately and we saw that elimination of the calcium-activated current was mainly due to the K70E mutation: V4-K70E had no calcium-activated current, while V4-M605T exhibited calcium-activated current. This was consistent with what was seen in the yeast assays, where K70E almost entirely eliminated rescue of low-potassium growth and eliminated salt sensitivity while M605T retained function in both assays, albeit at a level slightly lower than that seen for wild-type TRPV4. In Chapter 4 we will discuss some ideas of how this N-terminal mutant might be disrupting channel activity.

Next, we tested the activity of V4-ET-M625I/H787Y, the double mutant that displayed the greatest degree of rescue of V4-K70E/M605T channel activity in the yeast assays: the clone rescued low potassium growth to a higher degree than wild type TRPV4, and caused salt sensitivity to the maximum level detectable by our assay. Either mutant alone rescued the low potassium growth, though at a lower level than the double mutant; in contrast, only the M625I mutation restored salt sensitivity. These oocyte recordings were in agreement with the yeast low potassium growth results: the double mutant M625I/H787Y strongly rescued the calcium-activated current in V4-K70E/M605T, and either mutant alone could also rescue the current but not to the same degree as the double mutant.

The N-terminal triple mutant from the yeast screen only weakly rescued function of V4-K70E/M605T in the yeast assays: V4-ET-R151W/P152S/L154F moderately rescued both low-potassium growth and salt sensitivity. When the individual mutants were tested separately, only V4-ET-L154F demonstrated rescue (this mutation was also independently isolated in the yeast screen as a single mutant) although an additive role for R151W and P152S was suggested by the
fact that the level of rescue for the triple mutant was slightly stronger than L154F alone. The TEVC recordings were in line with these data: the triple mutant V4-ET- R151W/P152S/L154F and the single mutant V4-ET-L154F each displayed calcium-activated current, but it was weak and quite variable; V4-ET-R151W and V4-ET-P152S did not have significant calcium-activated currents.

The mutagenesis screen picked up two C-terminal truncations, V4-ET-Q790Δ and V4-ET-N789Δ, with a unique activity profile. Whereas all the other V4-K70E/M605T mutants mostly showed agreement in the two yeast assays – if the mutant rescued low-potassium growth, it also rescued salt sensitivity – the C-terminal truncations exhibited very strong rescue of low-potassium growth but did not rescue salt sensitivity at all. Moreover, when the truncations were introduced into wild type TRPV4 they actually abolished the salt sensitivity of the channel, although they increased the level of low-potassium growth. The results of the TEVC recordings are in agreement with the salt sensitivity yeast phenotype: the C-terminal truncations have no effect on V4-K70E/M605T (that is, there is still a lack of calcium-activated current), but when introduced into TRPV4 they completely abolish calcium-activated current of wild type channel. Possible roles of the C-terminus on channel activity will be discussed further in Chapter 4.

It is noteworthy that H787Y, one of the mutants in the double-mutant V4-ET-M625I/H787Y, is located just one residue upstream from these truncations. The H787Y single mutant only moderately rescues low-potassium growth in V4-K70E/M605T and does not rescue salt sensitivity – in this respect, it is similar to the truncations in that there is only rescue in one assay and not the other. However, when the H787Y mutation is introduced into TRPV4 it does not abolish salt sensitivity the way the truncations do – therefore, these mutants must be affecting
the channel in different ways. In Chapter 4 we will elaborate on the functions of the C-terminal tail of TRPV4.

While the original mutagenesis screen was designed to capture mutants that could restore the ability of V4-K70E/M605T to rescue the growth of the trk1A trk2A yeast strain on low potassium media, the yeast salt sensitivity assay and the electrophysiological analysis of the channels in the Xenopus oocyte system have added additional levels of insight as to how these mutants alter channel function. In the following chapter, we will use these combined data to speculate on the possible roles of each channel region affected in this study.

3.4.4 Electrophysiological analysis of similar mutants from a recent finding

Recently, another group obtained gain-of-function alleles of TRPV4 through a yeast mutagenesis screen (Loukin et al., 2010a); some of the mutations they isolated were very similar to ours, confirming the importance of the channel regions we uncovered in this screen. Although the majority of the mutations found in our study were distinct from theirs, it appears as though they tend to cluster in very similar regions of the channel (refer to Chapter 2, Figure 2.4.6, for comparison of topographical location of mutants).

Loukin et al. analyzed the electrophysiological behavior of wild type TRPV4 versus some of their GOF mutants, using TEVC of Xenopus oocytes. As we did, they find that wild-type TRPV4 could be toxic to the oocytes; their GOF alleles were highly toxic. To combat this they included 1 µM of the channel blocker ruthenium red to the incubation buffer (ND96). Since ruthenium red is very difficult to eliminate from the oocyte and blocks inward currents we avoided using it, instead preventing oocyte death by diluting the injected channel cRNAs and also by eliminating calcium from the ND96 buffer as soon as we became aware of the robust
calcium-activated current of the wild-type channel. Their bath solution contains no sodium, using potassium instead as the major permeant ion; they use barium chloride instead of calcium chloride, thereby avoiding the calcium-activated chloride channel issue (66 mM KCl, 100 mM sorbitol, 1.8 mM BaCl₂, 5 mM K⁺-HEPES, pH 7.2).

As we did, they saw minimal baseline TRPV4 channel activity but enough that they could see outward rectification in the IV relation. As mentioned earlier in this chapter, they were able to elicit large TRPV4 currents with 4αPDD but only after a long pre-incubation with the agonist. They were also able to demonstrate robust hypotonic stimulation (HTS) of TRPV4 currents, which we did not see. However, HTS experiments were performed only during our initial studies (in standard bath solution) that exhibited a large basal current that turned out to be polluted by calcium-activated chloride current. It is possible that any HTS that was occurring was masked in this preparation.

In contrast to wild-type TRPV4, the GOF alleles isolated by Loukin et al. appeared to be pre-stimulated, demonstrating large currents in bath solution alone that were not further stimulated by 4αPDD application or HTS. As they point out, this suggests that the mutants induce a general intracellular gating defect as 4αPDD and HTS have been previously shown to activate gating through distinct pathways (Vriens et al., 2004). The GOF alleles retain outward rectification, leading them to conclude that TRPV4 contains a distinct voltage-dependent gating mechanism in addition to the main intracellular gate. They also measured the activation and deactivation kinetics and found no difference in activation time constants between wild type and GOF alleles, but the GOF alleles demonstrated a 3-fold larger deactivation time constant.

Loukin et al. isolated GOF alleles of TRPV4 that led to hyperactive channels. Our alleles had a less straightforward phenotype, not appearing to cause an increase in the basal activity of
either the polymorphic V4-K70E/M605T or wild type TRPV4. Rather, most mutations restored a calcium-activated sodium current to the V4-K70E/M605T channel – with the notable exception of the C-terminal truncations, which had no effect on calcium-activated current in V4-K70E/M605T but abolished said current in wild type TRPV4.

In the next chapter we will provide more in-depth speculation concerning the phenotypes of our alleles and what they may teach us about the workings of the TRPV4 channel at the subcellular level, as well as in the context of the physiological relevance of this channel.
Figure 3.1.1 Representative modes of TRPV4 activation. Left panel: time-course of whole-cell currents in HEK cells expressing wild type TRPV4, right panel: current-voltage (I-V) relationships obtained at time points indicated, for channels activated by a) heat, b) hypotonic stimulation, (HTS) or c) 1µM 4αPDD. Reproduced with permission from: (a) Güler et al. Heat-evoked activation of the ion channel, TRPV4. J Neurosci 2002; 22:6408-14. © 2002 Society for Neuroscience. (b and c) Vriens et al. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. Proc Natl Acad Sci 2004; 101:396-401. © 2002 National Academy of Sciences, USA.
Figure 3.1.2 Regions of TRPV4 implicated in activation and calcium regulation. Mutations shown to impair channel activation by heat and 4αPDD are shown in red; red barrels indicate the ankyrin repeat domain, also necessary for activation by heat. Mutations shown to impair calcium inhibition are shown in green. Orange barrels indicate positions of the calmodulin (CaM)-binding domain in C-terminus, and a region in N-terminus (overlapping with first ankyrin repeat), that interact to form channel auto-inhibitory structure: binding of Ca\(^{2+}\)-calmodulin to CaM domain disrupts auto-inhibitory structure leading to channel potentiation.
Figure 3.3.1.1  Basal activity seen in TRPV4 but not V4-K70E/M605T; restored in V4-ET-M625I/M787Y. a) Representative current-voltage (IV) relations of TRPV4, V4-K70E/M605T (V4-ET), and V4-ET-M625I/H787Y (ET-IY) in standard bath solution (NaCl as major permeant ion) with 0.1 mM CaCl₂. Oocytes incubated in 3 µM ruthenium red. b) Location of mutations in proposed channel topography.
Figure 3.3.1.2  Response of channels to hypotonic stimulation. a) Representative time course of experiment; application of hypotonic stimulation (HTS) indicated by line, circles indicate points at which data collected to compare channel activity; b) Average peak current of TRPV4, V4-ET, V4-ET-M625I/H787Y in iso-osmolar (white bar: -100 mV, grey bar, +100 mV) or hypo-osmolar (blue bar: -100 mV, purple bar: +100 mV) solution. Uninj: uninjected control oocytes. Oocytes incubated in 3 µM ruthenium red. No statistically significant increase seen after HTS.
Figure 3.3.2.1 Calcium-activated sodium current: present in wild type TRPV4, but not V4-K70E/M605T (V4-ET); mutation of M625I/H787Y restores calcium-activated current to V4-ET. a) Representative IV relations of the channel types at rest (blue trace) or during calcium activation (red trace). b) Representative time-course, circles indicate where data is taken for (c). c) Average peak current in presence of 0 mM Ca\(^{2+}\) (white bar: -100 mV, grey bar: +100 mV) versus 1 mM Ca\(^{2+}\) (blue bar: -100 mV, purple bar: +100 mV). Uninj: uninjected control oocytes. Star (*) indicates pairwise significance of \( p < 0.001 \).
Figure 3.3.2.2  Calcium is acting intracellularly. Pre-treatment of oocytes with calcium chelator BAPTA (a), or application of calcium during an IV protocol that does not allow inward current flow (b) eliminates calcium-activated current in TRPV4 or V4-ET-M625I/H787Y; V4-K70E/M605T (V4-ET) does not respond to calcium. Average peak current in presence of 0 mM Ca$^{2+}$ (white bar: -100 mV, grey bar: +100 mV) versus 1 mM Ca$^{2+}$ (blue bar: -100 mV, purple bar: +100 mV). Uninj, uninjected control oocytes. As n=1 for each condition, significance was not measured. Key in (a) applies to (b) as well.
Figure 3.3.2.3  Calcium-activated chloride current is contaminating the recordings. a) Activity of P/Q channel in presence of Ba$^{2+}$ versus Ca$^{2+}$. b) IV relation of TRPV4 in presence of NaCl versus Na$_2$SO$_4$. c) In new bath solution containing Na$_2$SO$_4$ instead of NaCl, we confirm that calcium is activating TRP channel currents. Average peak current in presence of 0 mM Ca$^{2+}$ (white bar: -100 mV , grey bar: +100 mV) versus 1 mM Ca$^{2+}$ (blue bar: -100 mV , purple bar: +100 mV), in perfusate containing chloride (NaCl) or sulphate (Na$_2$SO$_4$). Uninj: uninjected control oocytes. Star (*) indicates pairwise significance of  p < 0.02.
Figure 3.3.3  Location of mutations in TRPV4 transmembrane topology. Refer to this figure for the location in the channel sequence of the mutations tested for calcium-activated sodium current in the following figures. K70E and M605T (purple) are the mutations present in the loss-of-function variant of TRPV4 (V4-K70E/M605T aka V4-ET); residues in green, orange, and blue are those isolated in the gain-of-function mutagenesis screen of V4-K70E/M605T (Chapter 2).
Figure 3.3.3.1 Mutation K70E completely abolishes calcium-activated current. Average peak current in presence of 0 mM Ca\(^{2+}\) (white bar: -100 mV, grey bar: +100 mV) versus 1 mM Ca\(^{2+}\) (blue bar: -100 mV, purple bar: +100 mV) compared for wild type TRPV4, V4-K70E, and V4-M605T. Uninj: uninjected control oocytes. Due to large variability of V4-M605T, calcium-activated current increase not statistically significant. Refer to figure 3.3.3 for location of mutations in proposed topology.
Figure 3.3.3.2 Double mutant M625I/H787Y strongly rescues calcium-activated sodium current in V4-K70E/M605T. a) Effect of calcium on TRPV4, V4-ET, and V4-ET-M625I/H787Y. b) Effect of calcium on double mutant V4-ET-M625I/H787Y and single mutants (ET-M625I or ET-H787Y). Average peak current in presence of 0 mM Ca\(^{2+}\) (white bar: -100 mV, grey bar: +100 mV) versus (a) 0.1 mM Ca\(^{2+}\) or (b) 1 mM Ca\(^{2+}\) (blue bar: -100 mV, purple bar: +100 mV). Uninj: uninjected control oocytes. Star (*) indicates pairwise significance of p < 0.02. Refer to figure 3.3.3 for location of mutations in proposed topology.
Figure 3.3.3.3 N-terminal mutations show little ability to rescue calcium-activated current in V4-K70E/M605T (V4-ET). a) Effect of calcium on V4-ET, N-terminal single mutants, or triple mutant V4-ET-R151W/P152S/L154F (ET-WSF), b) Effect of calcium on TRPV4, V4-ET, and ET-WSF. Average peak current in presence of 0 mM Ca\(^{2+}\) (white bar:-100 mV, grey bar:+100 mV) vs. 1 mM Ca\(^{2+}\) (blue bar:-100 mV, purple bar:+100 mV). Uninj: uninjected control oocytes. Only triple mutant exhibited statistically significant rescue of Ca\(^{2+}\)-activated current (b, p<0.003). Refer to figure 3.3.3 for location of mutations in topology. Key in (a) applies to (b).
Figure 3.3.3.4  C-terminal truncations have no effect on calcium-activated current in V4-K70E/M605T, but abolish current in wild type TRPV4. a) neither C-terminal truncation has an effect on calcium-activated current in V4-K70E/M605T (V4-ET). b) Calcium-activated current in wild type TRPV4 is completely abolished by introduction of the C-terminal truncations (p<0.001 for both truncations compared to TRPV4). Average peak current in presence of 0 mM Ca$^{2+}$ (white bar: -100 mV, grey bar: +100 mV) versus (a) 1 mM Ca$^{2+}$ or (b) 0.1 mM Ca$^{2+}$ (blue bar: -100 mV, purple bar: +100 mV). Uninj: uninjected control oocytes. Refer to figure 3.3.3 for location of mutations in topology.
exhibits no basal activity in any of the solutions tested; b) V4-K70E/M605T (V4-ET) reversal potential could not be measured as channel of I-V; right panel: expanded view of -50 mV to +50 mV interval to show detail at reversal point.

Figure 3.3.5 Comparison of ion selectivity profile of channels. a) No change in reversal potential between wild type TRPV4 and V4-ET-M625I/H787Y (ET-IY). Left panel: full range of I-V; right panel: expanded view of -50 mV to +50 mV interval to show detail at reversal point. b) V4-K70E/M605T (V4-ET) reversal potential could not be measured as channel exhibits no basal activity in any of the solutions tested; uninj: uninjected.
Table 3.4.3  Summary of effects of mutants across all assays.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>YEAST SPOT ASSAYS</th>
<th>TEVC</th>
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<tbody>
<tr>
<td></td>
<td>Low potassium</td>
<td>Salt sensitivity</td>
</tr>
<tr>
<td></td>
<td>growth (trk1Δ trk2Δ)</td>
<td>(BY4741)</td>
</tr>
<tr>
<td>TRPV4 (wild type)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>V4-K70E/M605T</td>
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<td>–</td>
</tr>
<tr>
<td>V4-K70E</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>V4-M605T</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Mutants from screen:</td>
<td>in V4-ET</td>
<td>in TRPV4</td>
</tr>
<tr>
<td>M625I</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>H787Y</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M625I/H787Y</td>
<td>++++</td>
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</tr>
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<td>+++</td>
<td>++++</td>
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<td>N789Δ</td>
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Topography provided as reference for location of mutations in channel structure.
Chapter 4:

Conclusions
4.1 FUNCTIONAL SIGNIFICANCE OF TRPV4

As discussed in Chapter 3, the TRPV4 channel is part of the ‘thermo-TRP’ subgroup of the TRPV4 channel family; the channel is activated by moderate heat, cell swelling (through the arachidonic acid signaling cascade but also possibly through mechanical force), and phorbol ester derivatives. The unique activation profile of this channel has led to important roles in processes of mechanosensation, osmosensation, and thermosensation, as revealed by knockout mouse models. Many studies have pointed to a conservation of TRPV channel function through evolution, including the work of Liedtke et al, who demonstrated that mammalian TRPV4 could functionally rescue the ability of the C. elegans osm-9 mutant strain to avoid hyperosmotic stimuli, aversive mechanical stimuli (nose touch), and aversive olfactory stimuli (Liedtke et al., 2003). Mutagenesis studies have pinpointed channel regions and even individual residues that are important for these various functions. Mutations of TRPV4 are also found to underlie two types of well-characterized skeletal dysplasia disorders, as well as a family of inherited neurodegenerative diseases. In this section, we explore what has been learned about the functional roles of the TRPV4 channel.

4.1.1 Thermosensation and Nociception

Characterization of TRPV4 in heterologous systems demonstrated that the channel was activated by moderate rather than noxious heat; studies of the TRPV4 knockout mouse confirmed that the channel functions in discrimination of innocuous warm temperatures but also pointed to a functional role in thermal hyperalgesia (increased sensitivity to pain). When placed on a thermal gradient, TRPV4<sup>−/−</sup> mice selected a warmer floor temperature than their wild type
littermates; when exposed to acute moderate tail heating, TRPV4<sup>−/−</sup> mice exhibited prolonged latency in withdrawing their tail (Lee et al., 2005a). While TRPV4<sup>+/−</sup> mice had no alteration compared to wild type in escape from noxious heat (paw withdrawal from a 35-50°C hotplate) under normal conditions, a significant delay in escape was observed when hyperalgesia was chemically induced by subcutaneous injection of carrageenan. Moreover, recordings of neuronal activity in the femoral nerve showed a decrease in electrical activity in response to warmth in TRPV4<sup>−/−</sup> mice compared to wild type. This suggested a role for TRPV4 in coding the sensitivity of noxious heat rather than the threshold (Todaka et al., 2004).

The mechanosensitive and osmosensitive properties of TRPV4 were also revealed to play a role in pain sensation. The channel is expressed in neurons of the dorsal root ganglion, which functions in pressure sensation. TRPV4<sup>−/−</sup> mice exhibited greatly reduced sensitivity to noxious pressure as measured on the tail (Suzuki et al., 2003) and paw (Liedtke and Friedman, 2003). An almost two-fold increase was observed in the threshold pressure required to activate the femoral nerve; the mice also had an impaired response to noxious pain induced by H<sup>+</sup> (Suzuki et al., 2003). Mechanical hyperalgesia is induced by hypotonic cell swelling, which activates the pressure-sensing peripheral nerves of the DRG; the effect can be enhanced by the inflammation mediator prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). For rats in which nociceptors had been sensitized by PGE<sub>2</sub>, the pain response elicited by hind-paw injection of hypotonic solution (flinching) was decreased when TRPV4 expression was knocked down with antisense oligonucleotides (Alessandri-Haber et al., 2003).
4.1.2 Hearing

Hearing impairment was also observed in TRPV4 knockout mice (Tabuchi et al., 2005), consistent with a previous finding that a form of autosomal-dominant delayed-onset hearing loss (DFNA25) had been mapped to a chromosomal region that contained the TRPV4 gene (Greene et al., 2001). It had already been observed that TRPV4 was expressed in the mechanically sensitive hair cells of the inner ear (Liedtke and Friedman, 2003). The hearing threshold of TRPV4−/− mice as measured by auditory brainstem response matched those of wild type at 8 weeks of age, but by 24 weeks of age the threshold of the TRPV4−/− mice increased significantly. In addition, the cochlea appeared more vulnerable to acoustic injury: the shift in hearing threshold after acoustic overexposure was larger in knockout than wild type mice, though there was no statistically significant difference in the number of hair cells lost between knockout and wild type (Tabuchi et al., 2005). Although TRPV4 undoubtedly plays a role in hearing, no direct evidence has emerged to implicate TRPV4 as the transduction channel of mechanical hair cell stimulation. Indeed, in Drosophila melanogaster, the TRPV homologs Nanchung (nan) and Inactive (iav) are required in the neurons of Johnston’s organ for both hearing and geotaxis, but they appear to function as downstream enhancers rather than direct mechanosensors (Göpfert et al., 2006; Sun et al., 2009). Recent evidence points to the TRPN-type NompC channel as the mechanical transducer of hearing in the fly (Effertz et al., 2011).

4.1.3 Vascular regulation

The maintenance of normal blood pressure and blood flow is crucial to the homeostasis of an organism, and is affected by vasodilation and vasoconstriction factors secreted by the endothelium. Multiple studies have demonstrated a role of TRPV4 in regulation of vascular
endothelial and smooth muscle cells; evidence exists to implicate the mechanosensitive, osmosensitive, and perhaps even thermosensitive properties of the channel in these functions (Yin and Kuebler, 2010; Baylie and Brayden, 2011).

In endothelial cells, TRPV4 channels are activated by shear stress, epoxyeicosatrienoic acids (EETs), and downstream of Gq-coupled receptor activation; they affect vasodilation through stimulation of nitric oxide (NO), prostacyclin (PGI2), and endothelium-derived hyperpolarizing factor (EDHF) production (Köhler et al., 2006; Willette et al., 2008; Earley et al., 2009; Mendoza et al., 2010). TRPV4-mediated vasodilation is absent in knockout mouse models, and the increases in whole-cell current and calcium influx seen upon TRPV4 activation in isolated endothelial cells are blocked by ruthenium red (Watanabe et al., 2002b; Köhler et al., 2006; Wu et al., 2006; Hartmannsgruber et al., 2007; Earley et al., 2009; Mendoza et al., 2010). The primary role of TRPV4 in vasodilation is most likely through its ability to sense the shear stress caused by blood flow: although activation of TRPV4 by both hypotonic stimulation and heat have been observed in isolated endothelial cells and are reduced in TRPV4−/− mice (Watanabe et al., 2002b; Vriens et al., 2005; Köhler et al., 2006), in vivo the temperature and tonicity of the vasculature generally isolated from perturbations; this response may play a role during exposure to extreme stress conditions.

In vascular smooth muscle cells, TRPV4 channels respond to EETs released from the endothelium and activate a signaling cascade to effect vasodilation. Patch-clamp of isolated cerebral myocytes demonstrated that EET application activated TRPV4, causing calcium influx which in turn activated ryanodine receptors on the sarcoplasmic reticulum, triggering a calcium release that activated nearby BKCa channels, resulting in K+ current increase to cause hyperpolarization and vasodilation (Earley et al., 2005). This same group went on to demonstrate
that elevations in blood pressure after a hypertensive challenge are increased in TRPV4−/− mice, evidence that the channel functions in a negative feedback mechanism to control response to hypertensive stimuli (Earley et al., 2009), a finding supported by a study in which hypotensive responses were elicited by activators of TRPV4 in both normal and hypertensive rats (Gao et al., 2009).

4.1.4 Osmotic regulation

Maintenance of osmotic homeostasis is important on both the cellular and organismal level, and the activation of TRPV4 by hypotonicity plays an important role in these processes.

On the cellular level, swelling caused by the influx of water in a hypotonic environment can have disastrous consequences – the increase in hydrostatic pressure could lead to physical damage, while the dilution of the cytoplasm disrupts macro-molecular structure and function. Through a process known as regulatory volume decrease (RVD), hypotonic stress triggers calcium entry that precipitates signaling events resulting in the efflux of ions (K+ and Cl−) and organic osmolytes (such as sorbitol) to trigger efflux of water and reestablish normal cell volume (Lang, 2007).

Initial characterization of TRPV4 in vitro established that channel activity increased with the decrease of extracellular osmolarity (hypotonicity) (Strotmann et al., 2000; Liedtke et al., 2000). Using cultured human airway epithelial cells Arniges et al confirmed that calcium entry through TRPV4 is what triggers the RVD response, as knockdown of TRPV4 expression with antisense oligonucleotides eliminated the RVD response to hypotonicity (Arniges et al., 2004). The reverse experiment was consistent in Chinese hamster ovary (CHO) cells: native cells lack TRV4 expression and do not respond to hypotonicity with a RVD response, but heterologous
expression of TRPV4 conferred the ability to regulate cell volume (Becker et al., 2005). Furthermore, Arniges et al found evidence for a role of TRPV4 in the pathology of cystic fibrosis (CF), a disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel. The airway epithelia of CF patients lacks the RVD response, and they demonstrated that in these cells TRPV4 failed to respond to cell swelling (as measured by lack of calcium efflux), though the channels did still respond to the activator 4α-PDD. They concluded that the hypotonic activation of TRPV4 channels is CFTR-dependent (Arniges et al., 2004).

On a systemic level, osmotic homeostasis of extracellular fluid (ECF) is maintained by a feedback loop of osmo-sensing neurons in the central nervous system and the endocrine system. Neurons in the circumventricular organs of the brain, such as the organum vasculosum of the lamina terminals (OVLT) and the subfornical organ (SFO), reside outside of the blood-brain barrier and can therefore react to electrolyte levels in the circulatory system. They project to magnocellular neurons in the hypothalamus, which synthesize and secrete antidiuretic hormone (ADH) to modulate the free-water reabsorption rate in the collecting ducts of the kidney (Bourque and Oliet, 1997; Sinke and Deen, 2011).

The TRPV4 channel was found to be highly expressed in the circumventricular organs (Liedtke et al., 2000), and a mouse knockout of TRPV4 confirmed an impairment of general osmotic regulation. TRPV4−/− mice drank less water and became more hyperosmolar than their wild type littermates, and had significantly lower plasma levels of ADH following hyperosmotic shock. Infusion of an ADH analogue (dDAVP) triggered significant systemic hypotonicity in TRPV4−/− mice while wild type mice remained isotonic; furthermore, the TRPV4−/− mice had an impaired inhibitory response to hypo-osmolarity – they failed to reduce their amount of drinking. Meanwhile, their renal reabsorption response to ADH was unaffected. All of this suggested a
defect primarily in the osmosensing circuits of the CNS (Liedtke and Friedman, 2003). Around the same time, another group generated a TRPV4 knockout mouse but obtained contradictory results – they saw no difference in basal water intake, osmolarity, or serum ADH levels; they also saw an increase rather than decrease of ADH levels after hyperosmotic shock, leading them to conclude that TRPV4 inhibits ADH secretion (Mizuno et al., 2003). The reason for these discrepancies is still not clear, though it has been suggested that the knockouts are not equivalent, each group having disrupted a different exon of TRPV4 (Todaka et al., 2004). More recently, a loss of function polymorphism of TRPV4 was associated with hyponatremia (a disruption in electrolyte balance resulting from excess water diluting the serum concentration of sodium) in humans (Tian et al., 2009).

The organ responsible for maintaining systemic isotonicity is the kidney. The filtrate continuously passed through the kidney undergoes a 100-fold volume reduction before it is released from the body as urine: the tubules of the kidney establish a driving force for filtered water to be reabsorbed through a combination of apical water permeability in some segments and the hyperosmotic interstitium of the medulla (Cohen 2007, Chapter 29 In (Liedtke, 2007)). The response is fine-tuned by the release of ADH, which binds and activates the vasopressin V2 receptor (V2R) expressed in the basolateral membrane of collecting duct cells, activating a phosphorylation cascade resulting in translocation of stored AQP2 water channels to the apical plasma membrane where they will increase water permeability (Sinke and Deen, 2011).

TRPV4 is expressed in the kidney (Strotmann et al., 2000; Liedtke et al., 2000; Wissenbach et al., 2000), and although no phenotype was uncovered in knockout mice the restricted expression pattern of TRPV4 is intriguing: it is expressed only in those regions of the tubule that are not constitutively permeable to water. As one follows the path of filtrate through
the tubules, TRPV4 expression is absent in the ‘descending’ tubule, which is water permeant, but emerges precisely at the Loop of Henle, a tight hairpin turn leading to the water impermeant ‘ascending’ tubule – this transition serves to prevent the concentrated filtrate to be diluted by passive water entry as it exits the hypertonic inner medulla. TRPV4 is expressed throughout the ascending tubule with the exception of the macula densa, a segment that is water permeable (Tian et al., 2004). This expression pattern suggests that TRPV4 plays a role in responding to osmotic gradients that could develop in these regions.

4.1.5 Involvement in skeletal disorders and neurodegenerative diseases

In the last few years, multiple studies have revealed that gain-of-function mutations in the human TRPV4 gene are responsible for skeletal dysplasia disorders as well as neurodegenerative diseases (Figure 4.1).

4.1.5.1 TRPV4 in human skeletal dysplasias

A role for TRPV4 in bone development was observed in a TRPV4 knockout mouse, which displayed increased bone mass due to reduced bone resorption. Masuyama et al determined that TRPV4 is necessary for proper differentiation of osteoclasts, by mediating basolateral calcium influx which leads to signaling events resulting in gene transcription of differentiation factors (Masuyama et al., 2008). At the same time, a study published by Rock et al revealed TRPV4 as a candidate gene in the pathogenesis of a human skeletal dysplasia (Rock et al., 2008).

Brachyolmias are skeletal dysplasias that primarily affect the spine and display phenotypes including a short trunk, scoliosis, and mild short stature (Shohat et al., 1989); Type 3
brachyolmia is an autosomal dominant form characterized by severe scoliosis and flattened, irregular cervical vertebrae (Gardner and Beighton, 1994). Genetic analysis of two families with the disorder revealed point mutations in TRPV4 leading to the amino acid substitutions in the pore-lining S5 transmembrane domain: R616Q in one family, and V620I in the other. Patch clamp analysis in transfected HEK cells revealed a gain-of-function phenotype, with the mutant channels displaying a much larger basal constitutive current than wild type, as well as a larger response to activation either by 4αPDD or hypotonic stimulation (Rock et al., 2008). Single-channel studies confirmed that the R616Q mutation increases basal activity while retaining the same unitary conductance and rectification as wild type (Loukin et al., 2010b).

Spondylometaphyseal dysplasias (SMDs) are distinguished by abnormalities in the vertebrae and the metaphyses of the tubular bones. Two forms of SMD have been shown to be caused by mutations in TRPV4: Kozlowski type (SMDK) is autosomal dominant and is characterized by short stature, significant scoliosis, and delayed carpal bone ossification (Kozlowski 1973, Hypochondroplasia, In (Kaufmann, 1973)); metatropic dysplasia presents both lethal and nonlethal forms – nonlethal phenotypes include shortened limbs and severe scoliosis (Geneviève et al., 2008). Mutations found in the TRPV4 gene of SMDK patients resulted in amino acid substitutions R594H (located at the interface of the S4 transmembrane region and the intracellular S4/S5 loop, and found in 4 of the 8 patients tested), D333G (in the ankyrin repeat domain of the N-terminus), and A716S (interface of the S6 transmembrane domain and the intracellular C-terminal domain). Two patients with metatropic dysplasia were analyzed and had mutations leading to the amino acid substitutions I331F (in the ankyrin repeat domain) and P799L (in the MAP7 binding domain in the C-terminal tail) (Krakow et al., 2009).
The properties of TRPV4 channels containing the SMDK mutations were analyzed in transfected HEK cells: channels containing either the D333G or R594H mutation exhibited much larger basal current than the wild-type channel, similar to the mutations seen in brachyolmia. While the response of the D333G mutant channel to the agonist 4αPDD showed a significant increase compared to wild type TRPV4, the R594 mutant showed no response; both channels actually showed a reduced response to hypotonic stimulation. In contrast, the basal activity of the A716S mutant channel was not significantly different than wild type, but the channels were completely unresponsive to 4αPDD or hypotonic stimulation (Krakow et al., 2009).

Recently, Loukin et al provided compelling evidence that the severity of a skeletal dysplasia is directly correlated with the degree of increase of basal activity of the mutant TRPV4 type found in that patient. They selected 14 TRPV4 mutant alleles that covered the full spectrum of disease severity, and analyzed their single channel properties when heterologously expressed in Xenopus oocytes. Wild type TRPV4 had a basal open probability ($P_o$) of about 0.01. All mutant channels tested had higher basal open probabilities, increasing to a $P_o$ of 1.0 for most of the channels isolated from the lethal forms of metatropic dysplasia. The higher basal open probability meant that little further increase could be elicited by agonist or hypotonic stimulation, resulting in a negative correlation between clinical severity and response to channel activation by the corresponding TRPV4 mutant. As regulated calcium influx by TRPV4 is important in terminal differentiation of osteoclasts, it is possible that the sustained calcium entry in these TRPV4 mutants could result in pathologies in bone development (Loukin et al., 2011).
4.1.5.2 TRPV4 in human neurodegenerative disorders

Spinal muscular atrophies (SMAs) are autosomal-inherited disorders involving degeneration of peripheral motor and sensory nerves. Examples are congenital distal SMA (CDSMA); scapuloperoneal SMA (SPSMA); and Charcot-Marie-Tooth disease (CMT), which is also known as hereditary motor and sensory neuropathy (HMSN) and is the most common inherited neurological disease. The defining characteristic of these disorders is progressive muscle weakness and atrophy. Scoliosis and abnormal bone development occur in some disorders, as well as sensory deficits and incontinence (Deng et al., 2010; Landouré et al., 2010). In three independent studies, mutations in the N-terminal ankyrin repeat domain of TRPV4 were identified in patients with CDSMA, SPSMA, and CMT Type 2C (CMT2C).

Deng et al. identified the amino acid substitutions R269H and R316C in two unrelated families suffering from CMT2C and SPSMA, respectively; in transfected HEK293 cells these mutant channels displayed increased basal activity compared to wild-type as well as an increased response to stimulation by 4αPDD, hypotonic stimulation, or increased heat (Deng et al., 2010). Landouré et al also identified the R269H mutation, as well as the variant R269C, from CMT2C patients – both mutations resulted in channels with elevated basal current and increased response to 4αPDD or heat compared to wild type. In addition, they demonstrated that the mutant channels caused cellular toxicity: transfection of the channel into either DRG neurons or HEK293 cells caused a marked increase in cell death associated with increased intracellular calcium levels, which could be blocked by the TRP channel inhibitor ruthenium red (Landouré et al., 2010).

In the third study, Auer-Grumbach et al. also isolated the R269H and R316C mutations, and in addition found the mutation R315W, in a family with members suffering from CDSMA,
SPSMA, or CMT2C. However, their results were in direct opposition to the other two groups: in transfected HeLa cells, the mutant channels demonstrated greatly reduced basal currents and reduced response to 4αPDD or hypotonic stimulation. They also observed greatly reduced surface expression of the mutant channels compared to the wild type, suggesting that the mutations were affecting channel assembly or trafficking as opposed to altering intrinsic channel activity (Auer-Grumbach et al., 2010). In the other two studies, channels containing these same mutations demonstrated normal plasma membrane localization, but it is possible that the discrepancy is due to differences between the cell line types (HEK versus HeLa).

A gain-of-function phenotype was confirmed by Fecto et al., who analyzed the TRPV4 mutants from the three above-mentioned studies in three different cell types (HEK293, HeLa, and Neuro2a) through patch clamp and single channel recordings. Across cell type, they found that the TRPV4 mutants had normal plasma membrane expression, but higher basal whole cell currents and increased response to 4αPDD compared to wild type; single channel recordings revealed that in the TRPV4 mutants single channel conductance was unchanged but open probability was increased due to a change in gating. Finally, the TRPV4 mutants caused significantly increased cytotoxicity in all three cell lines – therefore, the likely pathology is that these mutants cause cation overload that leads to cell death (Fecto et al., 2011).
4.2 MUTATIONAL ANALYSIS OF TRPV4

4.2.1 Experimental design: analyze loss-of-function allele of TRPV4

As detailed in the preceding chapters, the major aim of this project was to investigate the properties of the mammalian TRPV4 channel through a mutagenesis screen and subsequent electrophysiological analysis. Expression of wild type TRPV4 (BC117426) can rescue the ability of a yeast strain lacking native potassium channels (trk1Δ trk2Δ) to grow on low potassium media, indicating that the channel must allow efflux of sufficient potassium to compensate for the lack of the normal potassium uptake system. In addition, TRPV4 expression induces cell death of a wild-type strain (BY4741) when exposed to high salt medium. Yeast can usually grow in a high-salt environment due to the low sodium permeability of the membrane and the presence of sodium pumps, but it would appear that the TRPV4 channel allows more sodium into the cell than it can handle, leading to cell death. In oocytes expressing wild type TRPV4, we observed significant and reproducible calcium-activated sodium currents.

We obtained a variant of TRPV4 (Open Biosystems clone LIFESEQ90218873), containing the two point mutations K70E in the N-terminal tail and M605T in the S4/S5 intracellular linker (V4-ET-K70E/M605T), that failed to exhibit any of the above-mentioned phenotypes: it did not rescue trk1Δ trk2Δ yeast growth on low potassium, did not induce salt sensitivity in wild-type yeast, and exhibited no calcium-activated sodium current in oocytes. We have been able to find no evidence in the literature or the NCBI GenBank repository for another occurrence of either of these amino acid substitutions. The cDNA clone comes from an Incyte cDNA collection, developed to generate full-open reading frame cDNA clones from a variety of tissues to complement the NIH Mammalian Gene Collection (MGC) consortium generated
largely by EST (expressed sequence tag) analysis (Baross et al., 2004). Given the complete failure of V4-K70E/M605T to function in our assays, it is hard to believe that these residues were present in the patient from whom the tissue was collected – perhaps the mutations were introduced during amplification. Regardless, the mutated channel provides an opportunity to explore the functional properties of TRPV4.

The underlying cause of the failure of V4-K70E/M605T to demonstrate the same activity as wild type TRPV4 was not clear. There could be problems with the processing of the channel: perhaps it was not trafficked to the plasma membrane (the mutations could create or destroy an ER retention or export signal), or perhaps it could not properly fold or insert in the membrane (the mutations could disrupt the tertiary folding, or disrupt necessary interactions with folding chaperones). Alternatively, the variant could create a channel that is functional but that has different conductance properties, so it may still function as a channel but not in a way that rescues our particular phenotype – for example, it may have lowered selectivity for potassium.

Our goal was to mutagenize V4-K70E/M605T to identify compensatory mutations that would allow the channel to rescue growth of trk1Δ trk2Δ yeast on low potassium medium. Next, we would determine if these mutants could also rescue the salt sensitivity phenotype in wild type yeast. Then, we expressed the channels in Xenopus oocytes and performed two-electrode voltage clamp (TEVC) recordings to determine if the mutants could rescue the calcium-activated sodium current. In addition, the mutations were engineered into wild type TRPV4 to see if they would affect activity of the true wild type channel. Taken together, these assays would build a functional picture of how the mutations affect channel activity and reveal the functional role of these channel regions (refer back to Table 3.4.3 for the combined results of the multiple assays).
Figure 4.1 shows the location of the mutations in the functional domains of TRPV4 in the context of other known gain-of-function mutations discussed throughout this chapter.

4.2.2 V4-K70E/M605T loss-of-function phenotype due to the N-terminal substitution

The channel encoded by V4-K70E/M605T showed no appreciable rescue of \( \text{trk}1\Delta \text{trk}2\Delta \) growth on low potassium. Upon first examination we assumed that this probably had less to do with the K70E, which is at the extreme N-terminus and is far upstream of the ankyrin repeats that functionally define that region, and more to do with M605T, located in the small intracellular loop linking the S4 and S5 transmembrane helices. The mutation from nonpolar methionine to polar threonine could possibly disrupt folding or disrupt the position of the pore-lining S5 helix, even if indirectly. However, when tested separately we saw that M605T only partially abrogated the ability of the channel to rescue low potassium growth or induce salt sensitivity, and still exhibited a calcium-activated current in oocytes; meanwhile, K70E completely eliminated low-potassium rescue, salt sensitivity, and the calcium-activated current. Therefore, the phenotype is primarily due to K70E, although M605T does somewhat impact function.

The most notable feature of the N-terminus of the TRPV channels is the stretch of ankyrin repeats (amino acids 111-358) that play important roles in channel assembly and maturation (Hellwig et al., 2005; Arniges et al., 2006). The K70E mutation is about 40 residues upstream of the repeats but it is possible that the mutation, which results in the replacement of a basic (positively charged) lysine with an acidic (negatively charged) glutamate, could interfere with the activities at this region. In studies of splice variants, those missing parts of the ankyrin repeats failed to oligomerize and were retained in the ER, but a splice variant missing residues 21-60 was trafficked to the membrane and responded to agonist at levels comparable to full-
length channel (Arniges et al., 2006). Given the types of compensatory mutations we found (discussed below), it seems unlikely that K70E is disrupting folding – it is hard to imagine how some of our mutations would restore proper folding, and many of the mutations can actually increase activity when introduced into the wild-type TRPV4 channel. Perhaps a more likely scenario is that K70E is disrupting the binding with certain intracellular partners that regulate channel activity. As discussed in Chapter 2, we were not able to quantitate the expression of channel on the yeast plasma membrane, as resolution could not be achieved between expression in the plasma membrane versus the underlying cortical ER.

4.2.3 Analysis of gain-of-function mutants of V4-K70E/M605T

In our screen, mutations of V4-K70E/M605T that allowed the channel to rescue the ability of the trk1Δ trk2Δ strain to grow on low potassium media were found in three regions of the channel: the N-terminus (R151W, P152S, L154F), the pore-lining helix S5 (M625I), and the C-terminus (H787Y, N789Δ, and Q790Δ).

4.2.3.1 N-terminal mutants

Two clones isolated in the V4-K70E/M605T screen contained mutations on the same spot, in the first ankyrin repeat in the N-terminal tail: V4-ET-L154F and the triple mutant V4-ET-R151W/P152S/L154F. The triple mutant weakly rescued the ability of V4-K70E/M605T to rescue growth on low potassium, as well as induce weak salt sensitivity. V4-ET-L154F also weakly rescued low-potassium growth and salt sensitivity, while neither V4-ET-R151W nor V4-ET-P152S alone had a measureable effect. However, the triple mutant rescued slightly better than L154F alone, indicating that the two additional residues are not merely an artifact of
mutagenesis. The three N-terminal mutations were introduced into wild type TRPV4: R151W, L154F, or the triple mutant slightly improved the ability of TRPV4 to rescue low potassium growth while P152S had no effect. Our TEVC recordings were in line with these data: in V4-K70E/M605T the triple mutant and single mutant L154F each displayed calcium-activated current, but it was weak and quite variable; R151W and P152S did not have measurable calcium-activated current. The limitations of our TEVC prevented us from deducing if the N-terminal mutations enhanced wild type TRPV4 activity.

These N-terminal mutants are close enough to K70E that it is possible they somehow counteract a disruption of channel assembly introduced by K70E, though it is hard to imagine how the bulky phenylalanine would compensate for a K to E substitution. Alternatively, the mutations could be disrupting an auto-inhibitory channel structure: a region in the N-terminus from residues 117-136 binds to the calmodulin-binding site on the C-terminus (residues 806-871), inhibiting the channel until calmodulin binds to the C-terminal domain and displaces the N-terminal domain (Strotmann et al., 2010). Our mutations are just downstream of the auto-inhibitory region, and the bulky phenylalanine (L154F) or tryptophan (R151W) residues could cause steric hindrance of binding.

This area of the N-terminus may play a role in response of the channel to agonist: R151 and E797 correspond to residues in the N- and C-termini of TRPV1 identified as the loci for binding of the agonist capsaicin (Jung et al., 2002). Watanabe et al. mutated these two regions: E797A or E797K resulted in a constitutively active channel but they saw no phenotype for mutations of R151 (R151D, R151A, or R151K did not induce any change in the response to 4αPDD) (Watanabe et al., 2003b). Given how slight our phenotype is for R151W, it may not have been detectable in their assay or may be specific to replacement with tryptophan. The
residue R151 was also isolated in the recent TRPV4 gain-of-function mutagenesis performed by Loukin et al, but they also saw no phenotype (Loukin et al., 2010a). It is notable that the N-terminal and C-terminal residues implicated in agonist binding lie just outside the respective N-terminal and C-terminal regions that form the auto-inhibitory structure.

As discussed earlier in this chapter, mutations in the ankyrin repeat domain (ARD) of TRPV4 underlie inherited human spinal muscular atrophies (SMAs). Single channel analysis revealed that the mutations affect channel gating, leading to an increase in open probability while single channel conductance remains unchanged (Fecto et al., 2011). The neuronal pathology is most likely due to cation overload from these overactive channels. It is attractive to hypothesize that our N-terminal mutations also affect open probability. This would be consistent with the overall gain-of-function – the mutants rescue low-potassium growth, cause salt sensitivity, and rescue the calcium-activated current in TEVC.

4.2.3.2 Mutation in S5 pore-lining TM domain

A double mutant consisting of M625I (in the pore-lining S5 transmembrane helix) and the H787Y (in the C-terminal tail) elicited the strongest response in our yeast assay, enabling V4-K70E/M605T to rescue low potassium growth to a level even higher than that of true wild type TRPV4 and causing severe salt sensitivity. Each mutant when tested alone in V4-K70E/M605T showed strong rescue of the low potassium growth, though not as strong as the double mutant, suggesting that they two mutations affect different gating processes. Introduction of either mutation into wild type TRPV4 resulted in an enhancement of low potassium growth; the double mutant could not be generated for testing, presumably because it increases activity of TRPV4 to such an extent as to be lethal to the yeast. In oocytes, the mutations (either mutation
alone, or the double mutant) rescued the calcium-activated current in V4-K70E/M605T; although
the number of oocytes tested was not enough for adequate statistical analysis, the trend appeared
to be that double mutant produced a stronger effect that either single mutant.

An important distinction was that M625I alone was responsible for the rescue of salt
sensitivity of V4-K70E/M605T: H787Y alone did not cause any salt sensitivity. The functional
significance of H787Y will be discussed in the following section, in the context of all of the C-
terminal mutations isolated in our study.

Perhaps M625I affects both activity and selectivity, while H787Y only increases activity.
M625I is located in S5 helix, which closely associates with the pore-forming S6 helix, and may
thereby influence the structure of the putative selectivity filter in the pore-loop (Voets et al.,
2002; Dodier et al., 2007). However, our preliminary data do not show a difference in selectivity
between wild type TRPV4 and V4-ET-M625I/H787Y.

M625I is proximal to other known mutations that also display a gain-of-function
phenotype: R616Q or V620I, isolated in patients with Type 3 Brachyolmia, when tested in
transfected HEK cells have been shown to result in a channel that displayed both higher basal
current and a higher degree of activation by hypotonic stimulation or chemical agonist (Rock et
al., 2008). M625I would be about one helical turn away from V620I, and is also a substitution to
an isoleucine.

A common mechanism in gating across TRP channels was suggested by the fact that
gain-of-function alleles leading to constitutive activity were traced to mutation of a cluster of
conserved phenylalanines in the C-terminal end of the S5 helix: F559I in Drosophila TRP (Hong
et al., 2002), F380L in yeast Yvc1p (Zhou et al., 2007; Su et al., 2007), and M581T in the FMF
sequence in mammalian TRPV1 (Myers et al., 2008). We do see a potential phenylalanine
cluster in TRPV4 – F615/R616/F617 – containing the residue R616 that is mutated in Brachyolmia patients.

4.2.3.3 C-terminal mutations

Interestingly, all three of the strongest rescuers in our screen contained a mutation essentially on the same spot in the C-terminus: H787Y from the double mutant described above, and the truncation mutants W788Δ and Q790Δ. This would suggest a mutational hotspot; indeed, this region has been shown to be very important in channel regulation: notably, the calmodulin-binding domain starts just downstream at residue 814 (Strotmann et al., 2003).

H787Y, W788Δ, and Q790Δ all produced strong rescue of low potassium growth in V4-K70E/M605T, but caused no salt sensitivity. In wild type TRPV4, all three enhanced the level of rescue of low potassium growth. Quite surprisingly, while the point mutation H787Y had no effect on the ability of wild type TRPV4 to induce salt sensitivity, both truncation mutants actually completely abolished the salt sensitivity response of the wild type channel. Similarly, in oocytes H787Y rescued calcium-activated sodium current in V4-K70E/M605T while the C-terminal truncations did not; when the C-terminal truncations were introduced into wild type TRPV4 they completely abolished the calcium-activated sodium current (H787Y had no affect).

Clearly, the point mutation H787Y in the C-terminus is acting in quite a different way than the C-terminal truncations. It may somehow affect channel gating or interaction with proteins that modulate channel activity. A previous study demonstrated that mutation of nearby residue E797 leads to increased spontaneous activity (Watanabe et al., 2003b) – as mentioned earlier, this residue corresponds to a residue in the C-terminus of TRPV1 that was implicated in binding of the agonist capsaicin (Jung et al., 2002). It is possible that mutations in this region of
the C-terminus could be disrupting the auto-inhibitory structure mentioned earlier in this chapter, in which a portion of the N-terminus is bound to a portion of the C-terminus (within residues 806-871) until it is displaced by calmodulin binding to the C-terminus (Strotmann et al., 2010). But if H787Y rescues the calcium-activated sodium current, why does it not cause cation overload leading to increased salt sensitivity? This is most likely explained by a technical consideration – the level of sensitivity of the two different assays. One could imagine that it would take relatively little influx of potassium to rescue the ability of yeast to grow on low potassium media; meanwhile, it would take quite a large influx of sodium to overwhelm the detoxification mechanisms to the point of cell death.

How to account for the seemingly contradictory phenotypes of the C-terminal truncations? Truncation of wild type TRPV4 results in stronger rescue of growth on low potassium media and therefore must be enhancing some aspect of channel activity. Perhaps elimination of the auto-inhibitory structure formed by the N- and C-termini causes an increase in the basal activity of the channel that is sufficient to increase low potassium growth. In the related channel TRPV1, C-terminal truncations have been associated with constitutive channel activity (Myers et al., 2008).

Meanwhile, the loss of wild-type TRPV4 channel activity that leads to abrogation of salt sensitivity in the C-terminal truncations, and loss of the calcium-activated sodium current, is most likely due to the loss of the calmodulin-binding site. It is known that intracellular calcium potentiates currents through TRPV4 by a calmodulin-dependent mechanism; the calmodulin binding site begins at residue 814, just downstream of our truncations, and previous study showed that truncation of the channel at residue 801 eliminated calcium potentiation and greatly slowed channel activation by the agonist 4αPMA (Strotmann et al., 2003). Our findings could
suggest that the potentiation of current by the calmodulin domain occurs by some mechanism that is distinct from its role in forming the autoinhibitory domain.

However, the possibility exists that our phenotype is produced by reduced surface expression of the channel. Previous studies have suggested that C-terminal truncations lead to increased ER retention: the Jendrach group in Germany observed that any truncation of the channel above residue 828 resulted in complete ER retention in either CHO cells or HaCaT keratinocytes (Becker et al., 2008). Although we did not perform surface expression analysis in our current study, we can infer that there are at least some channels getting to the surface, as evidenced by the rescue of low potassium growth.

### 4.2.4 Comparison of our mutants to a similar mutagenesis study

Our mutagenesis screen was designed to find mutations that restored functionality of V4-K70E/M605T (NM_021625), a variant of the human TRPV4 channel (BC_112972). Our assay system was to measure the ability of a channel to support growth of a yeast strain lacking native potassium channels (trk1Δ trk2Δ) to grow on low potassium media. Therefore, our screen was one in which gain of function was measured by growth of yeast compared to a baseline of lack of growth.

In contrast, Loukin et al. mutagenized wild-type rat TRPV4 (NM_023970) and screened for gain-of-function alleles by their ability to block yeast proliferation, presumably because increased channel activity would create increase in leak ions that would be toxic to the yeast. Therefore, their screen measured gain of function by lack of growth of the yeast strain (Loukin et al., 2010a).
Our approach had the advantage that we were looking for rescue of growth, and therefore might be able to pick up more subtle alleles. On the other hand, their approach enabled them to study mutations that had a direct impact on a wild type channel – we were looking for mutations that were compensatory to mutations in V4-K70E/M605T, so many of these mutations had no effect when introduced into wild type TRPV4. From a practical standpoint, their approach necessitated initial growth on a repressive media followed by replica-plating to an expressive medium; since we were looking for a rescue of growth we plated yeast transformants directly on a selective medium, eliminating the chance of losing positive colonies due to errors in replica plating.

Despite these differences, and despite the fact that the scale of their screen was much larger than ours was, Loukin et al. isolated a comparable number of mutants (6 to our 7) that appeared to cluster in very similar regions of the channel (Figure 4.1).

Loukin et al. isolated two mutations in the S5 helix: L619P and L623P, which they note are probably one full turn apart in the helix; both are prolines which would be predicted to put a kink in the helix. Just two residues upstream in the S5 helix is our mutant M625I, which was our strongest yeast growth rescue allele. The location of all of these mutants in the pore-forming S5 helix suggests that they play a role in formation of the intracellular gate of the channel.

They also isolated a C-terminal mutant, W733R, although our C-terminal mutants are farther down on the C-terminal tail (from 787-789).

They isolated a double mutant with a weaker phenotype consisting of D546G in the S2/S3 loop and R151Q in the N-terminus; they attribute the phenotype solely to D546G since they see no inhibition of yeast growth when they test R151Q alone. We did not isolate any mutations in the S2/S3 loop in our study, but we did isolate a mutation in the same N-terminal
residue: R151W. Coincidentally, we also found this mutation only as part of a multiply-mutated allele – the triple mutant R151W/P152S/L154F – and while we did not see an enhancement of our yeast growth phenotype when testing R151W alone, a functional role of the residue was hinted at by the fact that the triple mutant rescued growth more strongly than L154F alone (which had been independently isolated in another allele). This is an example of how different types of yeast assays can pick up more subtle phenotypes.

Similar to our study, Loukin et al. followed up with TEVC in *Xenopus* oocytes to study the altered properties of the mutant channels. They found, as we did, that wild-type TRPV4 could be toxic to the yeast; their gain-of-function alleles were highly toxic. As we did, they saw minimal baseline TRPV4 channel activity in a calcium-free bath solution, but enough that they could see outward rectification in the I-V relation. They were able to elicit large TRPV4 currents with 4αPDD, but only after a much longer pre-incubation with the agonist than we attempted. They were also able to demonstrate robust hypotonic stimulation (HTS) of TRPV4 currents, which we did not see. However, our HTS experiments were performed during our initial studies (in standard bath solution) in which we were seeing a large basal current that turned out to be polluted by calcium-activated chloride current. It is possible that any HTS that was occurring was masked in our preparation.

In contrast to wild type TRPV4, the gain-of-function alleles isolated by Loukin et al. appeared to be pre-stimulated, demonstrating large currents in bath solution alone that were not further stimulated by 4αPDD application or HTS, leading them to conclude that the mutants induce a general intracellular gating defect. The gain-of-function alleles retain outward rectification, suggesting that TRPV4 contains a distinct voltage-dependent gating mechanism in addition to the main intracellular gate. They also measured the activation and deactivation
kinetics and found no difference in activation time constants between wild type and gain-of-function alleles, while the gain-of-function alleles demonstrated a 3-fold larger deactivation time constant.

Loukin et al. were able to isolate gain-of-function alleles of TRPV4 that led to hyperactive channels. Our alleles had a less straightforward phenotype. Our alleles did not appear to cause an increase in the basal activity of either V4-K70E/M605T or wild type TRPV4. Rather, most of our mutations restored a calcium-activated sodium current to the V4-K70E/M605T channel – with the notable exception of the C-terminal truncations, which had no effect on calcium-activated sodium current in V4-K70E/M605T but abolished said current in wild type TRPV4.
4.3 FINAL THOUGHTS AND FUTURE DIRECTIONS

In these chapters we have detailed the identification, via mutagenesis screen, and analysis of function, through two-electrode voltage clamp electrophysiology, of compensatory mutations that rescue the loss-of-function phenotype of the variant TRPV4 channel V4-K70E/M605T. We isolated point mutations in the N-terminus, S5 transmembrane helix, and C-terminus; as well as C-terminal truncations; enabling V4-K70E/M605T to rescue low-potassium growth of the \textit{trk1}Δ \textit{trk2}Δ yeast strain (a phenotype of wild type TRPV4). The N-terminal and S5 point mutations also restored the ability of V4-K70E/M605T to induce salt sensitivity in wild-type yeast, and restored a calcium-activated sodium current in \textit{Xenopus} oocytes. The C-terminal point mutation rescued the calcium-activated sodium current, but not the ability to induce yeast salt sensitivity. The C-terminal truncations, meanwhile, could knock out the calcium-activated sodium current and yeast salt sensitivity phenotypes when introduced into wild-type TRPV4.

From our results, it would appear that the S5 point mutation causes an intracellular gating defect in the pore region, while the N-terminal and C-terminal point mutations likely disrupt the gating regulated by the autoinhibitory structure formed between these two regions. The C-terminal truncations also may affect gating through the autoinhibitory structure, as evidenced by the increased activity that allows rescue of low-potassium yeast growth; meanwhile, the loss of function evidenced by loss of salt sensitivity and calcium-activated current in wild type TRPV4 could be explained either as loss of potentiation by the calmodulin domain, or reduced channel expression due to ER retention.

An important next step in characterization of our mutants is to determine the membrane expression levels of the channels. Due to the presence of cortical ER just under the plasma
membrane of the yeast, and the lack of a reliable ER-specific marker, fluorescence microscopy could not be used to determine the membrane fraction of channel. Quantitation of membrane expression levels, through such means as surface biotinylation or immunoprecipitation of membrane fractions, could help us begin to clear up some ambiguities in our data. For example, do the mutations in the channel V4-K70E/M605T lead to a nonfunctional channel in the membrane, or is the loss of function due to the channel no longer being properly folded or trafficked to the membrane? We suspect the former, as it would be difficult to explain how the various compensatory mutations we isolated would be involved in overcoming a trafficking defect, but the possibility does need to be addressed. Another important issue to clear up is whether the C-terminal truncations cause increased ER retention of the channel, or if their loss-of-function phenotype is a result of altered channel activity.

The yeast expression system could be utilized to further explore the role of the calmodulin-binding (CaM) domain of TRPV4. As discussed previously, binding of calcium-calmodulin to this C-terminal domain disrupts the autoinhibitory structure formed with the N-terminal tail. We hypothesize that our N- and C-terminal point mutations cause increase activity by disrupting this domain in the absence of calcium-calmodulin, while the full loss of the domain in the C-terminal truncations leads to loss of a further potentiative role of the CaM domain. We could repeat our growth assays in a strain lacking the endogenous yeast calmodulin protein (Cmd1p) to test this hypothesis. One would expect the wild type TRPV4 to lose the ability to induce salt sensitivity in this strain, and the N- and C-terminal point mutations to be able to rescue salt sensitivity of wild type TRPV4.

One shortcoming of our electrophysiological data is that many of our results indicate trends rather than solidly statistically significant changes. This is mainly due to an insufficient
number of oocytes tested per condition to allow us to group significant numbers of oocytes together. For example, in oocytes the calcium-activated sodium current may have been evoked by 0.1 mM Ca\textsuperscript{2+}, 1 mM Ca\textsuperscript{2+}, or another concentration, depending on when the experiments were performed and the quality of the oocytes at that time. Some mutations were tested in one condition but not another, making global analysis difficult. Much time was spent obtaining recordings in the chloride-containing solutions before the contaminating calcium-activated chloride current was identified; those recordings were rendered mostly unusable.

To fully understand the effects of these channels, the next step would be to perform more thorough electrophysiological analysis. In TEVC or macropatch recordings, we could analyze the activation and deactivation kinetics of the channels. The most exact measurements would come from single-channel recordings, which would reveal if the mutant channels have altered open probability, conductance, etc.

We are also interested in more thoroughly characterizing the response of our mutated channels to agonists. We were not successful in showing TRPV4 activation by hypotonicity, heat, or 4αPDD. As discussed earlier, we now know that activation by 4αPDD is possible in *Xenopus* oocytes, given a longer incubation time. Loukin et al. also saw a response to hypotonic stimulation in oocytes – it may be that we simply did not study this under the proper conditions. It might have been helpful to perform our activation experiments under moderate heat, as this increases the response of TRPV4 to hypotonicity or phorbol esters (Gao et al., 2003). Establishing protocols for agonist stimulation of TRPV4 would allow us to more fully characterize the V4-K70E/M605T channel, to determine if it retains any activity at all, as well as the properties of the mutants from our screen.
In conclusion, our study has identified residues of TRPV4 that are important for proper channel activity; further analysis of these mutants can reveal more about the mechanisms of channel function.
Figure 4.1  Diagram of TRPV4 protein with location of gain-of-function mutations. Above channel: mutations found in human patients with skeletal dysplasias BO (brachyolmia), SMDK (spondylometaphyseal dysplasia, Kozlowski type), and MD (metatropic dysplasia). * Denotes common recurrent mutations. Below channel: mutations found in human neuropathies including SMA (spinal muscular atrophy) and CMT (Charcot-Marie-Tooth disease); gain-of-function mutations isolated in our screen (blue) that rescue function of V4-K70E/M605T (red), and mutations from the screen of Loukin et al. 2010 (black). ANK, ankyrin repeats; S1-S6, transmembrane domains 1-6; NT autoinhib, N-terminal auto-inhibitory domain; yellow stars indicate the residues (R151, E797) homologous to the capsaicin-binding loci in TRPV1. Adapted with permission from: Dai et al. Novel and recurrent TRPV4 mutations and their association with distinct phenotypes within the TRPV4 dysplasia family. J Med Genet 2010; 47:704-9. © 2010 BMJ Publishing Group Ltd.
Chapter 5:

Appendices
Appendix I:

A single amino acid mutation attenuates rundown of voltage-gated calcium channels

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Note added in dissertation:
My contribution to this work was to perform two-electrode voltage clamp recordings of the mutated channels developed by Dr. Zhen and Dr. Xie; Dr. Jian Yang wrote the manuscript.

ABSTRACT

The activity of voltage-gated calcium channels (VGCCs) decreases with time in whole-cell and inside-out patch-clamp recordings. In this study we found that substituting a single amino acid (I1520) at the intracellular end of IIIS6 in the $\alpha_1$ subunit of P/Q-type Ca$^{2+}$ channels with histidine or aspartate greatly attenuated channel rundown in inside-out patch-clamp recordings. The homologous mutations also slowed rundown of N- and L-type Ca$^{2+}$ channels, albeit to a lesser degree. In P/Q-type channels, the attenuation of rundown is accompanied by an increased apparent affinity for phosphatidylinositol-4,5-bisphosphate, which has been shown to be critical for maintaining Ca$^{2+}$ channel activity (Wu et al., 2002). Furthermore, the histidine mutation significantly stabilized the open state, making the channels easier to open, slower to close, harder to inactivate and faster to recover from inactivation. Our finding that mutation of a single amino acid can greatly attenuate rundown provides an easy and efficient way to slow the rundown of VGCCs, facilitating functional studies that require direct access to the cytoplasmic side of the channel.
1. INTRODUCTION

Voltage-gated Ca\(^{2+}\) channels (VGCCs) are subject to extensive regulation in cells (Catterall, 2000). Their activity decreases spontaneously over time in whole-cell or cell-free recordings. This phenomenon, called “rundown”, is a common feature shared by a number of ion channels, including certain types of Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) channels and NMDA and GABA\(_A\) receptors (Hille, 1992; Becq, 1996). Except for the low voltage-activated T-type Ca\(^{2+}\) channels, all high voltage-activated Ca\(^{2+}\) channels, including L-, N-, P/Q- and R-type channels, show rapid rundown in whole-cell and cell-free configurations (Plummer et al., 1989; Williams et al., 1992; McDonald et al., 1994; De Waard and Campbell, 1995; Mougnot et al., 1997; Martini et al., 2000). Extensive efforts have been devoted to studying the mechanisms of rundown of these channels, since a better understanding of this phenomenon could greatly facilitate experimental studies of these channels and help delineate how they are regulated in intact cells. Several mechanisms have been suggested for rundown of VGCCs, including proteolysis (Chad and Eckert, 1986; Kameyama et al., 1988; Belles et al., 1988; Romanin et al., 1991; Kameyama et al., 1998) and dephosphorylation of PKA-mediated phosphorylation (Armstrong and Eckert, 1987; Ono and Fozzard, 1992; Yazawa et al., 1997; Costantin et al., 1999). Recent work show that phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) can greatly stabilize the activity of P/Q-type, and to a lesser degree, N- and L-type Ca\(^{2+}\) channels (Wu et al., 2002; Gamper et al., 2004). This finding has been capitalized to perform functional studies on VGCCs that require direct access to and extensive perfusion of the cytoplasmic side of the channel (Zhen et al., 2005; Xie et al., 2005). In this study, we report another strategy to dramatically attenuate rundown of VGCCs by mutating a single amino acid in the \(\alpha_1\) subunit.
2. MATERIALS AND METHODS

2.1. Molecular biology and oocyte expression

Rabbit brain Ca\textsubscript{v}2.1, Ca\textsubscript{v}2.2, cardiac muscle Ca\textsubscript{v}1.2 and rabbit skeletal muscle $\alpha_2\delta$ were cloned in an oocyte expression vector, pGEMHE, or one of its variants. Rat brain $\beta_4$ subunit was cloned in the Bluescript SK vector. Site-directed mutations were generated by PCR mutagenesis and were confirmed by sequencing. cRNA for all constructs (WT $\alpha_1$, mutant $\alpha_1$, $\alpha_2\delta$ and $\beta_4$ subunits) were transcribed \textit{in vitro} using T7 polymerase after linearization. The $\alpha_1$, $\alpha_2\delta$ and $\beta_4$ cRNA were injected into \textit{Xenopus} oocytes with a total concentration of ~0.5µg/µl (ratio $\alpha_1:\alpha_2\delta:\beta_2a = 5:5:4$) and a volume of ~50nl per oocyte. Oocytes were prepared and maintained as described (Lu et al., 1999).

2.2. Electrophysiology

Whole-cell currents recorded with two-electrode voltage-clamp with the OC-725C oocyte clamp amplifier (Warner Instruments) were used mainly for checking the functional expression of channels in oocytes. Procedures and protocols were as described (Wu et al., 2002). All patch-clamp recordings were performed with the Axopatch 200A amplifier (Axon Instruments, Sunnyvale, CA). Currents were filtered at 2 kHz, digitized at 10–50 kHz. Oocyte was bathed in a control solution containing (in mM) 125 KCl, 4 NaCl, 10 HEPES, 10 EGTA (pH 7.3 with KOH). In all run-down and MARCKS peptide experiments, macroscopic currents were recorded using the inside-out patch configuration. Recording glass pipettes pulled from pyrex glass tubes (Corning, Acton, MA) were filled with a solution of (in mM) 85 BaCl\textsubscript{2}, 10 HEPES (pH 7.3 with KOH) and had a resistance of 0.2–0.3 MΩ. Currents were evoked from a holding potential of
−80 mV every 1.5 s by depolarizations of 6–8 ms from −30 mV to +100 mV in 10-mV increments, followed by a 30-ms repolarization to −30 mV. When some channel properties (activation, inactivation and deactivation) of the WT and I1520H mutant channels were examined, recording pipettes were filled with a solution containing (in mM) 45 BaCl₂, 80 KCl and 10 HEPES (pH 7.3 with KOH). In these experiments, the currents were obtained from the cell-attached configuration. To eliminate contamination from the Cl⁻ current mediated by the endogenous Ca²⁺-activated Cl⁻ channels, 50 nl of 50 mM BAPTA (Sigma, St. Louis, MO) was injected into the oocyte 10–30 min prior to the experiment. MARCKS peptide was synthesized by Global Peptide Services (Ft. Collins, Co). Its sequence is NH₂-KKKKKRFSFKSFKLSGFS-FKKNKK-COOH. 10 mM stock solution of MARCKS peptide was aliquoted and kept at −20°C and was diluted in the control solution before every application. MARCKS peptide was applied until its inhibition could not be distinguished from the intrinsic rundown of the current. Experiments were carried out at 21–23 °C.

2.3. Data analysis

Data acquisition and analysis were performed using pClamp8 (Axon Instruments) on a PC through a Digidata 1200 interface. The time course of decay of the tail currents after patch excision was fitted with a single exponential function. $T_{50}$ was calculated based on the fitting and defined as the time for the current to decay to 50% of its starting value. Speed of rundown was also quantified as the remaining tail current after 10-minute perfusion for P/Q-type channels and 3-minute perfusion for N-type and L-type channels. The effect of MARCKS peptide was calculated from the tail currents after +100 mV depolarization obtained before application and after washout of the peptide. Data are represented as mean ± s.d. (number of observations).
Significance was determined using the Kruskal-Wallis Test with Bonferroni correction. No statistical analysis was performed if the number of observation was less than 5. No corrections were made for leakage current, which was negligible in macropatch recordings.

3. RESULTS

3.1. Mutations of a single residue in IIIS6 reduce rundown of P/Q-type channels

The activity of P/Q-, N- and L-type Ca\(^{2+}\) channels stayed constant in the cell-attached mode but underwent rapid rundown upon inside-out patch excision. In our previous study of P/Q-type channel inner pore (Zhen et al., 2005), we found a cysteine mutation in the Ca\(_V\)2.1 \(\alpha_1\) subunit that could greatly reduce rundown in inside-out patches. This amino acid, I1520, is located at the intracellular end of IIIS6 (Fig. 1A), one amino acid below the putative membrane/cytoplasm interface (Fig. 1B). To obtain better mechanistic understanding of why and how the I1520C mutation affected rundown, we systemically mutated this isoleucine to all the other 18 amino acids and examined their effects on channel rundown. All mutant channels, except I1520K and I1520R, were functional.

Using the tail current after a depolarization to +100 mV (\(I_{\text{tail} (+100 \text{ mV})}\)) as a readout of the total number of functional channels, we could faithfully measure the time course of rundown (Fig. 2 A–C), as reported previously (Wu et al., 2002; Gamper et al., 2004). The extent of rundown was quantified as the remaining \(I_{\text{tail} (+100 \text{ mV})}\) after 10-minute perfusion with a control solution. The current of the WT channels decayed quickly, to 38.2% of its starting value (i.e., current obtained immediately after patch excision) 10 minutes after patch-excision (Fig. 2 A and
D). In contrast, the current of the I1520H mutant channels decayed much more slowly, to 84.0% of its initial value (Fig. 2 A and D).

Figure 2D shows the relative extent of rundown of all functional mutant channels compared with that of the WT channel. With the exception of the leucine and methionine mutations, all mutations appeared to attenuate rundown by varying degrees. We focused on the histidine and aspartate mutations in later studies because they were among the two most effective mutations and their side-chains carry opposite charges.

We next examined the effect on channel rundown of the histidine mutation at the homologous positions (S360, V714 and M1820, Fig. 1A) in the S6 segment of other three repeats. Interestingly, S360H, V714H and M1820H had no significant effect on rundown of P/Q-type channels (data not shown). Thus, the effect of I1520H appears to be specific for IIIS6.

### 3.2. Homologous mutations in N- and L-type channels also reduce rundown

We also examined whether histidine and aspartate mutations at the homologous positions of N- and L-type channels also retarded rundown of these channels (Fig. 1A). Because N- and L-type channels rundown much faster than P/Q-type channels do, their rundown was quantified as the remaining $I_{\text{tail}(+100 \text{ mV})}$ after 3-minute perfusion, or as $T_{50}$, the time required for $I_{\text{tail}(+100 \text{ mV})}$ to decay to 50% of its starting value immediately after patch excision. The aspartate mutation, but not the histidine mutation, reduced N-type channel rundown (Fig. 2 E and F). On the other hand, both mutations attenuated L-type channel rundown (Fig. 2 G and H).
3.3. Mutations that reduce rundown increase the apparent affinity for PIP$_2$

We then explored possible mechanisms of attenuation of rundown caused by I1520 mutations in P/Q-type channels. Because phosphatidylinositol-4,5-bisphosphate (PIP$_2$) is essential for maintaining Ca$^{2+}$ channel activity (Wu et al., 2002; Gamper et al., 2004), we examined the effect of removing PIP$_2$ on the WT and I1520H and I1520D mutant P/Q-type channels by applying a PIP$_2$-sequestering reagent to the intracellular side of the channels. This sequestering reagent is a peptide from the protein “myristoylated alanine-rich protein kinase C substrate” (MARCKS), which has been shown to sequester PIP$_2$, mainly through nonspecific electrostatic interactions between negatively charged PIP$_2$ and a basic region that contains a large number of positively charged amino acids. A peptide corresponding to this basic domain, residue 151 to 175 (NH2-KKKKKRFSFKKSFKLSGFSFKKNKK-COOH), has the same function as the whole protein (McLaughlin et al., 2002). We thus used this MARCKS peptide to sequester PIP$_2$ in the membrane. As expected, the MARCKS peptide accelerated the decay of $I_{tail(+100\,mV)}$ of the WT channels (Fig. 3A). However, while 3 µM peptide inhibited 74.1 ± 8.8% (n=10) of the WT channel current, 100 µM peptide inhibited only 38.6 ± 12.1% (n=8) of the I1520H channel current. The dose-response curve of the mutant channel was greatly shifted rightward compared with that of the WT channel (Fig. 3B), indicating that the apparent PIP$_2$ binding affinity was markedly increased in the mutant. Similar results were obtained for the I1520D mutant (Fig. 3B). These results indicate that the mutant channels still need PIP$_2$ to maintain their activity, but they have a higher apparent affinity for PIP$_2$.

The attenuation effect on rundown of the histidine and aspartate mutations and PIP$_2$ appears non-additive, since application of PIP$_2$ to the mutant channels did not further decrease rundown of N-, L- as well as P/Q-type channels (data not shown).
3.4. I1520H mutation changes channel biophysical properties

I1520 is strategically situated at the intracellular end of the IIIS6 segment, which together with the S6 segment of the other three repeats forms the inner pore (Zhen et al., 2005). Single mutations at IIIS6 as well as at other S6 segments have been shown to greatly affect inactivation (Hering et al., 1996; Kraus et al., 1998; Hering et al., 1998; Kraus et al., 2000; Berjukow et al., 2001; Stotz and Zamponi, 2001; Zhen et al., 2005). Furthermore, the activation gate is located at or near the membrane/cytoplasm interface (Xie et al., 2005). We therefore examined the effect of the I1520H mutation on a host of activation and inactivation properties (Fig. 4). Not surprisingly, there were indeed significant changes. The half activation voltage ($V_{1/2}$) was shifted from $13.8 \pm 2.4$ mV (n=10) in the WT channel to $0.6 \pm 3.9$ mV (n=8) in the mutant channel, while the slope factor ($k$) remained similar ($7.9 \pm 0.6$ mV (n=10) in WT and $6.1 \pm 0.8$ mV (n=8) in I1520H, respectively). Although the activation kinetics at a given voltage is the same (Fig. 4B), the deactivation kinetics is slower in the mutant channel (Fig. 4C). The mutant channels also became more resistant to inactivation (Fig. 4D and E). The mid-point voltage and slope factor of steady-state inactivation were $-24.1 \pm 4.8$ mV and $-8.5 \pm 2.5$mV (n=10) for the WT channel and $-20.4 \pm 4.5$ mV and $-4.9 \pm 1.0$ mV (n=10) for the I1520H mutant channel, respectively (Fig. 4D). At $+20$ mV, the time for 50% inactivation was $0.4 \pm 0.1$ s (n=10) for WT and $1.4 \pm 0.7$ s (n=10) for I1520H (Fig. 4E). Correspondingly, the mutant channels recovered more quickly from inactivation than did the WT channels (Fig. 4F).
4. DISCUSSION

4.1. Mechanism of attenuation of rundown

Several mechanisms have been suggested to contribute to Ca\(^{2+}\) channel rundown, including loss of unidentified cytoplasmic factors, reversal of PKA phosphorylation and Ca\(^{2+}\)-dependent proteolysis (McDonald et al., 1994). Studies on cardiac as well as neuronal L-type Ca\(^{2+}\) channels show that exposure of the cytoplasmic side to reagents that promote PKA phosphorylation, such as cAMP, Mg-ATP and the catalytic subunit of PKA, greatly attenuates rundown (Kostyuk et al., 1981; Doroshenko et al., 1982; Chad and Eckert, 1986; Armstrong and Eckert, 1987; McDonald et al., 1994). In some instances, addition of protease inhibitors such as leupeptin or calpastatin, in combination with Mg-ATP and/or an unknown cytoplasmic factor, further stabilizes Ca\(^{2+}\) channel activity and prevents rundown (Chad and Eckert, 1986; Romanin et al., 1991; McDonald et al., 1994; Hao et al., 1999). However, the stabilizing effect of Mg-ATP and PKA as well as protease inhibitors is highly variable and is usually short-lived in inside-out patches. Indeed, results from a number of studies suggest that neither PKA phosphorylation nor proteolysis plays a critical role in the rundown process (Byerly and Yazejian, 1986; Belles et al., 1988; McDonald et al., 1994; Kameyama et al., 1997).

Very little is known about the mechanism of rundown of P/Q- and N-type channels. In general, the molecular and structural mechanisms of Ca\(^{2+}\) channel rundown at the channel level are essentially unclear. Three possibilities can be considered, which are not mutually exclusive. First, the channel enters a permanent but existing inactivated state. Second, a new “rundown” gate is formed along the ion conduction pathway. Third, the voltage sensor and the activation gate are decoupled. Our results do not directly address or distinguish among these possibilities.
Nevertheless, based on our recent finding (Xie et al., 2005) that the activation gate of VGCCs is located at the intracellular end of the S6 segments, at or near the membrane/cytoplasm interface where I1520 is situated, we speculate that some mutations of I1520, such as the histidine mutation, probably help stabilize the open conformation. Furthermore, a previous study has shown that the gating current does not change when the ionic current undergoes rundown, suggesting a disruption of the linkage between the voltage sensor and activation gate after rundown (Costantin et al., 1999). Thus, strengthening and/or stabilizing the coupling between the voltage sensor and activation gate could be a mechanism for the I1520 mutations to counter rundown. Consistent with this idea, we find that the I1520H mutant channel becomes easier to open (Fig. 4A), slower to close (Fig. 4C), harder to inactivate (Fig. 4 D and E), and faster to recover from inactivation (Fig. 4F). These changes of biophysical properties are not surprising given the strategic location of I1520.

At first glance, there seems no mechanistic picture emerging from the amino acid substitutions at position 1520 since residues that appear able to attenuate rundown can be either large or small, hydrophilic or hydrophobic, negatively charged or positively charged (Fig. 2D). But if one looks at the results from a different perspective, one finds that three residues at this position, isoleucine (WT), leucine and methionine, tend to make the channels rundown faster (Fig. 2D). A common property of these three residues is that they are hydrophobic. It is therefore tempting to speculate that hydrophobic interactions formed between the amino acid at position 1520 and its yet unknown partners play a key role in facilitating channel rundown, and such interactions can only be produced by a hydrophobic residue of certain size at this position.

Another possible mechanism for some I1520 mutations to attenuate rundown is to increase the actual affinity of the mutant channels for PIP₂, which is required for maintaining the
activity of VGCCs (Wu et al., 2002; Gamper et al., 2004). This possibility, however, seems unlikely since, as a hydrophobic residue, I1520 is unlikely to be directly involved in binding PIP$_2$. Furthermore, substitutions with different types of amino acids, including not only histidine, but also cysteines, alanine and even negatively charged aspartate (which would repel the negatively charged PIP$_2$), were able to reduce channel rundown (Fig. 2D). The apparent enhanced interaction with PIP$_2$ for the I1520H and I1520D mutants is thus most likely an allosteric effect.

4.2. Usefulness of the slow rundown mutant channels

For P/Q-type channels, the attenuation of rundown by the I1520H and I1520D mutations is much stronger and more reliable than that achieved by bath application of PIP$_2$ and Mg-ATP (Wu et al., 2002). The effect of analogous mutations in N- and L-type channels is less dramatic, but it is still much more robust than that of PIP$_2$. Furthermore, addition of PIP$_2$ can produce significant shift in the voltage-dependence of activation, especially in the absence of reagents promoting PKA phosphorylation (Wu et al., 2002). In practice, introducing a single amino acid mutation is more convenient and reliable than applying Mg-ATP and PIP$_2$ in every patch and is also more economical. Thus, these mutant channels could be useful for functional studies on VGCCs that would benefit from a direct access to the cytoplasmic side of the channels, especially in inside-out membrane patches. Admittedly, the utility of these channels is complicated and limited by the fact that they not only display much slower rundown but also exhibit significantly altered biophysical properties. However, the histidine and aspartate mutant channels still demonstrate more or less proper voltage-dependent gating, so for those studies where the absolute value of the activation and inactivation properties is not the primary concern,
such as studies of channel modulation by intracellular proteins, factors and second messengers, these mutant channels could prove to be highly beneficial.

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We thank Y. Mori for Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 cDNAs, T. Tanabe for Ca\textsubscript{v}1.2 and \(\alpha_2\delta\) cDNAs, and E. Perez-Reyes for \(\beta_4\) cDNA. This work was supported by National Institutes of Health grant NS045819.
Figure 1. Amino acid sequences and transmembrane topology of the α₁ subunit of voltage-gated Ca\textsuperscript{2+} channels. (A) Amino acid sequences of the S6 segments in the four repeats of Ca\textsubscript{v}2.1 subunit and the S6 segment in the third repeat of Ca\textsubscript{v}2.2 and Ca\textsubscript{v}1.2 subunit. I1520 in Ca\textsubscript{v}2.1 and homologous residues in Ca\textsubscript{v}2.2 and Ca\textsubscript{v}1.2 are shown in italics. (B) Putative transmembrane topology of the α₁ subunit of voltage-gated Ca\textsuperscript{2+} channels. The black dot at the intracellular end of IIIIS6 shows the location of I1520.
Figure 2. Mutations of I1520 in P/Q-type or homologous positions in N- and L-type channels affect their rundown. (A) Representative time course of rundown of $I_{\text{tail}}(+100\,\text{mV})$ of the WT P/Q-type channel (•) and I1520H mutant channel (○) in a control solution. Currents are normalized to that obtained immediately after patch excision. (B), (C) Representative current traces for the WT and I1520H mutant channel, taken at the time points indicated in (A). In this and the following figures, channels were activated by depolarization to $+100\,\text{mV}$ from a holding potential of $-80\,\text{mV}$. Tail currents were recorded at $-30\,\text{mV}$ and were shown on an expanded time scale. (D) Remaining $I_{\text{tail}}(+100\,\text{mV})$ of the WT and all functional mutant channels after 10-min perfusion in a control solution. Error bar represents S.D. and the number in parenthesis represents number of recordings for each channel type. The histidine, aspartate, alanine and cysteine mutants are significantly different from the WT ($P<0.01$) but the leucine mutant is not. No statistical analysis was performed on the other mutants due to the small number of observations. (E), (F) Remaining $I_{\text{tail}}(+100\,\text{mV})$ of the WT and mutant N-type (A) and L-type (C) channels after 3-min perfusion in a control solution. (G), (H) $T_{50}$ for decay of $I_{\text{tail}}(+100\,\text{mV})$ of the WT and mutant N-type (B) and L-type (D) channels (** $P<0.001$).
Figure 3. Inhibition of WT and I1520 mutant P/Q Ca\(^{2+}\) channels by MARCKS peptide. (A) Inhibition of \(I_{\text{tail}} (+100 \text{ mV})\) of the WT channel by intracellular application of 3 µM MARCKS peptide. (B) Representative current traces for the WT and histidine and aspartate mutant channels. Traces a and b were taken immediately before and after application of MARCKS peptide, respectively, as exemplified in (A). (C) Remaining currents of the WT and I1520H and I1520D mutant channels after steady-state inhibition by different concentrations of MARCKS peptide.
Figure 4. Comparison of biophysical properties of WT and I1520H mutant P/Q Ca^{2+} channels (see figure legend, next page)
**Figure 4 Legend:** Comparison of biophysical properties of WT and I1520H mutant P/Q Ca\(^{2+}\) channels. (A) Voltage-dependence of activation. Currents were evoked from a holding potential of −80 mV every 3 s by 10-ms depolarizations ranging from −40 mV to +100 mV in 10-mV increment, followed by a 15-ms repolarization to −30 mV. Data points represent normalized tail currents recorded at −30 mV. All activation curves were well fitted by a Boltzmann function. (B) Activation time constants at different test potentials, obtained by fitting the activation phase with a single exponential function (n=10). (C) Deactivation kinetics. Channels were activated by a 20-ms prepulse to +100 mV and deactivation was measured by the decay of the tail current recorded at various repolarizing test potentials, ranging from −80 mV to 0 mV in 10-mV steps. The decay phase was fitted with a single exponential function. Deactivation time constants at different test potentials are plotted. (D) Voltage-dependence of inactivation. Steady-state inactivation was determined by a three-pulse protocol in which a 20-ms normalizing pulse to +20 mV (pulse A) was followed sequentially by a 25-s conditioning pulse (ranging from −60 mV to +40 mV) and a 20-ms test pulse to +20 mV (pulse B). The holding potential was −100 mV and interval between each protocol was 2 min. Peak current evoked by pulse B was normalized by that evoked by pulse A and was plotted against the conditioning potentials. (E) Inactivation kinetics. Current was evoked by a 25-s step depolarization to +20 mV from a holding potential of −100 mV. (F) Kinetics of recovery from inactivation. To obtain the time course of recovery, a control test pulse to +30 mV (pulse #1) was followed by a 4-s conditioning pulse to +30 mV. After varying durations ranging from 10 ms to 96 s at −80 mV, a test pulse to +30 mV (pulse #2) was given again. Recovery was quantified by normalizing the peak current evoked by pulse #2 with that evoked by pulse #1 and plotting it against the recovery duration. The time course was generally fitted well by the sum of two exponential functions. \(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\) are 0.11 ± 0.01 s and 7.3 ± 0.8 s (n=10) for the I1520H channel, and 0.47 ± 0.05 s and 12.5 ± 0.7 s (n=10) for the WT.
Appendix II:

Regulation of voltage-gated calcium channels by PKA and PIP$_2$
1. INTRODUCTION

Voltage-gated calcium channels (VGCCs) translate electrical signals on cell membranes into chemical signals through an influx of calcium ions, which act as second messengers to activate a variety of cellular processes (Catterall, 2000). The subtypes of VGCCs (L-, N-, P/Q-, R, and T-type) can be characterized by differential physiological properties, subcellular localization, and specific blockers. The molecular architecture of these channels consists of a pore-forming $\alpha_1$ subunit and a variety of regulatory subunits ($\beta$, $\gamma$, $\alpha_2\delta$).

Synaptic transmission is mediated by the action of N- (Cav2.2) and P/Q-type (Cav2.1) channels (Takahashi and Momiyama, 1993; Wheeler et al., 1994). Depolarization after an action potential triggers the opening of these channels in the pre-synaptic nerve terminal, leading to an influx of calcium ions that trigger transmitter release. To ensure temporal and spatial control of synaptic transmission, calcium channels are modulated through interactions with synaptic vesicle-release proteins, G-proteins, phosphatidylinositol-4,5-bisphosphate (PIP$_2$), and by protein phosphorylation, to name a few (Sheng et al., 1994; 1996; Catterall, 2000; Spafford and Zamponi, 2003).

Crosstalk between these pathways results in a regulatory network that gives rise to the fine-tuned response of a neuron to different stimuli. For example, N- and P/Q-type channels are inhibited by direct binding of the G-protein $\beta\gamma$ subunit; protein-kinase C (PKC) activity up-regulates the channel by phosphorylating the G$\beta\gamma$ binding site (Stea et al., 1995; De Waard et al., 1997; Zamponi et al., 1997). In addition, N- and P/Q-type channels are not necessarily modulated in the same way by these mechanisms; for instance, the channel subtypes respond differently to various G$\beta$ subunits (Zamponi and Snutch, 2002).
Our focus was on the regulation of the P/Q-type channel by cAMP dependent protein kinase (PKA) phosphorylation and PIP$_2$ binding, and the mechanism of the interplay between these two regulatory elements.

Presynaptic calcium influx activates Ca$^{2+}$/calmodulin-dependent adenylyl cyclase, which increases the concentration of cAMP and activates PKA; PKA can then phosphorylate VGCCs. PKA has been shown to increase excitatory synaptic transmission, and is required for long-term depression and long-term potentiation (Chen and Roper, 2003). Although direct evidence of the P/Q channel as a substrate for PKA phosphorylation did not exist, it was known that activity of P/Q channels expressed in *Xenopus* oocytes was increased by injection of cAMP; this could be reversed with a peptidic inhibitor of PKA (Fournier et al., 1993). This increase was accompanied by a negative shift of the activation curve, indicating that the channels are in a mode in which they are easier to open, because they can be opened at lower potentials (Mogul et al., 1993; Tamse et al., 2003). This was similar to the effect of PKA on the L-type channel, for which PKA phosphorylation sites had been isolated on the on both the $\alpha_1$ and $\beta$ subunits (Curtis and Catterall, 1984; 1985), suggesting a similar mode of action. Therefore, our first goal was to determine the sites of PKA phosphorylation on the P/Q-type channel.

Another important regulator of ion channels is phosphatidylinositol 4,5-bisphosphate (PIP$_2$), an integral membrane phospholipid that, in addition to being the precursor of important second messengers (IP$_3$ and DAG), directly regulates many types of channels, including K$^+$, TRP, and ENaC (Hilgemann et al., 2001). Work in our laboratory revealed an important role of PIP$_2$ in VGCCs: Wu et al demonstrated that that PIP$_2$ plays two opposing roles in the modulation of N- and P/Q-type VGCCs (Wu et al., 2002). On one hand, PIP$_2$ is necessary to maintain channel activity: activation of PLC, which hydrolyses PIP$_2$, causes channel inhibition in whole-
cell recordings, and application of PIP$_2$ greatly slows current rundown in inside-out patches. However, PIP$_2$ also alters the voltage dependence of channel activation, making the channels more difficult to open. Interestingly, this effect is reversed by PKA phosphorylation (Figure 1). These data led to a model postulating that VGCCs have two PIP$_2$ binding sites: a high-affinity S (stabilization) domain and a low-affinity R (reluctant) domain. When PIP$_2$ is bound to both sites, the channel is in the ‘reluctant’ state, opened only by strong depolarization. Phosphorylation of the R domain by PKA shifts the channels into the ‘willing’ state, now sensitive to weak depolarization. When neither site is bound by PIP$_2$, the channel is inactive (Figure 2).

Although multiple channel types have been shown to be regulated by PIP$_2$, and it is believed that this occurs through direct physical interaction with the channel (namely, an electrostatic interaction between the negatively charged inositol ring and positively charged amino acid residues in the channel), there is no consensus binding sequence that would facilitate identification of the interacting site (Suh and Hille, 2005). Therefore, the majority of functional studies rely on measurement of changes in function as PIP$_2$ is depleted from, or added back to, membranes. Despite the difficulties, direct binding has been demonstrated in other channels, such as the inward-rectifier K$^+$ channels Kir1.1 (ROMK1) and Kir2.1 (Huang et al., 1998; Liou et al., 1999). Given our model, in which PKA phosphorylates the ‘R domain’ of PIP$_2$ binding, we felt that identification of the PKA phosphorylation site could lead us to the location of the ‘R domain.’ We could then test our model of the mechanism of crosstalk between these two regulatory pathways, in which PKA phosphorylates the R domain, and this negative phosphate blocks the electrostatic interaction between PIP$_2$ and the channel. Unfortunately, due to technical difficulties that will be discussed below, this project was not successful.
2. METHODS AND RESULTS

2.1 Determine sites of PKA phosphorylation in vitro

First, an in vitro approach was used to narrow down which of the intracellular loops of the P/Q channel might serve as intracellular substrates for PKA phosphorylation. A member of the lab had generated GST-fusion peptides corresponding to the intracellular loops of the rabbit P/Q-type channel by inserting peptide sequences downstream of the glutathione S-transferase sequence in the pGEX expression vector (Figure 3a). Consensus PKA sites (RRXS/T) had been identified in the N-terminal tail, the intracellular loop between repeat domains I and II (1/2 loop), the loop between repeat domains II and III (2/3 loop), and the C-terminal tail (Mori et al., 1991). We performed in vitro phosphorylation to determine which of the intracellular domains might be phosphorylated by PKA. For each reaction, 150 pmol of GST-fusion protein was incubated with PKA catalytic subunit (Upstate Biotech) along with radio-labeled ATP (10 µCi γ-32P-ATP and 100 µM cold ATP), run on SDS-PAGE gel, and visualized for ATP incorporation through autoradiography (Figure 3b).

The strongest signal was seen in the C-terminal tail; the 1/2 loop and the N-terminal half of the 2/3 loop also gave a positive results. No ATP incorporation was seen for the N-terminus or the 3/4 loop. This largely correlated with the predictions of the program NetPhosK (Blom et al., 2004) (Figure 3c): no sites were predicted for the N-terminus or the 3/4 loop, while multiple sites were predicted for the C-terminus, 1/2 loop, and 2/3 loop. In addition, a site with a very high score was predicted in the small intracellular linker between the S4 and S5 transmembrane helices in repeat II (II S4/II S5).
2.2 Characterization of putative PKA phosphorylation sites in vivo

To determine if the predicted PKA sites are actually phosphorylated in vivo and produce a change in channel activity, we utilized the *Xenopus* heterologous expression system. Wild type and mutated rabbit P/Q-channel subunits were subcloned into a variant of the oocyte expression vector pGEMHE; cRNA was synthesized in vitro and injected into *Xenopus laevis* oocytes. Functional channels are expressed on the cell surface and can be used for recordings after 4-10 day incubation (see Chapter 2 Materials and Methods for more details).

Macropatch recordings were carried out using pipettes of 13-20 µm filled with a solution of 45 mM BaCl$_2$, 80 mM KCl, and 10 mM HEPES (pH 7.3). The oocyte bath solution contained 125 mM KCl, 4 mM NaCl, 10 mM HEPES and 10 mM EGTA (pH 7.3). Macroscopic currents were evoked every 3 seconds from a holding potential of -80 mV, by 10 msec depolarizations to test potentials ranging from -10 mV to +100 mV in 10 mV increments, followed by 15 ms repolarization to -20 mV. The patch of membrane was excised, revealing the intracellular side to the bath solution, where reagents such as PKA can be experimentally applied (inside-out macropatch method).

As previously mentioned, PKA phosphorylation results in an increased P/Q-type current and negative shift of voltage dependence of channel activation; one could test which mutations abolish these effects. However, these changes have been shown to not be large enough to be an effective assay. Instead, a more robust effect to study is if a mutation abolishes the ability of PKA to prevent PIP$_2$ inhibition. Voltage-dependent inhibition by PIP$_2$ can be seen as a 15-20 mV positive shift in the activation curve. Application of PKA before and during PIP$_2$ application prevents this shift (Figure 1). Therefore, if a mutation or combination of mutations still exhibits a
positive shift in activation when PKA is applied before PIP$_2$, the mutation has abolished PKA phosphorylation. Using standard site-directed mutagenesis techniques (Sarkar and Sommer 1990), we could construct P/Q-type $\alpha_1$ subunits in which the sites determined to be phosphorylated \textit{in vitro} were mutated to alanine, and test if these channels lose the response to PKA application.

We first tested a channel constructed by a former lab member, in which the five putative consensus PKA sites had been mutated: T58A (N-terminus), T95A (N-terminus), T425 (1/2 loops, NetPhosK score 0.53), T1043 (2/3 loop), and T2407A (C-terminus, NetPhosK score 0.61) (Figure 3a). We also constructed a channel in which we mutated the IIS4/IIS5 residue with the highest NetPhosK score: S599A (NetPhosK score 0.78). However, for both of these channels, preliminary recordings indicated that PKA application still blocked the shift in activation curve by PIP$_2$ (data not shown).

Unfortunately, it became apparent during these experiments that the response of the channels to PIP$_2$ application had become quite variable: sometimes PIP$_2$ could elicit the robust shift in the activation curve as normal, but often the shift would be minimal. This made it difficult to quantify if PKA was actually blocking the shift in activation. This could have been due to the quality of the particular batches of oocytes. The problem persisted for a long enough period that the project was put on hold; ultimately I was not able to revisit this project during the remainder of my graduate studies as other projects took priority. However, it remains an interesting approach for the lab to consider in the future.
Figure 1. PKA phosphorylation prevents PIP$_2$-induced inhibition of P/Q channels in inside-out patches. a, c: time course of $I_{\text{tail}}(+10)$ (filled circles) and $I_{\text{tail}}(+100)$ (open circles) after patch excision (time 0). Horizontal bars indicate application of specified reagents. b, d: voltage dependence of activation before (open squares) and 8-10 min after (filled squares) PIP$_2$ application. Application of PIP$_2$ inhibits $I_{\text{tail}}(+10)$ (a) and shifts activation curve (b) in the presence of protein kinase inhibitor (PKI). In presence of PKA catalytic subunit, PIP$_2$ no longer inhibits $I_{\text{tail}}(+10)$ (c) or shifts activation curve (d). In a channel with a mutated PKA site, we expect that PIP$_2$ will still be able to inhibit current and shift activation curve in the presence of PKA. Reproduced with permission from: Wu et al. Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P$_2$. Nature 2002; 419:947-52. © 2002 Nature Publishing Group, a division of Macmillan Publishers Ltd.
Figure 2. Model of P/Q-type VGCC channel modulation by PIP$_2$ and PKA. There are two distinct PIP$_2$ binding domains: a high-affinity S (‘stabilization’) domain and a low-affinity R (‘reluctant’ or ‘regulatory’) domain. PIP$_2$ binding to S domain shifts channel from an inactive to a willing state (transition 4), PIP$_2$ binding to R domain shifts channel from willing to reluctant (transition 2). Phosphorylation of the R domain by PKA dislodges PIP$_2$ binding, shifting channel to willing state (transition 1). Removal of PIP$_2$ from the membrane also keeps channel in willing state (transition 3), as R domain has lower affinity than S for PIP$_2$. Reproduced with permission from: Wu et al. Dual regulation of voltage-gated calcium channels by PtdIns(4,5)P$_2$. Nature 2002; 419:947-52. © 2002 Nature Publishing Group, a division of Macmillan Publishers Ltd.
Figure 3. In vitro phosphorylation assay of P/Q channel. a) Transmembrane topology of P/Q channel. GST fusion peptides constructed for each of the intracellular regions (orange lines). For longer loops, multiple overlapping peptides were constructed as full-length was not soluble (orange lines bounded by light green). Green dots indicate approximate locations of PKA consensus sites; red dot indicates approximate location of residue S599 in IIS4/IIS5 linker that had highest NetPhosK score. Size of fragments indicated; GST tag adds 26 kDa to size of each peptide. b) Autoradiography shows incorporation of radiolabeled ATP to indicated peptide in presence of PKA. GST alone, negative control; PKAce, positive control (Novagen). No ATP incorporation seen in absence of PKA (data not shown). c) Number of predicted PKA phosphorylation sites per intracellular region according to NetPhosK; scores are percent likelihood of a residue being a PKA phosphorylation site.
Appendix III:

Expression of voltage-gated calcium channels in yeast
1. INTRODUCTION

The regulation of ion channel activity is not dependent just on the activity of the channels as they reside in the plasma membrane, but also control of the numbers of channels on the membrane at any given time. Therefore, regulation of the rate of translation of channel subunits, and their trafficking to the cell membrane, are key components of cell excitability.

The trafficking of voltage-gated calcium channels (VGCCs) to the cell surface is controlled by multiple factors including interactions with regulatory subunits, calmodulin, G-protein coupled receptors, and modifications such as ubiquitination (Simms and Zamponi, 2011). The most well studied has been the interaction of the $\alpha_1$ pore-forming subunit with the regulatory subunits $\beta$ and $\alpha_2\delta$ - although the $\alpha_1$ subunit can be trafficked to the membrane alone, surface density is greatly increased by the presence of the regulatory subunits. The $\beta$ subunit binds to a region of the intracellular linker (AID – alpha interaction domain) between the first and second transmembrane domains of $\alpha_1$, possibly thereby blocking an ER retention motif. There is also evidence that the $\beta$ subunit protects the channel from ER-associated proteasomal degradation by blocking ubiquitination. Alternatively, a host of factors control the internalization and recycling of calcium channels; again, the $\beta$ subunit seems to play a role in this process.

We wanted to utilize the yeast *Saccharomyces cerevisiae* to study the mechanisms that effect trafficking of VGCCs. This is an ideal system in which to study protein folding, assembly, and transport – many of these pathways have been deduced in depth, and are homologous to the systems of higher organisms (Miller and Barlowe, 2010). In addition, yeast are an ideal system for genetic manipulation and biochemical analysis, given their well-characterized genome, fast
generation time, and wealth of existing experimental tools such as expression vectors for heterologous expression of target proteins of interest.

The yeast CCH1 gene encodes a putative voltage-gated calcium channel most similar to the L-type channel – although the overall sequence homology is only 24%, CCH1 shows high conservation of positively charged residues in the voltage-sensing S4 transmembrane domains; furthermore, Cch1 channel activity can be inhibited by L-type channel blockers nifedipine and verapamil but not by known blockers of P/Q-, N-, R-, and T-type channels (van der Aart et al., 1996; Fischer et al., 1997; Teng et al., 2008). Cch1 forms a high-affinity calcium influx system together with Mid1 (a stretch-activated calcium channel) that is necessary in mating: response of the yeast to mating pheromones (α- and α- factors) requires calcium influx, and deletion of either channel leads to cell death when exposed to mating pheromone (Iida et al., 1994; Paidhungat and Garrett, 1997; Fischer et al., 1997).

Therefore, our goal was to heterologously express mammalian VGCC subunits in a yeast strain lacking Cch1, confirm functional expression by growth assays (ability of the channel to rescue growth on low calcium media, or prevent mating pheromone-induced death), and then use this system to study the factors necessary for proper channel trafficking.
2. METHODS AND RESULTS

Two standard growth phenotypes were known for a Δcch1 strain: reduced survival on low-calcium media, and greatly reduced survival when exposed to α-factor (Fischer et al., 1997). The survival rates were comparable for the double mutant Δcch1 Δmid1 or either mutant alone; therefore we focused on Δcch1 as it is homologous to the VGCC α1 subunit.

Deletion strains were constructed in both the α-mating type strain BY4742 (MATα) and the a-mating type BY4741 (MATa) by utilizing standard PCR-generated deletion strategy to replace the open reading frame of Δcch1 with a KanMX gene cassette (Wach et al., 1994). Calcium channel α1 subunits (P/Q-type and L-type) were cloned into the p426GPD yeast expression vector p426GPD (see Chapter 2 Materials and Methods); the auxiliary subunit β2a was cloned into p425GPD which differs from p426GPD only in the auxotroph gene (leucine instead of uracil) so that it could be co-expressed with the α1 subunits by removing both leucine and uracil from the growth media.

To assay growth on low-calcium media, spot assays were performed (four-fold serial dilutions of yeast culture spotted on permissive versus selective media, see Chapter 2 Materials and Methods). Although previously published assays were performed on standard yeast SC media containing high calcium (10 mM) versus low calcium (100 μM), in our hands we could not establish a strong growth phenotype. To further reduce calcium concentration, we replaced agar with agarose and supplemented with the calcium chelator EGTA to fine-tune calcium concentration: 0 mM EGTA for permissive ‘high-calcium’ media, 2 mM EGTA for selective ‘low-calcium’ media. Under these conditions, we could establish a positive control: growth of Δcch1 (MATα) on low-calcium media could be rescued by expression of the CCH1 gene on a
plasmid (pBCS-CCH1-HA4, generous gift of Hidetoshi Iida). However, expression of P/Q- or L-type channel along with β2a subunit did not show any measurable rescue of growth (Figure 1a, L-type assay; P/Q-type, data not shown).

To assay rescue of growth in presence of α-factor, halo assays were performed. Saturated cultures of Δcch1 (MATa) expressing L-type VGCC (with β2a) or CCH1 on a plasmid were diluted to 1 x 10^6 cells/mL in low-calcium top-agarose and plated. Different concentrations of α-factor (Zymo Research, Orange, CA) – 0 µg, 5 µg, 10 µg – were spotted on filter paper discs and placed on the top-agarose. Plates were incubated at 30ºC for 2-3 days, and the diameter of the zone of growth inhibition surrounding the disc was measured. Although CCH1 expression could reduce the zone of inhibition caused by α-factor, the L-type channel had no effect (Figure 1b).

Although it is possible that the VGCCs were just unable to functionally rescue the Cch1 channel, we feel it is more likely that heterologous expression of these very large mammalian channels was not successful. We were never able to confirm expression of the VGCC proteins through western blot (data not shown). We also performed pulse-chase experiments to determine if the protein was being degraded: yeast cultures expressing HA-tagged VGCCs (P/Q- or L-type) co-expressed with β2a were pulsed with radiolabeled ^{35}S-Metione/Cysteine, then chased with cold Met/Cys. In this way, a population of newly translated proteins would be radiolabeled. Aliquots of the culture were taken at time points during an hour of incubation, to monitor if the protein was being degraded: the VGCC channel was immunoprecipitated with an antibody against the HA tag, run on an SDS-PAGE gel, and imaged through autoradiography. Despite multiple attempts, no trace of the VGCC protein was detected – although it is possible that this very large protein was just not soluble in our conditions, it is unlikely as we were able to detect Cch1-HA which is of similar size (Figure 2).
It was at this point that we decided to switch our focus to mammalian TRP channels. From a practical standpoint, we thought heterologous expression would be more successful: while the entire $\alpha_1$ pore-forming subunit (consisting of four transmembrane domains) of a VGCC is encoded by a single large transcript, TRP channel sequences encode a single transmembrane domain which then assembles into tetramers to form a channel. Therefore, it would probably express in yeast with fewer problems. In addition, the wide variety of TRP channel subtypes would increase our chances of finding a channel type that could functionally rescue a yeast channel phenotype (see Chapters 1-4).
Figure 1. Mammalian VGCCs do not functionally rescue Cch1. a) Spot assay of Δcch1 (MATα) expressing empty vector (negative control), CCH1 (positive control), or L-type VGCC with β2a subunit. While CCH1 rescues growth of Δcch1 on low-calcium (2 mM EGTA), L-type channel does not. b) Halo assay of Δcch1 (MATa) expressing empty vector, CCH1, or L-type VGCC with β2a subunit exposed to discs of α-factor (0 µg, 5 µg, 10 µg). Expression of L-type channel does not reduce the zone of inhibition caused by α-factor.
Figure 2. Pulse-chase experiment does not pick up any detectable expression of L-type channel protein. Radiolabeled Cch1 (Cch1-HA) or L-type (L-HA) channels immuno-precipitated from aliquots of Δcch1 (MATa) culture over time. Positive control, Gas1-HA. Faint band is seen for Cch1-HA, but no expression is seen for L-type channel (should appear near or above 220 kDa band of protein standard ladder).
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