Functional diversification of the yeast telomere protein, Rif1, to an anti-apoptotic factor in *Drosophila melanogaster*

A Thesis submitted to the University of Hyderabad for the award of a Ph. D. degree in Department of Biochemistry, School of Life Sciences

By

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DECLARATION

I, E. Sreesankar, hereby declare that this thesis entitled **"Functional diversification of the yeast telomere protein, Rif1, to an anti-apoptotic factor in *Drosophila melanogaster*"** submitted by me under the guidance and supervision of Dr. Krishnaveni Mishra, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Functional diversification of the yeast telomere protein, Rif1, to an anti-apoptotic factor in *Drosophila melanogaster*"** is a record of bonafide work done by E. Sreesankar, a research scholar for Ph.D. programme in Department of Biochemistry, School of Life Sciences, University of Hyderabad under my guidance and supervision.

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List of abbreviations

53BP1	p53 binding protein 1
5-FOA	5-Fluoroorotic acid
AMP	Ampicillin
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-related
BLM	Bloom syndrome
BSA	Bovine serum albumin
CDC	Cell Division Cycle
CY3	Cyanine 3
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	Double Strand Break
dsRNA	double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ES cells	Embryonic Stem cells
ESC1	Establishes silent chromatin1
EST	Ever Shorter Telomeres
HCl	Hydrochloric acid
HipHop	HP1-HOAP-interacting protein
HOAP	HP1/ORC-associated protein
HP1	Heterochromatin Protein 1
hRif1	human Rif1
HRP	Horseradish peroxidase
HTT	HeT-A, TART, TAHRE
kb	kilobase
KOH	Potassium hydroxide

LB	Luria-Bertani broth
LiAC	Lithium Acetate
LTR	Long Terminal Repeat
Mec1	Mitosis Entry Checkpoint
MMS	Methyl methane sulfonate
MRE11	Meiotic Recombination 11
mRif1	mouse Rif1
MRN	Mre11-Rad50-Nbs1
mRNA	Messenger RNA
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NHEJ	Non-homologous end joining
ORC	Origin recognition complex
ORF	Open reading frame
PCR	Polymerase chain reaction
Pol	Polymerase
PVDF	Polyvinylidene Fluoride
RAD	RADiation sensitive
Rap1	Repressor activator protein 1
Rif1	RAP1-interacting factor 1
Rif2	RAP1-interacting factor 2
RNA	Ribonucleic acid
RNAi	RNA interference
SC	Synthetic complete
scRif1	*Saccharomyces cerevisiae* Rif1
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SIR	Silent Information Regulator
siRNA	small interfering RNA
SOB	Super optimal broth
spRap1	*Schizosaccharomyces pombe* Rap1
spRif1	*Schizosaccharomyces pombe* Rif1

ssDNA	single stranded DNA
Stn1	Suppressor of cdc ThirteeN
TCA	Trichloroacetic acid
Tel1	TELomere maintenance
TPE	Telomere position effect
Tris	Tris(hydroxymethyl)-aminomethan
URA3	Orotidine-5'-phosphate (OMP) decarboxylase
WT	Wildtype
YPD	Yeast extract peptone dextrose
yRif1	yeast Rif1

CHAPTER 1

Introduction

1.1 Concept of telomere and role of telomere

Telomeres are the ends of linear eukaryotic chromosomes and contribute in multiple ways to genome stability. The concept of telomere has evolved through several studies beginning with the observations of Muller and McClintock that ends of chromosomes respond differently to chromosomal breaks compared to internal regions (Reviewed in Louis, 2002). Later on Elizabeth Blackburn, working with *Tetrahymena* chromosomes, identified DNA sequences that was repeated several times at the ends of the chromosomes. Jack Szostak fused these repeats isolated from *Tetrahymena* to yeast minichromosomes and found that it can protect the minichomosomes from degradation in yeast (Szostak & Blackburn, 1982). As telomere DNA from *Tetrahymena* protected chromosomes in an entirely different organism, yeast, this demonstrated the importance of this repeat sequence at the end of the chromosomes. Greider and Blackburn went on to identify telomere specific reverse transcriptase enzymatic activity which add the telomeric repeats and purified the RNA and protein components (Greider & Blackburn, 1985; Greider & Blackburn, 1989).

The ends of most eukaryotic chromosomes consist of simple repeat sequences that are added by a specialized reverse transcriptase, telomerase, that are usually variations of TTAGGG on the 5′ to 3′ strand. DNA binding proteins that specifically recognize this sequence bind to it and these proteins in turn recruit a variety of different proteins that together participate in a) replicating the telomere sequence during cell division and also maintaining the length to a genetically determined level, b) protect the chromosome from nucleolytic attacks and end to end fusion, c) maintain a unique structure that is refractile to both transcription and recombination (Shore & Bianchi, 2009; Smogorzewska & de Lange, 2004).

1.1.1 Telomere length regulation and telomerase

Conventional DNA polymerases are unable to replicate the very end of linear DNA because they cannot initiate synthesis de novo and instead require short primers. In

order to maintain these sequences, eukaryotes use a specialized self-templated reverse transcriptase called telomerase. The G-rich strand is synthesized by telomerase and the C-strand is elongated by the conventional lagging strand synthesis. Telomerase binding and activation is cell cycle dependent and is regulated by several interacting proteins (Fisher et al, 2004). Additionally, these proteins, through a complex interplay of interactions between them and the telomerase, ensure that shorter telomeres are preferentially elongated and long telomeres go through the cell cycle without elongation. In many higher eukaryotes, including mammals, the very end of the telomere consists of short single stranded stretches that are bound by single strand DNA binding proteins and folds into a specialized t-loop (Griffith et al, 1999). Lower eukaryotes, which usually have shorter stretches of telomere repeat sequences, do not form t-loops but their ends are also protected by ssDNA binding proteins. Thus the basic mechanism of capping ends of chromosomes is conserved across species; however, there are differences in details.

1.1.2 Telomere and DNA damage response

Although telomeres can be considered as double strand break they evade the double strand break response by the action of many proteins which bind to the telomere and prevent the DNA damage induced cell cycle arrest. There is increasing evidence that many DNA damage response proteins, particularly those involved in repairing double strand breaks, are involved in telomere maintenance. Cells defective in DNA double strand break repair proteins including Ku, DNA-PKC, ATM/ATR kinases, RAD51 and the MRN-complex show loss of telomere capping function.

1.1.3 Telomere and Telomere Position Effect (TPE)

Another conserved property of telomeres is gene silencing, initiated by telomeric repeats, often called Telomere Position Effect (TPE). Genes placed proximal to

telomere repeats are transcriptionally silenced by the binding of heterochromatin proteins (Reviewed in Ottaviani et al, 2008). In yeast, Sir-complex is recruited to the telomere through the combined action of the repeat-binding protein Rap1 and Yku70/80, which directly interact with Sir4. Sir4 recruits Sir2, an NAD-dependant histone deacetylase, that deacetylates the histones. Deacetylated histones are bound by Sir3-Sir4 and this action leads to more Sir2 being recruited leading to further deacetylation and propagation of this complex. This structure is refractory to transcription and thus establishes silent chromatin. TPE has been observed in several organisms including parasites (Freitas-Junior et al, 2005) *Drosophila* (Gehring et al, 1984; Hazelrigg et al, 1984; Levis et al, 1985), human (Baur et al, 2001; Koering et al, 2002). There are functional homologues of Sir proteins present in other organisms to maintain the TPE such as Heterochromatin Protein 1(HP1) in fission yeast and *Drosophila* (Kanoh & Ishikawa, 2001; Wallrath & Elgin, 1995).

Thus telomeres have three distinct properties associated with them that are conserved from yeast to mammals – a) a special reverse transcriptase based mechanism to maintain telomere, b) involvement of DSB-repair proteins to protect the telomere ends and finally c) transcriptional silencing.

1.2 Different types of telomere

Though the concept of telomere is common and all the linear chromosomes essentially protect the chromosome end, there are variations with the canonical telomerase mediated mechanisms. As shown in figure: 1, canonical telomeres have a G-rich telomere repeat sequences added by telomerase and most of the organisms including humans follow this mechanism to maintain the telomere.

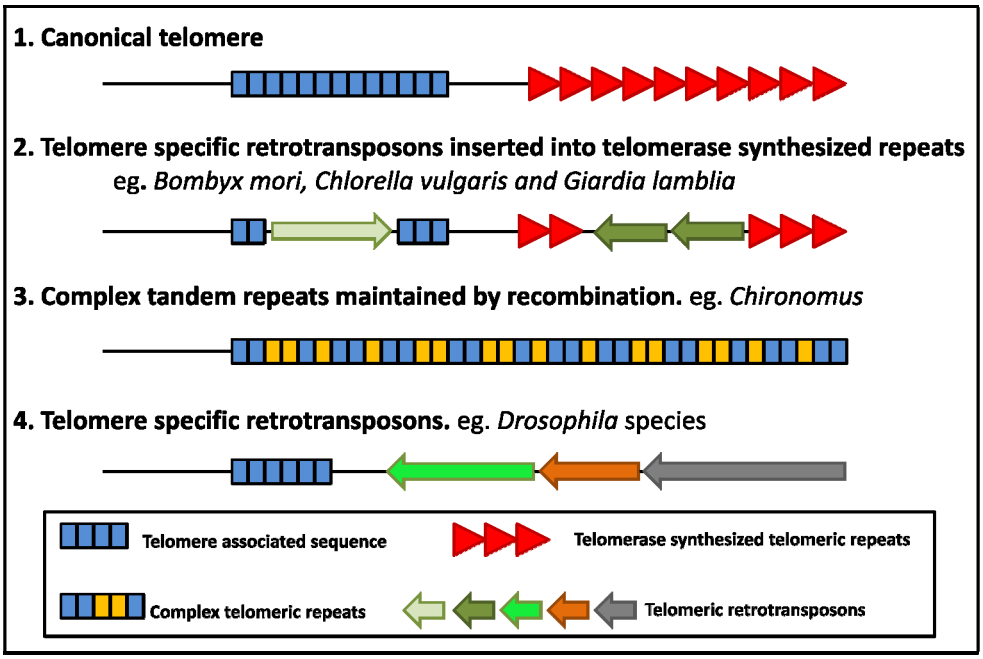


Figure 1: **Types of telomere sequences.** Schematic representation, of different telomeric sequences, showing differences in the type of telomeric repeat sequences in different organisms.

Some organisms have additional telomere specific retro-transposons which transpose to the telomerase added telomere sequences and the two sequences together constitute the telomeres eg. *Bombyx mori*. Another group of organisms like *Chironomus* synthesise or maintain their chromosome end by specific complex tandem repeats that are extended and maintained by recombination. Mosquitoes and *Drosophila sp.* maintain the chromosome end by transposition of telomere specific non-LTR retrotransposons to the chromosome ends whenever the telomere length shortens beyond the limits (Reviewed in Louis, 2002; Villasante et al, 2008). Even though the telomeres are different in their sequences, many conserved and specific proteins binds to them and have evolved to do the common function of protecting the very end of the chromosome.

1.2.1 *Saccharomyces cerevisiae* telomere

Saccharomyces cerevisiae has served as a good model system to study telomeres. Rap1, repressor activator protein 1, is a multifunctional protein which also binds to yeast telomeres. Rap1 recruits two more proteins – Rif1 and Rif2, which function to negatively regulate telomere length (Wotton & Shore, 1997). Rif1 specifically inhibits access to telomerase when telomere sequences are long and thereby directs telomerase to the short telomeres that need to be elongated and thus maintains telomere length homeostasis. Rif2 plays a minor role in this process. However, Rif2 plays an additional role in preventing end-joining (seeing the chromosome end as a double strand break) by preventing the action of NHEJ (non homologous end joining) proteins Yku70 and Yku80, which paradoxically, also localize to telomeres. Other than NHEJ, Yku70/80-complex is also involved in telomere length maintenance and in TPE (Mishra & Shore, 1999). End protection is carried out by ssDNA binding protein Cdc-13 and its interacting partners (Pennock et al, 2001). Along with Cdc-13/Est4, Est1-3 and Tlc1 proteins are required for the telomerase activity and mutations in these leads to progressive reduction in telomere length and hence the name, Ever Shorter Telomeres (Li et al). CDC-13/EST4 gene is known to be essential where as others are not (Smogorzewska & de Lange, 2004; Taggart & Zakian, 2003). The capping function of Cdc-13 is through its interaction with Stn1 and Tel1 and these are also essential genes (Grandin et al, 2001; Grandin et al, 1997; Petreaca et al, 2006). In their absence telomeres are longer with a significant amount ssDNA indicating that the protection of telomere sequences from nucleases is lost. Cdc-13 not only cooperates with Stn1 but also with Ku complex in telomerase recruitment (Grandin et al, 2000). Telomerase recruitment domain of Cdc13p is also regulated by phosphorylation and hence Cdc-13 is an important telomere-specific target of Mec1p/Tel1p (Tseng et al, 2006). At the end of the telomere, nucleosomes are replaced by all these proteins that act as the protective capping complex and the structure can be called as a “telosome” to differentiate it from the other part where nucleosomes bind and compact the long DNA sequences (Wright et al, 1992) (Figure: 2).

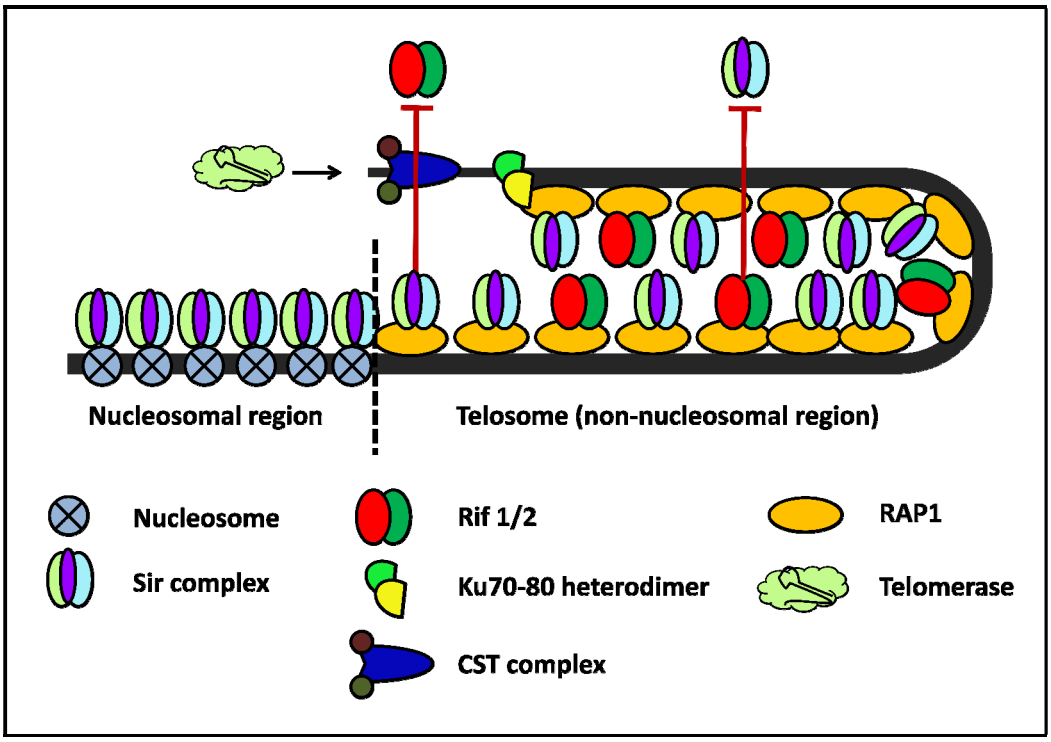


Figure 2: Structure of yeast telomere. Model of a typical budding yeast telomere showing both the telomeric double strand binding proteins and the single strand over-hang binding proteins.

Also Sir-proteins bind to the telomeric and sub-telomeric regions and establish transcriptional silencing in this region, a property known as TPE.

1.2.2 *Drosophila* telomere

Drosophila lack telomerase and maintain their telomeres by transposition of three non-LTR retrotransposons namely HeT-A, TART and TAHRE (Abad et al, 2004; Mason & Biessmann, 1995; Pardue et al, 1996). TART and TAHRE have two ORFs – ORF1 and ORF2 similar to GAG and POL gene of retrovirus. ORF2 encodes a protein which has both endonuclease and reverse transcriptase domain and is absent in HeT-A and so HeT-A needs a reverse transcriptase in trans for its transposition. When the HeT-A/TART/TAHRE array is relatively short, more and more transposition events take place to maintain the telomere length. The telomere specific retrotransposons specifically transpose to the end of the chromosome by

reverse transcription using their poly(A)+RNA. One of the GAG element from these retrotransposons gets transcribed within the nucleus and is transported to the cytoplasm to make the Gag protein which is believed to help the sense strand to reverse transcribe on to the end of the chromosome (Pardue & DeBaryshe, 2003) (Figure: 3).

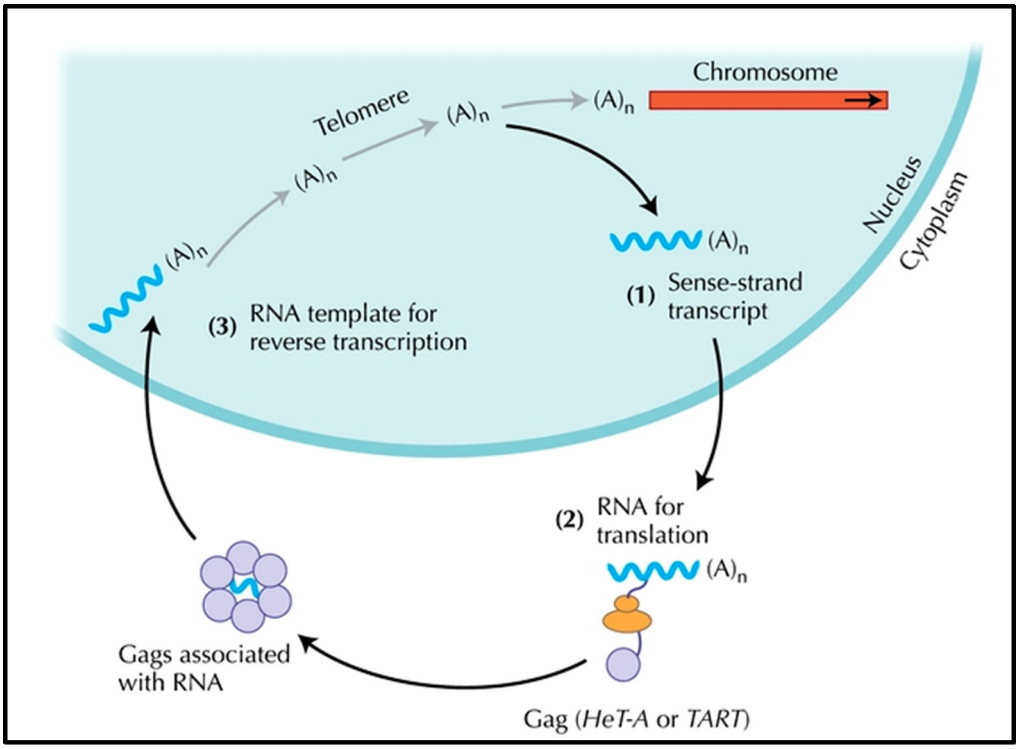


Figure 3: *Drosophila* telomere maintenance by retrotransposons. Model for telomere elongation in *Drosophila* by retrotransposition mediated mechanism.

Drosophila telomeres are bound by a number of proteins (Figure: 4). These include heterochromatin protein 1 (HP1) (Fanti et al, 1998), HP1/ORC associated protein (HOAP) (Cenci et al, 2003; Shareef et al, 2001), HipHop (Gao et al, 2010), ATM kinase (Bi et al, 2004; Oikemus et al, 2004; Silva et al, 2004; Song et al, 2004), MRN-complex (Bi et al, 2004; Ciapponi et al, 2004) and Ku70/Ku80 heterodimeric

proteins. Ku70/Ku80 heterodimer and HP1 act as negative regulators of telomere length in fly (Melnikova et al, 2005; Savitsky et al, 2002). Decrease in Ku70/Ku80 protein dosage leads to increased transposition of retrotransposable elements. But all the terminally deleted chromosomes lack these retrotransposon sequences and the terminal sequences vary in different telomeres suggesting that such cells maintain their telomeres in a sequence independent manner. Both terminally deleted and normal telomeres recruit the capping proteins - HP1, HOAP, HipHop, ATM/ATR and Mre11-Rad50-Nbs (MRN) complex proteins. The MRN complex is required to cap telomeres during *Drosophila* embryogenesis and hypomorphic mutation of *mre11* and *nbs* leads to maternal effect lethality because of telomere fusions and consequent mitosis defects. This may be because the depletion of maternal pool of NBS protein causes exclusion of Mre11 and Rad50 (MR) from chromatin (Gao et al, 2009). HipHop and HOAP shares functional homology to capping proteins of other organisms in that these proteins binds to the telomere double-stranded DNA and prevent telomere fusion events; the loss of these proteins leads to increased frequency of fused telomeres (Gao et al, 2010).

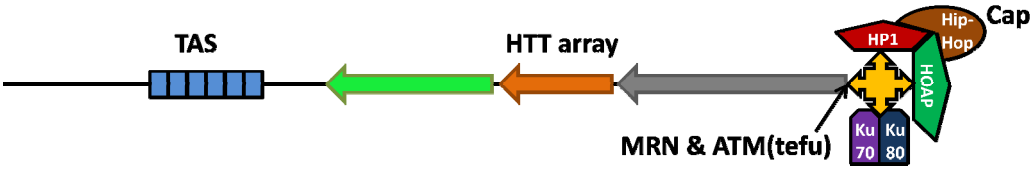


Figure 4: *Drosophila* telomere capping. *Drosophila* telomere represented shows the different regions of *Drosophila* telomere and telomere capping complex along with its interactions to proteins involved in DNA damage responses.

1.3 Rif1, a negative regulator of telomere length in yeast

Rif1 was first identified in budding yeast as an interactor of Rap1 and hence the name Rap1 interacting factor1 (Rif1). Rap1 protein binds to the telomeric DNA specifically and recruits both Rif1 and another Rap1 interacting factor, Rif2 through its C-terminus interaction. Rif1 is a negative regulator of telomere length in *Saccharomyces cerevisiae* and maintains telomere length homeostasis by

preventing access of long telomeres to telomerase. *rif1* mutants have telomeres that are longer than the wild type. Rif2, which also interacts with Rif1, works in parallel with Rif1 to maintain telomere length. The double mutant, *rif1rif2* has telomeres that are much longer than the wild type. Rap1 also recruits Sir proteins and both Sir3/4 and Rif1 bind to the same region of Rap1 (C-terminus), therefore, there is an underlying competition for Rap1 binding between Sir and Rif complexes leading to indirect influence of Rif1 on heterochromatin formation at the telomeres. Rif1 is localized predominantly to the telomere and its binding is increased during S-phase, however, it also associates with multiple other sites in the genome (Bianchi & Shore, 2007; Mishra & Shore, 1999; Smith et al, 2003) (Figure: 5).

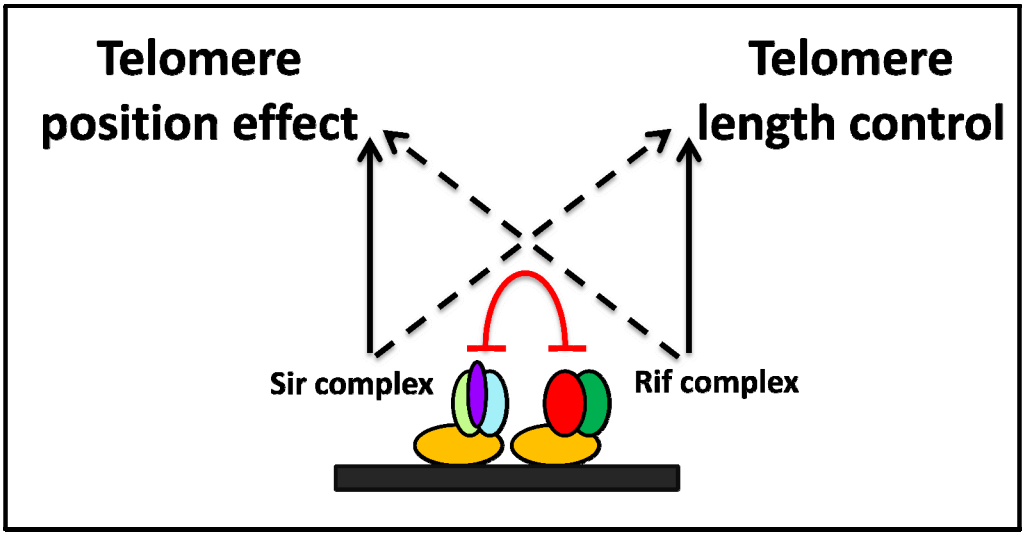


Figure 5: Role of Rif1 in *Saccharomyces cerevisiae*. Rif1 recruitment to telomeres through its Rap1 interaction, and direct and indirect roles of Rif1 is depicted as a model. Dashed line represents the indirect role where as the solid line represents the direct role of Rif proteins in budding yeast.

The mechanistic basis of how Rif1 negatively regulates telomerase at the yeast telomeres is still unclear. A few recent studies provide some understanding towards its function. Rif1 (along with Rif2) seems to inhibit telomerase association by

restricting the access of the positive regulator of telomerase, Tel1, to the telomeres (Hirano et al, 2009).This seems to be dosage dependent as those telomeres that have more Rif1 (longer telomeres) inhibit association more strongly. Therefore, the number of Rif1 molecules bound to the telomere repeats acts as a sensor for length and fewer Rif1 molecules bound to the telomeres makes them more likely to be extended thus regulating telomere length. Very recently Rif1 was shown to be involved in anti-check point activity by preventing RPA (Replication Protein A) binding to uncapped telomere ends and attenuate the checkpoint activation at telomeres. Even short telomeres can evade the check point function, in trans, by virtue of its association to the long telomere present in the same telomere cluster (Ribeyre & Shore, 2012; Xue et al, 2011).

Given the important roles of Rif1 at the yeast telomeres, homologues of Rif1 in fission yeast, human, mouse have been recently identified (Adams & McLaren, 2004; Kanoh & Ishikawa, 2001; Silverman et al, 2004; Xu & Blackburn, 2004).

1.4 Rif1 in *Schizosaccharomyces pombe*

In *S. pombe* both spRap1 and spRif1 (orthologues of scRap1 and scRif1) are independently recruited to the telomeres by Taz1, the telomere DNA binding protein. Both Taz1 and spRap1 are negative regulators of telomere length and have important roles in transcription repression at telomeres. $Rif1^{+}$ deletion also increases the telomere length as in *S.cerevisiae* but not as much as $Taz1^{+}$ and $Rap1^{+}$ deletions (Kanoh & Ishikawa, 2001; Miller et al, 2005). The localization of SpRif1 is not only at telomeres but is localized throughout the nucleus in both wild type and *taz1*Δ cells but prominent telomere localization is observed in the *rap1*Δ cells (Kanoh & Ishikawa, 2001).

1.5 Mammalian homologues of Rif1

Mouse Rif1 (mRif1) expresses highly in totipotent and pluripotent cells in early mouse development and in the germ cells in adult (Adams & McLaren, 2004). mRif1 is rapidly down regulated upon differentiation of embryonic stem(ES) cells under in vitro conditions. The protein is not expressed in most of the adult

tissues/organs. Before fertilization, mRif1 is present in germinal vesicle stage oozytes in the nucleus but not in the nucleolus. After ovulation it is largely excluded from the condensed meiotic chromosomes but appears to be dispersed throughout the cell in metaphase oocytes. Also mRif1 was excluded from condensed chromosomes during nuclear division in case of mitosis. It was shown that mRif1 can associate with telomeres as it physically interacts with Trf2 and mRif1 also can be cross-linked to telomeric DNA of ES cells.

Human homologues of Rif1 (hRif1) were identified by two different groups (Silverman et al, 2004; Xu & Blackburn, 2004). Their results suggest that hRif1 has diverged from its telomeric function in yeast counterpart in course of evolution. Human Rif1 does not normally associate with telomeres and binds only to aberrant telomeres and is instead involved in DNA damage response or DNA repair. The human protein functions as a DNA-damage-response factor required for cell survival after radiation damage. It is also required for the proper execution of the intra-S-phase checkpoint that serves to slow down DNA synthesis when DNA damage has occurred, giving sufficient time for the DNA repair machinery to act on the damage. hRif1 localizes to DSBs in an ATM- and 53BP1-dependent manner (Silverman et al, 2004). Since hRif1 is recruited to the repair foci upon DNA damage, it may either participate in DNA damage checkpoint signalling or DNA repair or both. Using hRif1 siRNA it has been shown that hRif1 is required for efficient DNA repair. Furthermore, hRif1 depleted cells showed defects in homology-mediated repair pathway. Another report suggests that hRif1 acts as an anti-apoptotic factor and is involved in both staurosporine (a general protein kinase inhibitor) induced apoptosis and DNA damaging agent induced apoptosis (Wang et al, 2009). Additionally, Xu, L. et al report that hRif1 aligns across midzone microtubules in early anaphase and this hints at some role of this protein in cell cycle beyond DNA damage response (Xu & Blackburn, 2004). Recently Rif1 was shown to interact with BLM-complex and work in tandem with BLM during DNA replication to promote reinitiation of replication at the stalled replication forks (Xu et al, 2010). Interestingly, Rif1 binds to the DNA and thus acing as an interface to recruit BLM helicase to DNA and maintain normal replication of stalled replication

forks (Buonomo et al, 2009; Xu et al, 2010) providing the first molecular clue to Rif1 activity.

mRif1 is essential for the embryonic development, as the Rif1 knockout mice are embryonic lethal unlike that of ATM and 53BP1 in which the mouse is viable. This implies that Rif1 protein will have additional roles than the downstream roles of the above two proteins at the DNA damage/repair foci (Buonomo et al, 2009).

1.6 Rif1 homologue in *Drosophila*

Based on our bioinformatic analysis and reports in literature a putative *Drosophila* homologue of Rif1 was identified. Though the structure of *Drosophila* telomere is different, there are a lot of analogies in the overall mechanism of telomere functions. The mechanism of telomere addition and protection seem to be similar in the sense that, in both cases, the template for the telomeric DNA addition is RNA; both are protected by capping proteins which include DNA damage response proteins and proteins that repair the DNA damage-among these Ku70/Ku80, ATM/ATR, MRN etc are also bound at all telomeres from yeast to mammals; both telomeres show telomere position effect (TPE). Additionally, we had preliminary evidence in the lab that yeast Rif1 interacts with yeast Ku protein that is involved in end protection in all organisms including yeast, *Drosophila* and humans.

In this context, the presence of dRif1 is interesting and raises several questions. What is the molecular role of Rif1? Has that role been conserved in *Drosophila*? Does it have any role at the *Drosophila* telomeres? Based on available literature, we hypothesized that Rif1 may be involved in genome protection in *Drosophila*. This work was initiated to study the function of dRif1.

Objectives

1. Detailed bioinformatic analysis of Rif1 homologue in *Drosophila* to identify evolutionarily conserved features of the protein

2. Investigating the function and functional conservation of *Drosophila* Rif1 using cell lines

3. Characterization of putative Rif1 homologue in *Drosophila melanogaster*

Approaches

1. Identification of *Drosophila* Rif1 by bioinformatics as a tool
2. Testing the conservation of function of putative *Drosophila* Rif1 in S2 cells
3. Testing the conservation of telomeric function in yeast by complementation assay
4. Testing conservation of DNA damage response function in HeLa by complementation assay
5. Expression of dRif1 in different developmental stages of fly - protein & RNA expression
6. Localization of dRif1 in developmental stages
7. Sub cellular localization of dRif1
8. Gain of function and loss of function studies of dRif1 by fly genetics

CHAPTER 2

Materials and Methods

2.1 Molecular biology methods

2.1.1 Polymerase chain reaction

The PCR (Polymerase Chain Reaction) was performed using Taq DNA polymerase supplied by Bioron/jump start Taq DNA polymerase from Sigma. Wherever the PCR products were used for cloning for protein expression, Vent DNA polymerase from NEB was used for error less amplification. A 20µl reaction volume was prepared in ddH2O with DNA (20-100ng), primers (5 pico moles), buffer (1X), dNTPs (0.5mM), MgCl2 (1.5mM) and Taq DNA polymerase (1Unit). The primer annealing temperature was identified by gradient PCR and the products were further amplified at the optimal temperature. The Eppendorff thermal cycler was used to amplify the product. The PCR products were resolved on the Agarose gel electrophoresis and confirmed. If required, the separated products were purified by using Qiagen gel extraction kit.

2.1.2 Molecular cloning

The cDNAs were either PCR-amplified or were digested by restriction enzymes from the donor plasmid. The digested cDNAs were run on an Agarose gel and purified using Qiagen gel extraction kit. The purified cDNAs were cloned into the vector digest and transformed into ultra competent bacterial cells. Each step followed in this procedure is described below.

2.1.2.1 Preparation of competent cells

Ultra competent cells were prepared from *E.coli* DH5α to transform plasmids or ligation mixture. The ultra-competent cells were prepared according to the protocol described (Inoue et al., 1990). The primary culture was prepared by inoculating single colony of E.coli DH5α in 10ml of LB medium and incubated at 37ºC overnight. The primary culture was further inoculated at different dilutions into fresh LB medium and incubated at 18ºC

until it reached an OD of 0.6 at a wavelength of 600nm. As soon as the OD turns 0.6, the culture was kept on ice for 15 minutes and centrifuged to collect the bacterial pellet at 3000g at 40C. The pellet was re-suspended in 32ml of ice cold inoue buffer (10 mM PIPES pH 6.7, 15 mM CaCl2, 250 mM KCl and 55 mM MnCl2) and incubated on ice for 15 minutes. The cells were centrifuged again and pelleted. This step was repeated once again and finally the cells were re-suspended in 8ml of inoue buffer containing DMSO. The resupended cells were aliquoted 100µl each in 1.5 ml tubes, snap frozen in liquid nitrogen and stored at -70ºC.

2.1.2.2 DNA transformation into bacterial cells

The ligation mixture or plasmid DNA was transformed into the ultra-competent cells. The 100µl aliquot of ultra-competent cells frozen at -70ºC was thawed on ice and the DNA was added and gently tapped for proper mixing. The mix was incubated on ice for 20 minutes and heat shock was given at 42ºC for 90 seconds in water bath and immediately transferred on to ice and maintained for 5 minutes. 900µl of LB medium was added to the cells and incubated at 37ºC for an hour. The cells were plated on a LB-Agar plate containing suitable antibiotic and incubated for overnight at 37ºC. The colonies were then streaked on a fresh LB-Agar plate containing suitable antibiotics for further screening.

2.1.2.3 Isolation of plasmid

Plasmids were isolated using different methods according to the requirement of downstream applications. Plasmids for screening clones by sequencing and restriction digestion were isolated by alkaline lysis or boiling lysis method. For transfection the plasmids were isolated using Qiagen plasmid purification kits. All three methods are explained below.

2.1.2.3.1 Isolation of plasmid by alkaline lysis method

The 3ml bacterial culture was centrifuged at 5000g for 2minutes to collect bacterial pellet and the pellet was resuspended in 100µl of Solution-1 (50 mM glucose+25 mM Tris.Cl, pH 8.0+10 mM EDTA autoclaved and store at 4°C) and 200µl of Solution-2 (0.2 N NaOH 1% (wt/vol) sodium dodecyl sulfate (SDS) freshly prepared) was added and mixed immediately by inverting it 4-5 times and kept on ice for 5 minutes. 150µl of an ice cold solution-3 (3M potassium acetate, pH-5.2) was added to the mixture and further mixed by inverting 4-5 times. The mixture (lysate) was centrifuged after keeping on ice for 5 minutes at 14000g for 15minutes. The supernatant was collected and the plasmid DNA was precipitated by adding equal volume of iso-propanol and centrifuging at 14000g for 20 minutes. The DNA pellet was washed with 70% ethanol. The pellet was dried and resuspended in water or 10mM Tris.Cl pH-8.0.

2.1.2.3.2 Isolation of Plasmid by boiling lysis method

A 2ml of bacterial culture was centrifuged at 5000g for 2 minutes. The bacterial pellet was resuspended in 100µl of water. Equal volume of lysis buffer (0.1N NaOH, 1%SDS, 10mM EDTA) was added to the mixture. The whole mixture was boiled at 100ºC for 1-2 minutes and kept on ice immediately for 5 minutes. 50µl of Magnesium chloride was added to the mixture and kept in ice. The lysate was centrifuged at 14000g for 5 minutes and supernatant was collected. Plasmid DNA was precipitated by adding 50µl of 3M potassium acetate (pH-5.2) and kept in ice for 5 minutes. The mixture was centrifuged at 14000g for 5 minutes. To the supernatant, two volumes of isopropanol was added and centrifuged at 14000g for 5 minutes. The DNA pellet was washed with 70% ethanol, air dried and resuspended in required amount of sterile milliQ water or 10mM Tris.Cl (pH-8).

2.1.2.3.3 Isolation of plasmid using column

For transfection into cell lines and for microinjection the plasmids were isolated using Qiagen plasmid purification kits by using the protocol recommended by the manufacturer.

2.1.2.4 Screening of the recombinant plasmids

The recombinant plasmids were screened and confirmed by various methods like colony PCR, assaying the size difference between positive and negative clones on Agarose gel and restriction digestion of the plasmids.

2.1.2.4.1 Colony PCR

An isolated colony of bacteria was resuspended in 50µl of sterile water by vortexing for 30 seconds. The resuspended bacteria were lysed by boiling for 5 minutes. The boiled mixture was centrifuged at 14000g for 1 minute and 5 µl of the supernatant was used in PCR reaction as a template to amplify the insert using either insert specific primers or standard primers present in the vector. The PCR product was confirmed on agarose gel.

2.1.2.4.2 Screening of the clones by restriction digestion

Plasmids were isolated using protocols as described below and presence of the insert was confirmed by restriction digestion. The restriction digestion protocol followed is described in this section. The recombinants were confirmed by electrophoresis on agarose gel by comparing the size difference between vector with insert and empty vector. Restriction digestions were performed to confirm the clones, sub clone the insert into another vector and to digest the PCR product flanking with restriction sites. One or two enzymes were used with the suitable buffers supplied by the manufactures. 5U/µg endo nucleases were used to set the digestion. BSA was added if required. The reaction mixtures were incubated at 37ºC for 1-

2hrs. Digests were run on agarose gel and the presence of DNA was confirmed or isolated for further use.

2.1.3 Vectors

The pET28a vector was used to clone the N-terminus and C-terminus regions of dRif1 for bacterial expression of the truncated protein which was purified and used to immunize the animal. The *Drosophila* expression vector pUAST was used to express both 3X FLAG tagged and untagged versions of full length dRif1 in the fly ectopic over expression experiments. Sym-pUAST vector was used for RNAi experiments. The mammalian expression vectors *pCMV-FLAG2A* (AF072538) was used for expressing 3XFLAG dRif1 in *HeLa* cell line. yEPLac181, pRS315 and pRS316 vectors were used to express dRif1 in yeast.

2.1.4 RNA isolation and Northern blotting

Total RNA from each sample was isolated using TRIzol (Invitrogen) reagent. Briefly the cells were pelleted and 600µl of trizol was added to the pellet and snap frozen in liquid nitrogen and stored at -80^{0}C until use. The cells were homogenized by using 1ml sterile RNase free tips with frequent freezing in liquid nitrogen and made up trizol volume to 1ml. 200 µl of chloroform was added to it and vortexed for 20minutes. After that the tubes were centrifuged at 13,000rpm for 15minutes at 4^{0}C, the aqueous phase was collected and added equal volume of isopropanol and kept on ice for 45minutes. After that the RNA was pelleted by centrifuging at 13,000rpm for 15minutes at 4^{0}C and the pellet was washed with 85% ethanol made in DEPC treated water. The pellets were air dried, dissolved in DEPC treated water, run on a formaldehyde gel in MOPS buffer and transferred to positively charged nylon membranes (IMMOBILON-NY+, Millipore) in 20X SSC by capillary transfer method. ^{32}P-labeled probes were generated by random priming the full length dRif1 using standard methods. Hybridization was carried out overnight at 65°C in buffer containing 0.5 M

Na-phosphate pH 7; 7% SDS. Following hybridization, the membranes were washed two times in 1XSSC and 0.5% SDS for 5minutes each and again in 0.2XSSC and 0.1% SDS for 15 minutes each.

2.2 Biochemical experiments

2.2.1 Protein extraction from cell lines and flies

The cells grown on 6 well plates were washed with 1xPBS and the cell lysate was prepared by scrapping the cells using 200 µl cell lysis buffer (2% SDS, 5% β-mercapto ethanol 50mM Tris.HCl (pH 6.8)). The DNA was sheared by sonication on a waterbath sonicator. The lysate was centrifuged at 10000g for 15 mins at 4ºC. The protein extracts were stored at -20 ºC.

The protein extracts from different developmental stages of fly were prepared by crushing the embryos/larvae/adult flies and homogenized in 2x Laemmli buffer by using hand held homogenizer. The lysate was boiled for 5 mins and centrifuged at 10000g for 15 mins at 4ºC. The supernatant (protein extract) was stored at -20 ºC for further use.

2.2.2 SDS PAGE gel electrophoresis

The protein extract obtained from the total lysate of cells or adult flies were resolved by denaturing Poly Acrylamide Gel Electrophoresis. This method was described by Laemmli 1970 (Laemmli, 1970). 5% Stacking gel and 10% resolving gel were cased generally using 1.5mm thick gel casting apparatus supplied by Hoefer. Protein samples were boiled in loading buffer or Laemmli buffer (50 mM Tris.HCl (pH 6.8), 100 mM DTT, 7% SDS, 0.1% bromophenol blue, 10% glycerol) for 5 minutes and loaded onto the gel and electrophoresed at 30mA on stacking gel and 40mA when it reaches resolving gel. Pre stained molecular weight markers obtained from Biorad was loaded along with the samples.

2.2.3 Coomassie staining of the PAGE gel

After the run, gel was removed and stained with coomassie brilliant blue stain (0.025% in Acetic acid:Water:Methanol v:v:v, 10:40:40) for 2-3 hours and destained in destaining solution (Acteic acid:Methanol:Water 10:40:40) by changing it several times until protein bands were clear against a clear background. If the protein has to be transferred onto membranes coomassie staining was avoided, but proteins were stained after transferring on to membrane using Ponceu-S.

2.2.4 Transfer of proteins to membranes by wet transfer method

The proteins resolved by SDS-PAGE were transferred into a membrane for western blotting. The gel was assembled with the transfer membrane (Immobilon-P) which was activated by dipping in 100% methanol. The membrane was equilibrated with transfer buffer before stacking into the wet transfer apparatus cassette. The order of assembly was "Sponge - three Whatmann papers – Gel – Membrane - three Whatmann papers- sponges". All the above mentioned materials were equilibrated in transfer buffer, (39mM Glycine, 48mM Tris.HCl, 0.037% (w/v) SDS & 20% (v/v) methanol) the cassette was assembled and kept in the transfer apparatus by keeping the gel towards cathode. Transfer was performed by applying 400mA for 2-3 hours/225mA for overnight under cold condition. The membranes were dipped in methanol, dried immediately and used for further studies.

2.2.5 Ponceu-S staining of the membranes

Proteins transferred on to the membrane were stained using Ponceu-S stain (0.5gm dissolved in 1ml acetic acid and then diluted to 100ml water) for 10 minutes. The membrane was washed in water by shaking until bands were clearly visible against the background. After recording the stain was completely removed by washing with water.

2.2.6 Immuno detection of proteins by western blotting

The protein transferred membranes were wetted in methanol and equilibrated in PBST buffer (1x PBS buffer prepared from 10XPBS with MilliQ water, Tween-20 was added to the final concentration 0.1% v/v). Membrane was first blocked with PBST buffer containing 5% BSA/milk powder for 1-2hours at room temperature with constant stirring. Membrane was incubated with primary antibody suspended in blocking solution in appropriate dilution at 4ºC for 8-10hours or for 1hour at room temperature with constant rotation. The membrane was washed with PBST buffer for 3 times with 7 minutes interval between each wash and constant rotation. The membrane was incubated with secondary antibody with appropriate dilution in PBST for 1hour in room temperature with constant rotation. The membrane was again washed with PBST buffer for 3 times as previous. Blots were detected by chemiluminescent methods. Luminol and the peroxide reagents were mixed in equal volume and added to the blot (125µl mixture per cm2 of membrane) and developed using X-ray film or by ChemiCapt/Versadoc chemiluminescene detector. The membrane was developed at different time points depending on the intensity of the signal.

2.3 Antibody against dRif1

The full-length cDNA clone (RE66338) was obtained from the Drosophila Genomics Resource Center (DGRC). Approximately 1.2kb region from the N (54-450 amino acids) and C (694-1094 amino acids) terminus of dRif1 were chosen (N-dRif1 and C-dRif1) for raising antibody. These two regions were amplified by PCR and cloned in-frame in pET-28a His-tag vector. Both of the clones were transformed in Bl-21 strain and expressed by IPTG induction. Since the N terminal region was making inclusion bodies and the purification was difficult, we have proceeded with the C terminal region for raising the antibody. C-dRif1 was purified using Ni-NTA His bind resin from QIAGEN (figure 2) and a New Zealand white rabbit was immunized

with ~300µg of purified C-dRif1 with Freund's complete adjuant. It was followed by 3 booster doses ~300µg along with Freund's incomplete adjuant with 15 days of interval period. Seven days after the 3rd booster a test bleed was taken and tested for the specificity and a 20 ml bleed was collected from the animal as test bleed picked a band at expected size. This was followed by a 4th booster and collected another 15ml bleed as in case of 3rd booster. The serum was prepared by clotting the blood at 37^0C for 60 minutes and centrifuged at 3000 rpm for 10 minutes and collected the supernatant serum, made aliquots and stored at -80^0C. A portion of the serum was affinity purified against purified CdRif1 protein bound to nitrocellulose and used for different experiments.

2.4 Yeast methods

2.4.1 High efficiency yeast transformation

Yeast transformations with plasmids were done based on high efficiency LiAc protocol (Gietz and Woods, 2002). $5x10^6$ cells from the overnight incubated primary culture were added to the 25ml broth and incubated for 4-5 hrs at 30°C with constant rotation. $1x10^8$ cells from the secondary culture were used for transformation. The cell pellet was washed in 1ml of 0.1 M LiAc and resuspended in 240µl of 50% Poly ethylene glycol (PEG). To this mixture, 36µl of 1M LiAc and 74 µl of transformation mix containing 40µl of salmon sperm DNA and 34µl of both plasmid/DNA fragment and sterile MilliQ water were added. Cells were vortexed briefly and incubated at 42°C for 40 minutes. Then cells were spun at 13k for 15 sec and cell pellet was resuspended in 200µl of sterile water and plated on selective dropout media.

2.4.2 Extraction of genomic DNA from yeast cells

Genomic DNA from yeast cells were isolated by using two methods in this study. Zymolyase method was used for telomere blot and rapid isolation method was followed for using the DNA as template for PCR.

2.4.2.1 Zymolyase method

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3 k rpm for 5 min. The cell pellet was resuspended in 0.5 ml of 1M Sorbitol and 0.1M Na_2 EDTA (pH 7.5) and transferred into 1.5 ml microfuge tube. Cells were speroplasted by incubating the cell suspension with 20 µl of Zymolyase 100,000U (2.5 mg/ml) at 37^0 C for 60 min. Cells were centrifuged for 1 min at 13 k rpm and the cell pellet was resuspended in 0.5 ml of 50mM Tris-Cl (pH 7.4) and 20mM Na_2 EDTA (pH 8.0). 50 µl of 10% SDS was added to the cell suspension, mixed well and then incubated at 65^0 C for 30 min. 200 µl of 5M Potassium Acetate was then added to the cell suspension and placed in ice for 60 min. Cells were centrifuged for 5 min at 13 k rpm and supernatant was tranferred to a fresh microfuge tube. One volume (0.75 ml) of 100% isopropanol was added to the supernatant, mixed and allowed to sit at room temperatue for 5 min. Then centrifuged very briefly for 2 min at 13 k rpm and supernatant was poured off. DNA pellet was air dried and resuspended in 0.3 ml of TE (pH 7.4) containing 20 µg/ml of Rnase A. DNA was incubatded at 37^0 C for 30 min for degrading RNA. Then 30 µl of 3M Sodium Acetate (pH 7.0) was added to the DNA solution and mixed. To this, 0.2 ml of 100% isoproponal was added and mixed once again. DNA was recovered by centrifuging at 13 k rpm for 2 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.4.2.2 Rapid isolation of genomic DNA from yeast cells

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3 k rpm for 5 min. The cell pellet was washed in 0.5 ml of sterile distilled water and resuspended in 200 µl of breaking buffer. Glass beads (~ 200 µl volume) were added to the cell suspension and then 200 µl of phenol/chloroform/isoamylalchohol (25:24:1) was added and mixed. Cells were vortexed at high speed for 2 min. 200 µl of TE (pH 8.0) was added and once again vortexed briefly for 10 to 15 sec. Then the sample was centrifuged at 13 k rpm for 5 min at room temperature. The aqueous layer was transferred to a clean microfuge tube and 1 ml of 100% ethanol was added and mixed by inversion. DNA was recovered by centrifuging at 13 k rpm for 5 min. The supernatant was poured off, DNA pellet was air dried and resuspended in 30µl of TE (pH 8.0).

2.4.3 Extraction of whole cell protein from yeast cells by Trichloro Acetic acid (TCA) method

Cells grown overnight in 5ml of YPD or in selective broth were harvested by centrifuging at 3 k rpm for 5 min. The cell pellet was resuspended in 200 µl of 20% TCA and glass beads were added up to the meniscus and then cells were lysed by vorteing for 1 min. Cell suspension was transferred into a new microfuge tube. Glass beads were washed twice with 200 µl of 5% TCA and the washes were added to the previous suspension. Cell pellet was collected by centrifuging at 3 k rpm for 10 min and resuspended in 200 µl of 1x laemmli buffer. The laemmli buffer turns red because of the low pH of cell pellet. Therefore, 50 µl of 1M Tris base (no pH adjustment) was added to turn blue. The sample was boiled for 3 min and centrifuged again at 3 k rpm for 10 min. Protein sample was transferred to a new microfuge tube and the pellet was discarded.

2.4.4 Telomere blotting

Yeast transformation was done using Lithium acetate mediated transformation and DNA was isolated using zymolyase method(both the methods as descrbed before). Approximately 1.5µg of genomic DNA was digested with Xhol and subjected to electrophoresis on a 0.8% agarose gel in TAE buffer for 18-20 hrs at 30 V. The gel was soaked in 0.4 N NaOH for 10 min, and capillary transferred to charged Nylon membrane (IMMOBILON-NY+, Millipore) using 0.4 N NaOH. The membrane was hybridized to the radio labeled dGT repeat (by nick translation) at 55°C. After hybridization washing was performed to remove the un-hybridized probes and the hybridization was detected in a phosphor-imager.

2.4.5 Silencing assay

Silencing assays were done to test the loss in silencing in yeast. For this, the yeast cells were initially grown in nutrient rich broth or selective broth dropped out for specific amino acids (for retaining plasmids) at 30^0C with appropriate rotation for overnight and then the culture was subjected to 10 fold serial dilution for 5 times. 5 µl of each dilution was spotted on complete medium to check the total number of cells grown and on selective medium for measuring the loss in silencing of reporter gene. For example, loss in silencing of *TRP1* reporter gene is tested by spotting on tryptophan dropout medium. In case, the reporter gene is *URA3*, then serially diluted cultures were spotted on the medium containing 1mg/ml of 5-FOA (5-Fluoro orotic acid). Expression of the *URA3* gene (Orotidine-5'-phosphate decarboxylase) leads to the conversion of 5-FOA into 5-fluorouracil, a toxic compound. This indicates that strains expressing *URA3* cannot grow in this medium and those repressing *URA3* can grow. Therefore, 5-FOA serves as a good indicator of the expression status of the *URA3* reporter gene. After spotting, plates are incubated at 30^0C for 2-3 days and analyzed loss in silencing by observing growth of cells.

2.4.6 Immunofluorescence

Immunofluorescence was done as described in (Gotta et al., 1996). Briefly, diploid yeast strain KRY109 (Sir4-13xmyc) was transformed with either empty vector/dRif1/yRif1 in single/multi copy vector and the transformants were grown in SC-Leu broth. Overnight grown 5ml cultures were fixed with 0.5 ml of formaldehyde and incubated at 30^{o}C in a shaker for 20 min. Cells were then washed thrice with sterile water and resuspended in 200 µl of 0.1M EDTA-KOH and 10mM DTT and incubated at 30^{o}C for 10 min. Cell suspension was centrifuged at 3 k rpm for 5 min. The cells were speroplasted by resuspending in 200 µl of YPD broth containing 1.2M sorbitol and one-tenth volume of zymolyase (2mg/ml) and incubated at 30^{o}C for 15 min. Speroplasts were washed thrice with 500 µl of YPD sorbitol and resuspended in 100 µl of YPD sorbitol. Speroplasts were spotted on multi-well slides coated with polylysine. They were further permeabilized with methanol and acetone by incubating for 5 min and 1 min respectively at -20^{o}C. Speroplasts were blocked with 1% ovalbumin/BSA and incubated with appropriate primary antibody dilutions (mouse FLAG-M2 antibody-SIGMA 1:500, rabbit myc antibody-Abcam 1:15,000) for overnight at 4^{o}C. Cells were then thoroughly washed thrice with PBST buffer for 5 min each and incubated with recommended dilutions of fluorescently labelled secondary antibody (alexa fluor-488 tagged secondary anti mouse antibody 1:500, Cy3 tagged secondary anti rabbit antibody 1:500) in dark at room temperature for 45 min. Cells were thoroughly washed thrice with PBST buffer for 5 min each. After washes, slides were mounted in mounting medium containing DAPI and then viewed and photographed in an LSM510meta multiphoton confocal microscope. Images were processed using the LSM software.

2.5 Cell culture experiments

2.5.1 S2 cellculture

S2 cells were cultured in Schneider's medium with 10% FBS containing pencillin-streptomycin (50 µg/ml Penicillin and 60 µg/ml Streptomycin). Cells were grown at 25^0C without CO_2 and maintained by splitting every 3-4days.

2.5.1.1 RNAi in S2 cells

We used double stranded RNA(dsRNA) to knock-down dRif1 levels in S2 cells. Three different primer sets were designed with no off target along with 5' T7 binding site. GFP dsRNA was used as control/mock experiment. MEGAscript T7 kit from ambion was used to make dsRNA according to the manufacturer's instructions and checked on gel for integrity of RNA made and stored at -20^0C until use. $1X10^6$ cells/ml of S2 cells were treated with ~30µgs of the dsRNA in serum free media for 30minutes and later supplemented with serum containing media. After 4days again one more round of treatment was given to completely knockdown dRif1.

Primer Name	Primer Sequences
dRif1_4E_F	taatacgactcactatagggGGGCACGGATCACACTTC
dRif1_4E_R	taatacgactcactatagggCTTGACGGGTTCTTCGGATA
dRif1_2E_F	taatacgactcactatagggGCAGGCGTTGTCCAACTATC
dRif1_2E_R	taatacgactcactatagggCAGCACTGGATGCCAGAAG
dRif1_DRSC_F	taatacgactcactatagggCGGCAAAACGAACTAATGGT
dRif1_DRSC_R	taatacgactcactatagggTGATGGGATGATCACGAAGA
GFP_F	taatacgactcactatagggCTACCTGTTCCATGGCCAAC
GFP_R	taatacgactcactatagggGGTAAAAGGACAGGGCCATC

Table 1: Primers used for making dsRNA. List of primers designed along with 5' T7 transcription site overhangs shown as lower in lower case

The level of dRif1 went down considerably in the 4th day but to complete reduction and thereby to know the effect of dRif1 knock-down to a longer period (telomere transcription) we treated the cells for a second round of dsRNA treatment for three days. After that the cells were harvested and made extracts from untreated and treated samples. To check the level of knock down the samples were run on 8% gel. Also cells were processed for RNA isolation, IF and DNA damage induction treatment.

2.5.1.2 DNA damage induction in S2 cells

2.5mM HU for 16hrs, 50µg/ml Bleomycin for 4 hrs were used for the DNA damage induction. To check the level of phosphorylated γH2AvD, 15% SDS PAGE was performed from these cell extracts and did western blot with γH2AvD antibody (Rockland immunichemicals).

2.5.1.3 Real time PCR to check the telomere transcription in S2 cells

Real time quantitative pcr was carried out to check the level of telomere transcription in both mock treated and dRif1-dsRNA treated (dRif1 knock down cells). RNA isolation, cDNA preparation and rt PCR steps are described below.

Primer Name	Primer sequences
RpS17-F	AAGCGCATCTGCGAGGAG
RpS17-R	CCTCCTCCTGCAACTTGATG
HeT-F	TTGTCTTCTCCTCCGTCCACC
HeT-R	GAGCTGAGATTTTTCTCTATGCTACTG
TAHRE-F	CTTCCCCTCCGCTCTCATC
TAHRE-R	CCTAGATCTGCATTTGTATTAGTAGCTG
TART-F	CAAAAAATCCTTTCCGAGATCC
TART-R	GGGCATCAATATTTAGAATGAACAG
Jockey-F	ACGACTCAATCTAGGGCTCGTG
Jockey-R	CGTCCATTCTCGTATTGATGG

Table 2: List of primers used for qRT PCR. Primers used for real time experiment to check the telomeric transcription of retrotransposons along with control primers

2.5.1.3.1 RNA isolation

RNA isolation was carried out using trizol method as described previously and the concentrations were analysed by taking the Nanodrop reading for each samples.

2.5.1.3.2 cDNA preparation

RNase free DNase treated 2µg of RNA was used to make cDNA using high capacity reverse transcription kit from Life technologies according to the manufactures instruction and stored at -20^{0}C until use for the real time PCR.

2.5.1.3.3 Real time PCR

Real time PCR was carried out in ABI-FAST 7500 real time machine by the Syber green Real time master mix method and the data was analyzed using comparative Ct method to get the fold change in the transcription levels of telomere transcripts compared to the Rps17 gene with respect to the mock treated.

2.5.1.4 Immuno fluorescence in S2 cells

S2 cells were grown at 25°C in Schneider's medium (GIBCO) with 10% heat-inactivated FBS. Confluent cells were harvested, washed once in 1XPBS and plated directly on microscope slides. Cells were fixed in 4% formaldehyde, washed with PBS plus 0.1% Triton (PBST), blocked in 1% BSA (Sigma) in PBST for 1 hr, and incubated with primary antibody for 1hr. Slides were washed and incubated with secondary antibody with proper dilutions for about 45 minutes, washed, mounted in mounting media containing DAPI and imaged in confocal microscope.

2.5.2 HeLa cell culture

HeLa cells were obtained from Dr. Veena K Parnaik, CCMB. Cells were cultured in DMEM with 10% FBS. All media were reconstituted with antibiotics (50 µg/ml Penicillin and 60 µg/ml Streptomycin) and supplemented with L-Glutamine (200mM). Cells were incubated at 37^0C in 5% CO_2 environment and detached from substratum using 1X Trypsin-EDTA (0.1% each, in PBS, for 2-3 min at 37^0C) following a quick wash in PBS to remove residual FBS, trypsin was subsequently inactivated with medium containing serum. Medium was replaced every alternate day. Cells were passaged for a maximum of 4-5 times after revival from frozen state. Stock cultures were never allowed to reach greater than 80% of confluence and were usually harvested at 70-80% confluence.

2.5.2.1 Cryopreservation of cell lines

Cells were harvested by trypsinization and centrifugation and the cell pellet was resuspended in freezing medium (DMSO diluted to 10% in a 1:1 mixture of growth medium and FBS) at a density of 0.5 $X10^6$ cells per ml. Cells suspended in freezing medium were aliquotted (1 ml/vial) into cryo vials, stored at -70^0C overnight in an insulated rack before long term storage in liquid nitrogen freezers.

2.5.2.2 Trasfection of cells

The cells were transfected using Effectene. HeLa cells were plated on 60 mm dish or on glass cover slips 18X18mm (Fisher). The cells were allowed to grow for 24 hrs. The transfection was done according to the manufacturer's protocol (Qiagen). Briefly, 1 µg DNA was diluted into the DNA condensation buffer, EC buffer, to a total volume of 100 µl. 3.2 µl of enhancer was added and incubated at the room temperature for 10 minutes. 4 µl of Effectene was added and incubated at room temperature for 15 minutes. The volume containing DNA:lipid complex was made up to 1ml

with 10% serum containing DMEM and cells were incubated with the complex for 12 hrs. The transfection mixture was removed and the cells were cultured for 24-48 hrs in growth medium.

2.5.2.3 Immuno staining and confocal microscopy

The cells plated on cover slips were fixed in 1% Formaldehyde (Sigma) for 45-60 minutes and permeabilized in PBS + 0.2% TritonX100 for 15 mins. The cells were incubated with blocking buffer for 1hour (1XPBS, 10% FCS, 0.2 % TritonX100). The Primary antibodies were diluted in blocking buffer and the cells were incubated with the primary antibody for an hour. The cover slips were washed thrice with PBS + 0.2% TritonX100. The secondary antibodies were diluted in 1XPBS with 0.2 % TritonX100 and incubated on the cover slip for 45 minutes. The cells were washed thrice with 0.2 % TritonX100 in 1XPBS and mounted on DAPI containing mounting medium. The imaging was done using LSM510meta multiphoton confocal microscopy and the images were processed using LSM image analysis softwares.

2.6 Fly genetics

2.6.1 Maintenance of flies

Flies are maintained at room temperature or at 25°C on standard cornmeal with sugar agar. Newly emerged female virgins were recognized by their pale body pigmentation and a presence of a dark spot (meconium) in their abdomen. Males, smaller than the female in size was easily separated based on dark pigmented abdomen and sex comb on the first pair of legs.

2.6.2 *Drosophila* transgenesis

The constructs were microinjected to *w1118* embryos along with Turbo-helper plasmid (source of transposase) in injection buffer (5mM KCl, 0.1mM Phosphate buffer, pH-7.8) and made transgenic lines. The flies emerging after injection were crossed with w^{1118} female virgins or young

male flies. The progenies of this cross having red eye color were crossed with double balancer female virgins or young male flies. This helped us to identify the chromosome of the transgene insertion and also one more round of cross with double balancer was done to establish the stock. All the transgenes having no visible phenotypic effect due to the insertion were used for further studies. Both pUAST and Sym-pUAST vectors are used in UAS-GAL4 based expression system. The expression is seen only when GAL4 binds to UAS which is upstream of the gene in the transgenic system. This GAL4 is obtained by crossing these transgenic lines to different GAL4 driver flies which expresses the protein in temporal and spatial specific manner. All the transgenes having no visible phenotypic effect due to the insertion were used for further studies.

2.6.3 *Drosophila* embryo staining

2.6.3.1 Drosophila embryo collection and staging

Flies were kept in embryo collection cages and the embryos laid on fly food plates are washed and collected on a sieve. To stage the embryos, the plated were kept for overnight (overnight collection), it was changed then and kept for 2 hr time. The first 2hr collection was discarded as it might contain some older embryos. Next 2hr collections are taken and labelled as 0-2hr. Several 0-2hr collection plates were staged by incubating for 2hr, 4hr etc to obtain 2-4hr(2hr staging of 0-2hr collection), 4-6hr(4hr staging of 0-2hr collection)etc upto 16-18hr. Staging was performed in 25^0C.

2.6.3.2 Immuno-staining of Drosophila embryo

Flies were kept in embryo collection cages and the embryos laid on fly food plates are washed and collected on a sieve. To stage the embryos, the plated were kept for overnight (overnight collection), it was changed then and kept for 2 hr time. The first 2hr collection was discarded as it might contain some older embryos. Next 2hr collections are taken and labelled as 0-2hr. Several 0-2hr collection plates were staged by incubating for 2hr, 4hr etc to obtain 2-

4hr(2hr staging of 0-2hr collection), 4-6hr(4hr staging of 0-2hr collection)etc upto 16-18hr. Staging was performed in 25^0C.

Embryos were dechorionated and fixed in formaldehyde and devitalized using methanol and stained with purified dRif1 and other antibodies with appropriate dilutions. The embryos were blocked for 1hr in 1X PBS + 0.1% TritonX100 + 0.1%BSA for 1hr. Primary antibody incubation was carried out overnight at 4^0C in cold room. Washing was performed as 3 quick washes followed by 3 washes for 20 mins each all in PBST and then incubated for 2-3hrs in secondary antibody. The washing steps are repeated and finally the embryos were mounted in DAPI containing mounting media from Vectashield and sealed the coverslips using nail polish. Images were taken in Zeiss LSM-510 Meta confocal microscope.

2.6.4 Imaginal Disc staining

Third instar larvae were dissected in PBS and fixed in 4% paraformaldehyde for 20 minutes. Blocking was done for 1hours in PBST +0.1% BSA. Primary antibody was diluted in PBST+0.1% BSA, and incubated over night at 4^oC or for 2-3 hrs at room temperature. Washing was performed for 3 times for 15 minutes each with PBST. Incubation with Secondary antibody diluted in PBST+0.1% BSA was performed for 1 hour at room temperature. Washing step was repeated as previously and discs were mounted with mounting media containing DAPI or TOPRO-3.

2.6.5 Ovary dissection and staining

Ovary was dissected from female flies fed with yeast for 2 days in 1X PBS and removed extra cuticle or other tissues attached to it. These ovaries were fixed in 1X PBS + 0.1 % Triton X-100 (PBST) with 4% Formaldehyde for 20 minutes. Washed 3-4 times and blocked in PBST + 0.1% BSA for 1hr. Incubated with primary antibody diluted in PBST+0.1% BSA, O/N at 4^oC , Washed 4 times for 1 minute and 1 time for 15 minutes in with PBST+0.1%

BSA and incubated with secondary antibody diluted in PBST+0.1% BSA for 2hrs at room temperature. Again washed as previously and spread the ovaries in mounting media containing DAPI on a slide and mounted with cover slip and sealed.

CHAPTER 3

Identification of *Drosophila* Rif1 and testing conservation of function of putative dRif1 in *Drosophila* S2 cells

3.1 Introduction

Rap1 Interacting Factor1, Rif1, protein was first identified in budding yeast and *rif1* showed increase in telomere length. Rif1 gets recruited to telomere through Rap1 C-terminus along with another Rap1 Interacting Factor, Rif2 and localises to the telomere as 4-6 clusters (Mishra & Shore, 1999) and Rif1 is involved in the heterochromatin dynamics of these clustered telomeres (Park et al, 2011). In fission yeast the Rif1 protein homologue, spRif1, is a negative regulator of telomere length and is recruited by Taz1 to the telomere (Kanoh & Ishikawa, 2001). Although Rif1 in mouse, mRif1, was seen to be physically associated with telomere so far no telomere specific role has been identified (Silverman et al, 2004). Human and mouse Rif1 localize to the DNA damage/ repair foci upon damage to DNA by various agents (Buonomo et al, 2009; Silverman et al, 2004; Xu & Blackburn, 2004). Human Rif1, hRif1 localizes to the telomere only when it is aberrant and is unlikely to have a telomere specific function. Preliminary bioinformatics analysis from our lab suggests that homologue for Rif1 exists in fly as well. And while our analysis was in progress, there were reports that plausible homologue of Rif1 exists in fly (Adams & McLaren, 2004; Kanoh & Ishikawa, 2001; Xu et al, 2010). The fact that *Drosophila* telomere is maintained by non-telomerase retrotransposition mediated mechanism and the homologues for the binding partners for Rif1 did not exist in flies induced us to ask the question why Rif1 is present in flies?

We hypothesised that the Rif1 has a unique conserved molecular function in these organisms though it has been recruited to different processes and characterizing the fly homologue would give important insights into its molecular function. It is important to point out that despite over a decade of studies in yeast the molecular mechanism of Rif1 action is not known even in yeast. It does not have any obvious motifs that provide clues to its roles. To understand the conservation of Rif1 protein function we carried out detailed bioinformatics analysis in order to identify Rif1 homologues across species and identify conserved features of this protein. Furthermore, in order to know whether dRif1 has a telomeric role (yRif1 function) or DNA damage/repair function (hRif1 function), we made use of *Drosophila* embryo derived S2 cell line system and addressed its role.

3.2 Results

The Rif1 protein sequence of human and yeast were used for finding the homologues in NCBI protein sequence database. By this approach we found Rif1 homologues in 92 different organisms, including 54 fungal species, 18 insects and 16 vertebrate species. In addition, we found the homologues in *Hydra magnipapillata* (Cnidarian), *Trichoplax adhaerens* (Placozoan) and *Saccoglossus kowalevskii* (Hemichordata). Phylogenetic tree constructed using the protein sequences of Rif1 shows an evolutionary pattern from lower to higher organisms and indicates that the insect homologues are closer to human than fungal Rif1(Figure: 6A). We did not find clear homologues of Rif1 in plants, although a related protein in a lycophyte, *Selaginella moellendorffii*, was detected. While search with this lycophyte protein sequence in plants returned several uncharacterized proteins showing reasonable similarity, these proteins lack the key conserved SILK/PP1 interaction domain (Figure: 6B). We therefore deemed the plant homologues to be too diverged for further analysis. The bioinformatic analyses were carried out with the help of R.Senthilkumar, Dr. Rakesh K Mishra's Lab, CCMB.

3.2.1 Conserved domains of Rif1 homologues

We found three motifs, namely, HEAT repeat, SILK motif and a domain present in the C-terminal end which was shown to have DNA binding property (Xu et al, 2010), that are conserved across the species from yeast to mammals in Rif1(Figure: 6B). In addition, previously predicted BLM helicase interaction domain is conserved only in the vertebrates. HEAT repeat spans ~1000 amino acids in Rif1 homologues (Xu et al, 2010). In our detailed analysis we found a highly conserved region of 101-149 amino acids present within the HEAT repeat that is Rif1 specific. This domain is also present in the putative homologues identified in plants.

Our analysis identified another novel feature, SILK motif or Protein Phosphatase1 (PP1) interaction domain**,** in all Rif1 homologues. The highly conserved residues RVxF were also detected along with the SILK motif, which is the docking motif

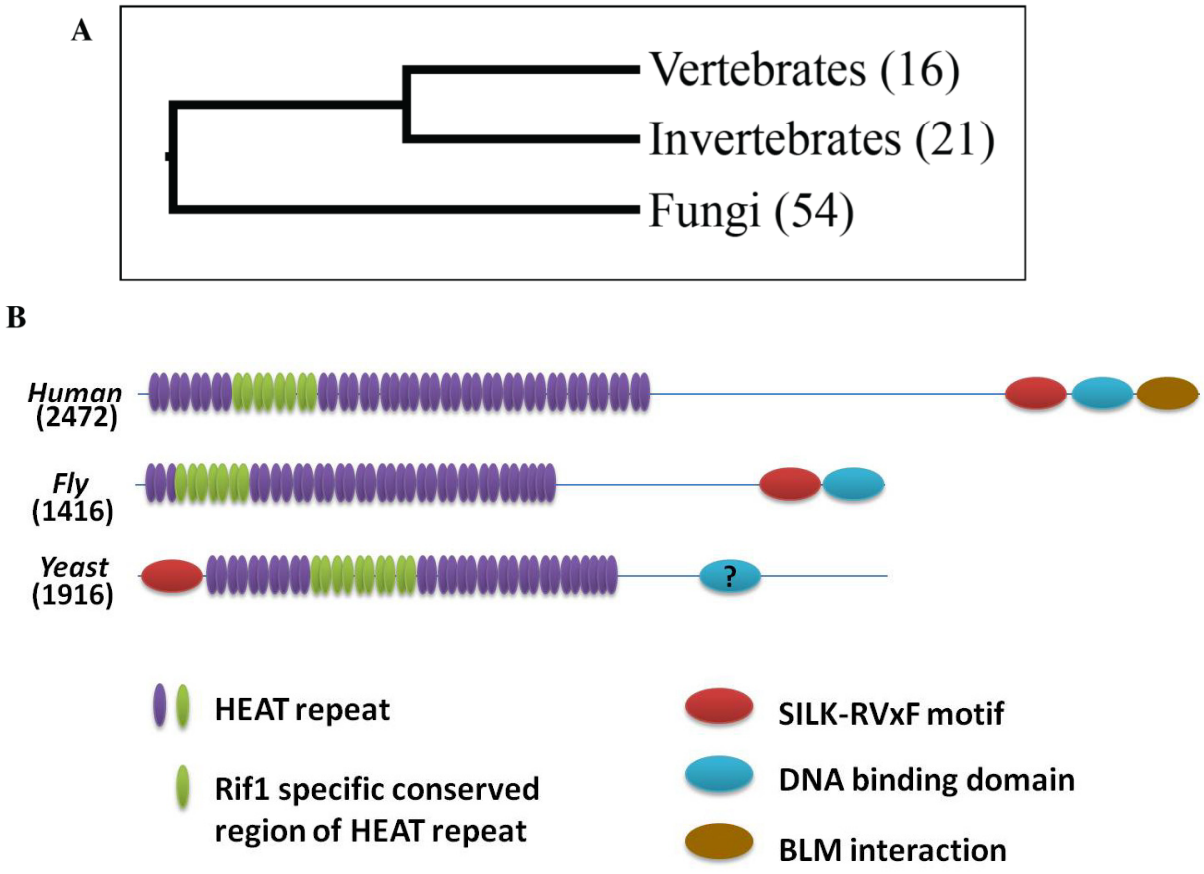


Figure 6: A) The phylogenetic tree of Rif1 homologues. The simplified version of the phylogenetic tree of Rif1 homologues (The detailed tree is shown in the Additional file 2). A common branching is seen in three major classes (Fungi, Invertebrates and Vertebrates) and the number of organisms from each branch having the Rif1 homologues is mentioned in the parentheses. **B) The conserved domains of Rif1 homologues**. The conserved domains of Rif1 homologues of human, fly and yeast are shown. The protein length is mentioned below the organism name. The conserved domains are highlighted in different shapes (SILK/PP1 interaction domain – diamond, DNA binding domain –oval (horizontal), BLM interaction domain –rectangle, HEAT repeat – oval (vertical) and the core conserved region of HEAT repeat is highlighted in grey). The motifs are mapped approximately to the scale.

essential for PP1 interaction (Egloff et al, 1997; Hendrickx et al, 2009). In all Rif1 homologues, we found SILK and RVxF combination to be present with varying length of amino acid sequences between them. Interestingly, the SILK-RVxF domain at the N-terminal end of Rif1 homologues of fungi is present at the C-terminal end of multi-cellular eukaryotes (Figures: 7 & 8). Thus there has been a swapping of SILK motif in Rif1 from N-terminal end to C-terminal end during the course of evolution. This shift is seen from placozoans onwards, which are the basal group of multi-cellular organisms. Additionally, in single cell organisms, when the SILK motif is seen in the N-terminus its architecture was 'SILK-RVxF'; but in multi cellular organisms the motif is shifted to C-terminus and the architecture is reversed to 'RVxF-SILK'. Based on the architecture and position of the SILK domain, we again find that the *Drosophila* homologue is closer to vertebrates than yeasts.

A unique DNA binding domain was reported in human Rif1 which helps in bringing the BLM helicase to the stalled replication forks (Xu et al, 2010). We found that this domain is conserved from yeast to human. Although the sequence homology of Rif1 is poor between unicellular and multicellular organisms, the profile based search strongly supports the conservation of this DNA binding domain between these two groups of organisms. BLM interaction domain was also reported in the study of human Rif1 (Xu et al, 2010). Our analysis shows that this domain is conserved only in vertebrates.

3.2.2 Raising specific antibody to characterize dRif1 function

The full-length cDNA clone (RE66338) was obtained from the Drosophila Genomics Resource Center (DGRC). Approximately 1.2kb region from the N (54-450 amino acids) and C (694-1094 amino acids) terminus of dRif1 were chosen (N-dRif1 and C-dRif1) for raising antibody (Figure: 9A). These two regions were amplified by PCR and cloned in-frame in pET-28a His-tag vector. Both of the clones were transformed in Bl-21 strain and expressed by IPTG induction. Since the N terminal region was making inclusion bodies and the purification was difficult,

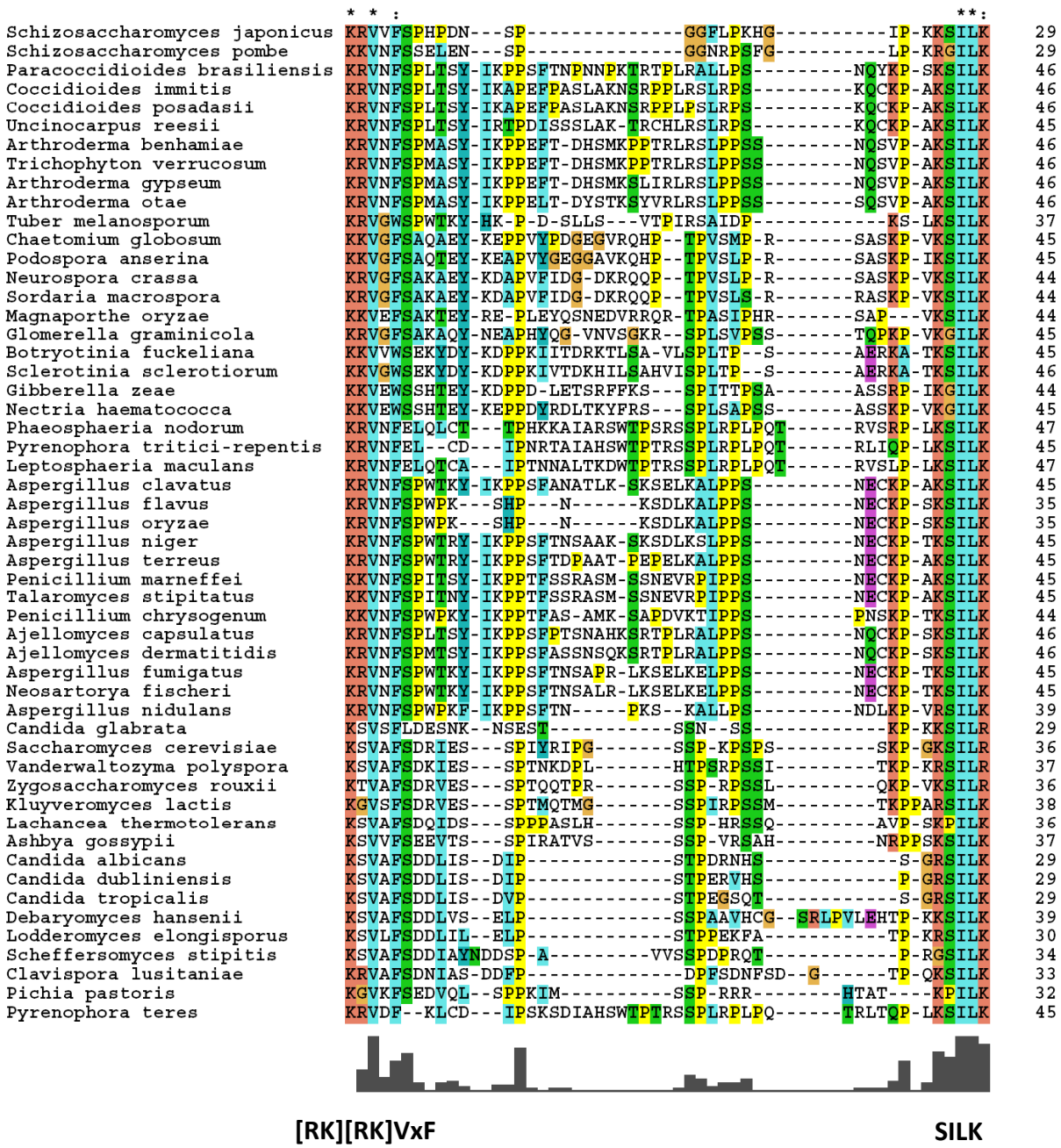


Figure 7: The N-terminal SILK/PP1 interaction domain of unicellular organisms. The organism name and the length of the domain for each sequence are shown to the left and right of the multiple sequence alignment, respectively. The amino acids are highlighted in different colours based on their property. The degree of conservation at each position in the alignment is represented as bar graph at the bottom of the alignment.

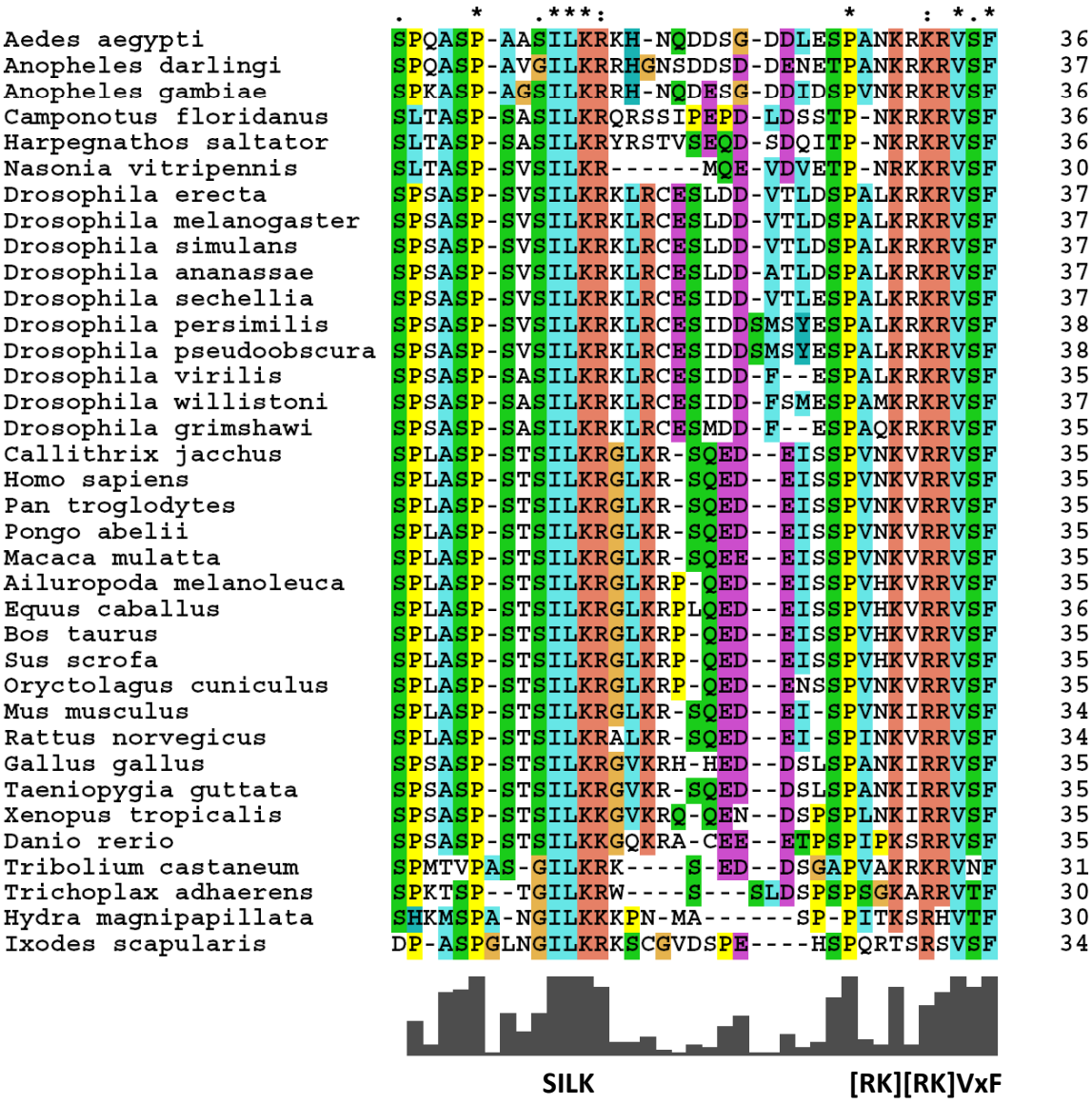


Figure 8: The C-terminal SILK/PP1 interaction domain of multicellular organisms. The organism name and the length of the domain for each sequence are shown to the left and right of the multiple sequence alignment, respectively. The amino acids are highlighted in different colors based on their property. The degree of conservation at each position in the alignment is represented as bar graph at the bottom of the alignment.

we used the C terminal region for raising the antibody. C-dRif1 was purified using Ni-NTA His bind resin from QIAGEN (Figure: 9B) and a New Zealand white rabbit was immunized with ~300μg of purified C-dRif1 with Freund's complete adjuvant. It was followed by 3 booster doses ~300μg along with Freund's incomplete adjuvant with 15 days of interval period. Seven days after the 3rd booster, a test bleed was taken and tested for the specificity and a 20 ml bleed was collected from the animal as test bleed picked a band at expected size. This was followed by a 4th booster and collected another 15ml bleed as in case of 3rd booster. The serum was prepared by clotting the blood at 37^0C for 60 minutes and centrifuged at 3000 rpm for 10 minutes and collected the supernatant serum, made aliquots and stored at -80^0C. A portion of the serum was affinity purified against purified CdRif1 protein bound to nitrocellulose and used for different experiments.

Mouse antibodies were raised against the same purified protein, CdRif1 and immunized two mice to raise antibody. BALB/c mice were immunized with ~100μg of purified C-dRif1 with Freund's complete adjuvant. It was followed by 3 booster doses ~100μg along with Freund's incomplete adjuvant with 10 days of interval period. 1.5ml bleeds were collected based on test bleed results after the 3rd, 4th and 5th booster doses and serum was prepared as previously.

All the antibodies gave a specific band ~160kDa as expected as shown in the western blot experiment (Figure: 9C) with fly embryo total protein extract and therefore we used these antibodies for all other experiments described hereafter.

3.2.3 dRif1 is localized to the nucleus and is prominently associated with heterochromatin in S2 cells

In order to understand the function of dRif1 we used S2 cell line system first to check which of the function dRif1 retained compared to its homologues in other organisms. Western blotting detected a specific band for dRif1 in the S2 whole cell extracts (Figure: 10A) .We performed immunolocalization in S2 cells using the antibody raised against dRif1 to see the subcellular localization, and found that dRif1 was nuclear localized (Figure: 10B). dRif1 stained the nucleus in a heterogeneous manner, with most nuclei showing one or two prominent dark

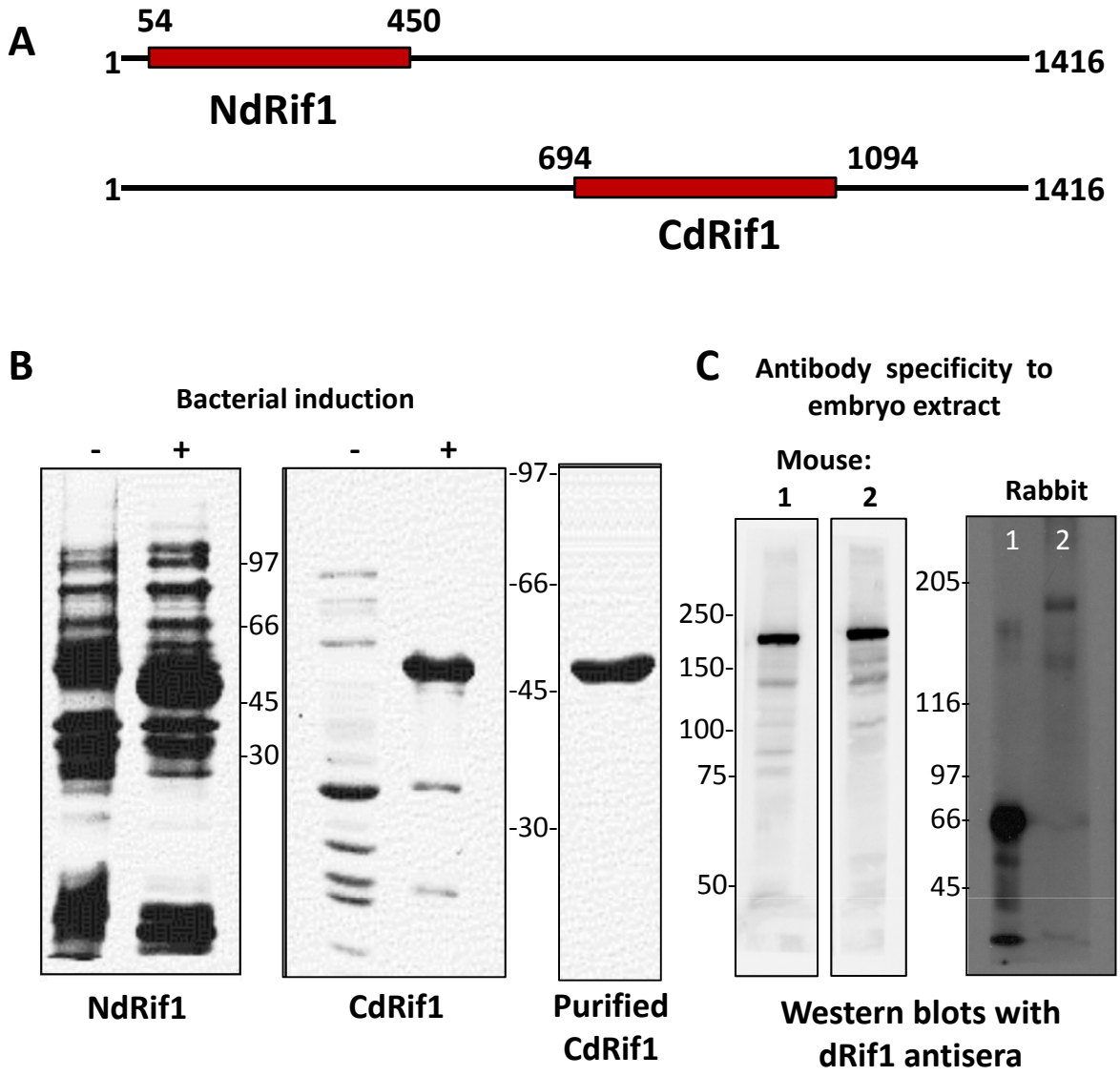


Figure 9: Antibody against dRif1. A) Regions of the protein expressed in bacteria are shown in red boxes along with the amino acid positions. B) Induction of NdRif1 and CdRif1 by IPTG (+) loaded along with un-induced extracts (-). The induced CdRif1 purification by Ni-NTA column showed single specific band on coomassie stained gels. C) Western blotting using antisera from different animals (Mouse1,2 and Rabbit) detected a band ~160kDa from fly embryo extracts. Rabbit antisera also detected the bacterially expressed CdRif1 protein as shown in lane.1.

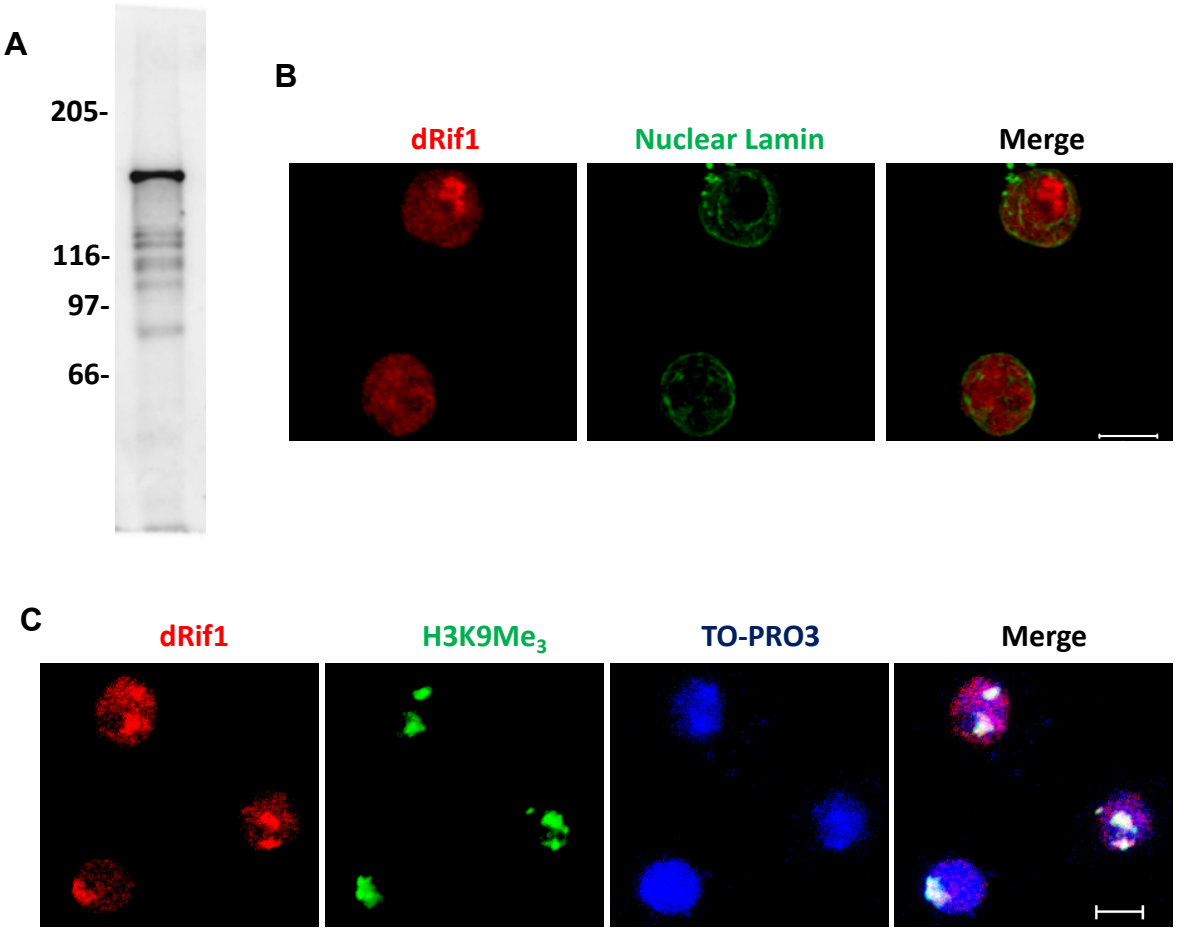


Figure 10: dRif1 localizes to the nucleus in unperturbed cells. A) Western blot of total protein extract from S2 cells was probed with antibody against dRif1. A 160kDa lights up prominently. **B)** S2 cells were immunostained with antibodies to dRif1 (red) and costained with antibodies to lamin (green) to mark the nucleus. **C)** S2 cells were immunostained with antibodies to H3K9 trimethyl (green) and dRif1 (red) and TO-PRO3 to mark the nucleus (scale bar, 5mm).

patches along with a diffuse nuclear staining. As the same regions also appeared to contain dense DNA staining, we tested if this patch corresponded to heterochromatin. We colocalized dRif1 with the heterochromatin marker, histone H3 trimethyl lysine 9. As shown in figure: 10C, we found that dRif1 associates with heterochromatin. This localization is similar to what is observed in yeast cells where yeast Rif1 is predominantly associated with telomeric heterochromatin, although it does not have direct roles in establishment or maintenance of telomeric heterochromatin.

3.2.4 Conservation of DNA damage function of dRif1 in *Drosophila* S2 cells

Human Rif1 is nuclear localized uniformly in unperturbed cells. Multiple forms of DNA damage, including ionising radiation, hydroxy urea, UV, etoposide, aphidicolin cause hRif1 to relocalize into foci, which often coincide with the damage sites (Buonomo et al, 2009; Silverman et al, 2004; Wang et al, 2009; Xu & Blackburn, 2004). To test if dRif1 also responded to damaged DNA in a similar manner, we treated S2 cells with hydroxy urea and aphidicolin and asked if dRif1 also relocalized to halted replication forks. Cells were also costained with γ-H2AvD antibodies to mark the sites of damaged DNA. In contrast to what has been observed in human cells, we did not see any major relocalization of dRif1 with either hydoxy urea or aphidicolin treatment (Figure: 11). DNA damage foci that showed strong γ-H2AvD staining were prominent in the treated cells showing that treatment did induce DNA damage. Therefore, dRif1, unlike hRif1, does not relocalize upon DNA damage.

3.2.5 Knock-down of dRif1 in S2 cells does not affect DNA damage response

The experiments described above showed that dRif1 does not respond to DNA damage by localizing to the repair sites. However, in order to test this more directly, we carried out knock down experiments using double-stranded RNA. Three different primer sets with no off targets were designed for Rif1. We used dsRNA of GFP for the mock treatment experiments. We did two successive rounds of dsRNA treatment and performed both western blot and immunofluorescence studies and confirmed that dRif1 protein levels went down to undetectable levels by the sixth

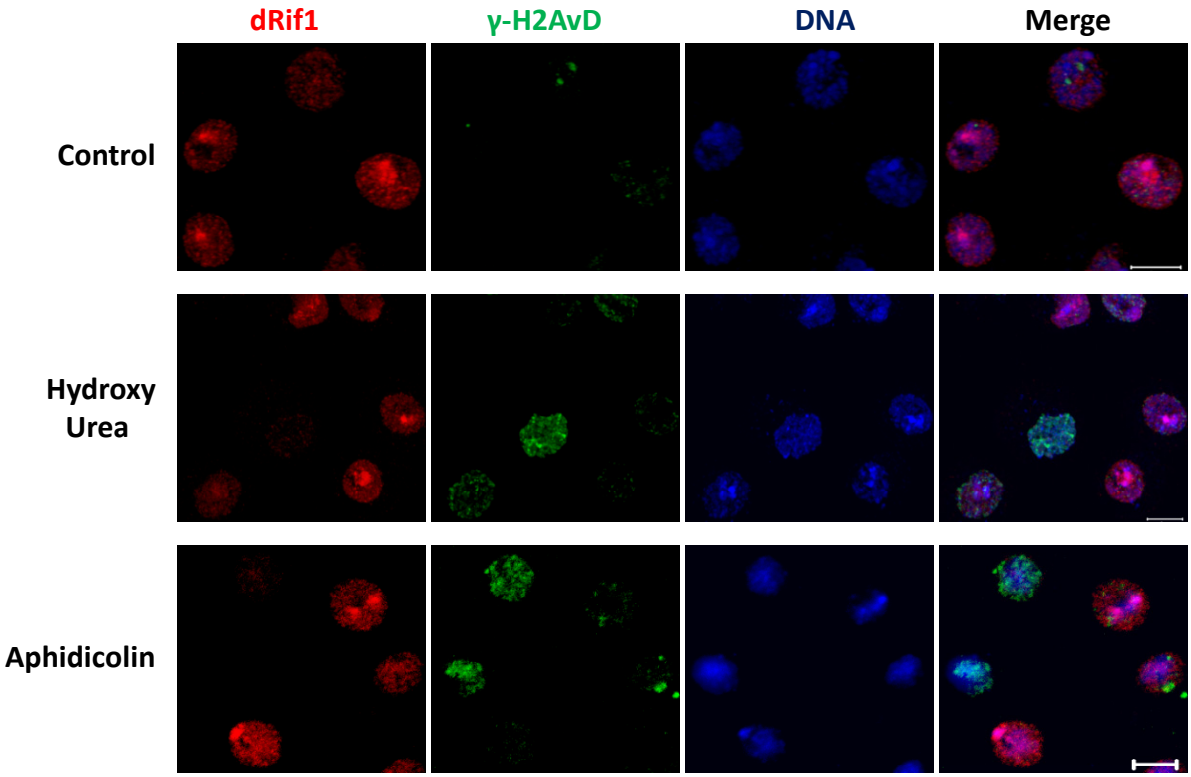


Figure 11: dRif1 does not colocalize to the DNA damage foci induced by HU and Aphidicolin. S2 cells were treated with either 2.5mM hydroxy urea (HU) or 25μM aphidicolin for 16hrs to induce DNA damage and then fixed, stained with antibodies to dRif1 (red) and γH2AvD (green). The slides were mounted in mounting media containing DAPI or TO-PRO 3 (blue) (Scale bar, 5μm).

day (Figures: 12 A and D). Cells remained healthy and continued to divide for several days after dsRNA treatment. These knockdown cells were treated with DNA damage inducing agents, HU and bleomycin. After treatment we stained the cells for γ-H2AvD and dRif1. First, we did not find any difference in viability between mock treated and dRif1-RNAi cells upon induction of DNA damage. Second, upon staining for γ-H2AvD, we found several spots come up on DNA damage induction (Figures: 12 A, B and C). We also performed western blots to compare the levels of γ-H2AvD between wild type and knock down cells (Figure: 12D). Our results show, both by immunofluorescence and by western blots, that repair foci (therefore, signaling of DNA damage) occurs normally in the absence of dRif1. These data further strengthen our conclusions that unlike human Rif1, *Drosophila* Rif1 does not participate in DNA damage response by translocating to sites of repair.

3.2.6 Knock-down of dRif1 does not affect telomere transcription in S2 cells

As yRif1 represses transcription of telomeric repeat containing transcripts (TERRA) (Greenwood & Cooper, 2011; Iglesias et al, 2011) and is involved in telomere position effect in *C. glabrata*, we asked if telomere specific transcript levels are under dRif1 control in *Drosophila*. *Drosophila* telomeres have retrotransposon elements that are generally transcriptionally suppressed and are activated by mutations that inactivate telomere position effect and rasiRNA pathway, indicating that they are under strict transcriptional repression (Reviewed in Capkova Frydrychova et al, 2008). We isolated RNA from wild type and knock down cells and performed quantitative reverse transcriptase PCR for transcripts from telomere associated retrotransposons (Torok et al, 2007).We do find low levels of transcripts in wild type cells and this level is not significantly affected by knock down of Rif1 (Figure: 12E). This result suggests that dRif1 is unlikely to regulate telomere retrotransposon transcript levels in S2 cells.

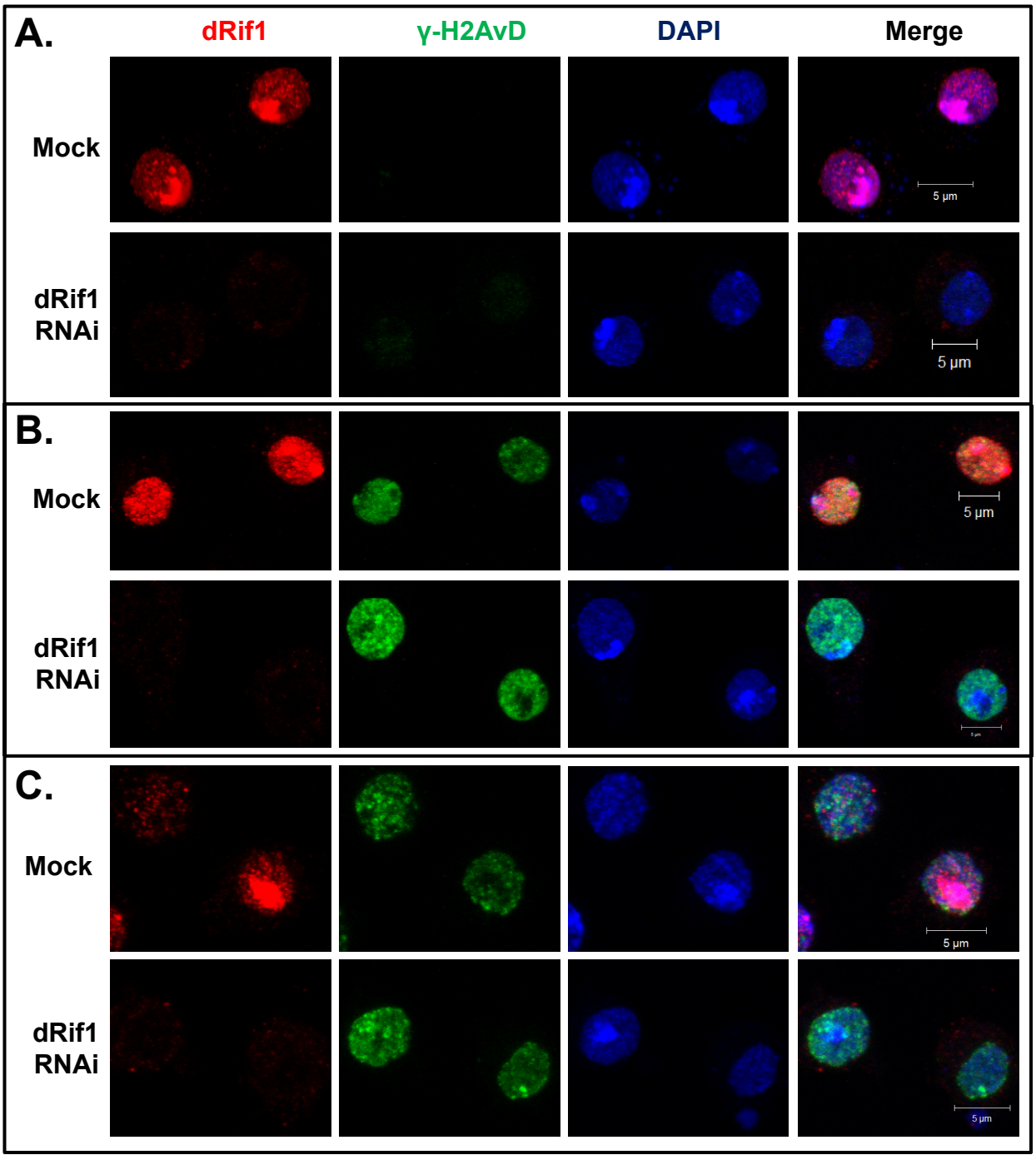


Figure 12: Knock down of dRif1 in S2 cells. S2 cells were treated with either dsRNA of GFP or dRif1. Each was further split into three parts and was either mock treated (A) or treated with hydroxyurea (B) or bleomycin (C). The above mentioned cells were stained with γH2AvD to detect DNA damage/repair foci. All the cells were fixed and stained for dRif1(red), γH2AvD (green) antibody and DAPI (blue).

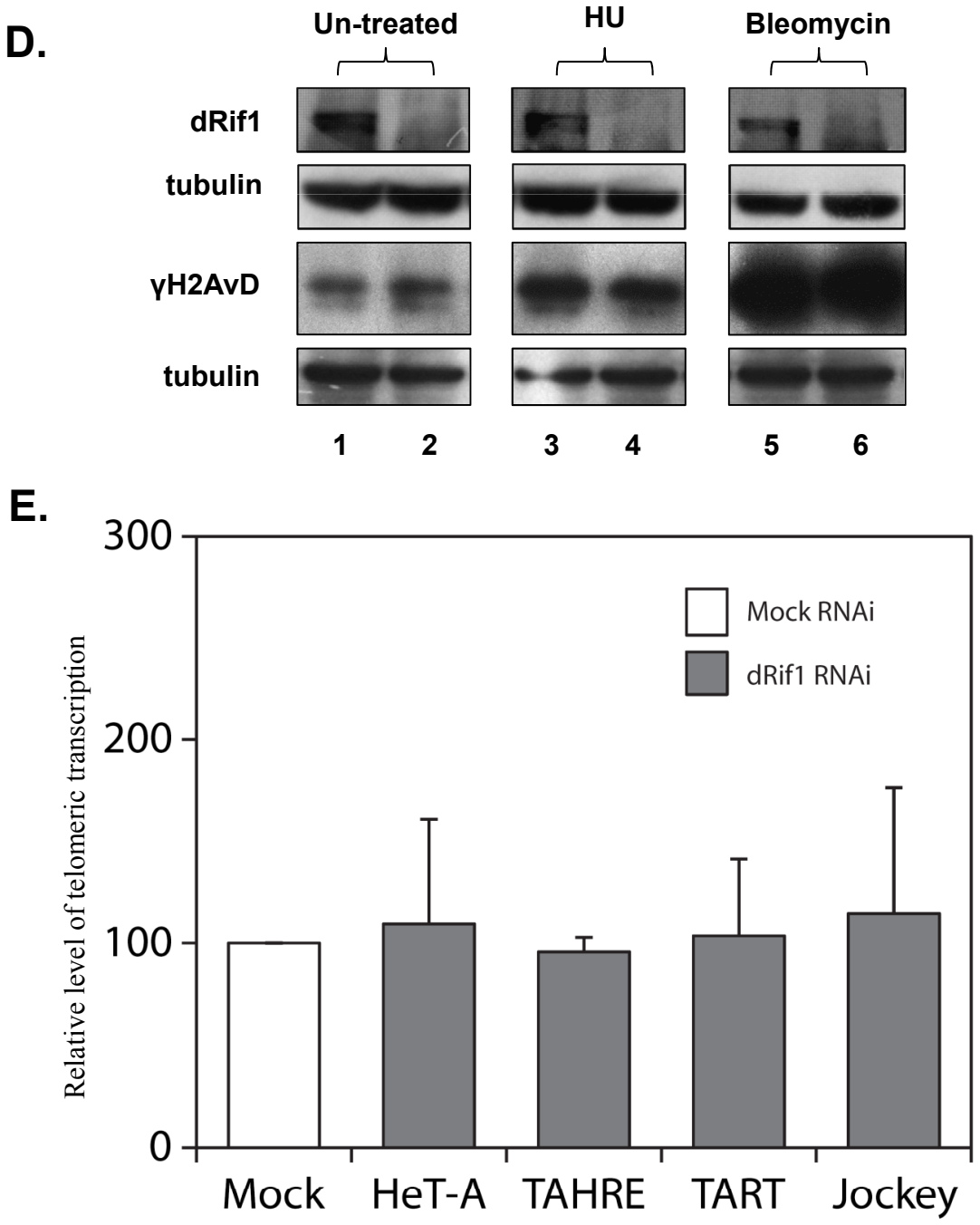


Figure 12: Knock down of dRif1 in S2 cells (Contd.) D) Protein extracts were tested for dRif1 and γH2AvD expression; tubulin was used as a loading control. dRif1 was undetectable in RNAi treated cells (Lanes 2,4,6). γH2AvD levels increase upon exposure to DNA damage. **E)** RT-PCR was performed in the above mentioned cells to detect levels of telomere associated retrotransposon transcripts, HeT-A, TAHRE, TART and the internal retrotransposon, Jockey. The relative level was obtained by normalizing mock treated of each to 100. Error bars indicate SD. Averages are from two independent experiments.

3.3 Discussion

Rif1 was identified in yeast almost two decades ago and genetic and biochemical studies have clearly shown that it is a negative regulator of telomerase. Emerging evidence shows that Rif1 in mammals has diverged from its primary role in telomere synthesis and maintenance to a broader role in response to DNA damage. Our detailed analysis of Rif1 from multiple organisms has identified several novel features. *Drosophila* Rif1 is evolutionarily closer to vertebrate Rif1 than yeast Rif1. All Rif1 homologues contain the conserved HEAT-repeats and this may carry out the core Rif1 activities. HEAT repeat is a structural domain with poor sequence homology and is present in several proteins (Andrade et al, 2001). As this domain has been implicated in interacting with proteins, it might recruit a variety of proteins to carry out its functions. Within these HEAT repeats, our studies identify a conserved Rif1 specific repeat and this might be useful in identifying the core conserved interacting partners. The more diverse repeats are likely to facilitate participation in other functions. Our analysis also identified a conserved SILK motif along with a highly conserved RVxF motif, again present in all organisms, from yeast to humans. Earlier studies have shown that the SILK motif is specifically associated with RVxF motif in certain class of PP1 interacting proteins (Hendrickx et al, 2009; Liu et al, 2010). Recently, a large scale proteomics study revealed that the mammalian Rif1 interacts with PP1 by affinity chromatography (Moorhead et al, 2008), indicating that Rif1 is a target of PP1. As this motif has been retained in all species, this is likely to participate in the core Rif1 functions. dRif1 lacks the C-terminal BLM interaction domain but contains all the conserved features associated with the N-terminal region.

dRif1 encodes for a 160kDa protein that is localized to the nucleus. We find that a large fraction of the protein is associated with heterochromatin. This is similar to the predominantly telomeric localization of Rif1 in yeast (Mishra & Shore, 1999; Smith et al, 2003). A very recent report implicates yRif1 in heterochromatin establishment at the silent mating type loci (Park et al, 2011) and genome wide chromatin immunoprecipitation studies also show that yRif1 is associated with the silent mating type loci (Smith et al, 2003). Rif1 in human cell lines were also shown

to be associated with arrested replication forks in the vicinity of pericentromeric heterochromatin although this was not observed in unperturbed cells (Buonomo et al, 2009). These results taken together implicate an evolutionarily conserved role for Rif1 at the heterochromatin.

We find that DNA damage induction did not change dRif1 staining pattern and also dRif1 did not colocalize to the damage foci. Knock down of dRif1 did not lead to any difference in response to DNA damage in S2 cells suggesting that dRif1 is unlikely to function at repair sites.

CHAPTER 4

Conservation of function of dRif1 in yeast and mammals by functional complementation study

4.1 Introduction

Rif1 function as a telomere length maintaining protein in yeast (Shore & Bianchi, 2009) and also has been implied to have roles in heterochromatin dynamics (Park et al, 2011). Mammalian Rif1 seems to be primarily involved in general DNA damage/repair function compared to the specific telomeric roles in yeast (Buonomo et al, 2009; Silverman et al, 2004; Wang et al, 2009; Xu et al, 2010; Xu & Blackburn, 2004). hRif1 does not interact with normal telomere and it associates to telomere only when the telomere is aberrant. Although expressing the hRif1 in *Saccharomyces cerevisiae* could not complement the scRif1 function, it perturbed the telomere length maintenance process (Xu & Blackburn, 2004). This could be due to the conservation of interaction with certain partners in these two organisms.

Therefore in order to elucidate the evolutionary conservation/divergence of the protein, we decided to complement the Rif1 functions in *Saccharomyces cerevisiae* and in HeLa cells to test whether dRif1 can either complement or perturb the roles of its homologues in the respective organisms. As we have already addressed the telomeric function (scRif1 function) and DNA damage/repair function (hRif1 function) in *Drosophila* embryo derived S2 cell lines, we decided to express the protein in budding yeast and check whether it can alter the length regulation function in it.

4.2 Results

4.2.1 Effect of heterologous expression of dRif1 protein in yeast on telomere function in yeast

The bioinformatic analyses show that Rif1 is conserved throughout eukaryotes. However, we found that dRif1 doesn't have roles in transcriptional silencing at telomeres though yRif1 antagonises transcriptional silencing at yeast telomeres. Functional conservation of interactions of dRif1 can interfere with the normal functioning of yRif1 in yeast and we hypothesised by expressing dRif1 in heterologous yeast system it could interfere with telomere length regulation or TPE.

4.2.1.1 Telomere length

To test how much of the core functional properties of yeast Rif1 are retained in *Drosophila,* we performed complementation assays. To this end the full length dRif1 was expressed under the control of yeast Rif1 promoter and transformed into wild type, *rif1*, *rif2* and *rif1rif2* mutant yeast strains and the telomere length was estimated. Yeast lacking Rif1p have much longer telomeres than wild type cells (Hardy et al, 1992; Wotton & Shore, 1997). As seen in Figure: 13, wild type cells have compact telomeres around 1.2kb (lanes 1, 3&11) *rif1* (lanes 6&8) and *rif2* (lanes 12&14) mutations increase the length of the telomeres considerably, with *rif1* mutants showing more pronounced effects. The double mutants have an additive effect and the telomeres are extremely long and disperse (lanes 17&19). Single copy expression of yeast Rif1 is able to complement *rif1* phenotype (lanes 5, 10, 16&21); however, dRif1 does not change the telomere length in any of these strains (wild type- lanes 2&4, *rif1*- lanes7&9, *rif2*- lanes 13&15 and *rif1rif2*- lanes 18&20).

4.2.1.2 Telomere position effect

Apart from telomere length, yeast Rif1 antagonizes telomere position effect (TPE). Therefore, we tested whether TPE in yeast is perturbed by dRif1 expression**.** Telomere position effect is the silencing of expression of genes placed proximal to the telomeres due to the assembly of heterochromatin initiated by telomere repeats. KRY12 contains a *URA3* gene at the left end of chromosome VII. Strains that express *URA3* gene cannot grow on plates containing 5-FOA, as the drug is converted to a toxin by the *URA3* gene product. However, wild type yeast strains silence the *URA3* marker placed adjacent to the telomeric repeats and hence can grow on plates containing the drug 5-FOA. As shown in figure: 14, KRY12 strain carrying empty vectors (pRS315 and yEpLac181) able to grow on plates containing 5-FOA showing that the telomeric *URA3* is repressed. Upon over expression of yRif1, silencing at telomere is reduced as expected, and the strain showed sensitivity to growth on 5-FOA containing plates. We checked whether TPE of yeast Rif1 can be complemented by its fly homologue by transforming and comparing the heterologous dRif1 expressing cells to the vector alone control cells.

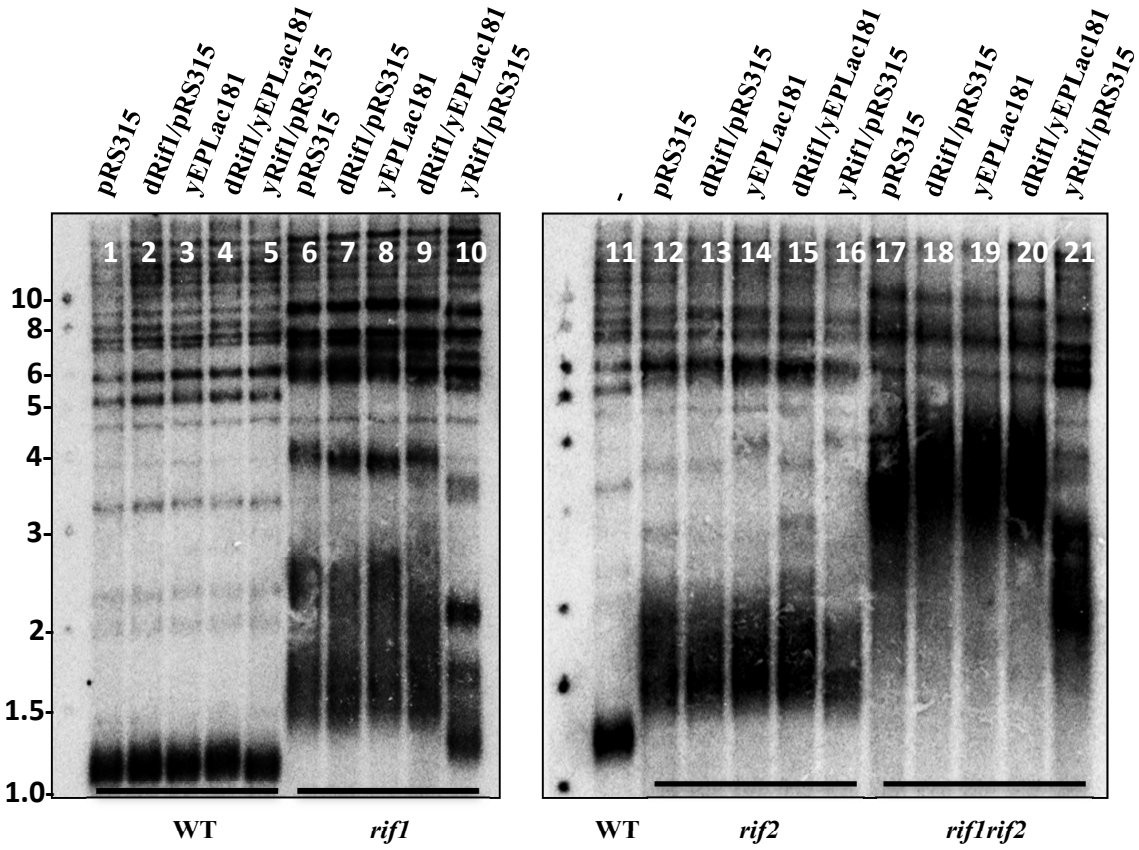


Figure 13: Expression of dRif1 protein in yeast does not interfere with telomere length maintenance. Southern blot of telomeric restriction fragments from wild type (KRY-12), *rif1*, *rif2* and *rif1rif2* double mutant yeast strains transformed with either empty vector (pRS315, yEpLac181), yRif1(positive control) or dRif1 in different vectors. XhoI digests of the genomic DNA probed with dGT repeat to identify the telomeric repeat length. The median length of wild type telomeres is approximately 1.2kb, *rif1* is 2kb, *rif2* is 1.6kb and *rif1rif2* is 4kb.

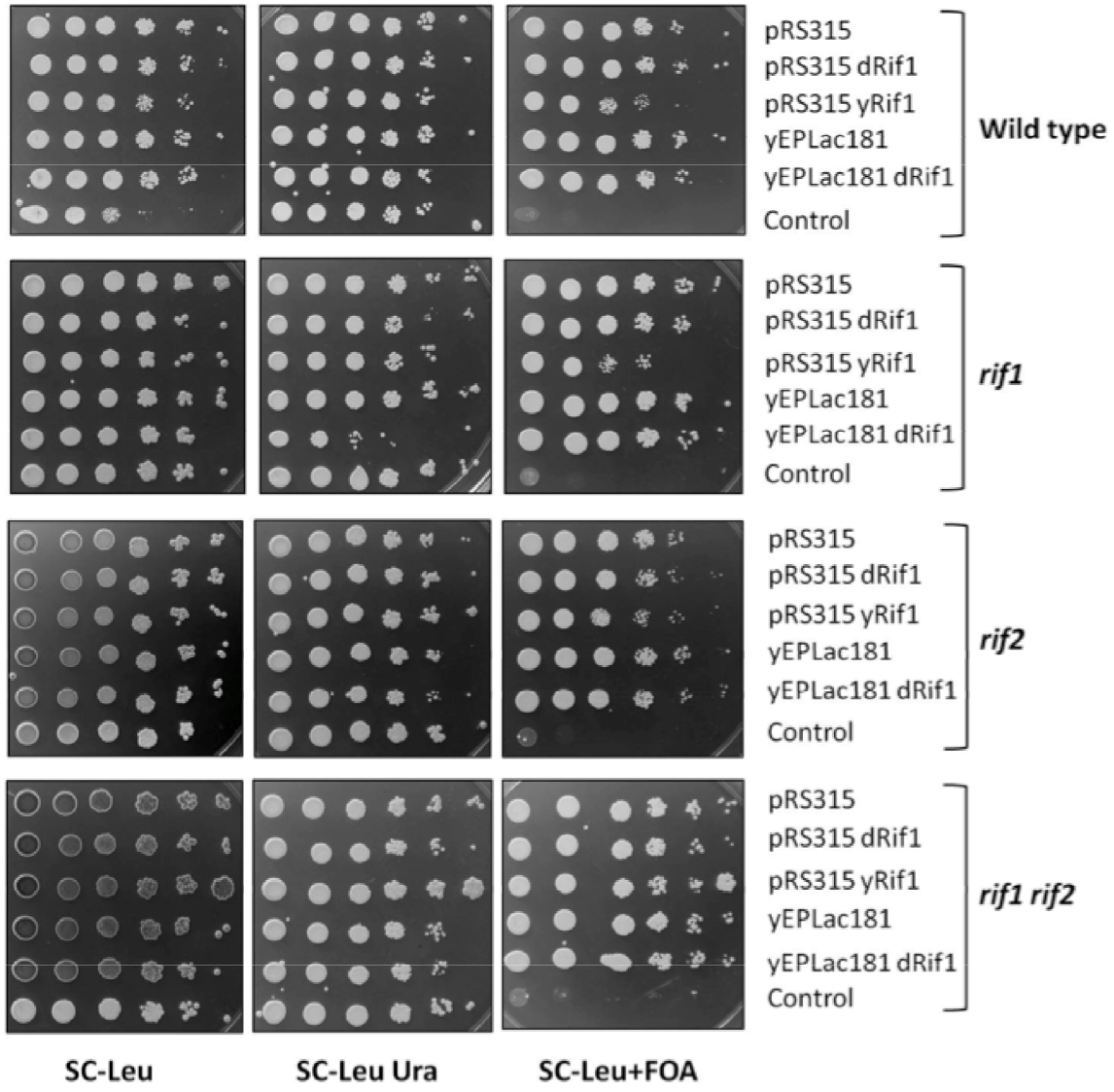


Figure 14: Telomere position effect is not altered by expression of dRif1 in yeast. Wild type, *rif1*, *rif2* and *rif1rif2* strains containing *URA3* gene at the telomere of chromosome VIIL were transformed with empty vectors (pRS315, yEPLac181), dRif1 (pRS315dRif1, yEPLac181dRif1) and yeast Rif1 (pRS315yRif1). WT grows in both plates lacking Uracil and contining 5-FOA, showing that the *URA3* is silenced. y*ku70* mutants show complete loss of gene silencing leading to expression of *URA3* and therefore lack of growth on FOA plate. The silencing on FOA plates with tenfold dilution and spotting assay do not show difference in growth compared to the corresponding vector alone control in wild type, *rif1*, *rif2* and *rif1rif2* double mutant strains.

We checked the complementation in wild type, r*if1*, r*if2* and *rif1rif2* double mutant strains of yeast. However, upon transformation of dRif1 plasmid the TPE did not alter as compared to the same strain transformed with empty vector control. This suggests that dRif1 might have diverged from its yeast counterpart as it does not interfere with telomeric position effect in yeast cells.

These two results suggest that the *Drosophila* protein does not retain much functional similarity to its yeast counterpart and therefore, cannot complement yeast Rif1 in telomere maintenance. This is in contrast to the humanRif1, which increases telomere length in *rif2* mutants (Xu & Blackburn, 2004). These data lead us to speculate that unlike hRif1, *Drosophila* Rif, has lost the ability to interact with and interfere with telomere length regulation in yeast.

4.2.2 dRif1 localized to the yeast nucleus but not to the telomeres

As a further test of functional conservation of dRif1, we determined the subcellular localization of dRif1 when expressed in yeast. We first confirmed by western blot analysis that dRif1 was expressed in yeast cells. As seen in figure: 15A, 3X FLAG-tagged Rif1 could be detected in yeast. Empty vector or 3X FLAG-tagged dRif1 was transformed into yeast strains that express myc-tagged Sir4 protein. Sir4, silent information regulator 4 protein, is localized to telomeric clusters and appears as 3 to 6 bright foci in the nuclei. These strains were fixed and immunofluorescence experiments were performed using FLAG (dRif1) and myc (Sir4) antibodies. We found that 20-30 percent of the cells have clear nuclear signal for dRif1, showing that dRif1 localizes to the nucleus in yeast (Figure: 15B; panels 2 and 4). Empty vector alone transformed control cells did not show any signal for dRif1. However, there was no colocalization with Sir4 protein which associates to the telomere. Therefore we conclude that dRif1 protein does not localize to the telomeres in yeast and it also explains the failure of dRif1 in complementing its yeast counterpart in our previous experiments. There were two interesting features about dRif1 localization in yeast. First, only a subset seemed to show dRif1 staining, suggesting that not all cells were expressing dRif1. Second, dRif1 localized to a distinct compartment within the nucleus, which is neither telomeres, nor nucleolus. This

novel site usually appeared as bright spot in the nucleus and sometimes appeared as a ring. Such novel site localization has recently been reported for Slx5, a ubiqutin E3 ligase that targets SUMO proteins and reported to have roles in DNA damage response (Darst et al, 2008)

Mammalian Rif1 is localized to the nucleus and relocalizes to the DNA damage/repair foci (Silverman et al, 2004; Xu & Blackburn, 2004). We induced DNA damage in yeast cells expressing dRif1 by incubating the overnight grown cultures with 0.05%MMS and checked for the dRif1 localization pattern upon DNA damage (Figure: 15B, panels 3 and 4). We found that the staining remained the same and Rif1 retained its unique pattern; although now most of the nuclei showed the more prominent ring like localization unlike the prominent spot or small ring staining in the untreated cell nuclei. As reported, the Sir4p spots became more diffuse (Kennedy et al, 1997; Martin et al, 1999). These data suggest that dRif1 does not relocalize to DNA damage sites.

4.2.3 dRif1 does not co-localize with DNA damage sites in human cells

The lack of relocalization of dRif1 to sites of damage in yeast could be either due to lack of conservation of the partners or pathways in yeast or alternatively might indicate lack of conservation of this function in dRif1. In order to distinguish between the two possibilities, we expressed 3X FLAG-dRif1 in HeLa cells. The full-length dRif1 along with 3XFLAG was cloned in pCMV vector and transfected into HeLa cells. Figure: 16A shows that the protein is expressed upon transfection. Since dRif11 colocalized with heterochromatin in S2 cells, we stained dRif1 using FLAG antibody to confirm transfection and also co-stained with H3K9Me3 antibody as heterochromatin marker. We did not find any significant colocalization of dRif1 with heterochromatin in HeLa cells (Figure: 16B). After 24hrs of transfection, the cells were treated with 2mM HU for 16hrs or 50µg/ml bleomycin and both treated and untreated cells were stained with antibodies to 53BP1 and FLAG. Untreated control cells showed clear nuclear localization on 3X FLAG tagged dRif1 where as 53BP1 showed one or two foci (Figure: 16C, row1 and 3).

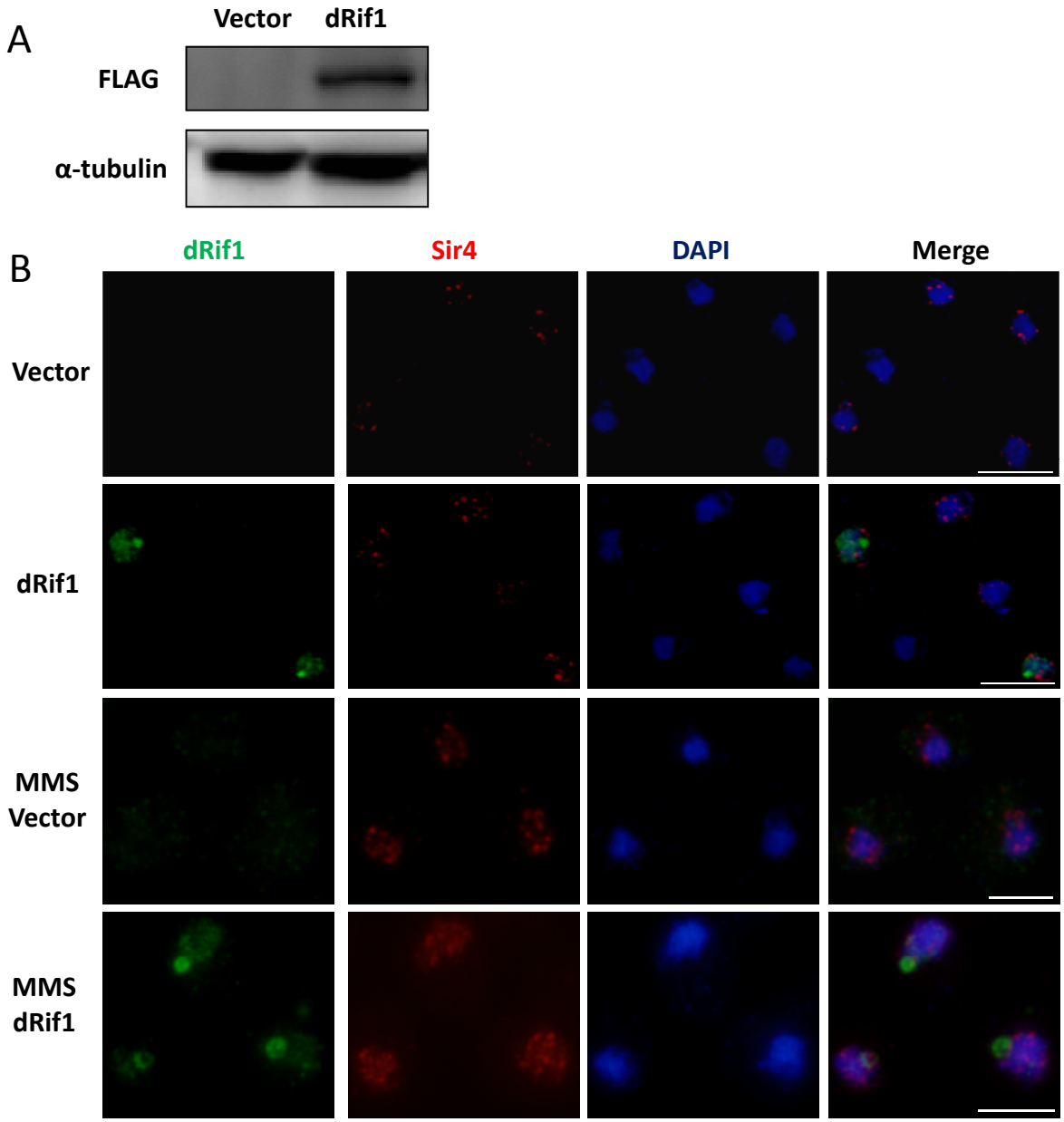


Figure 15: A) FLAG tagged dRif1 is expressed in *S.cerevisiae.* Total cell extracts of yeast transformed with empty vector(lane 1) or 3XFLAG tagged dRif1(lane 2) were probed with antibodies to FLAG and tubulin. Lanes 2 shows 3XFLAG dRif1 expression. **B) dRif1 introduced in yeast localizes to the nucleus but not to yeast telomeres.** Yeast cells transformed with empty vector or 3X FLAG dRif1 were strained for 3XFLAG dRif1 (green) and 13Xmyc Sir4 (red). Nuclei were stained with DAPI (blue). Empty vector transformed cells didn't show FLAG staining but dRif1 showed nuclear localization (PanelB, raw 1&2). DNA damage induction with 0.05%MMS for 90 minutes treatment has no effect on dRif1 (green, rows 3,4) but Sir4 shows diffusion as expected (red, row 3,4). (Scale bar, 5μm)

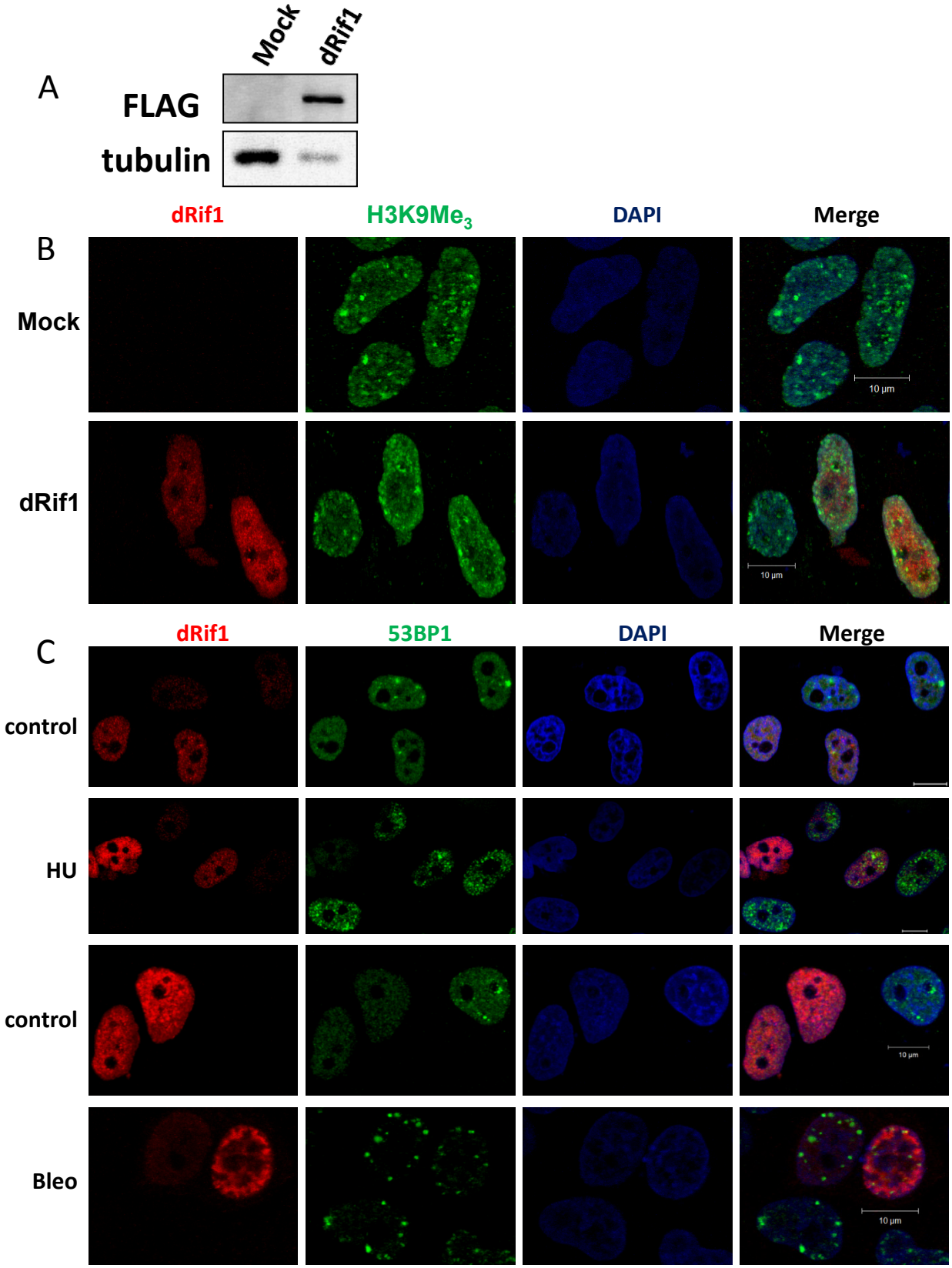


Figure 16: Heterologous-expression of dRif1 in HeLa. HeLa cells were transfected with full length dRif1 with a N-terminal 3XFLAG tag. A) 3XFLAG dRif1 expression in transfected HeLa cells. B) transfected HeLa cells did not colocalize with $H3K9Me_3$ antibody (green) C) Cells were either mock treated (row 1,3) or treated with 2mM HU (row 2) or 50mg/ml of bleomycin (row 4) for 4hrs, fixed and stained for dRif1(red) and 53BP1 (green). DAPI (blue) was used to mark the nuclei. (Scale bar, 10µm).

When HeLa cells were treated with HU (row2) or bleomycin (row4), we found that damage sites were marked with 53BP1. Eventhough hRif1 has been shown to accumulate at such damage sites; we observed that dRif1 did not accumulate in these sites (Buonomo et al, 2009; Silverman et al, 2004; Xu & Blackburn, 2004). An interesting feature of expressing dRif1 protein in HeLa cells was that when a larger amount of protein was expressed, the nuclei appeared deformed and additionally, did not show large 53BP1 spots or foci upon HU or bleomycin treatment. However, in cells not expressing dRif1, or expressing low levels of dRif1, prominent 53BP1 spots were observed, even though dRif1 did not colocalize with these damage spots, indirectly suggesting that dRif1 protein interfered with the normal DNA damage response of HeLa cells. These data along with our previous result showing that dRif1 does not accumulate at DNA damage sites in *Drosophila* cells as well, suggest that the *Drosophila* homologue may not respond to DNA damage in the same manner as the human homologue. This could be due to the poor conservation of the C-terminal domain in *Drosophila* which is essential for interacting with BLM complex in human cell lines (Xu et al, 2010).

4.3 Discussion

Drosophila and human Rif1 behave differently in yeast; whereas human Rif1 interferes with telomere length in yeast (Xu & Blackburn, 2004), dRif1 does not. This suggests that hRif1 has retained its ability to interact with telomeric partners of Rif1, most likely Rap1 and *Drosophila* Rif1 has lost that ability possibly because unlike yeasts and vertebrates, *Drosophila* does not have Rap1. In this context, it is important to note that out of the 325 genomic targets identified for Rif1 in yeast, only about 88, mostly telomeric, colocalize with Rap1, suggesting there are a large number of Rap1 independent targets for Rif1 even in yeast (Smith et al, 2003). We speculate that telomere independent functions of Rif1 are conserved in *Drosophila* and yeast and need to be explored.

Interestingly, upon DNA damage, dRif1 does not associate with the DNA repair foci although hRif1 does. In fact, presence of dRif1 reduces the formation of DNA

repair foci in HeLa cells. The C-terminus of vertebrate Rif1 has now been shown to interact with the BLM complex and also contain a DNA binding domain. However, the *Drosophila* homologue does not have the extended C-terminus that carries out the critical functions of association with BLM protein. This suggests that *Drosophila* Rif1 may not have the ability to associate with replication forks and the differential response of dRif1 and human Rif1 to the presence of stalled replication forks are consistent with this.

The retention of Rif1 homologue in *Drosophila* raises an important question as to when and how Rif1 diversified its function. As telomerase based telomere maintenance was replaced by alternate mechanisms of maintenance in many insects including *Drosophila*, telomerase and associated proteins have no counterparts in these organisms (Villasante et al, 2007). However, presence of Rif1 in *Drosophila* suggests that the recruitment of Rif1 to non-telomere based roles happened before *Drosophila* lost telomerase. Alternatively it might mean that Rif1 has both a telomeric and an evolutionarily conserved non-telomeric role in yeast. Even in yeast, only the C-terminus of Rif1 has been shown to interact with Rap1 and Rif2 proteins. The conserved N-terminal domain containing HEAT repeats has so far not been implicated in any function. Could this domain hold the clue to the evolutionarily conserved role of Rif1? Even though Rif1 was found as a negative regulator of telomerase, it has now been implicated in many more (previously unanticipated) functions like in telomere protection, recombination mediated telomere maintenance and repression of telomere specific transcripts in yeast (Anbalagan et al, 2011; Hirano et al, 2009; Iglesias et al, 2011). However a molecular or biochemical basis underlying these functions is lacking. Studies in a genetically and developmentally tractable system like *Drosophila* will give us an additional important handle to understand the function of this conserved protein.

CHAPTER 5

Characterization of dRif1 protein in *Drosophila melanogaster*

5.1 Introduction

Telomeres in *Drosophila* are maintained by a unique retrotransposon mediated mechanism. When the telomeres are short, retrotransposons specific to telomeres in fly, HTT (HeT-A, TART and TAHRE), transpose to the telomeres and maintain the telomere length. Presence of a yeast telomere specific protein, Rif1, homologue in fly was intriguing because of the differences in fly telomere maintenance and absence of many of the telomere binding protein homologues especially Rif1 recruiting protein Rap1. The mode of action of this protein remains unclear in these organisms. Presence of Rif1 in many evolutionarily distant organisms hints towards an important role for this protein in these organisms. The function of Rif1 protein also has diverged from yeast to mammals from a telomeric function to DNA damage/repair function. Therefore we set out to analyze the function of Rif1 in *Drosophila*.

5.2 Results

5.2.1 Expression of dRif1 protein in different developmental stages of fly

In order to understand dRif1 function, we first studied the expression profile of dRif1in different developmental stages of fly. We carried out the RNA and protein profiling of dRif1 in different developmental stages of fly. We followed this with immunostaining to check the subcellular localization of the protein.

5.2.1.1 RNA expression in different developmental stages of fly

In order to understand dRif1 function we set out to characterize the expression profile of dRif1. We first tested the expression of RNA in all stages of *Drosophila* development. Total RNA was extracted from various stages of *Drosophila* development including embryos, larvae and adult male and female flies and northern blots were performed using dRif1 probe. As shown in figure:17A, dRif1 was expressed in embryos. No expression was detected in adult males although adult females expressed dRif1 at significant levels. The blots were probed with actin to indicate amount of RNA loaded in each of these lanes.

5.2.1.2 Protein Expression in different developmental stages of fly

We have carried out western blotting with protein extracts from embryos, larvae and adult flies (male and female) to check the expression of the protein in different developmental stages of the *Drosophila.* Total protein extracts were separated on SDS-PAGE and transferred to PVDF membranes and probed with anti Rif1p antibody and later tubulin antibody to indicate protein loaded in each lane. It is evident from Figure:17B, that a band at expected size was detected in the embryonic protein extract. In larval and adult tissues (both male and female) the protein expression was negligible or nil as compared to the embryonic extract. These studies show that the protein is present only in embryos and embryo derived S2 cells and not in later stages of development.

5.2.1.3 Sub-cellular localization of dRif1 in different fly tissues

We next determined the subcellular localization of this protein by immunofluorescence. The specific antibody raised in rabbit/mouse was used for the localization studies. The protein was found to be localized to the nucleus in embryos (Figure: 18). Panel.A shows a whole syncytial embryo with nuclei marked by nuclear lamin which stains the rim of the nuclei. And the panel.B shows higher magnification image showing dRif1 localization within the nuclei. As the early embryos, before cellularization sets in, undergo synchronized nuclear divisions in *Drosophila*, it is possible to get embryos which are at different mitotic stages. We observed a clear absence of dRif1 localization in some embryos. Also we observed a difference in the levels of the protein with age of the embryo, with decrease in amounts at the later stages. To confirm these observations we decided to carry out localization studies with different cell cycle markers in *Drosophila* embryo.

5.2.2 Localization of dRif1 in different developmental stages of embryo

In order to understand the development stage wise localization dRif1- whether the protein is expressed throughout all the nuclei or is there any pattern in its expression, we collected the embryos and staged it so as to obtain 0-2, 2-4, 4-6 like

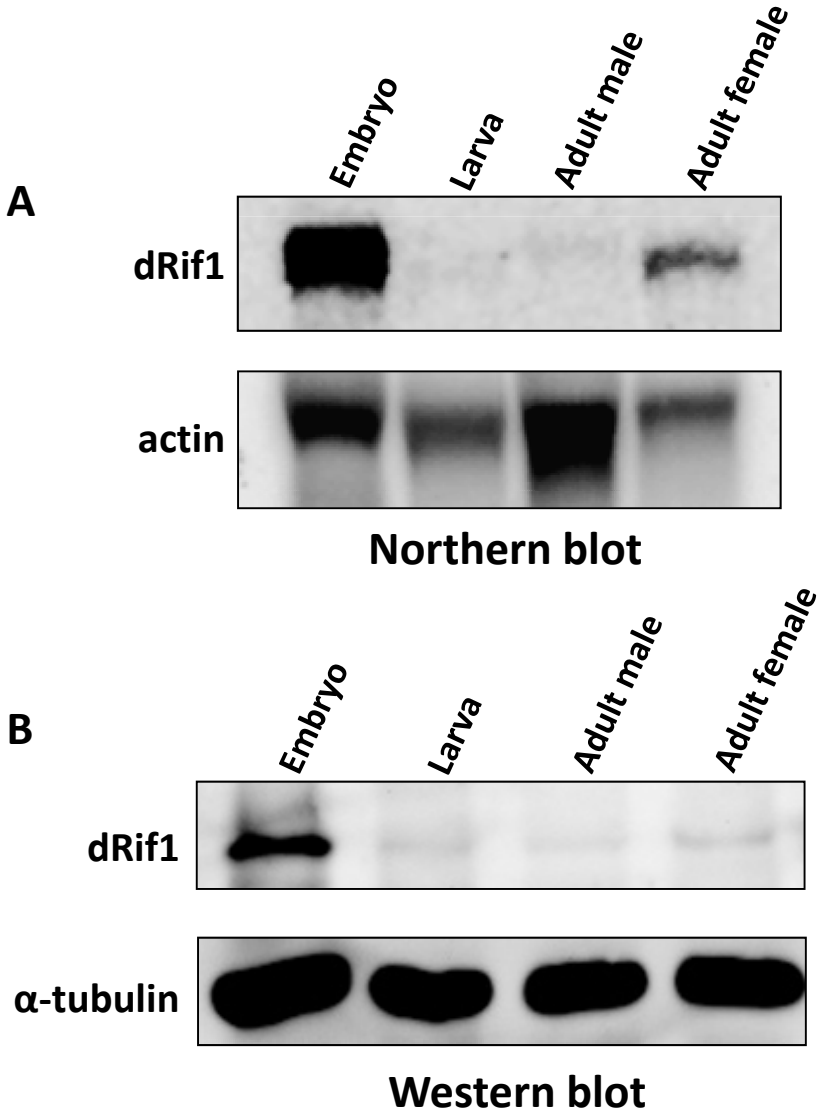


Figure 17: Expression of dRif1 in different developmental stages of fly. A) Expression of dRif1 mRNA - Total RNA from the indicated tissues were separated in agarose gels, transferred to nylon membrane and probed with full length dRif1. The blot shows that dRif1 mRNA is expressed in embryo and adult female tissues and is absent in larvae, pupae and adult males. The same blot was probed with actin to indicate amount of RNA loaded on the gel. **B) Expression of dRif1 protein –** Proteins from different developmental stages were extracted and separated on SDS-PAGE and blotted onto PVDF membranes and developed with dRif1 antibody. Blot shows that the protein is expressed in embryos abundantly compared to other developmental stages**.** α-tubulin was used as a loading control

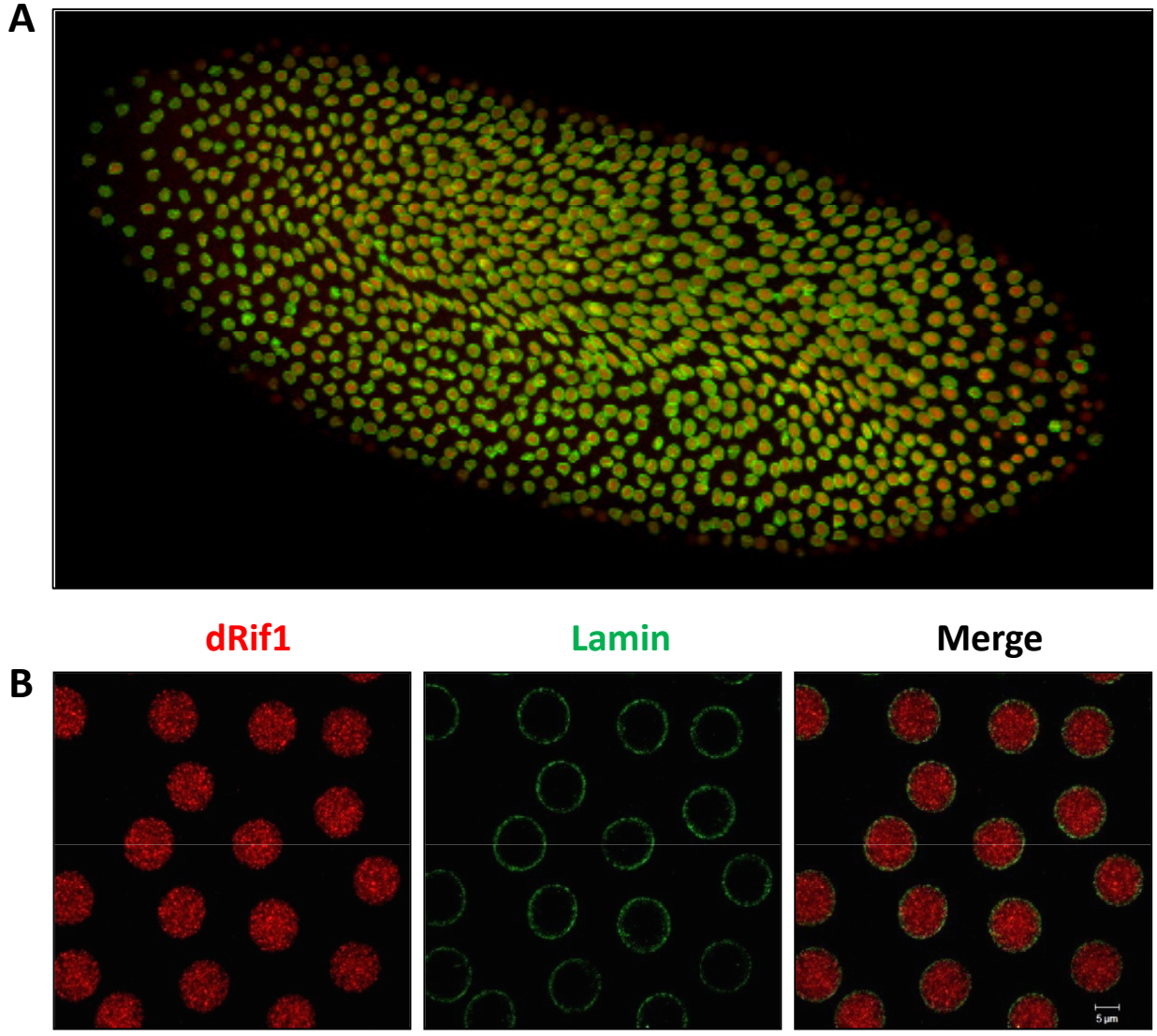


Figure 18: Subcellular localization of dRif1 in *Drosophila* embryos - Confocal images showing the localization of dRif1(red) in (A) a whole *Drosophila* embryo (20X magnification) and (B) higher magnification (63X) images shows its expression in nuclei. Lamin(green) stains the nuclear rim and dRif1 stains heterogeneously within the nucleus

up to 16-18hrs old embryos and carried out staining with dRif1 along with H3PS10 which is a mitotic marker. Up to 4hr old embryonic stage (Figure: 19, 2-4hrs), dRif1 was found to be localized to all nuclei in the embryo with difference in only cell cycle stages as described later in detail but after this stage up to 8hr stage the intensity of dRif1 was found to be high in domains/regions of the embryos where H3PS10 also found to be staining (Figure: 19, 4-6hrs and 6-8hrs). Thereafter up to 14hrs the embryonic CNS was intensely stained with dRif1 and also the CNS of these embryos was stained for H3PS10 (Figure: 19, 8-10hrs, 10-12hrs and 12-14hrs) and as it progressed beyond 14hrs the protein was not present in any of the nuclei in all the embryos (Figure: 19, 14-16hrs). Localization of dRif1 through different developmental stages shows that the protein is present in mitotic domains during development of embryo and the protein level reduces in later stages of development.

5.2.3 Localization of dRif1 in *Drosophila* female specific tissue – ovary

Ovary staining was performed to confirm whether any adult female specific tissues express dRif1 as we could detect some levels of mRNA in the northern experiment with RNA from different developmental stages were loaded. From figure: 20, the panel.A shows a bunch of ovarioles staining for both Rif1 and tubulin antibody. Panel.B shows follicle cells and the internal optical sections which show the much bigger nurse cells. It is clear that dRif1 localizes to the nuclei in both nurse cells and follicle cells of the developing egg chambers as both these cell types shows dRif1 signal within their nuclei. This suggests that not only the mRNA is expressed but also the protein expresses in the female.

5.2.4 Localization dRif1 across cell cycle

In the embryos, since there were marked differences in the localization of dRif1 in different nuclei and they appeared to be because of difference in mitotic stages, we colocalized dRif1 with different cell cycle markers to test whether the dRif1 localization oscillates in a cell cycle stage dependent manner. H3PS10 antibody was used to mark the mitotic cells (Figure: 21A) and PCNA was used as an early G1-S phase marker (Figure: 21B). We found that in G1-S phase, abundant amounts of

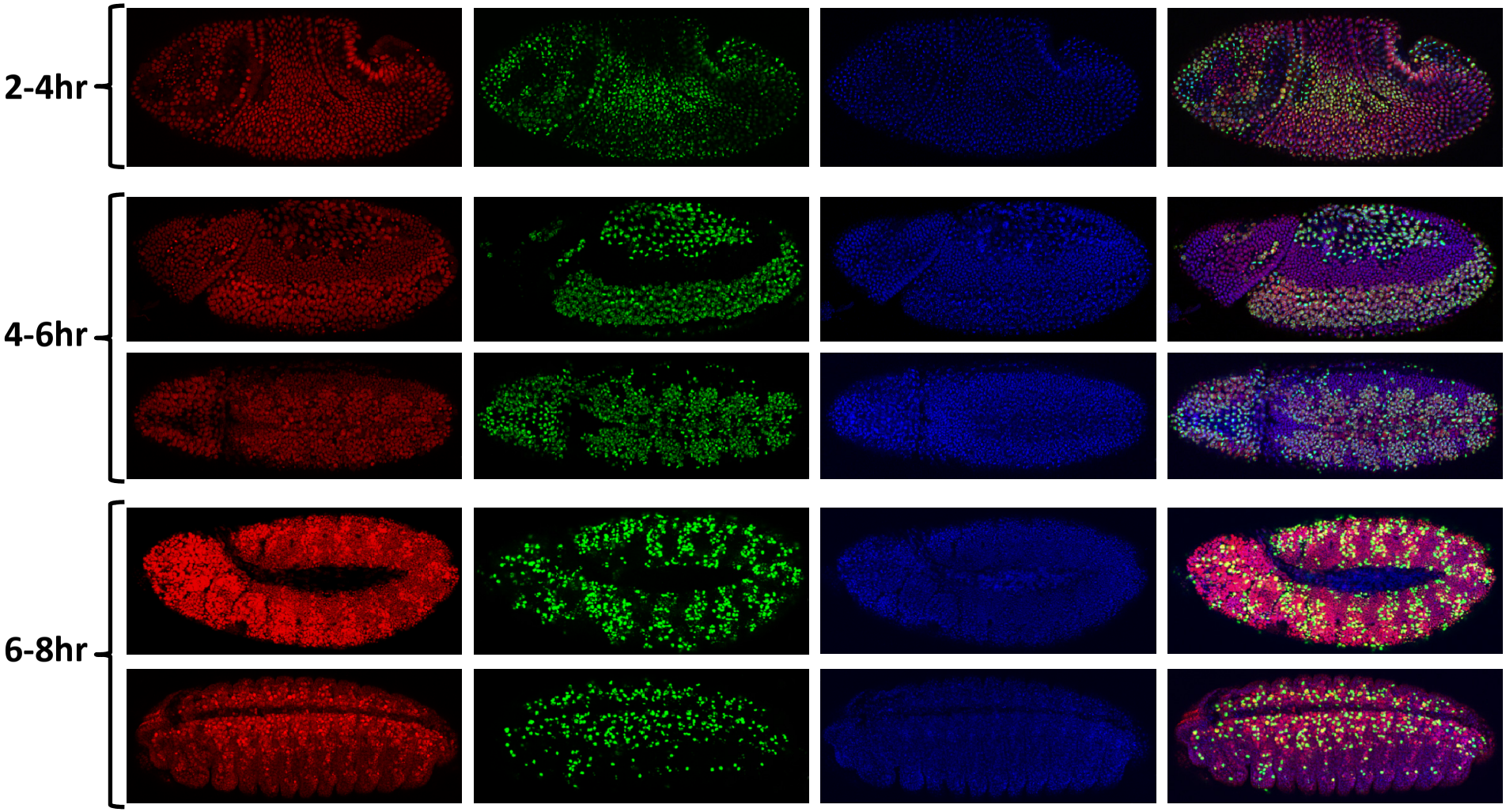


Figure 19: Localization of dRif1 through different developmental stages of embryos. All the embryos were collected as 0-2hr old and staged as labelled and stained separately. Embryos are shown as anterior to the left**.** Up to 4hr old embryonic stage, dRif1 (red) was localized to all nuclei with similar intensity but after this stage up to 8hr stage the intensity of dRif1 was found to be high in domains/regions of the embryos where H3PS10 (green) also was detected, ie the dividing or proliferating cells and dRif1 appeared to be lower in the non-dividing cells. Nuclei were stained with DAPI (blue).

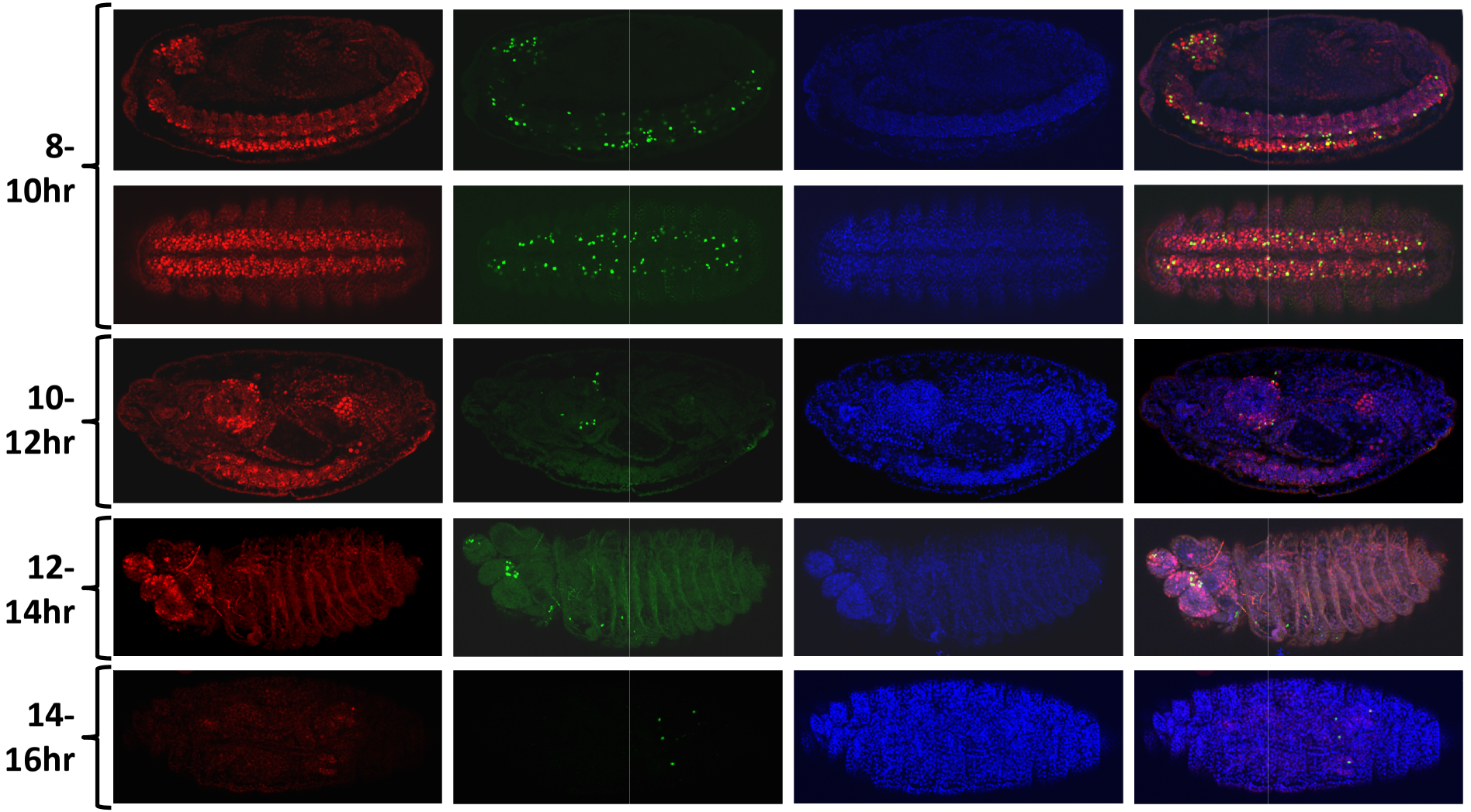


Figure 19. Localization of dRif1 through different developmental stages of embryos(contd.). After 8hrs up to 14hrs, the embryonic CNS was intensely stained with dRif1 (red) and also the CNS of these embryos was stained for H3PS10 (green). As it progressed beyond 14hrs the protein could not be detected in any of the nuclei in all the embryos. Nuclei were stained with DAPI (blue). All the embryos were collected as 0-2hr old and staged as labelled and stained separately. Embryos are shown as anterior to the left.

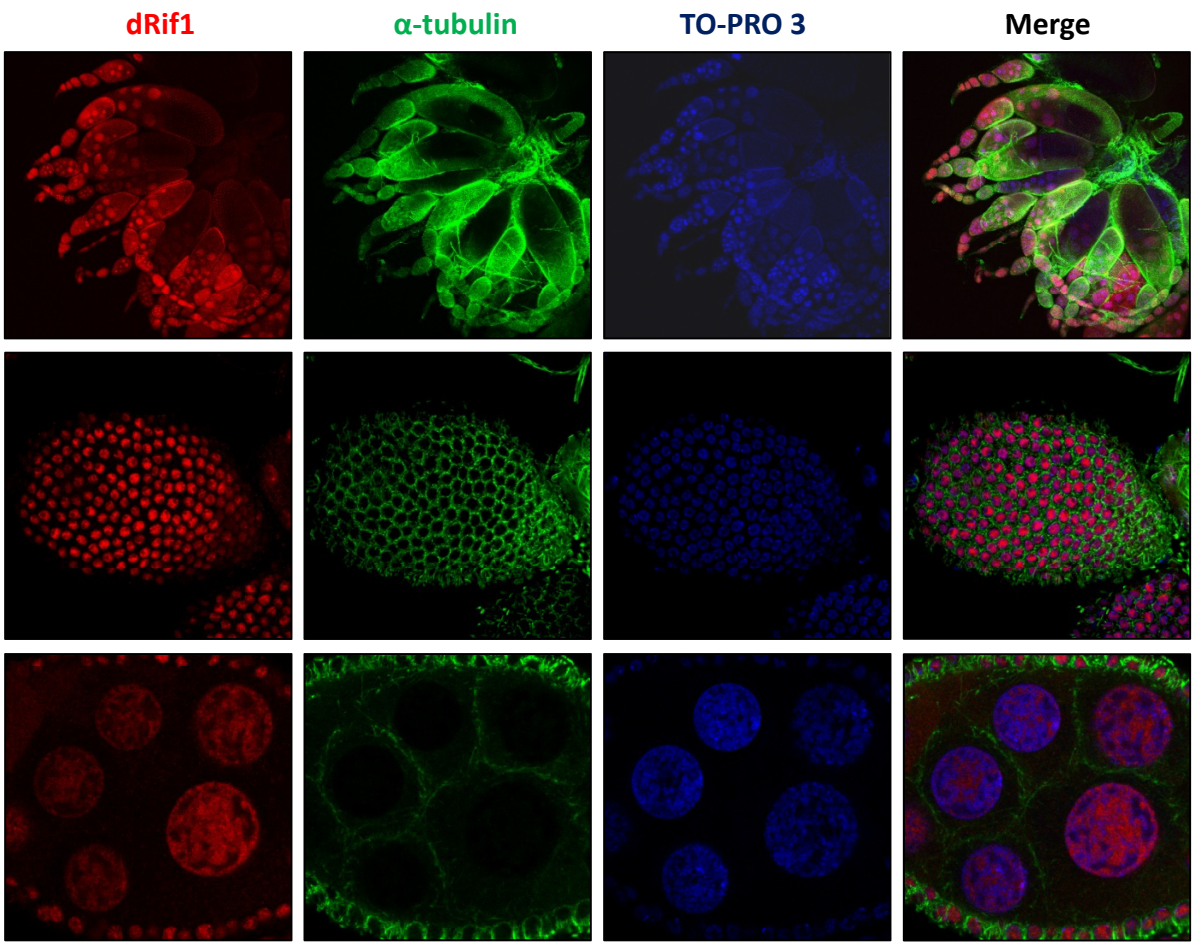


Figure 20: dRif1 is expressed in adult ovary of *Drosophila melanogaster* – Panel A- shows a bunch of ovarioles stained for dRif1 (red) and α-tubulin (green). B and C – higher magnification images of an ovariole. It is clear from panel B that dRif1 localizes to the nuclei in nurse cells which is the outer most layer and from panel C that it localizes to the nurse cell nuclei as well which is located interior to the follicle cells shown above. Topro (in blue) marks the nuclei.

dRif1 could be detected. However, when we stained the multiple stages of mitosis, we found that in the metaphase stage of mitosis, when the chromosomes are at the metaphase plate, dRif1 was not localized to the fully condensed chromosome. But towards the end of the anaphase, when the chromosomes are pulled towards the pole, dRif1 was found to be associated with the chromosome (Figure: 21A, compare panel 1 and 2 of row2). The localization studies show that a) the protein is nuclear, b) it is not associated with the metaphase chromosome and c) post metaphase, the protein re-associates with the condensed chromosome. We investigated this dynamic association in further detail.

5.2.5 Dynamic association of dRif1 to chromatin

Figure 22 shows a single embryo with multiple stages of mitosis. After dividing synchronously for a while, *Drosophila* embryos undergo quick cycles of mitosis in what is referred to as the "mitotic wave". In this stage, as mitosis progresses we see, in a single embryo, the various stages of mitosis. Such an embryo was stained with dRif1, tubulin to indicate spindle microtubules and H3P10 to mark the condensed chromosomes. The regions of the embryo that have been observed in detail at higher magnification are indicated as squares. The first portion of the embryo, magnified in the first panel of second row, contains both the metaphase and anaphase nuclei. In the metaphase nuclei, no red signal can be detected, but the spindle (green) and chromosomes (blue) are clearly visible with the chromosomes aligned along the metaphase plate. But Rif1 is found to be associated with the chromatin in the adjacent nuclei (in the same panel), where the nuclei are in late anaphase. This is more clearly visible in the next panel as the chromosomes have moved to the poles; reduction in blue staining of H3PS10 can be indicating the onset of anaphase and consequent dephosphorylation of H3PS10. Another magnified view in panel2 of row1 shows mitotic nuclei with H3PS10 and spindles but no dRif1.

This observation was consistent with the hRif1 localization studies that showed that hRif1 is not associated with chromosomes during metaphase. But there is a difference in that we did not observe any midzone microtubule localization of dRif1

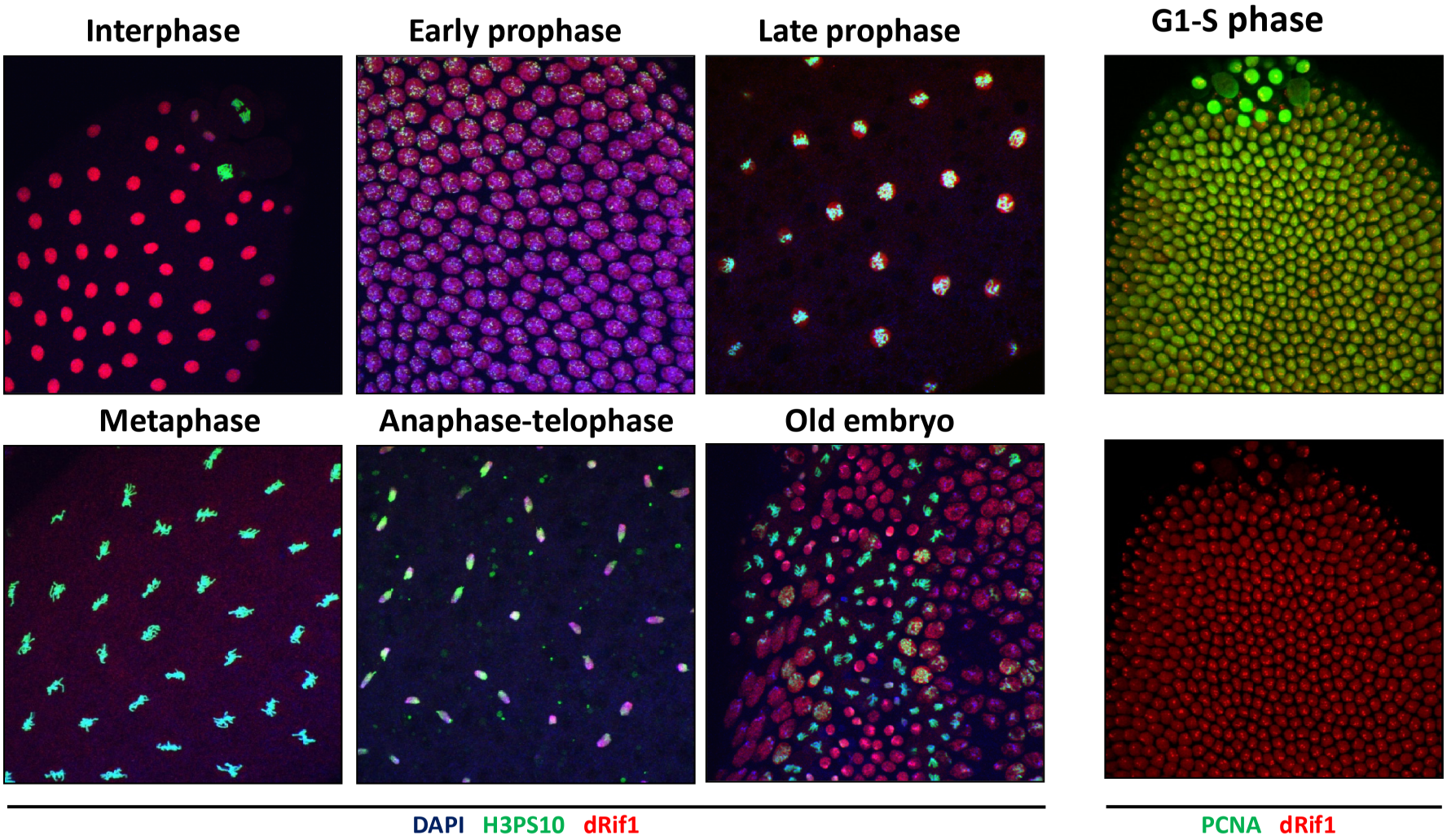


Figure 21: dRif1 localization across cell cycle – Whole embryos were stained with anti-dRif1 antibody (red) along with mitotic marker antibody, H3PS10 (green) in the first panel and with G1-S phase marker, PCNA (green) in the second panel**.** All cells with PCNA staining also showed dRif1 staining. In the metaphase cells, no Rif1 could be detected. In the whole embryo picture of an older embryo, clear patches of nuclei with intense dRif1 coexist with areas of complete absence of Rif1 which in turn show strong H3PS10 staining..

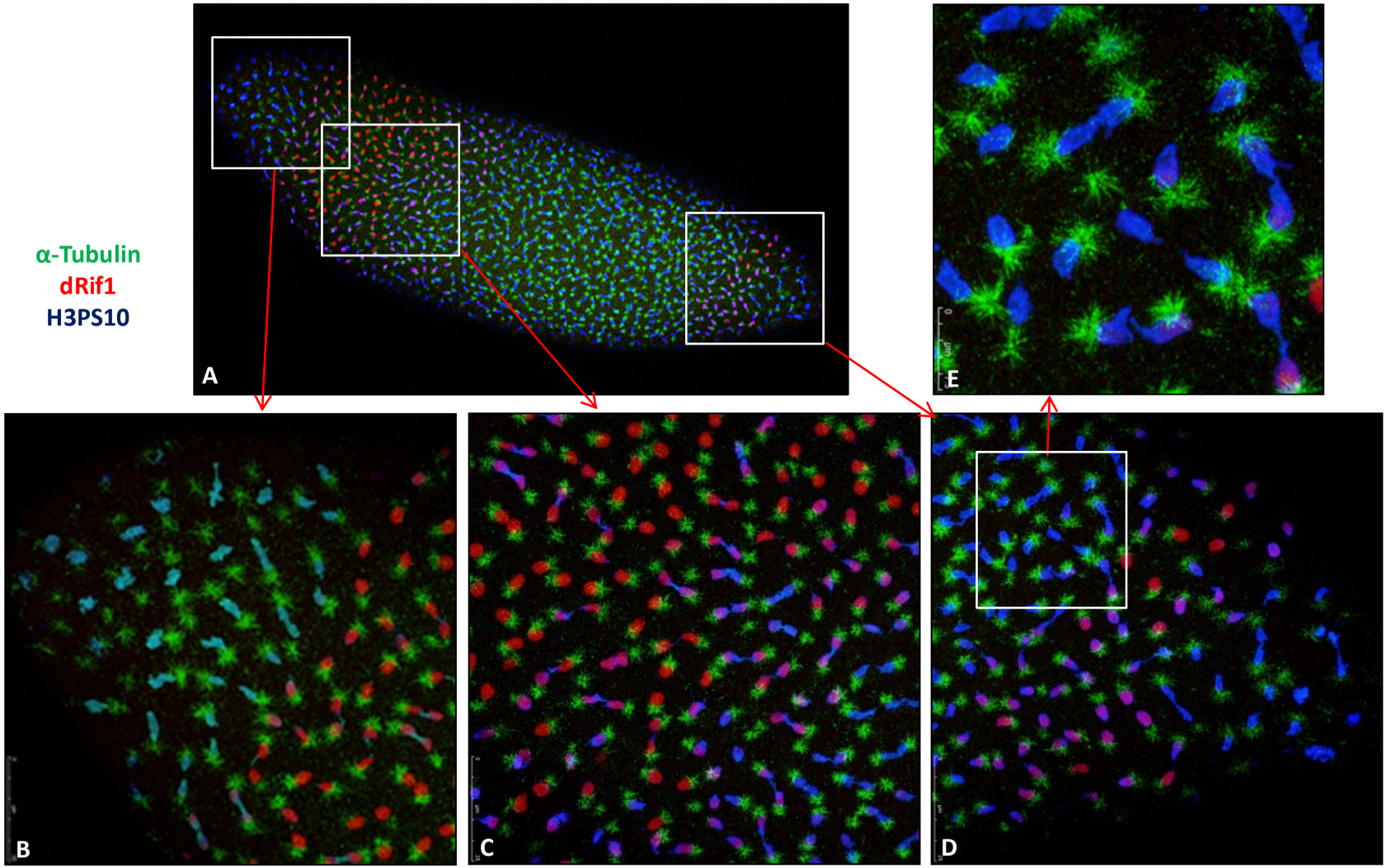


Figure 22: dRif1 localization across the "mitotic wave" in early embryo – Whole embryos were stained with antibodies against dRif1(in red), H3PS10 (Mitotic marker in blue) and tubulin (stains mitotic spindle in green). A) A whole embryo showing multiple stages of mitosis. The marked areas were imaged at higher magnification. B) Absence of dRif1 on metaphase chromosome can be clearly observed C,D and E) Regions showing chromosomes without (metaphase) dRif1 and adjacent nuclei with reassociated dRif1 during late anaphase.

as in case of its human counterpart. hRif1 stained heterogeneously in the interphase nuclei and in metaphase, hRif1 was notably absent from the condensed chromosomes but in telophase cells it localized to the still-condensed chromosomes. This relocalization coincides or is in concert with the loss of H3PS10 mark. We investigated this observation further.

5.2.6 Dynamic association of dRif1 to chromatin coincides with the H3 dephosphorylation at S10

To further confirm the observation from the previous section, we obtained higher magnification images and looked at single nuclei at different mitotic stages from the same embryo (Figure: 23). As shown in figure: 23, dRif1(red) is not found on condensed chromosomes (blue) that are still around the metaphase plate (1,2,3) although strong H3PS10 signal can be detected (green) that colocalizes with the DNA staining. However, in later stages, (4,5,6) dRif1 begins associating with the chromosomes (red) and it coincided with the movement of chromosomes (blue) away from the metaphase plate and towards the poles and the dephosphorylation of H3 at the Ser10 (diminishing green signal). Therefore these observations clearly indicate that dRif1 is not associated with the chromosomes during metaphase and associates with the chromosomes at the onset of anaphase.

5.3 Discussion

In *Drosophila* the mRNA of dRif1 was found to be expressed in embryonic stages and in adult female but the protein could be detected only in the embryonic stages. It is possible that small amounts or specific tissues express dRif1 in the later stages that were not detected in the western blot. We think that the significant amount of RNA seen in adult females is for the maternal contribution to the oocyte. In addition we found that protein is expressed in the nuclei of both follicle cells and nurse cells in the ovary. The protein expression in ovary can be either for the maternal deposition or it may have some unknown role to play in ovary development. During the cell cycle, we found

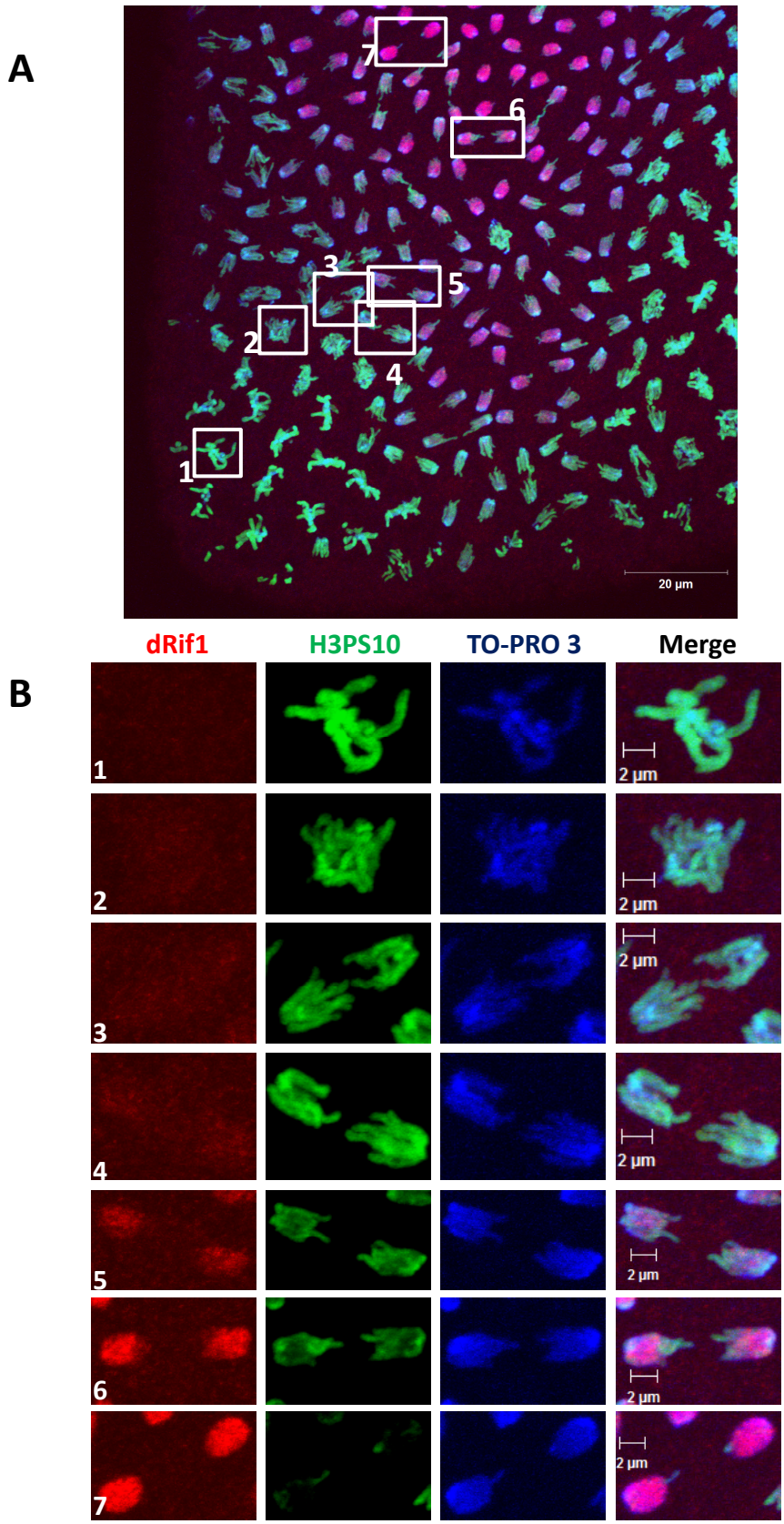


Figure 23: dRif1 association to chromosome coincides with the dephosphorylation of H3 at S10. Higher magnification images of a *Drosophila* embryo (A) and insets are magnified as marked (B). Panel B shows the absence of dRif1 in metaphase (1,2,) and that the association of dRif1 to chromatin (red channel) in anaphase (3-7) and this coincides with the diminishing of S10 phospho-specific H3-antibody (H3PS10, green). TO-PRO 3 stains the DNA(blue).

interesting correlations of dRif1 association and concurrent dephosphorylation of H3PS10 from the condensed chromosomes. We speculate that the interaction of dRif1 via its SILK/RVxF motif with PP1 might have roles in this process, as PP1 is the main dephosphorylating enzyme for chromosomes. Also presence of this protein very early in the embryonic development, even before maternal transcription begins, and the localization pattern through development suggests that it has very important roles in the early development in fly.

CHAPTER 6

Gain of function and loss of function studies of dRif1 in *Drosophila melanogaster*

6.1 Introduction

The localization and expression studies suggest that this protein has chromosome associated function and is possibly required in early development. However, it is present abundantly in cultured cells as well. In order to identify the molecular pathways that dRif1 is involved, loss and gain of function strains will be useful. Induction of gene expression ectopically/gain of function is a well established way to determine the function of a gene. Ectopic expression of a gene can be done by introducing a transgene with a modified promoter into the target organism (transient or stable transfection) or by using the Gal4-UASsystem. Another important way to identify the function is by systematically knocking out genes. This is done by either deletion or disruption of function (such as by insertional mutagenesis) and the resulting organisms are screened for phenotypes that provide clues to the function of the disrupted gene. Also knock-down can be made by using RNAi mediated methods.

6.2 Results

6.2.1 Ectopic expression of dRif1

For over expression studies, full length dRif1 was cloned with a 3X FLAG tag and without any tag in pUAST fly expression vector. Both these constructs were microinjected into fly embryos to make transgenic flies and transgenic fly stocks obtained are shown in Table: 3&4. These transgenic flies were crossed with different GAL4-driver flies to bring the transgene and driver together in a fly to bring about tissue-specific conditional expression of the transgene. The Gal4 driver flies express GAL4 protein in tissue specific conditional manner and the GAL4p binds to UAS and expresses the transgene in those tissues whenever the GAL4p is present. Thus we obtained lines where the transgene can be induced to express in tissues of our choice.

Vector: pUAST; **Insert:** CG30085 (dRif1)

S.No.	Stock name	Chromo some	Eye Colour P/+ Male	Female	P/P Male	Female	Genotype
1	dRif1 5.1	II	Yellow	Yellow	Lethal		*P/CyO*
2	dRif1 5.4	II	Yellow	Yellow	Red	Red	*P/P*
3	dRif1 18.4	II	Yellow	Yellow	Red	Red	*P/P*

Table 3: List of full length dRif1 stocks made. Table showing different full length dRif1 transgenic stocks made along with their features and phenotype

Vector: pUAST; **Insert:** 3X FLAG- CG30085 (dRif1)

S.No.	Stock name	Chromo some	Eye Colour P/+ Male	Female	P/P Male	Female	Genotype
1	3XFLAG dRif1 10.2	II	Yellow	Yellow	Red	Light Red	*P/P*
2	3XFLAG dRif1 10.3	III	Red	Yellow	Dark Red	Dark Red	*P/P*
3	3XFLAG dRif1 10.4	II	Yellow	Yellow	Lethal		*P/CyO*
4	3XFLAG dRif1 11.1	II	Red	Red	Lethal		*P/CyO*
5	3XFLAG dRif1 18.2	III	Red	Yellow	Red	Red	*P/P*
6	3XFLAG dRif1 29.1	II	Yellow	Yellow	Dark Red	Red	*P/P*

Table 4: List of full length 3XFLAG dRif1 stocks made. Table showing different full length 3XFLAG dRif1 (with an N-terminal 3XFLAG tag) transgenic stocks made along with their features and phenotype

All pUAST lines (pUAST dRif1 and pUAST 3XFLAG dRif1) were crossed to actin-GAL4, tub-GAL4, omb-GAL4, ey-GAL4, neuralized-GAL4, tsh-GAL4 and GMR-GAL4 driver lines. actin-GAL4 & tub-GAL4 express GAL4 ubiquitously in the fly. ey-GAL (express GAL4 in the eye-antennal disc) & GMR-GAL4 (causes high-level expression in the eye imaginal discs in cells posterior to the morphogenetic furrow) expression can be visualised in the eye. omb-GAL4 and tsh-GAL4 express GAL4 in the pattern of optomotor blind(omb) and teashirt(tsh) gene expression pattern respectively. neuralized-GAL4 expresses in the nervous system, sensory organ and eye-antennal discs.

The expression of dRif1 transgenic protein was detected by western blotting using different transgenic lines and different GAL4 driver lines (Figure: 24A). All drivers showed expression of Rif1 protein which is normally not detected in the larval stages. Out of these, over expression of dRif1 by actin-GAL4 and tub-GAL4 was found to be lethal and others did not show any visible phenotype. Flies over expressing dRif1 develop into pupae but failed to transform into adult flies. Also at this metamorphosis stage we found that the fly organ developments were severely affected (Figure: 24B). We could see that the abdomen is deformed and bulged, organs like wing, leg and eyes were also not developed fine compared to the wild type. Even though 100% of the 3rd instar larvae make pupal case similarly to wild type fly, at the metamorphosis stage from pupae to adult is probably affected as no adult fly emerges. Later we repeated the experiment with the EP-line (stock number: 27427) crossing to tub-GAL4 and actin-GAL4

6.2.2 Over expression of dRif1 lead to enlargement in wing imaginal disc size in the larvae

In an effort to understand the reason for early pupal lethality, we examined the tissues in the 3rd instar larvae. In order to check the fate of these tissues which undergo transformation to develop into adult organs, we checked wing imaginal discs which will transform into adult wing structures. Looking and comparing the discs with wild type wing discs it was evident that the over expression of dRif1 caused enlargement of disc size and morphology tremendously as shown in figure: 25A&B. We find that both the size and thickness of the discs is enlarged when dRif1 is over expressed. We dissected several age-matched larvae and confirmed this phenotype. Therefore, to know whether the protein is present in these important precursor cells for the organ development, we have carried out western blotting with wing imaginal discs and could detect dRif1 protein (Figure: 25 C). However, around 20 discs equivalent protein had to be loaded to detect the protein. As stated earlier, we could not detect protein in whole larvae and think that it is expressed in very small amounts or could be present in very few cells of the disc.

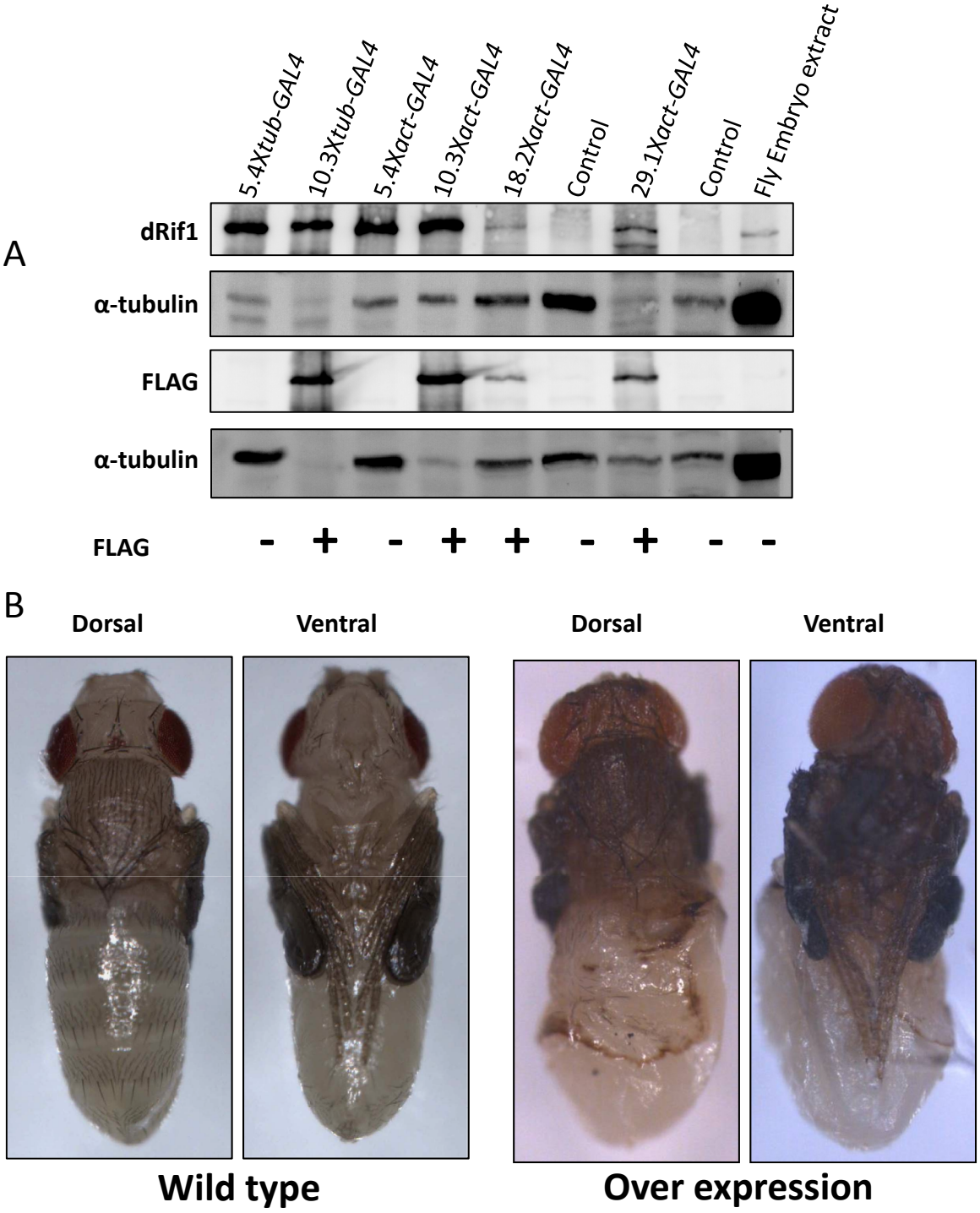


Figure 24: Ectopic expression of dRif1. A) Expression of dRif1 from transgenic flies. Larval total protein was extracted from the indicated genotypes and probed with both dRif1 and FLAG antibody. α-tubulin was used as a loading control. B) Dorsal and ventral view of pupae from wild type and dRif1 overexpressing animals. Defects in multiple organ including head, eyes, abdomen, legs can be seen

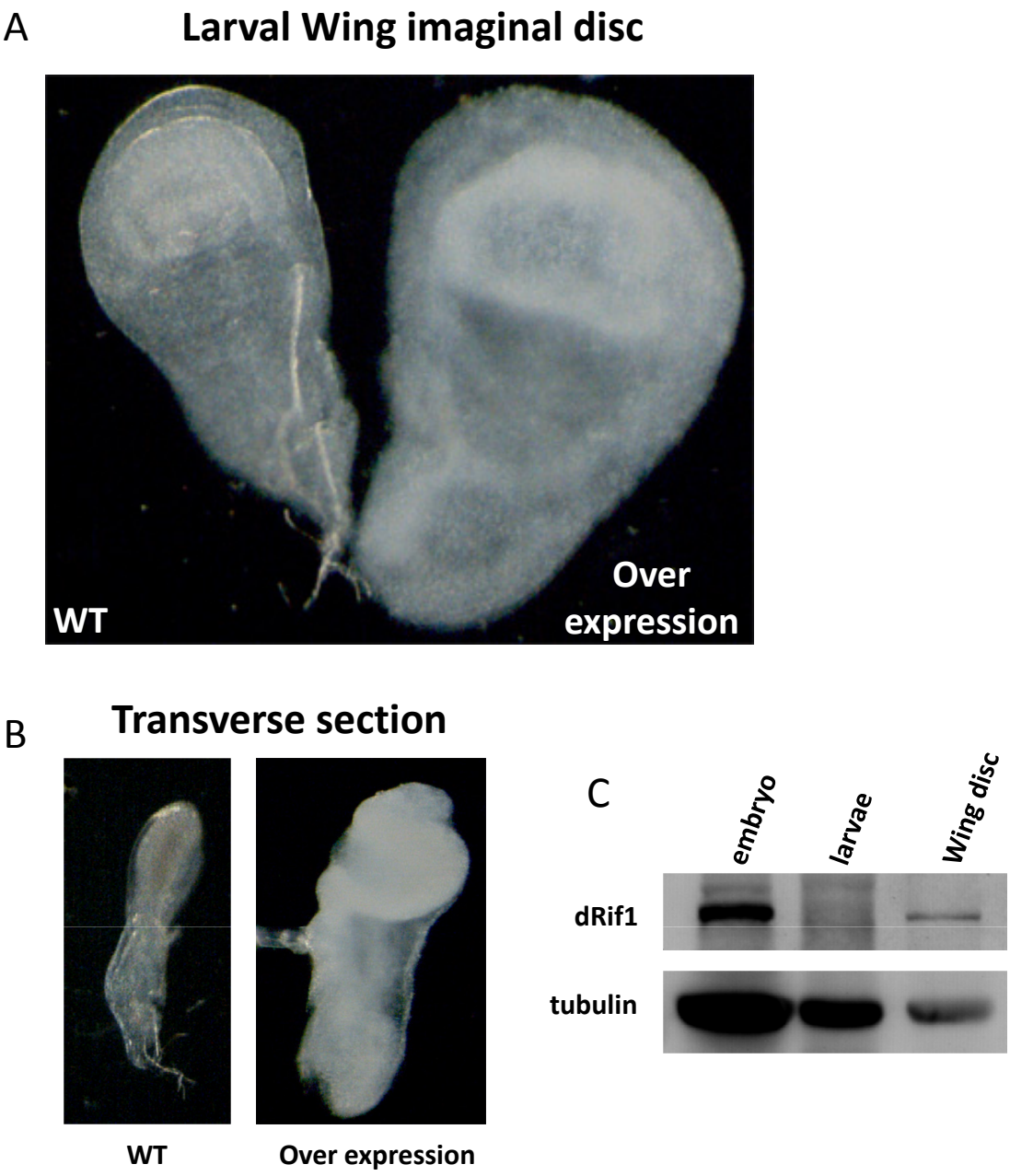


Figure 25: Over expression of dRif1 leads to enlargement in wing imaginal disc size in the larvae. A) wing imaginal disc dissected from 3rd instar larvae of wild type and over expression condition are captured side by side to show the difference in the size of these discs. B) a transverse view of wild type and over expression disc showed clear increase in the size of these discs C) western blot shows that the wing imagianl disc express dRif1 in wild type conditions though total protein from larvae failed to pick up the signal. α-tubulin was used as a loading control.

6.2.3 Knock down of dRif1 by UAS-GAL4 based RNAi method

For knock down, two parts of dRif1, ~1.2kb each, towards N-terminus (NdRif1) and C-terminus (CdRIf1) were cloned in Sym-pUAST fly RNAi vector. Both these constructs were microinjected into fly embryos to make transgenic flies and transgenic fly stocks obtained are shown in Table: 5. These transgenic flies were crossed with different GAL4-driver flies to bring the transgene and driver together in a fly to bring about tissue-specific conditional knock down of the transgene.

S.No.	Stock name	Chromosome	Eye Colour		Genotype
			P/+	P/P	
			Male Female	Male Female	
1	CdRif1 2.1	II	Yellow	Lethal	*P/CyO;TM2/TM6*
2	CdRif1 2.2	II	Yellow	Red	*P/P;TM2/TM6*
3	CdRif1 2.3	I	Light Red	Red	*P/P*
4	CdRif1 7.1	III	Red Yellow	Dark Red Red	*Pin/CyO: P/P*
5	CdRif1 7.2	III	Yellow	Lethal	*Pin/CyO: P/TM2*
6	CdRif1 7.3	III	Red	Lethal	*Pin/CyO; P/TM2*
7	NdRif1 1.1	II	Yellow	Red	*P/P;TM2/TM6*
8	NdRif1 1.2	III	Yellow Light Red	Lethal	*Pin/CyO; P/TM2*

Table 5: List of dRif1 RNAi stocks made. Table showing different transgenic stocks made for RNAi with NdRif1 and CdRif1 along with their features and phenotype

All the RNAi lines (of both Sym-pUAST NdRif1 and Sym-pUAST CdRif1) were crossed to *actin-GAL4*, *tub-GAL4*, *omb-GAL4*, *ey-GAL4*, *neuralized-GAL4*, *tsh-GAL4* and *GMR-GAL4* driver lines. None of these GAL4 induced knock down showed any visible phenotype. As these crosses result in heterozygous condition and also there is no transcription till 13th stage of the embryo, the RNAi may not be knocking down the expression. Therefore we decided to make a stock which will be homozygous for both the p-element (a. for the transgenic RNAi construct and b. for

the gal4-driver if viable) and check the progenies of this fly. We made a stock of *P/P; daGAL4/daGAL4, P/P; tubGAL4/TM3* and viability, fecundity and fertility of these flies were compared to the wild type stock. But we did not find any difference in those parameters. We finally tested if the knock-down was efficient by western blot with dRif1 antibody in the homozygous stocks. We found that the level of dRif1 protein is not reduced compared to the wild type (Figure: 26). Also the transgenic RNAi line obtained from VDRC, V33672, also failed to give any phenotype and reduction in protein levels. This led us to conclude that the RNAi mediated knock down was not effective for dRif1.

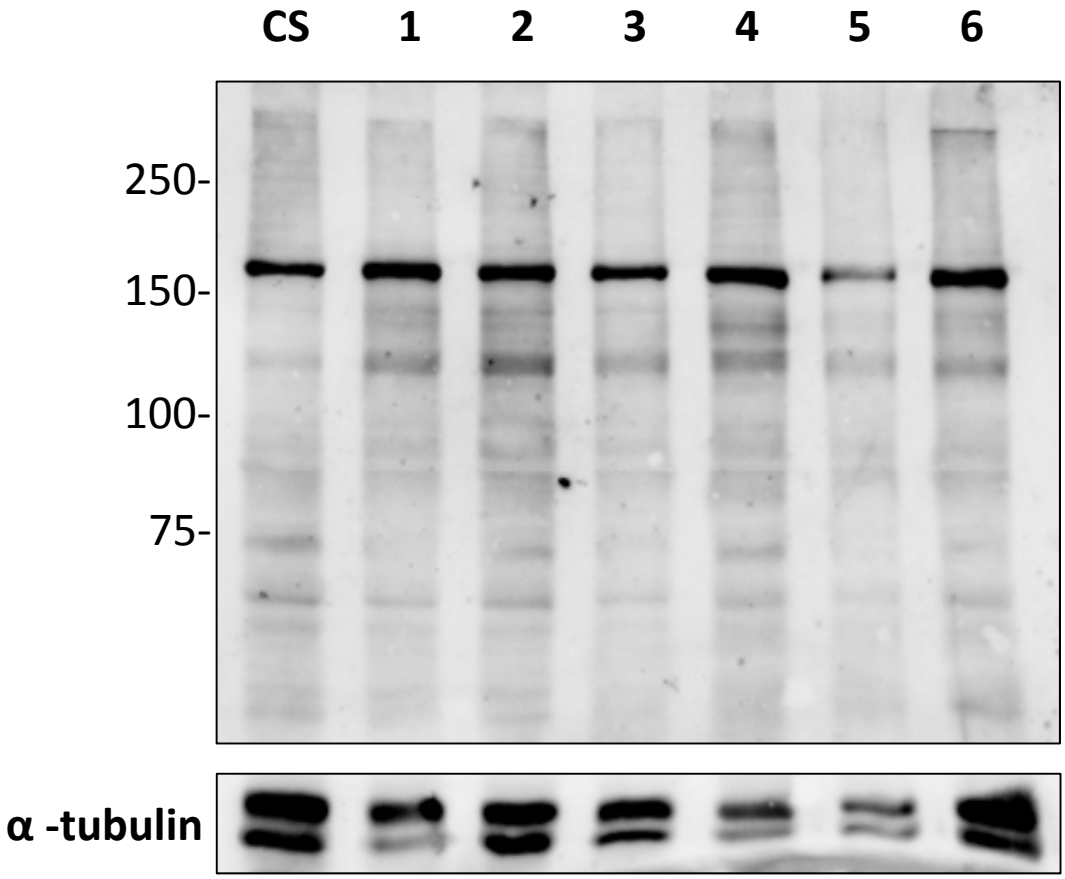
6.2.4 dRif1 mutation and mutant phenotype

Different RNAi transgenic lines were ineffective in knocking down dRif1 even with ubiquitous GAL4 driver lines. Therefore in order to obtain a mutation in dRif1 we performed P-element excision mediated mutagenesis. For that we have obtained an EP-line (stock number: 27427) inserted in the 5' UTR of dRif1 from the Bloomington stock centre and carried out the mutagenesis. The mutant was generated in Dr. Rakesh K Mishra's lab, CCMB and we carried out further experiments using these mutants.

Briefly, out of the three mutants obtained, two of them were homozygous lethal and one was partially homozygous viable (*m-28*). The *m-28/ m-28* homozygous flies emerged with very low frequency and the female flies were all sterile. The homozygous flies emerged show hallmarks of apoptosis all through its body like serrated wing, loss of ommatidia & fused ommatidia which lead to reduction in eye size, necrotic patches on the abdomen and on the wing (Figure: 27A&B). Also many of the homozygous larvae turn to black in color and die (Figure: 27C).

6.2.5 dRif1 mutation showed elevated apoptosis in the wing imaginal discs.

The over expression of dRif1 showed enlargement in wing imaginal disc size in 3rd instar larvae and since the mutant showed increased manifestation of apoptosis, we



Sl.No.	Transgenic Line	Genotype
1	CdRif1 2.2	P/P; daGal4/daGal4
2	VDRC	P/P; daGal4/daGal4
3	NdRif1 1.1	P/P; daGal4/daGal4
4	NdRif1 1.1	P/P; tubGal4/TM2
5	VDRC	P/P; tubGal4/TM2
6	CdRif1 2.2	P/P; tubGal4/TM2

Figure 26: *UAS-GAL4* based RNAi was not effective in knocking down the dRif1 protein levels. Western blotting from embryo extracts from different genotypes of fly, as shown in the table, shows that the level of dRif1 did not altered by the RNAi transgenic lines carrying the *GAL4*. α-tubulin was used as a loading control.

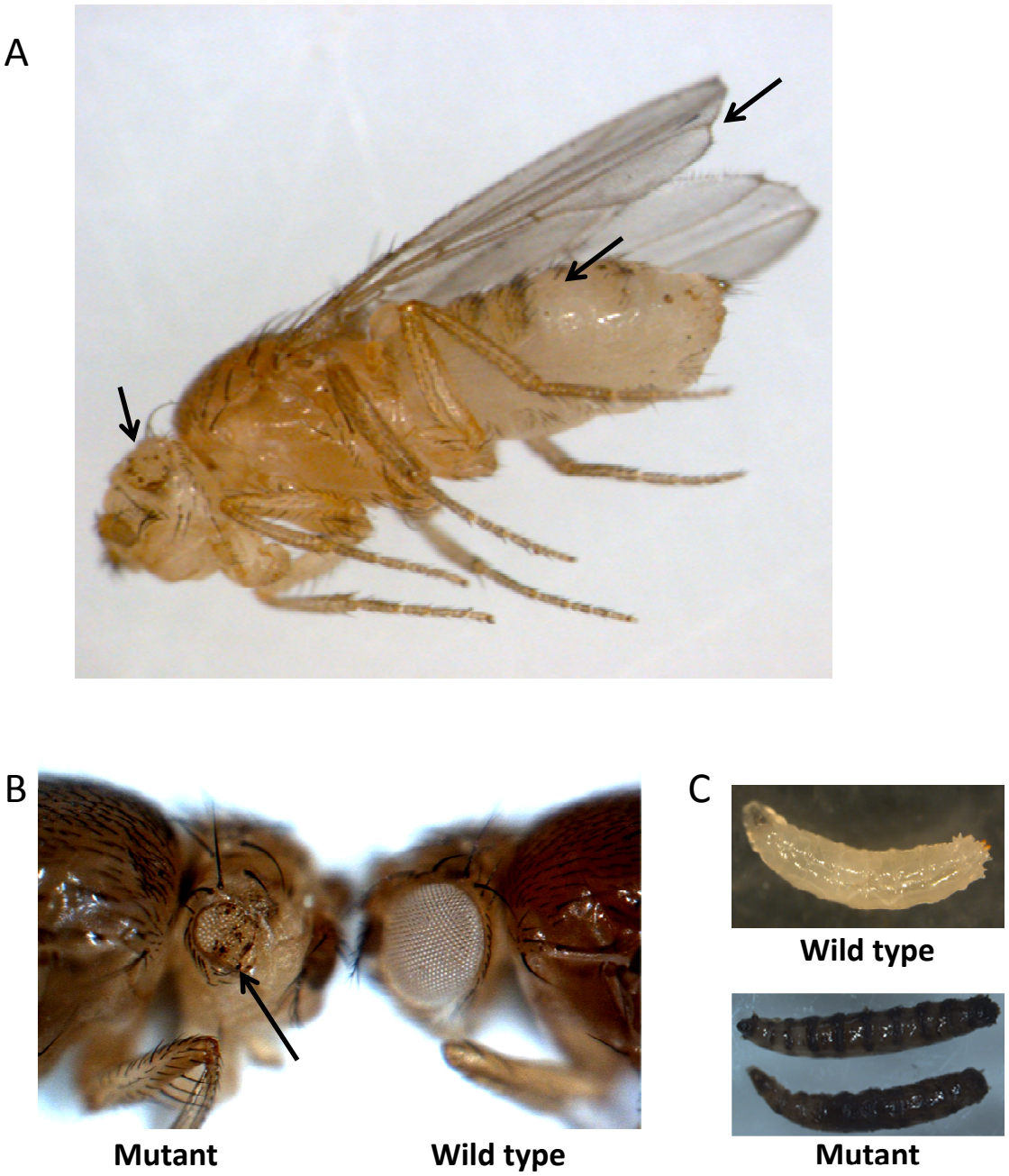


Figure 27: dRif1 mutation phenotype. The homozygous *m-28/m-28* mutant fly showed several apoptotic symptoms through out its body(A). Necrotic pathches were seen on abdomen, wings were serrated and the eye, as showed here enlarged(B), lost ommatidia, fused ommatidia and thereby reduced in the size itself as compared to the wild type eye (C) many of the homozygous mutant larvae turned to black in colour due to necrosis and died.

checked whether knocking down dRif1 reduces the disc size, which is the opposite effect of over expression.

Dissecting out the wing imaginal disc from the *m-28/m-28* homozygous 3rd instar larvae showed a significant reduction in the size of the imaginal disc as expected (Figure: 28A). Again multiple discs were dissected to confirm this phenotype. In order to obtain some molecular evidence of apoptosis to establish that elevated apoptosis is the reason for reduced size, we used two different well established techniques to identify cell death: by acridine orange staining which detect dead cells as bright green spots (Figure: 28B) and by detecting caspase activation by immunostaining with cleaved caspase-3 antibody. Cleavage of caspase-3 is the final step in the apoptosis activation pathway and is a hallmark of apoptotic cells. Both these techniques showed elevated levels of cell death/ apoptosis in these tissues as shown in figure: 28B&C when compared to wild type cells. Therefore, we conclude that in dRif1 mutants there is a reduction in the size of imaginal discs with a concomitant elevation in apoptotic cells.

6.3 Discussion

Functional characterization of dRif1 in *Drosophila* was addressed using two approaches- A) ectopic/over expression and B) loss of function. Using *Drosophila* genetics we could express the protein spatial and temporal specific manner and discovered that the expression of the protein ubiquitously led to early pupal lethality preventing metamorphosis of larva to adult. The over expression of this protein also lead to increase in imaginal disc size though the larvae were heading towards death. This was confounding and we hypothesized that the over expression might be preventing the developmentally programmed apoptosis. We speculate that as there is a massive developmentally programmed apoptosis in the larval tissue in order to transform the crawling larvae to the flying adult, the ectopic over expression of dRif1 might perturb this. This is the reason for the enlarged disc morphology.

The hypothesis can be supported if the knock down of the protein gives an opposite effect since the absence of the protein may lead to early onset of programmed apoptosis or there by the program itself may be altered. Therefore we went ahead to

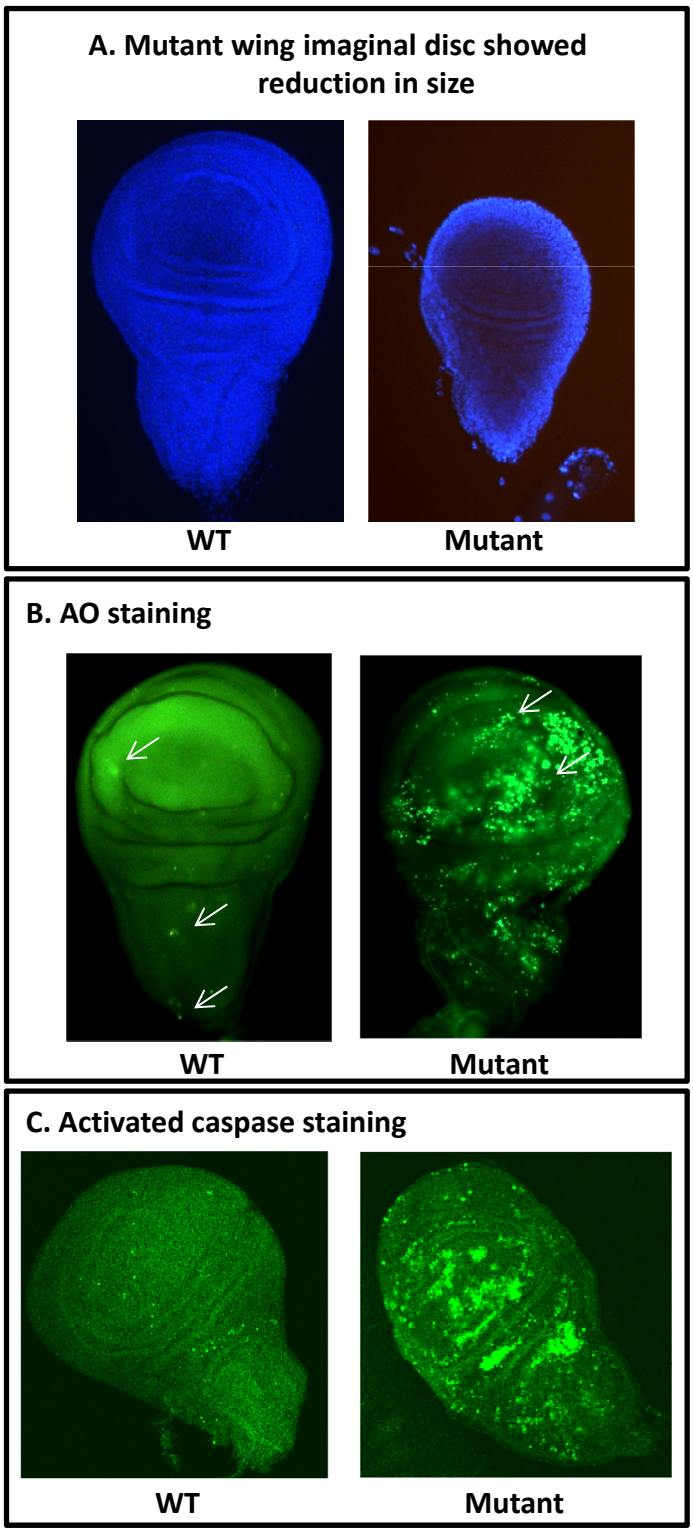


Figure 28: dRif1 mutation showed elevated apoptosis in the wing imaginal discs. A) DAPI staining of wild type and mutant wing imaginal discs showing the reduction in size in mutant dRif1 condition. B) Acridine orange staining shows that the mutant wing imaginal discs with increased acridine orange signals compared to the wild type disc C) Immunostaining with cleaved caspase-3 antibody (green) showed elevated apoptosis in wing imaginal discs.

do knockdown the protein by making use of the well established UAS-GAL4 mediated knock down of dRif1 but unfortunately did not knock down the protein. This could be because in the UAS-GAL4 mediated knock down, the dsRNA transcription begins late in the embryo after 2hrs as the zygotic transcription in fly starts then only and the fly uses the maternally deposited RNA/protein for its development till then. Also the transgene cannot knockdown the maternal transcription using the GAL4 system since the promoter used in pUAST,hsp70 , cannot fire in the oozyte tissues to knock down the RNA there to prevent maternal deposition. dRif1 protein and/or RNA is deposited to the embryo maternally. Very high expression of dRif1 mRNA in the adult female and protein expression in the female specific tissue well attribute to this maternal deposition of RNA or also protein as well.

The mutant generated in Rakesh K Mishra's lab, CCMB were used for further characterization. One homozygous viable line could be obtained. It is probably a hypomorph as the females are sterile. This line has phenotypes that show elevated apoptosis like serrated wings, dark spots on the abdomen and necrotic patches in the eye. Additionally, when the larval imaginal discs were dissected, they were much smaller than wild type and discs showed elevated apoptosis as indicated by increased presence of apoptotic markers.

The loss or function and gain of function reinforce our hypothesis that dRif1 might be a developmentally regulated anti-apoptotic factor.

CHAPTER 7

Discussion

Discussion

Rif1 was identified in yeast almost two decades ago and genetic and biochemical studies have clearly shown that it is a negative regulator of telomerase. Emerging evidence shows that Rif1 in mammals has diverged from its primary role in telomere synthesis and maintenance to playing a broader role in response to DNA damage. In this work we initiated a study on the *Drosophila* homologue of Rif1. Presence of Rif1 in *Drosophila* was intriguing since the fly maintains its telomere by noncanonical retrotransposition mediated mechanism.

7.1 Novel features of Rif1 protein

Our detailed analysis of Rif1 from multiple organisms has identified several novel features. *Drosophila* Rif1 is evolutionarily closer to vertebrate Rif1 than yeast Rif1. All Rif1 homologues contain the conserved HEAT-repeats and this may carry out the core Rif1 activities. As this domain has been implicated in interacting with proteins, it might recruit a variety of proteins to carry out its functions. Within these HEAT repeats, our studies identify a conserved Rif1 specific repeat and this might be useful in identifying the core conserved interacting partners. The more diverse repeats are likely to facilitate participation in other functions. Our analysis also identified a conserved SILK motif, again present in all organisms, from yeast to humans. As this motif has been retained in all species, this is likely to participate in the core Rif1 functions. SILK motif has been shown to involved in PP1 recruitment (Hendrickx et al, 2009). Another PP1 Recruitment motif, RVxF (Moorhead et al, 2008; Wakula et al, 2003) is also present in all the Rif1 homologues. Together these two motifs can interact with PP1 and though the consequence of this docking is unknown, it implies either it could be for dephosphorylating dRif1 itself or dRif1 might act as an adapting molecule of PP1 to its targets/substrates. Very recently, genome wide pull down studies showed that dRif1 interacts with PP1-87B, a PP1, physically (Guruharsha et al, 2011) and *Drosophila* embryo whole phospho-proteome analysis showed that dRif1 is phosphorylated protein (Zhai et al, 2008). All these observations throw light to its mode of action even though the details are not clear and even though the protein has diverged in its function from yeast to

mammals. dRif1 lacks the C-terminal BLM interaction domain which was present in the human homologue (Xu et al, 2010) but contains all the conserved features associated with the N-terminal region.

7.2 dRif1 diverged in its function from its homologues

Complementation studies along with its functional conservation studies in S2 cells shows that the protein in *Drosophila* has diverged in its function. Rif1 was identified and known to be involved in regulating telomere length in yeast. But we found that the fly homologue does not retain this function and it also was not able to perturb the telomere length regulation in yeast, unlike hRif1, which when expressed in yeast exacerbates the *rif1*Δ effect on telomere length (Xu & Blackburn, 2004). hRif1 is involved in general DNA damage or repair function.. Therefore we assessed this function in fly by inducing DNA damage and performing colocalization experiments in *Drosophila* S2 cells. We found that it doesn't colocalize to DNA damage or repair markers suggesting that dRif1 has unique function which other homologues might also have but are presently unidentified. yRif1 localizes to the heterochromatin as it is primarily associated with the telomere (Mishra & Shore, 1999; Smith et al, 2003). In a partly similar manner, we find a strong association of dRif1 to heterocromatin in S2 cells though the heterochromatin in S2 cells. In *Drosophila*, heterochromatin is predominantly centromeric, unlike yeast where the telomeres are the major heteochromatic loci. Our cross-complementation study, by expressing dRif1 in HeLa cells, perturbed the 53BP1 foci formation i.e., the DNA damage response, suggesting that dRif1 might retain some of its interaction even though it diverged from the function per se. hRif1 binds to DNA and thus helps to recruit BLM helicase, through its C terminal interaction, to stalled replication forks. dRif1 does not have the extended C-terminus that carries out the critical functions of association with BLM protein giving logic to its divergence in function.

7.3 dRif1 shows interesting pattern of expression during development

We see the dRif1 expression very early, as early as when the nuclei start to divide. At this stage there is no zygotic transcription in fly. Also we observed that the

protein is expressed in nurse cells and follicle cells. These are the tissues which are involved in maternal contribution. By the northern blot experiment, we see that the RNA also expressed in the adult female in significant amount. Together these results indicate that the dRif1 RNA/protein is being deposited maternally. Also the maternal contribution and its early expression show that the protein has important roles to play in early embryonic development. Our subcellular localization studies through embryonic developmental stages found that the protein follows the dividing or proliferating cells and begins to reduce in differentiated cells. And it diminishes to undetectable levels when the embryo reaches 14hrs of development. This again shows that the protein is important in early embryonic development.

7.4 dRif1 localization is cell cycle dependent and associated with chromatin in anaphase

Drosophila embryos are very good tool to study cell cycle dynamics. This is because of several reasons: a. the embryos are transparent making microscopy easy, b. early embryos are in a syncytium with no cellularization and the nuclei in the whole embryo undergo division in highly synchronised manner and c. after the synchronised divisions it loses synchrony and progresses in mitosis from one end of the embryo giving a unique opportunity to get progressive stages of mitosis in a wave like fashion. Taking advantage of this we found that dRif1 localization is dynamic through mitosis. In interphase and prophase the protein is localised to the nuclei but is found dispersed in metaphase, with no staining on the condensed metaphase chromosome. When the nuclei reached late anaphase or telophase the protein associated with the chromosome. Though we do not know the significance of this unique localization pattern directly, it could have important roles to play in these stages. We find an important characteristic of the dRif1 association with chromatin and this coincides with the diminishing H3PS10 staining i.e., dephosphorylation of H3 at S10. The fact that dRif1 has PP1 interacting motifs, and can interact with PP1 physically (Guruharsha et al, 2011), gives some clues to its function. The interactor of Rif1, PP1, has important roles to play in mitosis like dephosphorylating moesin which couples with the anaphase polar relaxation leading to mitotic exit (Axton et al, 1990; Dombradi et al, 1990; Kunda et al, 2012).

Also loss of PP1 leads to abnormal mitosis due to improper chromatin condensation and segregation (Axton et al, 1990).Either dRif1 can adapt PP1 to PP1-substrates or dRif1 itself gets dephosphorylated to activate or deactivate some pathways which can be studied further. The HEAT repeat domain may give substrate specificity to dRif1 and we imagine that dRif1 acts as an adapter: Recruiting PP1 through its SILK-RVxF domain and an additional substrate(s) via the HEAT repeat so that it can take PP1 to different proteins.

7.5 dRif1 functions as an anti-apoptotic factor in balancing the developmentally programmed apoptosis

Loss of function and gain of function studies are very useful tools to find function of a gene. Ectopic expression of dRif1 ubiquitously was early pupal lethal but tissue specific expression did not give any visible phenotype. The larvae are transformed to adult through a transitional stage known as pupae during which the larvae metamorphose to an adult fly. At this stage, apoptosis has very important roles to play in development of organs and other tissues in fly which it is programmed developmentally. Examining the larval organs showed that the wing imaginal disc was enlarged. This led us to think that the dRif1 expression might impair the programmed cell death/apoptosis leading to altered disc morphology and failure to metamorphose into the adult form. Using a generated dRif1 mutation we observed the opposite effect. Most of the mutant lines were lethal showing that the gene is essential and one line, which survived up to adult stage but infertile, showed severe manifestation of apoptosis. Also the disc size/morphology was reduced, the opposite effect to the over expression of dRif1. Together, the loss of function and gain of function study show that dRif1 acts as an anti apoptotic factor essential for normal fly development. Interestingly, the interactor of dRif1, PP1, knock down also gives similar phenotype - lethality at larval to pupal boundary with little or no imaginal cell proliferation (Axton et al, 1990; Dombradi et al, 1990). Therefore to conclude, we propose that dRif1 has an anti apoptotic role in *Drosophila* development and PP1 interaction might be important for its function.

Future Prospects:

In this study, functional analysis of the *Drosophila* homologue of an ancient protein was initiated and has provided important new information about the possible role of this protein. First, as our analysis suggests that this protein is a developmentally regulated putative anti-apoptotic factor, further investigation of this phenotype should be done. This can be achieved by performing detailed analysis of cells destined for programmed apoptosis in the over expression condition to see if dRif1 indeed suppresses the programmed cell death. Second, the link between PP1 and dRif1 has to be investigated directly by performing both genetic and physical interaction studies. Third, the localization of dRif1 in such a prominent manner to heterochromatin also suggests that it might have special roles at the heterochromatin. This idea can also be further explored by first testing if dRif1 has any role in heterochromatin establishment via PEV assays. In summary, this work has opened multiple new avenues to investigate the molecular contribution of Rif1 to cellular function.

APPENDIX

A1. Construction of truncated 6X His tagged dRif1 clone in pET28a vector

The full-length cDNA clone (RE66338) was obtained from the Drosophila Genomics Resource Center (DGRC). Approximately 1.2kb region from the N (54-450 amino acids) and C (694-1094 amino acids) terminus of dRif1 were chosen (N-dRif1 and C-dRif1) for raising antibody. These two regions were amplified by PCR and cloned in-frame in pET-28a His-tag vector at EcoRI and HindIII site. The clones were confirmed by both restriction digestion (Figure: A1) and by sequencing.

Primer Name	Primer Sequences
RifNL	G**GAATTC**ACGGATGACGAACTGGTGGA EcoRI
RifNR	CCC**AAGCTT**CCGGAACTCGTTCTGATTGT HindIII
RifCL	G**GAATTC**CTCTTCGTGATCATCCCATC EcoRI
RifCR	CCC**AAGCTT**CGGCGAGTCCAGAGTAACAT HindIII

Table A1: Primers used to amplify parts of dRif1. Restriction sites added with the primers are indicated were used for cloning.

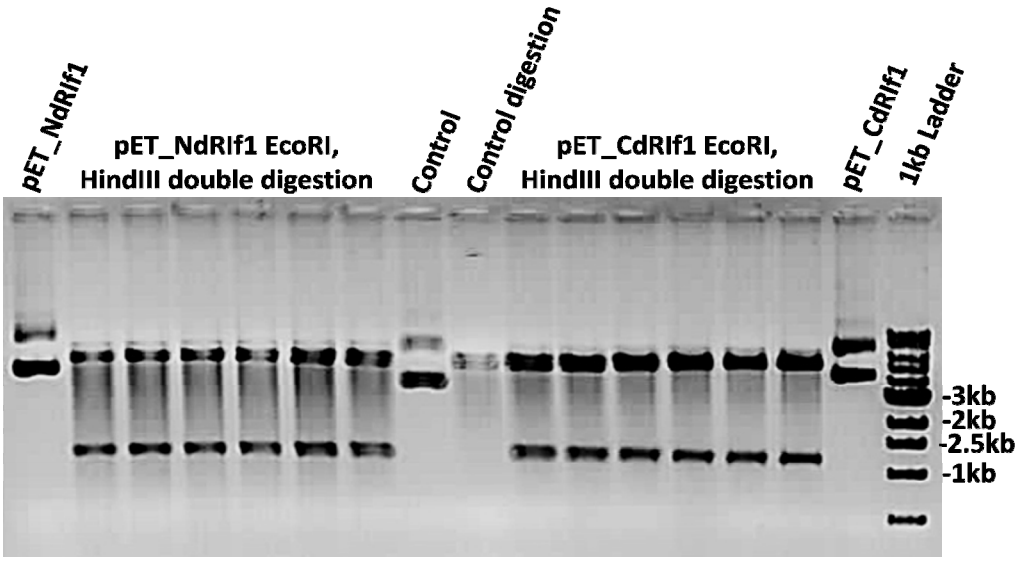


Figure A1: Restriction digestion to release the cloned fragment confirming the clones obtained. Both CdRif1 and NdRif1 released ~1.2kb fragments confirming the clones where as the control, vector alone, did not show any release.

A2. dRif1 full length constructs for expressing dRif1 in yeast

Different constructs were made in yeast expression vectors for expressing the full length dRif1 in yeast. dRif1 was cloned in both single copy vectors(pRS313, pRS315) and in multi-copy vector(yEPLac181). The yeast Rif1 promoter was PCR amplified and cloned towards 5' of the gene using the primer mentioned below. The PCR product was digested using SacI and EagI enzyme, the vectors were digested using SacI and XbaI, full length dRif1 was obtained as a NotI and XbaI fragment from RE66338 cDNA clone by restriction digestion followed by gel elution. All these fragments were ligated together to get the final construct in all these three vectors (Figure: A2).

Primer Name	Primer Sequences
RifPL	GCAT**GAGCTC**TACAAATCGTTAAAAAAGGGCC SacI
RifPR	GCAT**CGGCCG**TGCAAATTGACCACAAAAACGA EagI

Table A2: Primers used to amplify the yeast Rif1 promoter. Primers are shown along with restriction sites added to clone the amplified promoter into yeast expression vectors.

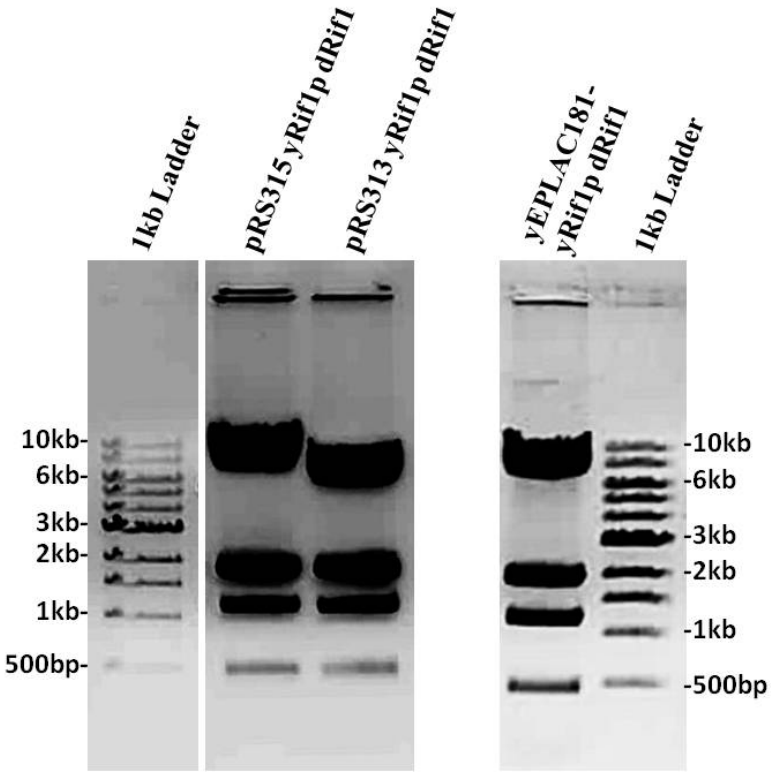


Figure A2: Confirmation of full length dRif1 constructs in yeast expression vectors. SacI restriction digestion gives four fragments if dRif1 and yRif1p are incorporated in all the vectors. All the clones gives three common bands of approximately 460bp, 1151bp, 1859bp and a specific band depending on vector size. It gave approximately 6570bp, 7621bp and 7344bp respectively in pRS313, pRS315 and yEPLac181 vectors respectively depending on the sizes of these three vectors.

A3. Addition of 3XFLAG tag upstream to dRif1 in-frame

In order to add a tag to the dRif1 for the later localization studies, we designed 3X FLAG oligos so that it will give NcoI overhang after annealing and which will be added at the 5' end and dRif1 continues after that in-frame. These oligos were annealed by mixing both the forward and reverse oligos in 1:1 proportion, heating to 94^0C and followed by cooling to room temperature slowly. These oligos were treated with polynucleotide kinase (PNK) and the RE66338 cDNA clone (in pFLC vector) was digested with NcoI and purified and dephosphorylated using shrimp alkaline phosphatise and used for ligating. The clones were checked by NcoI digestion and checked the 72bp drop-out band by running a 2 % gel (Figure: A3). The orientation and number of copies of 3X FLAG insertions were identified by sequencing. This tagged dRif1 was gel purified after NotI and XbaI digestion and used for ligation same as mentioned previously.

Primer Name	Primer Sequences
FTOP	CATGGACTACAAAGACCATGACGGTGATTATAAAGA TCATGACATCGATTACAAGGATGACGATGACAAGCC
FBOT	CATGGGCTTGTCATCGTCATCCTTGTAATCGATGTCA TGATCTTTATAATCACCGTCATGGTCTTTGTAGTC

Table A3: 3X FLAG tag for dRif1. Oligo nucleotides designed to incorporate 3XFLAG tag which upon annealing the two gives an NcoI over hangs for cloning.

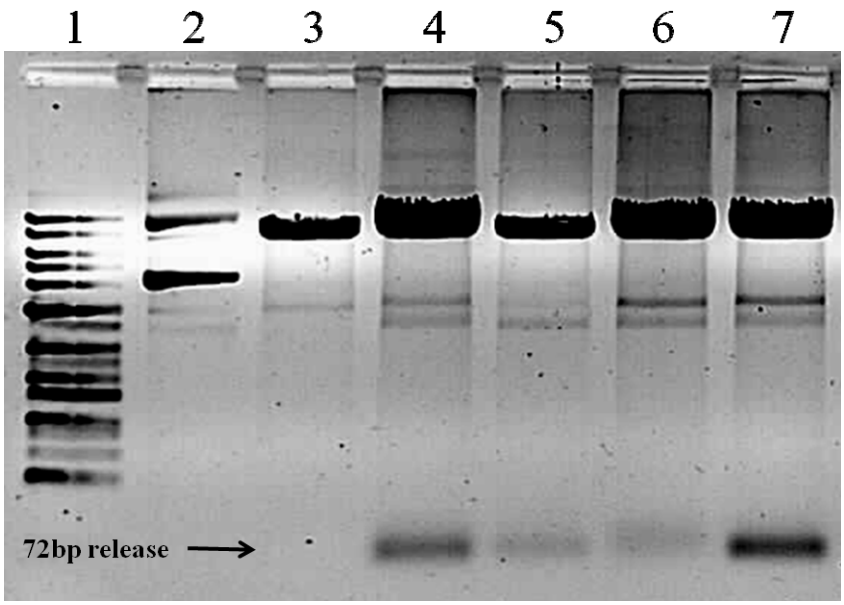


Figure A3: Incorporation of 3XFLAG (72bp) tag upstream to dRif1 in RE66338. Annealed oligoes were cloned in pFLC vector as NcoI fragment and released by NcoI digestion for conformation**.** 1. 1kb ladder, 2. RE66338 cDNA, 3. RE66338 cDNA clone digested with Nco1, 4-7. RE66338 cDNA clone incorporated with 3XFLAG.

A4. Constructs for knock-down

Two constructs in Sym-pUAST RNAi vector were made to knock down the RNA. 1191bases (160 to 1331base) towards N & 1203 bases (2080 to 3263 base) towards C-terminal of dRif1 (Sym-pUAST N-dRif1 & Sym-pUAST C-dRif1) were used. These were obtained as EcoRI-NotI fragment from pET 28a vectors (described in A.1) and ligated to EcoRI-NotI digested Sym-pUAST vector (Figure: A4).

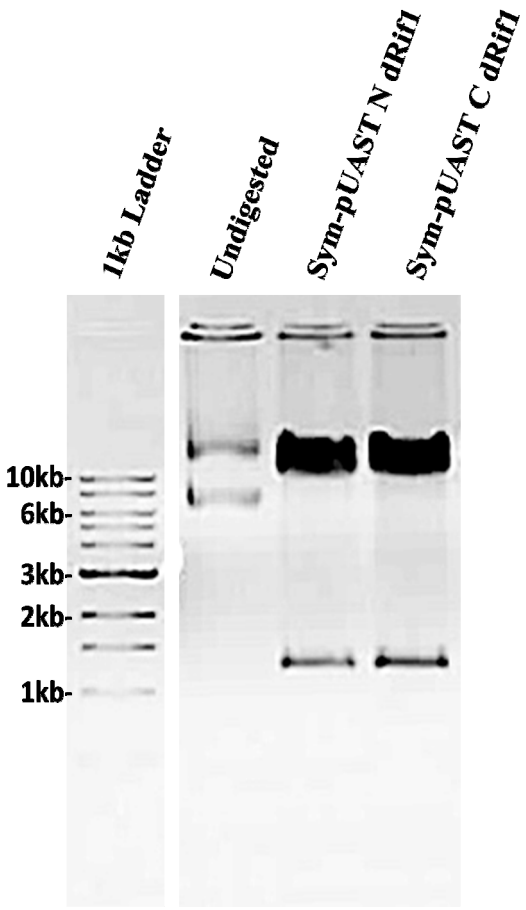


Figure A4: Confirmation of RNAi constructs. Double digestion with EcoRI and NotI releases 1.2kb fragments of both N-terminal (NdRif1) and C-terminal parts of dRif1 incorporated in Sym-pUAST vector.

A5. Constructs for over expression in fly

The full-length cDNA was cloned in pUAST expression vector (pUAST-dRif1) for the over expression studies. dRif1 with an N-terminal 3X FLAG tag was also cloned in pUAST vector. Both were obtained as a NotI-XbaI fragment from pFLC vector without tag and with 3XFLAG tag (described in section A.3) respectively and cloned in pUAST vector digested with the same restriction sites. The clones were confirmed using EcoRI restriction digestion (Figure: A5)

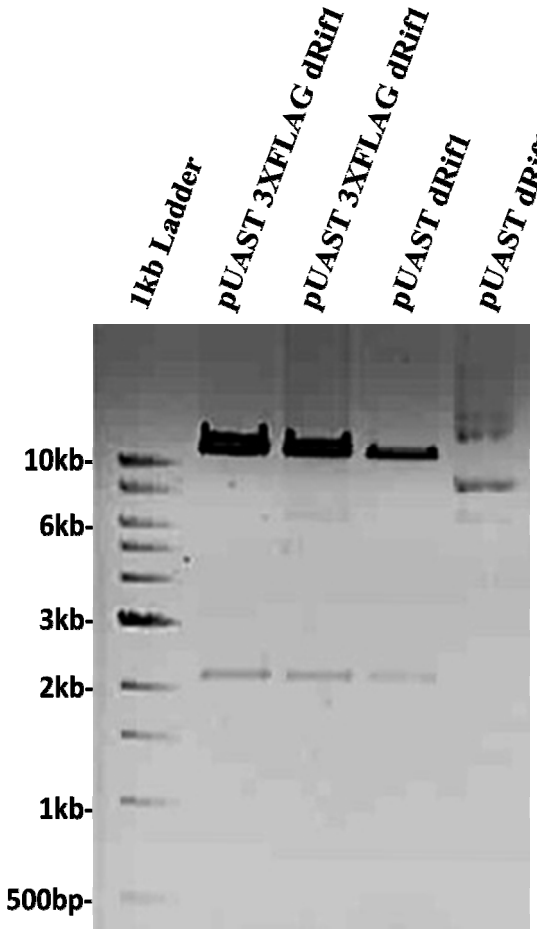


Figure A5: Confirmation of full length dRif1 constructs in pUAST vector. Restriction digestion with EcoRI gives two band of ~11.4kb and ~2.2kb in both 3XFLAG tagged and untagged full length dRif1.

A6. Construct for HeLa cell expression of dRif1

The full-length cDNA along with 3XFLAG tag was gel eluted after digesting it from pRS315-3X FLAG dRif1 using NotI ApaI double digestion and ligated to the *pCMV-FLAG-2A* vector digested using the same restriction enzymes so as to remove the FLAG present in the pCMV vector. The clone was confirmed by restriction digestion using NotI and XbaI restriction digestion (Figure: A6).

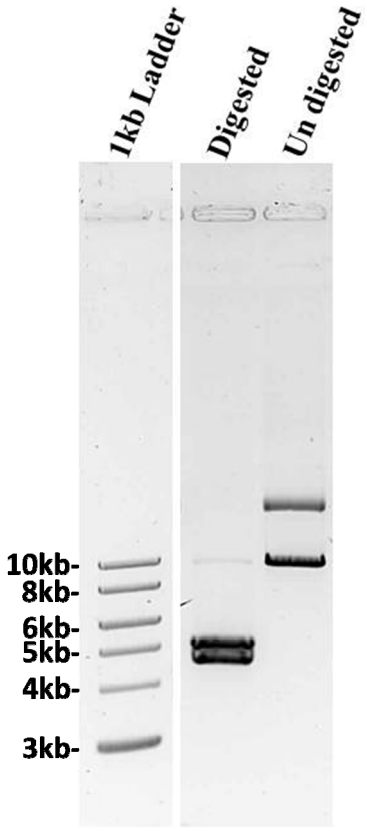


Figure A6: Confirmation of pCMV-3XFLAG dRif1 construct. The construct was digested using NotI and XbaI restriction sites to release 3X FLAG dRif1 (~4.7kb). The pCMV vector being 4.3kb in size, the gel was run for a long time to resolve these two bands.

List of publications

1. **Sreesankar E**, Senthilkumar R, Bharathi V, Mishra RK, Mishra K (2012) Functional diversification of yeast telomere associated protein, Rif1, in higher eukaryotes. *BMC Genomics* **13**(1)**:** 255. (From the thesis work)

2. Pasupala N, **Easwaran S**, Hannan A, Shore D, Mishra K (2012) The SUMO E3 ligase Siz2 exerts a locus-dependent effect on gene silencing in Saccharomyces cerevisiae. *Eukaryot Cell* **11**(4)**:** 452-462. (Apart from the thesis work)

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