



Department of Biology

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# Heterologous expression and functional characterization of human TRPA1 in *Saccharomyces cerevisiae*

Masters degree project in Molecular Biology: Molecular Genetics and  
Biotechnology (45 Credits)

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# **Abstract**

## **Background**

TRPA1 belongs to the superfamily of Transient Receptor Potential (TRP) ion channels that are expressed in nociceptive neurons. They are present in the membrane of mammalian somatosensory system cells. TRP superfamily discoveries and studies have changed the understanding of sensory biology, disease processes and biological mechanisms. Human TRPA1 has been implicated in some disease and thus it is a target in drug development in the treatment of pain, inflammation, diabetics among others. Yet there is more to be known in how these ion channels function and behave, especially their activation, modulation and regulation by agonists. Hence TRPA1 was cloned, expressed and studies were done to investigate its function and characteristics with mustard oil and cinnamaldehyde, which has been widely used in studies, as agonists.

## **Results**

TRPA1 was successfully sub cloned into the p425GPD vector and transformed into *Saccharomyces cerevisiae*. As judged by growth inhibition, 25  $\mu$ M mustard oil and 100  $\mu$ M cinnamaldehyde in growth media activated TRPA1 *in vivo* in yeast cells expressing the protein heterologously.

## **Conclusion**

Yeast growth assay is a useful tool in studying TRPA1 functions and characteristics thus providing a background for further studies using PCR mutagenesis to map binding sites for the agonists.

## Background

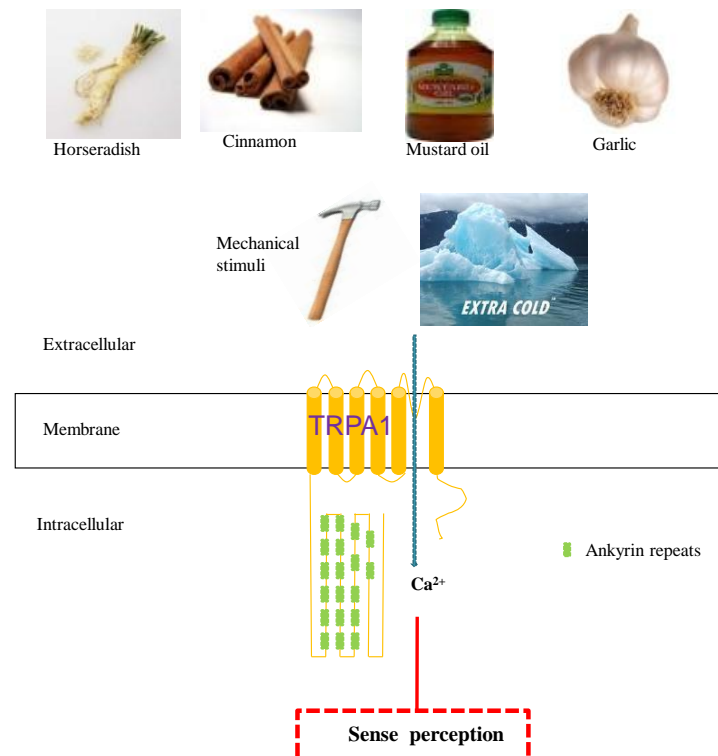
Transient receptor potential (TRP) ion channels is a superfamily of cation channels [1] which can be classified into two broad groups according to sequence and topological differences [2], and seven subfamilies based on their amino acids sequence homology [1-3]. Group one includes TRPC (classical or canonical), TRPV (vanilloid), TRPM (melastatin), TRPN (no mechanoreceptor potential C; NOMPC) and TRPA (ankyrin), while group two has TRPP (Polycystin) and TRPML (mucolipin). They are tetramers [1, 4] where each monomer have six putative transmembrane domains [1, 3, 4] forming a cation selective pore between domains five and six [1] in centre helices of the tetramer.

Structurally, the length of these intrinsic proteins varies in their intracellular C- and N- termini among the groups and subfamilies [5], this plays an important role in modulation and regulation of the channels trafficking and function. The list of domains and functions includes: transmembrane span, most preserved structure in TRP channels responsible for channel formation and integration into the membrane, some residues of which has been identified as agonist binding site in TRPV1 [6, 7]; the pore region allows flow-through and selection of cations in biological membranes [8]. Except for TRPA and TRPP, they have a region of 23-25 conserved amino acid residues known as the TRP domain, just after the sixth transmembrane domain [3, 4] inside which are the TRP box one and two; box one is highly conserved and made up of “EWKFAR” motif, while box two is less conserved but proline rich [1]. TRP domain is the putative signature region of the superfamily; located near to the C-terminal, the TRP box is highly conserved in TRPC, TRPV, and TRPM but less conserved in TRPP, and TRPML subfamilies, it has been shown to be important in interaction with PIP<sub>2</sub> (phosphatidylinositol-4,5-bisphosphate).

Ankyrin repeats appear in most TRP channels on the N-terminal, they are involved in protein-protein interactions and interconnection of membrane proteins [8]. Coiled-coil domain functions mainly in oligomerization of proteins, it is made up of two or more alpha helices from a monomer wrapped around one another to form a super coil, and can interact with helices from other monomers to form tetramers [1]. PDZ domains are found in some TRPs; they bind to protein scaffold and function in regulating cell surface expression. Deletion of this domain changes the general distribution of the protein in plasma membrane [9]. There are calmodulin binding sites in some TRPs where they control calcium influx, which is regulated in relation to cellular processes [5] and localization of the protein [10]. Lastly there are phosphorylation sites, which can be phosphorylated by protein kinases, thereby regulating the properties of the ion channels by a recurring and reversible post-translational modification. It has been suggested as a negative feedback mechanism that controls  $\text{Ca}^{2+}$  entrance into cells [11]. These and more structural features, such as  $\text{PIP}_2$  binding site and unique fusion of cytosolic kinase domains [12] (in TRPM7 and TRPM2), of TRP superfamily determines the functionality, classification and characteristics of the proteins.

Human TRPA1, the only member of the TRPA subfamily in mammals, it is so-called because of its extensive ankyrin repeats (8-18) on the N-terminal domain, these repeats are proposed to interact with cellular cytoskeleton or control ligand binding [13-15]. It is expressed in neurons of the dorsal root, nodosa and trigeminal ganglia, it is also found in hair and skin cells. More than one member is found in insects with high chemosensitivity [16]. In mammals, it functions as chemo- and thermo- sensors being activated by noxious cold of about  $17^\circ\text{C}$  [17], oxygen [18], pungent agonists such as mustard oil (MO), cannabinoids, cinnamaldehyde, ligustilide and icilin; [4] and generally electrophilic compounds. Recently, a class of pyrimidines was reported as superior agonist to MO in activation of human and rat

TRPA1 [19], it is stable in aqueous solution at about neutral pH, non-volatile, soluble at room temperature and non-electrophilic. It may replace MO as agonist in future studies. These agonists are said to activate TRPA1 by covalently binding with cysteine residues on the intracellular N-terminal [19] resulting in alkylation of the residues [20].



**Figure 1:** Schematic diagram of TRPA1 with natural sources of common agonists. The agonists cause activation of the channel which leads to opening of the pore located between 5 and 6 transmembrane domain of the ion channel. This leads to influx of  $Ca^{2+}$  and action potentials in the membrane, which follows a cascade of event that leads to perception of sense by transmission of signals to the brain via the sensory nerves.

The mechanism for activation of TRPA1 can be direct or indirect. Direct activation is common mechanism use by chemical compounds, protons and calcium; excess oxygen has been proposed to activate the protein by oxidation of cysteine residues to sulfenic acids which leads to formation of disulfide bonds [21], mustard oil and cinnamaldehyde activates, by Micheal adducts formation, basically with cysteines at the N-terminal of the protein [22].

Indirect activation is common by pro-inflammatory agents [21], through the phospholipase C pathways [23]. This mechanism is reported to be involved in store-operated calcium entry activation of the channel in which transient release of calcium into the cytosol triggers intracellular activation of TRPA1 and continuous influx of the divalent cation [4].

TRPA1 has been implicated in some diseases; it has been associated with chronic pain states such as inflammation, diabetics, bronchitis, fibromyalgia and emphysema among others [24]. A gain of function mutation in the gene has been implicated in familial episodic pain syndrome [25], in a reverse genetic study the mutation was mapped down N855S of the S4 transmembrane residue. It has also been reported that TRPA1 is involved in GPCRs mechanisms that leads to coughing [26], it was shown that TRPA1 and TRPV1 are involved downstream in the event that triggers sensory nerves activation and coughing. Thus TRPA1 is an interesting protein for understanding these diseases processes and treatments.

In this project, a yeast growth assay for functionality investigation of TRPV1 [7] was set up, optimized and applied to study TRPA1. This assay is based on fact that activation of the TRP channels will cause an inflow of  $\text{Ca}^{2+}$ , excess of this cation is lethal to yeast in which the channels are been expressed. Thus by spotting the yeast cells, the effect of the influx provides useful information on the activity of the channel *in vivo* in growing yeast cells. Also, cobalt assay, which is more quantitative, was carried out. In this assay, cobalt is expected to flow into the cell, from the assay buffer, through activated channel. The cation can then be stained with ammonium sulphide, which gives black precipitate when spun down [7, 27]. The gene was cloned on the *S. cerevisiae* expression vector, p425GPD, for heterologous expression of the encoded protein to allow functional and characterization studies. This was used for optimization of the assay method to suite agonists for TRPA1. Selected compounds, mustard

oil and cinnamaldehyde, were used in this assays at different concentrations and serially diluted inocula. This was to determine concentration of agonist that is required to activate the channel to the point of lethality to the yeast cells. These agonists were chosen so that mutants that would be insensitive to MO but could be activated by cinnamaldehyde, and vice versa, could be identified in screens.

Establishing these assays in our laboratory will be useful in generating random mutations in certain cassettes of the gene, which are important for modulating and regulating its functionality and mapping of agonist binding sites on structural relation of the protein. Hence, a better understanding of the mechanisms of activation by agonists may result from these studies in future.

# Results

## Plasmids constructions

Figure 2 below summarises the cloning strategy.

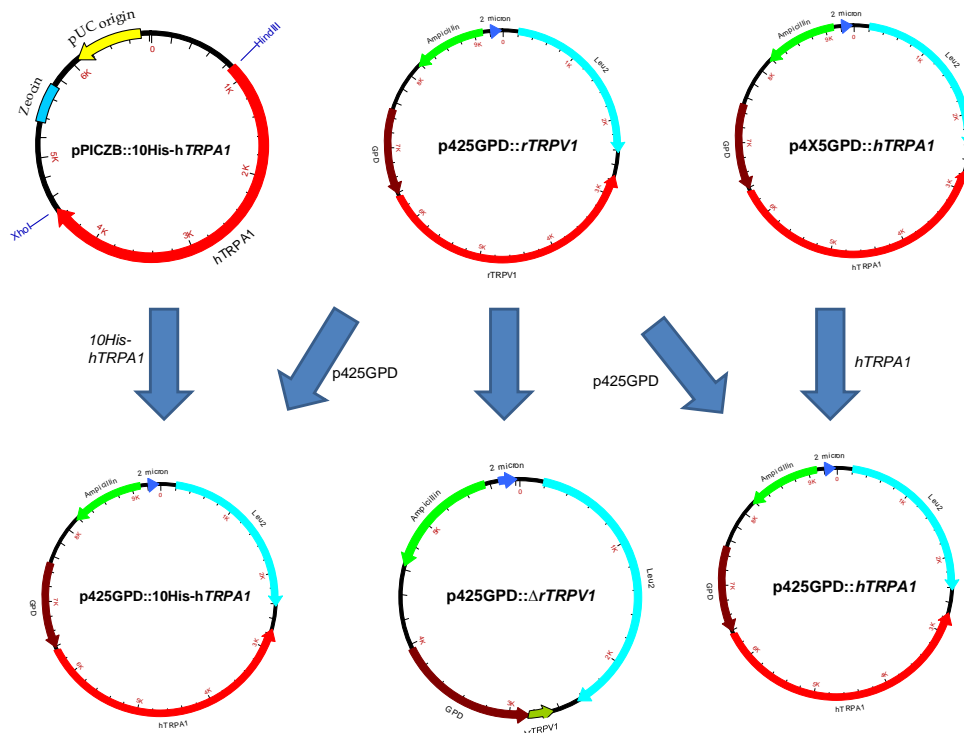
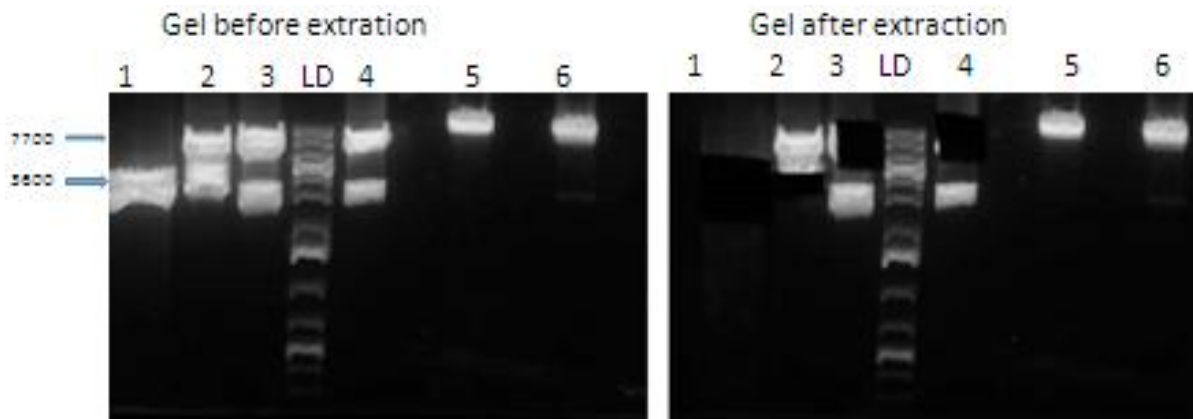


Figure 2: Three plasmids were constructed; p425GPD::10His-hTRPA1, p425GPD::ΔrTRPV1 and p425GPD::hTRPA1. p425GPD::10His-hTRPA1 and p425GPD::hTRPA1 were constructed by exchanging rTRPV1 in p425GPD::ΔrTRPV1 with 10His-hTRPA1 and hTRPA1 from p425GPD::10His-hTRPA1 and p425GPD::hTRPA1 respectively using XhoI and HindIII restriction sites. Deletion of most the sequence of rTRPV1 was also made using BamHI, leaving few base pairs leftover fragment.

In order to sub clone *TRPA1* into p425GPD vector, p425GPD::rTRPV1 and 10His-pPICZB::hTRPA1 were digested; the restriction digestions of the plasmids with XhoI and HindIII, resolved into two separated bands on agarose gel (Figure 3). Desired DNA fragments were excised from the gel; 1- 10His-hTRPA1, 2- hTRPA1; 3 and 4 – p425GPD, in addition, 5 and 6 was included as controls using each of the restriction enzyme to digest 10His-



pPICZB::*hTRPA1*, that is in lane 5 has the plasmid digested with *Hind*III and lane 6 has the plasmid digested with *Xho*I.



**Figure 3:** 1% agarose gel on the left side shows the separation of the gene fragment from vector backbone, (1)10His-pPICZB::*rTRPV1* digested with *Xho*I and *Hind*III; (2) p4X5::*hTRPA1*; (3) and (4) p425GPD::*rTRPV1*; LD- lane for GeneRuler 1 kb plus DNA ladder. Gel picture on the right show the gel after excision of the fragments.

The fragments were purified and ligated in ratio 1:1 and 3:1, insert to vector, and controls positive (Commercial plasmid, pUC18; fermentas) and background (linearized vector only) controls using T4 DNA ligase (fermentas), then transformed into *E. coli*, there were transformants after incubation at 37°C for 18-24 hours. Ten transformants were picked from each of the plates and screened for inserted gene by colony PCR using gene specific primers. The PCR products were ran on agarose gel to screen for correct sizes.

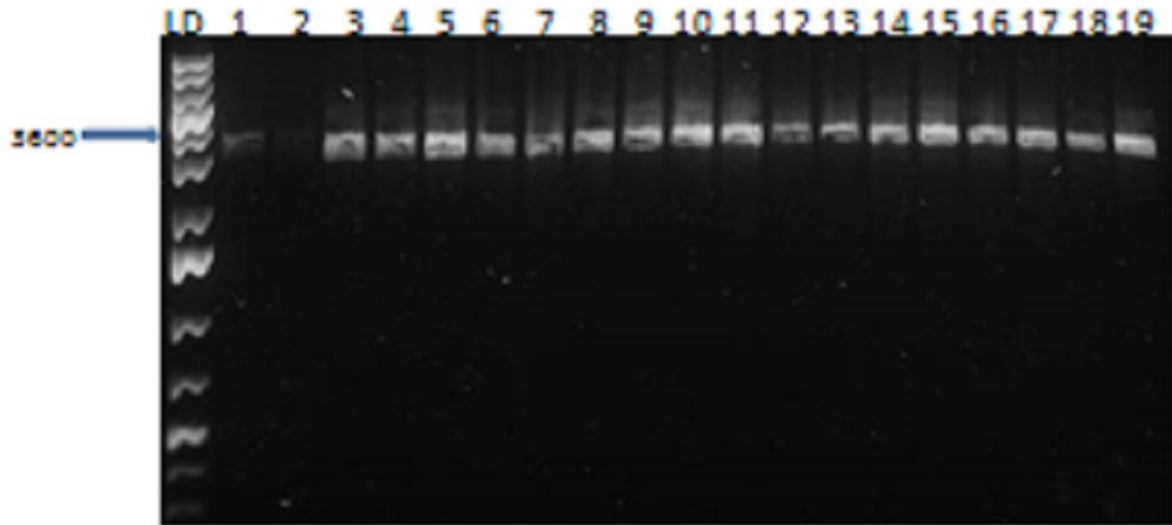


Figure 4: Colony PCR products ran on 1% agarose gel, expected band of about 3.6 kb was observed in almost all the screened colonies. LD- GeneRuler 1kb plus ladder, 1-10 are PCR products from colonies of constructs without His-tag while 11-19 are from colonies of constructs with His-tag.

Four colonies were selected, two from each construct, and two plasmids of p425GPD::*rTRPV1* as positive control. Plasmids were extracted from these colonies and confirmed by restriction digestion with *Bam*HI. They were then checked on agarose gel, on which they resolved into two clear bands of about 3 kb and 8 kb as expected (Figure 5). Two of the plasmids, one from each construct were isolated from transformants and further confirmed by sequencing.

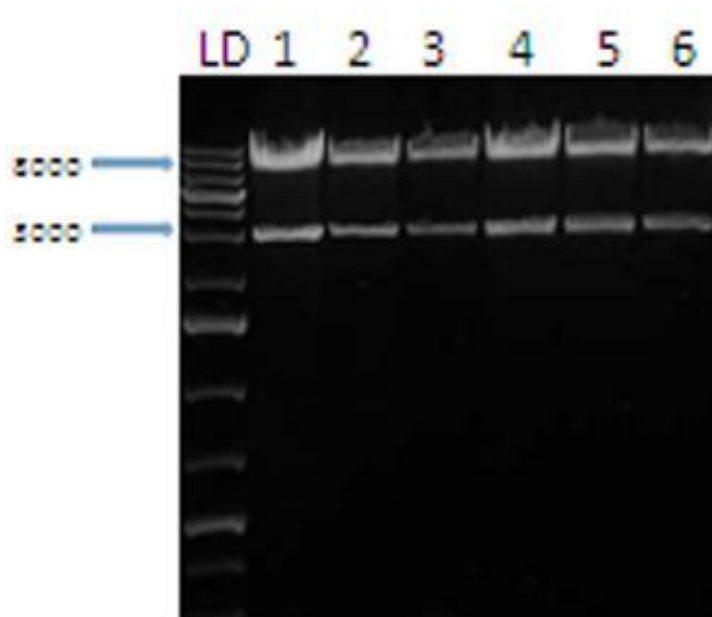


Figure 5: 1% agarose gel with two resolved bands of about 3 kb and 8 kb, after restriction digestion control of constructs with *Bam*HI. Lane 1 and 2 has construct with p425GPD::*hTRPA1*, 3 and 4 Histag-p425GPD::*hTRPA1* and in lane 5 and 6, p425GPD::*rTRPV1*.

Both confirmed plasmids were transformed into *S. Cerevisiae*. The transformation was confirmed by extracting the plasmids and transforming them into *E. coli* for amplification. Then restriction digestion control with *Bam*HI carried out as described earlier; and sequenced before the clone without His-tag was used for functional assay.

To construct a truncated insert plasmid, which can grow on LeuDO but expressing no functional TRP and to be used as negative control in assay, p425GPD::*rTRPV1* was digested. The digestion of p425GPD::*rTRPV1* with *Bam*HI, when ran on 0.8 % agarose gel resulted in two separate bands. The upper band containing the vector backbone and few base pairs from the *rTRPV1* gene, were re-ligated and transformed into *E. coli*. Five of the transformants were selected for plasmid preps and confirmed by restriction digestion control. Two of the correct plasmids were sequenced and transformed into *S. cerevisiae*.

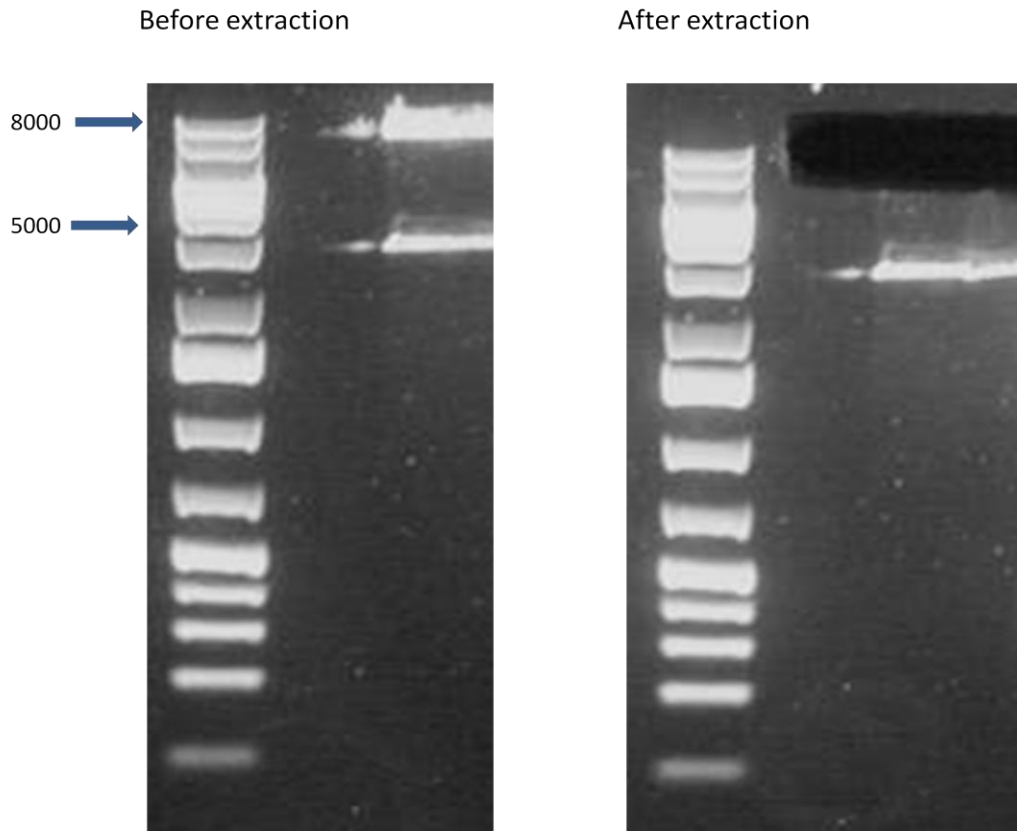
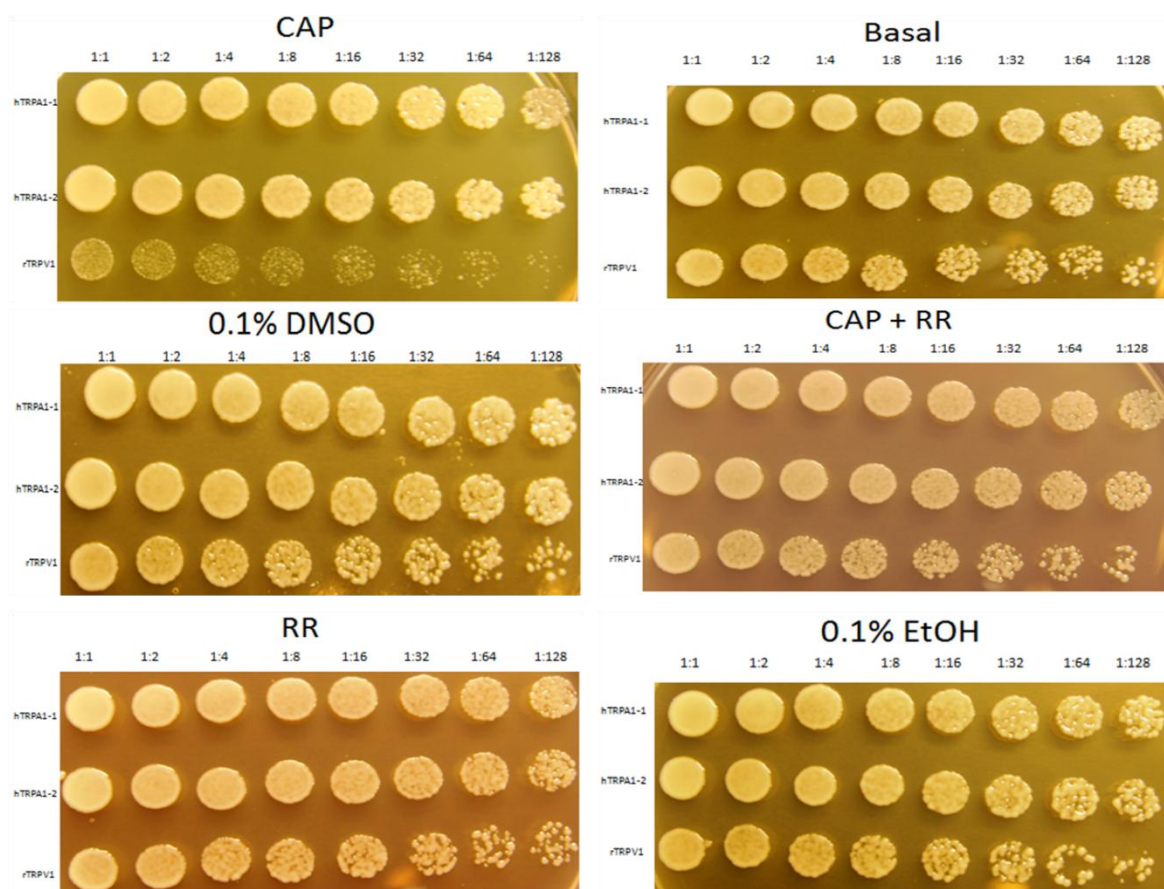


Figure 6: Gel picture of extraction of truncated construct before and after excision, run on 0.8 % agarose gel after digestion with *Bam*HI.

### Yeast assays

Activation of rTRPV1 was lethal to the growth of yeast as seen on plate (Figure 7), this is a confirmation of a previous study [7] with extended dilution series. In addition, two clones of *hTRPA1* were used as controls in the experiment; it was found that capsaicin only activates TRPV1 and not TRPA1 as seen on the plates in Figure 7. Controls were used to ascertain that growth lethality was due to the activation of the gene and not by presents of other chemicals. Ethanol, solvent for capsaicin, to final concentration of 0.1%; DMSO, solvent used for MO and cinnamaldehyde (also to final concentration of 0.1%), ruthenium red, the channel blocker (final concentration of 10  $\mu$ M) and LeuDO with no supplement (basal) all showed no activation of the expressed channel; as there was no inhibition to the growth of the yeast. To

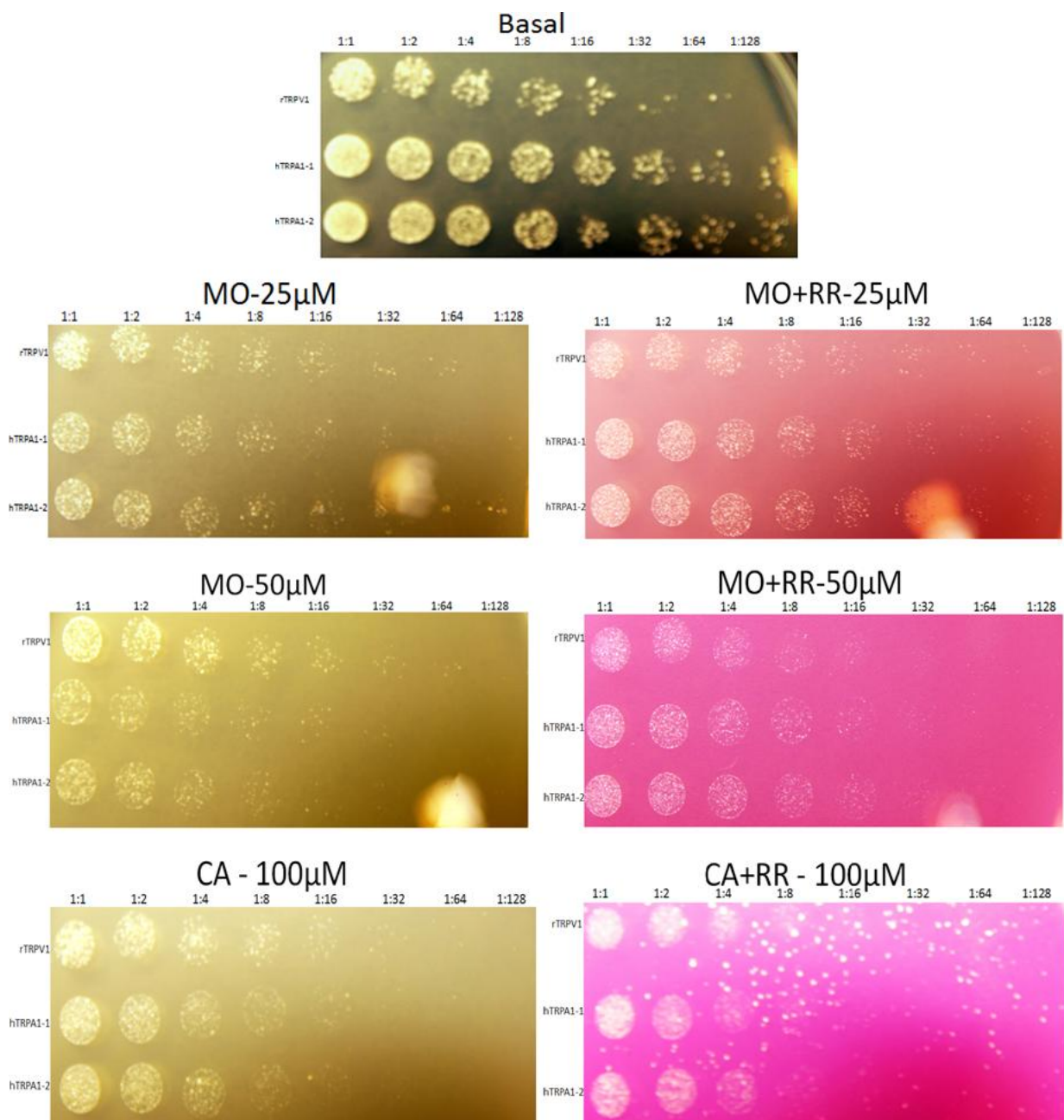
demonstrate that the channel can be blocked to prevent the influx of  $\text{Ca}^{2+}$ , ruthenium red was added to the assay medium with capsaicin, both to final concentration of  $10\ \mu\text{M}$  (CAP+RR) and another plate with only ruthenium red (RR). It was evident that the blockage of the channel prevents cation inflow into the cells because there was growth of the yeast cells, even in the presence of  $10\ \mu\text{M}$  capsaicin when channel was blocked. The growth is comparable to the growth on basal medium.



**Picture 7: Yeast spot growth assay for functional characterization of rTRPV1 using capsaicin as assay agonist. Basal is the base medium without additional or assay supplement; CAP contains  $10\ \mu\text{M}$  capsaicin, CAP+RR has capsaicin and ruthenium red in equal final concentration of  $10\ \mu\text{M}$ ; 0.1% EtOH and 0.1% DMSO has ethanol and DMSO to 0.1 % final concentration in medium respectively and RR has  $10\ \mu\text{M}$  ruthenium red. Assays were done on rTRPV1 using two clones of hTRPA1 as controls with initial  $\text{OD}_{600}$  0.80 for all innocula.**

The same assay procedure was also carried out on TRPA1 clones using cinnamaldehyde and mustard oil as agonists at different concentrations. In this experiment, there was evidence of

activation of TRPA1 by growth inhibition of the yeast due to activation of the channel. Comparing the assay plates to the basal plate, Figure 8, there is evidence of a specific growth inhibition of yeast cells carrying *hTRPA1* as compared to the ones with *rTRPV1*. For instance, *hTRPA1* clone 1 shows little tiny colonies at dilution 1:16 on plate with 25 $\mu$ M MO, while it grows very well on the basal medium.



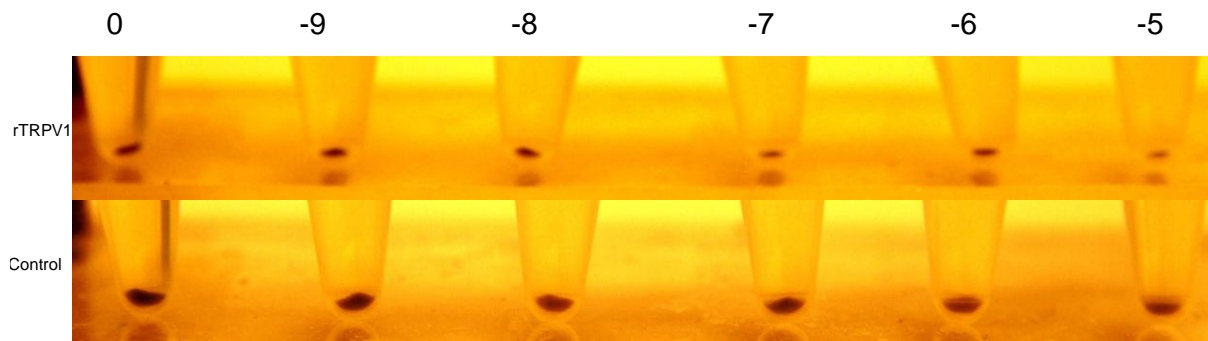
**Figure 8: Yeast growth assay with concentration of mustard oil (MO) at 25  $\mu$ M and 50  $\mu$ M; also cinnamaldehyde at 100  $\mu$ M, on the right hand side plates with ruthenium red and agonist at the same concentrations. Pictures of plates were taken after 48-72 hours of incubation at 28°C with initial OD<sub>600</sub> of 0.21.**

However, lesser growth of all constructs can be seen on plates with MO (25  $\mu$ M), more convincingly, at 50  $\mu$ M final concentration of MO. The inhibition also appears to be rescued on plates with ruthenium red although not growing as fast as it appears on basal plates. Still, it is evident that there are better growths on these plates. On the plate with 100  $\mu$ M cinnamaldehyde, there is growth inhibition of the yeast cells when compared to the basal medium. Implying that there is activation of TRPA1 channel by this agonist, but also the inhibition is comparable to what one can observe on TRPV1. In this case, there seems not to be any effect of the channel blocker, ruthenium red because there are little or no differences on the assay plates.

### **Cobalt assay**

There were efforts to repeat the cobalt assay experiments as reported by Myers et al.(2008)[7]; it proves abortive as there are black precipitates in all tubes, both controls and samples. Figure 9 shows the assay with varying concentration of agonist, capsaicin, ranging from  $10^{-9}$  to  $10^{-5}$ , as seen in the picture below there are precipitates in the control tubes without capsaicin and cells that are not expressing the protein which means that there is a very high background of the black clumped cells. The experiment was repeated with less amount of ammonium sulphide, 100  $\mu$ l as against 200  $\mu$ l that was used initially and less incubation time 2 minutes as against 5 minutes but these did not reduce the background.

# Cobalt Assay



**Figure 9: Photographs of ammonium sulphide stained precipitates of yeast cells in cobalt chloride assay. On top is the concentration (M) of capsaicin in each tube to the power of 10, first panel contains yeast cells expressing rTRPV1 while the bottom panel is the non transformed auxotrophic yeast strain.**



## Discussion

### Expression of recombinant plasmids

The expression plasmid p425GPD, is a shuttle vector developed for the expression of proteins in *S. cerevisiae* [28], it belongs to the p4X5prom series that has *LEU2* gene as a selection marker hence useful tool for nutrient deficient selection on LeuDO medium. The cloning cassette is located next to a glyceraldehydes-3-phosphate dehydrogenase (GPD) promoter; it is a strong constitutively active expression promoter [28, 29]. *TRPA1* was cloned in the multiple cloning sites in between *HindIII* and *XhoI* restriction sites thus under directed control of the promoter. In addition, the plasmid has ampicillin resistance gene which allows selection in *E. coli* and a 2 micron origin of replication which gives high copy number (10-30) of the plasmid per cell [30] in yeast. This plasmid has been a useful tool as vector for heterologous expression of eukaryotes proteins by several authors, it was used in functional studies of TRPV1 [7], STT3 [29], CDR1 and CDR2 [31] among other studies. Also a construct with His-tagged TRPA1 was made to check for the level of expression of the gene and a truncated construct to be used as a negative control (no functional protein is expected to be express in this construct because most part of the gene has been deleted down to about 200 bp, however, it has *LEU2* locus and thus make the yeast grow on LeuDO) in functional and characterization studies; although they were not used in this study, they will be valuable resources in further studies.

### Yeast growth lethality for in-vivo functional assay of the TRPs

The growth response of yeast expressing membrane protein has been used in studying protein functions and activities [7, 32-34], especially exploring cell death due to function of the

expressing transmembrane protein [7, 34]. It is a valuable tool in genetic studies such as analyzing mutants [7], protein-protein interactions [35] and protein identification [36]. In Figure 7 and Figure 8, this technique was used in to investigate the function and characteristics of hTRPA1 and rTRPV1 channels, based on the fact that there is influx of calcium ions from the culture medium into the cells in the presence of agonists; capsaicin, mustard oil and cinnamaldehyde, which becomes lethal to the yeast cells. Hence, less or no growth was observed on yeast spots with activated channels.

### **Agonists' activation of hTRPA1 and rTRPV1**

There was an obvious growth inhibition of yeast carrying rTRPV1 in Figure 7 in response to capsaicin as agonist while there was no effect on hTRPA1, this findings affirms the specificity of TRPV1 as capsaicin receptor [37, 38]. On the other hand, mustard oil activates TRPA1 but seems to also have some effects on TRPV1; however, the response is minimal when compare basal plate with growth on plates with MO, clearly seen at 50  $\mu$ M MO concentration. This lent credence to earlier report that TRPV1 is a mediator for MO [39], where they demonstrated that MO can activate mouse and recombinant human TRPV1. Higher concentration (100  $\mu$ M) of cinnamaldehyde was required to activate TRPA1, previous studies showed that similar amount of cinnamaldehyde is required to activate TRPA1 [40], this shows that MO is a more efficient agonist for the channel than cinnamaldehyde.

## **Conclusions**

*TRPA1* was successfully cloned into p425GPD expression vector; the protein was heterologously expressed and assayed for functional and characteristic studies *in vivo* in *S.*

*cerevisiae*. The confirmation of functional studies of TRPV1 serves as a positive control for the assays of TRPA1 and impetus into the further studies. MO activates TRPA1 at 25  $\mu$ M and cinnamaldehyde at 100  $\mu$ M in culture media. However, attempts to establish cobalt assay on TRPV1 in our laboratory as reported in previous study failed [7].

Further studies should be carried out on generating mutants' library, by unbiased PCR mutagenesis and yeast in-vivo recombination in selected cassettes and screening for clones which cannot be activated by 50  $\mu$ M MO and can be activated by 100  $\mu$ M cinnamaldehyde and vice versa. This will be interesting in mapping the agonists binding sites and provide insights in to the regulation and modulation of the protein, these sites can also be mapped on topology structure of related channels.

## **Materials and Methods**

### **Molecular cloning and Plasmids constructions**

All general molecular biology methods are according to Sambrook et al, 1990[41].

Two constructs of human *TRPA1* (His-tagged and without His-tag) were cloned on expression vector; p425GPD. These were done by double restriction digestion of p425GPD::*rTRPV1* (obtained from Julius Lab.)[7] with *HindIII* and *XhoI* restriction enzymes (Fermentas) and run on one percent agarose gel to separate the vector backbone from the gene; the vector was extracted and purified using gel purification kit (Omega). Similarly, 10His-pPICZB::*hTRPA1* with 10 His-tags at N-terminal was restricted and purified to obtain insert with His-tag. Also, PCR product, amplified with primers designed to exclude the His-tag sequence (Phusion High Fidelity DNA polymerase) and confirmed by sequencing, was restricted with *HindIII* and

*XhoI* to obtain insert without His-tag (this was cloned into a vector, p4X5GPD, designated with the series name because the sequences could not be confirmed and has unexpected restriction pattern). Both gel purified DNA fragments were ligated onto the purified backbone vector using T4 DNA ligase (Fermentas) and transformed into *E. coli* (XL-1 Blue) and cultures on LB agar plates with 100  $\mu\text{gml}^{-1}$  ampicillin.

Transformants were picked and checked for inserts by colony PCR; plasmids were then isolated from positive clones using mini plasmid prep kits (Omega) digested with *BamHI* and run on agarose gel to check for the fragments sizes. Right plasmids were further confirmed by sequencing and transformed into *S. cerevisiae* strain BY4741 [7] with lithium acetate/polyethylene glycol and selected on LeuDO medium [35]. A truncated plasmid was also constructed by deleting most of the gene from p425GPD::*rTRPV1* using *BamHI* which cut the plasmid at two sites. The band containing vector backbone and few leftover sequences (*BamHI* restriction site is about 200 bp into the gene), was extracted from gel, purified, re-ligated and transform into *E. coli*. All constructs were confirmed by sequencing before transforming into *S. cerevisiae*.

### **Yeast Growth Assays**

Assay media were prepared by adding agonist or blocking agents into molten leucine drop-out (LeuDO) agar medium, made up of Yeast Nitrogen Base (YNB), ammonium sulphate and amino acids without leucine, to desired concentration and allow solidify. YNB contains  $\text{Ca}^{2+}$  in form of calcium chloride (0.8M) and calcium panthothenate (0.04M)[35]. Inocula were prepared by growing the assay yeasts in LeuDO (liquid medium) overnight,  $\text{OD}_{600}$  were taken and all inocula diluted to the same OD; they were then serially diluted in double fold

dilutions in 8 series. 10  $\mu$ l of each dilution were spotted on assay plates, allowed to dry and incubated for 48-72 hours at 28°C.

### **Yeast Cobalt Assays**

The assays were carried out as described in [7], Yeast cells carrying the of p425GPD::*hTRPVI* was cultured overnight to logarithmic phase, it was spun down and resuspended in yeast assay buffer [7] and 200  $\mu$ l was aliquoted into 6 eppendorf tubes. Capsaicin was added in a series of concentrations and incubated at 30°C for 5 minutes, then resuspended in 5 mM CoCl<sub>2</sub> and washed twice in yeast assay buffer; lastly, stained with ammonium sulphide. The tubes were observed for black precipitate.

### **Acknowledgements**

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