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Abstract	<p>The transient receptor potential (TRP) superfamily is one of the largest families of cation channels. The metazoan TRP family has been subdivided into major branches: TRPC, TRPA, TRPM, TRPP, TRPV, TRPML, and TRPN, while the TRPY family is found in fungi. They are involved in many physiological processes and in the pathogenesis of various disorders. An efficient high-yield expression system for TRP channels is a necessary step towards biophysical and biochemical characterization and structural analysis of these proteins, and the budding yeast, <i>Saccharomyces cerevisiae</i> has proven to be very useful for this purpose. In addition, genetic screens in this organism can be carried out rapidly to identify amino acid residues important for function and to generate useful mutants. Here we present an overview of current developments towards understanding TRP channel function and structure using <i>Saccharomyces cerevisiae</i> as an expression system. In addition, we will summarize recent progress in understanding gating mechanisms of TRP channels using endogenously expressing TRPY channels in <i>S. cerevisiae</i>, and insights gained from genetic screens for mutants in mammalian channels. The discussion will focus particular attention of the use of cryo-electron microscopy (cryo-EM) to determine TRP channel structure, and outlines a "divide and conquer" methodology for combining high resolution structures of TRP channel domains determined by X-ray crystallography with lower resolution techniques including cryo-EM and spectroscopy.</p>	
Keywords (separated by '-')	Cryo-electron microscopy - Protein expression - TRP channel - X-ray crystallography	

Chapter 2

Functional and Structural Studies of TRP Channels Heterologously Expressed in Budding Yeast

Vera Moiseenkova-Bell and Theodore G. Wensel

Abstract The transient receptor potential (TRP) superfamily is one of the largest families of cation channels. The metazoan TRP family has been subdivided into major branches: TRPC, TRPA, TRPM, TRPP, TRPV, TRPML, and TRPN, while the TRPY family is found in fungi. They are involved in many physiological processes and in the pathogenesis of various disorders. An efficient high-yield expression system for TRP channels is a necessary step towards biophysical and biochemical characterization and structural analysis of these proteins, and the budding yeast, *Saccharomyces cerevisiae* has proven to be very useful for this purpose. In addition, genetic screens in this organism can be carried out rapidly to identify amino acid residues important for function and to generate useful mutants. Here we present an overview of current developments towards understanding TRP channel function and structure using *Saccharomyces cerevisiae* as an expression system. In addition, we will summarize recent progress in understanding gating mechanisms of TRP channels using endogenously expressing TRPY channels in *S. cerevisiae*, and insights gained from genetic screens for mutants in mammalian channels. The discussion will focus particular attention of the use of cryo-electron microscopy (cryo-EM) to determine TRP channel structure, and outlines a “divide and conquer” methodology for combining high resolution structures of TRP channel domains determined by X-ray crystallography with lower resolution techniques including cryo-EM and spectroscopy.

2.1 Introduction

Ion channels regulate the flow of ions across the plasma membrane in response to a variety of chemical, electrical, temperature, or mechanical signals. Determining ion channel structure is essential for understanding mechanisms of channel gating,

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46 modulation, ion selectivity and permeation. TRP channels display multifunctional
47 and polymodal behavior in their regulation and interactions with proteins, lipids,
48 and other small molecules and ions, and with electric fields. They thus present
49 themselves as intriguing candidates for structural analysis. Detailed structural infor-
50 mation on TRP channels will allow development of new strategies for drug design
51 targeting these channels. Several research groups have expended considerable effort
52 towards understanding TRP channel structure, and structure-function relationships.
53 In this chapter, we will review the current progress in understanding TRP chan-
54 nel structures through structural analysis of both full-length proteins and channel
55 fragments.

57 2.2 The TRP Channel Family

59 The TRP family of channels derive their name from a *Drosophila trp* mutant with
60 defective vision characterized by a transient receptor potential that was reported 40
61 years ago [1, 2]. Twenty years later, molecular cloning and functional analysis led
62 to the discovery that the defect lies in a gene encoding a cation channel, known as
63 TRP in *Drosophila* [3]. In the last decade, subsequent investigations identified over
64 70 homologues to the original TRP channel in invertebrates and vertebrates. To date,
65 a total of 27 mammalian genes belonging to the TRP family have been reported and
66 are subdivided into six major branches [4]: TRPC (canonical), TRPA (ankyrin),
67 TRPM (melastatin), TRPP (polycystin), TRPV (vanilloid), and TRPML (mucol-
68 ipin). The TRPN (NOMP-C homologues) sub-family of proteins are not found in
69 mammals, but they are expressed in invertebrates such as flies and worms [5], and in
70 cold-blooded vertebrates [6, 7]. Yeast and other fungi express TRP channels known
71 as the TRPY sub-family [8, 9].

73 Based on sequence comparison and structural prediction algorithms, TRP chan-
74 nels are related to the superfamily of voltage-gated cation channels. Typically, TRP
75 channels are predicted to have 6 transmembrane helices (TM1-6) per subunit, with
76 varying sizes of cytoplasmic amino- and carboxy-termini, and are thought to form
77 tetrameric assemblies [10, 11]. Depending on the TRP family branch, the cytoplas-
78 mic amino-terminal domain contains different number of ankyrin repeats, ranging
79 from zero to twenty nine, which have been proposed to be involved in a range of
80 interactions (reviewed in [12]), including activating ligands, protein-protein inter-
81 actions, and gating by voltage and temperature [13]. Recently it was discovered that
82 mutations in the ankyrin domain of TRPV4 underlie autosomal dominant disorders
83 of the peripheral nervous system [14, 15], including Charcot-Marie-Tooth disease
84 type 2C, the most common inherited neurological disease [16]. The carboxy-termini
85 of most contain a signature “TRP box” motive and coiled-coiled regions important
86 in protein assembly. The majority of functionally characterized TRP channels are
87 permeable to Ca^{2+} with the exception of TRPM4 and TRPM5, which are perme-
88 able to monovalent cations [17]. Ca^{2+} selectivity is poor for many TRP channels
89 but TRPV5 and TRPV6 are highly permeable Ca^{2+} channels [18]. These channels
90 function as polymodal sensors and are gated by diverse stimuli that include the

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91 binding of intracellular and extracellular messengers; changes in temperature, and
92 chemical or mechanical stress [19].

93 TRP channels are widely distributed through the body, expressed in a vast num-
94 ber of different cell types and have numerous splice variants. TRP channels are
95 particularly abundant in sensory receptor cells, and play a critical role in vision,
96 touch, hearing, taste, pain and temperature sensation. The importance of determin-
97 ing TRP channel structure is highlighted by their emerging roles as major drug
98 targets for the treatment of pain, inflammation, and a range of disorders [20–26], and
99 by the association of genetic defects in TRP channels with a number of devastating
100 diseases, ranging from the most common single-gene neurological defect to poly-
101 cystic kidney disease [27], to night blindness [28–33]. The long-term hope is that
102 understanding TRP channel structures, the structural determinants of ligand bind-
103 ing, and the effects of disease mutations on structure, will aid in the development of
104 new therapeutics.

106 2.3 Ion Channel Structural Biology

109 In the past several years, considerable progress has been made in the field of
110 membrane protein structural biology. High-resolution structures for the pharmaceu-
111 tically relevant eukaryotic membrane proteins, such as G protein-coupled receptors
112 [34–37], transporters [38] and ion channels [39–45], have been obtained and pro-
113 vided very valuable information about mechanism of action for these proteins. Still,
114 membrane protein structure determination remains a difficult task. Despite exten-
115 sive efforts in many laboratories, the number of solved membrane protein structures
116 remains small because of the many challenges presented by membrane proteins.
117 Early success in crystallization of eukaryotic membrane proteins came from the use
118 of the native sources that provide a large amount of protein, for example, bovine
119 retinas providing a milligram of rhodopsin per cow [34]. These problems have been
120 especially challenging for eukaryotic ion channels among which only five high-
121 resolution structures have been determined. Because of the low levels at which
122 ion channels are typically expressed endogenously, structural studies of TRP chan-
123 nels and others require efficient heterologous systems for high level expression and
124 purification. It is important that the cells used for expression are capable of properly
125 folding and assembling the multiple subunits, and of producing them in stable and
126 active form.

127 *E. coli* expression has been widely used for soluble eukaryotic proteins and for
128 bacterial membrane proteins, as well as for soluble domains of transmembrane pro-
129 teins. In the case of TRP channels, soluble fragments successfully produced in
130 high yields from bacteria include the ankyrin repeat domains of proteins in the
131 TRPV family [46–50], domains of TRPM7 including the C-terminal cytoplasmic
132 coiled-coil assembly domain [51] and the α -kinase [52], and the coiled-coil region
133 of the TRPP2 C-terminal domain [53].

134 Unfortunately, methods have not been found for routine expression in functional
135 form of multi-pass eukaryotic membrane proteins such as ion channels in bacteria,

136 likely as a result of lack of appropriate chaperones or other components of the fold-
137 ing and assembly machinery associated with the endoplasmic reticulum. No success
138 has been achieved with full length TRP channels or their fragments containing
139 transmembrane domains.

140 Baculovirus-mediated expression in insect cells offers another useful tool for
141 generating recombinant membrane proteins [54]. In 2009, several new eukary-
142 otic ion channels structures were solved using insect cell expression [42, 44, 45].
143 However, the high cost of this methodology represents a drawback. Expression
144 of some TRP channels using baculovirus has been reported, including overexpres-
145 sion of TRPV1 [55] and TRPV4 [56]. In the case of TRPV4 ~~sufficient~~ protein ~~was~~
146 ~~purified for structure determination by in~~ insect cells showed the possibility of using
147 this system for structural biology of TRP channels, ~~and~~ the TRPV4 structure was
148 determined using electron microscopy [56].

149 Although even more expensive, mammalian tissue culture cells offer a native
150 folding and assembly environment for mammalian membrane proteins. COS-1 cells
151 have been used to express sufficient amounts of a rhodopsin mutant engineered for
152 enhanced thermal stability [57]. Mammalian cells have been used extensively for
153 expression and functional studies of TRP channels, but only in a few cases have TRP
154 channels been purified from them. TRPC3 [58, 59] and TRPM2 [60] were expressed
155 in mammalian cells, and used for structural studies by electron microscopy and
156 single particle analysis.

157 Yeast is another traditional and powerful tool for the expression of eukaryotic
158 recombinant proteins [54]. The advantages include relatively low cost, rapid cell
159 growth and ease of producing large volume cultures. Although distant in evolution
160 from mammals, yeast possess conserved protein folding and assembly machinery
161 that can be exploited to produce mammalian membrane proteins in large amounts.
162 Most commonly, *Pichia pastoris* and *Saccharomyces cerevisiae* are used for the
163 overexpression of membrane proteins. *P. pastoris* can achieve exceptionally high
164 cell densities, that in favorable cases can provide high levels of protein production
165 for structural biology. The first atomic structure of a mammalian potassium channel
166 was possible only after the methodology for the overexpression of functional chan-
167 nel using *P. pastoris* was published [61]. Since that published work, the *P. pastoris*
168 system has been used to obtain structures for a number of ion channels, including
169 aquaporin [62] and potassium channels [39, 41, 43, 63, 64]. Although attempts have
170 been made, there has been no success reported in overexpression in functional form
171 of TRP channels in *P. pastoris*.

172 173 174 175 **2.4 TRP Channels Expression in *Saccharomyces*** 176 ***cerevisiae* and Functional Analysis**

177
178 One of the most characterized methods for the overexpression of recombinant
179 membrane proteins has been budding yeast, *S. cerevisiae*. As eukaryotic organ-
180 isms, yeast contain the machinery necessary for overexpression of eukaryotic

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181 membrane proteins, including rough endoplasmic reticulum and Golgi apparatus
182 with associated molecules required for translocation, folding, and post-translational
183 modifications, in addition to membrane trafficking machinery [65]. They also have
184 the advantage of easy plasmid and genetic manipulation for protein expression. A
185 multitude of different strains including protease-deficient strains as well as a variety
186 of expression vectors comprising yeast episomal plasmids (Yeps) and yeast inte-
187 grating plasmids (Yips) are commercially available and allow genetic manipulation
188 [66]. In addition, yeast express an endogenous TRP channel, known as TRPY1 or
189 YVC1 in *S. cerevisiae* [8, 67], so they have the necessary factors for folding and
190 assembling channels of this family.

191 The earliest example of heterologous expression of a mammalian TRP channel
192 in yeast was that of TRPV1 [68]. The approach was based on a method that was
193 published for the overexpression of P-glycoprotein (MDR1) from the Al-Shawi lab-
194 oratory [65]. The method is simple, and incorporates important steps that help to
195 increase the expression of the protein by several-fold. High levels of expression of
196 the protein were obtained using a maximally active *PMA1* promoter. Although high
197 levels of expression of a Ca^{2+} -permeable channel, even under conditions of low
198 activity, might be expected to be toxic to yeast; however, by transient expression pro-
199 duces large amounts of protein. Protein folding and stability are improved by addi-
200 tion of glycerol, which apparently acts as a sort of chemical chaperone. The optimal
201 concentration of glycerol was determined for each construct, and was found to vary.

202 For heterologously expressed protein to be useful for structural studies, it needs
203 to be functional. Ca^{2+} flux studies using Fura-2 in yeast confirmed that mammalian
204 TRPV1 expressed in *S. cerevisiae* conducts Ca^{2+} in response to its well known
205 agonist, capsaicin. A comparison of different detergents suggested that good sol-
206 ubilization could be obtained using either 1% egg L- α -lysophosphatidylcholine or
207 50 mM *n*-dodecyl- β -D-maltoside; subsequent studies (see below, and VYM and
208 TGW unpublished observations) suggest that related detergents may be more suit-
209 able. Although TRPV1 expressed with a C-terminal His₁₂ tag was purified to about
210 80% purity using nickel-chelate affinity chromatography, the yield was only about
211 1 mg per 16 L of yeast culture.

212 In order to obtain higher yields of functional and highly purified protein, a tech-
213 nique widely used for purification of rhodopsin and related visual pigments [69, 70]
214 was used [71]. An epitope from the C-terminus of rhodopsin, recognized by a mono-
215 clonal antibody 1D4 [72], was engineered into the C-terminus of TRPV1, allowing
216 purification to homogeneity in a single step using an affinity column of immobi-
217 lized 1D4 antibody, and elution with a peptide corresponding to the epitope. For
218 this purification, *n*-decyl- β -D-maltoside was found to be the most useful detergent
219 among those tried.

220 In this preparation, some heterogeneity in subunit molecular weight was revealed
221 by SDS PAGE. A band appearing at a lower molecular weight than full length
222 TRPV1 was consistently observed, but in varying relative amounts. Enzymatic
223 de-glycosylation and stringent disulphide reduction did not eliminate the het-
224 erogeneity, suggesting that the smaller fragment results from proteolysis. The
225 smaller fragment is recognized by the C-terminal 1D4 antibody, so presumably a

226 piece of the cytoplasmic N-terminus is removed. A construct lacking part of the
227 N-terminal domain does not display the lower band (VYM and TGW, unpublished
228 observations).

229 Gel filtration in detergent revealed that purified TRPV1 migrated predominantly
230 as a monodisperse homotetramer. Thus the mammalian protein assembled in yeast
231 appears to have the appropriate stoichiometry for a native TRP channel.

232 Although the presence of functional protein in the yeast membrane had been
233 demonstrated, it was possible that only a small fraction of the total expressed pro-
234 tein was functional, or that detergent extraction disrupted the native structure and
235 function. To address this question, the purified TRPV1 was reconstituted by dial-
236 ysis in the presence of phospholipids into phospholipid bilayers under conditions
237 that yielded, on average, less than one tetramer per vesicle. This ratio can be deter-
238 mined accurately by measurement of protein-to-lipid ratios in the final vesicles, and
239 by using electron microscopy to determine the vesicle size distribution. When the
240 reconstitution was carried out in the presence of 5 mM Ca^{2+} , and the external Ca^{2+}
241 removed by chelate chromatography, the fraction of Ca^{2+} released by a TRPV1 ago-
242 nist, resinaferatoxin, provided an estimated lower limit for the fraction of protein
243 that was in functional form. The estimate is a lower limit because of the likeli-
244 hood that more Ca^{2+} leaks out of the TRPV1-containing vesicles than out of the
245 protein-free vesicles used as controls. This method provided a lower limit of 72%
246 active protein, consistent with nearly all of the purified protein being in active form.
247 This method does not distinguish among channels with cytoplasmic domains out-
248 side vs. inside the vesicles, due to the membrane permeability of the agonist. Future
249 studies using antibodies, lectins, and/or cytoplasmic ligands such as calmodulin,
250 will be needed to determine whether vesicle insertion happens with a preferential
251 orientation, and to optimize conditions for achieving preferential orientation.

252 Other TRP channels have been expressed at high levels in *S. cerevisiae* and puri-
253 fied, using the same approaches as for TRPV1. These include TRPV2, TRPY1–4,
254 TRPM8, and TRPA1 (unpublished data). They are all behaving well in this system,
255 allowing purification of sufficient quantities of protein for functional and struc-
256 tural work. Thus the strategy of transient over-expression in budding yeast and
257 epitope affinity chromatography in detergent appears to be one of general utility
258 for members of the TRP family.

259 260 261 **2.5 Cryo-EM Structures of TRP Channels**

262
263 Single-particle EM can provide structural information for a large variety of bio-
264 logical molecules without the need to produce crystals [73]. Very little sample is
265 required [74]. Single particle cryo-EM [75] is a method in which the specimen, typi-
266 cally a protein embedded in vitreous ice, is held at cryogenic temperatures while
267 images are obtained by the electron microscope [76]. After completing the imaging,
268 single-particle reconstruction methods are used to align and classify the individ-
269 ual particle images, and solve the structure of the protein [77–79]. Because the
270 individual molecules (particles) are randomly oriented in the ice layer, the particle

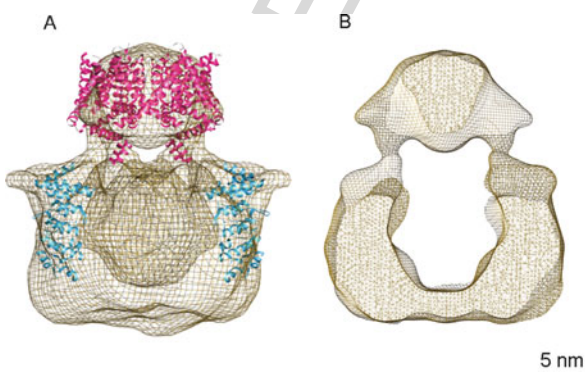
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271 images can be classified into groups representing distinctive views of the original
 272 3-D particle. Particle images in each group can then be aligned with each other and
 273 a consensus shape determined. Once these averaged views are obtained, a “map”
 274 of density throughout the volume of the particle can be calculated to complete the
 275 reconstruction process.

276 Ion channels are excellent candidates for single-particle analysis work [76].
 277 Several channel structures have also been determined by cryo-EM, including a the
 278 voltage gated sodium channel [80], inositol triphosphate receptor [81, 82], muscle
 279 L-type voltage gated calcium channel (dihydropyridine receptor) [83, 84], muscle
 280 calcium release channel/ryanodine receptor (RyR1) [85–88], voltage-gated channel
 281 KvAP [89], large-conductance calcium- and voltage-activated potassium channel
 282 (BK) in a lipid environment [90] and others.

283 In the last few years, several TRP channel structures have been studied by elec-
 284 tron microscopy [91]. These include TRPV1 [71], TRPV4 [56], and TRPC3 [59],
 285 imaged in ice, and TRPC3 [58], and TRPM2 [60] imaged in negative stain.

286 The structure of TRPV1 using a cryo-EM approach was solved recently using
 287 preparation affinity purified from budding yeast and tested for tetrameric structure
 288 and ligand-gated ion flux as described above [71]. Cryo-EM images and single par-
 289 ticle reconstruction revealed that the structure is fourfold symmetric and consists of
 290 two well-defined domains (Fig. 2.1). The more compact has the right dimensions to
 291 correspond to the membrane-spanning domain, likely composed of six transmem-
 292 brane segments per subunit. This domain is 40 Å in the dimension thought to be
 293 normal to the membrane surface, and about 60 Å in diameter. This domain fits well
 294 with the high-resolution structure of the voltage-gated potassium channel Kv1.2 [39].
 295 The other domain, which contains the majority of the mass, as expected for the



310 **Fig. 2.1** Structure of TRPV1 determined by electron cryo-microscopy and single particle analysis
 311 [71]. On the *left* is an iso-dense surface in transparent mesh representation. Superimposed are rib-
 312 bon diagrams of the *x-ray* structures of the transmembrane domain of Kv1.2 [39], 2A79 (magenta),
 313 and the ankyrin repeats of TRPV1 [48], 2PNN (cyan). On the *right* is a similar representation cut
 314 through a center plane perpendicular to the proposed plane of the membrane, showing the large
 315 space within the basket-like cytoplasmic domain of TRPV1

316 N- and C-terminal cytoplasmic domains, is an open, basket-like structure connected
 317 by thin densities to the putative transmembrane domain. There is a region near these
 318 connecting densities, and therefore relatively near to the proposed membrane sur-
 319 face region, that fits well with the high-resolution structure of the ankyrin repeat
 320 domain of TRPV1 [48].

321 Another structure of the TRPV sub-family has been determined using electron
 322 microscopy in ice [56]. The structure of TRPV4 revealed considerable similarity
 323 to the TRPV1 structure, and contains two-distinct regions, likely corresponding to
 324 the transmembrane and cytoplasmic domains of the channel respectively (Fig. 2.2).
 325 The results from these studies provided insight into structural organization of TRPV
 326 sub-family of ion channels and can be used as an initial testable structural template
 327 for studying full-length TRPV channels at higher resolution.

328 As described above, there is reason for some confidence in the published struc-
 329 tures for the TRPV sub-family, given their self-consistency and the extensive
 330 characterization of the TRPV1 preparation. From the reported structures for the
 331 TRPC and TRPM sub-families, many questions remain [91] (Fig. 2.3). Structures
 332 for TRPC3 [58] and TRPM2 [60] determined in negative stain indicated a bullet-
 333 like shape for each, with the dense bullet-head region proposed to be the channel
 334 domain with its transmembrane segments, and a more open and larger domain pro-
 335 posed to be the cytoplasmic regions. There are some qualitative features of these
 336 structures reminiscent of the TRPV family structures, but the detailed structures are
 337 quite distinct. Limitations on the interpretation of these structures arise from the
 338 lack of functional characterization, concerns about the presence of lipid aggregates,
 339 and the inherent limitations and artifacts associated with negative stain. A very
 340 different structure of TRPC3 was reported by the same group, based on images
 341 collected in ice [59]. The ice structure is lace-like and very open, with a very
 342 large overall volume. No regions of appropriate size and continuous density for a
 343 membrane-spanning domain are obvious.

344 The major strength of single-particle reconstruction method is its ability to
 345 produce structural information about proteins that are especially challenging for
 346 X-ray crystallography. These proteins include ones for which high-yield expression
 347 systems are not yet available, as well as large multi-domain proteins, which are too
 348 flexible and too large [92], and membrane proteins for which suitable crystallization
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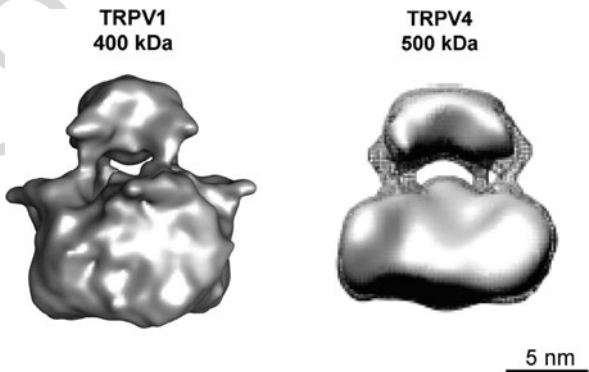
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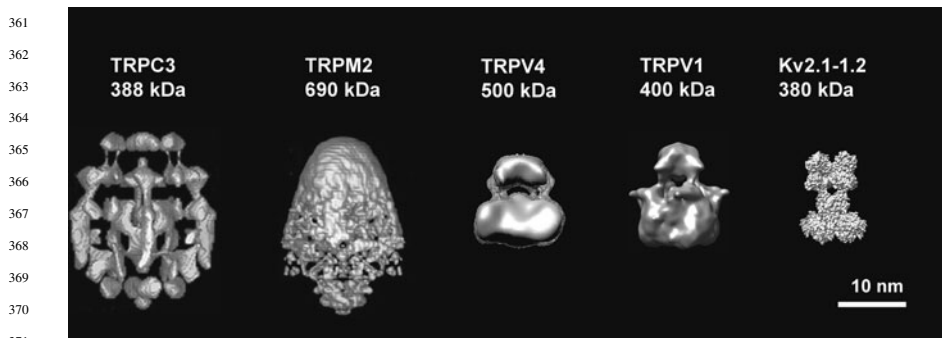
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Fig. 2.2 Similarity of structures of TRPV1, *left* [71], and TRPV4, *right* [56]; (figure reproduced with permission) determined by electron cryo-microscopy



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372 **Fig. 2.3** A comparison of reported channel structures. Images reproduced by permission from
373 [59], TRPC3 [60], TRPM2 [56, 71], TRPV1. The Kv2.1-1.2 chimera structure is from coordinates
374 of 2R9R [41], and reproduced by permission from [91]
375

376 conditions have not yet been found. Cryo-EM allows proteins to be imaged in their
377 native aqueous environment, and in a variety of functional states, without constrains
378 of crystal contacts. In addition, EM data can be collected and structural information
379 extracted as soon as purified protein is available, whereas for X-ray crystallography,
380 considerable effort must be expended in finding suitable crystallization conditions.
381 The major traditional disadvantage of single-particle electron microscopy has been
382 the limitations on resolution. There have been improvements on this front in recent
383 years, especially for large complexes and those with high symmetry, so that for the
384 most favorable cases, near atomic resolution can be achieved, and some side chains
385 can be visualized as well as accurate peptide backbone traces [93–95]. However,
386 for smaller molecules, such as TRP channels, achieving atomic resolution by this
387 method is not feasible with current methods, so that other methods such as homol-
388 ogy modeling, mutagenesis and spectroscopy are needed to answer questions about
389 positions of specific amino acid residues within the low resolution maps. Another
390 limitation, which may come into play with some preparations of TRP channels, is
391 that images of individual particles have low signal-to-noise, so it is possible to select
392 objects other than the protein of interest, such as lipid aggregates or protein-free
393 micelles, or to include degraded, aggregated or denatured forms of the protein in
394 the data set. If care is not taken to avoid such “bad” particles, bizarre results can be
395 obtained. More work will be needed, using more well-characterized TRP proteins
396 and higher resolution cryo-EM data, to resolve the discrepancies among published
397 TRP channel structures.
398
399

400 2.6 Divide and Conquer Approach: Combining X-Ray and EM 401 Data with Computational Modeling 402

403
404 Given recent advances in electron microscopy and image processing [73, 74, 96], it
405 is likely that structures of resolutions approaching 10 Å can be obtained for at least
some TRP channels using electron cryo-microscopy. At this resolution, secondary

406 elements, especially α -helices, can be identified to generate sequence-based models
407 for understanding relationships between channel structure and function. However,
408 in the absence of high-quality two-dimensional crystals, it is unlikely that atomic
409 resolution structures will be obtained directly from electron microscopy, and such
410 structures are needed to visualize such features as the conductance pore, selec-
411 tivity filter, gate, and ligand-binding sites. While determining x-ray structures of
412 full-length TRP channels remains a goal worth pursuing, success has come, and
413 will likely continue to come, more quickly from crystallization of cytoplasmic
414 domains of TRP channels. It should then be possible to fit these high resolution
415 structures into lower resolution structures of complete channels determined by EM,
416 to obtain high resolution models. For this purpose, in addition to x-ray (or perhaps
417 NMR) structures of the domains of interest, homology models based on high resolu-
418 tion structures of homologous proteins (e.g. transmembrane domains of potassium
419 channels) can also be very useful.

420 High resolution structures of fragments of TRP channels have included the
421 α -kinase domain of TRPM7 [52], ankyrin repeats from TRPV subfamily of pro-
422 teins [46–50], the C-terminal cytoplasmic coiled-coil domain of TRPM7 [51], and
423 C-terminal cytoplasmic coiled-coil domain TRPP2 [53]. As an example of combin-
424 ing these with EM structures, the ankyrin repeat domain of TRPV1 could be
425 readily fit into density near the membrane surface in the cytoplasmic region of the
426 TRPV1 structure determined by cryo-EM [71]. As an example of using structures
427 of homologous proteins for this purpose, the structure of Kv1.2, was readily fit into
428 the transmembrane region of the same TRPV1 map.

430 2.7 Biochemical Studies with TRP Channels Purified from Yeast

431
432
433 In addition to structural analysis, purified channels can be used to measure inter-
434 actions with other proteins and regulatory small molecules without the confounds
435 arising from studies in cell membranes containing many other proteins. For exam-
436 ple, recently, several members of TRP family have been proposed to be regulated
437 by intracellular Ca^{2+} , and/or by calmodulin (CaM). Using calmodulin labeled with
438 a fluorescent dye and measurements of emission anisotropy, it is possible to monitor
439 calmodulin binding to TRPV1, TRPV2, and TRPA1 at nanomolar concentrations.
440 Because the measurements can be carried out in real-time using T-format instrumen-
441 tation, in which the light intensities for parallel and perpendicular components are
442 detected concurrently using two independent detectors, both kinetics and thermody-
443 namic parameters can be reliably measured (VYM and TGW, unpublished results).

445 2.8 Functional Studies and Genetic Screens

446 of TRP Channels in Yeast

447
448
449 Once it was found that channels of other species could be expressed in functional
450 form in budding yeast [68] it became possible to use the power and ease of yeast
genetic screens to study structure-function relationships in TRP channels. This

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451 approach was initially applied to the endogenous channel of *S. cerevisiae*, TRPY1
452 (YVC1) [97, 98]. Heterologous expression studies revealed that TRP channels from
453 other fungal species are also functional in *S. cerevisiae*, so they can be studied
454 conveniently in that system [9].

455 TRPY1 is a large conductance (~300 pS) channel expressed in vacuolar mem-
456 branes in yeast. Gain-of-function mutagenesis analysis revealed that aromatic
457 residues on the fifth (phenylalanine) and six (tyrosine) transmembrane segments
458 of the channel control the gating of the TRPY1 channel [98]. Alignment of all the
459 TRP channels revealed that the phenylalanine on the intracellular base of fifth trans-
460 membrane segment of the channel is conserved and may be part of a generic gating
461 mechanism for TRP channels. A very similar approach was used to investigate
462 the TRPV4 gating mechanism, and revealed not only the importance of the main
463 intracellular gate as reported for TRPY1, but also a new voltage-dependent gating
464 mechanism for TRPV4 [99]. Genetic screens for functional changes in mammalian
465 TRP channels have been successfully carried out in *S. cerevisiae* as aids to under-
466 standing structure-function relationships [100]. Results of this study, which used
467 gain-or loss-of-function phenotypes, revealed that pore helix of TRPV1, TRPV2 and
468 TRPV3 play an important role in gating mechanisms of these mammalian channels
469 in addition to the intracellular gate [100].

470 In summary, the use of mutagenesis and the robust *S. cerevisiae* system to study
471 functional and structure-function mechanisms of activation and gating for TRP
472 channels will likely continue to gain popularity and produce very valuable results
473 for understanding TRP channel biology.

2.9 Conclusion

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478 As with any eukaryotic ion channels, studies of TRP channels at the molecular level
479 remain challenging. However, the emergence of new tools, such as expression and
480 genetic screens in *S. cerevisiae*, functional reconstitution protocols, cryo-electron
481 microscopy, and x-ray crystallography of soluble fragments, will likely be combined
482 in the next few years with the huge collection of data from functional studies in
483 vertebrate cell membranes to provide a comprehensive picture of the structures and
484 functional mechanisms of TRP channels.

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