# The use of the Yeast Two Hybrid system to detect direct binding partners of Hs-SDE2

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## Certificate of Examination

This is to certify that the dissertation titled **“The use of the Yeast Two Hybrid system to detect direct binding partners of Hs-SDE2”** submitted by **Ms. Sukriti** (Reg. No. MS10059) 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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| | | (Supervisor) |

Dated : 24 April 2015

## Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Shravan Kumar Mishra 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.

Sukriti
(Candidate)

Dated : 24 April 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.

Dr. Shravan Kumar Mishra
(Supervisor)

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# ABSTRACT

SDE2 [silencing defective 2] protein in the fission yeast *Schizosaccharomyces pombe* emerges to be conserved among various eukaryotic organisms, and is weakly homologous to ubiquitin family proteins (see below). The absence of SDE2 in *S. pombe* causes defective silencing at the telomeres. SDE2 mutant phenotypes also indicate that the protein could also contribute to the preservation of genome integrity by participating to respond and repair DNA double-strand breaks. Biochemically the protein also co-purifies with a wide range of splicing factors, suggesting that it is associated with RNA splicing. Ongoing studies in SK Mishra's lab suggest that SDE2 is a Ubl and seems to be associated with RNA splicing.

The covalent attachment of ubiquitin-like proteins (Ubls) confers diverse functions on their target proteins. The modified substrates might be involved in various biological processes, which include DNA replication, signal transduction, cell cycle control, embryogenesis, cytoskeletal regulation, metabolism, stress response, homeostasis and RNA processing. The splicing reaction that assembles exonic (protein-coding) regions in eukaryotic mRNAs from their much longer precursors provides a uniquely flexible means of genetic regulation. Thus, resolving mechanism of RNA splicing at the molecular level is not only important for understanding gene expression, but it is also of medical importance, as aberrant pre-mRNA splicing is the basis of many human diseases or contributes to their severity.

The identification of protein-protein interactions (PPIs) is essential for a better understanding of biological processes, pathways and functions. The aim of my thesis was to detect interactions between *Homo sapiens* (Hs) SDE2 and other unknown proteins using yeast two hybrid system using HIS3 reporter gene so that function of Hs-SDE2 in eukaryotes can be deciphered. Yeast two hybrid assay was performed using the cDNA library screen approach. Two separate screens were performed for Hs-SDE2 full length protein and the ubiquitin fold region containing N-terminus of Hs-SDE2 protein. Each part of SDE2 sequence was fused into the pGBKT7 vector, generating bait clones. The expression of vector constructs were verified through sequencing, and the constructs were transformed into Y2HGold yeast strain. The high-complexity cDNA libraries made from 3 different tissues or cells, expressed in yeast strain Y187 were used for the screening. The binding partners of SDE2 reported in this study belong to various pathways like

signaling and growth regulation. Thus results shown in this thesis indicate that SDE2 might have additional functions other than RNA splicing.

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# Chapter 1

# Introduction

## 1.1 Biological background

### 1.1.1 Ubiquitin and Ubiquitin-like proteins (Ubls)

Ubiquitin is a highly conserved 76 amino acid protein consistently present throughout all eukaryotes. It is a typical example of a protein that modifies other proteins. Via a complex enzymatic cascade, ubiquitin becomes attached to a multitude of targets through an isopeptide bond between its C-terminal glycine residue and one or more accessible lysines in its targets. Ubiquitin consists of two α-helices and five ß-sheets in a ßßαßßαß arrangement. This structural fold (known as the ubiquitin globular ß-grasp fold) is conserved in other proteins with no obvious sequence identity [2]. These proteins are known as ubiquitin-like (Ubl) modifiers. Although Ubls do not share high sequence similarity with ubiquitin, all members of this superfamily are characterized by the presence of this same three-dimensional structure ß-grasp fold. These Ubls function as critical regulators of many cellular processes including transcription, DNA repair, signal transduction, autophagy, and cell-cycle control [1].

### 1.1.2 The RNA splicing process

Splicing is carried out by the spliceosome, a large macromolecular complex that assembles onto RNA sequences and catalyzes the two transesterification steps of the splicing reaction. The excision of the introns from a pre-mRNA and the joining of the exons are directed by special sequences at the intron/exon junctions called splice sites. The 5'splice site marks the exon/intron junction at the 5'end of the intron (Figure 1.1). This includes a GU dinucleotide at the intron end encompassed within a larger, less conserved consensus sequence. At the other end of the intron, the 3'splice site region has three conserved sequence elements: the branch point, followed by a polypyrimidine tract, followed by a terminal AG at the extreme 3' end of the intron [3].

In the first step, the 2'-hydroxyl group of a special A residue at the branch point attacks the phosphate at the 5'splice site. This leads to cleavage of the 5'exon from the intron and the ligation of the intron 5'end to the branch-point 2'hydroxyl. This step produces two reaction intermediates, a detached 5'exon and an intron/3'-exon fragment in a lariat configuration containing a branched A nucleotide at the branch point [3]. The second transesterification step is the attack on the phosphate at the 3' end of the intron by the 3'-hydroxyl of the detached exon. This ligates the two exons and releases the intron, still in the form of a lariat (Figure 1.1).

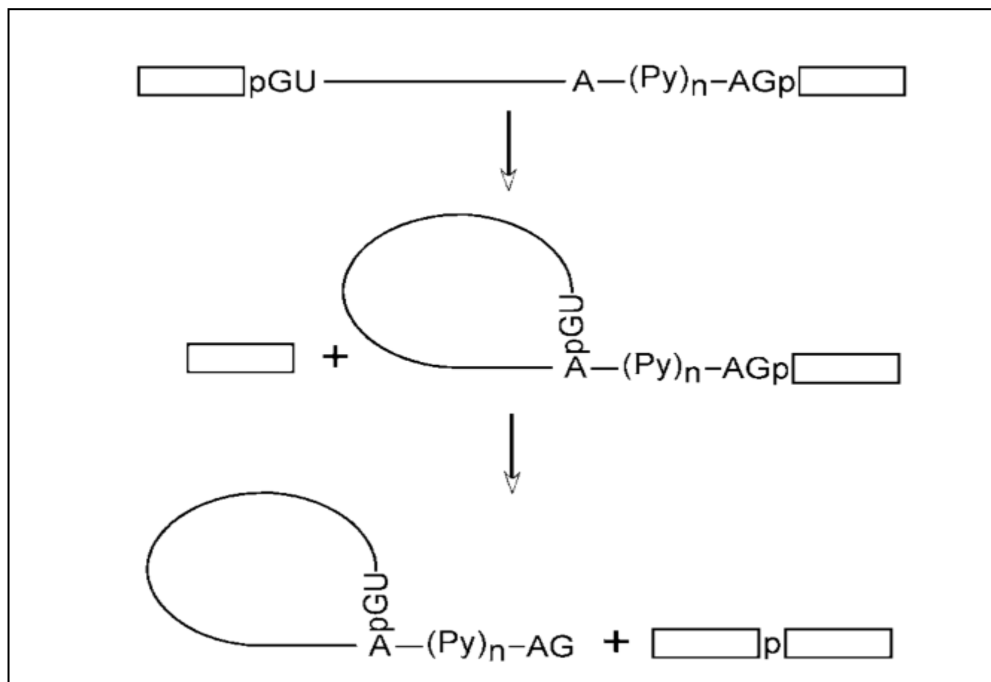


**Figure - 1.1 -Two transesterification steps in splicing reaction [3].**

The first step in splicing is typically the ATP-independent recognition of the 5' splice site by the U1 snRNA and the association of the U1 snRNP with this region, which results in the formation of the commitment (E) complex. Proteins [branch point bridging protein (BBP) and Mud2] bind to the branch site in E (early) complex. In an ATP-dependent reaction, U2 displaces BBP/Mud2 and binds to the branch site in A complex. A key role of the U1 snRNP is to promote the association of the U2 snRNP with the branch point region of the intron. The association of the U2 snRNP with the branch point region is an ATP- dependent process in which at least six proteins, components of the essential splicing factors SF3 complex, bind to the branch point region. The association of both the U1 and U2 snRNPs defines complex A (the pre-spliceosome).

Association of the tri-snRNP complex containing the U4, U5 and U6 snRNPs with the pre-spliceosome is required to form the B complex. Subsequent to assembly of B complex, catalytic activation requires several additional rearrangements. These include departure of U1 and U4 to form B act complex, formation of catalytic structures between the pre-mRNA, U2, and U6 snRNAs and destabilization of several U2 snRNP proteins (the SF3 complex) from the rest of the machinery to form B* complex. Tri-SnRNP addition initiates a series of RNA-RNA arrangements, most notably the displacement of

U1 snRNA from the 5’ splice site by U6 snRNA, which create the catalytically active component C complex [fig.1.2]. It is the C complex that catalyzes the two chemical steps of splicing. C1 complex is formed after 5' SS cleavage. For exon ligation, the spliceosome undergoes a conformational change into C2 complex. After the two chemical steps of splicing are complete, the spliceosome enters a disassembly and recycling pathway in which the spliced exons are released and the post-spliceosomal intron product complex (I) is disrupted [5].

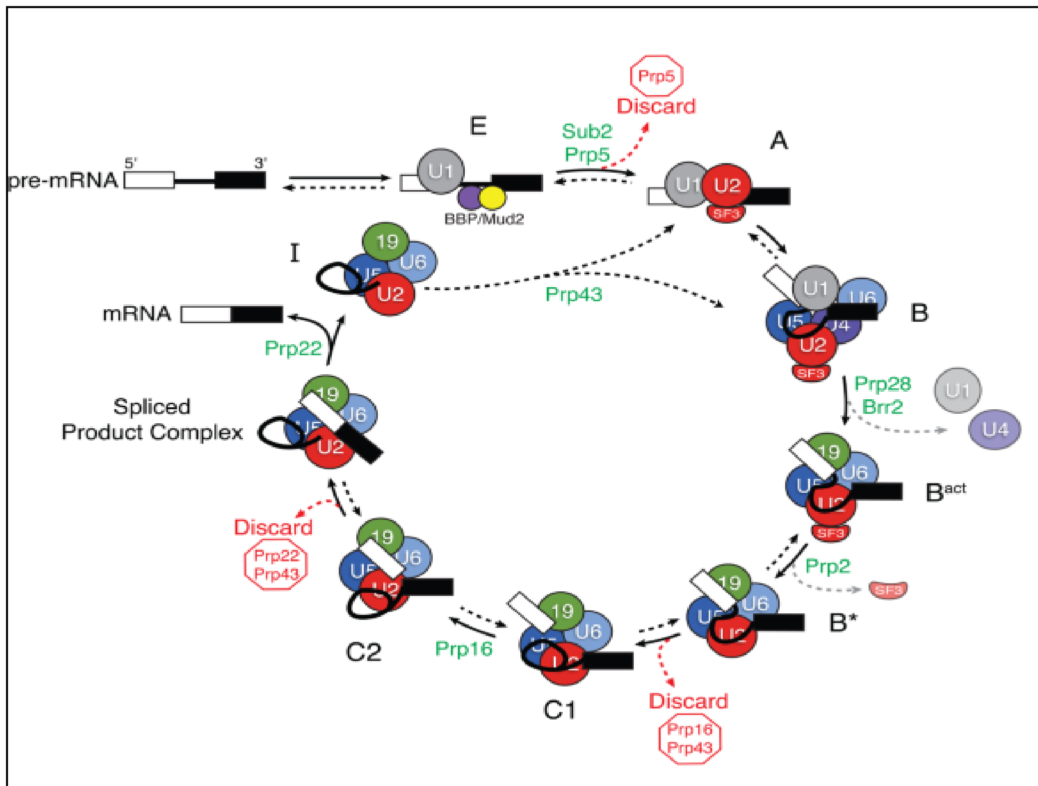


**Figure - 1.2 - A model for step-wise spliceosome assembly and catalysis [5].**

### 1.1.2 Structural and functional properties of SDE2 protein

SDE2 [silencing defective 2] or gene SPAC31G5.18C of Schizosaccharomyces pombe encodes a 263 amino acid protein called SDE2. BLAST search indicates that SDE2 is homologous to recognized proteins in various eukaryotic species including human, mouse, rat, chicken, zebrafish, fly, worm, and plants, which suggests that it is conserved to some extent in eukaryotes. Alignment of the amino acid sequences of SDE2 and its homologs has disclosed that the N- and the C-terminal regions of SDE2 are partially conserved [4]. A homology search of the C-terminal domain of human SDE2 shows that a similar domain is present in human splicing factor SF3a60, which functions in splicing during the formation of pre-splicing complex A [6] implying that the C-terminal region also has some role in process of splicing. g. Even though SDE2 lacks previously characterized domains by a conserved domain search, it shows weak homology to ubiquitin-related proteins [4].

Earlier studies have shown that SDE2 plays an important role in telomeric and centromeric silencing. It has also been suggested that SDE2 is also required for the maintenance of chromosome integrity and could contribute to the preservation of genome integrity by participating in the response to and repair of DNA double-strand breaks. [4] More recent studies have made known the fact that SDE2 physically associates with splicing factors and the deletion of SDE2 results in accumulation of un-spliced transcripts. These observations strongly implicate SDE2 in splicing [7].

### 1.1.4 Research objective

It is still not known whether Sde2 is also a bonafide splicing factor and if so what the mechanism by which it regulates splicing is. Homology of SDE2 to ubiquitin related proteins raises the possibility that it has some important role. Further studies are needed to resolve what kind of proteins SDE2 interacts with or covalently attaches to, and what conserved role SDE2 plays in higher eukaryotes. Enduring research in SK Mishra's lab suggests that HS-SDE2 is a ubiquitin like modifier (Fig.1.3) and is associated with RNA splicing. The primary objective of this thesis was to utilize the Y2H system using the HIS3 reporter gene, to explore the interactions between Hs-SDE2 and other known proteins. It was also of interest to see whether interactions of SDE2 with splicing factors could be confirmed, as the findings are not well established in the literature. We also aimed to find out the protease which might be causing the split of SDE2 full length protein.

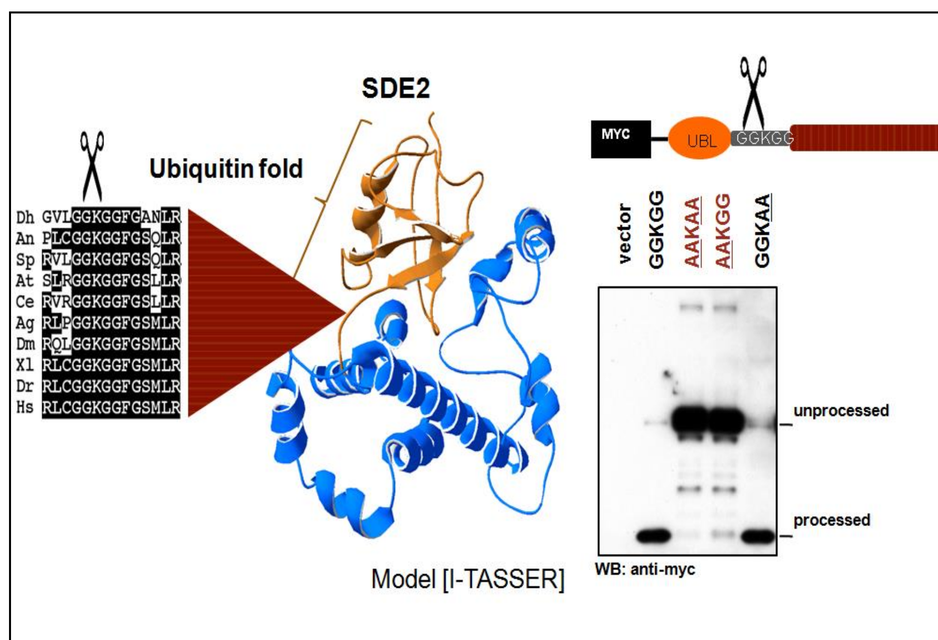


**Figure - 1.3 – Regulation of Hs-SDE2 :** Structure prediction by I-TASSER has revealed that SDE2 contains ubiquitin fold. Sequence analysis across various species shows that GGKGG is the conserved sequence within SDE2.Western blot analysis performed by Dr. SK Mishra suggests that GG in conserved sequence GGKGG is responsible for the formation of processed form of HS-SDE2.

## 1.2 Methodical background

### 1.2.1 The yeast two hybrid system

Identification of protein-protein interactions (PPIs) is essential for a better understanding of biological processes, pathways and functions. Several techniques have been developed to study protein-protein interactions, the yeast two-hybrid system is one such powerful tool for proteomic-based investigations. The yeast two-hybrid system originally created by Fields and Song more than two decades ago is a genetic system in which the interaction between two proteins of interest is detected by the reconstitution of a transcription factor and the consequent activation of reporter genes under the control of this transcription factor. In this technique, a protein X is expressed as a fusion to a DNA binding domain (DBD). The DBD–X fusion is commonly termed the "bait." Because of the affinity of the DBD for its operator sequences the bait is bound to a promoter element upstream of a reporter gene but does not activate it because it lacks an activation domain. A second protein Y is expressed as a fusion to an activation domain (AD) of the same transcription factor and is commonly termed the "prey." The prey is capable of activating transcription but usually does not do so because it has no affinity for the promoter elements upstream of the reporter gene. If bait and prey are co expressed and the two proteins X and Y interact, then a functional transcription factor is reconstituted at the promoter site upstream of the reporter gene (Fig.1.5). Thus, in a yeast two-hybrid assay a protein–protein interaction is measured through the activation of one or several reporter genes in response to the assembly of a transcription factor by the said protein–protein interaction [11].

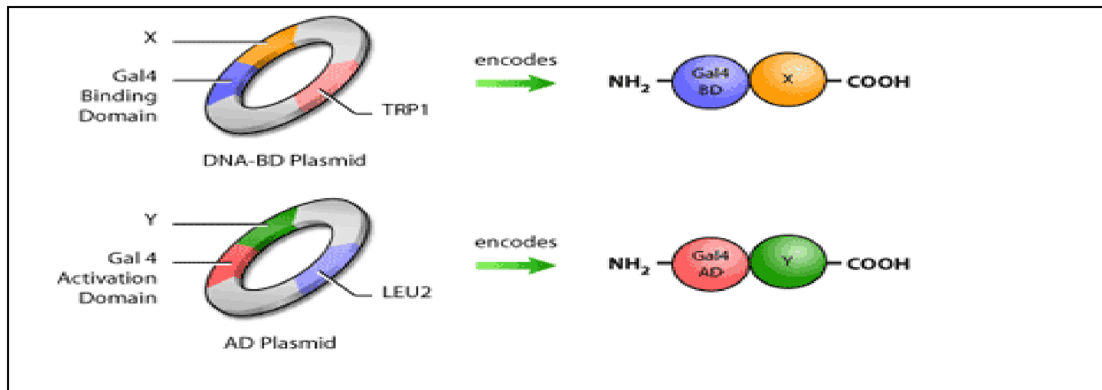


**Figure - 1.4 - Bait and prey plasmids:** The yeast two-hybrid assay uses two plasmid constructs: the bait plasmid, which is the protein of interest fused to a GAL4 binding domain, and the prey plasmid, which is the potential binding partner fused to a GAL4 activation domain [11].

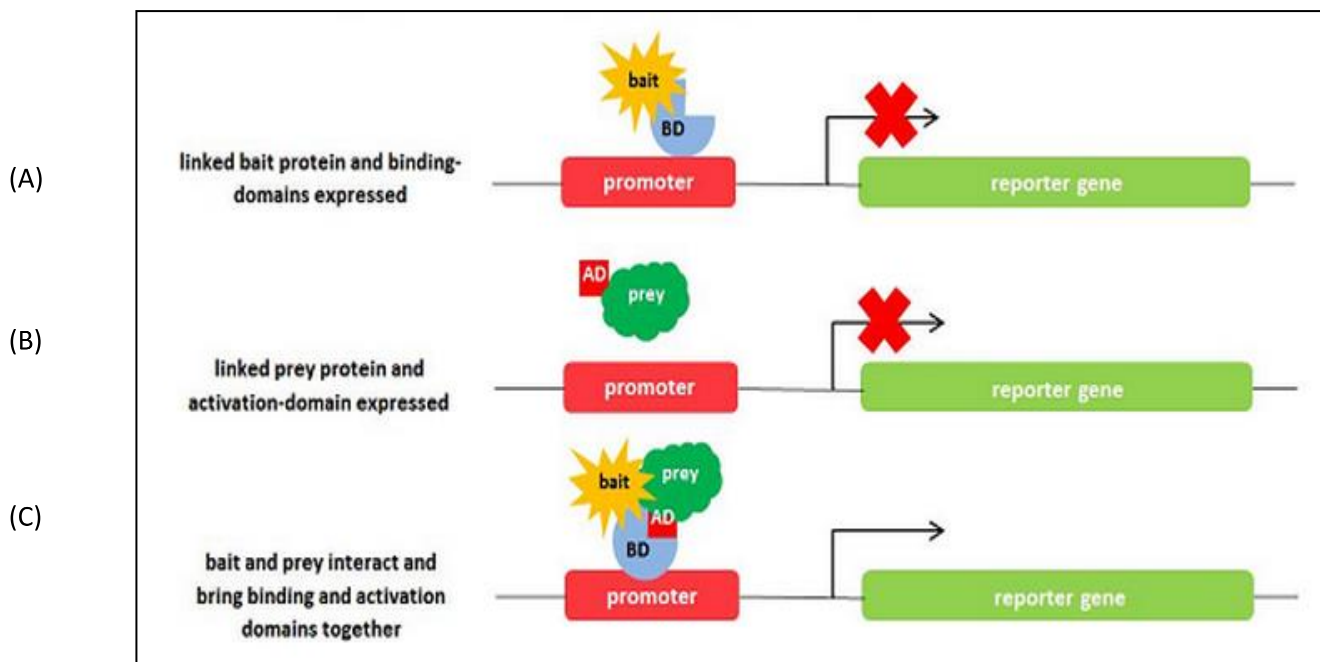


**Figure – 1.5 - The yeast two-hybrid system :** (A) A bait is expressed as a fusion to a DNA binding domain (DBD), The DBD–bait binds to the operator sequences present in the promoter region upstream of the reporter gene but does not activate its transcription since the DBD–bait does not contain an activation domain. (B) A prey is expressed as a fusion to an activation domain (AD), The AD–prey fusion has the capability to activate transcription in yeast but because it is not actively targeted to the promoter it does not activate transcription of the reporter gene. (C) The interaction between bait and prey targets the AD–prey fusion protein to the promoter, thereby reconstituting an active transcription factor. The hybrid transcription factor is bound to the promoter upstream of the reporter gene and therefore activates transcription. The readout of the activated reporter gene is measured as growth on selective medium (auxotrophic selection markers, such as HIS3, URA3,or ADE2). Yeast expressing only the DBD–bait or the AD–prey on its own do not grow on selective medium (HIS-) whereas yeast harboring an interacting DBD–bait and AD–prey display growth on it [12].

### 1.2.2 Advantages and limitations with the yeast two hybrid(Y2H) system

The yeast two hybrid system has a clear advantage over classical biochemical or genetic methods, in the way that it is an in vivo technique that uses the yeast cell as a living test-tube. The use of the yeast host can be considered an advantage since it bears a greater resemblance to higher eukaryotic systems than a system based on a bacterial host. With regards to classical biochemical approaches, which can require high quantities of purified proteins or good quality anti-bodies, the two hybrid system has minimal requirements to initiate screening, since only the cDNA of the gene of interest is needed. A major drawback of testing protein-protein interactions in a heterologous system such as the yeast is that interactions may depend on certain posttranscriptional modifications, such as disulfide bridge formation, glycosylation, or phosphorylation,which may not occur properly or at all in the yeast system. Since the fusion proteins in the two hybrid system must be targeted to the nucleus, extracellular proteins or proteins with stronger targeting signals may be at a disadvantage. Given that the two hybrid assay measures reporter activity, it cannot be excluded that a third protein may perhaps be bridging the bait and prey [12].

### 1.2.3 Large-scale screens using the cDNA library approach

I used the cDNA library approach to find direct binding partners of HS-SDE2 protein. In cDNA library screen approach the bait is expressed in a yeast reporter strain of the mating type a, whereas a collection of preys (the cDNA library) is transformed into a yeast reporter strain of the mating type α. The bait bearing strain is then mated with the mixture of library strains, and clones expressing an interaction pair are isolated on selective media. To determine the identity of the interacting prey, the library plasmid encoding it has to be isolated from the yeast strain and amplified in Escherichia coli. The region encoding the prey is then sequenced [12].

# Chapter 2

# Materials and Methods

## 2.1 Experimental outline

Following are the major steps in the experimental work of the thesis:

Step 1 – Design primers for PCR and amplify Hs-SDE2 gene by PCR.

Step 2 – Generation of bait clone.

Step 3 – Test bait for autoactivation.

Step 4 – Screen Mate & Plate library

Step 5 - Confirm and interpret results.

## 2.2 Amplification of genes by polymerase chain reaction

The polymerase chain reaction (PCR) is a molecular biology technique to amplify a single or a few copies of a piece of DNA up to several orders of magnitude.

Materials : DNA template used – prep81×Hs3MYC-SDE2-3FLAG ,

The domain specific primers :

1. for HS-SDE2 full length

   BamH I RP (5' CCAGGATCCTCATTTTTTCTTCCCTTTCAAAGG 3' ) and

   EcoR I FP (5' CGTGAATTCATGGCGGAGGCCGCGGCGC)

2. for HS-SDE2 N-term

   EcoR I FP(5' CGTGAATTCATGGCGGAGGCCGCGGCGC) and

   BamH I RP (5'CCAGGATCCGCAAAGTCTGGGTTCCAAAC 3')

Protocol for PCR of Hs-SDE2 gene: To prepare master mix for a 50µl reaction, combine the following in an eppendorf tube in order :

**Table - 2.1 –Reaction mix for PCR**

| Component | Working | Reaction vol.(µl) |
|---|---|---|
| HF phusion buffer (5x) | 1x | 10 |
| EcoR I FP (100µM) | 10µM/mL | 5 |
| BamH I RP(100µM) | 10µM/mL | 5 |
| dNTPs 25Mm | 10Mm | 1 |
| DNA (100ng) | 2ng/µL | 1 |
| Phusion HF DNA polymerase | -- | 1 |
| $dH_20$ | | 27 |
| | TOTAL | 50 |

This reaction mix is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time.

Thermal Cycler Conditions :

**Table - 2.2 – Thermal Cycler conditions in PCR**

| Step | Time/cycles | Temperature[in degrees Celsius] |
|---|---|---|
| Initial denaturation | 3’ | 97 |
| 3 step cycling<br><br>Denaturation | 30” | 97 |
| Annealing | 30” | 55 } 35 cycles |
| Extension | 1.3’ | 72 |
| Final extension | 5’ | 72 |
| Hold | Infinity | 4 |

PCR reactions were then analyzed on 1.5% agarose gels in 1X TAE buffer [50X TAE buffer : Tris base = 242g , Glacial acetic acid = 57.1mL, 0.5M EDTA (pH 8) = 100mL ; Total volume = to 1000mL ], containing 1 μg/mL ethidium bromide. 10 μL of PCR sample was mixed with 2 μL of 6X DNA loading dye in an eppendorf tube, centrifuged briefly, and mixed by pipeting up and down before loading onto the gel. Gel electrophoresis was carried out at 80 V for 1 hour. Gels were visualized under a UV trans-illuminator.

## 2.3 Purification of PCR products

The advantages of purifying PCR products is that it ensures removal of Primers, nucleotides, and buffer components, non target amplification products, Compounds that may inhibit digestion reaction [10].

Protocol for PCR cleanup:

(using bioneer PCR purification kit)

1. Add 5 volumes of PB Buffer to 1 volume of the PCR reaction. Mix them completely. Place a Binding column tube in a 2 ml tube.
2. Apply the sample to the Binding column tube to bind DNA.
3. Centrifuge for 30-60 sec to make the sample pass through the Binding column tube.
4. Discard flow-through and place the Binding column tube in the same tube.
5. Add 500 µL of WB Buffer to the Binding column tube and centrifuge for 30-60 sec to wash. This step removes salts and soluble impurities in the Binding column tube
6. Discard flow-through and place the Binding column tube in the same tube again.
7. Repeat the washing procedure using 500 µL of WB Buffer. Centrifuge the Binding column tube for an additional 2 min for drying.
8. Place the Binding column tube in a clean 1.5 ml tube.Add 20 µL of $dH_20$ to the center of the Binding column filter, and let the column stand for 1 min. 12. Centrifuge for 1 min to elute.

## 2.4 Restriction digestion

Restriction Digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction endonucleases. These special enzymes recognize specific sequences in the DNA molecule and then cleave the phosphodiester bonds between the nucleotides at that site wherever that sequence occurs in the DNA. After PCR, DNA fragment needs to be digested with the appropriate restriction enzymes. Vector also needs to be digested with the appropriate enzymes in preparation for ligation with PCR product (insert-to-be).

Method:

- Add in appropriate amount of insert, vector, water, restriction enzymes in an autoclaved micro-centrifuge tube.
- Incubation at 37˚C for 3 hrs.
- Check for digested fragments using gel electrophoresis.

1.Restriction digestion of PCR product :

**Table - 2.3-Restriction digestion of PCR product**

| Component | Reaction vol.(µL) [HS-SDE2 N-term] | Reaction vol.(µL) [HS-SDE2 FL] |
|---|---|---|
| Cutsmart buffer | 3 | 3 |
| PCR product (DNA)(1µg) | 8.5 | 7 |
| EcoR1-HF | 1 | 1 |
| BamH1-HF | 1 | 1 |
| $dH_20$ | 6.5 | 8 |
| Total | 20 | 20 |

2. Restriction digestion of vector :

**Table - 2.4 - Restriction digestion of vector**

| Component | Reaction vol.(µL) |
|---|---|
| Cutsmart buffer | 8 |
| (DNA)(1 µg ) | 5 |
| EcoR1-HF | 1 |
| BamH1-HF | 1 |
| $dH_20$ | 5 |
| Total | 20 |

## 2.4 Ligation

The final step in the construction of a recombinant plasmid is connecting the insert DNA (gene or fragment of interest) into the compatibly digested vector backbone. This is accomplished by covalently connecting the sugar backbone of the two DNA fragments. This reaction, called ligation, is performed by the T4 DNA ligase enzyme. The DNA ligase catalyzes the formation of covalent phosphodiester linkages, which permanently join the nucleotides together.

For a typical ligation reaction digested insert, digested vector, T4 ligase buffer and T4 ligase is required. 100ng of digested vector was used and the amount of digested insert added was calculated using following formula:

Amount of insert = Amount of vector X (size of insert/size of vector) X MR

{MR = Molar ratio of vector & insert (1:3 to 1:8)}

Method:

- Add in appropriate amount digested insert, digested vector, water,T4 ligase buffer, T4 ligase in an autoclaved micro-centrifuge tube.
- Incubation at room temperature for 1-2 hrs.

**Table - 2.5 – Ligation reaction of digested insert and vector**

| Component | Reaction vol.(μl) |
|---|---|
| Insert | 1 |
| Vector | 5 |
| Ligase buffer | 2 |
| T4 DNA Ligase | 1 |
| $dH_20$ | 11 |
| Total | 20 |

## 2.6 Plasmid transformation

Once recombinant plasmid is constructed, it is introduced into recipient cells. Introduction of recombinant DNA into recipient cells is called transformation. Special treatment makes cells competent - capable of accepting foreign DNA. When competent cells are mixed with DNA some cells become transformed and acquire recombinant vector DNA.

***E. coli* DH5α Transformation**

100µL of competent *E. coli* DH5α was transformed with 10µL of ligation reaction. Incubation was carried out on ice for 30min. The cells were then heat-shocked at 42ºC for 45sec, and incubated on ice for 2min. 500µL of LB media was added and incubation at 37ºC, with shaking, was carried out for 1hr. The cells were then centrifuged at 5000rpm for 2min. 250- 400µL of supernatant was removed and the pellet was resuspended in the remaining supernatant. This was plated onto LB+Kan (for pGBKT7-insert), and incubated overnight at 37ºC.

**Transformation of yeast**

Mix 1µl of the isolated plasmid with 10 µl of yeast competent cells. Add 66 µl of 40% PEG [100 mM LioAc, 10 mM Tris-HCl, and 1mM EDTA].Incubate at 30˚C for 30 minutes. Give heat shock at 42˚C for 25 minutes. Keep on ice for 5 minutes. Add 100 µl sterile water. Plate whole solution on appropriate dropout plate and incubate at 30˚C for 3-4 days.

## 2.7 Restriction enzyme analysis of positive expression constructs

Restriction analysis was also performed on positive expression constructs following the cloning, to confirm the presence of the genes in the destination vectors. Overnight cultures of transformed DH5α cells containing LB medium with ampicillin (100µg/ml) were incubated at 37˚C.The plasmid DNA was purified from them using the alkaline lysis (miniprep) method and digested with BamH I and EcoR I enzyme for 3 hours as described earlier. The enzyme digested fragments were analyzed under a UV transilluminator after EtBr staining, following gel electrophoresis.

## 2.8 Sequencing

To ascertain the presence and correct orientation of the insert into the destination vectors, the clones that appeared correct in the restriction enzyme analyses were subjected to nucleotide sequencing by 1st BASE DNA Sequencing Services in Malaysia. The nucleotide sequences received in return from 1st BASE, were compared with the correct nucleotide sequences of the Hs-SDE2 gene by using the internet based alignment tool; Basic Local Alignment Search Tool (BLAST) provided by NCBI.

## 2.9 SDS-PAGE

SDS-PAGE is widely used to analyze the proteins in complex extracts. The system actually consists of two gels - a resolving gel in which proteins are resolved on the basis of their molecular weights and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their molecular weights.

Preparation of resolving gel (12%):

**Table - 2.6-Preparation of resolving gel (12%)**

| Component | Volume (ml) |
|---|---|
| $dH_2O$ | 1.65 |
| 1.5 M Tris-Cl | 1.25 |
| 10% SDS | .05 |
| 30% acrylamide | 2 |
| 10% APS | .05 |
| TEMED | .005 |

First resolving gel is put in apparatus. Immediately, a layer of water is also put on it to prevent the evaporation. Once the resolving gel gets solidified in 20-30 minutes, layer of water is thrown and stacking gel is poured.

Preparation of stacking gel (4%)

**Table - 2.7 - Preparation of stacking gel (4%)**

| Component | Volume (ml) |
|---|---|
| $dH_2O$ | 2.80 |
| 0.5 M Tris-Cl | 1.26 |
| 10% SDS | .05 |
| 30% acrylamide | 0.83 |
| 10% APS | .05 |
| TEMED | .005 |

Method:

- Prepare the required percentage of gel according to the size of the proteins.
- Add 50 µl of the HU + 15%DTT (9:1) buffer to the sample proteins and heat them at 65°C for 10 minutes.
- Load the sample and relevant protein ladder onto the gel and let it run.

## 2.10 Western blot analysis

After SDS-PAGE gels are transferred to nitrocellulose membrane in transfer buffer (500mM Glycine, 50mM TrisHCl, 0.01% SDS, and 20% methanol) at 120mA for 1h. Membranes are washed in TBS-T (10mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and then blocked with non-fat milk extract in TBS-T for 30min to 1h. Membranes are exposed to primary antibody in skim milk for 1.5 hours at room temperature. Membranes are washed with TBS-T three times for ten minutes. Then are incubated with appropriate secondary antibodies for 1h at room temperature. After washing the membranes three times for ten minutes, signals were visualized using Western Pico Super ECL reagent (Pierce).

## 2.11 Yeast two hybrid system

In this study, the Matchmaker™ GAL4-based yeast two-hybrid system (Clontech, Mountain View, CA) was used. The eukaryotic transcription factor, GAL4, can bind to GAL4-responsive elements located in the UAS. The Matchmaker™ system consists of two plasmids: pGBKT7 and pGADT7, which can be cloned to contain the bait and prey proteins, respectively. pGBKT7 has a TRP1 gene and Kan resistance marker while pGADT7 has a LEU2 gene and Amp resistance marker (Yeast Protocols Handbook, 2009). A yeast strain that is auxotrophic for tryptophan and leucine is chosen, and this allows selection of yeast colonies that have been successfully transformed with both plasmids. There are several possible yeast strains that can be used in conjunction with pGBKT7 and pGADT7, for this study, the Y2HGold strain was chosen. For thesis experiment, mate and plate cDNA library from hela, bone marrow and brain tissues of humans expressed in Y187 yeast strain were used. These three cDNA libraries were mixed as 1:1:1.

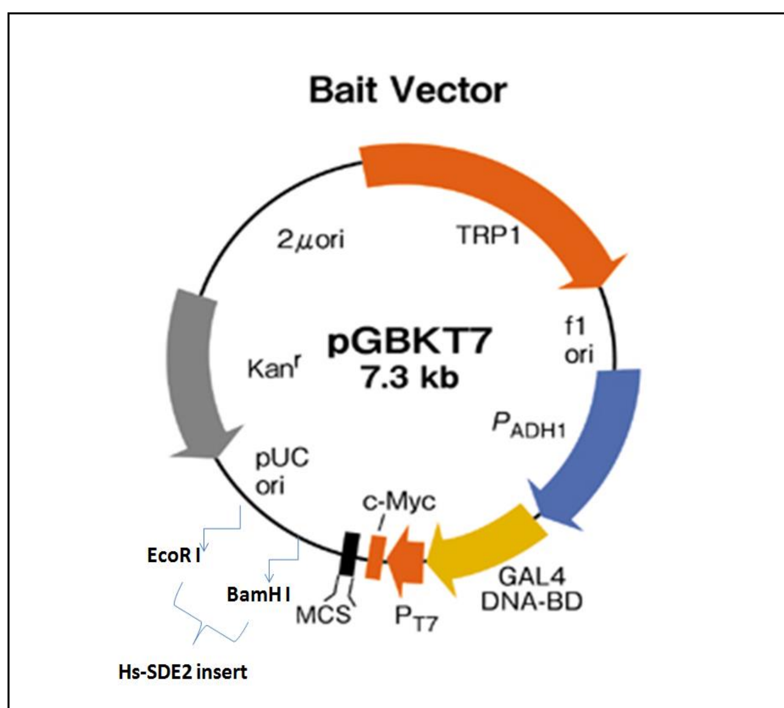


**Figure – 2.1 – Features of pGBKT7 DNA-BD plasmid** : pGBKT7 is a plasmid that encodes a fusion of the Hs-SDE2 protein and the GAL4 DNA-BD.Hs-SDE2 gene was cloned into pGBKT7 at the EcoR I and BamH I sites.

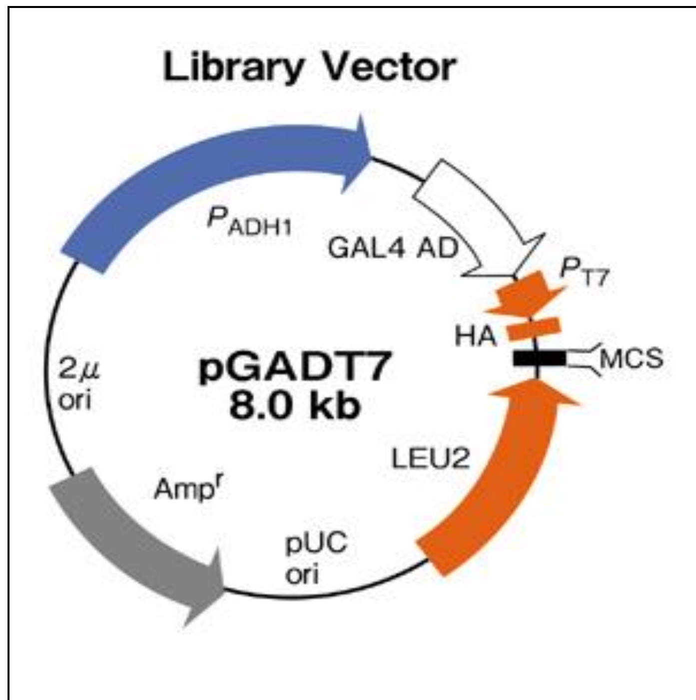


**Figure – 2.2 - Features of pGADT7 AD plasmid:** pGADT7 is used to introduce the prey protein. It encodes the cDNA library fused with GAL4 activation domain. The ampicillin resistance gene allows selection in *E. coli*. DH5α strain.

**List of additional Materials required and their preparation**

a) <u>YPDA (2X)</u>

| Bacto peptone | 10 g |
|---|---|
| Bacto yeast extract | 20 g |
| Adenine sulphate | 40 mg |

} in 250 mL $H_2O$

| Glucose | 20 g |
|---|---|

} in 250 mL $H_2O$

Above mentioned ingredients were dissolved fully and sterilized by autoclaving at 15psi (1.05kg/cm²) for about 20min.They were mixed thoroughly before usage.

b) Synthetic complete (SC) medium

This is a mixture that has all of the following mixed together in a powder in the right ratio. Media that contain this are thus non-selective (nearly all yeast will have everything they need to grow).

| Component | Amount (mg/10L of media) |
|---|---|
| Adenine hemisulfate | 180 |
| Alanine | 760 |
| Arginine HCl | 760 |
| Asparagine monohydrate | 760 |
| Aspartic acid | 760 |
| Cysteine HCl monohydrate | 760 |
| Glutamic acid | 760 |
| Glutamine | 760 |
| Glycine | 760 |
| Myo-inositol | 760 |
| Isoleucine | 760 |
| Leucine (leu) | 3800 |
| Lysine | 760 |
| Methionine | 760 |
| Aminobenzoic acid | 80 |
| Phenylalanine | 760 |
| Proline | 760 |
| Serine | 760 |
| Threonine | 760 |
| Tryptophan (trp) | 760 |
| Tyrosine disodium | 760 |
| Uracil | 760 |
| Histidine (his) | 760 |
| Valine | 760 |

c) Dropouts

These are a whole series of other mixes that are derived from SC, but from which certain nutrients have been omitted. Such media is thus called a "dropout" (it has everything except the nutrient that has been dropped out). For example, SC-trp has all elements of SC except tryptophan.

d) SC-trp medium

| Ingredient | Amount | |
|---|---|---|
| YNB | 3.35g | in 200 mL $H_2O$ |
| SC-trp dropout | 1 g | |

| Ingredient | Amount | |
|---|---|---|
| Glucose | 10 g | in 100 mL $H_2O$ |

Above mentioned ingredients were dissolved fully and sterilized by autoclaving at 15psi (1.05kg/cm²) for about 20min.They were mixed with 200 ml of autoclaved water before usage.

e) Selection plates

SC-leu / SC-leu-trp / SC-trp / SC-leu-trp-his

| Ingredient | Amount | |
|---|---|---|
| YNB | 7 g | in 400 mL $H_2O$ |
| Dropout | 2 g | |

| Ingredient | Amount | |
|---|---|---|
| Agar | 20 g | in 400 mL $H_2O$ |

| Ingredient | Amount | |
|---|---|---|
| Glucose | 20 g | in 200 mL $H_2O$ |

Above mentioned ingredients were sterilized separately by autoclaving at 15psi (1.05kg/cm²) for about 20min. Later, they were mixed and plates were poured.

## 2.12 Testing bait for autoactivation

As a first step for any two-hybrid screen, it is imperative to confirm that the bait does not autonomously activate the reporter genes in Y2HGold, in the absence of a prey protein.

Materials: pGBKT7 containing SDE2 gene cloned in frame with the *GAL4* DNA-BD (pGBKT7), competent PJ697a Gold cells.

Method: Transform 100 ng of pGBKT7+Bait construct in PJ697a strain. Spread 100 µl of a the transformation mixture onto SC-leu-trp-his,SC-leu-trp,SC-leu-trp-his + 5mM 3AT plate and incubate at 30˚C for 3-4 days.

## 2.13 Preparation of competent yeast cells:

Inoculate overnight at 30°C single colony of *S. cerevisiae* PJ697a/ *S. pombe* Y2H Gold strain in 5ml of YPAD medium. Transfer it to either 50 ml or 100 ml YPAD medium and inoculate at 30°C till the $O.D_{600}$ reaches 0.6 – 1.0.Centrifuge at 3000 rpm for 5 minutes at RT and discard supernatant. Wash the pellet with sterile 25ml water and centrifuge again. Wash the pellet with sterile SORB with 1\10th of culture volume. Add 360 µl of SORB for 50 ml culture. Add 40-50 µl denatured single stranded Salmon sperm DNA to the culture. Aliquot 100 µl of competent cells in 1.5 ml micro centrifuge tubes and freeze them in 80°C.

## 2.14 Protocol for carrying out yeast two-hybrid cDNA library screening using yeast mating

Protocol :

1. Prepare a concentrated overnight culture of the bait strain (Y2HGold [pGBKT7+Bait]) as follows: Inoculate one fresh, large (2–3 mm) colony of bait strain into 50 ml of SD/–Trp liquid medium. Incubate shaking (250–270 rpm) at 30°C until the $OD_{600}$ reaches 0.8 (16–20 hr).Centrifuge to pellet the cells (1,000 g for 5 min), discard the supernatant. Resuspend the pellet to a cell density of $>1x10^8$ cells per ml in SC–trp (4–5 ml).
2. Combine the library strain with the bait train as follows: thaw a 1 ml aliquot of the library strain in a room temperature water bath. Combine 1 ml of the Mate & Plate Library with 4–5 ml Bait Strain (from Step 4) in a sterile 2 L flask. Add 45 ml of 2xYPDA liquid medium (with 50 µg/ml kanamycin).Rinse cells from the library

vial twice with 1 ml 2xYPDA and add to the 2 L flask. Incubate at 30°C for 20–24 hr, slowly shaking (30–50 rpm).

3. After 20 hr, check a drop of the culture under a phase contrast microscope (40X). If zygotes are present, continue to next step, if not, allow mating to continue, incubate for an additional 4 hr.
4. Centrifuge to pellet the cells (1,000 g for 10 min) and discard the supernatant. Resuspend all pelleted cells in 10 ml of 0.5X YPDA/Kan liquid medium. Measure the total volume of cells + medium.
5. From the mated culture, spread 100 µl of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on each of the following 100 mm agar plates and incubate at 30°C for 3–5 days SC–trp, SC–leu,SC-leu-trp.Plate the remainder of the culture, 200 µl per 150 mm on SC-leu-trp-his (50–55 plates). Incubate at 30°C for 3–5 days.
6. Calculate the number of screened clones (diploids) by counting the colonies from the DDO plates after 3–5 days.

   Number of Screened Clones = cfu/ml of diploids x resuspension volume (ml)
7. Determine the Mating Efficiency.

   No. of cfu/ml on SC-leu = viability of the Prey Library

   No. of cfu/ml on SC-trp = viability of Bait

   No. of cfu/ml on SC-leu-trp = viability of diploids

   Calculate Mating Efficiency (percentage of diploids) as follows :

   No. of cfu/ml of diploids/ No. of cfu/ml of limiting partner x 100 = % Diploids
8. Patch out all the pink colonies that grew on SC-leu-trp-his plates onto higher stringency plates using a flat sterile toothpick or yellow pipette tip.
9. All positive interactions must be further analyzed to identify duplicates and to verify that the interactions are genuine [13].

## 2.15 Predicting protein-protein interactions by bio - computational tool

Results from yeast two hybrid analysis were analyzed using PSOPIA [Prediction Server Of Protein-Protein Interactions].PSOPIA is an Averaged One-Dependence Estimators (AODE) for predicting protein-protein interactions using three sequence based features; (I) sequence similarities to a known interacting protein pair, (II) statistical propensities of domain pairs observed in interacting proteins and (III) a sum of edge weights along the shortest path between homologous proteins in a PPI network [9].

# Chapter 3

# Results

## 3.1 Cloning of bait into pGBKT7 Vector

Prep81×HS3MYC-Hs-SDE2-3FLAG was used as a PCR template for amplification of Hs-SDE2 N-term and Hs-SDE2 full length. Following PCR, the amplified DNA products were analyzed qualitatively by electrophoresis on 1.5% agarose gel and the fragment size of approximately 1300 bp and 230 bp , corresponding with the expected sizes were obtained as in following figure.

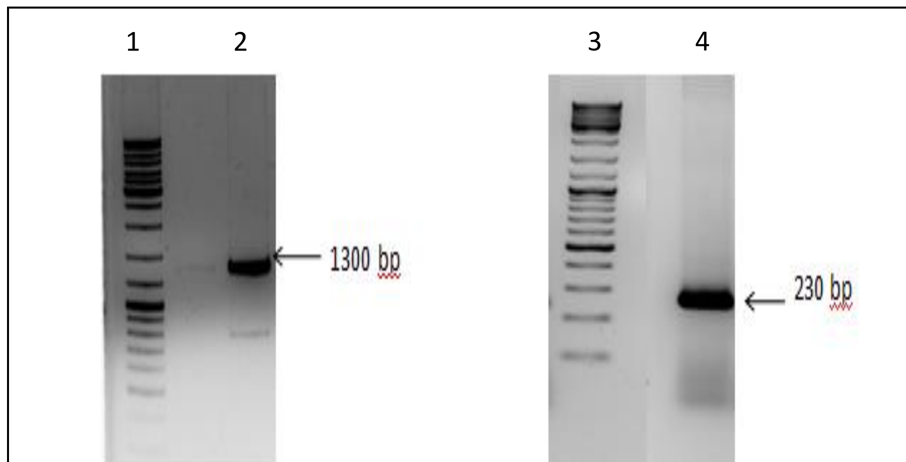


**Figure - 3.1 - PCR amplification of Hs-SDE2 :** Amplification of pREP81×Hs-3MYC-SDE2-3FLAG was done using specific primers. Gel on left shows amplification for Hs-SDE2 [full length] and gel on right shows amplification for Hs-SDE2 [N-term]. Lane 1,3 - DNA molecular weight marker ( bands are in kilo base pairs (kb)) ; lane 2 and 4 - PCR amplification products.

The PCR product was then extracted and ligated into the pGBKT7 vector followed by transformation of competent E. coli Dh5α cells. Colonies on LB-Kan plates were screened after plasmid isolation and subjected to restriction digestion to verify the presence of HS-SDE2 in the recombinant plasmids.

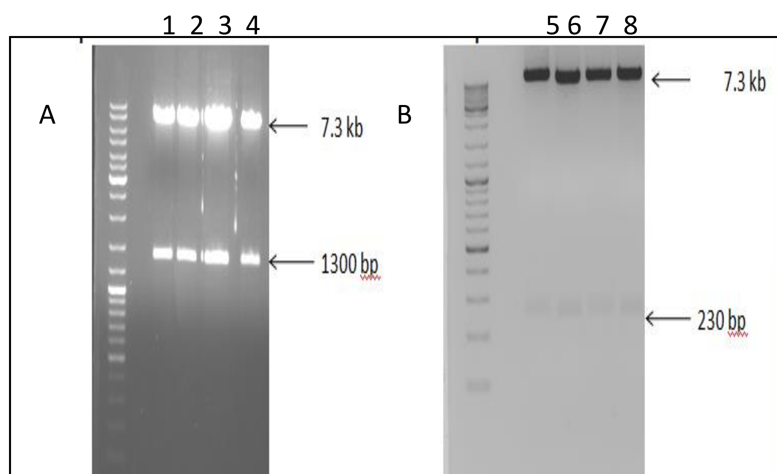

**Figure - 3.2 - Screening of recombinant plasmids with restriction endonucleases** : The leftmost lane shows the DNA marker , and the next lanes show pGBKT7-HS-SDE2 clones (panel A shows positive clones of pGBKT7-Hs-3MYC-SDE2 full length and panel B shows positive clones of pGBKT7-Hs-3MYC-SDE2 N-term. Lanes 1-8 show bait plasmid DNA double digested with EcoR I and BamH I and was expected to yield two bands (7.3 kb -pGBKT7 vector , 1300 bp- Hs-SDE2 full length , 230 bp- Hs-SDE2 N-term )

Since PCR might introduce mutations, plasmid DNA obtained from the positive clones was sequenced and analyzed. The cloned HS-SDE2 fragments were identical to the one available on the NCBI database and it contained no mutations. The results also confirmed that the inserted HS-SDE2 encoding fragment was in frame with the coding region of GAL4 DNA Binding domain.

## 3.2 Detecting bait expression

Y2H Screens often fail due to lack of interactions, which can be caused if the bait is not expressed appropriately for an interaction to occur in yeast. By inappropriate expression I mean, poor expression, incorrect localization, or degradation of the bait fusion protein (a protein of interest fused to a transcription factor binding domain). Often Western blotting is used to test the competency of a bait fusion before screening, allowing authentication that the protein is correctly expressed in the yeast cells.

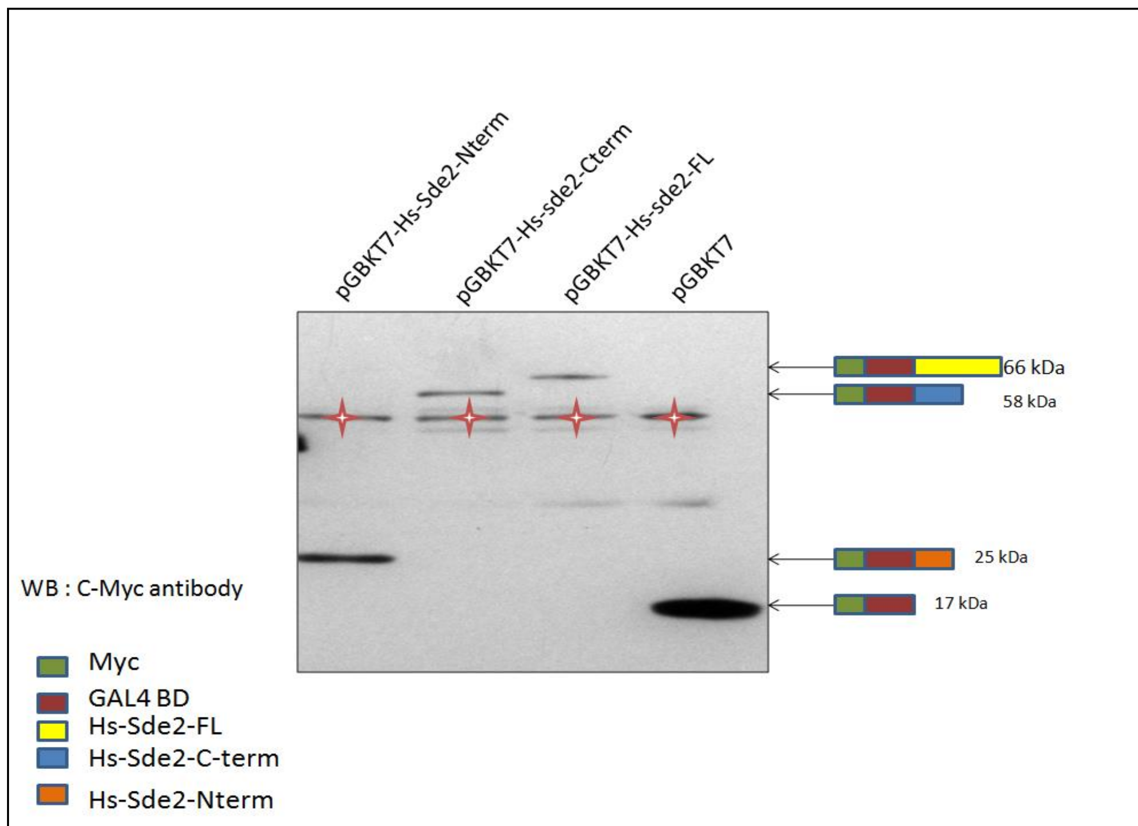


**Figure - 3.3 – Detection of bait expression:** to determine whether or not the bait is expressed well in yeast, bait proteins in yeast containing pGBKT7-based bait plasmids were detected by c-Myc monoclonal antibody via Western blot. pGBKT7 was used as control which expresses a 17 kDa protein. Yeast protein extracts were prepared by harvesting *S.cerevisiae* PJ697a cells [grown in SC-trp medium] at $O.D_{600}$ = 2 which contained Hs-SDE2 fused in frame with GAL4 BD and Myc epitope tag in pGBKT7 plasmid.

### 3.3 Testing bait for autoactivation

Before starting a screen, it is important to check that bait does not autonomously cause the activation of reporter gene in the absence of prey. *S. cerevisiae* PJ697A contains the auxotrophic mutations that are complemented by the bait and prey vectors (*leu2* and *trp1*) and is unable to synthesize histidine and is therefore unable to grow on media that lack this essential amino acid. When bait and prey proteins interact, Gal4-responsive His3 expression permits the cell to biosynthesize histidine and grow on –His minimal medium. In my experiment none of the two baits were found to be autoactivating.

**Table - 3.1 – Autoactivation test for bait**.

| leu2 plasmid | trp1 plasmid | Expected interaction |
|---|---|---|
| pGADC1 | pGBKT7 | No interaction |
| pGADC1 | pBGKT7-HS-HS-SDE2-Nterm | No interaction |
| pGADC1 | pGBKT7-HS-HS-SDE2-FL | No interaction |
| pGADC1 | pGBKT7-HS-HS-SDE2-Cterm | No interaction |
| pSEM3 | pGBKT7-HS-HS-SDE2-Cterm | Positive control |

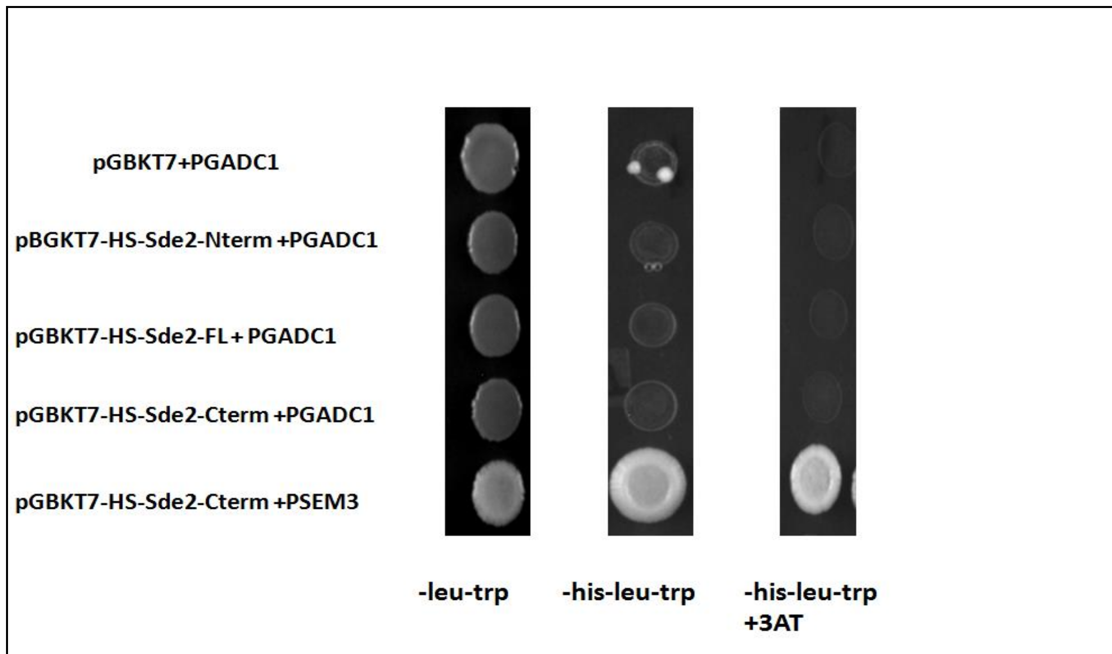


**Figure - 3.4 -Testing bait for autoactivation :** As desired, *S. cerevisiae* PJ697a cells containing the bait and prey vectors (*leu2* and *trp1*) were able to grow on –leu-trp medium but were unable to grow on media lacking histidine. This confirms that none of the two baits – Hs-SDE2 [Full length] and Hs-SDE2 [N-term] were found to be autoactivating. 3-AT [which limits histidine biosynthesis] was also added to yeast media to fine tune the leaky expression of the HIS3 reporter gene.

## 3.4 Selection plates

Four different synthetic complete (SC) selection plates were used in the Y2H analysis, and all four were lacking the amino acids leucine and tryptophan. Omitting these verified that both bait and prey were present in the yeast cell, as the bait transcribe an enzyme in the tryptophan biosynthesis pathway, and the prey will synthesize an enzyme in the leucine pathway. If only one of the expression constructs were present, the cell should not be able to grow. The first plate SC (-leu-trp) was the control plate, merely indicating that the cells contained both bait and prey plasmids. The SC (-leu-trp-his+10 mM 3-AT) indicates interactions using the *HIS3* reporter gene, and cells on it should only grow if bait and prey interacts, thereby able themselves to produce histidine. 3-AT was added as a supplement to prevent false positives. The SC (-leu-trp-ade) plate shows interactions using the *ADE2* reporter gene.

**Table - 3.2 - The selections plates and their purposes.**

| Selection plate | Purpose |
|---|---|
| SC-leu-trp | Transformation control |
| SC-leu-trp-ade | ADE2 activation (positive selection) |
| SC-leu-trp-his | HIS3 activation (positive selection) |
| SC-leu-trp-his+ 3AT | HIS3 activation (positive selection) |

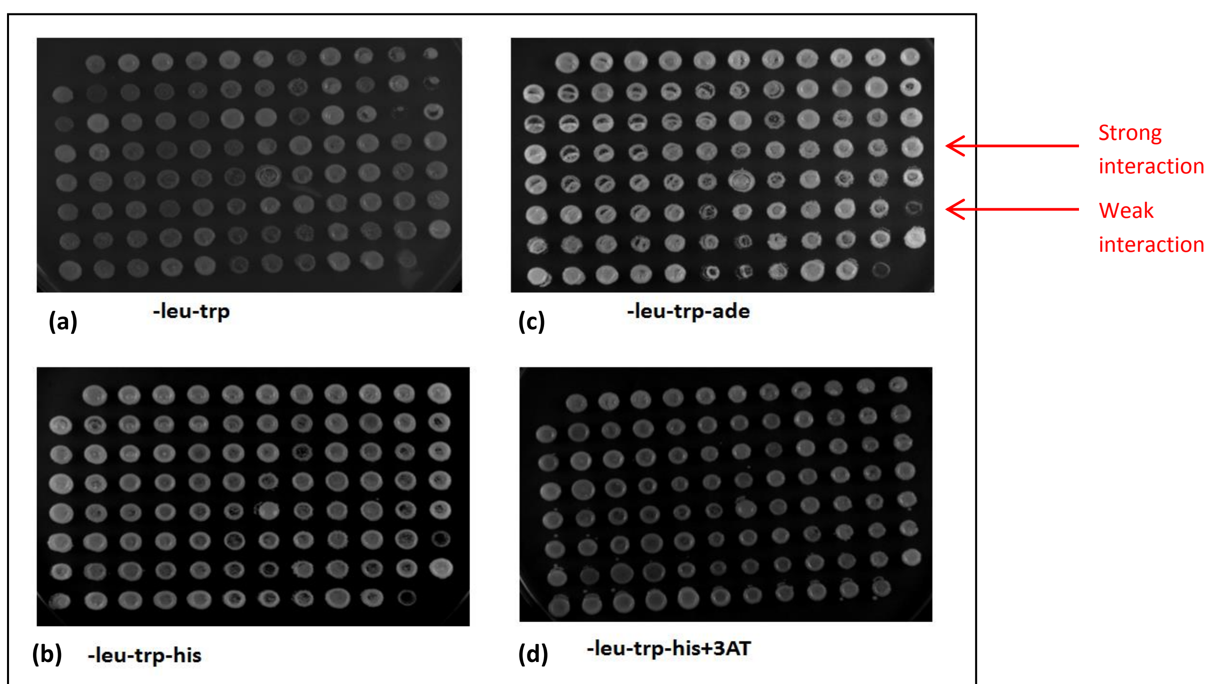


**Figure - 3.5- Y2H analysis on selection plates :** The first plate (a) was a positive transformation control to show that both bait and prey plasmids are present in the cell. The three next plates performed the test for reporter gene activation. In plate b, *HIS3* reporter activation was tested. (d) 3-AT was added to reduce auto activation. In plate (b), *ADE2* reporter activation was tested. Comparison of the experimental interactions with the controls were used to evaluate results (activation of *HIS3* and *ADE2* reporter), and assess the strength of the interaction being tested (strong, weak or no interaction).

## 3.5 Calculation of number of screened clones and mating efficiency

Number of screened clones can be calculated by counting the colonies from the double drop-out plates after 3–5 days. It is necessary that at least 1 million diploids are screened, since using less than this will result in less chance of detecting genuine interactions . Also with mating efficiency of less than 2% , 1 million diploids cannot be achieved and the screen will obviously not be very prolific.

**Formulae :**

Number of clones screened = cfu/ml of diploids × resuspension volume

Number of cfu/ml on SC-leu = viability of prey library

Number of cfu/ml on SC-leu-trp = viability of diploids

Mating efficiency = (Number of cfu/ml of diploids ÷ Number of cfu/ml of prey library) × 100

[cfu:colony forming unit]

**<u>Library screen for HS-HS-SDE2 [N-term] :</u>**

Resuspension volume = 15 ml

Number of colonies on 1/1000 dilution on SC-leu-trp plate = 34

Number of colonies on 1/1000 dilution on SC-leu plate = 57

Plating volume = 100 μl

Number of cfu/ml of diploids = $34\times10\times1000$

$= 34\times10^4$

Number of cfu/ml of prey library = $57\times10\times1000$

$= 57\times10^4$

Mating efficiency = $[(34\times10^4) \div (57\times10^4)] \times 100$

$= 59.6\ \%$

Number of screened clones = $34\times15\times10\times1000$

$= 5.1\times10^6$

**Library screen for HS-SDE2 [Full length] :**

Resuspension volume = 15 ml

Number of colonies on 1/1000 dilution on SC-leu-trp plate = 23

Number of colonies on 1/1000 dilution on SC-leu plate = 153

Plating volume = 100 µl

Number of cfu/ml of diploids = $23 \times 10 \times 1000$

$= 23 \times 10^4$

Number of cfu/ml of prey library = $153 \times 10 \times 1000$

$= 153 \times 10^4$

Mating efficiency = $[(23 \times 10 \times 10^4) \div (153 \times 10^4)] \times 100$

$= 15\ \%$

Number of screened clones = $23 \times 15 \times 10 \times 1000$

$= 3.45 \times 10^6$

## 3.6 Distinguishing genuine positives from false positives

With every two-hybrid screen there is a chance of detecting false positives and it is important to confirm that the interactions are genuine. An interaction is Genuine Positive if Both Bait and Prey are required to activate the Gal4-responsive reporters. And the interaction is False Positive if Prey can activate the Gal4-responsive reporters in the absence of the bait.

Protein interactions in yeast were confirmed using selective media.100 ng of each of the following pairs of vectors were co transformed into Y2HGold Competent Cells:

1.pGBKT7/Bait + Prey (in pGADT7)

2.Empty pGBKT7 + Prey (in pGADT7)

Spread 100 µl of the transformation mix on the SC-leu-trp ; SC-leu-trp-his ; SC-leu-trp-his + 3AT ; SC-leu-trp-ade plates

Expected results after 3–5 days at 30 °C :

1.Genuine positive

**Table-3.3-Expected result for a genuine positive**

| Sample | Growth |
|---|---|
| Bait + candidate prey | Yes |
| Empty pGBKT7 + candidate prey | No |

2. False positive

**Table 3.4-Expected result for a false positive**

| Sample | Growth |
|---|---|
| Bait + candidate prey | Yes |
| Empty pGBKT7 + candidate prey | Yes |

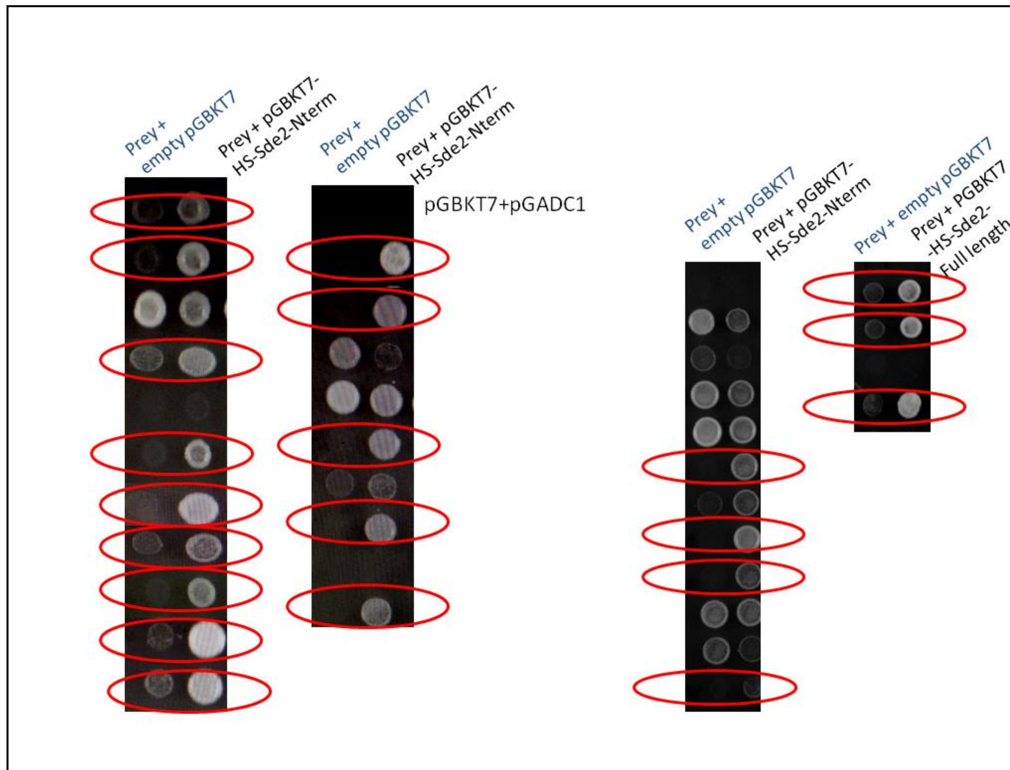


**Fig – 3.6 - Distinguishing genuine positives from false positive interactions.**
Figure shows yeast growth on SC-leu-trp-his + 3AT selection plate. Each putative interaction was checked against empty vector (not fused with bait). Red circles indicate genuine positives in which prey do not activate the Gal4-responsive reporters in the absence of the bait.

## 3.7 Sequence Analysis of a Genuine Positive

Once an interaction has been verified as being genuine, the prey insert can be identified by sequencing. DNA isolated from *E.coli* is used for this procedure. Library inserts were sequenced using T7 promoter. These sequences were compared to those in NCBI database and following results were obtained.

**Table - 3.5 – Interactors of HS-SDE2 [N-term]**

| S.no | Predicted protein homologue | Additional remarks on protein |
|---|---|---|
| 1. | 5-Methyltetrahydrofolate-homocysteine methyltransferase reductase | It is a member of the ferredoxin-NADP(+) reductase (FNR) family of electron transferases. |
| 2. | Opsin 3 | It has the canonical features of a photoreceptive opsin protein. |
| 3. | Growth differentiation factor 11 (GDF11) | It is a regulator of cell growth and differentiation in both embryonic and adult tissues. |
| 4. | Mitogen-activated protein kinase 6 (MAPK6), transcript variant X4 | It is a member of the Ser/Thr protein kinase family. |
| 5. | Bone morphogenetic protein 6 (BMP6) | It induces ectopic bone growth. |
| 6. | La ribonucleoprotein domain family, member 1 | It specifically recognizes the 3' terminus of poly(A) Mrna,key regulator of mRNA translation. |
| 7. | Homo sapiens DKFZp686F03189 | It is a member of the Ser/Thr protein kinase family. |
| 8. | Mitogen-activated protein kinase kinase 1 (MAP2K1), transcript variant X3 | It is a member of the dual specificity protein kinase family and forms essential component of MAP kinase signal transduction pathway. |
| 9. | Homo sapiens HSPC145 | It is a mammalian mitochondrial ribosomal protein which help in protein synthesis within the mitochondrion. |
| 10. | Dynactin 6 (DCTN6) | It contains an RGD (Arg-Gly-Asp) motif in the N-terminal region, which confers adhesive properties to macromolecular |

| | | |
|---|---|---|
| | | proteins. |
| 11. | Pongo abelii NADH dehydrogenase (ubiquinone) 1 beta subcomplex | It is an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase . |
| 12. | SATB homeobox 1 (SATB1) | It is a matrix protein which binds nuclear matrix and scaffold-associating DNAs through a unique nuclear architecture. The protein recruits chromatin-remodelling factors in order to regulate chromatin structure and gene expression. |
| 13. | RBM39 | It co localizes with core spliceosomal proteins in nucleus. |

**Table-3.6-Interactors of HS-SDE2 full length**

| S.no. | Predicted protein homologue | Additional remarks on protein |
|---|---|---|
| 1. | Zinc ribbon domain containing 1 [ZNRD1] | It is a DNA-directed RNA polymerase I subunit which contains two potential zinc-binding motifs, involved in cancer and HIV. |
| 2. | Phosphodiesterase 4D interacting protein | It serves to anchor phosphodiesterase 4D to the Golgi/centrosome region of the cell. |

## 3.8 Computational analysis of genuine interactions

Genuine interactions were analyzed by using PSOPIA [Prediction Server Of Protein Protein Interactions] software. PSOPIA works on the principle that a protein pair would have more potential to interact if their homologous proteins exist in proximity of each other in a known Protein-Protein interaction network. Once we input the sequences of two proteins, PSOPIA assigns a score corresponding to the interaction between two proteins. And if this score is greater than the threshold score of 0.293, the protein pair is said to be interacting. This threshold score has been assigned after training the software on various datasets using three features: (a) sequence similarities to known interacting proteins (b) statistical propensities of domain pairs observed in interacting proteins and (c) a sum of edge weights along the shortest path between homologous proteins in a PPI network.

**Table - 3.7 – Analysis of genuine interactions using PSOPIA tool:** Red colored numerical indicate that the score is more than the threshold and the corresponding protein has more potential to interact with Hs-SDE2.

| S.no. | Predicted protein homologue | PSOPIA Score |
|---|---|---|
| 1. | 5-Methyltetrahydrofolate-homocysteine methyltransferase reductase | 0.3537 |
| 2. | Opsin 3 | 0.3537 |
| 3. | Growth differentiation factor 11 (GDF11) | 0.3727 |
| 4. | Mitogen-activated protein kinase 6 (MAPK6), transcript variant X4 | 0.3727 |
| 5. | Bone morphogenetic protein 6 (BMP6) | 0.3537 |
| 6. | La ribonucleoprotein domain family, member 1 | 0.4187 |
| 7. | Homo sapiens DKFZp686F03189 | 0.3727 |
| 8. | Mitogen-activated protein kinase kinase 1 (MAP2K1), transcript variant X3 | 0.3727 |
| 9. | Homo sapiens HSPC145 | 0.3537 |
| 10. | Dynactin 6 (DCTN6) | 0.3537 |

| 11. | Pongo abelii NADH dehydrogenase (ubiquinone) 1 beta subcomplex | 0.000 |
|---|---|---|
| 12. | SATB homeobox 1 (SATB1) | 0.3537 |
| 13. | RBM39 | 0.3436 |
| 14. | Zinc ribbon domain containing 1 [ZNRD1] | 0.3613 |
| 15. | Phosphodiesterase 4D interacting protein | 0.3231 |

## 3.9 Discussion

This study was undertaken to investigate the association between human SDE2 and other proteins. Hs-SDE2 was inserted into a yeast expression plasmid pGBKT7 as a GAL4 DBD fusion construct. The recombinant plasmid molecule was confirmed by sequencing to be in-frame with the GAL4 DNA binding region. The construct was used to transform yeast cells and selected using auxotrophic markers. Yeast cells containing the bait plasmid were further mated with the yeast containing mammalian cDNA library fused to the activation domain of GAL4.

Approximately $5.1\times10^{6}$ clones for Hs-SDE2 N-term and $3.45\times10^{6}$ clones for Hs-SDE2 full length were screened. Sequence analysis verified the presence of an open reading frame (ORF) fused in frame to the GAL4 AD sequence in the genuine positives obtained through yeast two hybrid assays. These results were further authenticated by using a computational server PSOPIA (Prediction Server Of Protein protein Interactions) which predicts interaction between two proteins on the assumption that homologous proteins share similar interaction patterns as well as similar functions.

Yeast two hybrid results in my project indicates that Hs-SDE2 may play a much wider role inside the cell since the proteins which were identified as interacting partners of Hs-SDE2 participate in various processes like signal transduction pathway, chromatin remodeling, cell growth.

Three out of 13 interactors found for Hs-SDE2 [N-term] are part of the MAP kinase signal transduction pathway. A defect in the MAP/ERK pathway leads to uncontrolled cell growth. GDF11, BMP6, ZNRD1 which are known growth regulators have also featured in this screen. Based on these results one can hypothesize that Hs-SDE2 might have a role in regulation of cell growth. Moreover, previous studies have shown that links do exist between the UBLs and MAP kinase pathways [14]. And, SDE2 interactions with MAP kinases found here also goes with this argument.

Though we could not find interactions between SDE2 and some other known splicing factors but another interactor found in this study RBM39 indicates that role of SDE2 in splicing cannot be neglected. RBM39 is actually found in the nucleus, where it co

localizes with core spliceosomal proteins. Another interactor found in the study is SATB1 which is a regulator of chromatin remodeling and gene expression. This interaction provides an interesting link to localization of SDE2 to the nucleus, and further analysis of it can potentially advance the knowledge of conserved role of SDE2.

Interactions of Hs-SDE2 with a variety of proteins like SATB1, DCTN6, opsin 3 etc. indicate its potential roles in many other pathways. However, these interactions need to be verified using approaches like FRET or co – immunoprecipitation. Further analysis can also be done to understand what may control these interactions such as phosphorylation or any other post-translational modifications.

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