# Redox Regulation of Yeast Vacuolar Channel Protein (YVC1) In *Saccharomyces cerevisiae*

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***A dissertation submitted for the partial fulfillment***

***of BS-MS dual degree in Science***


**Indian Institute of Science Education and Research Mohali**

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# Certificate of Examination

This is to certify that the dissertation titled "Redox Regulation of Yeast Vacuolar Channel (YVC1) in *Saccharomyces cerevisiae*" submitted by Ms. Krishna K. Das (Reg. No. MS10051) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted**.**

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Dated: April 24, 2015

# Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Anand K. Bachhawat at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography**.**

Krishna K. Das

(Candidate)

Dated: April 24, 2015

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand K. Bachhawat

(Supervisor)

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**Krishna K. Das**

# List of Figures

**Introduction**

***Figure 1.1:*** *$Ca^{2+}$ regulates many cellular processes.*

***Figure 1.2:*** *Basic structure of TRP channels.*

***Figure 1.3:*** *$Ca^{2+}$ level in a normal yeast cell.*

***Figure 1.4:*** *$Ca^{2+}$ channels and transporters in S.cerevisiae.*

***Figure 1.5:*** *Different kinds of modifications of proteins in response to oxidative stress.*

**Results and discussions**

***Figure 3.1:*** *MSA of YVC1 with homologues in fungi.*

***Figure 3.2:*** *MSA of YVC1 with homologues in higher organisms.*

***Figure 3.3:*** *Predicted structure of transmembrane domains of YVC1.*

***Figure 3.4:*** *yvc1Δ shows enhanced resistance to oxidizing agents.*

***Figure 3.5:*** *Intracellular $Ca^{2+}$ measurement using aequorin based luminescent assay.*

***Figure 3.6:*** *YVC1 is glutathionylated under oxidative stress.*

***Figure 3.7:*** *YVC1 glutathionylation is affected by NEM and IAM.*

***Figure 3.8:*** *A functional plate assay using $H_2O_2$ (selection plate SD- His). C17A and C191A show resistance to oxidizing agent.*

***Figure 3.9****: The image shows the localization of Mutants.*

***Figure 3.10:*** *The mutants YVC C17A, YVC C61A and YVC C191A are showing lower level of glutathionylation compared to control.*

***Figure 3.11:*** *C17A and C191A mutants are showing lower level of cytosolic $Ca^{2+}$.*

# List of Tables

**Introduction**

***Table 1.1:*** *The table shows all the* $Ca^{2+}$ *channels of yeast and their function.*

**Materials and methods**

***Table 2.1:*** *Details of the strains used in this study.*

***Table 2.2:*** *Various plasmids used in this study.*

***Table 2.3:*** *The list shows all primers used in this study.*

**Results and discussions**

***Table 3.1:*** *Homologues of YVC1.*

# Abbreviations

| | |
|---|---|
| **A** | Alanine |
| **Ala** | Alanine |
| **C** | Cysteine |
| **Cys** | Cysteine |
| **APS** | Ammonuim per sulfate |
| **ATP** | Adenosine Triphosphate |
| **dNTPs** | 2'- deoxyadenosine 5'- Triphosphate |
| **ER** | Endoplasmic Reticulum |
| **GSH** | Reduced glutathione |
| **GSH1** | GSH synthesizing enzyme |
| **GSSG** | Oxidized glutathione |
| **$H_2O_2$** | Hydrogen Peroxide |
| **IAM** | Ido- acetamide |
| **MSA** | Multiple Sequence Alignment |
| **$Na^{2+}$** | Sodium |
| **NEM** | N'- Ethylmaleimide |
| **PEG** | Poly Ethylene Glycol |
| **RNS** | Reactive Nitrogen Species |
| **ROS** | Reactive Oxygen Species |
| **SDS** | Sodium Dodecyl Sulphate |
| **SDS PAGE** | Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis |
| **SOCE** | Store- operated $Ca^{2+}$ entry channels |
| **TEMED** | N,N,N',N'- tetramethylethylenediamine |
| **TM** | Trans- membrane |

# Contents

**List of Figures** i

**List of Tables** ii

**Abbreviation** iii

**Contents** iv

**Abstract** vii

**Chapter 1 Introduction** 1

1.1 $Ca^{2+}$ homeostasis inside the cell …….. 2

1.2 Yeast and $Ca^{2+}$ homeostasis……. 4

1.3 $Ca^{2+}$ channels and transporters in yeast …….. 5

1.4 Redox regulation of $Ca^{2+}$ channels …….. 6

1.5 Yeast Vacuolar Channel Preotein (YVC1) …….. 7

**Chapter 2 Materials and methods** 9

2.1 Chemicals and reagents ……. 10

2.2 Strains and Plasmids …….. 10

2.3 Primers …… 11

2.4 Media …….. 12

2.4.1 LB …… 12

2.4.2 YPD ……. 13

2.4.3 SD ……. 13

2.4.4 SOB …….. 13

2.5 Buffers, Stock solutions and other chemicals …….. 13

2.5.1 Ampicillin stock solution …….. 13

2.5.2 GSH stock solution ……. 14

2.5.3 TERNAse …… 14

2.5.4 50% Glycerol …… 14
2.5.5 Alkaline lysis for plasmid isolation …… . 14
2.5.6 Agarose gel electrophoresis reagents …… 15
2.5.7 Yeast transformation solutions ……. 15
2.5.8 SDS PAGE solutions and reagents …… 16
2.5.9 Western blotting reagents …….. 16
2.5.10 Hydrogen peroxide …… 17
2.5.11 Diamide ……. 17
2.5.12 *N*- Ethylmaliemide ……. 18
2.5.13 Ido- acetamide …… 18
2.5.14 Coelentrazine …….. 18

Methods

2.6 Growth and maintenance of bacteria and yeast strains ……... 18
2.7 Recombinant DNA methodology …… 18
2.8 Tagging the protein with His tag ……. 19
2.9 Construction of site directed mutants of YVC1 ……... 19
2.10 Transformation of yeast …… 19
2.11 Functional plate assay by dilution spotting …….. 20
2.12 Protein purification from the yeast whole cell extract …….. 20
2.13 Protein electrophoresis and western blotting …… 20
2.14 Aequorin based Ca2+ luminescent assay ……. 21
2.15 Sequence analysis ……. 21

**Chapter 3 Results and Discussions 22**

3.1 Sequence analysis …….. 23
3.1.1 Homologues of Yvc1 ……..... 23
3.1.2 Multiple sequence alignment using Clustal W ……... 23
3.1.3 Transmembrane prediction using Topcons ……. 26
3.2 A functional plate assay using oxidizing agent …… 26
3.3 Intracellular $Ca^{2+}$ measurement of *yvc1*Δ starin during oxidative stress ……. 27

3.4 Glutathionylation analysis …… 28
3.4.1 *In vivo* assay using diamide …….. 28
3.4.2 *In vitro* assay using diamide …….. 28
3.5 Analysis of all Cys → Ala mutations …… 29
3.5.1 A functional plate assay using $H_2O_2$ …….. 29
3.5.2 Checking Localization of all Cys → Ala mutants …….. 30
3.5.3 Glutathionylation assay for all Cys → Ala mutants …… 30
3.5.4 $Ca^{2+}$ measurement using aequorin based luminescent assay ….. 31

Conclusion 32

References 33

# Abstract

**YVC1 is yeast vacuolar channel that effluxes $Ca^{2+}$ from the vacuole to the cytosol. Previous studies in this lab have shown that, there is an increase in the levels of cytoplasmic $Ca^{2+}$ and YVC1 contributes to this increase. YVC1 contains 9 cysteine residues and since these cysteines are expected to play a role in its activation, we mutated 8 of the 9 cysteines to alanines. These Cys → Ala mutations were evaluated for protein expression, localization and function. C343A mutant was hyperactive. We also observed that C61A was defective in localization. Among the other mutants, we observed that C17A and C191A were nonfunctional and also these mutants were partially defective in glutathionylation. Functionality of C17A and C191A were also defective as detected by aequorin based assay for $Ca^{2+}$. The results suggest a role for the two cysteine residues in YVC1 activation.**

# Chapter 1

## *Introduction*

## 1.1 $Ca^{2+}$ homeostasis inside the cell

Calcium ($Ca^{2+}$) is an important regulator of many cellular processes in eukaryotic organisms, which includes cell division, cellular motility, neural activity, protein trafficking, gene expression, muscle contractility, developmental regulation and apoptosis. $Ca^{2+}$ is a carrier of signals from the beginning of a cell's life to its end. Function of $Ca^{2+}$ in apoptosis is particularly important, when we consider its role in the cardiovascular, immune and nervous system disorders. Moreover during oxidative stress, $Ca^{2+}$ can alter the physiological condition of the cell via influx of the ion from external environment and storage organelles. $Ca^{2+}$ can also regulate the modification of proteins as well as modulate signal transduction pathways. The rise in cytoplasmic $Ca^{2+}$ beyond a certain level thus disrupts the normal metabolic processes, leading to cell death.

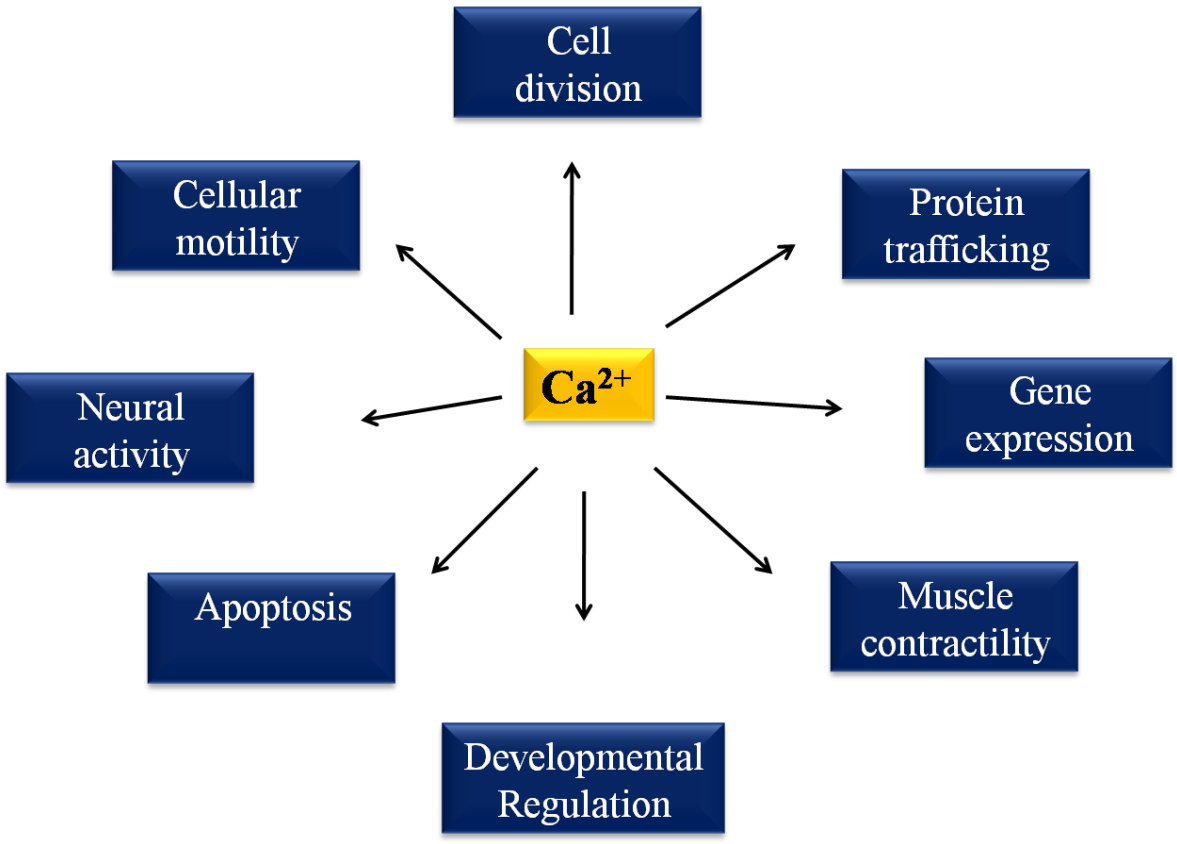


***Figure 1.1:*** *$Ca^{2+}$ regulates many cellular processes.*

Since $Ca^{2+}$ is an important messenger, its levels are tightly regulated inside the cell. There is a large $Ca^{2+}$ gradient across the cellular organelles. In a typical cell the intracellular $Ca^{2+}$ level is 100

nM. But it is subject to 10 to 100 fold increase during various cellular processes. This $Ca^{2+}$ comes from external as well as from its main storage organelle ER, where the concentration is in mM range. So maintaining this $Ca^{2+}$ homeostasis is very important for the survival of the cell. This is done by the $Ca^{2+}$ channels present on the plasma membrane, ER, Golgi apparatus and mitochondria.

There are three major groups of channels; (a.) the voltage-gated channels (VOCs) (b.) the receptor-operated channels (ROCs), which are activated by the interaction with the ligand (c.) the store-operated $Ca^{2+}$ entry channels (SOCEs), which require the presence of $Ca^{2+}$ for its activation. These channels are present on the plasma membrane. Among the three groups, the SOCEs are present on normal cell lines as well as neuron and skeletal muscle cells [1]. All the three groups can be further divided into subfamilies based on their function. All these channels are selectively permeable to $Ca^{2+}$ even if other ions are present in higher concentration.

Other than these three groups, there are transient receptor potential channels (TRP), intracellular $Ca^{2+}$ channels and $Ca^{2+}$ pumps also present to maintain the $Ca^{2+}$ homeostasis inside the cell. There are 3 $Ca^{2+}$ ATPases pumps in animal system, which can be present on the PM, ER and in the Golgi membrane. All of these help the cell to reduce the cytosolic $Ca^{2+}$ level to normal state by exporting the excess amount of $Ca^{2+}$ to external environment or to internal spaces. The pumps need energy for moving $Ca^{2+}$ through their hydrophobic channel. They are gaining this energy by splitting ATP molecule. We can say that they are the fine tuners of cellular $Ca^{2+}$ level as they have a high affinity interaction with $Ca^{2+}$ [1]. Also most of the animal cells have a low affinity $Ca^{2+}$ pump which ejects $Ca^{2+}$ in exchange with $Na^{+}$, termed as $Ca^{2+}/Na^{+}$ exchanger.

The TRP family constitute a large family of cation-conducting channels. They are expressed in both excitable and non-excitable tissue types. There are 6 different subfamilies of TRP channels in mammals, according to their homology: TRPC (canonical), TRPV (Vanilliod), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycistine). So far, 28 TRP channels have been found in mammals. The TRP channels are non- selective cationic channels with six transmembrane domains. Another common feature of TRP channels is their pore forming region between the fifth and the sixth transmembrane domain [1]. Some of the TRP channels are localized to internal organelles also. Thus TRPV and TRPP can be found on ER and Golgi membranes too. A class of TRP channel has been reported to be modulated by ROS/RNS and to control cellular responses [2].

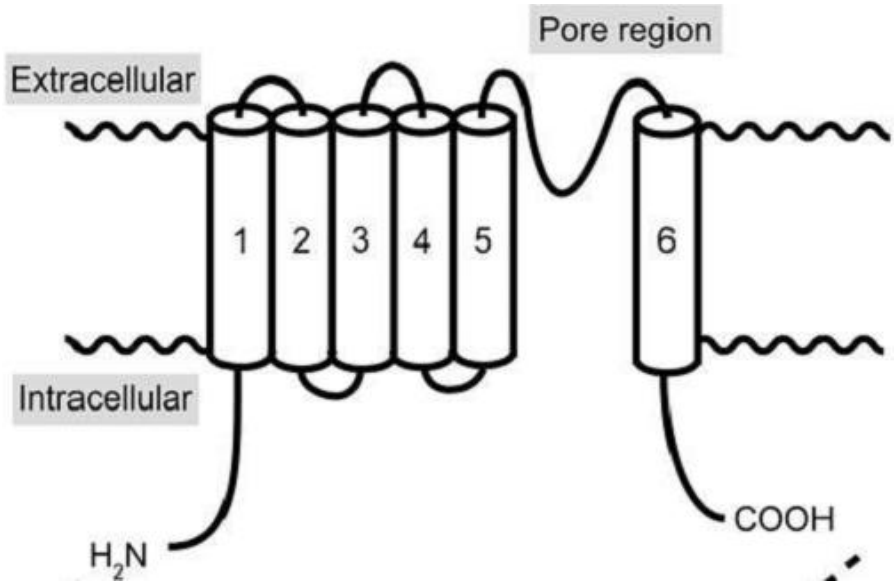


***Figure 1.2:*** *Basic structure of TRP channels [3].*

## 1.2 Yeast and $Ca^{2+}$ homeostasis

In budding yeast, the intracellular $Ca^{2+}$ concentration is in the range of 50- 200 nM range. Previous studies have shown that under oxidative stress the cytoplasmic $Ca^{2+}$ level increased to millimolar range with the activation of a number of $Ca^{2+}$ channels and transporters of *Saccharomyces cerevisiae*. The major source of $Ca^{2+}$ in yeast cells is their vacuolar rather than ER. More than 90 percent of $Ca^{2+}$ is stored in the vacuole (2mM).

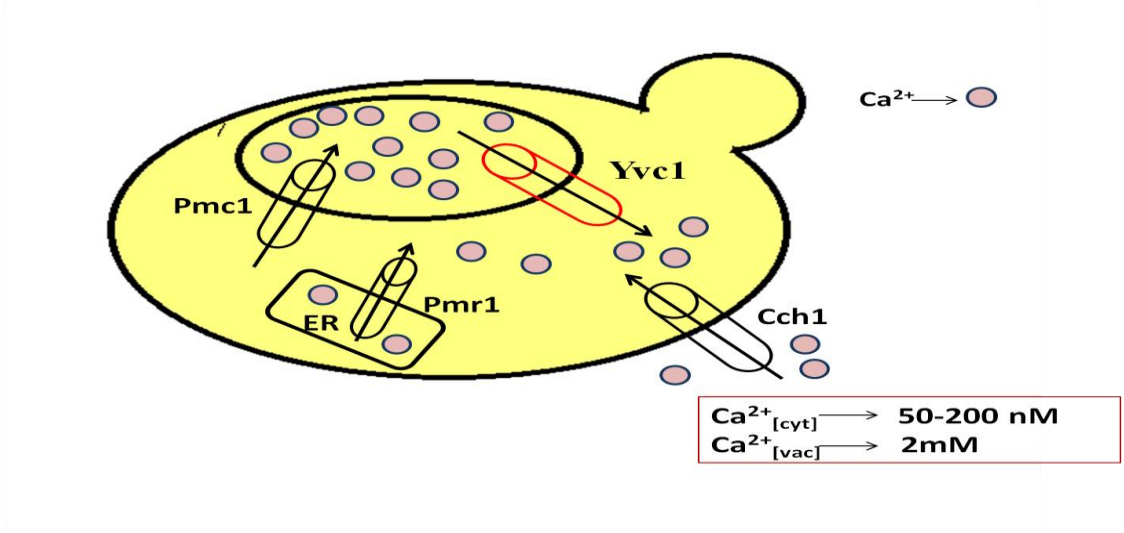


***Figure 1.3****: $Ca^{2+}$ level in a normal yeast cell.*

As in other eukaryotic organisms, $Ca^{2+}$ has a very important role in cellular and physiological processes of a yeast cell. These signal transduction pathways are regulated by the changing amplitude of $Ca^{2+}$ level inside the cytoplasm. Most of the factors in these pathways are conserved among the eukaryotes. One such factor is the $Ca^{2+}$ channels and transporters. We can find the homologues of yeast $Ca^{2+}$ channels and transporters in higher eukaryotic organisms including humans.

## 1.3 $Ca^{2+}$ channels and transporters in yeast

A large number of $Ca^{2+}$ channels and transporters are present in yeast cells which have an important role in $Ca^{2+}$ influx and regulation of intracellular processes. Excessive quantities of $Ca^{2+}$ are toxic to the cell and it is regulated with the help of these channels.

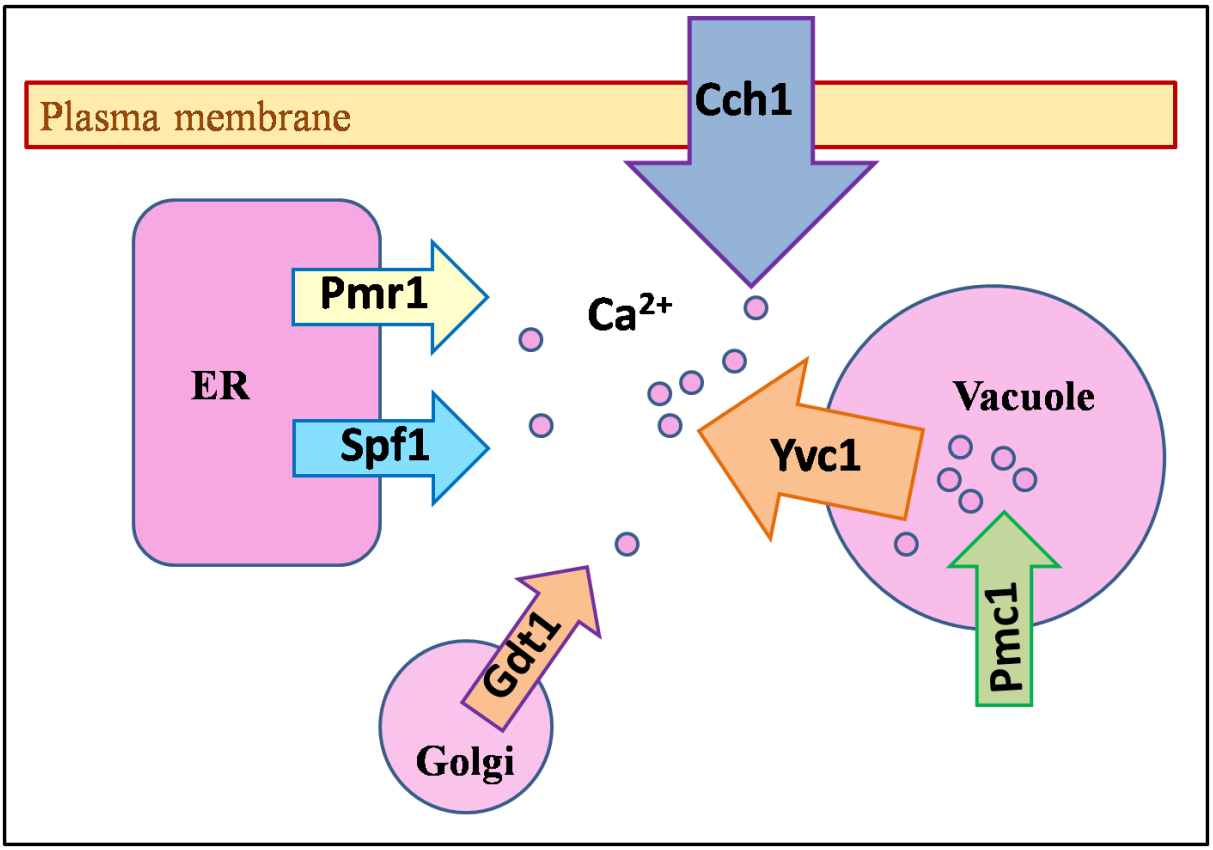


***Figure 1.4**: $Ca^{2+}$ channels and transporters in S.cerevisiae.*

One of the high affinity $Ca^{2+}$ channels is Cch1, which is present on the plasma membrane. Another important channel present on the vacuolar membrane is Yvc1 which belongs to the family of human TRP channels. Previous studies have shown that both these channels get activated under oxidative stress leading to an increase in the level of cytoplamic $Ca^{2+}$. The redox regulation of these channels is of extreme important for the survival of cell. ***Table 1.1*** explains all the $Ca^{2+}$ channels/ transporters present in *S.cerevisiae*.

| Protein Name | Type | Localization | Function |
|---|---|---|---|
| Cch1 | Channel | PM | Influx of $Ca^{2+}$ from external environment under oxidative stress |
| Yvc1 | Channel | Vacuole | Mediates release of $Ca^{2+}$ from Vacuole to cytosol in response to hyperosmotic shock |
| Pmc1 | ATPase | Vacuole | Involved in depleting cytosolic $Ca^{2+}$ level |
| Pmr1 | P- type ATPase | ER | $Ca^{2+}$, $Mn^{2+}$ transport to golgi, $Ca^{2+}$ dependent protein sorting& processing |
| Spf1 | P- type ATPase | ER | Ion transporter of ER membrane |
| Gdt1 | Not Known | Golgi | Involved in $Ca^{2+}$ homeostasis, function unknown |

***Table 1.1****: The table shows all the $Ca^{2+}$ channels of yeast and their function.*

## 1.4 Redox regulation of $Ca^{2+}$ channels

Reactive oxygen species (ROS) are oxygen molecules in different reduction states. They primarily induce cell death. So the understanding of how $Ca^{2+}$ signaling is altered by redox control will help us to know the mechanism of activation and regulation of these

channels. The following schematic diagram shows the possible mechanism for the activation of $Ca^{2+}$ channels under oxidative stress.

Glutahione (GSH) is an antioxidant present in cells. Under oxidative stress GSH can either be converted to its oxidized form, glutathione disulfide (GSSG) or form mixed disulfides with proteins. S- glutathionylation is the reversible addition of glutathione to thiolate anions of cysteines in target proteins [4]. Thus S- glutathionylation can modify protein structure and function.

Another effect that oxidative stress can have on protein is to cause disulfide bond formation that can also alter protein structure.

Another way that protein can be altered is by ROS which directly interacting with the exposed thiol groups of proteins and change their functionality. One such modifications is sulfenylation (SOH), induced by hydrogen peroxide ($H_2O_2$) and superoxide ($O^{2-}$). It can be reversible or irreversible depending on the local redox environment of the cell.

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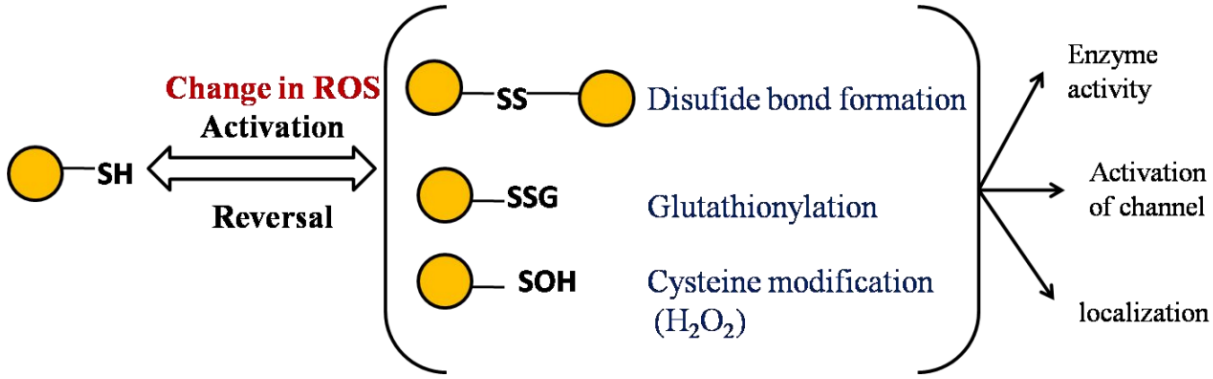


***Figure 1.5**: Different kinds of modification of proteins in response to oxidative stress.*

## 1.5 Yeast Vacuolar Channel Protein (YVC1)

The most abundant store of $Ca^{2+}$ in a yeast cell is the vacuole. YVC1 is $Ca^{2+}$ permeable channel present on the vacuolar membrane. The channel contributes to an influx of $Ca^{2+}$ from vacuole to cytosol under oxidative stress. But the mechanism of activation and regulation of the channel is not known yet. Excessive amount of $Ca^{2+}$ flux followed by the activation of YVC1 is toxic to the cell.

In *Candida albicans*, a common opportunuistic pathogen, the YVC1 homologue was found to play a key role in hyphal polarized growth and re- orientation to host signal. The study showed that YVC1 homologue was essential for the fungi during infection and survival in host tissue [6]. Similarly the YVC1 homologue in *Aspergillus fumigatus* has an important role in $Ca^{2+}$ homeostasis and the pathogens virulence [7].

YVC1 belongs to the TRP family of proteins. The human homologue of YVC1 is TRPV1 proteins. It is a plasma membrane channel involved in pain and heat sensation. The previous studies have reported covalent cysteine modification as a mechanism for the activation of TRPV1 [8].

The vacuolar cahnnel YVC1 found to be mechanosensitive under osmotic upshock from some previous studies [9]. But the mechanical activation occurs regardless of the $Ca^{2+}$ concentration, and is independent of its known $Ca^{2+}$ activation [9]. As $Ca^{2+}$ is a regulator of many cellular processes, to get a clear image about the role of YVC1 in $Ca^{2+}$ homeostasis is important. Studies have to be done to know how YVC1 is regulated under oxidative stress.

# Chapter 2

## *Materials and methods*

# Materials

## 2.1 Chemicals and Reagents

All chemicals were purchased from commercial sources and were of analytical grade. Media components, other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, U.S.A.), HiMedia (Mumbai, India) and Merck. India. Ltd(Mumbai, India). Primers were designed using SnapGene software and purchased from IDT. Enzymes including all restriction enzymes, *Vent* DNA polymerase, *Taq* DNA polymerase, Phusion High Fidelity DNA polymerase, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antartic Phosphatase and their buffers, dNTPs, DNA and protein molecular weight markers were purchased from NEB. Gel-extraction kits and plasmid mini prep columns were obtained from Thermo. Chip immunoprecipitation kit was purchased from Millipore. Anti-his mouse monoclonal antibody, anti-glutathione moue monoclonal antibody, anti-mouse HRP linked polyclonal antibody were purchased from Cell signaling and Abcam. The HRP substrate was bought from Millipore. Coelentarazine for the luminescent assay was purchased from Promega.

## 2.2 Strains and Plasmids

For all cloning we used *Escherichia coli* DH5α cells as a host. The only yeast strain used in this study was *yvc*Δ. The genotype of the strain is given below.

| S.no | Strain no | Source | Genotype | Remarks |
|---|---|---|---|---|
| 1 | **ABE 3796** | Euroscarf | BY4741; *MAT a his3Δ1 leu2Δ0 met15Δ0 ura2Δ0 Yvc1Δ::kanMX* | *Yvc1Δ* strain |

***Table 2.1****: Details of the strains used in this study.*

The details of the plasmids used in this study are given in ***Table 2.2*****.**

| S. no | Strain no | Remarks |
|---|---|---|
| 1 | **ABE 4149** | Complete Yvc1 gene with N- terminus His tag cloned in |

| | | |
|---|---|---|
| | | pRS313 TEF Vector at XbaI and XmaI sites. |
| 2 | ABE 4235 | Yvc1 gene with mutation of **Cys17 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 3 | ABE 4236 | Yvc1 gene with mutation of **Cys61 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 4 | ABE 4237 | Yvc1 gene with mutation of **Cys61 to Ala** and **Cys79 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 5 | ABE 4147 | Yvc1 gene with mutation of **Cys106 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 6 | ABE 4238 | Yvc1 gene with mutation of **Cys132 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 7 | ABE 4239 | Yvc1 gene with mutation of **Cys179 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 8 | ABE 4240 | Yvc1 gene with mutation of **Cys191 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 9 | ABE 4241 | Yvc1 gene with mutation of **Cys343 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |
| 10 | ABE 4244 | Yvc1 gene with mutation of **Cys624 to Ala** cloned in pRS313 TEF vector at the sites of XbaI and XmaI with His tag. |

***Table 2.2****: Various plasmids used in this study.*

## 2.3 Primers

The list of all primers used in this study is given in ***Table 2.3.***

| **S. no** | **Name** | **Nucleotide sequence(5'→ 3')** |
|---|---|---|
| 1 | YVCXbaI Fw | TGTGTGTCTAGACCCATTTATGTTAGCTAC |
| 2 | YVCXmaI Rw | ACACACCCCGGGTTAGTGGTGATGGTGATGATGCTCTTTCTTATCCTTTATGTCTAATTTTTC |
| 3 | YVC17 Fw | ATTTCTAACGAACAGGCAATGCCGGAAAACAAT |
| 4 | YVC17 Rw | ATTGTTTTCCGGCATTGCCTGTTCGTTAGAAAT |

| | | |
|---|---|---|
| 5 | YVC61 Fw | CCCAATGATATTGTTGCCGACCATTCTGAGATTT |
| 6 | YVC61 Rw | AAATCTCAGAATGGTCGGCAACAATATCATTGGG |
| 7 | YVC79 Fw | CTGGCTTATGAAGCAGCGGGCGGGAACCCTAAA |
| 8 | YVC79 Rw | TTTAGGGTTCCCGCCCGCTGCTTCATAAGCCAG |
| 9 | YVC106 Fw | TCACTTCTTAAAGTTGCTGAGTGGTATTCC |
| 10 | YVC106 Rw | GGAATACCACTCAGCAACTTTAAGAAGTGA |
| 11 | YVC132 Fw | GCCTCACAACAGTTAGCGAAATTGTTAATTGAA |
| 12 | YVC132 Rw | TTCAATTAACAATTTCGCTAACTGTTGTGAGGC |
| 13 | YVC179 Fw | GCCACAGATATGCACGCGACTACAGTGATAGGA |
| 14 | YVC179 Rw | TCCTATCACTGTAGTCGCGTGCATATCTGTGGC |
| 15 | YVC191 Fw | AGTGGATTTCAGCGTGCTTTAAAATGGATATGG |
| 16 | YVC191 Rw | CCATATCCATTTTAAAGCACGCTGAAATCCACT |
| 17 | YVC343 Fw | TATAGAGTCTTATCCGCAGCAGCACCATTT |
| 18 | YVC343 Rw | AAATGGTGCTGCTGCGGATAAGACTCTATA |
| 19 | YVC624 Fw | AAAAACGTTAAAAAAGCGAGTCCCTCCTTCGAA |
| 20 | YVC624 Rw | TTCGAAGGAGGGACTCGCTTTTTTAACGTTTTT |

***Table 2.3****: The list shows all primers used in this study.*

## 2.4 Media

All media, buffers and stock solutions were prepared using Millipore elix 3 deionized water. They were sterilized by autoclaving at 15psi pressure and $121^0$C for 15- 20 minutes. Additional nutrients and amino acids were prepared as per the requirement. Agar was added at a final concentration of 2.5% if required.

| | | |
|---|---|---|
| **2.4.1 YPD (Yeast extract-Peptone-Dextrose) Medium** | Yeast extract | 10g/l |
| | Peptone | 20g/l |
| | Dextrose | 20g/l |

| | |
|---|---|
| **2.4.2 SD (Synthetic Defined) Medium** | Yeast nitrogen base 1.7g/l<br>Ammonium sulfate 5g/l<br>Glucose 20g/l<br>Supplementary amino acids 80mg/l |
| **2.4.3 SOB (Super Optimal Broth) Medium** | Tryptone 20g/l<br>Yeast extract 5g/l<br>NaCl 0.5g/l<br>1M KCl 1.25 mL<br>1M $MgCl_2$ 5 mL<br>1M $MgSO_4$ 5 mL<br>pH of the medium was adjusted to 7 using 1N NaOH |

## 2.5 Buffers, Stock solutions and other chemicals

### 2.5.1 Ampicillin stock solution

A 50mg/ml stock of ampicillin was prepared in deionized water. The stock keeps at -$20^0$C in a tight container.

### 2.5.2 GSH stock solution

A 100 mg/ml stock of glutathione was dissolved in required amount of deionized water and keeps at -$20^0$C in a tight container.

### 2.5.3 TERNAse

A 10 mg/ml RNAse stock was prepared in TE buffer (pH 8). The final working concentration was 10 µg/ml.

### 2.5.4 50% Glycerol

The required amount of glycerol was dissolved in deionized water. After autoclaving, kept at room temperature. It was used in making -80$^0$C stock of *E.coli*

### 2.5.5 Alkaline lysis for plasmid isolation

| | |
|---|---|
| **a. Solution I (Resuspension)** | 50 mM Glucose<br>25 mM Tris-HCl (pH 8)<br>10 mM EDTA (pH 8)<br>Auticlaved and stores at 4$^0$C. |
| **b. Solution II (Lysis)** | 0.2 N NaOH (diluted from 5 N NaOH)<br>1% SDS (diluted from 20% stock)<br>Freshly prepared when required. |
| **c. Solution III (Neutralization)** | Potassium acetate 294.5 g/l<br>Glacial acetic acid 110 mL/l<br>Make up the solution to 1000 mL and<br>keep at 4$^0$C after autoclaving. |
| **d. PCI<br>(Phenol Chlorofom Isoamyl Alcohol) Solution** | Purchased readymade PCI solution from Sigma- Aldrich and stored at 4$^0$C. |

### 2.5.6 Agarose gel electrophoresis reagents

| | |
|---|---|
| **a. 1X TAE<br>(Tris- Acetate- EDTA)<br>Buffer** | 40 mM Tris-acetate<br>1 mM EDTA (pH 8) for a 1000 mL<br>solution. 50X stock was prepared and<br>keep at room temperature after autoclaving. |
| **b. Orange G dye 6X** | 0.25% Orange G |

| | |
|---|---|
| | 30% Glycerol |
| **c. 0.8% agarose gel** | 0.8 g in 100 mL of 1X TAE buffer. |
| **e. Ethidium bromide** | A final concentration of 0.5 µg/mL from the stock of 10 mg/mL. |

### 2.5.7 Yeast transformation solutions

| | |
|---|---|
| **a. TE (Tris- EDTA) Buffer** | 10 mM Tris- HCl (pH 8)<br>1 mM EDTA |
| **b. Lithium Acetate** | A final concentration of 0.1 M.<br>10M stock is prepared in TE buffer and<br>Keep at room temperature. |
| **c. PEG (Polyethylene glycol)** | PEG 3350 500g/l dissolved in $dH_2O$. |

### 2.5.8 SDS PAGE solutions and reagents

| | |
|---|---|
| **a. 30% Acrylamide mix** | 29.2% Acrylamide<br>0.8% N,N'- methylenebisacrylamide<br>Filtered before use. |
| **b. Resolving gel buffer** | Tris- HCl 184.5g/l |

| | |
|---|---|
| **(4X stock)** | Adjust the pH to 8.8 using 6N HCl and keep at $4^0$C. |
| **c. Stacking gel buffer (4X stock)** | Tris- HCl 60.5g/l<br>Adjust the pH to 6.8 using 6N HCl and keep at $4^0$C. |
| **d. SDS** | 0.4% SDS was used in the buffer. |
| **e. TEMED** | Added according to the gel concentration |
| **f. Ammonium per sulfate (APS)** | 10% solution was used in the buffers. |
| **g. Gel running buffer** | 25 mM Tris base (pH 8.3)<br>250 mM Glycine<br>0.1% SDS |

### 2.5.9 Western blotting reagents

| | |
|---|---|
| **a. Transfer buffer** | 48 mM Tris- base<br>39 mM Glycine<br>20% Methanol |
| **c. Washing buffer** | 0.1% Tween 20 in 1X PBS |
| **d. Blocking agent** | 5% BSA (Bovine Serum Albumine) in 0.1% PBST |

| | |
|---|---|
| **e. HRP substrate** | |

**Other Chemicals**

### 2.5.10 Hydrogen peroxide ($H_2O_2$)

$H_2O_2$ is a strong oxidizing agent with an oxygen- oxygen single bond. We used it as an agent to induce oxidative stress in both plate based assay and Ca2+ luminescent assay. It was added in each experiment as per the requirement. We purchased a 10 M stock from commercial sources.

### 2.5.11 Diamide

Diamide is reported to be a thiol oxidizing agent. It can increase the protein glutathionylation in a concentration dependent manner. We used this chemical in the immunoprecipitation based glutathionylation assay. The final working concentration was determined as per the requirement.

### 2.5.12 *N*- Ethylmaleimide (NEM)

NEM is a organic compound derived from maleic acid. It can irreversibly bind to the thiol groups because of the presence of an alkene. We used this chemical to block the cysteine residues of YVC1 in the glutathionylation experiments.

### 2.5.13 Ido-acetamide (IAM)

IAM is an alkylating agent which can covalently bind to the thiol groups of cysteien residues. As it is an irreversible reaction, we used this to block the cysteine residues of YVC1. The chemical was added as per the concentration of protein.

### 2.5.14 Coelenterazine

Coelenterazine is a light- emitting molecule found in many aquatic organisms. It is a substrate for many luciferases like aequorin. The molecule absorbs light in the

ultraviolet and visible spectrum. We used it as a luciferase for aequorin in the $Ca^{2+}$ luminescent assay.

## Methods

### 2.6 Growth and maintenance of bacteria and yeast strains

The *E. coli* straine were grown in LB medium at $37^0$C. The *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The yeast strains were grown on YPD medium at $30^0$C. The yeast transformants were selected and grown on SD medium with the required supplements.

### 2.7 Recombinant DNA methodology

All molecular techniques used in this study in both bacterial and yeast cells were according to the standard protocol.

### 2.8 Tagging the protein with His tag

PCR has been done using the primers, YVC XbaI forward and YVC XmaI reverse to construct the His tag on the N-terminal of the protein YVC1. This was done to check whether the mutants are localizing properly. I have used the His tagged proteins throughout my experiments.

His tag was encoded by the sequence- CAT CAT CAC CAT CAC CAC

### 2.9 Construction of site directed mutants of YVC1

I separately mutated at 8 cysteine residues of YVC1 to alanine residues using splice overlapping extension method. All the mutants were of His tagged at the N-terminal as explained before. I used 4 different primers for creating these point mutations. For creating C17A mutation, I created two constructs using the following set of primers;

(1) YVC XbaI Fw & YVC17 Rw

(2)YVC17 Fw & YVC XmaI Rw

Then I fused these two templates using YVC XbaI Fw and YVC XmaI Rw. In this manner I created the other Cys to Ala mutations of YVC1. The mutations were confirmed by sequencing.

## 2.10 Transformation of yeast

All the mutant plasmids of YVC1 were transformed into *S. cerevisiae* of *yvc1*Δ background using the standard lithium acetate method. *S.cerevisiae* culture was grown in YPD medium for 16 hours at $30^0$C. Reinoculated the culture into fresh YPD medium with a starting OD of 0.1. The culture was allowed to grow for 5-6 hours. The cells were harvested by centrifuging at 6000 rpm for 5 min followed by washing with sterile distilled water and 0.1 M lithium acetate. The cells were incubated at $30^0$C for 30 minutes in 0.1 M lithium acetate to get a cell density of $10^9$cells/ml. After spun down suspended the cells in 0.1 M lithium acetate and made 100 µl aliquots. Approximately 50 µg of heat denaturated single stranded DNA followed by 0.3- 0.7 µg of plasmid were added to each aliquot and incubated for 10 minutes at $30^0$C. 0.3 ml of 50% PEG was added to each tube and mixed well. After incubation of 45 minutes at $30^0$C the samples were subjected to heat shock at $42^0$C for 10 minutes. After cooling pellet down the cells at 6000 rpm for 5 minutes followed by 2 washing with sterile distilled water. The culture was suspended in appropriate amount of water and spread on selection plates.

## 2.11 Functional plate assay by dilution spotting

For growth assay, *S.cerevisiae* strains with YVC cysteine to alanine mutants were grown in SD medium without histidine (the mutant gene was cloned downstream of TEF promoter in pRS313 vector, which has a histidine marker). A secondary culture was inoculated at an OD of 0.1 and grown at $30^0$C for 6 hours. These were serially diluted to the concentrations of 0.1, 0.01, 0.001 and 0.0001. The cell suspensions were spotted on SD-His plates with different concentrations of $H_2O_2$. The plates were incubated at $30^0$C for 3 days and photographs were taken.

## 2.12 Protein purification from the yeast whole cell extract

All the mutant cells were grown in SD-His medium. A secondary culture was inoculated in 100 ml of medium and allowed to grow at $30^0$C at 30 minutes until the growth reaches the exponential phase ($OD_{600}$ 0.6- 0.8). For glutathionylation *in vivo* assay the culture was treated with 1.8 mM diamide for 10 minutes at $30^0$C. The cells were harvested by centrifugation and resuspended in yeast breaking buffer along with protease inhibitor and phenylmethanesulfonyl fluoride. Acid-washed glass beads were added to the cell suspension and gave vigourous vortexing. The whole cell extract were collected by centrifugation. For selectively getting YVC1 protein, added anti-his mouse monoclonal antibody (as per the amount of whole cell extract) and incubated at $4^0$C overnight. We used an immuniprecipitation kit (from Millipore) to purify the YVC1 protein alone.

## 2.13 Protein electrophoresis and western blotting

I used a 5% non- reducing gel to run my samples. Equal amount of proteins were loaded in two gels without β- mercaptoethanol (after heating at $90^0$C for 10 minutes with the loading buffer). Both the gels were blotted on nitrocellulose blotting papers. Immediately after blotting, blocking was done using 5% BSA. One blot was incubated with anti-His mouse monoclonal antibody and the second one with anti-GSH mouse monoclonal antibody at $4^0$C overnight with gentle shaking. Anti- mouse HRP linked polyclonal antibody was added and incubated overnight at $4^0$C with gentle shaking. Washing was done using 0.1% PBST 3 times. The signal was detected using HRP substrate.

For *in vitro* glutathionylation assay, proteins were purified from the yeast whole cell extract and given *N*-Ethylmaleimide/Ido-acetamide treatment for 1 hour at $30^0$C in Tris buffer (pH 8). After washing, 100 nM diamide along with 10mM GSH for 10 minutes at $30^0$C was added. This was followed by the method for checking glutathionylation.

## 2.14 Aequorin based $Ca^{2+}$ luminescent assay

Real time cytosolic $Ca^{2+}$ level were measured using this assay. All the cells having the mutant plasmids were first transformed with aequorin plasmid (Leucine marker). The transformed mutants were grown in SD-His-Leu medium at $30^0$C until the OD reached 1.5. The cells were harvested by centrifugation at 6000 rpm for 5 minutes. The cells were suspended in minimal medium (200 µl) and incubated with 100 mM coelenterazine for 30 minutes at $30^0$C. After washing with sterile distilled water, resusupended the cells in 200µl of SD-Leu-His medium. To each of the sample added 500 nM EGTA and incubate for 30 minutes at $30^0$C. Luminescence was measured in 96 well white plates by inducing oxidative stress using 4 mM $H_2O_2$.

## 2.15 Sequence analysis

The sequences were retrieved from *S.cerevisiae* Genome Database (SGD). Protein sequences of homologues of YVC1 were retrieved. Conserved amino acids among these homologues were found by multiple sequence alignment using the Clustal W software. The topology and transmembrane domains were predicted using Topcons consesnsus method.

# Chapter 3

## *Results and Discussions*

## 3.1 Sequence analysis

### 3.1.1 Homologues of Yvc1

| Organism | % similarity with Yvc1p | Size of the protein(aa) | Location | Function |
|---|---|---|---|---|
| *H. Sapiens* - TRP V 1 | 23 | 838 | Plasma membrane | Pain and heat sensation |
| *D. melanogaster*- TRP isoform a | 22 | 1128 | unknown | unknown |
| *M. musculus* – TRP channel 4 isoform 1 | 22 | 974 | unknown | unknown |
| *C. albicans*- Hypothetical protein Cao | 45 | 675 | Vacuole | Predicted to be $Ca^{2+}$ transporter |

***Table 3.1:*** *Homologues of Yvc1.*

### 3.1.2 Multiple sequence alignment using Clustal W

MSA of YVC1 with fungi is shown in ***Figure 3.1***. The result shows that cysteine 106, 191 and 343 are conserved among the homologues in different fungi.

MSA with the homologues of *H. sapiens, M.musculus, D. melanogaster* shows that cysteine 17 and 79 are conserved. The data is shown in ***Figure 3.2.***

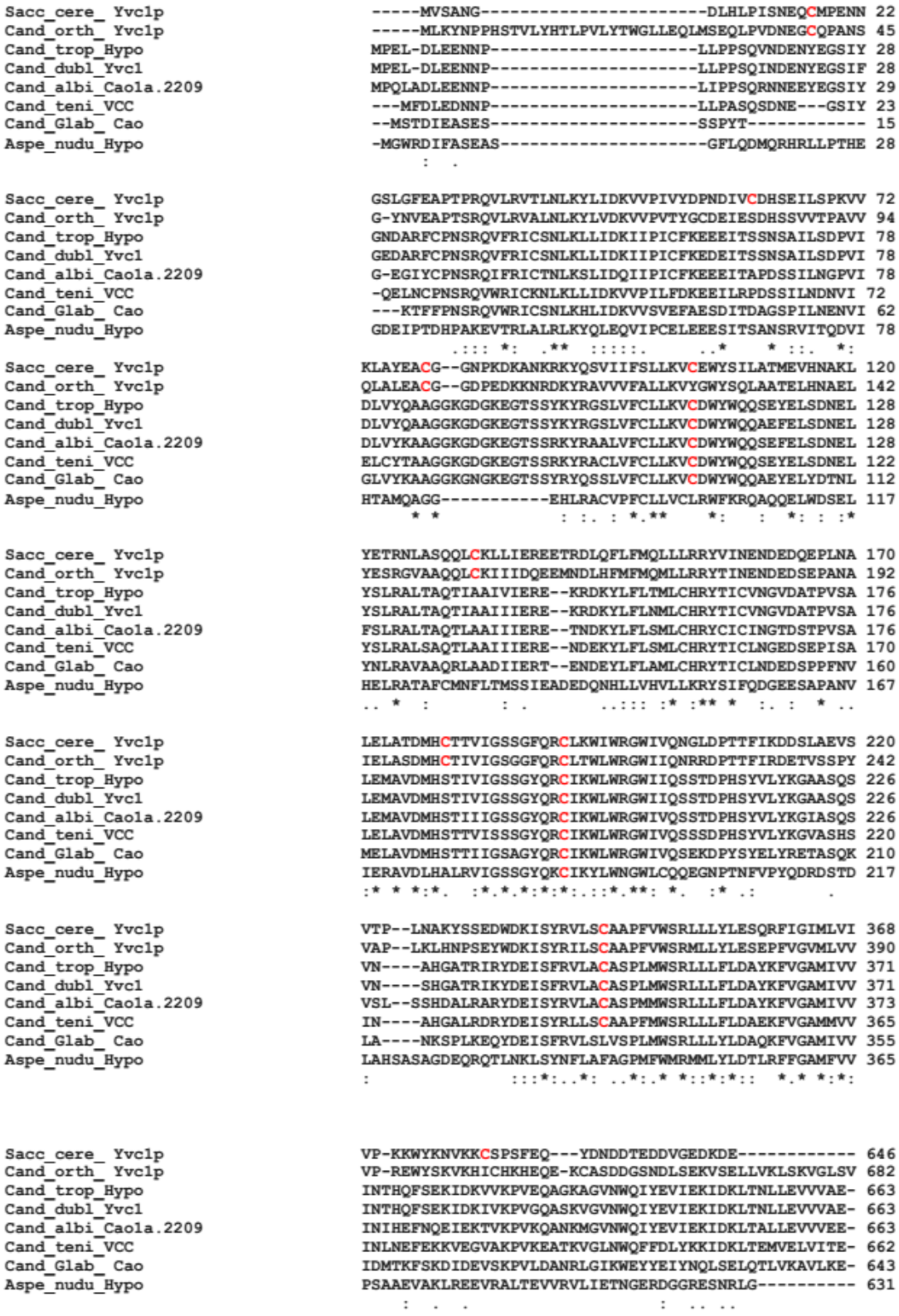


***Figure 3.1:*** *MSA of YVC1 with homologues in fungi.*

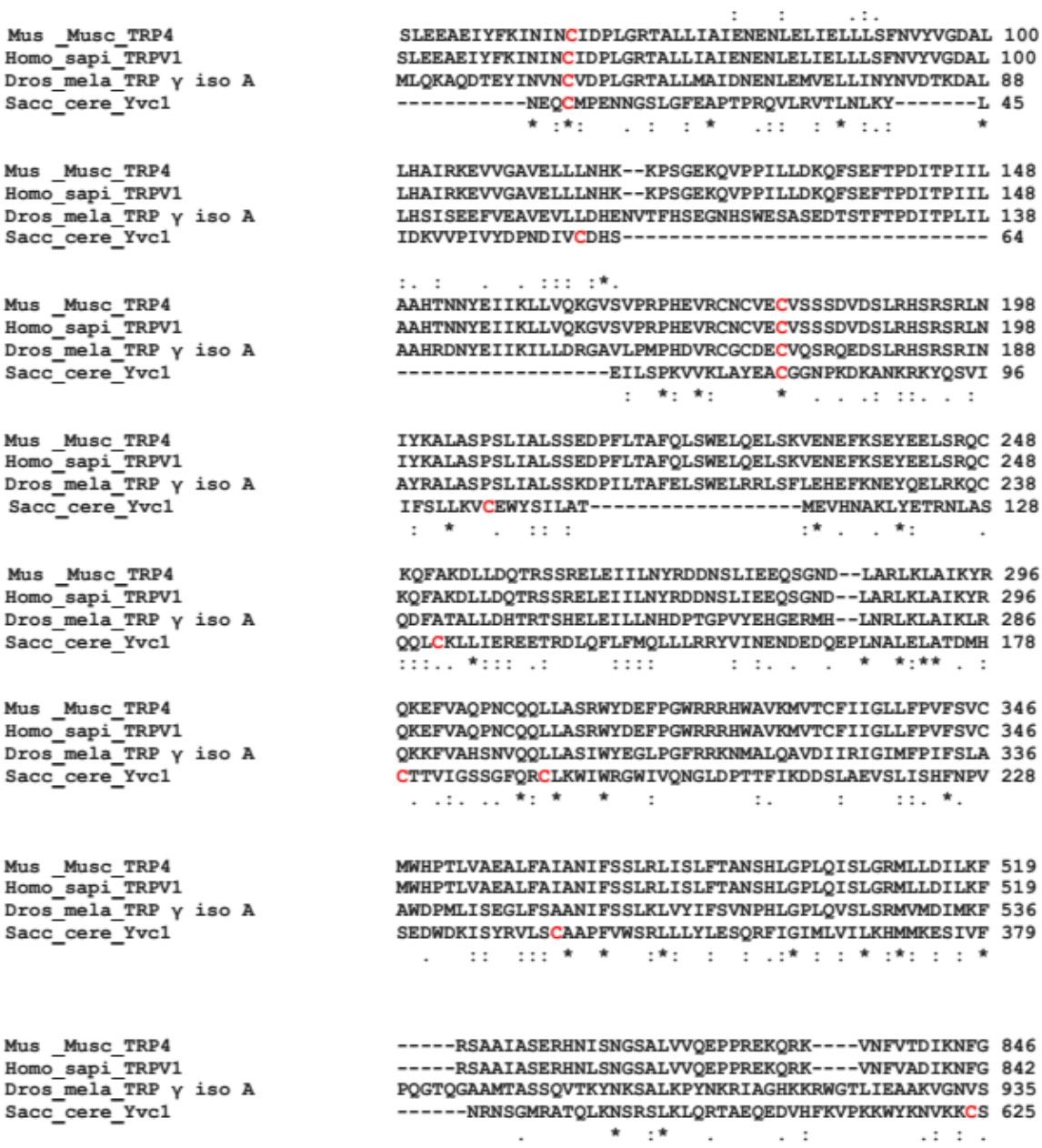


***Figure 3.2:*** *MSA of YVC1 with homologues of higher organisms.*

### 3.1.3 Transmembrane prediction using Topcons

The Topcons consensus method predicted that YVC1 is a protein with 6 transmembrane domains. I also looked at the position of each of the cysteine residues of protein using the predicted transmembrane structure. ***Figure 3.3*** shows the TM structure of YVC1 made by me as per the result from Topcons method.

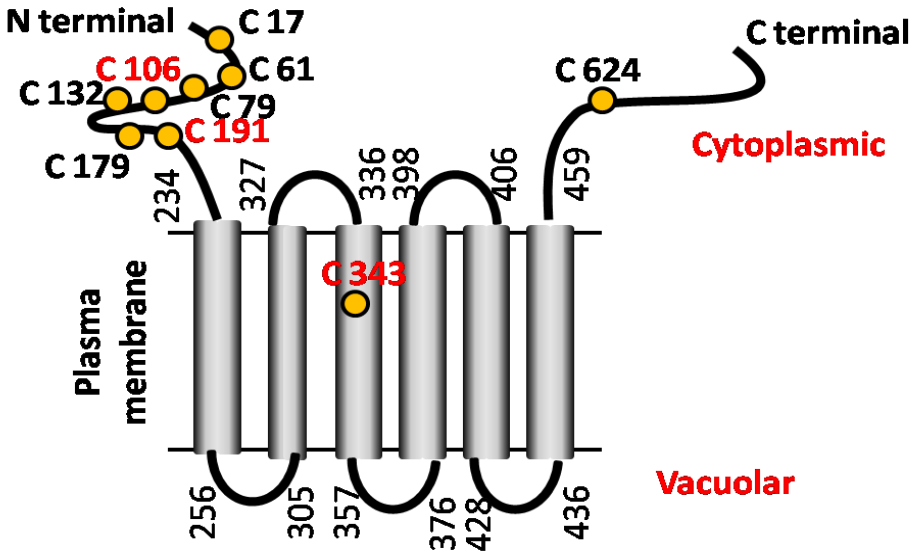


***Figure 3.3:*** *Predicted structure of transmembrane domains of YVC1.*

## 3.2 A functional plate assay using oxidizing agents

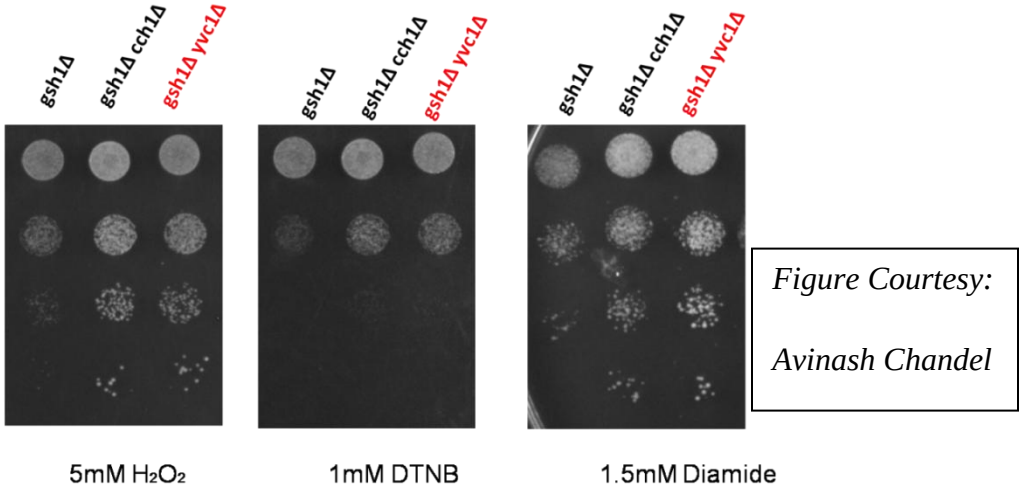


***Figure 3.4:*** *yvc1Δ strain shows enhanced resistance to oxidizing agent.*

GSH1 is the first enzyme in glutathione biosynthesis. The *gsh1*Δ strains are sensitive to oxidative stress. The same cell lines show enhanced resistance to oxidizing agents in *yvc1*Δ background. This indicates Yvc1 contributes to the sensitivity of yeast cells under oxidative stress. This work is done previously in the lab and the data is not published (Chandel & Bachhawat, unpublished).

### 3.3 Intracellular $Ca^{2+}$ measurement of yvc1Δ strain during oxidative stress

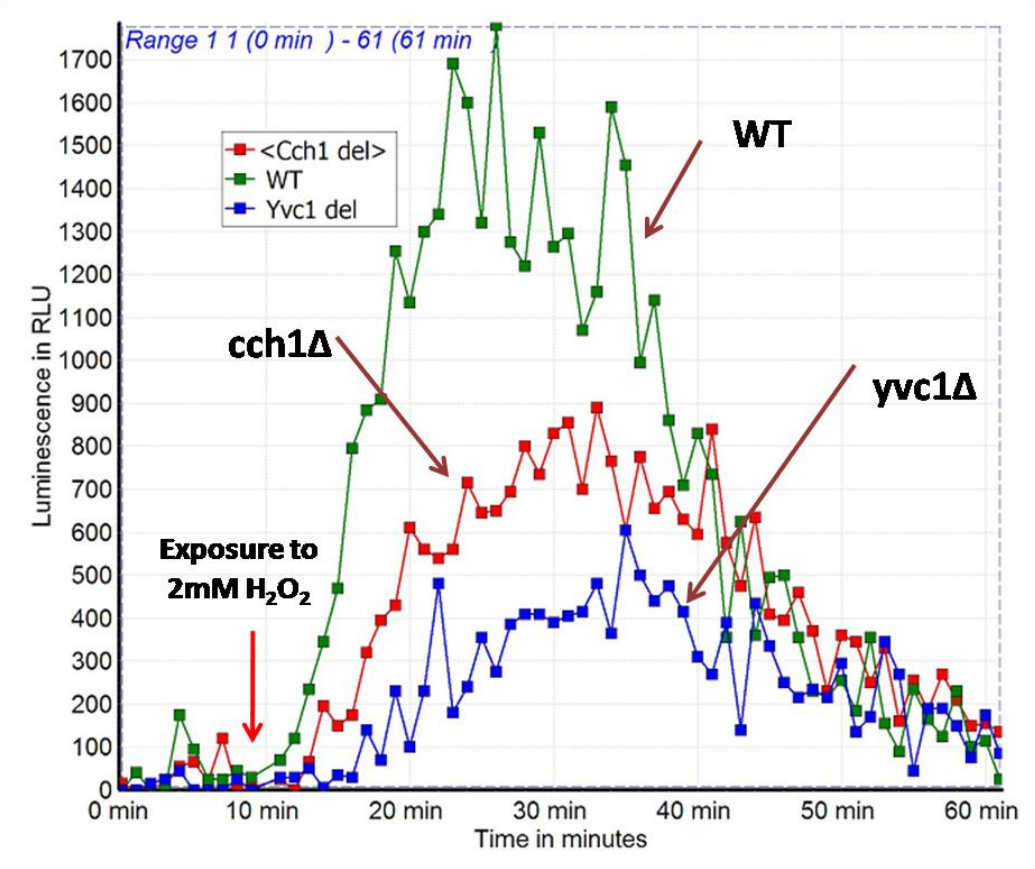


***Figure 3.5:*** *Intracellular $Ca^{2+}$ measurement using aequorin based luminescent assay.*

As the *yvc1*Δ strain showed resistance to oxidizing agents in the plate assay, I measured the cytosolic $Ca^{2+}$ level using aequorin based luminescent assay. The result shows that $Ca^{2+}$ level is low in *yvc1*Δ cells compared to control and *cch1*Δ cells after exposing to $H_2O_2$. This implies that the enhanced resistance of *yvc1*Δ cells is due to decrease in the level of $Ca^{2+}$ as the channel is not active in these cells.

## 3.4 Glutathionylation analysis

### 3.4.1 *In vivo* assay using diamide

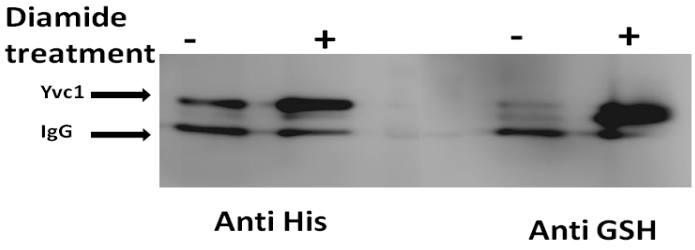


***Figure 3.6:*** *YVC1 is glutathionylated under oxidatitive stress.*

An immunoprecipitation based assay was done to check whether the protein YVC1 is glutathionylated or not. To induce such a condition, treated the cells with diamide. The western blotting results show that in the diamide treated cells, YVC1 showed a thick band at the required length using anti- GSH antibody. Whereas the non- treated cells showed a very faint band. The control blot showed band at the required size using anti- His antibody. So analysis of the results leads me to the conclusion that YVC1 is glutathionylated.

### 3.4.2 *In vitro* assay using diamide

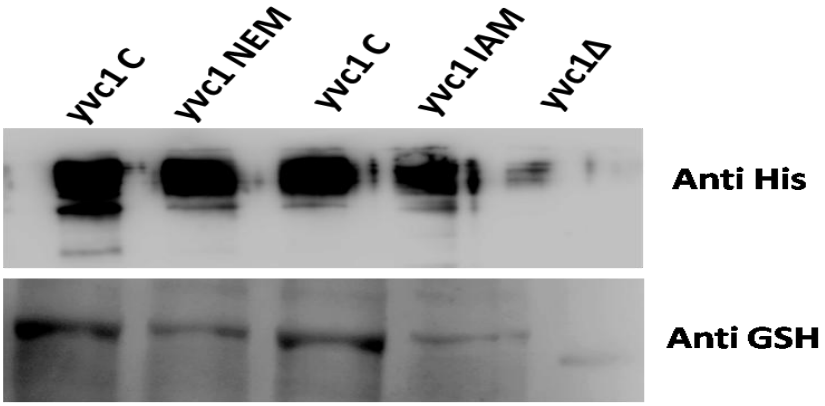


***Figure 3.7:*** *YVC1 glutathionylation is affected by NEM and IAM.*

AS YVC1 seems to be glutathionylated, I treated the protein with *N*- ethylmaliemide and Ido- acetamide which will irreversibly block the thiol groups of cysteine residues.

Then I gave NEM and IAM treatment to the protein to see whether it is affecting the glutathionylation of YVC1. The western blotting results show that the level of glutathionylation has decreased in the samples treated with NEM and IAM. Whereas, the control shows good level of glutathionylation. By this assay it is confirmed that YVC1 is a protein which is prior to get glutathionylated under oxidative stress.

## 3.5 Analysis of all Cys → Ala mutations

There are 9 cysteine residues present in YVC1. To check which of these cystienes are glutathionylated I created all Cys → Ala mutations- C17A, C61A, C106A, C132A, C179A, C191A, C343A, C624A and a double mutant C61A C17A. C79A is a mutation which is under construction.

### 3.5.1 A functional plate using $H_2O_2$

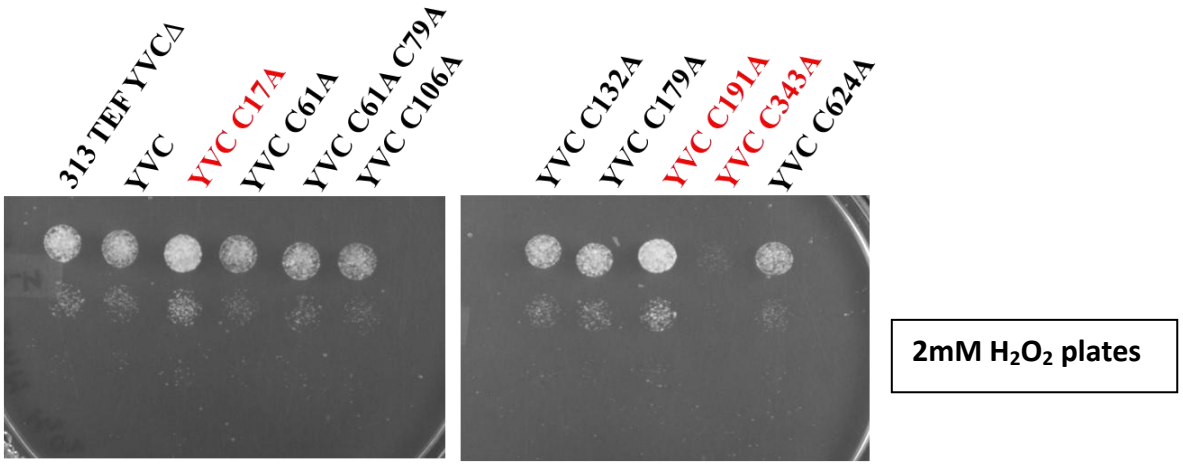


***Figure 3.8**: A functional plate assay using $H_2O_2$ (selection plate SD-His).C17A and C191A show resistance to oxidizing agent.*

The spotting assay of mutants in 2 mM $H_2O_2$ plates show that YVC C17A and YVC C191A mutants are growing more than the control cells. This implies that these mutants can be loss function mutants. Another important observation here is YVC C343A mutant is showing very less growth compared to control. This can be a gain of function mutant.

### 3.5.2 Checking localization of all Cys → Ala mutants

Localization of mutants reveals that except YVC C61A, all other mutants are localizing properly to the vacuole. Localization was checked by Mr. Avinash Chandel.

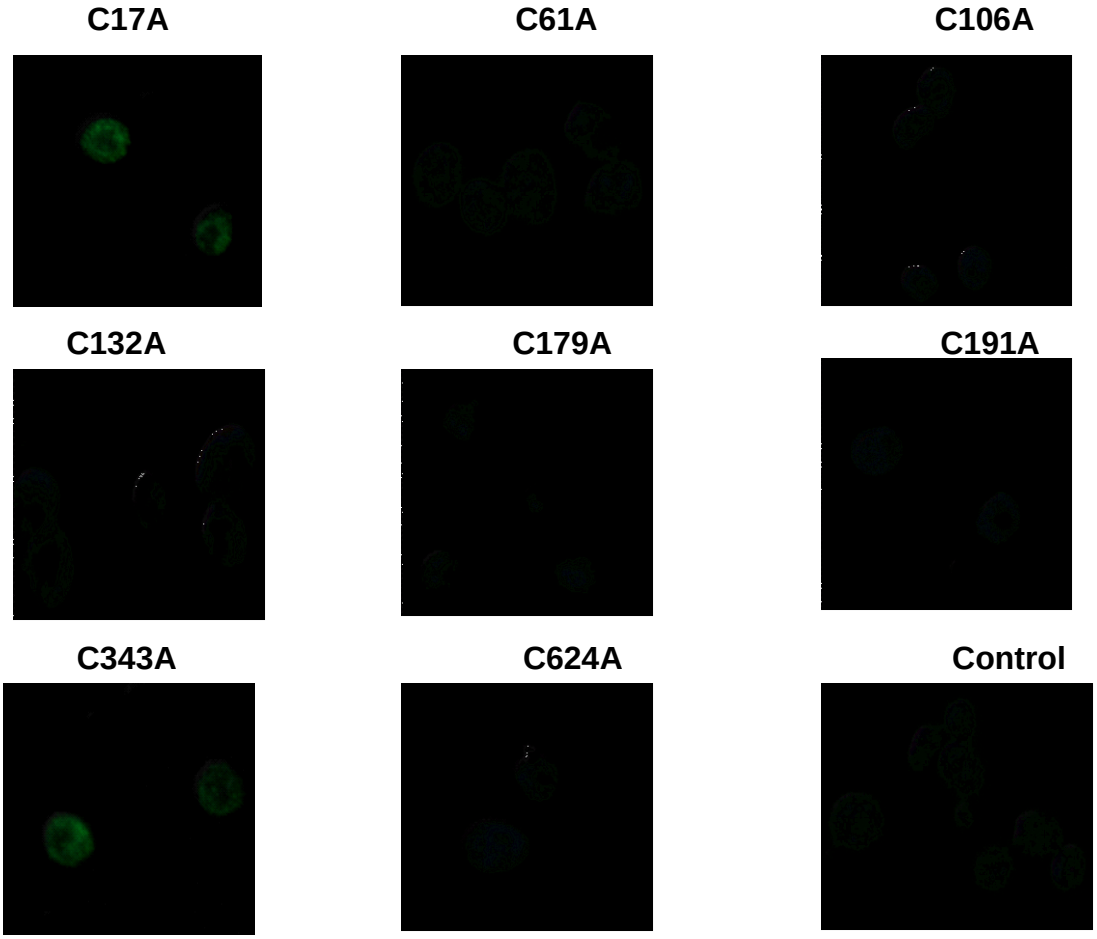


***Figure 3.9****: The image shows the localization of Mutants.*

### 3.5.3 Glutathionylation assay for all Cys → Ala mutants

The glutathionylation assay for the mutants shows YVC C17A, YVC C61A and YVC C191A are having lower level of glutathionylation. Whereas, YVC C343A shows similar band as control. Here C61A is a mislocalized protein.

The lower level of glutathionylation of YVC C17A and YVC C191A indicates that these can be the cystienes which are getting glutathionylated and leads to the activation of the channel YVC1 under oxidative stress.

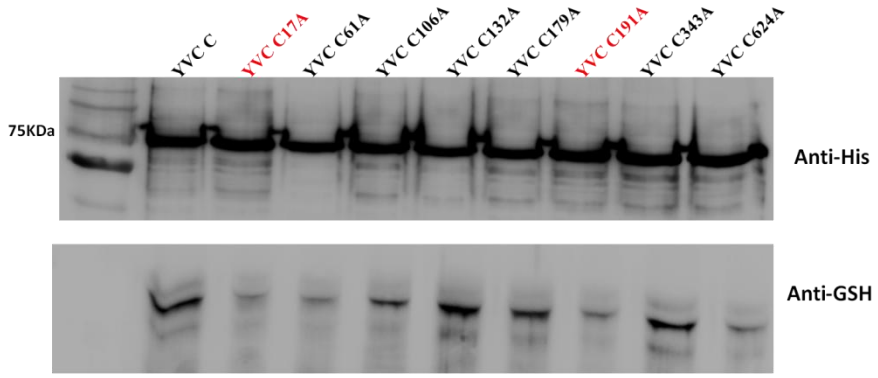


***Figure 3.10:*** *The mutants YVC C17A, YVC C61A and YVC C191A are showing lower level of glutathionylation compared to the control.*

### 3.5.4 $Ca^{2+}$ measurement using aequorin based luminescent assay

YVC C17A and YVC C191A mutants show lower amount of $Ca^{2+}$ as compared to control. This is a clear implication that cys17 and cys191 are the most critical cysteines that are glutathionylated under oxidative stress. The higher amount of $Ca^{2+}$ in YVC C343A mutant confirms that the mutant is hyper active.

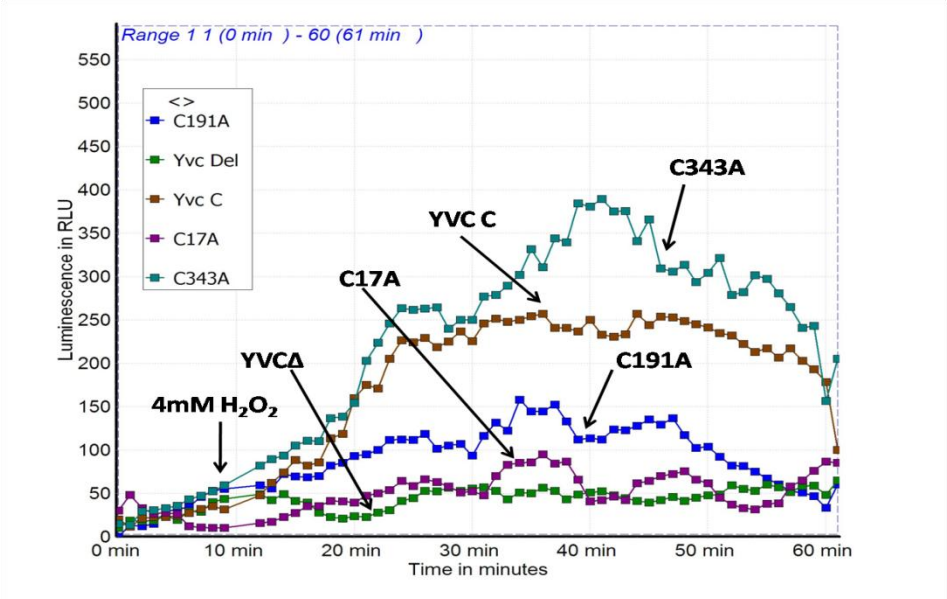


***Figure 3.10:*** *C17A and C191A mutants are showing lower level of cytosolic $Ca^{2+}$.*

# Conclusion

The Yeast Vacuolar Channel protein, YVC1 has an important role in maintaining $Ca^{2+}$ homeostasis inside the cell. The analysis of *yvc1*Δ strain yielded clue about the regulation of YVC1. As the response of yeast cells towards oxidative stress is rapid, the most probable mechanism of channel activation is some kind of post- translational modifications. Our study reveals that glutathionylation is one of the post translational modification involved in YVC1 regulation. As the important targets of glutathionylation are the thiol groups of cysteine residues, we have created Cys→ Ala mutations of 8 of the 9 different cystiene residues of the protein YVC1. From the functional plate assay using $H_2O_2$, C17A and C191A were showing more growth than control whereas C343A mutation was showing increased sensitivity towards oxidative stress. By checking glutathionylation, C17A and C191A were showing lower level compared to control. Further analysis is needed for the C61A mutation which shows mislocalization to ER. The aequorin based calcium assay shows lower levels of cytoplasmic $Ca^{2+}$ burst by the C17A and C191A mutants, which further confirms their loss in activity. On the other hand higher calcium burst in case of C343A suggests a constitutive gain of function by the C343A mutant. The data suggests that YVC1 is activated by the glutathionylation of Cys 17 and 191 under oxidative stress and Cys 343 plays a role in calcium channel activation

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