

A NOVEL INTERACTION BETWEEN MENIN AND THE PAF COMPLEX

**IDENTIFICATION OF A NOVEL INTERACTION BETWEEN MENIN AND
THE PAF COMPLEX**

By

JENNIFER ERICKSON, BSc

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AUTHOR: Jennifer Erickson, B.Sc. (Dalhousie University)

SUPERVISOR: Dr. Ana Regina Campos and Dr. André Bédard

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Abstract:

Multiple Endocrine Neoplasia Type 1 (MEN1) is an inherited cancer syndrome characterized by tumour formation in two or more endocrine tissues. Tumours arise due to a biallelic loss of the *MEN1* tumour suppressor gene, consistent with the “two hit” loss of function, in which the first “hit” occurs in the germline and the second in somatic cells. *MEN1* encodes a 67kDa protein, menin, which functions in a variety of cellular processes including transcription regulation, cell cycle control and genome stability. The *Drosophila melanogaster* homolog of menin is an 83kDa protein sharing many of the amino acids that routinely exhibit mutations in MEN1 patients. To identify novel menin-interacting proteins, a mass spectrum analysis was performed and Atu was identified. Atu (dLeo1) is the *Drosophila melanogaster* homolog of Leo1, a member of the RNA Polymerase II associated factor (PAF) complex. PAF functions as a platform in transcription elongation, recruiting proteins to RNA Polymerase II facilitating such processes as histone modification, 3' end formation and mRNA surveillance. Studies were undertaken to verify the interaction between menin and dLeo1 in the *Drosophila melanogaster* model system, and determine the role of the interaction in maintaining genome stability in response to stress. The menin-dLeo1 interaction was confirmed, observed following non-genotoxic stress. dLeo1 and dPaf1, and additional member of PAF, were in turn found to play a role in maintaining genome stability. Loss of dLeo1 in particular results in increased genome instability even under physiological conditions. Thus a novel menin-interacting protein was identified, which in turn will contribute to the understanding of menin as a tumour suppressor.

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List of Abbreviations:

<i>18w</i>	<i>18 wheeler gene</i>
6AU	6-azauracil
AEL	after egg laying
AP-1	activator protein 1
APC	adenomatous polyposis coli
AP ^r	ampicillin resistance
ASK	activator of S-phase kinase
Atms	antimeros
ATP	adenosine triphosphate
Atu	another transcription unit
BDGP	Berkeley Drosophila Genome Project
BLAST	Basic Local Alignment Search Tool
BM-TNE	Bart's Modified TNE buffer
BRCA1	breast cancer 1, early onset
BSA	bovine serum albumin
CAM ^r	chloramphenicol resistance
Ches1	checkpoint suppressor 1
COMPASS	complex proteins associated with Set1
CPF	cleavage and polyadenylation factor
CPSF	cleavage and polyadenylation specificity factor
CRM1	chromosome region maintenance 1
CstF	cleavage stimulation factor
dCBP	<i>Drosophila</i> CREB-binding protein
DGRC	Drosophila Genomics Resource Center
DRB	5,6-Dichlorobenzimidazole 1- β -D-ribofuranoside
dsDNA	double stranded DNA
DSIF	DRB sensitivity inducing factor
ECL	enhanced chemiluminescence
ER α	estrogen receptor α
FA	Falconi anemia
FACT	facilitates chromatin transcription
FANCD2	Fanconi anemia, complementation group D2
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GST	glutathione S-transferase
HCE	human capping enzyme
HDAC	histone deacetylases
HMTase	histone methyltransferase
Hox	homeobox
HPT-JT	hyperparathyroidism jaw tumour syndrome

HRP	horseradish peroxidase
<i>HRPT2</i>	<i>hereditary hyperparathyroidism type 2</i>
hs-GAL4	heat shock GAL4
Hsp	heat shock protein
hTERT	human telomerase reverse transcriptase
IGFBP-2	insulin-like growth factor-binding protein 2
IL-8	interleukin 8
IR	ionizing radiation
lacZ	β -galactosidase
LB	Luria broth
LEDGF	lens epithelium-derived growth factor
LOH	loss of heterozygosity
LEDGF	lens epithelium-derived growth factor
MCM	minichromosome maintenance complex
MEF	mouse embryonic fibroblasts
MEN1	Multiple Endocrine Neoplasia Type 1
<i>MEN1</i>	<i>Multiple Endocrine Neoplasia Type 1 gene (human)</i>
<i>Men1</i>	<i>MEN1</i> homologous gene (murine)
MEN2	Multiple Endocrine Neoplasia Type 2
MAPK	mitogen-activated protein kinase
MLL	mixed lineage leukemia
<i>Mnn1</i>	<i>MEN1</i> homologous gene (<i>Drosophila</i>)
mwh	multiple wing hair
NCBI	National Center for Biotechnology Information
NELF	negative elongation factor
NES	nuclear export signal
NF κ B	nuclear factor κ B
NLS	nuclear localization signal
Nrd1	nardilysin
PAF	Polymerase II associated factor complex
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	P element transposon used as part of the BDGP Gene Disruption Project to disrupt gene function. Contains <i>white</i> ⁺ gene and exhibits kanamycin resistance.
PlacW	P element transposon used as part of the BDGP Gene Disruption Project. Contains <i>white</i> ⁺ gene, ampicillin resistance, <i>lacZ</i> and an <i>Hsp70</i> promoter.
P-TEFb	positive transcription elongation factor b
PZ	P element transposon used as part of the BDGP Gene Disruption Project. Contains <i>rosy</i> ⁺ gene, kanamycin resistance, <i>lacZ</i> and an <i>Hsp70</i> promoter.
qPCR	quantitative polymerase chain reaction
Ras	rat sarcoma

RNAi	RNA interference
RNA Pol II	RNA Polymerase II
RPA	replication protein A
RTK	receptor tyrosine kinase
SII	transcription elongation factor A
SBF	Swi4/Swi6 transcription factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser2 RNA Pol II	Serine 2 phosphorylated RNA Polymerase II
Ser5 RNA Pol II	Serine 5 phosphorylated RNA Polymerase II
shRNA	short hairpin RNA
siRNA	small interfering RNA
SKI	super killer complex
snoRNA	small nucleolar RNA
SOC	super optimal broth with catabolite repression (SOB media + glucose)
SOS	Son-of-sevenless
T _A	annealing temperature
TAC1	Trithorax acetylation complex 1
TBS	Tris buffered saline
TFIIB	transcription factor II B
TFIIF	transcription factor II F
TFIIS	transcription Factor II S
TGF-β	transforming growth factor type β
Trx	trithorax
TSA	trichostatin A
UAS	upstream activating sequence
UTR	untranslated region
UV	ultraviolet
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor

Introduction

1.0 Menin – Human Disease and Gene:

1.1 Multiple Endocrine Neoplasia Type 1

Multiple Endocrine Neoplasia Type 1 (MEN1) is a hereditary tumour syndrome characterized by tumour formation in two or more endocrine tissues, such as the parathyroid, pituitary and adrenal glands. MEN1 can be described as an autosomal dominant syndrome. Though it is a relatively rare disease, penetrance approaches 100% in patients inheriting a mutant allele (Dreijerinki et al, 2006). There are six multiple endocrine neoplastic syndromes that, like MEN1, manifest in predominantly endocrine tissues. Multiple Endocrine Neoplasia Type 2 (MEN2) is one such neoplastic syndrome that arises as a result of gain of function mutations in the rearranged during transfection (RET) protooncogene (reviewed by Marx, 2005). MEN1 differs from MEN2 and other neoplastic syndromes in that there are no cures or preventative treatments (Brandi et al, 2001).

MEN1 tumours can be categorized as hormone-producing and hormone-nonproducing tumours. Hormone-producing tumours account for the widest variety of tumour types, and include enteropancreatic tumours, including gastrinomas, and insulinomas, foregut carcinoids, anterior pituitary tumours and prolactinomas (Agarwal et al, 2004). Among MEN1 patients, the most common tumour type observed is parathyroid adenoma, exhibiting a 90% penetrance among patients, and generally the first clinical manifestation of the disease appearing between 20 and 25 years of age (Brandi et al, 2001). Gastrinomas, exhibiting a 40% penetrance by age 40, have the highest malignant potential of the hormone-producing tumours. Hormone-nonproducing tumours

include facial angiofibromas, truncal collagenomas, lipomas and meningiomas (Agarwal et al, 2004). Patient mortality generally arises due to excess secretion of several hormones, including gastrin, insulin and prolactin. In some cases, it may also be due to secondary effects of tumour mass and malignancy (Brandi et al, 2001).

1.2 Structure of the MEN1 gene

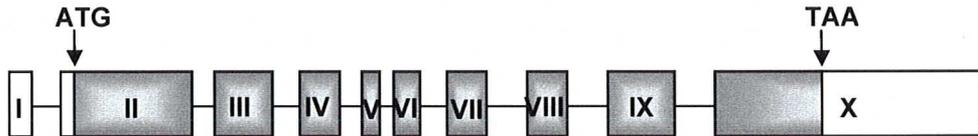
The first genetic information about MEN1 came about in 1988, when through genetic linkage analysis of four families with a history of MEN1, Larsson et al (1988) localized the gene responsible for MEN1 to chromosome 11q13. It would be another decade until the *MEN1* gene was identified (Chandrasekharappa et al, 1997). Through positional cloning, Chandrasekharappa et al (1997) identified a gene extending 9kb yielding a 2.8kb transcript. The gene, *MEN1*, consists of 10 exons, the first of which is non-coding. It encodes a 610 amino acid protein, menin, with a molecular weight of 67kDa (**Fig. 1.1A**). The protein did not demonstrate similarities to any other protein, providing few clues as to its normal function or subcellular localization.

Analysis of subcellular fractions and immunofluorescence demonstrated menin to be a primarily nuclear protein. Sequence analysis revealed two nuclear localization signals at the C-terminus of the protein, NLS1 (amino acids 479-497) and NLS2 (588-608) (Guru et al, 1998). Each signal appears to act independently, as menin continues to localize to the nucleus even with the deletion of a single NLS. However deletion of both NLS1 and NLS2, while reducing menin localization to the nucleus, was not sufficient to completely abolish it (La et al, 2004). Further sequence analysis revealed an accessory

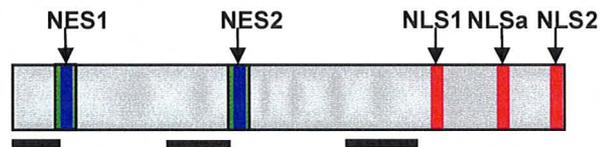
Figure 1.1: The human *MEN1* gene and its *Drosophila melanogaster* homolog *Mnn1*. (A) *MEN1* is a 9kb gene consisting of 10 exons, the first of which is non-coding. The 2.8kb transcript of *MEN1* encodes a 610 amino acid protein, menin, with a molecular weight of 67kDa. Menin consists of 3 NLSs (NLS1, NLS2 and NLSa) at the C-terminus of the protein and 2 NESs (NES1 and NES2) located in the leucine-zipper like regions towards the N-terminus. (B) The *Drosophila melanogaster* homolog of *MEN1*, *Mnn1*, is a 7kb gene consisting of 5 exons, the first of which is non-coding. The 4.3kb transcript encodes a 763 amino acid protein measuring 83kDa. Menin shares a 46% identity with MEN1. The second NLS, NLS2, is conserved in menin as is the 2nd of 3 JunD binding sites from MEN1. Genomic organization: coding regions are shaded in gray, and the start (ATG) and stop (TAA) codons are indicated. Protein schematics: NLSs are represented by red lines, NESs by blue lines and leucine-zipper like regions by green lines. JunD binding sites are indicated by black lines beneath the schematic.

A Human MEN1:

Genomic organization:

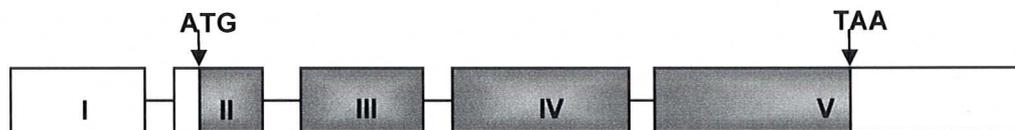


Protein:

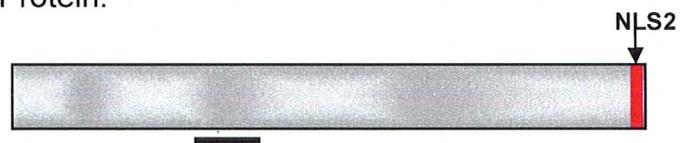


B Drosophila melanogaster Mnn1:

Genomic organization:



Protein:



NLS, NLSa, consisting of four basic residues between amino acids 546 and 572 (La et al, 2006). Point mutations in individual NLSs were not sufficient to affect nuclear localization; however mutations in all three prevented it. Thus, each NLS functions redundantly (La et al, 2006).

A role apart from nuclear localization has been implied for the NLSs. They may also have a role in positively and negatively regulating transcription. La et al (2006) demonstrated that loss of any NLS, through incorporation of point mutations, resulted in menin's inability to reduce both IGFBP-2 mRNA and protein levels in the cell, likely via its interaction with the *IGFBP-2* promoter region. Conversely, expression of *caspase 8*, a gene demonstrated to be induced by menin, was not activated when NLS sequences were mutated. Thus the authors suggested that the NLSs of menin may aid in the recruitment of positive and negative transcriptional regulators, stabilizing interactions between menin and other proteins with gene promoters by virtue of their ability to bind dsDNA (La et al, 2006; La et al, 2004).

Recently, two nuclear export signals (NES) were identified in the leucine zipper-like regions of the *MEN1* gene, NES1 (amino acids 22-41) and NES2 (amino acids 258-267). Analysis of menin homolog sequences revealed these regions to be conserved between species (Cao et al, 2009). Homologous sequences have not yet been identified in *Drosophila*. Menin exhibited a primarily cytoplasmic localization in NES-GFP transfected cells, and heterokaryon formation assays fusing HeLa cells expressing menin-GFP to *Men1*^{-/-} MEFs demonstrated GFP-tagged menin migrating to MEF nuclei. Treatment with a nuclear export inhibitor prevented menin migration to the cytoplasm.

Thus, NESs appear to work in conjunction with NLSs to facilitate nuclear-cytoplasmic shuttling of menin, confirmed by fluorescence recovery after photobleaching (FRAP) analysis of GFP-menin expressing *Men1*^{-/-} MEFs (Cao et al, 2009).

1.3 Mutations of the MEN1 gene

MEN1 tumour formation arises following a biallelic loss of *MEN1*, consistent with the two-hit loss of function model proposed by Knudson (1971) following his study of retinoblastoma patients. The first “hit” occurs as a mutation of *MEN1* in germinal cells, rendering one copy of the gene nonfunctional. These mutations include nonsense mutations, frameshift deletions and missense mutations (Pannett and Thakker, 1999). The second “hit” involves the loss of the remaining copy of the gene in somatic cells. The second “hit” generally occurs as a loss of heterozygosity (LOH) involving the region of chromosome including region 11q13 or as somatic point mutations (Larsson et al, 1988; Pannett and Thakker, 2001). Sporadic development of MEN1, in which a germline mutation of the *MEN1* gene is not inherited, is a result of both mutations occurring in somatic cells.

In the decade following discovery of the *MEN1* gene, 1,336 mutations have been identified, of which 1,133 are germline and 203 are somatic (Lemos and Thakker, 2008). The mutations were scattered throughout the coding region, although there appears to be a cluster of mutations in the second exon (Agarwal et al, 2004). Many of these mutations are predicted to truncate the menin protein, resulting in the loss of NLSs and other functional domains in the C-terminus, or reduce protein levels due to nonsense-mediated

mRNA decay (Lemos and Thakker, 2008; Khajavi et al, 2006). There is no genotype-phenotype correlation between the various mutations and disease development.

1.4 MEN1 is specific to endocrine tissues

Menin is a ubiquitously expressed protein, however its tumourigenic effects are observed only in endocrine tissues. Non-endocrine tissues, such as the liver, can withstand the biallelic loss of *MEN1* without undergoing tumourigenesis (Scacheri et al, 2004). The specificity of MEN1 to endocrine tissues is not well understood, however several mechanisms have been proposed. Menin may influence the function of nuclear receptors, such as estrogen receptor α (ER α) and vitamin D receptor (VDR), involved in endocrine processes, or via its association with mixed lineage leukemia (MLL) and involvement in the canonical Wnt signaling pathway (reviewed by Gracanin et al, 2009). Sierra et al (2006) demonstrated that the MLL-menin complex is recruited to β -catenin, resulting in histone H3 lysine 4 methylation at Wnt target genes, such as *c-Myc*. Furthermore, Wnt signaling has been implicated in the regulation of cell proliferation and hormone secretion in several endocrine tissues, including pancreatic β cells, the pituitary gland and enteroendocrine tissues (Rulifson et al, 2007; Davis et al, 2009; García-Martínez et al, 2009). Thus it is possible that the tissue-specific nature of MEN1 may in part be due to the association of the MLL-menin complex with the canonical Wnt signaling pathway.

2.0 Model Systems of MEN1 Loss-of-Function

2.1 The murine *MEN1* model system

Since the identification of the *MEN1* gene, homologs have been identified in a variety of organisms, including: mouse, rat, zebrafish and *Xenopus laevis* (Chandrasekharappa and Teh, 2003). Mouse, rat and zebrafish menin homologs exhibit the highest similarity to human menin, sharing 96.7%, 97.2% and 67% sequence similarity respectively. However, homologous sequences have not been identified in the less complex model organisms *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Chandrasekharappa and Teh, 2003).

To enable further study on the menin protein and its role in tumorigenesis, a murine model was generated (Crabtree et al, 2001). A number of similarities exist between human and murine menin, suggesting that mice would make an ideal model system in which to study tumorigenesis. The murine homologue, *Men1*, has a similar construction to *MEN1* in that both consist of 10 exons, with the first being non-coding. The cDNA demonstrates an 89% sequence similarity across the coding region, and the 611 amino acid protein exhibits a 97% amino acid identity with human menin (Stewart et al, 1998).

Crabtree et al (2001) attempted to construct conditional knockout mice through homologous recombination. Two *Men1* loss-of-function alleles were created, *Men1^{TSM}* and *Men1^{ΔN3-8}*. *Men1^{TSM/+}* mice carry a neomycin cassette inserted in the second intron of *Men1* that renders the gene nonfunctional. *Men1^{ΔN3-8/+}* mutants were created by crossing heterozygous mice carrying the TSM allele to a line ubiquitously expressing Cre

resulted in excision of the region spanning from the neomycin cassette to a loxP site in the eighth intron, encompassing exons 3-8. Heterozygous mice, *Men1*^{TSM/+}, exhibited tumour formation similar in both incidence and distribution to MEN1 patients. Tumourigenesis in heterozygous mice results in pancreatic, pituitary and parathyroid adenomas and adrenal cortical tumours, all of which contribute to the MEN1 phenotype in patients. Homozygous null mice were found to be embryonic lethal. *Men1*^{TSM/TSM} and *Men1*^{ΔN3-8/ΔN3-8} mice were lethal at E14.5 and E10.5-11.5 respectively (Crabtree et al, 2001). Thus, despite similarities between human and murine genes encoding menin, embryonic lethality associated with homozygous loss of *Men1* create difficulties in studying the role of menin in promoting tumourigenesis.

2.2 Drosophila melanogaster as a MEN1 model system

The *Drosophila melanogaster* genome was published in its entirety in 2000 (Adams et al, 2000). Within the genome, homologs of mammalian oncogenes and tumour suppressors have been identified, and similarities exist between *Drosophila* and human regulatory pathways and cell cycle control (reviewed by Potter et al, 2000). This, coupled with their small genome size, has made *Drosophila* a useful model organism to study human diseases. In the case of MEN1, they are a suitable model to study the tumour syndrome.

Drosophila mutants to study MEN1 were constructed by several labs through P element imprecise excision (Busygina et al, 2004; Papaconstantinou et al, 2005; Cerrato et al, 2006). *Drosophila* mutants differ from murine models in that, unlike mice, flies homozygous for the mutation are both viable and fertile, permitting loss-of-function

studies (Busygina et al, 2004; Papaconstantinou et al, 2005; Cerrato et al, 2006). Unlike mammals, menin does not seem to be required for proper development in flies.

In addition to the viability of null mutants, *Drosophila* is an excellent model to study the gene behind MEN1 because of the range of tools available to researchers. One such tool is the Gal4-UAS system, originally derived from yeast (Fischer et al, 1988). Through the use of this system, gene expression can be manipulated in a spatial and temporal manner. Ectopic expression of UAS-containing constructs permits gene overexpression or reduction of protein levels through RNAi or dominant negative mutant expression. This system also permits protein visualization through lacZ or GFP production (reviewed by Phelps and Brand, 1998).

2.3 Drosophila as a model to study human tumour suppressors

Drosophila has proven to be an effective model organism in elucidating the function of proteins relevant to human disease, in particular the tumour suppressor p53. Human p53 is a tumour suppressor protein, lost in most human cancers that functions by inducing apoptosis and growth arrest (reviewed by Levine, 1997; Hainaut et al, 1998). Two *p53*-related genes have been identified in vertebrates, *p63* and *p73*, both of which contain domains similar to those in p53. Some isoforms of these proteins are capable of transactivating p53 target genes and inducing apoptosis (Kaelin, 1999).

In 2000, three groups simultaneously identified the *Drosophila* homolog of human *p53*, *dmp53* (Brodsky et al, 2000; Jin et al, 2000; Ollman et al, 2000). *Dmp53* demonstrates several similarities with its human counterpart with respect to its functional domains and its role in the cell. Both proteins consist of a well-conserved central DNA

binding domain, and less conserved N-terminal transactivation domain and C-terminal oligomerization domain (Ollman et al, 2000). Within the central binding domain of p53 there are mutational hotspots that result in a dominant negative form of the protein that prevent it from binding to DNA, as commonly seen in human tumours. The corresponding mutations in *dmp53* yield a similar fate, transforming the protein into a dominant negative unable to bind DNA (Brodsky et al, 2000; Jin et al, 2000).

Dmp53 functions like its human counterpart by inducing apoptosis after overexpression and in response to DNA damage following X-ray and UV radiation (Brodsky et al, 2000; Jin et al, 2000, Ollmann et al, 2000; Jassim et al, 2003). Research of *dmp53* also extends to the p53-related proapoptotic genes *Ark*, *hid* and *reaper*, and their expression in response to DNA damage. For instance, *hid* expression is induced following UV and IR exposure, however *Ark* and *reaper* expression were dependent on the type of DNA damage, UV and IR respectively (Ujfaludi et al, 2007). One important difference between human and *Drosophila* p53 is its role in promoting growth arrest. Overexpression of *dmp53* does not influence progression from G₁ to S phase, however expression of *decapo*, the *Drosophila* homolog of p21, blocks DNA synthesis. This suggests that p21 induction by p53 evolved later and the role of p53 in growth arrest is a more recently acquired function than its role in promoting apoptosis (Ollmann et al, 2000). In terms of complexity, *Drosophila* expresses one p53-like protein rather than three like humans. Thus, *Drosophila* is an excellent model with which to study p53 and its function, in part due to similarities in both protein structure and function.

Drosophila has also proven to be an effective model in the study of receptor tyrosine kinase (RTK) signaling, in particular the role of Son-of-sevenless (SOS). The importance of the Ras cascade *Drosophila* eye development has been established, in particular with respect to photoreceptor development (Simon et al, 1991). *SOS* encodes a guanine nucleotide exchange factor (GEF) homologous to yeast CDC25, which activates Ras and Rac (Bonfini et al, 1992; Chardin et al, 1993; Nimual et al, 1998). Ras in turn activates Raf which then initiates the mitogen-activated protein kinase (MAPK) cascade (reviewed by Avruch et al, 2001). *Drosophila* SOS is similar to the human protein with respect to the functional domains of the protein, including 5 of 6 putative MAPK phosphorylation sites at the C-terminus of mammalian SOS (Silver et al, 2004).

3.0 The *Drosophila melanogaster* MEN1 homolog, *Mnn1*

3.1 The *Mnn1* gene

The *Drosophila* homolog of *MEN1*, *Mnn1*, was simultaneously identified by two groups (Maruyama et al, 2000; Guru et al, 2001). The *Mnn1* gene consists of 5 exons, producing a 4.3kb transcript (**Fig. 1.1B**). *Mnn1* encodes a 763 amino acid protein with a molecular weight of 83kDa. *Mnn1* differs from other *MEN1* homologous genes in that it contains fewer exons, however the first exon is non-coding which is conserved amongst species. The *Drosophila* menin protein is larger than human menin (Guru et al, 2001). Difference in size between menin and other homologs can be attributed to non-homologous regions (amino acids 554-644 and 692-737) in the C-terminus of the protein (Maruyama et al, 2000).

Human and *Drosophila* menin display a 46-48% sequence identity, which at first glance appears low. In MEN1 patients, 90 amino acids routinely exhibit mutations, such as in-frame deletions and missense mutations. Of these, 62 are identical and a further 14 are conserved amino acid residues in *Drosophila*. A NLS homologous to NLS2 in human, rat and mouse menin was identified between amino acids 758-762. The second of three JunD binding sites in menin is also conserved in *Drosophila*, however *Drosophila* JunD (DJun) does not appear to interact with menin (Guru et al, 2001). In addition to the conserved NLS and JunD binding site, highly conserved regions spanning amino acids 123-146 and 267-296 were also identified, with identities between human, rat, mouse, zebrafish and *Drosophila* sequences surpassing 80% (Maruyama et al, 2000).

4.0 The Role of Menin in the Cell

Menin protein does not exhibit similarities to other proteins, making it difficult to elucidate its function in the cell and ultimately its role in controlling tumourigenesis (Chandrasekharappa et al, 1997). A number of interacting partners of menin have been identified, and through these interactions menin has been implicated as playing diverse roles in the cell (**Table 1.1**).

4.1 Menin as a tumour suppressor

Several lines of evidence indicate that menin is a tumour suppressor. In MEN1 patients, germline and somatic mutations are observed in the *MEN1* gene resulting in a loss of protein function often through truncation or absence altogether (Pannett and Thakker, 1999; Kikuchi et al, 2004). Both hereditary and sporadic tumours demonstrate LOH of the *MEN1* gene, in which the wild-type allele is lost (Larsson et al, 1988;

Table 1.1: Proteins demonstrated to interact with menin in both vertebrate and invertebrate systems. Analysis of the menin sequence reveals it to be unlike any other known proteins, thus its function is difficult to discern. Experimental evidence has identified a number of menin-interacting proteins, and through these interactions the function of menin is indicated. Menin-associated proteins are listed alongside the function implicated by the interaction, in addition to the primary literature in which the interaction was published.

Table 1: Proteins demonstrated to interact with menin in both vertebrate and invertebrate systems

Protein	Function	Reference
JunD	Transcription regulation	Yazgan and Pfarr, 2001
NFκB	Transcription regulation	Heppner et al, 2001
Smad3	Transcription regulation	Kaji et al, 2001
Pem	Transcription regulation	Lemmens et al, 2001
Rpb1 (Ser5P CTD)	Transcription regulation	Hughes et al, 2004
FANCD2	DNA repair	Jin et al, 2003
RPA2	DNA repair	Sukhodolets et al, 2003
ASK	Cell proliferation	Schnepp et al, 2004
dsDNA	Cell proliferation	La et al, 2004
Ches1	Cell proliferation	Busygina et al, 2006
MLL	Chromatin modification	Hughes et al, 2004
mSin3A	Chromatin modification	Kim et al, 2003
GFAP	Cytoskeletal protein	Lopez-Egido et al, 2002
Vimentin	Cytoskeletal protein	Lopez-Egido et al, 2002
β-Catenin	Nuclear-cytoplasmid shuttling	Cao et al, 2009
nm23	GTP hydrolysis	Yaguchi et al, 2002

Heppner et al, 1997). This is confirmed through immunohistological analysis of tumours from MEN1 patients and murine models demonstrating the loss of menin (Crabtree et al, 2001; Crabtree et al, 2003; Milne et al, 2005). In cell culture, cell proliferation is reduced upon overexpressing menin in RAS-transformed NIH3T3 cells and BON1 cells (Kim et al, 1999; Stålberg et al, 2004).

4.2 Menin plays a role in transcription regulation

Menin interacts with a variety of transcription factors, including JunD, NF κ B and Smad3, indicating that it plays a role in regulating transcription. JunD, a member of the AP-1 family of proteins, was identified as interacting with menin from a yeast two-hybrid screen (Agarwal et al, 1999). This interaction was confirmed *in vivo* through coimmunoprecipitation experiments and mammalian two-hybrid analysis in both yeast and mammalian cells. The interaction requires the N-terminus of menin, particularly residues 8-70, but not the C-terminus (Agarwal et al, 1999). Menin's ability to bind to other members of the AP-1 family of transcription factors was investigated, however none were found to interact. By binding to JunD menin represses transactivation by JunD, a response unique to JunD. Though menin does not directly interact with c-Jun, another member of the AP-1 family, coexpression of menin and c-Jun result in an increase of c-Jun-mediated transcription (Agarwal et al, 1999). The effect of menin on c-Jun is likely indirect, acting by influencing the action of activator proteins or through competition for such limiting factors (Ikeo et al, 2004). Two JunD isoforms have been isolated, a full length and a short isoform, expressed equally in terms of cell types, tissues and stoichiometry (Okazaki et al, 1998). Interestingly, menin only binds to and

suppresses the transactivation of the full length JunD, not the truncated form. The difference in binding capabilities is attributed to the menin binding domain of JunD is located in the first 48 amino acids, that is absent in the truncated form (Yazgan and Pfarr, 2001).

There is evidence that transcriptional repressors associate with histone deacetylases, repressing transcription by facilitating nucleosome formation (reviewed by Kuo and Allis, 1998). In fact, following treatment with the histone deacetylase inhibitor trichostatin A (TSA), menin was unable to repress JunD-mediated transcription. Thus, menin's repression of JunD activity may occur through recruiting HDACs (Gobl et al, 1999). Kim et al (2003) confirmed that menin binds to HDAC1, recruiting the complex through an interaction with mSin3A, a general transcriptional corepressor.

p50, p52 and p65, members of the NF κ B family of transcription factors, were identified as binding partners of menin through coimmunoprecipitation experiments. Similar to JunD, menin repressed transactivation of p65, and amino acids 305-381 of menin were essential for this interaction (Heppner et al, 2001). As menin may bind to both JunD and NF κ B proteins, menin could function as a bridge during transcription to promote or repress activation (Dreijerink et al, 2006).

Several genes have been identified containing both AP-1 and NF κ B binding sites, including: *vascular endothelial growth factor (VEGF)*, *IL-8*, *cyclin D1* and *hTERT*, which may be a target of JunD and NF κ B activity (Lin and Elledge, 2003; Bobrovnikova-Marjon et al, 2004; Dreijerink et al, 2006; Toualbi-Abed et al, 2008). The hTERT promotor contains JunD and NF κ B binding sites, and chromatin immunoprecipitation

demonstrated menin binding to those regions of the promotor. Downregulation of menin by shRNA led to increases in both hTERT expression and telomerase activity. Phenotypically, cells transfected with menin shRNA continued to proliferate and in essence became immortalized (Lin and Elledge, 2003).

A role for menin in transforming growth factor type β (TGF- β) signaling was identified with the discovery of the interaction between menin and Smad3 (Kaji et al, 2001). Knockdown of menin through antisense RNA resulted in an inhibition of TGF- β -induced transcription and increase in cell proliferation (Kaji et al, 2001; Ratineau et al, 2004). Transcription activation by TGF- β is mediated by phosphorylation of Smad2 or Smad3, which in turn associate with Smad4 and translocate to the nucleus, targeting specific genes (reviewed by Hendy et al, 2005). Through immunoprecipitation experiments an interaction was identified between menin and Smad3. Interactions were not observed between menin and the remaining Smad proteins, Smad2 and Smad4. The importance of this interaction lies in the association of the complex with DNA. Menin was found to enhance DNA binding of the Smad3 complex, as a reduction in binding was observed when menin was knocked down by antisense RNA (Kaji et al, 2001).

4.3 Menin may play a role in DNA repair

Menin has been shown to interact with FANCD2 and RPA2, and through these interactions has been implicated as having a role in DNA damage repair. This role in DNA repair is implied through its interaction with these proteins coupled with demonstrated sensitivity to various mutagens, and a direct role has not yet been demonstrated. An interaction between menin and FANCD2 was identified through

coimmunoprecipitation experiments (Jin et al, 2003). FANCD2 is a member of the Falconi anemia (FA) family of genes that function together to promote DNA damage repair (Timmers et al, 2001). When at least one of these genes is mutated, the result is hypersensitivity to DNA damage, in particular intrastrand DNA crosslinking. Clinically, this manifests itself as Falconi anemia, a disease characterized by birth defects, bone marrow failure and increasing susceptibility to cancer (Grompe and D'Andrea, 2001). Treatment of cells with γ -irradiation results in increasing FANCD2-menin interactions, peaking 4 hours after exposure. This suggests that menin functions in DNA damage repair through its interaction with FANCD2. When cultured with an intrastrand DNA crosslinker, *MEN1*^{-/-} cells exhibit decreased survival and a higher number of chromosomal aberrations similar to those in FA cells, when compared with cells expressing endogenous levels of the protein. This provides evidence the menin and FANCD2 interaction could take place as part of a common DNA repair pathway (Jin et al, 2003).

Evidence obtained from *Mnn1* and *FANCD2* *Drosophila* mutants subjected to various DNA repair assays indicate that *Mnn1* and the FA genes (including FANCD2) appear to regulate different mechanisms of DNA repair. In response to mutagen exposure, differences were observed in the scale of DNA damage. *Mnn1* mutants experience small deletions while FANCD2 mutants experience large-scale rearrangements. Thus with respect to interstrand crosslink repair, menin may function as a regulator of error-prone polymerases. Marek et al (2008) identified a number of deletions within regions of 4 identical nucleotides that could reflect an error-prone

translesion synthesis pathway, important for the successful repair of interstrand crosslinks. It is also possible that menin and FANCD2 co-operatively function in the S-phase checkpoint, as both have been identified as functioning in the IR-inducible S-phase checkpoint, and not in the physical repair of interstrand crosslinks (Taniguchi et al, 2002; Busygina et al, 2006; Marek et al, 2008).

RPA2, a subunit of the RPA complex, was identified as interacting with menin through yeast two-hybrid screening (Sukhodolets et al, 2003). The replication protein A (RPA) complex plays a role in DNA replication and repair, homologous recombination and telomere maintenance (reviewed by Sakaguchi et al, 2009). Interaction between menin and other members of the complex, RPA1 and RPA3, were tested but not observed to interact. Coimmunoprecipitation with menin-specific antibodies pulled down both RPA1 and RPA2, and RPA1, RPA2 and menin exhibit similar intranuclear localization. Thus menin may influence the ability of the RPA heterodimer to bind to DNA through its interaction with RPA2 (Sukhodolets et al, 2003). RPA has been demonstrated to bind to dsDNA, and RPA acts as a repressor protein at the promotor of certain genes (Luche et al, 1992; Singh and Samson, 1995; Tang et al, 1996; Treuner et al, 1998). Thus, RPA may also function in menin-mediated repression of JunD and NF κ B activity at the promotor of certain genes, suggesting a connection between the roles menin plays in transcription regulation and DNA repair, as (Sudhodolets et al, 2003).

4.4 Menin plays a role in cell proliferation

In addition to its roles in transcription regulation and DNA repair, menin also plays a role in cell proliferation. Menin has been implicated as functioning in the

canonical Wnt signaling pathway through its interaction with β -catenin. Analysis of the menin sequence revealed two functional NESs towards the N-terminus of the protein. Further analysis demonstrated that menin shuttles between the nucleus and cytoplasm, likely through the nuclear pore complex-dependent nuclear export receptor CRM1. The relationship between menin and β -catenin was further explored by examining β -catenin accumulation in *Men1*^{-/-} and *Men1*^{+/+} MEFs. In the absence of *Men1*, β -catenin accumulated mainly in the nucleus, whereas in control cells it was localized at the cytoplasmic membrane. This reiterates what was observed in *Men1*^{-/-} β cells of *Men1*-null mice. Menin overexpressing results in reduced endogenous levels of nuclear β -catenin, however analysis of whole cell lysates indicate overall β -catenin levels remained constant. This implies that the canonical Wnt signaling pathway could be activated following loss of menin, resulting in β cells proliferation, and that menin inhibits transcriptional activation of Wnt signaling (Cao et al, 2009). Nuclear-cytoplasmic shuttling regulates several tumour suppressors, including p53 and BRCA1, both of which may restrict tumourigenesis by accumulating in the nucleus as a result of NES masking (reviewed by Fabbro and Henderson, 2003).

Activator of S-phase kinase (ASK) is another protein that interacts with menin, binding at the C-terminus of menin (amino acids 396-610). Expression of ASK alone in menin-null cells results in a moderate increase in cell proliferation, and expressing menin alone inhibited cell proliferation. However, coexpressing both menin and ASK resulted in a repression of cell growth, suggesting that menin can function to repress ASK-mediated cell proliferation (Schnepf et al, 2004). Many mutations identified in MEN1

patients result in a truncated form of menin that is without the C-terminus (Pannett and Thakker, 1999). Coinfection of ASK with either wild-type menin or menin harbouring mutations rendering the C-terminus nonfunctional, demonstrate that without the ASK-interacting domain located in the C-terminus, menin is unable to repress ASK-induced cell proliferation. Thus menin may act as a tumour suppressor by repressing cell proliferation through its interaction with ASK (Schnepf et al, 2004). In fact, it was previously reported that overexpressing menin resulted in reduced cell proliferation of Ras-transformed NIH3T3 cells both *in vitro* and *in vivo* (Kim et al, 1999). ASK is a component of the Cdc7/ASK kinase complex, which plays a role in DNA replication in part by activating the minichromosome maintenance (MCM) protein complex, which acts as a eukaryotic replicative helicase (Masai and Arai, 2002; reviewed by Bochman and Schwacha, 2009).

Through a series of shift assays, menin was demonstrated to bind dsDNA in a sequence-independent manner. Analysis of GST-menin fusion proteins spanning the N-terminus (amino acids 1-218), C-terminus (amino acids 396-610) or central region (219-395) of menin revealed that menin binds dsDNA via its C-terminus. In particular, NLS1 and NLS2 are crucial in enabling the interaction. Menin binds to a variety of DNA structures, including linearized DNA, Y-structures, branched structures and 4-way structures. This interaction may play a role in repressing cell proliferation. Expression of wild-type menin represses cell proliferation in menin-null cells, however expressing menin harbouring mutations that inhibit the interaction cannot recapitulate suppression of

cell proliferation (La et al, 2004). However, it may be difficult to distinguish between the effects on DNA binding and nuclear localization.

Examination of cell cycle phases revealed that in the presence of menin, the number of cells in G₁ decreased but those in G₂/M increased, suggesting a block in G₂/M phase. Thus menin may influence cell proliferation through cell cycle progression (La et al, 2004). This is similar to what has been observed for BRCA1, another tumour suppressor. BRCA1 also binds dsDNA in a sequence-independent manner; however BRCA1 demonstrated a preference to bind to branched DNA structures, in particular 4-way junctions (Paull et al, 2001). Like menin, BRCA1 is also been implicated in regulating gene transcription, genome stability and cell proliferation (reviewed by Scully and Livingston, 2000).

4.5 Menin plays a role in the stress response and maintaining genome stability

Menin plays an important role in the stress response following a variety of stressors. *Mnn1 Drosophila* mutants are sensitive to various forms of stress, including heat shock, hyperosmolarity, hypoxia and oxidative stress as well as γ -irradiation and the crosslinking agents nitrogen mustard and cisplatin. Lethality is observed as a result of exposure to the various stressors, and reintroduction of *Mnn1* through *UAS-Mnn1*, *hs-GAL4* transgenes almost completely rescues the radiation hypersensitivity phenotype (Busygina et al, 2004; Papaconstantinou et al, 2005). Mutation frequency is significantly higher in *Mnn1* mutants than control flies, and this further increases following exposure to nitrogen mustard, suggesting that menin is required for repair of interstrand crosslinks, perhaps through a pathway leading to nucleotide excision repair (Busygina et al, 2004).

With respect to the cell cycle, in response to ionizing radiation the dynamics of G₂-M arrest were unchanged in *Mnn1* mutants, suggesting that menin is not required for the G₂-M arrest following DNA damage. However, an error was detected in the S-phase arrest following irradiation, as DNA synthesis continued while decreasing in wild-type flies. This was also observed in *Men1*^{-/-} MEFs following irradiation, indicating a defect in the G₁-S checkpoint (Busygina et al, 2004). *Ches1*, a member of the forkhead/winged helix transcription factors, was identified as a menin-interacting partner through immunoprecipitation experiments. Overexpressing *Ches1* in a *Mnn1* mutant background resulted in a return to wild-type levels of S-phase arrest and viability following irradiation. Together, these indicate that menin and Ches1 may function in the same complex or pathway, perhaps acting downstream of menin in repairing DNA damage (Busygina et al, 2006).

Misexpression of *Mnn1* through overexpression or repression results in lethality following heat shock. Further examination of the relationship between *Mnn1* and the heat shock genes, specifically *Hsp70* and *Hsp23*, revealed that menin influences activity of the *Hsp70* promoter and *Mnn1* expression is itself regulated during heat shock. *Hsp70* is induced during heat shock, but unable to reach wild-type levels in *Mnn1* mutants. Interestingly, a 70kDa isoform of Mnn1 was observed in Mnn1-overexpressing embryos subjected to heat shock. It accumulated during heat shock and declined during a 1-3 hour recovery. It was not observed in embryos expressing the *Mnn1* shRNA construct. Menin and Hsp70 do not interact physically, suggesting that menin may act in a complex recruited to the *Hsp70* promoter following stress (Papaconstantinou et al, 2005).

4.6 *Menin plays a role in facilitating histone methyltransferase activity*

The association between menin and a Set1-like histone methyltransferase (HMTase) complex was simultaneously identified by two groups (Hughes et al, 2004; Yokoyama et al, 2004). Menin interacts with MLL2 and Ash2L, acting as a member of a Set1/Ash2/Trithorax-like HMTase complex that methylates histone H3 lysine 4. Analysis of patient tumours reveals that in some cases HMTase activity is disrupted, suggesting that perhaps the HMTase activity of menin is related to its tumour suppressor role. The large subunit of RNA Pol II, Rpb1, interacts with menin. Specifically, menin interacts with RNA Pol II when phosphorylated on Ser5, indicating that the menin HMTase complex associates with transcriptionally active genes in the promotor-proximal region. However, a direct interaction between menin and RNA Pol II has not been identified during transcription elongation (Ser2 RNA Pol II) or when RNA Pol II is unphosphorylated. This is similar to the yeast Set1 complex, involved with transcription activation. Perhaps menin recruits the HMTase complex to certain genes resulting in gene activation (Hughes et al, 2004). This is true in the regulation of Hox genes *Hoxc6*, *Hoxc8*, *Hoxa9*, and *p27^{Kip1}* and *p18^{Ink4c}*, two cyclin dependent kinase inhibitors that control cell proliferation through cell cycle regulation in neuroendocrine tissues and the central nervous system (Hughes et al, 2004; Yokoyama et al, 2004; Milne et al, 2005). Expression of *p27^{Kip1}* and *p18^{Ink4c}* was reduced in *Men1^{-/-}* and *MLL*-null MEFs. Menin bound to the coding regions of *p27^{Kip1}* and *p18^{Ink4c}*, and MLL was not required for this to occur. Decreased MLL binding was observed in *Men1*-null cells, indicating that menin

recruits MLL to target loci thus influencing transcription regulation. Increased binding was observed when menin was expressed in these cells (Milne et al, 2005).

5.0 The PAF Complex

5.1 Identification of the PAF complex in yeast

In a survey of RNA Pol II-associated factors in yeast, a number of proteins were identified, including yPaf1 and yCdc73 (Wade et al, 1996). yPaf1 is a nuclear protein that when deleted results in pleiotropic effects including delayed doubling time at 30°C and a temperature-sensitive phenotype at both warmer (36°C and 38°C) and colder (14°C and 25°C) temperatures. yPaf1 mutants exhibited an altered morphology, in that cells were larger and round. Expression levels of certain genes were affected by the yPaf1 mutation, indicating that yPaf1 may influence transcription as a general transcription factor or act as a mediator for a subset of genes (Shi et al, 1996). yCdc73 is a nuclear protein that directly interacts with RNA Pol II. Deletion of yCdc73 results in a slightly increased doubling time and a temperature sensitive lethality phenotype at 38°C. *yPaf1-yCdc73* double mutants exhibit similar traits as yPaf1 mutants with respect to growth rate and morphology, suggesting that both yPaf1 and yCdc73 function in the same pathways. In fact, yPaf1 and yCdc73 associate with each other, as well as with RNA Pol II, indicating that they exist in a complex with RNA Pol II (Shi et al, 1997).

yCtr9 was identified in a survey of proteins regulating SBF-dependent gene expression. Immunoprecipitation of yCtr9 revealed two interacting proteins, yPaf1 and yCdc73, which associate and function as part of a larger multiprotein complex. The authors hypothesized that this complex plays a role in transcription based on an

association with RNA Pol II and that *yCtr9* and *yPaf1* affect the G₁ cyclin *CLN2* transcription. Thus *yPaf1*, *yCdc73* and *yCtr9* represented a novel transcription factor complex (Koch et al, 1999). Further characterization of the complex confirmed *yCtr9* as a member of the RNA Pol II-associated complex and identified two additional components, *yRtf1* and *yLeo1* (Mueller and Jaehning, 2002). It had previously been described that *yPaf1*, *yCdc73* and *yCtr9* mutants exhibited a range of phenotypic changes, *yPaf1* and *yCtr9* mutations yielding the most severe (Shi et al, 1997; Koch et al, 1999). *yRtf1* and *yLeo1* mutants did not demonstrate phenotypes similar to *yPaf1*, *yCdc73* and *yCtr9*. *yRtf1* mutants experienced slightly slower growth and mild temperature sensitivity, while *yLeo1* mutants grew like wild-type strains. However, *yRtf1-yPaf1*, *yRtf1-yCtr9* and *yLeo1-yPaf1* double mutants exhibited phenotypes less severe than *yPaf1* or *yCtr9* mutants alone. Thus, loss of *yRtf1* and *yLeo1* suppressed the phenotypes observed through the loss of *yPaf1* or *yCtr9* (Mueller and Jaehning, 2002).

5.2 The PAF complex in *Drosophila melanogaster*

Since its discovery in yeast, PAF has been identified in humans and *Drosophila*. PAF is similar to the yeast complex in that it consists of 5 subunits: dPaf1, dLeo1, dCdc73, dCtr9 and dRtf1 (**Fig. 1.2**). dLeo1, dPaf1, dRtf1 and dCdc73 were identified through BLAST searches of the *Drosophila* genome using *S. cerevisiae* sequences (Zhao et al, 2005; Adelman et al, 2006). Though the PAF subunits are considered nonessential in yeast, *Drosophila* carrying mutations for any of the PAF subunits are recessive lethal, indicating that in higher eukaryotes they have gained additional functions making them essential for survival (Adelman et al, 2006; Flybase, 2010). *Drosophila* PAF differs from

that of yeast in that the dRtf1 subunit is not stably associated with the rest of the complex (Adelman et al, 2006).

5.3 The PAF complex in humans

Parafibromin, the protein product of the *HRPT2* tumour suppressor gene, was identified as the human ortholog of yCdc73. A survey of hCdc73-interacting proteins revealed the human homologs of hPaf1, hLeo1 and hCtr9. Reciprocal coimmunoprecipitation experiments confirmed these interactions. The interaction between hCdc73 and RNA Pol II was confirmed when immunoprecipitation experiments identified the interaction between hCdc73 and Rpb1, the largest subunit of RNA Pol II, regardless of its state of phosphorylation, suggesting involvement in both initiation and elongation (Rozenblatt-Rosen et al, 2005; Yart et al, 2005).

Interestingly, the human homolog of yRtf1 was not identified from immunoprecipitation experiments, suggesting that human PAF did not contain the hRtf1 subunit (Rozenblatt-Rosen et al, 2005; Yart et al, 2005). hRtf1 was not believed to associate with PAF in humans until recently, when Kim et al (2010) identified the human homolog of yRtf1 associated with the remaining members of the complex. Interestingly, several isoforms of hRtf1 were identified from HeLa cell extracts, possibly the result of alternative splicing. Only the short form of hRtf1 associated with members of the complex (Kim et al, 2010). Human PAF is unique from the homologous complexes in yeast and *Drosophila* due to the incorporation of an additional subunit, hSki8. Zhu et al (2005) identified hSki8, a member of the human SKI complex, as a novel PAF subunit in

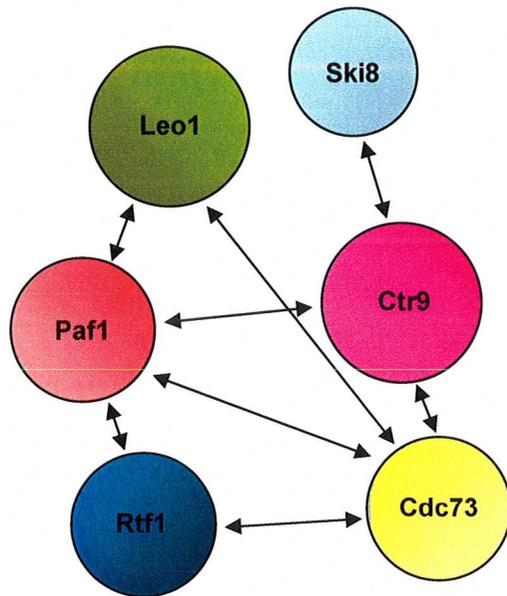
higher eukaryotes. Thus, the complete PAF complex consists of 6 subunits: hPaf1, hLeo1, hCtr9, hSki8, hRtf1 and hCdc73 (**Fig. 1.2**) (Kim et al, 2010).

6.0 Functions of the PAF Complex

6.1 PAF functions in transcription elongation

PAF participates in a number of processes in the cell, the best studied of which is its role in transcription elongation. In yeast, the complex was originally identified through its association with RNA Pol II at transcriptionally active genes. Initially, the yPaf1/yCdc73 complex was thought to function in transcription initiation because glutathione-agarose chromatography revealed yCdc73 and yPaf1 to be in a complex with RNA Pol II and the general initiation factors TFIIB and TFIIF. TFIIS, an elongation factor, was not detected as a member of this complex (Shi et al, 1997). However, Squazzo et al (2002) found that deletion of the yPaf1 or yRtf1 subunits rendered yeast sensitive to 6-azauracil (6AU), resulting in defects in *PUR5* induction in response to 6AU treatment. This suggested that the complex functions in transcription elongation rather than initiation. 6AU is an assay used to determine mutations affecting transcription elongation. It works by interfering with nucleotide synthesis, resulting in alterations to those available to facilitate elongation. In wild-type yeast 6AU stimulates transcription of the *PUR5* gene, and conversely yeast harbouring mutations affecting the elongation machinery exhibit impeded *PUR5* induction. Thus, monitoring *PUR5* induction in response to 6AU treatment indicates the state of transcription elongation (Shaw and Reines, 2000). They further identified both physical and genetic interactions between

Figure 1.2: The PAF complex. The PAF complex was originally identified in yeast and consists of 5 subunits: Paf1, Cdc73, Rtf1, Ctr9 and Leo1. It has since been identified in an assortment of organisms, including *Drosophila melanogaster* and humans. *Drosophila* PAF is quite similar to the yeast complex. They consist of the same 5 subunits, although dRtf1 does not stably associate with the remaining subunits of the complex. In humans, an additional subunit is incorporated into the complex, hSki8. Interactions among the subunits are indicated by black arrows.



(Adapted from Jaehning, 2010)

members of the complex and Spt5 and Spt16-Pob3 elongation factors (Squazzo et al, 2002; Krogan et al, 2002). A previous study had demonstrated a genetic interaction between yRtf1 and Spt4-Spt5 and TFIIIS, known elongation factors, supporting PAF's role in transcription elongation (Costa and Arndt, 2000). The first molecular evidence demonstrating its role in transcription elongation came about through the use of an *in vitro* transcription elongation assay. Elongation efficiency was determined by measuring transcription of two G-less cassettes transcribed from the hybrid *GAL4-CYCI* promotor. PAF was required for efficient transcription elongation, and this was repeated *in vivo*. Functionally, two types of subunits within PAF were hypothesized, yPaf1-yCdc73 and yLeo1-yRtf1, based on differences in the severity of the transcription phenotype. yPaf1 and yCdc73 resulted in a more severe phenotype, while yRtf1 and yLeo1 demonstrated wild-type transcription elongation efficiencies (Rondón et al, 2003).

Krogan et al (2003) provided evidence that the PAF complex promotes transcription elongation via histone methylation. A Global Proteome analysis of *S. cerevisiae* looking for defects in methylation of histone H3 lysine 4 revealed *yPaf1*, *yRtf1* and *yCtr9* mutants to be deficient in this regard. Genetic and physical interactions were observed between PAF and COMPASS, a HMTase complex that methylates histone H3 lysine 4. A further interaction between COMPASS and Ser5 RNA Pol II was identified, suggesting that the COMPASS-RNA Pol II interaction is focused at the promotor during early elongation. The interaction was absent in *yCtr9* mutants, indicating its dependence on the presence of PAF. This was not true for the PAF-RNA Pol II interaction, in which COMPASS was not required. The importance of this interaction in early elongation was

confirmed when an absence of PAF prevented COMPASS from localizing to the 5' region of the gene. Therefore, PAF is required for the recruitment of COMPASS to RNA Pol II. PAF was also required for methylation of histone H3 lysine 79, although this appears to be specific to the yPaf1 and yRtf1 subunits, as methylation occurred in the absence of yCtr9 and yLeo1. An interaction between PAF and Dot1p is thought to mediate this process (Krogan et al, 2003).

In addition to facilitating methylation of lysines 4 and 79, PAF also plays a role in monoubiquitinating histone H2. Specifically, the yRtf1 subunit was essential for the ubiquitination of histone H2, perhaps through an interaction with Rad6-Bre1 ubiquitinating activity and histones (Ng et al, 2003). Its importance in ubiquitinating histone H2B was confirmed through deletion of either the yRtf1 or yPaf1 subunit, which resulted in a loss of ubiquitination of histone H2B. A functional and physical interaction between COMPASS, RNA Pol II and Rad6 was demonstrated requiring the presence of PAF. In the absence of yRtf1, the interaction between Rad6 and COMPASS-RNA Pol II was abolished, however Rad6 was still observed to localize to the promotor in the absence of yRtf1. Thus a link was established between the ubiquitination and methylation machinery at the promotor region, in that PAF modulates ubiquitination of histone H2B lysine 120 via the Rad6-Bre1 complex and this then influences the methylation of lysines 4 and 79 of histone H3 (Wood et al, 2003; Zhu et al, 2005b).

The importance of PAF and its role in ubiquitinating histone H2B lysine 120 in promoting transcription elongation was reinforced through its association with the FACT (Facilitates Chromatin Transcription) complex (Pavri et al, 2006). FACT acts as a

histone chaperone that both destabilizes and reassembles nucleosomes during transcription, and interactions between PAF and the Spt16 subunit of FACT had previously been reported (Krogan et al, 2002; Squazzo et al, 2002; Belotserkovskaya et al, 2003). FACT is necessary for transcription elongation modulated by PAF and ubiquitinated histone H2B lysine 120, and in fact recruits PAF which in turn recruits the H2B monoubiquitination machinery (Pavri et al, 2006). The association between Rad6 and PAF is mediated by the Bre1 complex, specifically via the hPaf1 subunit (Kim et al, 2009). The association between FACT, PAF and ubiquitinated histone H2B lysine 120 functions throughout the transcription unit following initiation. FACT is recruited to RNA Pol II upon the first nucleosome, which in turn recruits PAF and then the ubiquitination machinery, resulting in the monoubiquitination of histone H2B lysine 120. The histone H2A/H2B dimer is removed, eliminating the nucleosomal barrier and permitting RNA Pol II to proceed with transcription (Pavri et al, 2006). Following passage of RNA Pol II, reduced levels of PAF result in improper nucleosome reassembly, suggesting that PAF is required both before and after RNA Pol II traverses the nucleosomal barrier. PAF binds nascent RNA *in vivo* through the yLeo1 subunit, and its association with the coding region is in part stabilized by this interaction (Dermody and Buratowski, 2010).

A link between histone H2B monoubiquitination and histone H3 lysine 4 methylation was drawn by Kim et al (2009), who observed that ubiquitinated histone H2B stimulates histone H3 lysine 4 methylation by the Set1 complex. PAF mediates both histone H2B ubiquitination and transcription, perhaps by allowing PAF-recruited

ubiquitination factors easier access to the histone H2B lysine 120 by virtue of its ability to enable chromatin transcription rather than directly stimulating activity of the Rad6-Bre1 complex. Also, histone H2B ubiquitination occurs as a result of ongoing transcription rather than directly affecting how the transcription machinery functions (Kim et al, 2009).

PAF works in conjunction with the transcription elongation factor A (SII) in terms of facilitating transcription elongation, binding to SII via the hPaf1 and hLeo1 subunits (Kim et al, 2010). PAF was also observed to accumulate in the coding region of genes more so than at the promotor, echoing both Pokholok et al (2002) and Adelman et al (2006) who observed that while PAF was identified in the promotor region, the most dramatic increases in localization following gene induction occurred in the coding region. In a survey of the global localization of PAF subunits dPaf1, dRtf1 and dCdc73 using *Drosophila* polytene chromosomes, all three subunits localized to the middle of the *Hsp70* gene, rather than the promotor-proximal region, following heat shock. Chromatin immunoprecipitation confirmed their recruitment downstream of the promotor, mirroring that of Spt6 and FACT. This was also observed for more constitutively active genes including *actin* and *tubulin*. Trimethylated histone H3 lysine 4 levels increased rapidly at the *Hsp70* gene as a function of the duration of heat shock. Furthermore, qPCR revealed significantly higher levels of histone H3 lysine 4 methylation in the promotor-proximal regions rather than in the coding regions. Treatment with *dPaf1* RNAi and subsequent heat shock revealed trimethylation levels of histone H3 lysine 4 of the *Hsp70* gene to be

similar to that observed prior to heat shock. However, unlike in yeast, PAF did not influence methylation levels at histone H3 lysine 79 (Adelman et al, 2006).

6.2 PAF functions in 3' end formation

In addition to its role in transcription elongation, PAF also functions in posttranscriptional processing. Mueller et al (2004) hypothesized that PAF may have a posttranscriptional role with respect to the formation and stability of poly(A) tails following their observation that dissociation of the complex from RNA Pol II did not influence the distribution of the Ser2 phosphorylated form across the gene. Yet loss of PAF has deleterious effects on the cell. They demonstrated that yeast mutant for the various PAF subunits displayed shorter poly(A) tails, however it was undetermined if this was a result of changes in poly(A) stability, formation, or the nuclear export pathway (Mueller et al, 2004).

Decreasing yPaf1 results in altered mRNA stability, suggesting the complex may influence posttranscriptional changes. Examination of genes whose expression levels are directly influenced by the presence of yPaf1 revealed that loss of the complex resulted in increased production of extended mRNA transcripts. Thus for genes with more than one poly(A) site, the distal sites were utilized, but the proximal ones were not. As a result of extended untranslated regions (UTRs), the unstable transcripts were targets of nonsense-mediated decay (NMD). Thus, PAF is required for proper mRNA cleavage (Penheiter et al, 2005). Through the use of the well defined GAL10-GAL7 model, both Spt6 and yCtr9 are recruited to the gene; however yCtr9 association with RNA Pol II decreases in intergenic regions. A genetic interaction was observed between Spt6 and yCtr9, and Spt6

is necessary for the association of yCtr9 with RNA Pol II. It was also observed that when the major *GALI0* poly(A) signal was mutated, yCtr9 association persists further downstream than in wild-type yeast. This suggests that yCtr9 disengages from the elongation complex downstream of functional poly(A) sites (Kaplan et al, 2005).

Loss of PAF subunits results in reduced Ser2 RNA Pol II levels, and these reductions correlate to the phenotypic severity of their loss. yPaf1 and yCtr9 exhibit the severest phenotype and demonstrate the most drastic reduction in Ser2 RNA Pol II levels, yCdc73 and yRtf1 demonstrate intermediate phenotypes and Ser2 RNA Pol II levels, and yLeo1 resembles the wild-type in both phenotype and Ser2 RNA Pol II levels (Nordick et al, 2008). It had been previously demonstrated that loss of yPaf1 resulted in longer, unstable mRNA transcripts (Penheiter et al, 2005). Using the same PAF mutants, it was determined that loss of PAF subunits results in increased readthroughs proportional to the reduction in Ser2 RNA Pol II, indicating that PAF plays an important role in mRNA 3'-end formation. PAF interacts with the cleavage and polyadenylation factor Cft1, a member of the yeast CPF complex homologous to the human CPSF complex, and through this interaction recruits Cft1 to Ser5 RNA Pol II early in transcription (Nordick et al, 2008).

The PAF subunit hCdc73 interacts with cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF), both required for determining where cleavage and polyadenylation will occur in the pre-mRNA (reviewed by Hirose and Manley, 2000; Rozenblatt-Rosen et al, 2009). In the absence of hCdc73, there is a reduction in both pre-mRNA cleavage and synthesis of polyadenylated RNA during

transcription. RNA Pol II association with the promotor-proximal 5' UTR is unchanged, as are histone H3 lysine 4 methylation levels. However, the association of CPSF-30, CPSF-73, CstF-64 and CstF-77 with this region decreases. This is also true in the coding and 3' UTR regions. Together, these suggest that hCdc73 recruits CPSF and CstF to the gene locus, thus regulating mRNA levels (Rozenblatt-Rosen et al, 2009).

6.3 PAF functions in 3' end formation of snoRNA

In addition to recruiting factors that promote mRNA 3' end formation, PAF also functions in the formation of non-polyadenylated 3' ends of small nucleolar RNAs (snoRNAs). Genetic interactions have been identified between the yRtf1, yCdc73, yPaf1 and yCtr9 subunits of the PAF complex with Nab3 and Nrd1, which function in the Nrd1 pathway responsible for 3' end formation of nonpolyadenylated transcripts (Conrad et al, 2000; Steinmetz et al, 2001; Sheldon et al, 2005). PAF defects result in accumulation of *SNR13* and *SNR47* readthrough products, which encode the snoRNAs snR13 and snR47. 3' end formation was not affected by defects in a number of other factors influencing transcription activation, histone modification and chromatin remodeling, indicating that PAF's function in 3' end formation is independent of its histone methylation function. Nrd1 and Nab3 appear to be recruited to elongating RNA Pol II by PAF, perhaps by affecting the phosphorylation of serine 2 (Sheldon et al, 2005).

6.4 PAF functions in mRNA surveillance

PAF has been demonstrated to play a role in transcription elongation, as well as mRNA processing and maturation. In addition to these, the complex has also been implicated in mRNA surveillance. In purifying the human PAF complex, Zhu et al

(2005) identified a novel subunit of the complex unique to higher eukaryotes, hSki8. hSki8 is also a member of the SKI complex that functions in 3'-5' mRNA decay (Masison et al, 1995; Brown et al, 2000). hSki8 exists in two distinct complexes, the PAF complex with hPaf1, hCdc73, hCtr9, hRtf1 and hLeo1 and the SKI complex with hSki2 and hSki3 (Zhu et al, 2005; Kim et al, 2010). Reciprocal immunoprecipitation experiments reveal that the PAF and hSKI complexes interact, as immunoprecipitating with α hSki3 antibodies revealed the association with hPaf1 in nuclear extracts.

hSKI localization resembles that of PAF in both the promotor and coding regions. Further examination of the *MAGE-A1* gene, induced following treatment with a DNA demethylating agent, revealed both PAF and hSKI to be absent when the gene is transcriptionally inactive. Following induction, both complexes were recruited to the promotor and coding regions, however the down-regulation of PAF by siRNA led to a reduction in hSKI recruitment. Thus the hSKI complex associates with transcriptionally active genes by virtue of its interaction with PAF, and in doing so promotes 3'-5' mRNA decay, possibly in conjunction with the exosome, whose association with transcriptionally active genes through its interaction with Spt6 was previously reported (Andrulis et al, 2002; Zhu et al, 2005).

6.5 PAF functions in cell cycle control

PAF plays a role in regulating cell cycle progression. Zhang et al (2006) demonstrated that overexpressing activated H-RasV12 alone in NIH3T3 cells resulted in increased cell proliferation. When hCdc73 was then overexpressed in conjunction with H-RasV12, the proliferation observed from H-RasV12 expression alone was inhibited.

Thus, overexpression of hCdc73 inhibits cell proliferation. This was confirmed using Dox-inducible cells also expressing hCdc73. An increase in G₁-phase cells and decrease in S- and G₂/M-phase cells suggest this was a result of cell cycle arrest resulting from overexpression of hCdc73. Similar findings were demonstrated with siRNA treatment of *hCdc73* and *hPaf1*. Cells in S-phase increased while cells in G₁ phase were reduced. In fact, knockdown of either *hCdc73* or *hPaf1* resulted in increased cell proliferation in both HeLa and primary human fibroblast cells. Examination of *c-myc* expression revealed that expression was up-regulated in response to *hCdc73* and *hPaf1* silencing. Thus endogenous hCdc73 appears to inhibit *c-myc* expression (Lin et al, 2008).

Differential display and microarray analyses identified cell cycle regulated genes dependent on PAF expression, including the G₁ cyclin *CLN1*, *RNR1*, *HTB1* and *STP4* (Koch et al, 1999; Porter et al, 2002). Another member of PAF, yCtr9, was demonstrated to be essential for the expression of the G₁ cyclin *CLN2* (Koch et al, 1999). Moniaux et al (2009) demonstrated that cells in mitosis exhibited little to no *hPaf1* expression. Examination of each phase revealed that *hPaf1* expression increases as the cell cycle progresses, flow cytometry demonstrating the relative fluorescence from FITC-labeled hPaf1 to be 69.79, 85.64 and 133.77 in G₁-, S- and G₂-phase cells respectively. Upon entering mitosis, hPaf1 levels experienced an abrupt decline as only trace amounts were detected during prophase. With entry into G₁ phase, hPaf1 expression increased. Thus, regulation of *hPaf1* expression seems to be regulated by the cell cycle. Interestingly, both 60kDa and 80kDa forms of hPaf1 were identified in cellular lysates. The 60kDa form of hPaf1 was observed in cytoplasmic extracts, and the 80kDa form in both

cytoplasmic and nuclear extracts. Cytoplasmic 60kDa and 80kDa hPaf1 were both observed to increase as the cell cycle progressed from G₁ to G₂, however the 60kDa form was not observed in asynchronously growing cells. It was hypothesized that the 60kDa form is an hPaf1 precursor that matures into the 80kDa protein which is then transported to the nucleus until late G₂ phase. The nature of the modification of the 60kDa form of hPaf1 has not been determined. *hLeo1* expression demonstrated oscillatory pattern of expression similar to that observed for *hPaf1*, indicating that hPaf1 is in fact functioning as part of the PAF complex. hPaf1 associates with the promotor regions of *cyclin A1* in the G₁/S phase, *cyclin B1* in the G₂/M phase and both *cyclin A2* and *Cdk1* in the G₁/S and G₂/M phases. Knockdown of *hPaf1* resulted in a reduction in cyclins A1, B1, D1 and E1. Thus, PAF functions in regulating cyclin transcription with respect to the cell cycle (Moniaux et al, 2009).

PAF also functions in cell cycle regulation independent from its role in transcription by acting in the DNA replication process. Both hPaf1 and hLeo1 interact with the phosphorylated and hypophosphorylated forms of Polymerase α , a heterotetrameric protein that plays a role in DNA replication, in both G₁/S and G₂/M. In cells treated with *hPaf1* siRNA, Polymerase α was observed to localize at replication sites on chromosomal DNA, suggesting hPaf1 functions by delaying the binding of hypophosphorylated Polymerase α to replicating DNA. hPaf1 may also function in tubulin polymerization and spindle formation (Moniaux et al, 2009).

6.6 FAF functions in the canonical Wnt signaling pathway

A role for PAF was proposed in Wnt signaling. Mosimann et al (2006) demonstrated that dCdc73 interacts with β -catenin and is required for Wnt signal transduction, acting downstream of Axin and APC. Interestingly, hLeo1 also coimmunoprecipitated with β -catenin, suggesting that β -catenin interacts with the PAF complex as a whole. The authors hypothesize that β -catenin recruits Cdc73, resulting in transcriptional upregulation of Wnt target genes via activation of the PAF complex (Mossimann et al, 2006).

7.0 Rationale

Mass spectrometric analysis was performed by a collaborator at Rutgers University, Dr. Thomas Kusch, and used to identify interacting partners of menin. From this screen, *Atu* was identified as a putative binding partner. *Atu*, Another transcription unit, is the *Drosophila* homolog of *Leo1* and a member of the PAF complex (Mueller and Jaehning, 2002; Zhao et al, 2005; Adelman et al, 2006). Investigation of the putative interaction was undertaken using a variety of molecular and genetic techniques, and focused in two main areas: confirming the interaction and identifying a genetic interaction between *Atu/dLeo1* and menin (*Mnn1*). In confirming the interaction, coimmunoprecipitation experiments were used to identify a physical interaction between menin and *dLeo1*. Genetic studies, including the LOH assay at the *multiple wing hair* (*mwh*) locus, were used to first identify a genetic interaction between the genes, and second to explore the role of this interaction in maintaining genomic stability. With the identification of this novel interaction, exploring the mechanistic and functional

relevance of the interaction with the PAF complex will shed a new light on menin's role in tumorigenesis.

Materials and Methods

1.0 DNA Constructs

1.1 Recovery of *dPaf1* and *dLeo1* cDNA

Full length cDNA's of the *Drosophila* orthologs of *Paf1* and *Leo1*, *Atms* (Stock No. 15537) and *Atu* (Stock No. 15423), referred to as *dPaf1* and *dLeo1* respectively, were obtained from the *Drosophila* Genomics Resource Center (DGRC) (Appendix A, **Fig.A1**). Because the DNA arrives dried onto a Whatman disc, the disc was washed with 50 μ L of TE buffer. The buffer was immediately removed and saved at -20°C until the success of the transformation into library efficiency DH5 α was confirmed. 50 μ L of library efficiency DH5 α cells (Invitrogen) was added to the disc and incubated on ice for 30 minutes, vortexing 15 and 30 minutes into the incubation. Following the incubation, cells were heat shocked at 42°C for 45 seconds and returned to ice for 2 minutes. Cells were transferred to a tube containing 950 μ L of SOC media (Invitrogen) and incubated 1 hour at 37°C and 225rpm in a shaking incubator. Bacterial growths were plated on Luria Broth (LB) agar plates containing chloramphenicol (34 μ g/mL) and incubated overnight in a 37°C warm room. Colonies were selected and grown overnight at 37°C in LB media and chloramphenicol (34 μ g/mL) at 225rpm in a shaking incubator. Minipreps of the bacterial cultures were performed using the PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. To verify the effectiveness of the transformation, *dPaf1* and *dLeo1* minipreps were digested with EcoRI and XhoI (New England Biolabs) and analyzed on a 1% agarose gel. Plasmids were sequenced by Mobix using the T7 sequencing primer (**Table 2.1**).

1.2 Construction of V5 epitope-tagged dPaf1 and dLeo1 expressing plasmids

As dPaf1 and dLeo1 antibodies were not immediately available, V5 epitope-tagged dPaf1 and dLeo1 expression constructs were created. Primers were designed placing the V5 epitope (GKPIP NPLLGLDST) at either the N- or C-terminus of the protein (**Table 2.1**). BclI restriction sites were incorporated at 5' and 3' ends of the fragment to facilitate cloning into the BamHI restriction site of the expression plasmid. dPaf1 and *dLeo1* cDNA was amplified from the plasmids obtained from the DGRC using a combination of ProofStart (Promega) and Taq (Invitrogen) as per the manufacturer's instructions. The PCR program conditions were: (i) *1 cycle of:* 95°C for 2 minutes (ii) *15 cycles of:* denaturation-94°C for 10 seconds, annealing-52°C for 1 minute and extension-68°C for 3 minutes (iii) *25 cycles of:* denaturation-94°C for 10 seconds, annealing 52°C for 1 minute and extension-68°C for 3 minutes plus an additional 20 seconds per cycle and (iv) *1 cycle of:* 68°C for 7 minutes. The annealing temperature of 52°C was applicable for all *dPaf1* and *dLeo1* primer sets. Amplification reactions were verified on a 1% agarose gel and cleaned using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Cleaned amplification products were digested with BclI (New England Biolabs) overnight in a 50°C water bath and extracted using phenol/chloroform/iso-amyl alcohol.

The expression vector pPacB, containing the Actin promotor (**Fig. A2**), was digested overnight in a 37°C warm room with BamHI (New England Biolabs) and subsequently extracted using phenol/chloroform/iso-amyl alcohol. Digested pPacB was then treated with rAPid Alkaline Phosphatase (Roche) as per the manufacturer's

Table 2.1: PCR and sequencing primers. Primers used in the amplification of V5 epitope-tagged *dPaf1* and *dLeo1* cDNA cloned into the pPacB expression vector, as well as those used in the subsequent sequence analysis of the constructs, are listed. Primers used to amplify sequences flanking P element insertion sites of *dPaf1* and *dLeo1* mutants are listed, in addition to those used in sequencing the amplification products.

Table 2.1: PCR and sequencing primers

Primer Name	Primer Sequence (5' – 3')
PCR Primers:	
<i>Construction of V5 epitope-tagged dPaf1 and dLeo1 expressing plasmids:</i>	
NPaf1 Forward (ML-09-0741)	TTT TTT TGA TCA ATG GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG ATG CCA CCC ACG
NPaf1 Reverse 2 (ML-09-2311)	TTT TTG ATC ATT TAT TAT GAA CGC CG
CPaf1 Forward (ML-09-0743)	AAA AAA TGA TCA ATG CCA CCC ACG AT
CPaf1 Reverse 2 (ML-09-2312)	AAA AAA TGA TCA TTA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC TTC ATT GAG GCG CT
NAtu Forward (ML-09-0745)	AAA AAA TGA TCA ATG GGC AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG AGC CAA AAC TCG GAC
NAtu Reverse 2 (ML-09-2309)	AAA AAT GAT CAT TAA TCG TTG TCA CTG CC
CAtu Forward (ML-09-0747)	AAA AAT GAT CAC GAA TTG TCT TGA TTG C
CAtu Reverse 2 (ML-09-2310)	AAA AAA TGA TCA TTA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC ATC GTT GTC ACT GCC
<i>Verification of P-element insertion sites in dPaf1 and dLeo1 mutants:</i>	
Plac4 (10-1114)	ACT GTG CGT TAG GTC CTG TTC ATT GTT
Plac1 (10-1115)	CAC CCA AGG CTC TGC TCC CAC AAT
Pry4 (10-1116)	CAA TCA TAT CGC TGT CTC ACT CA
Pry1 (10-1117)	CCT TAG CAT GTC CGT GGG GTT TCA AT
Plw3-1 (10-1118)	TGT CGG CGT CAT CAA CTC C
Sequencing Primers:	
<i>Construction of V5 epitope-tagged dPaf1 and dLeo1 expressing plasmids:</i>	
T7	AAT ACG ACT CAC TAT AGG
PM001	CGT TAG AAC GCG GCT ACA AT
Actin promotor A	AGA GCA TTG CGG C
<i>Verification of P-element insertion sites in dPaf1 and dLeo1 mutants:</i>	
SeqSp1 (10-1119)	ACA CAA CCT TTC CTC TCA ACA A
SeqSp3 (10-1120)	GAG TAC GCA AAG CTT TAA CTA TGT
Splac2 (10-2277)	GAA TTC ACT GGC CGT CGT TTT ACA A
Spep1 (10-2278)	GAC ACT GAC AAT ACT ATT C
Sp5 (10-2279)	GCA TCA CAA AAA TCG ACG CTC AAG T

instructions and cleaned using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Both *dPaf1* and *dLeo1* cDNA sequences contain BamHI restriction sites, thus BclI restriction sites were incorporated into the V5 epitope-tagged cDNA to facilitate cloning into the BamHI site of the expression vector. Digestion with BclI produces cohesive ends compatible with those resulting from BamHI digestion.

Vector and insert were ligated in ratios of 1:1, 1:2 or 1:5. Ligations were performed either overnight using T4 DNA Ligase in ligation buffer (New England Biolabs) or for two hours using T4 DNA Ligase in Quick Ligation Buffer (New England Biolabs). Overnight ligations were incubated at 14°C in a water bath or in an Applied Biosystems GeneAmp thermocycler. Ligation reactions were further incubated for 2 hours at room temperature following the addition of 1mM ATP and 0.5λ of T4 DNA Ligase (New England Biolabs). Quick ligations were incubated at room temperature on the bench for 2 hours. Ligations were transformed into 50μL or 100μL of DH5α cells (Invitrogen) and grown overnight on LB agar plates containing ampicillin (50μg/mL). Bacterial cultures from resulting colonies were grown overnight using LB media and ampicillin (50μg/mL) at 37°C and 225rpm in a shaking incubator. Minipreps of the resulting bacterial growth were performed using the EZ-10 Spin Column Plasmid DNA Kit (Bio Basic Inc.) per the manufacturer's instructions.

To determine insert orientation, diagnostic restriction enzyme digests were performed. V5-dPaf1 and dPaf1-V5 constructs were digested with EcoRI and EagI (New England Biolabs), and V5-dLeo1 and dLeo1-V5 constructs were digested with EcoRI and

SacI (New England Biolabs). Digests were analyzed on 1% agarose gels. Those identified in the correct orientation were sequenced by Mobix using the Actin promoter A sequencing primer. Upon sequence verification, maxipreps were performed using the Plasmid Maxi Kit (QIAGEN).

1.3 Tissue culture of S2 cells

Immortalized S2 cells, derived from late-stage *Drosophila melanogaster* embryos, were cultured in 25cm² flasks (Sarstedt). Cells were detached from the flask by pipetting up and down and then split 1:5 and 1:10 in Insect Serum-Free Media (HyClone) containing 1% penicillin and streptomycin (Invitrogen) every two to three days. Cells were maintained in a room temperature incubator.

1.4 Transfection of S2 cells

To verify expression of the constructs, V5 epitope-tagged dPaf1 and dLeo1 constructs were transfected into S2 cells and analyzed by Western blotting. S2 cells were split 1:10 into 60mm plates (Corning) and grown overnight in a room temperature incubator. The following day, cells were transfected with the constructs containing the V5 epitope at either the N- or C-terminus, or pPacB alone as a negative control. Solution A (7.5µg of DNA added to 300µL of Insect Serum Free Media (HyClone)), and Solution B (10 µL of Cellfectin II (Invitrogen) added to 300µL of Insect Serum Free Media (HyClone)) were prepared for each 60mm plate of cells to be transfected in the tissue culture hood. Solution A was added to Solution B, mixed and incubated at room temperature for 20 minutes. 900µL of Insect Serum Free Media (HyClone) was then added and mixed gently. Cells were washed with 4mL of Insect Serum Free Media

(HyClone) and the DNA solution added to the cells. Cells were incubated 3-4 hours in a room temperature incubator and then 5mL of fresh media was added to the cells. Cells were incubated for 3 days in a room temperature incubator prior to preparation of cell lysate.

1.5 S2 cell lysate preparation

Cells were detached from plates using a scraper and transferred to a 15mL conical tube, then pelleted by centrifuging at 1000 rpm for 5 minutes at 4°C. The cellular pellet was washed once with cold 1x Phosphate Buffered Saline (PBS) and the 5 minute centrifugation at 4°C was repeated. SDS sample buffer (2% SDS, 10% glycerol, 5% β -mercaptoethanol, 60mM Tris pH 6.8, 1mM NaVO₄, 1mM NaF, protease inhibitor cocktail (Roche)) was added to cells and incubated at 100°C in a heating block for 5 minutes and then centrifuged at maximum speed for 15 minutes at 4°C. The supernatant was then transferred to a new tube.

1.6 Bradford Assay

Protein concentrations were determined using the Bradford Assay. To generate a protein standard curve, 7 standards containing increasing amounts of BSA (0, 1, 2, 4, 6, 8 and 10 μ g) and 2 μ L of SDS sample buffer were prepared to a total volume of 12 μ L. Lysate samples were prepared by adding 2 μ L of lysate to 10 μ L of ddH₂O. 990 μ L of diluted Bradford Reagent was added to each sample and incubated at room temperature 5 minutes. Absorbance was read at 595nm in an Ultraspec 2100 pro spectrophotometer and the resulting standard absorbance readings plotted against protein concentrations. Using the resulting standard curve, the concentration of the lysate samples was calculated.

1.7 Western blotting

50 μ g and 100 μ g of protein were measured and resolved by SDS-PAGE. Samples were loaded into a 10% bis-acrylamide gel (Stacking Gel: 10.8mL ddH₂O, 11.2mL 4x separating buffer pH 8.8, 8mL glycerol, 15mL 30% bis-acrylamide, 75 μ L 10% APS and 30 μ L TEMED - Separating Gel: 10.8mL ddH₂O, 4.5mL 4x stacking buffer pH 6.8, 2.7mL 30% bis-acrylamide, 120 μ L 10% APS and 45 μ L TEMED) and run overnight at 80V. Proteins were transferred from the gel onto a PROTRAN nitrocellulose transfer membrane (Whatman) at 60V for 3 hours in 1x transfer buffer (300mL 10x transfer buffer, 600mL methanol and 2100mL ddH₂O) in a 4°C cold room. Membranes were blocked with 5% milk in 1x Tris Buffered Saline (TBS) for at least one hour at room temperature and then incubated overnight at 4°C with monoclonal α V5 1° antibody (Invitrogen, Catalog number R960-25) (1:2500). Blots were washed twice with cold 1x TBS, twice with 1x Tris Buffered Saline with 0.1% Tween 20 (TBST) and once with 1x TBS, and then incubated with α mouse HRP-conjugated 2° antibody (1:10,000) (Cell Signalling) for 1.5 hours at 4°C. TBS and TBST washes were repeated and the blot was incubated with Enhanced Chemiluminescent (ECL) Western Blotting Substrate (Pierce) for two minutes. The blot was then exposed to high performance chemiluminescence film (GE Healthcare) for 1-5 minutes and developed.

2.0 Confirming the Interaction between Menin and the PAF Complex

2.1 Antibodies

The antibodies used in the following experiments include those against the V5 epitope, menin and trithorax (Trx). The monoclonal V5 antibody is commercially

available and recognizes the 14 amino acid V5 epitope (GKPIPPLLGLDST) (Invitrogen, Catalog number R960-25). It is used at a concentration of 1:2500 followed by incubation with α mouse secondary antibody (1:10,000). Mnn1 9562 (2nd bleed) is a polyclonal antibody raised against the full length of *Drosophila* menin. Specifically, antibody derived from the second bleed was used. It is used at a concentration of 1:2500 followed by incubation with α rabbit secondary antibody (1:10,000). Trx N1 (1241) is a polyclonal antibody raised against the N-terminus of the *Drosophila* Trx protein. It is used at a concentration of 1:1000 followed by incubation with α rabbit secondary antibody (1:10,000).

2.2 Coimmunoprecipitation of menin and members of the PAF complex

Upon expression verification of V5-tagged dPaf1 and dLeo1 constructs, co-immunoprecipitation experiments were performed. Two-60mm plates of S2 cells were transfected with V5 epitope-tagged constructs per immunoprecipitation sample. Transfections were performed as previously described. Three days post-transfection, cells were exposed to either heat shock or no heat shock. Those exposed to heat shock were sealed with Parafilm and submerged in a 37°C water bath for 15 minutes. Cells were then collected as previously described, without the 1x PBS wash, and lysed in 500 μ L of Bart's Modified TNE (BM-TNE) buffer (50mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP40, 10mM NaF, 2mM EDTA pH 8.0, 1mM EGTA pH 8.5, 1mM NaVO₄, protease inhibitor cocktail (Roche)) by incubating on ice for 30 minutes. Samples were then centrifuged for 15 minutes at maximum speed at 4°C and the supernatant transferred to a new tube. Prior to lysing in BM-TNE, a 1mL aliquot of the cellular suspension was

taken as a control to assess transfection efficiency. Lysate was collected from this 1mL aliquot in 300 μ L of SDS sample buffer as previously described.

Protein lysates were divided into two samples of 500 μ L to ensure equal amounts of protein in each immunoprecipitation sample, roughly 4-4.5 μ g per sample. 2 μ L of menin 9562 rabbit polyclonal antibody (2nd bleed) was added to one sample, and menin 9562 preimmune serum was added to the second sample as a negative control. Immunoprecipitation samples were mixed and incubated on ice at 4°C for 3 hours. Approximately 40 μ L of Protein G Sepharose beads (GE Healthcare) per immunoprecipitation sample were aliquotted and pulsed at 3000 rpm for 30 seconds. Beads were washed three times with 300 μ L BM-TNE and blocked in 1mL of BM-TNE with 1 μ g/ μ L BSA for at least 1 hour by rotating at 4°C. The amount of BSA added was dependent on the volume of beads being blocked. Beads were then washed four times with 300-400 μ L BM-TNE and two volumes of BM-TNE were added to the beads creating a slurry. Following the 3 hour incubation at 4°C, 60 μ L of the bead slurry was added to each immunoprecipitation sample and incubated at least 1.5 hours by rotating at 4°C, ensuring that the beads move freely. Beads were then collected and washed 5-6 times with 400-500 μ L BM-TNE. Following the final BM-TNE wash, 50 μ L of SDS sample buffer was added to each sample to elute the immune complex. 2 μ L of 0.25% bromophenol blue was added to each sample, then briefly vortexed to help separate proteins from the beads.

Immunoprecipitation samples were then stored at -80°C or analyzed immediately by SDS-PAGE. Samples were boiled for 5 minutes, returned to ice and centrifuged

briefly to eliminate any insoluble material prior to loading onto a 7 or 10% bis-acrylamide gel as previously described and run overnight at 80V. In addition to immunoprecipitation samples, 50µg of total cell lysate was loaded as a positive control to ensure the effectiveness of the transfection. Proteins were transferred to a PROTRAN nitrocellulose transfer membrane (Whatman) as previously described.

2.3 Coimmunoprecipitation analysis by Western blotting

After blocking for at least 1 hour in 5% milk/TBS, membranes were incubated with monoclonal α V5 1^o antibody (1:2500) (Invitrogen) or polyclonal α Trx N1 (1241) (1:1000), overnight, rocking at 4°C. Blots were washed and incubated with 2^o antibody as previously described. Those probed with α V5 were incubated with α mouse HRP-conjugated antibody (1:10,000) and those incubated with α Trx N1 1241 were incubated with α rabbit HRP-conjugated antibody (1:10,000). Blots probed with α V5 were incubated with ECL Western Blotting Substrate (Pierce) or Immobilon Western Detection Reagent (Millipore) for 2 or 5 minutes respectively, and those probed with α Trx N1 1241 were incubated with ECL Western Blotting Substrate (Pierce) for 2 minutes. Immuno-reactive protein species were revealed on high performance chemiluminescence film (GE Healthcare) for varying lengths of time before development. After washing with 1x TBS, the blot was incubated overnight with α Mnn1 9562 (2nd bleed) at 4°C to ensure the immunoprecipitation of menin was successful. Washes were performed as previously described, and the blot was incubated with α rabbit HRP-conjugated 2^o antibody (1:10,000). The membrane was washed, incubated with

ECL Western Blotting Substrate (Pierce) and exposed to hyperfilm as previously described.

3.0 Determining the Role of Menin and PAF in Genome stability:

3.1 Fly stocks

Fly stocks used to determine the role of the PAF complex in maintaining genomic stability include: Oregon R, $P\{PZ\}atms^{rk509}ry^{506}/TM3,Sb$ (Bloomington No. 12114), w^{1118} ; $P\{lacW\}Atu^{s1938}/TM3,Sb$ (Bloomington No. 10217), y^1w^{67c23} ; $P\{lacW\}18w^{k02701}/CyO$ (Bloomington No. 10518) and mwh^1 (Bloomington No. 549). Fly stocks were maintained on standard yeast-agar media at room temperature.

3.2 Verification of P element insertion sites in *dPaf1* and *dLeo1* mutants

To determine the functional interaction between menin and the PAF complex, loss-of-function *Drosophila* mutants for the *dPaf1* and *dLeo1* subunits were obtained from the Bloomington Stock Center. These mutants, created as part of the Berkeley *Drosophila* Genome Project Gene Disruption Project (Spradling et al, 1999), exhibit gene function disruption as a result of a P element insertion in the 5' UTR regions of the *dPaf1* and *dLeo1* genes. As these mutants have not been described in previous literature, the P element insertion sites were verified using the “Inverse PCR and Cycle Sequencing of P Element Insertions” protocol developed by the Berkeley *Drosophila* Genome Project (Rehm, 2008) to identify sequences flanking the insertion sites of PZ, PlacW and PEP elements.

dPaf1, *dLeo1*, *18w* (positive control) and *Oregon R* (negative control) adult flies were anesthetized using CO₂, placed in 1.5mL tubes, flash frozen by placing in an

ethanol/dry ice bath and stored at -80°C . *18w* flies were chosen as a positive control because the gene is interrupted by insertion of a PlacW element. Flies were ground in $200\mu\text{L}$ of Buffer A (100mM Tris-HCl pH 7.6, 100mM EDTA pH 8.0, 100mM NaCl, 0.5% SDS). An additional $200\mu\text{L}$ was added to the tube and the grinding continued until only cuticles were observed, creating a fly slurry. This slurry was incubated in a 65°C water bath for 30 minutes. $800\mu\text{L}$ of LiCl/KAc Solution (1 part 5M KAc: 2.5 parts 6M LiCl) was added to the slurry and incubated on ice for 15 minutes. The mixture was then centrifuged at maximum speed at room temperature for 15 minutes and 1mL of the supernatant transferred to a new tube. DNA was precipitated by adding $600\mu\text{L}$ of isopropanol to the supernatant, mixing and centrifuging at maximum speed at room temperature for 15 minutes. The supernatant was removed and the DNA pellet washed with 70% ethanol. After a quick spin, the ethanol was removed and the pellet air dried on ice for 30 minutes. The DNA pellet was resuspended in $150\mu\text{L}$ of TE buffer overnight at 4°C .

The genomic DNA was then digested with either Sau3A I or HinP1 I restriction enzymes (New England Biolabs) by combining $10\mu\text{L}$ of genomic DNA, the appropriate 10x digestion buffer, $100\mu\text{g}/\text{mL}$ RNase and 6 units of Sau3A I or 10 units of HinP1 I, and incubated 2.5 hours in a 37°C warm room. $10\mu\text{L}$ of the digested genomic DNA was ligated using T4 DNA Ligase (New England Biolabs) in a volume of $400\mu\text{L}$ overnight at 4°C . DNA was then isolated by ethanol precipitation. DNA was precipitated by adding 0.2M NaCl and 2 volumes of 100% ethanol and incubating on ice 10 minutes, then centrifuging at maximum speed for 15 minutes at 4°C . The supernatant was removed and

the DNA pellet dried 1 minute in a speed vacuum. The DNA pellet was then resuspended in 150 μ L of TE buffer.

PCR amplification of ligated genomic DNA was performed using primers specific to the P element (**Table 2.1**). In the case of *dPafI* mutants, which contain a PZ element, the Plac4/Plac1 primer pair was used to amplify the 5' end of the sequence and the Pry4/Pry1 pair used to amplify the 3' end. *dLeo1* and *18w* mutants, each containing a PlacW element, necessitated the use of the Plac4/Plac1 primer pair to amplify the 5' end and the Pry4/Plw3-1 pair to amplify the 3' end. All three primer pairs were used with *Oregon R* ligated genomic DNA as a negative control. Amplification reactions using primer sets Pry4/Pry1 and Pry4/Plw3-1 ($T_A=55^\circ\text{C}$) contained 10 μ L of template DNA, and in reactions using primer set Plac4/Plac1 ($T_A=60^\circ\text{C}$) the template DNA was increased to 20 μ L because no amplification product was obtained with the use of 10 μ L of template. The amplification reactions were performed using Taq polymerase (Invitrogen) as recommended by the manufacturer. The PCR amplification conditions were: (i) 1 cycle of: 95 $^\circ\text{C}$ for 5 minutes, (ii) 35 cycles of: denaturation-95 $^\circ\text{C}$ for 30 seconds, annealing-55 $^\circ\text{C}$ or 60 $^\circ\text{C}$ for 1 minute and elongation-68 $^\circ\text{C}$ for 2 minutes (iii) 1 cycle of: 72 $^\circ\text{C}$ for 10 minutes. Amplification products were resolved on a 1.5% agarose gel. Amplification products were cleaned using the illustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare). Though genomic DNA was digested with *Sau3A* I and *HinP1* I, the best quality amplification products were obtained from *HinP1* I digested-genomic DNA. Thus, these amplification products were sequenced by Mobix

and the appropriate sequencing primers, listed in **Table 2.2**. The sequences were blasted and the positions of the P element insertions were confirmed.

3.3 Loss of heterozygosity (LOH)

Using standard genetic crosses, flies were created heterozygous for the *mwh* and *dPaf1* or *dLeol* mutations on the third chromosome: *dPaf1/mwh* and *dLeol/mwh*. As a control, flies heterozygous for the *mwh* mutation alone were created, *mwh/+*. Prior to collections, flies were placed in a food tube for 1-2 hours at 25°C to improve egg yield. Flies were allowed to lay 2 hours at 25°C then transferred to a fresh tube and the embryos aged 3 hours at 25°C. 3-5 hours after egg laying (AEL), embryos were subjected to a 20 minute heat shock treatment in a 37°C water bath then returned to 25°C. Subsequently, heat shock treatments were repeated after 24 and 48 hours during first and second instar larval stages. Flies were allowed to develop and eclose at 25°C. Embryos resulting from overnight collections were raised at 25°C and used as non-heat shock controls. All collections were performed in 16x100mm tubes containing 2mL of yeast-agar media and 3-10 yeast pellets (Papaconstantinou et al, 2010). Prior to use, food on the sides of the tube was removed using a cotton swab to prevent flies from laying eggs on the side of the tube.

After eclosing, flies from both heat shock and non-heat shock treatments of *dPaf1/mwh*, *dLeol/mwh* and *mwh/+* were selected and anesthetized using CO₂. Flies were transferred to a glass tube containing 50% ethanol for approximately 2 minutes. The ethanol was removed and the flies transferred to a tube containing 70% ethanol for 2 minutes. This was repeated in 80%, 90% and 100% ethanol, and repeated twice more in

Table 2.2: Inverse PCR primer pairs and the associated sequencing primers.

Sequences flanking the P-element insertion sites of *dPaf1* and *dLeo1* mutants were amplified using one of three primer sets: Plac4/Plac1, Pry4/Plw3-1 or Pry4/Pry1 based on the type of element inserted. The resulting amplification products were sequenced with primers also dependent on the P-element inserted. PCR and sequencing primers were defined as part of the Inverse PCR and Cycle Sequencing of P Element Insertions protocol by the Berkeley Drosophila Genome Project (Rehm, 2008). Refer to Table 1.1 for the sequence of the primer pairs used in this experiment.

Table 2.2: Inverse PCR primer pairs and the associated sequencing primers

<i>Mutant</i>	<i>Primer Set</i>	<i>Sequencing Primer</i>
<i>dLeol</i>	Plac4 and Plac1	Sp1
	Plac4 and Plac1	Splac2
<i>dLeol</i>	Pry4 and Plw3-1	Spep1
	Pry4 and Plw3-1	Sp5
<i>dPaf1</i>	Plac4 and Plac1	SeqSp1
	Plac4 and Plac1	Splac2
<i>dPaf1</i>	Pry4 and Pry1	Spep1
	Pry4 and Pry1	SeqSp3

100% ethanol. Flies were then transferred to a glass-welled plate with 100% ethanol and their wings removed using fine forceps. Wings were transferred to a tube containing methyl salicylate where they remained until sinking, approximately 30 minutes to 1 hour, or a maximum of 30 days. Wings were mounted on glass slides by placing individual wings in drops of Permount (Fisher) and covering with a cover slip. Wings were observed on a Zeiss axioskop microscope at 40x and 63x magnifications. Wings were divided into 7 sections based on wing vein morphology and each counted one plane at a time. Slides were independently labeled by another member of the lab and the wings observed blindly. For each genotype and treatment, 30 wings were examined and cells exhibiting the *mwh*⁻ phenotype counted. The average number of *mwh*⁻ cells per wing was calculated and analyzed by performing unpaired T-tests.

Results

1.0 Characterization of Epitope-Tagged Expression Constructs

Antibodies were not readily available against both dPaf1 and dLeo1, thus expression plasmids were constructed in which the V5 epitope (GKPIPPLLGLDST) was affixed to the N- or C-terminus of the dPaf1 or dLeo1 protein (**Fig. 3.1**). The epitope was affixed to the *dPaf1* and *dLeo1* cDNA by PCR amplification and the resulting inserts cloned into the BamHI restriction site of the expression vector pPacB. *Drosophila melanogaster*-derived S2 cells were transfected with these constructs such that the cells overexpressed the V5 epitope-tagged dPaf1 or dLeo1 protein, facilitating co-immunoprecipitation experiments using menin or commercially available V5 antibodies. Of the 5 subunits comprising *Drosophila* PAF, the research focused on two in particular: dLeo1 and dPaf1. As previously stated, dLeo1 was identified from the mass spec analysis. dPaf1 was selected for study because it was the first subunit of the complex identified to interact with elongation factors. Thus, interaction with both dPaf1 and dLeo1 would suggest that menin interacts with the PAF complex.

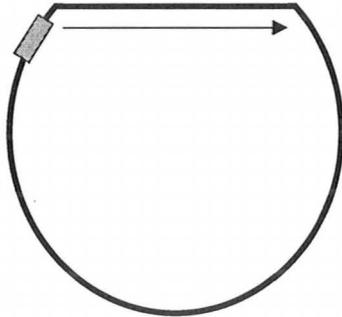
1.1 Verification of V5 epitope-tagged dPaf1 expression

The orientation of the V5 epitope-tagged *dPaf1* cDNA in the constructs was verified through a combination of diagnostic restriction enzyme digests and sequence analysis. Proper expression of the constructs was confirmed by Western blotting analysis. S2 cells were transfected with V5-dPaf1, dPaf1-V5 or pPacB alone, and 50 μ g of cell lysate was resolved by SDS-PAGE. Blotting with α V5 monoclonal antibody at a dilution of 1:2500 revealed a band of an approximate molecular weight of 75kDa in the V5-dPaf1 transfected cell sample (**Fig. 3.2**, Lane 1). In contrast, no expression was

Figure 3.1: Constructs expressing V5 epitope-tagged dPaf1 and dLeo1. The V5 epitope was incorporated onto the (A) 5' or (B) 3' end of the *dPaf1* cDNA, referred to as V5-dPaf1 or dPaf1-V5 respectively, and the (C) 5' or (D) 3' end of the *dLeo1* cDNA, referred to as V5-dLeo1 or dLeo1-V5 respectively, by PCR amplification. The resulting amplification product was cloned into the BamHI restriction site of the pPacB expression vector. Diagnostic restriction enzyme digests and sequence analysis confirmed the insert orientation. The arrow indicates the direction of transcription.

V5-dPaf1

A BclI – V5 epitope – *dPaf1* cDNA – BclI



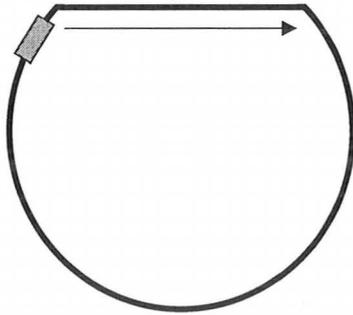
dPaf1-V5

B BclI – *dPaf1* cDNA – V5 epitope – BclI



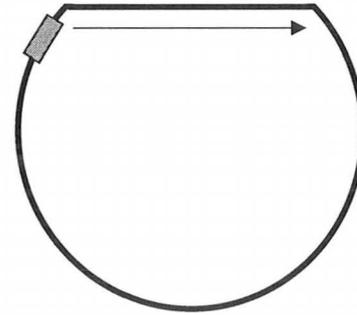
V5-dLeo1

C BclI – V5 epitope – *dLeo1* cDNA – BclI



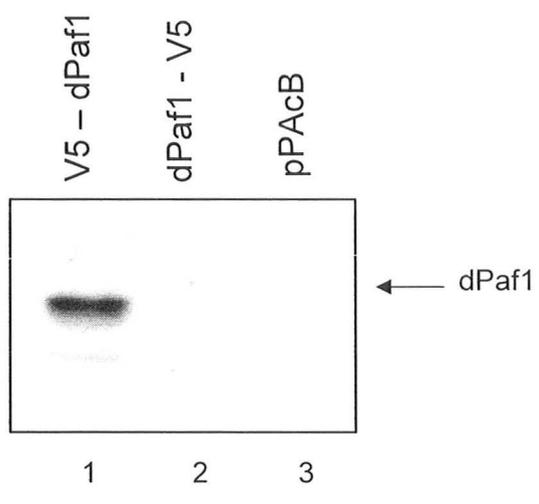
dLeo1-V5

D BclI – *dLeo1* cDNA – V5 epitope – BclI



 Actin 5C promoter

Figure 3.2: Confirmation of V5 epitope-tagged dPaf1 expression in S2 cells. V5-dPaf1, dPaf1-V5 or pPacB alone were transfected into S2 cells. 50µg of lysate was resolved by SDS-PAGE and analyzed by Western blotting, revealing a protein of 75kDa in V5-dPaf1 transfected cell samples. No expression was detected from dPaf1-V5 transfected samples. 100µg of total cell lysate was also analyzed; however expression of the dPaf1-V5 construct was again not detected.



detected from the dPaf1-V5 transfected sample (Lane 2). The amount of protein analyzed was doubled to 100µg and resolved by SDS-PAGE. As before, expression of the construct was not observed from the dPaf1-V5 transfected sample. Thus in subsequent co-immunoprecipitation experiments testing for an interaction between menin and the dPaf1 subunit of the PAF complex, the V5-dPaf1 construct alone was used to transfect cells.

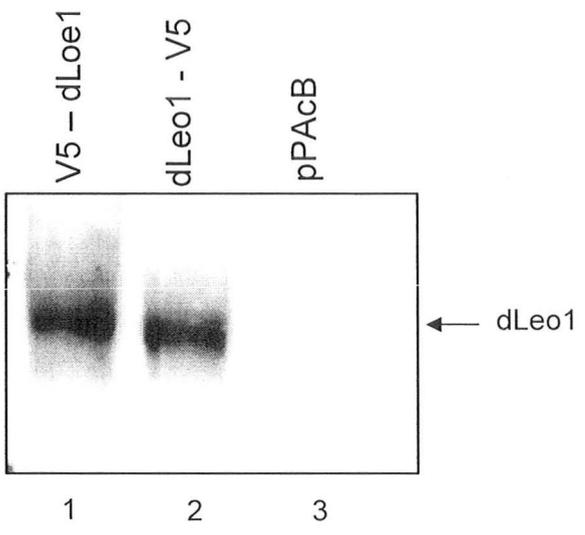
1.2 Characterization of V5 epitope-tagged dLeo1 expression

Similar to the procedure used to characterize the V5 epitope-tagged dPaf1 construct, diagnostic restriction enzyme digests and sequence analysis were used to verify the orientation of V5 epitope-tagged *dLeo1* cDNA in the pPacB expression vector. Following orientation confirmation, the expression of these vectors was confirmed by Western blotting analysis. V5-dLeo1, dLeo1-V5 and pPacB alone were transfected into S2 cells and 50µg of cell lysate was resolved by SDS-PAGE. Blotting with α V5 monoclonal antibody at a dilution of 1:2500 revealed a band of approximately 120kDa in both the V5-dLeo1 and dLeo1-V5 transfected samples (**Fig. 3.3**, Lanes 1 and 2). As the V5-dLeo1 and dLeo1-V5 expression constructs were demonstrated to express the protein, subsequent coimmunoprecipitation experiments testing the interaction between menin and dLeo1 were performed with both the N- and C-terminal tagged constructs.

2.0 Identifying a Physical Interaction between Menin and the PAF Complex

The PAF complex was originally discovered in *S. cerevisiae*, and has since been identified in a variety of organisms, including *Drosophila melanogaster* and

Figure 3.3: Confirmation of V5 epitope-tagged dLeo1 expression in S2 cells. V5-dLeo1, dLeo1-V5 or pPacB alone were transfected into S2 cells. 50µg of cell lysate was resolved by SDS-PAGE and analyzed by Western blotting, revealing a protein of approximately 120kDa in both V5-dLeo1 and dLeo1-V5 transfected cell samples.



humans (Wade et al, 1996; Shi et al, 1997; Mueller and Jaehning, 2002; Squazzo et al, 2002; Rozenblatt-Rosen et al, 2005; Adelman et al, 2006; Kim et al, 2010). An interaction between menin and the PAF complex has been proposed, but has not been observed to date (Agarwal et al, 2005; Rozenblatt-Rosen et al, 2005; Dreijerink et al, 2009). Hyperparathyroidism jaw tumour syndrome (HPT-JT), an inherited syndrome resulting in tumour formation in parathyroid tissue, arises following mutations in the *HRPT2* gene (Carpten et al, 2002; Cetani et al, 2004). The protein product of *HRPT2*, parafibromin, is the human homolog of γ Cdc73 and a member of the human PAF complex (Rozenblatt-Rosen et al, 2005). Several parallels can be drawn between parafibromin and menin. Both function as tumour suppressors that when lost result in tumour formation in parathyroid tissues. Both also interact with Set1-like HMT complexes methylating histone H3 lysine 4 (Hughes et al, 2004; Rozenblatt-Rosen et al, 2005).

Mass spectrum analysis of menin complexes performed by Dr. Thomas Kusch, a collaborator at Rutgers University, identified Atu as a candidate menin-interacting protein. Atu, referred to as dLeo1 is the *Drosophila* homolog of γ Leo1 and a subunit of the PAF complex in *Drosophila*. Thus, the interaction between menin and the PAF complex was investigated via two of its subunits, dPaf1 and dLeo1.

2.1 Menin does not interact with the PAF complex subunit dPaf1

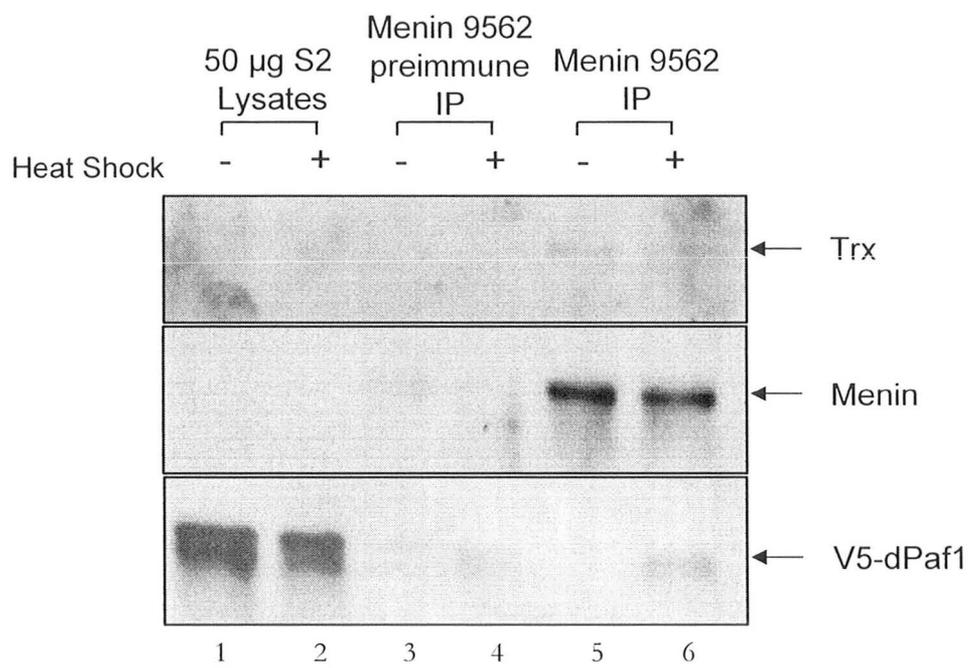
To investigate a putative interaction between menin and the PAF complex via the dPaf1 subunit, co-immunoprecipitation experiments were performed using S2 cells overexpressing the V5-dPaf1 protein. S2 cells were subjected to either a 15 minute heat

shock treatment at 37°C or left at room temperature, and menin 9562, an antibody targeted to full length menin, was used to precipitate menin along with any associated proteins from the S2 cell lysates. Western blotting did not reveal an interaction between menin and dPaf1 in either V5-dPaf1 sample (**Fig. 3.4**, Lanes 5 and 6). Rather, weak and non-specific binding of protein to the Sepharose beads was observed for both immune- and preimmune serum-immunoprecipitated samples (Lanes 3-6).

The effectiveness of the immunoprecipitation was confirmed by probing for menin. Menin was observed in both heat shock and non-heat shock treated menin 9562 immunoprecipitation samples (Lanes 5 and 6), demonstrating that menin was in fact precipitated successfully. As an additional positive control to verify that menin-associated proteins were also pulled down, the interaction between menin and Trx was sought. Trx is a protein that has previously been demonstrated to interact with menin (A. Pepper, unpublished results). The interaction was observed in the V5-dPaf1 sample not exposed to heat shock (Lane 5), and the interaction is reduced following the 15 minute heat shock treatment (Lane 6). This concurs with what has previously been reported (A. Pepper, unpublished results), demonstrating that menin-associated proteins were indeed precipitated. As a negative control, immunoprecipitations were performed using menin 9562 preimmune serum. Menin, Trx and V5-dPaf1 were not detected in these samples (Lanes 3 and 4). As a control, 50µg of lysate was resolved via SDS-PAGE to demonstrate the expression of the V5 epitope-tagged dPaf1 protein in transfected cell samples (**Fig. 3.4**, Lanes 1 and 2).

Figure 3.4: Menin does not interact with the PAF complex subunit dPaf1.

Following a 3 hour immunoprecipitation with the menin 9562 antibody, an interaction was not observed between menin and dPaf1. As a confirmation that menin-associated proteins were precipitated, the interaction between menin and Trx was observed in menin 9562 immunoprecipitation samples. As an additional positive control the blot was probed for menin to ensure the effectiveness of the immunoprecipitation, and menin was observed to precipitate in menin 9562 samples. Menin, Trx and V5-dPaf1 were not observed in menin 9562 preimmune samples.



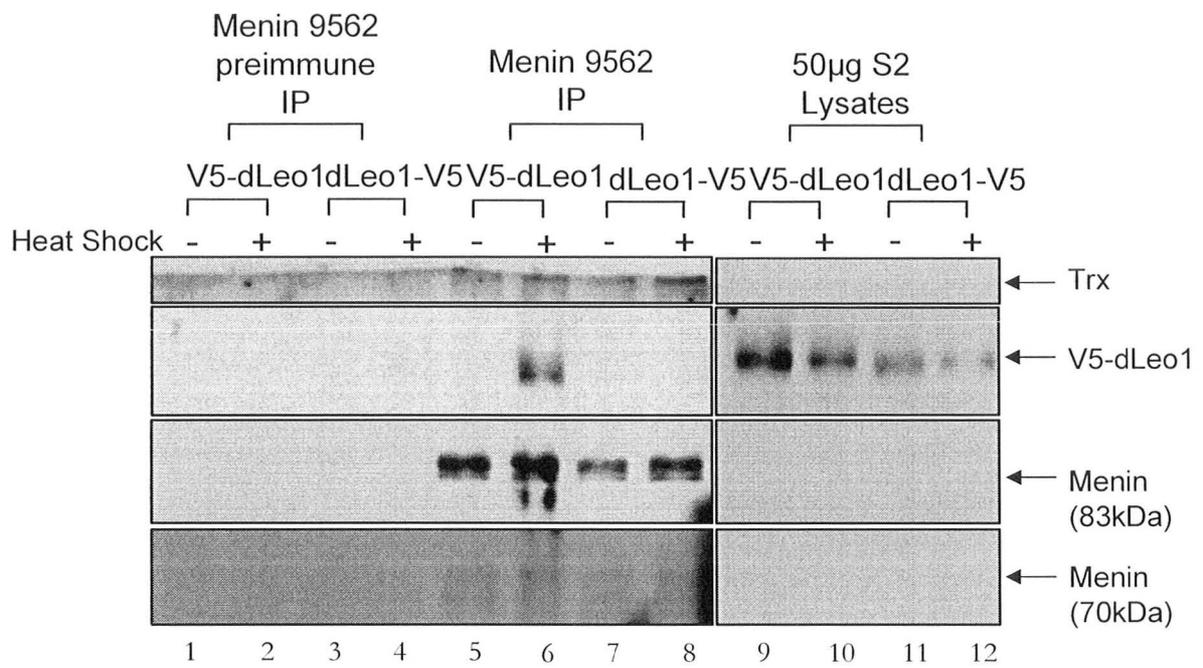
2.2 Menin interacts with the PAF complex subunit dLeo1

A putative interaction between menin and dLeo1, a subunit of the PAF complex homologous to yLeo1, was indentified through mass spectrometry analysis of menin complexes. To verify this interaction, co-immunoprecipitation experiments were performed. S2 cells were transfected with either V5-dLeo1 or dLeo1-V5 and subjected to a 15 minute heat shock treatment at 37°C or left at room temperature. Three hour immunoprecipitations were performed using the menin 9562 antibody and analyzed by Western blotting (**Fig. 3.5**).

Probing with the V5 antibody revealed an interaction between menin and V5-dLeo1 (Lane 6) following the 15 minute heat shock treatment. An interaction was not observed in cells retained at room temperature (Lane 5). It should also be noted that the interaction between menin and dLeo was not observed when the V5 epitope was located at the C-terminus of the protein (Lanes 7 and 8), suggesting that the interaction may occur at the C-terminus of the dLeo1 protein. However, the expression of the dLeo-V5 construct was low, particularly in heat shock treated samples, indicating that the lack of interaction may be determined by the low abundance of the tagged dLeo1 protein (**Fig. 3.6**, Lanes 11 and 12).

As a confirmation of the effectiveness of the immunoprecipitation, the blot was probed for menin. The 83kDa form of menin was observed in all menin 9562 immunoprecipitation samples (Lanes 5-8) indicating that the immunoprecipitation was successful. Interestingly, the 70kDa form of menin may have also been precipitated (Lanes 5-8). The 70kDa form of menin was previously reported to accumulate in

Figure 3.5: Menin interacts with the PAF complex subunit dLeo1. Following a 3 hour immunoprecipitation with menin 9562 antibody, an interaction was observed between menin and dLeo1 in S2 cells subjected to a 15 minute heat shock. The interaction was observed only in N-terminal tagged samples and following heat shock. As positive controls, the effectiveness of the immunoprecipitation was confirmed by probing for menin and Trx, a protein with which menin has previously been demonstrated to interact. Menin, Trx, V5-dLeo1 and dLeo1-V5 were not observed in Mnn1 9562 preimmune samples.



response to heat shock (Papaconstantinou et al, 2005). However, because it is observed in both heat shock and non-heat shocked samples, this may instead represent a degradation product. The blot was also probed for Trx to confirm the precipitation of menin-associated proteins, and the interaction was observed in both V5-dLeo1 and dLeo1-V5 menin 9562 immunoprecipitation samples (Lanes 5-8). As a negative control, mirror immunoprecipitations were performed using menin 9562 preimmune serum in place of the menin antibody. Menin, V5-dLeo1, dLeo1-V5 and Trx were not detected in these samples (Lanes 1-8). 50µg of lysate was resolved via SDS-PAGE to confirm the transfection (Lanes 9-12).

3.0 Functional Interaction between Menin and the PAF Complex

3.1 Confirmation of P element insertion sites in dPaf1 and dLeo1 mutants

To further study the interaction between menin and the PAF complex, functional studies were undertaken utilizing the model organism *Drosophila melanogaster*. *dPaf1* and *dLeo1* mutants were created as part of the Berkeley Drosophila Genome Project Gene Disruption Project (Spradling et al, 1999), however they had not been characterized in previous reports. Thus the position of the P element insertions was verified using the “Inverse PCR and Cycle Sequencing of P Element Insertions” protocol proposed by the Berkeley Drosophila Genome Project (Rehm, 2008). The P element insertion sites of *dPaf1* and *dLeo1* mutants were confirmed through sequence analysis of regions flanking the insertions sites. The flanking regions were amplified using primer sets specific to the P element (PlacW or PZ) and analyzed on a 1.5% agarose gel, revealing products ranging from 300 – 900 base pairs in length. Sequence analysis of these fragments against those

listed at the National Center for Biotechnology Information (NCBI) confirm the insertion sites listed both on Flybase (Flybase, 2010) and by the Berkeley Drosophila Genome Project Gene Disruption Project (Spradling et al, 1999) (**Appendix B**). The PZ insertion site in the *dPaf1* mutant was confirmed to be in the 5' UTR of the gene 74 nucleotides upstream of the initiating methionine, specifically the 601,354th nucleotide of the third chromosome (Accession No. NM_141236; Flybase ID FBtr0078868) (**Fig. 3.6A**). Similarly, the PlacW insertion site in the *dLeol* mutant was located 54 nucleotides upstream of the initiating methionine, specifically the 1,439,130th nucleotide of the third chromosome, the 5' UTR of the *dLeol* gene (Accession No. NM_079516; Flybase ID FBtr0078704) (**Fig. 3.6B**).

3.2 dPaf1 and dLeol play a role in maintaining genome stability

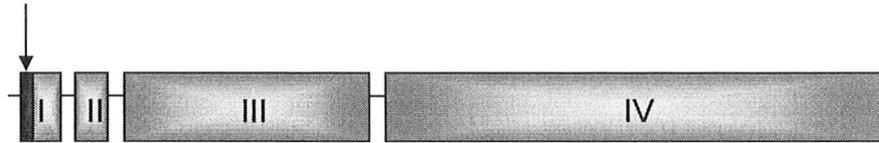
To determine the roles *dPaf1* and *dLeol* play in maintaining genomic stability, flies heterozygous for the *mwh* and *dPaf1* or *dLeol* mutations on the third chromosome were constructed using standard genetic crosses. The LOH assay utilizes the *mwh* gene, and is used as a gauge of genome stability in post-mitotic cells in response to stress (Baker et al, 1978; Brodsky et al, 2000; Papaconstantinou et al, 2010). In heterozygous progeny (*dPaf1/mwh*, *dLeol/mwh* or *mwh/+*), the wild-type copy of the *mwh* gene can get lost due to chromosomal breakage, chromosome loss, nondisjunction or a point mutation following exposure to a stressor (Brodsky et al, 2000). Here, chronic 20 minute heat shock treatments at 3-5 hours AEL (stage 6-10 embryos), 27-29 hours AEL (1st instar larvae) and 51-53 hours AEL (2nd instar larvae) constituted a non-genotoxic stress

Figure 3.6: Confirmation of P element insertion sites in *dPaf1* and *dLeo1* mutants. Inverse PCR was used to amplify regions flanking P element insertion sites in *dPaf1* and *dLeo1* mutants. Sequence analysis of the amplification products confirmed the insertion sites to be (A) 74 nucleotides upstream of the initiating methionine in the first exon (601,354th nucleotide of the 3rd chromosome) of *dPaf1* mutants (Accession No. NM_141236; Flybase ID FBtr0078868) and (B) 54 nucleotides upstream of the initiating methionine in the first exon (1,439,130th nucleotide of the third chromosome) in *dLeo1* mutants (Accession No. NM_079516; Flybase ID FBtr0078704). Both P elements are inserted in the 5' UTR of the genes.

A P{PZ}atms^{rk509}ry⁵⁰⁶
3R: 601,354



B P{lacW}Atu^{s1938}
3R: 1,439,130



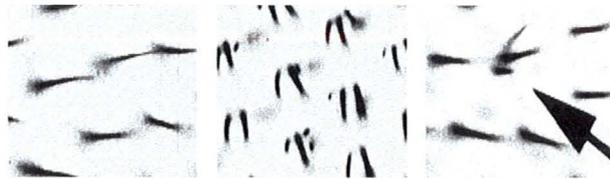
previously demonstrated to induce genome instability in *Drosophila melanogaster* *menin* mutants (Papaconstantinou et al, 2010). After eclosing, the wings were dissected from these flies and non-heat shock controls and examined for the *mwh* phenotype. On each wing there are approximately 20,000 cells, each of which has a single hair in *mwh* heterozygous flies. Following loss of the wild-type copy of the *mwh* gene, the *mwh*⁻ phenotype is observed, such that 2 or more hairs protrude from a single cell (**Fig. 3.7A**). Thus, 30 wings were examined from each genotype and treatment (exposure to chronic heat shock or maintained at 25°C) for cells expressing the *mwh*⁻ phenotype. It should be noted that experimental flies were heterozygous for the *dPaf1* and *dLeol* mutations. *dPaf1* and *dLeol* are both homozygous lethal, thus homozygous flies could not be constructed.

Flies heterozygous for the *mwh* mutation alone, *mwh*/+, demonstrated an average of 0.83 *mwh*⁻ cells per wing, regardless of heat shock treatment. This coincides with that previously described in *mwh*/+ flies (Papaconstantinou et al, 2010). Introduction of a heterozygous mutation in the *dPaf1* gene resulted in an average of 1.03 *mwh*⁻ cells observed, similar to that observed from *mwh*/+ mutants. This almost doubles to 1.9 *mwh*⁻ cells in flies subjected to chronic heat shock. This represents a significant increase from non-heat shocked control *dPaf1*/*mwh* flies ($p=2.5 \times 10^{-3}$) and *mwh*/+ heat shocked flies ($p=1.7 \times 10^{-3}$) (**Fig. 3.7B**).

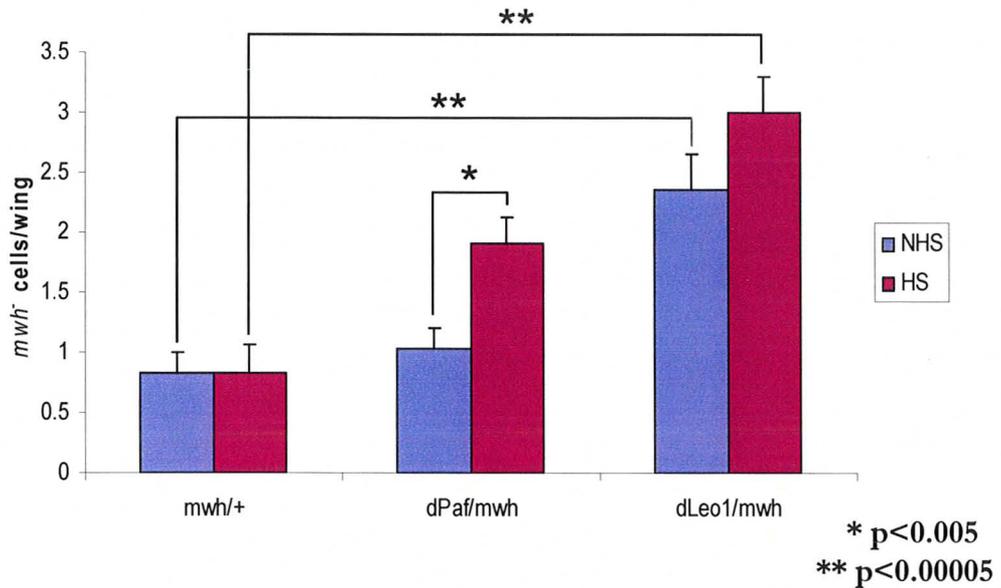
The most dramatic levels of genome instability were observed in *dLeol*/*mwh* heterozygous flies (**Fig. 3.7B**). Flies maintained at 25°C demonstrated an average of 2.37

Figure 3.7: dPaf1 and dLeo1 play a role in maintaining genome stability in response to heat shock. Following exposure to chronic heat shock treatment, genome stability was assessed (* $p < 0.005$, ** $p < 0.00005$). (A) The *mwh*⁻ phenotype is defined as two or more hairs protruding from a single cell on the *Drosophila melanogaster* wing. Cells with one functional copy of the *mwh* gene have one hair per cell. Loss of the functional copy of the *mwh* gene results in the *mwh*⁻ phenotype. (B) Flies carrying one copy of the *mwh* gene demonstrated an average of 0.83 *mwh*⁻ cells per wing, with and without heat shock treatment. Mutants heterozygous for *dPaf1* and *mwh* displayed a similar level of stability to *mwh*^{+/+} flies not exposed to heat shock. Exposure to chronic heat shock resulted in an increase to 1.9 *mwh*⁻ cells/wing ($p = 2.5 \times 10^{-3}$). An average of 2.37 and 3 *mwh*⁻ cells /wing were observed in *dLeo1/mwh* flies raised solely at 25°C or exposed to chronic heat shock respectively. This represents a significant increase from *mwh*^{+/+} control ($p = 2.4 \times 10^{-5}$) and heat shocked ($p = 3.7 \times 10^{-7}$) flies. Error bars represent standard error.

A



B



mwh⁻ cells, three times higher than *mwh*/+ flies ($p=2.4 \times 10^{-5}$) and more than double that observed in *dPaf1/mwh* control flies ($p=1.5 \times 10^{-4}$). *dLeo1/mwh* flies exposed to chronic heat shock demonstrated an increase to 3 *mwh*⁻ cells per wing, significantly higher than that observed in both *mwh*/+ (3.7×10^{-7}) and *dPaf1/mwh* ($p=4.2 \times 10^{-3}$) heat shocked flies. However, the increase in *mwh*⁻ cells following chronic heat shock was not significant ($p=0.13$). This differs from that observed in *dPaf1/mwh* mutants ($p=2.5 \times 10^{-3}$) as well as *Mnn1;mwh*/+ mutant flies (Papaconstantinou et al, 2010), both of which observed significant increases in *mwh*⁻ cells following heat shock. In fact, the level of instability observed in both control and heat shocked *dLeo1/mwh* flies is reminiscent of *Mnn1* mutant flies following chronic heat shock, which demonstrate nearly 4 *mwh*⁻ cells (Papaconstantinou et al, 2010).

The goal of this experiment was to explore a genetic interaction between menin and the PAF subunits *dPaf1* and *dLeo1*, and what effect this interaction may have in maintaining genome stability. Using standard genetic crosses, flies were constructed homozygous for the *Mnn1* mutation and heterozygous for both *dPaf1* or *dLeo1* and *mwh*. Two *Mnn1* deletion mutants were previously created in the lab, *Mnn1*^{e30} and *Mnn1*^{e173} (Papaconstantinou et al, 2005). Thus flies were homoallelic (*Mnn1*^{e30}/*Mnn1*^{e30} or *Mnn1*^{e173}/*Mnn1*^{e173}) or heteroallelic (*Mnn1*^{e30}/*Mnn1*^{e173}) for the *Mnn1* mutation. Flies were subjected to heat shock and collected following the same protocol used for *mwh*/+, *dPaf1/mwh* and *dLeo1/mwh* flies and the *mwh*⁻ cells counted. Unfortunately, contamination was identified in the original *Mnn1*^{e30} and *Mnn1*^{e173} stocks used to create the experimental flies, and the contamination was confirmed in all stocks subsequently

created from these stocks. Thus, although the experiment was performed using flies consisting of both *Mnn1* and *dPaf1* or *dLeol* mutations, the results are unusable as *Mnn1* mutant stocks were contaminated in flies with a functional *Mnn1* allele.

Discussion

The PAF complex plays diverse roles in transcription ranging from elongation to 3' end formation, posttranscriptional processing and mRNA surveillance (Krogan et al, 2003; Rondón et al, 2003; Penheiter et al, 2005; Zhu et al, 2005; Nordick et al, 2008; Rozenblatt-Rosen et al, 2009). The PAF complex in yeast and *Drosophila* consists of 5 subunits: dPaf1, dCdc73, dCtr9, dRtf1 and dLeo1 (Wade et al, 1996; Koch et al, 1999; Mueller and Jaehning, 2002; Adelman et al, 2006). Human PAF differs from other organisms with the incorporation of a sixth subunit, hSki8, unique to higher eukaryotes (Zhu et al, 2005; Kim et al, 2010).

An interaction between menin and the PAF complex has been hypothesized but to date has not been identified. The proposed interaction is based on several parallels drawn between menin and parafibromin, the protein product of the human homolog of Cdc73, *HRPT2* (Agarwal et al, 2005; Rozenblatt-Rosen et al, 2005; Dreijerink et al, 2009). Both function as tumour suppressors whose loss of function results in tumour formation in endocrine tissues. Loss of menin results in MEN1 syndrome, characterized by tumour formation in two or more endocrine tissues, and loss of parafibromin results in HPT-JT, which manifests itself as tumour formation in parathyroid tissues (Brandi et al, 2001; Carpten et al, 2002; Cetani et al, 2004). Both proteins also interact with Set1-like HMTase complexes that methylate histone H3 lysine 4 (Hughes et al, 2004; Rozenblatt-Rosen et al, 2005). A mass spectrometry analysis of menin complexes by Dr. Thomas Kusch at Rutgers University identified dLeo1, the *Drosophila* homolog of yLeo1, as a candidate interacting partner with menin.

Leo1 is perhaps the least characterized of the PAF subunits. The gene was originally identified in *S. cerevisiae*, and encodes an extremely hydrophilic and polar protein that is highly positively charged (Magdolen et al, 1994). yLeo1's role as a PAF subunit was later identified in yeast and humans (Mueller and Jaehning, 2002; Rozenblatt-Rosen et al, 2005). In yeast, yLeo1 is a nonessential protein as gene disruption results in a wild-type phenotype and normal growth (Magdolen et al, 1994; Mueller and Jaehning, 2002). However, *Leo1* mutations in *Drosophila* and zebrafish result in a recessive lethal phenotype indicating that in higher eukaryotes Leo1 has evolved to play an essential role in viability (Nguyen et al, 2010; Flybase, 2010). The same is true for the remaining members of the PAF complex: dPaf1, dCdc73, dCtr9 and dRtf1, mutations of which are homozygous lethal (Flybase, 2010). In fact, a functional study of its function in zebrafish suggests that zLeo1 is important in development, specifically cardiac differentiation and development of neural crest cells (Nguyen et al, 2010). Recently, the importance of yLeo1 to the PAF complex in transcription elongation was highlighted by Dermody and Buratowski (2010). Via the yLeo1 subunit, PAF binds RNA *in vivo*. Though both yRtf1 and yLeo1 were found to bind RNA, *yRtf1* mutations did not yield a reduction in the PAF-RNA association whereas *yLeo1* mutations resulted in a dramatic reduction. Furthermore, yLeo1 functions in maintaining the PAF complex at actively transcribed genes through its association with the nascent transcript (Dermody and Buratowski, 2010).

Coimmunoprecipitation experiments were performed using S2 cells overexpressing one of two PAF subunits, dPaf1 or dLeo1. An interaction between menin

and dPaf1 was not observed, and this was not a surprise as dPaf1 was not identified as a menin-interacting partner in the mass spectrum analysis. However, an interaction was identified between menin and dLeo1, confirming the results of the mass spectrum analysis. Because a dLeo1 antibody was not available, overexpressed dLeo1 was tagged with the V5 epitope at either the N- or C-terminus of the protein to allow use of the commercially available V5 antibody for detection. The interaction between the proteins was observed only in N-terminal tagged samples and not those harbouring the C-terminal epitope, suggesting that perhaps the interaction occurs at the C-terminus of the dLeo1 protein. It is also possible that the C-terminal-tagged dLeo1 protein does interact with menin, however lower expression of dLeo1-V5 may not have yielded sufficient protein to visualize the interaction (**Fig. 3.5**). The Leo1 protein is not well characterized. However, an analysis of human, mouse, zebrafish, *Drosophila* and yeast Leo1 reveal a conserved Leo1 domain and NLS at the C-terminus of the protein (Zhao et al, 2005; Nguyen et al, 2010). Thus, it is possible that menin interacts with the C-terminus of dLeo1, and that this interaction masks the V5 epitope of the dLeo1-V5 construct. Alternatively, the V5 epitope may disrupt the interaction of menin with dLeo1.

The menin-dLeo1 interaction was observed following a 15 minute heat shock at 37°C but not in unexposed cells, suggesting that the interaction occurs in response to stress. Menin was previously demonstrated to regulate the stress response in *Drosophila*, particularly in response to environmental stresses including: hypoxia, oxidative stress, hyperosmolarity and heat shock, γ -irradiation and the cross-linking agents nitrogen mustard and cisplatinum (Busygina et al, 2004; Papaconstantinou et al, 2005).

Papaconstantinou et al (2005) demonstrated that menin is a positive regulator of the heat shock proteins Hsp70 and Hsp23 in *Drosophila*. Heat shock of *Mnn1* null mutants during embryonic stages resulted in developmental arrest and lethality. Analysis of Hsp70 expression revealed that *Mnn1* overexpression leads to elevated levels of Hsp70 that do not decrease during recovery, and reduced expression when *Mnn1* was down-regulated by shRNA or absent in *Mnn1* null mutants (Papaconstantinou et al, 2005).

In a survey of PAF complex localization, Adelman et al (2006) subjected *Drosophila* larvae to heat shock treatments of varying lengths and examined dPaf1, dCdc73 and dRtf1 localization at the *Hsp70* gene. Prior to heat shock, PAF subunits and Ser2 RNA Pol II were not detected at the gene locus. Following a 5 minute heat shock PAF translocated to the gene and after 15 minutes both were observed throughout the coding region of the gene. Specifically, they were recruited downstream of the promoter region. Down-regulation of dPaf1 and dRtf1 via dsRNA resulted in reduced *Hsp70* RNA production. Histone H3 lysine 4 methylation of promoter-proximal nucleosomes following heat shock was also found to require PAF, specifically the dPaf1 subunit, consistent with the idea that recruitment of Set1-like HMTases on RNA Pol II depends on Paf1 (Adelman et al, 2006).

The pattern of PAF complex localization and histone H3 lysine 4 trimethylation on *Hsp70* observed by Adelman et al (2006) is strikingly similar to that of the TAC1 complex following heat shock (Smith et al, 2004). The TAC1 complex is a IMDa complex consisting of the Trx, dCBP and Sbf1 subunits (Petruk et al, 2001). TAC1 promotes chromatin remodeling via acetylation through its dCBP subunit and HMTase

activity via Trx at histone H3 lysine 4 (Petruk et al, 2001; Milne et al, 2002). Menin directly interacts with Trx, and in humans menin has been demonstrated to associate with mixed lineage leukemia, MLL, the human homolog of Trx (Milne et al, 2005; Caslini et al, 2007; Jin et al 2010). In fact, it has been suggested that menin functions as a scaffold, binding proteins such as lens epithelium-derived growth factor (LEDGF) to MLL promoting gene activation. Yokoyama and Cleary (2008) demonstrated that LEDGF colocalizes with menin and MLL on genes that play a role in MLL-associated leukemogenesis, such as *HOXA* and *CDK1*, and that the association of LEDGF with the menin-MLL complex is essential for sustained expression of these genes. They also found that a subset of mutations derived from MEN1 patients inhibited the interaction between menin and LEDGF. The menin-MLL association was maintained, however processes associated with the menin-MLL complex were hindered. Thus, a link can be drawn between menin's role as both a tumour suppressor and oncogene through its scaffolding function, in this case facilitating the binding of LEDGF to MLL (Yokoyama and Cleary, 2008). Recently, PAF was shown to function with MLL in promoting leukemogenesis. A direct interaction was observed between MLL and the hPaf1, hCtr9 and hCdc73 subunits, and this interaction was crucial for transcriptional activity and leukemogenesis. In fact, this interaction occurred at the CxxC-RD2 region of the protein, which is always observed in MLL-rearranged oncoproteins (Muntean et al, 2010).

PAF appears to play a role in maintaining genome stability following stress. LOH assays utilizing the *mwh* gene revealed that following heat shock, instability increased 2-3 times that observed in *mwh/+* control flies when *dPaf1* or *dLeo1* are mutated. Even

more striking are the differences observed in non-heat shock control flies. *Paf/mwh* mutants demonstrated low levels of instability similar to that of *mwh/+* and *Mnn1;mwh/+* mutants (Papaconstantinou et al, 2010). In contrast, *Atu/mwh* mutants demonstrated a significantly higher level of instability, 2.5-3 times what was observed in other genotypes in the absence of heat shock. It should also be noted that due to the recessive lethal nature of the *dPaf1* and *dLeo1* genes, all flies assayed for LOH were heterozygous, making the observations even more salient. Though one functional copy of the gene remained, the level of instability observed in *dLeo1/mwh* mutants is similar to what Papaconstantinou et al (2010) observed in *Mnn1*-null mutants following heat shock. Together, this suggests that the dLeo1 subunit is crucial in facilitating PAF's role in maintaining genome stability even in the absence of stress. Unfortunately, due to contamination issues with the *Mnn1* stocks the functional interaction between menin and PAF in terms of maintaining genome stability could not be determined. However, with the reinstatement of purified *Mnn1^{e30}* and *Mnn1^{e173}* fly stocks this can be further explored.

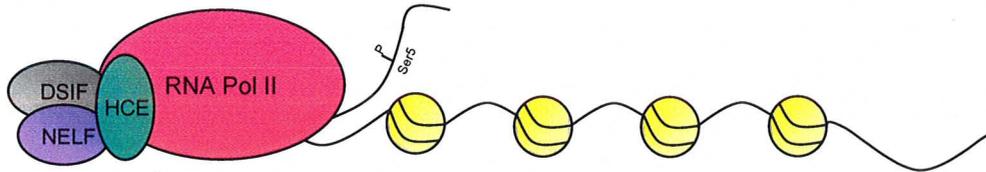
Thus, identification of the novel interaction between menin and dLeo1 provides evidence that menin promotes chromatin modification through facilitating HMTase activity. Observation of the interaction following heat shock further suggests that their association occurs in response to stress. A possible model for the interaction can be demonstrated using the *Hsp70* gene (**Fig. 4.1**). Under normal physiological conditions, RNA Pol II is stalled at the promotor-proximal end of a wide variety of genes, including the heat shock gene *Hsp70* (Rougvie and Lis, 1990). Under inducible conditions, such as heat shock, the positive elongation factor P-TEFb overcomes the negative effects of DRB

sensitivity inducing factor (DSIF), human capping enzyme (HCE) and negative elongation factor (NELF) allowing transcription to proceed (Lis et al, 2000; Renner et al, 2001). Approaching the first nucleosomal barrier in the promotor-proximal region, PAF is recruited to RNA Pol II which goes on to recruit the HMTase machinery via menin-Trx. Menin further binds proteins that enhance HMTase activity, such as LEDGF (Yokoyama and Cleary, 2008). The histone H2A/H2B dimer is disassembled and histone H3 lysine 4 is methylated, resulting in disassembly of the nucleosome permitting RNA Pol II to traverse the DNA and transcription to proceed. *Mnn1*-null mutants exhibit normal *Hsp70* expression in the first 15 minutes of heat shock, and it is after this point that the abnormal stress response is observed (Papaconstantinou et al, 2005). It is possible that menin is not essential for the initial recruitment of the HMTase machinery to stalled RNA Pol II. Menin's role may lie in recruiting and stabilizing the association between the PAF-Trx/TAC1 complex and RNA Pol II during re-initiation. Thus the PAF-menin interaction is not required for the induction of transcription in response to stress, but is instead necessary for maintaining transcription of stress-induced genes.

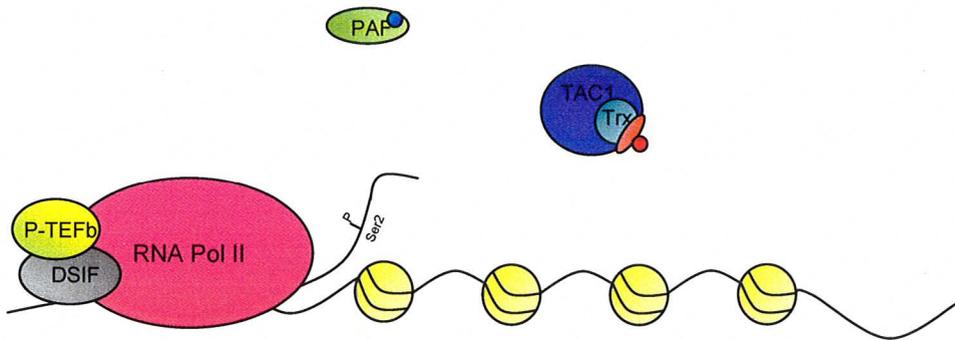
The identification of a novel menin-binding protein opens several doors of exploration. Due to antibody limitations, the interaction was detected using S2 cells overexpressing tagged versions of *dPaf1* and *dLeo1*, thus it is important to verify the interaction using endogenous protein. The production of a dLeo1 antibody will facilitate this, as well as allow for reciprocal coimmunoprecipitation experiments that were cost-prohibitive with the commercially available V5 antibody. Upon confirmation of the interaction at physiological levels, the domains of interaction should be determined. The

Figure 4.1: Model for the role of the menin-dLeol1 interaction following stress. (A) Under normal physiological conditions, RNA Pol II is stalled at the promotor-proximal region of the gene under the influence of DSIF, NELF and HCE. During this time PAF, TAC1 and menin are functioning elsewhere in the genome. (B) Under inducible conditions, P-TEFb suppresses the inhibitory effects of DSIF, RNA Pol II is released and transcription proceeds. PAF and TAC1-menin are recruited to the gene from other loci. (C) PAF and TAC1-menin are recruited to RNA Pol II upon the first nucleosomal barrier. Through this interaction, histone H3 lysine 4 is methylated as the histone H2A/H2B dimer is disassembled and RNA Pol II traverses the DNA and transcription proceeds. Thus menin and PAF function as a scaffold and platform to facilitate chromatin modification via HTMase activity.

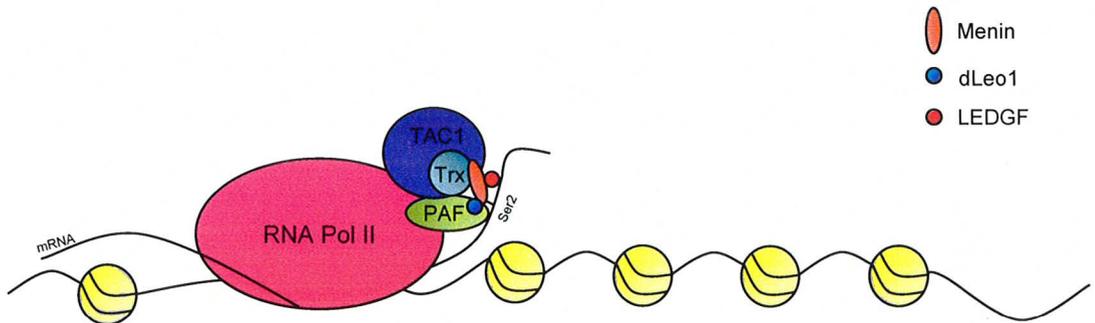
A Normal physiological conditions RNA Pol II is stalled:



B Transcription resumes during stress:



C Recruitment of PAF, TAC1 and menin promote elongation:



interaction was only observed in N-terminal tagged samples, suggesting that the interacting domain of dLeo1 is located towards the C-terminus. As of yet, the interacting domain of menin is unknown. This may be accomplished with the use of GST fusion proteins carrying mutations or deletions in the various domain regions.

As the interaction was observed following heat shock, examining the kinetics of the interaction would be informative. Subjecting cells to varying lengths of heat shock and demonstrating the state of the interaction at these time points would be useful in determining if the interaction is prolonged for the duration of the stress. It would also be prudent to examine the interaction during recovery from heat shock, and how this correlates with the expression of various stress induced genes, such as *Hsp70*.

In addition to further confirming the kinetics of interaction, the functional relationship between menin and dLeo1 should be explored. The LOH assay can be used to determine their role in maintaining genome stability using double mutants. This can also be tested using shRNA/RNAi. Flies carrying a *UAS-dsRNA-dLeo1* construct were obtained and tested for lethality (**Appendix C**). Driving the construct with ubiquitous GAL4 drivers resulted in 100% lethality, however survival was observed with the use of tissue-specific GAL4 drivers. Thus with the use of a wing disc-specific GAL4 driver, the shRNA/RNAi construct could be expressed solely in the developing wing permitting a more accurate demonstration of how menin and dLeo1 interact to influence genome stability. The experiments performed here have used heat shock as a stressor. If in fact the menin-dLeo1 interaction does play a role in maintaining genome stability, it would be interesting to see if this is conserved across several types of stress and DNA damage.

Mnm1 mutants are sensitive to a variety of stressors, including: heat shock, hyperosmolarity, hypoxia and oxidative stress (Papaconstantinou et al, 2005), as well as γ -irradiation and the cross-linking agents nitrogen mustard and cisplatin (Busygina et al, 2005). By exposing *Drosophila* to a variety of stresses and determining genome stability, it could be determined if their role in the stress response is global or specific to certain types of DNA damage.

Should the interaction not play a role in the stress response and genome stability, it may be worthwhile to investigate alternative pathways. Both menin and PAF, particularly dCdc73 and dLeo1, have been demonstrated to interact with β -catenin in the canonical Wnt pathway. Mosimann et al (2006) suggest a model in which β -catenin recruits PAF through dCdc73, thus promoting the upregulation of Wnt target genes. The identification of NESs in the *MEN1* gene, coupled with the observation that menin loss results in nuclear β -catenin accumulation, suggests that menin participates in the canonical Wnt pathway by exporting β -catenin to the cytoplasm (Cao et al, 2009). Thus, an alternative avenue of study could involve the menin-dLeo1 interaction and its role in the canonical Wnt signaling pathway.

A novel interaction was identified between menin and the PAF complex via the dLeo1 subunit. Initially detected in a mass spectrum analysis, the presence of a menin-dLeo1 complex was confirmed through coimmunoprecipitation experiments and observed following heat shock, suggesting the interaction is dependent on stress. Its function in maintaining genome stability is undetermined due to complications with *Mnm1* mutant stocks; however dLeo1 appears to function in maintaining genome stability

even under physiological conditions. Thus, identification of this interaction offers new insights into how the menin protein functions in promoting tumour suppression in endocrine tissues.

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Appendix A:
Cloning and Expression Vectors

Figure A1: *dPaf1* and *dLeo1* cDNAs were received from the BDGP in the pOT2 vector. *dPaf1* and *dLeo1* cDNAs were received from the BDGP cloned into the XhoI and EcoRI restriction sites of the pOT2 vector. The plasmid exhibits chloramphenicol resistance (CAM^r). The recommended sequencing primers, *PM001*, *PM002* and *T7*, are indicated with italics in the multiple cloning region of the vector. The restriction sites into which *dPaf1* and *dLeo1* cDNAs were cloned into pOT2, **XhoI** and **EcoRI**, are indicated in bold. *dPaf1* and *dLeo1* cDNAs measure 1771bp and 2354bp in length, and the pOT2 vector measures 1665bp.

dPaf1 cDNA (1771bp) or *dLeo1* cDNA (2354bp)

1	53	73	79	90	102	108	114	120	142	176	195	214	222
PM001	Sp6	XhoI	PvuII	BstXI	XbaI	BamHI	EcoRI	BstXI	BglII	T7	PM002	PstI	SmaI

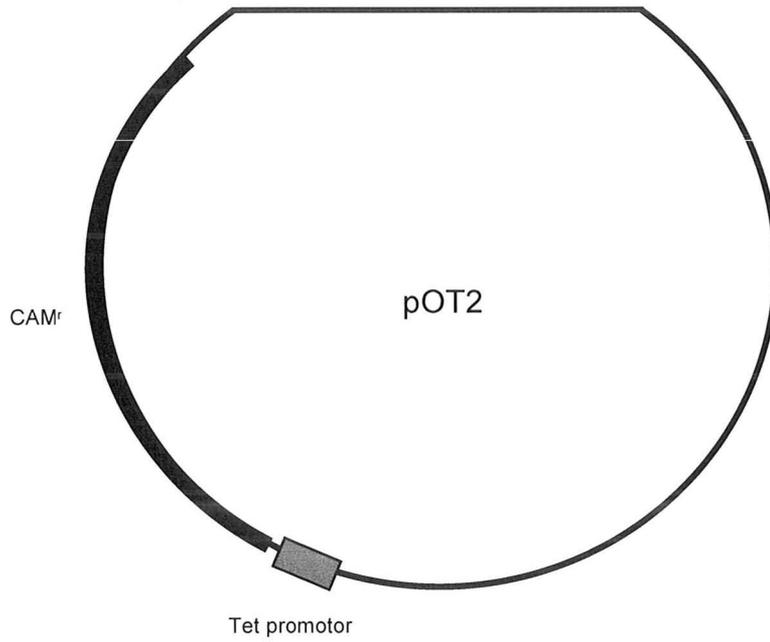
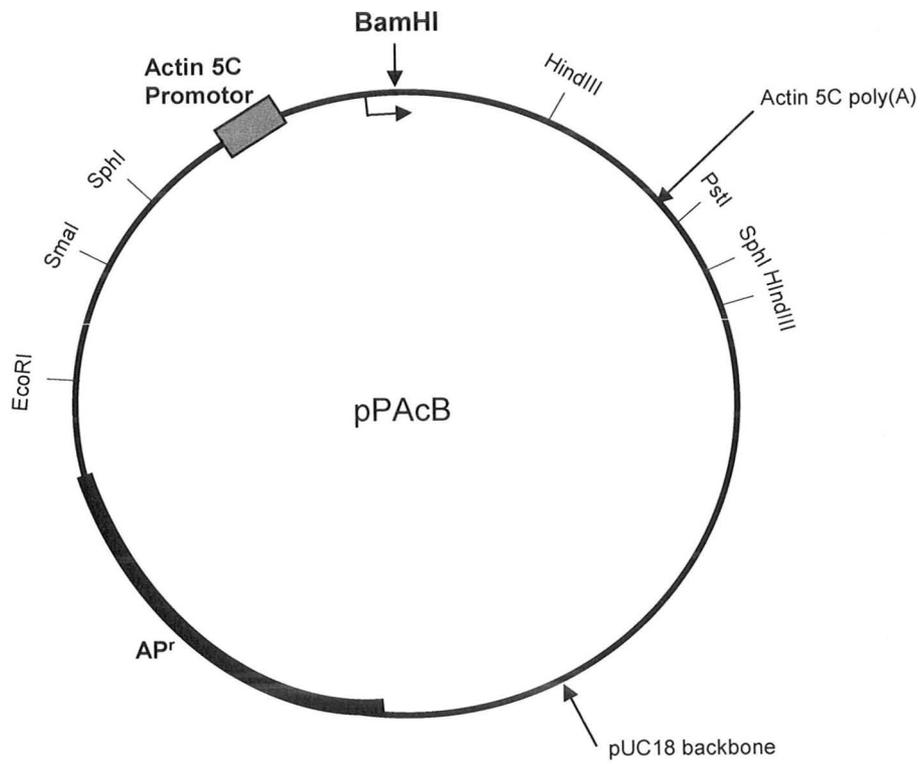


Figure A2: V5 epitope-tagged *dPaf1* and *dLeo1* are cloned into the pPacB expression vector. V5 epitopes were incorporated onto the 5' or 3' end of *dPaf1* and *dLeo1* cDNA by PCR. The fragment was then cloned into the **BamHI** restriction site, indicated in bold, of the pPacB expression vector. The actin 5C promoter is located upstream of the insertion site, and the direction of transcription is indicated. The vector is constructed with a pUC18 backbone and exhibits Ampicillin resistance (AP^r). The vector measures 6400bp.



Appendix B:
Sequences Confirming P Element Insertion Sites
in *dPaf1* and *dLeo1* *Drosophila* mutants

Figure B1: Sequences flanking the P element insertions in *dPaf1* and *dLeo1* mutants. Regions flanking the P element insertions were amplified using primers specific to the P element (PZ or PlacW) and sequenced. Amplification and sequencing primers are listed in Tables 2.1 and 2.2. Sequences were blasted and the insertion sites confirmed. For each sequence, the following information is provided: (i) the mutant (eg. *dPaf1*), (ii) the primer set used to amplify the region flanking the P element (eg. Pry4 and Pry1), and (iii) the primer used to sequence the fragment (eg. SeqSp3). All sequences were obtained from genomic DNA originally digested with the HinP1 I restriction enzyme.

A. *dPaf1* - Pry4 and Pry1 - SeqSp3

NNNNNNNCTANNNNNTCGTTATCTTCGGANCGATGTGGCGCTTCTAGTCNTNAAGAG
GCCNAATCATGATGAAATAACATAAGGTGGTCCCGTCGGCAAGAGACATCCACTTAA
CGTATGCTTGCAATAAGTGCAGTGAAGGAATAGTATTCTGAGTGTTCGTATTGAGT
CTGAGTGAGACAGCGATATGANN

B. *dPaf1* - Pry4 and Pry1 - Spep1

NNNNNNNANTGNNGNNTACGTAAAGTGGATGTCTCTTGCCGACGGGACCCCTTATG
TTATTTNATCATGATTTGGCCTCTTGATGGACTAGAAGCGCCACATCGCGTCCGAAGT
AACGAAGCTCTATGGGAGATTAACATAGTTAAAGCTTTGCGTACTCGCAAATTATT
AAAAATAAACTTTAAAAATAATTTTCGTCTAATTAATATTATGAGTTAATTCAAACC
CCACGGACATGCTAAGG

C. *dPaf1* - Plac4 and Plac1 - SeqSp1

NNNNNNNTGNNNNNNNNAAGTGTATACTTCGGTAAGCTTCGGCTATCGACGGGACC
NCCTTATGTTATTTTCATCATGGGCCNAATCTAGAAATCCTCAAATCTGCGCAACTGTT
GGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGA
TGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCAGTCACGACGTTGT
AAAACGACGGCCAGTGAATTCGGCTGCTGCTCTAAACGACGCATTTTCGTAATCCAAA
GTACGAATTTTTCCCTCAAGCTCTTATTTTCATTAACAATGAACAGGACCTAACGC
ACAGTA

D. *dPaf1* - Plac4 and Plac1 - Splac2

NNNNNNTGNNGGGAANCCTGGCGTTNCCNNCTTAATCGCCTTGCAGCACATCCCCCT
TTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTG
CGCAGATTTGAGGATTTCTAGATTTGGCCCATGATGAAATAACATAAGGTGGTCCCG
TCGATAGCCGAAGCTTACCGAAGTATACTTAAATTCAGTGCACGTTTGCTTGTGA
GAGGAAAGGTTGTGTGCGGACGAATTTTTTTTTGAAAACATTAACCCTTACGTGGAA
TAAAAAAAATGAAATATTGCAAATTTTGCTGCAAAGCTGTGACTGGAGTAAATTA
ATTCACGTGCCGAAGTGTGCTATTAAGAAATTTGTGGGAGCAGAGCCTTGGGTG

E. *dLeo1* - Plac4 and Plac1 - SeqSp1

NNNNNNNNNNNNNNNAAGTGTATACTTCGGTAAGCTTCGGCTATCGACGGGACCCC
TTATGTTATTTNNTGGCCCGCCGCAATTTGTCTTGATTGCAGATTTTCCGATTTT
AAACAAAATTTACTTGGCATTATGGGCAGCCAAAACCTCGGACGACGACAGTGGTGA
GTGGCTAATGGCTAAGTCCAGGCCTGAGTAACTAATTTTACACTGTTACATATGT
ATGTAGGTTTCATCCGGTTCGAGTTCGAAGTGGAAAGCCGACGTGTCACGCCGAAGGAG
GTAGTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAG
CTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCC
CAGTCACGACGTTGTAACGACGGCCAGTGCCAAGCTCTGCTGCTCTAAACGACGC
ATTTTCGTAATCCAAAGTACGAATTTTTTCCCTCAAGCTCTTATTTTCATTAACAATG
AACAGGACCTAANNNNCAGTAANNNNNNNNNGN

F. *dLeo1* - Plac4 and Plac1 - Splac2

NNNNNNNGNNGNANNCCCTGGCGTTNCCNNCTTAATCGCCTTGCAGCACATCCCCCT
 TTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTG
 CGCACTACCTCCTTGCGGCGTGACACTGCGGCTTCCACTTCGACTCGAACCGGATGA
 ACCTACATACATATGTGAACAGTGTAATATTAGTTACTCAGGCCTGGACTTAGCCA
 TTAGCCACTCACCCTGTCGTCGTCGAGTTTTGGCTGCCATAATGCCAAGTAAATT
 TTGTTTAAAATCGGAAAATCTGCAATCAAGACAATTCGCGGCCGGGCCATGATGAAA
 TAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAAGTATACACTTAAATTCA
 GTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTGTGCGGACGAATTTTTTTTTGAAAAC
 ATTAACCCTTACGTGGAATAAAAAAAAAAATGAAATATTGCAAATTTTGCTGCAAAGCT
 GTGACTGGAGTAAATTAATTCACGTGCCGAAGTGTGCTATTAAGAGAAAATTGTGG
 GAGCANNNNNNNGGGNGG

G. *dLeo1* - Pry4 and Plw3-1 - Sp5

NNNNANTGGCNAACCCGANGGNNNTAAAGANACCNGGCGTTTCCCCCTGGAAGCTC
 CCTCGTGCGCCCGAAATGGCTTTAAATTGTACACAAAAAATGTTCCGCAAAAACTT
 TGTGGCTGTCACGAATACAAACACACAAGAATTAGGGATGTA AAAATCTGCTATCGA
 GACAGCGTTGGTATCGATAGTAGTTAGTTTGCAACGAGAGGGTTTGGTGCATTGATT
 ACTGTATATTTCAAAGAATATTCGAATTGTTTTCTTTAAAGACAACAATTTTATTCGC
 CTTTGCGATTGGTAAAATAGTTGTTTACTTTTAATCGATAGACAAAAACAGTTATTGG
 GCTGCCATCTGGGGCGTCTATGCGAAGACCTCTTTGCGTATTCAGTTATCGATTACT
 GTGACAGTGCCTAGACCTCCTAAACTCAACTTTATTGCCCGGCCATGATGAAATAA
 CATAAGGTGGTCCCGTCGGCAAGAGACATCCACTTAACGTATGCTTGAATAAGTGC
 GAGTGAAGGAATAGTATTCTGAGTGTGCTATTGAGTCTGAGTGAGACAGNNNNNN
 GATTG

H. *dLeo1* - Pry4 and Plw3-1 - Spep1

NNNNNNNNNANTGNAGCNTACGTTAAGTGGATGTCTCTTGCCGACGGGACCCCTTAT
 GTTATTTNATCATGGGCCGGGAATAAAGTTGAGTTTAGGAGGTCTAGGCACTGTCA
 CAGTAATCGATAACTGAAATACGCAAAGAGGTCTTCGCATAGACGCCCCAGATGGC
 AGCCCAATAACTGTTTTTGTCTATCGATTAAAAGTAAACA ACTATTTTACCAATCGCA
 AAGGCGAATAAAATTGTTGTCTTTAAAGAAAACAATTCGAATATTCTTTGAAATATA
 CAGTAATCAATGCACCAAACCCTCTCGTTGCAA ACTA ACTACTATCGATACCAACGC
 TGTCTCGATAGCAGATTTTACATCCCTAATTCTTGTGTGTTTGTATTTCGTGACAGCC
 ACAAAGTTTTTTGCGGAACATTTTTTGTGTACAATTTAAAGCCATTTCCGGCGCACGA
 GGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTGCGGTTTCGCCACC
 TCTGACTTGAGCGTTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAA
 ACGCCAGCAACGCAAGCTTCTAGAGATCTGCAGGTCGACGGATCCGTCGAGCGTATA
 ACCATCTGTACAAAAAAGGATTTCTTTGCCAGTCGTACGACTTTGTACAGATGGTT
 ATCAGATGTGGACATAAAAAGAGGATGTTTGGATGTGGTCATAGACCTAATGGACA
 GTGATGGAGTTGNNNNNNNCCNACA

Appendix C:
Lethality of the *UAS-dsRNA-dLeo1* Construct

Materials and Methods:

Fly stocks:

Fly stocks used to determine the lethality of the *dLeo1* dsRNA construct were: *UAS-dsRNA-dLeo1* (CG 1433), *TubGAL4* (Bloomington No. 5138), *ActinGAL4* (Bloomington No. 4414), *daGAL4* (Bloomington No. 8641), *Dmef-2-GAL4* (Bloomington No. 27390), *w;D/TMS,Sb* (Bloomington No. 560), *w;S/CyO* (Bloomington No. 102716) and *yw;pin/CyO;CD8-GFP* (Bloomington No. 5130).

Determination of dLeo1 dsRNA construct lethality:

Prior to testing the lethality of the *dLeo1* dsRNA construct, spatial expression of the *GAL4* drivers was verified. *TubGAL4*, *ActinGAL4*, *daGAL4* and *Dmef-2-GAL4* flies were crossed to flies carrying the CD8 membrane GFP at 25°C. Early stage larvae were observed using a dissecting microscope (Zeiss) with a GFP filter (Biological Laboratory Equipment) for the GFP expression patterns.

Following verification of *GAL4* driver expression, *UAS-dsRNA-dLeo1* flies were crossed to flies carrying the ubiquitous *TubGAL4*, *ActinGAL4* or *daGAL4* drivers, or the tissue-specific *Dmef-2-GAL4* using standard genetic crosses. Parents were transferred to new tubes every 2-3 days and the progeny raised at either 25°C or 29°C. After eclosing, adult progeny were counted according to phenotype and the lethality of the construct determined by comparing the observed phenotypic ratios with the expected phenotypic ratios.

Results:***Lethality of the UAS-dsRNA-dLeo1 construct***

The lethality of the *UAS-dsRNA-dLeo1* construct was determined by crossing flies carrying the *UAS-dsRNA-dLeo1* construct with several GAL4 drivers, both ubiquitous and tissue-specific, and raising the progeny at 25°C and 29°C. When the construct was driven by the ubiquitous drivers *TubGAL4*, *ActinGAL4* and *daGAL4*, the construct was 100% lethal at both 25°C and 29°C (Table C1). However, when driven by the tissue-specific *Dmef-2-GAL4* driver different degrees of survival were observed at the two temperatures. At 25°C, 53 of the 151 adult flies counted were of the genotype *UAS-dsRNA-dLeo1/Dmef-2-GAL4*, indicating 100% survival. At 29°C, fewer *UAS-dsRNA-dLeo1/Dmef-2-GAL4* flies were observed than at 25°C. Of 206 adult flies counted, 50 contained the *UAS-dsRNA-dLeo1* construct driven by the *GAL4* driver, indicating a 72% survival rate. Thus when ubiquitously expressed in the fly, the dsRNA construct is 100% lethal but the flies are viable when the construct is expressed in a tissue-specific manner.

Table C1: Lethality of the *UAS-dsRNA-dLeo1* construct when driven by ubiquitous or tissue-specific GAL4 drivers. Progeny were raised at 25°C or 29°C and the observed phenotypic ratio was compared to the expected phenotypic ratio. When the construct was driven by the ubiquitous *TubGAL4*, *ActinGAL4* and *daGAL4* drivers at 25°C and 29°C, 100% lethality was observed. When driven with the tissue-specific *Dmef-2-GAL4* driver, 100% and 72% survival was observed at both 25°C and 29°C respectively.

Table C1: Lethality of the *UAS-dsRNA-dLeo1* construct when driven by ubiquitous or tissue-specific *GAL4* drivers.

GAL4 Driver	Progeny		Total
	<i>UAS-ds-RNA-dLeo1/GAL4</i>	<i>RNAi</i> or <i>GAL4/Balancer</i>	
25°C			
<i>TubGAL4</i>	0	123	123
<i>ActinGAL4</i>	0	138	138
<i>daGAL4</i>	0	137	137
<i>Dmef-2-GAL4</i>	53	98	151
29°C			
<i>TubGAL4</i>	0	80	80
<i>ActinGAL4</i>	0	171	171
<i>daGAL4</i>	0	264	264
<i>Dmef-2-GAL4</i>	50	156	206