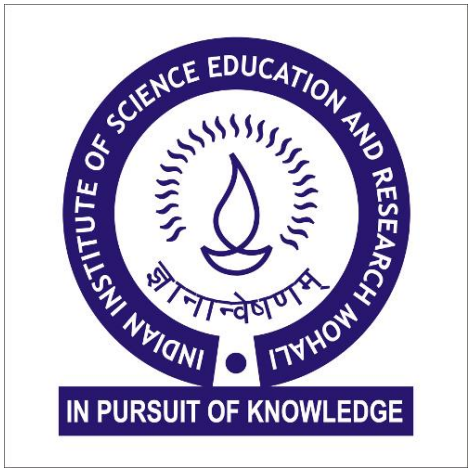
Role of an alternatively spliced form of the telomeric regulator Rap1 in *Schizosaccharomyces pombe*

PRAVER GUPTA

A dissertation submitted for the partial fulfilment of MS degree in Science



Indian Institute of Science Education and Research Mohali
April 2018

Certificate of Examination

This is to certify that the dissertation titled "**Role of an alternatively spliced form of the telomeric regulator Rap1 in *Schizosaccharomyces pombe***" submitted by Mr. Praver Gupta (Reg. No. MP15004) for the partial fulfillment of MS 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. Shravan Kumar Mishra (Supervisor)

Dr. Rajesh Ramachandran

Dr. Shashi Bhushan Pandit

Dated: April 20, 2018

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.

Praver Gupta
(Candidate)
Dated: April 20, 2018

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)

Acknowledgment

I would take this opportunity to express my sincere gratitude to my thesis supervisor Dr. Shravan Kumar Mishra who has been the biggest support to this project. He has been very patient and enthusiastic all through this time and has always encouraged me to push my limits.

A token of appreciation to my thesis committee members Dr. Shashi Bhushan Pandit and Dr. Rajesh Ramachandran for their invaluable suggestions and support.

A lot thanks to Prashant, Poonam, Poulami, Rakesh, Kiran, Karan, Anupa, Sumanjit, Bala and Asis, who have always been fun to work with while also always up for critical and insightful discussions. Special thanks to Prashant Pandit who was my very first mentor in the lab. He has fantastically taught me to produce quality results. I would also like to thank Vidya Bhaiya who silently works at the back and ensures that we are always full of clean and autoclaved supplies.

A special mention to MS lab members who have been always there to hangout and have fun. A separate mention ought to be for Rituraj, who has been one of the biggest supports in IISER. She has taught me a multitude of things which would be very difficult to mention in this short communication.

My bhangra team needs a special account to have enabled me to explore one of my best and exciting sides a.k.a Bhangra. Aashke rocks!

An informal mention to Happy Club, which has made the time spent in IISER a lot joyous. Hunar, Bhumi and Noor deserve a separate mention for being there at any time required.

There are no words to entail my Mom and Di's emotional support. Thanks for making me who I am.

I would like to thank IISER Mohali to have blessed me with this opportunity to work and learn in such an exciting research environment and for the financial support.

Praver Gupta

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Abstract

Eukaryotic chromosomes, being linear, are prone to hazardous consequences of chromosomal shortening, chromosomal circularization, chromosomal loss or non – homologous inter chromosomal recombination events with each cell cycle owing to the inherent nature of DNA replication and Repair process. In order to maintain proper chromosomal fidelity, cells employ intricate mechanisms to maintain telomeres which are complex in both space and time. Rap1 has been extensively studied as one of the most conserved telomeric factor which is necessary to maintain telomere heterochromatization, telomere position effect (TPE) and telomere identity by recruiting the Shelterin complex in eukaryotic cells. Here we report Rap1_N as an alternatively spliced form of the telomeric regulator, Rap1 in *Schizosaccharomyces pombe*. This isoform was first observed in the mutant of an intron – specific splicing factor, Sde2. Interestingly, Rap1_N became apparent in wild type scenario under heat stress at 37°C. On further functional analysis, it seems that Rap1_N acts as an independent backup telomeric silencing factor which becomes critical in times of stress when other factors might be compromised.

CHAPTER 1

1. INTRODUCTION

1.1. Precursor-messenger RNA (pre-mRNA) Splicing

Eukaryotes have been evolved with a "specialty" genome characterized by discontinuous protein coding sequences termed exons interspersed by non-protein coding sequences termed introns. Consequently, a given set of ∑ genes constituting the eukaryotic genome translate to a N + ∑ (generally spliced proteins) where N is the number of alternatively spliced protein products from the same set of genes. This N addition in the proteome leads to functional diversity as well as complexity in eukaryotes. Nevertheless, introns ought to be removed either fully or in combination to ensure the fidelity of cellular functions. This energy dependent enzymatic excision of individual introns is undertaken by the assembly, reaction and disassembly cycle of a large and highly dynamic RNA-protein complex termed the Spliceosome.

Alternative pre-mRNA splicing is the preferential removal of selected intron/s from a single mRNA molecule and is the central mechanism responsible for increasing the complexity in the protein repertoire of eukaryotes. This is a highly guided and regulated process which employs several spliceosomal factors for the splicing reactions to take place, while at the same time, several non-spliceosomal factors necessary to dictate splice site usage/selection or act as splicing enhancers or silencers. This complex network of regulation is what determines the outcome of a splicing event (Lee and Rio, 2015; Mishra and Thakran, 2018).

S. pombe Sde2 (Silencing Defective 2) had been characterized to be a nuclear protein essential for telomeric silencing and genomic stability (Sugioka-Sugiyama & Sugiyama, 2011). Recently, it has been shown to be an intron – specific splicing regulator required for the alternative splicing of the telomeric regulator, Rap1 (Thakran *et al.*, 2017).

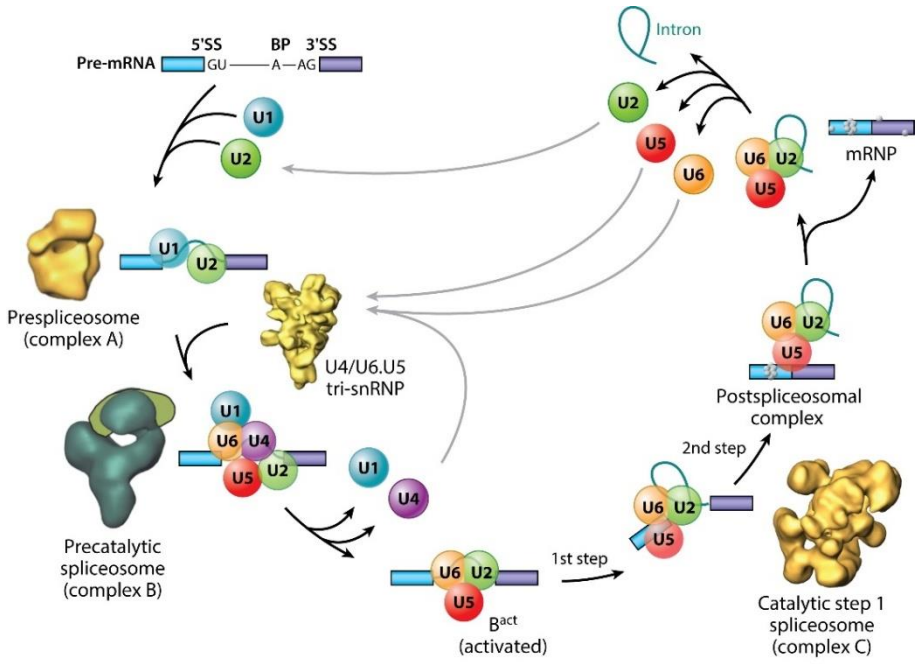


Figure 1.1: A detailed schematic of the Splicing Process. (Adapted from Lee and Rio, 2015)

1.2. Telomeres and significance of Telomeric Silencing

Telomeres constitute nucleoprotein structures which are constitutively heterochromatized chromosome end caps and are central in protecting linear chromosome ends in Eukaryotes. Since these ends carry 3’ overhangs, they are highly prone to DNA Damage Response (DDR) in accordance to these being corroborated for Double Strand Breaks (DSB) by the DNA repair machinery. Cells are thus protected from chromosome end degradation, erroneous recombination events or cell cycle arrest. Moreover, since eukaryotic chromosomes are linear, they impose the end replication problem wherein their replicative fidelity will be compromised at the chromosomal ends rendering them shorter with every replication cycle. Telomeric heterochromatin tricks the cell in not triggering a such a drastic and detrimental response. Several proteins and enzymes take part in maintaining telomere length and integrity. One of them viz. Telomerase is a reverse transcriptase which circumvents the end replication problem, supplementing lost telomeric repeats by synthesizing them over its integral RNA subunit as template and 3’ end of

chromosome as primer. In the absence of these mechanisms, chromosomes erode causing significant gene loss, priming them as sites of damage, massive genome instability, inappropriate chromosomal segregation and cellular senescence. Rap1, our protein of interest also is an integral part of telomeric module and is a negative regulator of telomere length (Kanoh and Ishikawa, 2001). Further details on Rap1 are elaborated in the next section.

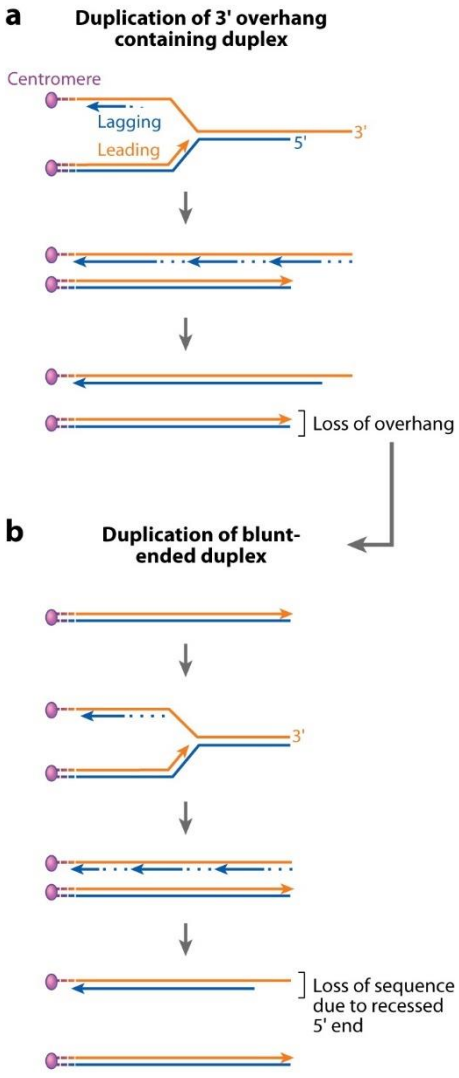


Figure 1.2 : The End Replication Problem. (Adapted from Jain and Cooper, 2010)

1.3. Repressor Activator Protein 1(RAP1)

Rap1(Repressor Activator Protein1) is a highly conserved telomere protein which was initially identified as a sequence specific transcriptional regulator in *Saccharomyces cerevisiae* that binds to the upstream activation site of the MAT alpha locus, $[(C)_{1-3}A]_n$ tracts at the telomeres and ribosomal protein genes. It can act as both an activator or a repressor depending upon its context of binding (Shore *et al.*, 1987; Kurtz *et al.*, 1991). In *S. cerevisiae*, Rap1p initiates transcriptional silencing at the telomeres and the mating type loci by recruiting Sir2/3/4 complex (Silent information Regulator) at the chromosome and is shown to maintain silent chromatin by stabilizing the Sir complex-nucleosome interactions (Moretti and Shore,2001). It also recruits Rap1 Interacting Factors, Rif1 and Rif2 to form the yeast shelterin like complex which protects the telomeres from DNA damage response processing and thus regulate telomere homeostasis (Wotton and Shore, 1997; de Lange T., 2009). *S. cerevisiae* Rap1p has also been shown to work in protecting the chromosome ends negatively regulating Non-Homologous End Joining (NHEJ) fusions at the telomere (Pardo *et al*., 2005). It has been shown in a *S. cerevisiae* senescence model strain (telomerase lacking) that the critical shortening of the telomeres causes Mec1 checkpoint kinase dependent Rap1 relocalization to the upstream promoter regions of many target genes including core histone coding genes driving the pace of senescence in addition to inducing global chromatin and gene expression changes (Platt *et al.,* 2013). Rap1p has been shown to bind DNA with its Myb-like domains with either high or low affinity modules in different stoichiometries modulated by Rap1 C-terminus (Feldmann *et al*., 2015).

Li *et al*. identified the human ortholog of scRap1p called hRap1 and showed that it shares three conserved sequence motifs with it, localizes to the telomeres via Trf2 and regulate telomere length (Li *et al.*, 2000). Moreover, Trf2 binding is crucial for endogenous hRap1 stabilization (Celli *et al.*, 2005). Additionally, mammalian Rap1 binds to telomeric and subtelomeric regions at $(TTAGGG)_2$ consensus motif (via Trf2) and is significantly involved in transcriptional regulation of subtelomeric loci genes (Martinez *et al.*, 2010). Teo *et al.* established a novel link between mammalian

Rap1 and NF-κB signaling reporting a cytoplasmic population of Rap1 devoid of Trf2 binding which was instead constitutively bound to I-κB kinases(IKKs), in turn activating NF-κB signaling (Teo *et al.*, 2010). One of the effects of this activation is positive regulation of Rap1 expression. A non-telomeric function of Rap1 was revealed in mice wherein it is required to control body weight and regulate metabolism which is independent of its binding to the telomeres via Trf2 (Yeung *et al*., 2013).

S. pombe ortholog of Rap1 has been described to be recruited to telomeres via Taz1 (ortholog of mammalian Trf1/2) and crucial for telomere length control, and meiotic telomere clustering towards the spindle pole body (Kanoh and Ishikawa, 2001; Chikashige and Hiraoke, 2001). *S. pombe* Rap1 has been shown to prevent chromosome end fusions by NHEJ, control telomeric 3' overhangs and telomerase inhibition in a Taz1 dependent manner (Miller *et al*., 2005). Fujita *et al.* identified the functional domains of spRap1 and established that all of the telomeric functions of Rap1 are via its RCT (Rap1 C-Terminus) domain which harbors the sites for its binding partners viz. Taz1 (required for Telomere length homeostasis), Poz1 (required for telomere end protection and gene silencing), Bqt1/2 (required for chromosomal bouquet formation during Prophase), Bqt4 (required for inner nuclear membrane tethering of chromosomes during interphase). Moreover, faithful segregation of chromosomes during closed mitosis has been shown to crucially depend on Rap1-phosphorylation (via Cdc2) dependent telomere dissociation from the nuclear envelope in fission yeast (Fujita *et al*., 2012; Kanoh J., 2013).

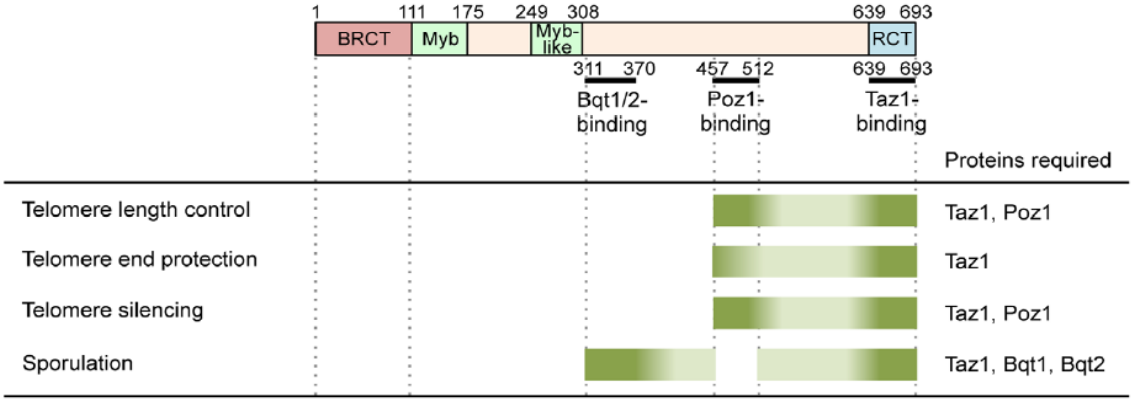


Figure 1.3: Functional domains of *S. pombe* Rap1. (Adapted from Fujita *et al.*, 2012)

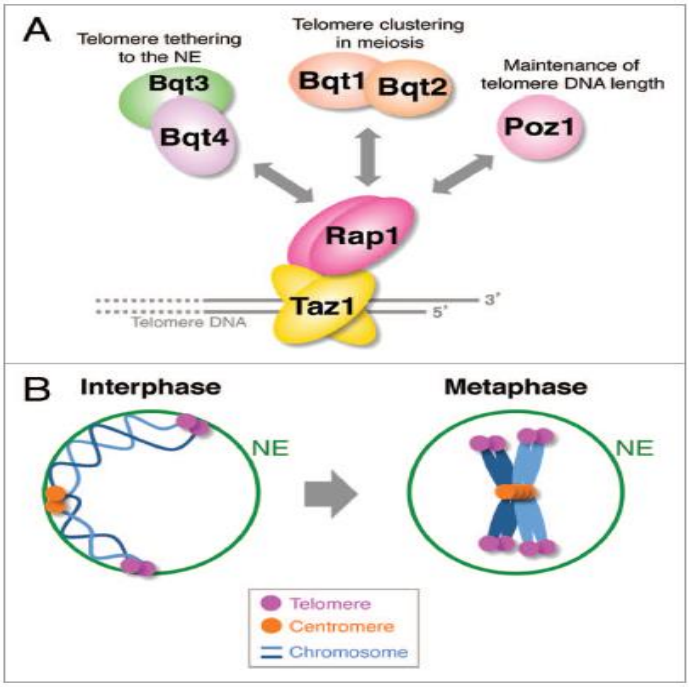


Figure 1.4: A) Physiological functions of Rap1 with its various protein partners in *S. pombe.* B) General chromosomal states in a fission yeast nucleus. (Adapted from Kanoh *et al.*, 2013)

By solving the crystal or solution structure of Rap1 RCT domain from diverse organisms with their binding partners and using comparative structural analysis, Lei and colleagues established RCT domain of Rap1 to be an evolutionarily conserved protein interaction domain which interacts with DNA binding proteins Trf2 in mammals and Taz1 in fission yeast to perform its telomeric functions while in budding yeast it is involved in recruiting Sir3 to the telomeres for transcriptional silencing; indicating the functional plasticity exhibited by this structurally conserved motif (Chen *et al*., 2011).

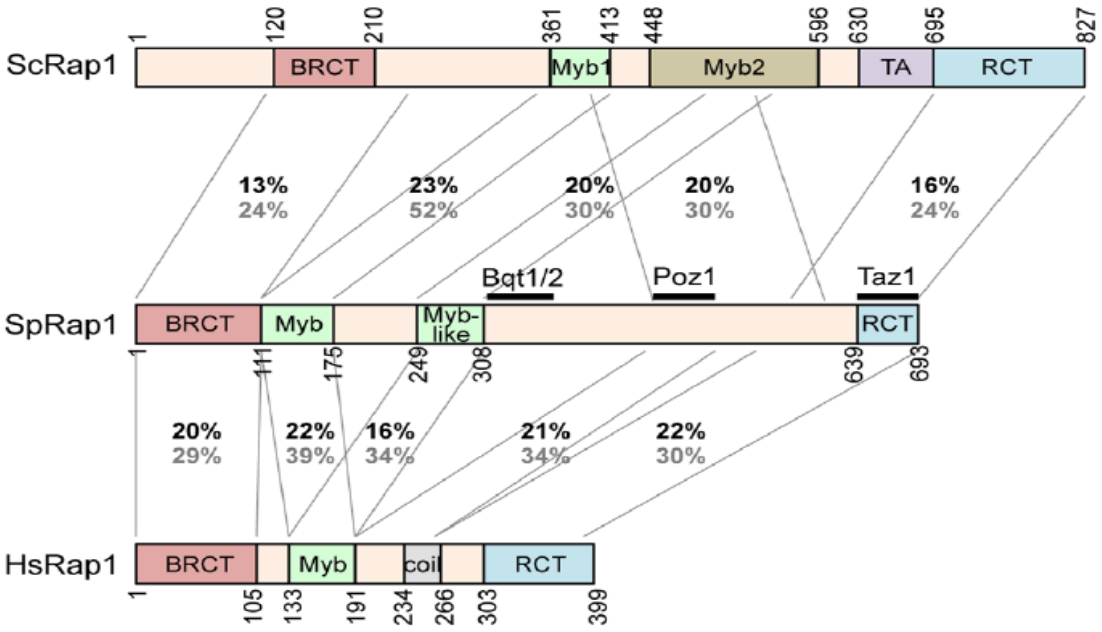


Figure 1.5: Sequence similarity amongst the different domains of Rap1 homologs. (Adapted from Fujita *et al.*, 2012)

1.4. Aim of the Study

Our lab recently reported *sde2*, a novel intron-specific pre-mRNA splicing factor which promotes the efficient splicing of a set of selective introns in *Schizosaccharomyces pombe* (Thakran *et al.*, 2017). It targets a subset of introns in a subset of genes which vary amongst their role in various cellular mechanisms. *S. pombe* Rap1 is one of the key targets of Sde2, the loss of which might result in the exhibition of *sde2* mutant phenotype. Intron specificity in Rap1 lies for intron-1 wherein when Sde2 is deleted, there is a preferable retention of intron-2 containing transcript. Interestingly, this particular transcript translates to a protein product. One of the most exciting observation was that this protein product also appears in wild type cells when they are given heat stress at 37°C. One would expect a wild type cell to not synthesize a stable protein in a non-permissive condition for no reason whatsoever. The protein ought to have a physiological relevance to the cell. That became the aim of this study.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Strains, plasmids and DNA techniques

Schizosaccharomyces pombe strains used in this study are listed in Appendix Table 3, and plasmids are listed in Appendix Table 1. Strains for telomeric silencing assays in Δ*sde2* strain (Sugioka-Sugiyama & Sugiyama, 2011) and Δ*rap1* (Kanoh and Ishikawa, 2001) were reported previously and obtained from NBRP-yeast, Japan. Preparation of *S. pombe* competent cells, transformation, chromosomal tagging, gene deletion and isolation of total proteins for Western blot (WB) assays by trichloroacetic acid (TCA) precipitation method was done following published protocols for S. cerevisiae (Knop *et al*., 1999; Janke *et al*., 2004). For growth and complementation assays, fivefold serial dilutions of cells were spotted on indicated agar plates until growth was seen. To shuffle-out ura4+ plasmids from *S. pombe*, 5-fluoroorotic acid (FOA; Zymo Research) (1.0 g/L of media) was used in agar plates.

2.2. Western Blotting

For immunoblot assays, total protein isolated from logarithmically growing 2.0 OD_{600} cells by either TCA precipitation or direct heating with 50µL HU buffer + 1µL β-mercaptoethanol, was separated on SDS–PAGE and transferred on PVDF membrane. For induction of proteins under *nmt1* promoter, primary cultures were set up in Synthetic Complete (SC) media (5µg/mL Thiamine) from which a secondary culture was grown in SC again until 0.7-0.9 OD_{600}. The required inoculum was washed twice with sterile water before inducing in EMM (Edinburgh Minimal Media, Formedium) media for indicated time points.

2.3. RNA isolation and RT–PCR (Reverse Transcriptase - Polymerase Chain Reaction)

RNA isolation and cDNA synthesis were done as described previously (Inada & Pleiss, 2010). Briefly, 2 OD_{600} cells (induced in EMM for time indicated) in log phase were harvested at 30°C by centrifugation. Total RNA was isolated by hot acid phenol method using 2-mL phase lock gel heavy tubes (5 prime), followed by DNase I (Promega) treatment for 15 min at 30°C. Zymo-Spin II column (Zymo Research) was used for clean-up of RNA. cDNA synthesis from 1µg total RNA was done using reverse transcriptase (RT) and random-hexamer primers (Invitrogen) at 42°C for 16 hours. PCR and the products were analyzed by agarose gel electrophoresis. Primers used in RT–PCR assays are listed in Appendix Table 2.

2.4. Genetic Assays

Yeast two hybrid screening was performed in a manner previously reported (Serebriiskii, 2010). Further details on the protocol are elaborated in the results section. The telomeric silencing assay is adopted from published methodology (Sugioka-Sugiyama & Sugiyama, 2011). Further details on the protocol are elaborated in the results section.

CHAPTER 3

3. RESULTS

3.1. Rap1 is alternatively spliced

In a splicing sensitive microarray, it was observed that rap1 intron-2 containing transcript is preferentially retained in Δ*sde2* strain relative of wild type both at 30°C and 37°C (Thakran *et al.,* 2017) which further gets translated to a 18.7 kDa protein (herein referred to as Rap1_N unless stated otherwise) stressing the fact that the aforementioned transcript ipso facto gets capped and polyadenylated. Intriguingly, Rap1_N becomes apparent in wild type scenario when the cells are given heat stress at 37°C from a tagged plasmid bound expression system (Karan, unpublished). To check whether Rap1_N also exists endogenously, we prepared an N-terminally 3xMyc tagged Rap1 (with introns) strain using an integrative vector, Infusion-pDUAL-nmt1. It was evidently observed that Rap1_N becomes apparent when the cells are heat treated. It is noteworthy to observe that the full length Rap1 decreases as Rap1_N increases with the increasing time of heat shock as compared to untreated control which suggests that the Rap1 intron-2 containing mRNA transcript (which translates to Rap1_N) is retained at the cost of the fully spliced intronless transcript (which translates to full length Rap1). However, it is fascinating to observe that towards the later time points (24 hours and 30 hours) of heat shock, full length Rap1 restores to its steady-state level. This may either be due to Rap1 full length protein stabilization or increased transcription of rap1 gene altogether that the cell might employ in order to retain its integrity and survive in such a limiting condition.

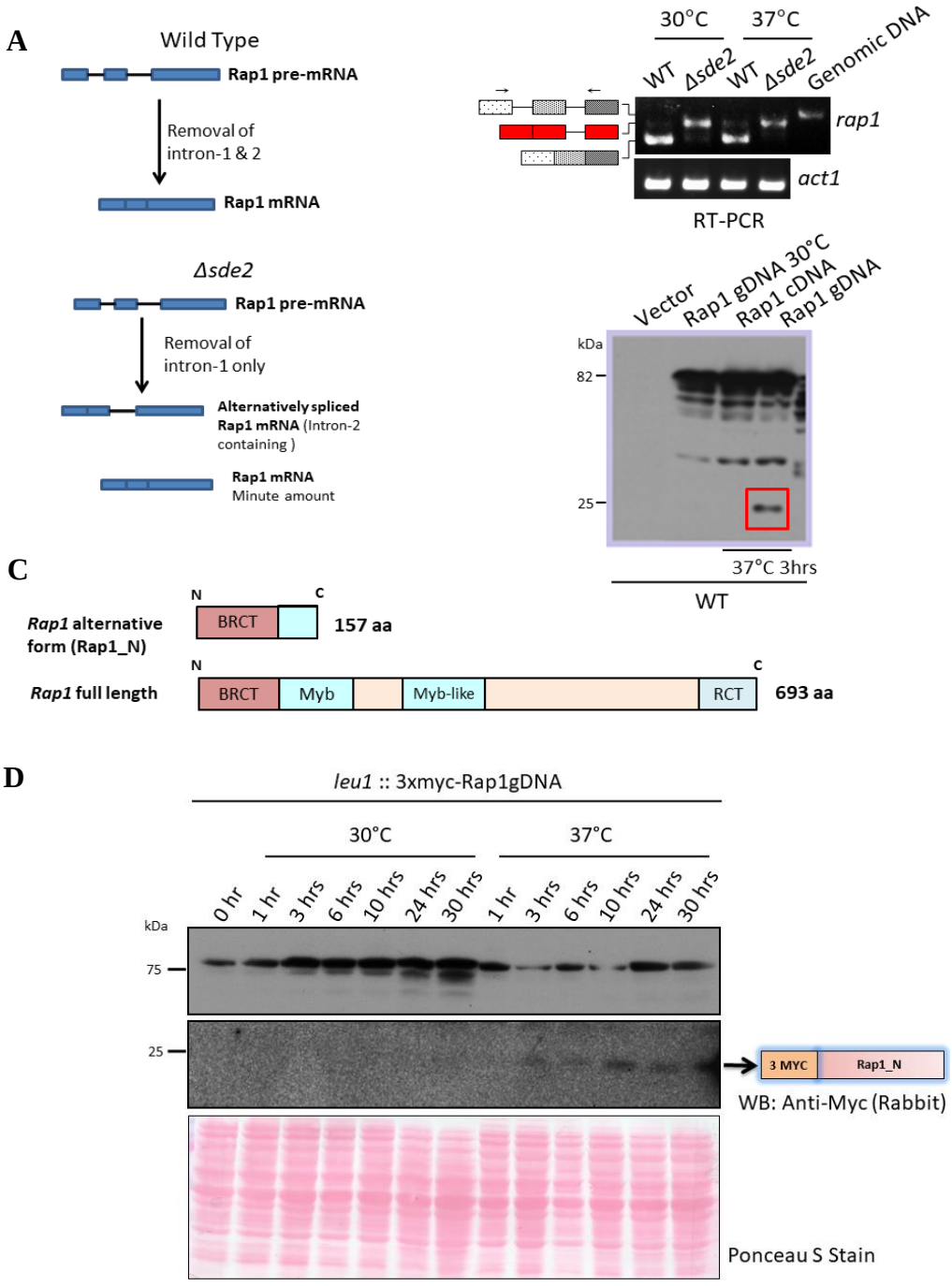


Figure 3.1: Rap1 is alternatively spliced. A) Schematic representing fundamental differences in splicing of *rap1* in WT and Δ*sde2* strains of *S. pombe.* B) Representative RT-PCR depicting the retention of Intron-2 containing transcript in Δ*sde2* (Courtesy Poonam Thakran) and Western Blot depicting the production of plasmid borne Rap1_N from the aforementioned transcript in WT at 37°C. C) Domain architecture of Rap1 alternative form and Rap1 full length. D) Representative immunoblot depicting alternatively spliced Rap1 under heat stress (37°C) at the indicated time points with 30° C as control. Ponceau S stained membrane as loading control.

3.2. Yeast two Hybrid with Rap1_N did not reveal any of its physical targets

In order to identify any physical interactor/s of Rap1_N, we performed a Yeast two hybrid screening with the chimera of Rap1_N covalently attached to the Gal4 Binding Domain (Gal4BD) as the Bait against an *S. pombe* two hybrid library (total protein attached to the Gal4 Activation Domain(Gal4AD)) as the prey, co-transformed in PJ69-7a two hybrid reporter strain. In a screen of more than 2 x 10^6 transformants, we obtained 384 putative positives which showed significant growth on 140mm SC-Leu-Ura-His selection plates. After shuffling out the bait vector by streaking the putative colonies on SC-Leu + 0.1% FoA (5-Fluroorotic Acid), the colonies were respotted on SC-Leu-Ura plates to ensure complete loss of the bait vector (Negative control), SC-Leu as Positive control and SC-Leu-His as an assay to eliminate the autoactivating preys as false positives. Out of the 384 putatives, only 8 colonies turned out to be putative true positives. We then isolated the prey plasmids from these putatives and electroporated in *E. coli* DH5α strain. Two clones from each transformed putative was then amplified and co-transformed individually with both pGBDU-C1 Empty and Rap1_N chimera vectors back in the yeast two hybrid reporter strain. The obtained co-transformants were subsequently dilution spotted on SC-Leu-Ura control as well as SC-Leu-Ura-His test plates to recheck their interaction capabilities. Surprisingly, both clones for each putative positive turned out to be false positive as we could not see any growth on the test plates and we could not recover any true physical interactors of Rap1_N in this screen.

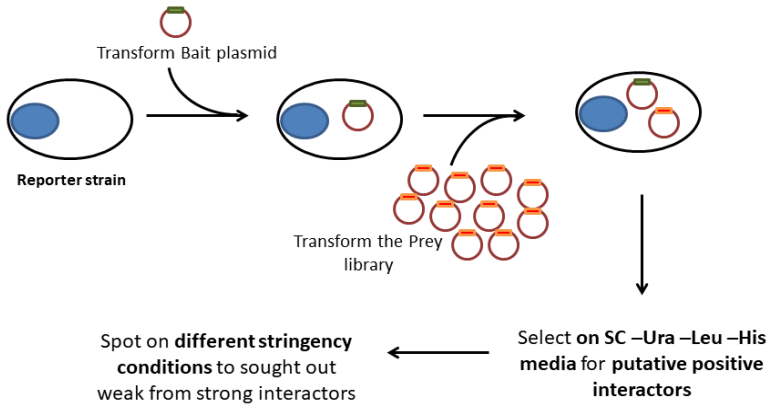


Figure 3.2: Schematic work flow of Yeast Two Hybrid screen.

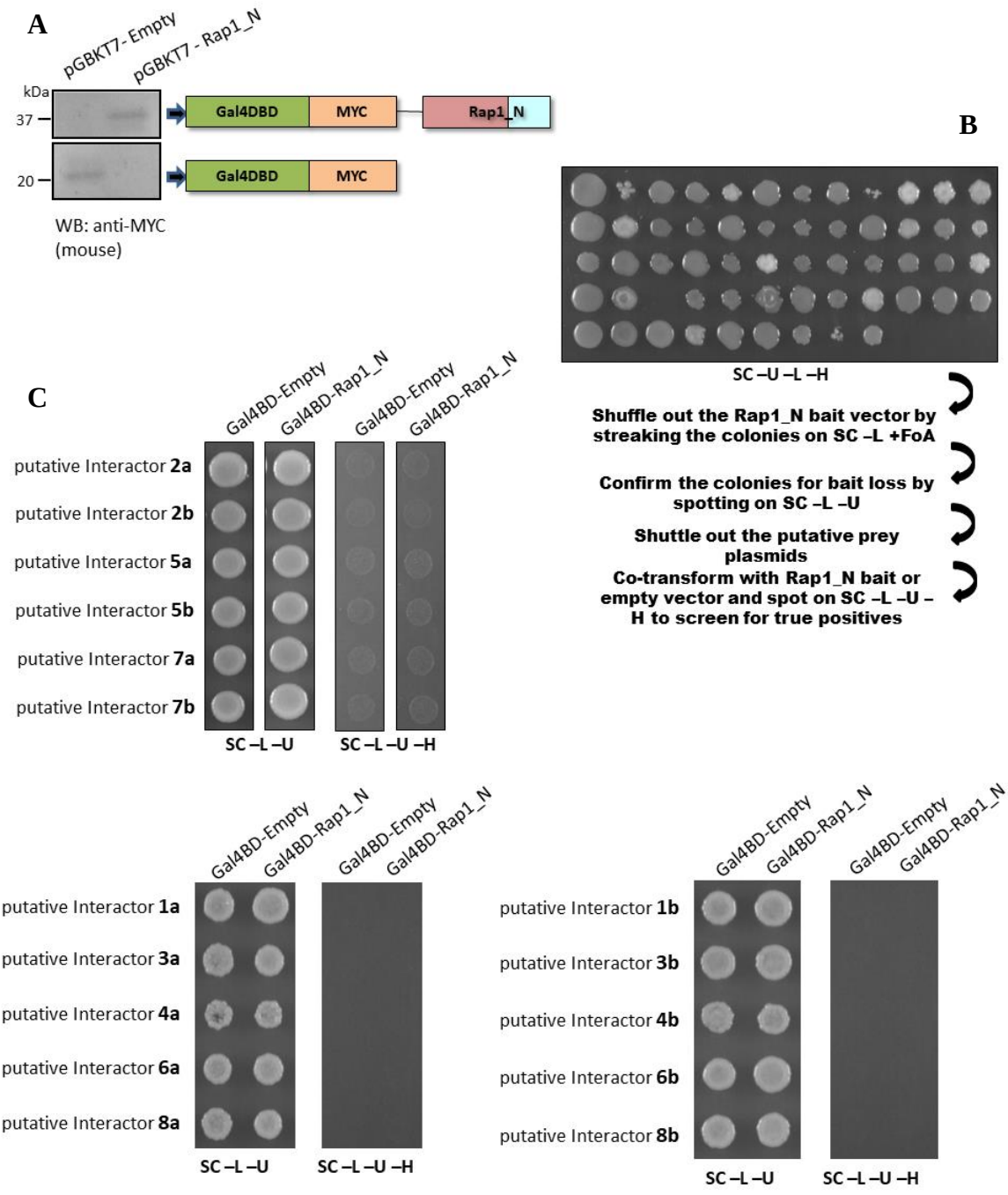
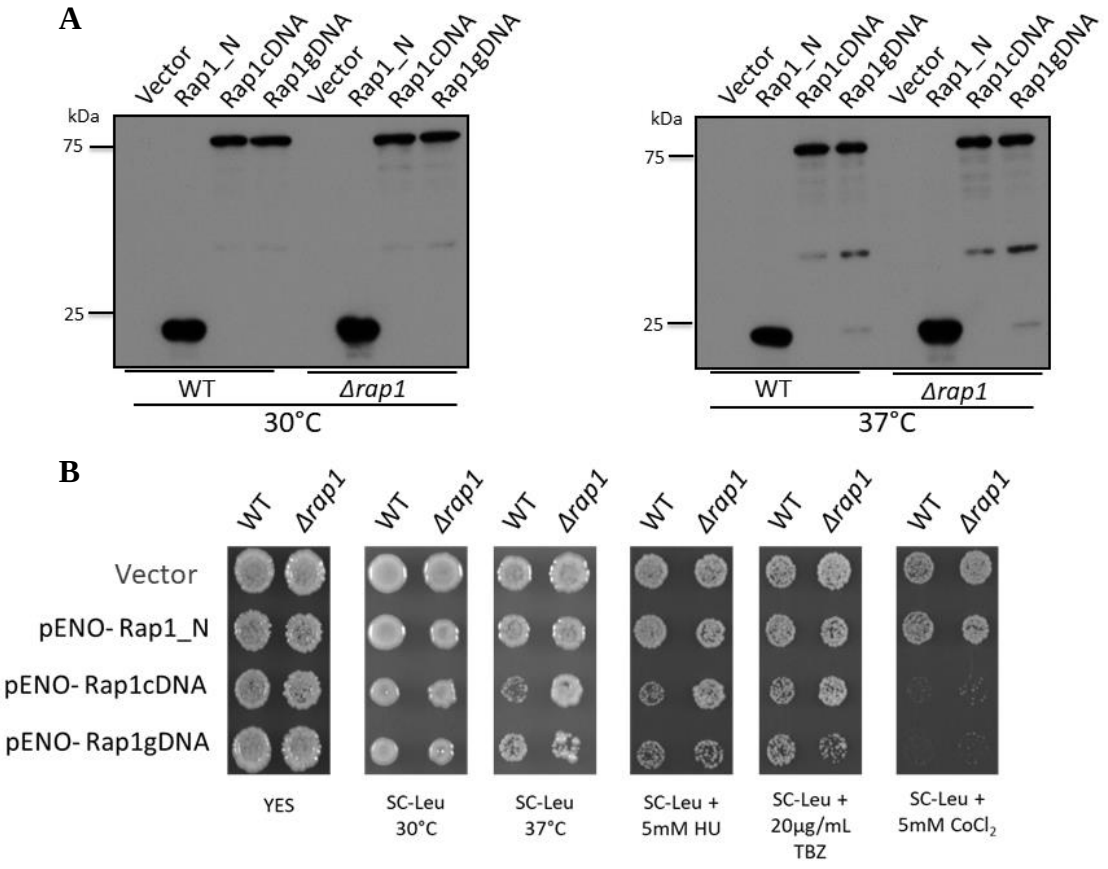


Figure 3.3: Yeast two hybrid screening with Rap1_N. A) Western blot depicting the expression of Gal4BD-Rap1_N chimera. B) Representative images of the putative positive transformants grown on selection plate (upper plate) and the putative positives spotted on SC-L-U after shuffling out the Ura positive bait plasmid (lower plate). C) Two clones of each of the non-autoactivating putative positive transformant was co-expressed with Gal4BD-Empty and Gal4BD-Rap1_N simultaneously and spotted on SC-L-U-H selection plates to check for true interaction.

3.3. Rap1 differential forms expressed in Δ*rap1* do not show a specific phenotype

In order to look for any *rap1* "form" specific phenotype, we overexpressed its various differential forms viz. Rap1_N, Rap1cDNA (full length Rap1), Rap1gDNA (genomic construct with both the introns; which is capable in supplementing both forms) in Δ*rap1* background. We employed two sets of scenarios: extreme overexpression with *eno101* promoter and mild overexpression with *rap1* endogenous promoter. We first checked the expression pattern for each clone in wild type control as well as Δrap1 by western blotting. Then, to obtain any form specific phenotype, we dilution spotted them on non-permissive conditions such as Hydroxyurea (HU) which is a DNA synthesis inhibitor, Thiabendazole (TBZ) which is a microtubule depolymerizing drug, $CoCl_2$ which is a hypoxia mimicking agent and heat stress at 37°C. As compared to wild type there was no apparent phenotype exhibited in response to any of the differential rap1 forms in the conditions tested.



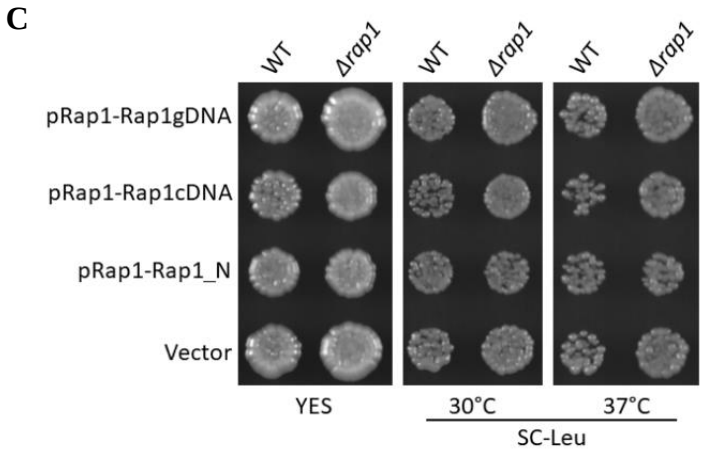


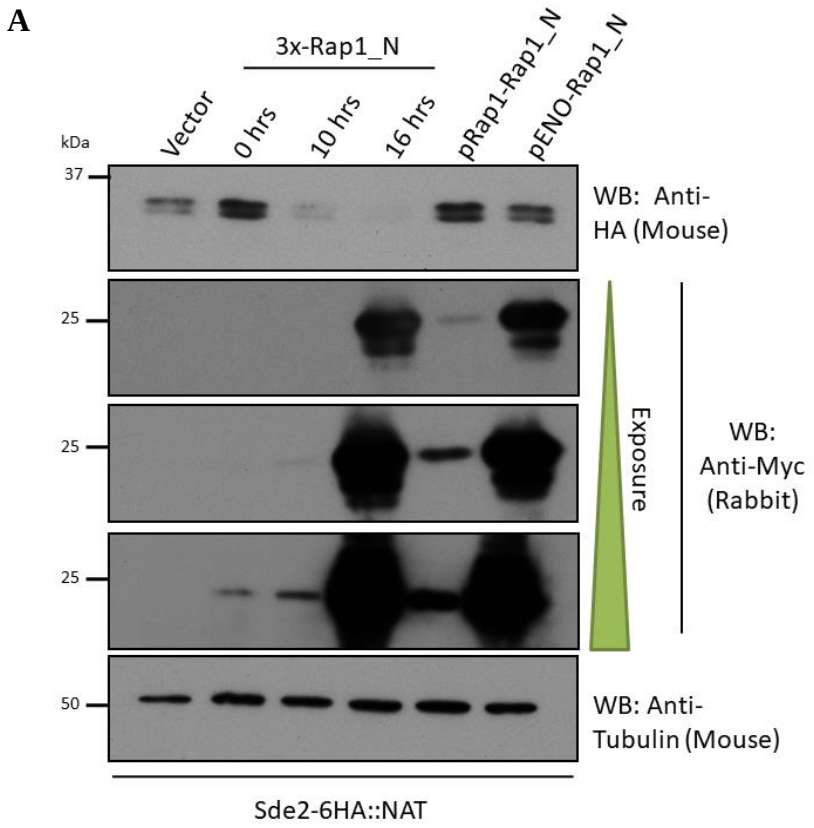
Figure 3.4: Rap1 differential forms expressed in Δrap1 do not show a specific phenotype. A) Western blot analysis to check the expression of indicated clones in WT and Δ*sde2* strains at 30°C and 37°C respectively. B) Dilution spotting of WT and Δ*rap1* strains transformed with indicated Rap1 forms under *eno101* promoter on indicated solid agar plates. C) Dilution spotting of WT and Δ*rap1* strains transformed with indicated Rap1 forms under *rap1* promoter on indicated solid agar plates.

However, it is noteworthy to observe that overexpression of both Rap1cDNA and Rap1gDNA constructs lead to lethality in the presence of $CoCl_2$ which is a hypoxia mimicking agent, irrespective of the genetic background. Further experiments need to be performed to explain this observation.

3.4. Rap1_N induction does not affect Sde2 levels

We speculated that Rap1_N might have a role in regulating Sde2 levels *in vivo.* To test this hypothesis, we expressed Rap1_N constitutively or under the thiamine repressible *nmt1* promoter in chromosomally C-term 6-HA tagged *sde2* wild type strain and followed Sde2 levels in response to Rap1_N induction in EMM (Edinburgh Minimal Media) by western blotting. It was surprising to see that as we induce Rap1_N expression, there is a concomitant decrease in Sde2 level which is intriguingly unchanged when Rap1_N is constitutively expressed. We wondered if this change in protein level, is a consequence of decreased transcription or decreased protein translation/stability. To answer this, we

synthesized cDNA from total RNA isolate of each sample and performed semi-quantitative PCR. It was surprising to see that even though Sde2 seems to decrease, *sde2* transcript level does not change as a function of Rap1_N induction time. This is an indication that Rap1_N effect on *sde2* whatever may be, is atleast not on its transcription. We speculated whether this decrease in Sde2 leads to any *sde2* null like phenotype since Δ*sde2* characterizes drastic growth phenotypes (Thakran *et al.,* 2017). To test this, we passaged the cells on EMM for maximal Rap1_N induction so as to see dramatic phenotypes if any. It was surprising to see that under any conditions where *sde2* deletion would show an intense phenotype, the Rap1_N induced cells fail to show a significant growth defect. This might be an indication that since both the detected proteins viz. 3xMyc-Rap1_N and processed Sde2-6HA have similar sizes, very high Rap1_N induction is resulting to a masking effect on Sde2 detection in the western blot analysis. Indeed, it turned out to be the case when we performed similar experiment but in a strain with Sde2 C-term EGFP (Enhanced Green Fluorescent Protein) tag.



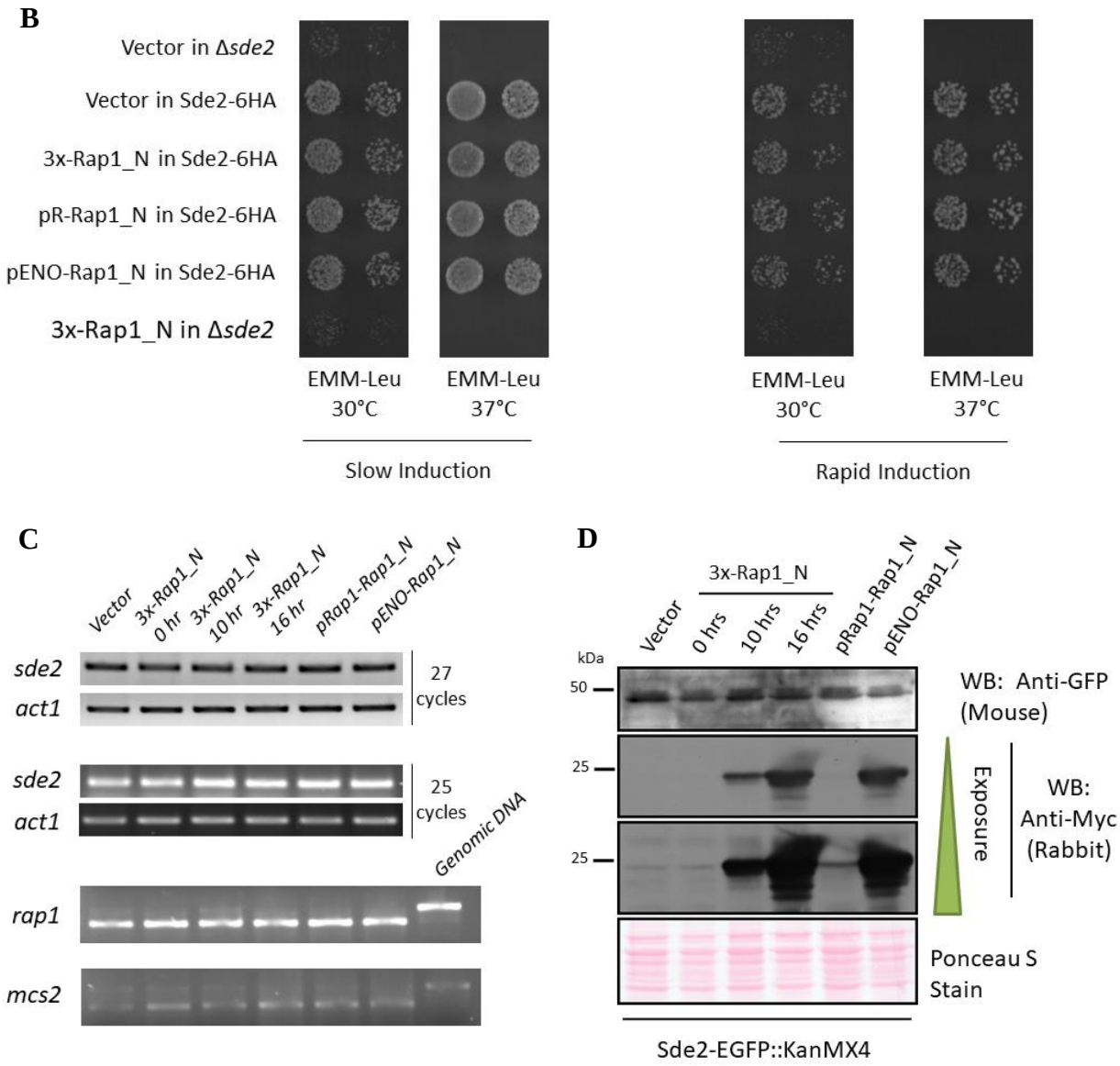


Figure 3.5: Effect of Rap1_N on Sde2. A) Representative western blots depicting Sde2-6HA levels in response to Rap1_N induction. 3xMyc-Rap1_N is expressed under *nmt81* promoter and induced in EMM for indicated time points. Tubulin is taken as loading control. B) Dilution spotting for the same transformants to assess if they show any of the *sde2* loss phenotype. Slow induction panel indicates that the cells are streaked on EMM solid media and then used for the spot analysis. Fast induction panel indicates that the cells were cultured in liquid EMM up till saturation and then used for spot analysis. Δ*sde2* row is taken as negative control. C) Semi-quantitative RT-PCR to check *sde2* transcript level and Sde2 specific splicing defects for *rap1* and *mcs2* in response to Rap1_N induction for indicated time points. Actin is taken as control. D) Representative western blots depicting Sde2-EGFP levels in response to Rap1_N induction. 3xMyc-Rap1_N is expressed under *nmt81* promoter and induced in EMM for indicated time points. Ponceau stained membrane is taken as control.

The result clearly indicates that Rap1_N has no effect on Sde2 levels when induced or otherwise. The earlier observation was a result of processed Sde2-6HA and Rap1_N being of almost equal sizes causing the inefficient detection of endogenous Sde2-6HA in response to Rap1_N induction.

3.5. Rap1_N shows a significant rescue of telomeric silencing defect in a sensitized background

Rap1 has been extensively studied and is shown to be a critical factor in telomere biology. It was nominal to speculate that Rap1_N might have a role along the same lines. To test this, we employed a telomere silencing reporter strain which has *ura4* cloned into the telomeric repeats and can be analyzed for any defects in telomeric silencing in response to a factor. We prepared different backgrounds for the strain and tested Rap1 differential forms in those backgrounds. In wild type and Δ*rap1* background, we do not see any significant change in telomeric silencing paradigm which indicates that in part, none of the Rap1 forms lead to silencing defect. In Δ*sde2* background, there is a dramatic silencing defect (Thakran *et al*., 2017) which is not rescued by any of the Rap1 constructs indicating the involvement of other telomeric factors regulated by *sde2* and consequently, the intensity of defect may not be rescued by Rap1 alone. The exciting observation is for the dramatic rescue for Δ*sde2* Δ*rap1* by Rap1_N, which is supported by its no growth on –Ura media while extensive growth on the counter selection on +FoA media plates. Δ*sde2* Δ*rap1* genetic background may very well be thought of as one which is sensitized to massive telomeric silencing defect since both Sde2 and Rap1 (both of which are critical in telomere maintenance in very different ways) are removed. Intriguingly, Rap1_N preferentially rescues this defect over full length Rap1 which indicates that there might be other *sde2* dependent factors that work coherently with Rap1 and that Rap1 might have a negative regulatory effect on Rap1_N. Nevertheless, this needs careful investigation and discussion.

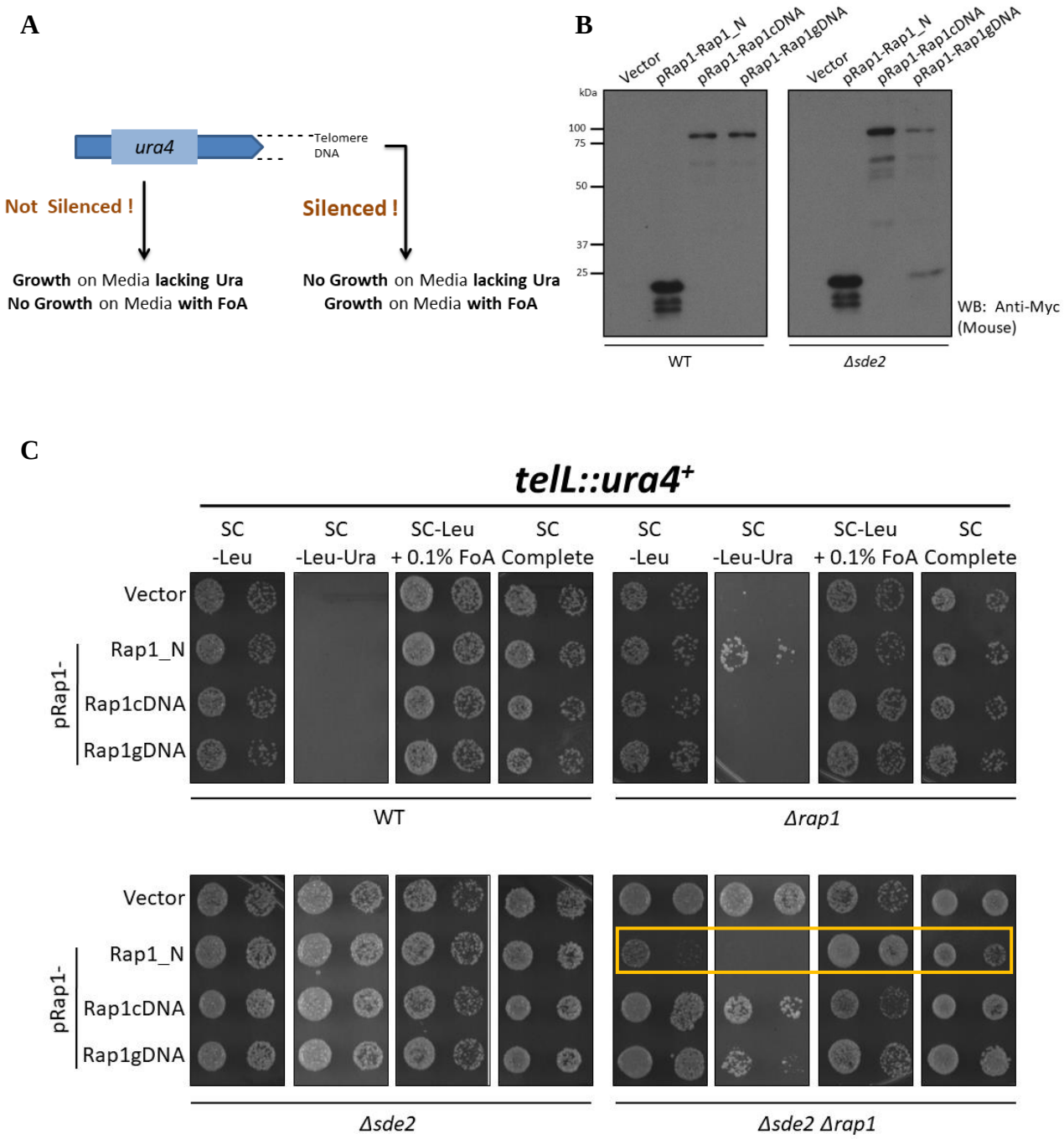


Figure 3.6: Rap1_N shows a significant rescue of telomeric silencing defect in a sensitized background. A) A schematic depicting the possible outcomes of a Telomeric Silencing Assay. B) Representative western blot to check the expression of indicated clones in the telomere silencing reporter strains. C) Telomeric silencing assay performed in four different genetic backgrounds carrying the indicated Rap1 forms expressed via a plasmid under *rap1* promoter. SC-Leu and SC-Complete plates serve as spotting and growth controls. Rap1_N rescues telomeric silencing defect in Δ*sde2* Δ*rap1* reporter strain indicated in the highlighted box.

CHAPTER 4

4. DISCUSSION

Telomeric structures are fascinatingly complex in their regulation and maintenance. Sde2 as a novel splicing factor required for the efficient splicing of many telomeric factors was recently reported (Thakran *et al.*, 2017). We are only beginning to comprehend its multitude of implications. Rap1, a bonafide telomeric regulator, is one of the strongly affected targets of *sde2* deletion, the result of which is a preferential retention of intron-2 in *rap1* mRNA transcript. This implies that there is a preference for *rap1* intron-1 over intron-2 for splicing via *sde2*, a phenomenon termed as Intron-specific pre-mRNA splicing. Fascinating is to observe that this "intron-2 retained" transcript is very well translated to protein product (Rap1_N) which is apparent in wild type cells under heat stress. To further analyze for Rap1_N function, we tried to find its protein interactor/s but it seems that it doesn't interact very strongly with a protein partner. Moreover, Yeast two hybrid analysis is inherently not a very sensitive assay and thus, its difficult to rule out the possibility that Rap1_N does interact with other factors.

Telomeric silencing assay with different genetic backgrounds suggests that in *Δsde2 Δrap1*, Rap1_N overexpression leads to efficient rescue of the silencing defect. One might ask at this point that why doesn't Rap1 itself rescue this defect in the first place. This discrepancy might be explained by the possibility that the telomeric silencing function of Rap1 inculcates other Sde2 dependent factors which are also compromised in *sde2* single deletion. Moreover, in Δ*sde2* it seems that the basal expression of Rap1 full length leads to a negative regulatory effect on Rap1_N. In consortium with this, *sde2 rap1* double deletion offers a clean background and consequently Rap1_N overexpression independently eases the telomeric silencing defect. In a cellular context, it makes sense that a structure as critical as the Telomere is under strict regulation. This regulation is complex in the involvement of different protein sets dedicated to maintain its identity. Nevertheless, in this regulatory network, it is enthralling to speculate the interplay of two protein isoforms of the famous telomeric regulator, Rap1.

Appendix

Table 1: List of Plasmids

S. No.	Plasmid
1	pREP81x-3Myc vector
2	pREP81x-3Myc-Rap1_N
3	pREP81x-3Myc-Rap1cDNA
4	pREP81x-3Myc-RapgDNA
5	pENO-3Myc vector
6	pENO-3Myc-Rap1_N
7	pENO-3Myc-Rap1cDNA
8	pENO-3Myc-Rap1gDNA
9	pRAP1-3Myc vector
10	pRAP1-3Myc-Rap1_N
11	pRAP1-3Myc-Rap1cDNA
12	pRAP1-3Myc-Rap1gDNA
13	pGBDU-C1 vector
14	pGBDUC1-Rap1_N
15	pGBKT7 vector
16	pGBKT7-Rap1_N
17	pREP3x-Rap1_N

Table 2: List of Primers used for RT-PCR

S. No.	Primers (5' - 3')	Description
1	CCCCTAGAGCTGTATTCCC	*act1* forward
2	CCAGTGGTACGACCAGAGG	*act1* reverse
3	CGTAGAAACGTTGTTAATGCACGTCCTGGGCGATTC	*sde2* forward
4	CTTTATTTAATTTCTGCCTTCTAGCTTCTTTCTTTGCG	*sde2* reverse
5	AAAAACTTTGAACATATTAGGGG	*rap1* forward
6	CTTATAATGTTGCCGCCAGG	*rap1* reverse
7	GCACTTTCTTCCGCTCTTTCC	*mcs2* forward
8	TTTCGGAAGCACTGTTTGACAATC	*mcs2* reverse

Table 3: List of Strains

S. No.	Strain	Organism
1	PJ69-7a	*S. cerevisiae*
2	h- *ade6*-M216 *leu1 ura4*-D18 (Wild type)	*S. pombe*
3	*leu1*::3xmyc-Rap1gDNA *Δrap1*::Nat-NT2	*S. pombe*
4	h-JY741 *sde2*–6HA::Nat-NT2	*S. pombe*
5	h+ *Δrap1*::KanMX4	*S. pombe*
6	h- *Δrap1*::Nat-NT2	*S. pombe*
7	h+ JY741 *Δsde2*:: Nat-NT2	*S. pombe*
8	h+ *leu1-32 ura4DS/E his3 ade6*-D1 *ade6*-M210 *otr1R*::*ade6+ tel1L*::*his3+ tel2L*::*ura4+ sde2Δ::*kan	*S. pombe*
9	h+ *leu1-32 ura4DS/E his3 ade6*-D1 *ade6*-M210 *otr1R*::*ade6+ tel1L*::*his3+ tel2L*::*ura4+ sde2Δ::*kan *sde2(WT)*::Nat-NT2	*S. pombe*
10	h+ *leu1-32 ura4DS/E his3 ade6*-D1 *ade6*-M210 *otr1R*::*ade6+ tel1L*::*his3+ tel2L*::*ura4+ sde2Δ::*kan *rap1Δ*::Nat-NT2	*S. pombe*
11	h90 *rap1*::kanr *ade6*-D1 *his3*-D1 *leu1*-32 *ura4*-D18 *otr1R(Sph1)*::*ade6+* TAS-*his3+-tel1(L)* TAS-*ura4+-tel2(L)*	*S. pombe*
12	h-JY741 *sde2*–EGFP::KanMX4	*S. pombe*
13	DH5α	*E. coli*

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