Tip60 and APP genetically interact to promote apoptosis-driven neurodegeneration

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Dedications

Dedicated to my loving parents who have supported me every step of the way.

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Abstract

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Chromatin packaging in the nucleus of eukaryotic cells is a dynamic process controlled by specific post-translational modifications of histone proteins. One such modification, acetylation, is catalyzed by histone acetyltransferase (HAT) enzymes, and serves to regulate chromatin condensation that promotes gene control. The HAT Tip60 plays a central role in developmental gene control, yet the specific cellular pathways that are regulated exclusively by the epigenetic based HAT activity of Tip60 remain to be identified. We have developed a system in transgenic *Drosophila* that allows for targeted and inducible production of dominant negative HAT defective Dmel\TIP60 in specific tissues and developmental stages. Such flies are a powerful experimental tool to exclusively explore the epigenetic dependency of cellular processes involving Tip60. Ubiquitous expression of dominant negative Tip60 results in lethality that is rescued by additional wild-type Dmel\TIP60, indicating that Tip60 HAT activity is essential for multicellular development, and specifically for nervous system function. We have exploited this system to identify novel gene targets that are controlled by Tip60 HAT activity using microarray analysis. Our results show that Tip60 HAT activity regulates genes involved in many cellular pathways previously unlinked to Tip60, and highlight a tissue-specific enrichment of genes involved in neuronal development. To further assess Tip60 HAT activity in neuronal development with a focus on disease, we have generated fly lines that produce varying levels of both Tip60 HAT activity and the Alzheimer's

Disease related amyloid precursor protein (APP). Ubiquitous and neuronal targeted expression of these constructs reveals a genetic interaction between Tip60 and APP in neuronal development and specifically in the regulation of apoptosis in the fly brain. This research should shed light on epigenetic based mechanisms underlying neuronal gene misregulation and neurodegeneration in human neurological disorders.

Chapter 1: Background and Significance

Epigenetics and Chromatin Packaging

Each of the cells composing a eukaryotic organism contain the same genomic contents, however individual cell types are able to maintain their distinction by expressing a tissue-specific subset of these genes. This precise regulation program can be maintained through successive cellular generations. Further, gene expression patterns are known to change both during development and in response to signaling pathways, indicating that these gene regulation programs are highly dynamic. This heritable yet dynamic mechanism of gene expression is commonly called epigenetics, and is largely based on alterations in the packaging of chromatin through post-translational histone protein modifications. Epigenetic modifications are versatile, spanning from patterns that persist throughout a cell's lifetime and on to the next generation, to marks that change rapidly and are short-lived. Epigenetics plays an intimate role in transcription, replication, and repair (Peterson and Cote 2004; Mellor 2005), implicating the processes governing chromatin regulation in many critical biological processes.

Chromatin Packaging

The expansive eukaryotic genome is precisely packaged within the nucleus through associations with histone proteins forming chromatin. Two copies each of histones H2A, H2B, H3 and H4 form an octomeric core which 147 base pairs of DNA wraps around forming a nucleosome. Nucleosomes form both on newly synthesized DNA strands and

on DNA of recently transcribed genes where they have been removed to allow transcription to proceed, (Boeger, Griesenbeck et al. 2003; Reinke and Horz 2003; Korber, Luckenbach et al. 2004; Shahbazian and Grunstein 2007). This deposition of new histone proteins onto DNA occurs in a very structured order beginning with two H3-H4 heterodimers followed by two H2A-H2B dimers (Worcel, Han et al. 1978; English, Adkins et al. 2006), and is facilitated by acidic histone chaperone proteins that neutralize the positive charge of the histones preventing non-sepcific DNA binding, (Verreault 2000; Loyola and Almouzni 2004). As the basic organizational units of eukaryotic genomes, nucleosomes form in regular intervals along the DNA strand with linker regions 8-100 base pairs long between, where linker histones including H1, H5, and H° associate and aid in packaging, (Robinson and Rhodes 2006; Godde and Ura 2008; Wood, Snijders et al. 2009). Through inter-nucleosomal interactions, the chromatin spirals into the highly compact 30nm fiber which is the form chromatin is commonly found in within the nucleus. Epigenetic mechanisms including histone modifying enzymes, chromatin remodeling complexes, and DNA methylation aid in the compaction and organization of distinct chromatin domains (Jenuwein and Allis 2001; Goll and Bestor 2005), which can affect the processes of transcription, recombination, and DNA repair, (Grewal and Jia 2007).

The degree of chromatin compaction is intimately linked to the rate of transcriptional activity of a gene or genomic region. Differential compaction of chromatin in the nucleus was first observed in 1928 (Heitz 1928) and was later attributed to loosely packaged chromatin regions termed euchromatin, and tightly condensed regions of chromatin termed heterochromatin, (Huisinga, Brower-Toland et al. 2006; Berger 2007).

The loose packaging of euchromatic regions promotes transcriptional activation because target promoters are more accessible to transcriptional machinery (Grunstein, Hecht et al. 1995; Yasuhara and Wakimoto 2008), however the majority of chromatin is packaged into heterochromatic regions where gene expression is suppressed largely because the tight packaging prevents transcriptional machinery from accessing target promoters, (Grewal and Jia 2007; Reddy and Jia 2008; Johnson, Li et al. 2009). The degree of chromatin compaction is regulated by post-translational modifications of the histone N-terminal tails. The N-terminal tails of the histone proteins extend out from the nucleosomal core and contain highly conserved amino acid residues which can be post-translationally modified by specific enzyme complexes. These residues can be acetylated, methylated, phosphorylated, ubiquitinated, or sumoylated, and often a single histone tail will contain multiple modifications, (Muller, Rieder et al. 2007; Gelato and Fischle 2008; Suganuma and Workman 2008; Scharf, Barth et al. 2009). Altogether there are over 60 different histone modifications that can occur on 31 residues between the four histone proteins as was determined using mass spectroscopy (Zhang, Eugeni et al. 2003), supporting the flexibility of chromatin packaging. These marks can act locally to regulate specific targets or can globally alter gene expression patterns over large chromatin regions, (Vermaak and Wolffe 1998).

Individual post-translational modifications sometimes directly affect gene regulation by altering the chromatin structure, and in other cases indirectly affect gene regulation by serving as marks to recruit other modifying complexes. The resulting combination of modifications results in differentially packaged chromatin regions. In general, specific modifications have been associated with specific transcriptional states. For example,

euchromatic regions have a characteristic histone profile of robust H4 acetylation and histone H3 lysine 4 methylation (Litt, Simpson et al. 2001; Noma, Allis et al. 2001; Cam, Sugiyama et al. 2005), while regions of heterochromatin have a characteristic histone profile of hypoacetylation and histone H3 lysine 9 methylation, (Grunstein 1998; Litt, Simpson et al. 2001; Nakayama, Rice et al. 2001; Noma, Allis et al. 2001; Cam, Sugiyama et al. 2005). This H3K9me mark is recognized by the highly conserved HP1-family proteins (Shimada and Murakami 2010), which aid in chromatin compaction, suggesting one mechanism for how epigenetic marks may alter chromatin states. These variations in characteristic histone profiling between differentially packaged chromatin regions underlie the connection between post-translational histone modifications and the compaction of chromatin.

Post-Translational Histone Modifications (PTMs)

The term epigenetics describes the precise regulation of gene expression through changes in the chromatin landscape. This phenomenon was first described by Conrad Waddington in 1959 in his observations that one genotype can lead to various phenotypes during development (Waddington 1959), suggesting that there was a code other than the genetic DNA code contributing to the expression of genes. The idea of epigenetics has been expanded upon and is commonly thought of as gene expression patterns that are heritable but separate from the to DNA sequence, (Holliday and Pugh 1975; Chambon 1978; Jaenisch and Bird 2003; Borrelli, Nestler et al. 2008). We now know that gene expression changes result from the dynamic activities of chromatin remodeling complexes, and the effects of the marks that they lay on the chromatin, (Cheung, Allis et

al. 2000; Strahl and Allis 2000). In some cases, these marks can be fast-acting and dynamic, reflecting responses to environmental changes and other stimuli, while in other cases they can be long-lasting and underlie the mechanisms in which cellular identity can be passed between generations. Thus, the flexibility provided by epigenetic gene regulation allows for gene expression patterns to be both transient and heritable.

The enzymes responsible for regulating chromatin remodeling have been sorted into three main functional groups, each of which plays a crucial role in gene expression, (Ruthenburg, Allis et al. 2007; Borrelli, Nestler et al. 2008). The most highly characterized of these groups is the "writers" which modify specific histone substrates by adding post-translational chemical marks. These marks include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and can be transient or maintained for long periods of time and even passed on during division, (Kouzarides 2007; Li, Carey et al. 2007; Margueron, Trojer et al. 2005). In some cases these marks directly affect transcription by altering the higher order chromatin structure, while other times they serve as docking sites for "readers". "Readers" are proteins with unique domains that associate with the marks left by the "writers", and histone modification reading activity often results in the recruitment of regulatory complexes for further remodeling, (Marmorstein and Berger 2001). Readers can be divided into two groups: "effectors" which either contain a second domain with catalytic activity to modify the site, or recruit other chromatin modifying enzymes to the site for further modification, and "presenters" which spatially hold the histone tail in the appropriate position for a second enzyme to modify. Lastly, there are "erasers" which remove the post-translational modifications laid by the "writers" from the chromatin. Less directly, an eraser may mask or otherwise

alter a mark without directly removing it. The majority of these histone modifying enzymes act within a larger protein complex which acts in a variety of cellular pathways, and their targets are often specific to the complex with which they are associated. The target residues affected by each enzyme and enzyme complex are highly selective both in terms of histone tail and amino acid residue selected. In addition to histone targets, most post-transcriptional modifying enzymes also modify specific residues on non-histone proteins as well. The following is a summary of only the most highly characterized remodeling enzyme classes.

Writers

Histone Acetyltransferases

Histone acetyltransferases or HATs remove the acetyl group from acetyl coenzyme-A and transfer it to highly conserved lysine residues on histone tails via an ordered sequential bi-bi kinetic mechanism, (Grunstein 1997; Wade, Pruss et al. 1997; Struhl 1998; Carrozza, Utley et al. 2003; Smith and Denu 2009). Histone lysines are generally protonated at biological pH values and are therefore unable to accept the acetyl group, and thus HATs contain an amino acid residue within their HAT domain which functions as a general base for catalysis. This residue is often a highly conserved glutamate that deprotonates the ε-amino group of the target lysine and facilitates the nucleophilic attack of this lysine by the acetyl-CoA substrate, (Tanner, Langer et al. 2000; Tanner, Langer et al. 2000; Roth, Denu et al. 2001). The deposition of the acetyl group to highly conserved lysine residues on histone tails is thought to mask the positive charge of their ε-amino group and weaken the electrostatic interaction between the histone tail and the negatively

charged phosphate backbone of the surrounding DNA, (Allfrey 1966; Kleff, Andrulis et al. 1995; Parthun, Widom et al. 1996; Workman and Kingston 1998; Cheung, Allis et al. 2000). This charge neutralization can also interfere with histone tail interactions between neighboring nucleosomes (Luger, Mader et al. 1997; Luger and Richmond 1998; Tse, Sera et al. 1998; Wolffe and Hayes 1999), and with linker DNA regions (Stefanovsky, Dimitrov et al. 1989; Mutskov, Gerber et al. 1998; Angelov, Vitolo et al. 2001), preventing chromatin packaging into the 30nm fiber and other higher order structures, (Annunziato, Frado et al. 1988; Tse, Sera et al. 1998; Shahbazian and Grunstein 2007). This acetylation induced loosening of higher-order chromatin folding allows transcriptional machinery to access the DNA (Vettese-Dadey, Grant et al. 1996) and is therefore associated with transcriptional activation (Lee, Hayes et al. 1993; Nightingale, Wellinger et al. 1998; Steger, Eberharter et al. 1998; Vignali, Steger et al. 2000; Roth, Denu et al. 2001), and is commonly associated with regions of euchromatin, (Turner and O'Neill 1995).

HAT enzymes can be found both in the cytoplasm where they play roles in nucleosome assembly, and in the nucleus where they activate transcription, (Roth, Denu et al. 2001; Shahbazian and Grunstein 2007). The cytoplasmic or B-type HATs immediately acetylate newly synthesized histones produced during S phase of the cell cycle when DNA is replicating, (Allis, Chicoine et al. 1985; Lucchini and Sogo 1995; Shahbazian and Grunstein 2007). The B-type HAT Hat1 also translocates into the nucleus with H3 and H4, presumably to ensure that they maintain their acetyl marks until deposition, (Ai and Parthun 2004; Poveda, Pamblanco et al. 2004). It is thought that the acetyl marks laid by B-type HATs on newly synthesized histones may determine the

chaperone protein they associate with, and thus their location of deposition in the genome, (Shahbazian and Grunstein 2007). This is supported by research showing that some histone chaperones selectively associate with newly synthesized but not old histone proteins, (Smith and Stillman 1991). Chaperones may also prevent deacetylation of histones until they reach their intended DNA site, at which point the original acetylation marks laid by cytoplasmic HATs are rapidly removed and new acetylation marks are laid by nuclear HATs, (Jackson, Granner et al. 1976; Annunziato and Seale 1983).

The deposition of acetyl marks on nucleosomal nuclear histones is catalyzed by the nuclear or A-Type HATs, and this activity is generally associated with transcriptional regulation, (Hassig and Schreiber 1997). Nuclear HATs activate transcription in two distinct mechanisms. First, HATs can be recruited to specific promoters by transcription factors to acetylate surrounding histones, fascilitating the expression of these targets. Secondly, HATs can acetylate in a global and untargeted manner where they act broadly and continuously across the genome, (Shahbazian and Grunstein 2007). Nuclear HAT activity is thought to either destabilize nucleosomes or allow their removal to promote transcription (Berger 2007; Govind, Zhang et al. 2007), and is therefore commonly associated with transcriptional activation.

Nuclear HATs can be classified into five distinct families, including the GNAT family, MYST family, p300/CBP HATs, general transcription factor HATs, and nuclear hormone-related HATs, (Marmorstein 2001). The GNAT and MYST families are among the best characterized for their roles in histone acetylation. Both families possess a highly conserved HAT domain with acetyltransferase activity and members with distinct

substrate specificities, which is thought to depend on their differing regions surrounding the conserved HAT domain, (Kimura and Horikoshi 1998). Despite these similarities, the GNAT and MYST families differ in conserved domains other than the HAT domain as well as by method of catalysis.

The GNAT family named for Gcn5-related N-acetyltransferase (Neuwald and Landsman 1997) and MYST named for founding members MOZ, Ybf2/Sas3, Sas2, and Tip60 (Borrow, Stanton et al. 1996; Reifsnyder, Lowell et al. 1996) are classified based on conserved domains, although both families display a highly conserved HAT domain which is responsible for the acetyltransferase activity. The GNAT family HATs play roles in transcriptional activation and DNA repair, and are essential for cell growth and development, (Carrozza, Utley et al. 2003). These HATs display up to four conserved domains, including an Arg/Gln-X-X-Gly-X-Gly/Ala sequence within the HAT domain which is involved in the acetyl-CoA substrate interaction, (Dutnall, Tafrov et al. 1998; Wolf, Vassilev et al. 1998). GNATs also have an amino-terminal domain for histone binding (Smith, Belote et al. 1998; Xu, Edmondson et al. 1998), and a carboxy-terminal bromodomain with "reader" activity that binds acetyl-lysines, (Pandey, Muller et al. 2002). In order to transfer the acetyl group from acetyl Co-A to histone lysine residues, GNAT family HATs form a ternary complex consisting of the HAT enzyme, acetyl Co-A substrate, and histone target, (Tanner, Trievel et al. 1999; Lau, Courtney et al. 2000; Tanner, Langer et al. 2000; Tanner, Langer et al. 2000). In the first step, the HAT binds acetyl-CoA inducing a conformational change in the histone binding pocket which in turn allows the HAT to bind to the target histone. In this way, the acetyl group is directly

transferred from acetyl-CoA to the histone without forming an intermediate complex with the HAT itself.

In addition to roles in transcription and DNA repair, the MYST family HATs play roles in a variety of other cellular functions. The highly conserved HAT domain is part of a larger MYST domain which often contains a zinc finger domain which is necessary for HAT function, (Utley and Cote 2003). Additionally, many MYST family members have an amino-terminal chromodomain with "reader" activity for methyl marks, a plant homeodomain (PHD) finger, and a second zinc finger, (Utley and Cote 2003). The catalytic activity of MYST family members acts in a "ping-pong" mechanism (Marmorstein 2001; Marmorstein 2001; Marmorstein and Roth 2001) where the HAT binds to acetyl Co-A and is modified by accepting the acetyl group, generating an acetyl-cysteine protein intermediate. The HAT then binds the target lysine, transferring the acetyl group.

In addition to these two main HAT families are the well characterized HATs p300 and CBP, which are very similar and often times interchangeable, (Arany, Sellers et al. 1994; Arany, Newsome et al. 1995; Shikama, Lee et al. 1999; Lundblad, Kwok et al. 1995; Eckner 1996). These enzymes have in addition to their conserved HAT domains three zinc fingers, two protein interaction domains, and a bromodomain with "reader" activity, (Janknecht and Hunter 1996; Shikama, Lee et al. 1999). Although once thought to be a completely independent HAT family, the HAT domains of p300 and CBP are actually quite similar to those of the GNAT family HATs, (Roth, Denu et al. 2001).

In addition to histone acetylation, most HATs are also capable of acetylating non-histone proteins which generally results in the activation of these substrates, (Glozak, Sengupta et al. 2005; Batta, Das et al. 2007). HATs are usually bound in multisubunit complexes, the other components of which are necessary for the acetyltransferase activity and which determines the substrate specificity. Many HATs associate with a variety of complexes, dicatating the substrate and therefore pathway affected by their catalytic activity.

Despite the traditional role in gene activation, histone acetylation has more recently been found at repressed targets, (Ura, Kurumizaka et al. 1997; Vogelauer, Wu et al. 2000; Deckert and Struhl 2001; Suka, Suka et al. 2001). In these cases the acetyl mark is not thought to play a direct role in the transcriptional repression of the target. Instead it is thought to "prime" the target for later expression, (Shahbazian and Grunstein 2007). However, some HATs have demonstrated a direct role in target silencing. The HATs SAS2 and SAS3 promote silencing of HML in yeast, (Carrozza, Utley et al. 2003; Reifsnyder, Lowell et al. 1996), and SAS2 promotes silencing at telomeres by maintaining the heterochromatin boundary, (Suka, Luo et al. 2002). Additionally, the *Drosophila* HBO1 homologue Chameau enhances HOX gene silencing, (Carrozza, Utley et al. 2003), and the HAT Tip60 also has been implicated in gene silencing through the recruitment of repressive modifiers to target promoters (Xiao, Chung et al. 2003; Gaughan, Logan et al. 2002; Doyon and Cote 2004; Achour, Fuhrmann et al. 2009).

Histone Methyltransferases

Histones can be methylated on either lysine or arginine residues. Arginine residues can be unmethylated, monomethylated, dimethylated symmetrically, or dimethylated asymmetrically (Cloos, Christensen et al. 2008) by protein arginine methyltransferases which transfer a methyl group from S-adenosyl-L-methionine (AdoMet) to the guanidine side chain of histone arginine residues, (Smith and Denu 2009). Lysine residues can be unmethylated or mono-, di-, or trimethylated at the ζ-amine of each of these sites by either SET domain histone lysine methyltransferases (Dillon, Zhang et al. 2005; Martin and Zhang 2005), the size and active domain binding specificity of which determines the degree of methylation, or Dot1/KMT4 histone lysine methyltransferases which specifically methylate core residue Lys-79 of histone H4, (Steger, Lefterova et al. 2008; Smith and Denu 2009). Histone methylation does not affect higher chromatin structure structure (Zhang and Reinberg 2001; Bannister, Schneider et al. 2002; Kouzarides 2002), it affects transcription solely through "effector" recruitment. For this reason while histone acetylation is generally associated with gene activation, histone methylation can be associated with either activation or repression depending on the associated "effector" complex. The level of methylation, as well as the location of the target residue, generates different outcomes on the chromatin state due to the different "reader" complexes that associate with each modification. For example, H3K9 and H3K27 monomethylation and H3K4 mono-, di-, and trimethylation are associated with transcriptional activation, while H3K9 and H3K27 di- and trimethylation are associated with repression, (Cloos, Christensen et al. 2008; Nottke, Colaiacovo et al. 2009).

DNA Methyltransferases

In addition to histone modifications, epigenetic modifications can also be made to DNA. In fact, histone and DNA modifications are tightly linked and recent evidence supports a role for histone methylation as an initiation step towards the more stable suppression induced by DNA methylation, (Fuks 2005). It has been proposed that H3K9 methylation could serve as a mark to recruit "effector" complexes containing DNA methyltransferases, generating CpG methylation marks in the same regions containing the lysine methylation marks, (Tamaru and Selker 2001; Cao and Jacobsen 2002; Jackson, Lindroth et al. 2002). Methyl-CpG-binding domain containing proteins are known to recruit "effector" complexes containing histone deacetylases which can further condense the chromatin as well as prepare H3K9 residues for methylation, (Rea, Eisenhaber et al. 2000).

DNA methylation is catalyzed by three members of the DNA methyltransferase family Dnmt1, Dnmt3a, and Dnmt3b which transfer a methyl group to deoxycytosine bases at the 5 position to form deoxymethylcytosine, (Miranda and Jones 2007). This process is generally associated with gene repression either directly by interfering with transcription factor binding (Watt and Molloy 1988) or indirectly through recruitment of histone deacetylase containing complexes, (Fan and Hutnick 2005). Approximately 70-80% of all CG pairs are methylated, excluding those found in the CG-rich CpG island regions which are generally unmethylated, (Law and Jacobsen 2010). These CG pairs can be demethylated either passively by preventing the methylation of newly synthesized DNA (Wolffe and Hayes 1999), or actively by removing existing methyl marks from the DNA,

(Jost, Siegmann et al. 1999; Jost, Oakeley et al. 2001; Morgan, Dean et al. 2004; Cloos, Christensen et al. 2008). DNA methylation patterns are generally laid *de novo* during differentiation by Dnmt3a and Dnmt3b (Okano, Bell et al. 1999) to suppress genes which are no longer needed. These DNA methyltransferases may also act in mature cells to maintain methylation states of pericentromeric heterochromatin, (Hansen, Wijmenga et al. 1999). During mitosis, Dnmt1 acts to replicate and thus maintain the methylation patterns previously laid by the other two DNA methyltransferases (Bird 2002), underlying the heritability of methylation patterns during replication. DNA methylation can suppress transcription by preventing transcription factor binding (Comb and Goodman 1990; Bird and Wolffe 1999), it can facilitate recruitment of histone deacetylase complexes which induce compaction of the surrounding chromatin regions (Bird and Wolffe 1999), and it an act directly in the silencing of gene expression, (Bachman, Rountree et al. 2001; Baylin 2002).

Readers: Effectors and Presenters

Methyl-lysine Readers

Methylated histone lysine modifications are recognized by chromodomain protein motifs which recruit the associated protein along with its complex members to the methylation site, (Berger 2007). Chromodomains are found in many chromatin regulatory enzymes including histone acetyltransferases (HATs), histone methyltransferases (HMTs), and ATP-dependent remodelers (Paro and Hogness 1991; Jones, Cowell et al. 2000; Eissenberg 2001; de la Cruz, Lois et al. 2005), where they play a role in protein-protein interactions (Ball, Murzina et al. 1997; Nielsen, Nietlispach et al.

2002) recruiting these effectors to specific methylated lysine sites on the chromatin. For example, the HAT Tip60 has a chromodomain which binds specifically to H4K9me3 and is essential for Tip60 HAT activation, (Sun, Jiang et al. 2009). Another chromodomain containing protein, Heterochromatin Protein 1 (HP1), recognizes different methyl marks depending on the protein complex with which it is associated, (Fanti and Pimpinelli 2008). In some cases the chromodomain of HP1 recognizes either a H3K9 trimethyl mark characteristic of heterochromatin (Cheutin, McNairn et al. 2003; Shimada and Murakami 2010), while in others it recognizes a dimethyl mark at this same residue which is more commonly found in regions of euchromatin, (Smallwood, Esteve et al. 2007; Tachibana, Sugimoto et al. 2002). HP1 binding directs chromatin regulators to further alter the chromatin structure near the target methyl mark, (Bannister, Zegerman et al. 2001; Lachner, O'Carroll et al. 2001; Nakayama, Rice et al. 2001).

Acetyl-lysine Readers

The only conserved domain shown to associate with acetyl lysines is the bromodomain, (Zeng and Zhou 2002). The bromodomain is highly conserved and found in many chromatin modifying enzymes and in proteins associated with various histone modifying complexes (Jeanmougin, Wurtz et al. 1997; Marmorstein and Berger 2001; Jeanmougin, Wurtz et al. 1997; Horn and Peterson 2001; Marmorstein and Berger 2001; de la Cruz, Lois et al. 2005). The binding of the bromodomain-containing proteins to specific acetylated lysines in active regions induces the recruitment of other protein complexes to these acetylated regions for further modification, (Marmorstein and Berger 2001; de la Cruz, Lois et al. 2005). Bromodomain containing proteins generally possess

only one bromodomain, though bromodomains can sometimes be found in pairs, (Marmorstein and Berger 2001). It is thought that proteins with multiple bromodomains may use them to recognize distinct combinations of acetyl lysines on the same target, (Yang 2004). For example, the RSC complex contains eight bromodomain proteins which may simultaneously contact acetyl marks on multiple histones, (Asturias, Chung et al. 2002). Some enzymes like human TAFII250 can bind multiple acetyl-lysines (Jacobson, Ladurner et al. 2000) which regulates the strength of the interaction, (Roth, Denu et al. 2001). Bromodomains found within the same protein generally do not have great similarity, suggesting that they are not redundant and that they in fact recognize different acetylated lysine residues, (Jeanmougin, Wurtz et al. 1997; Winston and Allis 1999; Marmorstein and Berger 2001).

Bromodomains are usually found in association with histone acetyltransferases, histone methyltransferases, and ATP-dependent remodeling enzymes, (Winston and Allis 1999). The recruitment of HATs to acetylated chromatin by self-contained bromodomains is thought to anchor them at active chromatin sites and suggests a role in self-perpetuation, (Winston and Allis 1999; de la Cruz, Lois et al. 2005). For example the bromodomain of the HAT Gcn5 is required to stabilize the SAGA complex on acetylated chromatin where it maintains acetyl marks, (Hassan, Prochasson et al. 2002). Bromodomain containing proteins can also be found in ATP-dependent chromatin remodeler complexes including SWI/SNF complexes and the RSC complex, (de la Cruz, Lois et al. 2005) and in some histone methyltransferases including Ash1, RIZ, and MLL, (de la Cruz, Lois et al. 2005). Bromodomains are also thought to recruit these enzymes to acetyled regions of chromatin for further modification.

Bromodomains were first shown to interact with acetyl-lysines *in vitro* (Dhalluin, Carlson et al. 1999; Ornaghi, Ballario et al. 1999), in an acetyl recognition mechanism that is similar to that between HATs and acetyl-CoA (Dutnall, Tafrov et al. 1998) and which involves invariant residues flanking the bromodomain binding pocket, (Winston and Allis 1999). Bromodomain specificity is based on the amino acids surrounding the acetyl lysine as well as those surrounding the bromodomain itself which allows for great diversity in acetyl lysine recognition, (Winston and Allis 1999; Ornaghi, Ballario et al. 1999). This variability of bromodomain binding may be used to target different writer enzymes to specific regions of chromatin.

Proteins with bromodomains can also use these regions to recognize acetylated non-histone proteins and acetylated DNA. For example, the HATs Gcn5, PCAF, TAF1, and CBP have bromodomains that in addition to acetylated histone lysines also recognize acetyl lysines on non-histone proteins including HIV Tat, p53, c-Myb, and MyoD, (Yang 2004). Additionally bromodomain containing proteins such as Bromodomain factor 1 (Bdnf1) and SWI/SNF preferentially bind acetylation rich DNA with their bromodomains, (Hassan, Prochasson et al. 2002; Kurdistani, Tavazoie et al. 2004).

Histone Tail Presentors

SANT domains are small motifs that are sometimes found in proteins involved in chromatin remodeling complexes and are structurally similar to the DNA-binding domains of c-Myc related proteins, (Aasland, Stewart et al. 1996). These domains generally associate with unmodified histone tails and "present" them to remodeling enzymes by stabilizing them in appropriate conformation for the associating enzyme to

recognize, (Boyer, Langer et al. 2002; Boyer, Latek et al. 2004). SANT domains may recruit chromatin modifying enzymes and present histone N-terminal tails to them to facilitate catalytic activity, (de la Cruz, Lois et al. 2005). Among the remodeling complexes containing SANT domain proteins are several HAT, HDAC, HMT, and ATP-remodeling complexes, (Fischle, Dequiedt et al. 2001; You, Tong et al. 2001; Boyer, Langer et al. 2002; Sterner, Wang et al. 2002; Yu, Li et al. 2003; de la Cruz, Lois et al. 2005). Additionally, SANT domain containing protein binding may block the region from other protein recognition, preventing the binding of antagonistic remodeling complexes, (Yu, Li et al. 2003).

Erasers

Histone Deacetylases

Histone deacetylases (HDACs) catalyze the removal of acetyl marks from histone tail lysines, generally found in upstream regulatory regions of target genes where they were laid by histone acetyltransferases (HATs), thus antagonizing or "erasing" the acetyltransferase activity of HATs, (Shahbazian and Grunstein 2007; Haberland, Montgomery et al. 2009). This activity unmasks the positively charged histone lysine residue, allowing charge interactions with the DNA backbone, which recondenses the chromatin while reducing the affinity for transcription factor and cofactor binding, (Shahbazian and Grunstein 2007). Histone deacetylation is therefore thought to facilitate transcriptional repression and is commonly associated with regions of heterochromatin, (Grewal and Jia 2007).

Like HATs, HDACs can be targeted to the chromatin in two ways; they can be specifically recruited to target promoters by transcriptional repressors, or they can broadly and unspecifically deacetylate large sections of the genome. For example, the deacetylase Rpd3 is targeted to sporulation genes in yeast by the transcriptional regulator Ume6 (Rundlett, Carmen et al. 1998; Roh, Kim et al. 2004) and histone deacetylase I (HDACI) is recruited to specific genes by the repressor Tup1 (Wu, Carmen et al. 2001), however both of these enzymes can also non-specifically deacetylate large euchromatic regions, (Vogelauer, Wu et al. 2000; Shahbazian and Grunstein 2007). Transcriptional repression ultimately results from both methods of histone deacetylation.

HDACs can be classified into four groups based on function and sequence similarity. Group I and Group II are considered the "classical" HDACs which utilize acive-site metal dependent catalysis, (Smith and Denu 2009). Group IV HDACs also use this means of catalysis, but are considered atypical and are classified based on sequence similarity. Group III HDACs, the sirtuins, utilize a nicotinamide adenine dinucleotide (NAD+) dependent catalytic mechanism, (Frye 2000; Smith and Denu 2009; Imai, Armstrong et al. 2000).

HDACs can be recruited to active genes for two reasons, (Shahbazian and Grunstein 2007). The first, although less understood, is to play a role in transcriptional activation by repressing aberrant initiation, (Carrozza, Utley et al. 2003; Joshi and Struhl 2005; Keogh, Kurdistani et al. 2005). Histone acetylation has very low thermodynamic stability and therefore is considered a transient chromatin mark, (Meaney and Ferguson-Smith 2010). As such, histone acetyl marks can rapidly be made to turn a gene on, and

just as rapidly removed to silence it. Such activities are particularly important for cellular activities such as neurotransmission where fast and distinct signals are necessary. Importantly, although acetylation is commonly associated with transcriptional activation, it is not necessarily the concentration of acetyl marks that induce gene expression. Rather, it is thougth that a high level of rapid acetylation in conjuction with equally high and rapid deacetylation which induces robust transcriptional expression, (Shahbazian and Grunstein 2007). In support of this concept, transcriptionally active regions show aceytlation turnover with half lives as short as 1-5 minutes while in transcriptionally silent regions this turnover can take 30-60 minutes, (Waterborg 2002). Therefore, the activities of HDACs may be just as critical for rapid gene activation as that of HATs. It is unclear whether acetylation turnover is mediated by global HAT and HDAC activity, or alternate recruitment of these enzymes with antagonizing activities, (Shahbazian and Grunstein 2007).

Alternatively, HDACs may be recruited to active genes to place these remodelers in a "priming" position to rapidly repress the active targets when their expression is no longer required, (Shahbazian and Grunstein 2007). For example, the immediate early target c-fos is rapidly induced upon exposure to growth factors and quickly repressed in less than 2 hours. Upon activation of this target, the transcription factor Elk-1 recruits HDAC-1 to the site to mediate this repression, (Yang, Vickers et al. 2001). Further, it is suggested that the deacetylase complex is recruited even during activation to prepare for repression, (Wang, Kurdistani et al. 2002). It has therefore been hypothesized that it is better to interfere with transcription than to have inefficient repression, (Shahbazian and Grunstein 2007).

Histone lysine deacetylation may further promote repression by promoting the binding of SANT domain containing transcriptional repressors which recognize unmodified histone tails (de la Cruz, Lois et al. 2005), and block other modifiers from accessing the region. For example, the corepressor SMRT contains two SANT domains: the first binds to deacetylated histone H4 while the second recruits the deacetylase HDAC3, while physically preventing HAT complexes from accessing the site (Yu, Li et al. 2003; Hartman, Yu et al. 2005), facilitating deacetylation. Other SANT domain containing protein such as CoREST and N-CoR function similarly by recruiting histone deacetylases (HDAC1 and HDAC2, and HDCA7 respectively) to histone targets as well, (de la Cruz, Lois et al. 2005).

Histone Arginine and Lysine Demethylases

Histone methylation was thought to be irreversible because of its high thermodynamic stability until the identification of petidylarginine deiminase 4 (PADI4) which converts methyl-arginine to citrulline, (Cuthbert, Daujat et al. 2004; Wang, Wysocka et al. 2004). Although PADI4 does not directly revert histone arginine methylation, it lent credibility to emerging hypotheses regarding histone demethylases enzymes, (Bannister, Schneider et al. 2002). The first true histone lysine "eraser", demethylase LSD1/KDM1, which had previously been identified as a corepressor (You, Tong et al. 2001), was the first enzyme shown to have mono and di-methylase activity, (Shi, Lan et al. 2004). LSD1/KDM1 was shown to remove mono- and dimethyl marks from H3K4 and H3K9 in a flavin adenine dinucleotide (FAD)-dependent amine oxidase reaction, (Shi, Lan et al. 2004; Lee, Wynder et al. 2005). LSD1/KDM1 can demethylate marks involved in both

transcriptional repression and transcriptional activation depending on the complex it associates with. For example, when in complex with the repressive CoREST complex, LSD1/KDM1 acts on mono- and dimethyl H3K4 marks resulting in target repression, (Shi, Lan et al. 2004). Alternatively, when in complex with the androgen receptor (AR), LSD1/KDM1 acts as a mono- and dimethyl H3K9 demethylase resulting in target activation, (Metzger, Wissmann et al. 2005).

Due to the requirement of nitrogen protonation, the demethylase activity of LSD1/KDM1 is unable to demethylate the most stable trimethyl lysine marks, however a mechanism in which this could be mediated had been proposed, (Trewick, McLaughlin et al. 2005). The first jumonji (JmjC) –domain-containing histone demethylase (JHDM) with trimethyl lysine demethylation potential identified was JHDM1A/KDM2A (FBXL11) (Tsukada and Zhang 2006), however this enzyme was shown to demethylate only mono- and dimethylaed H3K36. Soon after, family member JMJD2 was shown to have this additional trimethyl lysine demethylation activity, acting on di- and trimethylated H3K9 and H3K36, (Cloos, Christensen et al. 2006; Fodor, Kubicek et al. 2006; Klose, Yamane et al. 2006; Tsukada and Zhang 2006; Whetstine, Nottke et al. 2006; Yamane, Toumazou et al. 2006). The identification of other family members included the characterization of JMJD1 (Yamane, Toumazou et al. 2006), JARID1 (Christensen, Agger et al. 2007; Iwase, Lan et al. 2007; Klose, Gardner et al. 2007; Tahiliani, Mei et al. 2007; Lee, Thompson et al. 2008), and UTX/JMJD3, (Agger, Cloos et al. 2007; De Santa, Totaro et al. 2007; Jepsen, Solum et al. 2007; Lan, Bayliss et al. 2007; Lee, Villa et al. 2007).

The JHDM family enzymes belong to the dioxygenase superfamily and have been shown to demethylate H3K36 (JHDM1) (Tsukada and Zhang 2006), H3K9 (JHDM2A) (Yamane, Toumazou et al. 2006), and H3K9 and H3K36 (JHDM3 and JMJD2A-D), (Cloos, Christensen et al. 2006; Fodor, Kubicek et al. 2006; Klose, Yamane et al. 2006; Whetstine, Nottke et al. 2006). The catalytic mechanism of these enzymes utilizes cofactors iron Fe(II) and α-ketoglutarate (αKG) in an oxidative reaction to remove histone lysine modifications, allowing these enzymes to demethylate mono-, di-, and trimethyl lysine marks, (Smith and Denu 2009; Ozer and Bruick 2007). Additionally, the JHDM family member JMJD6 possesses histone arginine demethylase activity instead of histone lysine demethylase activity (Chang, Chen et al. 2007; Smith and Denu 2009), adding to the variability of functionality in this group.

Substrate recognition is complicated for the demethylases because mono-, di-, and tri-methyl marks require very different binding conformations, yet each enzyme can recognize multiple levels of methylation. Instead of relying on the binding domain itself, the specificity of demethylase enzymes may be reliant on "reader" domains that the demethylases themselves or their binding partners contain to recruit demethylase complexes to specific histone marks and chromatin regions, or to spread to neighboring regions. For example, the PHD domain of JMJD2A preferentially binds trimethylated H3 K4, K9, and K20 marks, (Huang, Fang et al. 2006; Lee, Thompson et al. 2008). Additionally, many histone demethylases complex with lysine methyltransferases (KTMs) and histone deacetylases (HDACs) (Cloos, Christensen et al. 2008), as possessing multiple modifying subunits allows the complex to rapidly regulate targets without leaving the site.

The Histone Code

This abundance of specific yet diverse post-translational histone modifications can regulate transcription independently has been described, however they can alternatively be considered in groups as highly complex patterns. This "histone code" was first proposed in 2001 (Jenuwein and Allis 2001) and has yet to be fully deciphered. The regulation of post-translational histone modifications relies on various signaling pathways that include many enzyme effector complexes which can generate various modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, as well as variations of each, (Cheung, Allis et al. 2000). Importantly, each of these enzymes is very specific in the marks that they "write", "read", or "erase", to both histone protein and amino acid residue altered, suggesting that it is not just the amount of each modification that is important, but also the precise location that plays a role in epigenetic regulation, (Strahl and Allis 2000; Turner 2000). Additionally, individual histone proteins can obtain many post-translational modifications on one tail, leading to infinite combinatorial possibilities when the eight histone tails composing a nucleosome are considered together. For example, as many as thirteen lysines have been shown to be acetylated on one nucleosome core, (Waterborg 2000). However, not all combinations have been observed in the nucleus suggesting that these combinations are also not random, (Jenuwein and Allis 2001). In support of this idea, histone H3 lysine 9 methylation and H3 serine 10 phosphorylation cannot occur on the same histone, (Rea, Eisenhaber et al. 2000). Alternatively, H3Ser10 is commonly found on the same histone as H3 lysine 9 and lysine 14 acetylation in transcriptionally active chromatin regions, (Cheung, Tanner et al. 2000; Clayton, Rose et al. 2000; Lo, Trievel et al. 2000). These

dual marks may necessitate strictly ordered deposition as H3K14 must be deacetylated prior to H3K9 methylation (Nakayama, Rice et al. 2001), suggesting that the first modification is required before the next can be added. The Histone Code hypothesis (Jenuwein and Allis 2001; Rice and Allis 2001) adds an additional level of complexity to transcriptional regulation based on post-translational modifications, and deciphering it will open a new realm to understanding transcriptional regulation.

The Histone Acetyltransferase Tip60

The MYST family histone acetyltransferase Tat interactive protein 60kD (Tip60) was originally identified in complex with the HIV-1 associated Tat protein, (Kamine, Elangovan et al. 1996). Human TIP60 has homologues with high conservation in many organisms including *G. gallus, M. musculus, D. melanogaster*, *C. elegans,* and *S. cerevisiae* (Esa1), (McAllister, Merlo et al. 2002; Ceol and Horvitz 2004; Doyon and Cote 2004; Kusch, Florens et al. 2004; Zhu, Singh et al. 2007). TIP60 is expressed ubiquitously and demonstrates cell type specific functionality, (Hlubek, Lohberg et al. 2001). Tip60 is predominantly found in the nucleus (Yamamoto and Horikoshi 1997; Gavaravarapu and Kamine 2000; Ran and Pereira-Smith 2000; Cao and Sudhof 2001) where it acetylates lysines on histone H4 and other non-histone proteins including transcription factors and kinases, but can be found in the cytoplasm where it associates with internalized membrane receptors to regulate kinase pathways and gene expression, (Sapountzi, Logan et al. 2006). *In vitro* Tip60 is shown to acetylate H2AK5, H3K14, and H4 K5, K8, K12, and K16, (Kimura and Horikoshi 1998). Tip60 is the only HAT known

to acetylate H2AK5, suggesting that this residue may serve as an endogenous marker for Tip60 activity, (Bhaumik, Smith et al. 2007).

TIP60 has three splice variants: TIP60 isoform 1, TIP60 isoform 2 (TIP60α), and TIP60 isoform 3 (TIP60β) (Ran and Pereira-Smith 2000; Sheridan, Force et al. 2001; Legube and Trouche 2003), although Tip60α is the most common. Tip60 has two functional domains: a C-terminal MYST domain and an N-terminal chromodomain. The catalytic MYST domain contains the HAT domain which binds acetyl coenzyme A and substrate, and also contains a zinc finger required for HAT activity and protein-protein interactions, (Nordentoft and Jorgensen 2003; Xiao, Chung et al. 2003). The function of the chromodomain has yet to be fully elucidated (Akhtar, Zink et al. 2000; Utley and Cote 2003), although chromodomains are known to bind methylated histone lysines, as well as to interact with RNA molecules. Recent findings demonstrate that Tip60 chromodomain binding to H3K9me3 activates its HAT activity in double stranded break repair, (Sun, Jiang et al. 2009).

The Tip60 Complex

Tip60 can be found in various transient protein complexes within the nucleus and cytoplasm of the cell, each with distinct cellular functions. Depending on the complex it is associated with, Tip60 is recruited to different promoters for activation. Tip60 can also acetylate non-histone proteins including transcription factors and kinases and this acetylation generally activates the catalytic activity of these proteins, (Sapountzi, Logan et al. 2006). Tip60 is an essential HAT (Smith, Eisen et al. 1998; Zhu, Singh et al. 2007; Hu, Fisher et al. 2009) which plays important roles in a variety of biological processes

(Sapountzi, Logan et al. 2006), so it is not surprising that mis-regulation of Tip60 often results in disease.

The complex that Tip60 is most commonly found in is highly conserved and contains at least 18 subunits, (Ikura, Ogryzko et al. 2000). In addition to Tip60's HAT activity, this complex also displays ATPase, DNA helicase, and structural binding activities, (Ikura, Ogryzko et al. 2000). The ATPase activity of the Tip60 complex is likely based on activities of the p400/Domino subunit, which is also known to remodel chromatin, as well as the actin and possibly RuvBL1 and RuvBL2 subunits, (Ikura, Ogryzko et al. 2000). The actin-related protein BAF53 recruits chromatin modifying enzymes to sites of DNA damage, supporting a role for Tip60 in the DNA damage response, (Ikura, Ogryzko et al. 2000). Also involved in the DNA damage response is the inhibitor of growth 3 (ING3) protein which is involved in the transcription of p53, supporting a role for the Tip60 complex in apoptosis, (Sapountzi, Logan et al. 2006). Additionally, mortality factor 4 related gene 15 (Mrg15) and mortality factor 4 related gene X (MrgX) are subunits involved in cell proliferation and senescence along with the subunit glioma amplified sequence 41 (Gas41) (Sapountzi, Logan et al. 2006), indicating that Tip60 may play a role in cell cycle regulation. Two subunits, Mrg15 and MrgX, contain chromodomains suggesting epigenetic roles for the Tip60 complex in addition to Tip60's HAT activity, (Ikura, Ogryzko et al. 2000). The subunit ING3 has plant homeodomain (PHD) fingers, commonly found in chromatin modifying complexes (Doyon, Selleck et al. 2004; Sapountzi, Logan et al. 2006), and the subunit bromodomain containing protein 8/thyroid receptor coactivator protein 120kDa (Brd8/TRCp120) contains a bromodomain, a domain shown to bind acetylated histones, (Cai, Jin et al. 2003; de la Cruz, Lois et al.

2005). These domains may play epigenetic roles by recruiting complex members to specific sites on the chromatin. The Tip60 complex also contains the histone tail binding DNA methyltransferase associated protein 1 (DMAP1) which is involved in DNA replication, (Cai, Jin et al. 2003; Doyon, Selleck et al. 2004). In *Drosophila*, the Tip60 complex also contains histones H2A.v and H2B, but this has not been confirmed in humans, (Kusch, Florens et al. 2004). The Tip60 complex is thought to facilitate transcriptional activation predominantly through acetylation of histone proteins, however Tip60 has also been shown to acetylate many non-histone proteins.

Tip60 in Apoptosis

Tip60 is recruited to target promoters through association with various transcription factors. One of these transcription factors is the tumor suppressor p53 which plays a critical role in the DNA damage response through transcriptional activation of cell cycle control, apoptosis, and DNA repair targets, (Legube and Trouche 2003). Defects in p53 lead to many human cancers, (Lane 1992; Levine 1997; Vogelstein, Lane et al. 2000; Vousden 2002). It is believed that in response to DNA damage, the decision between entering a pro-apoptotic program to eliminate the damaged cell or entering growth arrest in G1 to repair DNA relies on p53 activation of the appropriate pathway, (Lane 1992; Levine 1997; Prives and Hall 1999; Michael and Oren 2002; Vousden 2002; Sengupta and Harris 2005). In response to cellular stress p53 levels are rapidly upregulated and the peptide is activated through various post-translational modifications including ubiquitination, phosphorylation, and acetylation, (Appella and Anderson 2001; Brooks and Gu 2003; Xu 2003; Farnebo, Bykov et al. 2010). p53 acetylation is catalyzed by the

acetyltransferas enzymes CBP/p300 (Avantaggiati, Ogryzko et al. 1997; Gu, Shi et al. 1997; Lill, Grossman et al. 1997), PCAF (Sakaguchi, Herrera et al. 1998; Liu, Scolnick et al. 1999), and Tip60 (Legube, Linares et al. 2004; Tang, Luo et al. 2006) in response to stress, (Colman, Afshari et al. 2000; Appella and Anderson 2001; Ito, Lai et al. 2001; Luo, Nikolaev et al. 2001; Gu, Luo et al. 2004; Lavin and Gueven 2006).

In order for p53 to induce cell-cycle arrest, it must first be methylated by Set7/9 methyltranserase, (Kurash, Lei et al. 2008). It has been proposed that this methyl mark may serve as a docking site for the chromodomain of Tip60, as methylation of p53 K369 in mouse is necessary for p53 acetylation, (Kurash, Lei et al. 2008). Tip60 specifically acetylates K120 of p53, and the abundance of this modification is increased in response to DNA damage, (Tang, Luo et al. 2006). Without this mark apoptotic pathways are unaltered but p53-induced cell cycle arrest is disrupted, (Berns, Hijmans et al. 2004; Legube, Linares et al. 2004; Tang, Luo et al. 2006; Tyteca, Legube et al. 2006). It has been proposed that the acetylation state of K120 may serve in p53 target preference (Kitayner, Rozenberg et al. 2006), where acetylation of this residue may determine whether targets are activated to induce apoptosis or cell cycle arrest, (Legube, Linares et al. 2004; Sykes, Mellert et al. 2006; Tang, Luo et al. 2006; Tyteca, Legube et al. 2006). K120 mutations are often found in cancer (Meyers, Chi et al. 1993; Hashimoto, Tokuchi et al. 1999; Hayes, Dirven et al. 1999; Deissler, Kafka et al. 2004; Leitao, Soslow et al. 2004), likely blocking p53 acetylation by Tip60 and inhibiting cell cycle arrest (Sykes, Mellert et al. 2006), and thus allowing cells with damaged DNA to survive and proliferate.

Programmed cell death 5 (PDCD5) has been identified as a novel Tip60 binding partner (Stelzl, Worm et al. 2005), and has been shown to stabilize Tip60 particularly in response to DNA damage and act as a Tip60 coactivator in p53 acetylation and in the regulation of apoptosis-inducing targets including Bax, (Xu, Chen et al. 2009). It is possible that other p53 acetyltransferases such as CBP/p300 or PCAF which contain bromodomains may "read" the acetyl marks left by Tip60 for recruitment to and further acetylation of p53. Alternatively, Set7/9 methylation may alter the conformation of p53 making it receptive to acetylation by these factors, (Kurash, Lei et al. 2008). In response to DNA damage it seems that acetylation of p53 by Tip60 indicates that apoptosis pathways will be induced, while a lack of Tip60 acetylation will induce cell cycle arrest, (Fischle 2009).

Tip60 in DNA Repair

DNA damage can result both endogenously from metabolic processes and exogenously from environmental reagents. Double stranded breaks are the most difficult to repair, and inaccurate repair at these regions may result in cancer or cell death. To avoid such genomic instability, eukaryotic cells posses tightly regulated mechanisms for both DNA damage detection as well as cell cycle arrest to allow time for repair before entering division, (van Attikum and Gasser 2009). Tip60 was first implicated in the process of DNA doublestrand break (DSB) repair when HeLa cells expressing a dominant negative Tip60 showed defects in DSB repair, (Ikura, Ogryzko et al. 2000). Research stemming from this initial investigation have shown Tip60 to play critical roles in damage sensing, signaling, and repair, (Squatrito, Gorrini et al. 2006).

In response to double stranded breaks, Tip60 associates with the highly conserved FATC domain of ATM kinase (Sun, Jiang et al. 2005), and these proteins are recruited in complex to the break site by the MRN (MRE11-RAD50-NBS1) complex, (Squatrito, Gorrini et al. 2006; Fischle 2009). At this site, trimethylated H3K9 marks laid by the histone methyltransferases Suv39h1 and Suv39h2 (Peters, O'Carroll et al. 2001) are normally masked by HP1 (Jacobs and Khorasanizadeh 2002; Nielsen, Nietlispach et al. 2002), but in response to damage HP1β is phosphorylated by casein kinase 2 (CK2) releasing it from the chromatin, (Ayoub, Jeyasekharan et al. 2008). The chromodomain of Tip60 binds the newly exposed trimethylated H3K9 in the damaged region, activating the HAT activity of Tip60 and inducing it to acetylate ATM at lysine 3016 (Sun, Jiang et al. 2005; Sun, Xu et al. 2007). This acetylation event alters the conformation of ATM and activates it to autophosphorylate, allowing it to bind to and phosphorylate other damage response targets (Sun, Jiang et al. 2005) including nbs1, p53, chk2, SMC1, brca1, and H2AX, (Lavin and Gueven 2006; Jackson and Bartek 2009). Interestingly, H3K9me3 is predominantly found in regions of heterochromatin (Jacobs, Taverna et al. 2001; Noma, Allis et al. 2001; Richards and Elgin 2002), suggesting that Tip60 may act in DNA repair preferentially in heterochromatic regions. In support of this, ATM is required to repair DSBs in regions of heterochromatin, (Goodarzi, Noon et al. 2008). Tip60 therefore plays a critical role in DNA damage repair and maintenance of genomic integrity, processes that may be involved in tumor suppression.

In addition to ATM activation, Tip60 plays a second role in DNA repair in histone variant exchange through acetylation of histones H2AX and H4 at double stranded breaks, (Ikura, Tashiro et al. 2007; Downs, Allard et al. 2004; Kusch, Florens et al. 2004;

Murr, Loizou et al. 2006; Jha, Shibata et al. 2008). The histone variant H2AX becomes rapidly phosphorylated at Ser139 in an area up to several megabases surrounding double stranded breaks, forming γH2AX, (Rogakou, Pilch et al. 1998; Kusch, Florens et al. 2004). Tip60 is recruited to DSBs through association with this modification, (Fischle 2009). Histone variant exchange allows the incorporation of histones that can be post-translationally modified for specific functions. For example, phosphorylated γH2AX provides docking sites surrounding the break site for DNA damage and double-stranded break repair factors including MRN, 53BP1, and BRCA1, (Bassing, Chua et al. 2002; Bassing and Alt 2004; Stucki and Jackson 2006). Tip60 has been shown to acetylate γH2AX at lysine 5 (Downs, Allard et al. 2004), prior to its ubiquitylation by a Tip60 containing UBC13 complex (Ikura, Tashiro et al. 2007), which is necessary for its eviction following repair, (Keogh, Kim et al. 2006; Ikura, Tashiro et al. 2007). Tip60 further catalyzes the replacement of γH2AX with unmodified H2A in a reaction catalyzed by the p400 Tip60 complex subunit that likely exchanges the H2Av-H2B dimer (Kusch, Florens et al. 2004), thus reversing the phosphorylation mark. Alternatively, the phosphorylation mark can be reversed by several phosphatases, (Chowdhury, Keogh et al. 2005; Keogh, Kim et al. 2006). Tip60 therefore plays a pivotal role in histone variant exchange at DNA lesions.

Additionally, H4 acetylation induced by Tip60 HAT activity is necessary at double stranded breaks to decondense the chromatin and allow repair machinery to gain access to the damaged DNA, (Squatrito, Gorrini et al. 2006; Fischle 2009). The Tip60 complex component TRRAP is necessary for Tip60 recruitment to the break site, and for subsequent H4 acetylation of surrounding nucleosomes, (Murr, Loizou et al. 2006).

TRAAP is required for both repair by homologous recombination (Murr, Loizou et al. 2006) and nonhomologous end joining, (Robert, Hardy et al. 2006). Although H4 acetylation is required for DSB repair, it does not appear to be dependent on acetylation of a specific target lysine, as acetylation of at least one lysine (K5, K8, K12, or K16) was sufficient to initiate repair, (Bird, Yu et al. 2002). This lack of specificity supports the concept that H4 acetylation in response to DNA damage is not "read" by specific effectors but instead serves to open the chromatin at damaged sites to allow repair machinery to access the lesion.

Tip60 in Cell Cycle Progression

Cell cycle progression is tightly regulated by the E2F transcription factors consisting of cell cycle activating factors E2F1/2/3 and cell cycle repressing factors E2F4/5. Activation of cell cycle promoting target genes such as pocket protein p107, proliferating cell nuclear antigen (PCNA), and minichromosome maintenance 3/4 (MCM3 and MCM4) require recruitment of the Tip60 complex to the promoter region of these target genes by E2F1, where Tip60 HAT activity is responsible for laying the activating acetyl marks on histone H4, (Taubert, Gorrini et al. 2004). Without the HAT activity of Tip60, cells show cell-cycle defects (Ikura, Ogryzko et al. 2000), suggesting that the HAT activity of Tip60 is essential for proper cell cycle regulation.

Tip60 in Transcriptional Repression

As the epigenetic code is unraveled, an emerging theme is our inability to classify epigenetic marks and chromatin modifying enzymes as strictly stimulatory or inhibitory as transcriptional regulation is often the result of a combination of marks laid by various

factors. Additionally, regions can be primed for regulation and complexes often contain counteracting subunits to allow for rapid changes in regulation. As such, many chromatin regulatory complexes have been shown to contain both activating and repressing components, such as HATs and HDACs. In support of this concept, in addition to its traditional classification as a transcriptional activator, Tip60 has also been shown to play a role in transcriptional repression based on the activity of its binding partners.

Tip60 has been shown to associate with the signal transducer and activator of transcription 3 (STAT3), a transcription factor which plays important roles in cell cycle progression, apoptosis, and cell motility, (Xiao, Chung et al. 2003). The STAT3-Tip60 complex is shuttled into the nucleus in response to interleukin-9 receptor (IL-9R) signalling, where Tip60 is thought to recruit HDAC7 to repress transcription of STAT3 target promoters in cytokine signaling, (Xiao, Chung et al. 2003). Such IL-9 related gene repression is shown to promote proliferation, regulate growth, and inhibit apoptosis. Tip60 has also been found in complex with UHRF1 (Ubiquitin-like containing PHD and RING domain 1), DNMT1 (DNA methyltransferase 1), and HDAC1 (histone deacetylase 1), (Achour, Fuhrmann et al. 2009). This complex has implications in heterochromatin replication (Bostick, Kim et al. 2007), and in silencing of tumor suppressor targets RB1 and $p16^{INK4A}$, (Jeanblanc, Mousli et al. 2005; Achour, Jacq et al. 2008). Microarray analysis in embryonic stem cells lacking Tip60 found a majority of targets upregulated (Fazzio, Huff et al. 2008), further supporting a role for Tip60 in transcriptional repression, although this role is commonly thought to be indirect.

Tip60 and Embryonic Stem Cell Self-Renewal

Embryonic stem cells (ESCs) are unique in that they are able to both self-renew and differentiate into any cell lineage. The self-renewal pathway of ESCs requires careful maintenance of a very unique gene expression profile containing both active and repressed targets. Interestingly, a subset of these genes contains both activating and repressing marks, H3K4me3 and H3K27me3 respectively, (Pan, Tian et al. 2007; Zhao, Han et al. 2007; Fouse, Shen et al. 2008). It has been proposed that such "bivalent" marking represses expression while allowing targets to be primed for rapid activation upon differentiation, (Mikkelsen, Ku et al. 2007; Fazzio, Huff et al. 2008). It has been shown that acetylation of these promoters by Tip60 promotes target silencing and ESC self-renewal (Fazzio, Huff et al. 2008), while Tip60 knockdown results in phenotypes consistent with differentiated cells, (Fazzio, Huff et al. 2008).

Tip60 and the Amyloid Precursor Protein

The amyloid precursor protein (APP) has been implicated in Alzheimer's disease (AD) pathogenesis due to the toxic peptides formed during its proteolytic processing, (Zheng and Koo 2006). APP is processed by two sequential cleavage events. The first event is catalyzed by either α- or β-secretase releasing different N-terminal regions extracellular and indicating whether the processing will be amyloidogenic or non-amyloidogenic, respectively. The second event is catalyzed by γ-secretase releasing the small intracellular C-terminus (AICD) into the cytoplasm, (Zheng and Koo 2006). In the amyloidogenic processing pathway, the peptide left between the β- and γ-secretase cleavage sites is termed Aβ and has been shown to aggregate into the toxic plaques of

AD, (Zheng and Koo 2006). APP over-expression as well as defects in its processing can lead to increased toxic Aβ formation contributing to AD, underscoring the importance for proper expression and processing of this peptide.

In addition to the neurotoxic effects of the Aβ plaque aggregations, the APP intracellular domain (AICD) which is released upon γ-secretase cleavage in both the amyloidogenic and non-amyloidogenic pathways has also been shown to be cytotoxic. Importantly, AICD over-expression has been shown to induce neurotoxicity in various AD models. *In vitro*, AICD has been implicated in cytotoxicity (Lu, Rabizadeh et al. 2000), apoptosis (Lu, Rabizadeh et al. 2000; Kinoshita, Whelan et al. 2002), and degeneration (Yoshikawa, Aizawa et al. 1992), and *in vivo* in *Drosophila* AICD-dependent phenotypes include axonal transport defects (Gunawardena and Goldstein 2001; Rusu, Jansen et al. 2007), neuronal apoptosis (Gunawardena and Goldstein 2001), synaptic plasticity defects (Rusu, Jansen et al. 2007), and defects in PNS development, (Merdes, Soba et al. 2004).

Although the topic remains controversial, one theory to explain the mechanism by which AICD induces neurotoxicity is through a role in transcriptional activation. Specifically, AICD has been shown through a heterologous reporter system to associate with the histone acetyltransferase Tip60 in a transcriptionally active complex, (Cao and Sudhof 2001). It has therefore been proposed that alterations in AICD production lead to transcriptional changes that ultimately result in neurotoxicity and may contribute to AD pathogenesis.

Tip60 and AICD are thought to form a complex through the adapter protein Fe65. Fe65 has a PTB2 domain which binds the NPTY sequence of AICD, and a PTB1 domain which binds the NKSY sequence of Tip60, (Muller, Meyer et al. 2008). There is much debate over how the cytoplasmic AICD comes in contact with the nuclear histone acetyltransferase Tip60 to regulate transcription. The original and perhaps most widely accepted model is that AICD is released from the membrane and translocates into the nucleus where it interacts with Tip60 and plays a direct role in transcriptional activation. In this model AICD, which has a very short half life, is stabilized by Fe65 which facilitates its translocation into the nucleus, (Muller, Meyer et al. 2008). In support of this, APP-citrine/Fe65/Tip60 co-transfected HEK293 cells show nuclear localization of APP, Fe65, and Tip60, while treatment with γ-secretase inhibitor prevents this localization, (von Rotz, Kohli et al. 2004). Other *in vitro* studies using cells transfected with just the AICD peptide support this finding that AICD can be translocated into the nucleus, (Gao and Pimplikar 2001; Kinoshita, Whelan et al. 2002; Kim, Kim et al. 2003). Nuclear localization of APP has also been shown *in vivo* in transgenic mice over-expressing APP, (Chang, Kim et al. 2006). Importantly, a small 100aa fragment of Fe65 is sufficient to fascilitate this nuclear localization, (Minopoli, de Candia et al. 2001).

Other models suggest that the translocation of AICD into the nucleus is not required for it to send its signal for transcriptional activation. For example, it has been suggested that the role AICD plays only requires Fe65 binding which can be accomplished without γ-secretase cleavage, inducing a conformational change in Fe65 which allows it to bind to Tip60 upon nuclear translocation, (Muller, Meyer et al. 2008). In this model, AICD may initiate transcriptional activation without ever leaving the cell membrane, (Cao and

Sudhof 2004). Alternatively, AICD may translocate into the nucleus independently of Fe65, with complex formation occurring entirely within the nucleus, (Nakaya and Suzuki 2006). AICD has been shown to have many binding partners, and any of these may be involved in its nuclear translocation. In another scenario, it has been proposed that Tip60 is actually recruited to the cytoplasm where it is tethered to the membrane through Fe65/AICD interactions and is stabilized and activated there by CDK phosphorylation, (Hass and Yankner 2005). Upon activation, Tip60 and Fe65 translocate into the nucleus to activate transcription. These differences observed in localization and activity of AICD are likely do to differences in the *in vitro* model used, however regardless of the mechanism of activation, there is very strong evidence supporting a role for AICD in transcriptional activation through associations with Tip60. Importantly, rat primary cortical neurons transfected with AICD show an increase in H3 and H4 acetylation that is dependent on the Fe65-bindingYENPTY motif (Kim, Kim et al. 2004), supporting an epigenetic role for AICD. Importantly, H4 is a known acetylation substrate of Tip60, further supporting a role for AICD in transcriptional activation through the HAT activity of Tip60.

Further supporting the role for AICD in transcriptional activation is the identification of AICD-dependent transcriptional targets. Unfortunately however these targets are just as controversial as the mechanistic model describing their regulation. Several targets have been identified that are specifically regulated by AICD, but only two of these have been attributed to Tip60 chromatin remodeling. The first, the tetraspanin KAI1 which plays roles in motility, invasion, signaling, and tumor suppression (Hemler 2005), and has been shown to be activated by the AICD/Fe65/Tip60 complex, (Baek, Ohgi et al.

2002). KAI1 protein expression was also shown to be elevated in AICD/Fe54/Tip60 transfected cells, (Bao, Cao et al. 2007). Perhaps most convincingly, Chip analysis performed in 293 cells using antibodies against Fe65, APP, and Tip60 identitied KAI1 promoter fragments, indicating that all of these proteins are present at the promoter of this target, (Telese, Bruni et al. 2005). The second, Lrp1 is a lipoprotein receptor involved in lipid and cholesterol metabolism. The AICD/Fe65/Tip60 complex was also shown to interact with the promoter of the target LRP1 (Kinoshita, Shah et al. 2003; Liu, Zerbinatti et al. 2007), and co-transfection of AICD, Fe65, and TIP60 increases expression and activity of neprilysin, (Pardossi-Piquard, Petit et al. 2005). It is clear that more work is needed to identify the transcriptional targets of the AICD/Fe65/Tip60 complex because they may have important roles in neuronal disease.

Tip60 is a Genetic Hub

Many epigenetic regulators have broad biological implications as there are a limited number of epigenetic modifiers involved in the regulation of the expansive eukaryotic genome, and often these diverse functions are based on a diverse group of interacting partners. Tip60 plays important and well described roles in DNA repair, cell cycle control, and apoptosis, and has been implicated in countless other biological processes including immunity, stem cell identity, development, metabolism, neuronal system processes, HIV, and cancer pathologies, (Sapountzi, Logan et al. 2006). Additionally Tip60 is found as a component of various protein complexes, and has a plentitude of both histone and non-histone protein substrates. Even among epigenetic regulators, this is an unusually broad span of unrelated activities. To explain these seemingly global

implications, recent work has identified Tip60 as a hub gene, underscoring its importance as an epigenetic regulator. Genetic hub genes are often more highly conserved and essential than other genes, and are hypothesized to regulate cellular fitness, (Jeong, Mason et al. 2001; Fraser, Hirsh et al. 2002; Bussey, Andrews et al. 2006; Lehner, Crombie et al. 2006).

A unique high-throughput RNAi assay in *C. elegans* was used to identify genetic interactions using RNAi phenotypes of "library" genes with phenotypes of animals with a lesion in a single "query" gene, (Lehner, Crombie et al. 2006). While most library genes only showed a genetic interaction with a few query genes, six library genes interacted with over one quarter of the query genes tested indicating their roles as genetic hubs. All of these hub genes where involved in chromatin remodeling activities, and the Tip60 homologue was among the six hubs identified as was the Tip60 complex protein TRRAP, (Lehner, Crombie et al. 2006). In support of this finding, a genome-wide SL-SGA analysis with five nonessential subunits of the NuA4 complex, the yeast homologue of the Tip60 complex, and identified 268 different genetic interactions with 204 genes, (Mitchell, Lambert et al. 2008). New and diverse roles were identified for the NuA4 complex including protein transport, arginine biosynthesis, stress response, and ubiquitination. Further, they identified the NuA4 acetyltransferase complex as a genetic hub with effects on a diverse range of cellular processes, (Mitchell, Lambert et al. 2008). The identification of Tip60 as a genetic hub explains its diverse roles in biological processes and underscores the importance of deciphering these roles. It has been suggested that genetic diseases may result from combinatorial mutations in both a pathway specific gene and a chromatin modifying hub gene (Lehner, Crombie et al.

2006), suggesting that investigating the many roles of Tip60 may be central in understanding human disease pathologies.

Epigenetic Mechanisms in the Nervous System

Chromatin modifications made early in development often must persist in the nervous system throughout the lifetime of the organism. All the while, new modifications are constantly being made in response to environmental stimuli that may be transient or may also be stabilized as long term memories. These chromatin modifications are the result of many neurotransmitter signals being received by a single neuron which processes them into chromatin modifications to regulate gene expression to ultimately alter synaptic activities. The regulation of these intricate and complicated processes are poorly understood but ongoing research aims to explore the mechanisms underlying neuronal function.

Neuronal Development

The cells of the nervous system are unique in the complex connections they make and their diversity of roles including sensory perception, neuromuscular activity, and cognition. To accomplish this diverse set of tasks, neuronal populations are differentiated through establishment of unique epigenetic patterns of cellular identity. Each independent cellular identity is generated by a unique and tightly regulated set of gene expression patterns that are based on epigenetic modifications. Once established these

patterns are maintained and passed on to the next generation, such that differentiated cells are able to pass on cellular identity to daughter cells.

Neuronal development begins in the neural tube during embryonic development. The anterior telencephalon contains rapidly dividing neuronal stem cells which proliferate to form the brain. These cells differentiate into neurons and glial cells which will become the predominant cells of the brain. Differentiated neuronal cells migrate to specific regions of the developing brain to form brain structures by extending axons and dendrites to communicate with other neurons. Through synaptic communication, neural circuits are established. Each of these steps requires tight epigenetic regulation of gene expression that is only beginning to be elucidated.

DNA methylation is unique in that it provides long lasting and relatively stable heritable changes of the epigenome and therefore plays a critical role in the establishment and maintenance of neuronal cellular identity, (Jenuwein and Allis 2001). The DNA methyltransferase 1 (Dnmt1) maintains DNA methylation patterns in dividing neuronal progenitor cells (Goto, Numata et al. 1994) ensuring that the appropriate patterns are passed on to the next generation. This process is very important which is reflected in the high expression of Dnmt1 in the embryonic nervous system.

Dnmt1has specifically been implicated in the regulation of the JAK-STAT pathway which regulates gliogenesis, (Bonni, Sun et al. 1997; He, Ge et al. 2005). Dnmt1 expression induces global CNS methylation patterns (Rampon, Tang et al. 2000) and specifically induces methylation at the promoter of the glial marker GFAP (Feng, Fouse et al. 2007) blocking STAT3 binding and repressing transcription of this target,

(Takizawa, Nakashima et al. 2001). Developmental demethylation at the GFAP promoter allows STAT3 binding and GFAP activation during astrogliogenesis, (Teter, Osterburg et al. 1994). It was once believed that DNA methylation patterns established in development were maintained into adulthood. Counterintuitive to this hypothesis, DNA methylation levels appear to increase in the mature brain more than in other tissues (Wilson, Smith et al. 1987; Tawa, Ono et al. 1990), indicating that this modification may continue to be written in the nervous system following the developmental establishment of cell fate.

More transient post-translational histone modifications such as histone acetylation also play important roles in neuronal differentiation, (Jenuwein and Allis 2001). Histone acetyltransferase (HAT) activity appears to promote differentiation while antagonizing proliferation in neural progenitor cells as treatment with non-specific HDAC inhibitors which enhance HAT activity also enhance these phenotypes, (Hsieh and Gage 2004). Alternatively, treatment of oligodendrocytes with HDAC inhibitor blocked differentiation of this cell type, (Hsieh, Nakashima et al. 2004). These results indicate that specific modifications can play very different roles in the establishment of different cell-type specific gene expression patterns.

Synaptic Plasticity

External stimuli, environmental enrichment, and learning have all been linked to dynamic changes within the nervous system in terms of the number and strength of synaptic connections between neurons. Neurons form intricate networks at synaptic junctions, where chemical signals sent from the axon of one neuron can send information

to countless other cells. These signals are sometimes repetitive, reinforcing the downstream effects, and other times are transient and fast-acting allowing the cell to adjust to a changing environment. These signals composed of a variety of neurotransmitters must be both released and up-taken rapidly in response to an array of stimuli. Instances of excitation and depression can cycle very rapidly and it is the role of the neuronal cell to respond quickly and appropriately to each change in stimulus. In response to activation, neurons can strengthen or weaken connections in what is termed synaptic plasticity. Although it was originally belived that synaptic strengthening played a more important role in the nervous system than synaptic weakening, investigations into the purpose of sleep have revealed that while learning and environmental stimulations increase synaptic connections during the day, sleep becomes a highly conserved and necessary behavior to refine these connections and conserve both space and metabolic resources within the brain during the night, (Maquet 2001; Tononi and Cirelli 2006). These dynamic states of neuronal connections are made possible by the dynamic nature of chromatin remodeling through post-translational histone modifications. These modifications can be maintained over long periods of time by stable marks such as DNA methylation, or rapidly cycled for succinct signals with more transient marks like histone acetylation.

The mechanisms underlying synaptic plasticity were first investigated in the sensory-motor synapses of the simple marine mollusk *Aplysia*. The regulation of synapse formation was observed both as an increase in synaptic plasticity and enhancement of synaptic transmission, termed long-term facilitation (LTF), and as a decrease in synaptic plasticity and suppression of synaptic transmission, termed long-term depression (LTD),

(Sun, Wu et al. 2001; Lee, Bailey et al. 2008). The presence of these contradictory effects occurring in the same synapses suggested for the first time that synaptic plasticity is regulated by a "reversible and bidirectional molecular switch", (Graff and Mansuy 2008).

It was later shown that induction of LTF and LTD was determined by differential activation of pathways in response to specific neurotransmitter signaling. When the facilitatory transmitter serotonin (5-HT) was received in the synapse, the transcription factor c-AMP response element binding protein 1 (CREB1) was activated recruiting the histone acetyltransferase Creb Binding protein (CBP) (Chrivia, Kwok et al. 1993; Lonze and Ginty 2002) to the promoter of the immediate early target CCCAAT/enhancer binding protein (C/EBP), (Alberini, Ghirardi et al. 1994). Acetylation of residues H3K14, H3K8, and H4K8 by CBP at the C/EBP promoter induced expression of this target, promoting enhanced synaptic transmission and resulting in LTF, (Guan, Giustetto et al. 2002; Lee, Bailey et al. 2008). Alternatively, when the inhibitory transmitter FMRFa was received, CREB1/CBP was displaced from the C/EBP promoter by CREB2, and the histone deacetylase HDAC5 was recruited to the site. Deacetylation at the C/EBP promoter by HDAC5 repressed expression of this target and resulted in suppressed synaptic transmission and LTD, (Guan, Giustetto et al. 2002). Further, the *Aplysia* model has shown that when a sensory neuron receives both of these signals simultaneously, CREB1-CBP is displaced by CREB2 and HDAC5 is recruited to silence targets, and thus decreased synaptic transmission or LTD resulted (Guan, Giustetto et al. 2002), suggesting that it is better to suppress synaptic transmission than to aberrantly enhance it. Taken together these data show that LTP and LTD induce antagonistic

effects on chromatin and result in opposing patterns of target gene expression that affect synaptic plasticity in different ways.

Similarly counteracting mechanisms were later described in the mammalian system. Excitatory synapses with increased synaptic transmission, termed long-term potentiation (LTP), and inhibitory synapses with decreased synaptic transmission, termed long-term depression (LTD) (Bliss and Lomo 1973), are conserved across species and may occur at every synapse in the brain. These mechanisms utilize various neurotransmitter signalling pathways which are thought to mediate the post-translational modifications of histone proteins that result in LTP and LTD induction. Although a neuron can rapidly cycle between LTP and LTD in response to differing stimuli, these states have also been shown to last for longer periods of time and may underlie memory formation.

Memory Formation

LTP was first observed in the mammalian brain in the hippocampus (Bliss and Lomo 1973), which is responsible for the processes governing learning and memory. In this tissue, these phenomena are dependent on activation of post-synaptic N-methyl-D-aspartate (NMDA) receptors in response to glutamate release, which allows CA^{2+} to enter the cell at the post-synaptic dendrite spine during postsynaptic depolarization, (Kauer, Malenka et al. 1988; Malenka and Nicoll 1993). Subsequent activation of the extracellular regulated kinase (ERK) and the mitogen-activated protein kinase (MAPK) signaling pathway (Harris, Ganong et al. 1984; Morris, Anderson et al. 1986; English and Sweatt 1997) was accompanied by an increase in histone H3K14 acetylation (Levenson, O'Riordan et al. 2004), and synaptic strengthening. LTP induction and the resulting

expression cascades increase with experience in the hippocampus (Foster, Gagne et al. 1996; Power, Thompson et al. 1997; Waters, Klintsova et al. 1997), and for this reason LTP has been proposed as a model to study the mechanisms of memory storage, (Bliss and Gardner-Medwin 1973). Studies of memory indicate that a similar pattern of expanding and refining synaptic connections underlies both short term memory formation and long term memory storage, although the details of these processes are less defined, (Martin, Grimwood et al. 2000; Martin and Morris 2002). It has long been accepted that epigenetic modifications underlie the changing of transcriptional patterns during development, and this has become a common theme to also describe the changing transcriptional patterns which underlie memory formation.

When neurons receive information in the form of neurotransmission, changes occur in the epigenetic landscape within the neuron, (Feng, Fouse et al. 2007; Roth and Sweatt 2009; Roth, Roth et al. 2010). This process is further complicated by the presence of multiple synapses which are all signaling to the same cell simultaneously. In mechanisms still not completely understood, the neuron is able to receive signals from multiple synapses and incorporate this information into the appropriate epigenetic changes to alter connectivity in one or all of its synapses, (Magee 2000; Allen and Barres 2005; Spruston 2008). These effects are generally short term and constantly altered in response to the changing cellular environment. Short term changes in synaptic plasticity generally affect only the stimulated synapse, while long term changes incorporate multiple signals from different synapses that affect the plasticity of all of the neuron's synapses, (Guan, Giustetto et al. 2002). Therefore activity at one synapse can affect activity at another synapse through epigenetic regulation occurring in the nucleus. The

transcriptional activation in the nucleus of the cell affects all synapses of that neuron, and this processing of various information inputs into one outcome is thought to be the mechanism underlying memory formation, (Guan, Giustetto et al. 2002).

Investigation of a signaling cascades underlying the conversion of short to long term synaptic plasticity was the first indication that synaptic plasticity may underlie memory formation and was originally observed in *Aplysia*, (Guan, Giustetto et al. 2002). It was found that when protein kinase A (PKA) was activated it translocated to the nucleus with p42 MAP kinase, resulting in CREB1 activation and expression of immediate early gene targets, which are thought to underlie memory formation because the expression of these targets leads to synthesis of proteins needed for synaptic growth and strengthening, (Kandel 2001; Guan, Giustetto et al. 2002). It was later shown that this cascade is highly conserved across species.

The complex processes of learning and memory are based on long-lasting synaptic changes. It seems that with age and cognitive disorders new memories can be made, however there is a deficit in either the maintenance or retrieval of these memories over hours, days, weeks, or longer, (Gazzaley, Sheridan et al. 2007). This implies that these defects do not lie in LTP induction so much as the "late phases" of LTP which require changes in gene and protein expression (Davis and Squire 1984; Abraham and Williams 2003; Pittenger and Kandel 2003; Lynch 2004), which are generated in a coordinated response to signals sent to the nucleus from various synapses.

The HAT activity of CREB binding protein (CBP) plays a critical and highly conserved role in LTF in *Aplysia* as well as in long-term memory formation in rodents,

(Guan, Giustetto et al. 2002; Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Wood, Kaplan et al. 2005; Miller and Sweatt 2007). CBP mutations in humans result in Rubenstein-Taybi syndrome (Petrij, Giles et al. 1995; Murata, Kurokawa et al. 2001) which is a rare condition characterized by mental retardation, and thus provide a useful model for human cognitive disease study. CBP knock-out mice were generated to investigate the role of CBP and its epigenetic HAT activity on memory formation. CBP inactivation in these mice lead to decreased acetylation of histone H2B and a loss of late phase LTP, although early phase LTP was unaffected, (Alarcon, Malleret et al. 2004). Unfortunately these mice displayed severe developmental abnormalities, (Tanaka, Naruse et al. 1997; Tanaka, Naruse et al. 2000) making it difficult to assay memory. To overcome this, three CBP defective mouse models were generated including an inducible dominant negative (Korzus, Rosenfeld et al. 2004), a heterozygous knock out (Alarcon, Malleret et al. 2004), and a forebrain targeted dominant negative (Wood, Kaplan et al. 2005), all of which showed severe memory deficits in both spatial memory in the water maze task and associative learning using contextual fear conditioning. Both forebrain targeted mutant CBP expression and heterozygous CBP knock-out mice showed normal short term memory formation but severe defects in long term memory formation as seen in various tasks including contextual fear conditioning, cued fear conditioning, and novel object recognition, (Korzus, Rosenfeld et al. 2004; Alarcon, Malleret et al. 2004).

Taken together these studies suggest that acetylation plays a critical role in memory formation. The heterozygous CBP knock out mice showed impaired induction of late phase LTP, presumably due to transcriptional defects. Mice haploinsufficient for CBP demonstrated impaired cognitive functions, altered neuronal plasticity, and changes in

histone acetylation at promoter regions of specific memory-related genes, (Korzus, Rosenfeld et al. 2004; Alarcon, Malleret et al. 2004). These studies suggest that the state of the epigenome affects induction of long term memory formation through target gene regulation. Enhancing CREB-dependent gene expression rescues memory deficits (Knoepfler and Eisenman 1999), indicating that CBP plays an important role in memory consolidation, (Knoepfler and Eisenman 1999; Kondo and Raff 2004; Seo, Richardson et al. 2005).

Contextual fear conditioning, in which animals receive an aversive stimulus in association with a novel context (Phillips and LeDoux 1992; Kim, Rison et al. 1993) is a common model to study hippocampus-dependent associative learning. In mice, contextual fear conditioning is associated with increased levels of histone H3 serine 10 phosphorylation and lysine 14 acetylation, (Levenson, O'Riordan et al. 2004; Levenson and Sweatt 2005; Chwang, O'Riordan et al. 2006). Contextual fear conditioning was also associated with increased levels of DNMT3a and DNMT3b expression, (Miller and Sweatt 2007). These results indicate that a variety of epigenetic modifications are involved in learning, including DNA methylation and histone phosphorylation and acetylation. Importantly, transcriptional silencing of the memory suppressor gene protein phosphates 1 (PP1), which is thought to play a role in memory and LTP, is induced by rapid methylation following fear conditioning, while this response induces decreased DNA methylation, methyltransferase, and demthylase activities of the synaptic plasticity related reelin gene promoter inducing its expression, (Miller and Sweatt 2007; Dong, Guidotti et al. 2007). It seems that both DNA methylation and demethylation are important in memory formation.

As memories are made with experience and not inherited, the epigenetic landscapes of neuronal cells are not inherited, they are instead shaped in response to the various environmental signals received. Recent evidence indicates that experiences an animal is exposed to early in development can be remembered and even passed on to its' own offspring in a simple form of memory termed "behavioral memory". This phenomenon has been demonstrated in rats which naturally vary in the amount of maternal grooming they give to their pups. It has been demonstrated that high levels of maternal pup licking and grooming induced DNA demethylation and histone acetylation at the glucocorticoid receptor (GR) which regulates the hypothalamic pituitary adrenal response to stress in the hippocampus (Weaver, Cervoni et al. 2004), inducing its activation (Liu, Diorio et al. 1997; Weaver, Cervoni et al. 2004). This increases the uptake of the neurotransmitter serotonin, which reduces the stress response of the pup. These gene expression changes are maintained throughout the organism's lifetime as well as the altered stress response. Less stressed animals spend more time grooming their offspring, and thus when these animals reach maturity they in turn spend more time grooming their own pups, altering their epigenetic landscapes and thus passing on the behavioral memory. Interestingly, the stress response of the rat depends on the mother raising the pups and not the birth mother (Liu, Diorio et al. 1997), further supporting that these epigenetic changes were not inherited but were dependent on behavior. This example demonstrates how experience can affect epigenetic regulation in the nervous system by altering target gene expression levels, and how this can contribute to memory formation and maintenance.

Memory formation requires synaptic remodeling which relies on the regulation of various signaling cascades in response to synaptic activation, which results in epigenetic

modifications that activate a particular set of genes while repressing others. It has previously been shown that learning and memory depend on changes in transcription levels in the hippocampus (Blalock, Chen et al. 2003; Small, Chawla et al. 2004; Rowe, Blalock et al. 2007), which are based upon rapid and dynamic changes in the epigenetic marks to regulate these targets, (Miller and Sweatt 2007). This tight regulation of transcriptional expression patterns is based on the regulation of intricate epigenetic programs, (Swank and Sweatt 2001; Guan, Giustetto et al. 2002; Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Levenson, O'Riordan et al. 2004; Huang, Fang et al. 2006; Kumar and Thompson 2005; Wood, Kaplan et al. 2005; Chwang, O'Riordan et al. 2006). The immediate early genes have been studied for their contributions to memory formation because of their rapid induction which is critical to alter synaptic plasticity in neuronal excitation pathways, (Guzowski 2002). Immediate early gene activation results in structural remodeling of the excited synapse such as formation of new dendritic spines or enlargement of old ones, enlargement of post-synaptic densities (PSDs), and PSD splitting which doubles the number of synaptic connections (Yuste and Bonhoeffer 2001; Abraham and Williams 2003) and stabilizing changes in synaptic weight (Malenka and Bear 2004), thus enhancing the sensitivity of the receiving neuron the next time the signal is received. Even when the brain is at rest, low levels of immediate early gene expression are observed, and are thought to support memory processing, (Marrone, Schaner et al. 2008). Immediate early gene targets play diverse roles in a variety of neuronal pathways making them ideal to support synaptic plasticity, (Lanahan and Worley 1998).

Among the immediate early genes involved in memory and synaptic plasticity, the most highly characterized in these processes include Arc (activity regulated cytoskeletal

gene), zif268 (nerve growth factor inducible A), and bdnf (brain-derived neurotrophic factor), (French, O'Connor et al. 2001; Hall, Thomas et al. 2001; Guzowski 2002; Steward and Worley 2001). If these targets are not activated, memory consolidation can not proceed, (Guzowski 2002). Interestingly, immediate early genes are downregulated with age making them strong candidates for the mechanisms underlying age-related cognitive decline (Blalock, Chen et al. 2003; Rowe, Blalock et al. 2007), however the mechanisms underlying the misregulation of immediate early genes with age remains unclear.

Age-related Cognitive Decline

In a recent study the gene expression profiles were determined for both young and old mice before and after contextual fear conditioning. Surprisingly, it wasn't the number of misregulated genes in the aged animals that was interesting, it was the lack of changes in gene expression patterns in response to fear conditioning. Young mice differentially expressed over 2000 targets one hour after fear conditioning, the vast majority of which were upregulated, while old mice only differentially expressed six targets, (Peleg, Sananbenesi et al. 2010). These results celarly demonstrate the defects of immediate early target expression with age. The differentially regulated targets in young mice presumably facilitated memory formation and the lack of expression of these targets in aged mice may explain their defects in memory formation. Nearly three quarters of the targets differentially regulated in young mice were involved in biological processes associated with learning such as transcription, protein modification, and intracellular signaling, further highlighting the importance in this cascade of transduction to generate

the proteins needed to form and maintain memory, (Peleg, Sananbenesi et al. 2010). Further, old mice lack initiation of these biological processes, mechanistically explaining how they are able to learn but not to remember.

Perhaps even more remarkably, a histone H4 lysine 12 acetylation modification in the coding region of these induced targets was associated with their upregulation in young mice, while this H4K12 acetyl modification was absent in aged mice suggesting that H4K12 acetylation plays a role in elongation, (Peleg, Sananbenesi et al. 2010). Interestingly, the baseline H4K12 acetylation was comparable between young and old mice prior to fear conditioning, suggesting that an increase of this mark is specific to learning and memory situations. Correspondingly, an increase in levels of the HATs MYST4 and GCN512 and the HDACs HDAC2 and HDAC4 was also observed following fear conditioning and memory formation initiation in young mice, while the levels of these chromatin modifiers stayed constant in old mice, suggesting that these epigenetic regulators may be responsible for writing the memory inducing H4K12 acetyl mark, (Peleg, Sananbenesi et al. 2010). This work suggests that the H4K12 acetyl mark is critical in activating the expression of targets necessary for forming and maintaining long term memories, and directly link histone acetylation with memory formation, but the writers, readers, and erasers involved are unknown.

Epigenetic patterns are established during development for long-term memories that must be maintained into adulthood, (Swank and Sweatt 2001; Guan, Giustetto et al. 2002; Huang, Holmes et al. 2002; Korzus, Rosenfeld et al. 2004; Levenson, O'Riordan et al. 2004; Alarcon, Malleret et al. 2004; Kumar and Thompson 2005; Levenson and Sweatt

2005; Wood, Kaplan et al. 2005; Chwang, O'Riordan et al. 2006). Traumatic injury, disease, and aging are among many factors that can permanently alter these long-lasting patterns of gene expression, causing detrimental effects on the cell by "reprogramming" its epigenetic landscape, (Borrelli, Nestler et al. 2008). It is believed that these alterations may underlie many age-related neurological dysfunctions. Recent discoveries into how epigenetic modifications affect neuronal differentiation, development, learning, and memory (Crosio, Heitz et al. 2003; Levenson and Sweatt 2005; Tsankova, Renthal et al. 2007) are beginning to describe how memories are formed and maintained within the nervous system, which helps us to understand how they are lost with age.

The nervous system is especially prone to the accumulation of epigenetic alterations because these cells are post-mitotic and are not subject to the repair and checkpoint mechanisms that keep new and dividing cells without mutations (Walter, Grabowski et al. 1997). Oxidative DNA damage is commonly observed with aging (Finkel and Holbrook 2000), and the high metabolic rate of the brain subjects it to reactive oxygen species, suggesting that the neurons are subjected to many DNA damage inducing agents, (Barja 2004; Lu, Pan et al. 2004). Epimutations occur at a higher frequency than genetic mutations, indicating that they may become increasingly detrimental with age, (Bennett-Baker, Wilkowski et al. 2003; Feil 2006; Glasspool, Teodoridis et al. 2006). It seems possible that alterations in epigenetic patterns that accumulate with age may result in a loss of these early established epigenetic memories particularly in neurons. To further explore this concept, researchers have been deciphering the "histone code for memory", (Levenson and Sweatt 2005; Wood, Kaplan et al. 2005; Chwang, O'Riordan et al. 2006; Chwang, Arthur et al. 2007; Borrelli, Nestler et al. 2008).

Aging is a dynamic process that can vary between cells or organisms. The effects of aging can be modulated through a variety of cellular pathways. The neuronal functions of learning and memory generally decline with age (Park and Reuter-Lorenz 2009) in association with changes in the nervous system, (Burke and Barnes 2006). Age-related neuronal changes therefore may underlie the loss of cognitive function observed with aging. These changes commonly include a decrease in interactions between brain regions resulting in defects in information integration. These defects may result from disruptions in the myelinated fibers that provide these connections (Andrews-Hanna, Snyder et al. 2007), a decrease in localization of neuronal activity in response to executive tasks (Cabeza 2002; Cabeza, Anderson et al. 2002; Park and Reuter-Lorenz 2009), and gene expression changes of synaptic genes, (Lu, Pan et al. 2004; Lee, Weindruch et al. 2000; Jiang, Tsien et al. 2001; Blalock, Chen et al. 2003; Erraji-Benchekroun, Underwood et al. 2005; Fraser, Khaitovich et al. 2005; Loerch, Lu et al. 2008).

Among the gene expression changes observed with age, expression of the immediate early genes which play important roles in synaptic transmission and learning and memory are the most relevant. Age-associated changes in immediate early gene expression have been detected in the hippocampus, (Yau, Olsson et al. 1996; Desjardins, Mayo et al. 1997; Small, Chawla et al. 2004). It is well-documented that these memory promoting genes are misregulated with age, (Blalock, Chen et al. 2003; Small, Chawla et al. 2004; Rowe, Blalock et al. 2007).

Since LTP induction is necessary to form new memories, it was hypothesized that LTP would decrease with age-dependent cognitive decline. Although LTP does not

appear to decrease with age, stronger stimulation as well as more instances of induction are required to achieve the same level of LTP in older animals when compared to younger animals. Therefore a common pattern observed with age is an increased LTP threshold and a decreased LTD threshold (Landfield, McGaugh et al. 1978; Barnes 1979; Deupree, Bradley et al. 1993; Moore, Browning et al. 1993; Diana, Domenici et al. 1994; Diana, Scotti de Carolis et al. 1994; Norris, Korol et al. 1996; Shankar, Teyler et al. 1998), which induces a shift from predominantly LTP to predominantly LTD, and thus a decrease in synaptic connectivity, with age. Additionally, while short term LTP lasting between 1 and 3 hours shows no difference between young and old rats (Landfield and Lynch 1977), long term LTP does decay more rapidly with age (Landfield and Lynch 1977; Landfield, McGaugh et al. 1978; Barnes 1979; Barnes and McNaughton 1985; Deupree, Turner et al. 1991; Deupree, Bradley et al. 1993; Moore, Browning et al. 1993; Shankar, Teyler et al. 1998), while LTD increases (Norris, Korol et al. 1996; Foster and Norris 1997) due to the reduced threshold. Thus with age, less activity is required to activate LTD, thus it becomes easier to initiate the cascades leading to reduction in synaptic strength, while at the same time it becomes more difficult to initiate cascades leading to increased synaptic strength. It is believed that long-lasting synaptic transmission achieved through LTP may underlie memory formation, and that the switch from LTP to LTD with age may underlie age-related cognitive decline, making these mechanisms paramount in understanding not only normal age-related cognitive dysfunction but also that associated with various neurological disorders.

LTP plays a well-characterized role in memory, and it was therefore tested whether these age-associated changes in LTP would affect memory in aged animals, (Barnes and

McNaughton 1979; Barnes and McNaughton 1980; Barnes and McNaughton 1985; de Toledo-Morrell and Morrell 1985; Bach, Barad et al. 1999). In aged animals, spatial memory performance correlated directly with the ability to enhance synaptic connections, (Barnes and McNaughton 1979). To specifically study memory and not the affects of motor ability, the water-escape task can be used on rodents for memory of novel spatial information, (Gallagher and Rapp 1997; Nunez 2008; Faes, Aerts et al. 2010). Studies show that while aged animals can learn spatial discrimination and maintain this information over short periods of time such as one minute, and only mild impairment when the task is repeated in short intervals within one day, this spatial discrimination memory decreases over hours and even more so when the interval lasts days, (Gage, Dunnett et al. 1984; Rapp, Rosenberg et al. 1987; Foster, Barnes et al. 1991; Mabry, McCarty et al. 1996; Norris and Foster 1999; Peleg, Sananbenesi et al. 2010). This shows that although the aged rodents are able to form new memories, they are unable to maintain this information over time compared to young animals. The slower learning observed in aged animals can be attributed to decreased LTP induction, and the memory deficits are likely linked to increased LTP degradation and LTD induction, (Barnes and McNaughton 1980; Barnes and McNaughton 1985; Barnes and McNaughton 1979; Martinez and Rigter 1983; Dunnett, Evenden et al. 1988; Winocur 1988; Dunnett, Martel et al. 1990; Foster, Barnes et al. 1991; Solomon, Wood et al. 1995; Mabry, McCarty et al. 1996; Colombo, Wetsel et al. 1997; Oler and Markus 1998; Norris and Foster 1999; Zornetzer, Thompson et al. 1982). This pattern of slower learning and faster forgetting holds true for a variety of tasks, (Dunnett, Evenden et al. 1988; Dunnett, Martel et al. 1990; Geinisman, Detoledo-Morrell et al. 1995; Martinez and Rigter 1983; Solomon,

Wood et al. 1995; Winocur 1988; Zornetzer, Thompson et al. 1982; Peleg, Sananbenesi et al. 2010). Interestingly, the same pattern is seen in humans with age-related memory loss that usually occurs after twenty-four hours, (Park, Royal et al. 1988; Huppert and Kopelman 1989; Mitchell, Brown et al. 1990). However, following traumatic brain injury or in dementias such as Alzheimer's disease memories often only last for minutes (Hart, Kwentus et al. 1988; Morrison and Hof 1997), indicating that other phenomena are affecting these pathways to enhance the effects of normal aging. All of these learning and memory deficits ultimately results from defects in induction and maintenance of LTP, suggesting that understanding these mechanisms will be central to understanding age-related memory loss.

Age-related Cognitive Disease

While there are many factors contributing to cognitive decline with age, the effects can be exacerbated when in conjuction with age-related cognitive disease. Disease-related factors contributing to cognitive decline generally vary by disorder, although many involve dietary, environmental, and genetic factors. Most cognitive disorders are genetically complex and rely on complex neuronal pathways so they have been difficult to decipher. Promising research into epigenetic mechanisms underlying these puzzling disorders have shed light on the underlying mechanisms resulting in disease, where immediate early gene regulation is likely involved. These studies have opened the door to novel treatment paths for these currently untreatable disorders.

Alzheimer's disease (AD) is the most common dementia, for which the highest risk factor is age. We know that there are changes in the epigenome with age indicating that

these effects may underlie the pathogenesis of AD, but currently the age-related changes in the epigenome that occur in AD are only beginning to be explored. For example, age-dependent drifts in DNA methylation have been observed in brains of AD patients which are more pronounced than those observed in other tissues, (Wang, Oelze et al. 2008; Mastroeni, Grover et al. 2010). However, DNA methylation patterns change both with AD and in normal aging (Siegmund, Connor et al. 2007), making it difficult to determine what is normal aging and what is disease-related.

In addition to changes in DNA methylation, changes in histone acetylation have also been observed in AD. Several of the mutations linked to the onset of AD include mutations in the presenilin 1gene (PS1) which make the catatlytic units of the γ-secretase cleavage complex that is critical in proper neuronal functioning, (Russo, Schettini et al. 2000). These mutations in PS1 have been linked to increased activity of the HAT CBP, suggesting that acetylation may play a role in AD, (Marambaud, Wen et al. 2003). These studies have been carried out in the mouse forebrain with PS1 and PS2 mutants which results in severe memory deficits, neurodegeneration, and decreases in CBP activity, (Saura, Choi et al. 2004). The decrease in CBP has further been linked to decreases in expression of the immediate early targets c-fos and Bdnf (Saura, Choi et al. 2004), supporting decreased LTP and further supporting a role for this modifier in memory defects in AD.

Another HAT, Tip60 has also been implicated in epigenetic mechanisms underlying AD. Tip60 has been shown to form a transcriptionaly active complex with the C-terminus of the AD related amyloid precursor protein (APP), (Cao and Sudhof 2001).

The transcriptional targets of this complex may be misregulated in AD patients, contributing to the observed pathologies. Currently these targets or the mechanism of their misregulation is not clear, but a better understanding of the underyling changes in transcriptional regulation may provide novel therapeutic avenues to treat this disease. Importantly, AD mediated cell death results in decreased acetylation of the the CBP substrate histone H3 and Tip60 substrate histone H4 (Ikura, Ogryzko et al. 2000; Rouaux, Jokic et al. 2003), further supporting a role for these histone acetyltransferas enzymes in the neuronal processes underlying AD related memory deficits.

Other neuronal diseases have also been attributed to epigenetic mechanisms. Rett syndrome which is an X-linked disease characterized by mutations in the methyl CpG-binding protein 2 (MeCP2), (Amir, Van den Veyver et al. 1999; Wan, Lee et al. 1999; Amir and Zoghbi 2000). MeCP2 binds methylated DNA and recruits modifying enzymes to suppress transcription, (Amir, Van den Veyver et al. 1999; Collins, Levenson et al. 2004). Over-expression of MeCP2 enhances long term memory formation and induces LTP in the hippocampus through transcriptional regulation, (Collins, Levenson et al. 2004). Fragile X retardation results from a trinucleotide repeat expansion in the fmr1 or fmr2 gene (Gatchel and Zoghbi 2005) both of which have polymorphic trinucleotide repeats in the 5'UTR which are responsible for loss of gene expression, (Cummings and Zoghbi 2000). Expansion of these repeats causes hypermethylation of regions flanking CpG islands silencing fmr and surrounding genes, (Knight, Flannery et al. 1993). These studies indicate that epigenetic mechanisms when misregulated may contribute to complex neuronal pathologies.

Epigenetically-based Treatment of Cognitive Dsyfunction

Currently there are very few effective treatments for cognitive dysfunction. One group of pharmacological compounds currently under consideration for their uses in improving cognitive deficits are the histone deacetylase inhibitors which promote HAT activity by inhibiting the activity of HDACs. It is thought that these compounds might promote immediate early gene expression as these targets are commonly reduced in cases of memory dysfunction. In a wide range of studies, broad-spectrum HDAC inhibitors have been shown to promote LTP and facilitate memory formation, (Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Chwang, O'Riordan et al. 2006; Levenson, O'Riordan et al. 2004; Bredy, Wu et al. 2007; Chwang, Arthur et al. 2007; Miller and Sweatt 2007; Lattal, Barrett et al. 2007; Lubin and Sweatt 2007; Vecsey, Hawk et al. 2007; Bredy and Barad 2008). Specifically, HDAC inhibitiors such as trichostatin A (TSA), sodium butyrate, and suberoylanilie hyroxamic acid (SAHA) have been shown to enhance LTP in mammalian neurons (Levenson, O'Riordan et al. 2004), rat brains (Levenson, O'Riordan et al. 2004), and CBP HAT defective mice, (Alarcon, Malleret et al. 2004). Importantly, these compounds rescue memory defects in CBP deficient mice which were unable to make long term memories, and even enhance long term memory formation in wild type mice, (Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Levenson, O'Riordan et al. 2004; Wood, Kaplan et al. 2005). These memory improvements may result from re-regulation of CRE-containing genes as was observed in hippocampal slices after fear conditioning when systemic administration of the HDAC inhibitor TSA induced upregulation of some of these targets, (Vecsey, Hawk et al. 2007). The rescue of memory defects by HDAC inhibitors may also be based on a re-

establishment of H4K12 acetylation by preventing its deacetylation, (Peleg, Sananbenesi et al. 2010). Recent studies have shown that treatment of aged mouse hippocampus with the HDAC inhibitor SAHA increased memory associated with H4K12 acetylation and rescued behavioral memory deficits of these animals, partially rescuing observed age-related defects in gene expression, (Peleg, Sananbenesi et al. 2010). These results suggest that HDAC inhibitors epigenetically treat memory defects by reverting gene expression patterns back to a younger state. Animal models of Huntington's disease show a suppression of motor deficits and neurodegeneration when treated with HDAC inhibitors, (Ferrante, Kubilus et al. 2003; Hockly, Richon et al. 2003). Although HDAC inhibitors have shown promising effects in the treatment of a variety of human diseases including Huntington's, Parkinson's, stroke, amyotrophic lateral scleroris, spinal muscular atrophy, and Alzheimer's diseases (Chuang, Leng et al. 2009), in clinical trials with cancer patients they have shown strong negative side effects, (Bruserud, Stapnes et al. 2007). The development of more specifically acting histone deacetylase inhibitors may provide a novel therapeutic alternative for these and many other diseases.

Conclusions

Neuronal functioning relies on the complex synaptic networks generated between neurons through which these neurons communicate. External stimuli, environmental changes, and learning are among the factors that affect the dynamics of these synaptic interactions by strengthening and weakening the connections in what is termed synaptic plasticity. These affects can either enhance synaptic plasticity in response to facilitatory

neurotransmitters in long-term potentiation (LTP), or decrease synaptic plasticity in response to inhibitory neurotransmitters in long-term depression (LTD). Short term changes in synaptic plasticity affect only the receiving synapse, while long term changes in synaptic plasticity involve the incorporation of all of the signals received and results in changes in the structure of all of the cell's synapses. These changes in synaptic plasticity have recently been show to rely on the epigenetic regulation of immediate early target gene expression. This regulation is initiated by neurotransmitter uptake which induces chemical changes within the receiving neuron which initiates signal cascades by activating enzymes such as kinases. These enzymes can in turn activate chromatin regulatory enzymes, which modify the N-terminal tails of histone proteins resulting in the altered regulation of chromatin packaging and thus transcription. The histone modifications made by these regulators can rapidly alter the expression of immediate early genes, facilitating dynamic changes in synaptic plasticity that may last briefly or be maintained over long periods of time.

It is well documented that deficits in neuronal behavior arise with age, including defects in learning and memory. These defects are directly linked to an age-associated increases in LTP and decrease in LTD thresholds, resulting in promotion of LTD and decay of LTP. These shifts are reflected in global decreases in synaptic plasticity. These alterations are likely based on changes in gene regulation resulting from defects in the activation or activity of epigenetic histone modifying enzymes. In support of this theory, age-associated changes in the memory promoting immediate early gene expression have been observed, (Lloyd, Hoffman et al. 1994; Blalock, Chen et al. 2003; Burke and Barnes 2006). Importantly, identifying the epigenetic modifiers responsible for these age-

associated gene regulation defects, and the pathways leading to the changes in their activation, is paramount in understanding memory formation and maintenance.

Although age-associated deficits in memory occur normally, these deficits are enhanced in age-related dementias such as Alzheimer's Disease. It is likely that similar pathways are misregulated in diseased brains as are in normal aging, however the alterations in modifier activation and thus gene regulation may be more severe. In support of this theory that epigenetic changes underlie dementia, shifts in histone acetylation patterns have been observed in Alzheimer's disease with decreased acetylation of histones H3 and H4. This activity has been linked to the histone acetyltransferase activity of the HAT CBP as mutations in the catalytic components of the APP cleaving γ-secretase complex which have been found in Alzheimer's disease result in memory deficits, neurodegeneration, and decreases in CBP activity, (Saura, Choi et al. 2004). Further, these effects have been linked to decreases in expression of the memory-promoting immediate early genes, (Saura, Choi et al. 2004). Taken together, these results indicate that histone aceylation by CBP, and possibly other HATs, is altered with age and age-related dementia, leading to defects in neuronal gene regulation.

Another HAT thought to play a role in neuronal gene regulation contributing to Alzheimer's disease pathogenesis and age-related memory deficits is the Tat-interactive protein, 60kD (Tip60). Tip60 is an essential HAT that has been identified as a genetic hub gene involved in the regulation of a wide span of biological processes. Tip60 plays important roles in apoptosis, DNA repair, and cell cycle progression making it an important target in various human diseases. Tip60 has been shown to interact with the C-

terminus of the Alzheimer's disease related APP protein (AICD) in a transcriptionally active complex, although the mechanism of their interaction as well as the transcriptional targets regulated are controversial. It is tempting to speculate that misregulation of Tip60 or AICD complex components could alter the histone modifying activity of the complex, resulting in inappropriate marks on the chromatin that may lead to misregulation of immediate early targets. It is possible that this happens normally with aging and is enhanced in disease states. Alternatively in disease, the marks that are made by this complex earlier in development that need to be maintained are lost, resulting in target misregulation. Elucidating the role that Tip60 plays in the nervous system may help us to better understand the epigenetic mechanisms underlying memory formation and maintenance, as well as the mechanisms underlying age-related memory deficits.

Summary of Following Chapters

Epigenetic regulation has been shown to play an important role in the neuronal processes of learning and memory however the connections between specific chromatin regulators and neuronal processes are only beginning to be elucidated. Additionally, epigenetic misregulation of targets has been oberved with age however the connections between these epigenetic changes and age-related neuronal deficits have yet to be explored. The goal of my thesis was to explore the role of the histone acetyltransferase Tip60 in neuronal development and in interaction with the amyloid precursor protein (APP) with which Tip60 is thought to play a transcriptionally active role. Aberrant APP expression and processing have been linked to Alzheimer's Disease pathology, indicating that this complex might play important roles in memory formation and maintenace, and age-related neuronal deficits. To explore the epigenetic roles of Tip60 in the nervous system, I have characterized a novel transgenic *Drosophila* line that is mutant specifically for the HAT activity of Tip60. I have utilized this line to investigate the role of Tip60 in multicellular development, and I have further exploited this model to generate an interaction model to explore a genetic interaction between Tip60 and APP in the nervous system. Further, my research utilizing the γ-secretase inhibitor DAPT in this system indicates that the defects resulting from misregulation of the Tip60/APP interaction may be suppressed with this compound. In addition to answering the questions posed in my thesis, I have also contributed significantly to two additional projects involving other chromatin regulators including the demethylase Dmel\Kdm4A and the histone acetyltransfersae Dmel\Elp3, discussed in the Appendix.

Chapter 2: Microarray analysis uncovers a role for Tip60 in the nervous system and in general metabolism. Meridith Toth Lorbeck, Keerthy Pirooznia, Jessica Sarthi, Xianmin Zhu, and Felice Elefant. Tip60 has recently been identified as a genetic hub implicating this chromatin modifier in a variety of biological proesses, however the multicellular developmental roles of Tip60 have not previously been investigated. This manuscript describes the characterization of a transgenic *Drosophila* line generated with a single amino acid substitution within the HAT domain of Tip60 that produces a dominant negative effect in these flies that are specifically defective for Tip60 histone acetyltransferase activity. This line was utilized in microarray analysis to explore the epigenetic roles of Tip60 in development. We found a strong enrichment of targets acting in metabolic pathways, indicating that Tip60 plays an important role in general metabolism. Additionally we observed a tissue-specific role for Tip60 in the regulation of neuronal targets, suggesting that Tip60 plays an important role in neuronal development. This manuscript is currently under review at PLOS One.

Chapter 3: Tip60 and APP genetically interact to promote apoptosis-driven neurodegeneration. Meridith Toth Lorbeck, and Felice Elefant. Upon identifying a tissue-specific role for Tip60 in the nervous system, we chose to begin investigating the processes affected by Tip60 in this tissue through a proposed interaction between Tip60 and the neuronally important amyloid precursor protein (APP) which had previously been identified *in vitro*. This manuscript describes the investigation of a genetic interaction between Tip60 and APP in the multicellular developmental system of *Drosophila melanogaster*. The Tip60 HAT mutant fly line described in the previous chapter was utilized to generate interaction fly lines expressing both the mutant Tip60 and either APP

or APP lacking the Tip60 interacting C-terminus (APPΔCT). We found a genetic interaction between these targets ubiquitously, in neuronal development, and specifically in the promotion of apoptosis in the central nervous system. Importantly, all of these interactions were dependent on the presence of the toxic C-terminus of APP.

Chapter 4: Pharmacological inhibition of AICD generation rescues APP-induced neuronal defects. Meridith Toth Lorbeck, Emily Mazanowski, Thomas Paul, Olga Yarychkivsak, and Felice Elefant. We have shown a dependence of Tip60 activity on AICD in the nervous system, and this prompted us to ask if pharmacological prevention of AICD production could suppress these negative phenotypes. This manuscript describes the use of DAPT, a γ-secretase inhibitor, in *Drosophila* to rescue APP-induced neuronal defects. We found that APP over-expression induced a locomotor deficit consistent with axonal transport defects, as well as target gene misregulation in *Drosophila* larvae, and that DAPT suppressed these defects. These are the results of an undergraduate project which I have been superivising for two years.

Chapter 5: Conclusions and Future Directions. This chapter includes a summary of the conclusions made in the previous chapters, and explores potential experimental avenues that could be taken from this work in the future.

Chapter 6: Tables and Figures. This chapter contains the tables and figures for Chapters 2, 3, and 4.

Appendix A: The histone demethylase Dmel/Kdm4A controls genes required for lifespan and male-specific sex-determination in *Drosophila*. Meridith Toth Lorbeck*, Neetu Singh*, Ashley Zervos, Madhusmitta Dhatta, Maria Lapchenko, Chen Yang, and

Felice Elefant. (*) These authors contributed equally to this work. This chapter describes the characterization of the histone demethylase Dmel/Kdm4A utilizing a p-element insertion line disrupting the Dmel/Kdm4A gene. We found that this disruption generated a twitching phenotype and increased male longevity, which resulted from misregulation of targets involved in lifespan and male-specific sex-determination. This manuscript was published in Gene, 2010.

Appendix B: The histone acetyltransferase Elp3 plays an active role in the control of synaptic bouton expansion and sleep in *Drosophila*. Neetu Singh, Meridith Toth Lorbeck, Ashley Zervos, John Zimmerman, and Felice Elefant. This chapter describes the investigation of the roles of the histone acetyltransferase Elp3 in sleep and activity, as well as in synaptic bouton formation at the neuromuscular junction utilizing a novel Dmel\Elp3/RNAi model. This manuscript was published in the Journal of Neurochemistry, 2010.

Appendix C: Tables and Figures. This chapter contains the tables and figures for appendices A and B.

Chapter 2: Microarray analysis uncovers a role for Tip60 in the nervous system and in general metabolism.

Abstract

Tip60 is a key histone acetyltransferase (HAT) enzyme that plays a central role in diverse biological processes critical for general cell function, however the chromatin-mediated cell-type specific developmental pathways that are dependant exclusively upon the HAT activity of Tip60 remain to be explored. Here, we investigate the role of Tip60 HAT activity in transcriptional control during multicellular development, *in vivo* by examining genome-wide changes in gene expression in a *Drosophila* model system specifically depleted for endogenous dTip60 HAT function. We show that amino acid residue E431 in the catalytic HAT domain of dTip60 is critical for the acetylation of endogenous histone H4 in our fly model *in vivo,* and demonstrate that dTip60 HAT activity is essential for multicellular development. Moreover, our results uncover a novel role for Tip60 HAT activity in controlling neuronal specific gene expression profiles essential for nervous system function as well as a central regulatory role for Tip60 HAT function in general metabolism.

Introduction

The Tat-interactive protein-60 KDa (Tip60) is one of the founding members of the MYST histone acetyltransferase (HAT) super family, (Yamamoto and Horikoshi 1997; Kimura and Horikoshi 1998; Sterner and Berger 2000; Marmorstein 2001; Marmorstein

and Roth 2001; Roth, Denu et al. 2001; Utley and Cote 2003), first identified based on its interaction with the human immunodeficiency virus, type 1-encoded transactivator protein Tat, (Kamine 1996). Tip60 has been reported to play essential roles in a wide variety of cellular processes based upon the different protein complexes it is transiently associated with. The majority of cellular Tip60 protein purifies as part of a stable and conserved multimeric Tip60 protein complex containing at least 18 subunits, (Ikura, Ogryzko et al. 2000; Sapountzi, Logan et al. 2006). Importantly, this Tip60 complex is evolutionarily conserved from *Saccharomyces cerevisiae* to *Drosophila* to humans (Cai, Jin et al. 2003; Ceol and Horvitz 2004; Doyon, Selleck et al. 2004; Kusch, Florens et al. 2004; van Attikum and Gasser 2005; Zhu, Singh et al. 2007), making it amenable for functional characterization using multiple model systems. Such studies have revealed that a number of the Tip60 interacting protein partners within the Tip60 complex are specifically required for the diverse and general cellular processes that Tip60 regulates, including cell cycle and checkpoint control, apoptosis, and DNA damage repair, (Ikura, Ogryzko et al. 2000; Yan, Barlev et al. 2000; Sheridan, Force et al. 2001; Kinoshita, Whelan et al. 2002; Cai, Jin et al. 2003; Berns, Hijmans et al. 2004; Downs, Allard et al. 2004; Doyon and Cote 2004; Kusch, Florens et al. 2004; Legube, Linares et al. 2004; Li, Cuenin et al. 2004; Taubert 2004; Sun, Jiang et al. 2005; Tamburini and Tyler 2005; Murr, Loizou et al. 2006; Sapountzi, Logan et al. 2006; Squatrito 2006; Tyteca, Legube et al. 2006; Tyteca, Vandromme et al. 2006). Tip60 can also be recruited to the promoters of specific target genes *via* its transient interaction with a variety of different transcription factors to either activate or repress gene expression, (Sapountzi, Logan et al. 2006; Squatrito 2006). Activation requires the epigenetic HAT function of Tip60, which

acts to acetylate the nucleosomal histones of target genes, (Sterner and Berger 2000; Strahl and Allis 2000; Turner 2002; Fischle, Wang et al. 2003). Acetylation promotes chromatin disruption that in turn facilitates additional factor binding and transcriptional activation, (Grunstein 1997; Struhl 1998; Sterner and Berger 2000; Strahl and Allis 2000; Turner 2000; Turner 2002). Repression is thought to be independent of Tip60 HAT activity, and may result from its interaction with transcriptional silencers and histone deacetylases, (Gaughan, Logan et al. 2002; Xiao, Chung et al. 2003; Doyon and Cote 2004). Tip60 HAT activity also functions to directly acetylate certain transcription factors (TFs), which serves to activate or repress their respective gene regulatory functions, (Brady, Ozanne et al. 1999; Sterner and Berger 2000; Gaughan, Brady et al. 2001; Gaughan, Logan et al. 2002; Halkidou, Logan et al. 2004; Patel, Du et al. 2004; Glozak, Sengupta et al. 2005; Sun, Jiang et al. 2005; Sapountzi, Logan et al. 2006; Squatrito 2006; Sykes 2006; Tang, Luo et al. 2006; Spange, Wagner et al. 2009).

Experiments coupling chromatin immunoprecipitation (ChIP) with hybridization of oligonucleotide arrays in *Saccharomyces cerevisiae* demonstrate that Esa1, the yeast Tip60 homolog, is recruited to the promoters of virtually all active protein-coding genes, (Robert, Pokholok et al. 2004). However, a similar role for Tip60 in general gene activation remains to be determined during metazoan development, where robust and preferential Tip60 protein localization profiles in the developing myocardium and brain in chicken and mouse (Lough 2002) have been observed, and Tip60 cell type specific activity and preferential brain and heart tissue-specific expression patterns have been reported, (Hlubek, Lohberg et al. 2001; McAllister, Merlo et al. 2002; Sykes 2006; Tyteca, Legube et al. 2006; Tyteca, Vandromme et al. 2006; Zhu, Singh et al. 2007).

Indeed, studies in mammalian cells have revealed that Tip60 transiently associates with a growing list of specific transcription factors with which it acts as a coactivator (Kamine 1996; Brady, Ozanne et al. 1999; Gaughan, Brady et al. 2001; Kinoshita, Whelan et al. 2002; Frank, Parisi et al. 2003; Halkidou, Logan et al. 2004; Panchenko, Zhou et al. 2004; Barron, Belaguli et al. 2005) or coreprepressor (Gavaravarapu and Kamine 2000; Nordentoft and Jorgensen 2003; Xiao, Chung et al. 2003) in the regulation of specific target genes. Notably, TIP60 was recently identified as one of the six 'hub' genes uncovered in a large-scale genetic interaction screen in *C. elegans*, which were characterized by their ability to interact with multiple other genes and with all of the developmental signaling pathways screened in the study, (Lehner, Crombie et al. 2006). Moreover, RNAi depletion studies of Tip60 in an embryonic stem cell (ESC) line demonstrated that Tip60 represses a large number of developmental genes essential for embryonic stem cell (ESC) differentiation, and as such, identified Tip60 as a regulator of ESC identity, (Fazzio, Huff et al. 2008). However, despite the undisputed central role that Tip60 plays in the regulation of general developmental gene control, the question of whether the epigenetic HAT activity of Tip60 is required for differential tissue-specific gene expression profiles essential for organismal development remains to be explored.

Here, we investigate the role of Tip60 HAT function in transcriptional control during multicellular development *in vivo* by examining genome-wide changes in gene expression in a *Drosophila* model system specifically depleted for endogenous dTip60 HAT function. Our results support a critical role for dTip60 catalytic HAT residue E431 in the acetylation of endogenous histone H4 in our *in vivo* fly model, and demonstrate that dTip60 HAT activity is essential for multicellular development. Moreover, our

results uncover a novel role for Tip60 HAT activity in controlling neuronal specific gene expression profiles essential for nervous system function as well as a central regulatory role for Tip60 HAT function in general metabolism.

Materials and Methods

Construct generation:

*Mutagenesis***:** Alignments were generated using the BLASTn algorithm from NCBI with sequences corresponding to dTIP60 (NP_572151.1) and ESA1 (NP_014887.1), and these alignments were used to identify dTIP60 amino acid position E431 as the residue corresponding to the catalytic core E338 residue in yeast ESA1. A single base substitution was sufficient to change the codon from GAG to CAG, converting this glutamate to a glutamine. This mutation was incorporated into dTIP60 cDNA construct (Zhu, Singh et al. 2007) using the PCR based Quickchange Site Directed Mutagenesis Kit, (Quigen, Almeda, CA, USA). Forward and reverse PCR primers used were 5'GGCAAGACGGGATCGCCG**CAG**AAACCATTGTCTGATC3' and 5'GATCAGACAATGGTTT**CTG**CGGCGATCCCGTCTTGCC3' respectively, with the mutated codon shown in bold. PCR reactions for the mutant strand synthesis reaction contained 25ng of dTip60 template DNA, 125ng each of forward and reverse primer, and *PfuTurbo* DNA polymerase, (Stratagene, La Jolla, CA, USA). The cycling parameters were 15 cycles of 95° for 30 seconds, 55° for 1 minute, and 68° for 12 minutes using an Eppendorf Mastercycler, (Eppendorf, Madison, WI, USA). Non-mutated methylated parental strands were digested using DpnI (New England Biolabs, Inc., Ipswich, MA,

USA) and nicks were repaired upon transformation into DH5α super competent cells, (Invitrogen Corporation, Carlsbad, CA, USA). The entire dTip60^{E431Q} construct was sequenced by the University of Pennsylvania DNA Core Sequencing Facility (Philadelphia, PA, USA) for verification of the final construct.

Cloning procedures: The dTIP60^{E431Q} construct was subcloned into the pUAST GAL4 inducible expression vector (Brand and Perrimon 1993) as follows. The full open reading frame (ORF) of dTIP60 containing the E431Q mutation was amplified by PCR using forward primer 5'-CGG C*GA ATT C*GC CAA CAT GAA AAT TAA CCA CAA ATA TGA G-3' containing an EcoRI site (italics), a KOZAC sequence (underlined), and a sequence corresponding to the first eight codons of dTIP60, and reverse primer 5'-GGT T*GG TAC C*TC ATC ATC ATT TGG AGC GCT TGG ACC AGT C-3' containing a Bam HI restriction site (italics), two in-frame stop codons (underlined), and the last eight codons of dTIP60, (Zhu, Singh et al. 2007). PCR reactions were carried out with the Expand High Fidelity PCR system (Roche, Nutley, NJ, USA) using 400nM of each forward and reverse primer and cycling parameters of 30 cycles of 95°C for 2 min, 55°C for 1 min, and 72°C for 4 min, using an Eppendorf Mastercycler (Eppendorf, Madison, WI, USA). After digestion and ligation into the pUAST vector, the entire dTIP60^{E431Q} insert was sequenced by the University of Pennsylvania DNA Core Sequencing Facility (Philadelphia, PA, USA) for verification of the final construct.

***Drosophila* stocks:** P-element germline transformations were performed by Rainbow Transgene (Newbury Park, CA, USA) to generate multiple independent fly lines containing either the dTip60^{E431Q} or dTip60^{WT} transgenes. The ubiquitous driver was

GAL4 line 337, (Elefant and Palter 1999). The neuronal drivers used were pan-neuronal drivers elav-GAL4, Bloomington Stock Center no. 8760 or 8765 (Jones, Fetter et al. 1995; Rebay and Rubin 1995; Berger, Renner et al. 2007) , and 179y-GAL4, Bloomington Stock Center no. 3733 (Manseau 1997; Gunawardena and Goldstein 2001), and CNS specific 60IIa-GAL4, Bloomington Stock Center no. 7029, (Shilova, Garbuz et al. 2006; Chan and Kravitz 2007; Zhu, Singh et al. 2007). All crosses were performed in triplicate using ten newly eclosed virgin females and five males in narrow plastic vials (VWR International, West Chester, PA, USA) with yeasted *Drosophila* media (Applied Scientific Jazz Mix *Drosophila* Food, Thermo Fisher Scientific, Waltham, MA, USA) at 25°C.

Quantitative Real Time RT-PCR: Total RNA was isolated from staged three day old larvae using Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and treated twice with Dnase II (Ambion, Austin, TX) to remove DNA. Complementary DNA (cDNA) was synthesized from 1ug total RNA and oligo-dT primers using Superscript II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Real-time quantitative PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Power SYBR Green PCR master mix (Applied Bioystems, Foster City, CA, USA). All PCR reactions were carried out in triplicate in 20ul reaction volumes containing 1ng cDNA template and 500nm each of forward and reverse primer designed using the NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). Forward and reverse primer sets designed to amplify a 97bp non-conserved region of dTIP60 were 5'GACGGCTCACAAACAGGC3' and 5'GGTGTTGCGGTGATGTAGG, respectively. Forward and reverse primers designed to amplify a 105bp region within the

5'UTR region of dTIP60 were 5’CAGTTGTGGTCACAATTACCC3’ and 5’GTGCGCAGAAAGTTATACAGC3’, respectively. PCR was carried out by 40 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min with plate readings recorded after each cycle. Threshold cycle (Ct) values were obtained, and the comparative Ct method was used as previously described (Bookout and Mangelsdorf 2003) to calculate the fold difference in transcript level of the sample relative to the control. RP49 which encodes the ribosomal protein L32 was used as an internal standard and reference gene using forward and reverse primer pairs 5’CTGCTCATGCAGAACCGCGT3’ and 5’GGACCGACAGCTGCTTGGCG3’, respectively.

Microarray:

Probe preparation and microarray experiment: Samples were collected from wild type w^{1118}, dTip60^{E431Q}B, and dTip60^{WT}B lines crossed to the ubiquitous 337-GAL4 driver. Two samples from each genotypic cross, each containing a pool of thirty-five staged three day old whole larvae, were collected and flash frozen in liquid nitrogen. Total RNA was extracted from each of these sample pools using Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and treated two times with Dnase II (Ambion, Austin, TX) to remove genomic DNA. Each of these sample pools was used to probe a separate microarray chip, and thus the mean expression values for each of the three genotypic groups analyzed is the average of 70 individual larvae. Complementary DNA (cDNA) was synthesized from 1ug total RNA using oligo-dT primers and Superscript II Reverse Transcriptase, (Invitrogen Corporation, Carlsbad, CA, USA). RNA quality check, target labeling, GeneChip hybridization, and oligonucleotide microarray scanning were carried

out at Seqwright (Houston, TX, USA) on the GeneChip *Drosophila* 2.0 Array (Affymetrix, Santa Clara, CA) following a standard Affymetrix protocol.

Data Analysis: Affymetrix GeneChip Operating Software (GCOS) was used to quantitate each GeneChip to produce a .CEL file. GeneChip .CEL files were loaded into DNA-Chip Analyzer (dCHIP) (Li and Wong 2001); http://www.dchip.org) for normalization to reduce technical variation between chips, standardization to reduce variance of expression level estimates by accounting for probe differences, and analysis using model-based expression indexes (MBEI). Correlation coefficients calculated in dCHIP showed significant agreement between duplicate samples for all three genotypes analyzed, validating the consistency of the microarray data for each of the three genotypes analyzed. The dCHIP t-test function was used to identify genes whose expression differed significantly ($p<0.05$) between either dTIP60^{E431Q}B or dTIP60^{WT}B compared to the wild type control, and these genes were then filtered to select for those that showed a twofold or greater change and a 90% confidence bound of fold change. Genes were annotated and biological processes were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID), (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). Significance of overrepresentation of Gene Ontology (GO) terms was determined by a p-value less than 0.05. A number of significantly misregulated gene targets identified by microarray analysis were validated using qPCR of selected genes using aliquots of the same sample pools prepared for probe labeling and primer sets designed by NCBI/Primer-BLAST, (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers are available upon request. All data is MIAME compliant and has been

deposited into the MIAME compliant data base Gene Expression Omnibus (GEO), accession number GSE25635.

Immunohisochemistry and confocal microscopy: w^{1118} embryos were collected and staged over 15-17 hours, dechorionated, fixed in 4% paraformaldehyde and devitellanized. The fixed embryos were incubated with primary antibody overnight. Stained embryos were washed with 1XPBS-T (0.1% Tween) six times over a three hour period (30 minutes each) and were next incubated with the appropriate secondary antibodies for 3 hours at room temperature. Embryos were washed six time over a 3 hour period (30 minutes each) with PBST. The embryos were next mounted onto slides and imaged using the FV1000 Laser Scanning Confocal Microscope. The following antibodies were used for staining: rabbit polyclonal anti-Tip60 (1:100) (custom made Tip60 peptide antibody generated by Strategic Diagnostics, Newark, DE, USA; www.sdix.com), FITC tagged goat anti-horse radish peroxidase (HRP) (1:25; Jackson Immunoresearch, West Grove, PA, USA). Anti-rabbit fluorescent antibody Alexa Flour 647 for Tip60 visualization was obtained from Invitrogen.

Western Blot: Histones were isolated from 50 staged second instar larvae using a modified acid extraction protocol, (Gorski, Romeijn et al. 2004). Larvae were homogenized in hypotonic buffer containing 10 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl in the presence of protease inhibitors and 10 mM Sodium butyrate. After 10 minutes on ice, 10 µl of 10% TritonX-100 was added and the solution was briefly vortexed. Following a 15 second centrifugation, the nuclei were resuspended in 40 µl of

nuclear wash buffer (15 mM Tris–HCl (pH 7.4), 60 mM KCl and 15 mM NaCl). Histones were extracted in the presence of 0.4 M HCl for 1 hour on ice with regular shaking. After centrifugation, acid-soluble proteins were precipitated with Trichloroacetic acid, washed twice with acetone, air-dried, and resuspended in 50 µl of SDS sample buffer. Equal amounts of protein as quantitated by using a protein assay kit (Thermo Scientific, Waltham, MA, USA) were loaded onto an 18% SDS PAGE gel (29:1 acrylamide/bisacrylamide). Protein samples were denatured at 95 °C for 15 minutes prior to loading. The fractionated proteins were electro-blotted onto a nitrocellulose membrane, (Biorad, Hercules, CA, USA). The membrane was blocked with 3% BSA for 2 hours at room temperature and then incubated overnight at 4^{o}C with a primary antibody (Abd Serotec, Raleigh, NC, USA, AHP418) that recognizes four acetylated lysine residues (K5, K8, K12 and K16) of histone H4. The membrane was washed three times with 0.1% TBST (50 mM Tris-Hcl (pH 7.4), 150 mM NaCl, 0.3% Tween 20) and incubated with secondary antibody for 1 hour at room temperature. The membrane was washed three times with 0.1% TBST. Western detection was done using chemiluminiscence, (ECL kit, Thermo Scientific, Waltham, MA, USA). Signals were quantitated using a Fluorchem imager, (Alpha Innotech, San Leandro, CA, USA).

Results

Expression of mutant HAT defective dTIP60^{E431Q} produces a dominant negative lethal effect during *Drosophila* multicellular development. We previously identified and cloned the human homologue of TIP60 in *Drosophila,* referred to as Dmel\TIP60

(Zhu, Singh et al. 2007; Tweedie, Ashburner et al. 2009) or dTip60 (Kusch, Florens et al. 2004) and demonstrated by GAL4 targeted RNAi knockdown technology (Brand and Perrimon 1993) that ubiquitous reduction of endogenous Dmel\TIP60/RNAi in the fly results in lethality, (Zhu, Singh et al. 2007). These results support an essential role for the dTip60 protein in multicellular development. To extend these studies, and investigate the epigenetic dependency of fly development on Tip60 HAT function, we set out to create a fly line producing GAL4 inducible dominant negative acting dTip60 proteins specifically defective in their catalytic HAT activity. The mutant dTip60 construct was created by introducing a specific amino acid substitution E431Q into the conserved enzymatic HAT domain of dTIP60 that corresponds to mutation E338Q in the yeast Tip60 homolog Esa1, (Figure 1, A and B). Importantly, the Esa1(E338Q) mutant protein has been shown to retain proper protein folding, exhibit substantially reduced HAT activity, and demonstrate a dominant negative effect in yeast cells, (Yan, Barlev et al. 2000). Flies were transformed with dTIP60^{E431Q} within a GAL4 inducible pUAST construct, and two independently derived transgenic fly lines were chosen for initial characterization. The insertions were homozygous viable, and did not cause any observable mutant phenotypes in the absence of GAL4 induction.

The amino acid residue E338 in the catalytic HAT domain of the yeast Esa1 protein is thought to function as a general base for catalysis, however a conserved function for this residue in the Tip60 protein of multicellular organisms was unknown. To determine whether dTip60^{E431Q} would cause a dominant negative effect during fly development, we induced expression of either mutant dTip60^{E431Q} (independent lines A and B) or exogenous wild-type dTip60 designated dTip60^{WT} (independent lines A and B) at 25^{o}C

using the GAL4 driver 337, (Elefant and Palter 1999). This driver produces robust and ubiquitous GAL4 production beginning during late embryonic development and continuing into adulthood. The w^{1118} fly line crossed to 337-GAL4 served as a control. We found that control flies as well as two independent fly lines each expressing exogenous wild-type $dTip60^{WT}$ all exhibited normal phenotypes. However, induction of $dTIP60^{E431Q}$ for both independent lines A and B reduced fly viability to 0%, (Table 1). The developmental stage when lethality occurred varied between individual fly lines, with the majority of lethality occurring during the late pupal stage for line A and during the late second instar larvae stage for line B. Such variation in developmental stages of lethality is presumably due to position effect variegation on expression levels due to random transgene insertion within the genome, (Figure 1 C). Similar results were obtained using the ubiquitous actin driver Act5c, confirming the dominant negative lethal effects observed. Taken together, these results demonstrate that production of $dTip60^{E431Q}$ produces dominant negative lethal effects during fly development, and that the HAT catalytic activity base residue E431 is essential for dTIP60 function in multicellular development.

The mutant $dTip60^{E431Q}$ protein theoretically produces a dominant negative effect in the fly by outcompeting endogenous wild-type dTip60 for binding to the dTip60 complex when over-expressed. To determine whether the severity of the dominant negative effect correlates with $dTIP60^{E431Q}$ expression levels and therefore its ability to outcompete the wild type, we used qPCR to compare the exogenous levels of $dTIP60^{E431Q}$ transgene expression between fly lines A and B, as they exhibited the greatest and least severe dominant negative phenotypes, respectively, using the ubiquitous GA4 driver 337 for

induction. Quantification of transgene induced exogenously by dTIP60^{E431Q} or dTIP60^{WT} for each line was accomplished by amplifying total dTip60 mRNA using primers designed to a non-conserved region and thereby amplifying both endogenously and exogenously induced dTIP60, and calculating the relative fold change in mRNA expression levels in comparison to endogenous dTIP60 mRNA levels which were identified using primers designed specifically to the endogenous 5'UTR dTIP60 region that is not present in the exogenous dTIP60 transgene and thereby only amplifies endogenously expressed dTIP60 mRNA. All samples analyzed were from early second instar larvae, as this is the stage directly before dTip60^{E431Q}B induced lethality occurs. We found that both lines robustly expressed exogenous dTip60^{E431Q}, with line B expressing almost twice the level of dTip60^{E431Q} than line A suggesting that the more severe dominant negative effect of line A is due to the greater level of dTip60^{E431Q} it produces, (Figure 1C). Of note, although comparably robust levels of exogenous wild-type dTip60 were observed for dTip60^{WT} fly lines A and B, unlike dTip60^{E431Q} fly lines, both dTip60^{WT} fly lines exhibited normal phenotypes. To determine whether induction of HAT defective dTIP60^{E431Q} leads to depletion of endogenous histone H4 acetylation levels *in vivo,* we carried out western blot analysis on equal amounts of endogenous histone proteins purified from each of the second instar larval samples using antibodies to acetylated histone H4, which is the preferential histone substrate of Tip60. Our results reveal that endogenous levels of acetylated histone H4 are significantly depleted in both independent fly lines dTip60 A and B when compared to control samples (Figure 3). Taken together, these results demonstrate that the dominant negative effect is dependent

upon the level of mutant dTip60^{E431Q} produced, and that the amino acid E431 in the catalytic HAT domain of dTip60 is critical for acetylating endogenous histone H4 *in vivo*.

To confirm that the lethal effects we observed were specifically caused by defective dTip60^{E431Q} function, we assessed whether an additional copy of wild type dTIP60 would rescue dTip60^{E431Q} induced lethality. Four independent fly strains were produced that were homozygous for different combinations of both the strongest or weakest expressing dTip60^{E431Q} transgene and the strongest or weakest expressing dTIP60^{WT} transgene in addition to the endogenous dTIP60 gene on the X chromosome. These fly lines were designated as independent rescue lines dTip60^{Rescue} A, B, C, or D, (Table 1). Each of these fly lines were crossed to the ubiquitous GAL4 driver 337 and the viability of the progeny was scored, (Table 1). The results revealed that in this genetic background, when an additional copy of wild type dTIP60 (dTip60^{WT}) was present in flies also expressing the dTIP60^{E431Q} construct, a significant number of flies were rescued with 100% rescue for two of the four rescue lines, (Table 1). Similar results were obtained using a second ubiquitous driver Act5c. These results demonstrate that dTIP60^{E431Q} induced lethality is specifically caused by over-expression of the mutant protein, as this effect can be rescued by additional expression of wild-type dTip60. These findings as a whole demonstrate that the HAT activity of Tip60 is essential for *Drosophila* multicellular development, and support our system as a valuable *in vivo* model for investigating the epigenetic based dependency of developmental processes on Tip60 HAT function.

dTip60 HAT activity is required for the transcriptional regulation of genes involved in a diverse array of metabolic and general cellular processes. To gain insight into the role of Tip60 HAT function in transcriptional control during multicellular development, we used microarray analysis to examine changes in gene expression in response to ubiquitous induction of either dTip60^{E431Q} or dTip60^{WT} in the fly. Our strongest expressing transgenic fly lines dTip60^{E431Q} line B, dTip60^{WT} line B, and w^{1118} control flies were each crossed to the ubiquitous GAL4 driver 337. As induction of dTip60^{E431Q} with the 337-GAL4 driver results in lethality during late second instar larval stage, RNA samples were isolated from thirty five three day old pooled larvae collected prior to lethality to enhance our opportunity to detect Tip60 related cellular changes and ensure that such changes were not linked to tissue necrosis. Microarray analysis was carried out in duplicate on these pooled biological replicate samples using the Affymetrix *Drosophila* Genome 2.0 Array. A correlation matrix generated using dCHIP software demonstrated that the correlation coefficients calculated for duplicate samples for each of the three genotypes analyzed showed significant agreement, indicating high reproducibility of the gene expression data we present in this study. Genes selected for misregulation were identified as those with a fold change of greater than 2 or less than -2 ($p \leq 0.05$) between the w^{1118} control and dTip60^{E431Q} or dTip60^{WT} fly lines after normalization and standardization using dChip programs.

We identified a total of 1756 genes that were significantly misregulated in response to dTip60^{E431Q} induction, with 1051 genes up-regulated and 705 genes down-regulated. In contrast, only 106 genes were identified that were significantly misregulated in response to dTip60^{WT} induction in comparison to control samples, with 55 genes up-regulated and

51 down-regulated. This minimal number of genes misregulated was not surprising as induction of dTip60^{WT} in the fly leads to no observable phenotypic effects (Table 1). Importantly, the comparable levels of expression that we observed for ubiquitous induction of exogenous dTip60^{E431Q} and dTip60^{WT} in the fly (Figure 1C) argue that the significantly larger number of misregulated genes we identify in response to dTip60^{E431Q} expression are specifically due to consequences of the amino acid substitution in the HAT domain of dTip60, rather than simply an artifact caused by over-expression of the transgene itself or other activities of Tip60.

To identify biological processes that were significantly affected as a result of gene misregulation, we utilized the DAVID Functional Annotational Clustering tool (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009) to group the misregulated genes into clusters by their gene ontology (GO) based on biological process. The genes up-regulated in response to dTip60^{E431Q} clustered into 5 significantly enriched groups ($p<0.05$) that represent lipid metabolism, carbohydrate metabolism, amine metabolism, cell death, and response to biotic stimulus (immune) processes**.** Down-regulated genes clustered into 12 significantly enriched groups ($p<0.05$) representing electron transport, cellular localization (protein), fatty acid metabolism, carbohydrate metabolism, amino acid metabolism, Golgi vesicle transport, biosynthetic processes (translation), glycoprotein metabolism, cellular respiration, larval chitin-based cuticle development, and protein retention in ER. Interestingly, although there were more genes up-regulated in response to dTip60^{E431Q}, fewer significantly enriched gene ontology clusters were identified, suggesting that these genes are more random and less specific to cellular process than the set of down-regulated targets. Of note, up-regulated genes in response to

dTip60^{WT} did not group into any significant clusters and down-regulated genes grouped into only one significant cluster that related to bacterium responses, consistent with the lack of phenotypic effects resulting from dTip60^{WT} over-expression in the fly. Taken together, our microarray results support a role for dTip60 in the control of target genes involved in a diverse array of metabolic and general cellular processes.

dTip60 HAT activity is required for neuronal gene expression profiles and is essential for nervous system function. The majority of significantly misregulated genes affected by depletion of Tip60 HAT activity grouped to clusters enriched for metabolic and general cellular processes. However, as the microarray analysis was carried out on a mixed population of cells extracted from developing, whole, second instar larvae, these *in vivo* samples gave us the opportunity to investigate whether depletion of Tip60 HAT activity also affected genes linked to tissue and cell type specific biological processes as well. Analysis of the DAVID-generated gene clusters that were not significantly enriched revealed clusters of cell cycle control regulators, genes involved in general cell development, and intriguingly genes enriched for 17 biological processes all relating to neuronal function and development (Table 3), with 7 clusters linked to down-regulated genes and 10 clusters linked to up-regulated genes. The neuronal processes identified were diverse, with functions linked to behavior, learning and memory, as well as sensory, neurogenesis and general neuronal system function. Importantly, aside from one muscle development related cluster, these neuronal categories were the only tissue-specific related clusters identified in our analysis. Of note, due to the mixed population of cells

used for sample preparation from whole larvae, although this allowed for an analysis of gene expression in a whole organism, genes involved in small tissues such as the nervous system may have been diluted out, suggesting one possible reason that the neuronal clusters were not significantly enriched.

To validate the microarray results and this neuronal tissue-specificity, we carried out qRT-PCR analysis on 11 neuronally relevant genes encoding proteins with known functions. The up-regulated and down-regulated genes selected for this analysis represented a wide range of neuronal functions including neuronal cell type differentiation, transmission of nerve impulses, locomotion and behavior, learning and memory, as well as sensory processes including sight and olfactory behavior. A comparison of the microarrray data and qRT-PCR of selected targets showed good agreement (Figure 4), indicating the reliability of our microarray data as well as supporting a role for dTip60 in the regulation of a wide variety of genes required for neuronal development and function.

Our microarray data supports a role for Tip60 in neuronal linked processes. This finding prompted us to ask whether dTip60 was produced in the nervous system of the developing fly. Examination of the spatial distribution of the dTip60 protein in the *Drosophila* embryo at high resolution using immunohistochemistry with antibodies specific for the dTip60 protein revealed that despite its low global protein expression pattern during late embryonic stages, Tip60 protein is preferentially localized in the brain and central nervous system (CNS), and more specifically found within the anterior neuroblast population known as the neuropil, median cells of the CNS, and possibly

within the ganglion cells. Consistent with our finding that dTi60 HAT activity regulates an array of nervous system specific genes, we found that dTip60 is localized to the nucleus within developing CNS cells, (Figure 5 A-E). Robust dTip60 production was also observed within adult fly brains, (data not shown). To directly test whether dTip60 HAT activity is essential for neuronal development and function, we targeted dTip60^{E431Q} specifically to the nervous system using three nervous system GAL4 drivers: *elav*- GAL4 (Jones, Fetter et al. 1995; Rebay and Rubin 1995; Berger, Renner et al. 2007) and 179y-GAL4 (Manseau 1997; Gunawardena and Goldstein 2001) which produce robust levels of GAL4 throughout the entire nervous system *(*pan neuronal expression patterns), and 60IIA-GAL4 shown by us and others (Shilova, Garbuz et al. 2006; Chan and Kravitz 2007; Zhu, Singh et al. 2007) to direct GAL4 specifically to the brain and CNS. For a control, w^{1118} flies were crossed to these three neuronal GAL4 driver lines and showed normal development and no observable phenotypes. However, induction of dTIP60^{E431Q} using our strongest expressing insertion line B caused a reduction in viability to 0% for all three GAL4 drivers while weaker line A reduced viability to approximately 25% for elav-GAL4 (Table 2), 30% for 179y-GAL4, and 40% for 60IIa-GAL4 crosses (data not shown). Such variability between independent lines is likely due to the varying levels of mutant dTip60 protein production as previously described due to transgene position effects and may indicate that a certain threshold level of dTip60 is required for normal nervous system function. Taken together, our data suggest that dTIP60 controls neuronal specific gene expression profiles that are required for appropriate development and function of the nervous system.

Discussion

To create a suitable *in vivo* model to exclusively explore the role of Tip60 HAT activity in developmental gene control during multicellular development, we set out to create transgenic flies exogenously producing a dominant negative HAT defective Tip60 protein by introducing the amino acid substitution E431Q into its conserved catalytic HAT domain. Although the corresponding mutation in the Tip60 yeast homolog EsaI (E338Q) was shown to retain proper folding, and display a dominant negative effect on yeast cell growth by specifically disrupting EsaI HAT activity *via* putative disruption of the proton extraction capability of the enzyme (Yan, Barlev et al. 2000), it was unknown whether the mutant dTip60 protein would display similar dominant negative effects in the multicellular model *Drosophila*. Here, we show that production of dTip60^{E431Q} in flies causes both a reduction in endogenous acetylated H4 histones *in vivo* and a dominant negative lethal effect with increasing severity correlating with higher levels of mutant dTip60 ^{E431Q}. Based on these results, we speculate that the mutant dTip60^{E431Q} protein may produce its dominant negative effect in the fly by outcompeting endogenous wild-type dTip60 for recruitment to chromatin when over-expressed, thus titrating out endogenous histone H4 chromatin acetylation, with resultant deleterious effects on gene expression. Taken together, our findings support a critical role for dTip60 catalytic HAT residue E431 in the acetylation of histone H4 *in vivo* and show that dTip60 HAT activity is essential for multicellular development. Moreover, these findings support our system as a novel and valuable model for investigating the epigenetic based dependency of developmental processes on Tip60 HAT function *in vivo*.

Our microarray analysis of the genome-wide gene expression changes that result in flies in response to HAT mutant dTip60^{E431Q} production revealed that the majority of misregulated genes clustered into 17 significantly enriched groups, with 8 that were linked to metabolic processes including amino acid, carbohydrate, lipid, glycoprotein and fatty acid metabolism. The significant enrichment of these Tip60 HAT affected metabolic genes supports a central role for Tip60 HAT function in general cellular metabolism. Our findings are consistent with previous studies directly linking Tip60 in the epigenetic based transcriptional control of the central metabolic regulator LRP1 (Liu, Zerbinatti et al. 2007), a lipoprotein receptor essential for lipid and cholesterol metabolism. Tip60 also serves as a co-activator for the regulation of transcription factor peroxisiome proliferator-activated receptor γ (PPARγ) target genes that play key roles in the regulation of lipid and glucose metabolism, (van Beekum, Brenkman et al. 2008). Importantly, a recent elegant study using protein acetylation microarray analysis in yeast demonstrated that the NuA4 complex (yeast homolog of the human Tip60 complex), and specifically EsaI (yeast Tip60 homolog), controls the activity of the central glucose metabolism regulator phosphoenolpyruvate carboxykinase (Pck1p) *via* its direct acetylation, (Lin, Lu et al. 2009). Based on this finding, we speculate that the Tip60 HAT metabolic associated direct and indirect target genes we identified may not only be controlled epigenetically by Tip60 HAT action, but may also represent indirect targets of central metabolic regulator proteins that are directly controlled *via* their acetylation by Tip60. Of note, the majority of misregulated genes we identified in response to dTip60 HAT depletion were upregulated (Figure 3), supporting a critical role for Tip60 HAT activity in the repression of target genes, possibly by the direct interaction of Tip60 with

transcriptional silencers and/or histone deacetylases or *via* specific Tip60 chromatin acetylation marks that promote recruitment of such silencers to these genes. Involvement of Tip60 in transcriptional repression is not unprecedented (Gavaravarapu and Kamine 2000; Nordentoft and Jorgensen 2003; Xiao, Chung et al. 2003; Qi, Jin et al. 2006; Ai, Zheng et al. 2007), with previous studies supporting a critical role for Tip60 in epigenetically repressing a large number of developmental genes essential for embryonic stem cell (ESC) differentiation, (Fazzio, Huff et al. 2008). Moreover, microarray analysis of RNAi induced Tip60 knockdown in *Drosophila* embryonic cell culture also revealed a significant portion of genes that were upregulated in response to dTip60 loss, (Schirling, Heseding et al. 2010).

Epigenetic regulation has been postulated to provide a coordinated system of regulating gene expression at each stage of neurogenesis, thus promoting brain and CNS development, neural plasticity, learning, and memory, (Guan, Giustetto et al. 2002; Cunliffe 2003; Mattson 2003; Levenson, O'Riordan et al. 2004; Colvis, Pollock et al. 2005; Hsieh and Gage 2005; Levenson and Sweatt 2005; Feng, Desprat et al. 2006; Levenson and Sweatt 2006; Oliveira, Abel et al. 2006; Wood, Hawk et al. 2006; Fischer, Sananbenesi et al. 2007; Kiefer 2007; Miller and Sweatt 2007; Oliveira, Wood et al. 2007; Reul and Chandramohan 2007; Borrelli, Nestler et al. 2008; Graff and Mansuy 2008; Jiang, Langley et al. 2008; Lubin, Roth et al. 2008; Mehler 2008; Miller, Campbell et al. 2008; Sunyer, Diao et al. 2008; Ma, Jang et al. 2009; Reul, Hesketh et al. 2009; Roth and Sweatt 2009; Sweatt 2009; Franklin and Mansuy 2010; Gupta, Kim et al. 2010; Miller 2010; Peleg, Sananbenesi et al. 2010; Sharma 2010; Sweatt 2010). The identification of a number of neurological disorders that result from HAT misregulation

underscores a crucial role for acetylation in proper CNS development. For example, missense mutations in the CBP and p300 genes or loss of a CBP allele cause Rubinstein-Taybi syndrome (RTS), a human disease that displays complex phenotypic abnormalities including retardation and neoplasia. Moreover, memory loss associated with RTS is specifically due to lack of CBP HAT activity which can be reversed by treatment with specific histone deacetylase inhibitors (HDACs), indicative of a critical role for appropriate histone acetylation in long-term potentiation, learning, and memory, (Steffan, Bodai et al. 2001; Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Rouaux, Loeffler et al. 2004; Vecsey, Hawk et al. 2007; Stefanko, Barrett et al. 2009; Gaub, Tedeschi et al. 2010). Consistent with these studies, here we provide evidence supporting a role for Tip60 HAT activity in regulating neuronal gene expression profiles required for nervous system function. We show that dTip60 protein is robustly produced in the embryonic nervous system, is localized in the nuclei of brain and CNS cells, and that depletion of Tip60 HAT activity in these tissues results in fly lethality. Importantly, our gene ontology (GO) analysis shows good correlation with these dTip60 protein localization studies in that a substantial number of dTip60 HAT dependent target genes are enriched for neuronal related processes, with 17 clusters linked to diverse nervous system processes and one cluster linked to muscle development. Intriguingly, these were the only tissue-specific related processes identified in our microarray analysis, although we are aware that some cell-specific processes may have been diluted out due to the mixed whole larvae sample preparations used for analysis. A role for dTip60 in neuronal specific function is not unprecedented, with a previous study identifying the dTIP60 gene through its accession number as a potential novel neural precursor gene in a *Drosophila*

differential embryonic head cDNA screen (Brody, Stivers et al. 2002), although its identity at the time remained uncharacterized. Moreover, preferential expression of TIP60 in the mouse brain has been reported, (Brody, Stivers et al. 2002). Taken together, our results demonstrate yet another example of the importance of HAT function during neurogenesis, and add dTip60 to the growing list of HAT chromatin regulators critical for nervous system function.

Recent studies support an emerging hypothesis that inappropriate changes of specific acetylation marks in chromatin in the adult brain leads to gene misregulation that drives cognitive decline and specifically, memory impairment, (Lee and Pixley 1994; Kuhn, Dickinson-Anson et al. 1996; Foster 1999; Levenson and Sweatt 2005; Fischer, Sananbenesi et al. 2007; Liu, Zerbinatti et al. 2007; Mangan and Levenson 2007; Siegmund, Connor et al. 2007; Borrelli, Nestler et al. 2008; Maurice, Duclot et al. 2008; Shen, Liu et al. 2008; Wang, Oelze et al. 2008; Calvanese, Lara et al. 2009; Peleg, Sananbenesi et al. 2010; Penner, Roth et al. 2010; Sweatt 2010). These studies demonstrate that in learning assays, aged mice show a specific deregulation of histone H4 lysine 12 (H4K12) acetylation that corresponds with the misregulation of hippocampal gene expression profiles associated with learning and memory, (Peleg, Sananbenesi et al. 2010). Importantly, these effects can be reversed by restoring physiological levels of H4K12 acetylation. Thus, it is postulated that as individuals age, the accumulation of inappropriate changes in H4K12 acetylation, as well as additional acetylation and methylation marks, lead to altered transcription of neurogenic genes with subsequent negative consequences on cognitive function, (Sweatt 2010). Although the HAT activity of CBP has been implicated in learning and memory linked gene regulation (Alarcon,

Malleret et al. 2004; Korzus, Rosenfeld et al. 2004; Martin and Sun 2004; Wood, Kaplan et al. 2005; Maurice, Duclot et al. 2008; Wang, Weaver et al. 2010), additional specific HATs important in these processes remain to be identified. Here, we show that Tip60 protein is produced robustly in specific cells of the brain and CNS (Figure 5), and that Tip60 HAT activity is essential for appropriate levels of endogenous histone H4 acetylation, *in vivo*, (Figure 3). Moreover, we show that Tip60 is essential for brain and CNS development (Table 2), and intriguingly, is linked to the regulation of certain neuronal genes associated with various forms of behavior, learning, memory and synaptic function processes. Based on these results, it is tempting to speculate that Tip60 HAT activity may be involved in marking CNS chromatin important for learning and memory linked gene regulation. Consistent with this concept, Tip60 HAT activity has been implicated in the age-related neurodegenerative disorder Alzheimer's disease (AD) *via* its HAT dependant complex formation with the C-terminal fragment of the amyloid precursor protein (AICD) and linker protein Fe65, (Cao and Sudhof 2001; Baek, Ohgi et al. 2002; Cao and Sudhof 2004; von Rotz, Kohli et al. 2004; Hass and Yankner 2005). Recruitment of this complex is critical for the epigenetic regulation of certain genes linked to AD progression, (von Rotz, Kohli et al. 2004; Cacabelos, Fernandez-Novoa et al. 2005; Scarpa, Cavallaro et al. 2006; Mastroeni, Grover et al. 2008; Wang, Oelze et al. 2008; Kilgore, Miller et al. 2010). Future investigation into the molecular mechanisms underlying Tip60 HAT function in specific neuronal processes in the fly, particularly those associated with learning and memory, should enhance our understanding into the link between acetylation, cognitive aging, and age-related neurodegenerative disorders.

Chapter 3: Tip60 and APP genetically interact to promote apoptosis-driven neurodegeneration.

Abstract

The histone acetyltransferase Tip60 has been shown to play a significant role in neuronal development, but the tissue-specific pathways regulated by this chromatin modifier have yet to be investigated. Here we identify a genetic interaction between Tip60 and the amyloid precursor protein (APP) *in vivo* using a novel transgenic *Drosophila* model. We show that Tip60 and APP interact specifically in the nervous system, and that one process affected by this interaction is neuronal apoptosis. Importantly, the activities of both APP and Tip60 in the *Drosophila* nervous system are dependent upon the presence of the C-terminus of APP which appears to play a toxic role in this system.

Introduction

Dynamic post-translational modifications of histone proteins regulate gene expression by altering the levels of chromatin packaging. Recently this epigenetic regulation has been linked to the neuronal processes underlying learning and memory, (Levenson and Sweatt 2005; Miller, Campbell et al. 2008; Borrelli, Nestler et al. 2008). Among the chromatin modifiers implicated in these processes are histone acetyltransferase (HAT) enzymes which transfer the acetyl group from acetyl Co-A to

highly conserved lysine residues on the histone N-terminal tails. Histone tail aceytlation is thought to disrupt chromatin packaging, thus promoting transcriptional activation of specific targets. For example, studies have identified reduced acetylation in the promoter regions of certain neuronal targets in response to CBP knock down, resulting in a drastic decline in long term memory retention associated with the CBP-linked nervous system disorder Rubenstein-Taybi syndrome (RTS), (Wood, Attner et al. 2006; Korzus, Rosenfeld et al. 2004; Barrett and Wood 2008). Furthermore, the acetylation of H4K12 has very recently been shown to play a critical role in the expression of targets necessary for formation and maintenance of long term memory, (Peleg, Sananbenesi et al. 2010). Taken together, these studies suggest that epigenetic regulation may underlie the complicated processes of memory formation and retention.

These findings have led us to believe that other chromatin modifiers may also play a role in neuronal processes with disease implications. One candidate is the HAT Tat-interactive protein 60kD (Tip60) which has been shown by our group to play a tissue-specific role in neuronal development, and has already been implicated in a variety of human diseases with well-characterized roles in DNA repair, cell cycle progression, and apoptosis, (Ikura, Ogryzko et al. 2000; Kusch, Florens et al. 2004; Sapountzi, Logan et al. 2006; Squatrito, Gorrini et al. 2006; Tyteca, Legube et al. 2006). Tip60 is highly conserved from yeast to humans (van Attikum and Gasser 2005), and functions primarily in transcriptional activation either through histone acetylation (Sapountzi, Logan et al. 2006), or through transcription factor co-activation through direct acetylation ((Brady, Ozanne et al. 1999; Sapountzi, Logan et al. 2006; Gaughan, Logan et al. 2002), although Tip60 has additionally been implicated in transcriptional silencing through interactions

with transcriptional repressors, (Hlubek, Lohberg et al. 2001; Xiao, Chung et al. 2003; Achour, Fuhrmann et al. 2009). Like most chromatin mofiying enzymes Tip60 must be in complex to perform histone acetyltransferase activities. Although predominantly found in the large multiprotein Tip60-complex that also exhibits ATPase, DNA helicase, and structural binding activities (Ikura, Ogryzko et al. 2000; Kusch, Florens et al. 2004; Sapountzi, Logan et al. 2006), Tip60 has also been implicated in a variety of additional roles due to transient association with various other protein complexes. Interestingly, Tip60 is thought to form one such interaction with the neuronally functioning amyloid precursor protein (APP), (Cao and Sudhof 2001). APP is a broadly expressed single pass transmembrane protein which is most abundant in neurons. This protein is proteolytically processed in two sequential cleavage events. The initial event is catalyzed by either α- or β-secretase and releases the large extracellular N-terminal domain outside of the cell, and the second cleavage event is catalyzed by γ-secretase and releases a small intracellular domain (AICD) into the cytoplasm. When APP is cleaved by both β- and γ-secretases, the peptide left between these two cleavages is termed amyloid beta (Aβ) and has been shown to aggregate into the hallmark toxic amyloid plaques of Alzheimer's Disease (AD), (Zheng and Koo 2006). Predominatly due to increases in toxic Aβ production, alterations in APP expression and processing have also been linked to AD pathogenesis.

Despite the well-defined role that Aβ plays in AD-related plaque formation, recent evidence focusing on AICD suggests that this peptide may also play an important role in AD pathology. For instance, the over-expression of AICD leads to neuronal cytotoxicity in several *in vitro* models (Yankner, Dawes et al. 1989; Yoshikawa, Aizawa et al. 1992;

Lu, Rabizadeh et al. 2000; Kinoshita, Whelan et al. 2002), and has been linked to various nervous system defects *in vivo* in *Drosophila*, (Gunawardena and Goldstein 2001; Greeve, Kretzschmar et al. 2004; Merdes, Soba et al. 2004; Rusu, Jansen et al. 2007). Mutations in the presenilin proteins of the AICD generating γ-secretase complex are also linked to degeneration and AD progression, (Wolozin, Iwasaki et al. 1996; Checler 1999; Guo, Sebastian et al. 1999; Araki, Yuasa et al. 2000; Alves da Costa, Paitel et al. 2002; Alves da Costa, Mattson et al. 2003). Importantly, AICD is thought to play a role in transcriptional activation through interactions with Tip60. This Tip60/AICD complex has been shown to induce transcriptional activation *in vitro* (Cao and Sudhof 2001), however only a handful of controversial targets have been proposed, (Muller, Meyer et al. 2008). It has been hypothesized that alterations in APP expression or AICD generation result in the mis-targeting of Tip60 to genes that are critical for proper neuronal function, resulting in transcriptional alterations. Importantly, these changes in gene expression patterns are anticipated to occur long before pathological signs of the disease manifest, and their identification may reveal early markers for AD. Despite the convincing evidence that both Tip60 and APP play important roles in neuronal processes, a specific role for Tip60 in APP-mediated neuronal processes has yet to be revealed, and an *in vivo* model to study their interactions has yet to be generated.

The *Drosophila melanogaster* model system is well suited to study APP-mediated neuronal processes due to the complex nervous system and the powerful genetic tools commercially available for use in this system. Many studies have been carried out using transgenic fly lines that mimic human AD by over-expressing human APP as this protein lacks homology with *Drosophila* dAPPL in the central Aβ region, (Gunawardena and

Goldstein 2001; Greeve, Kretzschmar et al. 2004). Importantly, hAPP and dAPPL are highly conserved in the C-terminal region and co-localize to synaptic terminals, (Yagi, Tomita et al. 2000). Futher, hAPP has been shown to rescue behavioral defects of APPL knock out lines suggesting functional overlap, (Luo, Tully et al. 1992). Fly models expressing variations of APP have been generated to successfully replicate all of the important aspects of AD pathology including tauopathy (Wittmann, Wszolek et al. 2001), Aβ plaque formation (Greeve, Kretzschmar et al. 2004; Crowther, Kinghorn et al. 2005), APP-processing (Greeve, Kretzschmar et al. 2004), age-related neurodegeneration (Greeve, Kretzschmar et al. 2004), and progressive loss of learning ability, (Finelli, Kelkar et al. 2004; Iijima, Liu et al. 2004). Importantly, it has not been determined whether any of these defects are associated with the epigenetic activity of Tip60.

Our group has previously characterized a dominant negative Tip60 mutant fly line which we have shown to be specifically defective for HAT activity. Here, we utilize this line along with lines elegantly shown to express hAPP and hAPP with a C-terminal deletion (APPΔCT) (Fossgreen, Bruckner et al., 1998; Gunawardena and Goldstein 2001) to generate a double transgenic fly model that can be used to study the interaction between Tip60 and APP *in vivo*. We demonstrate that Tip60 and APP interact genetically in *Drosophila* development, and more specifically in neuronal development. Importantly, we identify an interaction between these proteins which promotes apoptosis within the developing central nervous system. An interesting finding from our studies is that the activity of Tip60 in the nervous system is specifically dependent on the C-terminus of APP, the portion of APP proposed to form a transcriptionally active complex with Tip60 *in vitro*, and the over-expression of which has been reported to be toxic both

in vitro (Yoshikawa, Aizawa et al. 1992; Lu, Rabizadeh et al. 2000; Kinoshita, Whelan et al. 2002) and *in vivo* in the *Drosophila* nervous system, (Merdes, Soba et al. 2004). Here we report a novel and tissue-specific genetic interaction between Tip60 and APP in neuronal development, and more specifically in the promotion of apoptosis in the larval brain. These results support our hypothesis that neurodegeneration underlying age-related dementia may be linked to the epigenetic activities of the histone acetyltransferase Tip60.

Materials and Methods

***Drosophila* Stocks:** UAS-695 (hAPP) and UAS-596DELCT lines (hAPPΔCT) (Fossgreen, Bruckner et al. 1998; Gunawardena and Goldstein 2001) were obtained from the Bloomington Stock Center numbers 6700 and 6703, and dTIP60^{E431Q} lines were previously generated by our group. For genetic interaction experiments, homozygous lines UAS-695;dTIP60^{E431Q} and UAS-596DELCT;dTIP60^{E431Q} were generated and termed hAPP;dTIP60^{E431Q} and hAPPΔCT;dTIP60^{E431Q}. Expression of transgenes was induced by 337-GAL4 (Elefant and Palter 1999) and 179y-GAL4 Bloomington Stock Center, no.3733 (Manseau, Baradaran et al. 1997; Gunawardena and Goldstein 2001) at 25°C. Control crosses were performed using w^{1118} crossed to the appropriate driver line. Homozygous 337-GAL4 males were crossed to virgin females homozygous for different UAS constructs such that all progeny carry 337-GAL4 and UAS constructs. Heterozygous 179y-GAL4 males with the transgene on the X were crossed to virgin females homozygous for different UAS transgenes such that all female progeny carry

both 179y-GAL4 and UAS constructs while all male progeny only carry the UAS constructs and therefore serve as an internal control. Control crosses were performed by crossing w^{1118} virgin females to GAL4 driver males. All crosses were performed in triplicate using ten newly eclosed virgin females and either seven 337-GAL4 or five 179y-GAL4 males.

Quantitative Real Time RT-PCR: More than one-hundred UAS-dTIP60^{E431Q} or UAS-695 (hAPP) virgin females were crossed to more than fifty 337-GAL4 males. Staged three day old dTIP60^{E431Q} larvae were collected immediately prior to lethality, and staged seven day old hAPP larvae were collected for RNA extraction. w^{1118} flies were crossed to 337-GAL4 and respecively staged larvae were collected for a control. Total RNA was isolated using Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and treated twice with Dnase II (Ambion, Austin, TX) to remove DNA. Complementary DNA (cDNA) was synthesized from 1ug total RNA and oligo-dT primers using Superscript II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Real-time quantitative PCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Power SYBR Green PCR master mix (Applied Bioystems, Foster City, CA, USA). Real time RT-PCR reactions were carried out in triplicate in 20ul reaction volumes containing 1ng cDNA template and 500nm each of forward and reverse primer. Primer sets were designed to amplify approximately a 100bp non-conserved region of each target using the NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) and are available upon request. PCR was carried out by 40 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min with plate readings recorded after each cycle. Threshold cycle (Ct) values were obtained, and

the ΔΔCT method (Bookout and Mangelsdorf 2003) was used to calculate the fold change in transcript level of the sample relative to the control. RP49 which encodes the ribosomal protein L32 was used as an internal standard and reference gene using forward and reverse primer pair 5'CTGCTCATGCAGAACCGCGT3' and 5'GGACCGACAGCTGCTTGGCG3'.

TUNEL Staining for Apoptosis: Third instar larval brains were carefully dissected and fixed in 2% Paraformaldehyde. Brains were washed 3 times in PBST (1% Triton X) for 15 minutes and incubated for 15 minutes in block solution (10% normal goat serum, 1% Triton X). Detection of apoptotic neuronal cells was performed using the Fluorescein Cell Death Kit (Roche) following the manufacturer's instructions. The reaction mixture was made using enzyme solution and label solution (1:9) and brains were incubated for 90 minutes at 37°C. Samples were then washed 3 times in PBST (1% TritonX) for 15 minutes each in the dark and transferred to PBS. Larval brains were then mounted in Vectashield mounting medium, and observed using the FV1000 Laser Scanning Confocal Microscope. All TUNEL-positive cells were counted three times each from the brains of 13 to 23 larvae per genotype. For a negative control, only label solution was used and no apoptotic cells were observed.

Results

Tip60 and APP genetically interact in *Drosophila* development. The human homologue of TIP60 in *Drosophila* (Dmel\TIP60) was previously cloned by our group (Zhu, Singh et al. 2007) and others (Kusch, Florens et al. 2004), and GAL4 targeting of dominant negative dTIP60^{E431Q} mutant lines led to defects in histone acetyltransferase

activity. Microarray analysis upon ubiquitous expression of this mutation highlighted a tissue-specific role for dTip60 in the nervous system. To extend our study of the epigenetic role of Tip60 in the nervous system we chose to investigate the interaction between Tip60 and the neuronally significant amyloid precursor protein (APP) to determine whether Tip60 and APP interact in the multicellular *Drosophila* system as they do *in vitro*. We set out to create GAL4 inducible fly lines carrying both our previously characterized HAT-defective dTIP60 transgene ($dTIP60^{E431Q}$) and either full-length human APP (hAPP) or human APP lacking the Tip60-interacting C-terminus (hAPPΔCT). The resulting double-transgenic lines are homozygous for hAPP or hAPPΔCT on the second chromosome and $dTIP60^{E431Q}$ on the third chromosome, with endogenous copies of dAPPL and dTIP60 on the X, and are termed hAPP;$dTIP60^{E431Q}$ and hAPPΔCT;$dTIP60^{E431Q}$, (Table 1). Double transgenic lines were made for both low ($dTIP60^{E431Q}$A) and high ($dTIP60^{E431Q}$B) expressing independent dTIP60 mutant lines with varying levels of expression due to position effect variegation, along with hAPP or hAPPΔCT to investigate whether Tip60 and APP interact in *Drosophila*, (Table 1).

A genetic interaction would be supported if the phenotype resulting from the simultaneous expression of two mutants is either more or less intense than would be expected by additively combining their individual phenotypes, (Hartman and Tippery 2004; Drees, Thorsson et al. 2005; Eddy 2006; Lehner, Crombie et al. 2006; Suzuki and Roth 2006). This accounts for the redundancy in phenotype due to an overlap in pathways affected. To test whether Tip60 and APP genetically interact in *Drosophila* development, we targeted expression of both $dTIP60^{E431Q}$ and hAPP individually at 25°C using the GAL4 driver line 337-GAL4 which induces robust and ubiquitous GAL4

expression from late embryogenesis into adulthood. The wild type w^{1118} fly line crossed to 337-GAL4 served as a control and showed no observable phenotypes. Upon ubiquitous targeting of hAPP, the majority of flies survived to adulthood although survivorship was only 60% of that of wild type, indicating some lethality, (Table 2). dTIP60^{E431Q} B induction caused 100% lethality, which predominantly occurred in the second instar stage, (Table 2). When these transgenes were expressed together using our hAPP;dTIP60^{E431Q} line we saw 100% lethality in the embryo stage which is slightly earlier in development than dTIP60^{E431Q} alone, indicating a phenotypic enhancement as was expected since both induced lethality individually, (Table 2). We then chose to co-express our dTIP60^{E431Q} mutants with hAPPΔCT, a version of hAPP lacking the proposed Tip60 interacting domain. Ubiquitous targeting of hAPPΔCT alone with the 337-GAL4 driver did not cause any observable phenotype (Table 2), consistent with previous studies. However, when the double transgenic hAPPΔCT;dTIP60^{E431Q} was expressed ubiquitously, 100% lethality was observed as expected, however surprisingly the lethality now occurred predominantly in the pre-pupal stage which is later in development, (Table 2). These results suggest a suppression of the dTIP60^{E431Q} induced lethal phenotype upon coexpression of APPΔCT. Thus dTIP60^{E431Q} co-expressed with hAPP enhanced the lethal phenotype while co-expression with hAPPΔCT suppressed this effect. The same pattern was observed upon co-expression of hAPP and hAPPΔCT with dTIP60^{E431Q} A, (data not shown). These results suggest that expression of hAPP lacking the C-terminus partially rescues the negative effects of dTIP60^{E431Q} upon ubiquitous expression. Taken together, these data suggest that there is a genetic interaction between Tip60 and APP *in vivo* in *Drosophila*, which occurs in an AICD dependent manner.

Tip60 and APP genetically interact within the nervous system of *Drosophila*. We have shown an interaction between Tip60 and APP upon ubiquitous targeting in *Drosophila*. This finding prompted us to ask whether this interaction also occurs tissue-specifically within the nervous system where APP is biologically most relevant and Tip60 has also been shown to play a significant role. Importantly, Tip60 has been hypothesized to play a role in the transcriptional regulation of APP targets in the nervous system (Cao and Sudhof 2001), but a model with which to address this question directly was previously unavailable. To investigate whether Tip60 and APP genetically interact in the nervous system, we targeted expression of dTIP60^{E431Q}A, chosen because the lethal phenotype induced by dTIP60^{E431Q}B was too strong to analyze, and hAPP using males from the pan-neuronal 179y-GAL4 driver line which induces robust GAL4 expression throughout the nervous system. The 179y-GAL4 insertion is located on the X-chromosome, so progeny were scored by sex with male offspring serving as an internal GAL4- control. Wild type w^{1118} crossed to 179y-GAL4 males was used as an external control and showed no observable phenotype. Targeted neuronal expression of either dTIP60^{E431Q} or hAPP alone resulted in a semi-lethal phenotype with approximately 50% lethality, (Table 3). Importantly, when the double transgenic hAPP;dTIP60^{E431Q} was neuronally targeted with 179y-GAL4, lethality only increased slightly to approximately 60% which was not significantly stronger than either construct expressed alone, (Table 3). This is far from the enhanced combined lethal phenotype expected, and is indicative of a strong genetic interaction between Tip60 and APP in the nervous system. These results suggest that Tip60 and APP are functioning in some of the same pathways in the nervous system as the observed lethal phenotypes upon dTip60^{E431Q} or hAPP expression

are predominantly due to an overlap of defects in the same neuronal processes. These data support the hypothesis that Tip60 and APP interact genetically in the nervous system.

To investigate whether this interaction depends on the C-terminus of APP, we expressed dTIP60^{E431Q} with hAPPΔCT using the 179y-GAL4 pan-neuronal driver at 25°C. hAPPΔCT targeting alone with 179y-GAL4 showed no observable phenotype (Table 3), consistent with previous findings suggesting that the lethality induced upon hAPP targeting in *Drosophila* is dependent on the C-terminus. Surprisingly however 100% of flies survived upon neuronal expression of the hAPPΔCT;dTIP60^{E431Q} double transgenic (Table 3), despite the 50% lethality observed upon expression of dTIP60^{E431Q} alone. These results suggest that the lethal phenotype induced upon dTIP60^{E431Q} targeting was completely rescued to that of wild type upon co-expression with hAPPΔCT, which was completely unexpected. This indicates that dTIP60^{E431Q} induced lethality in the nervous system is completely dependent on the presence of AICD. From this data we can hypothesize that the interaction between Tip60 and APP within the nervous system is dependent on the C-terminus of APP, as dTIP60^{E431Q} expressed with hAPP did not alter the lethal phenotype while co-expression with hAPPΔCT completely rescued this effect, (Table 3). This pattern mimics that observed between Tip60 and APP in general development, however the effects are greatly enhanced upon neuronal targeting, highlighting the tissue-specificity of this interaction in the nervous system.

These results have highlighted a strong overlap in the pathways affected by TIP60^{E431Q} and hAPP neuronal expression contributing to the lethal phenotypes. As an

indication of which neuronal processes may be affected by both dTIP60^{E431Q} and hAPP, we chose to examine whether APP over-expression alters the expression patterns of neuronal targets that our group has shown previously to be epigenetically regulated by Tip60. hAPP was ubiquitously expressed using the 337-GAL4 driver and mRNA levels of six dTIP60 neuronal targets involved in a variety of neuronal processes were detected in whole third instar larvae using qRT-PCR analysis. Control w^{1118} flies were crossed to the 337-GAL4 driver and age-matched larvae were used as a comparison for expression levels. Our analysis surprisingly revealed that two of the six dTIP60 neuronal targets tested were also expressed at altered levels upon hAPP expression compared to controls, (Figure 1). These targets included no optic lobe which has been proposed to play a role in the reactivation of quiescent neurons, and odorant binding protein 99b which acts in odorant perception. Since expression of either the Tip60 HAT mutant or hAPP alters the expression of these targets, we hypothesize that Tip60 and APP may act together in neuronal development and sensory perception. Taken together, our results indicate that Tip60 and APP genetically interact tissue-specifically within the nervous system of *Drosophila*, and support a role for their interaction in the pathways of sensory perception and neuronal development.

Tip60 and APP genetically interact to promote apoptosis within the central nervous system of *Drosophila*. We have shown that Tip60 and APP genetically interact within the nervous system, prompting us to ask what specific processes this interaction might be involved in. The over-expression of hAPP has been shown to induce neuronal apoptosis in *Drosophila,* (Gunawardena and Goldstein 2001), however the mechanisms underlying this phenotype have not been explored. Additionally, Tip60 is well known for its role in

the regulation of apoptotic pathways (Ikura, Ogryzko et al. 2000) (Tang, Luo et al. 2006), but the tissue-specificity of this activity has not been examined. To first investigate whether Tip60 plays a role in neuronal-specific apoptosis, dTIP60^{E431Q} flies were crossed to female 179y-GAL4 pan-neuronal driver flies at 25°C and third instar larval brains were carefully dissected to observe apoptotic cells through TUNEL analysis. TUNEL staining of third instar larval brains from wild type w^{1118} flies crossed to 179y-GAL4 at 25°C were used as a control. We found a dramatic increase in the total number of apoptotic cells that was 2.4 times that of wild type with significant increases in both the ganglion and the optic lobes of the brains of dTip60 HAT mutants (Figure 2, A and B), indicating that the HAT activity of Tip60 does play an important role in the regulation of apoptotic pathways within the nervous system. hAPP flies were also crossed to female 179y-GAL4 driver flies at 25°C and a dramatic increase in the number of apoptotic cells was observed that was 3.4 times that of controls (Figure 2, A and B), with significant increases in both the ganglion as has previously been described (Gunawardena and Goldstein 2001), and in the optic lobes. These results indicate that both Tip60 and APP play important roles in apoptosis in the central nervous system of *Drosophila*.

To examine whether Tip60 and APP act together in apoptosis promotion within the nervous system, we targeted expression of the double transgenic line hAPP;dTIP60^{E431Q} pan-neuronally using 179y-GAL4 females at 25°C and performed TUNEL staining to observe apoptotic cell number. Surprisingly, we found that when both dTIP60^{E431Q} and hAPP were expressed, a significant increase in apoptosis was observed that was only 2.3 times that of controls (Figure 2, A and B), which is less than when hAPP was expressed alone and nearly identical to dTIP60^{E431Q} induced levels. This was not the enhanced

effect that would be expected as an additive result of the two transgenes expressed together. These results indicate that Tip60 and APP genetically interact in the promotion of neuronal apoptosis, suggesting that Tip60 and APP induce apoptosis in the central nervous system through some of the same pathways.

To further investigate this interaction, hAPPΔCT was expressed in the nervous system using pan-neuronal expression with the 179y-GAL4 driver at 25°C and apoptotic cells were observed with TUNEL staining. We found a significant increase in apoptosis in the brain that was 2.5 times that seen in controls (Figure 2, A and B), which is less than the 3.4 increase observed when full length APP was expressed. These results indicate that APP-induced apoptosis is not completely dependent on the AICD region. However, since hAPPΔCT induced less neuronal apoptosis than full length APP, these results also suggest that AICD does contribute to the observed neuronal apoptosis. To determine whether mutant Tip60-induced neuronal apoptosis relies on the C-terminal region of APP, the double transgenic hAPPΔCT;dTIP60^{E431Q} was expressed with the 179y-GAL4 driver at 25°C and TUNEL analysis was performed. Surprisingly we observed a complete rescue in apoptotic cell number, with no significant difference compared to controls (Figure 2, A and B), despite the significant increases in apoptosis observed upon expression of either hAPPΔCT or dTIP60^{E431Q} alone. Thus, dTIP60^{E431Q} induced neuronal apoptosis is not affected by hAPP expression, but is completely rescued by hAPPΔCT expression, indicating that the C-terminus of APP plays a very important role in promoting neuronal apoptosis through the HAT activity of Tip60. These results support our hypothesis that Tip60 and APP genetically interact in the regulation of

neuronal apoptosis. Importantly, these results indicate that AICD plays a central role in this interaction, although further research is needed to elucidate the nature of this role.

We have demonstrated that Tip60 and APP genetically interact to promote neuronal apoptosis, which prompted us to ask which apoptosis-inducing pathways are affected by this interaction to induce this phenotype. To address this question, we used qRT-PCR analysis to examine the expression levels of five apoptosis targets, all of which were identified in our dTIP60^{E431Q} microarray analysis, and which are involved in different apoptosis-inducing pathways. Both hAPP and dTIP60^{E431Q} were expressed ubiquitously at 25°C using the 337-GAL4 driver. The stronger Tip60 HAT mutant line B was used to allow for the greatest likelihood of mis-regulated targets. dTIP60^{E431Q}B, hAPP, and appropriately age-matched w^{1118} controls were ubiquitously expressed using the 337-GAL4 driver and the mRNA levels of the five apoptosis targets were detected in whole larvae using qRT-PCR analysis. Our results indicate that both Tip60 and APP are involved in the regulation of programmed cell death 5 (PDCD5), a well-known regulator of apoptosis, (Chen, Sun et al. 2001; Chen, Wang et al. 2006; Bannai, Nishikawa et al. 2008). This target was upregulated upon hAPP over-expression and downregulated upon loss of Tip60 HAT activity, (Figure 3). These results suggest that Tip60 and APP interact in transcriptional regulation within the nervous system in promoting neuronal apoptosis, possibly through pathways involving PDCD5.

Discussion

Tip60 has recently been shown by our group to play a critical epigenetic based role in neuronal development, but the specific neuronal processes affected by Tip60 HAT activity have yet to be explored. Importantly, Tip60 has been shown to form a transcriptionally active complex with the C-terminus of APP (AICD) *in vitro* (Cao and Sudhof 2001) suggesting that the HAT activity of Tip60 may be important in the regulation of APP-associated processes within the nervous system. APP over-expression has been shown to cause various neuronal defects (van Dooren, Dewachter et al. 2005; Crowther, Page et al. 2006) and it has been hypothesized that some of these changes may result from transcriptional mis-regulation through association of AICD with Tip60. In this report, we investigate the genetic interaction between Tip60 and APP *in vivo* during multicellular development using a HAT defective dTIP60 mutant and well-characterized lines over-expressing hAPP and hAPPΔCT lacking the AICD region, (Gunawardena and Goldstein 2001). We show that Tip60 and APP genetically interact to tissue-specifically regulate neuronal processes, in an AICD-dependent manner. Moreover, our results uncover a novel role for Tip60 HAT activity in regulating neuronal apoptosis, and show that this activity relies on a genetic interaction with AICD. Our genetic analysis in *Drosophila* strongly supports the hypothesis that Tip60 and AICD interact in neuronal processes through transcriptional regulation, and that the mis-regulation of this interaction may lead to serious neuronal defects such as cell death.

To create a model with which to explore the interaction between Tip60 and APP *in vivo*, we set out to create transgenic fly lines carrying both a Tip60 HAT-defective

mutation dTIP60^{E431Q} and either hAPP, termed hAPP;dTIP60^{E431Q}, or hAPPΔCT lacking the Tip60 interacting C-terminal region termed hAPPΔCT;dTIP60^{E431Q}, (Table 1). Although Tip60 and APP have been shown to interact in a transcriptionally active complex *in vitro* (Cao and Sudhof 2001), it was unknown whether they would act similarly in a multicellular *Drosophila* model. Here, we show that Tip60 and APP do genetically interact in organismal development, as co-expression of APP increases dTIP60^{E431Q} induced lethality while co-expression of hAPPΔCT suppresses this effect, (Table 2). Further, this same interaction is observed upon neuronal targeting, however the interaction is dramatically enhanced in this tissue indicating the tissue-specific importance of this interaction in the nervous system, (Table 3). Importantly, when specifically targeted to the nervous system, hAPPΔCT completely rescues dTIP60^{E431Q} induced lethality, indicating a dependence of the dTIP60^{E431Q} induced phenotype on the generation of AICD in the nervous system. Further, we can infer that since Tip60 mutant induced lethality is dependent on AICD production, AICD may act upstream of Tip60 in this system.

To shed light on the neuronal processes affected by the interaction between APP and Tip60 in the nervous system, we investigated whether APP over-expression led to changes in the expression levels of neuronal targets previously shown to be mis-regulated by aberrant HAT activity of Tip60 with the thought that the neuronal processes affected by these targets may be affected by the Tip60/AICD interaction. We surprisingly identified two neuronal targets whose regulation was affected by both the Tip60 HAT mutant and APP overexpression, (Figure 1). These targets included *obp99b* involved in odorant perception (Hekmat-Scafe, Scafe et al. 2002), and *nol* involved in the

reactivation of quiescent neurons, (Guan, Prado et al. 2000). Considering the roles of these identified targets, we hypothesize that the Tip60/AICD interaction may be involved in pathways of sensory perception and neuronal development.

One pathway that has been shown to be affected by APP over-expression in the *Drosophila* nervous system is neuronal apoptosis. APP over-expression has been shown to dramatically increase the number of apoptotic cells in the *Drosophila* larval brain, and we hypothesize that this effect may contribute to the lethality induced by this transgene. This finding prompted us to ask whether this APP-induced neuronal phenotype was mediated by Tip60 activity, particularly since Tip60 has been well-characterized in apoptosis pathways. Importantly however, Tip60's role in apoptosis *in vivo* has not previously been investigated. To investigate whether the HAT activity of Tip60 is important in neuronal apoptosis pathways in *Drosophila*, we chose to express our HAT mutant dTIP60^{E431Q} specifically in the nervous system using the pan-neuronal 179y-GAL4 driver. We found that our Tip60 HAT mutant induced a dramatic increase in neuronal apoptosis in third instar *Drosophila* larval brains (Figure 2, A and B), suggesting that Tip60 plays an important role in apoptosis pathways *in vivo* as it has been shown to previously *in vitro*. Expression of full-length hAPP in the nervous system resulted in even higher levels of neuronal apoptosis than were observed with the Tip60 HAT mutant, confirming previous findings, (Gunawardena and Goldstein 2001). To determine if Tip60 and APP genetically interact in this promotion of neuronal apoptosis, we expressed our double transgenic hAPP;dTIP60^{E431Q} line neuronally and found increased levels of apoptosis similar to that of the Tip60 mutant alone and somewhat less than hAPP alone, (Figure 2, A and B). These levels are drastically less than would have

been expected from an additive phenotype, suggesting that Tip60 and APP do genetically interact in the promotion of neuronal apoptosis in this system.

To investigate whether this interaction is dependent on the C-terminus of APP, we expressed hAPPΔCT neuronally and assayed the number of apoptotic cells. Interestingly, APP lacking the C-terminus did increase neuronal apoptosis at levels less than hAPP and similar to the Tip60 HAT mutant alone, (Figure 2, A and B). These findings suggest that APP-induced neuronal apoptosis is not entirely dependent on the C-terminus, however the AICD region contributes to this phenotype. Thus it seems that APP overexpression induces neuronal apoptosis in at least two mechanisms, one involving AICD and the other related to another portion of APP. This hypothesis is consistent with previous findings indicating that both the Aβ and C-terminus regions of APP are involved in neuronal apoptosis induction, (Gunawardena and Goldstein 2001). To investigate whether Tip60-induced neuronal apoptosis depends on AICD, the double transgenic hAPPΔCT;dTIP60^{E431Q} was expressed neuronally and neuronal apoptosis in the third instar larval brain was quantified. Very surprisingly, we found that co-expression of mutant Tip60 with APP lacking the C-terminus completely rescued the robust neuronal apoptosis induced by each individually, (Figure 2, A and B). This result highlights a genetic interaction between dTip60 and hAPPΔCT that is drastically different from the interaction observed with full-length hAPP, suggesting an AICD specific effect. These results suggest that Tip60-mediated neuronal apoptosis relies on the AICD region of APP. Taken together, these results suggest that Tip60-induced neuronal apoptosis is completely dependent on AICD production, while the apoptosis induced by AICD is also dependent on functional Tip60 enzyme. These findings support the theory that Tip60 and

AICD interact in a transcriptionally active complex, and suggest that this complex may be involved in regulating neuronal apoptosis.

Importantly, our results in this interaction study highlight a common theme in the dependence of Tip60-induced neuronal phenotypes on the presence of AICD. One possible explanation is that robust expression of APPΔCT out-competes the endogenous APPL homolog for membrane space and cleavage complexes. Therefore, when APPΔCT is expressed, there is little or no AICD produced to interact with Tip60, thereby limiting potential negative cell signaling effects. This explanation is consistent with previous reports indicating a toxic nature of increased AICD expression (Yoshikawa, Aizawa et al. 1992; Lu, Rabizadeh et al. 2000; Kinoshita, Whelan et al. 2002; Merdes, Soba et al. 2004). Alternatively it could be argued that dTIP60^{E431Q} is not lethal when co-expressed with hAPPΔCT because although the AICD signal is depleted, Tip60 is also HAT defective and therefore unable to relay this deficiency into mis-expression of targets. Although our findings suggest that dTIP60^{E431Q} induced lethality is suppressed upon the loss of AICD, further investigation is still needed to decipher the mechanisms leading to this suppression.

Transcriptional mis-regulation caused by alterations in Tip60/AICD activities have been hypothesized to contribute to neurodegeneration, although the specific pathways leading to this pathology are unknown. The mis-regulation of *no optic lobe* by expression of both a Tip60 HAT mutant and hAPP is interesting because this target is thought to play a role in the reactivation of quiescent neurons, (Guan, Prado et al. 2000). It has been shown that when cell-cycle markers are re-expressed in post-mitotic neurons

they undergo apoptosis rather than cell division, (Nagy 2000; Copani, Uberti et al. 2001; Vermeulen, Berneman et al. 2003; Herrup, Neve et al. 2004; Becker and Bonni 2005; Copani, Caraci et al. 2007). This progression underlies the programmed cell death common to many neurodegenerative disorders including AD, (Busser, Geldmacher et al. 1998; Copani, Condorelli et al. 2001; Yang, Geldmacher et al. 2001; Yang, Varvel et al. 2006; McShea, Lee et al. 2007; Copani, Guccione et al. 2008; Varvel, Bhaskar et al. 2008; Lee, Casadesus et al. 2009; Varvel, Bhaskar et al. 2009). When cell-cycle re-entry is prevented by blocking cell cycle machinery (Copani, Uberti et al. 2001) or G1/S and G2/M transitions (Khurana, Lu et al. 2006), neuronal apoptosis can be prevented. These studies suggest that neurodegeneration associated with AD and other neurodegenerative disorders may be due to aberrant cell-cycle re-activation in post-mitotic neurons through mis-expression of cell cycle genes. E2F and Cdc2 are cell-cycle regulators shown to activate neuronal apoptotic machinery, (Liu and Greene 2001; Konishi, Lehtinen et al. 2002; Konishi and Bonni 2003; Becker and Bonni 2004). Interestingly, Tip60 plays a well-characterized role in cell-cycle regulation as E2F transcription factors recruit Tip60 to target gene promoters where acetylation of histone H4 activates the cell-cycle, (Taubert, Gorrini et al. 2004). It is tempting to speculate that when mis-directed, Tip60-dependent activities may play a role in aberrant re-expression of cell-cycle markers in post-mitotic neurons.

Another way that both APP and Tip60 play a role in cell death is through p53-dependent apoptosis, (Alves da Costa, Paitel et al. 2002). AICD has been shown to enhance both the transcriptional and pro-apoptotic functions of p53 through direct interactions, (Ozaki, Li et al. 2006) and additionally AICD is involved in the activation of

pro-apoptotic targets such as Gsk3β which promotes p53-mediated neuronal apoptosis, (Alves da Costa, Paitel et al. 2002; Watcharasit, Bijur et al. 2002; Kim, Kim et al. 2003; Ozaki, Li et al. 2006). APP over-expression has been shown to induce neuronal apoptosis in *Drosophila* in an AICD dependent manner (Gunawardena and Goldstein 2001), however an epigenetic basis for this phenotype has not been explored. Importantly, Tip60 also plays an important role in apoptosis regulation both through direct p53 association, and through p53 co-activation, (Legube, Linares et al. 2004). Tip60 has been shown to acetylate p53 at residue K120 and the presence of this mark promotes p53 induction of apoptosis, (Tang, Luo et al. 2006); additionally Tip60 can be targeted to apoptosis-promoting genes for activation, (Aylon and Oren 2007).

This information supports a common role for Tip60 and AICD in p-53 mediated apoptosis. In support of this hypothesis, we found that expression of the apoptosis inducing target PDCD5 was decreased upon dTIP60^{E431Q} targeting and increased upon hAPP targeting (Figure 3), although we must keep in mind that this may be due to indirect mechanisms. PDCD5 is an apoptosis related protein shown to enhance TAG/TROY-induced parapoptosis-like cell death, (Wang, Li et al. 2004). PDCD5 is upregulated in apoptotic cells (Chen, Sun et al. 2001) and has been identified as a novel Tip60 binding partner through a large scale yeast 2-hybrid screen, (Xu, Chen et al. 2009). Further, PDCD5 was shown to be necessary for Tip60 protein stabilization, and for facilitation of Tip60 dependent acetylation of p53 at K120 and of apoptosis related targets including Bax, (Xu, Chen et al. 2009). Our results support previous findings that PDCD5 is regulated by Tip60 (Xu, Chen et al. 2009), and suggest that this regulation may also be mediated by APP. Interestingly, PDCD5 rapidly translocates into the nucleus of

apoptotic cells where it binds to Tip60, (Chen, Sun et al. 2001). It is tempting to speculate that factors such as PDCD5 may stabilize and transport the AICD peptide into the nucleus where it is thought to associate with Tip60, especially considering that AICD has many binding partners. Further research is needed to investigate whether the Tip60/AICD interaction in the promotion of apoptosis in the nervous system is based on defects in p53-dependent pathways, aberrant cell-cycle re-entry, TAG-TROY pathways, or another apoptosis-inducing pathway.

A surprising finding from our studies is the suppression of TIP60 HAT mutant induced phenotypes by genetic reduction of AICD. Without AICD, neither APP over-expression nor the loss of dTIP60 HAT activity are lethal or induces apoptosis in the nervous system, indicating that these phenotypes directly rely on AICD production. These results suggest that pharmacologic reduction of AICD production may provide a novel therapeutic avenue to treat AD and other neurodegenerative disorders that may be caused by APP over-expression or alterations in Tip60 HAT activity. Interestingly, γ-secretase inhibitors which prevent the proteolytic cleavage event that releases AICD from the cell membrane have been tested for their application in such therapeutic settings, (Roberts 2002). *In vitro* studies indicate that γ-secretase inhibition successfully blocks Aβ production (Dovey, John et al. 2001), and such therapeutics have been shown to reduce plasma Aβ levels in AD patients (Siemers, Skinner et al. 2005; Siemers, Quinn et al. 2006; Fleisher, Raman et al. 2008), however these drugs have not been examined for their utility in treating AICD-induced neurotoxicity. Unfortunately, these drugs are not specific to APP and the γ-secretase complex also plays an important role in the release of the intracellular domain of Notch (NICD) which is also a critical event. Future studies

into the specific pathways leading to neuronal apoptosis as well as more specific inhibitors of AICD production may provide more effective treatment options for the complex pathologies of the nervous system.

Chapter 4: Pharmacological inhibition of AICD formation rescues APP-induced neuronal defects.

Abstract

Proteolytic processing of the amyloid precursor protein (APP) has been associated with Alzheimer's Disease (AD) through generation of the central toxic Aβ peptide (Atwood, Obrenovich et al. 2003), however the γ-secretase cleavage event contributing to the generation of this peptide also releases the APP C-terminus (AICD) which has also been shown to play a role in neuropathology, (Muller, Meyer et al. 2008). Importantly, AICD has also been shown to interact with the histone acetyltransferase Tip60 in a transcriptionally active complex (Cao and Sudhof 2001), suggesting that AICD induced neuronal defects may result from transcriptional misregulation via the epigenetic activities of Tip60. Several γ-secretase inhibitors have been developed to treat AD by preventing Aβ cleavage, but we hypothesize that they may also suppress AD neuropathology by preventing AICD formation. Here, we utilize one of these compounds, DAPT, in a *Drosophila* model overexpressing human APP. Our results support previous findings that DAPT interacts with the *Drosophila* γ-secretase complex, (Micchelli, Esler et al. 2003). We show that γ-secretase inhibition can suppress AICD dependent locomotor deficits in *Drosophila*. Further, our results support the hypothesis that these defects result from transcriptional misregulation as DAPT also suppresses AICD-induced gene expression changes. Taken together, our results support the use of *Drosophila* as a model to study the biological effects of γ-secretase inhibitor compounds designed to treat human neuropathologies. Our results here indicate that γ-secretase inhibitors can suppress neuronal defects induced by APP over-expression *in vivo*.

Introduction

The amyloid precursor protein (APP) is a single pass transmembrane protein predominantly expressed in the nervous system which has been well studied for its implications in Alzheimer's Disease (AD) pathology. Although the role of APP in the nervous system is unclear, it has been implicated in a variety of processes including cell growth, cell adhesion, apoptosis, axon and dendrite formation, synaptic differentiation, and general neuron viability, (Torroja, Packard et al. 1999; De Strooper and Annaert 2000; Takahashi, Dore et al. 2000). APP is proteolytically cleaved in two sequential events; the first is catalyzed by either α- or β- secretase releasing the large N-terminus extracellularly, and the second is catalyzed by γ-secretase releasing the small C-terminus (AICD) into the cytoplasm, (Zheng and Koo 2006). The transmembrane region between these sites is termed Aβ and has been shown to aggregate into the toxic plaques characteristic of AD (Gouras, Almeida et al. 2005), underscoring the importance of the proper regulation of APP expression and processing.

Interestingly, the intracellularly released C-terminus of APP has also recently received attention for its cytotoxic effects. *In vitro*, AICD has been shown to be cytotoxic, (Yankner, Dawes et al. 1989; Yoshikawa, Aizawa et al. 1992; Lu, Rabizadeh et al. 2000; Kinoshita, Whelan et al. 2002). Additionally, over-expression of APP in *Drosophila* results in various neurological defects including neurotoxicity, age-related neurodegeneration, behavioral defects, and axonal transport stalling (Gunawardena and Goldstein 2001; Greeve, Kretzschmar et al. 2004; Merdes, Soba et al. 2004; Rusu, Jansen et al. 2007), all of which are dependent on AICD expression. Although the mechanisms underlying the importance of AICD are not clear, one hypothesis is that this peptide acts

as a signal for transcriptional activation through interactions with the epigenetic modifier Tip60. Tip60 is a histone acetyltransferase enzyme responsible for laying post translational acetyl group modifications onto histone protein tails which are thought to facilitate transcription, (Sapountzi, Logan et al. 2006). It has been proposed that the over-expression of APP in addition to increasing toxic Aβ production may also increase AICD production and thus induce misregulation of AICD/Tip60 transcriptional targets. It is thought that these gene expression changes may also play an important role in AD pathogenesis.

Due to its role in AD pathology, pharmacological treatments have been generated to reduce Aβ production by suppressing the cleavage events releasing this peptide. For this reason several potent and specific γ-secretase inhibitors have been developed as potential AD therapeutics, (Wolfe 2001). Importantly, while preventing Aβ production these compounds also prevent AICD release. One of these compounds is *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) (Dovey, John et al. 2001) which has been shown to reduce Aβ levels *in vitro* and *in vivo* in mouse models (Dovey, John et al. 2001; Lanz, Himes et al. 2003; Takuma, Yan et al. 2005), but has not been studied for its use in reducing AICD production. DAPT has been tested in *Drosophila* where it shows strong phenotypes consistent with γ-secretase inhibition indicating that this compound interacts with the *Drosophila* enzyme (Micchelli, Esler et al. 2003), and importantly suggesting that the *Drosophila* developmental model system can be used as a model to study the effects of this compound *in vivo*.

A commonly used *Drosophila melanogaster* model for studying the neuronal implications of APP requires the overexpression of the human APP protein (hAPP) due to a lack of homology within the Aβ region between hAPP and the *Drosophila* homolog dAPPL, despite good conservation in the C-terminal region, (Gunawardena and Goldstein 2001; Greeve, Kretzschmar et al. 2004). Importantly, the γ-secretase complex is highly conserved from flies to humans including the catalytic presenilin subunits, (Fossgreen, Bruckner et al. 1998; Ye and Fortini 1999). In *Drosophila*, γ-secretase inhibitors as well as β-secretase inhibitors have been shown to suppress age-related neurodegeneration and lethality resulting from overexpression of APP, BACE, and the presenilins, (Greeve, Kretzschmar et al. 2004). These studies suggest that *Drosophila* provides a good model to investigate the utility of γ-secretase inhibitors in treating AICD-dependent neuronal defects.

One neuronal defect caused by APP over-expression that is dependent on AICD production is axonal transport stalling, which has been observed in mouse and *Drosophila* models of AD with overexpression of APP, (Kamal, Stokin et al. 2000; Gunawardena and Goldstein 2001; Mudher, Shepherd et al. 2004). Axonal transport mutants, including kinesin (Gindhart, Desai et al. 1998) and dynein (Martin, Iyadurai et al. 1999) mutants as well as mutants for kinesin and dynein associated proteins (Bowman, Kamal et al. 2000; Bowman, Patel-King et al. 1999), display well-established phenotypes that can be easily quantified in *Drosophila* through larval locomotor assays. These defects commonly include posterior segment paralysis, sluggish locomotion, and posterior curling (Gindhart, Desai et al. 1998; Martin, Iyadurai et al. 1999; Bowman, Kamal et al. 2000; Saxton, Hicks et al. 1991; Hurd, Stern et al. 1996).

To determine if neuronal defects resulting from APP overexpression can be rescued by pharmacological inhibition of AICD generation, we chose to administer the γ-secretase inhibitor DAPT to *Drosophila* larvae over-expressing hAPP. We found that overexpression of hAPP caused defects in locomotor ability that were dependent on AICD expression and which could be suppressed by DAPT. To further investigate whether these locomotor defects were due to transcriptional mis-regulation based on the interaction between AICD and the HAT Tip60, we measured the expression level of no optic lobe (*nol*), a neuronal target previously identified by our group to be regulated by both Tip60 and APP. We found that *nol* was mis-regulated upon overexpression of hAPP in an AICD dependent manner, and that this misregulation could be suppressed by DAPT. Taken together our findings suggest that treatment with the γ-secretase inhibitor DAPT may alleviate AICD induced neuronal defects by restoring gene expression patterns that were altered by aberrant AICD production. Our study highlights the utility of the *Drosophila* model to explore the biological effects of pharmacological compounds in an intact organism, and supports previous findings suggesting that AICD plays an important role in APP-related neuropathologies.

Materials and Methods

***Drosophila* Stocks:** Lines carrying a full-length human APP construct UAS-695 termed hAPP as well as lines carrying a human APP construct with a C-terminal deletion UAS-596DELCT termed hAPPΔCT (Fossgreen, Bruckner et al. 1998; Gunawardena and Goldstein 2001) were obtained from the Bloomington Stock Center, Bloomington Stock

Center, nos. 6700 and 6703. Expression of the UAS constructs was induced using the 337-GAL4 driver line for ubiquitous expression (Elefant and Palter 1999) and the elav-GAL4 driver line for pan-neuronal expression, Bloomington Stock Center, no. 8760 or 8765, (Jones, Fetter et al. 1995; Rebay and Rubin 1995). All crosses were performed at 25°C. Control crosses were performed using the w^{1118} line. For all crosses homozygous 337-GAL4 males were crossed to virgin females homozygous for different UAS constructs such that all progeny carry 337-GAL4 and UAS constructs. Homozygous elav-GAL4 virgin females with the transgene on the X were crossed to males homozygous for the UAS transgenes such that all progeny carry both elav-GAL4 and UAS. Control crosses were performed by crossing w^{1118} virgin females to 337-GAL4 driver males, or w^{1118} males to elav-GAL4 virgin females.

γ-secretase inhibitor administration: Appropriate amounts of a 50mM stock solution of DAPT prepared in ethanol were added to 3mL of water to achieve the desired concentration, and dried potato food was slowly added until the food became solid as has previously been described, (Micchelli, Esler et al. 2003). All vials were kept overnight to allow the ethanol to evaporate, and the following day crosses were set up and permitted to lay eggs for five days before the adults were removed. All crosses contained at least ten virgin females and at least five young males.

Larval Locomoter Assay: Wandering third instar larvae were selected and placed on Petri dishes containing 3% agarose for 120 seconds to acclimate. A $1cm^2$ grid was placed below the dish, and as each larvae crawled in a straight line the number of lines crossed

by the head were counted for 30 second intervals. This assay has previously been described, (Mudher, Shepherd et al. 2004; Ubhi, Shaibah et al. 2007).

Quantitative Real Time RT-PCR: UAS-695 and UAS-695DELCT virgin females were crossed to 337-GAL4 males at 25°C and staged seven day old wandering instar larvae were collected for RNA extraction. w^{1118} virgin females crossed to 337-GAL4 males provided a control. Total RNA was isolated using Trizol (Invitrogen Corporation, Carlsbad, CA, USA), treated twice with Dnase II (Ambion, Austin, TX, USA) and complementary DNA (cDNA) was synthesized from 1ug total RNA using oligo-dT primers and Superscript II Reverse Transcriptase, (Invitrogen Corporation, Carlsbad, CA, USA). Real-time quantitative PCR was performed using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Real time RT-PCR reactions were carried out in 20ul reaction volumes containing 1ng cDNA template and 500nm each of forward and reverse primer in triplicate. Primers amplifying a 102bp non-conserved region of no optic lobe were designed using the NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer pair chosen to amplify no optic lobe included forward primer 5'AGACGCCGCACGATCCGC3' and reverse primer 5'GCCTGGGGTATTCGCAATGGGG3'. The housekeeping RP49 gene was used as a reference using the forward primer 5'CTGCTCATGCAGAACCGCGT3' and reverse primer 5'GGACCGACAGCTGCTTGGCG3'. PCR was carried out by 40 cycles at 95°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min with plate readings recorded after each cycle. Cycle threshold (Ct) values were analyzed using the ΔΔCT method (Bookout and Mangelsdorf 2003) to calculate the fold change relative to the control.

Results

Administration of DAPT alters survival rates dose-dependently in *Drosophila.* γ-secretase inhibitors have been designed to prevent the generation of the neurotoxic Aβ peptide (Dovey, John et al. 2001), however inhibition of this cleavage event also precludes AICD release. We hypothesize that pharmacological inhibition of AICD generation may also suppress AICD-dependent neurotoxicity. The γ-secretase inhibitor DAPT was designed to inhibit human γ-secretase but has also been shown to interact with the *Drosophila* γ-secretase complex, (Micchelli, Esler et al. 2003). To verify that the DAPT compound had an effect in our *Drosophila* system, we used various concentrations and determined if this modulated *Drosophila* development. Full-length hAPP and C-terminally truncated hAPPΔCT constructs were expressed ubiquitously at 25°C using the 337-GAL4 driver line which induces robust ubiquitous GAL4 expression from late embryogenesis into adulthood. The wild type w^{1118} fly line crossed to 337-GAL4 served as a control. For each DAPT concentration, ten female UAS flies were crossed to seven 337-GAL4 males to induce ubiquitous expression and eggs were laid for five days on specially prepared food containing the appropriate amount of DAPT. Larvae were allowed to develop while injesting only the DAPT-containing food. Control larve were raised on food lacking DAPT. To assess survivorship, the number of adult flies that eclosed was counted from each vial. We found that at lower DAPT concentrations a large number flies survived, indicating that the concentration was not high enough to induce an effect, (Figure 1). As the dosage was increased, we observed a decrease in survivorship. At 0.5mM DAPT a great amount of lethality was induced, and the weakest line hAPP showed few flies surviving to adult with lethality occurring predominantly in

the pupal stage. These results indicate that at 0.5mM DAPT there is enough compound ingested by *Drosophila* larvae to elicit a strong developmental response. Further, this response resulted in nearly complete lethality for the hAPP line, predominantly occurring in the pupal stage. These results suggest that by utilizing third instar larvae in our following experiments we can ensure that these organisms have ingested a sufficient amount of compound to induce an effect however not so much that it has induced lethality. We therefore chose to conduct the following experiments on third instar larvae raised on 0.5mM DAPT. Taken together these results indicate that increasing the dosage of DAPT modulates *Drosophila* lethality, supporting previous studies suggesting that DAPT inhibits γ-secretase activity in *Drosophila,* (Micchelli, Esler et al. 2003).

APP over-expression results in larval locomotor defects that can be rescued by inhibiting γ-secretase activity. APP overexpression has been shown to induce axonal transport defects that are dependent on expression of AICD, (Torroja, Chu et al. 1999; Gunawardena and Goldstein 2001). We chose to investigate whether administration of the γ-secretase inhibitor DAPT could rescue these AICD-dependent defects in *Drosophila*. Axonal transport defects can be easily observed and quantified using larval locomotor assays. One such assay is the line crossing assay used to quantify the sluggish locomotion of wandering third instar larvae which is commonly associated with defects in axonal transport (Mudher, Shepherd et al. 2004; Ubhi, Shaibah et al. 2007). We first chose to examine whether this behavior was affected by the over-expression of APP in *Drosophila* larvae. To assess this, hAPP males were crossed to elav-GAL4 driver line virgin females which express GAL4 pan-neuronally. Control w^{1118} males crossed to elav-GAL4 virgin females were used as a wild type comparison. We found that

neuronally targeted expression of hAPP significantly decreased the speed with which larvae crawled compared to controls, as these larvae only crossed 4-5 lines in the time allowed while wild type larvae crossed 8, (Figure 2). These results demonstrate that neuronaly targeted hAPP expression induces locomoter defects in *Drosophila* consistent with defects observed in axonal transport mutants.

Previous studies have shown that the APP-induced axonal transport defects in *Drosophila* are dependent on AICD generation, (Gunawardena and Goldstein 2001). To determine if the locomotor defects observed upon APP over-expression were also dependent on AICD, we expressed the hAPPΔCT construct specifically in the nervous system and assayed the larval crawling ability. Female virgin hAPPΔCT flies were crossed to elav-GAL4 males at 25°C and the line crossing assay was performed on the progeny once they reached the third instar larvae stage. These larvae showed no defects in line crossing compared to wild type controls, (Figure 2). These results indicate that the larval locomoter defects observed by hAPP overexpression are dependent on AICD generation.

We have shown that over-expression of hAPP leads to defects in larval locomotion in an AICD dependent manner. Next we wanted to determine if pharmacological inhibition of AICD production could suppress these defects. To test this, we crossed elav-GAL4 virgin females to hAPP male flies at 25°C and raised the progeny on 0.5mM DAPT food. We then assessed the locomotor ability of wandering third instar larvae raised on food containing DAPT using the line crossing assay. We found that 0.5mM DAPT significantly suppressed the locomotor defects of the hAPP larvae. Without the drug,

hAPP larvae were able to cross only 4-5 lines in the time allowed compared to the wild type larvae which crossed 8 lines. With DAPT however, hAPP larvae were able to cross nearly 7 lines, which is very close to the results observed with wild type larvae, (Figure 2). To test whether this effect was related to inhibition of AICD production, we expressed hAPPΔCT lacking the AICD region in the nervous system using the elav-GAL4 driver, raised the progeny on 0.5mM DAPT, and assayed the locomotor ability of wandering third instar larvae using the line crossing assay. We found that DAPT had no affect on the locomotor ability of hAPPΔCT larvae lacking the AICD region, indicating that the observed results were not due to non-specific activity of DAPT and were specific to prevention of AICD production. Taken together these results indicate that the overexpression of hAPP induces AICD-dependent defects in *Drosophila* locomotion which can be rescued by inhibition of γ-secretase activity (Figure 2). These results suggest that pharmacological prevention of AICD production with the γ-secretase inhibitor DAPT can rescue AICD-dependent locomotor deficits induced upon hAPP overexpression.

DAPT can suppress transcriptional alterations resulting from APP overexpression. We have shown that APP overexpression induces locomotor defects in *Drosophila* larvae in an AICD-dependent manner and that these defects can be suppressed by administration of the γ-secretase inhibitor DAPT. It is unclear however whether this effect is due to epigenetic activity of AICD in transcriptional regulation in coordination with the HAT Tip60. To further support our hypothesis that the suppression of APP-induced phenotypes by inhibiting γ-secretase activity is due to a suppression of transcriptional misregulation induced by aberrant targeting of Tip60, we chose to assess the expression

of a target previously shown to be misregulated by both mutant Tip60 and APP over-expression in *Drosophila*, using qPCR. Previously we have shown that no optic lobe (*nol*) is upregulated in response to both Tip60 HAT mutant expression and hAPP over-expression. We chose to investigate whether inhibition of γ-secretase activity, and thereby prevention of AICD formation, could suppress the misregulation of *nol* upon APP overexpression. To test this, we crossed hAPP virgins with 337-GAL4 males which express GAL4 ubiquitously from late embryogenesis into adulthood, and collected seven day old wandering third instar larvae for RNA extraction. For a control wild type w^{1118} virgins were crossed to 337-GAL4 males at 25°C and seven day old wandering third instar larvae were collected for an expression level comparison. We found that hAPP overexpression increased expression of *nol* significantly by 7.6 fold when compared to wild type controls, (Figure 3). Our results support our previous findings that *nol* expression is upregulated in response to APP overexpression. To determine if that this effect is dependent on the C-terminus of APP, hAPPΔCT virgin females were crossed to 337-GAL4 males at 25°C and the expression of *nol* in seven day old wandering third instar larvae was analyzed. We found that ubiquitous expression of hAPPΔCT did not alter expression of *nol* compared to wild type controls (Figure 3), indicating that the regulation of this target is dependent on the C-terminus of APP. These results support the hypothesis that AICD plays a role in transcriptional regulation *in vivo*.

We have shown that the Tip60 neuronal target *nol* is misregulated when APP is overexpressed in an AICD dependent manner. We next wanted to investigate whether this misregulation could be suppressed by pharmacological prevention of AICD formation with the γ-secreatase inhibitor DAPT. We first tested whether DAPT altered

expression levels of *nol* in wild type flies. Wild type w^{1118} virgin females were crossed to 337-GAL4 males at 25°C and progeny were raised on 0.5mM DAPT for seven days, and qPCR was performed on wandering third instar larvae. Analysis of *nol* expression showed that DAPT did not affect the regulation of this target significantly in wild type flies, (Figure 3). These results indicate that this concentration of DAPT does not induce *nol* misregulation in wild type flies. We then wanted to know if the upregulation of *nol* upon APP overexpression could be suppressed by DAPT. To address this, hAPP was expressed ubiquitously and the progeny were raised on food containing 0.5mM DAPT. Seven day old wandering third instar larvae were collected for qPCR analysis. We found that when treated with 0.5mM DAPT, *nol* overexpression in hAPP larvae was significantly decreased from 7.6 fold to 3.3 fold compared to wild type larvae (Figure 3), which is more than 2 fold less than *nol* expression in hAPP larvae without DAPT, and significantly closer to the expression level in wild type. Since DAPT did not alter *nol* expression in wild type flies, these results indicate that 0.5mM DAPT was enough to suppress the misexpression of *nol* in hAPP expressing larvae. To determine if this effect was dependent on the C-terminus of APP, we crossed hAPPΔCT virgin females with 337-GAL4 males and raised the progeny at 25°C on food containing 0.5mM DAPT, and seven day old wandering third instar larvae were collected for qPCR analysis. We found that DAPT also reduced the expression of *nol* slightly in hAPPΔCT expressing larvae to -1.7 fold compared to wild type larvae raised on control food (Figure 3), suggesting that the normal expression levels of *nol* observed in hAPPΔCT larvae raised on control food are perturbed when DAPT is present. Taken together these results suggest that APP overexpression leads to the misexpression of *nol* and that this misregulation can be

suppressed by the γ-secretase inhibitor DAPT which prevents AICD production. These results suggest that γ-secretase inhibition may be a useful treatment for transcriptional misregulation induced by APP overexpression.

Discussion

APP overexpression leads to various neurological defects, many of which are C-terminally dependent. Drugs that inhibit the activity of the γ-secretase complex have been developed to treat Alzheimer's disease because they prevent the generation of the toxic plaque forming Aβ peptide (Dovey, John et al. 2001), but we hypothesize that these drugs may also be effective because they prevent the generation of the AICD peptide as well. To test this hypothesis we chose to adminster the γ-secretase inhibitor DAPT to flies overexpressing hAPP to determine if this compound could rescue neuronal defects induced by APP overexpression.

We chose to utilize the γ-secretase inhibitor DAPT because this compound has been shown to inhibit γ-secretase activity in *Drosophila* despite its formulation as an inhibitor for human γ-secretase, (Micchelli, Esler et al. 2003). To show that this compound acts on the *Drosophila* γ-secretase complex in our transgenic model, we administered DAPT in increasing dosages and observed a dose-dependent lethal phenotype, (Figure 1). These results indicate that DAPT acts to inhibit γ-secretase activity in our system. While the mechanism underlying this lethal phenotype cannot be interpreted by these results, there are several possible explanations. Lethality was seen in a previous study at concentrations producing full penetrance of a wing phenotype upon administration of

DAPT, (Micchelli, Esler et al. 2003). As noted in this report, the lethality could be due to uptake, metabolism, or distribution of DAPT in flies, or other unknown toxicities of this compound. Alternatively, it is important to note that DAPT inhibits γ-secretase cleavage of not only APP but also other proteins including Notch, (Fortini 2002; De Strooper 2003). Notch functions as a receptor and like APP, the same or a very similar γ-secretase complex releases the Notch intracellular domain (NICD) into the cytoplasm where it functions in signalling, (De Strooper, Annaert et al. 1999; Struhl and Greenwald 1999; Huppert, Le et al. 2000; Struhl and Adachi 2000). Notch signaling is essential for cell-fate determination and tissue patterning, and is required for viability, (Shellenbarger and Mohler 1978). Additionally, mutants of the catalytic presenilin components of the γ-secretase complex result in embryonic lethality in both *Drosophila* and mouse models, (Shen, Bronson et al. 1997; Ye, Lukinova et al. 1999). Therefore it is likely that the lethal phenotype we have observed may reflect defects in Notch signaling.

As our results support that DAPT sufficiently acts on γ-secretase activity in our *Drosophila* system, we next wanted to determine the appropriate dosage of DAPT to use to elicit a response without inducing lethality. Since many neurological phenotypes in *Drosophila* are easily assayed in the third instar larvae developmental stage, we chose to use a DAPT concentration that resulted in lethality in the pupal stage. This would ensure that a sufficient amount of compound was provided to generate an effect, while allowing for us to utilize larvae immediately prior to lethality in the third instar larval stage. We therefore chose to administer DAPT at the 0.5mM concentration in the following investigations.

APP overexpression leads to many AICD-dependent neuronal defects including axonal transport stalling. Specifically, APP has been identified as a kinesin receptor (Kamal, Stokin et al. 2000; Kamal, Almenar-Queralt et al. 2001), and dAPPL overexpression leads to axonal transport stalling in *Drosophila*, (Torroja, Chu et al. 1999). Axonal transport defects are easily observed as larval locomotor defects in *Drosophila*. During larval locomotion, the body wall muscles must contract in a highly co-ordinated fashion which requires proper functioning at the neuromuscular junction. Compromised locomotion may indicate defects in synaptic function (Chee, Mudher et al. 2005) due to the depletion of neurotransmitters and other materials in reaching the neuromuscular junction, (Ubhi, Shaibah et al. 2007). Since the axonal transport defects observed upon APP overexpression appear to be dependent on AICD, we hypothesized that the γ-secretase inhibitor DAPT could suppress these defects by decreasing AICD production. We found that hAPP overexpression in *Drosophila* resulted in severe locomotor defects consistent with axonal transport stalling phenotypes, (Figure 2). Importantly, hAPPΔCT larvae lacking AICD did not demonstrate these locomotor defects, indicating that this effect is dependent on AICD production. In further support that these defects are dependent on AICD production, we found that DAPT significantly suppressed the APP-induced locomotor defects (Figure 2), presumably by decreasing AICD generation. Further, our results indicate that this concentration of drug does not affect APPΔCT larvae locomotion, further supporting that this concentration is not strong enough to induce adverse affects. From these results we can suggest that increased expression of AICD induces locomotor defects indicative of axonal transport stalling, and

it is possible that DAPT may reduce this excess AICD production, suppressing this defective phenotype.

APP has been proposed to play a role in transcriptional regulation through interactions with the epigenetic regulator Tip60. To further investigate a role for γ-secretase in AICD-dependent transcriptional regulation *in vivo*, we chose to examine whether DAPT could suppress target misregulation induced upon hAPP overexpression. We chose to examine the expression levels of the target no optic lobe (*nol*), which we have previously identified as a target of Tip60 HAT activity through microarray analysis and qPCR validation, and which we have additionally shown to be misregulated upon ubiquitous overexpression of hAPP. Here we further support that *nol* is misregulated upon hAPP overexpression, and show that this effect is dependent on AICD as hAPPΔCT expression does not significantly alter *nol* expression levels, (Figure 3). Upon administration of DAPT to hAPP expressing larvae, we observe a suppression of this *nol* misregulation with the level reduced significantly, bringing it closer to that of wild type, (Figure 3). Additionally upon DAPT administration to hAPPΔCT expressing larvae, we observe a slight but significant downregulation of *nol*, (Figure 3). Taken together these results indicate that *nol* expression is increased when hAPP, and specifically AICD, are overexpressed, possibly by increasing complex formation with the histone acetyltransferase Tip60. By inhibiting AICD formation, DAPT is able to reduce this increase in activity, and therefore reduce this aberrant transcriptional expression. It is possible that when this same concentration of DAPT is given to hAPPΔCT expressing larvae which do not have the additional AICD production, these effects may be deleterious resulting in the observed reduction of expression compared to wild type. One

explanation for the reduction of *nol* expression observed upon hAPPΔCT expression in larvae raised on DAPT is that although hAPPΔCT is robustly overexpressed in these larvae, some endogenous APPL may still be generated resulting in appropriate regulation of *nol*, and resulting in the observed *nol* expression levels that are no different than wild type. Administration of DAPT to these larvae might prevent the small amount of AICD production generated during APPL cleavage, and thus reduce *nol* expression to a level that is less than that of wild type. Taken together, these results support our hypothesis that APP-induced transcriptional regulation defects can be suppressed by γ-secretase inhibitors, and demonstrate the sensitivity of gene regulation to the concentration of modifying complex components, highlighting the importance of determining a proper dosage of epigenetic-based therapeutic compounds.

Although further analysis into the mechanism of this suppression must be performed, these results support a growing body of evidence suggesting that γ-secretase activity is needed to release AICD, allowing it to translocate into the nucleus where it acts in transcriptional regulation. Following this model, APP overexpression would increase the amount of AICD generated, thereby increasing AICD/Tip60 activity, which is reflected in an increase in expression of *nol*. Inhibition of the γ-secretase cleavage event by DAPT we hypothesize may prevent production of AICD, reducing AICD/Tip60 activity, and reverting expression of *nol* to less than that of wild type. These results suggest that DAPT can be used to suppress defects in transcriptional regulation induced by APP overexpression, however there is much work still to be done before these therapies may have clinical applications.

Taken together, our results suggest that γ-secretase inhibitors might provide a novel therapeutic avenue to treat AICD dependent neuronal defects *in vivo*. Further, our data support the hypothesis that AICD overproduction leads to defects in transcriptional activation, and suggest that these defects can be suppressed by inhibiting γ-secretase activity with compounds like DAPT. One caveat of this work is the unspecific activity of DAPT and other γ-secretase inhibitors to inhibit proteolytic processing of not only APP but also Notch and other proteins cleaved by this complex. The development of specific inhibitors of APP cleavage are needed to prevent unwanted side-effects resulting from the inhibition of the cleavage of unintended targets.

Chapter 5: Conclusions and Future Directions

The histone acetyltransferase Tip60 has recently been identified as a genetic hub gene, supporting a role for this essential chromatin modifier in a wide variety of cellular processes. While the role that Tip60 plays in DNA repair, apoptosis, and cell cycle progression are among the most highly characterized, the diversity of cellular processes affected by Tip60 HAT activity in multicellular development have yet to be explored. It has been suggested that mutations in hub genes may be linked to various complex human diseases when in conjunction with mutations in pathway specific targets, suggesting that the investigation of the roles Tip60 plays in an organismal setting may be important in understanding a variety of human diseases.

Here, we have described a multicellular *Drosophila* model that we have used to study the effects of Tip60 HAT activity during development. A specific amino acid substitution converting the highly conserved catalytic core glutamate to glutamine demonstrated a dominant negative lethal effect when expressed ubiquitously using the inducible GAL4 system in *Drosophila*. These results indicate that this highly conserved residue is necessary for catalysis in dTIP60 as it was shown to be in the yeast homologue Esa1 (Yan, Barlev et al. 2000) and other MYST HATs (Trievel, Rojas et al. 1999; Clements, Rojas et al. 1999). Importantly, this model is specifically defective for HAT activity, allowing us to examine exclusively the epigenetic activities of Tip60.

Tip60 has been characterized *in vitro*, but its multicellular implications have yet to be explored. The GAL4 system provides robust inducible expression of our mutant protein

tissue-specifically (Brand and Perrimon 1993), and a large number of tissue-specific enhancers are commercially available such that our mutant Tip60 can be specifically expressed in any tissue of choice. Our model thus provides a basis to examine the specificity of Tip60 in development. We have shown that Tip60 plays an important role in neuronal development, but targeted expression in other tissues may lead to additional findings.

To investigate the roles that Tip60 plays in multicellular development, we performed microarray analysis on whole larvae targeting our mutant dTIP60 expression ubiquitously. Considering its role as a genetic hub, we were not surprised to find that a reduction of Tip60 HAT activity induced misregulation of a large number of targets. We were surprised however to find that the majority of these targets were upregulated, which is counterintuitive considering that HAT activity is usually associated with transcriptional activation. This is not unprecedented however as similar results have previously been reported when Tip60 was knocked down using RNAi in embryonic stem cells, (Fazzio, Huff et al. 2008). Interestingly, although there were fewer downregulated targets, as a whole they clustered better in DAVID analysis than the upregulated targets, indicating that they are involved in a more specific group of biological processes while the upregulated targets were more random in the processes affected. These results are the first to describe the transcriptional regulatory role of Tip60 in an intact organism.

The information obtained from our microarray analysis of targets regulated by the HAT activity of Tip60 provides the basis for a plentitude of future studies. Our analysis revealed a variety of biological processes significantly affected by Tip60 HAT activity,

including several previously unreported processes. Among the processes affected were cell death, response to biotic stimulous, electron transport, cellular localization, golgi vesicle transport, cellular respiration, and protein retention in the ER, however the majority of significantly affected biological processes were involved in metabolism. Tip60 has previously been reported to play a role in metabolism through regulation of the lipoprotein receptor LRP1 (Liu, Zerbinatti et al. 2007) which is involved in lipid and cholesterol metabolism, and co-activation of the peroxisome proliferator-activated receptor γ (PPARγ) targets involved in lipid and glucose metabolism (van Beekum, Brenkman et al. 2008), and the yeast Tip60 homologue has been reported to acetylate phosphoenolpyruvate carboxykinase (Pck1p) (Lin, Lu et al. 2009), regulating glucose metabolism. Further investigation into the specific roles that Tip60 plays in these pathways may uncover important information regarding a variety of diseases such as diabetes.

One caveat of our microarray analysis is that we cannot identify targets directly regulated by Tip60 acetylation from those indirectly regulated. One way to identify direct targets of Tip60 is through chromatin immunoprecipitation (ChIP). This process involves cross-linking DNA bound proteins to DNA, shearing the DNA, and identifying transcripts bound to the protein of choice using appropriate antibodies. The transcripts for specific targets of choice can be quantified, indicating the affinity of the protein for that specific promoter. This technique would allow for us to determine if Tip60 was bound to the promoters of specific targets, indicating direct transcriptional regulation.

While ChIP anlaysis of specific targets would provide useful information regarding the regulation of specific genes of interest, this method would not be efficacious to investigate the large number of targets identified by our microarray analysis as a whole. In a more encompassing approach, our lab is currently planning a ChIP- sequence (ChIP-Seq) experiment where the transcripts bound by the protein of choice are analyzed using high-throughput DNA sequencing. This technology obtains information regarding all genes bound by the protein of interest in one run, making it efficient and thorough for investigation of Tip60's roles in target regulation at the organismal level. Utilizing commercially available RNAi or P-element insertion *Drosophila* stocks, knock-down lines for specific genes could be investigated to better understand how transcriptional regulation by Tip60 results in associated phenotypes.

In addition to the most highly enriched biological processes regulated by Tip60 HAT activity in *Drosophila* development, our microarray analysis also revealed a tissue-specific enrichment of targets affected in neuronal development. Among the neuronal processes affected were differentiation and development, axonogenesis and dendrite formation, synaptic transmission, sensory perception, and behavior. Neuronal targeting of our mutant induced a lethal effect, supporting a previously uncharacterized role for Tip60 HAT activity in the regulation of neuronal development. Members of our group are currently expanding on this information by utilizing this model to investigate the roles of Tip60 in axonal transport, synaptic transmission, circadian behavior, and learning and memory.

Neuronal development is precise and tightly regulated and the involvement of Tip60 in this process could be investigated starting with the developmental and differentiation targets identified. Neuroblasts in the *Drosophila* larval brain are commonly used to study proliferation and differentiation in neuronal development. One way to determine if neuronal development is altered is through clonal analysis using the MARCM (Mosaic Analysis with a Repressible Cell Marker) technique. This technique would ultimately generate a HAT specific Tip60 mutant neuroblast in a heterozygous background. GFP tagging of the neuroblast allows tracing of all cells generated from it as the GFP is passed down through divisions. This technique can be used to obtain information about proliferative potential of neuroblasts by examining the number of cells of each cell type generated, division defects, and cell size, by highlighting defects in neuronal differentiation.

The identification of a tissue-specific epigenetic role for Tip60 in the nervous system was not unexpected considering the previously identified transcriptionally active complex containing both Tip60 and the neuronally significant amyloid precursor protein (APP). The C-terminus of APP (AICD) is thought to be cleaved from the cell membrane and translocate into the nucleus where it signals through Tip60 to induce expression of specific neuronal targets. Overexpression of AICD has been shown to induce neurotoxicity through a variety of biological pathways. Importantly, the over-expression of APP as well as defects in its proteolytic processing have been implicated in Alzheimer's Disease pathology. This interaction between AICD and Tip60 was previously restricted to *in vitro* analysis as an *in vivo* Tip60 model was not available, although an APP model has been well-established in *Drosophila*. To determine whether

the neuronal specific role of Tip60 HAT activity was linked to AICD-induced transcriptional activation, we developed an interaction model in *Drosophila* where transgenic flies express both our Tip60 HAT mutant and hAPP or hAPP lacking the Tip60 interacting C-terminus using the GAL4 inducible system. We found that both our Tip60 HAT mutant induced lethality and lethality induced by hAPP in the nervous system were dependent on AICD expression, and their co-expression indicated a genetic interaction. These results were the first to suggest that Tip60 and AICD interact within the nervous system *in vivo*.

To further support that this interaction has a basis in transcriptional regulation, we identified two neuronal transcriptional targets regulated by both our mutant Tip60 and hAPP. These targets are *odorant binding protein 99b* and *no optic lobe*. Odorant binding proteins are responsible for binding odorants and shuttling them to olfactory receptor neurons for recognition. *Drosophila* have olfactory receptor neurons in both the antennae and the maxillary pulp which project to the antennal lobe of the brain for processing. The identification of *odorant binding protein 99b* as a target of Tip60 and APP suggests that Tip60 and APP may interact specifically in olfactory perception. To further investigate this, a Tip60/APP interaction analysis of olfactory perception could be performed. Several behavioral assays have been established to determine the olfactory perception of *Drosophila* third instar larvae. For an example, wandering third instar larvae can be placed on a petri dish equidistant from a good smell and a bad smell. The amount of time it takes for the larvae to reach the good smell can be quantified as an indicator of olfactory perception. Interestingly, olfactory deficits have been linked to various neuronal defects. Anosmia is common in dementias and the olfactory centers of

the brain seem to be the first to accumulate Aβ plaques and to induce neurodegeneration in AD (Hawkes 2006), and for these reasons olfactory perception may serve as an early biomarker for AD diagnosis. Examining the antennal lobe for cell death in staged larvae might indicate if this region is the first in induce cell death in the fly in response to Tip60 mutant or APP expression.

No optic lobe is a particularly interesting target because it is proposed to induce the reactivation of quiescent neurons. In *Drosophila* neuronal development, the neuronal stem cells or neuroblasts divide asymmetrically to generate a large self-renewing daughter cell and a smaller ganglion mother cell. The ganglion mother cell then divides symmetrically into two terminally differentiated neurons or glial cells. Thus the majority of neurons post-development are post-mitotic and do not enter cell-cycle. In many neurodegenerative disorders, aberrant reactivation of cell-cycle markers have been observed which induces cell death rather than division and have been linked to neurodegeneration, however the mechanisms underlying this effect have not been investigated. Our results here suggest that cell-cycle reactivation may be involved with Tip60/AICD induction of cell-cycle promoting targets. To determine if there is an increase of neurons entering cell cycle in our Tip60 HAT mutant lines, immunohistochemical analysis can be performed using cell-cycle markers for different phases of cell-cycle such cyclin A for G2 and M phases, BrdU for S phase, and cyclin B and phosphohistone-3 (PH3) for M-phase. Markers for G2 would indicate that cell-cycle had been reactivated, S-phase would indicate that DNA replication was occurring, and M phase markers would indicate that the cells were dividing. We would expect that the number of neuronal cells entering cell cycle would increase, but instead of dividing they

might enter apoptotic pathways. To assess this, TUNEL analysis and caspase 3 antibodies could be used in conjunction with antibodies for the cell cycle markers in our Tip60/APP interacting lines to determine if the cells aberrantly entering cell cycle are also initiating apoptosis. This information could mechanistically support a role for Tip60 in neurodegeneration.

The Tip60/AICD interaction model we have generated can be used to determine if Tip60 and AICD genetically interact in a variety of neuronal processes. One of these pathways is apoptosis which may be linked to neurodegeneration. AICD has been linked to apoptosis through p53 dependent pathways as well as apoptosis-inducing target regulation. Tip60 also plays an important role in regulating apoptosis through acetylation of p53 as well as promoters of apoptosis targets. Here we describe a genetic interaction between Tip60 and APP in the regulation of neuronal apoptosis that is dependent on AICD through TUNEL analysis. In an attempt to shed light on the pathways misregulated and thus possibly contributing to this phenotype, we have found through qPCR the apoptosis-promoting target *programmed cell death 5* (PDCD5) to be is misregulated by both mutant Tip60 expression and APP over-expression. PDCD5 was previously shown to be necessary for Tip60-induced acetylation of p53 at lysine 120 which is required for induction of cell cycle arrest, (Xu, Chen et al. 2009). It is therefore likely that both Tip60 and APP mutants may affect p53 acetylation levels and K120. To investigate this, p53 acetylation levels could be analyzed in Tip60/APP interaction lines in conjunction with the ability of cells to arrest cell cycle using cell cycle markers and TUNEL analysis. The regulation of p53 regulated apoptosis and cell cycle targets could also be investigated using this model.

One limit of TUNEL staining is that it only detects cells in the short late stage of programmed cell death. To confirm the TUNEL results, anti-cleaved caspase 3 antibodies could be used to detect *Drosophlia* caspases such as ICE and DCPI. Additionally, condensed nuclei could be detected with TOTO3. Although our results show an increase in neuronal apoptosis, it cannot be determined which cell types are affected. Immunohistochemical analysis using a variety of neuronal markers could be used in conjuction with apoptosis assays to determine the cellular identity of the dying neurons. For example, Miranda can be used to identify neural stem cells and Repo is a commonly used glial marker. The neuronal marker Elav and the GMC/neuronal marker Prospero can be used to differentiate between ganglion mother cells and differentiated neurons. Prospero protein is inherited and expressed in the cytoplasm of ganglion mother cells, then it translocates into the nucleus to initiate division into daughter cells that upon differentiation express the neuronal marker Elav. Therefore ganglion mother cells should be Prospero positive and Elav negative, while differentiated neurons should be Prospero positive and Elav positive, (Callan, Cabernard et al. 2010).

Our TUNEL results also cannot specify the pathways by which apoptosis is induced. There are several pathways by which apoptosis can be initiated and markers for a number of them could be tested. For example, *Head Involution Defect* (HID) dependent induction of apoptosis is negatively regulated by the RAS1/MAPK pathway through direct phosphorylation by *Rolled* which is regulated by Ras1 and Raf1. In another pathway, p53 binds to and activates another apoptosis inducing target *Reaper* (RPR), which can also be activated by the EcR signaling cascade. The target *Grim* (GRM) regulates apoptosis in a third pathway. Each of these targets, HID, RPR, and GRM,

contain a Death Domain and are individually sufficient to induce apoptosis. Our transgenic fly model could be utilized to determine if each of these pathways is acting normally in Tip60/AICD interaction lines by looking at the regulation of each of these three factors and their downstream targets. ChIP analysis could be used to determine if APP and Tip60 regulate any of the factors involved in these pathways to determine which is leading to the observed apoptosis regulation defects.

We have identified a genetic interaction between Tip60 and AICD, however we cannot deduce information regarding the mechanism of their interaction from our current findings. Importantly, we observe a phenotype where the C-terminus of APP seems to be toxic and induces both APP- and Tip60-induced toxicity. In future experimentation, we could focus on this toxic fragment and see if expression of AICD alone can induce the same toxic effects. To further investigate the effects of APPΔCT expression, we could look at AICD protein levels in each of the interaction fly lines used and determine if the phenotypes observed correlate with the levels of AICD. This would also help us to understand whether APPΔCT induces a dominant negative effect on APPL as we anticipate it does. Additionally, to avoid confounding effects of endogenous APP expression, these experiments could be repeated in an APP null background.

To determine if Tip60 and AICD regulate transcription, sequential chips for targets using antibodies for both Tip60 and AICD to see if they are both at the promoter of specific neuronal targets would be pertinent. Additional interaction lines carrying Tip60 over-expressor and APP knock-down could be generated to better understand the interactions observed. Further, the binding domains required for the Tip60/AICD

interaction could be mutated to see if transcription is affected, and other neuronal processes affected by the Tip60/AICD interaction should be investigated. Lastly, acetylation patterns could be investigated using a variety of mutant lines to determine if histone acetylation is altered and if so which specific residues are affected.

All of our findings presented here demonstrate an AICD dependent effect where overexpression of APP lacking the C-terminus is not toxic and rescues TIP60 HAT mutant induced neuronal defects. This work suggests that if AICD generation could be prevented, this may rescue defects resulting from APP over-expression or defects in Tip60 HAT activity, and therefore may provide novel therapeutic strategies for a variety of disorders. To investigate this hypothesis, we have chosen to administer the γ-secretase inhibitor DAPT, a compound that prevents the cleavage event releasing AICD from the cell membrane. We found that DAPT rescued larval behavioral defects and suppressed the misregulation of target genes induced by APP overexpression. These results support the theory that AICD must enter the nucleus in order to regulate transcription. We are currently continuing this study to determine if Tip60 plays a role in these defects and if this occurs through an interaction with AICD. We will then determine if DAPT rescue of APP-induced neuronal defects occurs in a Tip60-dependent manner. Importantly, it would be interesting to investigate if DAPT could also rescue the neuronal apoptosis that was induced by the Tip60 HAT mutant and APP, or other specific neuronal processes regulated by the Tip60/AICD interaction. Alternatively, repeating these experiments using a model where APP is mutated at the cleavage site might confirm these findings that AICD must be cleaved to induce neurotoxicity. Other drugs that could be tested are

HDAC inhibitors that might enhance Tip60 target activation and HAT inhibitors that might suppress the upregulation of APP targets.

One area of recent interest that is linked to Tip60's role with AICD is age-related defects in memory. Interestingly, a histone acetylation mark located on H4K12 has been shown to decrease with age, and is intimately linked with age-related memory decline, (Peleg, Sananbenesi et al. 2010). Our HAT defective Tip60 model could support the investigation of Tip60's role in the addition of acetylation patterns at this site or the identification of new age-related acetylation changes. These studies could begin in the promoter region of the immediate early gene targets as this group of genes is tightly linked to memory formation. The fly model is advantageous because we can age the organism and see if this mark is changed with age, both globally and at specific target promoters. Additionally, learning and memory assays could be performed in conjuction with these assays to support of the neuronal implications of these epigenetic changes. In addition to the HAT activities of Tip60, the HAT CBP has also been implicated in Alzheimer's disease pathology as presenilin mutations are linked to decreases in CBP activity. Cell death in AD is linked to decreased acetylation of histone H3 and H4. Interestingly, CBP predominantly acetylates H3 while Tip60 prefers H4. Future investigations into the regulation of H3 and H4 acetylation with age in association with cell death in the nervous system of the fly using our Tip60 HAT mutant and APP interaction lines, possibly in conjunction with a CBP mutant fly line, may further contribute to the field of age-related epigenetic defects.

In conclusion, we have presented two very useful transgenic *Drosophila* models. The first is a Tip60 mutant line specifically defective for HAT activity which can be used to investigate the epigenetic role of Tip60 in multicellular development. Although this model could be used to explore a variety of Tip60 interactions, we have utilized it to generate a second interaction model modulating levels of Tip60 HAT activity and APP expression which can be used to investigate the interaction between Tip60 and APP in transcriptional regulation in the nervous system. We have already utilized this model to begin to explore the role that Tip60 plays in the nervous system, and we have shown a dramatic interaction between Tip60 and APP in the regulation of neuronal apoptosis. These results open the door to future investigations of Tip60s roles in age-related memory deficits and dementias.

Chapter 6: Tables and Figures

Chapter 2 Tables

Table 1. Ubiquitous expression of dTIP60^{E431Q} produces a dominant negative lethal effect.

Test Cross Fly Lines[a]	Number of Surviving Adult Flies[c]	
w^{1118}	99±3	
dTip60^{E431Q}A	0±0*	
dTip60^{E431Q}B	0±0*	
dTip60^{WT}A	120±28*	
dTip60^{WT}B	107±18	
Rescue Cross Fly Lines[b]		
dTip60^{Rescue}A	65±23*	
dTip60^{Rescue}B	97±9	
dTip60^{Rescue}C	110±10	
dTip60^{Rescue}D	56±7*	*p≤0.05

[a] **Test Cross Fly Lines.** Ten flies homozygous for either dTip60^{E431Q} or dTip60^{WT} P-element insertions or control w^{1118} were mated to seven flies homozygous for the ubiquitous 337-GAL4 driver. For independently derived fly lines dTip60^{E421} A and B, the P-element insertions are located on chromosome 3 and for independently derived fly lines dTip60^{WT} A and B, the P-element insertions are located on chromosome 2.

[b] **Rescue Cross Fly Lines.** Four independent rescue lines were generated, each homozygous for dTip60^{WT} (line A or B) on the second chromosome and dTip60^{E431Q} (line A or B) on the third chromosome. Rescue lines are designated as follows: line A is dTip60^{E431Q} B/dTip60^{WT}A, line B is dTip60^{E431Q} B/dTip60^{WT}B, line C is dTip60^{E431Q} A/dTip60^{WT}A, line D is dTip60^{E431Q} A/dTip60^{WT}B. Ten homozygous flies for each of the independent rescue fly lines were crossed to seven flies homozygous for the ubiquitous 337-GAL4 driver.

[c] **Total Number of Surviving Adult Flies.** Adult progeny were counted over an eight day period and the total number scored. Both dTip60^{E431Q} lines when crossed to the

Table 1 (continued)

ubiquitous 337-GAL4 driver reduced viability to 0% that of w^{1118} control flies. For $dTIP60^{WT}$ line A, there was a significant increase in survivorship when compared to control w^{1118} flies, and for $dTIP60^{WT}$B there was no affect. Each rescue line (A-D) crossed to the ubiquitous 337-GAL4 driver showed significant rescue of the observed lethal phenotype, with rescue lines A and D exhibiting greater than 50% rescue and rescue lines B and C exhibiting 100% rescue. The results are reported as mean ± SD (n=3); * $p \leq 0.05$.

Table 2. Neuronal expression of dTip60^{E431Q} promotes lethality.

Fly Lines[a]	GAL4-(♂)[b]	GAL4+(♀)[c]	
w^{1118}	100±4	76±18	
dTip60^{E431Q}A	86±2	25±14*	
dTip60^{E431Q}B	91±5	0±0*	
dTip60^{WT}A	67±3	69±4	
dTip60^{WT}B	43±10	54±6	*p≤0.05

[a] **Fly Lines.** Five male flies homozygous for either dTip60^{E431Q} or dTip60^{WT} P-element insertions or control w^{1118} were mated to 10 female virgin flies homozygous for the pan-neuronal elav-GAL4 driver located on chromosome X. Adult progeny were counted over an eight day period and the total [b] male (GAL4-) and [c] female (GAL4+) numbers were scored.

[b] **GAL4-.** Males were scored as a GAL4- internal control for GAL4+ comparison.

[C] **GAL4+.** Females were scored as GAL4+ and compared to the number of GAL4- males from the same vial to determine lethality. Both dTIP60^{E431Q} lines showed significant lethality, with 29% survival for line A and 0% survival for line B. Both dTIP60^{WT} lines showed no observable phenotypic effects. The results are reported as mean ± SD, (n=3); * p≤0.05.

Table 3. Additional gene ontology clusters of genes significantly misregulated in response to dTIP60^{E431Q}.

Biological Processes	Number of Targets Significantly Upregulated
Response to Stress	9
Growth	14
Lipid Biosynthetic Process	11
Response to External Stimulus[c]	11
Behavior (Chemosensory, Learning and Memory)[c]	23
Pigment Biosynthetic Process	7
Membrane Lipid Metabolic Process	9
Positive Regulation of Growth	4
Cell Adhesion	18
Cellular Process (Protein Metabolic Process)	328
Sensory Perception[c]	18
Amino Acid Derivative Metabolic Process	5
Apoptosis	11
Cellular Catabolic Process	14
Amine Transport (Amino Acid Catabolic Process)	14
Amino Acid Transport	4
Regulation of Hydrolase Activity	5
Circadian Rhythm[c]	4
Reproductive Process (Reproductive Developmental Process)	11
Reproductive Process (Mating Behavior)[c]	11
Embryonic Development	23
Aging	5
Muscle Development[d]	8
Neurological System Process[c]	36
Regulation of Programmed Cell Death	8
Cellular Homeostasis	7
Vesicle-Mediated Transport	22
Locomotory Behavior[c]	7
Protein Complex Assembly	9
Dorsal Closure	7
Catabolic Process (Alcohol, Glucose, Carbohydrate)	17
Developmental Process	98
Cofactor Metabolic Process	9
Ion Transport	17
Reproduction (Gamete Production)	24
Localization	89
Cell Communication (Signal Transduction)	74
Post-Embryonic Development (Sensory Organ Development)[c]	25
Nervous System Development[c]	21
Protein Modification Process (Phosphorylation)	26
Microtubule-Based Process	6
Cell Communication (Gene Expression)	74
Imaginal Disc Development	15
Chromosome Organization and Biogenesis	7
Cell Cycle	8
Cellular Localization (Protein Localization)	17

Biological Process	Number of Targets Significantly Downregulated

Protein Processing	5
Mitochondrion Organization and Biogenesis	7
Metabolic Process (Protein Metabolic Process)	257
DNA Metabolic Process	12
Aromatic Compound Metabolic Process	9
Heterocycle Metabolic Process	9
Response to Abiotic Stimulus	9
Vesicle-Mediated Transport	24
Secondary Metabolic Process (Pigment Biosynthetic Process)	6
Behavior (Courtship Behavior)[c]	11
Regulation of Gene Expression	14
Cell Differentiation (Cell Death)	26
Aging	4
Oogenesis	14
Amino Acid Biosynthetic Process	3
Cellular Homeostasis	5
Salivary Gland Development	6
Transcription (Reproductive Process)	9
Nervous System Development (Apoptosis)[c]	10
Response to Abiotic Stimulus	9
Sensory Perception[c]	10
Response to Biotic Stimulus (Immune System Process)	5
Embryonic Development	11
Cellular Developmental Process (Cell Differentiation, Gamete Generation)	26
RNA Processing	7
Cell Cycle	15
Protein Modification Process	35
Oogenesis	14
Polysaccharide Metabolic Process	6
Neurological System Process (Sensory Perception)[c]	13
Chromosome Organization and Biogenesis	9
Cell-Cell Signaling (Synaptic Transmission)[c]	5
Cytoskeleton Organization and Biogenesis (Actin Filament-Based Process)	9
Nervous System Development (Axonogenesis, Generation of Neurons)[c]	10
Biological Regulation (Regulation of Cellular Metabolic Process, Regulation of Gene Expression)	40
Cell Communication	28
Multicellular Organismal Process (Developmental Process/Neuronal Process)[c]	63

Table 3 (continued)

Table 3 (continued)

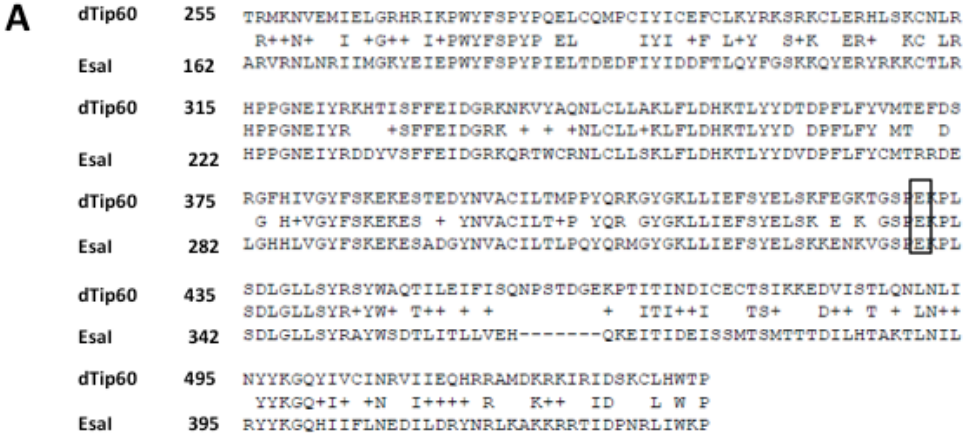
[a] **Biological process clusters of significantly upregulated genes.** In addition to the significantly enriched biological processes, DAVID analysis identified many biological process clusters of significantly upregulated genes. Gene ontology analysis shows their linkage with general and diverse biological processes.

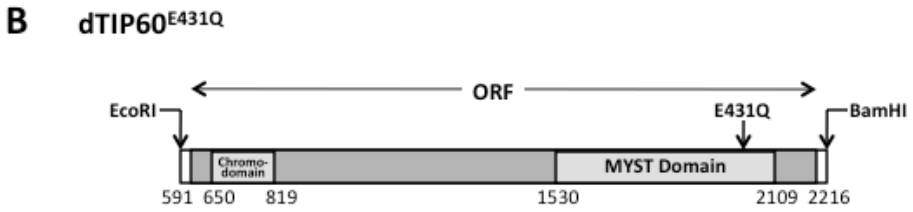
[b] **Biological processes clusters of significantly downregulated genes.** In addition to the significantly enriched biological processes, DAVID analysis identified many biological process clusters of significantly downregulated genes. Gene ontology analysis shows their linkage with general and diverse biological processes.

[c] **Neuronal linked biological processes.** Gene ontology analysis reveals an enrichment of genes involved in neuronal processes. These clusters are also highlighted.

[d] **Muscle linked biological process.** Gene ontology analysis reveals that the only other tissue-specific cluster identified was involved in muscle development.

Chapter 2 Figures





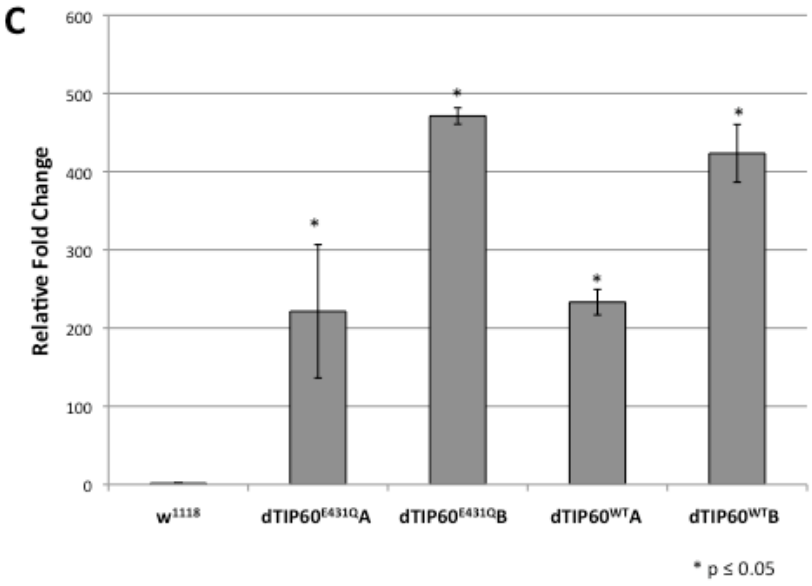


Figure 1 (continued)

Figure 1. Generation and characterization of transgenic dTip60^{E431Q} and dTip60^{WT} flies. **A.** Sequence alignment of the HAT domain between yeast EsaI and *Drosophila* dTip60. Boxed amino acid residue indicates the catalytic glutamate that is conserved between yeast and *Drosophila*. **B.** Schematic of the dTIP60 open reading frame. Shown is the location of the conserved regions encoding for the N-terminal chromodomain and the C-terminal MYST functional domain. An arrow denotes the position site of amino acid substitution E431Q. **C.** Exogenous expression levels of dTip60^{E431Q} and dTip60^{WT} in independent fly lines. Shown is a histogram depicting qPCR analysis of exogenous levels of dTip60 in staged three day old second instar larvae progeny resulting from a cross between ubiquitous GAL4 drive 337 and either dTip60^{E431Q} (lines A and B), dTip60^{WT} (lines A and B) or control w^{1118} flies. Determination of transgene induced exogenous dTip60^{E431Q} or dTip60^{WT} for each line was accomplished by amplifying total dTip60 mRNA using primers designed to a non-conserved region within both the endogenous and exogenous transgene induced dTip60, and calculating the relative fold change in mRNA expression levels in comparison to endogenous dTip60 mRNA levels using primers designed specifically to the endogenous 5'UTR dTip60 region that is lacking in the exogenous transgene induced dTip60. The relative fold change in mRNA expression levels between exogenous and endogenous dTip60 was measured using the comparative Ct method with RP49 as the internal control, and these results are summarized in the histogram. Asterisks (*) indicates significant fold changes between the respective genotype and control flies with values of $p \leq 0.05$; n=3. Error bars represent plus and minus the standard error of the mean.

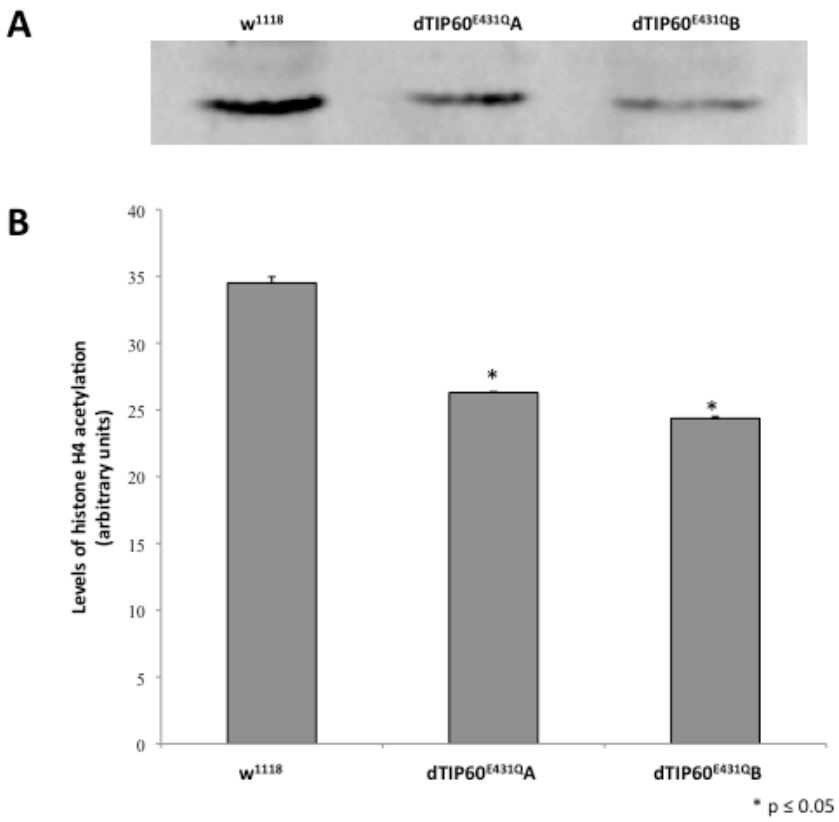


Figure 2. Expression of dTip60^{E431Q} in flies significantly depletes endogenous levels of histone H4 acetylation. **A.** Equal amounts of core histones isolated from from 50 three day old staged second instar larvae for each genotype crossed to GAL4 line 337 were resolved by 18 % polyacrylamide gel electrophoresis, Western-blotted, and immunostained with antibodies that recognize four acetylated lysine residues (K5, K8, K12 and K16) of histone H4. **B.** Western blot signals were quantitated using Fluorchem imager (Alpha Innotech) and the results are summarized in the histogram depicting arbitrary units of endogenous histone H4 acetylation for each of the three genotypes analyzed. Asterisks (*) indicates significant fold change in acetylation in relation to control w^{1118} flies where $p < 0.05$, n=3. Error bars represent plus and minus the standard error of the mean. **This experiment was performed by Keerthy Piroosnia.**

A

Number of Misregulated Targets	
dTIP60^{E431Q}	**1756**
Up-Regulated	1051
Down-Regulated	705
dTIP60^{WT}	**106**
Up-Regulated	55
Down-Regulated	51

B

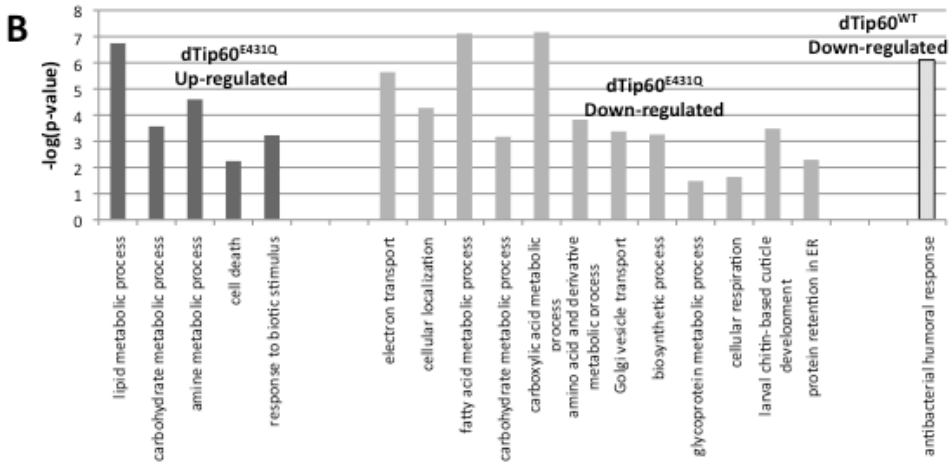


Figure 3. Microarray analysis reveals a central role for Tip60 in the transcriptional control of genes linked to diverse metabolic and general cellular processes. A. Total number of significantly misregulated genes in response to dTip60^{E431Q} or dTip60^{WT}. The dCHIP t-test function was used to identify genes whose expression differed significantly ($p<0.05$) and these genes were then filtered to select for those that showed a twofold or greater change and a 90% confidence bound of fold change. **B.** Significantly enriched gene ontology (GO) groups representing dTip60^{E431Q} and dTip60^{WT} misregulated genes. Genes were annotated and biological processes were analyzed using the Database for Annotation, Visualization, and Integrated Discover (DAVID). Significance of overrepresentation of Gene Ontology (GO) terms was determined ($p<0.05$). The genes up-regulated in response to dTip60^{E431Q} clustered into 5 significantly enriched groups, and down-regulated genes clustered into 12 significantly enrichment groups, with 8 of these groups enriched for metabolic processes. Genes misregulated in response to dTip60^{WT} grouped to one significantly enriched cluster.

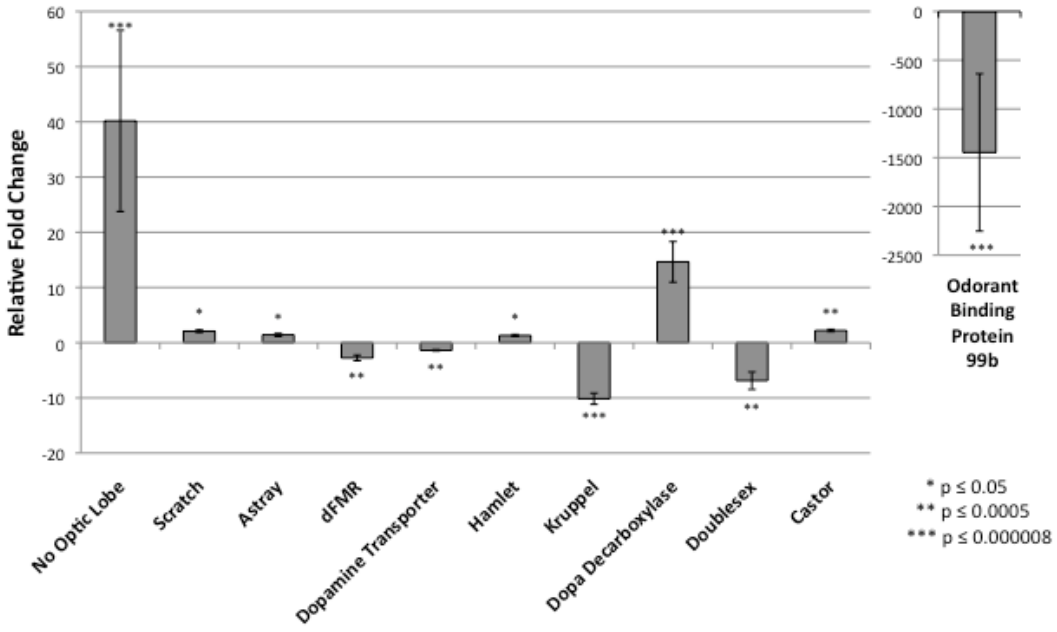


Figure 4. qRT-PCR validation of selected neuronal target genes identified through microarray analysis. Shown is a histogram depicting qPCR analysis of the expression of selected neuronal target genes identified by microarray using aliquots of cDNA pools prepared for microarray analysis. The relative fold changes in mRNA expression levels were measured using the comparative Ct method with RP49 as the internal control gene. Asterisks (*) indicates significant fold change where * is $p \leq 0.05$, ** is $p \leq 0.0005$ and *** $p \leq 0.000008$, n=3. Error bars represent plus and minus the standard error of the mean.

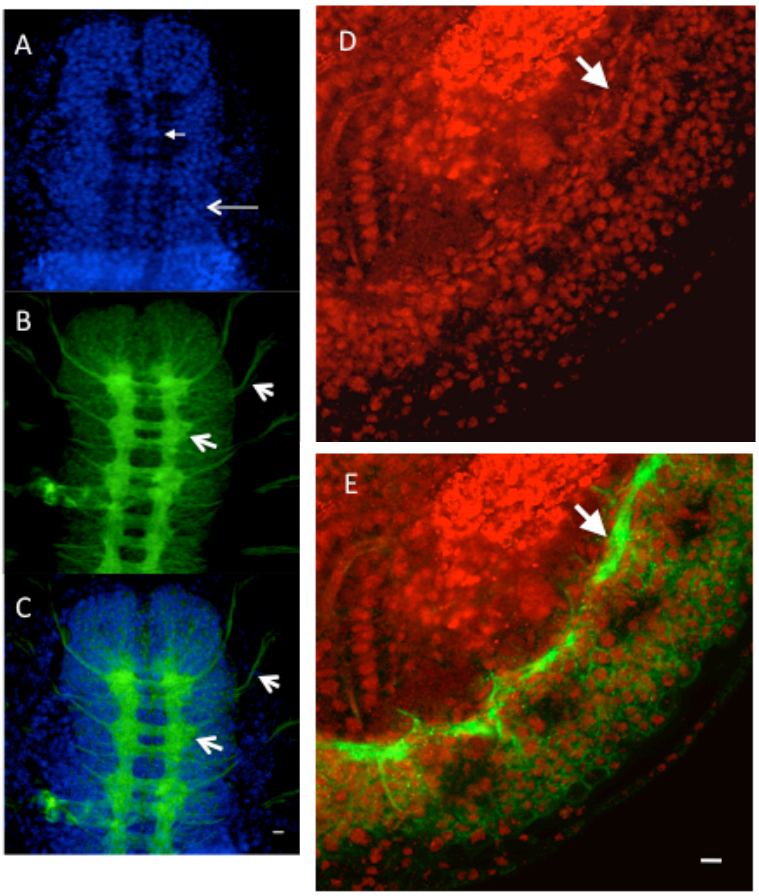


Figure 5. Tip60 localizates to the nervous system in *Drosophila* embryos. Confocal microscopic dorsal view of a w^{1118} wild-type embryo (stage 15) double labeled with dTip60 antibody (blue) and horseradish peroxidase (HRP) (green) that labels the surfaces of all neurons. **A.** dTip60 antibody staining in the anterior portion of the embryo. dTip60 is present in the central nervous system, and is localized within the anterior neuroblast population known as the neuropil (anterior cells shown), median cells of the CNS (small thin arrow), and possibly within the ganglion cells (thin long arrow). **B.** HRP labeled anterior portion of the nervous system. **C.** dTip60 and HRP confocal images merged image. dTip60 is not observed in the growing intersegmental nerve axons (thick arrows; **B, C**) as visualized by confocal imaging of merged HRP and dTip60 immunostaining at 60X magnification. **D.** Stage 15 embryo double labeled with dTip60 antibody (red) and HRP antibody (green). Lateral view of the ventral nerve cord showing presence of dTip60 in the CNS. **E.** Merged image of dTip60 and HRP antibodies showing dTip60 localization in the nucleus of these CNS cells. dTip60 is absent in the segmental and intersegmental axons (thick arrowheads). Scale bar: 10 um. **This experiment was performed by Jessica Sarthi.**

Chapter 3 Tables

Table 1. Summary of single and double UAS lines analyzed.

Fly Line	Description
Single Transgenic Lines[a]	
w^{1118}	Wild type control
$dTIP60^{E431Q}$	dTIP60 HAT-specific mutant
hAPP	Human amyloid precursor protein
hAPPΔCT	Human amyloid precursor protein with C-terminal deletion
Double Transgenic Lines[b]	
hAPP;$dTIP60^{E431Q}$	Homozygous for both hAPP and $dTIP60^{E431Q}$
hAPPΔCT;$dTIP60^{E431Q}$	Homozygous for both hAPP and $dTIP60^{E431Q}$

[a]**Single Transgenic Lines.** $dTIP60^{E431Q}$ lines are homozygous for *Drosophila* Tip60 containing a single nucleotide substation within the catalytic HAT domain on chromosome 3. hAPP and hAPPΔCT lines are homozygous for human APP and human APP lacking the Tip60-interacting C-terminus on chromosome 2, (Fossgreen et al.,1998; Gunawardena and Goldstein, 2001). w^{1118} flies were used as a control for all experiments.

[b]**Double Transgenic Lines.** Double transgenic lines were generated to be homozygous for hAPP or hAPPΔCT on chromosome 2 and $dTIP60^{E431Q}$ on chromosome 3 for genetic interaction studies.

Table 2. Tip60 and APP genetically interact in multicellular development.

Fly Line	Developmental Stage of Lethality	Approximate Day of Lethality
Single Transgenic Crosses[a]		
w^{1118}	Not Lethal	Day 12++
hAPP	Adult	Day 12+
hAPPΔCT	Not Lethal	Day 12++
$dTIP60^{E431Q}A$	Mid-Pupae	Day 9
$dTIP60^{E431Q}B$	2nd Instar	Day 4
Interaction Crosses[b]		
$dTIP60^{E431Q}A$;hAPP	Prepupae	Day 8
$dTIP60^{E431Q}A$;hAPPΔCT	Mid/Late Pupae	Day 10
hAPP;$dTIP60^{E431Q}B$	Embryo	Day 1
hAPPΔCT;$dTIP60^{E431Q}B$	Prepupae	Day 8

[a]**Single Transgenic Crosses.** Ten female virgins from each single transgenic line were crossed to seven males from the ubiquitous 337-GAL4 driver line and surviving progeny were counted for eight days. Remaining dead larvae and pupae were counted and scored by stage. hAPP was 40% lethal with flies surviving to adulthood but dying soon after eclosion (Day 12+) while hAPPΔCT showed no lethality compared to wild type w^{1118} controls, both of which lived well after eclosion (Day 12++). Two independent $dTIP60^{E431Q}$ lines showed 100% lethality that occurred during the mid-pupal stage (Day 9) for the weaker line and the second instar stage (Day 4) for the stronger line.

[b]**Interaction Crosses.** Two double transgenic lines were generated, each homozygous for $dTip60^{E431Q}$ (A or B) on the third chromosome and either hAPP or hAPPΔCT on the second chromosome. Ten female virgins from each double transgenic line were crossed to seven males from the ubiquitous 337-GAL4 driver line and surviving progeny were counted for eight days. Remaining dead larvae and pupae were counted and scored by stage. For each $dTIP60^{E431Q}$ line (A and B) co-expression with hAPP resulted in 100% lethality occurring earlier in development than the $dTIP60^{E431Q}$ line alone, and co-expression with hAPPΔCT resulted in 100% lethality occurring later in development than the $dTIP60^{E431Q}$ line alone.

Table 3. Tip60 and APP genetically interact in neuronal development.

Fly Lines[a]	Surviving Flies (GAL4-)[b]	Surviving Flies (GAL4+)
Single Transgenic Crosses		
w^{1118}	59±13	62±7
hAPP	63±17	29±8*
hAPPΔCT	56±14	56±6
$dTIP60^{E431Q}$	60±10	31±7*
Interaction Crosses		
$hAPP;dTIP60^{E431Q}$	63±25	25±7*
$hAPP\Delta CT;dTIP60^{E431Q}$	64±14	56±13
		*p≤0.05

[a]**Fly Lines.** Ten virgin females from each of the single transgenic lines or double transgenic interaction lines were crossed to five males from the pan-neuronal 179y-GAL4 driver line. Surviving progeny were counted for eight days and scored as either male (GAL4-) or female (GAL4+).

[b]**Surviving Flies (GAL4-).** Males were scored as a GAL4- internal control for GAL4+ comparison.

[c]**Surviving Flies (GAL4+).** Females were scored as GAL4+ and compared to the number of GAL4- males from the same vial to determine lethality. In the single transgenic crosses, both hAPP and $dTIP60^{E431Q}$ induced significant lethality while hAPPΔCT showed no effect compared to wild type w^{1118}. In the double transgenic interaction crosses, hAPP co-expressed with $dTIP60^{E431Q}$ slightly reduced hAPP induced lethality and did not affect $dTIP60^{E431Q}$ induced lethality significantly, while hAPPΔCT co-expression with $dTIP60^{E431Q}$ surprisingly rescued $dTIP60^{E431Q}$ induced lethality completely. The results are reported as mean ± SD (n=3).

Chapter 3 Figures

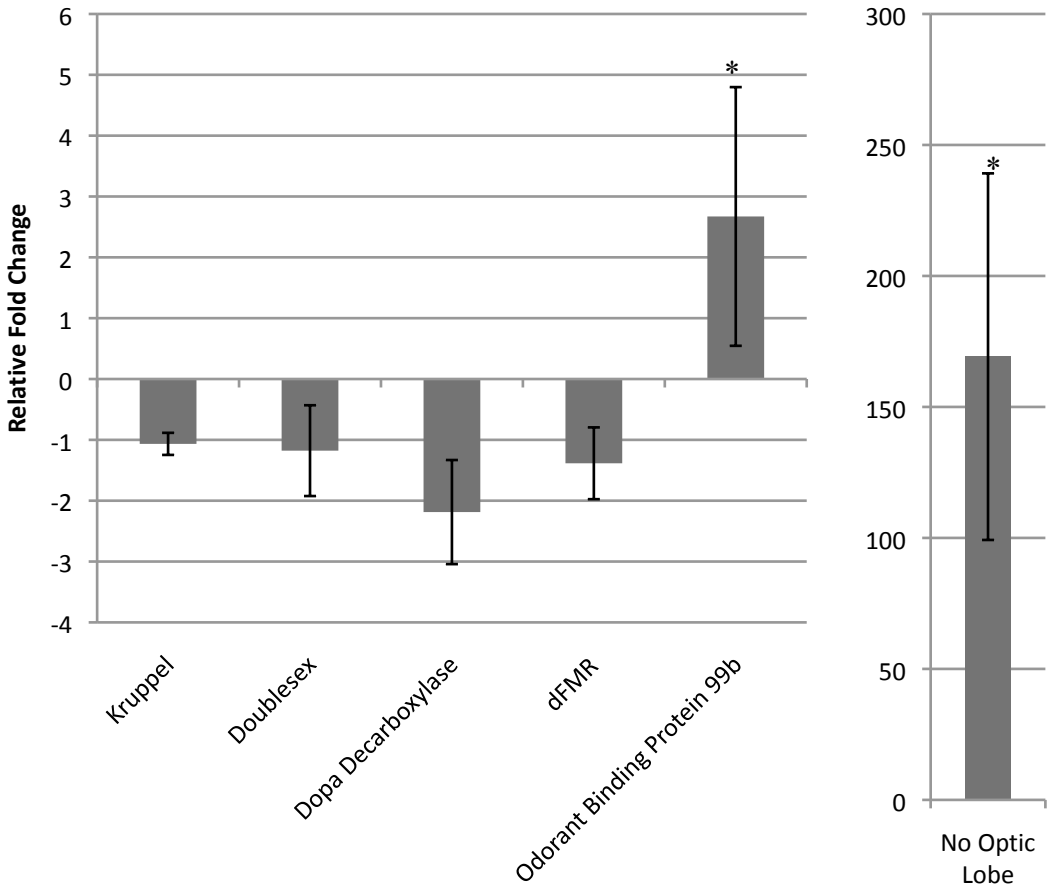
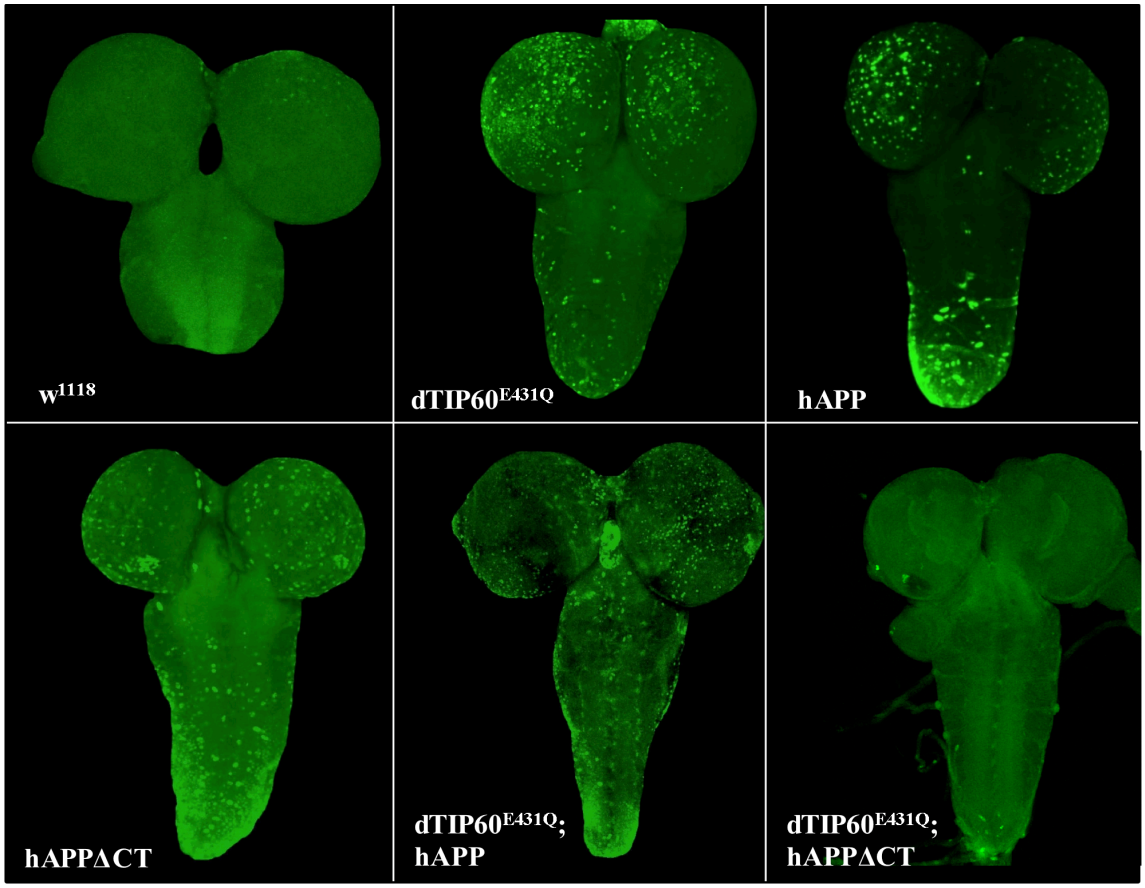


Figure 1. *Nol* and *obp99b* are misregulated upon overexpression of hAPP. Six genes regulated by Tip60 HAT activity were analyzed with qPCR to determine if APP over-expression also induced their misregulation. hAPP virgins were crossed to 337-GAL4 males to induce ubiquitous expression and third instar larval progeny were collected for RNA extraction. The relative fold change in mRNA levels was calculated compared to wild type using the comparative CT method with RP49 standardization. Two of the six genes tested, Odorant Binding Protein 99b and No Optic Lobe were significantly misregulated when hAPP was overexpressed. The results are reported as mean ± standard error (SEM), (n=3); $p \leq 0.05$.


w1118
dTIP60E431Q
hAPP
hAPPΔCT
dTIP60E431Q; hAPP
dTIP60E431Q; hAPPΔCT

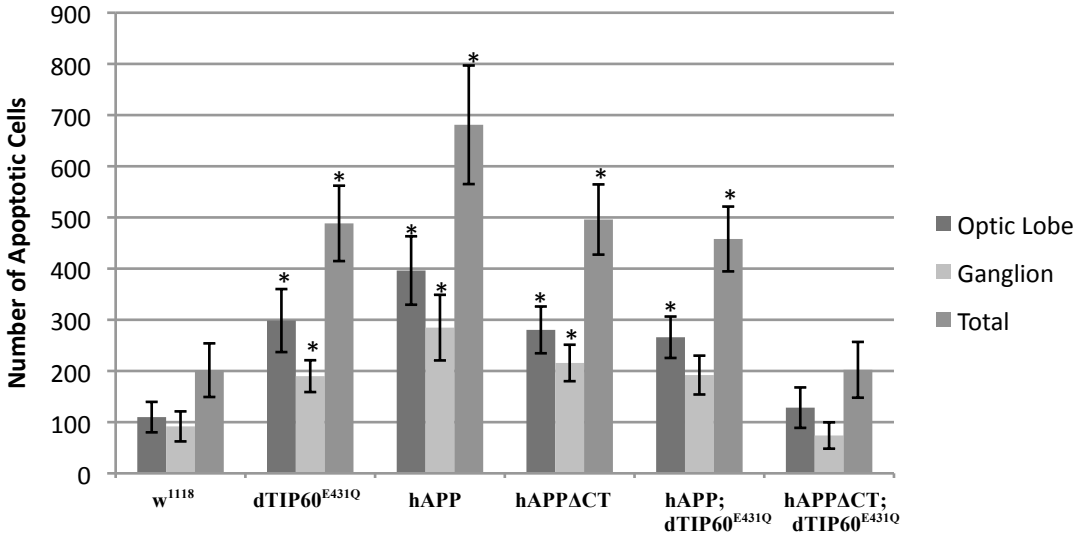

Number of Apoptotic Cells
900
800
700
600
500
400
300
200
100
0
w1118
dTIP60E431Q
hAPP
hAPPΔCT
hAPP; dTIP60E431Q
hAPPΔCT; dTIP60E431Q
Optic Lobe
Ganglion
Total

Figure 2 (continued)

Figure 2. TUNEL analysis reveals a dependence for dTip60^{E431Q} induced apoptosis on AICD production. **A.** TUNEL staining was used to identify apoptotic neurons in third instar larval brains. dTip60^{E431Q}, hAPP, hAPPΔCT, and double transgenics carrying both the dTip60 mutation and either hAPP or hAPPΔCT, were crossed to 179y-GAL4 drivers for neuronal expression. TUNEL analysis of the brains of third instar larvae shows that dTIP60^{E431Q}, hAPP, and hAPPΔCT all promote apotosis individually in the central nervous system, while coexpression of dTIP60^{E431Q} and hAPP does not result in an additive increase of apoptotic cells and coexpression of dTIP60^{E431Q} and hAPPΔCT completely rescues the neuronal apoptosis induced by each individually. **B.** The number of apoptotic neurons in TUNEL stained brains were quantified by manual counting of 15-20 brains for each genotype, three times each. These results support our observations. The values are reported as mean ± standard error (SEM).

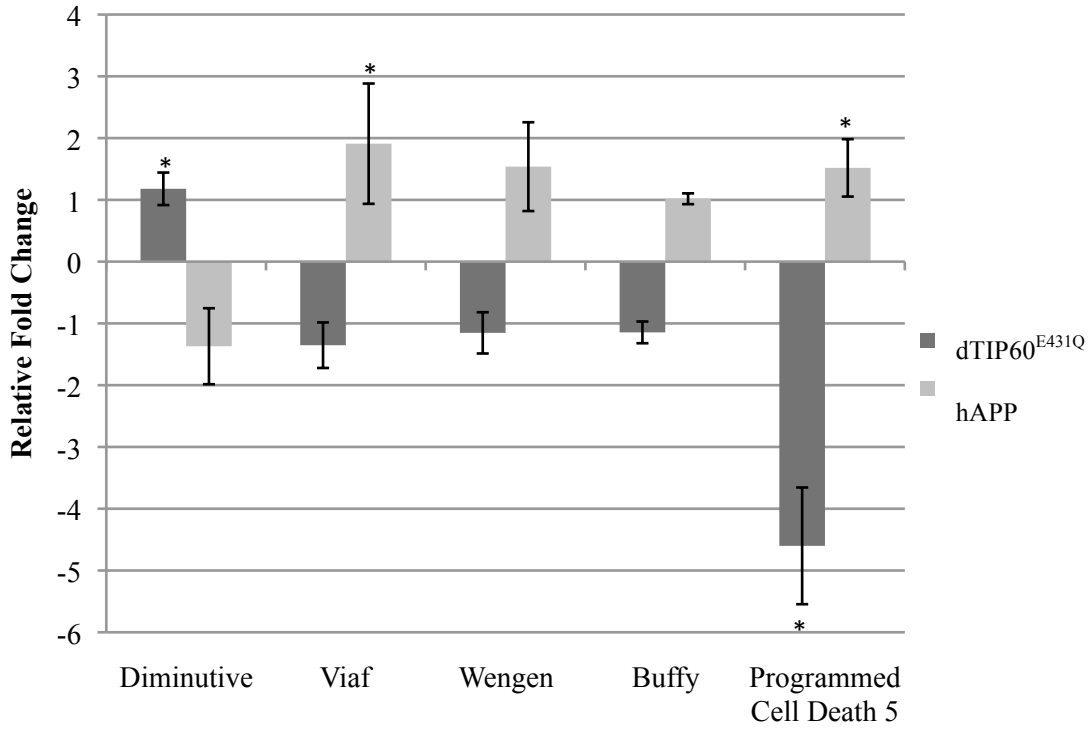


Figure 3. PDCD5 is misregulated upon expression of both dTip60^{E431Q} and hAPP. qPCR was used to examine the expression levels of apoptosis-related genes upon the loss of Tip60 HAT activity or APP over-expression to determine if Tip60 and APP are involved in the regulation of common apoptosis targets, as an indication of an overlap in pathways affected by these proteins. Of the five targets tested, only Programmed Cell Death 5 was misregulated by both the Tip60 HAT mutant and APP overexpression. The relative fold change in mRNA levels was calculated compared to wild type expression levels using the comparative CT method with RP49 standardization. The results are reported as mean ± standard error (SEM), (n=3).

Chapter 4 Figures

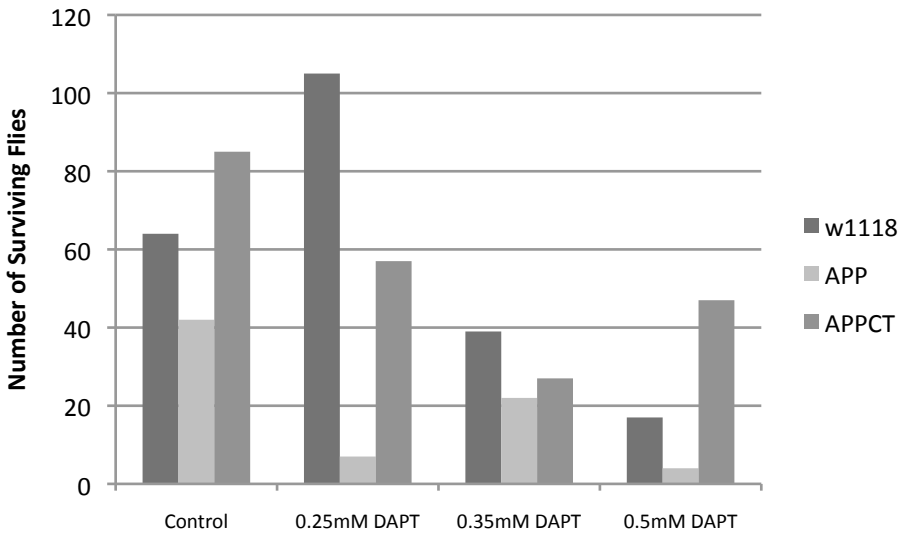


Figure 1. The γ-secretase inhibitor DAPT modulates lethality in flies overexpressing APP. Shown is a histogram depicting the number of surviving adult flies when hAPP and hAPPΔCT were expressed ubiquitously and larvae were raised on the γ-secretase inhibitor DAPT from embryo to adult. Increasing concentrations of DAPT resulted in a decrease in the number of surviving adult flies for hAPP, hAPPΔT, and wild type lines. When raised on 0.5mM DAPT the majority of hAPP flies died in the pupal stage.

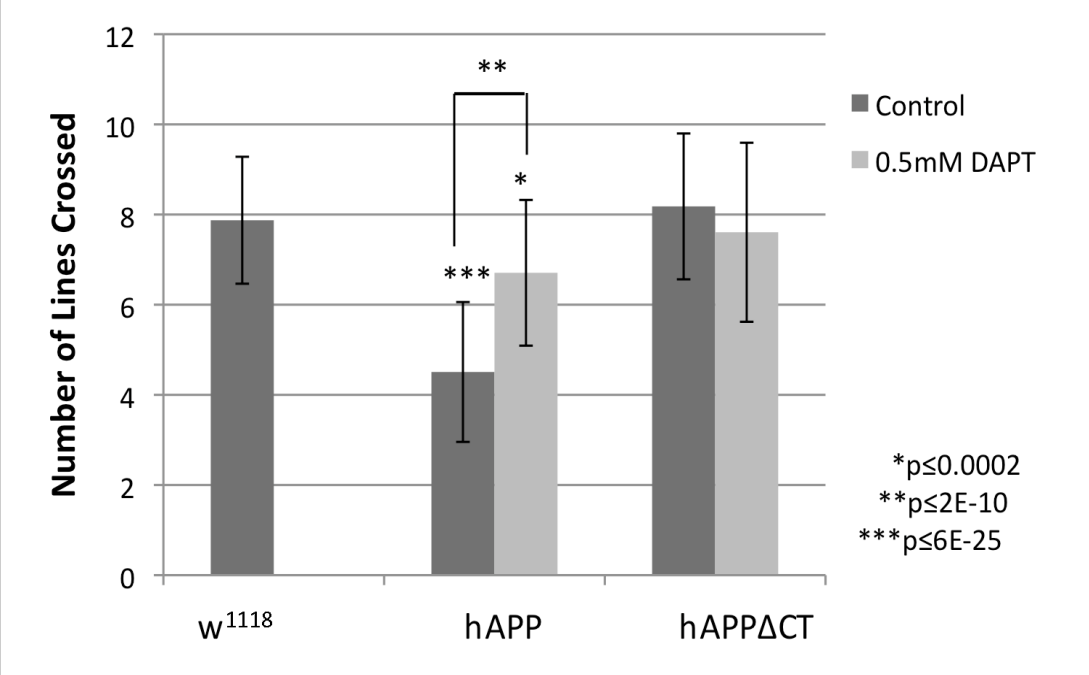


Figure 2. Locomotor defects induced by AICD overexpression can be suppressed with the γ-secretase inhibitor DAPT. Shown is a histogram depicting the number of lines crossed by wandering third instar larvae as an indication of locomotor ability. Larvae expressing hAPP in the nervous system demonstrated defects in locomotor ability that were not seen in hAPPΔCT expressing larvae. Treatment with DAPT significantly suppressed this defect, nearly to that of wild type. Asterisks (*) indicate significant fold changes where * is $p \leq 0.0002$, ** is $p \leq 2E\text{-}10$, and *** is $p \leq 6E\text{-}25$. Results are reported as mean ± standard deviation, ($n < 40$).

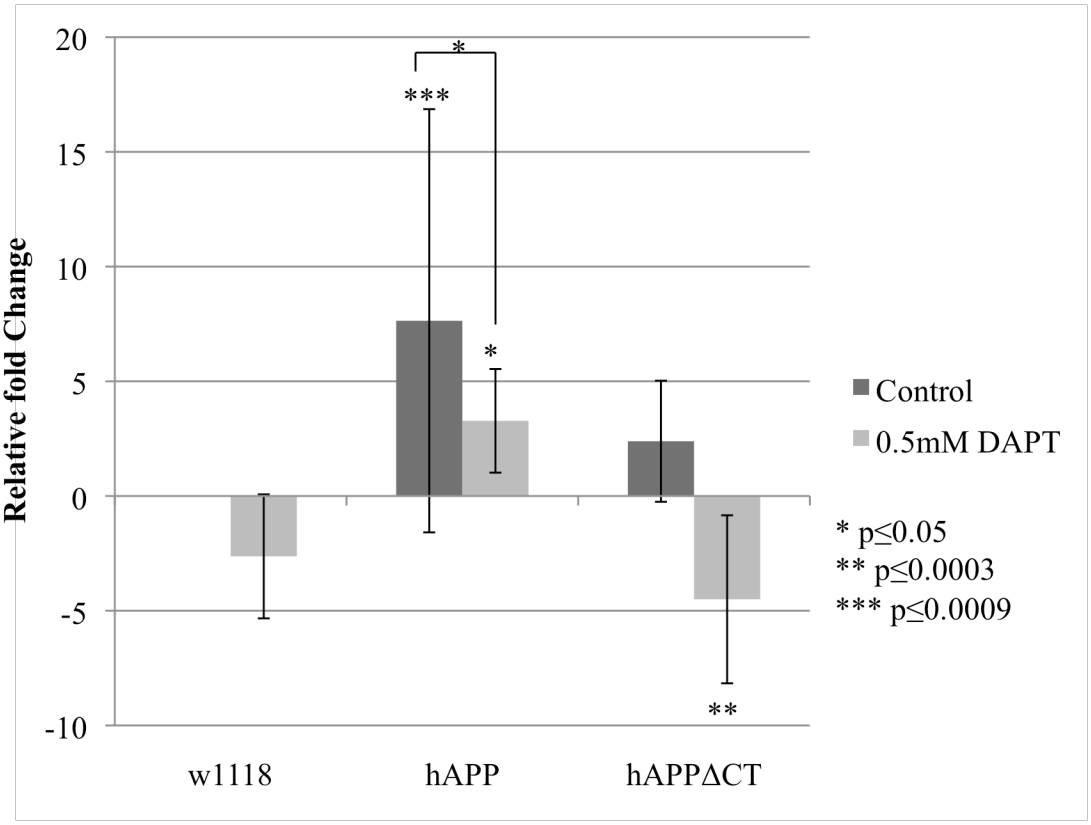


Figure 3. The γ-secretase inhibitor DAPT suppresses transcriptional misregulation of *nol* which is induced by AICD overexpression. Shown is a histogram depicting the results of qPCR analysis of the target *nol* which is regulated by both Tip60 and APP. The relative fold changes in mRNA expression levels were measured using the comparative Ct method with expression levels standardized to RP49. *Drosophila* larvae expressing hAPP ubiquitously showed significant upregulation of *nol,* and this effect was significantly suppressed by 0.5mM DAPT. *Drosophila* larvae expressing hAPPΔCT did not show misregulation of *nol*, however DAPT significantly reduced *nol* expression in hAPPΔCT expressing larvae. Asterisks (*) indicate significant fold changes where * is $p \leq 0.05$, ** is $p \leq 0.005$ and *** $p \leq 0.0009$. Results are reported as mean ± SEM, (n=3).

Appendix A: The histone demethylase Dmel/Kdm4A controls genes required for lifespan and male-specific sex-determination in *Drosophila*.

Abstract

Histone methylation plays an important role in regulating chromatin-mediated gene control and epigenetic-based memory systems that direct cell fate. Enzymes termed histone demethylases directly remove the methyl marks from histones, thus contributing to a dynamically regulated histone methylated genome, however the biological functions of these newly identified enzymes remains unclear. The JMJD2A-D family belongs to the JmjC domain-containing family of histone demethylases (JHDMs). Here, we report the cloning and functional characterization of the *Drosophila* HDM gene Dmel\Kdm4A that is a homolog of the human JMJD2 family. We show that homologs for three human JHDM families, JHDM1, JHDM2 and JMJD2 are present in *Drosophila* and that are each expressed during the *Drosophila* lifecycle. Disruption of Dmel\Kdm4A results in a reduction of the male lifespan and a male-specific wing extension/twitching phenotype that occurs in response to other males, and is reminiscent of an inter-male courtship phenotype involving the courtship song. Remarkably, certain genes associated with each of these phenotypes are significantly downregulated in response to Dmel\Kdm4A loss, most notably the longevity associated *Hsp22* gene and the male sex-determination *fruitless* gene. Our results have implications for the role of the epigenetic regulator Dmel\Kdm4A in the control of genes involved in lifespan and male-specific sex-determination in the fly.

Introduction

Histones are chromatin proteins that play an important role in DNA packaging and gene regulation. The initial level of chromatin packaging consists of the nucleosome, made up of DNA wrapped around two copies each of core histone proteins H2A, H2B, H3 and H4. Histones are subjected to a wide variety of covalent modifications that include acetylation, phosphorylation and methylation, (Berger 2002; Felsenfeld and Groudine 2003; Fischle, Wang et al. 2003; Martin 2005; Luger 2006). Distinct combinatorial patterns of such modifications are believed to serve as epigenetic marks that control chromatin packaging and subsequent gene expression by providing recognition sites for downstream chromatin regulatory factors, (Nowak and Corces 2000; Rice and Allis 2001; Fischle, Wang et al. 2003; Bottomley 2004).

Histone methylation plays an important role in many biological processes including heterochromatin formation, homeotic gene silencing, X-chromosome inactivation, genomic imprinting and transcriptional regulation (Lachner, O'Carroll et al. 2001; Feinberg, Cui et al. 2002; Santos-Rosa, Schneider et al. 2002; Margueron, Trojer et al. 2005; Martin and Zhang 2005; Martin 2005) and may exist on both the lysine (K) and arginine (R) residues of histones. Lysine methylation can occur on a variety of specific sites on histone H3 (K4, K9, K27, K36, and K79) and histone H4 (K20), thus allowing for the generation of distinct histone methylation patterns that directly influence chromatin regulated cellular processes. Importantly, lysine residues can also be mono-, di-, or trimethylated, and such differential methylation states serve to diversify the docking sites for effector chromatin proteins and modifiers, thus underscoring the complexity of histone methylation in regulating biological processes, (Zhang and

Reinberg 2001; Santos-Rosa, Schneider et al. 2002; Wang, Wysocka et al. 2004). Histone methylation had long been considered an irreversible epigenetic mark, however this viewpoint was challenged with the discovery of the first H3-K4 and K9 specific histone demethylase (HDM) LSD1, (Metzger, Wissmann et al. 2005; Shi, Matson et al. 2005). Since then, numerous different HDMs have been discovered that display distinct substrate and methylation conversion state specificity, supporting the concept that histone methylation, like acetylation, is a reversible and dynamically regulated process, (Chang, Chen et al. 2007; Shi 2007). Investigation of the specific biological roles of these newly identified HDMs will undoubtedly contribute to our understanding of histone methylation regulated cellular processes in development and disease.

The JmjC-domain-containing histone demthylases (JHDMs) represent the largest class of HDMs, (Wang, Wysocka et al. 2004; Tsukada, Fang et al. 2006; Shin and Janknecht 2007). These HDM enzymes are characterized by containing a conserved JmjC domain that catalyzes lysine demethylation of histones via an oxidative reduction reaction that requires iron Fe(II) and alpha- ketoglutarate (aKG) cofactors. Unlike LSD1, that reduces only mono- and dimethyl lysine modifications (Shi, Matson et al. 2005), certain JHDM family members can also reduce tri- histone lysine-methylation states, (Tsukada, Fang et al. 2006; Whetstine, Nottke et al. 2006; Shi 2007). Additionally, different JHDM families display distinct substrate specificity. For instance, the JHDM1 family reduces H3K36, the JHDM2 family reduces H3K9 while certain members of the JHDM3/JMJD2 family can reduce both H3K9 and H3K36. There are four JHDM3/JMJD2 genes within the human genome, designated JHDM3/JMJD2A-D (here, they will be referred to as JMJD2A-D for simplicity) (Tsukada, Fang et al. 2006; Whetstine, Nottke et al. 2006). It

has been suggested that JMJD2D gave rise to two additional human genes, JMJD2E and JMJD2F via local retrotransposition. Genes JMJD2A-C encode for proteins containing N-terminal JmjC and JmjN domains, followed by two C-terminus PhD domains and two Tudor domains, (Klose, Kallin et al. 2006; Whetstine, Nottke et al. 2006). In contrast, the JMJD2D family member encodes for a shorter protein product that lacks the C-terminal PHD and Tudor domains (Klose, Kallin et al. 2006).

Prior *in vitro* analysis of the catalytic activity of the four human JMJD2A-D protein family members reveals differences in both their substrate specificity and their ability to mediate different degrees of demethylation, supporting distinct biological functions for these family members. For example, while all JMJD2 family members can reduce H3-K9Me1, only JMJD2A and C have the capacity to also reduce H3-K36Me3. Additionally, while JMJD2A-D can all convert H3-K9Me3 to H3-K9Me2, only JMJD2D can reduce H3-K9Me3 to H3-K9Me2 and H3-K9Me1, (Whetstine, Nottke et al. 2006; Shin and Janknecht 2007; Shin and Janknecht 2007). However, although these newly identified HDMs are now well characterized in terms of their enzymatic specificity and activity, the biological role of these proteins during multicellular development remains to be elucidated.

Here, we report the cloning and functional characterization of the *Drosophila* HDM gene Dmel\Kdm4A that is a homolog of the human JMJD2 family. We show that homologs for the three main human JHDM families, JHDM1, JHDM2 and JMJD2 (Klose, Kallin et al. 2006; Tsukada, Fang et al. 2006), are each present in *Drosophila* and that each is expressed during the *Drosophila* lifecycle. Disruption of Dmel\Kdm4A in the fly results in a reduction in the male lifespan and a male-specific wing

extension/twitching phenotype that occurs in response to the presence of other males, and is reminiscent of an inter male courtship phenotype involving the courtship song, (Certel, Savella et al. 2007). Remarkably, certain genes associated with each of these phenotypes are significantly downregulated in response to Dmel\Kdm4A loss, most notably the longevity associated heat shock protein 22 (Hsp22) gene (Morrow, Battistini et al. 2004) and the male sex-determination *fruitless* gene (Dickson 2002; Dickson 2008), in which mRNA levels in male flies is almost undetectable. Taken together, our results support an essential role for epigenetic regulator Dmel\Kdm4A in the transcriptional activation of genes required for lifespan control and male-specific sex-determination and courtship behavior.

Materials and Methods

Identification of *D. melanogaster* histone demethylases, isolation of cDNA clones, and DNA sequencing. BLAST searches were carried out using the BLAST algorithm at both FLYBASE (1999) and NCBI with sequences corresponding to either JHDM1B, JHDM2B and JHDM3/JMJD2A. *Drosophila* sequences were identified that displayed high homology to each of these sequences (CG11033 for Dmel\JHDM1, CG8165 for Dmel\JHDM2 and CG15835 for Dmel\Kdm4A). As we failed to identify a cDNA clone corresponding to Dmel\Kdm4A in the expressed sequence tag (EST) database at the time we began this work, we cloned a cDNA using RT-PCR. Total RNA was isolated from *Canton S. D. melanogaster* pupae or adult flies using TRIzol (Invitrogen) and treated twice with DNA-free^{TM} (Ambion) to remove DNA. First strand cDNA was prepared

using the SuperScript™ II reverse transcriptase kit (Invitrogen) according to the manufacture's instructions with 1μg total RNA and 15 ng/μL of random hexamer primers (Roche). The full ORF for Dmel\Kdm4A was amplified by PCR using the forward primer, 5'-GATATAAA**GCGGCCGC**GCC*ATCA**ATG***TCCACGAGATCTTCATTCGCC3' containing eight additional base pairs to aid in restriction enzyme digest (underlined), a NotI site (bold), followed by a KOZAC sequence (in italics), and sequence corresponding to the first 7 codons of Dmel\Kdm4A. The reverse strand primer, 5'-GCTCTAGA**TCATCATCA***ATCCTC*GTCGTCAAGTGTGAG-3' contained eight additional base pairs to aid in restriction digest, a *XbaI* site (bold), followed by two in frame stop codons (italics), and the last five codons of Dmel\Kdm4A. PCR reactions were carried out using Expand™ High Fidelity PCR System (Roche) according to the manufactures instructions using 400 nM of each forward and reverse primers. The cycling parameters were 30 cycles of 95° for 2 min, 55° for 1 min, and 72° for 3 min, using Mastercycler (Eppendorf). The correct sized PCR amplification products were cloned into the TOPO pCR2.1 vector (Invitrogen) according to the manufacture's instructions. The entire insert DNA sequence for each of these constructs was determined by the University of Pennsylvania DNA Core Sequencing Facility, Philadelphia, PA.

Semi-Quantitative RT-PCR of staged *Drosophila* RNA. Total RNA was isolated from staged *Canton S. D. melanogaster* (12-24h embryo, 1st instar larvae, 2nd instar larvae, 3rd instar larvae, pupae, and adult fly) using TRIzol (Invitrogen) and treated twice with DNA-free™ (Ambion) to remove DNA. First strand cDNA was prepared using the

SuperScript^{TM} II reverse transcriptase kit (Invitrogen) according to the manufacture's instructions with 1μg total RNA and 15 ng/μL of random hexamer primers (Roche). Primer sets for Dmel\JHDM1 (forward primer: 5'-CGCGTGAAACAGGAGATAAAG3', reverse primer: GCTGGTGGCAATCACACTAATAG3') amplified a 464-bp fragment, Dmel\JHDM2 (forward primer: 5'-GTTTTCAGTGCATGACCAAG-3', reverse primer: 5' GGCAACGAGCTCTAGTGATG-3') amplified a 417-bp fragment and Dmel\Kdm4A (forward primer: 5'-GTTTCCAGCCAGAGCGATAC-3', reverse primer: GACAGGGCAGTTCATTCCATAG3) amplified a 401-bp fragment and RP49 (forward primer: 5'-5`GCCCAGCATACAGGCCCAAG3`3', reverse primer: 5' CGTTCTCTTGAGAACGCAGG3 3') amplified a 402-bp fragment. All PCR reactions were carried out in triplicate in 40 μl total reaction volumes containing: 0.5 U Taq (Qiagen), 1 μl cDNA (from the RT reaction described above), 250 μM dNTPs (Amersham Pharmacia Biotech), and 10 μM each of forward and reverse primer. The PCR cycling conditions were: 34 cycles at 95° for 3 min , 55° for 1 min, and 72° for 1 min with a 7 min. extension after each cycle.

qPCR analysis. Total RNA was isolated from 21 day old male Dmel\Kdm4A $^{P\text{-}supp}$ and Dmel\Kdm4A^{REV} flies using TRIzol (Invitrogen) and treated twice with DNA-free (Ambion) to remove DNA. cDNA was prepared using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions with 1 μg total RNA and 0.2μg/mL random hexamer primers (Roche Applied Science). PCR reactions were performed in a 20 μL reaction volume containing cDNA, 1x *Power* SYBR® Green PCR Master Mix (Applied Biosystems), and 10μM both forward and reverse primers,

(primer pairs available upon request). PCR was performed using an ABI 7500 Real-Time PCR system (Applied Biosystems) following the manufacturer's instructions. Fold change in mRNA expression were determined by the ΔΔCt method, (Livak and Schmittgen 2001; Yuan, Reed et al. 2006).

Immunohistochemical staining of embryos. The antibodies used in immunohistochemical staining of embryos were as follows: mouse anti-ELAV (Developmental Studies Hybridoma Bank, University of Iowa); mouse anti-REPO (Developmental Studies Hybridoma Bank, University of Iowa); mouse 22C10 (Developmental Studies Hybridoma Bank, University of Iowa); biotin-conjugated anti-mouse secondary antibody (Vectastain ABC Elite kit; Vector Laboratories); biotin-conjugated anti-rat secondary antibody (Vector Laboratories). Embryos collected from grape-agar plates (Flystuff) were dechorionated with 50% Clorox bleach, rinsed with 0.1% Triton-X solution in water, then transferred to eppendorf tubes. To fix eggs, the Triton-X solution was removed and equal volumes of 4% paraformaldehyde in PBS and heptane (1ml) were added. The tubes were gently and continuously shaken for 2 minutes by hand before removing first the bottom paraformaldehyde phase and then the top heptane phase. Eggs were devitellinized in a 1:1 heptane/methanol mixture (1ml), rinsed once with methanol, and then washed twice with PBT (PBS, 0.1% Tween-20).

Antibody staining was performed by first washing the embryos in phosphate-buffered saline (PBS) every 30 minutes with 0.1% Tween (PBT) over a 3 hour period at room temperature. Embryos were incubated with primary antibody (diluted 1:500 in PBT) overnight at 4°C in 1.5 ml microcentrifuge tubes with constant rotation. Embryos were washed with PBT every 30 min for 3 h at room temperature. Biotinylated anti-mouse

secondary antibody (Vectastain ABC Elite kit; Vector Laboratories) diluted 1:400 in PBT was added to the embryos and incubated overnight at 4°C. Embryos were then washed with PBT every 30 min over a 3 hour period at room temperature and incubated in biotin–streptavidin–horseradish peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) at room temperature for 1.5 h. Embryos were then washed eight times in PBT for 2 h. The signal was developed by incubation with 500 μl ImmPACT DAB (Vector Laboratories) in the presence of 1 μl of 10% H_2O_2. The reaction was terminated by washing the embryos with PBT and then with ethanol. The embryos were mounted in methyl salicylate and viewed with Zeiss Axioplan2 optics.

Identification of Dmel\Kdm4A$^{P\text{-}supp}$ and revertant fly lines. The P-element suppressor of Dmel\Kdm4A was identified by searching for stocks under the gene accession number for the gene: CG15835 at Flybase.org. The Bloomington fly stock number is 13828 and the genotype is: y^1 w^{67c23}; P{SUPor-P}CG15835^{KG04636}. These flies were designated as line Dmel\Kdm4A$^{P\text{-}Supp}$. To create Dmel\Kdm4A revertant fly lines, the P-element was remobilized and excised as described, (Palladina et al., 2002). Such excision flies were examined for precise excision of the P-element by single fly genomic PCR. Briefly, single male and female flies from the 56 potential P-element excision lines were collected. Genomic DNA was extracted by homogenizing flies in SB buffer (10 mM Tris-Hcl pH 8.0, 1mM EDTA, 25 mM NaCl, 200 g/ml Proteinase K) and the crude DNA extract was directly used for PCR reactions. To verify the presence of a single P-element insertion in Dmel\Kdm4A$^{P\text{-}Supp}$, sequencing was carried out on genomic DNA using primers P2 and P3, corresponding to the 3' and 5'ends of the P-element, respectively. Precise excision was verified using PCR with primer sets P1 and P2, corresponding to the

5’ genomic insertion site and 3’ end of the P-element, respectively and primer sets P3 and P4 corresponding to the 5’ end of the P-element and 3’ end of the genomic insertion site sequence. The primers were: P1 (5’-GAGATTCGTTTCGCTTGCTT-3’),

P2(5’-GGCAAGAAAGTAGGTTGATAAAGC-3’), P3(5’-GTCTGACCTTTTGCAGGTGC-3’), and P4 (5’-GCTGGATGTTGATTTGCTGG-3’). All PCR fragments were sequenced to confirm their correct identity.

Climbing assay. The climbing assay was performed as follows. Twenty female flies and twenty male flies (forty flies total) were placed in plastic vials. The number of flies at the top of the vial were counted after either 7s or 18s of climbing over a period of 14 days. Each time point was repeated a minimum of five times and a maximum of ten times. The experiment was repeated three independent times with similar results obtained from each experiment.

Longevity assay. Staged male and female flies were collected at 0-24 hours and grown separately after eclosion to eliminate the affect of mating on longevity. 113 Canton S males, 112 Canton females, 222 Dmel\Kdm4A$^{REV\ A}$ male, 196 Dmel\Kdm4A$^{REV\ A}$ females, 226 Dmel\Kdm4A$^{P\text{-}supp}$ males and 194 Dmel\Kdm4A$^{P\text{-}supp}$ females were maintained in embryo collection chambers capped with grape juice plates. The plates were applied with fresh yeast paste and changed every day and the number of dead flies was recorded. The data was analyzed using 2-way ANOVA with SAS programming and Microsoft Excel.

Twitching Assay. Staged 0-24 hour Dmel\Kdm4A$^{P\text{-}Supp}$ and Dmel\Kdm4A$^{REV\ A}$ male and female flies were collected in separate vials and allowed to acclimate for 4 days. 10

vials containing 3 male flies and 10 vials containing 3 female flies (60 flies total) were observed and the number of times the flies twitched was counted over 5 minutes. The number obtained was divided by 3 to calculate average number of twitches per fly.

Results

Identification and cloning of the *Drosophila* Dmel\Kdm4A gene that is a homolog of the human JMJD2 gene family. To identify human homologs of the JMJD2 gene family in *Drosophila*, conserved sequences within the JMJD2 genes were used to query the *Drosophila* Genome database for genomic DNA encoding homologous sequences. Two homologous genes (accession numbers: CG15835 and CG33182) were identified that were both located on chromosome 2, arm 2R: 3810274 to 3812488 and 9073721 to 9035781 respectively. Both proteins displayed the greatest structural similarity to family member JMJD2D in their protein structure, as both of them lacked the C-terminal PHD and Tudor domains. As the conceptual protein product encoded by gene CG15835 displayed a greater homology to the JMJD2 family due to its longer N-terminus, it was chosen for further analysis (Figure 2). Because no EST cDNA clones were available for CG15838 at the time we began this work, we cloned the gene using an RT-PCR based strategy on RNA isolated from Canton S pupae. The expected 1487 bp PCR product was cloned into the TOPO vector and the full sequence was determined and aligned to the CG15835 gene sequence at FlyBase. Four nucleotide differences were identified and these same base pair changes were also found to be present in cDNA clones prepared from RNA isolated from adult Canton S flies, (Figure 1). Importantly, these changes did

not alter the amino acid sequence of the CG15835 conceptual translation product posted at FlyBase.

Analysis of the conceptual translation product for the CG15835 gene (designated Dmel\Kdm4A) indicated that this isolated *Drosophila* gene is the homolog of the human JMJD2 family. First, an alignment between the Dmel\Kdm4A and each of the human JMJD2D proteins demonstrated significant homology over their entire coding sequences, (Figure 2). Structural protein data obtained using the conserved domain architecture retrieval tool (CDART) at NCBI demonstrated that the predicted protein domains specific for Dmel\Kdm4A and their locations within the protein are highly conserved between the human JMJD2D protein and Dmel\Kdm4A; both human family member JMJD2 and Dmel\Kdm4A each contain JmjN and JmjC domains within their N-termini and do not contain the C-terminal PhD and Tudor domains that JMJD2A-C contain. However, despite the strong structural similarity between human JMJD2D and Dmel\Kdm4A, Dmel\Kdm4A displays the highest amino acid conservation with JMJD2A: 63% identity and 80% similarity and JMJD2C: 63% identity and 81% similarity, (Figure 2). Taken together, our data strongly indicate that the Dmel\Kdm4A gene is homologous to the human JMJD2 family.

***Drosophila* homologs of the three main JHDM families are each expressed during *Drosophila* development.** To determine whether additional JHDM family members are also present in *Drosophila*, we carried out data base searches which revealed the presence of *Drosophila* homologous sequences corresponding to JHDM1 and JHDM2. Analysis of the conceptual protein sequence of each gene indicated the presence of the distinct

conserved domains specific for their classification, (Figure 2). To determine whether these genes were expressed during the *Drosophila* lifecycle, RNA was isolated from staged *Drosophila melanogaster* (12-24 h staged embryos, first, second and third instar larvae, pupae, adult flies) and DNaseI treated. cDNAs were generated for each developmental stage by RT priming with random hexamers and the RT products were amplified using PCR with primer pairs specific for each HDM. Importantly, primers amplifying the gene for ribosomal protein RP49 were used as an internal control. In general, we found that each of the three HDM transcript levels were present during the *Drosophila* lifecycle, (Figure 3). These data demonstrate that *Drosophila* contains actively transcribed homologous genes for each of the human Jmc family member homologs analyzed, and that each of these JHDM genes are expressed during the *Drosophila* life cycle.

Disruption of the Dmel\Kdm4A gene causes a male-specific wing extension/twitching phenotype. To decipher the cellular function of Dmel\Kdm4A during *Drosophila* development, we identified a P-element insertion fly line from the Flybase collection that contained a single 11.4 kb P-element inserted 76 bp downstream of the Dmel\Kdm4A start codon (Figure 4A). Sequence analysis confirmed that this was a single P-element insertion that disrupted only the Dmel\Kdm4A ORF and that no other genes were located in close proximity of the insertion site (Figure 4B). RT-PCR analysis using RNA isolated from male and female adult flies with primers spanning the entire Dmel\Kdm4A ORF demonstrated that Dmel\Kdm4A transcripts were completely absent in female flies and were significantly reduced in males (Figure 4B). These results confirmed that this P-element insertion disrupted Dmel\Kdm4A gene transcription and

we designated this fly line Dmel\Kdm4A$^{P\text{-}Supp}$. The characterization of this fly line provided us with the opportunity to study the biological function of Dmel\Kdm4A in the *Drosophila* multicellular developmental model setting.

Initial characterization of the Dmel\Kdm4A$^{P\text{-}Supp}$ flies revealed that the flies displayed a twitching of their wings when compared to wild-type Canton S flies. The Dmel\Kdm4A$^{P\text{-}Supp}$ flies extended and shook their wings in quick succession, making it appear as if their wings were twitching. This phenotype appeared at approximately five days of age and appeared to become more apparent as the flies aged. To confirm that the twitching phenotype we observed was specifically due to lack of Dmel\Kdm4A transcript, we re-mobilized the P-element to excise it from the Dmel\Kdm4A gene. Two independent lines of flies, shown by sequence analysis to carry precise excisions (designated Dmel\Kdm4A$^{REV\ A}$ and Dmel\Kdm4A$^{REV\ B}$) restored Dmel\Kdm4A transcript levels back to those comparable to wild-type flies, (Figure 4C). Initial observation revealed that both the precise excision independent fly lines showed no evidence of the twitching phenotype. Importantly, a quantitative twitching assay of staged 5 day old flies (the stage when the phenotype is first readily observable) revealed that the average number of wing extension/twitches per Dmel\Kdm4A$^{P\text{-}Supp}$ male flies was significantly higher than that of female Dmel\Kdm4A$^{P\text{-}Supp}$ flies and both male and female Dmel\Kdm4A$^{REV\ A}$ control flies, (Figure 5). Remarkably, this careful behavioral analysis revealed that the male-specific Dmel\Kdm4A$^{P\text{-}Supp}$ wing extension/twitching we observed was identical in appearance to the previously described unilateral wing extension and vibration used to produce the ritualistic courtship song (Billeter, Rideout et al. 2006; Certel, Savella et al. 2007; Dickson 2008) and did not occur randomly, but almost

exclusively as a result of male specific interaction. This behavior did not appear to be aggressive in nature, as no fencing, indicative of aggressive behavior, was observed, (Certel, 2007). Importantly, when 10 males and 10 females were observed in a vial, the male wing extension/twitching behavior was almost exclusively directed in response to male and not female flies, indicating male preference in this behavior. These results indicated that the male-specific reciprocal wing extension/ twitching phenotype we observed was similar to an inter-male wing extension/singing courtship behavior, and resulted directly from the P-element disruption of the Dmel\Kdm4A gene as the phenotype could be rescued by precisely excising the P-element to restore wild-type Dmel\Kdm4A transcript levels, (Figure 5).

Normal *Drosophila* display a negative geotactic response in that when they are tapped to the bottom of a vial, they rapidly climb to the top and remain there, (Feany and Bender 2000). This natural response is compromised due to aging and defects in neurological and muscle processes. The climbing assay is widely used to quantitate the severity of defects in such processes as well as to monitor the progression of severity in a number of *Drosophila* neurological disease models (Chan and Bonini 2000; Chen and Feany 2005) including Alzheimer's (Crowther, Kinghorn et al. 2004), Parkinson's (Feany and Bender 2000) and Huntington's (Agrawal, Pallos et al. 2005) diseases. To test the climbing ability of the Dmel\Kdm4A$^{P\text{-}Supp}$ flies, 40 male or female Dmel\Kdm4A$^{P\text{-}Supp}$ flies and 40 male or female Dmel\Kdm4A$^{REV\ A}$ control flies were placed in separate plastic vials and gently tapped to the bottom of each vial. The number of flies at the top of the vial was counted after 18 seconds of climbing. This climbing assay was performed at 3, 5, 8, 10 and 14 days of age. The results revealed that Dmel\Kdm4A$^{P\text{-}Supp}$ flies showed no

significant loss of climbing ability for male or female flies when compared to the revertant flies at all time points (our unpublished results). Immunostaining of mutant and wild-type embryos with antibodies specific for either the differentiated neuronal marker protein elav or the glial cell marker protein repo revealed a normal neuronal staining pattern for all embryonic stages observed, indicating normal embryonic neuronal formation in the Dmel\Kdm4A$^{P\text{-}Supp}$ flies (our unpublished results).

Disruption of the Dmel\Kdm4A gene leads to a reduction in the male lifespan. We did not observe a significant decrease in Dmel\Kdm4A $^{P\text{-}supp}$ viability when compared to Dmel\Kdm4A^{REV} flies. Therefore, we assessed the effects of the Dmel\Kdm4A mutation on fly lifespan. An equal number of staged 0-24 hour Dmel\Kdm4A$^{P\text{-}Supp}$ and control Dmel\Kdm4A $^{REV\ A}$ flies were transferred to grape juice agar plates in a collection chamber. Importantly, male and female flies used in this experiment were grown separately directly after their eclosion to eliminate the affect of mating on longevity. The plate was changed daily with fresh yeast paste over a period of 87 days with the number of dead flies per day recorded. The results of this assay were graphed as survival curves for each of the fly lines (Figure 6). We observed a significant reduction in the lifespan for male flies and no significant reduction for female flies, indicating that disruption of the Dmel\Kdm4A gene reduces the fly life-span in a male specific fashion.

Specific genes associated with mutant Dmel\Kdm4A$^{P\text{-}Supp}$ phenotypes are significantly downregulated in response to Dmel\Kdm4A loss. Histone methylation patterns within the genome play an important role in establishing and maintaining specific gene expression profiles required for proper cell function, (Lachner, O'Carroll et

al. 2001; Fischle, Wang et al. 2003). To investigate a potential molecular basis underlying the twitching and longevity defects we observed in the Dmel\Kdm4A$^{P\text{-}Supp}$ flies, we asked whether loss of Dmel\Kdm4A resulted in misexpression of genes known to be associated with such phenotypes. The mRNA levels of 16 specific genes from Dmel\Kdm4A$^{P\text{-}Supp}$ and Dmel\Kdm4A$^{REV\ A}$ A flies was assessed by quantitative real time PCR and the fold change in gene expression levels between the two fly lines was determined (Figure 7*)*. As the twitching and longevity phenotypes we observed were male specific and intensified with age, we chose to use staged 21 day old adult males for RNA analysis to enhance our opportunity to detect potential changes in gene expression associated with these defects. The putative Dmel\Kdm4A target genes chosen to be assessed were: Shaker (Sh) (Wang, Humphreys et al. 2000; Cirelli, Bushey et al. 2005), Hyperkinetic (Hk) (Ueda and Wu 2008), and ether a go-go (Zhong and Wu 1993) chosen for their involvement in K+ channel function and shown to display a shaking leg phenotype when mutated; park and pink1, involved in Parkinson disease (Greene, Whitworth et al. 2003; Tan and Dawson 2006); *Drosophila* Nicotinamidase (D-NAAM), Silent information regulator 2 (Sir2), and rpd3, selected for their involvement in a deacetylase-mediated longevity pathway (Rogina and Helfand 2004); bent (bt) and myosin heavy chain (Mhc), each involved in muscle function (Redowicz 2002); defective in the avoidance of repellents (dare) (Freeman, Dobritsa et al. 1999), Vap-33-1 (DVAP-33A) (Chai, Withers et al. 2008)}, and survival motor neuron (SMN) (Chan, Miguel-Aliaga et al. 2003) each involved in appropriate neuromuscular junction (NMJ) function; Heat shock protein 22 (Hsp22), the mitochondrial small heat shock protein involved in stress and aging (Morrow, Battistini et al. 2004); and *fruitless* (*fru*) involved in male-

specific neuron formation that promotes masculinization (Dickson 2002; Dickson 2008; Yamamoto 2008). The results of our analysis demonstrated that out of the 16 genes assessed, two of the genes (Hsp22 and *fru*) were significantly affected, with a marked decrease in mRNA levels for each of them. Of note, the *fru* gene was the most significantly downregulated (Figure 7A), with *fru* mRNA levels so low in the Dmel\Kdm4A$^{P\text{-}Supp}$ flies, that they were undetectable in our qPCR assay and thus their downregulation was confirmed using RT-PCR and gel electrophoresis analysis, (Figure 7B). That only certain genes were affected indicate that the gene changes we observed were specific. Additionally, Dmel\Kdm4A target gene downregulation in response to Dmel\Kdm4A loss is consistent with the function of Dmel\Kdm4A as an enzyme with potent histone demethylase activity for the removal of specific methyl groups from chromatin environments marked for repression, (Shi 2007; Lin CH 2008; Wallrath and Elgin 2008). Significantly, each of the affected genes was associated with the wing twitching and longevity phenotypes we observed in our Dmel\Kdm4A$^{P\text{-}Supp}$ mutant fly line. For example, Hsp22 has been proposed to be involved in the aging process, (Morrow, Battistini et al. 2004). Quite notably, the most downregulated gene, *fru,* functions in male specific neuronal processing involved in masculinization, with *fru* fly mutants displaying inter-male courtship behaviors consistent with the inter-male courtship song behaviors we observed in our Dmel\Kdm4A$^{P\text{-}Supp}$ mutant flies, (Billeter, Rideout et al. 2006). Taken together, our results support an essential role for Dmel\Kdm4A in the transcriptional activation of genes involved in the aging process and male- specific neuronal formation and courtship behavior.

Discussion

Using *Drosophila*, we describe the consequences of eliminating Dmel\Kdm4A function in an animal model. Our results help to place the previously described biochemical activities and certain functional activities of JMJD2 into a developmental context. To investigate the role of Dmel\Kdm4A during development, we identified a P-element insertion fly line in the FlyBase collection (designated Dmel\Kdm4A$^{P\text{-}Supp)}$, and confirmed that it disrupted Dmel\Kdm4A expression (Figure 4A). Our creation of two precise P-element excision lines (designated Dmel\Kdm4A^{REVA} and Dmel\Kdm4A^{REVB}) restored transcripts to wild-type levels, making our Dmel\Kdm4A$^{P\text{-}supp}$ and Dmel\Kdm4A^{REV} fly lines a powerful multicellular model system to explore developmental Dmel\Kdm4A function. Importantly, while this work was in progress, other groups also identified the Dmel\Kdm4A gene and demonstrated by overexpression assays the ability of this enzyme to specifically demethylate H3-K36 *in vivo* in flies and in *Drosophila* S2 cell lines (Lin CH 2008; Lloret-Llinares, Carre et al. 2008) and specifically demethylate H3K36me2 and H3K36me3 both *in vitro* and *in vivo*, (Lin CH 2008). These studies confirm the demethylation activity of Dmel\Kdm4A in *Drosophila* and strongly indicate that the cause of the phenotypes we describe here for the Dmel\Kdm4A$^{P\text{-}supp}$ fly line is due to an imbalance of histone methylation in tissues and developmental stages where Dmel\Kdm4A transcripts are lacking.

When first characterizing the Dmel\Kdm4A$^{P\text{-}supp}$ fly line, we observed that the Dmel\Kdm4A$^{P\text{-}Supp}$ flies exhibited a wing extension/twitching phenotype. Quantitative analysis of this phenotype revealed that the average number of twitches per

Dmel\Kdm4A$^{P\text{-}Supp}$ male fly was significantly higher than that of female Dmel\Kdm4A$^{P\text{-}Supp}$ flies and both male and female Dmel\Kdm4A$^{REV\ A}$ control flies, indicating that the twitching phenotype was male specific and caused by disruption of the Dmel\Kdm4A gene. Previous studies had demonstrated that mutations in genes that encode voltage gated ion channels and are associated with electrical signal transmission, display a twitching phenotype (Wang, Humphreys et al. 2000) and thus we reasoned that the Dmel\Kdm4A$^{P\text{-}supp}$ twitching phenotype may have originated from similar neurological defects. However, unlike these mutant fly lines, we observed no significant defects in the ability of either Dmel\Kdm4A$^{P\text{-}supp}$ male and female flies to perform the climbing assay, a test used to monitor neurological defects, (Chan and Bonini 2000; Chen and Feany 2005). We also did not detect any gross abnormalities in embryonic CNS and PNS development as assessed by immunohistochemical staining of embryonic glial cells, neuronal cells and embryonic axonal cytoskeleton formation, consistent with the defect being observed in the adult fly. Moreover, qPCR analysis revealed that expression levels of the major genes involved in voltage-gated ion-channel formation were unaffected in the Dmel\Kdm4A$^{P\text{-}Supp}$ mutant flies when compared to revertants (Figure 7). Taken together, these results indicated that the wing extension/twitching phenotype in Dmel\Kdm4A$^{P\text{-}Supp}$ flies was reminiscent of another biological pathway.

A detailed behavioral analysis of the mutant flies revealed that the male-specific Dmel\Kdm4A$^{P\text{-}Supp}$ wing extension/twitching we observed was not random, but occurred almost exclusively in response to the presence of other males and not females, demonstrating an inter-male preference for this behavior. Further observation revealed that the behavior was identical in nature to a central component of the courtship ritual, the

courtship song, which is produced by a visible unilateral wing extension and vibration and is commonly used as a measurable readout of the male's decision to court, (Dickson 2008). This behavior was often reciprocal in nature between the males, did not appear to be aggressive, as absolutely no fencing, indicative of aggressive behavior, was observed (Certel, Savella et al. 2007) and was identical in nature to previous studies describing inter-male wing extension courtship behaviors, (Certel, Savella et al. 2007; Clyne and Miesenbock 2008). Phenotypes involving male-male courtship preference have been well characterized in the fly and predominantly result from disruption of the *fruitless* (*fru*) gene, shown to play a prominent role in the development of appropriate male sexual behavior. The transcriptional regulation of the *fru* gene is complex, in that the single *fru* gene contains four different promoters, P1, P2, P3, and P4 that each encode closely related BTB/POZ (Broad complex, Tramtrack, and Bric-a-brac/Poxvirus and Zinc finger)-Zn finger (ZnF) proteins, that likely act as transcription factors, (Song, Billeter et al. 2002). The function of *fru* in directing male-specific sex determination depends on transcripts initiated from the P1 promoter, (Song, Billeter et al. 2002). These transcripts are sex-specifically spliced, and subsequently translated into male-specific Fru^{M} proteins that directs the formation of the masculinized P1 neuronal cluster in male flies, (Kimura, Hachiya et al. 2008; Yamamoto 2008). Transcripts produced from promoters P2-P4 function in sex-nonspecific roles in axonal pathfinding, (Song, Billeter et al. 2002). Remarkably, qPCR analysis using primers designed to detect *fru* transcripts revealed almost a complete absence of these transcripts in the male Dmel\Kdm4A$^{P\text{-}Supp}$ flies (Figure 7 B). Our finding that *fru* transcripts are almost absent in male Dmel\Kdm4A$^{P\text{-}Supp}$ flies, and that these flies exhibit inter-male courtship behavior is consistent with

previous studies demonstrating that mutations at the *fru* locus that lead to inter-male courtship behavior are always associated with a global reduction in the levels of *fru* gene expression, (Billeter, Rideout et al. 2006). Interestingly, the inter-male courtship behavior we observed was confined to the wing extension/courtship song stage of the well characterized courtship repertoire, (Billeter, Rideout et al. 2006). Moreover, although the flies distinctly exhibit male preference in performing this step of the courtship sequence, Dmel\Kdm4A$^{P\text{-}Supp}$ flies do produce offspring, indicating that male-female mating does take place. This observation is consistent with previous studies demonstrating that different *fru* mutant flies exhibit courtship abnormalities to different degrees and at separate stages of the courtship sequence depending on the mutant allele, (Villella, Gailey et al. 1997). Importantly, although the molecular steps leading to the production of male specific Fru^{M} proteins *via* sex-specific differential splicing of *fru* P1 transcripts is well characterized, the molecular mechanism(s) underlying how different P1-P4 initiated *fru* isoforms are spatially and temporally regulated remain unclear. Our findings have important implications for Dmel\Kdm4A in the control of *fru* gene expression, possibly by controlling certain regulators of the *fru* gene, or by directly modulating histone methylation levels at P1-P4 gene regulatory regions in certain cell types that results in the initiation and/or maintenance of the differential production and levels of different *fru* transcripts, a model we can now explore with the use of our characterized Dmel\Kdm4A fly lines.

Our longevity assays revealed that the lifespan of the male and not female Dmel\Kdm4A$^{P\text{-}supp}$ flies was significantly reduced. Intriguingly, this male-specific reduction in lifespan is consistent with studies of the HDM LSD1 in *Drosophila*

demonstrating that reduction of LSD1 leads to a reduction in fly viability that is more severe in male flies, (Di Stefano, Ji et al. 2007; Stefano 2007). It is known that lifespan in *Drosophila* is influenced by a number of factors including temperature, starvation and caloric restriction (Rogina, Helfand et al. 2002), oxidative stress (Mourikis, Hurlbut et al. 2006), mating (Aigaki and Ohba 1984), and certain gene mutations (Rogina, Reenan et al. 2000). Moreover, a number of studies support a role for the epigenetic regulators Sir2 and Rpd3 in controlling longevity. These histone deacetylases (HDACs) influence longevity through a pathway related to calorie restriction, (Rogina, Helfand et al. 2002; Rogina and Helfand 2004). Calorie restriction triggers downregulation of Rpd3 and upregulation of Sir2 activity, leading to the extension of lifespan in *Drosophila*, presumably *via* changes in HDAC production that influences gene expression profiles that control longevity. Here, we show that although levels of Rpd3 and Sir2 gene expression are unaffected in response to Dmel\Kdm4A loss, there is a significant reduction in mitochondrial Hsp22 transcript levels. Notably, disruption of the mitochondrial Hsp22 gene in flies results in a decrease in longevity while overexpression of the gene in all cells or motoneurons increases fly lifespan. Thus, our results suggest that JMJD2 is directly or indirectly involved in regulating the aging process *via* Hsp22 controlled pathways, (Morrow, Battistini et al. 2004).

Several studies have demonstrated connections between regulation of histone methylation and neurological disorders. Specifically, SMCX, a member of the H3K4me3-specific demethylase family, has been linked to X-linked mental retardation (XLMR), (Tzschach, Lenzner et al. 2006). Morevover, neuron specific genes are misexpressed due to histone demethylase LSD1 knockdown, (Di Stefano, Ji et al. 2007).

Intriguingly, the *fru* gene, also significantly down-regulated in response to Dmel\Kdm4A loss (Figure 7), plays an essential role in neurogenesis by directing the correct formation and positioning of a male-specific neuronal cluster termed P1, that is located in the dorsal posterior brain and directs typical male fly behavior and courtship, (Yamamoto 2008). It has been recently postulated that *fru* determines the level of masculinization of these neurons by regulating the transcription of a set of downstream target genes. Thus, it is tempting to speculate that in our Dmel\Kdm4A$^{P\text{-}Supp}$ flies there are additional neuronal genes, particularly those involved in P1 neurite formation, that are also misexpressed, resulting in disruption of the masculinized P1 cluster (Yamamoto 2008). Our development and characterization of the Dmel\Kdm4A$^{P\text{-}supp}$ and Dmel\Kdm4A^{REV} fly lines now provide a powerful multicellular model system to further explore the biological function of JMJD2 in controlling such gender-specific behavioral and neuronal processes.

Appendix B: The histone acetyltransferase Elp3 plays an active role in the control of synaptic bouton expansion and sleep in *Drosophila*.

Abstract

The histone acetyltransferase Elp3 (Elongator Protein 3) is the catalytic subunit of the highly conserved Elongator complex. Elp3 is essential for the complex functions of Elongator in both the nucleus and cytoplasm of neurons, including the epigenetic control of neuronal motility genes and the acetylation of α-tubulin that affects axonal branching and cortical neuron migration. Accordingly, misregulation of Elp3 has been implicated in human disorders that specifically affect neuronal function, including Familial Dysautonomia (FD), a disease characterized by degeneration of the sensory and autonomic nervous system, and the motor neuron degenerative disorder amyotrophic lateral sclerosis (ALS). These studies underscore the importance of Elp3 in neurodevelopment and disease, and the need to further characterize the multiple nuclear and cytoplasmic based roles of ELP3 required for neurogenesis in animal models, *in vivo*. In this report, we investigate the behavioral and morphological consequences that result from targeted reduction of Elp3 HAT levels specifically in the developing *Drosophila* nervous system. We demonstrate that loss of Elp3 during neurodevelopment leads to a hyperactive phenotype and sleep loss in the adult flies, a significant expansion in synaptic bouton number and axonal length and branching in the larval neuromuscular junction as well as the misregulation of certain genes known to be involved in these processes. Our results uncover a novel role for Elp3 in the regulation of synaptic bouton expansion during neurogenesis that may be linked with a requirement for sleep.

Introduction

The generation of complex synaptic regulatory networks and diverse cell types during neurogenesis is achieved, in large part, by precisely coordinated and tightly controlled gene expression profiles distinct for each neuronal cell lineage, (Orphanides and Reinberg 2002). Maintenance of such differential gene control is largely dependent on the way that DNA and its associated histone proteins are packaged into a highly organized chromatin structure in the nucleus of eukaryotic cells, (Elefant, Su et al. 2000; Orphanides and Reinberg 2002; Kiefer 2007; Reik 2007). Activation of gene expression profiles requires that chromatin condensation be disrupted to allow for transcription factor binding and RNA polymerase assembly and passage. Control of such chromatin reorganization is achieved in large part by specific patterns of covalent modifications on the N-terminal tails of histone proteins that include acetylation, methylation, and phosphorylation, (Elefant, Cooke et al. 2000; Strahl and Allis 2000; Berger 2001; Jenuwein and Allis 2001; Fischle, Wang et al. 2003). These distinct histone modification motifs serve as signals for the recruitment of chromatin organizational proteins, which influence chromatin structure and subsequent epigenetic gene control, (Strahl and Allis 2000; Jenuwein and Allis 2001; Berger 2002; Felsenfeld and Groudine 2003; Fischle, Wang et al. 2003; Margueron, Trojer et al. 2005).

Histone acetylation is one of the best characterized of the histone modifications and is carried out by a family of enzymes termed histone acetyltransferases (HATs). The HAT Elp3 (Elongator Protein 3) is the catalytic subunit of the highly conserved Elongator complex, which consists of six subunits, ELP1-6. Elp3 contains conserved motifs

characteristic of the GNAT family of HATs and acetylates histone H3 both *in vitro* and *in vivo*. The Elongator complex was initially identified as a mutisubunit complex that copurifies with the hyperphosphorylated form of the RNA polymerase II (RNAPII) holoenzyme in yeast and human cells, (Winkler, Petrakis et al. 2001; Hawkes, Otero et al. 2002; Kim, Lane et al. 2002). Support for a direct role for Elp3 in transcriptional regulation includes genetic studies revealing defective phenotypes for yeast elp3 nulls including slow activation of certain genes, defects in global histone H3 acetylation patterns essential for gene activation (Kristjuhan, Walker et al. 2002; Winkler, Kristjuhan et al. 2002; Kristjuhan and Svejstrup 2004), and the finding that Elp3 is essential for the association of Elongator with nascent RNA *in vivo*, (Petrakis, Wittschieben et al. 2004; Svejstrup 2007). The Elongator complex has also been reported to play a variety of different roles in distinct regions of the cell in addition to its nuclear role in transcriptional elongation including the formation of modified wobble uridines in tRNAs in the cell cytoplasm (Huang, Johansson et al. 2005; Esberg, Huang et al. 2006), and polarized exocytosis, (Rahl, Chen et al. 2005).

Misregulation of Elp3 has been implicated in a number of human disorders that specifically affect neuronal function, including Familial dysautonomia (FD), an autosomal recessive neurodevelopmental disease characterized by degeneration of the sensory and autonomic nervous system (Slaugenhaupt and Gusella 2002; Axelrod 2004; Gardiner, Barton et al. 2007; Simpson, Lemmens et al. 2009), and the motor neuron degenerative disorder amyotrophic lateral sclerosis (ALS) (Wallis, Russell et al. 2008). Accordingly, studies in mammalian cells reveal that Elp3 is essential for promoting histone H3 acetylation throughout the coding regions of certain neuronal cell motility

genes that is linked to their transcriptional activation (Close, Hawkes et al. 2006), supporting a nuclear-based epigenetic role for Elp3 in neuronal gene regulation. However, the role of Elp3 in neuronal function is complex, as more recent studies support a cytoplasm-based role for Elp3 in the acetylation of α-tubulin required for the migration and differentiation of projection neurons in cultured mouse cortical neurons (Gardiner, Barton et al. 2007; Creppe, Malinouskaya et al. 2009; Wynshaw-Boris 2009), and in motor neuron axonal branching and length in zebra fish, (Simpson, Lemmens et al. 2009). Taken together, these studies underscore the importance of Elp3 in neurogenesis and disease, and the need to further characterize the multiple nuclear and cytoplasm-based roles of ELP3 in the developing nervous system using effective *in vivo* multicellular model systems.

Here, we explore the behavioral and morphological consequences of targeting Elp3 HAT reduction specifically in the developing *Drosophila* nervous system, *in vivo*. We demonstrate that loss of ELP3 HAT activity during neurodevelopment leads to a hyperactive phenotype and sleep loss in adult flies, a significant expansion in synaptic bouton number and axonal length and branching in the larval neuromuscular junction as well as the misregulation of certain genes known to be involved in these processes. Our results provide insight into a novel role for Elp3 in the regulation of synaptic bouton formation during neurogenesis that may be associated with a requirement for sleep.

Materials and Methods

Behavioral Assays:

Activity Assay: Individual progeny from crosses Dmel\ELP3/RNAi/B x y^1w^*; P{GawB}60IIA (RNAi) and w^{1118} x y^1w^*;P{GawB}60IIA (control) were collected upon eclosion and allowed to acclimate to a 12:12 hour light/dark cycle at 29°C for 4 days after eclosion. Locomotor activity was monitored with the *Drosophila* Activity Monitoring System (Trikinetics) at 29°C, as per manufacturer's instructions. Activity counts were recorded every 30 minutes for 24 hours. The significant difference observed between ELP3/RNAi and control groups for total activity was determined using a Student's t- tests for each time point (n=32). The experiment was carried out three independent times with consistent results.

Digital Video Monitoring: Individual progeny from crosses Dmel\ELP3/RNAi/B x y^1w^*; P{GawB}60IIA (RNAi) and w^{1118} x y^1w^*;P{GawB}60IIA (control) were collected upon eclosion and allowed to acclimate to a 12:12 hour light/dark cycle at 29°C for 72 hours, beginning 4 days after eclosion. At day 3, individual flies were anesthetized and transferred to Corning Pyrex Glass tubes (65mm length, 5mm diameter) containing *Drosophila* media at one end. Movements were monitored at 29°C and recorded every 5 seconds by use of digital video recording as previously described, (Zimmerman, Raizen et al. 2008).

Analysis of video data: Total sleep, sleep bout number and mean sleep bout duration were calculated from video data using custom software as previously described, (Zimmerman et al. 2008b). The significance of differences observed between RNAi and control groups (see above) for total sleep, sleep bout number and mean sleep bout duration for 24

hours was determined using Student's t-tests for each sex (5 and 6 day old flies, n=32) or for each day (15 and 16 day old females, n=56).

Real-time RT-PCR: Total RNA was isolated from early pupae of the following crosses in quadruplicate: Dmel\ELP3/RNAi/B x da-GAL4, w^{1118} x da-GAL4 using TRIzol (Invitrogen) and treated twice with DNA-*free* (Ambion) to digest DNA. Total RNA was also isolated from 25 heads of 15 day old flies from either a Dmel/ELP3/RNAi/B or w^{1118} x GAL4 60IIA cross. cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions with 1 μg total RNA and 0.2μg/mL random hexamer primers (Roche Applied Science). PCR reactions were performed in a 20 μL reaction volume containing 1:4 dilution of 10ng cDNA, 1x *Power* SYBR® Green PCR Master Mix (Applied Biosystems), and 10μM both forward and reverse primers (primer pairs available upon request). PCR was performed using ABI 7500 Real-Time PCR system (Applied Biosystems) following the manufacturer's instructions. Fold change in mRNA expression were determined by the ΔΔCt method, (Livak and Schmittgen 2001; Yuan, Reed et al. 2006). Cycling parameters: 95°C for 3 min, 45-50 cycles of: 95°C for 15 sec, 60°C for 45 sec, followed by dissociation curve step.

Larval NMJ Preparations: 3rd instar larvae were filleted in HL-3 saline, pH7.2 and pinned out on Sylgard dishes with guts removed. Preps were then fixed in 3.5% paraformaldehyde 1° antibody csp (1.5 μg/μl) incubation overnight, 4°C. They were then washed 6 times in PBS-T (1x phosphate buffered saline +0.1% Triton), incubated in 2° antibody for 1 hour, washed twice in 1x PBS-T, then once in 1x PBS, then mounted onto

slides in Vectashield antifade mounting media. Confocal microscopy was performed using Olympus Microscope with fluoview software. Synaptic boutons were manually counted. In the analyses, each genotype is represented by 13 larval preparations (n=13). The significant difference observed in total bouton number and muscle surface area between ELP3/RNAi and control groups was determined using Student's t-tests.

Results

Ubiquitous reduction of ELP3 in *Drosophila* results in lethality. We previously cloned the human homologue of ELP3 in *Drosophila,* referred to as Dmel\ELP3, (Zhu, Singh et al. 2007; FlyBase 2009). To explore developmental ELP3 function *in vivo*, we generated Dmel\ELP3 knockdown flies to assess potential phenotypes resulting from ELP3 loss. GAL4 targeted RNAi knockdown technology (Brand and Perrimon 1993) was used to create transgenic flies capable of inducible reduction of endogenous Dmel\ELP3 in specific cell and tissue-types of choice. This strategy has been successfully used for functional analysis of numerous genes in *Drosophila*, (Cerrato, Parisi et al. 2006; Zhu, Singh et al. 2007; Rushton, Rohrbough et al. 2009). The Dmel\ELP3/RNAi construct was created by selecting a 650 bp RNAi non-conserved target sequence specific for Dmel\ELP3 (Figure 1A and B) shown by BLAST searches to exhibit non-redundancy within the genome. Three independently derived transgenic fly lines with insertions for this construct were chosen for use. Importantly, the insertions were homozygous viable, and did not cause any observable aberrant phenotypes in the absence of GAL4 induction.

To test for efficient ELP3 knockdown and to determine whether ELP3 is essential for multicellular development, we induced Dmel\ELP3/RNAi expression in the fly using the robust ubiquitous GAL4 daughterless (*da*) driver, (Bloomington stock no. 5460). Higher levels of GAL4 induction can be obtained at 29^{o}C when compared with 25^{o}C, presumably due to higher activity of the yeast GAL4 transcription factor. Induction of RNAi at 29^{o}C is commonly and successfully used for functional analysis of many genes in *Drosophila*, (Fortier 2000; Duffy 2002). Induction of Dmel\ELP3/RNAi using the *da*-GAL4 driver for each of the three independently derived Dmel\ELP3/RNAi insertion lines at 29^{o}C (Supplemental Table 1), but not 25^{o}C (data not shown), revealed that fly viability was significantly reduced for each of the three independent lines tested. In each case, no defects in development were observed until the late pupal stage, which is the stage at which the majority of lethality occurred. The variation in lethality between the Dmel\ELP3/RNAi line A when compared to lines B and C is likely due to position effect variegation on expression due to random transgene insertion.

To verify that endogenous Dmel\ELP3 was down-regulated, progeny resulting from a cross between Dmel\ELP3/RNAi line B or w^{1118} control fly lines and the *da* GAL4 line were allowed to develop to the pre-pupal stage and the fold change in gene expression between the two lines was assessed using real-time PCR, before lethality in the late pupal stage occurred. Analysis of RNAi induced Elp3 knockdown using real-time PCR revealed significant reduction of ELP3 (Figure 1C) at 29^{o}C but not at 25^{o}C (data not shown), supporting a role for ELP3 in fly viability and making it necessary to maintain flies at 29^{o}C for efficient ELP3 knockdown. Our results revealed that at 29^{o}C, endogenous Dmel\ELP3 transcript levels were significantly reduced in the

Dmel\ELP3/RNAi line B sample by 45 fold (Figure 1C), indicating that GAL4-induced Dmel\ELP3/RNAi expression is robustly silencing endogenous Dmel\ELP3 expression. These results were confirmed using a second ubiquitous GAL4 driver 337 (data not shown). Taken together, our results demonstrate that Dmel\ELP3 is essential for *Drosophila* multicellular development, and support our system as a valuable *in vivo* model for the functional analysis of Dmel\ELP3 during development.

Targeted reduction of Dmel\ELP3 in the nervous system causes an increase in climbing and locomotor activities and a loss of sleep in flies. Analysis of temporal and spatial patterns of Dmel\ELP3 expression during embryogenesis utilizing *in situ* hybridization experiments (our unpublished data; BDGP gene expression report – accession # CG15433) reveal that high levels of Dmel\ELP3 are detected in the nervous tissues, and specifically in the central nervous system of the fly. Moreover, reduction in ELP3 production has been implicated in neuronal degeneration associated with amyotrophic lateral sclerosis (ALS) (Simpson, Lemmens et al. 2009) and familial dysautonomia, (Slaugenhaupt and Gusella 2002; Axelrod 2004; Gardiner, Barton et al. 2007). To investigate Dmel\ELP3 in the nervous system of the fly, we targeted silencing of endogenous Dmel\ELP3 using GAL4 fly line y^1w^*;P{GawB}60IIA, shown by our laboratory and others (Chan and Kravitz 2007) to induce GAL4 preferentially in the brain and central nervous system (CNS). Induction of our two strongest independent Dmel\ELP3/RNAi fly lines B and C at 29^{o}C resulted in no significant reduction of viability when compared to control progeny from a w^{1118} and 60IIA-GAL4 cross. However, visual assessment of the adult Dmel\ELP3/RNAi flies from independent lines

B and C revealed that they were far more active than control flies in that they exhibited continuous movement, including circling the top of the vial and rapid jumping. To further explore this phenotype, we utilized the climbing assay, commonly used to validate and quantitatively assess behavioral manifestation of nervous system dysfunction. Wild-type *Drosophila* display a negative geotactic response such that when they are tapped to the bottom of a vial, they immediately climb to the top and remain there, (Feany and Bender 2000). This natural response is compromised as a result of aging and defects in neurological and muscle processes, and thus climbing ability has been successfully used to monitor and quantitate the progression of severity in *Drosophila* age-related neurological disease models, (Chan and Bonini 2000; Chen and Feany 2005). Dmel\ELP3/RNAi male and female flies consistently climbed more rapidly than the control w^{1118} flies after day 15 to end of period tested, (Supplemental Figure 1 A and B). Induction of Dmel\ELP3/RNAi using the well characterized pan-neural GAL4 driver $elav^{C155}$ also resulted in a similar hyperactive phenotype, (data not shown). Our results indicate that loss of Dmel\ELP3 in all neurons, and specifically in the brain and CNS of the fly, results in the flies having an accelerated rate of climbing in later adulthood.

Another characteristic of flies expressing Dmel\ELP3/RNAi preferentially in the brain and central nervous system using GAL4 driver 60IIA and $elav^{C155}$ was defects in their locomotor ability. Dmel\ELP3/RNAi flies that climbed to the top of the vials continuously circled the upper portion of the tube, and often executed rapid high jumps back down below the 9cm mark of the vial side shortly after climbing past it. To determine whether this observed hyperactivity of the Dmel\ELP3/RNAi flies was significant, we monitored their locomotor activity utilizing the *Drosophila* Activity

Monitoring System (DAMS), a powerful assay used to study and quantify gross activity. The results showed that the Dmel\ELP3/RNAi flies broke the beam a significant total number of times more than the control flies, indicating that loss of Dmel\ELP3 in the brain and CNS caused a significant increase in the total activity of the flies, (Figure 2). Our results demonstrate that loss of ELP3 in the nervous system, and specifically in the brain and CNS results in a significant increase in gross locomotor activity.

The hyperactivity of the Dmel\ELP3/RNAi flies prompted us to ask whether loss of Dmel\ELP3 in the nervous system also leads specifically to loss of sleep in these flies. The DAMS assay is a powerful tool to investigate and quantify changes in gross locomotor activity for different fly genotypes, though it has certain limitations for specifically studying sleep. Such limitations include insensitivity to small fly movements which occur outside of the path of the infrared beam which affects the identification of actual quiescent sleep behavior, as non-detection of beam breaks in the DAMS assay may not be associated with sleep but rather with the fly not being in the vicinity of the infrared beam path, (Zimmerman, Naidoo et al. 2008; Zimmerman, Raizen et al. 2008). To overcome these issues we used digital video analysis to determine whether loss of ELP3 also induced a lack of sleep in the flies. Single, staged, 4-day-old Dmel\ELP3/RNAi and control w^{1118} progeny from a GAL4-60IIA cross were transferred to 6cm glass tubes and behavior recordings of fly sleep carried at 5 second intervals over a 72 hour total, 12:12 hour light/dark time course. We quantified total sleep, sleep bout number and mean sleep bout duration. Female Dmel\ELP3/RNAi flies slept significantly less that the control flies both during the day and nighttime periods, (Figure 3A). Additionally, female Dmel/ELP3/RNAi flies have significantly shorter sleep bouts, (Figure 3B). Male flies do

not have significantly different total sleep (Figure 3A) but do show a small but significant increase in bout duration (Figure 3B). Neither male nor female Dmel\ELP3/RNAi flies had significantly different sleep bout numbers than the appropriate gender control flies, (data not shown). In certain strains, male *Drosophila melanogaster* sleep more than female flies, having pronounced daytime sleep (Shaw, Cirelli et al. 2000; Huber, Hill et al. 2004; Andretic and Shaw 2005; Zimmerman, Raizen et al. 2008), and have been shown to respond to sleep deprivation much less strongly than females of the same strain, (Shaw, Tononi et al. 2002; Huber, Hill et al. 2004; Andretic and Shaw 2005; Zimmerman, Naidoo et al. 2008). Indeed, males have been shown to have increased wakefulness after 6 hours of sleep deprivation, which is opposite the response of females, (Shaw, Cirelli et al. 2000; Hendricks, Lu et al. 2003; Huber, Hill et al. 2004). Therefore, having a sex dependent sleep phenotype in response to the induction of the ELP3 RNAi is not without precedent. In addition, both male and female Dmel\ELP3/RNAi flies show significantly greater distance moved per movement compared to control flies by video which confirms the DAMS data and explains the greater number of beam breaks observed, (data not shown). Similar sleep loss results were obtained using the elav^{C155} pan-neuronal GAL4 driver, (data not shown). Taken together, our results demonstrate that loss of Dmel\ELP3 in the nervous system reduces the amount of sleep and increases the amount of activity in the fly, implicating Dmel\ELP3 in sleep and activity related neuronal pathways.

Knockdown of Dmel\ELP3 results in the misregulation of genes involved in sleep, vesicle transport and fusion, and protein chaperone activity. ELP3 is implicated in

facilitating the transcriptional activation of genes, (Svejstrup *et al.,* 2007). To investigate a potential molecular basis underlying the increase in activity and loss of sleep phenotypes in the Dmel\ELP3/RNAi flies, we asked whether loss of Dmel\ELP3 leads to misexpression of genes known to be associated with such phenotypes. For this analysis, we induced ubiquitous silencing of Dmel\ELP3 using the *da*-GAL4 driver as we had demonstrated that this driver induces robust levels of Dmel\ELP3 knockdown in flies, (Figure 1C). mRNA was prepared from early pupae, directly before the lethal pupal stage (Suplemental Table 1), to ensure potential gene changes were not due to cell death. The mRNA levels of 6 specific genes from the progeny of a Dmel\ELP3/RNAi or control w^{1118} with da-GAL4 was assessed by quantitative real-time PCR and the fold change in gene expression levels between the two fly lines was determined, (Figure 4*)*. The putative target genes chosen for this analysis were: heat shock chaperone genes HSC70-3 (homolog of the mammalian endoplasmic reticulum chaperone BiP) and the cytoplasmic resident chaperone HSC70-4 (Elefant and Palter 1999), selected based on their involvement of the stress response linked with sleep loss (Naidoo, Casiano et al. 2007), the synaptobrevin (SYB) gene (VAMP2 homolog), an ADHD candidate gene and a vesicle-associated membrane protein (VAMP) that is part of the SNAP-receptor (SNARE) complex and mediates exocytotic vesicle fusion by interacting with specific plasma membrane proteins that allow for either cell growth or fusion of neurotransmitter containing vesicles required for neuronal firing (Bhattacharya, Stewart et al. 2002; Davids, Zhang et al. 2003; Wallis, Russell et al. 2008), SLEEPLESS, chosen for its role as a sleep-promoting factor (Koh, Joiner et al. 2008), gelsolin, a cytoskeleton modulator found to be downregulated in FD patient fibroblasts (Close, Hawkes et al. 2006), and

superoxide dismutase (SOD1) in which particular mutations result in ALS cases with a family history, (Simpson, Lemmens et al. 2009). The results of our analysis demonstrated that out of the 6 genes assessed, three of the genes (HSC3/BiP, SYB and SLEEPLESS) were significantly affected, with a marked increase in mRNA levels for BiP and SYB and a marked decrease in mRNA levels for SLEEPLESS, (Figure 4). Consistent with findings that ALS is linked to Elp3 loss in a SOD1 independent manner (Simpson, Lemmens et al. 2009), we observed that SOD1 levels were unaffected in response to ELP3 loss and internal control RP49 levels were also unaffected. That only certain genes were affected indicate that the gene changes we observed were specific. Surprisingly, loss of Dmel\ELP3 resulted in the up-regulation of certain genes which is not consistent with the recognized role of ELP3 as a transcriptional activator, suggesting that these genes are either indirect ELP3 targets, or that in some instances, ELP3, like other HATS, is also involved in repression of certain genes. Notably, each of the affected genes was associated with the increase in activity and decrease in sleep phenotypes we observed in our Dmel\ELP3/RNAi flies. Induction of BIP is shown to be associated with sleep loss and up-regulation of the SYB gene has been shown to be associated with attention deficit hyperactivity disorder (ADHD). Finally, SLEEPLESS was specifically identified in a screen for genes associated with sleep and downregulation of SLEEPLESS (Koh, Joiner et al. 2008) results in a reduction of sleep in flies. To ask whether these gene changes correlated with the behavioral changes we observed for adult ELP3/RNAi flies (Supplemental Figure 1, Figures 2 and 3), we carried out qPCR analysis using RNA isolated from the heads of 15 day old control flies or flies expressing Dmel\ELP3/RNAi specifically in the CNS using GAL4 driver 60IIA, (Chan and Kravitz 2007). Day 15 was

chosen as it corresponds to the beginning of the period of significantly increased climbing ability of Dmel\ELP3/RNAi flies, (Supplemental Figure 1). The results revealed that although the trend in gene expression profile levels for SYB, HSC3 and SLEEPLESS in these adult heads was similar to that of the *da* GAL4 induced ELP3/RNAi pre-pupae staged flies, the changes were not statistically significant, (Supplementary Figure 2). The differences in gene expression profiles between the two samples may reflect the more restricted and less robust expression pattern of the CNS GAL4 driver 60IIA than the *da* GAL4 driver, leading to dilution of significant gene expression changes in the whole adult heads assayed or that significant gene misregulation in response to ELP3 loss occurs earlier in development, leading to early physiological changes in the nervous system that are maintained into adulthood. Taken together, these results support an essential role for Dmel\ELP3 in the regulation of genes in the early pupal stage of the fly that may be involved in the behavioral phenotypes we observe in the adult flies.

Older Dmel\ELP3/RNAi flies respond normally to sleep deprivation. A P-element insertion in SLEEPLESS has been shown to have no effect upon normal sleep but demonstrates a significant loss of the homeotic sleep drive, i.e. this mutant is severely impaired in the ability to recover from lost sleep, (Koh, Joiner et al. 2008). Therefore, we examined the sleep phenotype of 15 and 16 day old control and Dmel\ELP3/RNAi females from a CNS GAL4 60IIA cross, which correspond to the beginning of the period of significantly increased climbing ability of Dmel\ELP3/RNAi flies, (Supplemental figures 1A and B). Females of this age show a significant loss of total sleep and have

significantly shorter sleep bouts, (Supplemental Figures 3A and B). We sleep deprived ELP3 RNAi flies and controls by gentle handling for 6 hours beginning at ZT 18. The flies were then allowed to recover for 24 hours. Unhandled controls were left undisturbed during the time of the deprivation. Sleep deprived ELP3 RNAi flies showed no significant differences in the amount of recovery sleep when compared to control flies deprived for the same amount of time in either the first 4 hours ($p=0.975$) or second 4 hours ($p=0.907$) following deprivation, (Supplemental Figure 4). The ELP3 RNAi and control sleep deprived groups slept significantly more than the respective control groups left undisturbed in both the first 4 hour period (ZT0 to ZT4) ($p>0.001$ and $p>0.001$, respectively) and second 4 hour period (ZT4 to ZT8) ($p>0.001$ and $p=0.004$, respectively) following the end of deprivation. These results show that loss of Dmel\ELP3, specifically in the CNS of 15 and 16 day old adult flies, does not appear to effect recovery sleep after sleep deprivation.

Loss of Dmel\ELP3 in the nervous system leads to an expansion of synaptic boutons in the larval fly neuromuscular junction. Chemical synapses transmit information directionally from a presynaptic cell to a target postsynaptic cell *via* the release of neurotransmitters from the presynaptic terminal or synaptic bouton. Such firing of distinct neuronal connections either initiates the muscle contractions associated with movement and activity or directly influences learning and behavior. Recent studies have demonstrated that sleep loss is associated with synaptic bouton expansion, (Gilestro, Tononi et al. 2009). Thus, changes in synaptic density, or synaptic plasticity, affect activity, sleep, learning, and memory processes. These studies prompted us to ask

whether there was an expansion in synaptic bouton formation resulting from Dmel\ELP3 loss, which would provide a potential mechanism underlying the behavioral changes we observed in the adult Dmel\ELP3/RNAi flies. To explore this possibility, we examined bouton morphology in the fly larval neuromuscular junction, as this system is extremely advantageous to the study of synaptic plasticity in that it is very well characterized and shows striking conservation of numerous key synaptic molecules identified in mammals, (Broadie and Bate 1993; Koh, Gramates et al. 2000; Collins and DiAntonio 2007). Dmel\ELP3/RNAi and control w^{1118} flies were crossed to the $elav^{C155}$ pan-neuronal GAL4 driver, and third instar progeny larvae were collected. Of note, we demonstrated that knockdown of Dmel\ELP3 using this driver results in an increased activity and reduction of sleep of the adult flies. To examine bouton morphology, boutons at muscles 6 and 7 at abdominal segment A4 were stained with anti-HRP that labels the entire presynaptic membrane, cysteine string protein (CSP) that is a specific marker of the presynaptic vesicles within boutons, and Phalloidin, a toxin that stains muscles, to identify and measure the surface area of the appropriate muscle groups and abdominal segments. The degree of bouton expansion at the NMJ was determined by counting the number of synaptic boutons. Remarkably, there was a dramatic increase (182%) of the total number of synaptic boutons in the Dmel\ELP3/RNAi larvae when compared with the wild type control (Figures 5A, B and C). Of note, there are two types of boutons that are found within larval NMJ muscles 6 and 7. These boutons are classified as type I small (Is) and type I big (Ib) by size. Type-Is boutons have larger stimulation thresholds and excitatory junctional currents of larger amplitude while type-Ib boutons exhibit more pronounced short-term facilitation, (Koh, Gramates et al. 2000). Intriguingly, although both type-Is

and type-Ib boutons in the Dmel\ELP3/RNAi lines were significantly increased when compared to the wild-type control, there was a substantially larger expansion of type-Is boutons when directly compared to Ib (329% increase of type Is to 129% increase of type Ib), supporting partial specificity in Dmel\ELP3 function in certain bouton types, (Figures 5A, B and C). In support of this concept, "satellite" bouton budding, a process that involves the budding of bouton(s) from one central bouton on the main branch, was indistinguishable in the Dmel\ELP3/RNAi flies when compared to the wild-type control, suggesting that Dmel\ELP3 does not affect this process. Of note, we also observed an increase in axonal arbor area in terms of length and branching relative to muscle surface area in the ELP3 RNA flies when compared to controls, (Figure 5D). Taken together, our results indicate that Dmel\ELP3 plays a role in controlling the degree of axonal arbor length and branching and synaptic bouton expansion, and displays at least some specificity in preferentially controlling type Is bouton formation.

Discussion

In this report, we investigate the behavioral and morphological phenotypes that result from targeted reduction of Elp3 HAT levels both ubiquitously and specifically in the developing *Drosophila* nervous system, *in vivo*. We demonstrate that targeted reduction of Elp3 in all tissues of the fly results in a significant reduction in fly viability, (Suplemental Table 1). Studies of Elp3 function in mammalian cell types indicate that loss of Elp3 indeed leads to severe defects in certain specific cellular processes. For example, loss of ELP3 in human HeLa cell lines leads to repression of certain genes that

encode proteins required for cell motility and cell migration. Accordingly, Elp3 depleted neuronal cells and fibroblasts from FD patients both display a significant reduction in cell motility, (Close, Hawkes et al. 2006). More recently, studies using mouse cortical neuronal cells directly implicate Elp3 in the acetylation of α-tubulin that controls the migration and differentiation of cortical neurons, (Gardiner, Barton et al. 2007; Creppe, Malinouskaya et al. 2009; Wynshaw-Boris 2009). Moreover, while this work was in progress, Simpson et al. identified a fly strain containing a lethal transposon that molecularly mapped to the ELP3 gene, confirming our findings that knockdown of ELP3 in all tissues has lethal consequences in the fly, (Simpson, Lemmens et al. 2009). Based on these findings, we speculate that as multicellular development in our Dmel\ELP3/RNAi flies proceeds, disruption of the cell specific processes that require non-redundant Dmel\ELP3 functions accrue over time, ultimately culminating in the phenotypes we observe.

We found that loss of ELP3 in the nervous system of *Drosophila* results in a significant increase in gross locomotor activity and a significant reduction in the amount of time the flies sleep. Consistent with these hyperactive behavioral phenotypes, our immunohistochemical staining of the larval neuromuscular junction (NMJ) using antibodies to synaptic markers HRP, that stains neuronal membranes and cysteine string protein, a pre-synaptic marker protein that regulates $Ca2^{+}$ channels and is essential for vesicle exocytosis and neurotransmitter release (Zinsmaier, Eberle et al. 1994; Dawson-Scully, Bronk et al. 2000; Bronk, Nie et al. 2005), show that flies depleted for ELP3 in the nervous system also display a significant increase in synaptic bouton number in the larval NMJ. Importantly, while our studies are the first to demonstrate a role for ELP3 in

synaptic bouton formation, while this work was in progress, Simpson et al demonstrated that flies containing a mutation in the GCN5-related acetyltransferase (GNAT) domain of ELP3 result in a disruption of photoreceptor projections into the fly medulla, indicative of defects in neuronal communication that may arise at least in part from altered axonal targeting and synaptic development, (Simpson, Lemmens et al. 2009). Importantly, although the actual bouton number in our ELP3 depleted flies is significantly higher than the wild-type controls, the actual boutons themselves exhibit a typical HRP and CSP staining pattern, suggesting that these boutons are functional for neuronal firing. Accordingly, in ELP3 depleted flies we observe a significant upregulation of synaptobrevin, a vesicle-associated membrane protein (VAMP) that is part of the SNAP-receptor (SNARE) complex and mediates exocytotic vesicle fusion that allows for either cell growth or fusion of neurotransmitter containing vesicles required for neuronal firing, (Bhattacharya, Stewart et al. 2002; Davids, Zhang et al. 2003; Wallis, Russell et al. 2008). Moreover, we observe down-regulation of *sleepless* (*sss*), a brain-enriched glycosyl-phosphatidylinositol (GPI)- anchored membrane protein proposed to enhance K^+ channel activity in restoring resting neuronal membrane potential, thus reducing neuronal excitability and inducing sleep, (Koh, Joiner et al. 2008). Together, a reduction in *sleepless* expression in conjunction with an increase in synaptic bouton number and concomitant increases in both CSP and synaptobrevin levels in ELP3 depleted flies suggest that neuronal firing may be occurring more readily, providing a potential molecular mechanism underlying their hyperactive behavioral phenotype.

Several important studies have recently been published on sleep processes in the fly, supporting a link between sleep need and synaptic bouton formation and function.

Gilestro *et al.* demonstrate that levels of several synaptic structural and secretory machinery protein markers in the fly increase during periods of wakefulness and decrease after sleep, with CSP showing a significant increase after 12 hours of continuous waking, (Gilestro, Tononi et al. 2009). Moreover, Donlea *et al.* show that the number of synaptic terminals in the brain of the fly after periods of social enrichment increases and that these numbers decrease after long bouts of sleep following social enrichment, (Donlea, Ramanan et al. 2009). Taken together, these studies support the synaptic homeostasis hypothesis, which claims that sleep is required to downscale synapse formation in the brain, (Tononi and Cirelli 2006). According to this model, potentiation of the synapses occurs while organisms are awake and increases during long durations of wakefulness. Downscaling of synapses during sleep may be necessary to lower energy consumption, free up space for synapses to grow during the next waking period, and decrease cellular stress caused by the synthesis and delivery of neurotransmitter containing synaptic vesicles. The significant increase in synaptic boutons, upregulation of BIP (Elefant and Palter 1999) that potentially counteracts the cellular stress associated with long bouts of wakefulness ((Naidoo, Casiano et al. 2007; Mackiewicz, Naidoo et al. 2008) and the reduction of total sleep we observe for the CNS expressing Dmel\ELP3/RNAi flies support the synaptic homeostasis hypothesis, although our data may also suggest that reduction in synaptic strength is a consequence rather than a drive for sleep in the fly.

How does ELP3 play an active role in the control of bouton formation and sleep in the fly? One possible explanation may be directly due to the recently discovered cytoplasmic-based function for ELP3 in the acetylation of α-tubulin that allows for their stable polymerization into microtubules. These studies demonstrate that loss of ELP3 in

cultured projection neuronal cells leads to severe defects in axonal branching, (Gardiner, Barton et al. 2007; Creppe, Malinouskaya et al. 2009; Wynshaw-Boris 2009). Moreover, these researchers demonstrate that purified ELP3 promotes α-tubulin acetylation of microtubules while specific blockage of this acetylation using a dominant negative version of α-tubulin that cannot be acetylated leads to axonal branching defects similar to those resulting from ELP3 loss, suggesting that Elp3-induced acetylation of α-tubulin is required for appropriate axonal branching. Transition to new axonal growth and branch formation has been shown to be accompanied by splaying of looped microtubules and formation of short microtubules that interact with the actin cytoskeleton to invade the lamellipodium, (Dent and Kalil 2001; Dent and Gertler 2003). Similarly, formation of synaptic boutons at axon terminals is achieved via looping of microtubules that invades and promotes bouton budding at the plasma membrane, (Collins and DiAntonio 2007; Colon-Ramos 2009). Such budding is believed to rely on destabilization of the cytoskeleton as indicated by studies demonstrating that disruption of dynamic actin filaments leads to blockage of long-term potentiation (LTP), a cellular correlate for memory formation indicative of synaptic bouton formation and function, (Halpain, Hipolito et al. 1998; Ramachandran and Frey 2009). Based on these observations, one hypothesis may be that loss of ELP3 promotes destabilization of microtubules *via* reduction of α-tubulin acetylation, leading to an increase in microtubule looping and splaying as well as destabilization of the cytoskeleton, ultimately enhancing axonal branching, length, and synaptic bouton budding. Alternatively, bouton expansion may arise from the epigenetic role of ELP3 in directly regulating *sleepless* gene expression. ELP3-induced disruption of the transcriptional activation of the *sleepless* gene in the

nucleus might, according to the synaptic homeostasis hypothesis, lead to long bouts of wakefulness and enhanced neuronal firing, thus triggering expansion of synaptic bouton formation via downstream pathways, (Tononi and Cirelli 2006).

We found that loss of Dmel\ELP3 in the CNS of 15 and 16 day old adult flies appeared to have no effect on recovery sleep after sleep deprivation, (Supplementary Figure 4). Our finding is in contrast to the lack of sleep homeostasis seen in a hypomorphic SLEEPLESS mutant which has a similar reduced sleep phenotype (Kho et al., 2008) to our ELP3/RNAi flies (Fig. 3A,B and Supplemental Figs. 3A and B) and less of a response than seen in transgenic flies over expressing BiP under the control of a heat shock promoter, (Naidoo et al., 2007). This normal homeostatic response could be attributable either to the contrasting effects of the concurrent changes of both of the SLEEPLESS and BiP genes or possibly to the more restricted expression of the CNS GAL4 60IIA promoter versus the global effect of the P element insertions in the SLEEPLESS gene and the heat shock promoter used in the BiP experiments. Additionally, as Dmel/ELP3 expression levels change during fly development (Zhu et al., 2007), it is possible that this may represent a changing role for ELP3 regulation upon these genes with increasing age of the fly, consistent with our observation that the climbing ability of Dmel/ELP3\RNAi flies is more significantly affected after day 15.

The identification of a number of neurological disorders that result from HAT misregulation underscores a crucial role for acetylation in neurogenesis. Missense mutations in the CBP and p300 genes or loss of a CBP allele cause Rubinstein-Taybi syndrome (RTS), a human disease that displays complex phenotypic abnormalities including mental retardation and neoplasia. Memory loss associated with RTS is shown

to be due to lack of CBP HAT activity, and treatment of an RTS mouse model with histone deacetylase inhibitors (HDACi) rescues RTS-associated memory deficits, indicating that appropriate histone acetylation is critical for long-term potentiation, learning, and memory, (Alarcon, Malleret et al. 2004; Korzus, Rosenfeld et al. 2004). Recent studies support the importance of selective HDACi design, as only specific HDACs appear to affect synaptic plasticity and memory formation, (Guan, Haggarty et al. 2009). For example, neuron-specific overexpression of HDAC2 and not HDAC1 in mice results in a decrease of synapse number and memory enhancement, and deficiency of HDAC2 in mice displays the converse of these effects. Importantly, WT-151, an HDACi that selectively inhibits HDAC6, a class II HDAC known to target K40 acetylation of α-tubulin does not increase memory formation in mice, inferring that stable ELP3-induced α-tubulin acetylation does not increase synaptic bouton formation, (Guan, Haggarty et al. 2009). Consistent with this finding, here we show for the first time that reduction of ELP3 HAT activity in the fly nervous system results in enhanced synaptic bouton formation and a decrease in sleep activity, supporting an active role for this HAT in the control of synaptic bouton formation. Further understanding of the molecular mechanisms underlying ELP3 in this process has the potential to pave the way for the design of selective epigenetic-based therapeutics for treatment of diseases affecting synaptic plasticity and degeneration.

Appendix C: Tables and Figures

Appendix A Figures

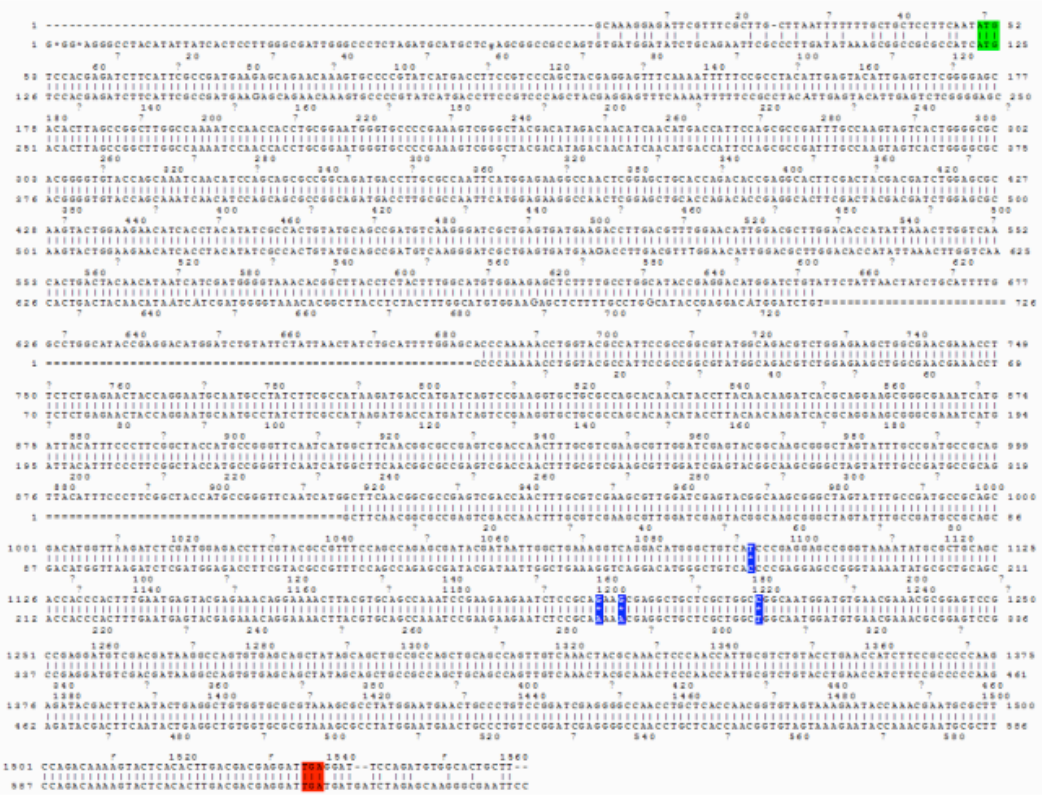


Figure 1. Dmel\Kdm4A DNA sequence. Alignment of the Dmel\Kdm4A cDNA and sequence found at NCBI. Mismatches are indicated in blue, start codon in green and stop codon in red.

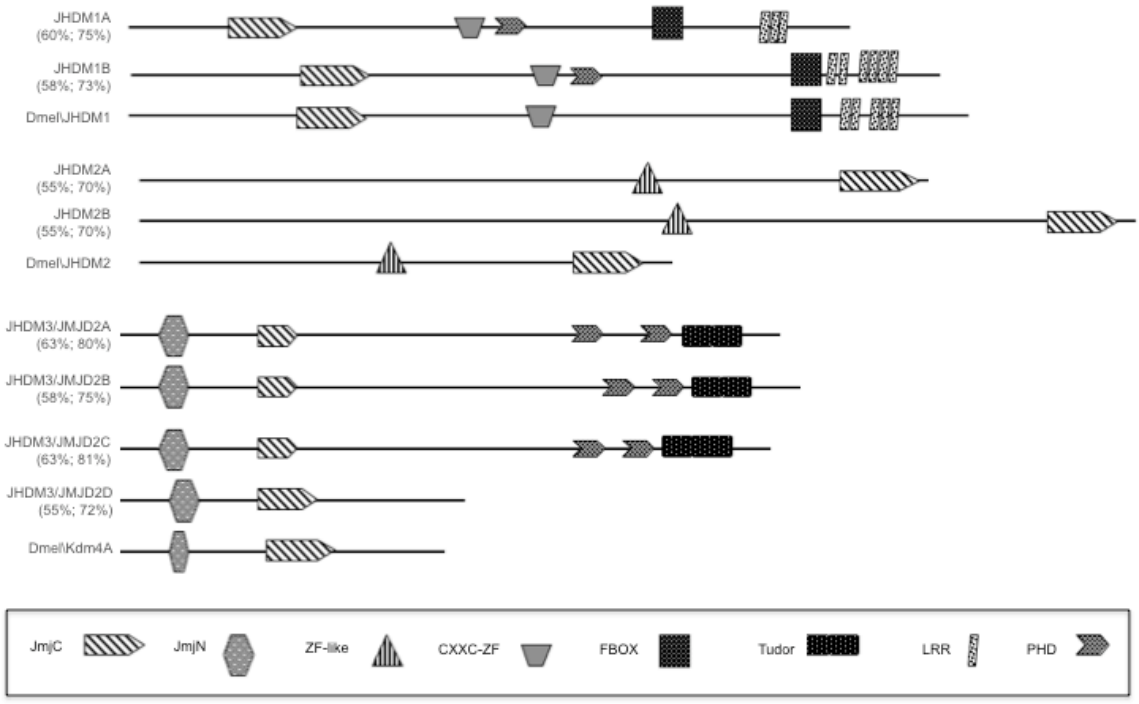


Figure 2. Schematic representation of histone demethylase families. Dmel/JHMD1, Dmel\JHMD2 and Dmel\Kdm4A are each highly conserved with their human homolog counterparts. Shown is a schematic representation (drawn to scale) of the conserved domains and their location within each of the JHMD1, JHMD2 and Dmel\Kdm4A family members. Structural domains and locations were obtained at CDD/NCBI. Numbers represent percentage identity and similarity with respect to the corresponding *Drosophila* homolog. The positions of the JumonjiC (JmjC) and JumonjiN (JmjN) domains are indicated. Zinc-like finger, CXXC-zinc finger, PHD and tudor domains and their locations are also shown.

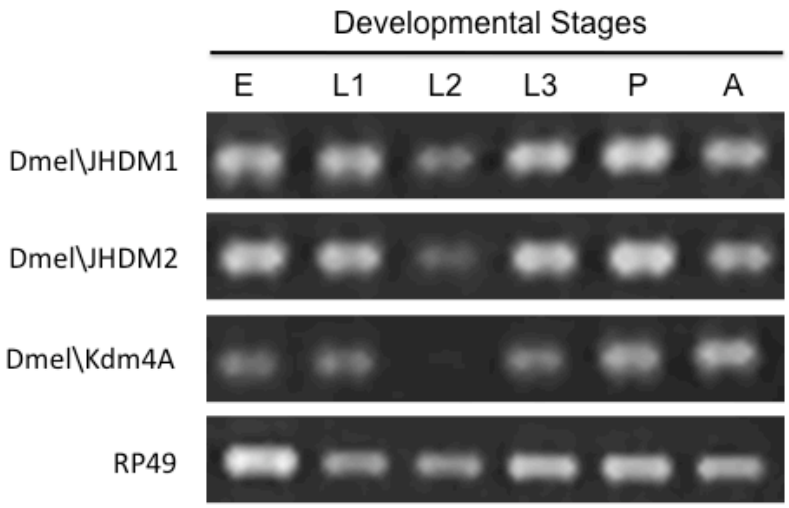


Figure 3. Dmel\JMHD1, Dmel\JMHD2 and Dmel\Kdm4A are each differentially expressed during *Drosophila* development. Semi-quantitative real-time PCR analysis of Dmel/JMHD1, Dmel\JMHD2 and Dmel\Kdm4A transcript levels using stage specific *Drosophila melanogaster* cDNAs (12-24 h staged embryos (E), first (L1), second (L2) and third (L3) instar larvae, pupae (P), adult (A) flies) prepared by RT priming of equal amounts of DNase treated RNA with random hexamers and PCR primer sets amplifying 400 bp regions specific for each HDMs. –RT controls were used for each sample. All experiments were repeated at least 3 independent times with consistent results.

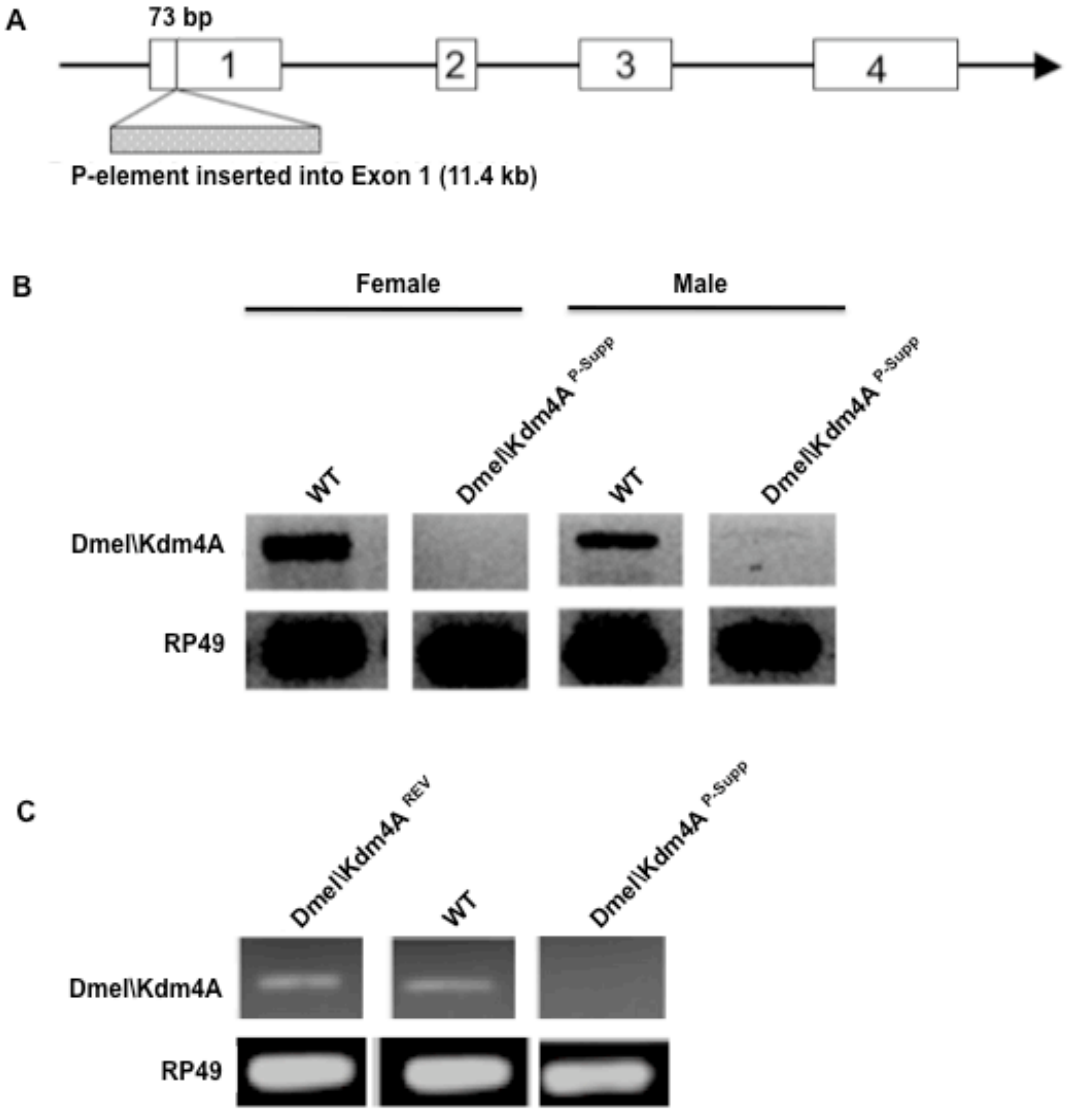


Figure 4. Characterization of Dmel\Kdm4A$^{P\text{-}supp}$ and Dmel\Kdm4A ^{REV} fly lines. A. Schematic representation of P-element location within the Dmel\Kdm4A locus. **B.** Semi-quantitative RT-PCR analysis of transcript levels in Dmel\Kdm4A$^{P\text{-}supp}$ and Canton S flies. RNA was isolated from either male or female adult flies and equal amounts of RNA for each sample was subjected to cDNA preparation using RT priming with random hexamers and PCR using primer sets spanning the Dmel\Kdm4A open reading frame (ORF). **C.** Semi-quantitative RT-PCR analysis of transcript levels in Dmel\Kdm4A$^{P\text{-}supp}$ and Dmel\Kdm4A $^{REV\ A}$ flies. All experiments were repeated at least 3 independent times with consistent results and similar results were obtained for Dmel\Kdm4A $^{REV\ B}$.

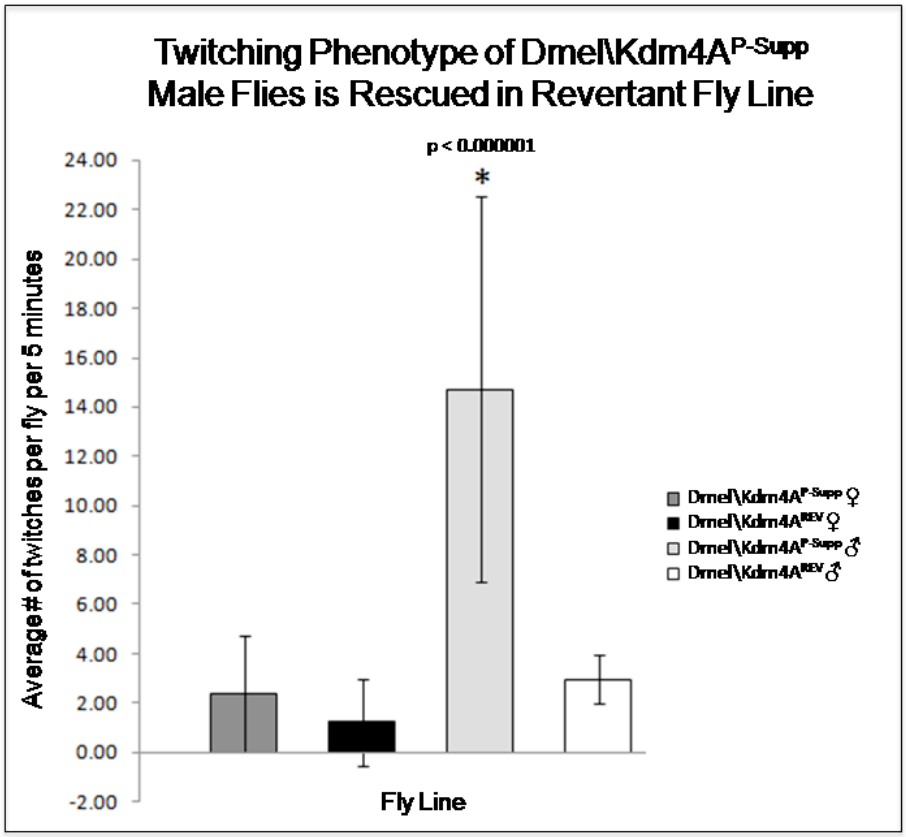


Figure 5. Disruption of Dmel\Kdm4A gene results in a twitching phenotype. Staged 0-24 hour Dmel\Kdm4A $^{P\text{-}Supp}$ and Dmel\Kdm4A $^{REV\ A}$ flies males and females were collected in separate vials and allowed to acclimate for 4 days. 10 vials containing 3 male flies and 10 vials containing 3 female flies were observed and the number of times the flies twitched was counted over 5 minutes. The number obtained in each vial was divided by 3 to calculate average number of twitches per fly.

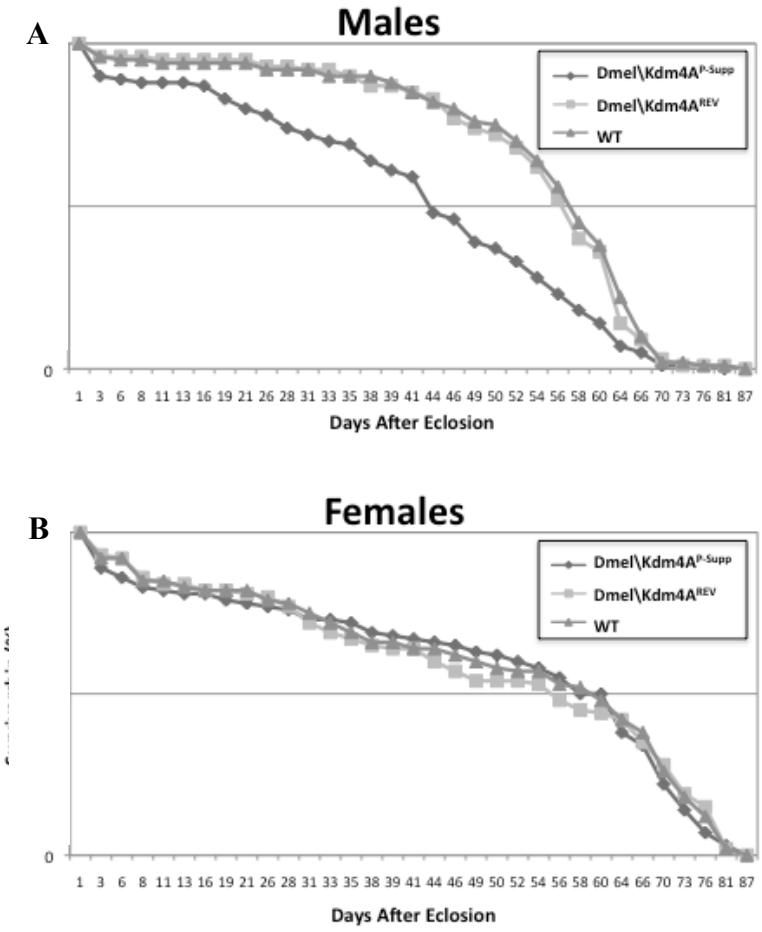


Figure 6. Disruption of Dmel\Kdm4A results in a reduction in male specific longevity. Survival curves of male **A** and female **B** flies that were separately reared after eclosion at 25°C. Dmel\Kdm4A $^{P\text{-}Supp}$ represents the mutant and Dmel\Kdm4A$^{REV\ A}$ represents the wild type control. Flies were maintained in embryo collection chambers at 25°C. The flies were changed each day and the number of dead flies was recorded. The data was analyzed using 2-way ANOVA with SAS programming and Microsoft Excel.

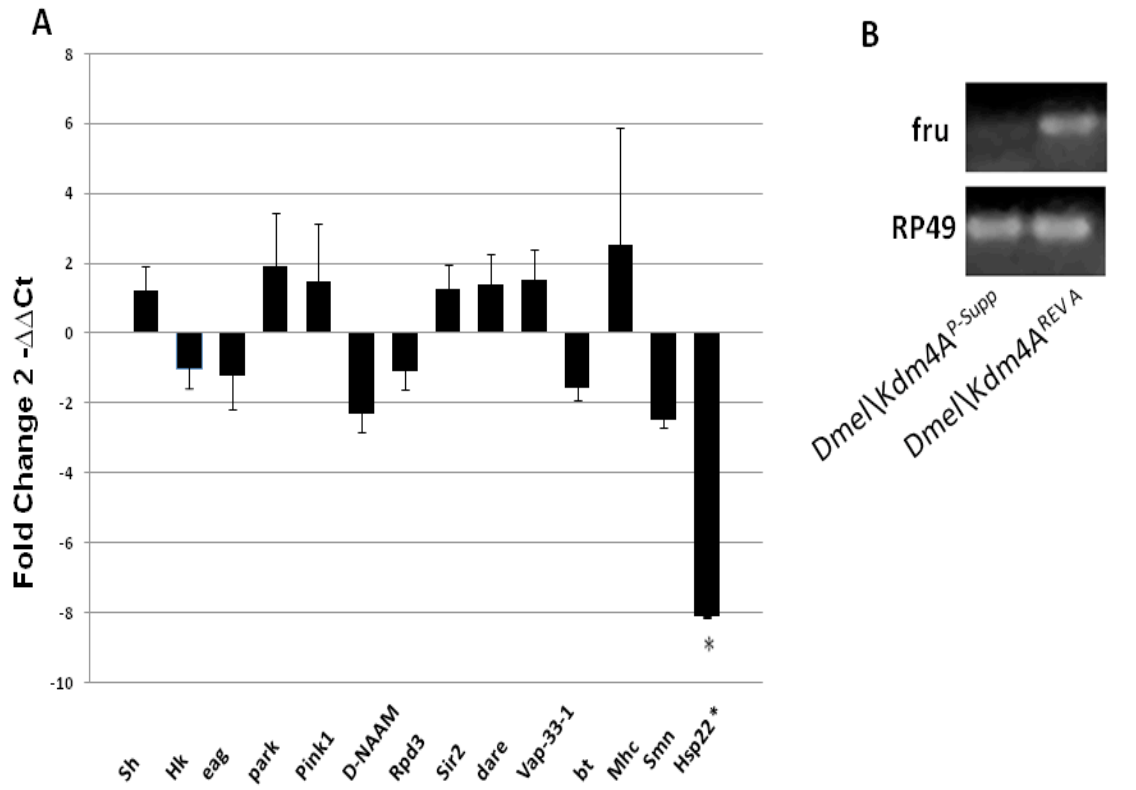


Figure 7. Specific genes associated with Dmel\Kdm4A $^{P\text{-}Supp}$ male-specific twitching and longevity pheonotypes are significantly downregulated in response to Dmel\Kdm4A loss. A. Shown is a histogram depicting qPCR analysis of the expressionof the indicated genes in staged 21 day old male Dmel\Kdm4A $^{P\text{-}Supp}$ and Dmel\Kdm4A ^{REV} flies. The relative fold change in mRNA expression levels were measured using the comparative Ct method with RP49 as the internal control gene. Error bars represent standard deviation. Astrics (*) indicate significant fold changes between Dmel\Kdm4A mutant and revertant flies with values of $p < 0.05$, (n=3). **B.** Semi-quantitative RT-PCR analysis of *fruitless* transcript levels in Dmel\Kdm4A$^{P\text{-}supp}$ and Dmel\Kdm4A $^{REV\ A}$ flies. All experiments were repeated at least 3 independent times with consistent results.

Appendix B Figures

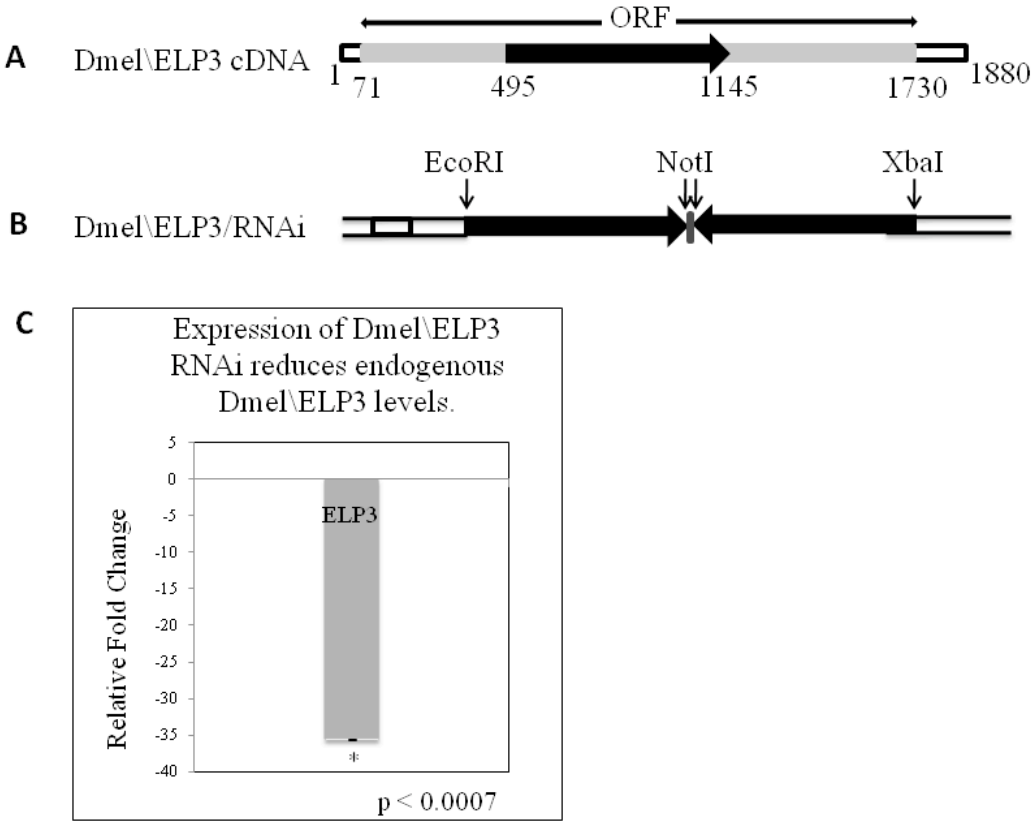


Figure 1. Expression of Dmel\ELP3 RNAi reduces endogenous Dmel\ELP3 levels. **A.** Schematic of the Dmel\ELP3 ORF. Solid arrow represents the location of the 650bp RNAi non-conserved target sequence chosen for use in creating the Dmel\ELP3/RNAi construct. **B.** Schematic of the Dmel\ELP3/RNAi construct. The 650bp RNAi target cDNA sequence was amplified by PCR using the cDNA Dmel\ELP3 clone as template and cloned into a sense–antisense inverted gene arrangement in the pUAST inducible expression vector, under the control of GAL4–UAS-binding sites. The inverted cDNA fragments are joined by a PCR generated short polylinker sequence and common NotI restriction sites, serving as the hinge region of the RNAi hairpin. **C.** Shown is a histogram depicting qPCR analysis of Dmel\Elp3 expression between Dmel\ELP3/RNAi and control w^{1118} samples. Progeny resulting from a cross between homozygous Dmel\TIP60/RNAi line B and daughterless w^{*}; P{GAL4-da.G32}UH1 were allowed to develop to the early pupal stage. The relative fold change in mRNA expression level was measured using the comparative Ct method with RP49 as the internal control gene. Error bars represent standard deviation. Asterisks (*) indicates significant fold change in relation to control where $p < 0.0007$. Error bar depicts standard deviation of the mean.

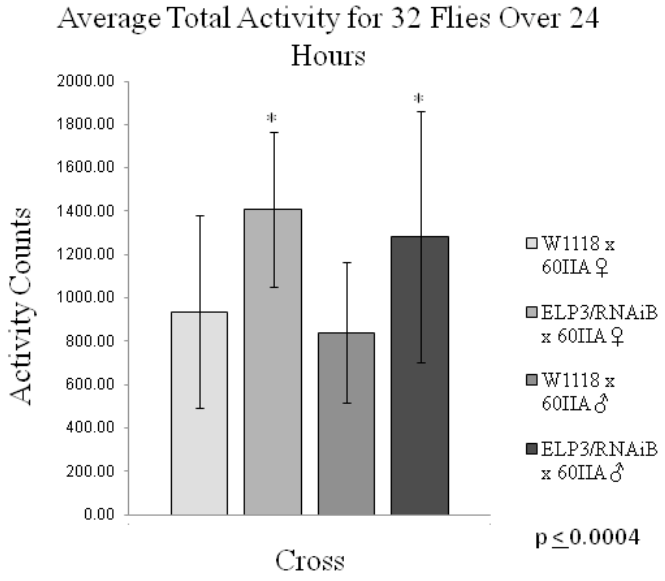


Figure 2. Expression of Dmel\ELP3/RNAi in the nervous system results in an increased locomotor activity in adult flies. Flies homozygous for Dmel\ELP3/RNAi line B or control w^{1118} were crossed to flies homozygous for CNS GAL4 driver 60IIA. Staged 4 day old male or female progeny from this cross were each placed in separate DAMS glass tubes (32 males and 32 females total per genotype) and the number of times the flies broke the infrared beam was recorded in 30 minute intervals over a 24 hour period with 12 hour light and dark cycles. Shown is a histogram depicting the total activity for adult flies measured over a 24-hour period. Asterisks (*) indicates significant fold change in relation to control where $p \leq 0.0004$. Error bars depict standard deviation of the mean.

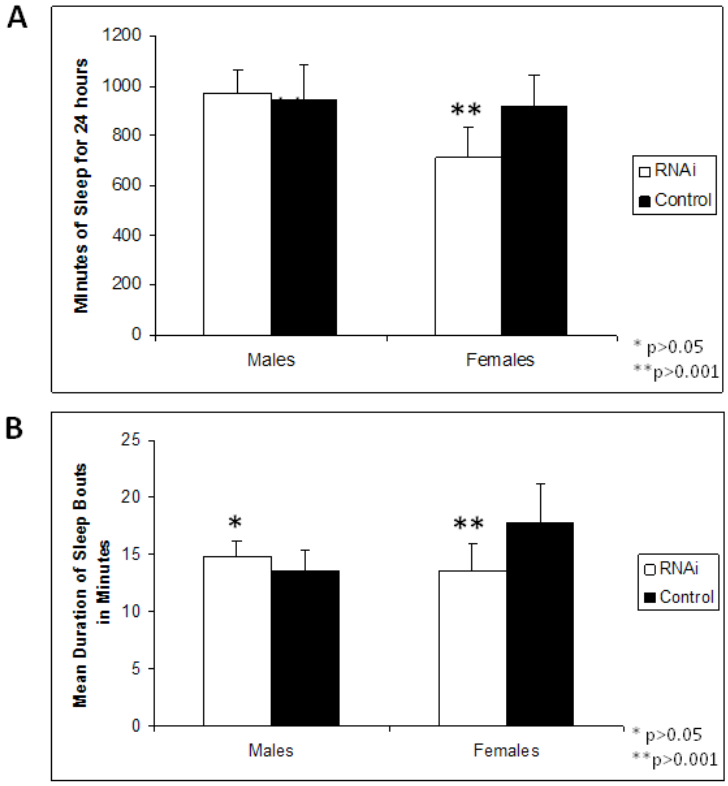


Figure 3. Expression of Dmel\ELP3/RNAi in the nervous system results in sleep loss in 5 and 6 day old adult flies. Single staged 4 day old Dmel\ELP3/RNAi and control w^{1118} progeny from a GAL4-60IIA cross were transferred to 6cm glass tubes and behavior recordings of fly sleep carried at 5 second intervals over a 72 hour total, 12:12 hour light/dark time course on days 5 and 6 dark periods only (n=32). **A.** Shown is a histogram depicting the averaged total sleep activity for adult male and female flies measured on days 5 and 6. **B.** Shown is a histogram depicting the mean sleep bout duration for males and females measured on days 5 and 6. Asterisks (*) indicates significant fold change in relation to control where single asterisks indicate $p \leq 0.05$ and double asterisks indicate $p \leq 0.001$. Error bars depict standard deviation of the mean.

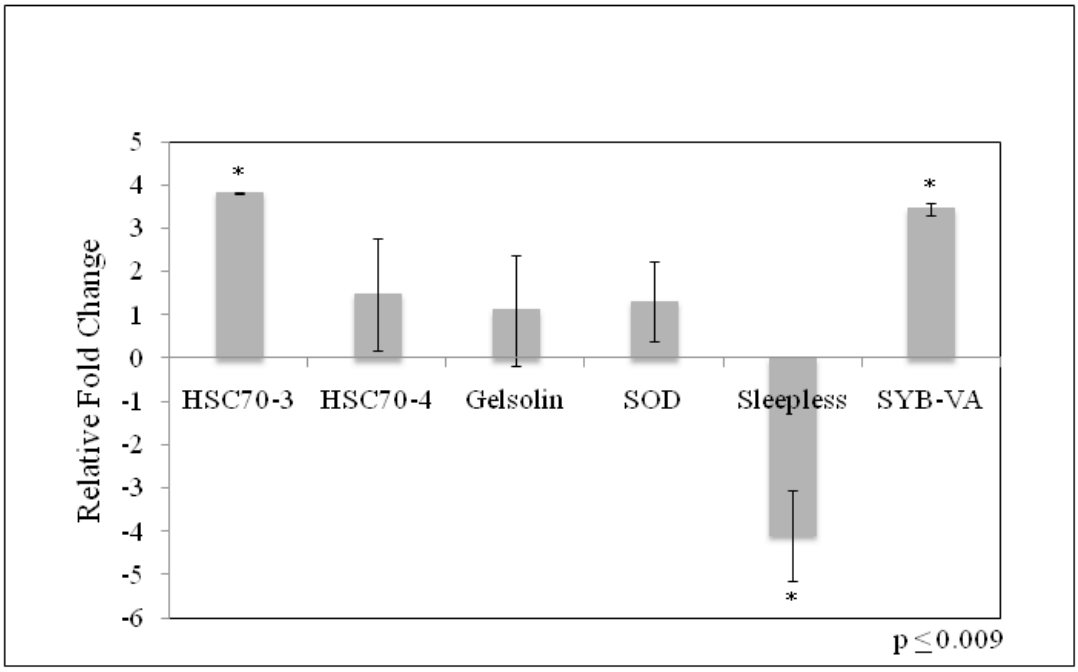


Figure 4. Specific genes associated with Elp3 phenotypes are misregulated in response to ubiquitous Elp3 loss in early pupal stages of the fly. Three female Dmel\ELP3/RNAi/B or w1118 flies were crossed to three *da*-GAL4 male flies and RNA was isolated from staged early progeny pupae. Shown is a histogram depicting qPCR analysis of the expression of the indicated genes in these staged early Elp3/RNAi or control pupal progeny samples. The relative fold change in mRNA expression levels were measured using the comparative Ct method with rp49 as the internal control gene. Error bars represent standard deviation. Asterisks (*) indicates significant fold changes between Dmel/Elp3 RNAi and control flies with values of $p < 0.009$. Error bars depict standard deviation of the mean.

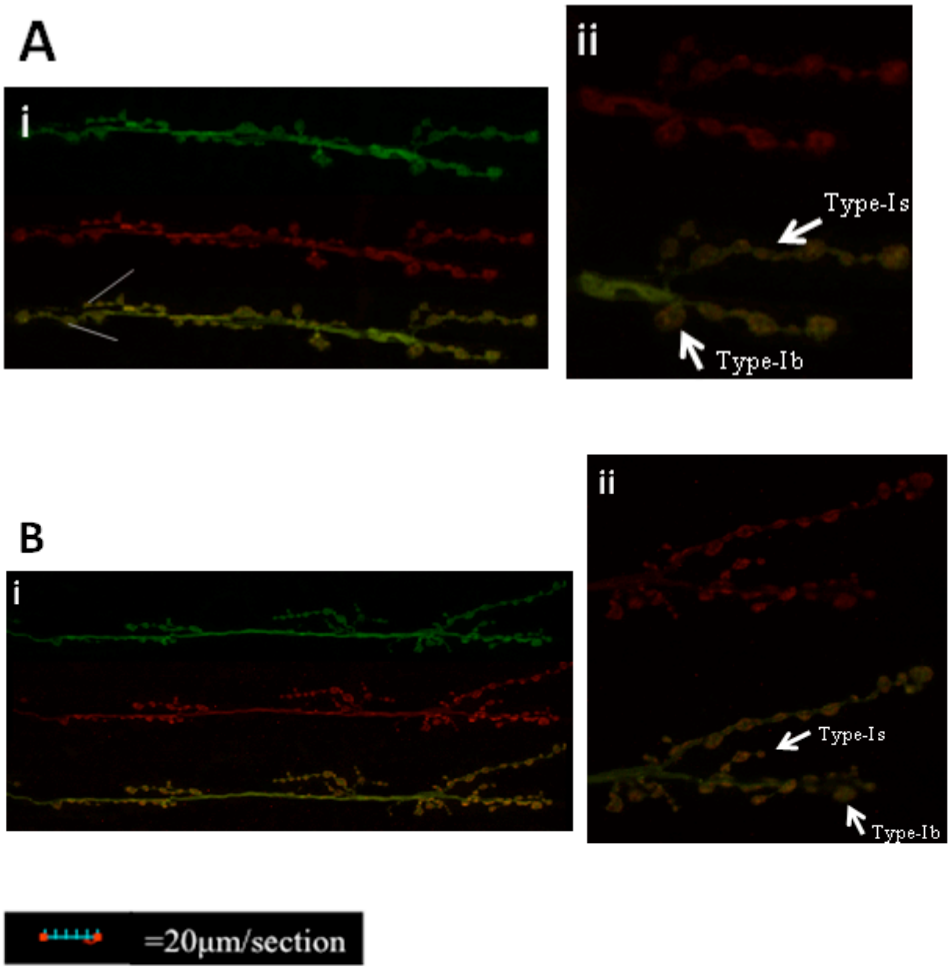


Figure 5 (continued)

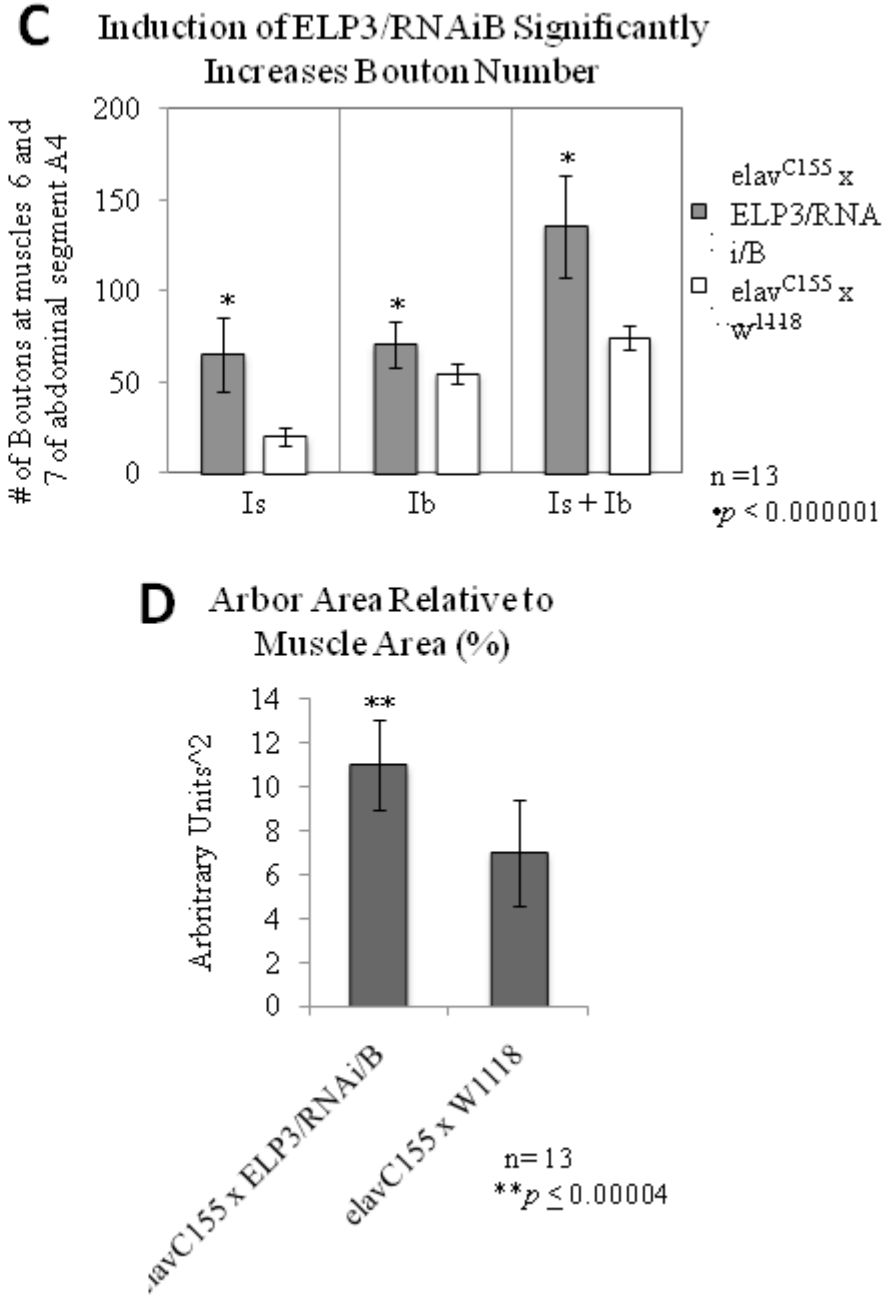


Figure 5 (continued)

Figure 5. Loss of Dmel\ELP3 in the nervous system leads to an expansion of synaptic boutons and axonal arbor area in the larval NMJ. Flies homozygous for either Dmel\ELP3/RNAi line B or control w^{1118} were crossed to flies homozygous for the nervous system $elav^{C155}$ pan-neuronal GAL4 driver, and staged third instar progeny larvae were collected. Confocal imaging analysis of boutons at muscles 6/7 at abdominal segment 4 immunohistochemical stained with anti-HRP (in green) that labels the entire presynaptic membrane, CSP (in red) that is a specific marker of the presynaptic vesicles within boutons, or merged HRP and CSP (in yellow). **A.** larva expressing Dmel\RNAi/B; i) is at 40x magnification and ii) is at 60x magnification **B.** control larva; i) is at 40x magnification and ii) is 60x magnification **C.** Histogram depicts quantitative analysis of Type Is and Ib bouton number at muscles 6 and 7 at abdominal segment 4, ($p < 0.000001$, $n \geq 12$). **D.** Muscles 6/7 at abdominal segment 4 were stained with Phalloidin, a toxin that stains muscles, to identify and measure their surface area. Histogram represents synaptic bouton arbor area relative to the muscle area in Elp3/RNAi and control larva. In the analyses, each genotype is represented by 13 larval preparations, (n=13). Asterisks (*) indicates significant fold change in relation to control where single asterisks indicate $p \leq 0.000001$ and double asterisks indicate $p \leq 0.00004$. All error bars depict standard deviation of the mean.

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Professional Experience

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Teaching Assistant: Physiology and Nutrition, Cells and Genetics, Essential Biology, Biology I: Cells and Tissues, Biology II: Growth and Heredity.

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Undergraduate Research: Social behavior of agouti and nonagouti *Peromyscus maniculatus*.

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Publications

Meridith Toth Lorbeck and Felice Elefant. Tip60 and APP genetically interact to promote apoptosis-driven neurodegeneration. In preparation.

Meridith Toth Lorbeck, Keerthy Piroosnia, Jessica Sarthi, Xianmin Zhu, and Felice Elefant. Microarray analysis uncovers a role for Tip60 in nervous system function and general metabolism. In review, Plos One.

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