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The use of yeast to understand TRP-channel mechanosensitivity

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Abstract Mechanosensitive (MS) ion channels likely underlie myriad force-sensing processes, from basic osmotic regulation to specified sensations of animal hearing and touch. Albeit important, the molecular identities of many eukaryotic MS channels remain elusive, let alone their working mechanisms. This is in stark contrast to our advanced knowledge on voltage- or ligand-sensitive channels. Several members of transient receptor potential (TRP) ion channel family have been implicated to function in mechanosensation and are recognized as promising candidate MS channels. The yeast TRP homolog, TRPY1, is clearly a first-line force transducer. It can be activated by hypertonic shock in vivo and by membrane stretch force in excised patches under patch clamp, making it a useful model for understanding TRP channel mechanosensitivity in general. TRPY1 offers two additional research advantages: (1) It has a large (~300 pS) unitary conductance and therefore a favorable S/N ratio. (2) Budding yeast allows convenient and efficient genetic and molecular manipulations. In this review, we focus on the current research of TRPY1 and discuss its prospect. We also describe the use of yeast as a system to express and characterize animal TRP channels.

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Introduction

Mechanical forces are universal and diverse; therefore, the ability for living organisms to sense and respond to them is fundamental and vital. The sensing of mechanical forces, known as mechanosensation, underlies such fundamental physiological processes as osmotic regulation as well as highly specified hearing, touch, balance, proprioception, blood-pressure monitoring, organ extension, weight load on bones, and so forth [14, 26]. Mechanosensation often involves converting different forms of mechanical forces into intracellular electrical or chemical signals. Mechanosensitive (MS) ion channels are such first-line transducers [22, 33, 50]. Currently, our understanding of eukaryotic force transducers is rather limited since their molecular identities remain largely unknown or unclear, let alone their working mechanisms. This is in great contrast to our knowledge on voltage and ligand transducers, where the molecular identities have been revealed, high-resolution structures have been solved, and detailed mechanistic models have been established [34, 45, 51].

In prokaryotes, MS channels of large conductance (MscL) and small conductance (MscS) protect bacteria from hypotonic shock. Comprehensive genetic, biophysical, structural, and biochemical analyses have revealed detailed gating mechanisms for MscL and MscS [4, 9, 42]. A key observation on these prokaryotic MS channels is that purified channel proteins reconstituted into artificial bilayers of defined lipids retain their mechanosensitivity, leaving no doubt that the forces that gate these MS channels

come from the lipid bilayer. Changes of the force acting at the lipid–channel interface, when the bilayer is stretched, are considered the ultimate drive for channel gating here [4, 9, 26, 42]. As reviewed by Kung [26], there are indications that the same principle may apply to eukaryotic MS channels. In eukaryotes, several members of transient receptor potential (TRP) ion channel family have been reported to be involved in mechanosensation and are recognized as promising candidate MS channels [6, 31, 41]. In the worm, Osm-9 is reported to be required for nose touch and hypertonic-stress response, first among MS-TRPs [8]. In the fly, *Nanchung* and *Inactive* of TRPV subfamily function in hearing [25], *Nanchung* and *water witch* of TRPA subfamily in hygrosensation [29]. These invertebrate MS-TRPs serve as nice models for mammalian MS-TRPs [54]. In rodents, rat TRPV4 responds to hypotonic swelling [27, 47], and mouse TRPML3 mutations result in vestibular defects and hearing loss [11]. In humans, TRPP1 and TRPP2 are proposed to sense fluid flow in kidney, and their mutations result in polycystic kidney disease [38]. Human TRPC1 and TRPC6 are also reported to be directly mechanosensitive [32, 46], although another report raises some concerns [16]. It remains interesting to define for some of the TRP channel candidates whether they serve as first-line force sensors or downstream amplifiers or play nonsensory roles [6].

TRPs are a loosely connected superfamily. Like other cation channels, a TRP channel likely comprises four subunits, each with six transmembrane α helical segments (S1–S6). Only the amino-acid sequence from S5 to just beyond S6 is conserved among the seven animal TRP subfamilies (TRP-A, -C, -M, -ML, -N, -P, and -V). Searches using this consensus sequence revealed TRP homologs in many unicellular eukaryotes such as *Paramecium*, *Dictyostelium*, *Trypanosome*, etc. [44]. TRP genes are also found in nearly all fungal genomes. The one in the budding yeast, *Saccharomyces cerevisiae*, called TRPY1, has been studied extensively. Note that yeast is an organism as well as a cell. This is like being a mouse and a HEK cell at the same time. As an organism, it exhibits sizes, shapes, growth rates, nutrient requirements, drug resistances, etc., offering diverse phenotype selections. As a cell, it proliferates more rapidly compared with cultured mammalian cells such as HEK, CHO, etc. It has a short doubling time (~2 h), requiring no special equipment to grow to very large population ($>10^8$) [5]. Also, the yeast genome was completely sequenced in 1996, first among eukaryotes. Many tools including bar-coded genomes and deletion–mutation collections have been developed. In addition, yeast can easily be cultured in defined media and allows the use of auxotrophic markers for selection and screen. High throughput screens based on phenotypes in vivo are routine and efficient. For example, plate replication can uncover mutations as rare as 10^{-8} with ease [20, 21]. Another advantage of the yeast system is efficient homologous recombination. Thus, a mutant gene can easily be integrated into the chromosome at its native locus. Homologous recombination is difficult, if not impossible, with multicellular animals. It is therefore no accident that the budding yeast, *S. cerevisiae*, remains one of the best experimental models to analyze the genetics and molecular and cellular biology of eukaryotes.

Electrophysiological studies of ion channels in yeast, though unfamiliar to most animal electrophysiologists, have been developed. Methods have been established to patch clamp its plasma membrane revealing the presence of a two-pore K^+ channel [61] and an MS channel of unknown molecular identity [19]. Interestingly, TRPY1 is expressed in the vacuolar membrane [39] but not the plasma membrane of yeast, an expression pattern reminiscent of that of animal TRPML. Methods have also been advanced to patch clamp its vacuolar membrane [39, 49, 60]. TRPY1 can clearly be activated by hypertonic shock in vivo (monitored with transgenic aequorin) [10] (Fig. 1a) and by membrane stretch in excised patches or whole vacuoles under patch clamp by directly applied pressure on the order of tens of millimeter Hg [60] (Fig. 1b). The combination of these two ways of observing TRPY1 activities makes it a useful model for MS-TRP channels and for eukaryotic primary force transducers. In addition, TRPY1 exhibits a large 300-pS signal over noise, requires no heterologous expression [39], and can easily be manipulated by molecular genetics [48, 59], facilitating the sorts of experiments that are practical with yeast genetics but difficult with animals.

Like its animal counterparts, TRPY1 is polymodal. Besides being an inward rectifier, it is activated by force and by cytoplasmic Ca^{2+} [3, 49, 56, 60]. Ca^{2+} activation likely amplifies the response to force through the positive feedback of Ca^{2+}-induced Ca^{2+} release [39, 49]. Sorting out how force and Ca^{2+} act on the same channel gate should help us understand TRPY1's mechanosensitivity. Ca^{2+} can activate TRPY1 without added stretch force. Strong stretch force can also induce channel activities in the virtual absence of Ca^{2+}, albeit weakly, indicating the mechanosensitivity of TRPY1 does not have an absolute dependence on the presence of Ca^{2+}. Tens of micromolar Ca^{2+} greatly enhance the observed force-induced activities, with open probabilities (P_o) following well the Boltzmann distribution (Fig. 2a, b). Formal analyses of P_o vs. gating parameters support a parallel model, in which the two gating stimuli are sensed and transmitted to the channel gate independently. The two gating energies are summed at the gate as Boltzmann factors to open the gate synergistically. Structurally speaking, mutant studies show that common Ca^{2+} binding proteins such as calmodulin or calcineurin are unnecessary for the Ca^{2+}

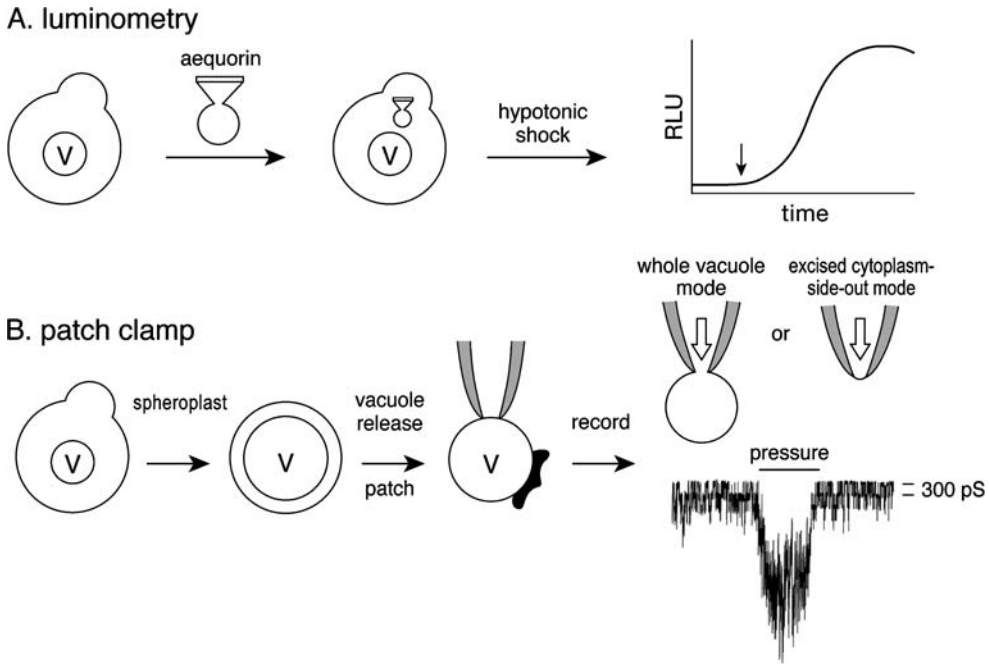

Fig. 1 Experimental procedures to examine TRPY1 activities in vivo and in vitro. **a** Monitoring of TRPY1's response to hypertonic shock in vivo. As described in [2, 10], yeast cells are transformed with plasmids bearing the apoaequorin gene. Transformed yeast cells are then challenged with hypertonic shocks and the Ca^{2+} release following TRPY1's activation is gauged by aequorin-Ca^{2+} relative luminescence units (*RLU*s). **b** Recording of TRPY1's current triggered by membrane stretch under patch clamp. Yeast cells are spheroplasted as described in [39, 60] before being broken by hypotonic swelling to release vacuoles (*V*). Released vacuoles are patch clamped in whole-vacuole mode or excised cytoplasmic-side-out mode. Membrane stretch forces are applied by directly blowing the patches with pressures of tens of millimeter Hg. A representative trace shows TRPY1's response to ~30 mmHg pressure stimulation

activation of TRPY1. However, removing a dense cluster of negative charges in the C-terminal cytoplasmic domain of TRPY1 greatly diminishes the Ca^{2+} activation. In addition, strategic insertions of amino acids in the C-terminal cytoplasmic domain upstream this negative-charge cluster selectively weaken the Ca^{2+} activation considerably but leave the mechanosensitivity nearly intact. This insertion phenotype correlates with the length but not the specific sequence of inserted amino acids. Based on these results and a low-resolution electron-cryomicroscopy structure of TRPV1 [35], it is modeled that TRPY1 senses membrane stretch force through membrane-embedded domains and senses Ca^{2+} via the cytoplasmic "hanging gondola" (Fig. 2c) [49].

The major obstacle of investigating detailed structure–function mechanism of TRPY1's mechanosensitivity is the lack of high-resolution TRP channel structure as a general guidance. Despite this difficulty, the powerful yeast genetics offers alternative tools to dissect TRPY1. Unbiased forward genetic screens of single-gene mutations in genomes have led to the identification of important channels, such as TRPs [37], *shaker* [40], *mec-4* [13], and cystic fibrosis transmembrane conductance regulator [24]. Forward genetic screens have also been successfully taken one step further to screens for single-residue mutations within a single gene to investigate TRP channel structure–function relationships in TRPY1 [48, 59], TRPV1 [36], TRPV3 [17], and TRPM8 [1]. In TRPY1, mutations isolated from a prospective mutagenesis screen, which is based on the in vivo hypertonic-shock-induced luminometric phenotype (Fig. 1a), indeed have pinpointed several crucial residues governing the channel's gating. For example, mutations at the intracellular base of S6 show severe defects of gating kinetics and indicate that TRP channel's gate has a similar functional organization to that of *shaker* [59]. Furthermore, pivotal residues discovered through TRPY1's screen can be generalized to other TRP members. For instance, one TRPY1's constitutively active gain-of-function (GOF) allele located at the intracellular base of S5 (F380L) amazingly aligns precisely with two other constitutively active GOFs in fly TRPC1 and rat TRPV1, which were discovered from independent forward mutagenesis screens. The same allele also resides near two other disease-causing GOFs in mouse TRPML3 and human TRPV4, respectively [37]. This uncanny convergence demonstrates that the intracellular base of S5 apparently tightly controls the gating of most, if not all, subtypes of TRPs.

Another interesting observation from TRPY1's forward mutagenesis screen so far is that mutations to conserved

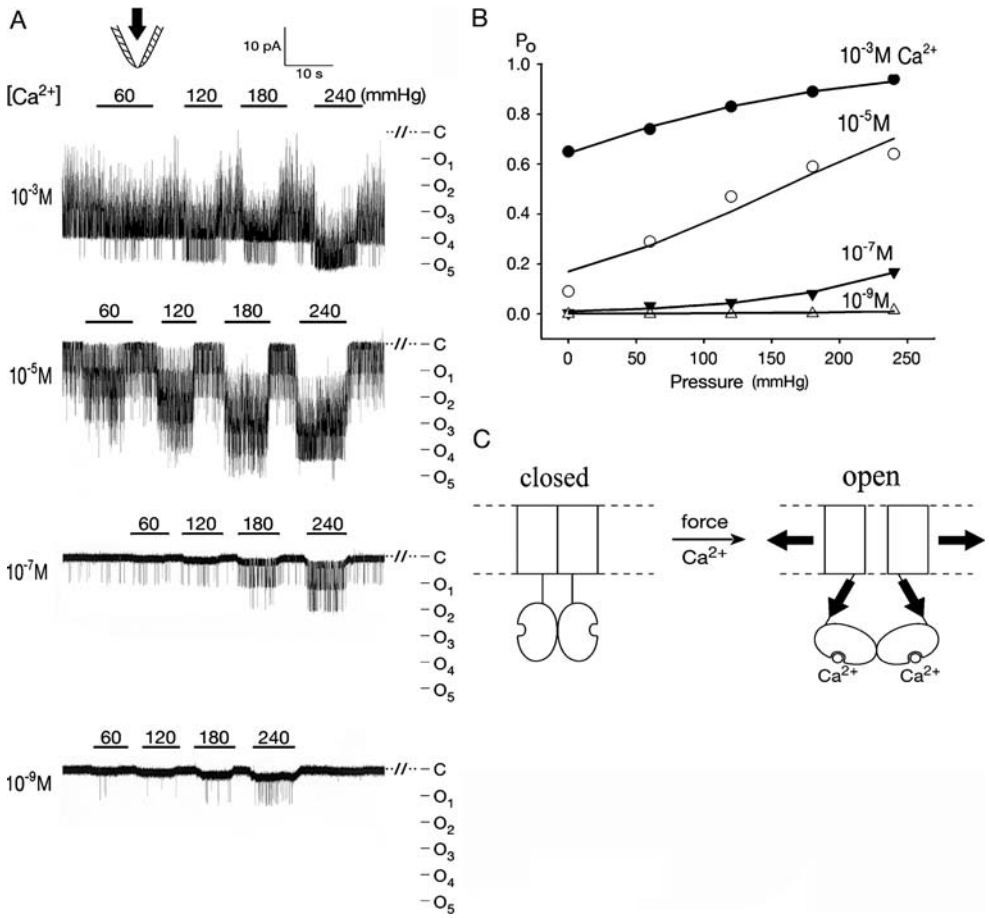

Fig. 2 Relationship between TRPY1's mechanosensitivity and Ca^{2+} sensitivity [49]. **a** One representative recording from repeated studies displays channel activities in response to four different pressures (60, 120, 180, and 240 mmHg, *left to right*) at four different [Ca^{2+}] (10^{-9}, 10^{-7}, 10^{-5}, and 10^{-3}M, *bottom to top*). All patch-clamp recordings were performed on isolated vacuoles in excised cytoplasmic-side-out configuration at −30 mV (vacuolar side positive). In the absence of Ca^{2+} (10^{-9}M), channels are weakly activated by strong pressure (240 mmHg; *bottom trace*). Above 10^{-7}M Ca^{2+}, force-induced responses become evident. At a high [Ca^{2+}] (10^{-3}M), robust basal Ca^{2+}-induced activities mask most of the responses to pressure. **b** P_o versus pressure at different [Ca^{2+}] from the data shown in **a** fitted with Boltzmann curves. **c** A mechanistic model of TRPY1 gating by force and by Ca^{2+}. This channel senses membrane stretch force through membrane-buried domains and senses Ca^{2+} via the cytoplasmic gondola. The two gating principles act on the gate in parallel

aromatic residues distributed in the lipid–water interface appear to significantly affect TRPY1's gating [48, 59]. One such aromatic residue at the intracellular base of S6, Y458, has been thoroughly studied. All functional channels among the 19 possible substitutions, except Y458F and Y458W, have severely abnormal gating kinetics, indicating that aromaticity, rather than size or shape, is the crucial parameter for normal gating [59]. The surprising S5 GOF allele described above also involves mutating away one phenylalanine, which resides within a phenylalanine cluster with members common among all TRPs [48]. The preference of aromatic residues at the two polar/nonpolar interfaces of the lipid bilayer is observed in many integral membrane proteins [52]. For instance, in the ground-breaking K^+ channel, KcsA, two aromatic belts are shown along the two interfaces, including the belt that forms the cuff around the selectivity filter and stabilizes it. Also, in KirBac1.1, a simulation study demonstrates that the interfacial aromatic belts may well contribute to its gating [12]. Interestingly, exogenously applied indole, the aromatic moiety of tryptophan, and many other aromatic compounds can activate TRPY1 both in vivo and under patch clamp [23]. Whether these added aromatic compounds exert their effects by specifically affecting the interfacial aromatic residues or by nonspecifically altering the force profile inside the bilayer remains unclear, though. The polar/nonpolar interface also harbors the strongest intrinsic force (surface tension) exerted by lipid to channel proteins [26, 43]. The role of interfacial aromatics in regulating TRPY1's mechanosensitivity remains to be defined.

Yeast can also be used to study animal TRP channels involved in mechanosensation. Indeed, rat TRPV4

(rTRPV4), which responds to hypotonic shock when heterologously expressed in cultured mammalian cells, has successfully been expressed in yeast and is shown to respond to hypotonic shock to release Ca^{2+} into cytoplasm (Fig. 3) [30]. Empty-plasmid control or a mutation in the filter [53] that eliminates rTRPV4's rescuing activity when expressed in Osm-9-deficient worms [28] does not give the rTRPV4's hypotonic response in yeast, indicating the response is due to TRPV4 channel activity. rTRPV1 has also been expressed in yeast [30, 36]. Therein, they faithfully reflect their molecular characteristics: TRPV1 responds to heat and capsaicin but not hypo-osmolarity, TRPV4 responds to hypo-osmolarity but not heat when expressed in yeast [30], despite previous claims in mammalian cells [7, 15, 18, 58]. Unexpectedly, rTRPV4, commonly expressed in plasma membrane in animals or cultured mammalian cells, traffics to internal locations when expressed in yeast, as the external application of EGTA or ruthenium red, does not eliminate its Ca^{2+} signal. This internal localization also makes the external applications of known rTRPV4's activators ineffective, presumably because they cannot access the internal membranes over the course of experiments. In addition, this internal location encumbers the electrophysiological study, since only one internal organelle, the vacuole, can practically be examined (Fig. 1b). It has been proposed that hypotonic swelling activates phospholipase A2, releasing polyunsaturated fatty acids (PUFAs) 5′-6′ EET to activate rTRPV4 [55, 57], thereby relegating the mechanosensitivity to enzymes and not rTRPV4. Interestingly, yeast cannot synthesize PUFAs. Thus, the hypotonic response of rTRPV4 in yeast does not require PUFAs. It is formally possible that hypotonic shocks in yeast produce other chemical elements to activate TRPV4 in place of PUFAs. Alternatively, TRPV4 itself may be mechanosensitive, at least in yeast. PUFAs may be required to remove some innate environmental inhibitions in mammalian cells which do not exist in yeast. Also, the dependence of PUFAs for TRPV4's hypotonic response in mammalian cells was tested at room temperature (25°C) [55, 57]. It might be interesting to further investigate whether TRPV4 can be directly opened by membrane stretch in excised patches at body temperature (37°C), at which the bilayer forces are expected to be different, since rTRPV4's activities dramatically increase from room to body temperature [7, 15, 18, 58].

In sum, yeast offers an alternative to cultured mammalian cells to study ion channels, especially MS-TRP channels. The electrophysiology of yeast has been advanced. This, coupled to the most advanced molecular genetics, makes yeast a novel and potentially powerful arena to study both the native TRPY1 as well as animal TRPs expressed therein, such as rat TRPV4. Expressing mammalian channels in cultured mammalian cells likely preserves much of the interactions between the channel and its native accessory subunits and modulators. Being evolutionarily distant from mammals, yeast is less likely

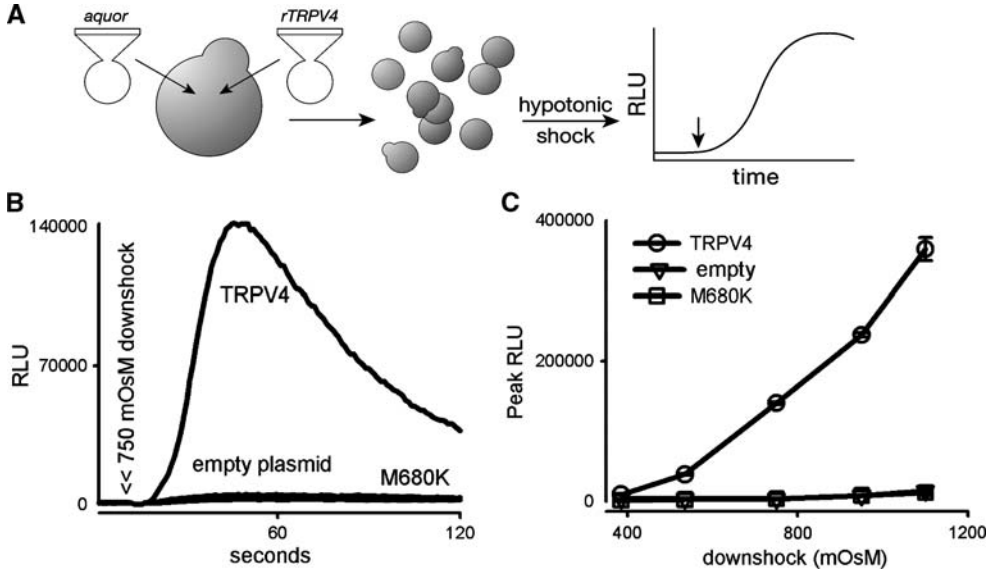

Fig. 3 Rat TRPV4 expressed in yeast respond to hypotonic shock [30]. **a** A diagram showing the experimental methods (modified from [2]). Yeast cells (in TRPY1 knock-out background, *yvc1Δ*) were first transformed with a plasmid that produces aequorin to monitor cytoplasmic Ca^{2+} by luminescence. They were then transformed with a *CEN* plasmid bearing the rat *TRPV4* gene. The transformed yeast culture was monitored with a luminometer and was hypotonically shocked by dilution. **b** A 750 mOsM hypotonic shock (*arrow heads*) triggers a large luminescence increase (in relative luminescence units, RLU) in TRPV4 transformants but not in transformants of an empty plasmid or plasmid bearing a TRPV4 with a mutation in its ion filter (M680K) [28]. **c** A dose–response relation between hypotonic shock and the peak response (mean±SD, $n=3$). Measurements from 2.4×10^6 cells each

to harbor such interacting proteins, making it easier to sort out properties native to the channel itself and those due to its partner proteins. Thus, the yeast system can be used to complement the studies in cultured mammalian cells towards a full understanding of TRPs in general and MS-TRPs in particular.

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