CHARACTERIZATION OF THE MENIN AND TIP60 INTERACTION

CHARACTERIZATION OF

THE MENIN-DTIP60 INTERACTION

IN

DROSOPHILA MELANOGASTER

By

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Abstract

Drosophila melanogaster (fruit fly) has been used as a model organism for almost a century. *Drosophila* shares key molecular pathways and regulatory proteins with humans, making it a useful organism to study human diseases. The disruption of these pathways often leads to similar defects in flies and humans. For example, the Fragile X mental retardation syndrome is caused by the disruption of *FMR1*, leading to intellectual and physical impairment from defects in synaptic transmission. Flies lacking dfmr1 show behavioural defects similar to humans, and also have impaired motor control. The Ras pathway is also well studied using the *Drosophila* model system and mutations in Ras1 show defects in cell proliferation in flies and humans. Multiple endocrine neoplasia type 1 is another syndrome that can be studied using *Drosophila* as a model organism.

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer syndrome predominantly characterized by tumour formation in various endocrine glands. Heterozygous patients for *MEN1* develop the disease following a loss of the wild type allele (loss of heterozygosity). Menin is encoded by *MEN1*, and has no obvious sequence homology to other proteins. In order to gain some insight on the role of menin, several studies have looked for proteins interacting with menin. Using a yeast two-hybrid screen, our laboratory identified Enhancer of Polycomb E(Pc), a component of the Tip60 histone acetyltransferase complex as a candidate, novel menin interacting protein. Closer investigation revealed dTip60 as an interacting partner of Menin in *Drosophila* S2 cells.

Menin was found to interact with dTip60 in S2 cells under optimal growing conditions and after 1hr of heat shock. However, co-immunoprecipitation after stress by γ-irradiation did not reveal an interaction. Additionally, the pattern of expression and role of dTip60 was examined in wild type Oregon R flies and in flies expressing RNAi

against dTip60. Heat shock lethality experiments were conducted to compare Tip60 RNAi flies to controls. dTip60 did not appear to play a role in the stress response at the larval developmental stages. The Tip60 RNAi flies showed robust expression of dTip60 throughout embryogenesis; therefore the maternal contribution of dTip60 was also investigated. The maternal contribution of dTip60 was significantly reduced in embryos from females harbouring the *UAS-dTip60* RNAi and daGAL4 constructs. This can be a valuable tool for future experiments with embryos into the function of dTip60.

Further experiments must be conducted to determine whether a dynamic interaction occurs after γ-irradiation using closer kinetics in S2 cells. Also, experiments using wild type flies showed that dTip60 is developmentally regulated. dTip60 is required for development since Tip60 RNAi flies do not survive past the pupal stage. Increased lethality is not seen in Tip60 RNAi flies compared to controls when subjected to heat shock, suggesting that dTip60 does not play a role in stress response at this time. A γ-irradiation lethality experiment should be conducted to determine if a complex containing menin and dTip60 is required for DNA damage repair. Lastly, to determine whether menin and dTip60 have a role in the same pathway, stress experiments must be conducted using *Mnn1*$^{-/-}$ flies as a comparison to phenotypes seen in Tip60 RNAi flies.

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List of Abbreviations

53BP1	p53-binding protein 1
AR	androgen receptor
ARP4	actin-related protein 4
ASK	activator of S-phase kinase
ATM	ataxia-talangiectasia, mutated kinase
ATR	ATM and Rad3 related kinase
CBP	CREB binding protein
CDC2	cell division cycle 2
CDK	cyclin-dependent kinase
CHES1	checkpoint suppressor 1
DEB	diepoxybutane
DSB	double strand break
dsDNA	double-stranded DNA
EGF	epidermal growth factor
EMSA	electromobility shift assay
E(Pc)	enhancer of polycomb
FACS	fluorescence-activated cell sorting
FANCD2	Fanconi anemia complementation group D2 protein
GAF	GAGA factor
GTP	guanosine-5'-triphosphate
GDP	guanosine-5'-diphosphate

GFAP	glial fibrillary acidic protein
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMTase	histone methyltransferase
Hox	homeobox gene
HP1	heterochromatin protein 1
HSF	heat shock factor
HSP	heat shock protein
iFRAP	inverse fluorescence recovery after photobleaching
IGFBP-2	insulin-like growth factor binding protein 2
ING3	inhibitor of growth 3
LOH	loss of heterozygosity
MDC1	mediator of DNA damage checkpoint 1
Mdm2	mouse double minute 2
MEF	mouse embryonic fibroblast
MEN1	multiple endocrine neoplasia type 1
MLL	mixed lineage leukemia
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end joining
NLS	nuclear localization signal
NM	nuclear matrix

NR	nuclear receptor
PKA	protein kinase A
RNAi	RNA interference
RPA2	replication protein A2
RTK	receptor tyrosine kinase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMC1	structural maintenance of chromosomes 1
Sos	Son of sevenless
TAC1	Trithorax acetylation complex
TGF-β	transforming growth factor-β
Tip60	HIV-1 tat-interactive protein 60kDa
TNF-α	tumour necrosis factor-α
TRRAP	transformation transactivation associated protein
UAS	upstream activating sequence
UBC13	ubiquitin conjugating enzyme 13

Chapter One: INTRODUCTION

This section will focus on the two proteins relevant to my project; the menin tumour suppressor and the Tip60 histone acetyltransferase. I will provide a brief overview on the use of *Drosophila melanogaster* as a model system, and continue with the literature on menin and Tip60.

1.1 The *Drosophila melanogaster* Model System

Drosophila melanogaster (fruit fly) has been used as a model organism for almost a century. *Drosophila* is a complex multicellular organism that shares many homologous proteins and signaling pathways with humans. The completion of the *Drosophila* genome project (Adams et al., 2000) allowed for the identification of many orthologous proteins that play a role in human pathology. Importantly, genes of these proteins can be examined using gain- or loss-of-function analysis to provide important insights in the understanding of human diseases. The fruit fly is small and inexpensive to keep in the lab with its simple diet, and has a short life cycle, reaching adulthood in only 11 days at 25°C. More importantly, it has several unique tools to perform the genetic analysis of previously uncharacterized genes.

1.1.1 Use of *D. melanogaster* to Study Human Disease

Closer study of *D. melanogaster* reveals that key molecular pathways required for developmental processes are evolutionarily conserved in humans. The disruption of protein in these pathways leads to similar defects in both flies and humans. For example, the Ras pathway is a well known signal transduction pathway conserved from flies to humans. Ras was first identified in murine retroviruses (Harvey, 1964), and is mutated in 20% of human tumours (Downward, 2003). *Drosophila* Ras shares 75% homology to human Ras (Neuman-Silberberg et al., 1984). Ras is a small GTP-binding protein in a signal transduction pathway initiated by receptor tyrosine kinases (RTKs).

The activation of Ras signals further downstream targets forming a Ras cascade (Wassarman, Therrien and Rubin, 1995). The initiating signal can be a growth factor such as epidermal growth factor (EGF). Inactive Ras is bound to GDP, and the activation of Ras is catalyzed by Son of Sevenless (Sos) which promotes the exchange of GDP to GTP in response to EGF signal (Simon et al., 1991; Rogge, Karlovich and Banerjee, 1991). The use of *Drosophila* genetics allowed for the identification of proteins involved in the Ras pathway such as Son of Sevenless (Sos) (Downward, 2003). In *Drosophila*, the Ras pathway is involved in eye development (Wassarman, Therrien and Rubin, 1995). Similar to humans, *Drosophila* Ras also has a role in cell proliferation and cell fate determination (Karim and Rubin, 1998). The constitutive coactivation of EGF and Ras in *Drosophila* leads to neoplastic tumour-like growths that mimic human gliomas (Read et al., 2009). Additionally, the Fragile X mental retardation syndrome is caused by a loss of FMRP and characterized by mental and physical impairment (Zalfa and Bagni, 2004). Similarly, the loss of dfmr1 (*Drosophila* homolog of FMRP) causes behavioural defects and impaired motor control (Zhang et al., 2001). Flies also exhibit synaptic structural defects that would alter neurotransmission.

Other genes involved in human pathologies (cancer, in particular) have been identified and studied successfully in *Drosophila* including p53 (Jin et al., 2000), ATM (Song et al., 2004), and ATR (LaRocque et al., 2007). p53 is a transcription factor and is considered a tumour suppressor involved in the regulation of DNA repair, cell cycle progression, and apoptosis (Lu and Abrams, 2006). p53 is mutated in over 50% of human cancers (Lu and Abrams, 2006). Use of *Drosophila* revealed that p53 is able to bind a radiation-induced enhancer sequence of the pro-apoptotic gene Reaper to control apoptosis in response to DNA damage (Brodsky et al., 2000). The identification of additional genes controlled by p53 was possible through genome-wide microarray analysis in *Drosophila* (Brodsky et al., 2004). For ATM, studies revealed that null mutants for dATM are pupal lethal or eclose with defects in the eyes and wings (Song et al.,

2004). These developmental defects and lethality suggest an important role for ATM in cell cycle checkpoint regulation in response to DNA damage that arises normally during cell proliferation or differentiation (Song et al., 2004). In contrast to the phenotype seen in ATM mutants, null mutants for the ATM related protein ATR (known as mei-41 in *Drosophila*) are not lethal (Song et al., 2004; LaRocque et al., 2007). Mei-41 is involved in cell cycle checkpoint between G2 and M phase, and DNA double strand break repair that is independent of the usual Chk1/Chk2-mediated checkpoint response (LaRocque et al., 2007). The initial identification of these proteins in *Drosophila* is a particularly good illustration of the use of *D. melanogaster* to study pathways and proteins of medical relevance.

The study of pathways led to the development of many genetic and molecular tools to study gene function and loss-of-function. A variety of *Drosophila* mutants for most proteins are also available for functional studies. Therefore, *D. melanogaster* is a good model to study proteins related to human disease. For my project, the UAS/GAL4 system was used as a tool to study my proteins of interest: menin and dTip60.

1.1.2 Use of the UAS/GAL4 System

GAL4 was originally identified in *Saccharomyces cerevisiae* (yeast) as a positive regulator of galactose induced genes (Laughon and Gesteland, 1984). GAL4 is composed of 881 amino acids and regulates the transcription of genes by directly binding to an Upstream Activating Sequence (UAS). The UAS element is analogous to an enhancer element in multicellular eukaryotes, and the binding of GAL4 to the UAS element is an essential prerequisite to the transcription of these genes (Laughon and Gesteland, 1984). Fischer and colleagues demonstrated in 1988 that GAL4 could be used to activate transcription in *Drosophila* using a reporter gene under UAS control (Fischer et al., 1988). There were no adverse phenotypic effects in *Drosophila* when GAL4 was

expressed. Therefore, Brand and Perrimon used the UAS/GAL4 system for targeted gene expression (Brand and Perrimon, 1993).

Importantly, the UAS/GAL4 system may be used to achieve spatially restricted gene expression or ubiquitous gene expression depending on the type of GAL4 driver used. This makes the UAS/GAL4 system a very powerful tool to address *in vivo* gene function. The timing of expression can be tightly controlled by selecting GAL4 drivers active at specific developmental stages. Also, different parental fly lines can be generated carrying either the UAS transgene, or the GAL4 transcriptional activator. This bipartite approach allows for the maintenance in viability of parental lines when working with a toxic gene product (Brand and Perrimon, 1993). Additionally, GAL4 expression is temperature dependent in flies, with minimal activity at 16°C and maximal activity at 29°C (Duffy, 2002). Therefore, expression levels can be altered simply by temperature shifting to bypass the detrimental effects of some transgenes encoding a deleterious protein.

Alternatively, this system can be used for RNA interference. The introduction of long dsRNA activates the RNAi pathway (Sharp, 2001). The dsRNA is cloned into a plasmid construct such as pUAST and controlled by the UAS element. Flies harbouring this *UAS-RNAi* construct are generated via P-element mediated germline transformation. The *UAS-RNAi* flies are transcriptionally silent because expression requires transcriptional activation by GAL4. Flies harbouring the *UAS-RNAi* construct are mated to flies that express GAL4 to activate transcription of dsRNA to activate the RNAi machinery. The gene of interest will be down-regulated in the resulting progeny through post-transcriptional gene silencing.

1.2 Studying Human Diseases

Human diseases (cancer, in particular) have been the subjects of intense focus and massive research effort for decades. This is due to their immense impact on human

health. Cancer, in general, is not an inherited disease that can be easily tracked, but a culmination of genetic alterations in a cell. The accumulation of genetic defects leads to tumour formation in response to uncontrolled cellular proliferation and lack of senescence. Finding the cure is a difficult task when malignant tumours can also acquire the ability to metastasize to distant sites, as in the case of many cancers.

Other diseases of medical relevance focus on the maintenance of genome stability which may also lead to cancer such as Ataxia Telangiectasia and Fanconi anemia. Ataxia Telangiectasia is characterized by cerebellar ataxia, neuro-degeneration (Boder and Sedgewick, 1958), genome instability and cell cycle checkpoint defects (Kastan and Lim, 2000). The gene responsible for Ataxia Telangiectasia, called Ataxia Telangiectasia mutated (ATM), was cloned and identified in 1995 (Savitsky et al., 1995). ATM is a serine/threonine kinase involved in cell cycle control, and DNA repair (Savitsky et al., 1995). The inability of Ataxia Telangiectasia patients to repair DNA leads to genome instability. The Fanconi anemia genome instability syndrome may also predispose patients to cancer. Cells from Fanconi anemia patients display chromosomal aberrations and increased sensitivity to DNA cross-linking agents (Moldovan and D'Andrea, 2009). Fanconi anemia is a heterogenous disease that can arise from mutations in at least 13 distinct genes (FANCA, FANCB, FANCD1, FANCD2, etc.) from the FA DNA repair pathway (Kee and D'Andrea, 2010).

In addition, syndromes have been characterized leading to an increased predisposition to cancer, such as Li-Fraumeni syndrome and Multiple Endocrine Neoplasia Type 1. Li-Fraumeni syndrome is caused by germline mutations in *TP53* (or *P53*) predisposing the patient to the formation of sarcomas, tumours and adenocarcinomas (Li et al., 1988). Another syndrome, Multiple Endocrine Neoplasia Type 1 (MEN1 syndrome), will be discussed in greater detail in the next section.

The study of these cancer syndromes employs forward genetics. Clinical studies are used to characterize the syndrome, before mapping of the altered genes. Interacting proteins in the affected pathways can be identified and studied using molecular techniques such as co-immunoprecipitation, yeast two-hybrid screens, GST-pull down assays. Lastly, model organisms such as *Drosophila melanogaster* can be used to observe interactions via genetic screens, and examine phenotypic defects when genes of interest are mutated. Molecular methods were used to study the functions of menin, a protein with a role in preventing the development of Multiple Endocrine Neoplasia Type 1.

1.3 Multiple Endocrine Neoplasia Type 1

Multiple Endocrine Neoplasia Type 1 is an autosomal dominant familial syndrome characterized by the development of tumours in various endocrine organs (Wermer, 1954). The tumours formed in MEN1 patients can be benign or malignant. Patients who carry a germline mutation for *MEN1* commonly develop tumours in the parathyroid, anterior pituitary glands and endocrine pancreas. Tumours form through a biallelic loss-of-function, the first of which is inherited, and the second of which is caused by a spontaneous mutation. This corresponds to Knudson's two-hit tumour suppressor model (Knudson, 1971). The second mutation in the wild-type allele of a *MEN1* heterozygote can be a missense, frameshift, or nonsense mutation, recognized as a loss of heterozygosity. Non-functional menin formed from a missense mutation is likely degraded by the ubiquitin-mediated proteosome degradation pathway (Yaguchi et al., 2004). To date, there have been no clearly defined mutation hotspots, as *MEN1* disease causing mutations have been found to occur over the whole *MEN1* gene (Balogh et al., 2006; Brandi, 2000). Heterozygous *Men1* mice are viable but also develop tumours in the various endocrine glands following a loss of heterozygosity of the wild type allele (Bertolino et al., 2003; Crabtree et al., 2001). *Men1* mice with a homozygous

deletion are embryonic lethal (Crabtree et al., 2001). Furthermore, homozygous mutations for *MEN1* in humans have never been found.

1.3.1 Menin

The *MEN1* gene was first identified and characterized by Marx and colleagues in 1997, which encodes for menin (Chandrasekarappa et al., 1997). *MEN1* contains ten exons that span over 9 kb of genomic DNA at chromosome 11q13 (Chandrasekarappa et al., 1997). Menin is a 67 kDa, 610 amino acid protein with no obvious sequence homology to other proteins.

Deletion analysis of the menin protein revealed two nuclear localization signals (NLS1 and NLS2) at the C-terminal end, which are functionally independent of one another as a single NLS is sufficient for proper targeting to the nucleus (Guru et al., 1998). Additionally, a third NLS sequence was identified but was not able to target GFP to the nucleus, therefore it is considered an accessory NLS (NLSa) (La et al., 2006). Menin also contains five consensus GTPase-like motifs that surprisingly have low binding affinity for GTP or GDP. Although menin alone has low binding affinity to GTP, it has only been shown to hydrolyze GTP through its association with a tumour metastasis suppressor nm23 (Yaguchi et al., 2002). A schematic representation of menin is shown in Figure 1.

Figure 1: Schematic representation of menin. Menin is a protein with no sequence homology to other protein families. Two nuclear localization signals (NLS1 and NLS2) reside at the C-terminus, and a third accessory nuclear localization signal (NLSa) aids in facilitating the nuclear localization of menin with NLS1 and NLS2. Menin also has two leucine zipper-like motifs, and 5 GTPase-like motifs (G1 to G5).

1.3.2 Regulation of *MEN1* Expression

Currently little is known about the regulation of *MEN1* gene expression. In non-tumour cell lines derived from MEN1 patients containing a germline mutation, levels of menin are comparable to those of healthy controls, indicating a compensatory mechanism for allelic loss (Wautot et al., 2000). Evidence suggests that *MEN1* gene promoter activity is regulated according to the amount of menin present in the cell at a given time, suggesting a feedback mechanism to ensure proper protein levels within the cell. In cell lines derived from human embryonic kidney cells (HEK293), Zablewska and colleagues showed that menin down-regulation via siRNA transfection activated the *MEN1* gene promoter using a reporter assay (Zablewska et al., 2003). Conversely, the overexpression of menin down-regulated *MEN1* gene promoter activity.

Tumours formed in *MEN1* patients affect the endocrine organs. Mensah-Osman and colleagues showed that somatostatin, a natural paracrine inhibitor of gastrin secretion increased menin expression through the inhibition of protein kinase A (PKA) using immuno-histochemistry in mice (Mensah-Osman, Zavros and Merchant, 2008). In human gastric adenocarcinoma (AGS) cells, the use of forskolin, an inducer of PKA, inhibited menin expression. Suppression of PKA using RNA interference also enhanced the expression of menin as a further line of evidence. A positive correlation exists between cells that have a higher expression of menin, and cells that have a higher expression of somatostatin receptors (Mensah-Osman, Zavros and Merchant, 2008). It is thought that activated PKA inhibits the expression of menin by phosphorylating the CREB transcription factor. Phosphorylated CREB binds the *MEN1* promoter to negatively regulate transcription, although this theory has not been tested to date. Closer analysis of the first 1000bp of the *MEN1* promoter did not reveal the presence of CREB binding sites, although the upstream promoter region has yet to be investigated (Zablewska et al., 2003).

In genetically altered mice that permitted conditional expression of menin in β-islet cells, lactogenic hormones such as prolactin were shown to decrease menin expression by activating the STAT5-Bcl6 pathway (Karnik et al., 2007). Activation of the STAT5-Bcl6 pathway results in the proliferation of pancreatic β-cells from the transcriptional inhibition of p27 and p18 (Karnik et al., 2007). Additionally, mice that were administered steroids such as progesterone and dexamethasone (inhibitors of prolactin) simultaneously with prolactin rescued the phenotype and normal menin expression (Karnik et al., 2007).

1.3.3 Dynamics of Menin

Evidence suggests that menin plays a role in many cellular processes depending on the interacting partner and context. Menin interacting proteins have been described and characterized in different systems. Menin can activate or inhibit transcription by its association with different transcription factors such as JunD/AP-1, Ches1 and NF-κB as well as chromatin modifying complexes such as mSin3a/HDAC (histone deacetylase), and the Trithorax/MLL (mixed lineage leukemia) histone methyltransferase complex (Agarwal et al., 1999; Busygina et al., 2006; Heppner et al., 2001; Hughes et al., 2004; Kim et al., 2003). Menin interacts with crucial proteins involved in different pathways, summarized in Table 1. At this time, it is unclear what role these interactions may play in the development of the MEN1 Syndrome.

Table 1: Summary of menin Interacting Proteins and Complexes

Interacting protein/complex	Function	Reference
JunD	Transcription factor	Agarwal et al., 1999
Ches1	Transcription factor	Busygina et al., 2006
NF-κB	Transcription factor	Heppner et al., 2001
Smad3	Transcription factor	Kaji et al., 2001
mSin3a/HDAC complex	Epigenetic regulator	Kim et al., 2003
Trithorax/MLL HMTase complex	Epigenetic regulator	Hughes et al., 2004 Yokoyama et al., 2004
FANCD2	DNA repair	Jin et al., 2003
RPA2	DNA repair and replication	Sukhodolets et al., 2003
ASK	Cell proliferation	Schnepp et al., 2004a
GFAP	Cytoskeleton	Lopez-Egido et al., 2002
Vimentin	Cytoskeleton	Lopez-Egido et al., 2002
NM23	Tumour metastasis suppressor	Yaguchi et al., 2002
Lamin C	Nuclear Lamina	Our unpublished observations
CP309	Centrosome	" "
E(Pc)	Epigenetic regulator	" "

1.3.4 Role of Menin in Transcriptional Regulation

The activity of menin on transcriptional regulation depends on the particular interacting partner, and context of the interaction. One earlier identified menin interacting protein is JunD, a member of the AP1 family of transcription factors (Agarwal et al., 1999). Menin represses JunD mediated transcriptional activation by recruiting and associating with the mSin3A histone deacetylase complex (Gobl et al., 1999; Kim et al., 2003). The inhibition of JunD activity by menin is abolished when an HDAC inhibitor is added (Gobl et al., 1999; Kim et al., 2003). Generally, the acetylation of histones yields an open chromatin conformation which activates transcription, while the deacetylation results in the repression of a gene (Jenuwein and Allis, 2001). Menin is also known to interact with NF-κB, and similarly represses NF-κB mediated transactivation activity via recruitment of HDAC's (Heppner et al., 2001).

Menin is also a repressor of hTERT, a component of telomerase. This activity of menin was revealed from a genetic screen performed with stably transfected HeLa cells and FACS analysis (Lin and Elledge, 2003). Chromatin immunoprecipitation showed that menin associates with the hTERT promoter to negatively regulate *hTERT* expression (Lin and Elledge, 2003). Menin also negatively regulates insulin-like growth factor binding protein 2 (IGFBP-2) and represses transcription by binding the *IGFBP-2* promoter (La et al., 2004a). The binding of menin to the *IGFBP-2* promoter requires the presence of intact NLS's. Mutations of the NLS domains result in menin being unable to bind the IGFBP-2 promoter (La et al., 2004a).

Menin promotes transcription through the interaction with a complex containing the histone methyltransferase MLL (Yokoyama et al., 2004). The histone methyltransferase complex containing MLL is known to methylate histone H3 lysine 4 (H3K4) and activate gene transcription (Yokoyama et al., 2004). The H3K4 epigenetic mark is typically associated with transcriptionally active chromatin. Microarray analysis

in MEF cells and mouse embryos revealed that menin is a positive regulator of *Hoxc6* and *Hoxc8* gene expression (Hughes et al., 2004). Chromatin immunoprecipitation in MEF cells showed that menin directly regulates Hoxc8 by associating with the enhancer, promoter and 5' coding region of the *Hoxc8* gene (Hughes et al., 2004). Specifically, the MLL complex together with menin is known to transcriptionally regulate clustered homeobox (*Hox*) gene expression (Yokoyama et al., 2004) which controls cell proliferation in this context. This will be discussed in the next section. Menin is subject to post-translational modification and is phosphorylated at Ser543 and Ser583 in 293T cells (MacConaill et al., 2006). Mutations at Ser543 and Ser583 do not affect menin's association with the HMTase complex containing MLL or menin's ability to bind the *Hoxc8* locus. The methyltransferase activity of the complex is also not affected due to these point mutations (MacConaill et al., 2006). Therefore, the role of these phosphorylation sites remains unclear.

Menin's interaction with the HMTase complex containing MLL can also upregulate the transcription of the cyclin-dependent kinase inhibitors $p18^{ink4c}$ and $p27^{kip1}$ (known as p18 and p27 respectively) in cells and murine pancreatic islets (Karnik et al., 2007; Milne et al., 2005). The maintenance of p18 and p27 expression through epigenetic regulation of H3K4 is required to prevent growth and proliferation of pancreatic islet β-cells (Karnik et al., 2007). Binding of menin to *p18* and *p27* loci was observed in chromatin immunoprecipitation experiments but this recruitment is not dependent on MLL. In fact, the reverse is true; recruitment of MLL to *p18* and *p27* loci is menin-dependent (Milne et al., 2005). Therefore, menin is required for MLL targeted gene expression (Yokoyama et al., 2004). Dr. Michael L. Cleary's group (Stanford University of Medicine) showed that menin associates with MLL-fusion proteins in human leukemia cells through co-immunoprecipitation experiments. The association of menin with the MLL-fusion oncoproteins is necessary for the maintenance of sustained *Hox* gene expression in myeloid progenitor cells derived from mouse bone marrow and

HeLa cells (Yokoyama et al., 2005; Yokoyama et al., 2004). Chromatin immunoprecipitation analysis revealed that *Hoxa9* is a direct target gene for menin in this context (Yokoyama et al., 2005).

1.3.5 Role of Menin in Cell Proliferation

Evidence suggests that menin associates with different proteins or DNA directly to control cell proliferation. Menin is considered a tumour suppressor repressing cell proliferation through interactions with different substrates. Menin deficient MEF cells and rat duodenal (IEC-17) cells show increased cell proliferation compared to wild type cells (Milne et al., 2005; Ratineau et al., 2004).

As mentioned earlier, menin interacts with the histone methyltransferase (HMTase) complex containing MLL to regulate the transcriptional activity of the clustered homeobox genes (*Hox*) (Yokoyama et al., 2004). Regulation of these specific *Hox* genes by the MLL HMTase complex promotes cell proliferation in this cellular context. Menin and MLL oncogenic fusions directly target *HoxA7*, *HoxA9*, and *HoxA10* promoters (chromatin immunoprecipitation) in human leukemia cells (Yokoyama et al., 2005). This data implicates menin as an essential cofactor in the transcriptional misregulation of *Hox* genes with MLL oncogenic fusions. *Hox* gene expression is aberrantly sustained, thereby promoting cell proliferation (Yokoyama et al., 2005).

Menin binds directly to dsDNA via the C-terminal region of the protein in a sequence-independent manner (La et al., 2004b). Loss of the menin C-terminus results in increased in MEF cell number specifically in G_2/M phase (La et al., 2004b). Therefore, the binding of menin to dsDNA may have a role in suppressing cell proliferation. Co-immunoprecipitation experiments in COS cells revealed an interaction of menin with Smad3 (Kaji et al., 2001). Smad3 is activated by transforming growth factor-β (TGF-β), and importantly plays a role in growth inhibition induced by TGF-β (Kaji et al., 2001). In this case, menin activates the DNA binding capacity of Smad3. Smad3 can then bind to

transcriptional regulatory sites to activate TGF-β signaling and repress cell proliferation. In rat intestinal epithelial cells (IEC-17), downregulation of menin led to the resistance of cells to TGF-β induced growth inhibition. Increase in cell proliferation was due to a decreased expression of TGF-β receptor II (as a result of menin downregulation) required for TGF-β signaling (Ratineau et al., 2004). Therefore, the presence of menin is required to inhibit proliferation in the TGF-β pathway. Menin also inhibits cell proliferation by interacting with, and repressing NF-κB activity (Heppner et al., 2001).

In a model using RAS-transformed murine NIH3T3 cells, the over-expression of menin attenuated the tumorigenicity of RAS (Kim et al., 1999). Menin overexpression blocks transformed cell morphology, anchorage independent growth, and also inhibits tumour formation in nude mice (Kim et al., 1999). Therefore in this context, menin also inhibits cellular proliferation.

According to mass spectrometry analysis and a yeast two-hybrid screen, menin was identified as an interacting partner with activator of S-phase kinase (ASK). ASK is a component of the cell division cycle cdc7/ASK kinase complex essential for cell proliferation in eukaryotic cells (Schnepp et al., 2004a). Menin inhibits the kinase activity of ASK *in vivo*, resulting in the inhibition of cell proliferation. GST-pull down assays determined that the carboxy-terminal end of menin interacts with ASK (Schnepp et al., 2004a). Furthermore, the downregulation of menin results in an overexpression of cyclins D1, D3 and Cdk4, altering the cell cycle and promoting cell proliferation (Ratineau et al., 2004).

In addition, the depletion of menin in BJ cells (human diploid foreskin fibroblasts) by shRNA leads to the reactivation of *hTERT* expression, and an increase in telomerase activity (Lin and Elledge, 2003). These menin-deficient BJ cells become immortalized as they surpass their normal lifespan by over 45 doublings with no signs of replicative senescence (Lin and Elledge, 2003).

1.3.6 Role of Menin in Apoptosis

Evidence of menin's involvement in apoptosis is seen in overexpression studies where the ectopic over-expression of menin in murine embryonic fibroblasts induces apoptosis (Schnepp et al., 2004b). The apoptotic activity of menin is dependent on the presence of two pro-apoptotic proteins: Bax and Bak (Schnepp et al., 2004b). Deletion of menin also increases the resistance of cells to UV and TNF-α induced apoptosis (Schnepp et al., 2004b). In addition, the expression of caspase 8 is down-regulated in menin null MEF cells. Consistent with this data, menin regulates caspase 8 expression by binding the 5'UTR as evidenced from reporter assays and chromatin immunoprecipitation (La et al., 2007). Importantly, point mutations in menin also failed to stimulate caspase 8 expression and TNF-α-mediated apoptosis (La et al., 2007).

1.3.7 Menin in Cell Cycle Regulation

Progression of the cell cycle is dependent on the activity of cyclin-dependent kinases (CDKs) which are negatively regulated by CDK inhibitors p18 and p27. The inhibition of CDKs results in termination of DNA synthesis in S-phase, and G1 arrest (Sherr and Roberts, 1999). The down-regulation of menin allows proliferation by preventing G1 arrest in quiescent cells (Ratineau et al., 2004). Menin can also prevent entry into S-phase by repressing ASK activity (Schnepp et al., 2004a). In response to DNA damage by ionizing radiation, menin is required to stimulate S-phase arrest by interacting with the transcription factor CHES1 which will be discussed in the next section (Busygina et al., 2006).

Menin also interacts with type III intermediate filament proteins GFAP and vimentin (Lopez-Egido et al., 2002) to regulate the cell cycle. Menin colocalizes with GFAP in glioma cells at the S-G2 phase of the cell cycle, and is thought to have an inhibitory role in the nucleus before the start of S-phase (Suphapeetiporn et al., 2002; Lin and Elledge, 2003). Menin must be transferred to the cytoplasm before S-phase can

proceed and it is sequestered to the cytoplasm by interacting with the cytoskeleton proteins (Lopez-Egido et al., 2002).

1.3.8 Role of Menin in DNA Damage Response

MEN1 patients show evidence of genome instability in peripheral leukocytes (Scappaticci et al., 1991). The presence of cellular machinery involved in monitoring and repairing DNA lesions is important to prevent genome instability. The defined function of menin in the DNA damage response has yet to be elucidated, but there is evidence of menin's involvement in this process. MEFs deficient of the *Men1* gene are hypersensitive to ionizing radiation and are defective for a DNA damage-activated checkpoint (Busygina et al., 2006). Specifically after DNA damage by ionizing radiation, menin-deficient MEF cells do not undergo S-phase arrest and enter G2 phase completely within 24 hours (Kottemann and Bale, 2009). In contrast, a number of menin-complemented MEF cells are retained in G1 and S phases in response to ionizing radiation in the same time course experiments. This suggests that menin has a role in the intra-S checkpoint, and the G1/S transition checkpoint (Kottemann and Bale, 2009). Chromatin immunoprecipitation experiments also revealed that menin could bind the promoter of *p21*, a cyclin-dependent kinase inhibitor in a DNA damage dependent manner (Kottemann and Bale, 2009). p21 is the primary target of p53 during G1- and S-phase checkpoints following DNA damage (Bartek and Lukas, 2001). The histone methyltransferase MLL is also recruited by menin in a DNA damage-dependent manner to the *p21* promoter, as shown in chromatin immunoprecipitation experiments (Kottemann and Bale, 2009).

Menin may also play a role in the BRCA1-mediated DNA repair pathway through an interaction with FANCD2, a well characterized protein in the BRCA1 pathway (Jin et al., 2003). The interaction between menin and FANCD2 is enhanced following the induction of double-stranded breaks via γ-irradiation (Jin et al., 2003). Menin also

interacts with replication protein A2 (RPA2), a subunit of the trimeric single-stranded DNA-binding protein involved in DNA repair and replication (Sukhodolets et al., 2003). Additionally, co-immunoprecipitation experiments confirmed an interaction between human menin and CHES1 (checkpoint suppressor 1) in HEK293 cells (Busygina et al., 2006). CHES1 is a member of the fork head/winged helix family of transcription factors that was first isolated in yeast for its ability to suppress DNA-damage activated checkpoint mutations (Pati et al., 1997). GST-pull down assays revealed the C-terminal region of human menin (428-610) interacts with CHES1 (Busygina et al., 2006).

Additionally, menin is a putative target of the kinases ATM and ATR which are central regulators of the DNA damage response (Matsuoka et al., 2007; Stokes et al., 2007). In response to DNA damage (γ-irradiation and UV radiation), menin is phosphorylated by ATM and ATR at Ser394 (Stokes et al., 2007). Levels of menin on chromatin and in the nuclear matrix are enhanced following DNA damage treatments (Jin et al., 2003; Farley et al., 2006). While these results suggest a role for menin in the DNA damage response, the function of the tumour suppressor in this process remains unclear.

1.3.9 Role of Menin in Genome Stability

MEN1 patients show increased chromosomal breakage in lymphocytes (Gustavson, Jansson and Oberg, 1983; Scappaticci et al., 1991). Chromosomal abnormalities in MEN1 patients include di- and tri-centric chromosomes, translocations, deletions and inversions. Skin fibroblasts from MEN1 patients also show similar defects (Scappaticci et al., 1991). Furthermore, peripheral blood lymphocytes from MEN1 patients accumulate extensive chromosomal breakage after treatment with diepoxybutane (DEB), a DNA cross-linking agent, compared to normal lymphocytes (Tomassetti et al., 1995).

The clinical evidence above suggests a role for menin in the control of genome stability. The key controls of genome stability include: effective DNA repair, the control of cell cycle checkpoints, and the control of apoptosis to eliminate damaged cells (Poisson, Zablewska and Gaudray, 2003). Menin is demonstrated to have a role in all of these processes, as outlined in previous sections.

1.4 *Drosophila* Menin

The gene for *Drosophila* menin is *Mnn1*, the homolog of human *MEN1*. *Drosophila* menin is composed of 763 amino acids and 83 kDa in size (Maruyama et al., 2000; Guru et al., 2001). *Drosophila* menin is 46% identical to human menin, but importantly, 65% of the mutations that occur in MEN1 patients correspond to conserved amino acids in both species (Lemos and Thakker, 2008). Translation initiation of menin occurs at an upstream methionine codon, extending *Drosophila* menin by 12 amino acids. There are also additional amino acids spanning the C-terminal region that are not present in vertebrates (Guru et al., 2001). Similar to human menin, *Drosophila* menin also has two putative NLS's at the C-terminal end of the protein according to sequence alignment analysis (Maruyama et al., 2000).

Mice lacking menin do not survive past early embryonic stages. In contrast, *Drosophila* lacking *Mnn1* are viable and fertile with no obvious phenotype (Busygina et al., 2004; Papaconstantinou et al., 2005).

Additional *Drosophila* menin interacting proteins were identified through a yeast two-hybrid screen by our lab. Briefly, a *Drosophila* Mnn1 bait fragment (1-752) was made and screened against a *Drosophila* whole embryo prey library at Hybrigenics. Lamin C (a protein of the nuclear lamina), CP309 (a centrosome protein), and Enhancer of Polycomb E(Pc), were among the clones identified with the highest confidence, with 30, 3, and 3 clones identified respectively. E(Pc), a component of the Tip60 chromatin modifying complex, will be discussed later.

1.4.1 *Drosophila* Menin in Transcriptional Regulation

Menin was found to genetically interact with members of the AP-1 family of transcription factors Jun and Fos. The localized over-expression of menin produces a defect in thoracic closure in flies reminiscent of JunD mutants (Cerrato et al., 2006). These genetic interactions are complex in a context dependent manner. Menin has been shown to interact with JunD in immortalized cell lines, and has an essential role in modulating JunD-mediated inhibition of cell proliferation (Agarwal et al., 2003).

1.4.2 *Drosophila* Menin in DNA Damage Response

In response to DNA damage by ionizing radiation, studies in *Drosophila Mnn1*$^{-/-}$ mutants show defects, failing to enter S-phase arrest (Busygina et al., 2006). This implicates menin to have a role in the S-phase cell cycle checkpoint, consistent with recent findings in MEF cells (Kottemann and Bale, 2009).

Busygina and colleagues identified Ches1 as an interacting partner of menin through a genetic screen whereby defects in phenotype due to the over-expression of *Mnn1* are modified when co-expressing an interacting partner. The defective thoracic cleft phenotype is rescued by over-expressing Ches1 in these flies (Busygina et al., 2006). Defects in incomplete S-phase arrest in *Mnn1*$^{-/-}$ mutants following exposure to ionizing radiation are also rescued by Ches1 over-expression (Busygina et al., 2006).

1.4.3 Role of *Drosophila* Menin in Stress Response

Menin is a global regulator of the stress response (Papaconstantinou et al., 2010). *Drosophila Mnn1*$^{-/-}$ mutants are viable and fertile with no obvious phenotype (Busygina et al., 2004; Papaconstantinou et al., 2005). Under different conditions of stress such as heat shock, hypoxia, hyperosmolarity and oxidative stress, *Mnn1* mutants cannot induce a proper stress response, leading to increased lethality

(Papaconstantinou et al., 2005). A detailed review of the heat shock response is covered in later sections.

Specifically for heat shock, the expression of certain heat shock proteins (HSPs) in *Mnn1*$^{-/-}$ early embryos is impaired after 15 minutes. After this time, the expression of Hsp70 cannot be sustained without the presence of menin. Conversely, the over-expression of menin leads to the inability to down-regulate Hsp70 following a return to normal temperatures after heat shock (Papaconstantinou et al., 2005). This data suggests that menin is required for the maintenance of proper Hsp70 expression but not the initiation of the heat shock response.

In *Drosophila*, the histone methyltransferase complex containing the MLL homolog Trithorax (Trx) is designated the Trithorax acetylation (TAC1) complex (Petruk et al., 2001). The TAC1 complex is required to regulate *Hox* gene expression. Trx is required for the maintenance, but not the initiation, of *Hox* gene expression throughout embryonic development (Hanson et al., 1999). In addition to modulating *Hox* gene expression, the TAC1 chromatin modifying complex is required for proper *hsp70* expression (Smith et al., 2004). The exposure of embryos to heat shock revealed that the TAC1 complex is recruited to the coding region of *hsp70*, according to chromatin immunoprecipitation experiments (Smith et al., 2004). Components of the TAC1 complex are also recruited to heat shock loci of polytene chromosomes. The role of menin in this process still remains to be elucidated; however, co-immunoprecipitation experiments revealed that menin is an interacting partner of Trx (A. Pepper, unpublished results).

1.4.4 Role of *Drosophila* Menin in the Maintenance of Genome Stability

Drosophila menin also has a role in the maintenance of genome stability. *Mnn1* loss-of-function mutants display a significant increase in loss-of-heterozygosity (LOH)

upon exposure to heat shock (1hr at 37°C) (Papaconstantinou et al., 2010). The lethality observed in menin mis-expression (over-expression or down-regulation by RNAi) and heat stress is partially suppressed by a dominant negative mutant of Chk2. This implicates the Chk2-p53 stress response machinery in the heat shock response of embryos with menin mis-expression (Papaconstantinou et al., 2010).

1.5 Association of Menin with Chromatin Remodeling Complexes

Menin modulates cellular function by recruiting chromatin remodeling complexes depending on the context. Menin can recruit histone deacetylase (HDAC) complexes such as mSin3A/HDAC complex to negatively regulate transcription of target genes (Kim et al., 2003). Menin also activates transcription of *Hox* genes by associating with the histone methyltransferase complex containing MLL (Yokoyama et al., 2004). It is possible that menin may modulate the activity of other known chromatin modifying complexes, such as Tip60, since E(Pc), component of the Tip60 complex was identified as a potential interacting partner of menin through a yeast two-hybrid screen (Hybrigenics) and our unpublished results.

1.6 Tip60

Previously, it was discussed that Enhancer of Polycomb E(Pc) is an interacting partner with menin, identified through a yeast two-hybrid screen (Table 1; Hybrigenics). E(Pc) is a component of the Tip60 complex (discussed in the next sections), providing a basis into the study of Tip60.

1.6.1 The Tip60 Protein

Tip60 is a protein that has been well characterized in yeast and flies. Tip60 is considered a tumour suppressor, and its pattern of expression is altered in both breast and prostate tumours (Gorrini et al., 2007; Kim et al., 2005; Halkidou et al., 2003).

Additionally, Tip60 expression is significantly downregulated in colon and lung carcinomas (LLeonart et al., 2006).

Tip60 was originally identified as the HIV-1 Tat interactive protein. It belongs to the highly conserved MYST family of histone acetyltransferases (Kamine et al., 1996; Squatrito, Gorrini and Amati, 2006; Utley and Coté, 2003). The MYST family, named for its founding members Moz, Ybf2/Sas3, Sas2, and Tip60 all share structurally similar catalytic HAT domains (Carrozza et al., 2003). Tip60 acetyltransferase activity is reduced after association with the viral HIV-1 Tat protein. The activation of Tip60 regulated genes is decreased due to reduced acetyltransferase activity (Creaven et al., 1999).

In humans, Tip60 is encoded by the *HTATIP* gene which is located at chromosome 11q13.1 and contains 14 exons (Kamine et al., 1996). Three isoforms of Tip60 exist and are formed through alternative splicing: Tip60 isoform 1, Tip60 isoform 2 (Tip60α), and Tip60 isoform 3 (Tip60β). The most characterized splice variant is Tip60 isoform 2, which is 513 amino acids long, 58 kDa in size (Sapountzi, Logan and Robson, 2006). Tip60 shares high sequence homology with *Gallus gallus* (chicken), *D. melanogaster* (fruit fly), and Mus *musculus* (mouse), ranging from 57-99% sequence homology (McAllister, Merlo and Lough, 2002). A schematic representation of the Tip60 protein is shown in Figure 2.

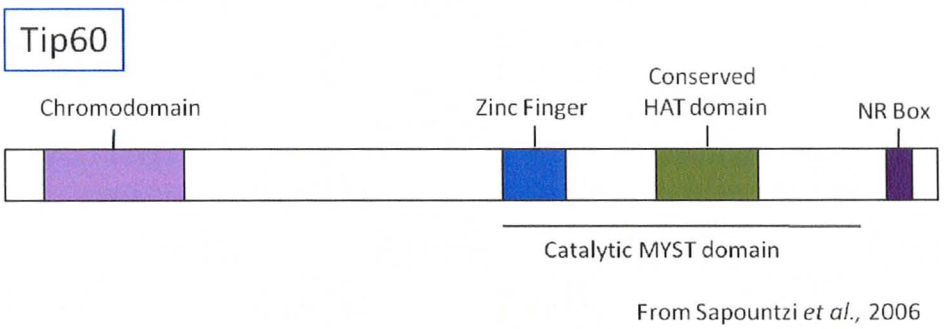

Figure 2: Schematic representation of the Tip60 protein. Tip60 has an N-terminal chromodomain known to interact with methylated histones. A nuclear receptor box (NR) resides at the extreme C-terminus to target Tip60 to the nucleus. A catalytic MYST domain at the C-terminal end contains a zinc finger motif, and a highly conserved histone acetyltransferase (HAT) domain.

The Tip60 protein contains an N-terminal chromodomain that functions as a methyl-lysine binding domain (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). The Tip60 chromodomain contains conserved aromatic amino acids similar to heterochromatin protein 1 (HP1) that are required for binding substrates such as tri-methylated histone H3 lysine 9 (Sun et al., 2009). A catalytic MYST domain is located at the C-terminal end and contains a conserved HAT domain and a zinc finger motif. The HAT domain can bind acetyl coenzyme A and another substrate such as a histone to facilitate in the transfer of acetyl groups. The zinc finger in the MYST domain is essential for proper protein-protein interactions, as well as acetyltransferase activity (Nordentoft and Jorgensen, 2003; Xiao et al., 2003). A nuclear receptor box is found at the extreme C-terminus of the protein (Gaughan et al., 2001). Tip60 is considered a nuclear protein, and is predominantly found in the nucleus (McAllister, Merlo and Lough, 2002; Schirling et al., 2010).

Functional dTip60 is required for survival. Evidence for this is seen in *Drosophila melanogaster*, where the complete knockdown of dTip60 by RNAi is lethal as flies do not survive past pupal stage (Schirling et al., 2010; Zhu et al., 2007). Also, there are no mutant alleles for dTip60 in flies because of the lethal phenotype. Mutant mice lacking Tip60 also die before implantation (Gorrini et al., 2007). The lethality in mice occurs after failure to pass the blastocyst stage of development. Therefore, Tip60 is essential for embryogenesis in mice during the blastocyst-gastrula transition (Hu et al., 2009).

1.6.2 Tip60 the HAT (Histone Acetyltransferase)

The Tip60 protein has been shown to possess acetyltransferase activity and recombinant Tip60 can preferentially acetylate histones H2A, H3, and H4, but not H2B, *in vitro* (Yamamoto and Horikoshi, 1997; Kimura and Horikoshi, 1998). Tip60 acetylates proteins by catalyzing the addition of an acetyl group from acetyl-CoA to specifically conserved lysine residues at the N-terminal tails of nucleosomal histones (Sterner and

Berger, 2000). This modification loosens chromatin compaction by weakening the interaction between histones and DNA, and histones with their neighbouring nucleosomal contacts. Additional factors can be recruited to the site of acetylation (Roth, Denu and Allis, 2001). In *D. melanogaster*, Kusch and colleagues showed that dTip60 can acetylate phospho-H2Av specifically on lysine 5 (Kusch et al., 2004). H2Av is a member of the H2AZ family of histone variants and is also a functional homolog of H2AX in humans (Kusch et al., 2004). Similar to observations of H2AX in humans, H2Av is also phosphorylated at sites of DNA damage. In yeast, the acetylation of histone H4 by the Tip60 homolog Esa1 is required for sufficient DNA double-strand break repair (Bird et al., 2002).

In addition to the role Tip60 plays in acetylating histones, other proteins were identified as substrates for Tip60. The kinase ATM is acetylated by Tip60 following DNA damage (Sun et al., 2005). Tip60 functions as a co-activator and acetylates a number of transcription factors such as: androgen receptor (AR) (Gaughan et al., 2002), NF-κB, and c-Myc (Patel et al., 2004). In addition, Tip60 can acetylate and activate p53 to modulate its function in cells between cell-cycle arrest and apoptosis (Sykes et al., 2006; Tang et al., 2006; Legube et al., 2004). Tip60 specifically acetylates p53 at K120, identified through mass spectrometry analysis (Tang et al., 2006). The acetylation of the K120 residue of p53 by Tip60 is a crucial step to the activation of *puma*, which is a key mediator of p53-dependent apoptosis.

1.6.3 Regulation of Tip60

The regulation of Tip60 acetyltransferase activity by the viral protein Tat has been well documented. The interaction between Tip60 and Tat inhibits Tip60 acetyltransferase activity, causing down-regulation of Tip60 controlled genes (Creaven et al., 1999).

Tip60 is also involved in a number of nuclear and cytoplasmic processes within the cell, therefore regulation can be quite complex. For this reason, the expression and stability of Tip60 is tightly controlled. Tip60 has a short half-life of 30-190 minutes depending on the cell type, and is targeted for proteosomal degradation through ubiquitination by mouse double minute 2 (Mdm2) (Legube et al., 2002). The degradation of mono- or polyubiquitinated Tip60 in the absence of stress maintains low protein levels. Tip60 is also regulated by phosphorylation via cyclinB/cell division cycle 2 (cdc2), causing phosphorylated levels of Tip60 to accumulate at the G2/M transition of the cell cycle (Lemercier et al., 2003). Tip60 is regulated by acetylation in the zinc finger motif at lysines 268 and 282 by p300/CREB binding protein (CBP), although the function for this acetylated Tip60 is unknown at this time (Col et al., 2005). Recently, it was shown that Tip60 is able to undergo autoacetylation in 293T and U2OS cells, and this autoacetylation is enhanced following damage by UV. This suggests that the active form of Tip60 required for proper DNA damage response is in fact acetylated (Wang and Chen, 2010). Furthermore, Tip60 was found to exist as an inactive dimer prior to autoacetylation. Lastly, the acetylation of Tip60 is negatively regulated through direct interaction by SIRT1 deacetylase, as observed from HDAC inhibitor experiments on cells, and co-immunoprecipitation experiments (Wang and Chen, 2010).

1.6.4 The Human Tip60 Complex

The human Tip60 complex containing the protein Tip60 is composed of over 18 subunits and is involved in transcriptional regulation and DNA damage repair. The Tip60 complex has histone acetyltransferase (HAT), as well as chromatin remodeling activities (Kusch et al., 2004). A schematic of the Tip60 complex is shown in Figure 3.

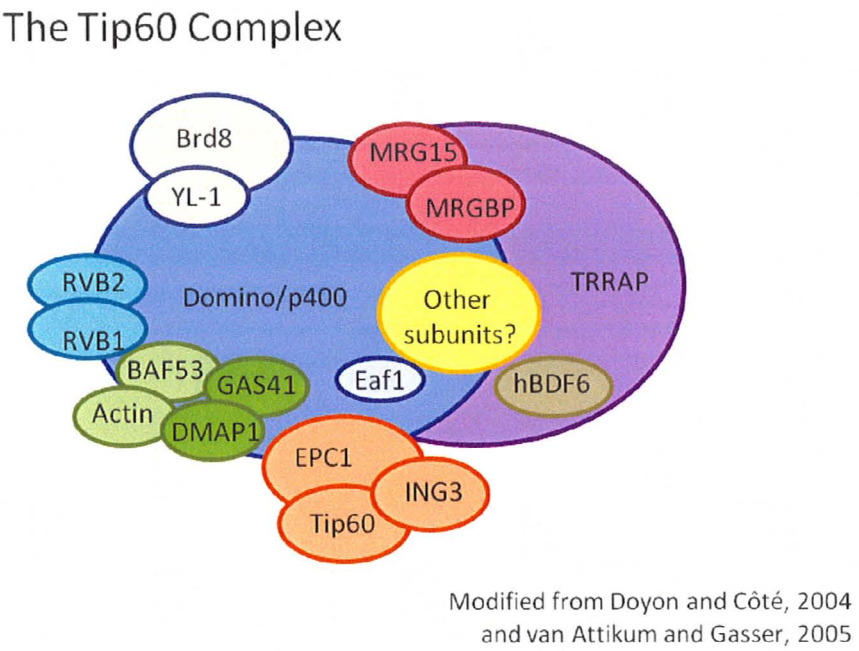

Figure 3: The Tip60 Complex. The Tip60 complex is a multi-subunit complex comprising of over 18 subunits with histone acetyltransferase and chromatin remodeling activities. Proteins that have been demonstrated to closely associate, or share similar function are grouped together by colour coding. The complex contains the ATPase Domino/p400, and the scaffolding protein TRRAP. Importantly, Tip60 closely interacts with EPC1 and ING3 forming the minimal Tip60 complex (orange).

One of the important components of the Tip60 complex is TRRAP (transformation/transcription domain-associated protein), a central scaffold protein with an inactive phophatidylinositol 3-kinase-related protein kinase (PIKK) domain (Ikura et al., 2000). The complex also contains the ATPase p400/Domino, which is essential for chromatin remodeling via histone variant exchange (Ikura et al., 2000). Enhancer of Polycomb (EPC1) and Inhibitor of Growth 3 (ING3) are located in the closest vicinity to Tip60 in the complex and they are involved in transcriptional control and growth inhibition/apoptosis, respectively. ING3 also has plant homeodomain (PHD) fingers, which are domains commonly found in chromatin modifying complexes with the ability to bind histone H3 tri-methylated at lysine 4 (H3K4me3) (Doyon et al., 2004). Additionally, the complex contains Rvb1 and Rvb2 helicases, homologous to RuvB helicase in *Escherichia coli* that actively promote branch migration of Holliday junctions during homologous recombination (HR) (Tsaneva, Muller, and West, 1992). *In vitro*, the Tip60 complex has also been shown to have ATPase, DNA helicase and DNA binding capacities (Ikura et al., 2000). Although the Tip60 complex is a large multi-protein complex of over 18 proteins, only three are required for sufficient HAT activity toward chromatin substrates: Enhancer of Polycomb (EPC1), Inhibitor of Growth 3 (ING3), and Tip60 itself (Doyon et al., 2004). Together, they are known as the minimal Tip60 complex (indicated by the orange subunits in Figure 3).

Most cellular Tip60 is associated in the stable Tip60 complex, although Tip60 can also form distinct transient complexes with other proteins upon stimulation of specific cellular processes. For example, Tip60 also exists in complex with ATM and activates ATM by acetylation in response to DNA damage (Sun et al., 2005).

1.6.5 Homologous Tip60 Complexes

Homologous complexes in *D. melanogaster* (flies) and *Saccharomyces cerevisiae* (yeast) have been characterized. The human Tip60 complex has both HAT and

chromatin remodeling activities. In yeast, these functions are performed by two distinct complexes, designated the NuA4 and SWR1 complexes, respectively (Doyon and Coté, 2004). Similar to humans, flies have one dTip60 complex. A study using *D. melanogaster* revealed that the dTip60 complex also has chromatin remodeling activity and facilitates histone variant exchange of phospho-H2Av with unmodified H2A in response to DNA damage by ionizing radiation (Kusch et al., 2004).

1.6.6 Role of Tip60 in DNA Damage Repair

The role of Tip60 in DNA damage repair has been extensively characterized. DNA damage, particularly DNA double-strand breaks (DSBs) can be caused by environmental stresses such as ionizing radiation and are considered the most deleterious type of DNA damage. Inefficient or erroneous repair of these breaks will cause genetic rearrangements, often leading to cancerous cells or cell death. Normally in cells, low Tip60 protein levels are maintained through ubiquitination by Mdm2 to target Tip60 for proteosomal degradation (Legube et al., 2002). The half-life of Tip60 is increased and rapidly stabilized in response to DNA damaging signals such as UV radiation. Ubiquitination by Mdm2 is inhibited by UV, allowing Tip60 to aid in DNA damage repair (Legube et al., 2002).

The tumour suppressor p53 is a critical substrate of Tip60 as different post-translational modifications infer the activity of p53 towards cell cycle arrest or apoptosis in response to DNA damage and stress (Kruse and Gu, 2009). Importantly, a large scale RNAi screen revealed that Tip60 is an activator of p53 in response to stress, and levels of p53 activation positively correlate with levels of p53 acetylation (Berns et al., 2004). The decision between cell cycle arrest and apoptosis depends on the activity of Tip60 on p53. Following DNA damage, Tip60 interacts with p53 and is recruited to p53 target promoters such as $p21^{Cip1}$. $p21^{Cip1}$ expression is promoted by Tip60 to mediate cell cycle arrest (Tang et al., 2006). Alternatively, the Tip60/p53 interaction may lead to the

specific acetylation of an evolutionary conserved lysine 120 (K120) residue on p53 to induced the expression of the pro-apoptotic genes *BAX* and *PUMA* (Sykes et al., 2006). Mutation of K120 in p53 blocks the transcription of *BAX* and *PUMA* and often leads to cancer.

Tip60 is a critical factor in the response to DNA double strand break repair, as DNA double strand breaks accumulate following γ-irradiation in cells expressing catalytically inactive Tip60 (Ikura et al., 2000). In addition, the same cells are resistant to apoptosis upon exposure to γ-irradiation, suggesting a role for Tip60 to signal the presence of DNA damage (Ikura et al., 2000). Similarly in yeast, mutations in the Tip60 homolog Esa1 results in hypersensitivity to DNA-damaging agents and an inability to repair DNA using non-homologous end joining (NHEJ) (Bird et al., 2002). The binding of Tip60 with a concomitant binding of TRRAP (a protein in the Tip60 complex) correlates with enrichment of histone H4 acetylation in the vicinity of a double strand break suggesting acetylation is a signal for the recruitment of repair proteins (Stante et al., 2009; Murr et al., 2006). Also, in mouse embryonic stem (ES) cells, the presence of TRRAP is essential to repair. The depletion of TRRAP results in an inability of ES cells to repair DNA double-strand breaks as observed in the alkaline comet assay (Murr et al., 2006).

The DNA damage sensor protein ATM normally exists in an inactive dimeric form bound to Tip60. Upon induction of DNA damage, Tip60 directly acetylates ATM, allowing ATM to efficiently autophosphorylate. The ATM dimer dissociates when phosphorylated, resulting in increased ATM kinase activity on specific substrates such as histone variant H2AX (phosphorylated form is γ-H2AX). The γ-H2AX mark on chromatin allows recruitment of more repair proteins to the site of DNA damage (Sun et al., 2005). γ-H2AX rapidly accumulates in the vicinity of DSBs spanning up to 50kb around the break with the highest level of phosphorylation present at 3-5kb surrounding the

double-strand break (Shroff et al., 2004). Recently, work in *S. cerevisiae* showed that actin-related protein 4 (Arp4), homologous to BAF53 of the human Tip60 complex binds to phosphorylated H2A following DNA damage *in vitro* (Downs, Lowndes and Jackson, 2000; Downs et al., 2004). The binding of Arp4 also correlates with increased acetylation of histone H4 at lysine 8 by Esa1 (yeast Tip60 homolog) surrounding the induced DSB (Bird et al., 2002).

In HeLa cells, Tip60 was shown to rapidly associate with H2AX immediately after induction of DNA damage from ionizing radiation in a process independent of histone phosphorylation (Ikura et al., 2007). Tip60 acetylates H2AX specifically at lysine 5 (K5) and also associates with the ubiquitin-conjugating enzyme UBC13. Together, the Tip60-UBC13 complex regulates the ubiquitination of H2AX following DNA damage. Ubiquitination of H2AX by UBC13 is acetylation-dependent and causes histone eviction/release of H2AX from chromatin as observed in iFRAP (*inverse*-fluorescence recovery after photobleaching) analysis. The eviction of histones allows for chromatin reorganization and repair (Ikura et al., 2007).

Interestingly, the *Drosophila* dTip60 complex co-purifies with histones H2B and the histone variant H2Av (Kusch et al., 2004). The copurification of the dTip60 complex with H2Av suggests a role dTip60-dependent acetylation of phospho-H2Av (functional homolog of γ-H2AX) for chromatin remodeling. Histone H2Av is rapidly phosphorylated upon induction of DNA damage, and dTip60 acetylates phospho-H2Av at lysine 5 (K5) to exchange it with unmodified H2Av using the p400/Domino ATPase subunit of the dTip60 complex (Kusch et al., 2004). The removal of phospho-H2Av reverses the mark imposed by DNA damage. Phospho-H2Av accumulates in the vicinity of DSBs in cells lacking Tip60 (Kusch et al., 2004). Similarly in HeLa cells, acetylation of histone H4 is required for the proper exchange of phosphorylated H2AX (γ-H2AX) with unmodified H2AX at sites of DNA damage induced by UV radiation (Jha, Shibata and Dutta, 2008). Yeast has a

comparable mechanism for chromatin remodeling. The NuA4 (HAT) dependent acetylation of nucleosomal histone H4 and H2A is a prerequisite for histone variant exchange with Htz1 (H2A.Z histone variant) into chromatin by the SWR1 (chromatin remodeling) complex (Altaf et al., 2010).

1.6.7 Role of Tip60 in Gene Regulation

Gene regulation is a complex process which requires access to genes by transcriptional machinery for proper expression. DNA within chromatin is tightly compact and requires large multi-protein complexes to modify and loosen chromatin. The nature of chromatin is highly dynamic. Histones are epigenetically modified to regulate chromatin structure through acetylation, methylation, and phosphorylation (Ehrenhofer-Murray, 2004).

dTip60 activity is also implicated in gene regulation through its localization on polytene chromosomes in *Drosophila*. Compact regions of polytene chromosomes are generally correlated with inactive genes, whereas less compacted interband or puffed regions correlate with actively transcribed genes. dTip60 localizes to interband regions of polytene chromosomes, suggesting a role in actively transcribed regions, but interestingly, it has also been implicated in gene repression in the same study (Schirling et al., 2010).

Recruitment of the Tip60 complex to chromatin can also be mediated by the TRRAP scaffolding protein (a component of the Tip60 complex). TRRAP associates with Myc which recruits the Tip60 complex to Myc targeted genes (Frank et al., 2003). The activation of these Myc targeted genes occurs after the acetylation of histone by Tip60 to open chromatin. Additionally in *Drosophila*, Pontin (a component of the Tip60 complex) shows a strong genetic interaction with dMyc to control development, survival, and size (Bellosta et al., 2005). The genetic interaction between Pontin and dMyc is implicated to have a role in cellular growth and proliferation.

In addition to Tip60 (HAT) involvement in gene activation, Tip60 can also function as a co-repressor independent of its HAT activity. Tip60 can indirectly repress genes by interacting with the DNA-binding factors KLF4 and STAT3, resulting in the recruitment of HDAC7 to gene promoters (Ai et al., 2007; Xiao et al., 2003).

1.7 Heat Shock Response

Recently, menin has been implicated as a regulator of the stress response in *Drosophila melanogaster* (Papaconstantinou et al., 2005). The misexpression of menin disrupts the embryonic response to heat shock and leads to increased lethality when embryos are subjected to a 1hr dose of heat shock at 37°C. The lack of menin leads to an inability to upregulate hsp70 for the heat shock response. Conversely, the overexpression of menin leads to the inability to downregulate hsp70 upon return to normal temperatures (Papaconstantinou et al., 2005). Menin is also recruited to the hsp70 promoter after heat shock in Oregon R embryos (A. Pepper, unpublished results). From these data, menin is a regulator of the heat shock response, and provides a basis into the study of this stress response.

The heat shock response is a highly regulated cellular response that is triggered in all organisms when exposed to suboptimal physiological conditions (Lindquist, 1986). The heat shock response is induced by high temperature stress, leading to the rapid production of heat shock proteins (HSPs) (Lindquist, 1986). Failure to respond to heat stress is a major factor in the process of cell death. In addition, heat shock causes an almost complete shutdown of major transcriptional or translational activities in cells (Shamovsky and Gershon, 2004). In eukaryotes, the transcription factor involved in this process, designated heat shock factor (HSF) is present in an inactive state which undergoes an activating conformational change after heat shock (Westwood, Clos and Wu, 1991; Wu, 1984). There are 4 HSF proteins in vertebrates (HSF-1 to HSF-4) and the most heat shock responsive form is HSF-1. In contrast, *Drosophila* only expresses one

HSF protein homologous to HSF-1 (Clos et al., 1990). A conformational change allows for trimerization of HSF-1 through integrating the leucine zipper domains to form a coiled coil domain (Morimoto, 1998). HSF-1 then binds to the heat shock elements (HSEs) in the promoters of heat shock responsive genes such as HSPs (Perisic, Xiao and Lis, 1989). Only HSF-1 trimers are competent to bind heat shock elements. In *Drosophila*, maximal heat shock response occurs at 36-37°c, and HSP transcripts can be detected 4 minutes after exposure (Lindquist, 1980). HSPs are the main product of protein synthesis during heat shock until the organism is returned to normal temperatures. Normal cellular processes resume after returning to normal temperatures (DiDomenico, Bugaisky and Lindquist, 1982a; DiDomenico, Bugaisky and Lindquist, 1982b). HSPs such as Hsp70, Hsp90, and Hsp27 act as chaperones to prevent aggregation of partially misfolded proteins within the cell (Lindquist and Craig, 1988). This prevents the vast degradation of proteins during heat stress. The chaperones can refold proteins for reuse upon return to normal temperatures.

1.7.1 HSP70

HSP70 is the most evolutionarily conserved HSP and human HSP70 is 73% identical to *Drosophila hsp70* (Lindquist, 1986). The *HSP70* gene is regulated in a complex manner and is poised to act quickly upon induction of heat shock. HSP70 is a chaperone designed to protect misfolded proteins in response to heat shock, and facilitate in their proper refolding upon return to normal temperatures. The chaperone function of HSP70 is critical in the protection against stress-induced apoptosis through the inhibition of procaspase activation (Mosser et al., 2000). When subjected to heat shock, the expression of HSP70 is induced by the binding of heat shock factors (HSFs) to the heat shock elements (HSEs) located 5'-upstream of the *HSP70* gene (Morimoto, 1998). Three proteins are known to occupy the promoter sequences of HSP70 under non-heat shock conditions: GAGA factor (GAF), TATA-binding protein, and RNA polymerase II (Lis, 1998). The RNA polymerase II associated with each HSP70 promoter

is paused at the transcriptional start site and is poised for elongation. RNA polymerase II has synthesized the beginning of the transcript about 25 nucleotides long at this site (Rougvie and Lis, 1988). HSP70 activation is also dependent on acetylation of histones, as the use of histone deacetylase (HDAC) inhibitors such as Trichostatin A and sodium butyrate enhances the transcription of the *hsp70* gene in *Xenopus* (Ovakim and Heikkila, 2003). Additionally, *Drosophila* third-instar larvae fed with histone deacetylase inhibitors show a "puff" structure on their polytene chromosome that correlates with higher transcriptional levels at position 87A-C where the *hsp70* gene is located (Chen et al., 2002).

The stability of Hsp70 in the cell is proportional to the severity of heat shock (Yost, Petersen and Lindquist, 1990). Hsp70 mRNA has a short half life in non-heat shock conditions fewer than 50 mins (Theodorakis and Morimoto, 1987). Hsp70 transcripts are stabilized during heat shock due to the retention of their poly(A)-tails. Deadenylation machinery is only active at lower temperatures (Dellavalle, Peterson and Lindquist, 1994). Other polyadenylated transcripts are also retained in the cell, but they are not translated (Theodorakis and Morimoto, 1987). *In-vitro* translation assays in 293 cells of HSP70 mRNA demonstrated that HSP70 is constitutively synthesized at a similar efficiency with or without heat shock (Theodorakis and Morimoto, 1987). In contrast, actin mRNA could not be translated during heat shock.

1.7.2 Regulation of HSF-1

An important part of the heat shock response is the proper regulation of HSF-1. HSF-1 expression is stimulated by elongation factor-1α (EF-1α), and a unique large non-coding RNA (HSR1), identified using an electrophoretic mobility shift assay (EMSA) (Shamovsky and Gershon, 2004). *In vitro* assays using purified EF-1α and HSR1 showed that HSF-1 could only be activated when both stimulating components were added. Luciferase assays using the Hsp70 promoter in HeLa cells revealed an inability of HSF-1

to bind Hsp70 during heat shock when HSR1 expression is reduced via siRNA (Shamovsky et al., 2006). Interestingly, functional mapping of HSR1 revealed specific domains required for the activation of HSF-1 at the 5' end. Functional domains of HSR1 are hidden under normal conditions in a closed conformation. The secondary structure of HSR1 changes upon sensing heat stress to expose the functional domains and activate HSF-1 together with EF-1α (Shamovsky and Nudler, 2008). In addition, cells lacking HSR1 become thermosensitive and also fail to acquire thermotolerance through heat shock pre-conditioning (Shamovsky et al., 2006). An HSF-1 inhibitory protein 30kDa in size was also found in a heat shocked baby hamster kidney fibroblast (BHK-21) cell fraction, which has yet to be characterized (Shamovsky and Gershon, 2004).

HSF-1 which binds *HSP70* is also regulated by another chaperone HSP90. HSF-1 monomers are kept inactive when bound to HSP90 in a complex. The addition of HSP90 inhibitors such as geldanamycin, or the induction by heat shock allows dissociation of HSF-1 from HSP90 and trimerization to bind heat shock responsive promoters (Zou et al., 1998). In addition, HSP90 is required for the dissociation of the HSF-1 trimer upon return to normal temperatures. HSF-1 levels are also regulated through feedback inhibition by HSP70. HSF-1 is rapidly phosphorylated and sumoylated in response to heat shock (Akerfelt, Morimoto and Sistonen, 2010). HSF-1 is sumoylated at lysine 298 and localizes to nuclear stress granules upon induction of stress (Hong et al., 2001). Acetylation of HSF-1 is another post-translational modification of HSF-1, but the kinetics of acetylation are slower and coincide with HSF-1 attenuation (Akerfelt, Morimoto and Sistonen, 2010). The state of HSF-1 acetylation is determined by a balance between acetyltransferase p300/CBP (CREB binding protein), and an NAD^+-dependent deacetylase SIRT1. The increased expression of SIRT1 stabilized the DNA-binding capacity of HSF-1 trimers, and also prolonged their presence on the *HSP70* promoter (Westerheide et al., 2009).

1.8 Rationale

Human menin associates with chromatin remodeling complexes such as the HMTase complex containing MLL (Hughes et al., 2004; Yokoyama et al., 2004), and the mSin3A/HDAC complex (Kim et al., 2003) to facilitate their recruitment to chromatin. The methylation of histone H3K4 by the HMTase complex containing MLL opens chromatin and allows for the recruitment of additional factors and complexes. In flies, menin associates with a homologous HMTase complex called TAC1, containing the MLL homolog Trx. Knowing that menin associates with chromatin remodeling complexes, and menin interacts with dTip60 (Appendix A), menin may also have a role in recruiting the Tip60 chromatin remodeling complex to chromatin.

E(Pc) was previously identified as a positive clone in the yeast two-hybrid screen using *Mnn1* as bait (Hybrigenics). We graciously received antibodies for E(Pc), Ing3, and dTip60 (minimal dTip60 complex; Figure 3) from Dr. Tom Kusch at Rutgers University, Piscataway, NJ, USA (Kusch et al., 2004). Ying Wu used these antibodies to identify a possible interaction between menin and E(Pc), Ing3, or dTip60, through co-immunoprecipitation. Co-immunoprecipitation did not reveal an interaction of menin with E(Pc). Instead, the experiment identified dTip60 as an interacting partner of menin through co-immunoprecipitation experiments in a menin overexpressing S2 cell line (Appendix A). Peptide antibodies for dTip60 were ordered and generated by New England Peptide (see Methods) for future experiments.

1.9 Main Objective

The main objective of my thesis is to characterize the interaction between menin and Tip60 in response to heat stress and γ-irradiation.

Co-immunoprecipitation experiments in *Drosophila* S2 cells were performed to confirm the previously identified interaction using the new dTip60 antibody from New

England Peptide. Menin was implicated as a requirement for proper heat shock response (Papaconstantinou et al., 2005), therefore the interaction was examined in S2 cells after heat shock. Tip60 is well characterized for its role in DNA damage repair in response to double-strand breaks. Co-immunoprecipitation was also used to investigate a potential interaction between menin and dTip60 in S2 cells in response to DNA double-strand breaks caused by γ-irradiation.

For the second part of the project, the role of the menin and dTip60 interaction was examined in *Mnn1* and dTip60 mutant flies. *UAS-dTip60* RNAi and the corresponding control flies were obtained from Dr. Felice Elefant at Drexel University in Philidelphia, Pennsylvania (Zhu et al., 2007), and their efficiency of knockdown was examined through SDS-PAGE and Western blotting analysis. Stress experiments (heat shock) were conducted comparing dTip60 RNAi flies to controls in their efficiency of stress response, and their lethality in response to stress.

Chapter Two: METHODS

2.1 Antibodies

Two rabbit peptide antibodies for dTip60 (3614, 3615) were designed against the first 11 amino acids of the N-terminal end of dTip60 generated by New England Peptide (MKINHKYEFDC). The rabbit anti-menin 5073 peptide antibody was designed against the last predicted 15 amino acids (C-terminal end) of *Drosophila* menin (GDSIAASRPKRTRRE) and generated by Sigma Genosys. The mouse monoclonal anti-actin antibody was from Millipore (MAB1501). The rabbit anti-acetyl histone H3 antibody was from Millipore (#06-599).

2.2 S2 Cell Culture

Schneider 2 (S2) cells were regularly cultured in Hyclone SFX Insect medium (catalog #SH30278.01) with penicillin/streptomycin (Gibco 15140-122) at room temperature (22°C) and split every 2-3 days into 25 mm^2 vented tissue culture flasks at 1:5 and 1:10 concentrations. For co-immunoprecipitation experiments, dense and actively dividing S2 cells were split from the 25 mm^2 flasks into 100 mm^2 tissue culture dishes as discussed below.

2.3 Lysate Preparation of S2 Cells

2.3.1 Cell Culture Conditions

S2 cells were split 1:8 into 100mm tissue culture dishes from a confluent tissue culture flask of S2 cells and allowed to grow at room temperature (22°C) for 48 hours until dense and actively dividing with minimal floating cells.

2.3.2 Heat Shock and Lysate Preparation

If a heat shock sample was required, the tissue culture dish was sealed with parafilm and heat shocked at 37°C by submerging in a water bath for the required amount of time (5min to 1hr) before lysate preparation.

S2 cells were collected and washed once with 4mL of 1xPBS pH 7.4 (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4). Cells were pelleted by centrifugation at 1000 rpm for 5 minutes. Cell media and PBS were removed using a Pasteur pipette and water aspirator. Cell lysates were made with 5 volumes of SDS sample buffer (2% SDS, 10% Glycerol, 5% β-mercaptoethanol, 60mM Tris-HCl pH 6.8, 1mM NaF, 100mM $NaVO_4$, complete mini protease inhibitor cocktail tablet Roche Diagnostics ref #11 836 170 001), and heated at 100°C for 5 minutes to denature proteins. Lysates were centrifuged for 10min at maximum speed (14000rpm) to pellet cellular debris, and the final supernatant was transferred to a new microcentrifuge tube. Lysates were stored at -80°C until ready for use. Before loading onto a gel, proteins were quantified using Bradford assay and 100µg of each lysate were aliquoted for loading.

2.4 Co-immunoprecipitation in S2 Cells

2.4.1 Cell Culture Conditions

S2 cells were split 1:8 into 100mm tissue culture dishes from a confluent tissue culture flask of S2 cells and allowed to grow at room temperature (22°C) for 48 hours. Dishes of dense and actively dividing S2 cells were used for co-immunoprecipitation.

2.4.2 S2 Cell Nuclear Extraction

S2 cells were collected and washed once with 1xPBS pH 7.4. Cells were centrifuged at 1000rpm for 5 minutes, and the media was removed using a Pasteur pipette and water aspirator. For nuclear isolation, cells were incubated in nuclear

isolation buffer (1.28M sucrose, 40mM Tris pH 7.6, 20mM $MgCl_2$, 4% Triton-X) for 15 minutes on ice. Nuclei were pelleted by centrifugation at 1000rpm for 5 minutes, and cell debris removed using a Pasteur pipette and water aspirator. Lysis of nuclei followed the same protocol as written in section 2.4.3.

2.4.3 Heat Shock, γ-Irradiation Treatments and Lysate Preparation

For experiments requiring heat shock conditions, S2 cell dishes were sealed with parafilm and submerged in a water bath at 37°C for durations ranging from 5 minutes to 1 hour prior to lysis.

For experiments requiring γ-irradiation treatments, 60mm S2 cell dishes were sealed with parafilm and placed in a GammaCell1000 ^{137}Cs irradiator. S2 cells were irradiated for 8min (25Gy), and allowed to recover for durations in time ranging from 15min to 45min prior to lysis. For co-immunoprecipitation experiments, 3x60mm dishes of cells were collected for each condition.

For lysate preparation, S2 cells were collected and washed once with 4 mL of 1x PBS pH 7.4. Cells were centrifuged at 1000 rpm for 5 minutes. Cell media and PBS were removed using a Pasteur pipette and water aspirator before cell lysis with 1 mL TNE buffer (50mM Tris-HCl pH 7.6, 150mM NaCl, 1% Nonidet P-40, 10mM NaF, 2mM EDTA pH 8, 1mM EGTA pH 8.5, complete mini protease inhibitor cocktail tablet Roche Diagnostics ref #11 836 170 001) on ice for 15 minutes. Lysates were centrifuged at maximum speed (13 000 rpm) for 10 minutes to pellet cellular debris before transferring to a new microcentrifuge tube. A Bradford assay was performed to quantify the amount of protein in each lysate before aliquoting equal amounts of protein (1000 – 2000 µg protein) into new microcentrifuge tubes for each immunoprecipitation. Protein samples were topped up to 1 mL total volume using TNE lysis buffer prior to immunoprecipitation.

2.4.4 Co-immunoprecipitation

For co-immunoprecipitation experiment, 1µL of menin antibody 5073 was added to each lysate and incubated at 4°C on a rotator for 3 hours. Pre-immune serum (1µL) was also incubated separately as a negative control.

For reverse co-immunoprecipitation experiment, 5µL of affinity purified dTip60 antibody 3615 was added to each lysate and incubated at 4°C on rotator for 3 hours. A pre-immune sample (5µL) was prepared as a negative control.

Protein-G Sepharose beads (GE Healthcare 17-0618-01) were blocked with BSA (1µg/µL) for 1 hour on 4°C rotator, and resuspended as bead slurry (1 part beads, 2 parts TNE lysis buffer). 60µL bead slurry was added to each IP and incubated on 4°C rotator for 1 hour. Following IP, washes were carried out by pulsing down beads, removing supernatant and adding cold TNE lysis buffer: 3 washes using high salt TNE lysis buffer (50mM Tris-HCl pH 7.6, 300mM NaCl, 1% Nonidet P-40, 10mM NaF, 2mM EDTA pH 8, 1mM EGTA pH 8.5), followed by 2 washes using TNE lysis buffer. After the final wash, TNE lysis buffer was removed and beads were resuspended in 60µL SDS sample buffer and 2µL of 0.15% bromophenol blue per sample. The eluted proteins were stored in -80°C freezer until ready for SDS-PAGE.

2.5 SDS-PAGE and Western Blotting Analysis

For the analysis of total S2 cell proteins, 100µg of protein was resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, BioScience). The products of immunoprecipitation were also resolved on a 10% SDS-polyacrylamide gel before transfer to nitrocellulose. Membranes were blocked with milk solution (5% skim milk powder, 0.02% Na Azide, dissolved in 200mL of 1xTBS pH 7.6 (50mM Tris-HCl, 150mM NaCl)) for 1 hour at room temperature. Blots were incubated in their respective primary antibody in blocking buffer overnight at 4°C. Antibodies used

were: rabbit anti-menin 5073 (1:2500 dilution), rabbit affinity purified anti-dTip60 3615 (1:100 dilution), mouse anti-actin (1:5000 dilution; Chemicon International MAB1501), goat anti-HSP70 (1:1000 dilution; Santa Cruz Biotechnology #sc-27028), and rabbit anti-acetyl histone H3 (1:1000 dilution; Millipore #06-599). After primary antibody incubation, blots were washed 4 times with 1xTBS pH 7.6, followed by incubation in the appropriate secondary antibody at 1:10,000 dilution for 1 hour at room temperature. Blots were washed 4 times with 1xTBS after secondary antibody incubation before ECL detection method according to the protocol of the manufacturer (GE Healthcare).

2.6 Fly Stocks

All fly stocks were maintained at room temperature (22°C) on standard yeast-agar media, and include Oregon-R, daGAL4 (Bloomington #8641).

The *Mnn1*^{e173} and *Mnn1*^{e30} lines were previously generated in the lab using imprecise P-element excision. *Mnn1*^{e30} contains a deletion spanning 2,096-bp from the beginning of the P-element insertion in exon 1 to the end of exon 4. *Mnn1*^{e173} harbours a larger 4,216-bp deletion, beginning 661-bp upstream of the published *Mnn1* cDNA sequence and ending in intron 4 (Papaconstantinou et al., 2005).

The *UAS-dTip60* RNAi A, B, and C lines, as well as the *UAS-dTip60* control A and B lines were a gracious gift from Dr. Felice Elefant (Drexel University, Philadelphia, PA, USA), made and characterized previously (Zhu et al., 2007). The *UAS-dTip60* RNAi constructs were made against a 613-bp unique sequence within the dTip60 coding region in a sense-antisense inverted gene arrangement separated by a "hinge" polylinker. The *UAS-dTip60* control constructs were made against the same unique 613-bp sequence in a sense-sense orientation. Constructs were inserted into flies via P-element germline transformation.

2.7 Embryo Collections

Embryos were collected on standard grape juice agar (For 5x100mm plates or 10x60mm plates: 45mL ddH_2O, 8mL grape juice, 1.8g agar) with added yeast paste for 2 hours at 25°C. Embryos were allowed to age at 25°C on the grape juice plate until the correct stage for the experiment up to 1st instar larval stage.

2.7.1 Collection of Larvae

For experiments that required 1st instar larvae, grape juice plates were pre-cleared of older larvae prior to collection. 1st instar larvae (25-26 hours AEL) were manually removed from grape juice plate and washed thoroughly with double distilled water before transferring to a microcentrifuge tube in a small amount of water. If larvae needed to be heat shocked, the microcentrifuge tube would be submerged in a water bath at 37°C for heat shock times ranging from 5 minutes up to 1 hour before lysis.

For experiments that required 2nd instar larvae, grape juice plates were pre-cleared of older larvae at 20 hours AEL prior to transfer. 1st instar larvae at 25-26 hours AEL were transferred into 60mm yeast agar food plates and allowed to age at 25°C for 24 hours. 2nd instar larvae (49-50 hours AEL) were manually removed from food plates and washed thoroughly with double distilled water before transferring to a microcentrifuge tube. If larvae needed to be heat shocked, the microcentrifuge tube would be submerged in a water bath at 37°C for heat shock times ranging from 5 minutes up to 1 hour before lysis.

For experiments that required 3rd instar larvae, grape juice plates were pre-cleared of older larvae at 20 hours AEL, prior to larvae transfer. 1st instar larvae at 25-26 hours AEL were transferred into 60mm yeast agar food plates and allowed to age at 25°C for 68 hours. 3rd instar larvae were manually removed from food plates and thoroughly washed with double distilled water. If larvae needed to be heat shocked, the

microcentrifuge tube would be submerged in a water bath at 37°C for heat shock times ranging from 5 minutes up to 1 hour before lysate preparation.

2.7.2 Lysate Preparation

Embryos were collected in scintillation vials with sieves and washed thoroughly with double distilled water before homogenizing in 5 volumes of SDS sample buffer. Lysates were heated at 100°C for 5 minutes, and centrifuged at maximum speed (13 000 rpm) for 10 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube. Protein concentrations were determined by Bradford assay before SDS-PAGE and Western blotting analysis.

2.8 Heat Shock Lethality Experiments

Embryos were collected on standard grape juice agar as previously mentioned. Embryos were manually transferred to standard yeast agar food vials, at 50 embryos per vial. Embryos or larvae were heat shocked at the specified developmental stage by submerging the vials in a water bath, and heat shocked at 37°C for 1hr. Controls (no heat shock) were used to compare rates of development and lethality.

2.9 Quantification of Relative dTip60 Levels

Relative amounts of dTip60 were quantified using Image J analysis software. JPEG images of scanned Western blots were uploaded into Image J. Gel lanes were defined using gel analysis menu and plotted. Quantities of actin and dTip60 were defined as the total area underneath the plotted curves. Relative quantities of dTip60 were calculated as a percentage of actin to correct for uneven loading in the respective lanes.

Chapter Three: RESULTS

3.1 Menin and dTip60 Interaction in S2 Cells

Preliminary results by Ying Wu had previously identified dTip60 as an interacting partner of menin through co-immunoprecipitation experiments in S2 cells overexpressing a Flag- and HA-tagged menin construct (Appendix A). While these results were promising, experiments to identify an interaction between menin and dTip60 using endogenous protein in S2 cells had not been attempted. Firstly, co-immunoprecipitation in S2 cell lysates proved difficult possibly because of the dynamic nature of this interaction. Menin and dTip60 are predominantly nuclear proteins; therefore the interaction was examined in S2 cell nuclear extracts. Co-immunoprecipitation of menin with dTip60 in S2 cell nuclear extracts confirmed that menin is present in a complex with dTip60, as shown in Figure 4.

After confirming the interaction of menin with dTip60, heat shock experiments were performed to determine whether the interaction also occurs after heat stress. Co-immunoprecipitation using total S2 cell lysates (not nuclear extracts) was performed to ensure accurate representation of heat shock times; the nuclear isolation step would add an extra 15 minutes to the protocol, therefore this step was omitted. In Figure 5, an interaction between menin and dTip60 was seen after 1 hour of heat shock at 37°C (Figure 5) in S2 cells. However, in the absence of stress, an interaction between menin and dTip60 was not evident in these conditions (Figure 5). Differences in the protocol may account for this difference. Much less protein (1mg collected from 100mm dishes) was used for these co-immunoprecipitations in Figure 5. In contrast, the interaction between menin and dTip60 in Figure 4 was seen after S2 cell collection from 150mm dishes, and nuclear enrichment prior to lysis.

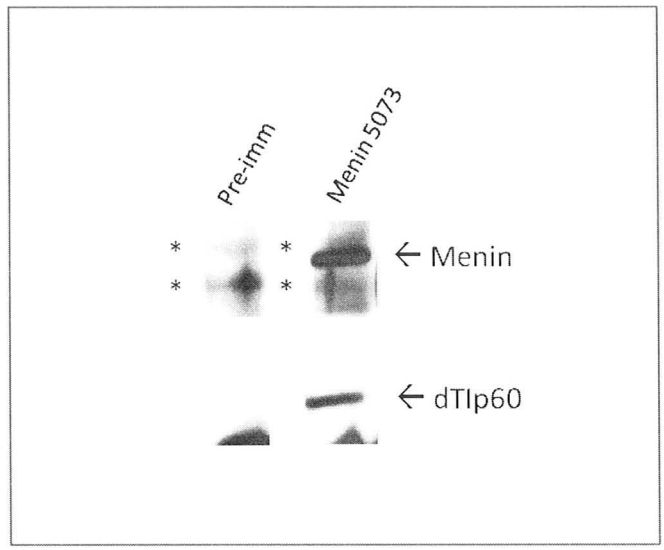

Figure 4: Menin Interacts with dTip60. S2 nuclear lysates were extracted from 150mm plates to enrich for nuclear proteins and co-immunoprecipitate endogenous menin (5073; 1µL) with dTip60. dTip60 interacts in a complex with menin in S2 cells, under growing conditions with no stress. Pre-immune (from 5073 rabbit) was used as a negative control. Asterisks (*) represent non-specific proteins pulled down in the immunoprecipitation.

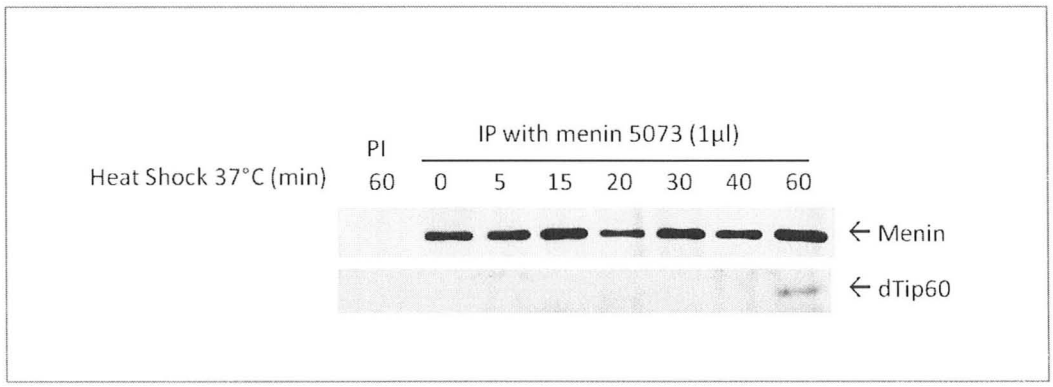

Figure 5: Menin interacts with dTip60 after Heat Shock. S2 cells were heat shocked at 37°C for the indicated duration of time. Total S2 cell lysates were collected and quantified by Bradford assay before co-immunoprecipitation with anti-menin 5073 (1µL) antibody. 1mg of protein was used for each co-immunoprecipitation. Menin is shown to interact with dTip60 after 1hr heat shock at 37°C. Pre-immune (PI from 5073 rabbit) was used as a negative control.

In Figure 5, cells for the negative control were heat shocked for 1hr as well before lysate preparation and incubation with pre-immune (PI) serum to ensure absence of non-specific interaction. Co-immunoprecipitation experiments were also performed using increased severity of heat shock at 39°C. An interaction between menin and dTip60 was also seen after 1hr of heat shock at 39°C (data not shown).

The interaction between menin and dTip60 occurring after heat shock prompted the investigation into whether these two proteins interact after other forms of stress such as DNA damage by γ-irradiation. S2 cells were subjected to 25Gy (Grays) of γ-irradiation and collected at various times of recovery for co-immunoprecipitation experiments. In Figure 6, co-immunoprecipitation failed to detect an interaction of menin and dTip60 in response to DNA damage caused by γ-irradiation.

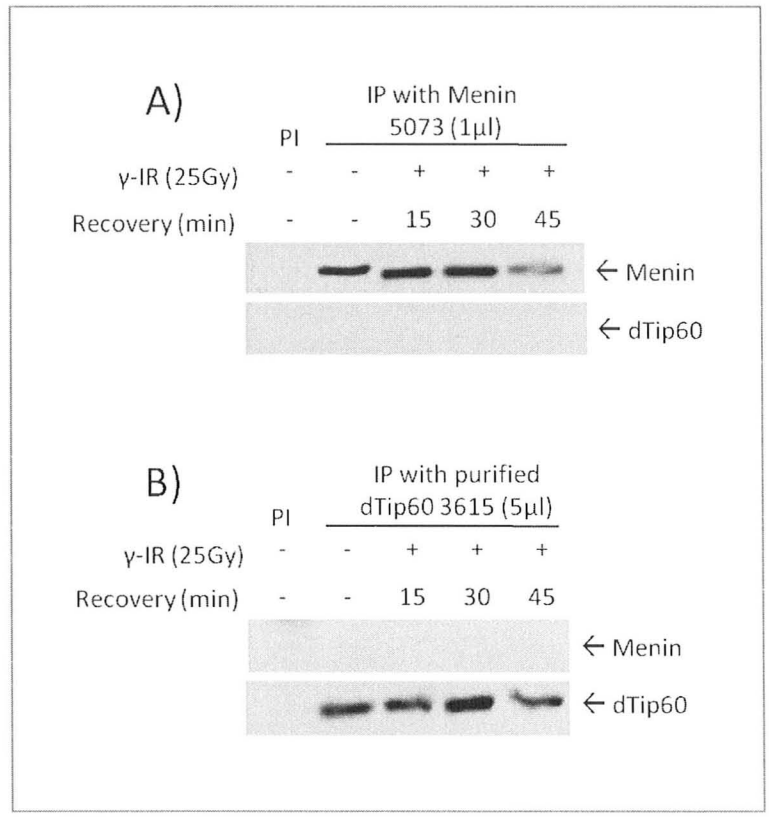

Figure 6: Menin does not interact with dTip60 after γ-irradiation. S2 cells were exposed to 25Gy of γ-irradiation and allowed to recover for the indicated durations of time. In (A), co-immunoprecipitation was carried out with anti-menin 5073 (1µL) antibody and blotted for dTip60. The corresponding reverse co-immunoprecipitation was carried out in (B). These experiments failed to show an interaction between menin and dTip60 in response to γ-irradiation. Pre-immune (PI from the respective rabbits) were used as negative controls.

DNA damage by γ-irradiation failed to show an interaction between menin and dTip60. However, another group showed that Tip60 is negatively regulated by a histone deacetylase (HDAC) SIRT1, and active Tip60 (with histone acetyltransferase ability) is autoacetylated (Wang and Chen, 2010). SIRT1 activity is inhibited by the use of HDAC inhibitor sodium butyrate. A possibility is that the use of sodium butyrate may stabilize active levels of dTip60 in S2 cells to facilitate the detection of a complex between menin and dTip60 in response to DNA damage caused by γ-irradiation. Preliminary experiments were performed to determine the concentration of sodium butyrate to be used, and the length of treatment before co-immunoprecipitation (Appendix C). A concentration of 10mM sodium butyrate for a 24hr treatment was decided as an appropriate treatment prior to co-immunoprecipitation. Initially, co-immunoprecipitation using anti-menin 5073 antibody showed a protein appearing in Ponceau stain of the membrane. The protein was about 17kDa in size and appeared specifically after the combination of sodium butyrate treatment and γ-irradiation. This observation prompted the investigation into whether menin is interacting in complex with a histone such as acetylated-histone H3 in response to γ-irradiation. In Figure 7, acetylated-histone H3 appears to interact with menin; however, the pre-immune negative control also shows presence of acetylated-histone H3. This demonstrates that the interaction between menin and acetylated-histone H3 is an artifact of the protocol. In addition, dTip60 was not shown to interact with menin despite the use of the HDAC inhibitor sodium butyrate, as seen in Figure 7.

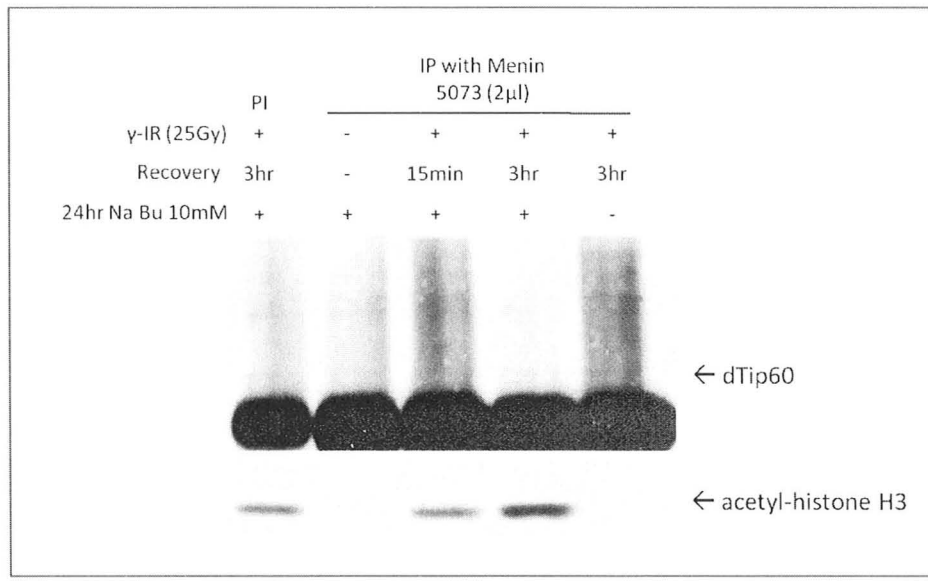

Figure 7: Menin does not interact with dTip60 after γ-irradiation and HDAC inhibitor treatment. S2 cells were treated with HDAC inhibitor sodium butyrate (Na Bu 10mM for 24hr) as indicated. Cells were also exposed (+) or not (-) to 25Gy of γ-irradiation and allowed to recover for the indicated durations of time. The appearance of acetylated-histone H3 is an artifact as indicated by the pre-immune (PI) negative control. Acetylated-histone H3 appeared only in lanes where cells were treated with both sodium butyrate and γ-irradiation. The experiment also failed to show an interaction between menin and dTip60 in response to γ-irradiation with sodium butyrate treatment.

3.2 Characterization of the Menin/dTip60 Interaction in *Drosophila*

In order to examine the role of the menin and dTip60 interaction in flies, null mutants were obtained for menin (Papaconstantinou et al., 2005) and UAS-dTip60 RNAi flies were obtained for dTip60 (Zhu et al., 2007). The menin flies were previously made and characterized in our lab, while the dTip60 RNAi flies were obtained from Dr. Felice Elefant's lab in Drexel University in Philadelphia, PA. The dTip60 RNAi flies have a pupa lethal phenotype when driven by a ubiquitously expressed GAL4 driver. However, their development is completely normal until the pupal stage, therefore I first examined the expression of dTip60 during development. Figure 8 shows dTip60 expression during development in wild type Oregon R flies. dTip60 levels are abundant throughout embryogenesis up to 1st instar larval stage in Oregon R. A modified form of dTip60 is detectable beginning in late stage embryos (16-18hr after egg laying) that migrates slightly slower than the original dTip60 protein as seen in Figure 8. Levels of dTip60 begin to decrease at 2nd instar larval stage to the lowest detection threshold in 3rd instar larvae.

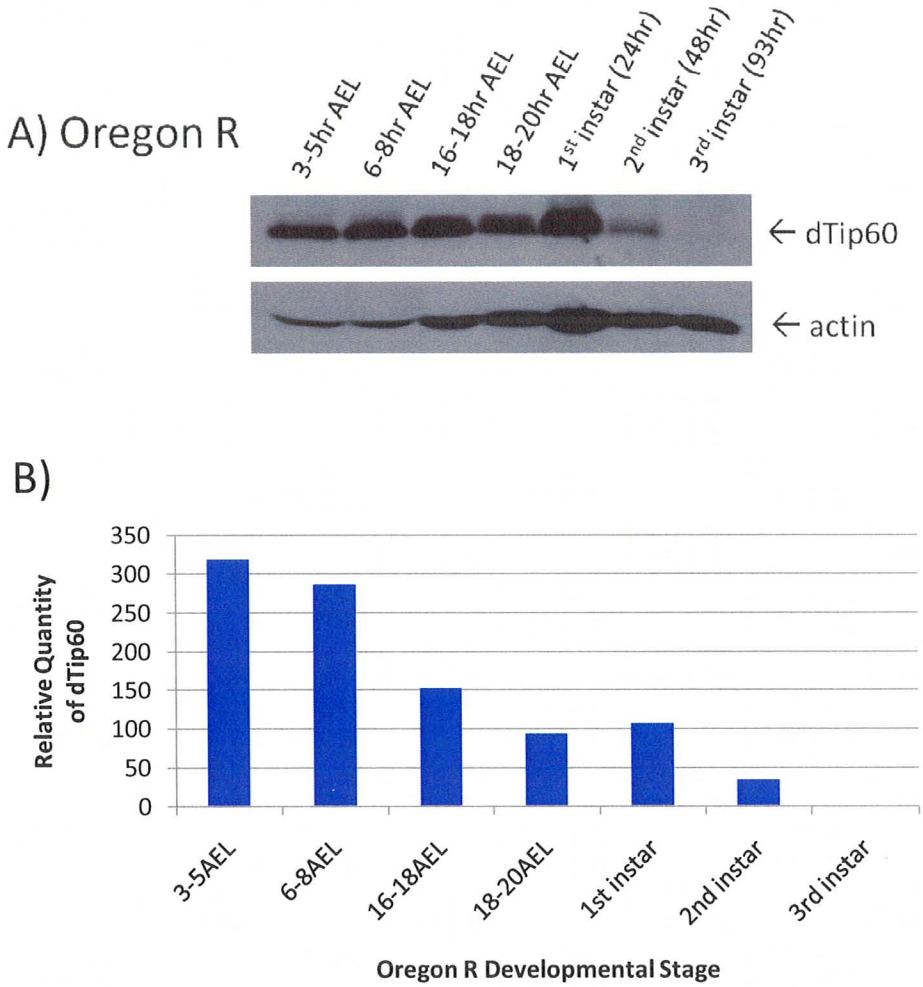

Figure 8: dTip60 is developmentally regulated. Protein samples from Wild type (Oregon R) embryos or larvae were made at the indicated stages of development. (A) 100µg of protein from each lysate was run on SDS-PAGE and visualized by Western blotting analysis. Endogenous levels of dTip60 decrease with development to almost non-detectable levels at 3rd instar larval stage. AEL = After egg laying. (B) Relative Quantity of dTip60 corrected to actin.

Next, the stage of development for use in the stress experiments was determined in the Tip60 RNAi line. Zhu and colleagues determined that dTip60 transcripts were not detectable in 3rd instar larvae of the RNAi lines compared to the control lines (Zhu et al., 2007). Other developmental stages were not examined for their efficiency of dTip60 knockdown in the RNAi line compared to the control in their experiments; therefore the knockdown of dTip60 was visualized using Western blotting analysis. Figure 9 shows the amount of dTip60 protein in the *UAS-dTip60* control line compared to the *UAS-dTip60* RNAi flies, both driven by the ubiquitously expressed *daughterless*-GAL4 (daGAL4) driver. The *daughterless*-GAL4 driver was used because it is ubiquitously expressed throughout development and is required for sex determination and oogenesis early in development. This ensures that the RNAi pathway is activated early in development. Also, daGAL4 was the only homozygous viable driver available in the lab at the time and I wanted to ensure that all progeny would receive one copy of the driver from a simple one step genetic cross.

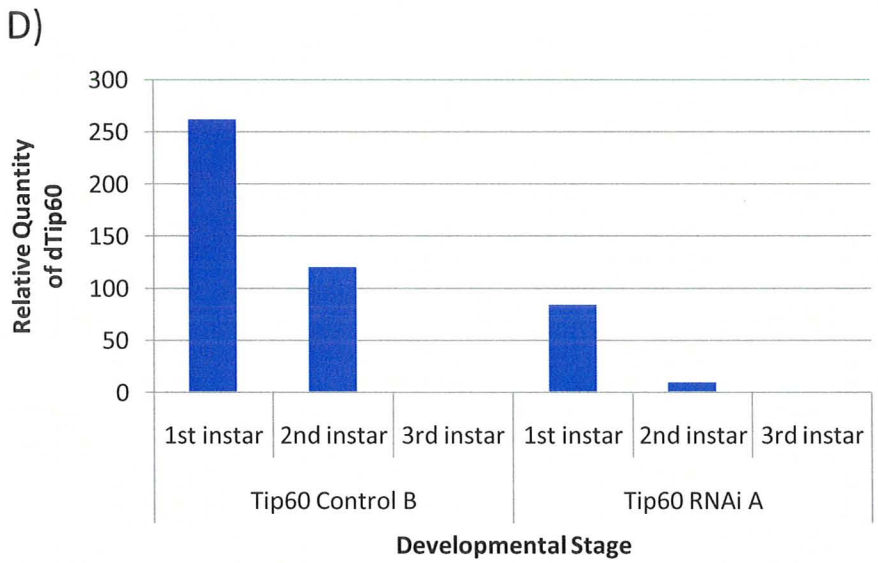

Figure 9: Control of dTip60 using RNAi. A comparison of dTip60 levels at different developmental stages up to 3rd instar larvae between the dTip60 control flies (A), and the dTip60 RNAi flies (B). Endogenous levels of dTip60 follow a decreasing pattern of expression with development. (C) Relative quantity of dTip60 for both lines corrected to actin. (D) Closer look at the quantification of dTip60 levels during larval stages. The levels of dTip60 follow the same pattern of expression in the dTip60 RNAi flies compared to the control, with an observable knockdown of dTip60 in the RNAi flies beginning at the first instar larval stage.

As shown in Figure 9, the expression pattern of *UAS-dTip60* Control B flies is comparable to wild type (Figure 8). The expression of dTip60 differs between the *UAS-dTip60* Control B flies (Figure 9A) and the *UAS-dTip60* RNAi A flies (Figure 9B), beginning in larvae. There is more than a 50% reduction of dTip60 in the *UAS-dTip60* RNAi flies at 1st and 2nd instar larval stages compared to the control (Figure 9D). In addition, there appears to be a 50% reduction of relative dTip60 levels in the *UAS-dTip60* RNAi early embryos (3-5AEL) compared to the control (Figure 9C). Other than the difference in early (3-5AEL) relative dTip60 levels, there is no reduction seen in relative dTip60 of other embryo stages (up to 18-20AEL; Figure 9C). Therefore, this difference may be attributed to uneven loading of proteins (Figure 9A, 9B). Previous groups have shown that dTip60 expression decreases approaching 3rd instar larval stage, until a subsequent increase in pupal stage that is maintained in the adult. The decreasing pattern of dTip60 expression to 3rd instar larval stage is consistent with previous findings. The lethality observed in dTip60 RNAi flies at 25°C suggests that dTip60 has a role in development at this stage, and lack of dTip60 is detrimental to the organism. Differences in the relative quantities of dTip60 are observed beginning at the 1st instar larval stage, as shown in Figure 9C and 9D.

The observation that the *UAS-dTip60* RNAi A flies die during pupal stage suggests a requirement for dTip60 at this stage of development. Although dTip60 levels are reduced in the RNAi line beginning 1st instar larval stage (Figure 9), development until pupal stage is normal. dTip60 may be dispensable for development at larval stages, but may still be required for DNA damage response if these larvae are stressed. A heat shock lethality experiment was performed to determine if a lethal dose of heat shock will cause the *UAS-dTip60* RNAi A flies to die prior to entering pupal stage, and thus have the inability to form pupae (Appendix D). Larvae were given a 1hr dose of heat shock (37°C) at the specified developmental stages and allowed to recover at 25°C. There is no marked difference between the percentage survival to pupal stage from the

heat shock treatments between all three lines: Oregon R, *UAS-dTip60* Control B, and *UAS-dTip60* RNAi A (Appendix D). As expected, survival of the *UAS-dTip60* RNAi A flies to adult stage was drastically reduced, with the exception of a few escapers.

Interestingly, the expression of dTip60 in the *UAS-dTip60* RNAi A line seen in Figure 9B is quite robust throughout embryogenesis despite the presence of RNAi driven by the ubiquitously expressed *daughterless*-GAL4 early in development. This prompted the investigation into the effect of maternal contribution of dTip60 in the embryos. Figure 10 shows the maternal contribution on *UAS-dTip60* RNAi A embryos driven by *daughterless*-GAL4. F1 generation females containing the *UAS-dTip60* RNAi A construct and a daGAL4 driver were crossed to a daGAL4 male. Lanes 4 and 5 of Figure 10 are embryo proteins collected from these F1 females. Lane 4 is a representation of embryo proteins collected at room temperature (21°C). For the proteins in lane 5, flies were temperature shifted up to 25°C for 2 days before embryo collections to observe the possibility of a stronger knockdown. Lanes 1 to 3 are early embryos from Oregon R, daGAL4, and *UAS-dTip60* RNAi A (no driver), serving as controls. Controls (1 to 3) have abundant amounts of dTip60 in early embryos as expected. As shown in Figure 10, embryos collected from females containing the *UAS-dTip60* RNAi A construct and daGAL4 have a marked decrease in dTip60 expression.

This interesting result in Figure 10 demonstrates that it is possible to significantly remove a large portion of maternally contributed dTip60. Knowing this, the embryos can be used as a powerful tool in future experiments. The effect of early dTip60 knockdown in embryos during stress responses can be examined.

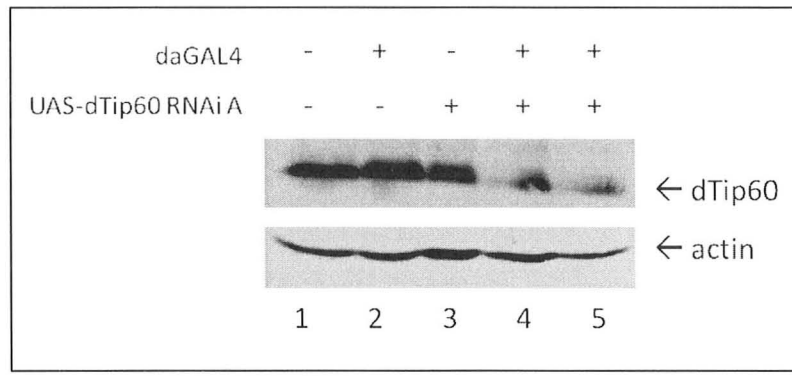

Figure 10: Maternal Contribution of dTip60. The maternal contribution of dTip60 was examined in the *UAS-dTip60* RNAi A line compared to controls: Oregon R (Lane1), daGAL4 (Lane 2), and *UAS-dTip60* RNAi A (no driver, Lane 3) using Western blotting analysis. All proteins were collected from early embryos (3-5AEL). *UAS-dTip60* RNAi A virgin females were crossed to daGAL4 males. Their F1 progeny should have one copy each of UAS and GAL4. Virgin females from the F1 progeny containing the UAS and GAL4 constructs were mated to daGAL4 males. Early embryo proteins were collected from these F1 females at room temperature (21°C, Lane 4), or 25°C (lane 5).

Chapter Four: DISCUSSION

Ying Wu revealed that menin is able to associate as part of the same complex as dTip60 in S2 cells. This prompted the investigation into the characterization of the menin and dTip60 interaction. The menin and dTip60 interaction may play a role in different cellular processes such as DNA damage repair and the response to stress by heat shock.

4.1 Menin Interacts in a Complex with dTip60 in S2 cells

Previously, menin was shown to interact with dTip60 in S2 cells overexpressing menin, but not with endogenous menin (Appendix A). Furthermore, these results were fleeting, as the interaction was difficult to repeat. This is suggestive of the dynamic nature of the interaction. Initial attempts to optimize the co-immunoprecipitation protocol did not allow for an easier detection of the menin/dTip60 interaction. Some of the variables manipulated were antibody incubation times and temperatures, as well as buffers used (Appendix B). In Figure 4, the interaction between menin and dTip60 was detected after harvesting a large amount of protein (150mm plate), and a prior enrichment of nuclear proteins for co-immunoprecipitation. This data suggests that menin is part of a complex with dTip60 in conditions where S2 cells are actively dividing in the absence stress.

4.1.1 Menin Interacts in a Complex with dTip60 after Heat Shock

The role of menin in stress responses have been observed in flies (Busygina et al., 2004; Papaconstantinou et al., 2005), but the mechanism by which this occurs remains to be elucidated. In humans, the recruitment of the methyltransferase complex containing MLL is dependent on menin (Yokoyama et al., 2004). In flies, menin also associates with the TAC1 complex containing the MLL homolog Trx (Petruk et al., 2001).

Additionally, the TAC1 complex is required for modulating *hsp70* expression during heat shock (Smith et al., 2004). Although Tip60 has been well characterized for its role in DNA damage repair (Ikura et al., 2000), Tip60 may also have a role in the stress response by acting together with menin. In Figure 5, and interaction between menin and dTip60 is seen after 1hr heat shock at 37°C in S2 cells.

Heat shock proteins such as HSP70 are induced within minutes in response to heat shock (Morimoto, 1998). In order for the menin and dTip60 interaction to have a role for the heat shock response, they must be poised to interact within minutes of exposure; an observation not seen in Figure 5. Also, the severity of heat shock (from 37°C to 39°C) does not appear to induce a stronger or quicker interaction between menin and dTip60 (data not shown for 39°C). Considering that the interaction occurs at such a late stage of heat shock (1hr), the interaction between menin and dTip60 may not play a role in the expression of heat shock proteins in the heat shock response. On the other hand, heat shock has been linked to other changes within the cell. Recent evidence links heat shock to the formation of γ-H2AX foci in human lung carcinoma (H1299) cells deficient of p53 (Takahashi et al., 2004), and MEF cells (Hunt et al., 2007). Foci formation in response to heat shock is ATM dependent but HSP70 independent (Hunt et al., 2007). Generally, acute heat shock has not been demonstrated to induce DNA double-strand breaks (Hunt et al., 2007; Wong et al., 1995), although this is a much debated topic. Exposure to heat may not induce DNA damage directly, but rather indirectly via protein damage (Takahashi et al., 2004). Levels of p53 are also stabilized in response to heat shock, and the phosphorylation of p53 is ATM dependent (Wang and Chen, 2003). While ATM kinase activity is known to be enhanced following autophosphorylation during DNA double-strand break repair, and the prior acetylation of ATM by Tip60 is required for this process, it is possible that Tip60 may also be required to activate kinase activity of ATM following heat shock. Menin's interaction with dTip60 may modulate chromatin remodeling activities in response to heat induced

chromosomal alterations at this late stage, although this theory remains to be tested. A schematic representation of this speculative model is shown later in Figure 11.

4.1.2 Menin does not interact with dTip60 after γ-irradiation

DNA damage repair is considered a stress response, and is activated by various forms of stress including oxidative stress, UV, or γ-irradiation. Menin is involved in stress responses, and the interaction with dTip60 was examined in response DNA damage caused by γ-irradiation. In Figure 6, the exposure to 25Gy of γ-irradiation at up to 45 minutes of recovery failed to generate a detectable interaction. DNA repair is known to be active hours after damage, and the interaction between menin and dTip60 may be transient, or occur after 45 minutes of recovery, but this has yet to be explored.

Recently, additional information showed that Tip60 is negatively regulated by the NAD^+-dependent deacetylase SIRT1 (Wang and Chen, 2010). Tip60 can exist as a dimer, and autoacetylate to dissociate from the dimer form and impart acetyltransferase activity to substrates. The autoacetylation of Tip60 is augmented by UV radiation, suggesting the Tip60 species involved in DNA damage repair requires the acetylated form. Inhibition of SIRT1 by sodium butyrate in S2 cells will stabilize the acetylated form of dTip60 within the cell. This will allow Tip60 to actively associate with other proteins involved in the process of DNA repair. Unfortunately, the treatment of S2 cells with sodium butyrate did not aid in revealing an interaction between menin and dTip60 (Figure 7). The signals in Figure 7 have a poor resolution and are the result of protein aggregation which generates background. Therefore an interaction was difficult to see in this case. The aggregating proteins are non-specific as the smears are also seen in the pre-immune. Attempts at detecting the interaction were limited to 15 minutes or 3 hours of recovery in this experiment. Considering that the interaction may be regulated, menin may only come together with dTip60 in a complex for a short period of time following damage. Closer studies of the kinetics of interaction have yet to be

completed. Interestingly, the initial co-immunoprecipitation involving the treatment of S2 cells with sodium butyrate and γ-irradiation revealed an unexpected protein of about 17kDa in size (Figure 7). Further investigation into this potential interacting protein with menin revealed a non-specific interaction with acetylated-histone H3, as shown in Figure 7. This was determined to be an artifact of the protocol. Histones are generally evicted from sites of DNA damage to allow for the DNA repair machinery to be recruited and repair the lesions. Histones are also acetylated prior to eviction, to signal the site of damage. This fact in combination with the presence of sodium butyrate, a histone deacetylase inhibitor, allowed for the eviction of a large portion of acetylated histones from chromatin. Histones have large hydrophobic domains and may be insoluble. Rather than a true interaction by binding in a complex to the antibody used for co-immunoprecipitation, the histones, largely insoluble, could have been trapped with the Protein-G sepharose beads, and were therefore visualized in these lanes (Figure 7). The main evidence for this artifact is the presence of a strong acetyl-histone H3 band in the pre-immune negative control, seen in Figure 7. Additionally, the histones were in such abundance that they were visually apparent on the nitrocellulose membrane after Ponceau stain, which is never observed for proteins pulled down in an immunoprecipitation.

4.2 dTip60 Expression during Development in Oregon R

In the second part of my project, the role of menin and dTip60 was examined in flies. The development of flies from a previously characterized *UAS-dTip60* RNAi fly line is normal until pupal stage, where lethality occurs. The loss of dTip60 is detrimental to the flies; therefore dTip60 is required for normal development. First, dTip60 expression was examined wild type Oregon R during development until 3rd instar larval stage (Figure 8) by Western blotting analysis. A large amount of dTip60 is expressed in early embryos, as shown in Figure 8, and decreases in larvae consistent with previous reports (Zhu et al., 2007). A second immunoreactive protein species of slower mobility was also

detected during development. The appearance of the slower migrating dTip60 begins in late embryos (16-18AEL) and is suggestive of a modified form of dTip60 required for development. The modified dTip60 may be acetylated or phosphorylated, as these two post-translational modifications are known to occur in dTip60. The decrease in both forms of dTip60 to undetectable levels in 3rd instar larvae (Figure 8) suggests that dTip60 is downregulated at this developmental stage. Although dTip60 is undetectable in 3rd instar larvae in the Western blot, small amounts of protein cannot be visualized in this manner. Therefore, there is a possibility that dTip60 is still expressed in a small subset of cells, which could be detected by immunohistochemistry at this stage.

4.2.1 Control of dTip60 by RNAi

Next, the developmental stage where Tip60 RNAi flies successfully down-regulate dTip60 was determined. Proteins were collected at different stages of development up to 3rd instar larval stage, and analyzed by Western blotting analysis. The dTip60 pattern of expression of the Tip60 Control B flies (Figure 9A, 9C) is comparable to wild type (Figure 8). Surprisingly for the Tip60 RNAi proteins, Figure 9B shows robust expression of dTip60 in embryos despite the presence of dsRNA that should activate the RNAi machinery. *daughterless*-GAL4 is expressed early in development and is required before gastrulation, therefore the *UAS-dTip60* RNAi construct should be expressed in early embryogenesis. One possibility is that the proteins in Figure 9B are maternally contributed. For this reason, the down-regulation may not be apparent until the 1st instar larval stage compared to the Tip60 Control B line. Maternal contribution will be discussed in section 4.2.3.

The comparison of relative dTip60 quantities shows that there is a reduction of 50% in Tip60 RNAi early embryos (3-5AEL) relative to the Tip60 Control B early embryos (Figure 9C). This reduction is not consistent with the relative dTip60 levels in other stages of embryogenesis. Therefore, the initial reduction of dTip60 in the Tip60 RNAi

embryos compared to the control may be attributed to uneven loading. This affects the calculation for relative quantities of dTip60 corrected to actin.

Additionally, a reduction of dTip60 levels of over 50% was seen in the Tip60 RNAi larvae compared to the control larvae (Figure 9D). The down-regulation of dTip60 is consistent in 1st and 2nd instar larvae (Figure 9D), therefore these stages should be used in future stress experiments to characterize dTip60 function, as this is the point where down-regulation becomes detectable. Interestingly, despite the knock down of dTip60 in the *UAS-dTip60* RNAi A line (Figure 9B) in larvae, their development is normal until pupal stage. Consistent with previous findings, the Tip60 RNAi flies do not eclose, with the exception of a few escapers. This finding demonstrates the need for functional dTip60 at pupal stage. The amount of transcript for dTip60 generally increases at pupal stage during normal development (Zhu et al., 2007). While dTip60 may not be required for development in larval stages, it may be required for a proper stress response at this time. These larvae (Tip60 RNAi A) may be sensitive to stress compared to Tip60 Control B larvae, as discussed in the heat shock lethality experiment.

4.2.2 Heat Shock Lethality

Tip60 RNAi A larvae driven by daGAL4 develop normally until pupal stage. While a reduction of dTip60 protein levels is seen at larval stages, dTip60 may not be required for development at this stage. To determine if dTip60 is required for the stress response, the survival of Tip60 RNAi A flies was investigated and compared to that of Tip60 Control B and Oregon R flies in response to heat shock (Appendix D). If dTip60 is required for the response to heat stress, or DNA damage in response to heat stress, there should be a decrease in the percentage of survival to pupal stage in the Tip60 RNAi A flies compared to controls. The developmental stages shown in Appendix D indicate the stage that larvae were subjected to heat shock (1hr at 37°C). When larvae were not subjected to heat shock (no HS), the percentage survival was similar in all

three lines (Tip60 RNAi A, Tip60 Control B, Oregon R) at a survival rate of over 90% (Appendix D). At the 2nd instar stage, exposure to heat shock did not affect viability of Oregon R, with 100% survival. Survival to the pupal stage of 2nd instar larvae subjected to heat shock dropped to 72% and 66% for the Tip60 Control B and Tip60 RNAi A lines, respectively. Lastly, the survival to pupal stage after subjecting 3rd instar larvae to heat shock was 86%, 86%, and 84% for Oregon R, Tip60 Control B, and Tip60 RNAi A respectively. According to the data, there appears to be greater lethality in the Tip60 RNAi A line when exposed earlier to heat shock at 2nd instar (66%, Appendix D), although statistical significance cannot be confirmed between rates of survival since the experiment was only performed once. The rate of survival to pupal stage of the Tip60 RNAi A flies is similar to controls (Appendix D), therefore, dTip60 does not appear to play a role in the stress response at this developmental stage.

4.2.3 Maternal Contribution of dTip60

The expression of dTip60 was surprisingly strong in the Tip60 RNAi A flies throughout embryogenesis in Figure 9B. This prompted the investigation into the effect of maternal contribution in these flies. The Tip60 RNAi A flies behave like temperature sensitive mutants, as the F1 progeny do not survive past pupal stage when driven at 25°C, but survive when driven at 22°C. This phenomenon is seen regardless of the driver being used and has been observed by two other groups (Zhu et al., 2007; Schirling et al., 2010). Perhaps at 22°C, the down-regulation is less efficient due to a lower expression of GAL4 and enough functional dTip60 remains to ensure viability. With this knowledge, it is possible to cross the Tip60 RNAi A virgins to daGAL4 males at room temperature (22°C), and obtain F1 progeny harbouring both the *UAS-Tip60* RNAi A and daGAL4 constructs. The protein from embryos of these females should have a decreased amount of dTip60. Figure 10 shows a decrease in the amount of dTip60 in embryos (3-5AEL) harvested from females harbouring the *UAS-Tip60* RNAi A and daGAL4 constructs at 22°C as well as 25°C. Downregulation is therefore occurring in these females

regardless of the temperature at which they are collected. This leads to the decreased protein seen in these embryos (lanes 4 and 5, Figure 10). Considering that the maternal effect can be decreased by obtaining F1 generation adults (containing *UAS-Tip60* RNAi A and daGAL4) with the ability to survive at 22°C, this may be a very powerful tool for use in future experiments.

4.3 Future Directions

Although the interaction between menin and dTip60 has been identified, many questions remain to be answered, and further experiments must be conducted to characterize the interaction.

Menin was shown to interact in a complex with dTip60, after 1hr of heat shock at 37°C (Figure 5). Their association is unlikely to reflect a role in the heat shock response, since the heat shock response occurs minutes after exposure. The cell undergoes many changes during times of stress. It is likely that prolonged heat stress causes the misfolding of many proteins that may indirectly affect chromatin structure. Heat shock leads to the formation of γ-H2AX foci in cells (Hunt et al., 2007), but these foci are different from those seen after ionizing radiation. Only a subset of key components related to DNA damage repair (MDC1, but not 53BP1 and SMC1) co-localize with γ-H2AX foci in response to heat shock, and the formation of these foci are ATM dependent (Hunt et al., 2007). Other researchers have found that acute heat shock (10 to 80min at 45.5°C) induces very few DNA double-strand breaks in CHO cells, which optimally grow at 37°C (Wong et al., 1995). From this, it is deduced that the formation of γ-H2AX foci in response to heat shock is not primarily due to DNA damage. This indicates that a different pathway is required to signal the presence of altered chromatin structure due to heat stress. The menin and dTip60 interaction may have a role in this pathway. In order to examine this, immunofluorescence can be performed on S2 cells to examine the co-localization of menin and dTip60 with γ-H2AX foci after heat shock exposure.

The interaction of menin with dTip60 appears after 1hr of heat shock (Figure 5), but experiments have not been performed to examine the persistence of this interaction after prolonged heat exposure. To examine the persistence of the interaction of menin and dTip60 after heat shock, S2 cells can be heat shocked for up to 2 hours. Co-immunoprecipitation experiments can be performed using anti-menin antibody, and the menin with dTip60 interaction can be visualized using Western blotting analysis.

Menin did not interact with dTip60 in response to γ-irradiation (Figure 6). Regardless, the interaction was not examined after longer recovery periods. Therefore, co-immunoprecipitation should be carried out after treating S2 cells with γ-irradiation (25Gy) and longer recovery times to determine whether an interaction appears at a later time, similar to what is seen after heat shock.

For the second part of my project, Tip60 RNAi A flies did not display increased lethality when subjected to heat shock (Appendix D). The heat shock lethality experiment should be repeated at least two more times to determine whether the modest increase in lethality (Tip60 RNAi A 2^{nd} instar heat shock) is statistically significant. Also, Tip60 has been well characterized for its role in DNA damage repair in response to UV radiation or DNA double strand breaks via γ-irradiation. For this reason, lethality should be examined in the Tip60 RNAi A flies compared to controls (Tip60 Control B, Oregon R) after exposure of larvae to γ-irradiation.

In Figure 10, the maternal contribution of dTip60 was examined. A decrease in dTip60 expression was seen in embryos (F2 progeny) from the F1 generation females harbouring the *UAS-dTip60* RNAi A and daGAL4 constructs. This reveals that dTip60 is maternally contributed. Experiments can be conducted to shed light on the effect of significant downregulation of dTip60 in early embryogenesis. Embryos from these females can be used for early stress response experiments caused by heat shock or γ-

irradiation. Cell death due to stress can be visualized by staining embryos with acridine orange. Also, the fate of these embryos (F2 progeny) past early embryogenesis has not been examined. It is possible that they may die early in development compared to the F1 progeny due to lower levels of dTip60 in early embryogenesis. Future experiments designed to examine this process will require an additional selection process for the F2 progeny; flies of the F2 generation will not all inherit the *UAS-dTip60* RNAi A and daGAL4 constructs together using the current method. After successfully decreasing the maternal contribution and obtaining F2 progeny with the *UAS-dTip60* RNAi A and daGAL4 constructs, development of these flies can be observed at 25°C. Additionally, stress experiments can be performed (heat shock lethality, γ-irradiation lethality) and quantified compared to the *UAS-dTip60* Control B flies. This data should be compared to the heat shock lethality data obtained previously from flies of the F1 generation (Appendix D).

Importantly, to genetically confirm an interaction between menin and dTip60, stress experiments must also be performed in $Mnn1^{-/-}$ flies ($Mnn1^{e30}$ and $Mnn1^{e173}$). Phenotypes from these stress experiments should be compared and the $Mnn1^{-/-}$ phenotype should be similar to what is observed in the Tip60 RNAi lines. Previously, heat shock lethality in $Mnn1^{-/-}$ flies was only performed by heat shocking embryos. The heat shock lethality experiment should be performed using the $Mnn1^{-/-}$ flies at the same developmental stage (2nd or 3rd instar larvae) as the Tip60 RNAi A flies and quantified for comparison. In addition, the proposed γ-irradiation lethality experiment for the *UAS-dTip60* RNAi flies should also be performed with the $Mnn1^{-/-}$ flies and the survival to pupal stage should be compared. The survival to adult stage cannot be compared in this proposed experiment since *UAS-dTip60* RNAi flies do not survive past pupal stage.

Interestingly in mouse models, Tip60 is recruited to the *Hsp70* promoter following heat acclimation at 34°C, while their optimal temperature is 24°C (Tetievsky

and Horowitz, 2010). Heat acclimated mice show cytoprotective memory, as significantly higher amounts of Tip60 and HSF1 are recruited to the *Hsp70* promoter compared to normothermic conditions (Tetievsky and Horowitz, 2010). Tip60 may also be recruited to the *hsp70* promoter in flies following heat shock. Chromatin immunoprecipitation experiments must be conducted to investigate the recruitment of dTip60 to the *hsp70* promoter and its role in heat shock/heat acclimation.

Additionally, it may be interesting to see if menin and dTip60 interacts in a Trx mutant background. In *C. elegans*, the MLL complex has been shown to cooperate with the Tip60 complex to attenuate LET-60 RAS signaling through positively regulating a common downstream effector: apical junction molecule-1 (AJM-1) (Fisher et al., 2010). This is direct evidence that the two chromatin modifying complexes (MLL and Tip60) are able to converge in the same pathway. In flies, perhaps the complex containing menin and dTip60 is formed after the tri-methylated H3K4 epigenetic mark is generated, which is dependent on the Trx methyltransferase. A speculative schematic model of this process is given in Figure 11. The interaction can be examined with co-immunoprecipitation experiments in Trx mutant embryos, compared to Oregon R. Also, Tip60 interacts with chromatin through its chromodomain which recognizes methylated histone marks. Tip60 can potentially interact with menin at this site. Therefore, the recruitment of dTip60 and menin to H3K4 tri-methylated histones may be examined through chromatin immunoprecipitation experiments in Trx mutant embryos and Oregon R embryos.

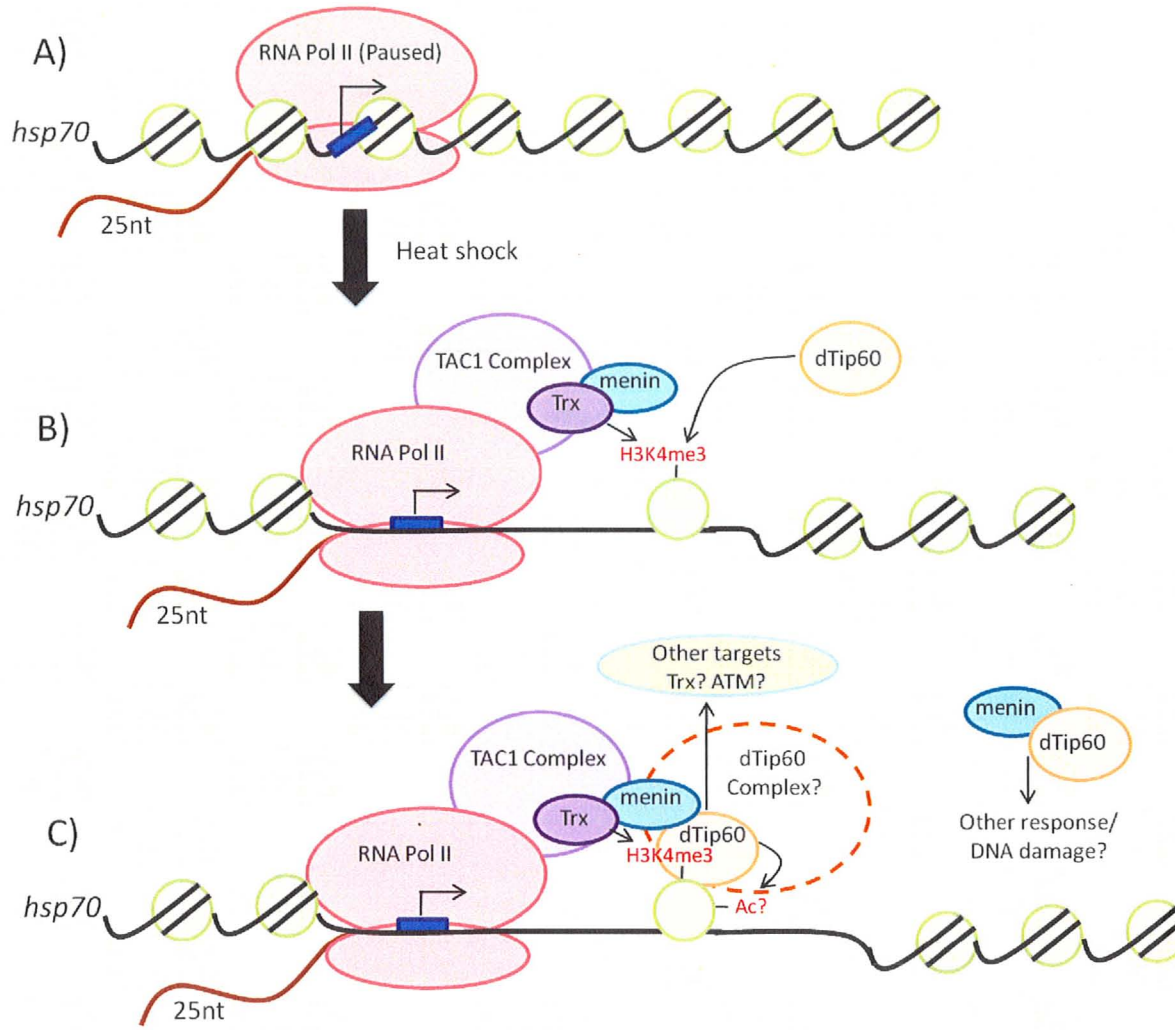

Figure 11: A schematic model of menin interacting with chromatin remodeling complexes at the *hsp70* promoter. (A) RNA polymerase II is engaged and paused at the transcriptional start site after synthesizing a short transcript of about 25nt. (B) After heat shock, the TAC1 complex is recruited to the *hsp70* promoter. Trx methylates histone H3K4 allowing for recruitment of dTip60. (C) dTip60 is recruited to chromatin and interacts with tri-methylated H3K4 through its chromodomain. dTip60 subsequently acetylates histones (H2A, H3, or H4) to open chromatin. Also, menin is proposed to function as a scaffolding protein by associating with Trx and dTip60. In addition, dTip60 may also function to acetylate other targets such as ATM, or participate in another response related to DNA damage together with menin.

4.4 Conclusions

There is increasing evidence on the convergence of pathways leading to the cooperative association of different chromatin modifying complexes, as shown by Fischer and colleagues (2010). Menin and Tip60 are both involved in a vast number of cellular processes and may also potentially interact in various cellular contexts. Futhermore, menin and Tip60 have common interacting partners. It was recently shown that Tip60 interacts with FANCD2 to mediate apoptosis in transformed human fibroblasts (Hejna et al., 2010). FANCD2 is also a menin interacting protein as mentioned earlier with a role in DNA repair from the BRCA1 pathway (Jin et al., 2003). From my data, I have shown that Tip60 can be part of the same complex as menin under normal replicating conditions as well as conditions of heat shock. Additional work using *D. melanogaster* mutants will allow for further characterization and functional significance of this interaction.

5.0 References

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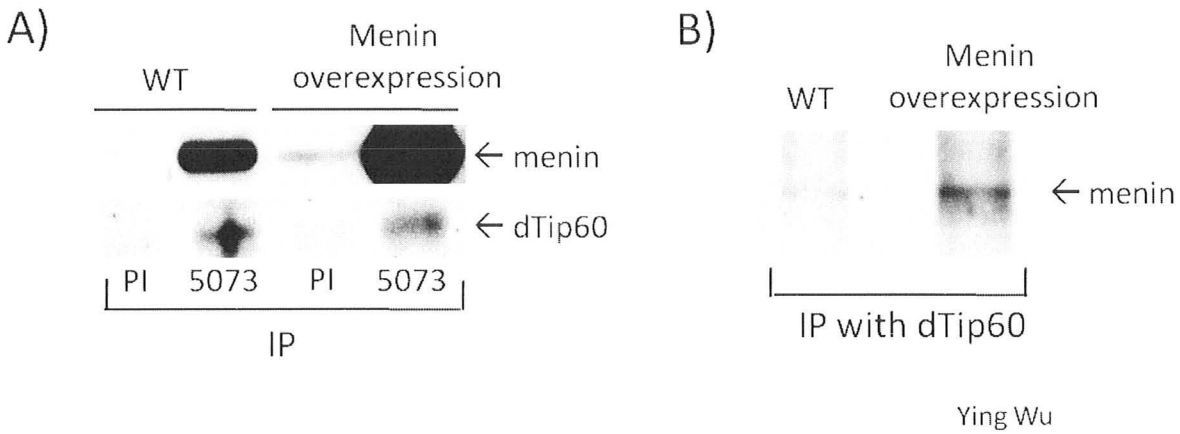
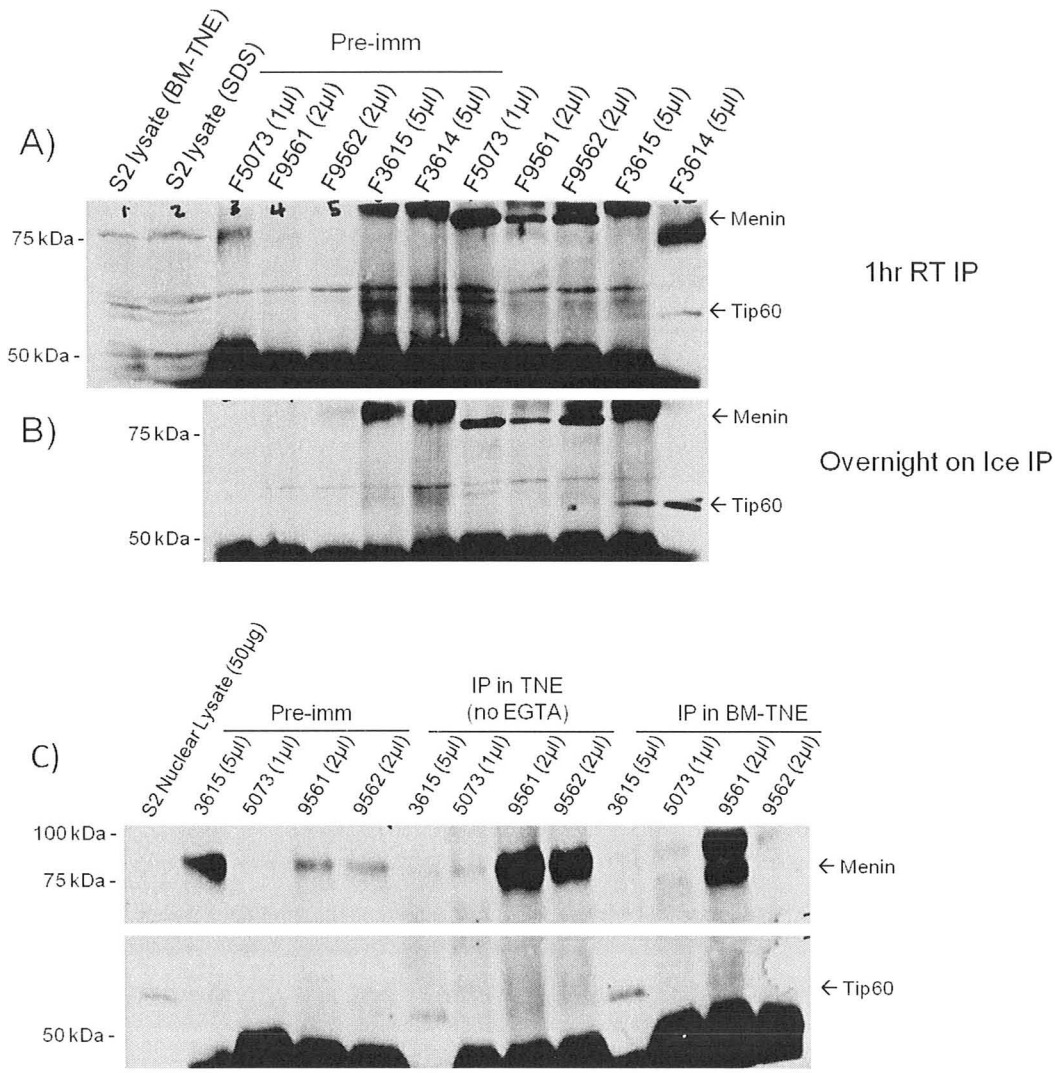
Appendix A



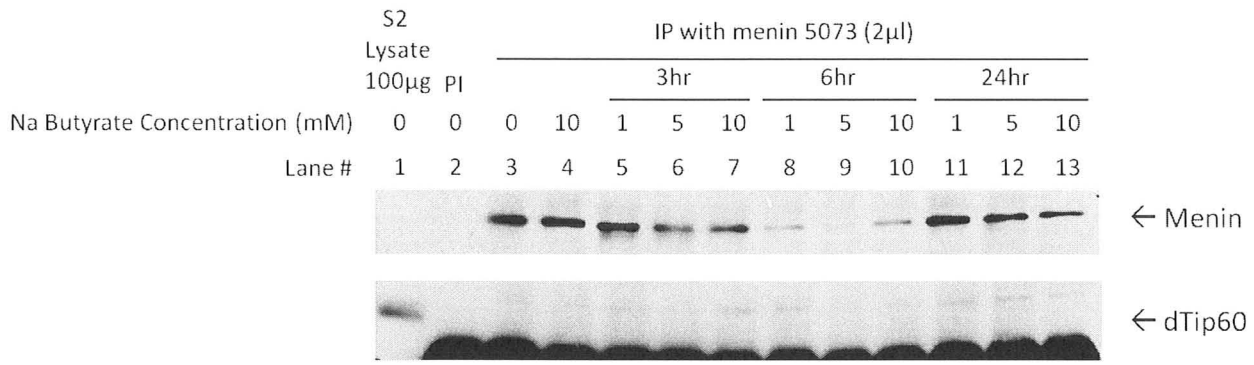
Menin associates in a complex with dTip60. S2 cells (wild type, WT) or a stable S2 cell line overexpressing menin were used to examine an interaction between menin and dTip60 in co-immunoprecipitation experiments. (A) Anti-menin antibody (5073) showed dTip60 present in a complex with menin in the WT S2 cell line as well as the menin overexpression cell line. (B) Reverse co-immunoprecipitation using anti-dTip60 antibody (Tom Kusch) revealed menin present in a complex with dTip60 in WT S2 cells and menin overexpressing S2 cells. PI = pre-immune. IP = immunoprecipitation.

Appendix B



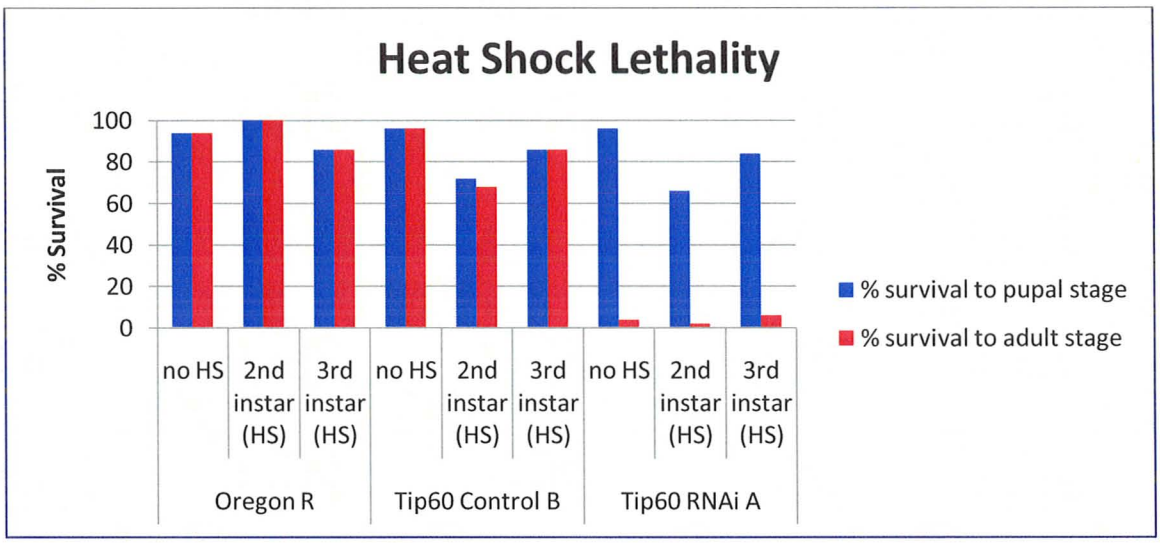
Optimizing the co-immunoprecipitation protocol. Antibody incubation times and temperatures were modified to optimize the co-immunoprecipitation protocol. (A) Immunoprecipitation experiments were carried out with anti-menin (5073, 9561, 9562), or anti-dTip60 (3615, 3614) antibodies for 1hr at room temperature (RT). (B) Same experiment as (A), except with an overnight incubation on ice. Other incubation conditions (3hr on ice, and 6hr on ice) were also conducted (not shown) and results were similar to the overnight on ice incubation. (C) S2 nuclei were isolated using nuclear extraction (see Methods), and proteins were prepared using different lysis buffers prior to immunoprecipitation with anti-menin and anti-dTip60 antibodies.

Appendix C



Sodium butyrate (Na Butryate) dosage conditions on S2 cells. Different concentrations of Na butyrate (1mM, 5mM, and 10mM) were added to growing S2 cells for differing amounts of time (3hr, 6hr, or 24hr) prior to immunoprecipitation with anti-menin antibody (5073), with the exception of lanes 1-4. In lane 4, the S2 cell proteins were prepared using TNE lysis buffer with the addition of 10mM Na butyrate prior to immunoprecipitation using anti-menin 5073 antibody. IP = immunoprecipitation. PI = pre-immune.

Appendix D



Heat Shock Lethality. Wild type (Oregon R), *UAS-dTip60* Control B, and *UAS-dTip60* RNAi A flies were subjected to lethal doses of heat shock (1hr at 37°C) at the indicated stages of development and allowed to recover at 25°C. Pupae and adults were counted to determine the percentage of survival between the three lines. The *UAS-dTip60* Control B and *UAS-dTip60* RNAi A lines were driven using daGAL4. Survival of the *UAS-dTip60* RNAi A line to pupal stage is comparable to controls. The majority of the *UAS-dTip60* RNAi A flies did not survive to adulthood, as expected, with the exception of a few escapers. For all samples, n=50.