Title
Identification and Characterization of the Drosophila Inverted Repeat Binding Complex

Author
Francis, Malik Joseph

Publication Date
2011

Peer reviewed|Thesis/dissertation

Identification and Characterization of the Drosophila Inverted Repeat Binding Complex

By

Malik Joseph Francis

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Donald C. Rio, Chair
Professor Kathleen Collins
Professor Stuart Linn
Professor David Wemmer
Professor Judith Campisi

Spring 2011

Abstract

Identification and Characterization of the *Drosophila* Inverted Repeat Binding Complex

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Malik Joseph Francis

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Professor Donald Rio, Chair

Efficient P element transposition is dependent on the expression of a transposon-encoded transposase and the recruitment of endogenous *Drosophila* DNA repair proteins to sites of transposase induced DNA breaks. Previous biochemical analysis of proteins bound to the 31 bp inverted repeats identified an 18 kDa basic leucine zipper protein (bZIP) (CG6272) termed Inverted Repeat Binding Protein 18 (IRBP18). IRBP18 forms a functional heterodimer with another bZIP protein Xrp1/CG17836, which is sufficient to bind the 31bp terminal inverted repeats *in vitro*. Together these proteins, in cell culture models, repress transcription from the P element promoter, as well as facilitate efficient DNA repair after transposase-mediated cleavage. Additionally flies homozygous for null mutations of IRBP18 show increased killing after somatic mobilization of P elements: consistent with an inability to repair post cleavage DNA breaks. In the absence of P elements, IRBP18-null flies are male sterile and display increased sensitivity to DNA damaging agents. IRBP18/Xrp1 heterodimer forms the site-specific binding core of a larger IRBP complex; of which several of the co-purifying proteins function in RNA interference (RNAi) pathways. Recently RNAi pathways have been shown to be integral components of general genome maintenance due to regulation of heterochromatin formation of repeated DNA elements, such as ribosomal DNA genes. In addition in this thesis Rm62, the novel role of the DEAD/H-box RNA helicase, in DNA repair is examined. Flies homozygous for a hypomorphic allele of Rm62 display similar sensitivity to DNA damaging agents as IRBP18 mutants. Interestingly, the majority of the DNA repair phenotype of Rm62 is caused by a nucleolar localized isoform of the protein. Furthermore RNAi-knockdown of the Rm62 interaction partner, Argonaute 2, also resulted in decrease in DNA repair efficiency. Taken together, these data implicate the IRBP complex as a critical component in maintaining genomic integrity and thereby connecting a new site-specific DNA binding protein complex to genome stability and DNA repair.

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Acknowledgements

My years at Berkeley were a mix of deep personal anguish, self-discovery, camaraderie, growth, and success. This milestone in my career is a culmination of the hard work and sacrifices of so many people. I am unsure that these words are sufficient to express my true feelings of love and gratitude, but I will attempt to come close.

To the GAO: thanks for all you patience with my pathological inability to stay organized. I am fairly sure that much of the administrative paper work in my student career has been completed after some deadline, so I thank you for not encircling and pelting me with insults or, worse, rocks.

I would be remiss if I did not thank the people who shaped my early professional career. To my first mentors, Larry Wetterau and Hassy Cohen: thank you for sparking my interest in research and giving me the opportunity to grow. I am extremely grateful for all the fundamentals I learned in the Cohen lab. Dan Rodgers helped give me the confidence and support that I needed to be successful. To Art Garfinkel: thanks for seeing my potential and playing tennis with me. I would not be here if not for your efforts. To Bob Goldberg: you taught me the fundamentals of teaching, challenged my thinking, saw in me talent that I was not sure existed, and encouraged me grow as a person. I just want you to know that I keep my promise. Thank you all so much for your early significant investment in me.

I would like to thank Tom Cline (fly genetics help and Cesium source), the Botchan Lab (reagents and AKTA time when our machine broke down), the Karpen Lab (Deltavision), David King (turbo FLAG), Lori Kohlstaedt and Daniela Mavrici (Mass Spectrometic analysis) and the Tjian Lab (reagents and the homogenizer).

Thanks to my committee (David Wemmer, Kathy Collins, Judith Campisi, Stu Linn), who are probably confused as to why my thesis came so late; so am I. Your mentorship and encouragement has sustained me over the years.

To Kathy: thank you for suggesting the key experiment in my thesis. During my second year, the purification scheme we were pursuing was not yielding usable data, or so we thought (more on that later). The TAP-Tag IRBP18 construct enabled us to purify the complex (in particular Xrp1) and also perform biochemical experiments. You have been an influential mentor and champion of my professional interests. Thank you.

I have to say that being a member of the Rio lab marked an enjoyable period in my life. I do not have the space to tell all the fun stories. My work is a continuation of the excellent work of Siobhan Roche. After many, many hours in the cold room she identified IRBP18 as the founding member of the IRBP complex. Further purification of this complex would not have been achieved without the head start you gave me. Thank You. I would like to thank Julie and Matt for carefully reading my manuscripts and this thesis. Aurora, thanks for cleaning up my mess before I ever got to it and for feeding me.

You've been like a second mom and everyone should be so lucky. Thank you Sharmistha for alerting me when you commenced eating your yogurt (my stomach thanks you). To Marco: my appreciation for your teaching me a tenth of your biochemical knowledge; I am now an expert for it. Julie and Marco were also my coffee partners everyday at 3:15pm. I should say, however, that conversations between Marco and me were completely different from those with Julie and…I'll leave it at that! So many pints, laughs, fights, meals and ultimately awesome memories! Thank you.

I also had the extreme pleasure of mentoring three off-the-charts undergraduate students. Ron Panganiban made important contributions to the Rm62 story of Chapter 4. He showed that Rm62 had DNA repair activity and the some of the isoforms were differentially localized. His extraordinary ability to out drink me on occasion earned my respect, but his love of science and ability to perform excellent experiments earned my gratitude. Michael Cho was instrumental in developing the Xrp1 story in Chapter 2. When I finally got mass spec data indicating that Xrp1 was the bZIP interaction partner for IRBP18, we went to work purifying the recombinant heterodimer. On 7/22/09, I showed that the purified complex reconstituted IRBP DNase I footprinting activity. That night we knew we solved a 22 year-old problem. And after testing several dsRNA constructs showed that Xrp1 also promotes DNA repair in concert with IRBP18. These two cloned many of the constructs employed in this thesis: in particular the TAP-Tag IRBP18, PEP-luciferase, and pET-Duet with Xrp1 and IRBP18. "Little" Sarah Chamanara kept the flies organized, and helped me count some tens of thousands of flies in experiments not presented here or detailed in Chapter 3. She was a ray of sunshine everyday with her infectious smile and home baked cookies. Everyone should have people so dedicated and hardworking around them.

From afar my interactions with Don could be described as anything from contentious to convivial. To Don: I am eternally grateful for your acceptance of me as a graduate student in your lab and your nurturing my transition from physiologist to a molecular biologist who can perform protein purification of multi-subunit complexes. I cannot imagine another individual who could have served as a better mentor for my growth as a scientist. You challenged me to think in critical ways and fostered an environment for me to grow. Thank You. (Since this will be for all time, please remind Anthony that the Celtics will always suck.)

I have had the great honor and distinct privilege of having a group of diverse, strong willed friends. Whenever I was depressed, down on myself, or feeling lonely they were there to pick me up. I cannot thank everyone individually because it would be a document half the size of thesis. Thank you to everyone who was so supportive of my main hobby/passion. Special thanks to Martin and Harumi for letting me crash at your place when I first moved to Berkeley and at various times when I needed a warm place to stay. If I have been to Chicken Town, Becketts (RIP), or the Trappist with you, then you are very, very special to me.

To Yuki: thank you so much for everything. You really have no idea how much your friendship means to me.

I would be dishonest to say that I am not fortunate to have grown-up in this United States versus the one in which my grandparents came of age. Although discrimination is still a real problem in this country, their personal achievements and the sacrifices of their generation is why I have so many diverse opportunities. I hope that by small measure I have made you proud.

To my brothers: thanks for always being there for me. So many times, for so many years I have dropped the ball or have been so focused on my career that I was not always there when needed. To my (not so) little brother Khalid and my older brothers Tony, Will, (and special thanks) Sean (for covering up my financial short comings): thank you for everything.

To my parents: I know you did the best you could, and I love you for that. To my mother especially, thank you for always being there for me; especially when I messed up so royally, so often. You will have that happy ending you so richly deserve.

Chapter 1

Introduction

Transpositional Recombination

The importance of transposable elements in genome organization and evolution is currently being revealed through modern genomics. Transposons comprise a significant portion of the genome: current estimates of transposon content of the *Drosophila melanogaster* genome is between 15-22% (Biemont and Vieira, 2006). Because many of these elements are mobile, host organisms have developed mechanisms to combat the deleterious effects of transposition events such as the creation of double-strand breaks (DSB), insertion into or near protein-coding genes, illegitimate recombination and genome rearrangement.

Transposition reactions can be broadly categorized into two types: DNA transposition and retrotransposition. DNA transposition occurs via a self-encoded transposase/recombinase which recognizes sequence elements at both the 5' and 3' end. Once these ends are cleaved the transposon inserts into a non-homologous region of the genome. In contrast, retrotransposition employs an RNA intermediate in addition to a self-encoded recombinase. DNA cleavage at a new insertion site generates a 3'-OH that serves as a primer for reverse transcriptase which makes a DNA copy of the element RNA into the genome.

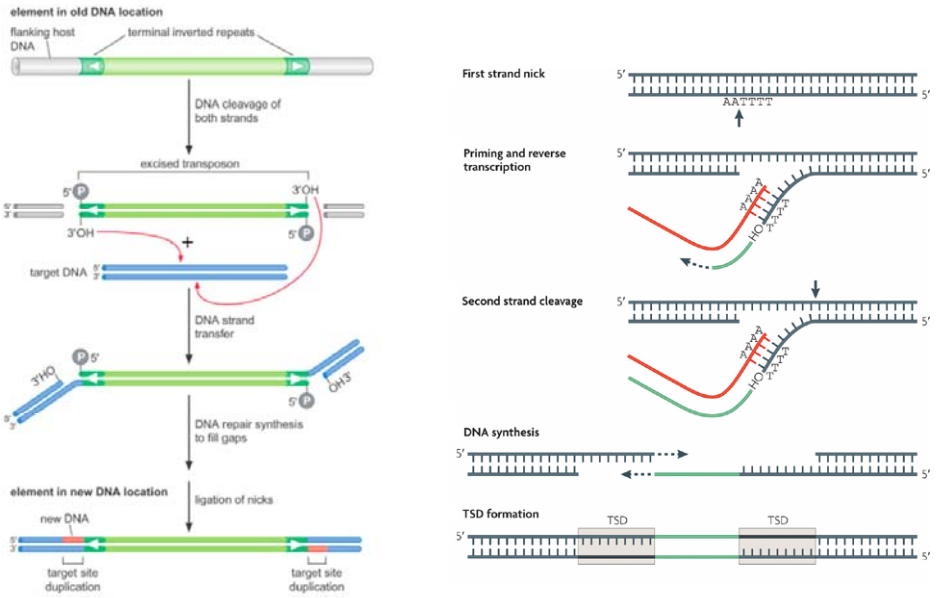


Figure 1 Overview of Transpositional Recombination. On the left is a generalized mechanism of "cut and paste" transposition from (Watson et al., 2004). The right panel is a schematic of a generalized mechanism of one mechanism of retro-transposition, called target-primed reverse transcription (TPRT) (Cordaux and Batzer, 2009). In each case target site duplications are created after DNA repair/synthesis.

DNA transposition can be further classified as replicative or non-replicative (cut and paste) reactions (Figure 1). In each case the host replication or repair machinery resolves intermediate DNA structures created by the transposition reaction. Replicative transposition reaction proceeds by initially by cleaving the 3' end of the element. The newly exposed end is in turn used to attack another site in the genome. The 5' end of the element remains attached to the donor DNA and the endogenous replication machinery generates a second copy of the element. This structure, the cointegrate, is resolved by recombination of the two homologous elements. Cut and paste transposition cleavage occurs at both ends of the element. Insertion of an excised transposon into a new target site results in the creation of target site duplication via staggered cleavages at the new insertion site which vary in length according to the transposase specificity. The direct consequence of the reaction is the creation of a DSB at the donor site (previous location of the excised site) and the robust activation of endogenous repair pathway to ensure genome stability

How do Drosophila maintain genome stability with a large portion of its genome comprised of mobile transposable elements?

Although transposons are found at high copy number, the majority of those are non-autonomous due to deletions at the terminal repeats or internal deletions that remove protein coding potential (Slotkin and Martienssen, 2007). Removal of these sequences impairs the ability of the transposase to recognize the sequence and perform accurate cleavage.

Transposable element activity can also be reduced by the expression of a repressor protein. In the case of the P element, the majority of protein expressed in germline and the all of the proteins in somatic cells are repressor proteins which lacks catalytic activity yet retains the same binding ability for the transposase binding site (see later in Chapter 1).

RNAi based mechanisms are also important is reducing transposable element mobility at both the pre and post-transcriptional levels. A diverse family of small RNAs (typically 21-30 nt) act to regulate mRNA stability, mRNA translation, transcription and genome stability. In the classical RNAi pathway, small RNAs are generated by the cleavage of longer dsRNA pre-cursors by dicer protein family members. The cleaved products are loading into another complex, RNA-induced silencing complex (RISC), by protein-RNA interaction with Argonaute (AGO) family of proteins. AGO proteins have a conserved ribonuclease H-like motif PIWI domain. This domain exhibits small RNA directed nuclease activity of RNA transcripts.

The Piwi clade of the argonaute protein family bind small RNAs termed piwi interacting RNA (piRNAs) that are larger (26–30 nucleotides) than most small RNAs (Aravin et al., 2007b; Brennecke et al., 2008). In contrast to siRNAs and miRNAs which are derived

from double-stranded and short hairpin RNA precursors, piRNA are strand-specific, suggesting that piRNA precursors are transcribed from only one strand (Chu and Rana, 2007; Thomson and Lin, 2009). piRNAs are derived from clustered regions of the genome devoid of known transposons . A sub set of *Drosophila* piRNA are termed repeat-associated siRNAs (rasi-RNA) and are derived from the *Flamenco* locus of clustered region enriched for repeats and transposable elements (Brennecke et al., 2007; Yin and Lin, 2007). Importantly production of piRNA and rasiRNAs is dicer independent.

Piwi proteins are localized to the nucleus and mediate cleavage of mRNA or induce histone methylation to silence a given locus (Pal-Bhadra et al., 2004). Methylation of histones is an important intermediate and marker of heterochromatin. The majority of transposons (both autonomous and non-autonomous) are localized to heterochromatin as indicated by the presence of Histone H3 lysine 9 methylation (H3K9me) (Ding et al., 2007; Klattenhoff et al., 2009; Slotkin and Martienssen, 2007). This repressive mark serves to the dual purpose of reducing transcriptional activity as well as reducing recombination between repeated DNA sequences.

In yeast, genetic deletions in the RNAi components were found to reduce centromeric heterochromatin silencing. Transcripts from the centromeric regions of chromosomes were processed and entered into the RNA induced transcriptional silencing (RITS) complex. Once on the RNA is loaded in the RITS complex, the homologous sequences within the genome are recognized and silenced by H3K9me. Mutations in *Drosophila* RNAi genes *piwi*, *aubergine*, and *homeless* disrupt HP1 (Pal-Bhadra et al., 2004) localization and suppress transgene silencing (Brennecke et al., 2008). The similarities in organization and transcriptional activities between yeast and *Drosophila* centrosomes imply a similar mechanism for RNAi-mediated heterochromatin assembly operates in *Drosophila* as well.

Surprisingly, one of three helicase implicated in heterochromatin formation, Rm62 is a member of the IRBP complex (Chapter 2 and 4). Rm62 was originally identified as one of the gene products of the Triplo-lethal locus (Dorer et al., 1990). Subsequent mutagenic screening demonstrated it to be a modifier of retrotransposon expression and suppressor of position effect variegation (PEV); thereby playing a role in heterochromatin formation (Csink et al., 1994). Consistent with a role in chromatin structure, Rm62 forms a RNA-dependent interaction with a *gypsy* insulator binding protein, Centrosomal protein 190 (CP190) (Lei and Corces, 2006). Disruption of this interaction resulted in nuclear reorganization of a compromised *gypsy* insulator. Additionally, Rm62 is required for gene deactivation in addition to its role in RNA export (Buszczak and Spradling, 2006). How the helicase is targeted to the element is unknown. One distinct possibility is that the targeting occurs via specific sequences within the element. This is an attractive model as methylation of the element at the terminal repeats would effectively insulate it from chromatin regulation and also keep transcript levels to a minimum.

P element Transposition

P element transposons are a class of mobile DNA that have integrated into the genome of *Drosophila melangaster* approximately 100 years ago (Engels, 1997). They were identified by studying a genetic phenotype called Hybrid Dysgenesis. Briefly males from wild populations were crossed to females from lab stocks; the resulting progeny had germline mutations, temperature sensitive sterility, and male recombination. The reciprocal cross yield phenotypically normal progeny. *Drosophila* strains were classified as either P (paternal contribution) or M (maternal contribution) strains. Genetic mapping experiments suggested that Hybrid dysgenesis phenotype was caused by a mobile element. The P element was confirmed as the mobile element that causes hybrid dysgenesis by DNA hybridization experiments revealed that P elements were present in variable locations in P strains yet absent from most M strains.

Their basic organization can be divided into two regions: a) cis-acting DNA sequences involved in the DNA cleavage and joining reactions that bind and assemble P element transposase to carry out transposition and b) the protein coding regions for the P element transposase enzyme. Mutational analysis of the P element ends demonstrated that approximately 150 bp of DNA are essential at each end for transposition *in vivo* (Mullins et al., 1989). Contained within these terminal sequences are the 31bp inverted repeats at the transposon ends, 11bp internal repeats which serve as enhancers of transposition (Mullins et al., 1989) and internal 10bp transposase binding sites (Kaufman et al., 1989)

Regulation of Transposase expression

Expression of the catalytically active transposase is restricted to the germ line via tissue specific splicing of the third intron (IVS3) (Laski et al., 1986; Laski and Rubin, 1989). IVS3 retention in somatic cells results in the production of an mRNA that contains an in-frame premature stop codon and encodes a truncated 66 kDa repressor protein (Laski et al., 1986; Laski and Rubin, 1989; Misra and Rio, 1990). This mechanism is regulated by at least three splicing factors: hrp48, PSI, and U1 snRNP.

PSI forms direct protein-RNA interaction with two pseudo-5' splice sites (F1 and F2) located upstream of the accurate 5' splice site (Adams et al., 1997; Siebel et al., 1995). Binding of PSI recruits U1snRNP to these pseudo-5'splice sites. The AB domain of PSI forms a direct protein-protein interaction with U1-70K subunit of the U1 snRNP (Labourier et al., 2001). Also bound to the F2 site is hrp48. Mutations within the F2 site, that decreased hrp48 binding *in vitro*, also relieves somatic inhibition of IVS3 splicing *in vivo*. Additionally flies homozygous for a hypomorphic allele of hrp48, somatic splicing of an IVS3-containing reporter RNA is activated (Hammond et al., 1997). Biochemical and genetic evidence suggest a clear role for the binding of hrp48 to the F2 site and inhibiting accurate splicing of ISV3.

To date, how these factors are loaded onto a single RNA and inhibit splicing is still unknown. The interaction between the AB domain of PSI and U1-70K suggest PSI may function to stabilize the binding of U1 snRNP to the relatively weak or inactive exonic F1 pseudo-5' splice site (Labourier et al., 2001). The direct predication of this model is steric hindrance of accurate 5' splice site such that U1cannot access it.

Mechanism of P element transposition

Transposase recognition of cognate DNA sequence

As determined by the 1.74-Å resolution co-crystal, the DNA binding domain of transposase uses a bipartite, major and minor groove binding mechanism to specifically recognize the 10bp TNP binding sequence (Figure 2) (Sabogal et al., 2010). Minor-groove recognition is achieved by a combination of direct base contacts and indirect sequence readout of DNA deformation through a variable, basic loop. By contrast, the adjacent major groove is sequence-specifically recognized by the central β-sheet of the domain. A β-sheet of the DNA binding domain interacts with the central GTGG sequence of the major groove. Two residues His18 and Gln42 from two β-strands, along with the N terminus, interact with both strands of the DNA duplex in the major groove. Additional contacts are made by the main chain atoms of Tyr3, Leu16 and Asn40, along with the side chain of Gln42. AT-rich minor groove contacts are made loop 4 via water mediated interactions. Main chain atoms make contacts with the phospodiester backbone.

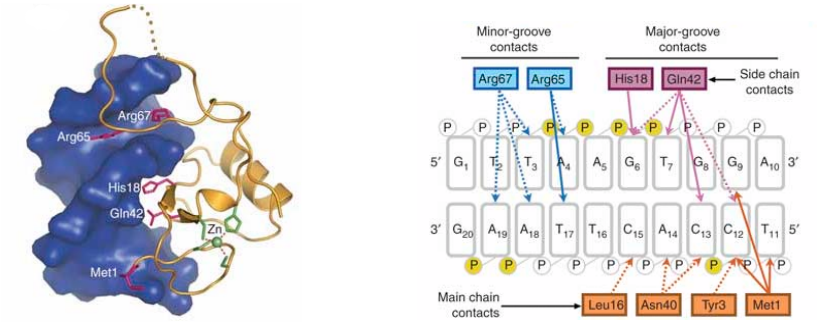


Figure 2 DNA sequence recognition by the THAP domain of the P element Transposase (Sabogal et al., 2010)

Oligimer formation, synapsis, and activation

Dimerization of transposase monomer occurs through a leucine zipper interaction located upstream of the THAP domain (Lee et al., 1996). A second interaction domain is thought to mediate tetramer formation of the active enzyme.

Other domains of the transposase protein that are essential for activity are a non-canonical GTP-binding domain and a putative acidic catalytic motif, common to most transposases/integrases (Kaufman and Rio, 1992; Mul and Rio, 1997). GTP is required for formation of synaptic complexes where both 5' (21 bp spacer) and 3' (9 bp spacer) P element ends are paired before DNA cleavage (Kaufman and Rio, 1992; Tang et al., 2005). The requirement of pairing the 9 and 21 bp spacers is mechanistically similar to the 12/23 rule of V(D)J recombination (van Gent et al., 1996a). The transposase protein, in addition to its cofactors GTP and Mg^{2+}, is the only protein required to catalyze the cleavage and strand transfer reaction steps of the transposition reaction (Kaufman and Rio, 1992; Tang et al., 2007; Tang et al., 2005).

Transposase Cleavage reaction

The transposition reaction occurs by "a cut and paste" mechanism in which the transposase catalyzes double-strand DNA cleavages within the 31 bp inverted repeats creating 17 bp 3' single-stranded extensions at each transposon end (Beall and Rio, 1997; Kaufman and Rio, 1992).

Both the strand cleavage and transfer reactions occur through two metal ion catalyzed SN2 reactions (Yang et al., 2006). P element transposase has a conserved RNase H fold that is interrupted by the GTP binding domain. Within the RNase H fold are several candidate Asp and Glu resides that can participate in a two metal ion catalysis. In this reaction a water molecule serves as the nucleophile and attacks the phosphodiester backbone. The resulting 3' hydroxyl groups serve as nucleophiles for strand transfer or integration into a target site at another genomic location (Kaufman and Rio, 1992).

During strand transfer of the excised P element intermediate, the transposase protein contacts the transposase binding site and both double- and single-stranded portions of the 31 bp inverted repeats and joins the 3' P element ends to an asymmetrically cleaved target site (Beall and Rio, 1998). Repair of the single-stranded gaps at the target site by the host DNA repair machinery results in an 8 bp duplication of the target site.

Repair of donor site

Repair of the donor DNA site following P element excision has been demonstrated to occur through two distinct DNA repair pathways: non-homologous end joining (NHEJ) and homologous recombinational repair (HR). A variant of classical DSB repair termed Synthesis dependent strand annealing (SDSA) is thought to account for the copying mechanism as the donor site is restored by copying the P element from a homologous sister chromatid. The SDSA model, much like HR, initiates repair of DSB by exonuclease-mediated resection of the 5' strand to generate 3' single-stranded tails. These tails are then free to invade homologous templates and prime DNA synthesis. Nascent DNA strands are displaced from the template and can then anneal with complementary DNA from the recipient chromosome (Nassif et al., 1994). Thus, SDSA

bypasses the generation of Holliday junctions and results in gene conversion without crossing over. NHEJ is an alternate repair pathway that does not employ a templating mechanism. DSBs are joined directly and processed without the use of a homologous template sequence. This repair pathway results in either gain or loss of nucleotides because joining occurs at regions of micro-homology. The NHEJ pathway repairs DNA breaks after mammalian V(D)J recombination. The major players involved in NHEJ are the Ku p70/80 heterodimer, XRCC4 and ligase IV. At present a detailed biochemical mechanism of P element DNA repair has not been elucidated.

General DSB response

The selection of which DNA damage response pathway to initiate is complex and dictated by several factors, most importantly cell cycle position, availability of substrates, activation of protein kinases and interplay of exonucleases and helicases. A detailed mechanistic discussion of each pathway is not within the scope of this section, rather an understanding of the conserved overarching themes and concepts that direct repair pathway choice. I will give a general overview of three pathways to repair DSBs in cells, NHEJ, MMEJ, and HR.

Early activation of the protein kinase ATM

Ataxia-telangiectasia mutated (ATM) is a highly conserved eukaryotic DNA repair kinase that becomes active upon early cellular recognition of DSBs. In non-stressed state ATM exists as an active monomer. One of the earliest events in DSB repair is a protein-protein interaction between ATM and the Mre11-Rad50-NBS1 (MRN) complex bound to broken DNA ends (Lee and Paull, 2004; Lee and Paull, 2005). This interaction promotes an autophosphorylation of S1981, which in turn disrupts the homodimeric interaction (Bakkenist and Kastan, 2003). Activated ATM can rapidly phosphorylate both chromatin associated and nucleoplasmic targets. Some critical ATM targets are Histone H2AX, p53, ATF2, and Chk2 kinase (Shiloh, 2001).

Activation of p53

In mammalian systems, the major regulatory mechanism of the steady levels of p53 is a protein-protein interaction with a E3 ubiquitin-ligase, Mdm2. In unstressed cells, Mdm2 can monoubiquinate p53, which allows p53 to be exported from the nucleus, or it can polyubiquinate p53, resulting in proteasomal degradation. In response to genotoxic stress, p53 is a directly phosphorylated by ATM (S15) and cell-cycle checkpoint kinase Chk2 (S20) (Canman et al., 1998; Shieh et al., 1997). The S20 phosphorylation of p53 destabilizes the interaction with Mdm2 and increases the steady state levels and S15 phosphorylation promotes p53 transcriptional activation.

p53 is a modular protein that is divided into four domains, an acidic amino acid rich N-terminal transactivation domain, a central DNA binding domain, regulatory domain and a

C-terminal tetramerization domain (Lu and Abrams, 2006; Mills, 2005; Sutcliffe and Brehm, 2004). The tetrameric form activates transcription in response to genotoxic and cellular stresses. The p53 DNA binding site consists of two degenerate half sites separated by a variable linker . Although additional functions of p53 have been reported, p53 primarily function is as a transcription factor that upregulates genes involved in cell cycle arrest, apoptosis, and DNA repair (Akdemir et al., 2007; Brodsky et al., 2004).

Drosophila p53 (dmp53) shares DNA binding affinity within the mammalian p53, but there are key functional differences between the two (Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000). Steady state levels of dmp53 remain unchanged through the cell cycle and in response to IR damage, due to the lack of both an Mdm2 binding site and an obvious ortholog Mdm2 encoded in the *Drosophila* genome. Several lines of experimental evidence suggest that dmp53 does not induce cell cycle arrest after DNA damage. The clearest and most direct explanation of this is that the p21 ortholog, decappo, is not transcriptionally upregulated in response to p53 (Brodsky et al., 2004). Additionally, in contrast to the vast array of reported post-translational modifications of mammalian p53, dmp53 as few described phosphorylation events, the most important is phosphorylation by chk2/mnk (Brodsky et al., 2004). Damage induced phosphorylation is one of the few functionally important modifications for transcriptional activation.

Non-homologous end joining (NHEJ)

The major repair pathway in G1 and G0 phases of the cell cycle is NHEJ. At a basic level NHEJ is four coordinated enzymatic activities: DNA end-binding, nucleolytic removal of damaged DNA, polymerases to aid in the repair, and ligation to restore the phosphodiester backbone.

The crystal structure of human Ku70/80 revealed a ring structure analogous to PCNA (Walker et al., 2001). The Ku heterodimer makes a few backbone and no base contacts, which is consistent with role in non-specfic DNA end binding. Ku70/80 binding to DNA coordinates the enzymatic activities of each of the core NHEJ subunits through protein-protein interactions.

Recent biochemical studies of purified mammalian K70/80 demonstrated evidence of 5′-deoxyribose-5-phosphate (dRP) and apurinic/apyrimidinic (AP) lyase activity (Roberts et al.). Similar to other lyases (DNA Polymerase β), Ku creates nicked DNA 3′ of an abasic site via a Schiff-base covalent intermediate with the abasic site. This activity is thought to be involved in the efficient end “cleaning” and joining. This observation explains how these activities occur in organisms without clear orthologs for DNA polymerase β, like *Drosophila* (Sekelsky et al., 2000).

At each break site there is non-sequential flexibility of each enzyme. This results in the stochastic nature of NHEJ repair of diverse DNA damage substrates. In vertebrates, the Artemis/DNA-dependent protein kinase catalytic subunit (DNA-PKcs) complex becomes active as a 5’- and 3’-endonuclease when DNA-PKcs binds to a DSB DNA end.

Polymerases μ and λ are two of the pol x family of polymerases known to play a role in NHEJ (Daley et al., 2005). A complex of XLF (Cernunnos), XRCC4, and DNA ligase IV forms the ligase activity for NHEJ. *Drosophila* lack obvious orthologs for pol X family members pol μ and λ, artemis and DNA-PK, so it is unclear how many of the catalytic steps in NHEJ occur in the absence of these proteins (Sekelsky et al., 2000).

Microhomology-mediated end joining (MMEJ)

A poorly understood alternate pathway end-joining pathway is MMEJ. It is a Ku70/80 independent and requires the presence of 5-25 nt terminal microhomology (McVey and Lee, 2008). The key difference between MMEJ and NHEJ is the role of end resectioning to find regions of microhomology. In yeast, Mre11-Rad50-Xbs1 and Exo1 have been shown to be required for MMEJ (Ma et al., 2003). In mammals, CtIP can also promote end resectioning (discussed further in next section). Creation of ssDNA long enough to promote MMEJ would then presumably disfavor binding of Ku subunits to the ends (Dynan and Yoo, 1998).

How a particular area of microhomology is chosen for the annealing is not well understood. In yeast, it appears that Rad51 can bind ssDNA ends created after end-resection and another protein Srs2 displaces Rad51 to promote the annealing steps. Once annealed the non-complementary DNA form 3' flaps that must be efficiently removed. Cleavage of these flaps is accomplished by xeroderma pigmentosum complementation group F (XPF)–excision repair cross complementation group 1 (ERCC1) (Ahmad et al., 2008).

In a fashion similar to NHEJ, error prone and translesion DNA polymerases play a role in nucleotide additions to fill in gaps at the repair sites. Ligase I, III and IV have all be implicated in final joining reactions (Lieber, 2010).

Homologous repair

Homologous recombinational repair (HR) takes place in both mitotic and meiotic cells to repair DSBs created or repaired in S and G2 phases of the cell cycle. Unresolved or misrepaired events lead to mutagenic events such as chromosome loss, deletion, duplication or translocation, which in turn lead to genomic instability. HR is dependent on the presence of a homologous DNA template to carry out repair of the broken chromosome; this is generally the sister chromatid in mitotic cells in late S and G2 phases.

One of the first mechanistic steps is the creation 3' ssDNA by 5'-3' exonuclease activity (Mimitou and Symington, 2009). This resectioning is accomplished by Mre11, Dna2, Sae2/CtIP, and Exo1 (Cejka et al., 2010; Eid et al., 2010
; Huertas et al., 2008; Limbo et al., 2010; Mimitou and Symington, 2009; Mimitou and Symington, 2010; Zhu et al., 2008). The resulting ssDNA is bound first by Replication

protein A (RPA) and accessory factors then bind to the ssDNA and later replaced by Rad51 (Seong et al., 2009).

The nucleoprotein filament formed by Rad51 searches a second DNA molecule for homology, resulting in pairing of the Rad51–ssDNA complex to a complementary ssDNA region within the homologous duplex (Krogh and Symington, 2004). The invading 3'end, from the broken chromosome, is used to prime DNA synthesis, templated by the donor duplex. The DSB repair (DSBR) model predicts that the other end of the break is captured by the displaced strand from the donor duplex (D-loop) and is used to prime a second round of leading strand DNA synthesis (Krogh and Symington, 2004). A double Holliday junction intermediate is formed that can be resolved to form crossover or non-crossover products (Preston et al., 2006).

During synthesis-dependent strand annealing (SDSA), the invading strand that has been extended by DNA synthesis is displaced and anneals to complementary sequences exposed by 5'–3' resection of the other side of the break (McVey et al., 2004; Nassif et al., 1994). The remaining gaps can be filled by DNA synthesis and the nicks ligated. The SDSA model forms only non-crossover products and there is no alteration to the donor duplex during this mode of repair (Nassif et al., 1994).

Interaction of host proteins with 31bp inverted repeats

In addition to containing sequences critical for transposase-mediated P element excision, the terminal 31bp inverted repeats have been shown genetically to be important for repair (Gloor et al., 2000; Staveley et al., 1995). The biochemical identity of these proteins remains undetermined. The Rio laboratory initiated biochemical studies to identify the cellular proteins that bind the inverted repeats (Beall et al., 1994; Rio and Rubin, 1988). Partially purified fractions from *Drosophila* nuclear extracts displayed DNase I footprinting activity on 5' or 3' P element DNA probes. Silver-stained SDS-gels of active fractions revealed the presence of an 18 kDa protein that co-purified with the DNase I footprinting activity. Microsequence analysis of that 18 kDa protein identified it as an uncharacterized *Drosophila* Basic region-leucine zipper (bZIP) protein (CG6272), homologous to the C/EBP family transcription factors, but lacking a transactivation domain. We termed this protein Inverted Repeat Binding Protein 18 (IRBP18). The human protein that is most homologous to IRBP18 is C/EBPβ (31% identical and 57% similar), particularly when only considering the DNA-binding domains (46% identity and 80% similarity). The closest *Drosophila* protein to IRBP18 is C/EBP (slbo) with the most homology when the basic domains are compared (44% identity and 76% similarity).

bZIP transcription factors form either hetero or homodimers to sequence-specific ally bind DNA for regulate gene expression (McKnight, 2001). Each monomer consists of a long α-helix and the DNA N-terminal half binds in the major groove in a dsDNA sequence-specific manner. The C-terminal half mediates dimerization to form a parallel leucine zipper coiled coil. The leucine zipper domain consist of typically four to five

heptad repeats labeled by position ‘a’, ‘b’, ‘c’, ‘d’, ‘e’, ‘f’, and ‘g’ (Fassler et al., 2002; Krylov et al., 1994; Krylov et al., 1995; Vinson et al., 1993). Positions ‘a’, ‘d’, ‘e’ and ‘g’, positions are critical for specifying dimerization partners (Fassler et al., 2002; Krylov et al., 1994).

C/EBP Family memebers

Although in humans there are only six C/EBP family members, the number of different sized polypeptides produced is much larger (Ramji and Foka, 2002). C/EBPα and β genes produce one mRNA but several polypeptides by use of alternative translation initiation codons in the same mRNA molecule via a leaky ribosome scanning mechanism, or regulated proteolysis (Hsieh et al., 1998; Muller et al., 2010; Ossipow et al., 1993; Xiong et al., 2001). Several polypeptodes are produced from the C/EBPε gene as a result of alternative promoter usage and alternative mRNA splicing (Yamanaka et al., 1997).

C/EBPβ gene produces three protein isoforms, 38 kDa (LAP*), 35 kDa (LAP) and 20 kDa (LIP). All three proteins possess a bZIP domain, but the LIP isoform does not contain an activation domain. LIP, in a similar fashion to small MAF proteins, can act as a dominant-negative inhibitor of C/EBP function by forming heterodimers that cannot activate transcription (Motohashi et al., 1997). At least four protein isoforms can be produced from the C/EBPε gene (32 kDa, 30 kDa, 27 kDa and 14 kDa), of which, the activation potential of the 30 kDa form is lower than the 32 kDa form, and the 14 kDa form lacks an intact transcriptional activation domain.

The genome of *Drosophila melanogaster* has two known C/EBP family members, slow border cells (slbo)/CG4354 and Inverted Repeat Binding Protein 18 (IRBP18)/CG6272. Unlike mammalian systems these proteins appear to play a more specialized role in *Drosophila* development. SLBO is expressed primarily in developing larvae and ovarian border cells. Mutant slbo flies appear to be normal except that the females are sterile due to slow migration of border cells in the developing egg chamber (Montell et al., 1992). SLBO regulates the mRNA levels of DE-cadherin, *breathless* (*btl*), jing, and FAK as these genes have been measured at lower steady-state levels in slbo mutants (Liu and Montell, 2001; Montell, 2001; Murphy et al., 1995)

The role of bZIP proteins in DNA Repair

Mammalian bZIP protein and damage response

A well characterized example of a bZIP protein involved in the DNA damage response is human Activating Transcription Factor 2 (ATF2). ATF2 exists as an auto-inhibited monomer and this inhibition is relieved by phosphorylation of T69 or T71 by stress response kinases p38 or JNK (Ouwens et al., 2002). Transcriptionally active ATF2 upregulates steady levels of genes involved in stress response, apoptosis and cell cycle

regulation. Additionally ATF2 functions as a DNA repair gene independent of transcriptional activation (Bhoumik et al., 2005).

Residues S490 and S498 of ATF2 are direct targets of the DNA repair kinase ATM (Bhoumik et al., 2005). The consequence of their phosphoylation is two-fold: 1) ATM activation plays a role in the intra-S phase checkpoint following IR. This checkpoint is critical for proper entry into the DNA replication phase of the cell. 2) ATF2 localizes to DSB foci with MRN complex. The role of ATF2 at the break site is still unclear. The DNA repair activities are distinct from the transcriptional activities, as mutations to T69 and T71 do not interfere with the ATF2 localization to DSB foci. It is unclear if the converse is holds true (Bhoumik et al., 2005; Ouwens et al., 2002). ATF2 was also shown to affect transcription of a large set of genes implicated in DNA repair, suggesting that its role in the DNA damage response requires its dual functions as a transcription factor and a DNA damage response protein (Bhoumik et al., 2008; Bhoumik et al., 2005; Hayakawa et al., 2004).

C/EBP family members have been implicated in various repair processes in diverse systems and can exert their physiological effects in both transcription dependent as well as independent fashions. In response to UVB irradiation, both C/EBP α and β are transcriptionally up-regulation in human keratinocytes, α >70-fold more so than β (Yoon and Smart, 2004). Upregulation of C/EBPα was not observed in p53 null cell lines, nor when the ATM inhibitor caffeine was added to cells suggestive of p53 dependent transcriptional activation. Loss of C/EBPα resulted in impaired G1 damage checkpoint and concomitant increased sensitivity to UVB-induced apoptosis. The G1 checkpoint is regulated by the interaction of C/EBPα with another mammalian p53 target, p21 (Harris et al., 2001; Timchenko et al., 1996). The direct consequence of this interaction is the stabilization of p21, and efficient induction of the G1 damage checkpoint.

Interestingly, human C/EBP family members are found to have interactions with repair proteins: RAD51, Ku70/80, PARP-1, FANCD2. The consequences of these interactions are diverse. The interaction of C/EBPβ and RAD51 results in a synergistic increase in transcription of the HIV-1 LTR (Chipitsyna et al., 2006). C/EBPα was also found to interact with the NHEJ members Ku70/80 and with poly (ADP-ribose) polymerase-1 (PARP-1) in prostate cancer cell lines (Yin and Glass, 2006). Cells exhibiting these interactions had increased radiation sensitivity and decreased ability to repair DSB as the addition of C/EBPα reduced the end joining efficiency *in vitro* (Yin and Glass, 2006). In response to DNA cross-linking agent mitomycin C, C/EBPδ interacts with the Fanconi anemia complementation group D2 protein FANCD2 and importin 4 via distinct domains (Wang et al., 2010). This interaction mediates nuclear import of FANCD2 where it is monoubiquitinated, which is required for replication-associated DNA repair (Wang et al., 2010).

Drosophila bZIP proteins and damage response

Oxygen-induced stress is a direct cause of brain degeneration in *Drosophila.* Numerous genes including IRBP18 are upregulated in response to hyperoxia (99.5% oxygen) as a stressor (Gruenewald et al., 2009). While many of the genes upregulated by this stress response are a part of metabolic pathways and help protect against this type of stress, IRBP18 was one of the few putative transcription factors. Presently it is unclear which genes are potential targets of IRBP18.

Dmp53 upregulates transcription of three bZIP protein (IRBP18, Xrp1 and CG15479) in response to ionizing radiation (Akdemir et al., 2007). Also deletion of Xrp1 resulted in loss of heterzygosity (LOH) in response to IR. However there is no functional data for IRBP18 or CG15479 as general repair proteins.

The convergence of the IRBP complex and DNA repair

As a first step in understanding the function of the cellular proteins that bind the 31 bp inverted repeats, we set about characterizing the biochemical composition of the protein complex. DNA affinity chromatography and UV photocrosslinking initially identified a 70 kDa protein that co-purified with DNase I footprinting activity (Rio and Rubin, 1988). Molecular genetic evidence suggested this protein was Ku70 and mapped cytologically to the mus309 region (Beall et al., 1994; Beall and Rio, 1996). Although Ku70 co-purified with footprinting activity, the addition of a Ku70 antibody to fractions failed to inhibit footprinting. Additionally, recombinant Ku70/Ku80 from a baculovirus expression system could not reproduce the footprint. These data indicate that the Ku70 was not the inverted repeat binding protein (IRBP) (Roche S and Beall E unpublished data).

Subsequent attempts to purify IRBP identified IRBP18. Antibody addition to active fractions containing IRBP18 blocked footprinting activity. Recombinant IRBP18 protein either from bacteria or baculovirus was unable to reconstitute activity due to sequence content of the inverted repeats. Interestingly RNAi knockdown of IRBP18 in a NHEJ cell culture assay revealed an increased frequency and extent of deletions flanking a P element-induced DNA break (Min, B unpublished). These data indicate that IRBP18 plays a role in the NHEJ repair at the donor site.

The important unanswered questions and the basis of this thesis are: 1) What are the additional proteins that constitute the IRBP complex 2) What are the *in vivo* roles of the IRBP complex? Here I will describe the purification of the IRBP complex, using a TAP-Tag approach. This led to the identification of Xrp1 as heterodimeric partner for IRBP18. This heterodimer represses transcription from the P element promoter and facilitates the repair of transposase induced breaks of P element donor site. Surprisingly, IRBP18 null flies are sterile. Additionally, IRBP18 directs DNA repair after ionizing radiation (IR) and Methyl methane sulfate (MMS) treatment, but not camptothecin. Lastly, the isoform specific DNA repair properties of Rm62, one of the other members of the DNA dependent IRBP complex will be explored.

Chapter 2

Purification of the *Drosophila* Inverted Repeat Binding Complex

Introduction

The *Drosophila* P element transposase is a GTP dependent site-specific endonuclease (Hickman et al., ; Kaufman and Rio, 1992; Tang et al., 2005). Transposition is regulated at the level of pre-mRNA splicing by restricting expression transposase to the germline due to somatic expression of the cellular splicing factor PSI (Adams et al., 1997; Siebel et al., 1995; Siebel et al., 1994). P element transposition can also be controlled by truncated repressor proteins, such as the 66 kDa or KP proteins (Gloor et al., 1993; Lee et al., 1998; Misra et al., 1993; Misra and Rio, 1990). Additionally piwi associated RNA (piRNA) pathway has been demonstrated to repress transposition in trans (Aravin et al., 2007a; Brennecke et al., 2007). Finally, cellular proteins must be involved in the repair of P element-induced DNA breaks and these proteins interact with sites on P element DNA (Preston et al., 2006; Rio and Rubin, 1988).

Mutational analysis of both 5' and 3' P element ends demonstrated that approximately 150 bp of DNA at each end is essential for transposition *in vivo* (Mullins et al., 1989). Contained within these terminal sequences are the 31 bp terminal inverted repeats, 11 bp internal repeats, which serve as enhancers of transposition, and internal 10 bp transposase binding sites (Beall and Rio, 1997; Kaufman et al., 1989; Mullins et al., 1989; O'Hare and Rubin, 1983). Once assembled on the synapsed transposon ends the P element transposase catalyzes double-strand DNA cleavages within the 31 bp inverted repeats, creating 17 nt 3' single-stranded extensions at the donor site and transposon ends (Beall and Rio, 1997). In order to maintain genome integrity the cell must keep transposition frequencies fairly low, and efficiently repair DNA breaks created at P element insertion sites. Somatic expression of P element transposase in the presence of 15-20 small non-autonomous P elements leads to a temperature-dependent lethality (Engels et al., 1987; Robertson and Engels, 1989).

Repair of P element DNA break sites can occur through two distinct DNA double-strand break (DSB) repair pathways: non-homologous end joining (NHEJ) and a variant of classical homologous recombinational repair, termed Synthesis-Dependent Strand Annealing (SDSA) (Engels et al., 1990; Gloor et al., 2000; Min et al., 2004; Preston et al., 2002; Weinert et al., 2005). The choice between these two pathways is thought to be dictated by cell cycle progression and the availability of pathway substrates and tissue-type (Johnson-Schlitz et al., 2007; Preston et al., 2006).

Additional clues about how the P element breaks might be repaired comes from genetic and biochemical studies of repair of another mobile element, the fish Sleeping Beauty (SB) transposon (Izsvak et al., 2004). A protein-protein interaction between the SB transposase and the NHEJ factor Ku70 has been observed, implying a physical link between the transposon-encoded protein and cellular DNA repair factors. Additionally, mutations in the NHEJ factor, DNA-protein kinase (DNA-PK), reduced the transposition frequency of SB. Although *Drosophila* lack an identifiable ortholog of DNA-PK, other repair genes Ku70/80, Bloom's helicase (mus 309) and Rad54 have been shown

genetically to be involved in P element DNA break repair (Adams et al., 2003; Beall and Rio, 1996; Kooistra et al., 1999; Min et al., 2004; Romeijn et al., 2005).

In addition to containing sequences critical for transposase-mediated DNA cleavage, the terminal 31 bp inverted repeats of the P element have been shown genetically to be important for repair (Staveley et al., 1995). To date the identity and the role of endogenous *Drosophila* proteins, bound to the 31 bp inverted repeats, on the regulation of P element transposition is undetermined. Previous genetic and biochemical data have implicated *Drosophila* Ku70 as a protein that binds to the P element 31 bp inverted repeats. Recombinant *Drosophila* Ku70/80 heterodimer, however, did not bind the P element 31 bp inverted repeats (E. Beall, unpublished data).

We have purified a multi-subunit Inverted Repeat Binding Protein (IRBP) complex that binds to the P element 31bp inverted repeats. The core DNA binding subunits consist of a basic leucine zipper (bZIP) heterodimer between Xrp1 (CG17836) and a previously uncharacterized 18 kDa bZIP protein that we term IRBP18 (CG6272). In this study, we demonstrate that the IRBP complex acts as to repress transcriptional activity from the P element promoter and also to facilitate DNA repair of double-strand breaks at sites of P element excision after transposase cleavage. Our data sheds light on the interplay between endogenous cellular DNA binding protein complexes and the regulation and genomic repair of host genome DNA damage caused by an active mobile element.

Results

IRBP18 is a member of the Inverted Repeat Binding Protein complex

To determine the identity of additional putative IRBP complex members, we used a combination of conventional chromatography and tandem affinity purification (TAP) outlined in Figure 1B. We cloned a ZZ-TEV-3X FLAG (two protein A modules-TEV protease site-3 tandem copies of the short FLAG antibody epitope) version of IRBP18 and created a stably transformed *Drosophila* cell line (Figure 1A). Protein expression of the ZZ-TEV-3XFLAG was similar to the expression level of the endogenous protein as assessed by immunoblotting (Figure 2A).

Nuclear extracts derived from 8-10L of ZZ-TEV-3X FLAG IRBP18 fusion protein-expressing cells were prepared and fractionated by gel filtration chromatography (Figure 1B). This size selection chromatography step helped select for larger protein complexes that incorporated TAP-tagged IRBP18 protein. Gel filtration column fractions containing an IRBP18 immunoblot signal were pooled, bound to IgG agarose and then cleaved with TEV protease (Figure 2B). The TEV protease-released 3X FLAG IRBP18 protein-containing complexes were then further purified by anti-FLAG epitope immunoaffinity chromatography in the presence and absence of ethidium bromide (Figure 4). The purified complex was eluted under native conditions with a 3X FLAG synthetic peptide or under denaturing conditions at low pH (sees Experimental Procedures). IRBP complex purified under native conditions retained DNase I footprinting activity similar to previous purification attempts (Figure 3) (Beall et al., 1994; Rio and Rubin, 1988). Silver stain analysis of the complex revealed the presence of several stoichiometric bands that were resistant to ethidium bromide treatment, suggesting that protein-protein, not DNA-protein interactions were involved (Figure 4). To identify these proteins, 2D LC MS/MS analysis was performed on both the protein mixture and individual proteins in SDS-gel slices (Figure 5A, B).

Most interestingly, mass spectrometric analysis of these tandem-affinity purified fractions identified the basic leucine zipper protein Xrp1/CG17836 as a potential b-ZIP partner for IRBP18 (Figure 5A). Xrp1 is a bZIP protein that also contains an AT hook DNA binding motif, similar to that found in the high mobility group (HMG) class of DNA binding proteins (Akdemir et al., 2007). There are 5 annotated Xrp1 mRNA isoforms, two leading to the synthesis of the same ~72 kDa protein and three yielding the identical protein sequence at ~45 kDa. Interestingly, we could only detect the presence of the 72 kDa isoform within the IRBP complex by immunoblotting (Figure 6). Immunoblot analysis of subcellular fractions of S2 cells, demonstrated that only the 72 kDa was nuclear (Figure 7, lane 3 and 4) while the 45 kDa isoforms were mostly in the cytosolic fractions (Figure 7, lane 2).

To investigate whether IRBP18 and Xrp1 also interact *in vivo* on the P element terminal 31bp inverted repeats, we performed chromatin immunoprecipitation (ChIP) experiments using chromatin isolated from the P element-containing Harwich P strain embryos (0-12 hour). These experiments indicated that IRBP18, Xrp1, as well as the 66 kDa P element repressor protein, are present on both the 5' and 3' P element ends compared to a negative rabbit IgG control (Figure 8).

Several RNA binding proteins including nucleolar proteins involved in rRNA processing (Fibrillarin, Nop56), and RNA interference pathways (RM62, Fmr1) were also detected (Figure 5B) (Buszczak and Spradling, 2006; Garcia-Planells et al., 2000; Ishizuka et al., 2002; Zinszner et al., 1997). Interestingly the 72 kDa Xrp1 isoform has two nucleolar localization signals (RRxR) (Figure 9A) (Muller et al., 2010). We next asked if there were sequences similar to the P element inverted repeats present in the *Drosophila* r DNA region. Two similar sequences were found; one in the non-transcribed intergenic spacer region (IGS) and another within the 18S rDNA gene (Figure 9B). ChIP analysis demonstrated that IRBP18 can bind 18S rDNA sequences but not the IGS (Figure 9C). Taken together, these data imply that Xrp1 and IRBP18 are bona fide interaction partners that exist within a larger IRBP complex.

IRBP18 and XRP1 form a heterodimer sufficient to reconstitute site-specific IRBP DNA binding activity

To determine if the IRBP18/ Xrp1 heterodimer is sufficient to reconstitute IRBP activity we co-expressed Xrp1 as an N-terminal fusion with maltose the FLAG epitope (FLAG-Xrp1) and IRBP18 with an N-terminal His_6 affinity tag (His-IRBP18) in bacteria. The recombinant heterodimer was purified first by Ni^{2+} affinity chromatography and second by gel filtration chromatography. Immunoblot analysis confirmed the presence of the heterodimer (Figure 10A, lanes 6-8). The recombinant IRBP18/Xrp1 heterodimer exhibited high affinity site-specific DNA binding to the P element inverted repeats, as determined by DNase I footprinting experiments, in a manner identical to native IRBP (Figure 10B lanes 4-6). By contrast, neither the purified IRBP18 or Xrp1 proteins alone exhibited footprinting to the P element repeats at any concentration tested (data not shown). Additionally, binding of the IBRP18/Xrp1 heterodimer to the P element inverted repeat was unaltered by the addition of purified P element transposase to the footprinting reactions (Figure 11, lane 6), indicating that both proteins can interact with P element DNA at the same time. Taken together, these experiments indicate that the IRBP18/Xrp1 b-ZIP heterodimer forms the sequence-specific DNA binding core subunits of the IRBP complex.

IRBP18 and Xrp1 are transcriptional repressors of transposase gene

The IRBP complex binding site is located within the 5' and 3' 31 bp terminal inverted repeats and at the 5' end of the P element, which are both in close proximity to the transposase promoter TATA box sequence (Kaufman et al., 1989). Therefore, we asked if IRBP18 and/or Xrp1 might function to regulate transcription from the P element promoter. Plasmids containing HA-tagged-Xrp1, FLAG-IRBP18, luciferase driven by wild-type P element promoter (1-152) (PEPr-luc) or mutant promoter (LS 4-15 and Δ1-19) and renilla luciferase were transfected into *Drosophila* S2 cells and protein extracts were prepared and measured for luciferase activity (Figure 12A) (Kaufman et al., 1989; Robertson, 1996). Expression of the HA-Xrp1 and FLAG-IRPB18 genes was driven by the copper-inducible metallothionein promoter. Copper-dependent expression of either Xrp1 or IRBP18 resulted in a 50% decrease in luciferase levels compared to the P element promoter-luciferase plasmid alone (Figure 13). Expression of both proteins did not result in further decrease in luciferase activity. In contrast the neither IRBP18 or Xrp1 could modulate the transcriptional activity from the mutant promoters (Figure 14).

As an independent means to investigate whether IRBP18 and Xrp1 are transcriptional repressors of the P element promoter, we used double-strand RNA interference (RNAi) knockdowns to deplete either IRBP18 and/or Xrp1 and the effects on P element promoter activity were then assayed. The efficiency of RNAi knockdown of IRBP18 and Xrp1 was determined by immunoblot analysis. A decrease in the steady-state protein levels of greater than 90% was observed in response to double-strand RNAi treatment against IRBP18 or Xrp1, but not with control RNAi duplexes derived from plasmid DNA sequences (data not shown). Additionally, we observed a decrease in the protein concentration of IRBP18 in the single double-strand RNA treatment for Xrp1, suggesting that the proteins form a functional heterodimer *in vivo* (data not shown). Knockdown of either the IRBP18 or Xrp1 proteins increased luciferase activity two-fold (Figure 15). There was no further increase in P element promoter-controlled luciferase activity in the double IRBP18/Xrp1 RNAi knockdown. Taken together, these data indicate that the IRBP18 and Xrp1 DNA binding proteins appear to act as transcriptional repressors of the P element promoter.

The IRBP complex directs donor site repair after P element transposase cleavage

It has been suggested that IRBP plays a role in protecting the P element ends following transposase-mediated DNA cleavage or to facilitate DNA repair (Rio and Rubin, 1988; Staveley et al., 1995). We therefore asked if another function of the IRBP complex binding to the 31 bp inverted repeats might be to play a role in DNA repair after P element excision. DNA repair efficiency at sites of P element-induced DNA breaks can be quantitated using a cell culture-based assay for P element excision and DNA repair (Beall and Rio, 1996; Min et al., 2004). RNAi knockdown of either IRBP18 or Xrp 1 resulted in a reduction of the normalized DNA repair efficiency to 35-50% of the control

level. The combination treatment of double-standed RNA against both IRBP18 and Xrp1 resulted in no further reduction in DNA repair efficiency (Figure 16A). The P element excision-DNA repair assay was also performed with cells that had been transfected with either anti-IRBP18 or non-immune rabbit IgG antibodies. This *in vivo* antibody blocking assay of the IRBP complex yielded a similar reduction in repair efficiency to that observed with the RNAi knockdowns, again suggesting a role for the IRBP complex in repair of P element-induced DNA breaks (unpublished data, Min, B).

One additional feature of the P element excision-DNA repair assay is the ability to isolate individual DNA repair events as plasmids in bacteria. Therefore, we examined the qualitative nature of deletions surrounding the donor site DNA repair events on the recovered plasmids under different conditions from the RNAi assays. We separated deletion sizes into three size classes: 0 bp deletion, small deletions (1–49 bp), and large deletions (≥50 bp) (Figure 16B). In the IRBP18 RNAi-treated samples, the 0 bp deletion population decreased to 54% of sequenced plasmids had 0 bp deletions compared to the control group (68%), with increases in small deletions (37.5% compared to 16.66%). The Xrp1 RNAi-treated samples, the 0 bp deletion population decreased to 33.33%, with increases in small deletions and a similar decrease in the large deletion population (58% and 8% respectively). The double IRBP18 and Xrp1 RNAi-treated samples, were similar to the Xrp1 alone-treated group. An increase in the frequency of deletions at the donor DNA site after IRBP18 and Xrp1 RNAi knockdown, along with the LM-PCR data and the ChIP analysis of IRBP18 on the P element ends, suggest that IRBP complex acts directly in donor site repair.

In a parallel experiment, the ability of IRBP18 to direct P element repair in vivo was tested in the intact organism. Somatic mobilization of P elements at 25°C results in complete larval and pupal lethality, so called “somatic dysgenesis”, where as at a lower permissive temperatures (18°C and 21°C) survivors can be obtained (Engels et al., 1987). Using an IRBP18/CG6272 mutant, the effect of IRBP18 on the somatic dysgenesis phenotype was tested. Flies homozygous for Birm2 (a second chromosome containing 17 non-autonomous P elements) and heterozygous on the third chromosome for the IRBP18 null allele (CG6272WHf05006 ; a transposon insertion in the coding sequence) were crossed with flies with wild type second chromosome and a recombinant third chromosome with CG6272WHf05006 and a somatically-expressed P element transposase source, P Δ2-3 (99B) (Figure 18) (Engels et al., 1987; Robertson et al., 1988). This transposase source is immobile due to two deletions with the 5’ P element inverted repeats (1-19 and 1-348) removing IRBP complex binding sites and potential transcriptional regulation; thus the steady state transposase levels should be comparable across the genotypes in this cross (Figure 14) (Robertson, 1996). This cross should yield progeny in a genotypic ratio of 1:1:1:1 (Figure 18). IRBP18 immunoblot analysis confirmed the null phenotype of the CG6272WHf05006 homozygous mutant flies (Figure 17A). Adult CG6272WHf05006 homozygous flies null for IRBP18 exhibit a significant decrease in viability compared to both sibling controls as well as control crosses (Figure 20 and 21), presumably attributable to defects in repair of chromosomal DNA breaks caused by P element transposase cleavage. This killing phenotype was

exacerbated at elevated temperatures (16% viability at 18°C compared to 6% at 21°C) (Figure 19). Taken together, the data from these two independent experiments, cell culture RNAi and in vivo somatic fly dysgenic crosses, correlate very well and indicate that IRBP18/CG6272 is a cellular protein that plays a role in facilitating DNA repair double-strand DNA breaks caused by P element transposase cleavage. It is also possible that the IRBP18 complex might also be more generally involved in DNA repair.

Discussion

The first report of IRBP activity in *Drosophila* extracts suggested the existence of cellular protein factors that might play a role in P element excision or donor DNA repair events. Fractionation of extracts led to the identification of *Drosophila* Ku70, but recombinant *Drosophila* Ku heterodimer did not exhibit *in vitro* binding to the P element terminal inverted repeats (unpublished data). Therefore, alternate and more extensive fractionation and purification schemes were devised resulting in the identification of one of the two *Drosophila* C/EBP bZIP proteins, IRBP18/CG6272. TAP-tagged purification of the IRBP18/CG6272 protein yielded a protein complex with at least eight members, including another bZIP DNA binding partner protein, Xrp1/CG17836. Here, we report that the site-specific DNA binding core of the IRBP complex is a bZIP heterodimer comprised of IRBP18/CG6272 and Xrp1/CG17836. We also demonstrate that this complex has dual functions: 1) to repress transcription from the P element promoter; and 2) to facilitate DNA repair of the P element break sites.

Amino acid sequence analysis suggests IRBP18 and the LIP isoform of C/EBPβ share similar domain structure. LIP has been demonstrated to inhibit transcription by the formation of non-productive transcriptional complexes (Descombes and Schibler, 1991). We demonstrate that IRBP18 and Xrp1 can both repress transcription activation from the P element promoter. The P element transposase (and presumably the 66 kDa and KP repressor proteins) also modulates transcriptional activity of the P element promoter (Kaufman and Rio, 1991). The P element promoter TATA box and the 5' transposase binding site overlap, thus the most likely mechanism of transcriptional repression is direct competition for DNA binding between transposase and/or IRBP complex and transcription initiation factors, TFIID (Kaufman and Rio, 1991).

Similar to other transposition reactions, the P element transposes via a two-step reaction in which initially the two ends of the transposon are cleaved from the flanking DNA (donor cleavage). Then in a second step the excised transposon intermediate captures and integrates into a target DNA site (strand transfer). ChIP and DNase I footprinting indicated that IRBP and transposase can co-occupy both P element ends. A potential role of transcriptional repression could be to give the transposase the necessary space to access its internal DNA binding sites, assemble the synaptic complex and then to access sequence specific-cleavage sites within the 31 bp inverted repeats (Kaufman et al., 1989; Tang et al., 2007; Tang et al., 2005). Another direct consequence of the IRBP complex might be rapid recruitment of cellular DNA repair proteins to these breaks. More information about the kinetics of DNA cleavage and repair of P element breaks *in vivo* are required to substantiate this model.

Surprisingly, our mass spectrometric data indicated that the IRBP complex also contained several nucleolar proteins. The nucleolar organizer region contains tandemly repeated ribosomal RNA genes (rDNAs) located within heterochromatin in most eukaryotes. In the course of validating our biochemical purification, we present preliminary evidence for

IRBP binding to the 18S rRNA gene. The ability to bind rDNA sequences appears to be a conserved feature of C/EBP proteins because it has been shown that mammalian C/EBPα and β repress rRNA transcription (Ali et al., 2008; Muller et al., 2010). Although sub-nuclear organization of the P element has not been thoroughly examined, recruitment of the P element to potentially repressive heterochromatin by IRBP18 and Xrp1 could provide an additional layer transcriptional repression of the P element promoter.

Once the P element ends are cleaved by P element transposase, the IRBP complex could then help to stabilize newly created double-strand DNA breaks that contain 17nt 3' single-strand extensions that could potentiate DNA repair. Two independent approaches showed that IRBP18 and Xrp1 can play a role in post cleavage DNA repair of P element breaks. Additionally, the loss of IRBP18 resulted in the destabilization of donor site repair intermediates. The putative molecular functions of IRBP18 and Xrp1 are potentially analogous to another mammalian b-ZIP protein ATF2. Transcriptional activation of ATF2 by stress response kinases p38 or JNK upregulates steady levels of genes involved in the stress response, apoptosis and cell cycle regulation (Lopez-Bergami et al.). Additionally human ATF2 functions in DNA repair, independent of its transcriptional activity (Bhoumik et al., 2005). Residues S490 and S498 of ATF2 are direct targets of the DNA repair signaling protein kinase, ATM (Bhoumik et al., 2005). ATF2 also localizes to DNA double-strand break foci along with the MRN complex. However, the exact role of ATF2 sites of DNA breaks remains unclear.

Several lines of evidence suggest that the IRBP18 and Xrp1 proteins function in concert in other biochemical pathways. Upregulation of each protein is thought be important for protection from hyperoxia-induced neurodegeneration (Gruenewald et al., 2009). Additionally, both IRBP18 and XRP1 transcript levels are induced in response to ionizing radiation (IR), which is depend upon activation by the *Drosophila*p53 homolog (Akdemir et al., 2007; Brodsky et al., 2004). Finally on another note, a recent genome-wide RNAi screen revealed that both IRBP18/CG6272 and Xrp1/CG17836 appear to play a role in both the miRNA and siRNA pathways (Zhou et al., 2008). Because small piwi-associated RNA (piRNA) pathways play a role in suppression of transposon mobility, there may be a logical role for IRBP18 and Xrp1 to play in this regard. Thus taken together, the IRBP18 and Xrp1 proteins appear to function in multiple pathways involving genome defense in *Drosophila* and may directly link DNA repair/damage response pathways to transposon mobility.

Experimental Procedures

Purification of TAP-tagged IRBP18

Nuclear extracts from 8-10L (3-4 X 10^{10} cells) were prepared and resuspended in 3-5ml of HGKNED (25 mM HEPES pH 7.6, 10% glycerol, 1 mM EDTA/EGTA, 0.01% NP-40 and 200mM KCl) supplemented with 0.2% PMSF and 0.5 mM DTT, .2mM Beta-Glycerophosphate, .2mM Sodium Fluoride and .2nM trichostatin A. Resuspended extracts were fractionation over 120ml Superdex S200 PG column. Fractions were immunoblotted for the presence of TAP-Tagged IRBP18 with an affinity purified IRBP18 antibody (1:2000). Fractions were pooled and incubated with rabbit IgG resin (Sigma) overnight. The IgG resin was washed 10ml of HGKNED, then incubated with TEV protease for a minimum of 4 hours. TEV eluted material was then put over FLAG antibody beads overnight, by washing, and eluting in 3X FLAG peptide or glycine (pH 2.5) for 30min. The glycine elutions were neutralized by the addition of Trizma.

Chromatin Immunoprecipitation

500 μg of chromatin was incubated with 1 μg of the indicated antibody. Antibody concentration was determined by Bradford protein assay. Purified DNA was incubated with TE and PCR amplified using standard PCR protocols.

DNaseI Footprinting Assay

DNase I protection experiments were performed as described (Kaufman et al., 1989). The pN/P175 plasmid was used for generating the 31 bp inverted repeat DNA probe used the DNase I protection experiments.

Subcellular Fractionation

S2 cells were prepared according to (Mendez and Stillman, 2000) and blotted with Fibrillarin (1:2000), Histone H2AvD (Rockland) (1:2000), IRBP18 (1:2000), Xrp1(1:2000), and Tubulin (1:5000).

Double-Stranded RNA Synthesis and RNAi

Synthesis of double-stranded RNA and treatment of S2 cells was performed exactly as described (Min et al., 2004) . The sequences of the IRBP18 coding sequence PCR primers, including T7 RNA polymerase promoter sequence were: forward 5’ CGG CCA GTG AAT TGT TTA ATA CGA CTC ACT ATA GGG CCG GCC AAA AAG AGA ACT GC and reverse 5’ CGG CCA GTG AAT TGT TTA ATA CGA CTC ACT ATA GGG TTA GTC ATT GTC CTT GGG AT. For Xrp1 forward 5’ TAA TAC GAC TCA CTA TAG GGA GGA CGA AGA GGA GAC TAC CAC CG and reverse 5’ TAA TAC

GAC TCA CTA TAG GGA GGA GTA AGT GCT CTT CTG CCG CT were used as a primer pair.

RNAi and P Element Excision and Repair Assay

2.5×10^6 L2 cells were plated in 60-mm tissue culture dishes and 1 hour in 5% FBS-M3 medium. The cells were washed with 2 ml of serum-free M3 medium and overlaid with 1 ml of serum-free M3 medium. Ten micrograms of dsRNA in ddH_2O was added. After a 1h incubation, 1 ml of 10% FBS-M3 medium was added to make final 5% FBS-M3. The cells then were kept in an incubator for 48h before another dsRNA treatment. Cells were then transfected 1 microgram each of the reporter and transposase source (Effectene transfection reagent, Qiagen). The number of ampicillin-resistant colonies per ml of bacteria in SOC medium gives the estimate of total plasmids collected, and the number of kanamycin- and ampicillin-resistant colonies per ml of bacteria in SOC medium provides an estimate of reporter plasmids that are excised and repaired. The ratio of repaired plasmids to total plasmids gives the excision and repair activity.

Luciferase assays

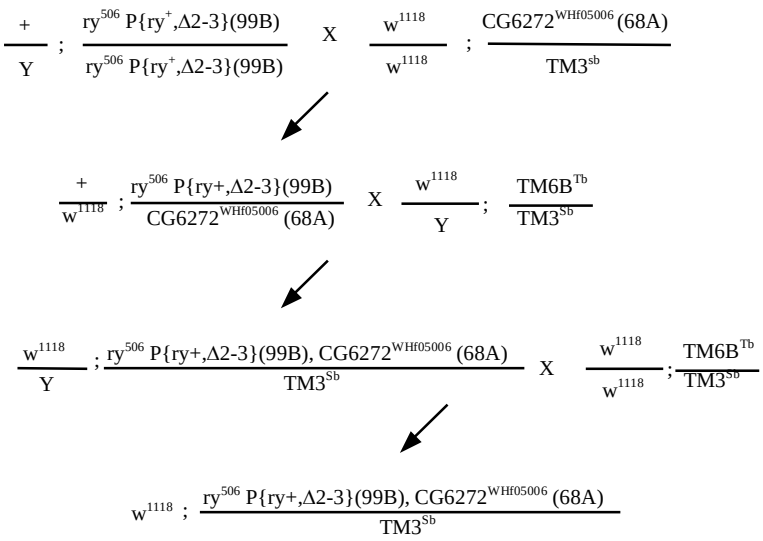
The firefly luciferase cDNA was subcloned into the NdeI and XbaI sites in pBluescript SK+. The P element promoters (WT 1-152; LS 4-15; Δ1-19) was PCR amplified and cloned into the XhoI and NdeI sites of pBluescript. HA-Xrp1 and FLAG-IRBP18 were cloned into the XhoI and XbaI sites of pUC-HygMT-SV40A. Renilla luciferase was used as the transfection control. A total of 300 ng of DNA were transfected into S2 cells grown in a 12 well plate, and after a 24h $CuS0_4$ was added. Luciferase activity was measured 24 hours after by the Dual-Glo® Luciferase Assay kit on the PerkinElmer VICTOR 3 1420 Multilabel Plate Reader. For experiments were dsRNA were used, S2 were treated with the indicated dsRNA for 48 hours. The cells were then washed with fresh media and transfected. Luciferase activity was scored 24 hours post transfection.

Drosophila strains

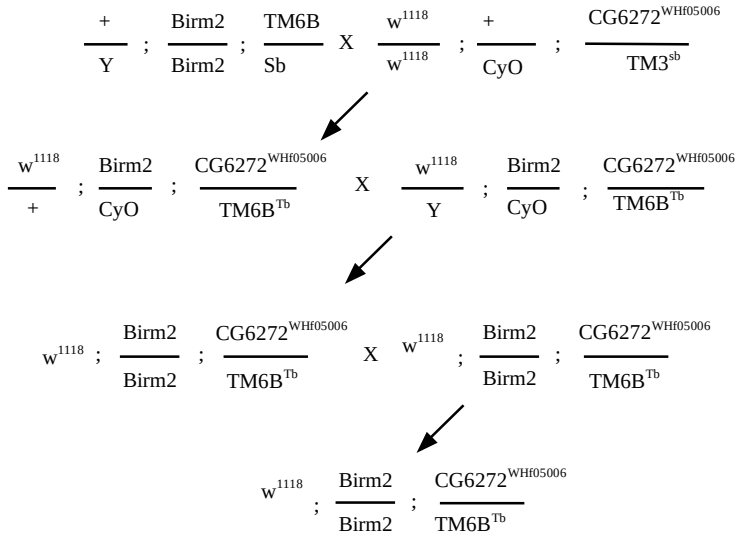
The *Drosophila* strains ry^{506} P{ry^{+},Δ2-3}(99B)/$TM6B^{Tb}$, Birm2; Sb/$TM6^{Tb}$, CyO/Sco, and w^{1118}; $CG6272^{WHf05006}$/$TM6^{Tb}$ (Exelixis Collection at the Harvard Medical School) (Thibault et al., 2004). The w^{1118}; $CG6272^{WHf05006}$/$TM3^{Sb}$ strain was made by crossing w^{1118}; $CG6272^{WHf05006}$/$TM6^{Tb}$ males with w^{1118}; $TM3^{Sb}$/CxD virgin females. Oranged-eyed, stubble bristle, wild-type winged males flies were selected, and then mated again to w^{1118}; $TM3^{Sb}$/CxD virgin females to obtain a balanced stock. All flies were raised on standard cornmeal, molasses, and yeast medium at 25°C.

Somatic dysgenesis experiment

Flies were maintained at 25°C unless noted in standard conditions. Null phenotype of IRBP18 mutant flies was validated by western blot analysis of 2 males from each genotype (Figure 6B). The recombinant 3rd chromosome was generated by the following set of crosses and screened for orange eye color:



The Birmingham2 strain was generated by the following crosses and screen by eye color:

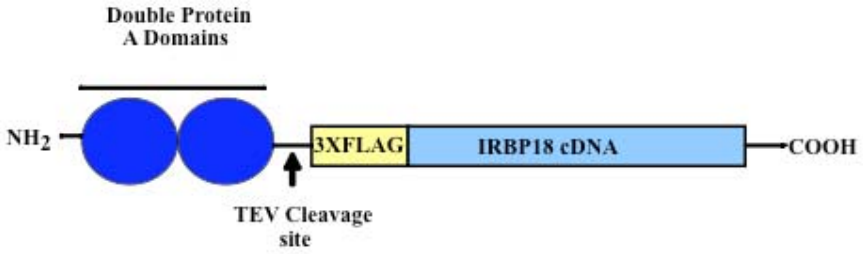


Flies for the somatic dysgenesis cross were allowed to mate at 25°C degrees for 2 days. The flies were then placed at 18°C (5 days) and 21°C for (2 days).

Figure 1 Purification Scheme of the IRBP complex.

A.) IRBP18 was fused to tandem protein A domains followed a TEV protease site and 3 copies of the FLAG epitope (ZZ-TEV-3XFLAG IRBP18). B.) IRBP complex purification scheme, was optimized to separate unincorporated ZZ-TEV-3XFLAG IRBP18 from functional complexes.

A.


Double Protein
A Domains
NH2
3XFLAG
IRBP18 cDNA
COOH
TEV Cleavage
site

B.

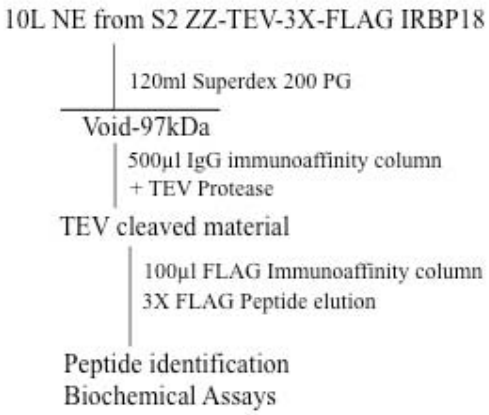
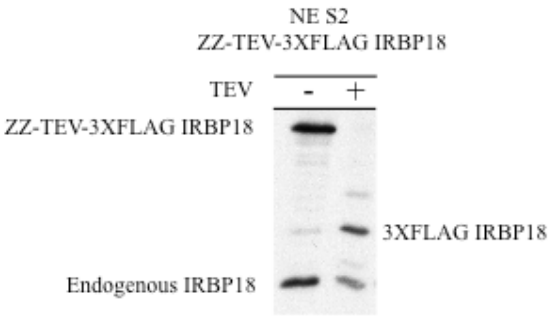

10L NE from S2 ZZ-TEV-3X-FLAG IRBP18
120ml Superdex 200 PG
Void-97kDa
500µl IgG immunoaffinity column
+ TEV Protease
TEV cleaved material
100µl FLAG Immunoaffinity column
3X FLAG Peptide elution
Peptide identification
Biochemical Assays

Figure 2 Characterizing ZZ-TEV-3XFLAG IRBP18 containing IRBP complexes. A.) Immunoblot analysis of TEV cleaved nuclear extract of S2 cells expressing of ZZ-TEV-3XFLAG IRBP18. ZZ-TEV-3XFLAG-IRBP18 is expressed at rough 1.5 more than the endogenous protein. B.) Previous characterization of the IRBP complex determined the endogenous complex migrates through the Superdex S200 column at an apparent molecular weight between 158 and 67 kDa. ZZ-TEV-3XFLAG IRBP18 is shifted in size by approximately 22kDa the size of the ZZ-TEV-3X-FLAG module.

A.


NE S2
ZZ-TEV-3XFLAG IRBP18
TEV
-
+
ZZ-TEV-3XFLAG IRBP18
3XFLAG IRBP18
Endogenous IRBP18

B.

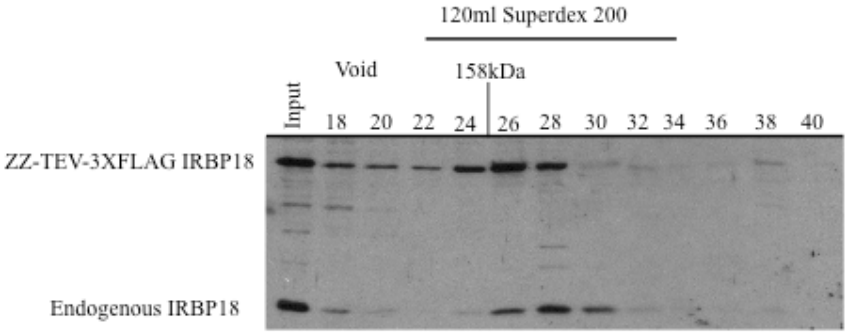

120ml Superdex 200
Void
158kDa
Input
18
20
22
24
26
28
30
32
34
36
38
40
ZZ-TEV-3XFLAG IRBP18
Endogenous IRBP18

Figure 3 Purified 3XFLAG IRBP18 containing IRBP complexes have robust footprinting activity.
A.) 3XFLAG eluted IRBP complex were assayed for DNase I footprinting activity on the 5’ end of the P element.


IRBP Complex
5' CATGATGAAATAACAT

Figure 4 Silver stain analysis of the IRBP complex
Silver stain analysis of 3XFLAG eluted complex separated by 12% SDS-PAGE. FLAG immunoaffinity chromatrography was performed in the presence and absence of Ethidum Bromide (150μg/ml)

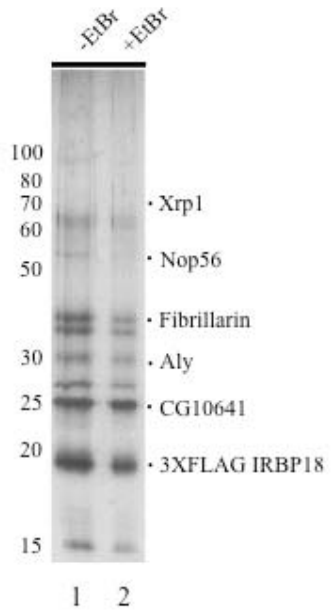

-EtBr
+EtBr
100
80
70
60
50
30
25
20
15
Xrp1
Nop56
Fibrillarin
Aly
CG10641
3XFLAG IRBP18
1
2

Figure 5 Mass spectrometric analysis of the IRBP complex.
A.) MudPit analysis of glycine eluted IRBP complexes. B.) Results of 2D LC-MS/MS analysis of gel slices purified after SDS-PAGE fractionation of the IRBP complex.

MudPit

CG Number	Gene Name	MW	Peptide #	Function
CG6272	IRBP18	13kDa	110	DNA binding
CG17836	Xrp1	44/72kDa	23	DNA binidng
CG7013	Manf	20kDa	23	unknown
CG12002	Peroxidasin	50/170kDa	12	oxidative stress
CG1890		12.9kDa	2	unfolded protein binding

Gel slice

(kDa)	CG number	Gene Name	Peptide #	Function
20	CG6272	IRBP18	15	DNA binding
	CG3595	spaghetti squash	4	myosin heavy chain binding
25-27	CG10641	epsilonCOP	3	calcium ion binding
30	CG1101	aly	3	nuclear mRNA splicing
35	CG17158	cpb	6	actin binding.
	CG9543		5	protein binding
	CG9888	Fibrillarin	8	rRNA processing
	CG10540	cpa	5	actin binding.
55	CG11100	Mes2	3	unknown
	CG13849	Nop56	5	unknown/nucleolar
	CG8280	Ef1alpha48D	3	translation elongation factor activity
80	CG6203	Fmr1	13	RNA binding
	CG4264	Hsc70-4	8	chaperone binding
	CG6944	Lamin	4	protein binding
	CG4147	Hsc70-3	2	ATPase activity.
	CG5119	pAbp	4	protein binding; mRNA 3'-UTR binding
190	CG15792	zipper	373	myosin light chain binding
	CG9155	Myosin 61F	11	ATPase activity,

Figure 6 The 72 kDa isoform is a confirmed member of the IRBP complex.
Eluates from both TEV and FLAG immunoaffinity chromatography were size fractionated by SDS-PAGE and immunoblotted for the presence of IRBP18 (1:4000) and Xrp1 (1:2000).

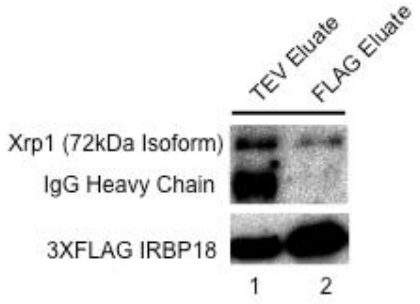

TEV Eluate
FLAG Eluate
Xrp1 (72kDa Isoform)
IgG Heavy Chain
3XFLAG IRBP18
1
2

Figure 7 Xrp1 isoforms are differentially localized.
Immunoblot analysis of subcellular fractionation of asynchronous S2 cells into whole cell extracts (WCE), nucleoplasm and chromatin pellet.

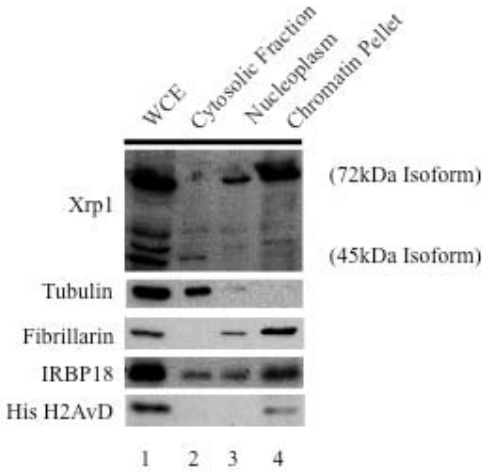

WCE
Cytosolic Fraction
Nucleoplasm
Chromatin Pellet
Xrp1
(72kDa Isoform)
(45kDa Isoform)
Tubulin
Fibrillarin
IRBP18
His H2AvD
1
2
3
4

Figure 8 Xrp1 and IRBP18 interact with both the 5’ and 3’ ends of the P element *in vivo*.
Chromatin immunoprecipitation (ChIP) was performed using the antibodies indicated at the top, and the isolated DNA was analyzed using PCR for enrichment of 5’ and 3’ ends of the P element. Shown is an Ethidium bromide-stained gel of PCR reactions performed with both gene-specific and F element primers simultaneously.

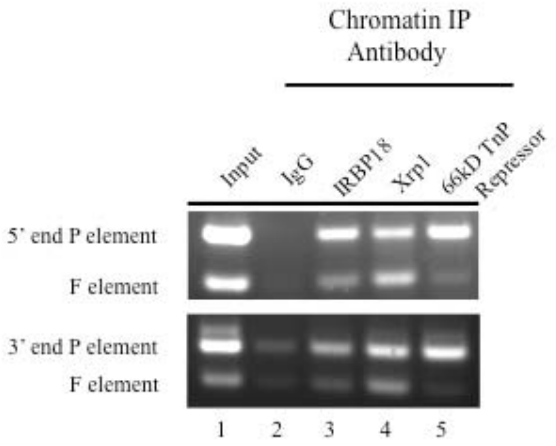
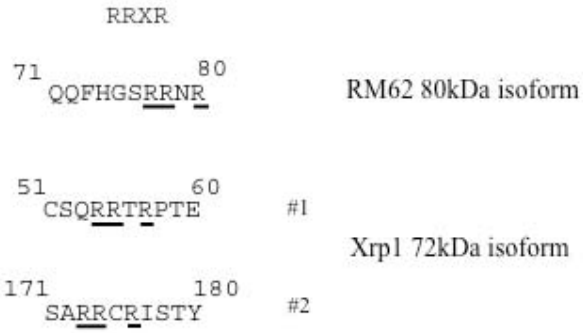

Chromatin IP
Antibody
Input
IgG
IRBP18
Xrp1
66kD TnP
Repressor
5' end P element
F element
3' end P element
F element
1
2
3
4
5

Figure 9 Nucleolar localization of IRBP18 and the 72 kDa isoforms Xrp1.
(**A.**) Identification of RRXR motifs within Rm62 and Xrp1 that mediating nucleolar localization of extended-C/EBPα and other proteins with known nucleolar localization (Muller et al., 2010). (B.) Alignments of sequences similar to the IRBP binding sequence. (C.) DNA gel of semi-quantitative PCR of the 18S rDNA and the Intergenic sequences (IGS) with input. ChIP assays were performed as in Figure, 8.

A.



B.

P element IRBP binding site	CATGATGAAATAAAC
Internal IRBP binding site	ATGGAATAAT
rDNA IGS	AATGTTGAAATATTC
18S rDNA locus	GATAATGAAATAAGA
Copia element	GATGATGAAAACTAC

C.

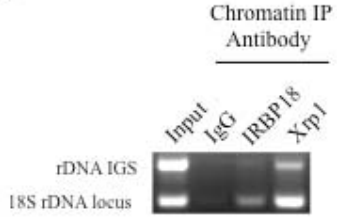
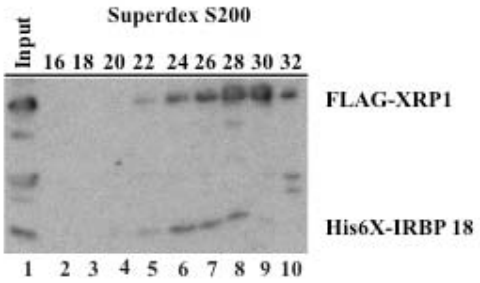


Figure 10 IRBP18/Xrp1 heterodimer is sufficient to reconstitute DNA binding.
(A.) Western Blot analysis of the recombinant His6X-IRBP18/FLAG-Xrp1 heterodimer separated by Superdex S200 gel filtrated chromatography. (B.) DNase I footprinting of recombinant heterodimer.

A.


Superdex S200
Input
16 18 20 22 24 26 28 30 32
FLAG-XRP1
His6X-IRBP 18
1 2 3 4 5 6 7 8 9 10

B.


Superdex S200
18 20 22 24 26 28 30 32
1 2 3 4 5 6 7 8

Figure 11 IRBP18 and Transposase can bind the same molecule of DNA.
DNase I footprinting of the 5’ end of the P element with recombinant heterodimer and titrated amounts of purified transposase.

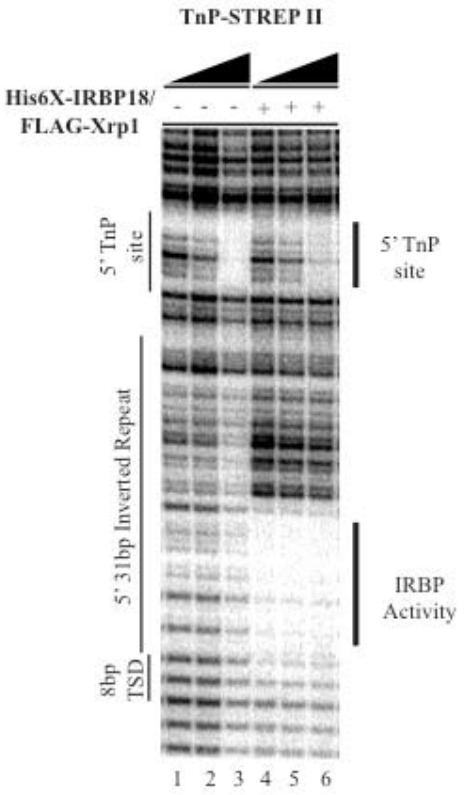
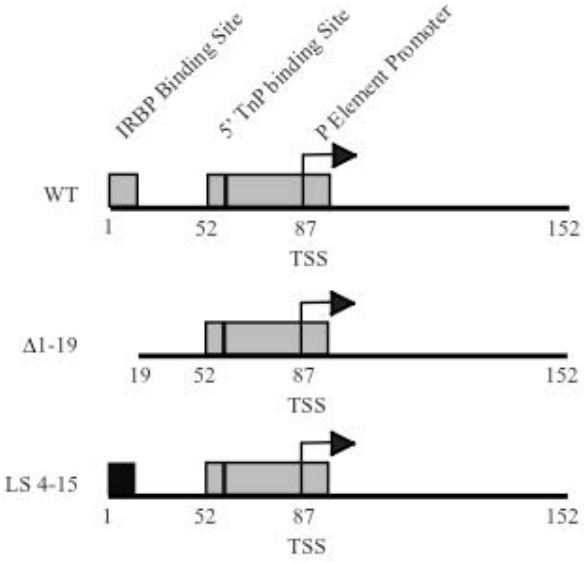

TnP-STREP II
His6X-IRBP18/
FLAG-Xrp1
- - - + + +
5' TnP
site
5' 31bp Inverted Repeat
8bp
TSD
5' TnP
site
IRBP
Activity
1 2 3 4 5 6

Figure 12 Design and characterization of the P element promoters.
A.) The P element promoter and 5'UTR (1-152) was ligated to the firefly luciferase cDNA. The IRBP binding site (position 1-16) is upstream of the Transposase binding site (position 52-62). The transposase binding site overlaps the TATA box. Mutant promoter LS 4-15 (Kaufman et al., 1989) has reduced IRBP18 binding (PhD thesis, Roche, S) due to mutation within the IRBP binding. Δ1-19 from the Δ2-3 transposase allele commonly used to mobilize P elements (Robertson, 1996). B.) Expression levels of each promoter construct.


IRBP Binding Site
5' TnP binding Site
P Element Promoter
WT
1
52
87
152
TSS
Δ1-19
19
52
87
152
TSS
LS 4-15
1
52
87
152
TSS

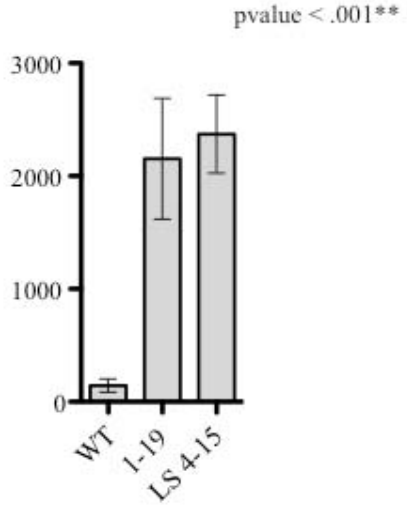
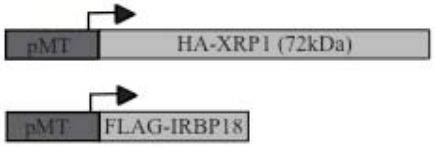

pvalue < .001**
3000
2000
1000
0
WT
1-19
LS 4-15

Figure 13 IRBP18 and Xrp1 repress P element transposase transcription.
A.) Expression of HA tagged Xrp1 (HA-Xrp1) and a FLAG tagged IRBP18 (FLAG-IRBP18) constructs were driven by the copper induced metallothionein promoter. B.) S2 cells were co-transfected with wild-type PEP-luciferase, renilla in combination with the indicated expression construct. Lucifierase activity was measured after a 24 h incubation in the presence or absence or 500 μM copper sulfate.

A.


pMT
HA-XRP1 (72kDa)
pMT
FLAG-IRBP18

B.

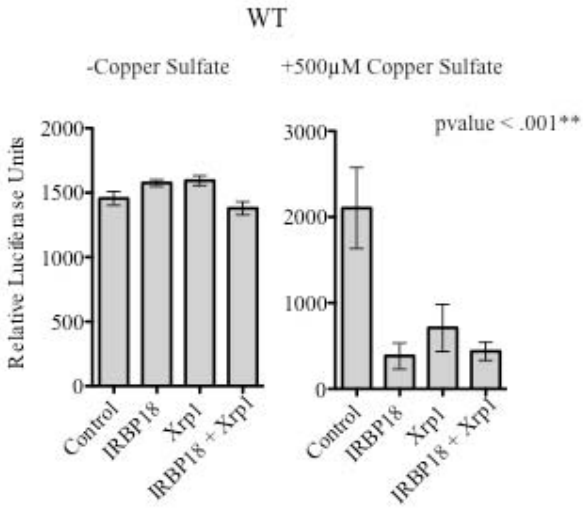
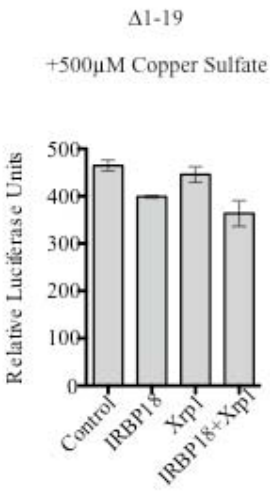

WT
-Copper Sulfate
+500µM Copper Sulfate
pvalue < .001**
Relative Luciferase Units
2000
1500
1000
500
0
3000
2000
1000
0
Control
IRBP18
Xrp1
IRBP18 + Xrp1

Figure 14 Mutant promoter removed IRBP18 and Xrp1 regulation.
S2 cells were co-transfected with A.) Δ1-19 or B.) LS 4-15 PEP-luciferase, renilla in combination with the indicated expression construct. As in figure 13, luciferase activity was measured 24 hours after the addition of 500 μM copper sulfate.

A.


Δ1-19
+500μM Copper Sulfate
Relative Luciferase Units
500
400
300
200
100
0
Control
IRBP18
Xrp1
IRBP18+Xrp1

B.

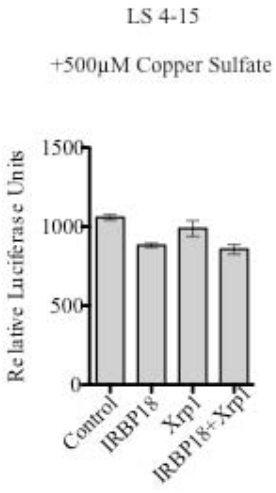

LS 4-15
+500μM Copper Sulfate
Relative Luciferase Units
1500
1000
500
0
Control
IRBP18
Xrp1
IRBP18+Xrp1

Figure 15 RNAi knockdown of both IRBP18 and Xrp1 relieves transcriptional repression.
Cells were treated with dsRNA for 48 hours before co-transfection of reporter plasmids. Luciferase activity was measured 24 h post co-transfection.

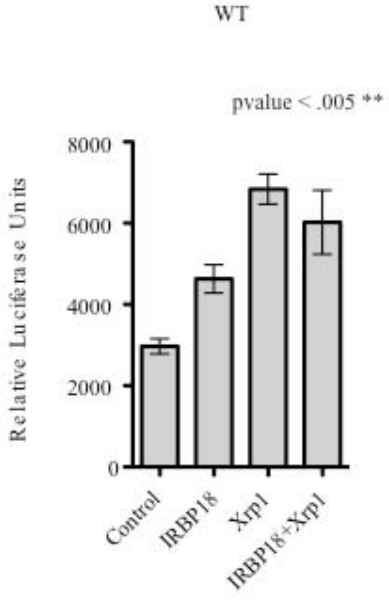
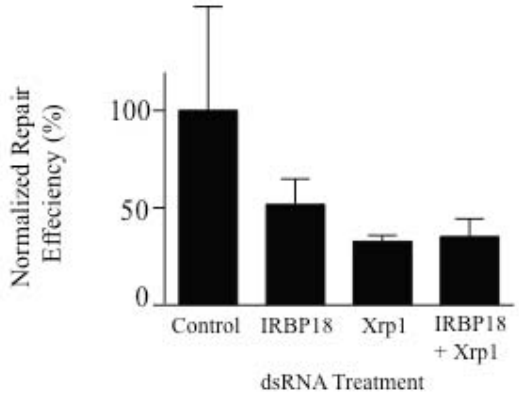

WT
pvalue < .005 **
Relative Luciferase Units
8000
6000
4000
2000
0
Control
IRBP18
Xrp1
IRBP18+Xrp1

Figure 16 RNAi knockdown of IRBP18 and Xrp1 reduces repair efficiency.
(A.) S2 cells were treated the dsRNA for 48 prior to transfection with a transposase source and the reporter assay. Recovered plasmids were counted and the normalized repair efficiency was scored as the number of colonies in the kanamycin + ampicillin divided by the number of colonies on ampicillin normalized to the control treatment. (B.) Sequence analysis of recovered clones. Sequenced clones were categorized into 3 bins: no deletion (0 bp, black bars) into the flanking donor DNA of pISP-2/K_m, small deletions (1–49 bp, gray bars) and large deletions (≥50 bp, silver bars). The fraction of total clones sequenced in each bin was expressed as the percentage of the total clones sequenced.

A.


Normalized Repair Effeciency (%)
100
50
0
Control
IRBP18
Xrp1
IRBP18 + Xrp1
dsRNA Treatment

B.

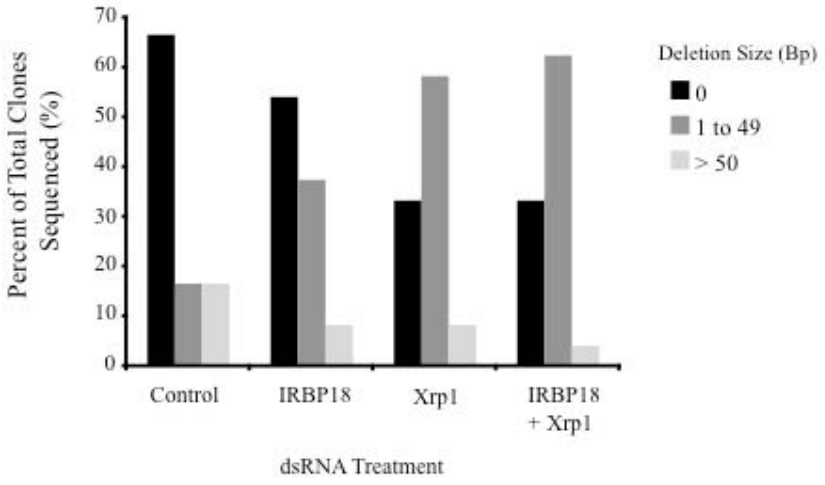
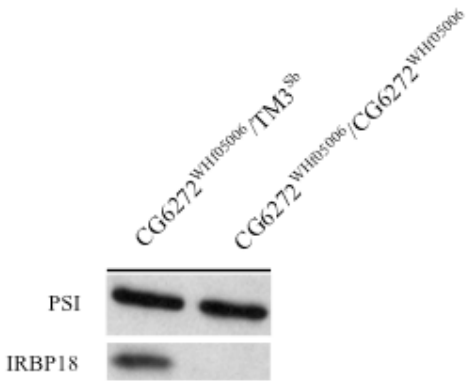

Percent of Total Clones Sequenced (%)
70
60
50
40
30
20
10
0
Control
IRBP18
Xrp1
IRBP18 + Xrp1
dsRNA Treatment
Deletion Size (Bp)
0
1 to 49
> 50

Figure 17 Validation of the mutant alleles
(A) Immunoblot analysis of adult male flies heterozygous or homozygous for a null allele of IRBP18 (See experimental methods). (B.) PCR analysis of genomic DNA isolated from the Birm2 and Δ2-3 strains. Primers designed to detect the full length transposase enzyme (Δ2-3) or the positive control 3'end of the P element were used in the PCR reaction.

A.


CG6272WH05006/TM3Sb
CG6272WH05006/CG6272WH05006
PSI
IRBP18

B.

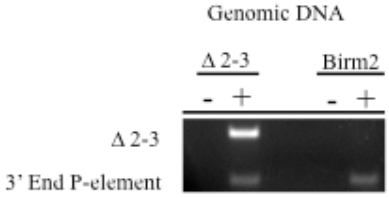

Genomic DNA
Δ 2-3
Birm2
- +
- +
Δ 2-3
3' End P-element

Figure 18 Crossing scheme of the somatic dysgenesis experiment.

A.

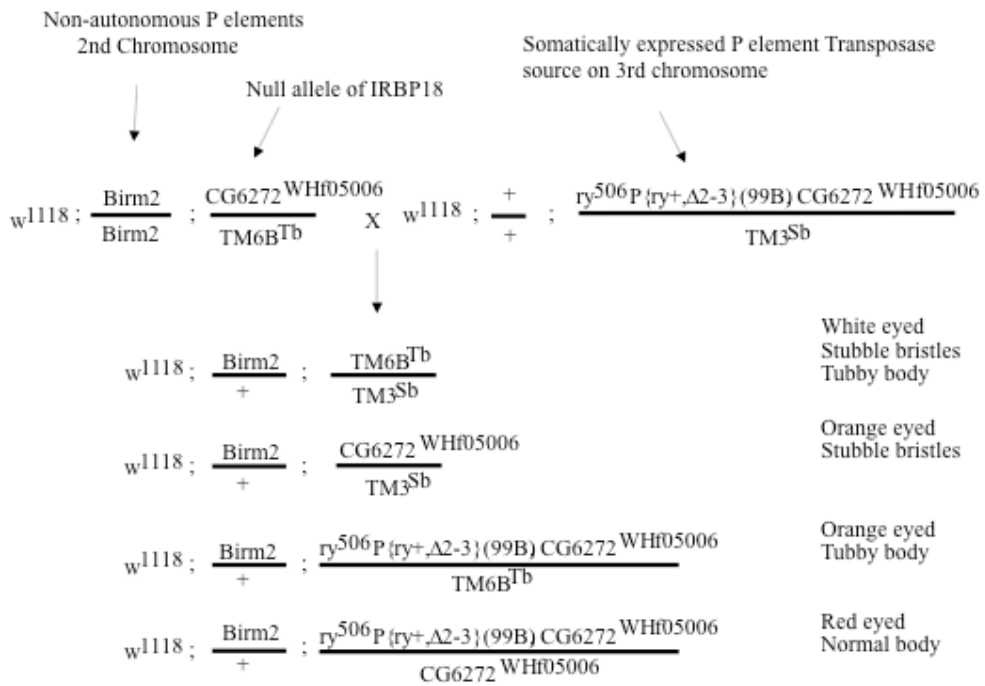

Non-autonomous P elements
2nd Chromosome
Null allele of IRBP18
Somatically expressed P element Transposase
source on 3rd chromosome
w1118 ; Birm2/Birm2 ; CG6272 WHf05006/TM6BTb X w1118 ; +/+ ; ry506 P{ry+,Δ2-3}(99B) CG6272 WHf05006/TM3Sb
w1118 ; Birm2/+ ; TM6BTb/TM3Sb
White eyed
Stubble bristles
Tubby body
w1118 ; Birm2/+ ; CG6272 WHf05006/TM3Sb
Orange eyed
Stubble bristles
w1118 ; Birm2/+ ; ry506 P{ry+,Δ2-3}(99B) CG6272 WHf05006/TM6BTb
Orange eyed
Tubby body
w1118 ; Birm2/+ ; ry506 P{ry+,Δ2-3}(99B) CG6272 WHf05006/CG6272 WHf05006
Red eyed
Normal body

Figure 19 IRBP18 null flies increases somatic dysgenesis phenotype.
Individual crosses were performed at two permissive temperatures (18°C and 21°C). The line indicates the expected percentage of each phenotype. In this case each phenotype should be presented at 25%.

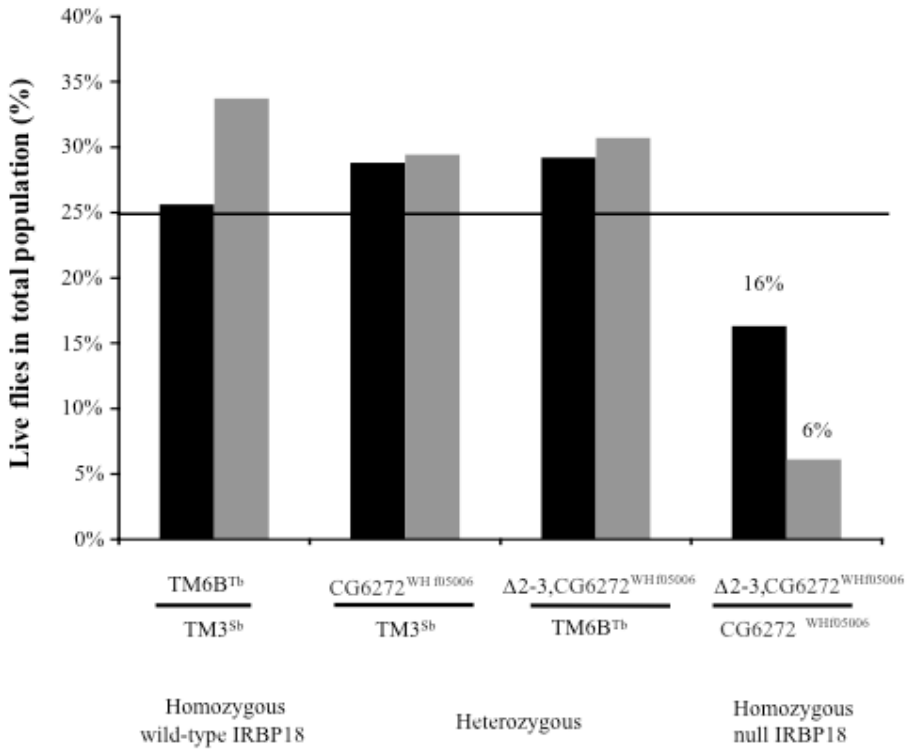

Live flies in total population (%)
40%
35%
30%
25%
20%
15%
10%
5%
0%
16%
6%
TM6B^Tb
TM3^Sb
CG6272^WHf05006
TM3^Sb
Δ2-3,CG6272^WHf05006
TM6B^Tb
Δ2-3,CG6272^WHf05006
CG6272^WHf05006
Homozygous wild-type IRBP18
Heterozygous
Homozygous null IRBP18

Figure 20 Control Cross without Birm2 chromosome.
A.) Crossing scheme. B.) Results of control cross performed at 21°C.

A.

w^{1118} ; $\frac{+}{+}$; $\frac{CG6272^{WHf05006}}{TM6B^{Tb}}$ X w^{1118} ; $\frac{+}{+}$; $\frac{ry^{506}P\{ry+,\Delta2\text{-}3\}(99B),\ CG6272^{WHf05006}}{TM3^{Sb}}$

w^{1118} ; $\frac{+}{+}$; $\frac{TM6B^{Tb}}{TM3^{Sb}}$	White eyed Stubble bristles Tubby body
w^{1118} ; $\frac{+}{+}$; $\frac{CG6272^{WHf05006}}{TM3^{Sb}}$	Orange eyed Stubble bristles
w^{1118} ; $\frac{+}{+}$; $\frac{ry^{506}\ P\{ry+,\Delta2\text{-}3\}(99B),\ CG6272^{WHf05006}}{TM6B^{Tb}}$	Orange eyed Tubby body
w^{1118} ; $\frac{+}{+}$; $\frac{ry^{506}\ P\{ry+,\Delta2\text{-}3\}(99B),\ CG6272^{WHf05006}}{CG6272^{WHf05006}}$	Red eyed Normal body

B.

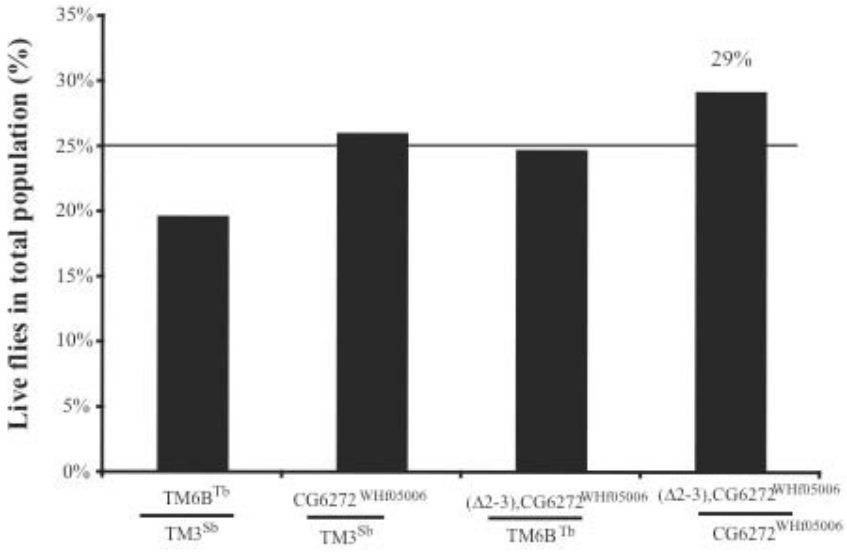


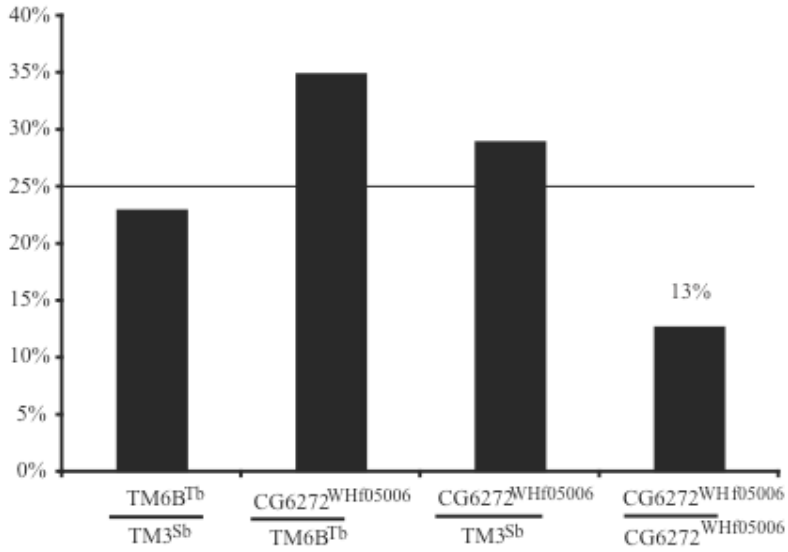
Figure 21 Control cross without transposase source.
A.) Crossing scheme. B.) Results of control cross performed at 21°C.

A.

w^{1118} ; $\frac{Birm2}{Birm2}$; $\frac{CG6272^{WHf05006}}{TM6B^{Tb}}$ X w^{1118} ; $\frac{+}{+}$; $\frac{CG6272^{WHf05006}}{TM3^{Sb}}$

w^{1118} ; $\frac{Birm2}{+}$; $\frac{TM6B^{Tb}}{TM3^{Sb}}$	White eyed Stubble bristles Tubby body
w^{1118} ; $\frac{Birm2}{+}$; $\frac{CG6272^{WHf05006}}{TM3^{Sb}}$	Orange eyed Stubble bristles
w^{1118} ; $\frac{Birm2}{+}$; $\frac{CG6272^{WHf05006}}{TM6B^{Tb}}$	Orange eyed Tubby body
w^{1118} ; $\frac{Birm2}{+}$; $\frac{CG6272^{WHf05006}}{CG6272^{WHf05006}}$	Red eyed Normal body

B.



Chapter 3

The Genetics of the *Drosophila* bZIP Protein CG672/IRBP18 and its Role as a General DNA Repair Factor

Introduction

CCAAT/enhancer binding proteins (C/EBP) are a subclass of basic-leucine zipper (b-ZIP) site-specific DNA binding proteins that play important roles in several developmental and metabolic pathways (Ramji and Foka, 2002). In the human genome, there are six highly related C/EBP genes that encode 12 protein isoforms. Both the C/EBP-α and C/EBP-β genes produce one mRNA, but multiple protein isoforms are produced by regulated alternative translation initiation (Calkhoven et al., 2000; Lin et al., 1993; Muller et al., 2010; Timchenko et al., 2002; Welm et al., 1999). The C/EBP-β mRNA, for example, produces three protein isoforms p38 (Liver Activating Protein* (LAP*)), p33 (LAP) and p20 (Liver Inhibitory Protein (LIP)).

The genome of *Drosophila* melanogaster has two known C/EBP family members, slow border cells (slbo)/CG4354 and CG6272/Inverted Repeat Binding Protein 18 (IRBP18), unlike mammals, these proteins appear to play more specialized roles in *Drosophila* development. SLBO is expressed primarily in developing larvae and in ovarian border cells (Montell et al., 1992) . Slbo mutant flies appear to be normal except that the females are sterile due to abnormal migration of border cells in the developing egg chamber. SLBO regulates transcription of the DE-cadherin, breathless (btl), jing, and FAK, since the steady-state mRNA levels for these genes are lower in slbo mutants (Liu and Montell, 2001; Montell, 2001; Murphy et al., 1995).

CG6272/IRBP18 is a ubiquitously expressed 113 amino acid b-zip protein that lacks a recognizable transactivation domain, similar to the LIP isoform of C/EBPβ and C/EBPγ (Ramji and Foka, 2002) IRBP18 forms a functional heterodimeric interaction with another bZIP protein Xrp1 (Chapter 2). Together these proteins form the DNA binding core of the Inverted Repeat Binding Protein complex, affect DNA repair following P transposable element excision and also repress transcription from the P element promoter. A role for the mammalian C/EBP genes in DNA repair has been observed in several instances. In human keratinocytes, UVB ultraviolet DNA damage induces p53-dependent transcriptional activation of both the C/EBPα and β genes (Yoon and Smart, 2004). The loss of C/EBPα, resulted in misregulation of the damage-induced G1 checkpoint; presumably through the loss of the interaction C/EBPα with p21/CIP promoter . C/EBPα is also expressed in prostate cancer cells where it interacts with the NHEJ DNA repair protein Ku heterodimer and the poly (ADP-ribose) polymerase 1 (PARP-1) proteins (Yin and Glass, 2006).

Both CG6272/IRBP18 and Xrp1/CG17836 are DNA damage-induced *Drosophila* p53 transcriptional targets (Akdemir et al., 2007; Brodsky et al., 2004). Genetic deletion of the Xrp1 gene resulted in loss in heterozygousity (LOH), indicated a role in chromosome instability or DNA repair (REF) (Akdemir et al., 2007). By contrast the over-expression of the nuclear-localized 72kDa isoform of Xrp1 resulted in decreased cell proliferation in *Drosophila* cell culture (Chapter 2), (Akdemir et al., 2007) The p53 DNA damage-induced apoptotic response was unaffected in the Xrp1 mutant flies, suggesting that Xrp1 functions to preserve genomic stability through a pathway independent from apoptosis.

While CG6272/IRBP18 functions in repair of DNA breaks caused by P element transposase (Chapter 2), it was not known if CG6272/IRBP18 functions as a general DNA double-strand DNA break repair protein.

In this report we demonstrate that CG6272/IRBP18 is a general DNA repair protein and is required for normal fertility, a property often associated with *Drosophila* DNA repair mutants (Banga et al., 1995; Boyd et al., 1987; Kusano et al., 2001) The male sterility of CG6272/IRBP18 mutants is more severe than mutant females. CG6272/IRBP18 flies are sensitive to both ionizing radiation (IR) and methyl methane sulfonate (MMS), but not to the topoisomerase I inhibitor campothecin. There is also preliminary evidence that the CG6272/IRBP18 protein and its bZIP partner Xrp1, exist in a damage-induced protein complex, whose composition changes upon ionizing radiation treatment. These data imply that CG6272/IRBP18 functions to recognize and repair specific types of DNA damage.

Results

IRBP18 null flies have reduced fertility

In order to determine an in vivo role for the CG6272/IRBP18 gene in Drosophila, we took advantage of a PiggyBac transposon inserted within the CG6272/IRBP18 locus. This allele $CG6272^{WHf05006}$ has an insertion within the second protein coding exon and disrupts the open reading frame (Thibault et al., 2004). Adult flies homozygous $CG6272^{WHf05006}$ have undetectable steady-state levels of CG6272/IRBP18 protein and are fully viable (Chapter 2). We found that both male and female $CG6272^{WHf05006}$ homozyous mutant flies have reduced fertility. We quantitated the fertility of individual mutant CG6272/IRBP18 females and observed a semi-sterile phenotype (Figure 1 and Table 1). Some mutant females had a small number of wondering larvae in their vials after 4 days of egg laying. By contrast, nearly all heterozygous control wild type sisters tested from the same crosses that had upward of 80 wandering larvae per vial, indicating the reduced fertility of the CG6272/IRBP18 females. The sterility of CG6272/IRBP18 homozygous mutant males was more severely affected. Only two of the vials produced viable larvae (4 in one and 2 in the other).

CG6272/IRBP18 mutants are sensitive to DNA damaging agents that create double-strand DNA breaks

To examine whether CG6272/IRBP18 might play a role as a general repair factor, we crossed heterozygous ($CG6272^{WHf05006}$/TM3,Sb), allowed the adults to lay for 24 hours and the treated the 3rd instar larvae with increasing amounts of methyl methane sulfonate (MMS) or IR (Figure 2). The expected ratio of progeny from this cross is 2:1 heterozygous to homozygous adults. If CG6272/IRBP18 mutant flies are sensitive to these DNA damaging agents, then ratio of heterozygous adults should increase as the homozygous population dies. Flies homozygous null for the CG6272/IRBP18 mutation show dose-dependent sensitivity to both MMS and IR, compared to observed ratios in the control groups (Figure 3A and B). The radiosensitivity observed in the CG6272/IRPB18 null mutant flies is more severe by comparison to the Dmp53 (Sogame et al., 2003) and DmRAD54 (Jaklevic and Su, 2004; Kooistra et al., 1999) but similar to the DNA ligase IV (lig IV) mutants (Gorski et al., 2003). In contrast to the results obtained with MMS and IR, no killing was observed after treatment with the DNA topoisomerase I inhibitor, camptothecin, which creates nicked DNA trapping covalent DNA-protein adducts with the enzyme (Pascucci et al., 2005).

CG6272/IRBP18 protein exists in a dynamic damage-induced protein complex

We next asked if the CG6272/IRBP18 protein was s a part of a protein complex whose composition was changed upon DNA damage. We had previously shown that the CG6272/IRBP18 complex was part of the protein complex that recognized the P transposable element terminal inverted repeats (Chapter 2). In order to explore this possibility, we prepared extracts from 8L of ZZ-TEV-3X FLAG IRBP18 fusion protein-expressing cells that were treated with IR (30Gy of γ-rays from a 137Cs source) and allowed to recover for 30min. post treatment. As a control, another 8L fusion protein-expressing cells were grown in parallel but not treated with ionizing radiation. Nuclear extracts derived from these two cell samples were prepared and the CG6272/IRBP18 containing complex was purified as described in chapter 2 (Figure 4). The IR-treated samples retained, or has possibly enhanced, DNase I footprinting activity on the P element terminal inverted repeat binding site (Figure 5). Following purification, analysis by SDS-polyacrylamide gel electrophoresis and silver staining, we observed at least 6 polypeptides that were present in the IR-treated samples, but were not apparent in the control group (Figure 6). These results suggest that upon DNA damage, by ionizing radiation, the CG6272/IRBP18 complex may undergo an alteration, whereby new protein components are brought into the complex. These observations, taken together with the IR- and MMS-sensitivity of the CG6272/IRBP18 mutants, suggest a novel role for this complex in either DNA damage signaling or repair.

Discussion

Proper maintenance of genomic integrity after DNA damage requires the coordination of multiple cellular responses. The recruitment and assembly of molecular complexes at both the sites of DNA damage and in distant cellular compartments, indicates that an integration of cellular components is required to execute an efficient DNA damage response. In response to DNA damage, gene expression profiling showed that Dmp53 activates transcription of 29 high-stringency target genes (Akdemir et al., 2007). Many of these genes are involved in either the pro-apototic response, DNA repair, or transcriptional regulation. Three of the transcription factors induced by Dmp53 are CG6272/IRBP18, CG15479, and Xrp1/CG17836 are b-ZIP proteins (Akdemir et al., 2007). We have previously demonstrated that CG6272/IRBP18 and Xrp1/CG17836 form a functional heterodimeric DNA binding complex that represses transcription at the P element promoter and plays a role in DNA of P element transposase-induced DNA cleavage events (Chapter 2).

The Dmp53 gene is a dispensable for normal development, however, it is an important transcriptional regulator of genes involved in DNA damage induced pro-apoptosis and DNA repair signaling (Brodsky et al., 2000; Sogame et al., 2003). The role of Dmp53 appears to be more specialized that its mammalian ortholog, in that is does not appear to effect cell cycle arrest upon DNA damage, suggesting that the ancestral function of p53 may be in apoptosis (Lu and Abrams, 2006; Lu et al., 2009). Mammalian p53 induces two sets of genes, rapidly activating genes involved in cell cycle arrest and later activating pro-apoptotic genes (Morachis et al.) This two-tiered system allows time for the cell to assess and repair damaged DNA sites, while maintaining the flexibility to turn later turn on the apoptotic cell death response. In contrast, Dmp53 does not induce cell cycle arrest after DNA damage, indicating an earlier role for p53 in apoptosis (Brodsky et al., 2004).

In mammalian cells, the coiled-coil leucine zipper region of C/EBP-α can interact with the cdk inhibitor p21 (Harris et al., 2001; Timchenko et al., 1996). This protein-protein interaction results in reduced cell proliferation, but is independent of the transcriptional activity of C/EBP. Additionally, C/EBP family members have been demonstrated to interact with the DNA repair proteins RAD51, Ku70/80, PARP-1, and the cell cycle protein kinase CDK2 (Chipitsyna et al., 2006; Harris et al., 2001; Wang et al., 2001; Yin and Glass, 2006) Mutations in the *Drosophila* orthologs of the cdk inhibitor p21, the cell cycle transcription factor E2F, the homologous DNA repair protein RAD51, and the cell cycle protein kinase CDK2 do not exhibit male sterility. Thus, if any of these C/EBP protein-protein interactions are conserved in Drosophila, they are at the very least dispensable for testes development. Whether the CG6272/IRBP18 protein acts as a transcriptional regulator downstream of Dmp53 could be addressed using mRNA-seq to compare the gene expression profiles of control versus CG6272/IRBP18 mutant or RNAi-knockdown cells following IR treatment.

In testing the sensitivity of CG6372/IRBP18 homozygous mutant flies, we found that DNA damage induced by IR or MMS, but not camptothecin resulted in killing of CG6272/IRBP18 mutant flies. An explanation of these results lies in the types of DNA breaks that are caused by the different agents (Helleday et al., 2008). Ionizing radiation creates replication-independent double-strand DNA breaks that thus can kill non-replicating cells. In cycling cells, IR activates cell-cycle checkpoints in order to avoid formation of toxic DNA replication lesions during S-phase or carry-over of mutation into the G2 and mitosis phases of the cell cycle. MMS is a direct alkylating agent that attacks nucleophilic groups on nucleic acids. Though this mutagen induces generally single-strand breaks, with increasing concentrations of MMS such breaks can appear on opposite strands of DNA in close proximity, so that double-strand breaks may occur (Pascucci et al., 2005). Camptothecin selectively traps topoisomerase I cleavage complexes, which creates nicked DNA strand leading to replication-dependent DNA lesions and cell cycle arrest in the S and G2 phases of the cell cycle (Hsiang et al., 1989a; Hsiang et al., 1989b). Perhaps the DNA repair functions of CG6272/IRBP18 are active during certain stages of the cell cycle. Nonetheless, our data indicate that CG6272/IRBP18 mutants are sensitive to DNA double-stand breaks and the finding that these mutants exhibit sterility is also a common phenotype of *Drosophila* DNA repair mutants (Boyd et al., 1987).

It would appear that the CG6272/IRBP18 protein exists in a protein complex whose composition changes upon ionizing radiation treatment. One CG6272/IRBP18 complex described in chapter 2 binds the 31bp terminal inverted repeats of the P transposable element and the one described here, which changes protein composition in response to DNA damage. Purification and SDS-polyacrylamide gel silver stain analysis of the complex purified after DNA damage demonstrated that the CG6272/IRBP complex has at least 6 additional protein components. The identities of these additional protein complex members could shed light on to the mechanism of how CG6272/IRBP18 functions in the DNA damage/repair response.

Experimental Procedures

Drosophila strains

The *Drosophila* strains used were Oregon-R and w^{1118}; $CG6272^{WHf05006}$/TM6,Tb (Exelixis Collection at the Harvard Medical School). The ^{w1118}; $CG6272^{WHf05006}$/TM3,Sb strain was made by crossing w^{1118}; $CG6272^{WHf05006}$/TM6,Tb males with w^{1118}; TM3,Sb/CxD virgin females. Oranged-eyed, stubble bristle, wild-type winged males flies were selected, and then mated again to w^{1118}; TM3, Sb/CxD virgin females to obtain a balanced stock. All flies were raised on standard cornmeal, molasses, and yeast medium at 25°C.

Fertility Assays

w^{1118}; $CG6272^{WHf05006}$/TM3,Sb flies were crossed with Oregon-R flies. Females were isolate for three days before mating. For female fertility, individual females were place into separate vials with two Oregon-R males. For male fertility each male was placed with two Oregon-R females. Flies were allowed to mate for 4 days and live flies were counted.

DNA Damage Agents and Treatments

w^{1118}; $CG6272^{WHf05006}$/TM3,Sb flies were self crossed and at the 3rd instar larva stage treated with MMS (Sigma), IR (γ-rays from a ^{137}Cs source; gift from T. Cline) or camptothecin (Sigma) were used at the indicated doses.

Purification of the Damage-Induced CG6272/IRBP complex

8L (3×10^{10} cells) of ZZ-TEV-3X FLAG IRBP18 fusion protein-expressing cells were harvested, washed and resuspended in 100ml of 1X PBS and then exposed to 30Gy of ionizing radiation (γ-rays from a ^{137}Cs source). In parallel, a second 8L culture was prepared and resuspended in 100ml of PBS but not treated with IR. The cells were allowed to recover for 30 min. post-IR treatment before nuclear extracts were prepared (Chapter 2). The concentrated nuclear extracts were resuspended in 3-5ml of HGKNED (25 mM Hepes-KOH pH 7.6, 10% glycerol, 1 mM EDTA, 1mM EGTA, 0.01% NP40 and 200mM KCl) supplemented with 0.2% PMSF and 0.5 mM DTT, 0.2mM β-glycerophosphate, 0.2mM sodium fluoride and 0.2nM trichostatin A. The extracts were fractionated by gel filtration chromatography over a120ml Superdex-200 PG column. Columns fractions were immunoblotted for the presence of TAP-tagged CG6272/IRBP18 with an affinity-purified rabbit polyclonal CG6272/IRBP18 antibody (1:2000 dilution). CG6272/IRBP18-positive fractions were pooled and incubated with rabbit-IgG resin (Sigma) overnight. The bound IgG resin was washed with 10ml of HGKNED, then incubated with recombinant TEV protease for a minimum of 4 hr at room temperature. The TEV-eluted protein material was then incubated with an anti-FLAG antibody resin

overnight, washed, and eluted with a 3X FLAG peptide (150μg/ml) at 4°C for 30 min. The native FLAG-eluted complexes were analyzed by SDS-PAGE followed by silver staining (Pierce Silver Snap Kit). SDS-gel bands were then excised and digested with trypsin prior to 2D-LC-MS/MS analysis.

Figure 1 Diagram of Fertility Crosses

Female

w^{1118} ; $\frac{CG6272^{WHf05006}}{TM3^{Sb}}$ X Oregon R

w^{1118} ; $\frac{CG6272^{WHf05006}}{CG6272^{WHf05006}}$ X Oregon R

Male

w^{1118} ; $\frac{CG6272^{WHf05006}}{TM3^{Sb}}$ X Oregon R

w^{1118} ; $\frac{CG6272^{WHf05006}}{CG6272^{WHf05006}}$ X Oregon R

Table 1. IRBP18 mutant fertility

The crosses performed were as follows: $CG6272^{WHf05006}/TM3^{sb}$ or $CG6272^{WHf05006}/CG6272^{WHf05006}$ males were crossed to Oregon R virgin female; $CG6272^{WHf05006}/TM3^{sb}$ or $CG6272^{WHf05006}/CG6272^{WHf05006}$ virgin females were crossed to Oregon R males. Fertility was scored by counting flies from the homozygous cross and normalized to flies from heterozygous cross

Table 1 *IRBP18 mutant fertility*

	Male	Females
CG6272 ^{WHf05006}/TM3^{sb}	100%	100%
CG6272 ^{WHf05006}/CG6272^{WHf05006}	1%	24%

Figure 2. Crossing Scheme for DNA damage
$CG6272^{WHf05006}/TM3^{sb}$ flies were self-crossed. 3^{rd} instar larvae were treated with ionizing radiation at rate of 1.68 Gy/min, MMS, or camptothecin.

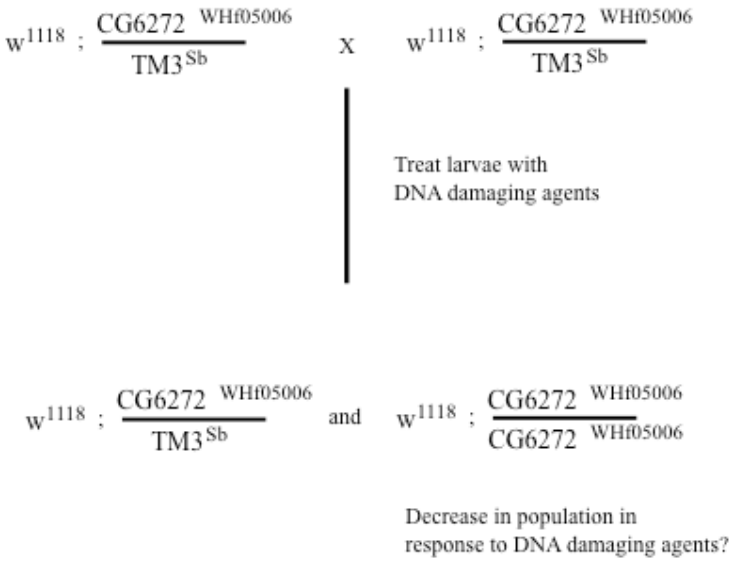
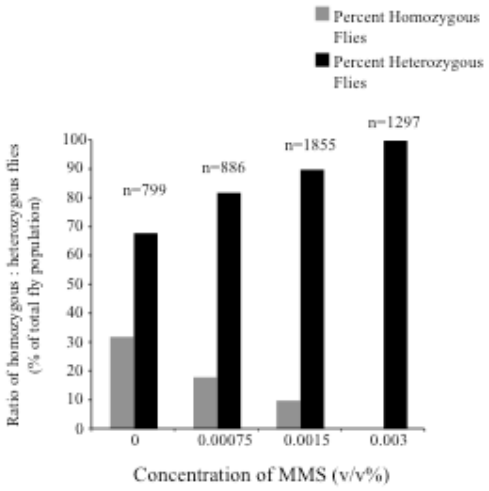

w1118 ; CG6272 WHf05006 / TM3 Sb
X
w1118 ; CG6272 WHf05006 / TM3 Sb
Treat larvae with
DNA damaging agents
w1118 ; CG6272 WHf05006 / TM3 Sb
and
w1118 ; CG6272 WHf05006 / CG6272 WHf05006
Decrease in population in
response to DNA damaging agents?

Figure 3. CG6272^{WHf05006}/CG6272^{WHf05006} flies are sensitive to IR and MMS. Shifts in the expected ratio of homozygous CG6272^{WHf05006} flies to heterozygous flies, as determined by the vehicle control, were measured. The total number of flies counted per concentration tested is indicated above the paired bars. A.) Increasing amounts of MMS decreased the number of CG6272^{WHf05006}/ CG6272^{WHf05006} flies in a dose dependent fashion (v/v). B.) The viability homozygous flies are reduced 50% at 5Gy compared to control.

A.


Percent Homozygous Flies
Percent Heterozygous Flies
Ratio of homozygous : heterozygous flies (% of total fly population)
n=799
n=886
n=1855
n=1297
0
0.00075
0.0015
0.003
Concentration of MMS (v/v%)

B.

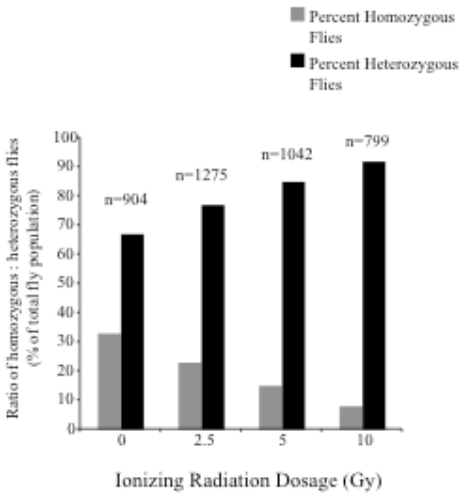
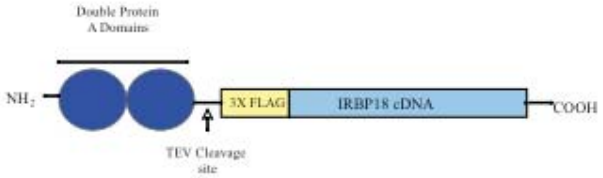

Percent Homozygous Flies
Percent Heterozygous Flies
Ratio of homozygous : heterozygous flies (% of total fly population)
n=904
n=1275
n=1042
n=799
0
2.5
5
10
Ionizing Radiation Dosage (Gy)

Figure 4 Purification scheme of damage induced IRBP complex.



+/- 30Gy 8L NE from S2 ZZ-TEV-3X-FLAG IRBP18

120ml Superdex 200 PG

Void-97kDa

500µl IgG immunoaffinity column

+ TEV Protease

TEV cleaved material

100µl FLAG Immunoaffinity column

3X FLAG Peptide elution

Peptide identification
Biochemical Assays

Figure 5 Validation of IRBP DNaseI footprinting activity.
Pairwise comparison of DNaseI footprinting activity of IRBP complex with and without 30Gy.

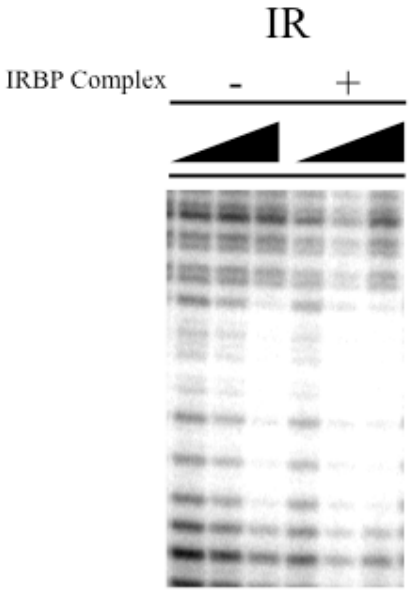

IR
IRBP Complex
-
+

Figure 6 Silver Stain analysis of damage induced complex.
5% of the eluted sample was size-fractionated by SDS-PAGE and silver stained. Bands present in the IR treated group are indicated by red bars.

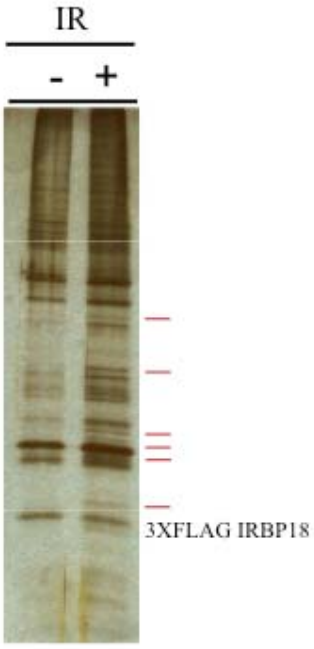
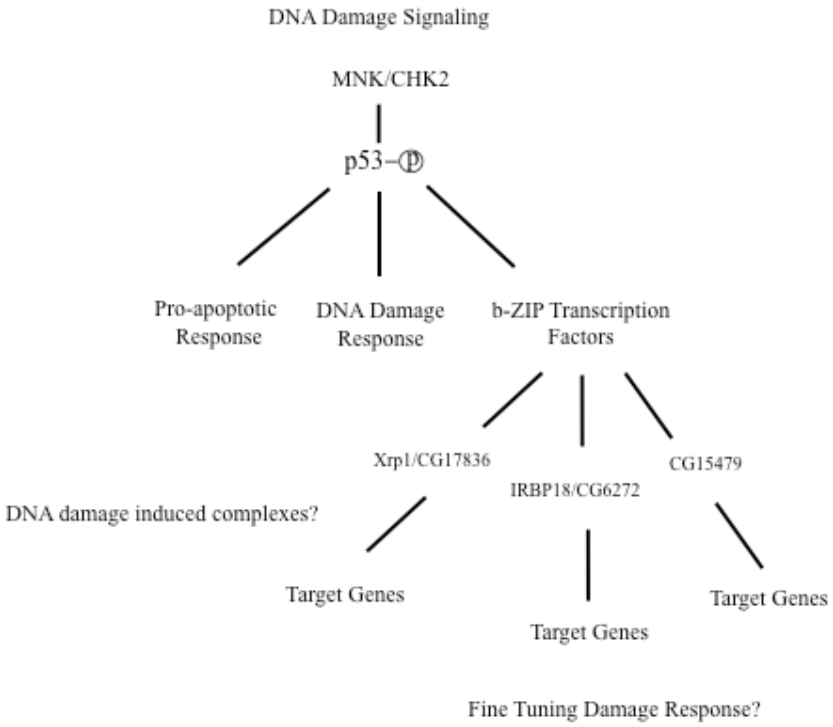

IR
-
+
3XFLAG IRBP18

Figure 7 Model for IRBP18 function


DNA Damage Signaling
MNK/CHK2
p53–Ⓟ
Pro-apoptotic Response
DNA Damage Response
b-ZIP Transcription Factors
Xrp1/CG17836
IRBP18/CG6272
CG15479
DNA damage induced complexes?
Target Genes
Target Genes
Target Genes
Fine Tuning Damage Response?

Chapter 4

IRBP Complex connects RNAi to DNA repair

Introduction

RNA binding proteins are emerging players in mediating DNA damage response (DDR) (Paulsen et al., 2009) and maintaining genome stability (Li and Manley, 2005). Purification of the *Drosophilamelanogaster* Inverted Repeat Binding Protein (IRBP) Complex revealed the presence of several RNA binding proteins including a RNA helicase (Rm62) (Chapter 2). RM62/dmP68/Lip is a multifunctional protein within the Asp-Glu-Ala-Asp (DEAD) box family of putative ATPases and helicases. Members of this family have established roles in almost every aspect of nucleic acid metabolism including regulating chromatin structure, transcription, RNA interference (RNAi), pre-mRNA splicing, translation, RNA degradation, and ribosome biogenesis (Fuller-Pace, 2006). Rm62 has nine annotated mRNA isoforms predicted to yield 7 proteins at 62kDa, one at 17kDa and another at 80kDa. The contribution of each of these isoforms to various biochemical processes that RM62 is involved in is not well understood. Accordingly Rm62 localizes to subcellular compartments throughout the cell (Buszczak and Spradling, 2006; Ishizuka et al., 2002; Lei and Corces, 2006).

Rm62 was originally identified as one of the gene products of the Triplo-lethal locus (Dorer et al., 1990). Subsequent mutagenic screening demonstrated it to be a modifier of retrotransposon expression and suppressor of position effect variegation (PEV) (Csink et al., 1994); thereby playing a role in heterochromatin formation. Consistent with a role in chromatin structure, Rm62 forms a RNA-dependent interaction with a *gypsy* insulator binding protein, Centrosomal protein 190 (CP190) (Lei and Corces, 2006). Disruption of this interaction results in nuclear reorganization of a compromised *gypsy* insulator.

Both Rm62 and its mammalian ortholog DDX5/p68 play a direct role in the maturation of small RNAs critical for efficient RNAi. Rm62 forms protein-protein interactions with the *Drosophila*Fragile X mental retardation protein (Fmr1) and Argonaut 2 (Ago2) (Ishizuka et al., 2002). DDX5 is in a stable complex with Drosha and p53, which is necessary for post-DNA damage induced maturation of miRNAs (Jaklevic et al., 2008). These observations are consistent with a conserved role in a range of RNAi pathways. Recently, *Drosophila*RISC members, Ago2 and dicer 2, have been identified has mediators of rDNA heterochromatin maintenance. Loss of these proteins results in decreased di-methylation of Lysine 9 of Histone H3 (H3K9me2) and subsequent nucleolar instability (Peng and Karpen, 2007; Peng and Karpen, 2009). The IRBP complex binds rDNA sequences and interacts with numerous nucleolar and RNAi proteins including Rm62 (Chapter 2); these observations imply a potential connection between this site specific DNA binding complex and heterochromatin formation.

In this report we demonstrate Rm62 is a gene important for DNA repair, surprisingly the majority of this activity is encoded within the 80kDa isoform. Moreover we demonstrate that AGO2 is also important for DNA damage response. Although the evidence for RNAi induced heterochromatin formation is strikingly clear, how these proteins find their target sequence is not well understood. Many of the subunits of IRBP complex have established roles in RNAi biochemistry. Additionally the DNA binding core, a b-Zip

heterodimer between IRBP18/CG6272 and Xrp1, localize to heterochromatic regions of the rDNA locus. We propose IRBP18 and Xrp1 provide a molecular bridge that localizes RNAi machinery to specific sites within the genome.

Results

The 80 kDa isoform of Rm62 localizes specifically to the nucleus

The 62 kDa (Rm62 M) and the 80 kDa (Rm62 80) isoforms share a helicase domain, ATP binding domain and the regulatory Q motif (Fig. S1). Rm62 80 has an additional 144 amino acid N-terminal extension of no known sequence homology (Figure 1A). Within the N-terminal extension is a nucleolar localization signal similar to the one found in the 72 kDa isoform of Xrp1 (Figure 1B).

We used two independent sub-cellular fractionation protocols (see methods and materials). In each case, tubulin was used to monitor the quality of the fractionation. Immunoblot analysis of enriched cellular fractions revealed the Rm62 M isoforms could be detected in both the cytosol and nucleus. This observation is consistent with demonstrated dynamic shuttling of the mammalian DDX5/p68 between the nucleus and cytosol (Figure 2A) (Wang et al., 2009). In contrast Rm62 could only be detected in the nucleus. Once localized to the nucleus, both Rm62 M and Rm62 80 can associate with chromatin. These data are consistent both previous immunofluorescence experiments (Figure 2B) (Buszczak and Spradling, 2006; Lei and Corces, 2006).

Rm62 is required for DSB repair

IRBP18 and Xrp1 are required for proper DSB repair. Since Rm62 is a member of the IRBP complex we next asked if Rm62 could also function to direct DNA repair after double strand breaks (DSB) induced by the radiomemic Methyl methanesulfonate (MMS). Many of the alleles in the Rm62 complementation group are homozygous or transheterozygous lethal. We used the hypomorphic allele $Rm62^{CB02119}$, which encodes an in-frame green fluorescence protein fusion with Rm62 80 . Flies homozygous for this allele are viable but display female sterility (Buszczak and Spradling, 2006). To determine a role for Rm62 in DNA repair, we crossed heterozygous siblings ($Rm62^{CB02119}/TM3^{sb, ser}$), allowed the females to lay for 24 hours and treated 3rd instar larvae with increasing amounts of MMS (Figure 3A). The expected ratio of this cross is 2:1 heterozygous to homozygous adults. If RM62 is sensitive to these DNA damaging agents then ratio of heterozygous adults should increase as the homozygous population dies. Homozygous $Rm62^{CB02119}$ flies are sensitive to MMS with maximum effect observed at an intermediate concentration of 0.0015% (v/v %) (Figure 3B). The null allele of IRBP18 displayed similar sensitivity at this concentration as the $Rm62^{CB02119}$ allele. We conclude from this experiment that Rm62 functions to promote efficient DSB repair. Additionally IRBP18 ($CG6272^{WHf05006}$) null allele displays partial synthetic lethality with $RM62^{CB02119}$ (Figure 4). This result suggests IRBP18 and Rm62 interact genetically for efficient DSB repair.

DNA repair properties is encoded in the Rm62 80 isoform

In a cell culture-based approach for assaying DNA repair we tested the contribution of the various isoforms of Rm62 by employing RNAi to knockdown either the Rm62 M or Rm62 80 isoforms (Min et al., 2004). We designed dsRNA against an exon (exon 7) shared between 8 of the 9 isoforms and an exon specific for the Rm62 80 isoform (Figure 5A) RNAi treatment of the shared exon resulted in a modest but significant decrease in the steady state levels of all isoforms. The isoform specific RNAi resulted in complete knockdown of Rm62 80 (Figure 5A). The shared isoform RNAi resulted in a marginal decrease in repair efficiency (65% of control), in contrast the 80kDa isoform had a more severe effect (33% of control) (Figure 5B).

The relative abundance of Rm62 80 is far less than the 62kDa isoforms. We next asked if the level of Rm62 80 is increased in response to DNA damage. We treated TAP-tagged IRBP18 S2 cells with 20 Gy of IR and allowed the cells to recover for 30 min after treatment (Figure 5C). We observed an increase in the steady state levels of Rm62 80. Consistent with observation is the presence of p53 response elements located upstream of both Rm62 and DDX5 genes (Figure 5D). In sum, we conclude that Rm62 is a gene required for DSB repair and majority of that function is encoded by the Rm62 80 isoform.

Involvement of Ago2 in DNA repair

Genome wide analysis of RNAi pathways revealed both IRBP18 and Xrp1 as putative modulators of miRNA, siRNA and endo-siRNA pathways (Zhou et al., 2008). Other members of the IRBP complex have established roles in RNAi summarized in Figure 6A. Specifically, cytosolic Rm62 is essential for RNAi as it is in complex with Argonaute2 (Ago2) and *Drosophila* fragile X mental retardation protein (dFmr1) (Ishizuka et al., 2002). RNAi knockdown of Rm62 reduced cellular RNAi efficiency. Additionally Ago2 and Dicer-2 play a critical role in genome stability (Peng and Karpen, 2007; Peng and Karpen, 2009). We therefore asked if knockdown of Ago2 could reduce repair efficiency. RNAi knockdown resulted in a two-fold decrease in repair activity (Figure 6B). These data are consistent with a larger role the IRBP complex subunits in a connection between RNAi and DNA repair pathways.

Discussion

Here we have demonstrated a role for the DEAD box RNA helicase Rm62 in DSB repair. Given the established roles for Rm62 in regulating chromatin structure, transcription, pre-rRNA production, mRNA splicing, and RNAi, our observation that it participates in DSB repair is not entirely unexpected. The novel and surprising aspect is the majority of the repair activity is encoded in the Rm62 80 isoform. A role in DNA repair by Ago2 suggests a larger role for the IRBP complex in connecting RNAi pathways, genome stability, and DSB repair.

Very little information is known about the molecular events that regulate the relative abundance of the Rm62 80 isoform. We have identified putative p53 response elements in the promoters of both Rm62 and DDX5/p68. We show evidence that Rm62 80 levels increase at least two fold. This could potentially place Rm62 within p53 signaling pathway along with Xrp1 and IRBP18 (Figure 6C). Additionally any increase in transcription from the Rm62 promoter would need to be coordinated with efficient splicing of the Rm62 80 isoform. The identity of these factors and the interaction of these two pathways is a critical next step in understanding how global DNA damage response integrates cellular signals both at the break site and distal locations throughout the cell.

Creation of small RNAs is an important aspect of the DDR. In neurospora crassa, creation of DSB induces the expression of an argonaute protein, QDE-2 and subsequent expression of 20-21nt RNAs called qiRNA (Lee et al., 2009). Disruption of this pathway results increased sensitivity to DSB agents. Interestingly 86% of qiRNA are derived from rDNA sequences. It is not clear if this pathway is conserved in *Drosophila*or what the downstream effectors are. Importantly, DDX5 is in a stable complex with Drosha and p53, this complex is necessary for post-DNA damage induced maturation of miRNAs (Suzuki et al., 2009).

A significant function of RISC is the maintenance of rDNA heterochromatin, by depositing H3K9me2 modifications. The role of rDNA organization and copy number has recently come to be appreciated as an important regulator of global genome stability. *Drosophila*Ago2 and dcr-2 have direct roles in regulating the depositing H3K9me2; loss of this heterochromatic mark increase incidents of spontaneous DSBs, illegitimate recombination, and number of nucleoli (Peng and Karpen, 2007; Peng and Karpen, 2009). In yeast decrease in rDNA copy number increase sensitivity to MMS, and in *Drosophila* global chromatin structure was altered in response to loss of rDNA copies (Ide et al., 2010; Paredes and Maggert, 2009). It would appear that RNAi pathways are at the nexus of chromatin regulation, genome stability and DNA repair.

The relationship between the IRBP complex and RISC is a direct one. First large scale genome screening have identified IRBP 18 and Xrp 1 as regulators of endogenous siRNAs and miRNAs abundance. Second, purification of the IRBP complex revealed the

presence several known subunits of RISC. Third, IRBP18 and Rm62 share a similar perinucleolar structure similar to the Xist non coding RNA involved in X chromosome silencing (Chapter 2) (Buszczak and Spradling, 2006; Zhang et al., 2007). Lastly, Dicer 2 mutants exhibit reduced H3K9me2 in a subset of loci, that correspond to IRBP18 and Xrp1 ChIP signals within the 18S rDNA gene (Peng and Karpen, 2007).

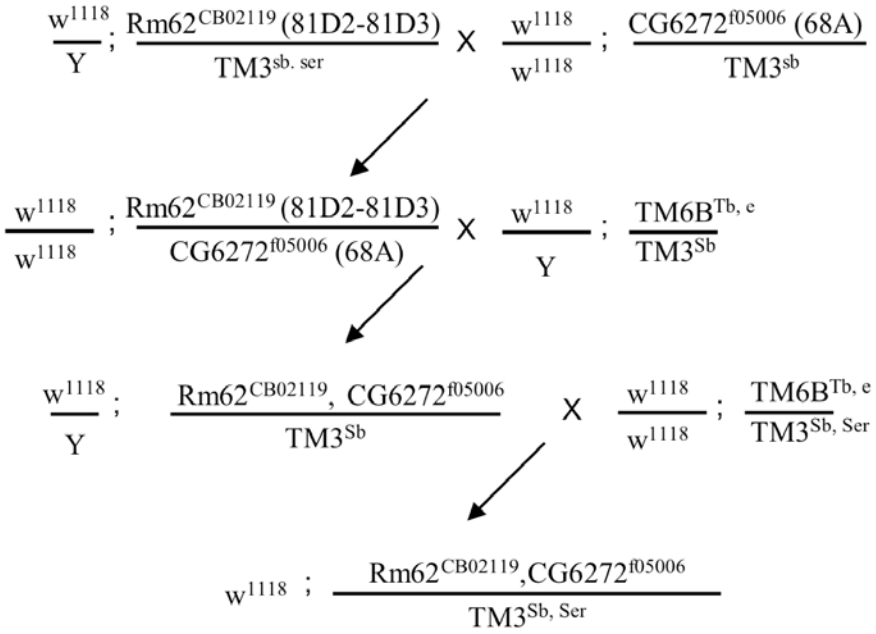
The evidence for RNAi as a critical player in maintaining genome stability in *Drosophila* is clear. What is not clear is how the RNAi machinery gets localized to sites in the genome. One of the surprising aspects of the IRBP complex is the presence of several proteins directly involved RNAi production and function. We propose that IRBP18 and Xrp1 anchor RNAi machinery to the specific sites in genome. The direct consequence of this would be coordinate heterochromatin formation at repeated sequences like the rDNA locus. More importantly this may be a coordinated with p53 function in response to DNA damage.

Experimental Procedures

Drosophila Strains

The *Drosophila* strains used were w^{1118}; $CG6272^{WHf05006}$/TM3, sb, w^{1118}; $Rm62^{CB02119}$/TM3, sb, ser (Bloomington Stocks), w1118; TM6B, tb, e/TM3sb, ser, w1118; TM6B, tb, e/TM3, sb.

The recombinant chromosome was created by the following crosses:



MMS test

w1118; Rm62CB02119/TM3 Sb, Ser flies were self crossed and at the 3rd instar larva stage treated with MMS (Sigma), at the indicated doses.

Nuclear/Cytosolic Fractionation

Whole-cell lysates were prepared by harvesting cells, washing once with 1× PBS, and lysing cells in 1× SDS sample buffer. Nuclear extracts were prepared as follows. Approximately 10^8 cells were harvested and washed once with 1× PBS. All subsequent steps were performed at 4°C or on ice. The cells were resuspended in 2 packed-cell pellet vol of buffer I (15 mM HEPES-KOH pH 7.6,10 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 350 mM sucrose, 1 mM DTT, 0.2 mM PMSF). Triton X-100 was added to 0.1%, and cells were processed with a Dounce homogenizer. Nuclei were then pelleted

at 7,650 × *g* and resuspended in 4 packed-cell pellet vol of buffer AB [15 mM Hepes pH 7.6, 110 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.2 mM PMSF,1× protease inhibitor. Pelleted nuclei were resuspended in 1X sample buffer.

Subcellular Fractionation Chromatin Fraction

S2 cells were prepared according to (Mendez and Stillman, 2000) or and blotted with Fibrillarin (1:2000), IRBP18 (1:2000), (Rm62 1:3000 Gift from Spradling Lab) and Tubulin (1:5000).

Double-Stranded RNA Synthesis and RNAi

Synthesis of double-stranded RNA and treatment of S2 cells was performed exactly as described (Min et al., 2004) . The sequences of the Rm62 coding sequence PCR primers, including T7 RNA polymerase promoter sequence were: Exon 7 forward 5'-TAA TAC GAC TCA CTA TAG GGA GGA CGA GGA ACC GAA TTC GGT GGC- 3' and reverse 5'-TAA TAC GAC TCA CTA TAG GGA GGA CCA GGA GCA CCC TAA CGT AGC-3' Rm62 80 Forward 5'-TAA TAC GAC TCA CTA TAG GGA GGA GAC GAG GTT AGG TAA GAC ACG-3' and Reverse 5'-TAA TAC GAC TCA CTA TAG GGA GGA GAG CTG TCT CGA AAC GCA CGC-3'

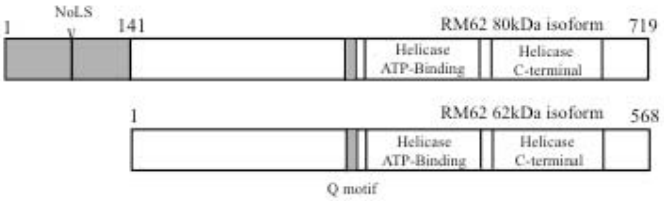
RNAi and P Element Excision and Repair Assay

2.5×10^6 L2 cells were plated in 60-mm tissue culture dishes and 1 hour in 5% FBS-M3 medium. The cells were washed with 2 ml of serum-free M3 medium and overlaid with 1 ml of serum-free M3 medium. Ten micrograms of dsRNA in ddH_2O was added. After a 1h incubation, 1 ml of 10% FBS-M3 medium was added to make final 5%FBS-M3. The cells then were kept in an incubator for 48h before another dsRNA tratment. Cells were then transfected 1 microgram each of the reporter and transposase source (Effectene transfection reagant, Qiagen). The number of ampicillin-resistant colonies per ml of bacteria in SOC medium gives the estimate of total plasmids collected, and the number of kanamycin- and ampicillin-resistant colonies per ml of bacteria in SOC medium provides an estimate of reporter plasmids that are excised and repaired. The ratio of repaired plasmids to total plasmids gives the excision and repair activity.

Figure 1 Rm62 80 has a nucleolar localization sequence

(A) Schematic of the Rm62 protein isoforms. Rm62 M and Rm62 80 share a regulatory Q motif, and helicase domains. Rm62 80 encodes and additional 144 amino acid extension that contains a NoLS. (B.) The sequence is similar to the two found in another IRBP complex member Xrp1 (72kDa isoform).

A.



B.

RRXR-Nucleolar Localization Sequence

71 80
QQFHGSRRNR RM62 80kDa isoform

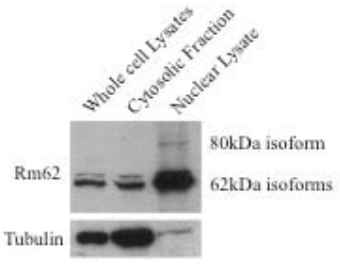
51 60
CSQRRTRPTE #1

Xrp1 72kDa isoform

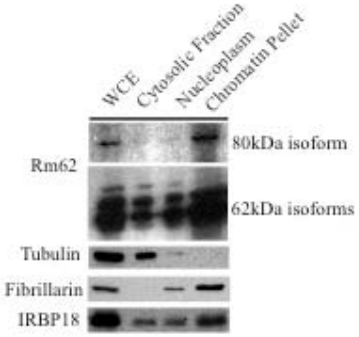
171 180
SARRCRISTY #2

Figure 2 Rm62 80 is localized to the nucleus.
(A.) Subcellular fractionation of S2 cells demonstrated that Rm62 80 is localized to the nucleus. (B.) In a separate approach Rm62 80 Rm62 is enriched in the chromatin fraction. (C.) summary of subcellular localization of Rm62 and DDX5/p68.

A.



B.

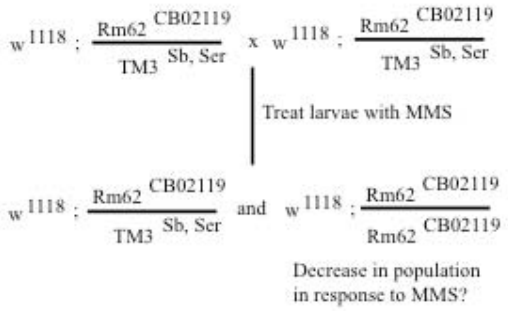


C.

	Human p68/DDX5	Rm62 (80kDa)	Rm62 (62kDa)
Nuclear	Yes	Yes	Yes
Nucelolar	Yes	Yes - perinucleolar	Not Known
Cytoplasm	Yes	No	Yes

Figure 3 Rm62 is a DNA repair gene
(A.) Rm62CB02119/TM3sb, ser siblings were self-crossed and treated with MMS at the 3rd instar larvae stage. (B.) Results of MMS treatment.

A.


w1118 ; Rm62 CB02119 / TM3 Sb, Ser x w1118 ; Rm62 CB02119 / TM3 Sb, Ser
Treat larvae with MMS
w1118 ; Rm62 CB02119 / TM3 Sb, Ser and w1118 ; Rm62 CB02119 / Rm62 CB02119
Decrease in population in response to MMS?

B.

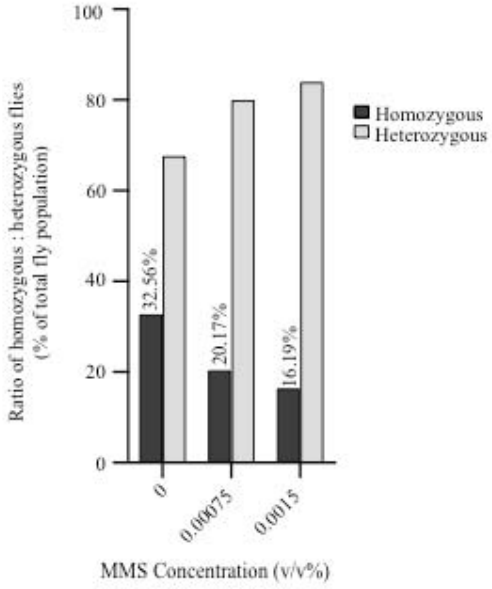
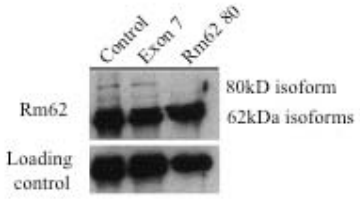

Ratio of homozygous : heterozygous flies (% of total fly population)
100
80
60
40
20
0
32.56%
20.17%
16.19%
Homozygous
Heterozygous
0
0.00075
0.0015
MMS Concentration (v/v%)

Figure 4 Rm62 mutant flies are partial synthetic lethal with IRBP18 null allele.

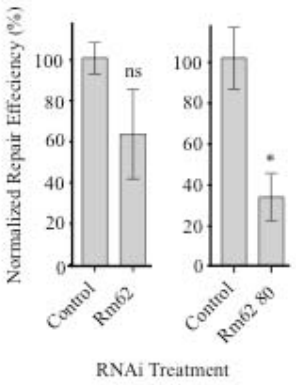
Genotypes	Viability
$Rm62^{CB02119}/Rm62^{CB02119}$	100%
$CG6272^{WHf05006}/CG6272^{WHf05006}$	100%
$Rm62^{CB02119}, CG6272^{WHf05006}/Rm62^{CB02119}, CG6272^{WHf05006}$	57%

Figure 5 DNA repair properties of Rm62 are encoded in the Rm62 80 isoform. (A.) S2 cells were treated with dsRNA for 96 hours followed by nuclear/cytosolic fractionation. Nuclear lysates were then immunoblotted. The dsRNA directed against a common (exon 7) or the Rm62 80 isoform was completely knocked down. (B.) S2 cells were treated the dsRNA for 96hours prior to transfection with a transposase source and the reporter assay. Recovered plasmids were counted and the normalized repair efficiency was scored as the number of colonies in the kanamycin + ampicillin divided by the number of colonies on ampicillin normalized to the control treatment. Knockdown of Rm62 80 significantly reduced DNA repair. (C.) Immunoblot analysis of nuclear extracts treated with IR. (D.) Sequence analysis of both DDX5 and Rm62 promoters revealed putative p53 response elements.

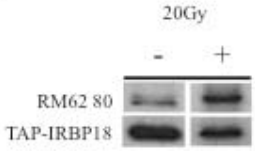
A.



B.



C.



D.

Putative p53 Response Elements

Drosophila RM62 -706

AGACTTGAAAT(n8)AAGCAAGTTC

Human DDX5/p68 -201

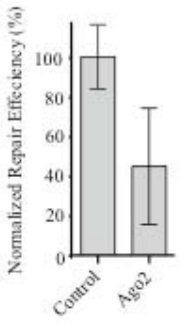
GGGCTTGGGA(n11)TGGCTTGCTT

Figure 6 IRBP complex connects RNAi to DNA repair.
(A.) Summary of IRBP complex members and known functions. Many members have well characterized roles in RNAi. (B.) Quantitation of DNA repair efficiency after knockdown of Ago2 (C.) Model for connecting p53 pathway to Rm62 function.

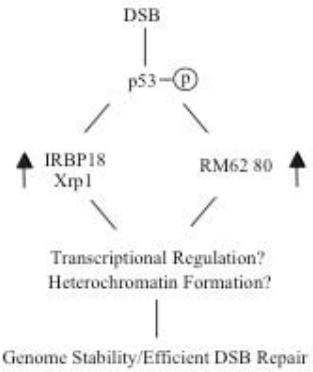
A.

IRBP Members	Nucleolar Localization	Pathway/Process: DNA Repair	Genome Stability	RNAi
IRBP18	Yes	Yes	Yes	Yes
Xrp1	Yes	Yes	Yes	Yes
RM62	Yes	Yes	Yes	Yes
HSC70-3	-	-	-	Yes
HSC70-4	-	-	-	Yes
Fmr1	-	-	-	Yes
Fibrillarin	Yes	-	-	-
Nop56	Yes	-	-	-
Nop60B	Yes	-	-	-
Nop5	Yes	-	-	-
Zipper	-	-	-	-

B.



C.



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