Studies on the regulatory domain of 5, 10-Methylene tetrahydrofolate reductase (MTHFR)

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Certificate of Examination

This is to certify that the dissertation titled **"Studies on the regulatory domain of 5, 10-Methylene tetrahydrofolate reductase (MTHFR)"** submitted by Ms. Swati Dhiman (MS14131) for the partial fulfillment 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.

Dr. Rajesh Ramachandran

Dr. Shashi Bhushan Pandit

Prof. Anand Kumar Bachhawat (Supervisor)

Date: April 26, 2019

Declaration

I hereby declare that the work presented in this dissertation entitled **"Studies on the regulatory domain of 5, 10-Methylene tetrahydrofolate reductase (MTHFR)"** is the result of investigations carried out by me under the guidance of Prof. Anand K Bachhawat at the Department of Biological Sciences, Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. In keeping with the general practice of reporting the scientific observations, due acknowledgement has been made wherever the work described is based on the finding of other investigators. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Swati Dhiman
Date: April 26, 2019

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

Prof. Anand Kumar Bachhawat (Supervisor)

Acknowledgement

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ABBREVIATIONS

Weights and measures

%	Percent
uM, nM, mM, M	Micro moles, nanomoles, millimoles, molar
°C	Degree centigrade
bp, kb	Base pair, kilo base
kDa	Kilo Dalton
OD	Optical density
Psi	Per square inch
rpm	Revolutions per minute
RT	Room temperature
sec, min, hr	second, minute, hour
ug, mg, g	micro grams, milligrams, grams
ul, ml, L	micro liter, milliliter, liter

Symbols

α	alpha
Δ	delta

Techniques

PCR	Polymerase chain reaction

Chemicals

Amp	Ampicillin
ATP	Adenosine triphosphate
dNTPs	2'-deoxyadenosine 5'-triphosphate
EDTA	Ethylenediamine-tetra-acetic-acid
GSH	reduced Glutathione
HCl	Hydrogen chloride
PEG	Poly Ethylene Glycol

Miscellaneous

DNA	Deoxyribonucleic acid
TE	Tris chloride and EDTA
WT	Wild type
LB	Lysogenic broth
SAM	S-adenosyl methionine
SBDs	SAM binding domain
GFP	Green fluorescent protein
DR	*Danio rerio*
CR	Conserved region
n	number
cDNA	complementary DNA

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Abstract

5,10-Methylene tetrahydrofolate reductase(MTHFR) is a cytoplasmic enzyme which catalyzes the conversion of 5,10-methylene tetrahydrofolate(CH2-THF) to 5,10-methyl tetrahydrofolate(CH3-THF) using NADPH as a reducing equivalent. This enzyme plays an important role in folate one carbon metabolism pathway. The polymorphisms of this enzyme associated with disruption of function cause neural tube defects and hyperhomocysteinuria in many individuals.This enzyme consists of N-terminal catalytic domain and C-terminal regulatory domain linked by a small stretch of amino acids called as linker region. The function is negatively regulated by the metabolite S-adenosyl methionine (SAM) produced downstream to methionine in the methionine cycle. It has been known that SAM binds to the regulatory domain and thus regulates the activity of the enzyme, but the exact SAM binding region is not known. The mutational studies on the conserved residues in the regulatory domain of *S. cerevisiae* MTHFR (MET13) show a deregulated phenotype. On the continuation of the previous finding of a deregulated mutant of MET13_R357A in the lab, the conservation of deregulation of MTHFR in higher organisms was examined in *Homo sapiens* MTHFR. The isoforms of *Homo sapiens* MTHFR shows interesting phenotype when supplemented with different sulphur sources. The second part of the work involves attempts at construction of a genetically encoded SAM sensor based on fluorescence. It has been shown that the insertion of an in sense molecular recognition domain in between the GFP responds on binding to the ligand. Based on this we are trying to create a GFP based SAM sensor by inserting the regulatory domain of the MTHFR protein in frame with the GFP protein.The response of GFP constructs to SAM on insertion of the regulatory domain of MTHFR shows a potential to be a SAM sensor.

Chapter 1

Introduction and review of literature

1.1 Folate one carbon metabolism

Folate, which is also known as folic acid, is a B vitamin. The assimilation of folate by the cell initiates a series of metabolic reactions involving the generation and transfer of one carbon units such as methyl group from one metabolite to another thus called as folate one carbon metabolism [1]. It is compartmentalized in both cytoplasm and nucleus. In nucleus, the condensation of tetrahydrofolate (THF) from folate and formate from serine, glycine forms methylene-THF, which in turn undergoes metabolic reactions to form (dTMP) thymidylate, a precursor for nucleic acids. The recycling of methylene THF to THF leads to its transport back to the cytoplasm. The cytoplasm mediated one carbon metabolism gives way to three anabolic pathways : (a) de novo purine biosynthesis, the conversion of THF to 10- formyl THF is required for purine biosynthesis, (b) de novo thymidylate biosynthesis, the conversion of dUMP to dTMP (thymidylate) requires methylene-THF, an intermediate in the folate cycle, (c) methionine biosynthesis, the methylation of homocysteine to methionine is mediated by the transfer of methyl group from 5-methyl THF when recycled back to THF. This particular intermediate 5-methyl THF formation is catalyzed by a cytoplasmic enzyme called as Methylene tetrahydrofolate reductase (MTHR). The impairment of this folate mediated one carbon metabolism gives rise to various defects in the body such as folate deficiency and secondary nutrient deficiencies. There will be decrease in the production of the precursor of purine biosynthesis, dTMP ultimately leading to the lesser production of dTTP, more incorporation of uracil in the DNA replication, making DNA strands more susceptible to double strand breaks and thus making the integrity of the genome vulnerable. The folate deficiency has also been linked to cell cycle disruptions suggesting an indirect link between folate deficiency and cancer. The secondary nutrient deficiencies involve the increase in the amount of homocysteine in the cell, a condition called as hyperhomocysteinuria, decrease in the amount of methionine and methylation levels. There is also significant decrease in the amount of methylation because of methyl group trap in the form of methyl-THF. The folate deficiency causes neural tube defect which is a developmental disorder characterized as defects in the closure of spinal cord. The MTHFR polymorphism at 677 residues is involved directly in neural tube defects [2] therefore this enzyme becomes a lot important to study.

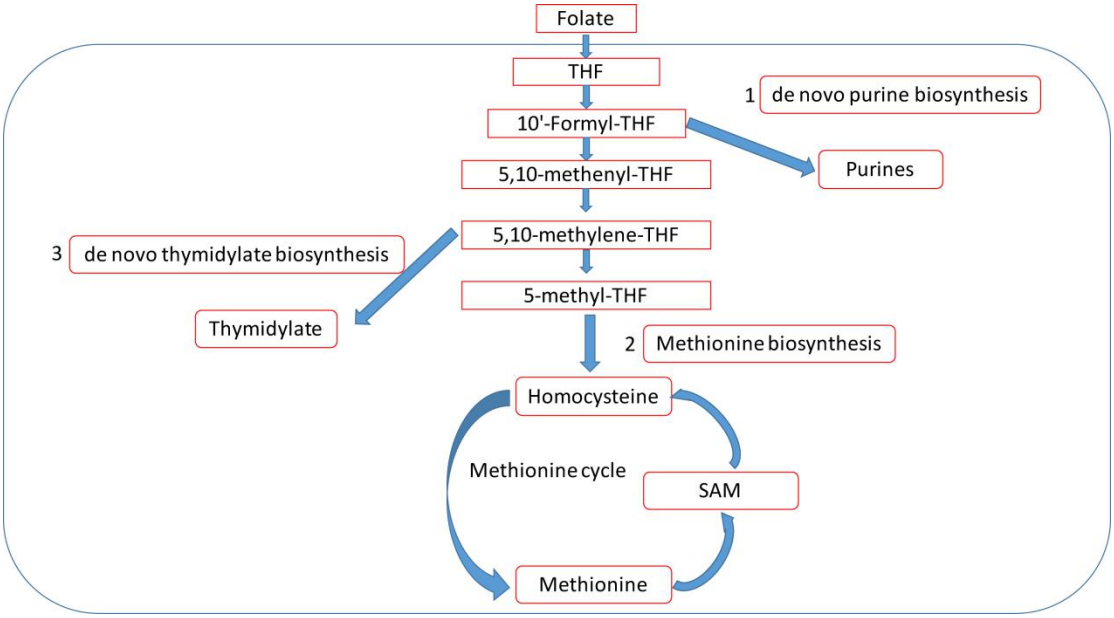

Fig. 1.1 Folate one carbon metabolism in cytoplasm[1]: the uptake of folate gives way to three anabolic reactions (1) de novo purine biosynthesis (2) methionine biosynthesis (3) de novo thymidylate synthesis

1.2 5, 10-Methylene tetrahydrofolate reductase (MTHFR)

5,10-Methylene tetrahydrofolate reductase is a cytoplasmic enzyme which catalyzes the conversion of 5'-methylene tetrahydrofolate (CH2-THF) to 5'-methyltetrahydrofolate (CH3-THF) in the folate one carbon metabolism pathway [3]. It is a dimeric flavoprotein which uses NADPH/NADP as a reducing equivalent for the reduction of methylene to methyl group attached to THF. It functions as a major methyl group donor in remethylation of homocysteine to methionine. The transfer of methyl group takes place from the folate cycle to the methionine cycle.

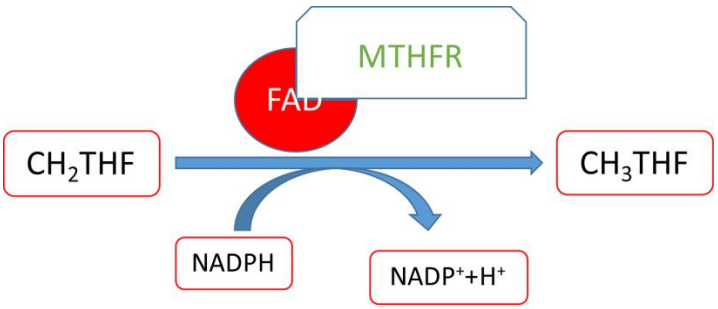

Fig.1.2 Schematic representation of the chemical reaction catalyzed by MTHFR: MTHFR is a flavoprotein which reduces the methylene group to methyl of THF using NADPH as a reducing equivalent.

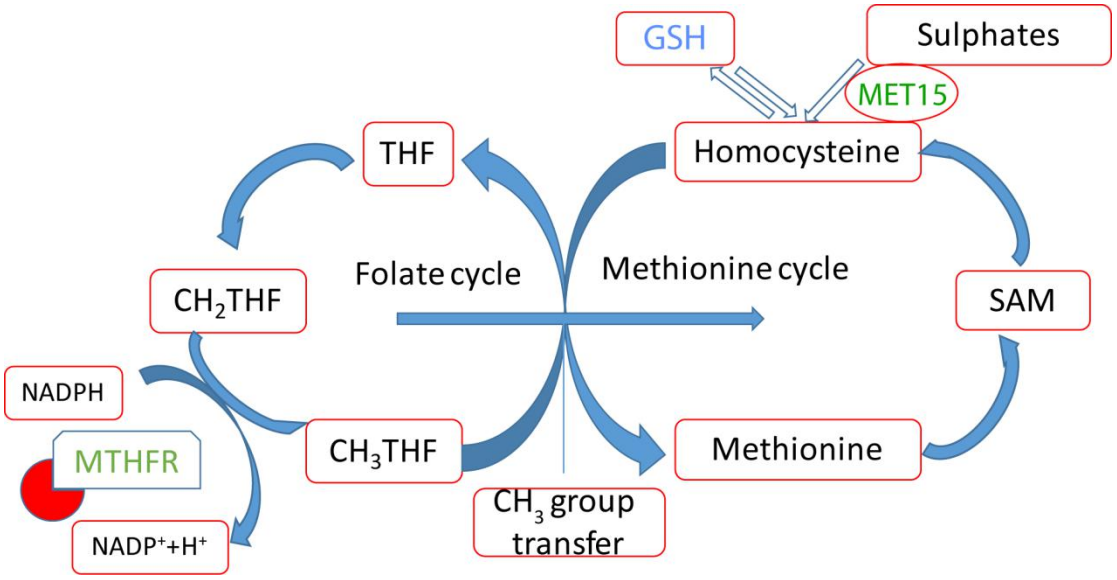

Fig. 1.3 Schematic representations showing the role of MTHFR in connecting folate cycle and methionine cycle: MTHFR ensures the continuous transfer of one carbon units from folate cycle to methionine cycle thus governing the amount of methionine pools in the cell.

1.3 Domain organization of MTHFR

MTHFR is an enzymatic protein which consists of an N-terminal catalytic domain and a C- terminal regulatory domain linked by a small stretch of amino acids called as the linker region. The catalytic domain contains NADPH/NADH binding domain [4], FAD binding domain [5] and CH2-THF substrate binding domain [6]. The regulatory domain contains S-adenosyl methionine (SAM) binding domain, but the exact residues involved in the SAM binding are still undiscovered. The regulatory domain in general is poorly studied. The protein sequence alignment of regulatory domain of *S. cerevisiae*, *Danio rerio*, *Homo sapiens* shows a lot of conserved regions (CRn) found across these taxa.

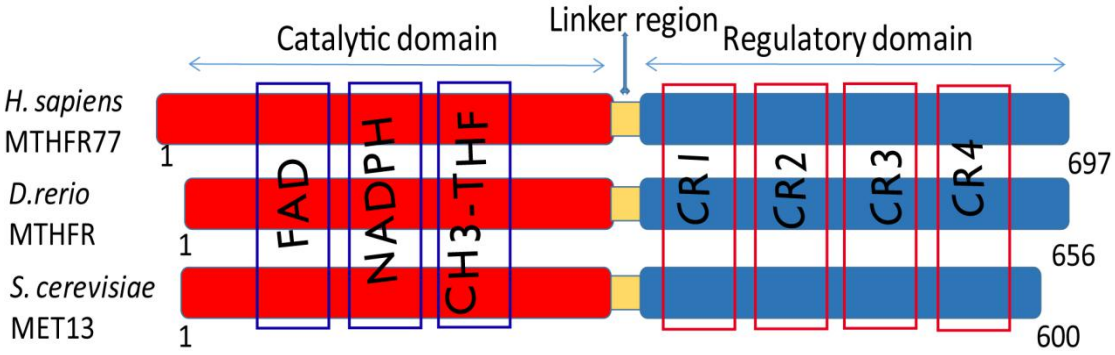

Fig. 1.4 Schematic representation of MTHFR protein domains in *S. cerevisiae, Danio rerio* and *Homo sapiens*: Red color represents the catalytic domain, blue color represents the regulatory domain, and yellow color means linker peptide. The catalytic domain binds to FAD, NADPH and the substrate. The regulatory domain is enriched with conserved regions (CRn).

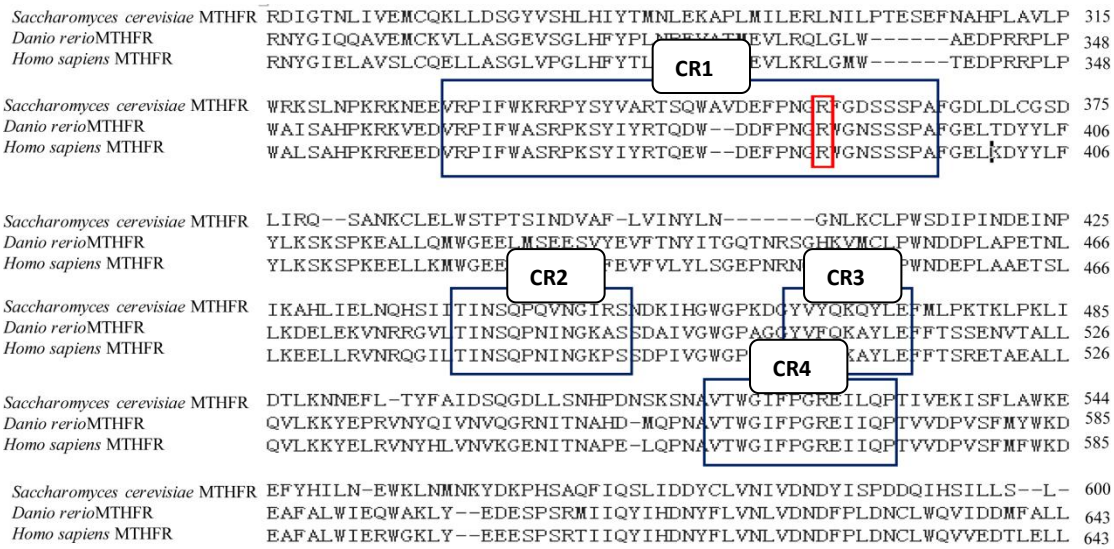

Fig. 1.5 Multiple sequence alignment using CLUSTAL OMEGA of regulatory domains of *S. cerevisiae, Danio rerio* and *Homo sapiens:* The blue colour box highlights the conserved regions in the regulatory domain of *S. cerevisiae, Danio rerio* and *Homo sapiens.*

1.4 S-adenosyl methionine (SAM)

SAM is a metabolite which is formed by the condensation of ATP and methionine. The adenosyl group is transferred from the adenosine triphosphate to methionine. This metabolite is produced in the downstream of folate one carbon metabolism. SAM is known to interact and alter the activity of various enzymes such as cystathionine β synthase, MTHFR etc. It is the second most abundant metabolite which acts as a methyl donor to various molecules.

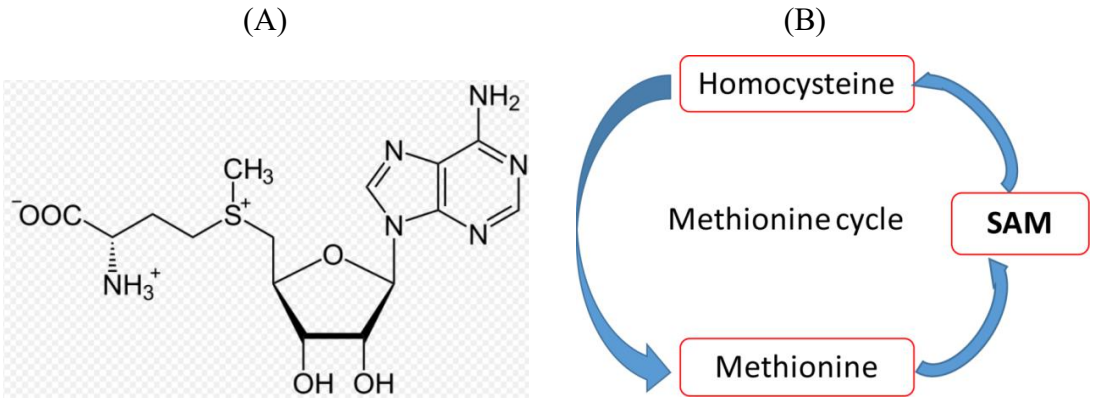

Fig. 1.6 (A) Chemical structure of S-adenosyl methionine: It consists of adenosyl group and methionine (B) Methionine cycle: It shows the production of SAM downstream to methionine in methionine cycle

1.5 *Homo sapiens* MTHFR

There are two isoforms of *Homo sapiens* MTHFR which are of 70 and 77 kDa. The 70 kDa isoform differs from the 77 kDa isoform in having extra 41 amino acid residues at the extreme end of the N terminal. The complete characterization of cDNA sequence indicates variable 5',3' UTR regions resulting in multiple transcripts [7]. It has also been shown that the mutation of ATG initiation codon to ATA leads to a biased expression of 77kDa isoform over 70 kDa isoform suggesting that multiple transcription start sites and alternative splicing in the methylene tetrahydrofolate reductase gene results in two enzyme isoforms.

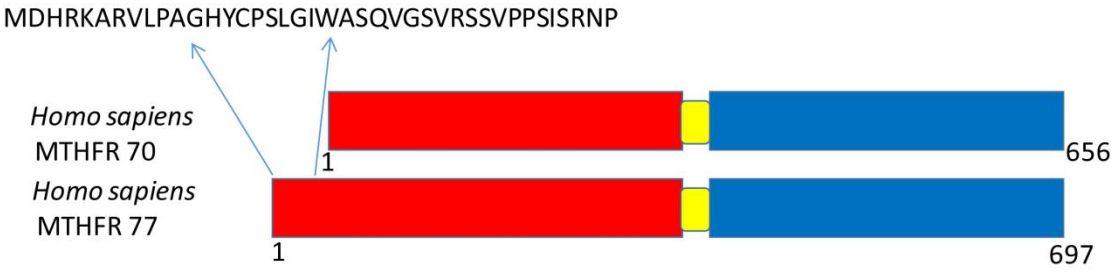

Fig. 1.5 Schematic representation of the N terminal differences of 41 amino acids between two isoforms of *Homo sapiens* MTHFR 70 and 77 kDa.

1.6 Regulation of enzyme MTHFR

Despite conservation of amino acid residues of the regulatory domain across various taxa, there are indeed a lot of ways in the way this enzyme is regulated.

In the simplest unicellular organism such as *E. coli*, the regulatory domain is missing and the function of this enzyme is regulated at the transcriptional level [8].

The unicellular eukaryote *S. cerevisiae* contains a regulatory domain which is known to be allosterically regulated by SAM [9].

The multicellular eukaryote, Arabidopsis thaliana MTHFR protein, consists of a regulatory domain whereas the feedback inhibition by SAM is not observed [10].

In *Homo sapiens* MTHFR, the regulation takes place at different levels. At the N terminal, the 34 threonine (Thr) residue undergoes phosphorylation which increases the sensitivity of protein to SAM and SAM inhibits the activity of this enzyme by binding to the regulatory domain forming a feedback inhibition loop [11].

1.7 Isolation of the deregulated mutant of MTHFR

MTHFR is an important enzyme and its regulation becomes an important part to study. Previous studies in the lab were focussed towards finding the residues in the regulatory domain involved in SAM binding. Mutational studies on *S. cerevisiae* MTHFR- (MET13) regulatory domains were carried out focussing on the conserved regions CR1, CR2, CR3, CR4. From those studies, a mutation R357A in CR1 was found to be deregulated for MTHFR activity.

The multiple sequence alignment shows that R357 is a conserved residue in the CR1 region. This was mutated to alanine (Ala) and then transformed in WT *S. cerevisiae* strain. The growth based spotting assay on methionine showed no growth that is methionine toxicity even in the presence of WT copy of MET13 gene. It infers that MET13_R357A is a dominant mutation. The activity of the WT protein decreases in the presence of SAM whereas the activity of this mutant was checked and found out to be unresponsive to SAM. This data suggests that this MET13_R357A is a dominant deregulated mutant (Unpublished data, Muskan Bhatia).

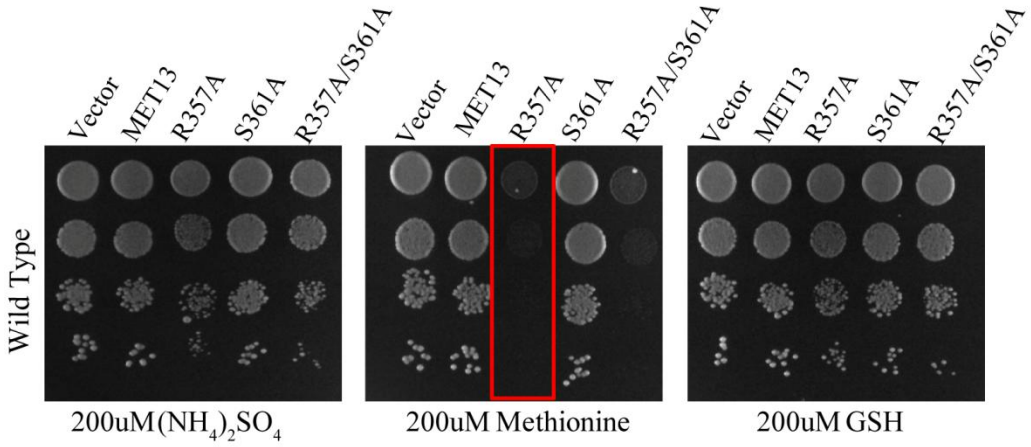

Fig. 1.6 Functional characterization of MET13 and its R357A, S362A mutants in *S. cerevisiae* regulatory domain. MET13 and its alanine mutants in the regulatory domain expressed under TEF promoter, and corresponding vector (p416TEF) were transformed into WT ABC733 strain and analyzed by Growth based spotting assay. Transformants were grown in minimal medium, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.002, 0.002, 0.0002 OD_{600} of cells. 10uL of these dilutions were spotted on various sulphur sources. The photograph was taken after 2 days of incubation at 30°C.

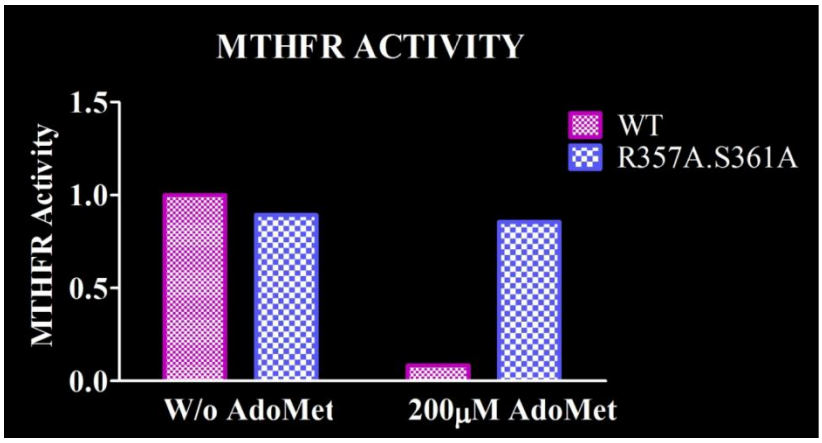

Fig. 1.7 Quantification of the activity of MET13 and its R357A mutant in the regulatory domain. The MET13 WT protein and its R357A mutant was expressed under *T7* promoter and transformed in *E. coli* , BL21(DE3) bacterial strain. The transformants were grown at 37°C and then induced with IPTG and grown at 18°C for 16hrs overnight. The protein was purified and then the MTHFR activity assay was performed. The activity of the WT protein is normalized to 1 and compared with its R357A mutant.

Section B: Aim and objective of the study

Based on these previous findings in the lab, the current work on MTHFR was planned to examine if there is conservation of deregulation in mutant MTHFR phenotype in higher organisms such as *Homo sapiens* and *Danio rerio*. The specific objectives were:

I) Clone the *Danio rerio* MTHFR, create and functionally evaluate deregulated mutant in *Danio rerio* MTHFR

II) Evaluate functionality of *Homo sapiens* isoforms of MTHFR (WT and mutant) on various sulphur sources

III) Create S-adenosyl methionine (SAM) sensors by MTHFR regulatory domain insertions into GFP

Chapter2

Materials and methods

Section A: Materials

2.1. Chemicals and reagents

All the chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma Aldrich, (St. Louis, USA), HiMedia, (Mumbai, India), Merck, India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Snapgene software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, Vent DNA polymerase, Taq DNA polymerase and other modifying enzymes), their buffers and dNTPs were purchased from New England Biolabs Inc, (Beverly, MA, USA). Gel-extraction kits and plasmid miniprep columns were obtained from Bioneer. Methionine, GSH and cysteine were obtained from Sigma-Aldrich, USA.

2.2. Strains and plasmids

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* strain and the yeast strains used in the study are given in Table 1. The list of various plasmids used in this study is given in Table 2.

2.3 Oligonucleotides

The list of various oligonucleotides primers used in this study is given in Table 3.

Table 1: List of bacterial and yeast strains used in the study

Organism	Strain	Genotype	Source
E. coli(DH5α)	AB 460	*F' gyr A(Nal) recA1 relA endA1 thi-1 Hsd 17(rk-mk+) gln V44 deoR Δ(lacZYA-argF) U169 [φ80dΔ(lacZ) M15]*	Lab strain
S. cerevisiae	ABC 733	*BY4741,MATa, his3Δ-1,leu2-Δ0, met15Δ0, ura3Δ0*	Lab strain
S. cerevisiae	ABC 2613	*MATa,his3Δ-1,leu20,lysΔ0,met15Δ0, ura3Δ0,met13Δ*	Lab strain
S. cerevisiae	ABC 2614	*MATa,his3Δ-1,leu20,lysΔ0,ura3Δ0, met13Δ*	Lab strain

Table 2: List of Plasmids used in the study

Clone	**Plasmid name**	**Description**
ABE 443	p416TEF	The CEN- vector bearing URA3 marker and TEF promoter - MCS terminator for expression in yeast and Amp^r marker for selection in *E. coli* [12]
ABE 5584	p416TEF_hMTHFR70 His	hMTHFR 70 with C terminal 6X His tag cloned in p416TEF
ABE 5598	p416TEF_hMTHFR77 His	hMTHFR 77 with C terminal 6X His tag cloned in p416TEF
ABE 5601	p416TEF_hMTHFR77 R392A His	hMTHFR 77 with R392A mutation and C terminal 6X His tag cloned in p416TEF
ABE 5605	p416TEF_hMTHFR77 S396A His	hMTHFR 77 with S396A mutation and C terminal 6X His tag cloned in p416TEF
ABE 5684	p416TEF_GFP	GFP cloned in p416TEF
ABE 5889	p416TEF_GFP_RS	GFP with restriction sites HindIII and SalI between 172 and 173 sites cloned in p416TEF

ABE 5890	p416TEF_GFP_SBDI	*S. cerevisiae* MET13 regulatory domain(299-400) cloned in the restriction sites HindIII and SalI between 172 and 173 sites within GFP cloned in p416TEF
ABE 5891	p416TEF_GFP_SBDII	*S. cerevisiae* MET13 regulatory domain(299-600) cloned in the restriction sites HindIII and SalI between 172 and 173 sites within GFP cloned in p416TEF
ABE 5892	p416TEF_GFP_SBDIII	*Homo sapiens* MTHFR regulatory domain(338-656) cloned in the restriction sites HindIII and SalI between 172 and 173 sites within GFP cloned in p416TEF

Table 3: List of Oligonucleotides and their sequences used in this study

Oligo name	**Sequence(5'-3')**
*mthfr*_ClaI_F	GATCCCATCGATATGGTGAACCAAAGAGC
*mthfr*_XhoI_R	TAGAGGCTCGAGCTAGTGATGGTGATGGTGATGCAATTCATTTGCCTCC
*mthfr*_R388A_F	TTCCCTAACGGAGCATGGGGGAACTC
*mthfr*R388A_R	AGAGTTCCCCCATGCTCCGTTAGGGAAATCATCCC
*mthfr*_HindIII_ClaI_F	GGAATATCCAAGCTTCCATCGATGGATGGTGAACCAAAGAGC
*mthfr*XhoI_ClaI_R	ATTACATGAGTCGACCTCGAGCTAGTGGTGATGATGGTGATGC
Sc_GFP_BamHI_FP	ATCACGGATCCATGAAAGGAGAAGAACTTTTCACTG
Sc_GFP_XhoI_RP	ATCATGAATTCCTATTTGTATAGTTCATCCATG
EGFP(173)_F	AAGCTTATCGAGATCGAGGTCGACGACGGCAGCGTGCAGC
EGFP(173)_R	GTCGACCTCGATCTCGATAAGCTTCTCGATGTTGTGGCGG

MET13(299-400)_HindIII_F	ATCGAGAAGCTTATTCTACCTACGGAATCAG
MET13(299-400)_SalI_R	GCCGTCGTCGACCAAGAAGGCGACGTCGTTG
MET13(299-600)_HindIII_F	ATCGAGAAGCTTATTCTACCTACGGAATCAG
MET13(299-600)_SalI_R	GCCGTCGTCGACTAGGCTTAGTAGGATGG
MET13(338-656)_HindIII_F	ATCGAGAAGCTTATGTGGACTGAGGACCCC
MET13(338-656)_SalI_R	GCCGTCGTCGACTGGAGCCTCCGTTTCTCTCT CG

2.4 Media

All the media, buffers and stock solutions were prepared using Millipore Elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch2 (psi) pressures at 121degreeC for 15 minutes, or by using membrane filters (Advanced Micro-devices Pvt. Ltd., India) of pore size 0.22 μm (for heat labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100 μg/ml.

2.4.1. LB

Media	**Reagents**	**Weight**
2.4.1 LB (Lysogenic broth)	Yeast extract Tryptone Nacl	5g/l 10g/l 10g/l
2.4.2 YPD (Yeast extract peptone dextrose)	Yeast extract Peptone Dextrose	10g/l 20g/l 20g/l
2.4.3 SD (Synthetic defined)	YNB(yeast nitrogen base) Ammonium Sulphate(w/o amino acids) Dextrose Amino acids(as required) pH	1.7g/l 5g/l 20g/l 80mg/l 6-6.5

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1. Ampicillin Stock Solution (100 mg/ml)

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and it was filter-sterilized using 0.2 µm filter membrane. Finally it was stored at -20°C in aliquots in micro centrifuge tubes.

2.5.2. GSH Stock Solution (200 mM)

The required amount of glutathione (reduced form) was dissolved in 1 ml of deionized water and was filter-sterilized using 0.2 µm filter membrane. It was used for further dilutions and was stored at -20°C.

2.5.3. Methionine Stock Solution (200 mM)

The required amount of methionine was dissolved in 1 ml of deionized water and was filter-sterilized using 0.2 µm filter membrane. It was used for further dilutions and stored at 4°C.

2.5.4. Cysteine Stock Solution (200 mM)

Fresh stock of cysteine was prepared by dissolving required amount of cysteine in1 ml of deionized water and was filter sterilized using 0.2 µm filter membrane.

2.5.5. 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

2.5.6 Solutions for plasmid extraction

A)	Solution-I(Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4oC.
B)	Solution-II (Lysis Solution) (freshly prepared)	0.2 N NaOH (freshly diluted from a 10 N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
C)	Solution-III (Neutralization	5 M Potassium acetate 60 ml

	Solution)	Glacial acetic acid 11.5 ml Deionized water 28.5 ml The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. It was stored at 4oC.
D)	PCI (Phenol-chloroform-isoamyl alcohol) Solution (100ml)	Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] Chloroform 48 ml Isoamyl alcohol 2 ml Stored at 4°C in dark brown bottle.
E)	TE Buffer (Tris-EDTA) (pH 8.0)	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
F)	TE-RNAse (stock prepared at 10 mg/ml)	Working stock 20 µg/ml in TE Buffer, pH 8.0.

2.5.7. Agarose Gel Electrophoresis Reagents

A)	1× TAE (Tris-acetate-EDTA) Buffer (per 1000 ml)(prepared from 50× TAE stock)	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
B)	Orange-G dye (Gel loading dye, 6X)	0.25% orange-G and 30% glycerol
C)	0.7-1% Agarose gel in 1× TAE	
D)	Ethidium Bromide (10 mg/ml) Stock	Final working concentration used at 0.5 µg/ml.

2.5.8. Solutions for preparation of chemical competent *E. coli* cells [13]

A)	SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g

		Above mentioned components were dissolved in 950 ml of water. 10 ml of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 ml and autoclaved. Just before use, 5 ml of filter sterilized 2 M MgCl2 was added.
B)	SOC	SOB + 20 mM Glucose
C)	10% glycerol	10ml glycerol in total 100ml solution and then autoclaved

2.5.9. Yeast Transformation Solutions (*S. cerevisiae*) [14]

a) 0.1 M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

SECTION B: Methods

2.6 Growth and maintenance of bacteria and yeast strains

The *Escherichia coli* strains DH5α was routinely grown at LB medium at 37℃. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 30℃. The yeast transformants were selected and maintained on SD medium with supplements as per the requirement.

2.7 Recombinant DNA methodology (restriction digestion, ligation, transformation of *E. coli*, PCR amplification etc.

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols (Kaiser et al., 2000; Sambrook et al., 1989) or as per manufacturers' protocol, unless specifically mentioned.

2.8 Growth assay by dilution spotting

For growth assay, the different *S. cerevisiae* strains containing the desired plasmid were grown on the selection media overnight and then reinoculated at 0.2 OD_{600} in 5/10ml of same selection media. As these cells reach the exponential phase, the cells

were pellet down at 5000rpm for 3min. and the supernatant was discarded. The cells were washed twice with autoclaved water and finally re suspended in 1ml of autoclaved water. Then the OD of these cells was measured and final OD_{600} of 0.2 is made in 1ml water. Further serial dilutions from 0.2 to 0.02, and 0.002 were made by adding cells: water in 1:10, 1:100, 1:1000 ratios. After the dilutions are made the 10ul is added in the desired selection media plate. The plates were incubated at 30 ºC for two days and observed regularly and the photographs were taken.

2.9 GFP Assay:

2.9.1 Fluorimetry

The primary culture of the yeast transformants was put in the SD media and grown overnight at 30°C and then reinoculated in fresh SD media to an initial OD_{600} of 0.1, cells were allowed to grow at 30ºC for 6-7 hrs with shaking. After growing cells up to the exponential phase, cells were harvested at 4°C, followed by washing with cold water and re suspended in water to an OD_{600} of 1.0 and GFP fluorescence was measured in a spectrophotometer at Excitation:488nm and Emission: 510nm.

2.9.2 Confocal microscope

The primary culture of the yeast transformants was put in the SD media and grown overnight at 30°C and then reinoculated in fresh SD media to an initial OD_{600} of 0.1, cells were allowed to grow at 30ºC for 6-7 hrs with shaking. After growing cells up to the exponential phase, the cells were harvested and washed with autoclaved water. The final OD of cells used was OD_{600} = 1. 10ul of these cells were taken with the help of a pipette and put on the slide. All images were acquired at 25 °C using cells mounted on a glass slide layered with a thin film of 1% agarose and a TCS Leica SP8 Confocal Microscope with 63X with immersion oil, 1.4NA objective (UV diode LASER light source). The images setup was optimized for minimal light exposure to yeast cells.

2.10 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method. *S. cerevisiae* cultures were grown in YPD at 30℃ with shaking for 16 hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.2, cells were allowed to grow at 30℃ for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with autoclaved water followed by subsequent wash with 0.1 M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution after two washes with lithium acetate-TE solution. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of 1 x 10^9 cells/ml and divided into 100 µl aliquots. Approximately 50 g (5 µl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA (ssDNA), followed by 0.3g- 0.7g of plasmid/DNA fragment were added to each aliquot. 0.3 ml of 50% PEG3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and all the components were mixed well using a vortex. The whole cell suspension was subjected to heat shock at 42℃ for 30 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7000 rpm for 3 min. The cell pellet was re-suspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

Chapter 3
Results and discussions

3.1 Attempts to clone *Danio rerio mthfr*

A previous study on regulatory domain of *S. cerevisiae* MET13 shows that MET13_R357A is a deregulated mutant that shows toxicity/ growth defects on the methionine medium. The MET13_R357 well aligns with *mthfr*_R392 and because of the high conservation in the regulatory domains of MTHFR, R392A was assumed to show the same deregulated phenotype as yeast. To examine the *in vivo* effects of such a deregulated mutant in multicellular system, we tried to clone the *Danio rerio mthfr* and the R392A in yeast vector p416TEF and zebra fish vector pCS2 (+).

The cDNA of *Danio rerio mthfr* was obtained from Dr. Rajesh Ramachandran lab. The cDNA obtained was amplified by PCR and cloned into XhoI and ClaI sites of yeast expression vector p416TEF. Ligated plasmids were transformed in DH5α bacterial strain. The positive clones were screened and confirmed by restriction digestion. Later the sequencing results showed a stop codon mutation towards the N terminal. Further multiple attempts were made to clone the *mthfr* but all the attempts were futile.

Fig. 3.1 The sequence alignment of *mthfr* and MET13 regulatory domains using CLUSTAL OMEGA show that MET13_R357 aligns with *mthfr* _R392 in the CR1.

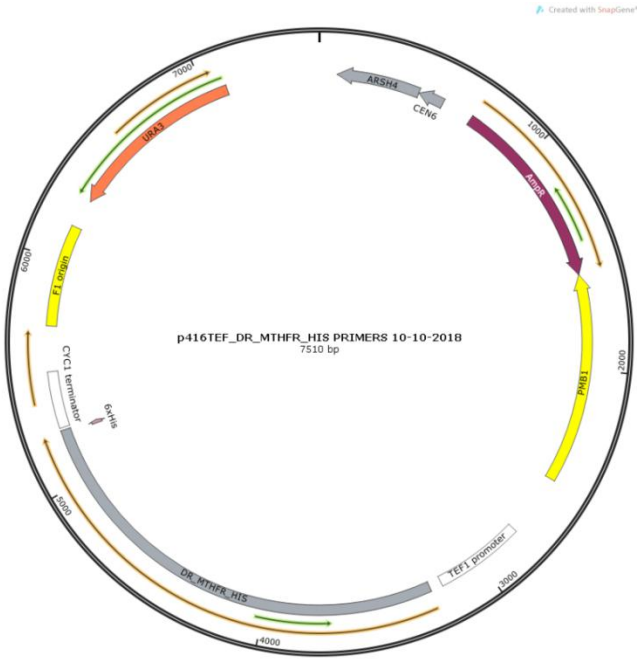

Fig.3.2 Clone map *mthfr*_His: The software Snapgene was used to create the clone map of *mthfr* cloned in yeast expression vector p416TEF. The *mthfr* was PCR amplified and cloned between the XhoI and ClaI restriction sites.

3.2 To check the functionality of *Homo sapiens* isoforms: Complementation assay

The *Homo sapiens* MTHFR isoform 70(hMTHFR 70) and *Homo sapiens* MTHFR isoform 77(hMTHFR 77) clones with 6X His tags were already cloned in the lab. (Credits: Varsha Jayaswal) These clones were freshly transformed into *met13Δ* and *met13Δmet15Δ* of *S. cerevisiae* strain background. *S cerevisiae* MET13 single deletion is a methionine auxotroph where MET13 encodes for *S. cerevisiae* MTHFR whereas MET15 deletion strain is an inorganic sulphur auxotroph where MET15 encodes for homocysteine synthase. So the double deletion background requires higher levels of sulphur source to grow well. The plasmids containing vector control, WT MET13, hMTHFR70, hMTHFR 77 were transformed in *met13Δ* and *met13Δmet15Δ* strain. Then the growth based spotting assay was done on methionine, homocysteine, ammonium sulphate. The hMTHFR77_HIS complements the MET13 gene in *met13Δ* strain whereas hMTHFR70_HIS was unable to complement on ammonium sulphate and Homocysteine. So the further experiments were done using hMTHFR 77kDa.

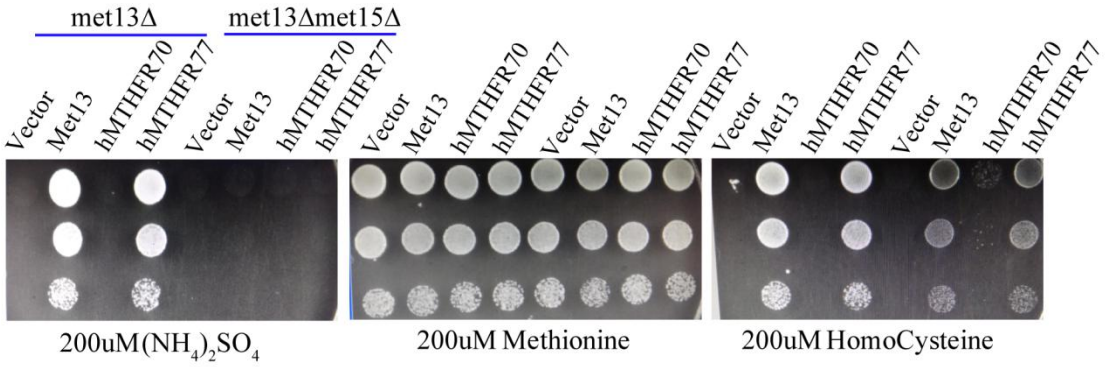

Fig. 3.2 Functional characterisation of hMTHFR 70 and 77 isoforms: MET13, hMTHFR 70 and hMTHFR 77 expressed under TEF promoter, and corresponding vector (p416TEF) were transformed into *met13Δ* and *met13Δmet15Δ* strain and analyzed by Growth based spotting assay. Transformants were grown in minimal medium with methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.002, 0.002, 0.0002 OD_{600} of cells. 10uL of these dilutions were spotted on various sulphur sources. The photograph was taken after 2 days of incubation at 30℃.

3.3 hMTHFR77_R392A does not show a phenotype similar to *S.cerevisiae* MTHFR_R357A

A previous study on regulatory domain of *S. cerevisiae* MET13 shows that MET13_R357A is a deregulated mutant that shows toxicity/ growth defects on the methionine medium. The MET13_R357 well aligns with hMTHFR_R392 and because of the high conservation in the regulatory domains of MTHFR, R392 was assumed to show same deregulated phenotype. The clones of hMTHFR were already cloned in the lab (Credits: Varsha Jaiswal). These clones were transformed in the *S cerevisiae met13Δ* strain background. The growth based spotting assay of the transformants was done on various sulphur sources. We observe that *Homo sapiens* MTHFR77_R392A, S398A does not show any growth defect on methionine plates indicating that these residues are most likely not involved in SAM binding in case of *Homo sapien*s.

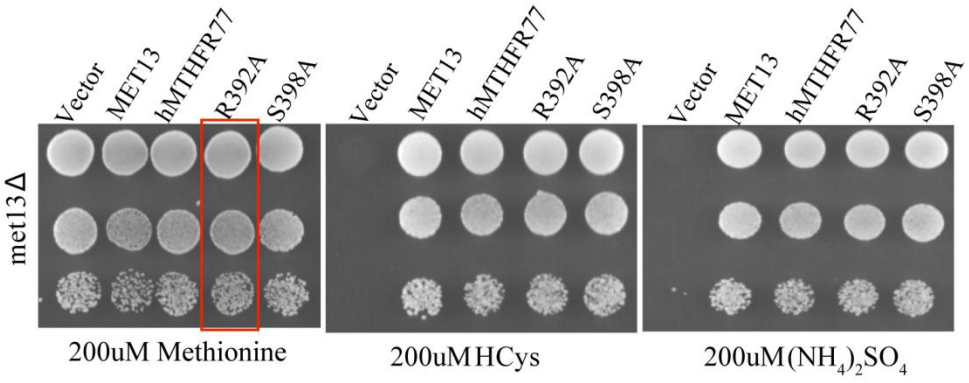

Fig. 3.3 Functional characterisation of hMTHFR 77 and its R392A, S398A mutant: MET13, hMTHFR 77 and R392A, S398A mutant expressed under TEF promoter, and corresponding vector (p416TEF) were transformed into *met13Δ* strain and analyzed by Growth based spotting assay. Transformants were grown in minimal medium with methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.002, and 0.002, OD_{600} of cells. 10ul of these dilutions were spotted on various sulphur sources. The photograph was taken after 2 days of incubation at 30°C.

3.4 hMTHFR 77 complements *met13Δmet15Δ* when grown on homocysteine but not on GSH and Cysteine

Glutathione is an organic sulphur source which is known to be taken up by the yeast cells by GSH transporter. The sulphur metabolism in *S. cerevisiae* involves the conversion of glutathione then cysteine and finally to homocysteine. In order to check the efficiency of hMTHFR77 in the presence of glutathione or cysteine, WT hMTHFR77, R392A and S398A mutant were transformed in *S. cerevisiae met13Δ* and *met13Δmet15Δ* double deletion strain and then growth based spotting assay was done. We observe that on Glutathione the WT MET13 and hMTHFR77 transformed strain grows well on *met13Δ* and *met13Δmet15Δ* strain, whereas the hMTHFR 77 transformed strain does not grow well in cysteine in *met13Δ*. In case of double deletion strain background, hMTHFR 77 does not grow well on either GSH or cysteine.

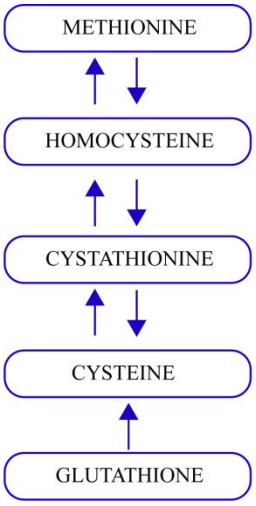

Fig. 3.3 The transsulphuration pathway of sulphur metabolism in *S. cerevisiae*: GSH is an organic sulphur source which is utilized by *S. cerevisiae* and converted to Homocysteine (HCys) and to methionine with intermediates formation such as cysteine and cystathionine by this transsulphuration pathway.

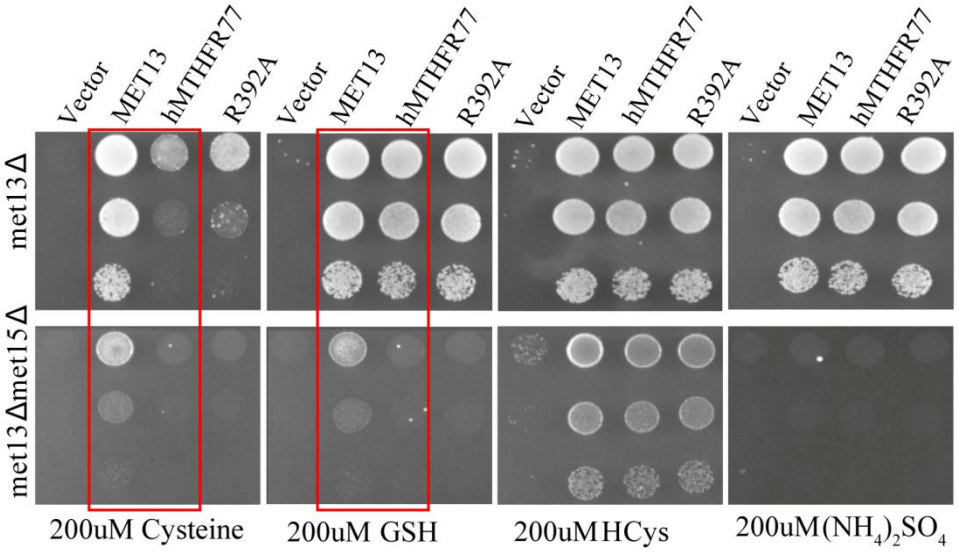

Fig. 3.4 Functional characterisation of hMTHFR 77 and its R392A mutant: MET13, hMTHFR 77 and its R392A mutant expressed under TEF promoter, and corresponding vector (p416TEF) were transformed into *met13Δ* and *met13Δmet15Δ* strain and analyzed by Growth based spotting assay. Transformants were grown in minimal medium with methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.002, and 0.002, OD_{600} of cells. 10ul of these dilutions were spotted on various sulphur sources. The photograph was taken after 2 days of incubation at 30℃.

3.5 Complementation of hMTHFR: effects of Homocysteine concentration

In order to explain the differential growth of MET13 and hMTHFR 77 on *met13Δ met15Δ* double deletion background we tried to see the effect of different concentrations of Homocysteine (2uM-500uM) on growth. We found that on lower concentrations of Homocysteine there is no growth even in MET13 transformed *S. cerevisiae* strain, as the concentration of Homocysteine increases both MET13 and hMTHFR77 shows growth which suggests that methionine formation in transsulphuration pathway is insufficient when complementation by *Homo sapiens* MTHFR is there, not efficient in transfer of methyl group to convert homocysteine to methionine and that is why we did not see the growth on GSH plate. The activity of MTHFR is independent of the concentration of Homocysteine in *met13Δ* strain but in *met13Δmet15Δ* strain, the conc. of Homocysteine is an important determining factor for growth.

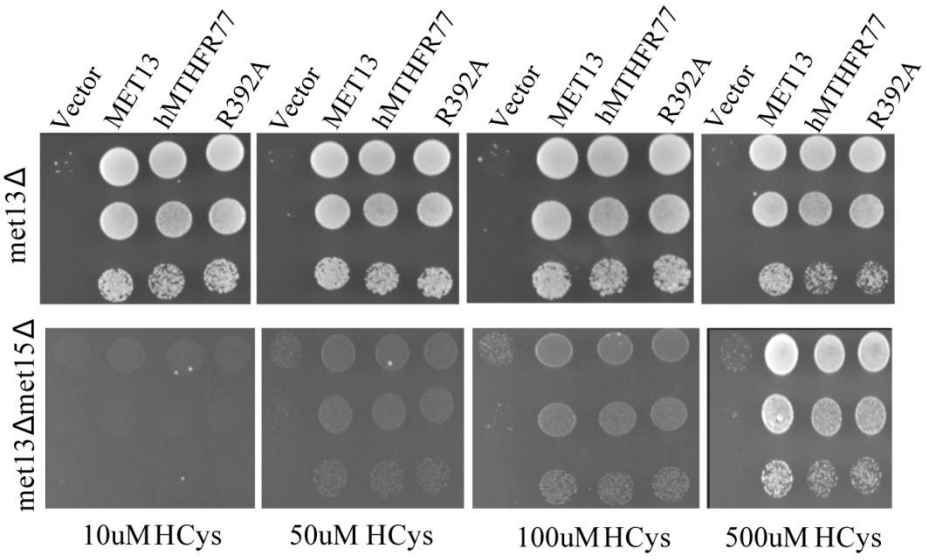

Fig. 3.5 Functional characterisation of hMTHFR77 on different conc. of Homocysteine: MET13, hMTHFR77 and its R392A mutant expressed under TEF promoter, and corresponding vector (p416TEF) were transformed into *met13Δ* and *met13Δmet15Δ* strain and analyzed by Growth based spotting assay. Transformants were grown in minimal medium with methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.002, and 0.002, OD_{600} of cells. 10ul of these dilutions were spotted on various sulphur sources. The photograph was taken after 2 days of incubation at 30°C.

Chapter 4

Genetically encoded GFP based SAM sensor

4.1 Attempts to create a SAM sensor by MTHFR regulatory domain insertions into GFP

The first step in order to create a fluorescence based SAM sensor was to identify the regions in the GFP where insertions of wide variety of sequences would not disturb the structure and function of GFP entirely. In a previous study, the insertions of 20 amino acids (peptide aptamers) at various places in GFP resulted in different amounts of fluorescence intensity with respect to the WT GFP protein [15]. The constructs containing insertions in the solvent loop of the GFP protein shows highest fluorescence intensity after insertion. It was concluded that the most tolerable insertion is between the residues 172 and 173 of the solvent interacting loops [15]. The insertion of molecular recognition domain in between these residues will lead to the formation of a GFP based sensor of that particular ligand [16]. The underlying principle involved is that the insertion of the molecular recognition domain will disrupt the fluorescence ability of GFP whereas in the presence of the ligand, the ligand will bind to the molecular recognition domain and this binding will lead to such conformation changes such that the GFP attains the ability to fluorescence again in the presence of the ligand [16]. Based on these established facts we tried to make SAM sensor based on fluorescence. The *S. cerevisiae* and *Homo sapiens* MTHFR regulatory domains are known to be negatively regulated by binding to SAM. So the aim was to insert the regulatory domains (SBD) of various lengths from different organisms in the GFP to make a SAM sensor. The first half of the GFP (1-172) containing HindIII and SalI restriction sites was PCR amplified and then the other half of GFP (173-238) containing the SalI and HindIII sites was amplified. Both of these were digested with HindIII enzyme and then third PCR was setup. The amplified PCR product was digested with BamHI and EcoRI restriction enzymes and simultaneously the yeast expression vector p416TEF was digested with same set of enzymes. The ligation was setup and transformed in DH5 bacterial strain. The resulting plasmid contains the linker insertions of HindIII and SalI restriction sites in between the 172 and 173 residues of GFP named as p416TEF_GFP_RS.

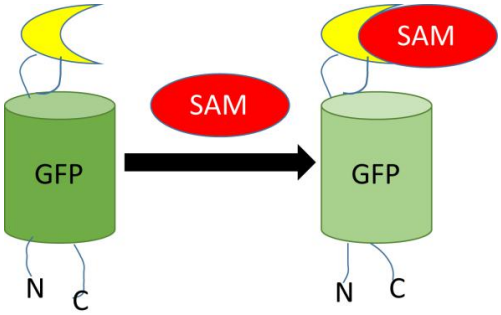

Fig. 4.1 Schematic representation of working mechanism of GFP based sensor: The insertion of a binding domain in frame with the GFP can be used to make flourescent protein based sensors. Yellow color represents the molecular recognition domain, green color is GFP whereas red color represents molecule, in this case SAM.

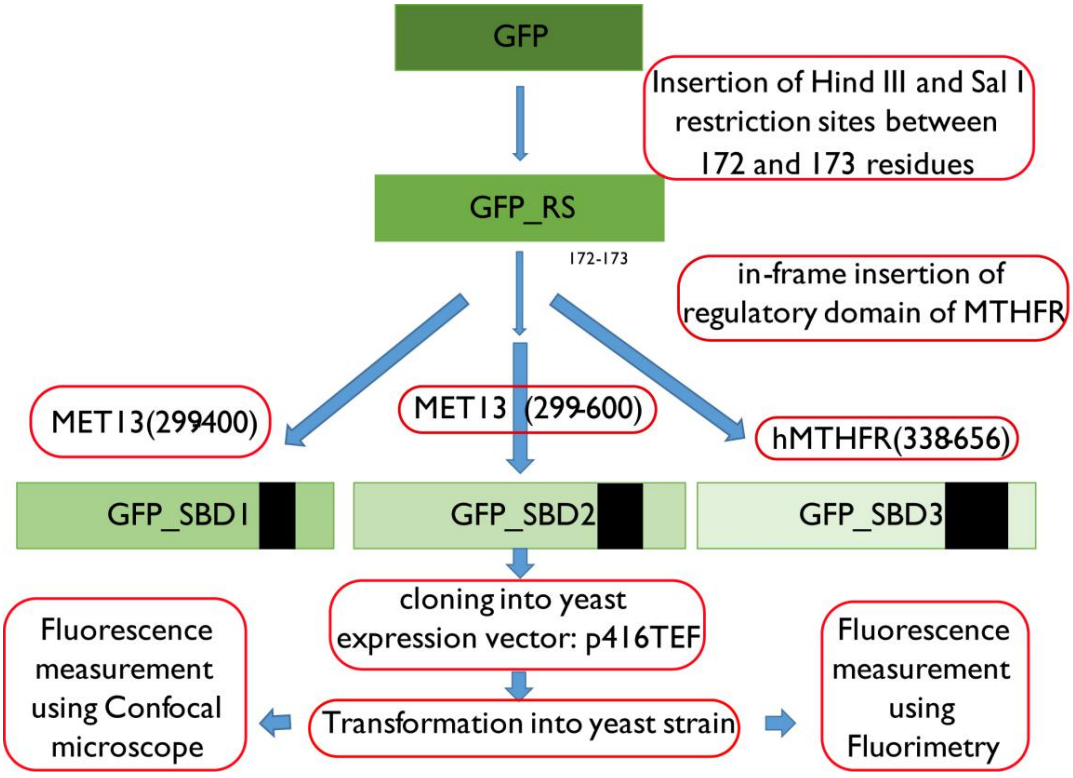

Fig. 4.2 The flow chart represents various steps involved in construction of a genetically encoded fluorescent protein based SAM sensor: The insertion of restriction site in between 172-173 residues of GFP. Further in frame insertions of SAM binding domains (SBD) in between these restriction sites. These constructs were made under TEF promoter of p416TEF vector and expressed in WT ABC733 yeast strain. The fluorescence measurement was done using single point measurements in Fluorimetry and confocal microscope.

4.1.1 Insertion of the molecular Recognition domain

To carry out insertions of putative SAM binding domains (SBD) of MTHFR in GFP at position 173-173, we chose various lengths of regulatory domain from both yeast and human. The regulatory domain of lengths of SBDI-300bp (299-400), SBDII-900bp (299-600) from *S. cerevisiae* and SBDIII-1000bp (338-656) from *Homo sapiens* MTHFR were PCR amplified. The SBDs and GFP_RS constructs were digested with HindIII and SalI restriction enzymes and ligation reaction was at 25°C for 2.5hrs. The clones obtained after transformation in *E. coli* were screened and the positive clones were confirmed by restriction digestion. The resulting plasmids contained the desired SAM binding domain insertions in the restrictions sites between the GFP.

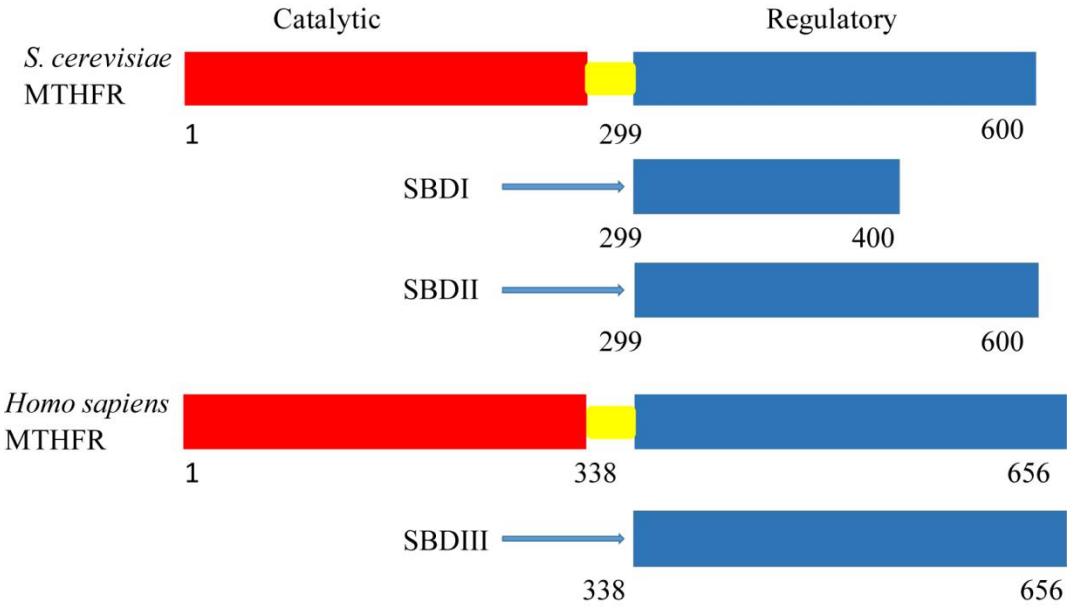

Fig.4.3 Schematic representation of MTHFR domains of *S. cerevisiae* and *Homo sapiens*: it shows the SAM binding domains from the regulatory domains, to be inserted in the GFP. SBDI, SBDII is a segment of *S. cerevisiae* MTHFR regulatory domain where as SBDIII is a part of *Homo sapiens* MTHFR regulatory domain.

(A) (B)

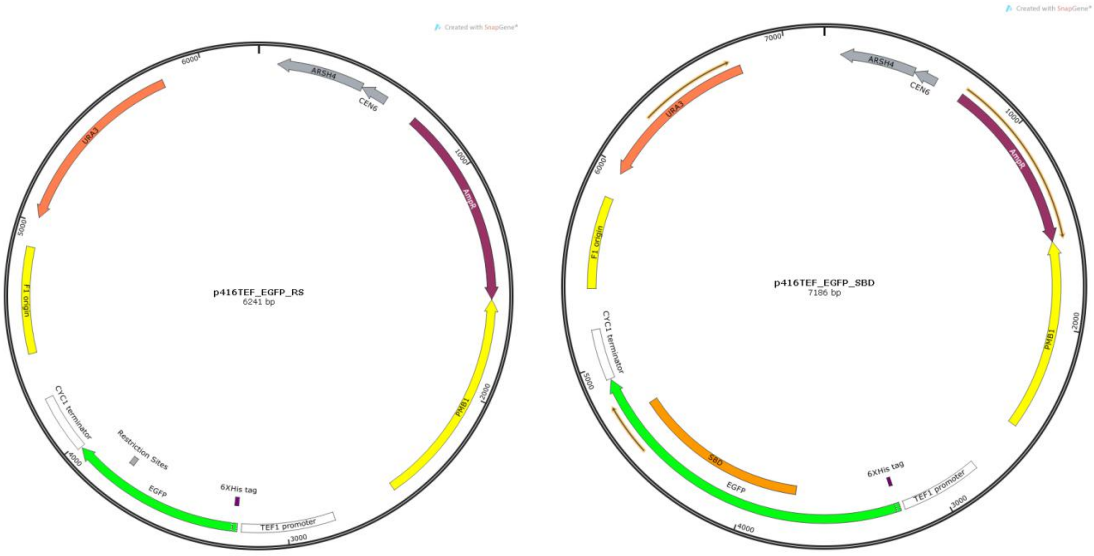

Fig. 4.4 (A) Clone map of p416TEF_GFP_RS (B) Clone map of p416TEF_GFP_SBD: The software Snapgene was used to create these clone map. The GFP with restrictions sites was made with splice overlap extension PCR and cloned in yeast expression vector p416TEF. After the addition of these restriction sites in between the GFP the SAM binding domains of various lengths were added.

4.2 Effects of insertion of regulatory domain of various lengths on GFP fluorescence

The insertion of such large domains might alter the folding of GFP thus interfering with the fluorescence. In order to check the activity of these constructs in terms of its fluorescence ability, fluorescence measurement was done. The plasmids containing these GFP_SBD constructs were transformed in the ABC733 WT strain of *S. cerevisiae*. The transformants were cultured in the minimal media with methionine. The secondary culture was put in 0.2 OD_{600} in the same selection media. The final OD_{600} of cells used for the fluorescence measurement was 1 OD_{600} cells. The fluorescence intensity was measured using Fluorimeter instrument as well as visually confirmed using confocal microscope. The quantitative and qualitative fluorescence measurement using Fluorimetry and confocal microscope suggests that with increase in the length of SBD inserted there is decrease in the amount of fluorescence.

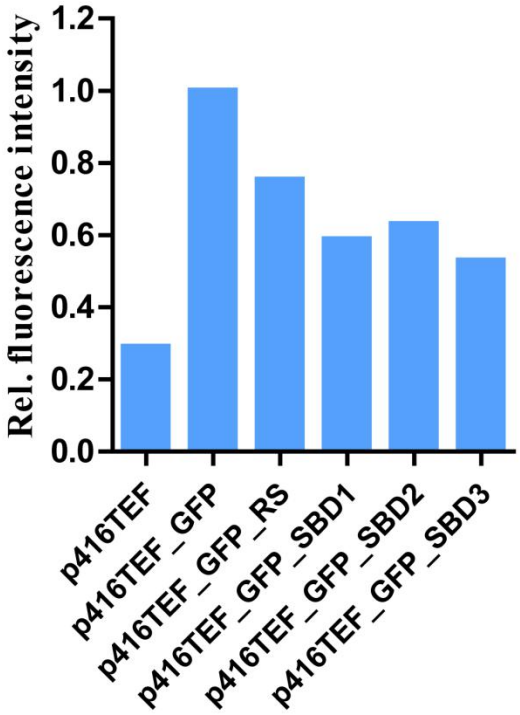

Fig.. 4.5 Fluorescence measurement using Fluorimetry: The fluorescence of intact GFP has been normalized to 1 and the intensity of all the constructs is calculated relatively to that, thus relative fluorescence intensity. The graph shows the relative fluorescence intensity of various constructs with respect to intact GFP fluorescence intensity.

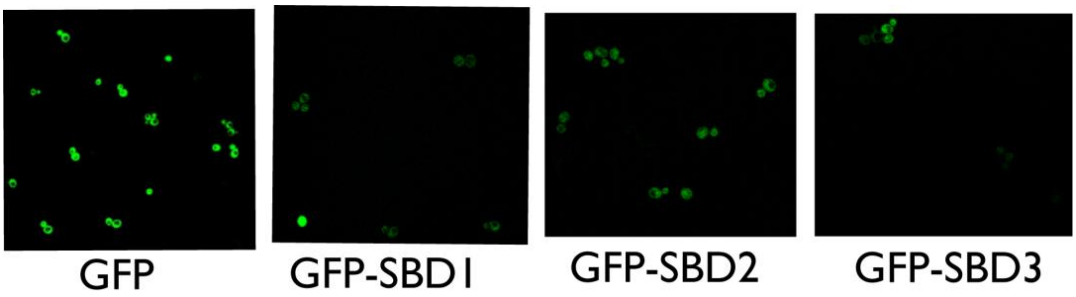

Fig. 4.6 Fluorescence measurement using confocal microscope: the confocal microscope images show the fluorescence intensity of various constructs (A) GFP (B) GFP_SBDI (C) GFP_SBDII (D) GFP_SBDIII.

Above data from confocal imaging and fluorescence measurement from Fluorimetry are indicative of decrease in fluorescence on addition of various SAM binding domains.

4.3 Response of MTHFR regulatory domain insertion constructs to SAM

After establishing the fact that the insertion of SBDs is indeed leading to the decrease in the relative fluorescence intensity but the total disruption of the protein function is not there as fluorescence is still observed after the insertions under the confocal microscope. Our next step was to check if these constructs are responsive to SAM. The GFP_ SBD transformants were grown in minimal media with methionine. The secondary culture was put in the same media in the presence of 1uM SAM and grown. The final OD_{600} of cells used was 1. The relative fluorescence intensity was measured using Fluorimeter and visualization using confocal microscope was done.

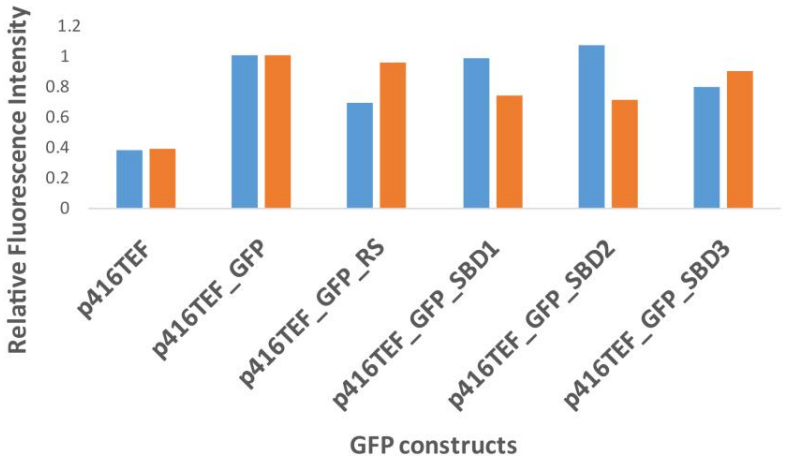

Fig. 4.7 Qualitative measurement of fluorescence in response to SAM: blue color bar represents the constructs grown in media without SAM and orange color bar represents the constructs grown in the presence of 1uM SAM.

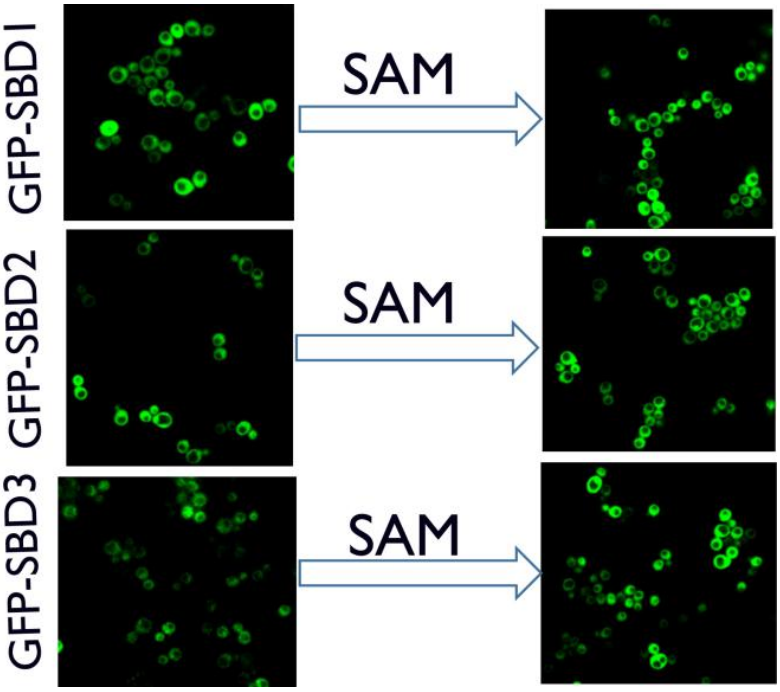

Fig. 4.8 Quantitative measurement of fluorescence in response to SAM: The confocal images shows fluorescence intensity in absence and presence of 1uM SAM.

Based on the above preliminary fluorescence measurement, we can say that the all these constructs appear to respond to SAM, since we observe either decrease or increase in fluorescence intensity in the presence of SAM. However, we are not sure if it is indeed because of SAM. Thus the validation of this preliminary SAM sensing capability and its subsequent improvement needs to be done by the experimental approach.

Discussion & Conclusion

The main findings of this thesis are that hMTHFR 70 isoform is unable to complement the MET13 gene in *met13Δ* strain whereas the hMTHFR 77 is able to complement the yeast mutant. The exact reason for these differences is not known. The hMTHFR77_R392A does not show any deregulation phenotype similar to its *S. cerevisiae* counterpart which indicates that in case of *Homo sapiens* this residue is not that important in SAM binding as it is in the case of *S. cerevisiae.* The differential growth of MET13 and hMTHFR 77 on supplementation of GSH and cysteine is an interesting result that we obtained, for which we currently have no explanation and further experiments needs to be done to explain this phenotype.

Attempts were made to design a genetically encoded SAM sensor (GFP-SBDn) using the regulatory domain insertions of MTHFR from *Homo sapiens* and *S. cerevisiae.* For this we made insertions of different SBD regions in GFP at a position that GFP is known to most tolerate insertion. Experimental evidences indicate that GFP-SBDn (hMTHFR_338-656) may have the potential to function as a putative SAM sensor however further validation is needed.

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