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Analyzing The Interactions Of Kdm5/lid And Sin3 In Drosophila Melanogaster

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ANALYZING THE INTERACTIONS OF KDM5/LID AND SIN3 IN *DROSOPHILA MELANOGASTER*

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DEDICATION

To my father: my inspiration, my mother: my strength and my loving husband: my pillar of support through this journey!

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CHAPTER 1

INTRODUCTION

Chromatin Packaging and Transcription Regulation

The term chromatin refers to eukaryotic DNA packaged with the aid of histone proteins. Packaging of eukaryotic DNA as chromatin is necessary to facilitate the long length (~ 2 m) of naked DNA to fit within the nucleus of a cell. ~147 bp of DNA wraps twice around a histone octamer, consisting of two units each of four core histone proteins, H2A, H2B, H3 and H4, forming a nucleosome (Kornberg, 1974; Luger et al., 1997). The DNA between two nucleosomes is named linker DNA and varies in length from 20-90 bp (Szerlong and Hansen, 2011). Histone H1 associates with linker DNA, interacting at the base of the nucleosome, near DNA entry and exit sites. A string of nucleosomes, referred to as the 'beads on a string' structure or the 10 nm fiber, forms the first level of chromatin compaction (Luger et al., 2012). Further coiling of chromatin results in a 30 nm fiber, which is considered the second level of compaction. Higher level DNA packaging results in the highly compact metaphase chromosomes observed during mitosis and meiosis (Woodcock and Ghosh, 2010).

DNA compaction, while being vital, hinders another necessity, which is DNA accessibility. To accommodate accessibility to DNA, packaging of DNA occurs as a highly dynamic process. Histone post-translational modifications by histone modifying enzymes, the role of histone chaperones and ATP-dependent histone remodeling enzymes and the incorporation of histone variants all contribute to nucleosome dynamics (Luger, 2006). These processes can thus contribute to removal and

reassembly of nucleosomes or repositioning of nucleosomes and recruitment of multiple trans acting factors, thereby regulating accessibility to RNA polymerase and other proteins that impact transcription.

Histone Modifications and Transcription

Of the multiple factors that regulate nucleosome dynamics, histone post-translational modifications (PTMs) play an important role in transcriptional regulation. A large number of histone modifications identified occur on amino acid residues of N-terminal tails of histones that protrude out of the nucleosome structure (Kouzarides, 2007). Multiple modified amino acid residues are, however, found within the globular domains of histones as well (Freitas et al., 2004). Acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination and proline isomerization are such modifications that contribute to transcriptional regulation (Kouzarides, 2007). To date, more than hundred distinct PTMs of histones have been discovered (Zentner and Henikoff, 2013). With the growing evidence associating histone modifications to transcription, the histone code hypothesis was proposed stating that histone modifications can act in sequence or in combination forming a histone code that can be read by chromatin reader proteins thus affecting transcriptional outcome (Strahl and Allis, 2000).

Traditionally, two distinct chromatin types have been defined; the transcriptionally active euchromatin, consisting of loosely compacted chromatin, and the silent heterochromatin, consisting of densely packaged chromatin (Kouzarides, 2007). Marks such as histone acetylation and methylation of H3K4, H3K36 and H3K79 are associated

with euchromatin while methylation of H3K9, H3K27 and H4K20 is associated with heterochromatin (Kouzarides, 2007). Recent studies analyzing genome-wide enrichment of transcription factors and histone modifications have resulted in the definition of multiple chromatin types or domains. Based on the factors and marks selected and the scale of sub classification anywhere from 3 – 51 chromatin domains have been defined (de Graaf and van Steensel, 2013). In *Drosophila*, analysis of genome-wide binding of 53 proteins in Kc cells using a DamID approach identified five principle types of chromatin (Filion et al., 2010). Three types of repressive chromatin and two types of active chromatin were identified. The repressive chromatin types included the classical heterochromatin, polycomb group (PcG) associated silenced chromatin and a novel type of repressive chromatin, which represents the largest type of heterochromatin encompassing the majority of silenced genes. The two active chromatin types share enrichment of many similar proteins, but show differences in multiple proteins, including the enrichment of H3K36me3, a mark generally associated with transcription elongation, and MRG15, which binds this mark. Another study utilized chromatin immunoprecipitation (ChIP) – array data from S2 and BG3 cells for 18 histone marks to identify 9 states of chromatin based on combinatorial histone modification patterns (Kharchenko et al., 2011). Multiple histone modification signatures corresponded to transcriptionally active regions, including promoter proximal regions (enriched for H3K4me2/me3 and H3K9ac), exons of transcribed genes (enriched for the transcriptional elongation mark H3K36me3), intronic regions (marked by H3K27ac, H3K4me1 and H3K18ac) and regions enriched for H3K36me1, but lacking H3K27ac. The X chromosome, distinguished by H4K16ac along with moderate enrichment of the

elongation mark, H3K36me3, forms another large domain of active transcription. Large domains of silent chromatin (depleted of active marks and moderately enriched for H3K27me3) or PcG mediated repressive regions (depleted of active marks and highly enriched for H3K27me3) separate the active regions. Two other repressive states include the pericentromeric heterochromatin (enriched for H3K9me2/me3) and heterochromatin-like regions (contain moderate levels of H3K9me2/me3). Analysis of genome-wide occupancy of 38 histone acetylation and methylation marks along with the occupancy of histone variant H2AZ, RNA polymerase II and CTCF in human CD4 T-cells by ChIPseq identified 5 categories of chromatin, which could be sub classified into 51 chromatin states (Ernst and Kellis, 2010). In this study, the authors identified 11 distinct promoter states, 17 transcription associated states, 11 active intergenic states, 6 large scale repressed states and 6 repetitive states. Together, a large amount of work has established the key association of histone modifications with transcriptional regulation.

Histone Acetylation and Methylation

Histone Acetylation

The discovery of histone acetylation in the early 1960s marked the first discovery of histone post-translational modifications (Allfrey et al., 1964; Phillips, 1963). Early work on this modification revealed an association of this mark with actively transcribed genes (Allfrey et al., 1964; Pogo et al., 1966). Histone acetylation occurs on lysine residues, where acetylation neutralizes the positive charge on lysines (Grunstein, 1997; Kuo and Allis, 1998; Struhl, 1998). This weakens the interaction of histones to DNA or

adjacent histones thereby loosening the compaction of the nucleosome template. For example, thermal denaturation studies revealed a reduced binding constant for acetylated histone H4 N-terminal peptides to double stranded DNA compared to non-acetylated counterparts (Hong et al., 1993). Further, hyper acetylation of reconstituted nucleosomal arrays resulted in complete inhibition of higher-order folding of these arrays (Tse et al., 1998).

Histone acetylation can thus allow for increased accessibility of RNA polymerase and other transcription factors to DNA, allowing for transcription activation. For instance, histone acetylation resulted in recognition of the 5S RNA gene in *Xenopus* by the transcription factor TFIIIA (Lee et al., 1993). In *HeLa* cells, the transcription factors USF and GAL4-AH displayed increased affinity to nucleosomal DNA (Vettese-Dadey et al., 1996). Further, the above described work by Tse et al., recorded a 15 fold increase in transcription by RNA polymerase III due to hyper acetylation of nucleosomal arrays (Tse et al., 1998).

Histone acetylation can also affect the chromatin template through its effect on chromatin remodeling. For example, acetylation of histone H4 inhibits chromatin remodeling through the ATPase imitation SWI (ISWI) (Clapier et al., 2002; Corona et al., 2002). The hydrophilic patch of amino acids (R17-H18-R19) on the histone H4 N-terminus is critical for substrate recognition by ISWI (Clapier et al., 2002). Acetylation of adjacent amino acids H4K12 and K16 drastically reduced the ATPase activity of ISWI. Moreover, overexpression of Males-absent on the first (MOF), an enzyme that acetylates H4K16, exacerbates the X chromosomal defects seen in male flies with partial loss of ISWI function (Corona et al., 2002). Blocking the acetylation of H4K16, in

contrast, suppressed loss of ISWI function defects. Further, histone acetylation also plays an important role in processes other than transcription, such as DNA replication and repair that require DNA access (Zentner and Henikoff, 2013).

Histone lysine acetyltransferases (KATs) are the enzymes that catalyze addition of acetyl groups to histones. Many transcription factors possess KAT activity and target ε-amino groups of lysine residues predominantly on N-terminal tails of histones. KATs often have broad specificity, modifying multiple histone lysine residues. Based on sequence similarity, KATs are grouped into several distinct families, such as GNATs (Gcn5-related acetyltransferases), MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) related KATs, p300/CBP KATs, general transcription factor KATs and nuclear hormone related KATs (Carrozza et al., 2003). KATs show high sequence similarity within families, while there is poor sequence homology between families (Kuo and Allis, 1998). KATs associate with other proteins forming large complexes, where these protein associations can dictate the histone substrate specificity, target gene specificity and functional effects of the KATs (Carrozza et al., 2003).

The GNAT family of KATs includes Gcn5, PCAF, Hat1, Elp3 Hpa2 and Nut1 (Lee and Workman, 2007). Apart from the catalytic domain, GNATs typically contain a bromodomain, which can target acetyl lysines (Marmorstein, 2001). GNATs mainly serve as co-activators for specific transcriptional activators and play roles in transcription and DNA repair (Carrozza et al., 2003; Marmorstein, 2001). GNAT family members have been implicated to be important for cell growth and development (Carrozza et al., 2003). The MYST family gains its name from the initially identified members MOZ, Ybf2/Sas3, Sas2 and Tip60 and includes other members such as Esa1,

MOF and HBO1 (Carrozza et al., 2003). In addition to the catalytic domain, many MYST family proteins contain a chromodomain and/or a small zinc binding domain (Marmorstein, 2001). These proteins are involved in diverse biological functions. Apart from their roles in transcription activation and DNA repair, they function in positive regulation of transcriptional silencing (Sas2 and 3), formation of leukemic translocation products (MOZ) and dosage compensation (MOF) (Borrow et al., 1996; Carapeti et al., 1999; Carrozza et al., 2003; Hilfiker et al., 1997; Reifsnyder et al., 1996).

CBP/p300 proteins contain a HAT domain and a bromodomain along with three cysteine-histidine rich domains (Marmorstein, 2001). Unlike the GNATs, the CBP/p300 family KATs act as global regulators of transcription. TAF1, a general transcription factor KAT, which is a subunit of the TFIID complex, has two kinase domains in addition to the HAT domain and a double bromodomain. These KATs are typically characterized by the occurrence of zinc fingers and chromodomains. SRC-1, ACTR and TIF-2 are examples of nuclear hormone related KATs, which function to coactivate genes that respond to nuclear receptors (Marmorstein, 2001).

Histone acetylation is highly dynamic, where the removal of the acetyl mark is brought about by histone deacetylases (HDACs). Rapid acetylation and deacetylation events at actively transcribed genes loosen the chromatin template allowing for passage of RNA polymerase, while resetting the chromatin template following passage of RNA polymerase during transcription (Waterborg, 2002).

HDACs, like KATs, can often target a broad range of lysine residues. The described HDACs fall into four classes (Bannister and Kouzarides, 2011). The class I HDACs are homologous to yeast RPD3 and include mammalian HDAC1, 2, 3 and 8.

Class II HDACs are homologs of yeast Hda1 and include mammalian HDAC4 - 7 and HDAC9 and 10. The class III HDACs include the sirtuins (SIRT1 – 7) homologous to yeast Sir2. Class IV comprises of a single HDAC, HDAC11.

Among the class I HDACs, HDAC1 and 2 share high similarity and are found together in multiple complexes, including SIN3, NuRD and CoREST (Hayakawa and Nakayama, 2011). They play important roles in cellular processes like cell proliferation, cell cycle regulation and apoptosis along with organismal development (Haberland et al., 2009; Lagger et al., 2002; Senese et al., 2007; Zupkovitz et al., 2010). HDAC3 is a part of the NCoR/SMRT complex and is involved in cell cycle regulation and the DNA damage response (Bhaskara et al., 2008; Eot-Houllier et al., 2008). HDAC8 is less characterized and is predominantly localized to the cytoplasm and thought to play an important role in smooth muscle cell function (Waltregny et al., 2005).

While most class I HDACs are ubiquitously expressed, class II HDACs are mostly tissue specific and associate with tissue specific transcription factors and co-repressors for their repressive functions in these tissues (Witt et al., 2009). These HDACs play significant roles in differentiation and development. Class II enzymes have been found to associate with NCoR/SMRT complexes (Fischle et al., 2002; Huang et al., 2000). The single class IV HDAC, HDAC11 is less characterized. It shows tissue specific expression and is shown to play a role in immune activation and tolerance and differentiation of oligodendrocytes (Liu et al., 2009b; Villagra et al., 2009). Class III enzymes or the Sirtuins differ in there catalytic activity from the other classes in requiring NAD^+ as a cofactor (Imai et al., 2000; Landry et al., 2000). Sirtuins are widely expressed and are involved in multiple biological processes including oxidative stress

response, DNA repair, regulation of metabolic processes and aging (Haigis and Guarente, 2006).

Dynamic modification of the chromatin template by the activity of HATs and HDACs allow for transcriptional regulation. Protein associations of these HATs and HDACs define the specificity of these enzymes, tightly regulating the functional outcomes. These protein associations also increase the biological complexity of the activity of these enzymes.

Histone Methylation

In contrast to histone acetylation, which opens up the chromatin and is thus predominantly associated with active transcription, histone methylation is linked to both activation and repression of transcription. Further, apart from lysine residues, methylation also occurs on arginine residues of histone tails. Methylation of histones can occur in mono-, di- or tri-methylated states. The transcriptional state is determined by the specific amino acid residue modified and the number of methyl groups attached (Martin and Zhang, 2005). Three major groups of enzymes bring about the methylation of histones. The SET (Su(var)3-9, E(z) and Trx) domain containing proteins and the non-SET domain containing Dot1 protein catalyze lysine methylation. The third group of enzymes, the protein arginine methyltransferases (PRMTs) catalyze arginine methylation.

Although a role for histone methylation was demonstrated in the early 60s, the first histone methyltransferases (HMT) was not discovered until 2000 (Allfrey et al., 1964; Murray, 1964; Rea et al., 2000). With the exception of Dot1, which methylates

H3K79, all lysine methyltransferases (KMTs) utilize a SET domain to catalyze the methylation reaction (Xiao et al., 2003). The SET domain and its flanking regions both appear to play an important role in the catalytic function. Both classes of KMTs, however, utilize the cofactor S-adenosyl-L-methionine (SAM) as the methyl donor to transfer one or more methyl groups to the histone lysine residues.

Much work has been done on lysine methylation and its role in chromatin dynamics. Five lysine residues in histone H3 (K4, K9, K27, K36 and K79) and a single residue in histone H4 (K20) have been well characterized as methyl targets (Martin and Zhang, 2005; Sims et al., 2003). While distinct enzymes are involved in bringing about these methylation events, the transcription status associated with these histone modifications also differs.

Of the active methyl marks, H3K4me3 demarcates promoter regions, where it is present near the transcription start site (TSS) of both active genes and inactive genes that are poised for activation (Barski et al., 2007; Santos-Rosa et al., 2002; Smith et al., 2009). Recognition of H3K4me3 by the PHD domain of transcription factors can recruit RNA polymerase II to promoters thus implicating this mark in transcription initiation (Black et al., 2012; Vermeulen et al., 2007). Apart from H3K4me3, regions flanking the TSS are enriched for H3K4me1/2 (Barski et al., 2007; Ernst et al., 2011; Filion et al., 2010; Gerstein et al., 2010; Kharchenko et al., 2011). H3K4me1 is enriched at enhancer sequences that act to regulate transcription (Hon et al., 2009; Rada-Iglesias et al., 2011). Multiple enzymes including the MLL proteins, SET1 proteins and ASH1 and their homologs are involved in the catalysis of H3K4 methylation (Allis et al., 2007; Black et al., 2012).

Active promoters are also often associated with gene bodies that carry H3K36me3 and H3K79me2/me3 catalyzed by SET2 and DOT1 enzymes and their homologs, respectively (Allis et al., 2007; Black et al., 2012). While H3K79me2/me3 peaks at the 5' end of genes and gradually decreases towards the transcription stop site, H3K36me3 peaks at the 3' end (Barski et al., 2007; Ernst et al., 2011; Filion et al., 2010; Gerstein et al., 2010; Kharchenko et al., 2011). H3K36me3 shows the most correlation with the level of transcription and is considered to play an important role in transcription elongation (Black et al., 2012).

Inactive genes on the other hand are enriched for H3K27me2/me3, H3K9me2/me3 and H4K20me3 (Allis et al., 2007; Black et al., 2012). H3K27me is catalyzed by EZH2 while SUV39, G9a and SETDB1 enzymes all methylate H3K9 and Pr-SET7/8 and SUV4-20 enzymes methylate H4K20 (Allis et al., 2007; Black et al., 2012). H4K20me3 is enriched near the TSS while H3K27me2/me3 and H3K9me2/me3 show broader distribution along the gene (Barski et al., 2007; Ernst et al., 2011; Filion et al., 2010; Gerstein et al., 2010; Kharchenko et al., 2011). H3K27me3 leads to repression by inhibiting transcription elongation (Chen et al., 2012). A special set of inactive genes carry both H3K27me3 and H3K4me3 at their promoters, which are termed bivalent promoters (Bernstein et al., 2006; Voigt et al., 2012). These marks, however, are thought to occur on opposing histone H3 copies of a nucleosome (Voigt et al., 2012). Such bivalent promoters are generally associated with developmental genes, where they function to poise gene expression (Voigt et al., 2013). They help maintain repression until differentiation signals are received.

The repressive marks H3K9me2/me3 and H4K20me3 are also enriched at the

highly condensed heterochromatin and regions with high repetitive sequences (Barski et al., 2007; Ernst et al., 2011; Filion et al., 2010; Gerstein et al., 2010; Kharchenko et al., 2011). Of interest is the enrichment of H3K9me2/me3 at active genes present within heterochromatic regions, which is distinct from euchromatic genes. The switch from active to repressive methylation marks, define the borders of heterochromatin and euchromatin. The conservation of such defined regions demarcated by methylation patterns highlight the importance of this chromatin modification.

In addition to methylation of lysine residues, histones are modified at specific arginine residues. Protein arginine methyltransferases (PRMTs) catalyze this reaction by transferring one or two methyl groups from S-adenosylmethionine (AdoMet) to guanidino nitrogen atoms of arginine residues (Di Lorenzo and Bedford, 2011). In mammals, eleven PRMTs named PRMT1 - 11 have been identified (Di Lorenzo and Bedford, 2011; Jahan and Davie, 2014). These fall into several categories based on the resulting methylation pattern. Type I enzymes (PRMT1, 2, 3, 4, 6, and 8) form a monomethyl intermediate and further result in asymmetrical dimethylation of arginine residues modified. Type II enzymes (PRMT5 and 9), similar to type I enzymes form a monomethyl intermediate, yet result in symmetrical dimethylation. PRMT7 results only in monomethylation of arginine thus classifying it as a type III enzyme, however, dimethylation activity has been reported in some instances. All three types of enzymes methylate terminal guanidino nitrogen atoms of histone arginine residues. PRMT10 and 11 were identified based on their sequence homology to PRMT7 and 9 respectively and are yet to be characterized. Monomethylation of an internal guanidine nitrogen was reported from yeast, which is categorized as type IV activity (Niewmierzycka and

Clarke, 1999; Zobel-Thropp et al., 1998).

Arginine residues in histone H2A, H3 and H4 have been identified to be methylated and are associated with transcription activation or repression. The enzymes PRMT1, 2 and 4 are generally characterized as transcription coactivators while PRMT5 and 6 are predominantly considered repressors (Di Lorenzo and Bedford, 2011). Histone arginine methylation can regulate transcription by the recruitment of activators or repressors to modified sites.

A further mode of regulation involves blocking the binding of effector proteins mediated by histone crosstalk. Crosstalk between neighboring arginine and lysine modifications is found to be a common phenomenon. A well-studied occurrence is the crosstalk between H3R2 and H3K4 methylation. Methylation of H3R2 by PRMT6 prevents methylation of H3K4 by the MLL1 complex, thus preventing the recruitment of activators to the H3K4 site (Hyllus et al., 2007). Apart from direct modification of histones, PRMTs also modify other transcription factors, coregulators, elongation factors and splicing factors thereby coupling the regulation of interrelated processes in transcription regulation (Wysocka et al., 2006a).

Similar to acetylation, methylation too is dynamically regulated in the cell. Direct removal of the methyl marks are catalyzed by histone lysine demethylases (KDMs). While several KDMs have been recently discovered, enzymes that directly remove methyl marks from arginine residues have not been convincingly demonstrated. The role of KDMs will be described in detail in the following section.

Histone Demethylases

KDMs are enzymes that catalyze the removal of methyl groups attached to amino acid residues of histone proteins. Unlike HDACs, which have been isolated and characterized over a long period of time, KDMs were only discovered quite recently (Mosammaparast and Shi, 2010). In fact, histone methylation was initially considered to be an irreversible reaction. This long-standing notion was based on the nature of the chemical bond forming methylated histones. Unlike the easily hydrolyzed amide bond formed upon histone acetylation, histone methylation results in a more stable C-N bond. Furthermore, in support of the idea of the irreversibility of methylation, experiments demonstrated that histones and methylated histones had equivalent half-lives (Byvoet et al., 1972; Thomas et al., 1972). Evidence for indirect histone demethylation through histone exchange, proteolytic cleavage of histone amino termini or conversion of methyl arginine to citrulline by protein arginine deiminases, however, was observed (Ahmad and Henikoff, 2002; Allis et al., 1980; Cuthbert et al., 2004; Wang et al., 2004b).

While many researchers suggested that it was theoretically possible to demethylate histones, the first real experimental evidence was obtained only in the past decade upon the identification of the lysine specific demethylase 1 (LSD1/KDM1) enzyme. KDM1 was initially identified as part of the CoREST HDAC containing complex (Hakimi et al., 2002; Hakimi et al., 2003; Humphrey et al., 2001; You et al., 2001). KDM1 was subsequently found to have the ability to catalyze histone H3K4 mono and dimethyl demethylation in a flavin adenine dinucleotide (FAD) dependent manner (Forneris et al., 2005; Shi et al., 2004). The amine oxidase domain of KDM1 was found to be important for the demethylase activity. KDM1 can catalyze the demethylation of

both H3K4 and H3K9 methylation.

Soon after, a second class of demethylases was discovered, which utilized the conserved Jumonji C (JmjC) domain to demethylate lysine residues in a Fe(II) and α-ketoglutarate dependent manner (Cloos et al., 2006; Fodor et al., 2006; Tsukada et al., 2006; Whetstine et al., 2006). These identified JmjC domain containing demethylases (JHDMs) contain specificity for various methyl lysine residues and methylation states. To date, demethylases that can remove most major histone methyl marks have been identified (Allis et al., 2007; Mosammaparast and Shi, 2010). Of the JHDMs identified to date, the KDM2s and KDM3s show specificity for mono- or di-methylated lysine residues. The KDM2s regulate H3K36 methylation, while KDM3s demethylate H3K9 residues. The KDM4, KDM5 and KDM6 classes of enzymes demethylate di- and tri-methylated lysine residues. The KDM4s demethylate H3K9 and H3K36 residues. KDM5s are involved in demethylation of H3K4 methylation. The KDM6s remove H3K27 methylation. Recently enzymes that can remove H4K20 methylation have also been reported (Liu et al., 2010; Qi et al., 2010; Stender et al., 2012). dKDM5/Little imaginal discs (LID) can demethylate H3K4 di and trimethylation in vitro, however, it is specific for H3K4me3 in vivo (Eissenberg et al., 2007; Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007).

Since the identification of KDMs, the important biological functions of these enzymes have come into light. The integral role of these proteins in many multi subunit complexes defines their biological significance in multiple processes. KDMs have been implicated in the regulation of chromatin packaging and transcription consistent with there chromatin modifying ability. Further, roles for these enzymes in cell cycle

progression, differentiation, stem cell pluripotency, epigenetic memory and developmental processes have been identified (Mosammaparast and Shi, 2010). The importance of KDMs is further highlighted by their key role in disease states including cancers and many neurological disorders (Black and Whetstine, 2013; Shi, 2007).

Complexes containing Deacetylases and Demethylases

Since the initial proposal of the existence of a histone code, it has now become undeniable that the coordinated action of multiple histone modifications often controls transcriptional outcome rather than a single modification by itself. Therefore, it is no surprise that multiple histone modifying enzymes would occur together in complexes. Since the discovery of histone demethylases, multiple protein complexes have been identified that include both histone deacetylases and histone demethylases. SIN3, NuRD (Nucleosome Remodeling Deacetylase), CoREST (Corepressor of REST) and NCoR (Nuclear receptor CoRepressor)/SMRT (Silencing Mediator for Retinoid and Thyroid receptor) are four such examples of complexes that incorporate both histone deacetylases and demethylases (Hayakawa and Nakayama, 2011).

The SIN3 protein acts as the scaffold for a multi subunit protein complex conserved across eukaryotes (Silverstein and Ekwall, 2005). The SIN3 complex incorporates the deacetylase RPD3 in yeast and *Drosophila* and the homologous proteins HDAC1 and 2 in mammals. Recent work has identified the association of the demethylase dKDM5/LID in *Drosophila* and KDM5A in mammals with the SIN3 complex (Hayakawa et al., 2007; Moshkin et al., 2009; Spain et al., 2010; van Oevelen et al., 2008). So far the yeast homolog of KDM5, Jhd2 has not been found to associate with

the SIN3 containing Rpd3S or Rpd3L complexes in yeast. While much work has been done to elucidate the role of the HDAC component in SIN3 complexes, the contribution of the KDM to the complex has not been well characterized. In mammals, KDM5A associates with an MRG15 containing mSin3B complex. MRG15 can recruit KDM5A to transcribed regions affecting their H3K4me states (Hayakawa et al., 2007). Further, a majority of the SIN3 target regions displayed binding of KDM5A and resulted in silencing of E2F target genes during differentiation (van Oevelen et al., 2008). In *Drosophila*, a SIN3, RPD3 and dKDM5/LID containing complex was demonstrated to bind to and silence Notch target genes (Moshkin et al., 2009). These findings highlight the coordinated action of the HDAC and KDM in the SIN3 complex in gene regulation.

The NuRD complex was initially characterized in mammalian and Xenopus cells, based on its nucleosome remodeling function (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998). Major components of the complex are also conserved in other organisms. The complex shares some components with the SIN3 complex, such as HDAC1/2 and RbAp48/46 (RBBP4/7). Other components of the complex include CHD3/4 (chromodomain helicase DNA binding proteins), MBD2/3 (methyl CpG binding proteins), MTA1/2/3 (metastasis tumor antigens) and p66α/β involved in transcription repression. Subsequently, a KDM1 interacting NuRD complex was purified, adding a demethylase component to the complex (Wang et al., 2009). The KDM1/NuRD complex acts to repress key genes involved in multiple signaling pathways. It is also implicated in the suppression of breast cancer metastasis. Recent work has identified the interaction of another demethylase, KDM5A, with the NuRD complex (Nishibuchi et al., 2014). The KDM5A/NuRD complex is implicated in regulation of developmental processes. In fact,

several NuRD complex components play important roles in development (Kaji et al., 2006; Kaji et al., 2007; Le Guezennec et al., 2006; Marino and Nusse, 2007). Differential interaction of the core proteins of the complex CHD3/4, MBD2/3, MTA1/2/3 and p66α/β result in multiple NuRD complexes, increasing functional diversity of the complex. For instance, MBD2 and MBD3 can associate exclusively with the NuRD complex forming two distinct complexes (Le Guezennec et al., 2006).

CoREST was initially found to associate with HDAC1/2, p80, Sox-like protein, ZNF217 and KDM1 (You et al., 2001). The demethylase activity of KDM1 on nucleosome substrates requires the CoREST complex, with higher demethylase activity observed on hypoacetylated nucleosomal templates (Shi et al., 2005; Yu et al., 2003). This suggests an important role for the HDAC in regulating KDM1 function. Other proteins such as G9a, EuHMT, HPC2, REBB-1, ZNF516, Gfi-1 and Gfi-1b have also been seen to associate with CoREST (Saleque et al., 2007; Shi et al., 2003). Like SIN3 and NuRD, CoREST too is thought to form distinct complexes to allow for different cellular functions.

NCoR and SMRT proteins form large complexes recruiting multiple proteins to nuclear hormone receptors thereby repressing downstream transcription (Ordentlich et al., 1999). NCoR and SMRT form distinct complexes but share many interacting partners, such as HDAC3, TBL1, TBLR1 and GPS2 (Guenther et al., 2000; Li et al., 2000; Yoon et al., 2003). Other HDACs, HDAC4, 5 and 7, and several transcription factors are also known to interact with NCoR and SMRT (Fischle et al., 2002; Glass and Rosenfeld, 2000; Jepsen et al., 2000). KDM4A and KDM5C interact with NCoR (Tahiliani et al., 2007; Zhang et al., 2005). No direct interaction of SMRT with KDMs is

known, however, SMRT represses the gene encoding KDM6B (Jepsen et al., 2007).

These complexes highlight the coordinated role of HDACs and KDMs where these proteins can form distinct complexes to achieve context specific cellular outcomes.

The SIN3 Complexes

SIN3, as mentioned above, is conserved from yeast to mammals and forms multiple complexes in these organisms (Silverstein and Ekwall, 2005). The SIN3 protein includes conserved domains such as, multiple PAH (paired amphipathic helix) domains, an HID (HDAC interaction domain) and a highly conserved region (HCR), which allow for protein-protein interactions (Wang et al., 1990; Wang and Stillman, 1993). Many of the core proteins in the multitude of SIN3 complexes are also conserved.

A SIN3 complex was first isolated in mammalian cells by three individual groups (Hassig et al., 1997; Laherty et al., 1997; Zhang et al., 1997). The histone deacetylases HDAC1 and HDAC2, retinoblastoma associated proteins RbAp46 and RbAp48 (RBBP4/7) that can associate with histone H4 and H2A thereby stabilizing nucleosome interaction of the complex and SIN3 associated proteins SAP18 and SAP30, which can stabilize the SIN3 HDAC interaction, were found as SIN3 complex components. Soon after SDS3 was also found to be a component of the complex important for stabilizing the SIN3 complex (Alland et al., 2002; Fleischer et al., 2003). Further characterization of the complex has identified components such as RBP1 (Retinoblastoma binding protein), SAP25, SAP130, SAP180, BRMS1 (Breast cancer metastasis suppressor), ING1/2 (Tumor suppressor protein), Pf1, a PHD zinc finger protein, and MRG15, a

chromodomain containing protein that can recognize H3K36me3 (Doyon et al., 2006; Fleischer et al., 2003; Kuzmichev et al., 2002; Lai et al., 2001; Meehan et al., 2004; Shiio et al., 2006; Skowyra et al., 2001; Yochum and Ayer, 2001, 2002). All components of the SIN3 complex do not exist together at all times, but form multiple subcomplexes.

In mammals two separate genes, *mSin3a* and *mSin3b* encode two distinct SIN3 proteins (Ayer et al., 1995). Splice variants of mSin3A and mSin3B have also been reported, increasing the complexity of complexes formed. mSin3A and mSin3B have been implicated in differing functions. The above described mammalian SIN3 complex corresponds to the mSin3A complex while an mSin3B complex that localizes to gene bodies was recently described comprising mSin3B, HDAC1, MRG15 and Pf1 (Jelinic et al., 2011). The histone demethylase KDM5A has also been shown to interact with mSin3B (Hayakawa et al., 2007; van Oevelen et al., 2008).

In *S. cerevisiae,* a single gene encodes Sin3, which is found in two distinct complexes, Rpd3L and Rpd3S (Carrozza et al., 2005; Keogh et al., 2005). Sin3, Rpd3 and Ume1 are common components of both complexes. Eaf3 and Rco1 uniquely associate with Rpd3S, which is recruited to coding regions of genes where it inhibits transcription from cryptic promoters. Rxt1, Rxt2, Dep1, Sds3, Pho23 and Sap30 associate with the Rpd3L complex, which is targeted to promoter regions of genes leading to repression of transcription. In *S. pombe*, three different genes encode SIN3 homologs, Pst1, 2 and 3, which form two known complexes, Complex I and Complex II (Nicolas et al., 2007). Complex I functions similar to Rpd3L and includes Pst1, Clr6 (the histone deacetylase), Sds3, Png2 (ING1 homolog) and Prw1 (RBBP4/7 homolog). Complex II, which is functionally similar to Rpd3S, comprises Pst2, Clr6 and Prw1 (in

common with Complex I), Cph1 and Cph2 (Pf1 homologs) and Alp13 (MRG15 homolog).

In *Drosophila*, a single gene encodes multiple alternatively spliced isoforms of SIN3, SIN3 187, 190 and 220 (Fig. 1.1). These isoforms are differentially expressed through development (Sharma et al., 2008). The predominant isoforms SIN3 187 and 220 form distinct complexes (Fig. 1.2) (Spain et al., 2010). SIN3, RPD3, SDS3, ARID4B (SAP180 homolog), ING1, SAP130, Pf1 and BRMS1 are shared between both complexes, while p55 (RBBP4 homolog), dKDM5/LID and EMSY uniquely associate with SIN3 220. The two complexes are recruited to common and distinct regions in polytene chromosomes and show differential ability to rescue viability in SIN3 deficient flies.

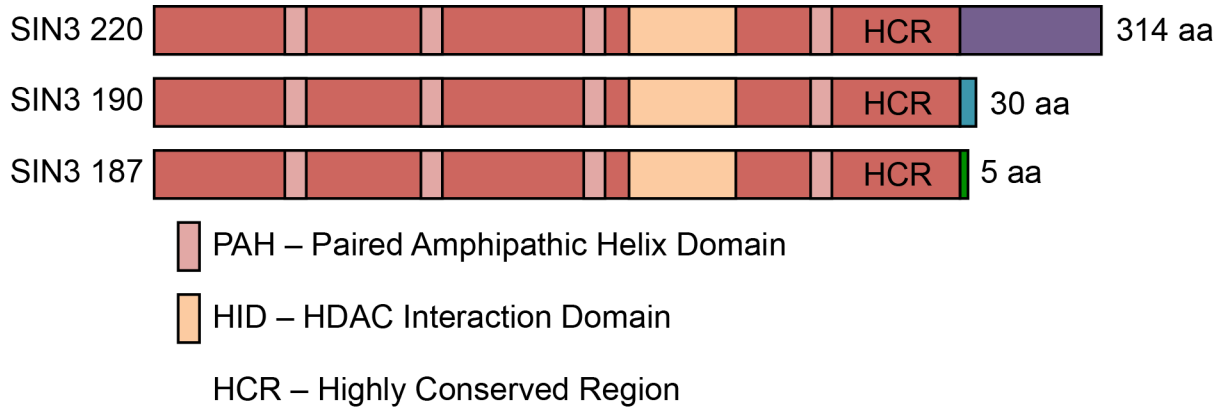

Figure 1.1: Schematic of the domain structure of *Drosophila* SIN3 isoforms. *Drosophila* SIN3 isoforms, SIN3 220, SIN3 190 and SIN3 187, named based on their kDa size share 1046 aa from the N-terminus differing only at the C-terminus. The lengths of the unique C-terminal regions are denoted on the right.

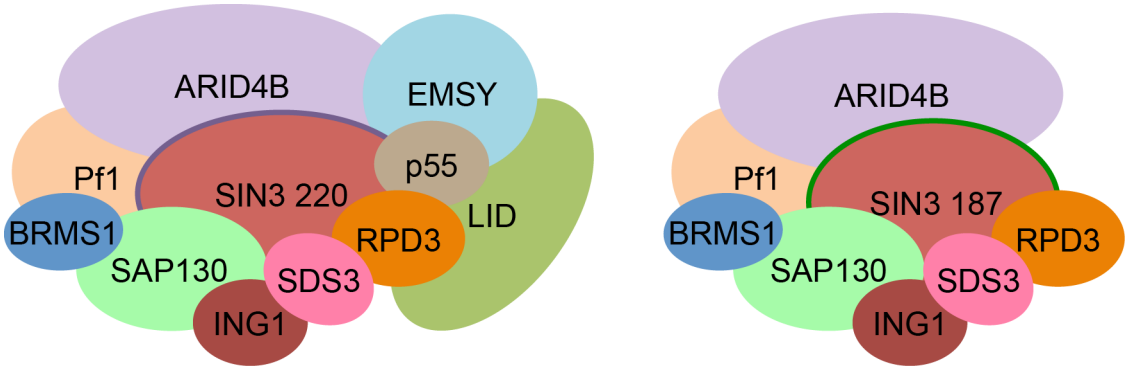

Figure 1.2: Schematic model of SIN3 isoform specific complexes in *Drosophila*. The protein associations are based on published LC/MS/MS analysis results of SIN3 isoform specific purifications (Spain et al., 2010).

The role of SIN3 as a global regulator of transcription can be attributed to the multiple complexes of SIN3 that exist. Further, due to these varying protein-protein interactions SIN3 plays key roles in regulating multiple cellular and developmental processes.

KDM5 and Interacting Proteins

The histone demethylase KDM5, like SIN3, is conserved across eukaryotes (Blair et al., 2011). In yeast, worms and flies, a single gene encodes the KDM5 homolog, while four genes in mammals encode the functionally overlapping KDM5 proteins KDM5A – D (Fig. 1.3). The KDM5s contain five conserved domains, the catalytic JmjC domain, JmjN, ARID (A/T rich interaction domain), C_5HC_2 zinc finger, and two to three PHD (Plant homeobox domain) fingers involved in protein-protein interactions, including recognition of methylated histones. KDM5 complexes have been purified in both *Drosophila* and mammals.

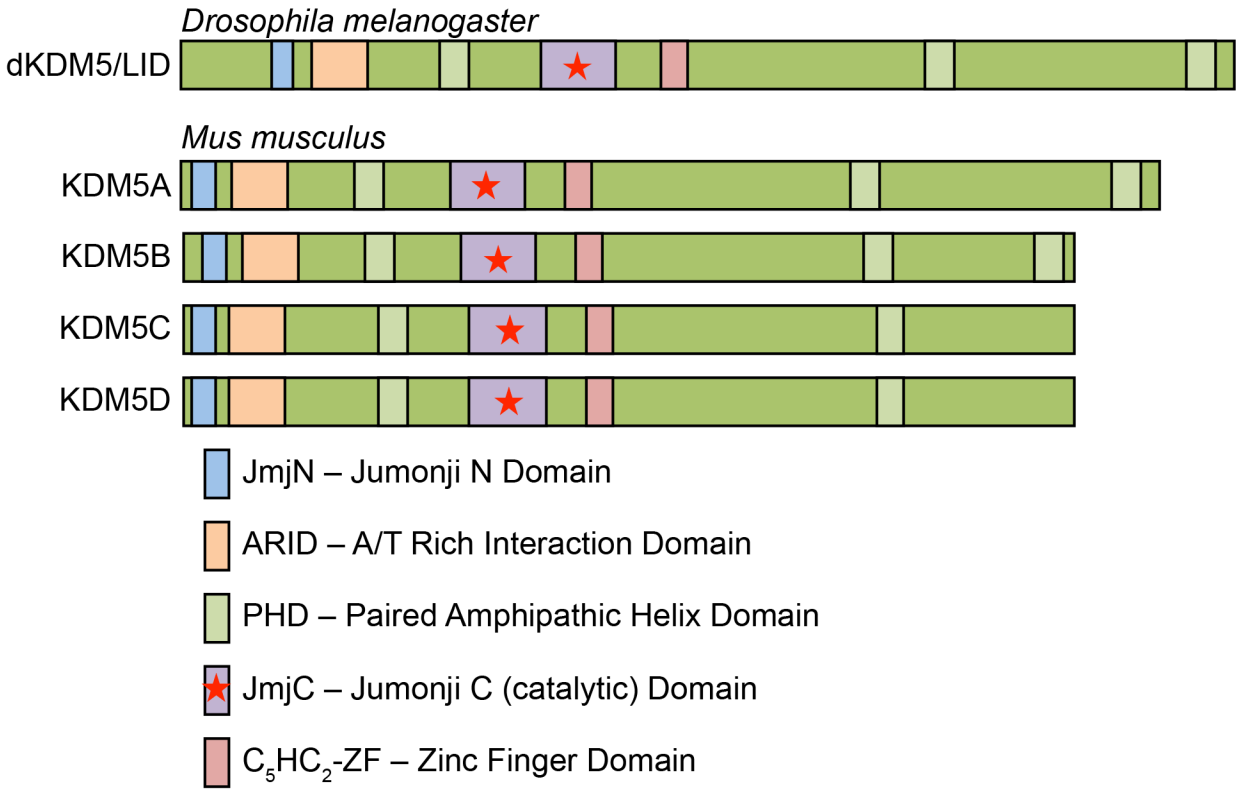

Figure 1.3: Schematic of the domain structure of *Drosophila* and mouse KDM5 proteins. A single gene in *Drosophila* and four distinct genes in mammals encode the KDM5 proteins.

In *Drosophila*, dKDM5/LID was identified in a genetic screen for novel trithorax group members (Gildea et al., 2000). Consistent with this, early work determined that dKDM5/LID interacts with dMyc and Ash2, a trithorax group protein (Secombe et al., 2007). Soon after, another group purified dKDM5/LID from embryo nuclear extracts (Lee et al., 2009). Mass spectrometry identified the association of RPD3, Pf1, MRG15 and CG13367 (Fig. 1.4A). Mammalian homologs of RPD3, Pf1 and MRG15 are all known to be part of a SIN3 containing complex. However, this initial dKDM5/LID purification did not identify SIN3 as an interacting partner (Lee et al., 2009). These authors further showed that the incorporation of RPD3 in this dKDM5/LID complex

diminished its deacetylase activity, which correlates with a role for dKDM5/LID in transcription activation. Subsequent work has identified a repressive role for dKDM5/LID complexes (Moshkin et al., 2009). Purification of dKDM5/LID in both S2 cells and embryo nuclear extracts by these researchers found an association of SIN3 and EMSY proteins, in addition to RPD3, Pf1 and MRG15 (Fig. 1.4B). These authors predict the occurrence of two dKDM5/LID containing complexes, LAF (LID and its associated factors), lacking RPD3, and RLAF, containing RPD3, which can associate with the histone chaperones ASF1 and NAP1, respectively. Interaction with the histone chaperones aids targeted demethylation of H3K4me3 by dKDM5/LID and is associated with silencing of Notch target genes. dKDM5/LID also interacts with Su(H) the DNA binding repressor, which mediates targeting of the LAF – ASF1 complex to Notch targets (Liefke et al., 2010). Recently dKDM5/LID was shown to interact with the transcription factor Foxo and regulate oxidative stress resistance genes (Liu et al., 2014). The interaction of dKDM5/LID with the SIN3 corepressor is further validated by *Drosophila* SIN3 isoform specific purifications, which showed interaction of dKDM5/LID with the large SIN3 isoform, SIN3 220 (Spain et al., 2010). The dKDM5/LID interacting proteins, RPD3, EMSY and Pf1 were also part of the described SIN3 220 complex.

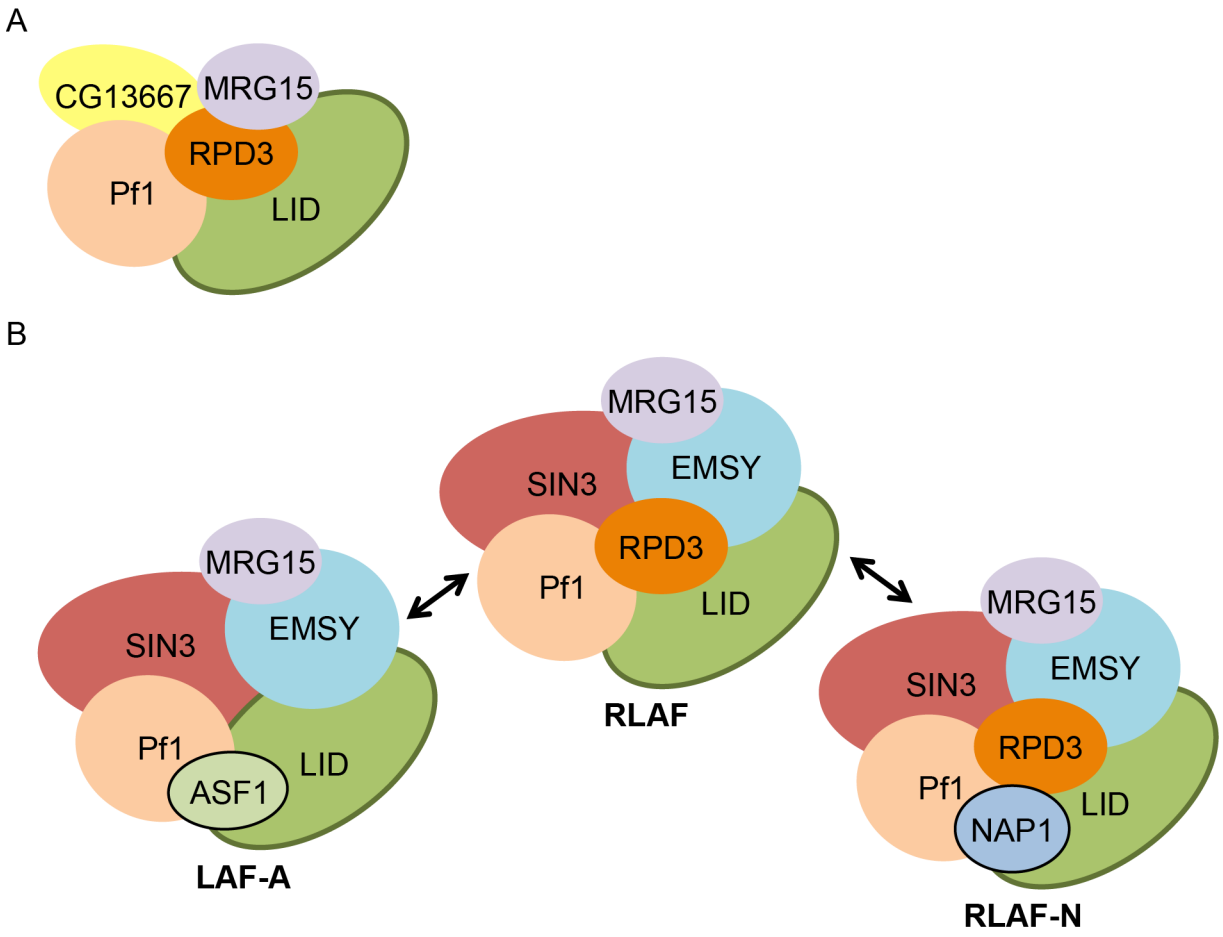

Figure 1.4: Schematic model of dKDM5/LID complexes in *Drosophila*. The protein associations are based on published mass spectrometric analysis results of dKDM5/LID purifications. (A) Based on (Lee et. al., 2009). (B) Based on (Moshkin et. al., 2009). LAF-A (LID and Associated Factors – ASF1), RLAF (RPD3, LID and Associated Factors), RLAF-N (RPD3, LID and Associated Factors – NAP1).

Of the mammalian KDM5s, KDM5A and KDM5B are the most characterized. KDM5A was initially isolated as a binding partner of pRB (Defeo-Jones et al., 1991). Further KDM5A associates with SIN3 complex proteins, mSin3B, MRG15 and HDAC2 (Hayakawa et al., 2007; van Oevelen et al., 2008). Recently KDM5A was also reported to interact with NuRD complex components, CHD4, MTA2 and GATAD2A (Nishibuchi et al., 2014). KDM5A interactions with multiple other proteins including Myc, Mad1, RBP-J, TBP, p107, rhombotin-2, and nuclear receptors have also been demonstrated (Chan

and Hong, 2001; Ge et al., 2010; Kim et al., 1994; Liefke et al., 2010; Mao et al., 1997; Secombe et al., 2007). Targeted immunoprecipitation assays demonstrated the interaction of KDM5B with multiple HDACs including HDAC1, 4, 5 and 7 and the corepressor NCoR (Barrett et al., 2007). Direct physical interactions were observed with the HDACs, while the NCoR interaction was likely indirect. KDM5B is also known to bind the developmental transcription factors BF-1 and PAX9 (Tan et al., 2003).

Like SIN3, the KDM5 proteins can form multiple context specific interactions, thus affecting diverse cellular and developmental processes.

SIN3 and KDM5 in Gene Expression Regulation

SIN3

The first reports of SIN3 function came from genetic screens in yeast studying mating type switching, where Sin3 and associated protein Rpd3 were found to be negative regulators of the *HO* endonuclease (Nasmyth et al., 1987; Sternberg et al., 1987). Confirming a role for Sin3 in gene repression, many more studies in yeast identified genes repressed by Sin3. A Sin3 mutant in yeast resulted in inappropriate expression of meiosis specific genes, *SPO11*, *SPO13* and *SPO16* during the vegetative phase (Strich et al., 1989). Sin3 and Rpd3 mutants lead to upregulation of *TRK2*, reducing potassium dependency of the cell (Vidal et al., 1990). Another genetic screen isolated Sin3 mutants that could not repress an *INO1-lacZ* reporter construct leading to constitutive expression of phospholipid genes (Hudak et al., 1994). Further, Sin3 along with Rpd3 and Sds3 were found to be involved in transcriptional silencing in yeast (Vannier et al., 1996). This early work in yeast demonstrated that Sin3 functions as a

global repressor of transcription.

Several genome wide studies have further established the role of SIN3 as a global regulator of transcription. In yeast, expression profiling found greater than 200 genes to be regulated by Sin3 (Bernstein et al., 2000; Fazzio et al., 2001; Watson et al., 2004). Microarray analysis of cultured cells identified 3% of the *Drosophila* genome to be regulated upon depletion of SIN3 by RNA interference (RNAi) (Pile et al., 2003). Genes regulated by SIN3 are involved in multiple processes, such as signal transduction, cell cycle regulation and transcription, with a significant proportion of genes involved in mitochondrial processes. Genome wide expression analysis of mammalian SIN3 regulated genes also found a large number of SIN3 targets, with significant enrichment of genes involved in cell cycle regulation, DNA repair and cytosolic and mitochondrial energy production (Dannenberg et al., 2005; van Oevelen et al., 2008).

Apart from the role in gene repression, SIN3 has also been implicated in activation of multiple gene targets. The first example of SIN3 in gene activation came from a study in yeast, where Sin3 positively regulated *GAM3*, which in turn lead to activation of the extracellular glucosamylase, *STA1* (Yoshimoto et al., 1992). The yeast Rpd3L complex components, including Sin3 has been shown to regulate transcription activation upon heat stress (Ruiz-Roig et al., 2010). In *Drosophila*, SIN3 was found to primarily activate genes involved in cell invasion or migration (Das et al., 2013). Mammalian Sin3A and Sin3B were found to positively regulate genes involved in muscle development (van Oevelen et al., 2010). The above mentioned genome wide expression studies too have identified many targets to be activated by SIN3, although

the majority of targets are negatively regulated.

Work analyzing the structure and protein associations of SIN3 has helped describe a mechanism for SIN3 mediated transcriptional repression. The SIN3 protein acts as a scaffold allowing the assembly of multiple HDAC containing complexes (Grzenda et al., 2009; Silverstein and Ekwall, 2005). SIN3 or the associated HDAC have no DNA binding activity. The SIN3 complexes are recruited to chromatin via association with DNA binding factors, co-repressors or chromatin associated proteins. Upon recruitment to chromatin, the associated HDACs can deacetylate histones in the surrounding area forming a transcriptionally repressive environment.

In yeast, two distinct mechanisms have been described for Sin3 dependent repression of targets. The first mechanism involves targeting of the Sin3 containing Rpd3L complex to target gene promoters by transcriptional repressors and DNA binding proteins like Mad1 and Ume6, enabling repression of these genes (Kadosh and Struhl, 1998; Kasten et al., 1996; Rundlett et al., 1998; Wang and Stillman, 1993). The second mechanism involves the Sin3 containing Rpd3S complex which is targeted to coding regions by Rco1 and Eaf3 proteins, which repress transcription initiation from internal cryptic promoters (Carrozza et al., 2003; Keogh et al., 2005; Li et al., 2007a; Li et al., 2007b; Nicolas et al., 2007). In mammals, SIN3, along with KDM5A, has been shown to bind downstream of the transcription start site (TSS) of E2F4 target cell cycle genes and spread further downstream to permanently silence these genes during differentiation (van Oevelen et al., 2008).

While growing examples suggest a role for gene activation by SIN3, the underlying molecular mechanism for such a role is still unclear. Much work needs to be

done to determine how SIN3 positively regulates transcription, a role contradicting its association with a HDAC and a KDM.

KDM5

In *Drosophila* the *lid* gene was first reported from a genetic screen identifying novel *trithorax* group genes (Gildea et al., 2000). This identification of *lid* as a trithorax group gene, involved in gene activation, is in contrast to its catalytic activity of removing H3K4me3, a mark associated with active transcription. Further work corroborated a *trithorax* function, where *lid* knockdown or mutant flies respectively resulted in decreased expression of the homeotic gene *Ubx* (*Ultrabithorax*) in S2 cells or its protein product in wing imaginal disc tissue (Lee et al., 2007; Lloret-Llinares et al., 2008). Moreover, *lid* was reported to antagonize heterochromatin mediated silencing, as *lid* mutants acted as enhancers of position effect variegation (PEV) (Lloret-Llinares et al., 2008). dKDM5/LID was also found to interact with dMyc and play a role in *dMyc* dependent activation of target gene *Nop60B* (Secombe et al., 2007). dMyc, however, inhibits the demethylase activity of dKDM5/LID by binding to the catalytic domain, giving a possible explanation for the role in activation. Another possible mode for gene activation by dKDM5/LID was observed wherein a dKDM5/LID containing complex inhibited the deacetylase activity of RPD3 in vitro (Lee et al., 2009). Further, the RPD3 target gene *Odd* was derepressed upon overexpression of dKDM5/LID. These initial studies established the role of dKDM5/LID in gene activation.

Consistent with its demethylase activity, dKDM5/LID has also been implicated in gene silencing. dKDM5/LID and SIN3 containing complexes were found to interact with

the histone chaperones ASF1 and NAP1, which associate with the sequence specific DNA binding corepressor complex Su(H)/H (Moshkin et al., 2009). Su(H)/H helps recruit dKDM5/LID containing complexes to Notch targets, silencing these genes. Knockdown of *lid*, *Sin3A* or other complex components resulted in upregulation of Notch targets E(spl)m4, E(spl)m7 and E(spl)m8.

Only recently studies have emerged analyzing genome wide expression changes upon depletion of dKDM5/LID. ChIPseq experiments from wing imaginal disc tissues identified over one thousand dKDM5/LID target genes, where dKDM5/LID binding at majority of the genes overlapped with RNA polymerase II binding and H3K4me3 mark enrichment (Lloret-Llinares et al., 2012). Gene expression profiling utilizing expression arrays upon *lid* knockdown from wing imaginal discs, however, identified only 11 genes to be significantly regulated. 10 of these genes were downregulated, suggesting a role for dKDM5/LID in gene activation. In contrast, another study performing microarray analysis from wing imaginal discs of *lid* mutants, however, identified a large number of genes (367 upregulated genes and 534 downregulated genes) to be regulated by dKDM5/LID (Liu et al., 2014). Gene ontology analysis of identified dKDM5/LID regulated genes showed significant enrichment of genes involved in stress response and oxidation reduction.

SIN3 and KDM5 in Cell Cycle Regulation

SIN3

As discussed above, one important category of genes regulated by SIN3 are the cell cycle regulators. In *Drosophila*, RNAi mediated knockdown of *Sin3A* leads to

reduced proliferating capacity in cultured cells, caused by a cell cycle arrest at the G2/M phase (Pile et al., 2002). *String* and *cyclin B*, genes that affect G2 to M transition of the cell cycle, are downregulated upon loss of SIN3. In mammals, mouse embryonic fibroblasts (MEFs) carrying a null mutation for *mSin3a* undergo cell cycle arrest (Cowley et al., 2005; Dannenberg et al., 2005). mSin3A is also essential for proliferation in embryonic stem (ES) cells, where it is important for expression of Myc and E2F target cell cycle regulators (McDonel et al., 2012). In contrast, mSin3B, while not essential for normal proliferation, affects differentiation by controlling cell cycle exit (David et al., 2008; van Oevelen et al., 2008). mSin3B helps recruit chromatin modifiers such as HDAC1 and KDM5A to E2F targets repressing these genes, thereby controlling cell cycle exit and differentiation.

KDM5

In mammals KDM5A has been linked to regulation of cell cycle progression. KDM5A interacts with pRB, an E2F binding partner that controls cells cycle progression (Benevolenskaya et al., 2005). Human SAOS-2 osteosarcoma cells depleted for *KDM5A* by siRNA stopped proliferating. Depletion of *KDM5A* in MEFs in the presence of MyoD promoted differentiation. ChIP experiments identified KDM5A targets that are differentiation dependent or independent, where cell cycle regulators were bound in a differentiation dependent manner (Lopez-Bigas et al., 2008). KDM5A cooperates with E2F to repress cell cycle regulators during differentiation (Beshiri et al., 2012). Increased cycling is observed in hematopoietic stem cells (HSC) and myeloid progenitors in KDM5A knockout mice (Klose et al., 2007). A possible link to cell

proliferation is observed in flies, where dKDM5/LID interacts with dMyc and is essential for dMyc dependent cell growth (Secombe et al., 2007).

SIN3 and KDM5 in Development

SIN3

SIN3 is an essential gene in multiple organisms. While SIN3 is not essential in the budding yeast *S. cerevisiae*, it is required for viability in the fission yeast *S. pombe* (Dang et al., 1999). *Sin3A* null mutants result in embryonic lethality in *Drosophila* (Neufeld et al., 1998b; Pennetta and Pauli, 1998). Similarly, in mice both mSin3A and mSin3B are essential during embryogenesis (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008).

In both flies and mammals, SIN3 plays important roles in multiple post-embryonic developmental processes. In *Drosophila*, SIN3 isoforms are differentially expressed along different developmental time points and in different tissues, while conditional knockdown of *Sin3A* at different developmental stages show a requirement for SIN3 at multiple stages (Barnes et al., 2014; Sharma et al., 2008). Early work identified a role for SIN3 in eye development. SIN3 was shown to enhance the rough eye phenotype resulting from mutation of *sina*, a gene involved in photoreceptor specification (Neufeld et al., 1998b). Further, genome wide RNAi screens identified SIN3 as a regulator of cardiac and neural development (Kim et al., 2004; Parrish et al., 2006; Sepp et al., 2008). Tissue specific knockdown of *Sin3A* in wing imaginal discs resulted in curved wings, possibly due to the regulation of the cell cycle regulator STG (Swaminathan and Pile, 2010). SIN3 is also implicated in multiple signaling pathways in flies. SIN3

associates with the steroid hormone corepressor SMRTER, implicating a role in development via ecdysone signaling (Pile and Wassarman, 2000; Tsai et al., 1999). RNAi screens have also implicated a role for SIN3 in Notch, ERK and JNK signaling pathways (Bond and Foley, 2009; Friedman and Perrimon, 2006; Mummery-Widmer et al., 2009).

In mammals, mSin3A was implicated in regulating development and homeostasis of lymphoid lineage cells, whereas conditional deletion of *mSin3a* affected T-cell development (Cowley et al., 2005). Further, deletion of *mSin3a* in mouse germ cells resulted in improper testicular development and infertility, highlighting a role in germ cell lineage development (Pellegrino et al., 2012). Deletion of *mSin3a* also regulates muscle development affecting proper formation of myotubes (van Oevelen et al., 2010). Deletion of *mSin3b* has been implicated in regulating development in multiple hematopoietic lineages (David et al., 2008).

KDM5

In *Drosophila*, similar to *Sin3A*, *lid* is an essential gene, where homozygous deletion results in lethality by early pupal development (Gildea et al., 2000). While dKDM5/LID is essential for organismal development, the demethylase activity of this enzyme seems dispensable for viability as *lid* mutants were restored to adulthood by expression of a catalytic mutant (Li et al., 2010). Mutants in the JmjN, C-terminal PHD and C_5HC_2 zinc finger domains of dKDM5/LID, however, could not restore viability in *lid* mutants, suggesting important roles for these domains in developmental functions. Mutants of *lid* were found to suppress the rough eye phenotype induced by

overexpression of dMyc, suggesting a role in eye development (Secombe et al., 2007). In this study, interaction of dKDM5/LID with dMyc was shown to be essential for dMyc regulated cell growth. dKDM5/LID has also been implicated in Notch gene silencing as part of a SIN3 associated complex and also associates with Su(H), a DNA binding repressor that targets Notch regulated genes (Liefke et al., 2010; Moshkin et al., 2009). The *C. elegans* homolog RBR-2 has been implicated in vulval development (Christensen et al., 2007; Nishibuchi et al., 2014).

In vertebrates, four genes KDM5A – D encode homologs of this demethylase. Deletion of a single gene does not affect organismal viability due to possible functional redundancies of the different KDM5 proteins (Benevolenskaya, 2007). Similar to *Drosophila*, both KDM5A and B affect Notch signaling. KDM5A interacts with the Su(H) homolog RBP-J to regulate Notch targets while KDM5B represses the Notch ligand *jagged*, which in turn represses Notch targets (Liefke et al., 2010; Roesch et al., 2010). KDM5A has also been implicated in the regulation of hematopoiesis (Klose et al., 2007). KDM5B interacts with the developmental transcription factors PAX9 and BF-1, also suggesting a role in developmental processes (Tan et al., 2003). KDM5C has been linked to neuronal development in both zebrafish and rat models (Iwase et al., 2007).

Histone Deacetylases and Demethylases in Cancer

High throughput sequencing of multiple tumor samples reveal that genes involved in epigenetic regulation are mutated at a high frequency (Hojfeldt et al., 2013; You and Jones, 2012). Further, many other epigenetic regulators show misregulation in multiple cancers.

HDACs are often aberrantly recruited to gene promoters via association with oncogenic DNA binding proteins resulting in repression of specific gene targets (Tang et al., 2013). Further, several HDACs, including HDAC1, 2, 3 and 6 are overexpressed in multiple cancers (Gupta et al., 2012; Jung et al., 2012; Kanno et al., 2012; Xie et al., 2012). RNA interference of individual HDACs mediated by siRNAs was shown to suppress tumor cell growth in several cell lines, highlighting the potential of HDAC inhibition in therapy (Hayashi et al., 2010; Kanno et al., 2012). HDAC inhibitors affect the dynamic turnover of acetylation, leading to hyperacetylation of targets, which can result in cytotoxicity in tumor cells (Leggatt and Gabrielli, 2012). HDAC inhibitors like Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA) and MS-275 have been shown to upregulate tumor suppressor genes (Banik et al., 2012; Glaser et al., 2003; Kurundkar et al., 2013; Pecuchet et al., 2010; Suzuki et al., 2011). They can further lead to cell cycle arrest and induction of apoptotic pathways (Al-Yacoub et al., 2012; Richon et al., 2000; Vrana et al., 1999). The HDAC inhibitor, SAHA (Vorinostat) has been approved for treatment of cutaneous T-cell lymphoma (CTCL) and Depsipeptide/FK228 (Romidepsin) for CTCL and peripheral T-cell lymphoma (PTCL) while others are in multiple stages of clinical trials (Hrabeta et al., 2014; Lane and Chabner, 2009; Smith and Workman, 2009). HDAC inhibitors are also being tested for combination therapy with other cancer therapeutic agents. A caveat of using HDAC inhibitors in treatment arises from the low specificity of these inhibitors and poor understanding of all cellular processes affected by these chemicals.

While the role of HDACs in cancer and the use of inhibitors for therapy has been a subject of interest for quite a while, the role of KDMs and targeting KDMs has gained

interest fairly recently. So far, only one gene, which encodes UTX/KDM6, has been shown to have recurrent inactivating mutations in multiple cancers, such as multiple myeloma, breast cancer, colorectal cancer, renal cell carcinoma, oesophageal squamous cell carcinoma and glioblastoma (Dalgliesh et al., 2010; van Haaften et al., 2009). Multiple KDMs, however, show increased expression in many tumors. For instance, KDM1 is overexpressed in multiple cancers and is necessary for the maintenance of MLL translocations in acute myeloid leukemia (AML) (Harris et al., 2012). Inhibition was seen to reactivate the all-trans retinoic acid (ATRA) differentiation pathway and also sensitize AMLs resistant to ATRA induced differentiation therapy (Schenk et al., 2012). Enzymes belonging to the KDM4 family of demethylases are also often overexpressed in cancer. In fact, genomic amplification of KDM4C is seen in breast cancer, medulloblastoma and squamous cell cancer (Ehrbrecht et al., 2006; Liu et al., 2009a; Yang et al., 2000). Depletion of KDM4B results in slower growth of multiple cancer cell lines (Fu et al., 2012; Kawazu et al., 2011; Shi et al., 2011; Yang et al., 2010). KDM5B was also found to be required for growth in breast cancer cells and melanoma (Roesch et al., 2010; Yamane et al., 2007). On the other hand KDM5A is silenced, downregulated or deleted in melanoma, while KDM5D is deleted in prostate cancer (Perinchery et al., 2000; Vogt et al., 1999). While development of inhibitors for KDMs are still in its infancy compared to HDAC inhibitors, several inhibitors of both KDM1 and JmjC family of demethylases have been and are being developed (Thinnes et al., 2014). A potential advantage of KDM inhibitors over HDAC inhibitors is the more specific catalytic activity of KDMs, which target highly specific histone modifications.

Apart from cancer, these enzymes are also implicated in other conditions such as

neurological and immune disorders, making these enzymes valuable targets for therapy (Black and Whetstine, 2013; Falkenberg and Johnstone, 2014). There is great potential for the use of both HDAC and KDM inhibitors in combinatorial therapy considering that these enzymes interact both physically and genetically. Both HDACs and KDMs, however, are often essential genes that are ubiquitously expressed and thus inhibiting these enzymes could result in side effects due to misregulation of essential processes. Therefore, further understanding of the biological roles of these enzymes is crucial for their efficient utilization in therapeutics.

Project Outline

Work in our laboratory and by others showed that *Drosophila* SIN3 associates with the histone demethylase dKDM5/LID (Moshkin et al., 2009; Spain et al., 2010). This finding is of interest as it adds a second catalytic component to the SIN3 complex, thus far considered primarily a histone deacetylase complex due to its association with RPD3. As discussed in the previous sections, roles for dKDM5/LID in histone demethylation, viability of flies and activation or silencing of specific genes is known. The functional roles of dKDM5/LID in specific cellular and developmental processes, however, have not been defined.

We validated the interaction of SIN3 and dKDM5/LID and went on to identify possible roles for dKDM5/LID in processes affected by SIN3. The biochemical validation of the interaction of SIN3 and dKDM5/LID and the characterization of the role of dKDM5/LID in SIN3 regulated processes in flies is described in Chapter 2. Our findings indicate a possibly important role for dKDM5/LID in the SIN3 complex.

The SIN3 complex is considered a global repressor of transcription. SIN3 is known to regulate about 3% of the *Drosophila* genome. Therefore, we sought to determine the contribution of dKDM5/LID, which associates with SIN3, to transcription regulation. Work addressing the role of SIN3 and dKDM5/LID in gene regulation is described in Chapter 3.

The SIN3 complex associates with a histone deacetylation module, RPD3, and a histone demethylation module, dKDM5/LID. Therefore, to understand the role of these histone modifying activities in SIN3 dependent regulation of transcription we studied the changes in histone modification patterns upon loss of *Sin3A* or *lid* at regulated genes. The work analyzing transcription factor binding and histone modification changes at target genes are described in Chapter 4.

This study has enhanced our understanding of the role of dKDM5/LID in multiple biological processes including gene transcription, cell proliferation and wing development. The molecular mechanism by which dKDM5/LID affects these multiple processes, however, is yet to be understood. Some of the questions that arise from this work and potential experiments to further dissect the functional roles of dKDM5/LID in the cell are discussed in Chapter 5.

CHAPTER 2

A ROLE FOR THE HISTONE DEMETHYLASE dKDM5/LID IN SIN3 DEPENDENT REGULATION OF CELL PROLIFERATION AND WING DEVELOPMENT

INTRODUCTION

Chromatin, composed of DNA wrapped around histone proteins, acts as the template for gene transcription in eukaryotes. The activity of nucleosome remodeling factors and histone modifying enzymes, as well as the incorporation of histone variants, regulates dynamics of chromatin packaging (Felsenfeld and Groudine, 2003). Dense packaging of chromatin is associated with transcription repression, while a more loose conformation is associated with activation. Histone modifying enzymes regulate transcription by modifying the N-terminal tails of histones, enabling or preventing the association of several distinct transcriptional activators and repressors (Bannister and Kouzarides, 2011). The histone code hypothesis states that existing histone modifications can affect subsequent modifications and collectively these modifications recruit chromatin binding proteins, influencing transcription regulation (Strahl and Allis, 2000). Histone modifying enzymes that coordinately affect transcription can associate at the chromatin template or interact with each other as part of regulatory complexes. Analysis of many immunopurified chromatin regulatory complexes indicates the presence of multiple histone modifying enzymes within the same complex. Two such enzymes found to occur together in chromatin regulatory complexes are HDACs and KDMs, reviewed by Hayakawa and Nakayama (Hayakawa and Nakayama, 2011). The SIN3, NuRD, CoREST and NCoR/SMRT complexes have all been shown to include both class I HDACs and a KDM.

The activity of HDACs typically leads to a transcriptionally repressive chromatin environment while the opposing activity of KATs results in an environment favorable for transcription (Bannister and Kouzarides, 2011). SIN3 acts as a scaffold protein for multiple HDAC complexes present in organisms from yeast to mammals and is thus generally associated with transcription repression (Silverstein and Ekwall, 2005). The distinct SIN3 complexes share much similarity in composition of proteins and biological functions. The HDAC RPD3, in yeast and *Drosophila*, and HDAC1 and 2, in mammals, render catalytic activity to the complex. Investigations using different model organisms has identified the interaction of SIN3 with a KDM, dKDM5/LID, in *Drosophila,* and the homolog KDM5A in mammals (Hayakawa et al., 2007; Moshkin et al., 2009; Spain et al., 2010; van Oevelen et al., 2008). This finding adds a second catalytic component to the SIN3 complex, which to date had been regarded as an HDAC complex. In *Drosophila*, a single gene encodes multiple isoforms of SIN3. Work in our laboratory has shown that dKDM5/LID predominantly associates with the largest SIN3 isoform, SIN3 220 (Spain et al., 2010).

Sin3A is an essential gene in both *Drosophila* and mammals (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008; Neufeld et al., 1998b; Pennetta and Pauli, 1998). SIN3 was initially identified in yeast as a global regulator of transcription (Nasmyth et al., 1987; Sternberg et al., 1987). SIN3 plays an important role in cell cycle progression. In *Drosophila*, knockdown of *Sin3A* by RNAi in cultured cells leads to a G2/M phase cell cycle arrest (Pile et al., 2002). In mouse embryonic fibroblasts (MEFs), deficiency of mSin3A leads to a reduction in proliferative capacity and an increase of cells in the G2/M phase of the cell cycle (Cowley et al., 2005; Dannenberg et al., 2005).

mSin3b deficient MEFs, however, continue to proliferate, but fail to exit the cell cycle (David et al., 2008). Further, SIN3 is known to be important for developmental processes. In *Drosophila*, SIN3 isoforms show differential expression in multiple tissues and life stages (Sharma et al., 2008). Conditional knockdown of *Sin3A* induced at different developmental time points indicates a requirement for SIN3 during multiple stages of development. SIN3 is also linked to key developmental and signaling pathways. SIN3 is associated with steroid hormone, Notch, ERK and JNK signaling pathways (Bond and Foley, 2009; Friedman and Perrimon, 2006; Mummery-Widmer et al., 2009; Pile and Wassarman, 2000; Tsai et al., 1999). SIN3 is further implicated in eye, wing, neural and cardiac development (Kim et al., 2004; Neufeld et al., 1998b; Parrish et al., 2006; Sepp et al., 2008; Swaminathan and Pile, 2010).

Similar to *Sin3A*, *lid* is an essential gene in *Drosophila,* first identified in a screen for *trithorax* group genes (Gildea et al., 2000). dKDM5/LID is a JmjC domain containing KDM, which specifically removes H3K4me3, a mark associated with active transcription (Eissenberg et al., 2007; Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007). In mammals, four paralogous genes encode *lid* homologs, KDM5A through KDM5D. KDM5A, KDM5B and KDM5C interact with SIN3 or HDAC complexes (Barrett et al., 2007; Hayakawa et al., 2007; Klose et al., 2007; Tahiliani et al., 2007; van Oevelen et al., 2008). KDM5A and KDM5B are known to regulate cell cycle progression (Benevolenskaya et al., 2005; Beshiri et al., 2012; Klose et al., 2007; Lopez-Bigas et al., 2008; Scibetta et al., 2007; Yamane et al., 2007). A likely involvement in cell proliferation is also noted in flies. dKDM5/LID is essential for dMyc dependent cell growth (Secombe et al., 2007). Additionally, dKDM5/LID is necessary for fly

development (Gildea et al., 2000; Li et al., 2010).

Taken together, research in *Drosophila* and mouse suggests that SIN3 and KDM5 have overlapping as well as distinct biological functions. In this work, we wish to further explore the potential intersection of functional activities of these two transcriptional regulators. The role of SIN3 in important biological processes such as cell cycle progression and development has been demonstrated. How SIN3 brings about these functions and which proteins of the SIN3 complex affect these specific functions, however, are not well understood. Here, we have focused on the role of dKDM5/LID in the context of the *Drosophila* SIN3 complex. We show that dKDM5/LID acts with similarity to SIN3 in affecting cell cycle progression and wing development, suggesting functional interaction of these proteins.

MATERIALS AND METHODS

Cell Culture

Drosophila Schneider cell line 2 (S2) cells were cultured at 27^0C in Schneider's *Drosophila* medium (1x) + L-glutamine (Life Technologies) with 10% heat-inactivated fetal bovine serum (Invitrogen) and 50 mg/ml gentamycin. 0.1 mg/ml penicillin/streptomycin and 0.1 mg/ml Geneticin for selection were added to cells carrying a stably integrated transgene with HA tagged SIN3 187 or SIN3 220. Construction of the HA tagged SIN3 187 and SIN3 220 expression cell lines has been previously described (Spain et al., 2010). The FLAG-HA tagged dKDM5/LID expressing cell line was generated by transfecting a pMK33 vector carrying FLAG-HA tagged *lid* cDNA using the Effectene transfection kit (Qiagen). 300 µg/ml Hygromycin B was added

to select for transfected cells carrying stable chromosomal insertions of the transgene. The FLAG-HA tagged *lid* construct inserted into a pMK33 vector (FMO08240) was obtained from the Berkeley *Drosophila* Genome Project, ORFeome collection (Yu et al., 2011). Expression of tagged proteins was induced by the addition of 1 µl/ml of 0.7 M $CuSO_4$ to relevant cultured cells.

Nuclear Extract Preparation and Co-immunoprecipitation

Nuclear extracts were prepared from both S2 control and dKDM5/LID FLAG-HA stably transformed cells and subjected to immunoprecipitation as previously described (Spain et al., 2010). In brief, approximately 900 µl of nuclear extract was incubated with 75 µl of anti-HA beads (monoclonal anti-HA agarose conjugate clone HA-7 (A2095, Sigma)). 150 µl of interaction buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 10% glycerol) was added to the extract and incubated with the antibody beads overnight at 4^0C. The beads were washed with radioimmune precipitation buffer (RIPA (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfide, 0.1% sodium deoxycholate)), Wash 2 buffer (20 mM HEPES (pH 7.4), 500 mM NaCl, 0.5 mM EDTA, 1.5% Triton X-100, 0.1% sodium deoxycholate, 10% glycerol) and Wash 3 buffer (20 mM HEPES (pH 7.4), 300 mM NaCl, 1mM $MgCl_2$, 0.1 mM EDTA, 10% Glycerol, 1.5% Triton X-100) for 10 min each. Bound proteins were eluted by incubation with 25 µl of Laemmli buffer (Bio-Rad) for 15 min at room temperature.

RNA interference

RNAi was performed based on modification of a published protocol (Clemens et al., 2000). In brief, 4 x 10^6 cells were plated in 4 ml of Schneider's *Drosophila* medium in a 60-mm-diameter dish. After 3 hr, FBS-containing medium was removed and replaced with 2 ml of serum-free medium. 50 µg of dsRNA was added per dish and mixed by swirling. After 30 min, 4 ml of Schneider's *Drosophila* medium was added. Cells were assayed four days following addition of dsRNA. RNAi was performed using dsRNA corresponding to *Sin3A* or *lid* mRNA. Construction of the *Sin3A* RNAi targeting sequence in pCRII-Topo vector and production of dsRNA is previously described (Pile et al., 2002). The sequences in the pCRII-Topo vector for *lid* knockdown were generated using the following primer set 5’ to 3’ (forward primer) CGA CAT GGC CGA AAT GGT and (reverse primer) GAT ACC CAG TTG CTG TAT GAC. dsRNA against GFP was used as a control. PCR templates for targeting the GFP gene were generated from template DNA (kindly provided by Dr. Russell L. Finley, Jr.) using the following T7 promoter sequence containing primer set 5' to 3’ (forward primer) GAA TTA ATA CGA CTC ACT ATA GGG AGA TGC CAT CTT CCT TGA AGT CA and (reverse primer) GAA TTA ATA CGA CTC ACT ATA GGG AGA TGA TGT TAA CGG CCA CAA GTT. Efficient knockdown of the target was routinely verified either at the protein level by western blotting or at the transcript level by real-time quantitative reverse-transcription PCR (qRT-PCR).

Western Blotting

Western blot analysis was performed in accordance with standard protocols

(Sambrook and Russell, 2001). For whole cell protein extract preparation, 1.5 x 10^6 cells were pelleted by centrifugation at 1250xg and lysed in 100 µl of Laemmli sample buffer (Bio-Rad). Protein concentrations were determined using the Bio-Rad DC protein assay reagent in accordance with the manufacturer's protocol. 15 – 20 µg of whole cell protein extracts, and 10 µl of nuclear extracts or entire eluate from immunoprecipitated samples were separated on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Scientific). Membranes were probed with various rabbit primary antibodies followed by incubation in donkey anti-rabbit HRP-conjugated IgG (1:3000; GE Healthcare, NA9340) secondary antibody where applicable. The antibody signals were detected using the ECL$^+$ or ECL prime western blot detection system (GE Healthcare). Primary antibodies used: HA-HRP (1:6000; Roche, 2013189), SIN3 (1:2000; (Pile and Wassarman, 2000)), RPD3 (1:3000; (Pile and Wassarman, 2000)), dKDM5/LID (1:5000; kindly provided by Dr. Julie Secombe (Secombe et al., 2007)), alpha-tubulin (1:1000; Cell Signaling, 2144).

Cell Proliferation Assay

Mock (GFP dsRNA) treated or RNAi treated cells were stained with Trypan Blue and cells were counted four days after RNAi treatment. Cell density of each sample was calculated as per hemocytometer standards.

Drosophila Stocks

Drosophila melanogaster stocks were maintained, and crosses were performed, according to standard laboratory procedures. The following stocks were used: UAS-

SIN3^{RNAi-I} (Sharma et al., 2008), SIN3 KD I (Swaminathan and Pile, 2010) and SIN3 KD II (Swaminathan et al., 2012); Act-GAL4 (4414), Ser-GAL4 (6791), Bx-GAL4 (8696), UAS-LID$^{RNAi-TRiP}$ (28944) and UAS-mCherry$^{RNAi-TRiP}$ (35785), obtained from the Bloomington Stock Center; UAS-LID$^{RNAi-KK}$ (103830) obtained from Vienna *Drosophila* RNAi Center; UAS-LID and UAS-LID-JmjC* (Secombe et al., 2007) kindly provided by Dr. Julie Secombe; hsFLP;Act5C>CD2>GAL4,UAS-EGFP kindly provided by Dr. Dirk Bohmann.

GFP clonal analysis

hsFLP;Act5C > CD2 > GAL4,UAS-EGFP flies were crossed to mCherry$^{RNAi-TRiP}$, UAS-SIN3^{RNAi-I}, UAS-LID$^{RNAi-TRiP}$ or UAS-LID$^{RNAi-KK}$ to generate random GFP positive clones. 0–4 hr embryos were collected and heat shocked 48–52 hr after egg laying. Wing discs from wandering third instar larvae were dissected and immunostained with anti-GFP as described below.

Immunostaining

Wing discs from wandering third instar larvae were dissected in 1 X PBS. Roughly 50 wing discs were fixed in 4% formaldehyde in 1 X PBS and stained as previously described (Sharma et al., 2008). Antibody against GFP (1:1000; Abcam, ab1218) followed by sheep anti-mouse Alexa 488 (1:2000; Life Technologies, A11001) was used for staining. Visualization and imaging was done using a Zeiss Axioscope 2 fitted with an Axio-phot photography system.

Imaging flies

Whole flies were imaged at 30x magnification using an Olympus DP72 camera coupled to an Olympus SZX16 microscope. Wings were imaged at 80x magnification using a SPOT RT color camera coupled to a Leica MZ125 microscope.

Statistical analyses

All significance values were calculated by the unpaired two sample Student's *t*-test using GraphPad. http://www.graphpad.com/quickcalcs/index.cfm.

RESULTS

SIN3 and RPD3 co-purify with dKDM5/LID

We previously identified components of *Drosophila* SIN3 187 or 220 isoform specific complexes by LC/MS/MS analysis and determined that dKDM5/LID co-purifies predominantly with the SIN3 220 complex (Spain et al., 2010). To build on that study, we sought to analyze the interaction of dKDM5/LID with SIN3 220 by western blot assay using dKDM5/LID specific antibody. Nuclear extracts were prepared from S2 cells and cells expressing HA tagged SIN3 187 or 220. Expression of the tagged SIN3 proteins was verified by western blot analysis of whole cell extracts prepared from above cells probed with antibody to the HA tag (Fig. 2.1). Nuclear extracts were subjected to immunopurification of SIN3 using anti-HA beads. Western blot with antibody to dKDM5/LID or antibody to SIN3 showed the association of dKDM5/LID predominantly with SIN3 220 (Fig. 2.2A). To validate the interaction of dKDM5/LID with SIN3, we performed the reciprocal experiment, where we immunopurified dKDM5/LID. We

generated a *Drosophila* S2 cell line carrying a transgene for expression of FLAG-HA tagged dKDM5/LID. The presence of the metallothionine promoter enables the induction of dKDM5/LID FLAG-HA by addition of $CuSO_4$. To verify the expression of dKDM5/LID, we prepared whole cell protein extracts from S2 cells and dKDM5/LID FLAG-HA expressing cells and probed with antibody against the HA tag (Fig. 2.1). To analyze putative dKDM5/LID interacting factors, we immunopurified proteins from nuclear extracts prepared from control S2 cells and dKDM5/LID FLAG-HA cells using anti-HA beads. A western blot of immunoprecipitated dKDM5/LID showed interaction of dKDM5/LID FLAG-HA with SIN3 and RPD3, two components of the SIN3 complex (Fig. 2.2B).

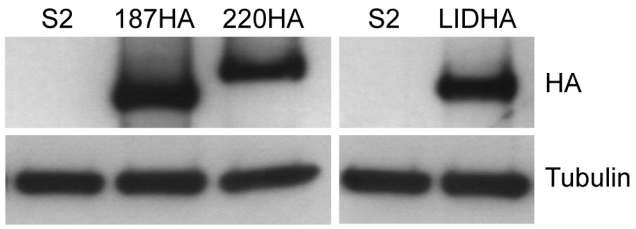

Figure 2.1: S2 cells express SIN3 187HA, SIN3 220HA or LID FLAG-HA. Western blot analysis of whole cell protein extracts prepared from indicated cells and probed with antibody to the HA tag and tubulin as a loading control.

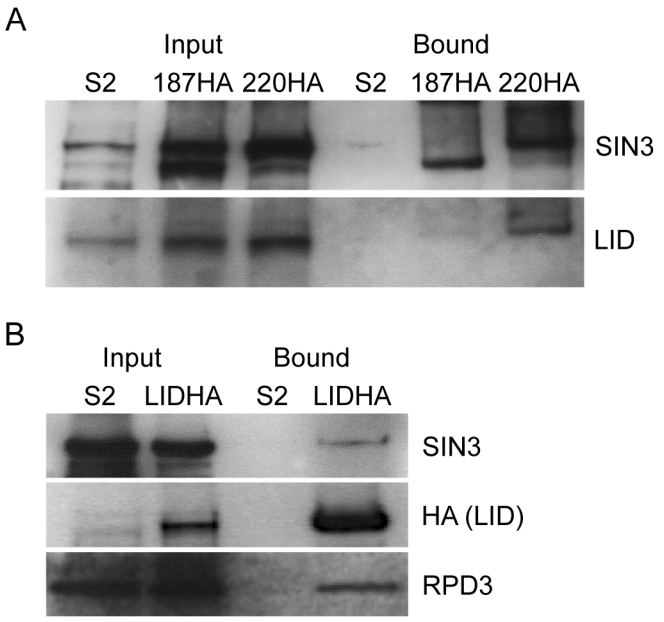

Figure 2.2: LID interacts with SIN3 complex components. Western blot analysis of input and bound fractions of nuclear extracts from S2, SIN3 187HA and SIN3 220HA (A) or S2 and LID FLAG-HA (B) cells. Antibody to the HA tag was used for immunoprecipitation. Blots were probed with the antibody listed to the right.

dKDM5/LID affects cell cycle progression

Having verified the interaction of dKDM5/LID with SIN3 and the catalytic component of the complex, the HDAC RPD3, we sought to determine the contribution of dKDM5/LID to SIN3 complex functions. Previous work in yeast, flies and mammals has shown SIN3 to play an important role in regulating progression through the cell cycle (Cowley et al., 2005; David et al., 2008; Pile et al., 2002; Stephan and Koch, 2009). In *Drosophila,* in addition to SIN3, other components of the SIN3 complex, such as RPD3 and p55 have also been shown to affect cell growth rates of cultured cells (Pile et al., 2002). We next investigated if dKDM5/LID also contributed to regulation of the cell cycle.

First, we checked for defects in cell proliferation by measuring cell density of

Drosophila S2 cells upon induction of RNAi of *Sin3A*, *lid* or both. S2 cells treated with dsRNA targeting GFP was used as a control. Verification of efficient knockdown of SIN3 by western blot analysis and dKDM5/LID by real time qRT-PCR or western blot analysis is routinely performed in the lab (Fig. 2.3A,B and data not shown). Antibody to α-Tubulin was used as a loading control for western blots. Gene expression studies described in Chapter 3 determined that knockdown of *Sin3A* or *lid* can alter expression of one of the α-Tubulin encoding genes (α-Tubulin at 84B) resulting in roughly 1.5 fold increase in gene expression. However, the level of decrease observed in the SIN3 or LID protein levels upon RNAi knockdown compared to the small changes in *Tubulin* gene expression and the fact that India ink staining of western blots showed comparable loading of samples indicates sufficient knockdown of these proteins.

Determination of cell density revealed that *lid* knockdown cells had decreased cell density, about 15% lower, compared to control cells treated with GFP dsRNA (Fig. 2.4A). *lid* knockdown, however, resulted in a less severe cell proliferation defect compared to *Sin3A* knockdown cells, which exhibited about a 35% decrease in cell density. Further, double knockdown of *lid* and *Sin3A* did not result in an additive effect on cell proliferation. The double knockdown cells showed densities comparable to single knockdown of *Sin3A*. These results suggest that multiple components of the SIN3 complex could contribute to the cell proliferation defect seen upon loss of SIN3. Loss of the scaffold protein, SIN3, may result in the disruption of function of these additional complex components, including dKDM5/LID, thereby resulting in a more pronounced proliferation defect relative to the other components. Apart from defective cell cycle progression, programmed cell death may also contribute to a decrease in cell density.

While Trypan blue staining of cells knocked down for *Sin3A* or *lid* did not display an increase in dead cells observed compared to control cells, it is not possible to conclusively exclude cell death as a contributor to the observed decreases in cell density.

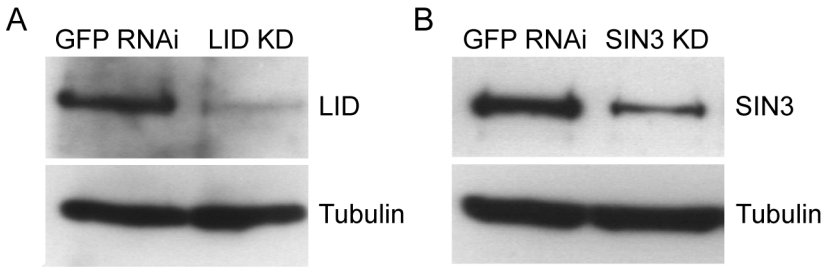

Figure 2.3: RNAi in S2 cells leads to efficient knockdown of SIN3 and LID proteins. Western blot analysis of whole cell protein extracts from control cells and cells treated with dsRNA targeting LID (A) or SIN3 (B) and probed with antibody to LID or SIN3 and tubulin as a loading control. KD – knockdown.

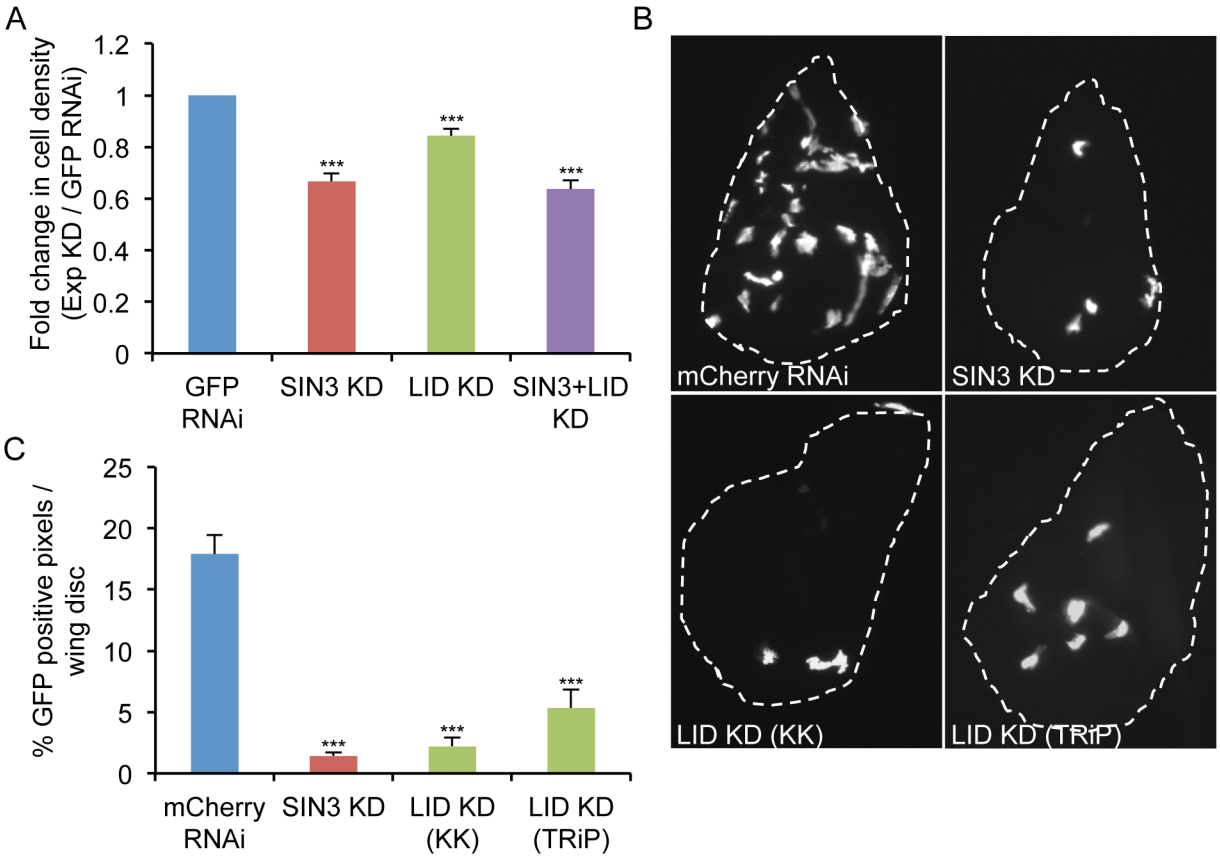

Figure 2.4: Knock down of *lid* leads to decreased cell proliferation in S2 cells and wing imaginal discs, similar to a reduction of *Sin3A*. (A) Quantification of cell density by cell counts of S2 cells treated with dsRNA targeting indicated proteins. Results are the average of five biological replicates. (B) Control and *Sin3A* or *lid* knockdown wing disc clones generated using the Flip-out GAL4 system and immunostained with antibody to GFP. (C) Quantification of GFP signal in wing imaginal discs. Results are the average pixel counts from 20 wing imaginal discs. KD – knockdown, KK – RNAi line from Vienna *Drosophila* RNAi Center, TRiP – RNAi line from Bloomington Stock Center, *** – $P < 0.001$.

To analyze the role of dKDM5/LID in cell proliferation in the context of fly development we looked at clonal cell growth in *Drosophila* wing imaginal discs. For this purpose, we utilized the heat shock Flip-out system to randomly generate EGFP-marked clones with or without *lid* knockdown. We previously showed that reduction of SIN3 results in small clones that are few in number (Swaminathan and Pile, 2010). Similar to our previous observation with SIN3 knockdown, we find that reduction of

dKDM5/LID also resulted in small EGFP positive clones that were few in number relative to the control (mCherry RNAi) (Fig. 2.4B). We utilized two separate RNAi lines to drive dKDM5/LID knockdown to eliminate the possibility that observed phenotypes are the result of off-target effects of RNAi. Quantification of the GFP positive pixels per disc shows a 3 to 7 fold reduction in the number of pixels compared to the control upon dKDM5/LID knockdown (Fig. 2.4C). Similar results were observed with both *lid* RNAi lines, strongly suggesting that the reduction in clonal cell growth is due to *lid* knockdown and not an off target effect. Data from both cell culture and developing flies demonstrate that dKDM5/LID plays an important role in regulating cell proliferation.

dKDM5/LID functions in wing development

Next we tested the role of dKDM5/LID in the regulation of developmental processes. *Sin3A* and *lid* are both essential genes in *Drosophila* and are implicated in the regulation of developmental processes (Gildea et al., 2000; Kim et al., 2004; Li et al., 2010; Neufeld et al., 1998b; Parrish et al., 2006; Pennetta and Pauli, 1998; Sepp et al., 2008; Sharma et al., 2008; Swaminathan and Pile, 2010). In *Drosophila,* the GAL4-UAS system can be used to induce RNAi of target genes. Crossing an Act-GAL4 driver line to a UAS-RNAi line results in progeny with ubiquitous knockdown of the gene of interest. Ubiquitous knockdown of *Sin3A* by RNAi results in lethality (Swaminathan and Pile, 2010). *lid* knockdown using a ubiquitous Act-GAL4 driver was shown to result in semi lethality (Eissenberg et al., 2007). In our hands, upon ubiquitous knockdown of *lid* using an Act-GAL4 driver, we observed varying degrees of lethality depending on the UAS-RNAi line and the temperature at which the flies are reared (Table 2.1). When

reared at 25^0C, crosses using the UAS-LID$^{RNAi\text{-}KK}$ line resulted in 50-70% lethality of the progeny. At 27^0C, however, very few flies survive compared to those reared at 25^0C. Use of the UAS-LID$^{RNAi\text{-}TRiP}$ line resulted in very few survivors even at 25^0C and complete lethality at 27^0C. The varying degrees of lethality is possibly due to the differences in RNAi efficiency of the dsRNA constructs utilized and the temperature dependence of GAL4 activity (Duffy, 2002).

		♂			♀		
		Adult Progeny Observed			**Adult Progeny Observed**		
Temp (^{0}C)	**UAS line**	**(UAS-X/ Act-GAL4)**	**(UAS-X/ CyO)**	**% Viable (UAS-X/ Act-GAL4)**	**(UAS-X/ Act-GAL4)**	**(UAS-X/ CyO)**	**% Viable (UAS-X/ Act-GAL4)**
25	UAS-LID$^{RNAi\text{-}KK}$ (2)	41	180	17.3 ± 13.3	90	165	49.6 ± 9.5
	UAS-LID$^{RNAi\text{-}TRiP}$ (1)	0	48	0	1	53	1.9
27	UAS-LID$^{RNAi\text{-}KK}$ (2)	0	54	0	5	63	13.5 ± 9.6
	UAS-LID$^{RNAi\text{-}TRiP}$ (3)	0	168	0	0	237	0
	UAS-LID (4)	0	228	0	0	194	0
	UAS-LID-JmjC* (1)	0	63	0	0	52	0

(n) Number of trials
X – Genotype, KD – knockdown, KK – RNAi line from Vienna *Drosophila* RNAi Center, TRiP – RNAi line from Bloomington Stock Center

Table 2.1: Ubiquitous knockdown or overexpression of dKDM5/LID results in a loss of viability. UAS-RNAi or UAS overexpression fly lines for *lid* were crossed with the Act-GAL4/CyO driver line and the progeny were analyzed and counted.

Such lethality observed upon knockdown of either SIN3 or dKDM5/LID proteins render it necessary to utilize conditional knockdown systems to study the functional roles of these proteins during development. We previously utilized the GAL4-UAS

system to conditionally knockdown *Sin3A* in a tissue specific manner (Swaminathan and Pile, 2010). Knockdown of *Sin3A* in wing precursor cells results in a curved rather than straight adult wing. Our data indicate that SIN3 and dKDM5/LID both play a role in cell proliferation. Since many cell cycle regulators are known to affect wing development (Herranz and Milan, 2008) and given the role of SIN3 in wing development, we tested if dKDM5/LID too can affect wing development. The Ser-GAL4 driver line can be utilized to conditionally knockdown a gene of interest in wing imaginal disc cells. We utilized both of the above mentioned UAS-RNAi lines to reduce expression of dKDM5/LID in wing imaginal disc cells using the Ser-GAL4 driver line. Knockdown of *lid* in wing discs using either RNAi line resulted in a curved wing phenotype similar to the phenotype observed upon *Sin3A* knockdown (Fig. 2.5A). Double knockdown of *Sin3A* and *lid* in wing discs resulted in a more severe curved wing phenotype than knockdown of either gene alone (Fig. 2.5A).

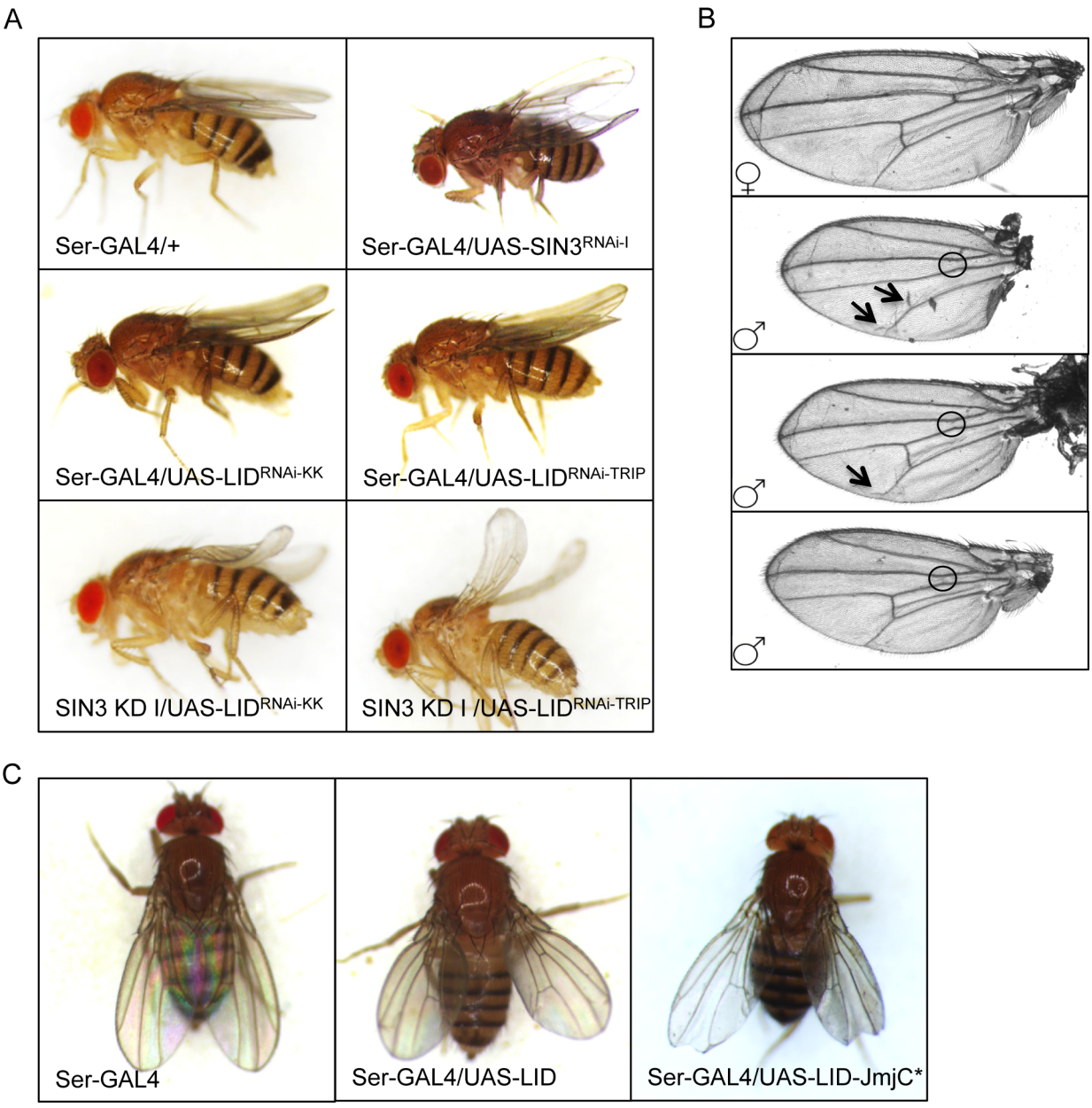

Figure 2.5: Reduction or overexpression of LID results in defects in wing morphology. (A) Micrographs of flies carrying the Ser-GAL4 driver and/or the indicated UAS-RNAi constructs. SIN3 KD refers to the presence of both the Ser-GAL4 driver and UAS-SIN3^{RNAi} construct. (B) Micrographs of wings from flies carrying the Bx-GAL4 driver and the UAS-LID$^{RNAi-KK}$ construct. Top left panel represents female wings showing wild type wing venation. Male wings show varying degrees of vein disruptions, indicated by circles and arrows. (C) Micrographs of flies carrying the Ser-GAL4 driver and the indicated UAS-overexpression constructs. KK – RNAi line from Vienna *Drosophila* RNAi Center, TRiP – RNAi line from Bloomington Stock Center.

Use of a second wing imaginal disc specific driver line, Bx-GAL4, also resulted in a curved wing phenotype upon knock down of *lid* (data not shown). Interestingly, in addition to the curved wing phenotype, additional vein defects were observed upon wing specific knock down of *lid* using the Bx-GAL4 driver (Fig. 2.5B). The differences in expression patterns of *Serrate* (*Ser*) and *Beadex* (*Bx*) in wing imaginal discs may contribute to the additional vein defects observed when *lid* was knocked down using the Bx-GAL4 driver. Wing vein disruptions have previously been observed in a *lid* mutant background upon additional mutation of *Su(H),* a regulator of Notch signaling (Liefke et al., 2010). Of note, the vein defects were only observed in male *lid* knockdown flies. One possible explanation for this observation is the fact that *Bx* is located on the X chromosome and thus can be subjected to regulation by the dosage compensation complex. Possible increased expression of the sequence targeting *lid,* due to dosage compensation, could thereby result in a more severe RNAi effect. Alternately it is possible that male specific developmental requirements for dKDM5/LID exist that can affect wing morphology. Published work suggests that male flies are more sensitive to mutations in *lid* (Li et al., 2010). These researchers found that male flies carrying a demethylase inactive *lid* gene were short lived and displayed increased sensitivity to paraquat. Similarly, RNAi mediated knockdown of a chromatin regulatory protein MRG15, which associates with dKDM5/LID, also resulted in shortened life span more prominently in male flies (Zhang et al., 2010).

Apart from testing the effect of reduced dKDM5/LID levels, we further analyzed the role of dKDM5/LID in development through overexpression. Similar to reduction of dKDM5/LID, ubiquitous overexpression of dKDM5/LID using the Act-GAL4 driver line

resulted in complete lethality when flies were reared at 27^0C (Table 2.1). This observation suggests that total levels of dKDM5/LID must be maintained; too much or too little is detrimental to fly development. Next, we overexpressed dKDM5/LID only in wing imaginal discs using the Ser-GAL4 driver. The resulting progeny had a held-out wing phenotype, sometimes with scalloped wing margins (Fig. 2.5C). Overexpression of a LID catalytic mutant resulted in more severe held-out wings with very distinctly scalloped wing margins. The held out wing and scalloped wing margin phenotypes were more pronounced in male flies.

Taken together, the phenotypes observed upon *lid* knockdown and overexpression show that LID is an essential factor for normal wing morphology. While the importance of dKDM5/LID in fly development has been observed previously, this is the first instance that a specific role for dKDM5/LID in wing development has been demonstrated.

Overexpression of dKDM5/LID partially rescues the *Sin3A* knockdown wing phenotype

Specific reduction of either SIN3 or dKDM5/LID in the wing imaginal disc resulted in a similar curved wing phenotype (Fig. 2.5A). We next wanted to test if overexpression of dKDM5/LID could rescue the wing defect caused by reduction of SIN3. Two fly lines previously generated in our laboratory, SIN3 KD I and SIN3 KD II, which have constitutive knock down of *Sin3A* in wing imaginal discs were utilized for this analysis (Swaminathan et al., 2012; Swaminathan and Pile, 2010).

SIN3 KD I and SIN3 KD II transgenic flies with constitutive knockdown of *Sin3A*

in wing imaginal discs display a curved wing phenotype with 100% penetrance. We crossed the SIN3 KD I or SIN3 KD II flies to flies carrying a UAS construct for overexpression of dKDM5/LID to determine if dKDM5/LID can rescue the *Sin3A* knockdown wing defect. To determine rescue we scored for straight wings, that is, decrease in the penetrance of the curved wing phenotype. Here we have only considered the decrease of the penetrance of the phenotype and not suppression of the phenotype itself. That is, even if the wings are somewhat less curved they were scored as curved and not straight. On average 95% of the female progeny resulting from the cross have straight wings, indicating rescue of the curved wing phenotype (Table 2.2). Such rescue of the curved wing, however, was not observed in male flies. This finding further indicates the possibility that male specific developmental requirements for SIN3 and or dKDM5/LID exist that can affect wing morphology. Male flies may require higher levels of SIN3 during wing development compared to females. The amount of dKDM5/LID overexpression obtained here may not be sufficient to compensate for such increased requirement for SIN3 in males. The rescue of the wing phenotype in females suggests that increased expression of dKDM5/LID can compensate for the reduction of SIN3 function, possibly due to overlapping roles of these proteins in wing development. Further, unlike knockouts, RNAi does not often result in complete loss of the proteins. Therefore, the SIN3 protein could still be expressed in low levels. It is possible that overexpression of dKDM5/LID results in the sequestering and efficient utilization of the small amounts of SIN3 available, thereby resulting in wild type wings. Introduction of an overexpressed catalytic mutant dKDM5/LID to the SIN3 KD I flies results in less than 5% decrease in the penetrance of the curved wing in female flies and no rescue in the

males. This lack of effect on female SIN3 KD flies suggests an important role for the demethylase activity of dKDM5/LID in the genetic interaction.

	♂		♀	
Genotype	**% Curly**	**Flies Scored**	**% Curly**	**Flies Scored**
SIN3 KD I	100		100	
SIN3 KD II	100		100	
SIN3 KD I / EGFP [(2)]	100	50	100	92
SIN3 KD I / LID [(3)]	100	134	4.5 ± 7.7	303
SIN3 KD II / LID [(2)]	100	122	3.8 ± 5.4	132
SIN3 KD I / LID-JmjC* [(2)]	100	100	97.5 ± 2.2	175

[(n)] Number of trials
KD – knockdown

TABLE 2.2: Overexpression of dKDM5/LID rescues the *Sin3A* knockdown curved wing phenotype in female flies. Flies constitutively knocked down for *Sin3A* (SIN3 KD) in wing imaginal discs were crossed to UAS overexpression fly lines for EGFP (control), *lid or* mutant *lid* and the progeny were analyzed and counted for curved wings.

DISCUSSION

In this study, we have addressed the question as to whether dKDM5/LID affects similar processes as SIN3. We verified that dKDM5/LID interacts with SIN3 and the complex component, RPD3. We find that both SIN3 and dKDM5/LID share similar roles in cell proliferation and wing development as flies with reduced dKDM5/LID phenocopied those with a reduction of SIN3. Our findings imply that dKDM5/LID is a key component of the SIN3 complex that contributes significantly to SIN3 complex function in cellular and developmental processes.

dKDM5/LID interacts with the SIN3 core complex

Consistent with previous work from our laboratory and others, immunopurification of FLAG-HA tagged dKDM5/LID revealed an interaction of dKDM5/LID with SIN3 and the HDAC component of the complex, RPD3. Two other dKDM5/LID purifications demonstrated that dKDM5/LID interacts with components of the SIN3 complex (Lee et al., 2007; Moshkin et al., 2009). Work by Lee et al., demonstrated an association of dKDM5/LID with RPD3 and Pf1, another component of the SIN3 complex, but did not find an interaction with SIN3 itself (Lee et al., 2007). However, Moshkin et al., isolated a dKDM5/LID complex that includes SIN3 and RPD3 proteins as well as Pf1 and EMSY, components of the SIN3 complex (Moshkin et al., 2009). Our work along with published data establishes dKDM5/LID as a key component of a SIN3 and RPD3 containing complex.

dKDM5/LID affects cell proliferation and wing development

Our results indicate that similar to SIN3, dKDM5/LID plays an important role during cell proliferation and wing developmental processes. The defects in cell proliferation seen in cell culture upon knock down of either *Sin3A* or *lid* is less severe than that observed in the developing wing imaginal disc cells. This is possibly due to the more significant roles of these proteins during development. Wing imaginal discs have previously been utilized to show the interconnection of pathways involved in growth, proliferation and developmental patterning (Neufeld et al., 1998a). In *Drosophila*, SIN3 affects expression of some cell cycle regulators (Pile et al., 2003; Swaminathan and

Pile, 2010). dKDM5/LID represses Notch target genes (Moshkin et al., 2009). Notch signaling, while being involved in wing development, also regulates cell proliferation in wing imaginal discs (Baonza and Garcia-Bellido, 2000). The combinatorial effects due to such gene expression changes seen upon reduction of SIN3 or dKDM5/LID could lead to the observed cell proliferation and wing developmental defects. Furthermore, in cell culture the double knock down of *Sin3A* and *lid* results in a cell proliferation defect comparable to single knock down of *Sin3A*. Double knock down of *Sin3A* and *lid* in the developing fly wing tissue, however, shows additive effects resulting in a more severe wing phenotype. This implies that dKDM5/LID may have additional roles in wing development that are distinct from the SIN3 complex function. This idea is further supported by the vein disruption phenotype observed upon *lid* knockdown and the held-out wing phenotype observed upon dKDM5/LID overexpression.

In conclusion, our findings demonstrate functional similarities of SIN3 and dKDM5/LID in regulating cell and developmental processes, thereby highlighting a key role for dKDM5/LID in the SIN3 complex. While we and others have shown the association of this KDM with SIN3, the SIN3 complex is still predominantly thought of as an HDAC complex. The results obtained emphasize the importance of further understanding the contribution of the KDM, dKDM5/LID, to the activity of the SIN3 complex.

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CHAPTER 3

GENOME WIDE ANALYSIS OF GENE EXPRESSION REGULATION BY SIN3 AND dKDM5/LID IN *DROSOPHILA* S2 CULTURED CELLS

INTRODUCTION

Regulation of gene transcription is amongst the most important of cellular processes. The activity of transcription factors, chromatin remodeling enzymes and histone modifying enzymes can all affect the regulation of transcription (Luger, 2006). The important role of epigenetic modifications of chromatin by the action of histone modifying enzymes has been well established since the discovery of histone acetylation in 1960s and the subsequent identification of the first HAT, Gcn5p (Allfrey et al., 1964; Brownell et al., 1996; Kuo et al., 1996; Phillips, 1963).

SIN3, a conserved protein from yeast to mammals, is the scaffold protein of one such histone modifying enzyme complex that incorporates a histone deacetylase and a demethylase (Hayakawa and Nakayama, 2011; Silverstein and Ekwall, 2005). In *Drosophila*, SIN3 interacts with the HDAC RPD3 and the KDM dKDM5/LID (Moshkin et al., 2009; Spain et al., 2010). Histone acetylation is a mark associated with transcription activation, while deacetylation leads to increased compaction of chromatin, reducing accessibility for the transcription machinery to the DNA template. Histone methylation, however, is associated with both transcription activation and repression. dKDM5/LID specifically removes H3K4me3, a mark associated with active transcription (Eissenberg et al., 2007; Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007). Therefore, the enzymatic activities of the associated histone modifying enzymes suggest a repressive role for the SIN3 complex.

Previous work in *Drosophila*, based on microarray expression analysis of S2 cultured cells upon *Sin3A* RNAi, determined 399 genes to be regulated by SIN3 (Pile et al., 2003). Of these, 364 genes were negatively regulated by SIN3, while the rest were positively regulated. The vast majority of genes are thus repressed by SIN3. This supports the predicted role of SIN3 as a repressor due its association with histone modifying enzymes that remove active marks of transcription.

Until recently, targeted gene expression analysis had been performed for only a few genes to understand the role of dKDM5/LID on transcription. These studies revealed that, consistent with its demethylase activity, Notch target genes are repressed by dKDM5/LID, while other genes are positively regulated (Lee et al., 2009; Lee et al., 2007; Lloret-Llinares et al., 2008; Moshkin et al., 2009; Secombe et al., 2007). Recently two groups published findings for genome wide changes in gene expression upon depletion of dKDM5/LID (Liu et al., 2014; Lloret-Llinares et al., 2012). These groups utilized expression arrays to identify dKDM5/LID regulated genes. The first group analyzed genome wide changes in gene expression upon *lid* RNAi in wing imaginal disc tissues (Lloret-Llinares et al., 2012). Although dKDM5/LID can remove H3K4me3, a mark associated with active transcription, they identified very few genes showing statistically significant changes in expression. The overall trend of gene expression changes, however, suggested a role in gene activation for dKDM5/LID. The second group, while also analyzing gene expression changes in wing imaginal disc tissues, utilized *lid* mutants to deplete dKDM5/LID (Liu et al., 2014). In contrast to the previous study, they reported a large number of genes (901) to be statistically significantly regulated by dKDM5/LID. Of the regulated genes, 367 were upregulated and 534 were

downregulated, suggesting a role in both gene activation and repression for dKDM5/LID.

In Chapter 2, we described phenotypes observed upon knockdown of *Sin3A* and *lid.* The observed phenotypes suggest that both SIN3 and dKDM5/LID affect cell proliferation and wing development. Another pathway that has been linked to regulation by both SIN3 and dKDM5/LID is tolerance to oxidative stress and determination of adult lifespan (Barnes et al., 2014; Li et al., 2010; Liu et al., 2014). SIN3 is critical for sensitivity to oxidative stress, where *Sin3A* knockdown flies reared on food treated with the oxidative agent paraquat show decreased tolerance compared to control flies exposed to treatment (Barnes et al., 2014). Glutathione supplementation, however, partially rescued this enhanced sensitivity to paraquat. The demethylase activity of dKDM5/LID too is important for tolerance to oxidative stress in adult male flies (Li et al., 2010). Expression of either wild type or a demethylase mutant of dKDM5/LID from a transgene can rescue lethality due to a *lid* null mutation. Male demethylase mutants are more sensitive to paraquat-induced oxidative stress and exhibit a reduced lifespan compared to the flies that express wild type dKDM5/LID. Recent work expanded on this finding, demonstrating that dKDM5/LID activates genes involved in regulation of cellular redox states by interacting and recruiting the oxidative stress transcription factor Foxo to target genes (Liu et al., 2014). These researchers further demonstrated that mutation of *lid* results in increased levels of oxidized proteins and DNA mutation in the cell and causes increased sensitivity to paraquat in flies.

While SIN3 and dKDM5/LID affect common processes, understanding the underlying transcriptional network is hampered by the poor understanding of

dKDM5/LID regulated genes. To overcome this, we utilized the highly sensitive RNAseq approach to determine genes that are commonly regulated by SIN3 and dKDM5/LID.

MATERIALS AND METHODS

Cell Culture

Drosophila S2 cells were cultured as described in Chapter 2.

RNA interference

RNAi methodology and dsRNA constructs are described in Chapter 2.

Western Blotting

Protein extract preparation, western blotting and antibody specifications are described in Chapter 2.

Gene Expression Analysis by RT-PCR

Total RNA was extracted from 1 x 10^7 S2 cells subjected to RNA interference and paraquat treatment using the RNeasy mini kit (Qiagen). Extracted total RNA was used to generate cDNA with random hexamers using the ImProm-II Reverse Transcription System (Promega). Generated cDNA was used as the template in a real-time quantitative PCR assay carried out in a Stratagene Mx3005P real-time thermocycler. Analysis was performed using Absolute SYBR Green ROX master mix (Fisher Scientific). Relative fold change in gene expression was determined by the comparative quantification ($2^{-\Delta\Delta CT}$) method of analysis (Livak and Schmittgen, 2001).

Taf1 was used to normalize cDNA amounts in the comparative analysis. The following primer sets (5' to 3' – forward and reverse) were used in the PCR reactions:

lid (TCG TGC GAA AAG ACA CAG AA and GCC CGA TCT GCT TCA CCA GC)

Taf1 (CTG GTC CTG GTG AGG TGA and CCG GAT TCT GGG ATT TGA)

Su(H) (CAT GGG TCC TGT GGC TTC and GCA GAT GCG GCG TAA AGT)

vari (ACG CTC AAG AAT CGG CTG and ACG GCC TCT TCC ATT TCC)

Gapdh1 (GCC CTG AAC GGC AAG CT and GTA AGA TCC ACA ACG GAG ACA TTG)

Cyt-c-p (GCT CGA CGT TTG TGT TCA AT and TTC CCT TCT CAA CAT CAC CA)

CG3476 (GCC ATC CAT TGG ACA CAA TA and GGC GGC ACA ATC TAT GAC T)

mRpL19 (CGA TCC CGA AAA CTA TTC AA and TTA CAT GTT CCG CAG TTT TG)

Thor (GCT AAG ATG TCC GCT TCA CC and CCC GCT CGT AGA TAA GTT TGG)

Reph (CTG ATG GTG GAG AAC CGC and TTT GGC TTG AAT GCC TCC)

Ssdp (GGA TTC CTG CAC ACC TGG and CCG TAG CCG GAG CTA ACA)

Sesn (GAG GAG CTC CAC CGG ACT and ATG CGC TCC ATT AGC GTC)

Mcm7 (ACC AAA TCC ACG AGC ACC and GGT CGG GCT TGA ATC CTT)

Sam-S (AAA CTT TGA CCT CAG GCC C and CGC TGG TAT ATC GGC TGG)

GstE6 (GAT CAG CGG CTG CAC TTT and TGG CAT CGT ATC GCT CCT)

ihog (GGA GGG GGC ACT GAA AAT and TAA CTG CGC AAA CGC AAA)

Statistical analyses for qRT-PCR results

All significance values were calculated by the two sample Student's *t*-test using GraphPad. http://www.graphpad.com/quickcalcs/index.cfm.

Gene Expression Analysis by RNAseq

RNA Isolation and Next Generation Sequencing

RNA isolation to next generation sequencing and initial quality control was performed at the Applied Genomics Technology Center, Wayne State University. Total RNA was isolated from S2 cells subjected to RNA interference and paraquat treatment using the EZ1® RNA Universal Tissue Kit (Qiagen). Cells were disrupted and homogenized in 750 µl QIAzol™ lysis reagent via bead-milling on the TissueLyser® II (Qiagen). RNA was collected from the homogenate by chloroform extraction and purified on the EZ1® Advanced (Qiagen) with additional DNase step to remove any residual DNA. Purified total RNA was quantified by UV spectrophotometry using the DropSense96® Microplate Spectrophotometer (Trinean) and purity was assessed based on the A260/A280 and A260/A230 ratios. RNA quality was assessed by microfluidics using the RNA R6K assay for the Agilent 2200 TapeStation. The electrophoretogram was examined to determine overall quality of the RNA.

The TruSeq RNA Sample Preparation Kit (Illumina) was used to prepare adapter ligated PCR fragments for sequencing. In brief, mRNA was purified from total RNA and fragmented. The cleaved mRNA was primed with random hexamers and reverse transcribed into first strand cDNA. The RNA template was then removed and a replacement, complementary strand was generated. The ends of the double stranded cDNA was repaired and adenylated. Then sequencing adapters were ligated to the prepared cDNA. PCR was used to selectively enrich the fragments containing the adapters. The PCR fragments were validated using Agilent 2200 TapeStation. Single indexed samples were multiplexed and sequenced on an Illumina HiSeq 2500

sequencing system in paired end mode with a read length of 2 x 50 bp. Samples were demultiplexed using Illumina bcl2fastq converter (v1.8.3). Read quality was assessed with FastQC. The RNAseq experiments were conducted in triplicate. Depth of coverage of ~25-30 million reads was obtained.

Bioinformatic Analysis

The Tuxedo pipeline was utilized for analysis (Trapnell et al., 2012). Reads obtained from RNA sequencing were aligned to the UCSC reference genome (dm3) using Bowtie/Tophat. Cufflinks was used to assemble the aligned reads into transcripts. The obtained reads were mapped to a total of 14,542 Refseq genes and FPKM (fragments per kilobase per million fragments mapped) values reflecting mRNA expression levels were generated through Cufflinks. Cuffdiff, an integrated package of Cufflinks, was used to identify statistically significant genes that were differentially expressed in treatment conditions compared to control. The default false discovery rate (FDR) of 5% was used for the differential expression analysis. The R statistics environment was used to visualize the data. The correlation plots were generated using the Lattice package. The volcano and scatter plots were generated using the CummeRbund package. The heatmap was generated using gplots package (heatmap.2). Gene ontology analysis was performed using DAVID (Huang da et al., 2009). The Functional Annotation Tool of DAVID was utilized where Gene Ontology term, GOTERM_BP_FAT was utilized to identify enriched biological processes and KEGG_PATHWAY was used to identify enriched pathways.

RESULTS AND DISCUSSION

Work in our laboratory determined that dKDM5/LID associates with the larger isoform of SIN3, SIN3 220 in *Drosophila* (Spain et al., 2010). We further find that knockdown of both *Sin3A* and *lid* result in similar phenotypes during cell proliferation and wing development (described in Chapter 2). To understand how SIN3 and dKDM5/LID affect these processes, it is necessary to understand the underlying transcriptional changes associated with reduced levels of *Sin3A* or *lid*.

RNAseq analysis identifies genes regulated by SIN3 and dKDM5/LID

To determine if SIN3 and dKDM5/LID can regulate common target genes, we utilized the highly sensitive RNAseq approach to identify genome wide changes in gene expression upon reduction of SIN3 or dKDM5/LID. As both SIN3 and dKDM5/LID are essential genes in flies we utilized RNAi to induce reduced expression of these two proteins. We treated *Drosophila* S2 cells with dsRNA targeting *Sin3A*, *lid* or both. S2 cells were treated with dsRNA targeting GFP as a control. Knockdown of SIN3 was verified by western blot analysis (Fig. 3.1A). Antibody to α-Tubulin was used as a loading control for western blots. RNAseq data analysis determined that knockdown of *Sin3A* or *lid* can alter expression of one of the α-Tubulin encoding genes (α-Tubulin at 84B) resulting in roughly 1.5 fold increase in gene expression. However, the level of decrease observed in the SIN3 or LID protein levels upon RNAi knockdown compared to the small changes in *Tubulin* gene expression and the fact that India ink staining of western blots showed comparable loading of samples indicates sufficient knockdown of *Sin3A*. *lid* knockdown was verified by real time qRT-PCR (Fig. 3.1B).

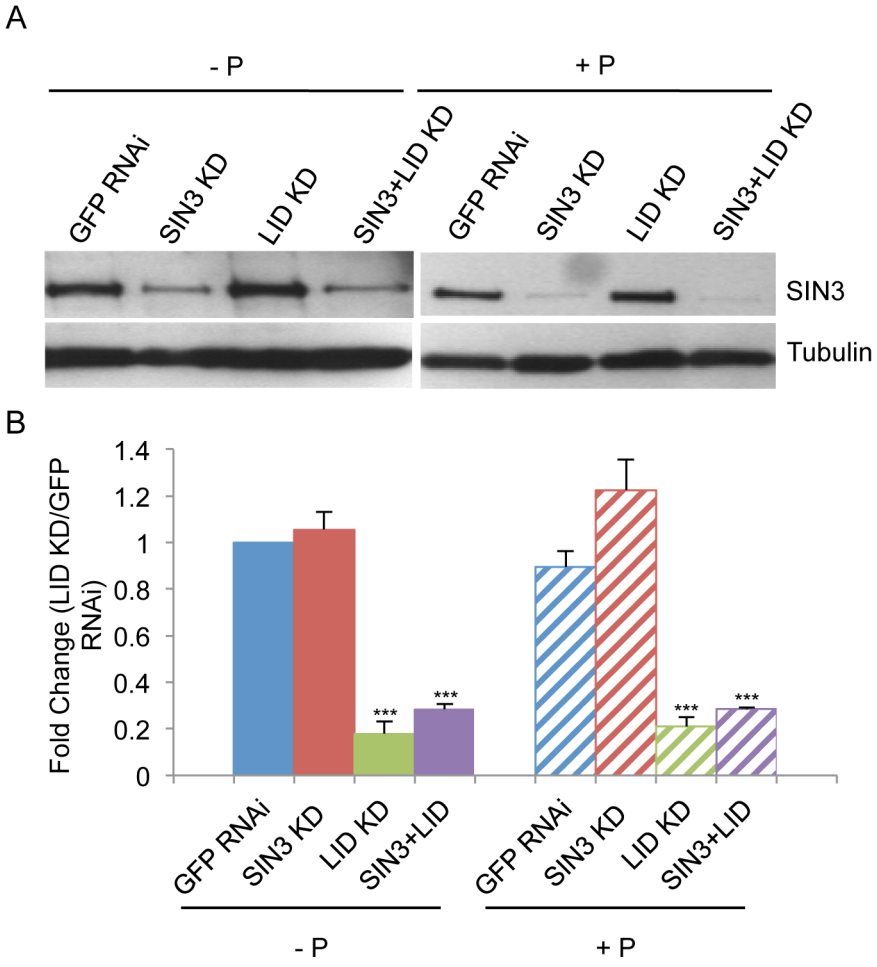
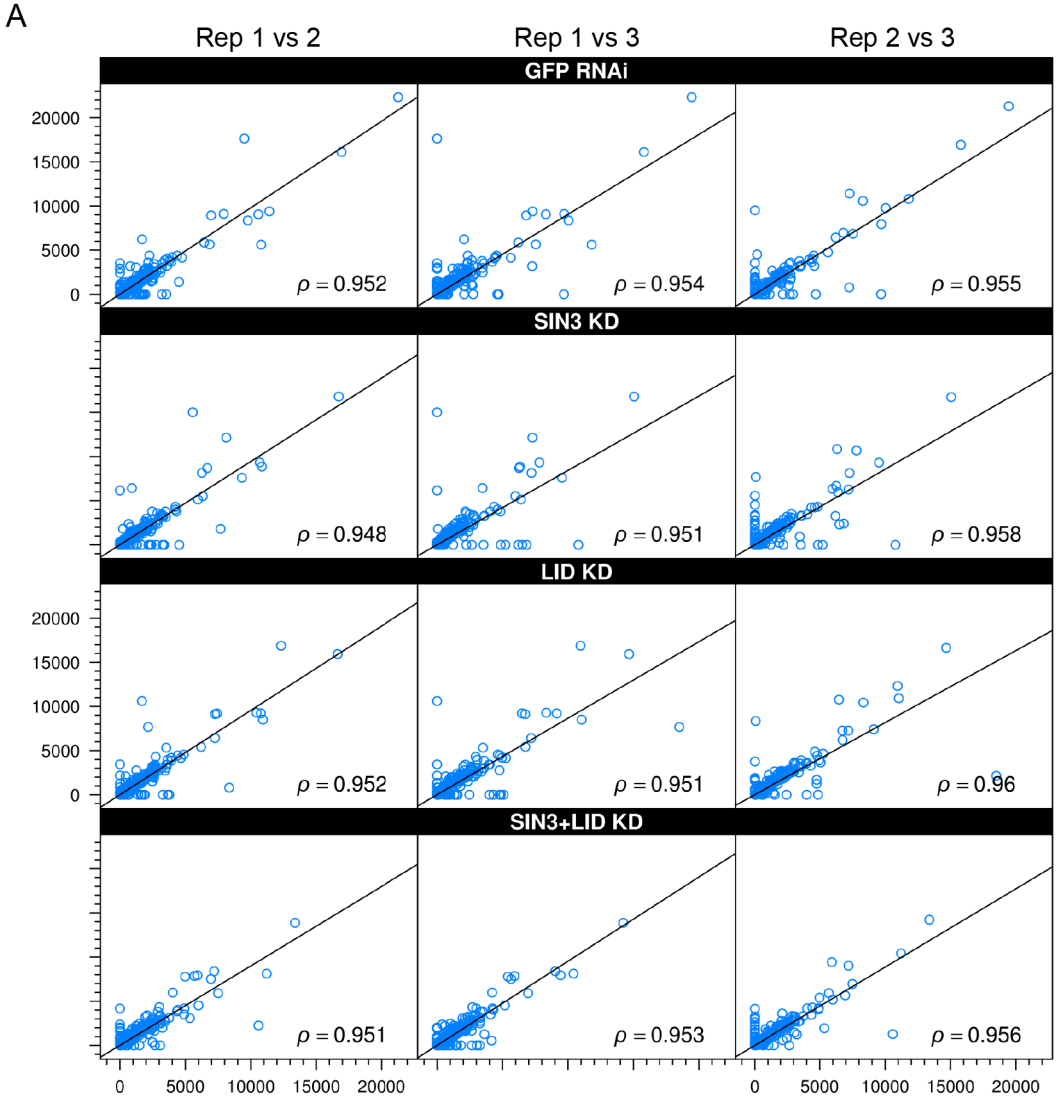


Figure 3.1: RNAi in S2 cells leads to efficient knockdown (KD) of SIN3 and LID. (A) Western blot analysis of whole cell protein extracts from cells treated with dsRNA targeting SIN3, LID or both and GFP as a control. Blots were probed with antibody to SIN3 and Tubulin as a loading control. (B) Real-time qRT-PCR analysis of *lid* transcript levels. *Taf1* was used to normalize expression levels. KD – knockdown, P – paraquat, *** – $P < 0.001$.

Isolated mRNA from RNAi treated cells was subjected to RNAseq analysis. Three biological replicates of the RNAseq data were obtained and Spearman's rank correlation analysis was performed to verify reproducibility of the data (Fig 3.2). Obtained Spearman's rank correlation coefficients (ρ) for all data sets were significant indicating reproducibility of the data.

A


Rep 1 vs 2
Rep 1 vs 3
Rep 2 vs 3
GFP RNAi
SIN3 KD
LID KD
SIN3+LID KD
ρ = 0.952
ρ = 0.954
ρ = 0.955
ρ = 0.948
ρ = 0.951
ρ = 0.958
ρ = 0.952
ρ = 0.951
ρ = 0.96
ρ = 0.951
ρ = 0.953
ρ = 0.956
0
5000
10000
15000
20000

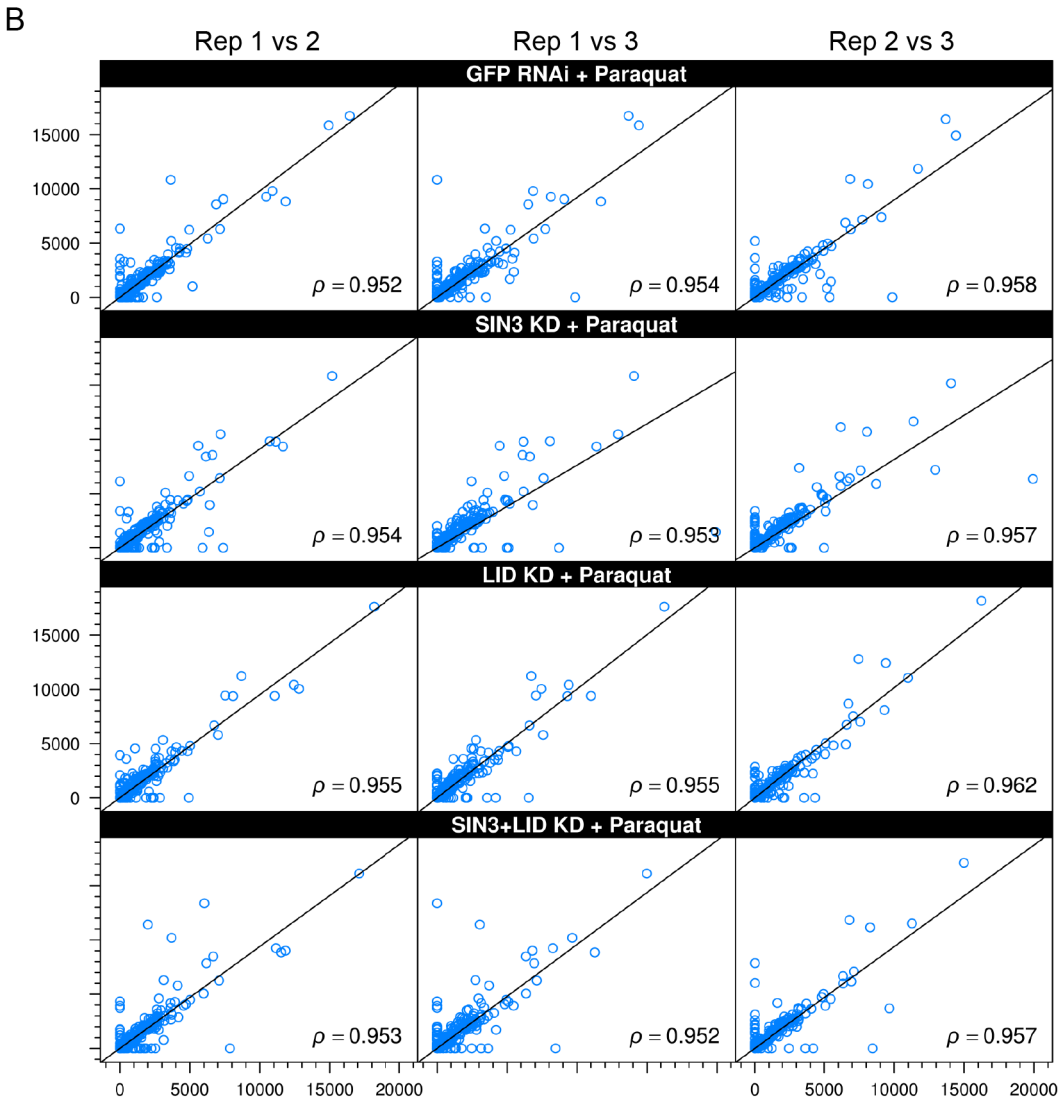


Figure 3.2: Biological replicates of RNAseq data correlate significantly. Correlation plots based on the FPKM values for the three replicates of RNAseq data from indicated samples under normal (A) and paraquat induced oxidative stress (B) conditions. The calculated Spearman's rank correlation coefficients (ρ) for all datasets are significant demonstrating reproducibility of the data. Rep – Replicate, KD – knockdown.

Differential expression analysis was performed comparing knockdown samples to control using an FDR cutoff of 0.05 to identify genes showing significant changes in expression level (Table 3.1, Fig. 3.3 and Supplementary Data 1). Greater than 1.4 fold

change in expression compared to control was observed for all genes identified as significantly regulated. 624 and 89 genes were determined as regulated by SIN3 and dKDM5/LID respectively. Dual knockdown of SIN3 and dKDM5/LID resulted in the misregulation of 849 genes. The increased number of genes that show significant regulation upon dual knockdown of *Sin3A* and *lid* compared to single knockdown suggests an additive role for these proteins in regulating gene expression of many gene targets.

	- Paraquat			**+ Paraquat**			
	SIN3 KD	**LID KD**	**SIN3+LID KD**	**GFP RNAi**	**SIN3 KD**	**LID KD**	**SIN3+LID KD**
All genes	624	89	849	212	1136	357	1308
Upregulated	271	27	378	95	514	141	622
Downregulated	353	62	471	117	622	216	686

KD – knockdown

TABLE 3.1: Whole genome expression analysis by RNAseq, identified genes regulated upon knockdown of *Sin3A*, *lid* or both. Only genes that displayed changes in expression with FDR < 0.05 and *P* < 0.05 were considered as differentially expressed.

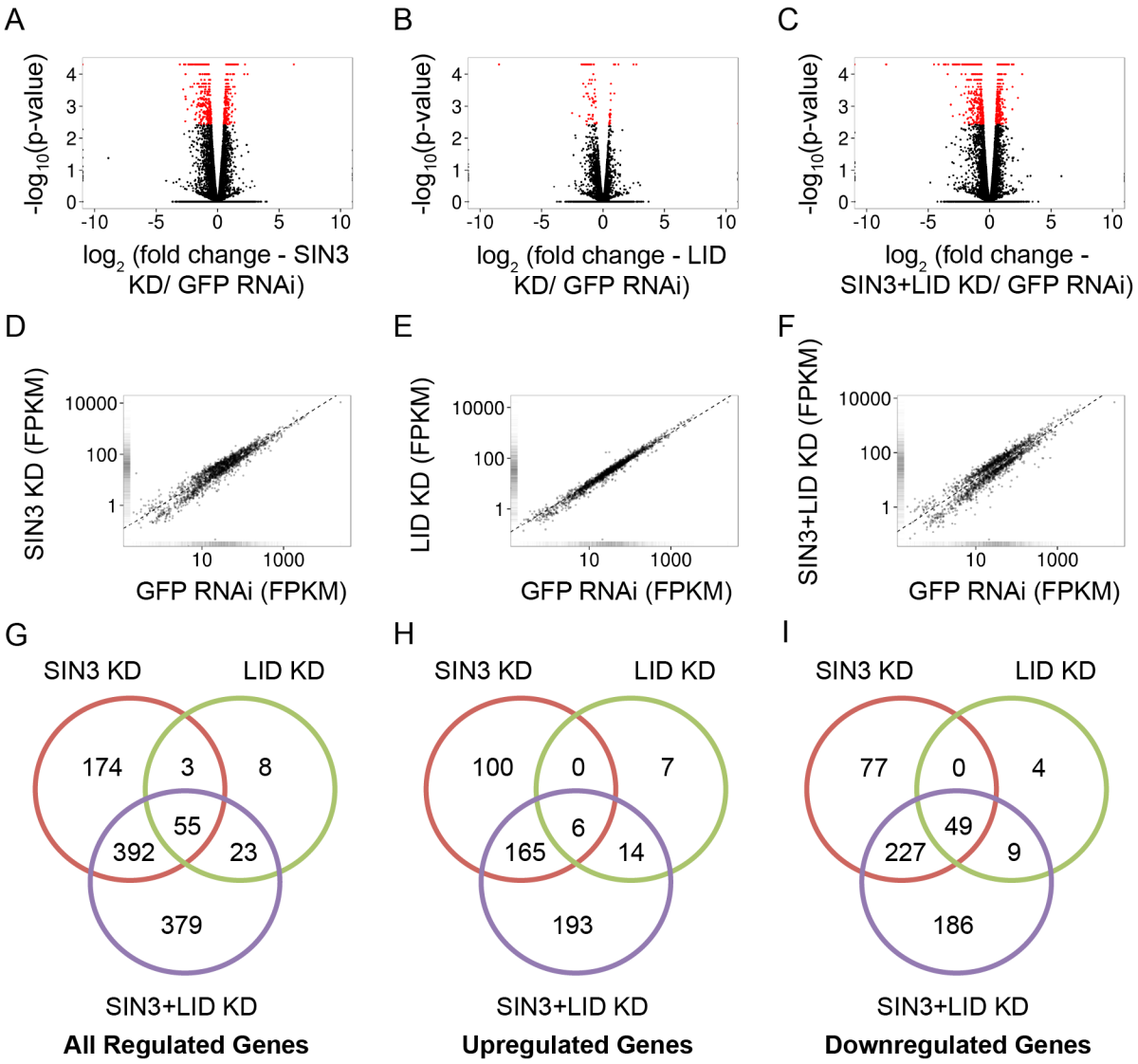


Figure 3.3: RNAseq analysis identifies genes regulated by SIN3, dKDM5/LID or both in S2 cells. (A-C) Volcano plots depicting log fold change in expression of indicated samples. Red – significant change in expression, Black – no significant change in expression. (D-F) Scatter plots depicting expression trends of all significantly regulated genes of indicated samples. (G-I) Venn Diagrams indicating significant overlap of genes regulated by SIN3 and dKDM5/LID. KD – knockdown.

The number of genes determined to be regulated by SIN3 or dKDM5/LID is comparatively higher than published genome wide studies utilizing microarray expression analysis (Lloret-Llinares et al., 2012; Pile et al., 2003). This may be

attributed to the increased sensitivity of RNAseq in quantifying expression levels, especially of low abundance transcripts. Further, while SIN3 regulated genes were previously determined in cultured cells, the reported gene list only included genes regulated in both S2 and Kc cell lines. LID regulated genes, however, were only reported from wing imaginal disc tissues and not cultured cells. Recent work, however, identified a large number of genes (901) to be regulated by dKDM5/LID in wing imaginal discs (Liu et al., 2014). The contrast between the two studies identifying dKDM5/LID regulated genes in wing imaginal discs may partly be due to differences in how dKDM5/LID was depleted in the tissue, where one group utilized RNAi while the other utilized mutant flies. Further, differences in the stringency of the bioinformatic analysis may have also contributed to this variability. Different from the previously published work, we analyzed changes in gene expression upon knockdown of *Sin3A* and *lid* in S2 cultured cells and have found a number of genes to change in expression levels. We therefore have identified a significant number of novel genes regulated by SIN3 and or dKDM5/LID in *Drosophila* S2 cultured cells.

Of the genes misregulated upon knockdown of *Sin3A*, 271 (43%) were upregulated and 353 (57%) genes were downregulated (Fig. 3.3G-I and Table 3.1). This somewhat equal distribution of genes that were upregulated or downregulated is in contrast to previous work indicating that SIN3 functions predominantly as a repressor (Pile et al., 2003). As mentioned above, the previously published microarray data used a combination of gene expression data obtained from both S2 and Kc cell lines. It is possible that downregulation of genes by SIN3 is more tissue and cell type specific compared to upregulation of target genes. Knockdown of *lid*, however, resulted in twice

as many genes to be downregulated, where 27 (30%) genes were upregulated and 62 (70%) were downregulated. This corroborates the published microarray expression data establishing a predominant role for dKDM5/LID in gene activation (Liu et al., 2014; Lloret-Llinares et al., 2012). Double knockdown of *Sin3A* and *lid* show a similar trend as *Sin3A* knockdown, where upregulated genes (378, 45%) were relatively equal in number to downregulated genes (471, 55%).

Considering the levels of gene expression changes, most genes did not show more than 3 - 4 fold changes in expression (Fig. 3.3A-F, Fig. 3.4 and Supplementary Data 1). This suggests that both SIN3 and dKDM5/LID act to modulate expression levels of transcribed genes rather than turn on or off target genes. This is consistent with the data showing that SIN3 and dKDM5/LID are enriched at actively transcribing genes (Filion et al., 2010; Kharchenko et al., 2011; Lloret-Llinares et al., 2012). Double knockdown of *Sin3A* and *lid* resulted in relatively higher expression changes at the majority of regulated genes, further corroborating an additive effect for these proteins in transcription regulation.

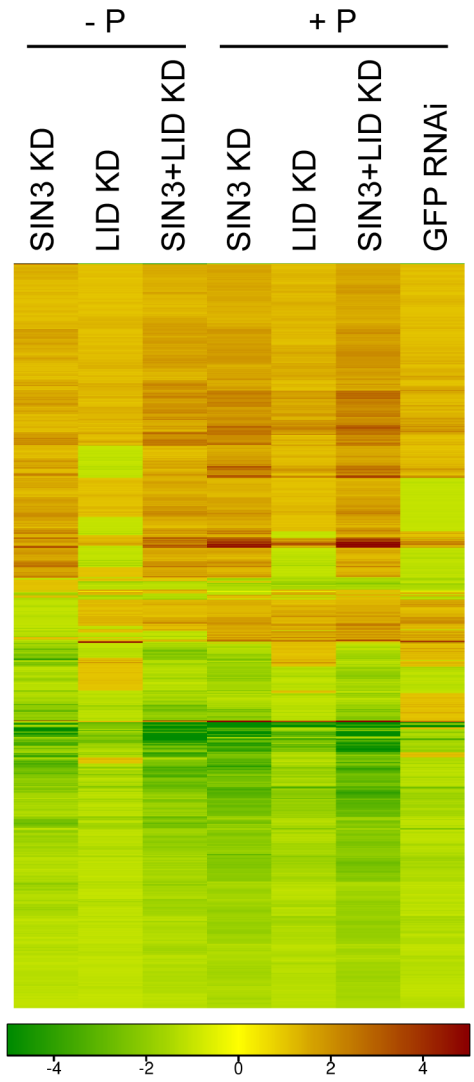


Figure 3.4: RNAseq identifies a role in both transcriptional activation and repression for both SIN3 and dKDM5/LID. Heatmap plotting fold change in expression of genes from indicated samples compared to GFP RNAi treated controls. All genes significantly regulated upon at least one of the indicated conditions were hierarchically clustered, where genes showing similar trends across samples are clustered together, to generate the heatmap. The scale bar depicts fold change in expression. KD – knockdown, P – paraquat.

SIN3 and dKDM5/LID regulate common gene targets

Of the 89 genes regulated by dKDM5/LID, a majority of genes (58, 65%) overlapped with SIN3 regulated genes (Fig. 3.3G). This result supports the idea that dKDM5/LID could function coordinately with SIN3 as part of a complex to regulate gene

transcription and downstream processes. Among the genes regulated by both SIN3 and dKDM5/LID only 6 genes were upregulated upon reduction of these proteins (Fig. 3.3H). The genes repressed by both SIN3 and dKDM5/LID are *fondue* (*fon*), *kraken*, α*-Tubulin at 84B* (α*Tub84B*), *suppressor of rudimentary* (*su(r)*), *Adenosylhomocysteinase at 13* (*Ahcy13*) and *Glutathione S transferase T4* (*GstT4*). These genes do not fall under any particular functional category and are involved in diverse processes. *fon* is implicated in metamorphosis and hemolymph coagulation (Scherfer et al., 2006). *kraken* is associated with digestion and detoxification (Edwin Chan et al., 1998). α*Tub84B* is a structural constituent of the cytoskeleton (FlyBase-Curators et al., 2004-). (*su(r)*) is involved in pyrimidine biosynthesis (Rawls, 2006). *Ahcy13* functions in the one carbon metabolic pathway (FlyBase-Curators et al., 2004-). *GstT4* is inferred to have glutathione transferase activity (FlyBase-Curators et al., 2004-). Repression of these genes by both SIN3 and dKDM5/LID is consistent with the associated enzymatic activity of these proteins. The large majority of genes (49), however, were downregulated upon decreased SIN3 or dKDM5/LID levels. The molecular mechanism for downregulation of gene expression by these two proteins is unclear. It is possible that the activating roles of SIN3 and or dKDM5/LID are indirect effects. It is also possible that SIN3 and or dKDM5/LID can recruit other proteins to these gene targets, which inhibit or oppose the HDAC and or demethylase functions. Three genes showed opposing directions of regulation by SIN3 and dKDM5/LID and therefore showed wild type levels of expression in the dual knockdowns. These genes are CG15117, involved in carbohydrate metabolism, CG8051, involved in transmembrane transport, and GstE2 involved in glutathione transferase activity (FlyBase-Curators et al., 2004-; Saisawang et al., 2012).

The differential mode of regulation of these three genes, along with the many identified genes that are regulated by only SIN3 or dKDM5/LID, indicate unique transcription regulatory roles for these proteins in addition to a common gene regulatory role as part of the SIN3 complex.

While the majority of genes regulated by SIN3 or dKDM5/LID were found to change in expression in the dual knockdowns, 31% of SIN3 regulated genes and 9% of dKDM5/LID regulated genes did not change in expression upon knockdown of both SIN3 and dKDM5/LID (Fig. 3.3G-I). As discussed previously most genes regulated by SIN3 and dKDM5/LID displayed small changes in gene expression levels. It is possible that while many of these small changes in gene expression were not statistically significant and not included in our list of genes that changed in expression, they may in fact be biologically relevant. While only three genes with statistically significant changes in gene expression were regulated in opposing directions by SIN3 and dKDM5/LID, several others (that were significant in one condition and not the other) displayed such trends and thus were not significantly regulated in the double knockdown. Many genes, however, display similar trends in gene expression changes in both the single and double knockdowns. The less than two fold changes in expression seen at majority of genes render them statistically significant in one condition versus the other.

SIN3 and dKDM5/LID regulate common and distinct functional categories of genes

To functionally annotate genes regulated by SIN3 and dKDM5/LID we utilized the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et

al., 2009). Gene ontology (GO) analysis of genes regulated by SIN3 identified many different biological processes. The top ten enriched processes were related to junction assembly, heat shock response and metabolism (Fig. 3.5A). While less enriched, genes regulated by SIN3 are also involved in multiple developmental and cell cycle related processes (Supplementary Data 2).

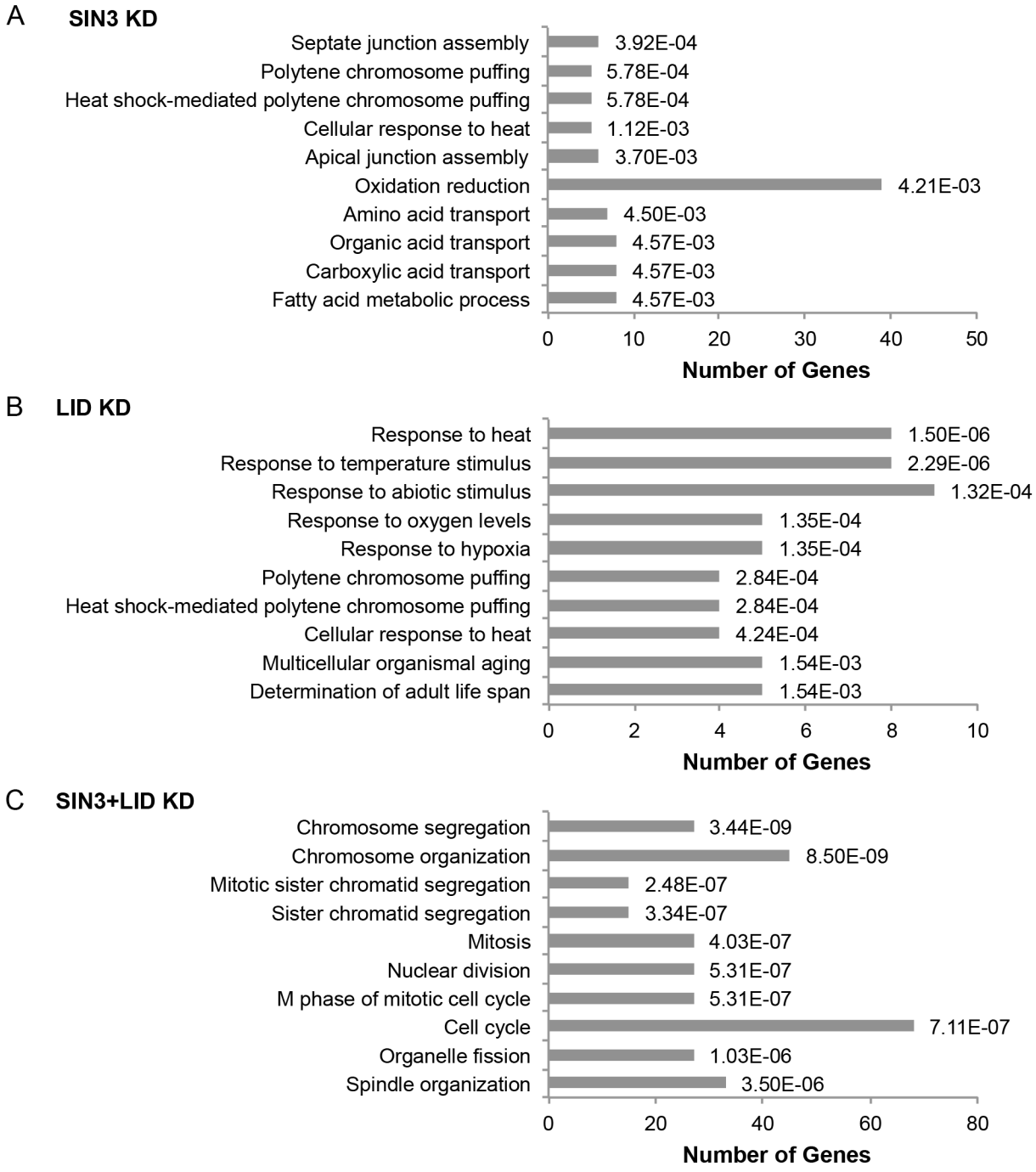


Figure 3.5: Genes regulated by SIN3, dKDM5/LID or both are enriched in similar and distinct biological processes. (A-C) Gene Ontology (GO) analysis of genes regulated by SIN3, LID or both as indicated. *P*-value for each enriched category is displayed on the right of the bars. KD – knockdown.

The SIN3 regulated genes *Neurexin IV* (*Nrx-IV*), *Transferrin 2* (*Tsf2*), *Contactin* (*Cont*), *varicose* (*vari*), *sinuous* (*sinu*) and *Gliotactin* (*Gli*) are linked to junction assembly processes. These septate junction assembly proteins have been implicated in maintenance of blood brain barrier, heart morphogenesis and open tracheal system development. *Nrx-IV*, *Cont*, *sinu* and *Gli* have been linked to maintenance of blood brain barrier and heart morphogenesis (Auld et al., 1995; Stork et al., 2008; Yi et al., 2008). *Nrx-IV*, *Tsf-2*, *vari* and *sinu* have been associated with open tracheal system development (Beitel and Krasnow, 2000; Paul et al., 2003; Tiklova et al., 2010). It is of interest that genetic screens have previously identified SIN3 as a regulator of cardiac and neural development (Kim et al., 2004; Parrish et al., 2006; Sepp et al., 2008). Identification of the above septate junction assembly genes as SIN3 targets could aid future work in determining how SIN3 regulates these developmental processes.

A large number of SIN3 regulated genes are involved in multiple metabolic processes. 39 genes were implicated in the functional category of oxidative reductive functions alone. The enrichment of genes involved in metabolic processes is consistent with published work from our laboratory, analyzing genes regulated upon SIN3 knockdown in S2 and Kc cultured cells (Pile et al., 2003). The published work discusses the enrichment of SIN3 regulated genes in mitochondrial energy metabolism. The current data adds to the previous work identifying novel genes involved in multiple metabolic processes. Apart from gene ontology analysis for enriched biological processes, we further performed pathway analysis using KEGG pathway analysis in DAVID. SIN3 regulated genes as could be expected were most enriched in metabolic pathways. Pathways enriched are glutathione metabolism (*P*-value: 0.0095), fatty acid

metabolism (*P*-value: 0.0183), fructose and mannose metabolism (*P*-value: 0.0313), pyrimidine metabolism (*P*-value: 0.0404), selanoamino acid metabolism (*P*-value: 0.0624), amino sugar and nucleotide sugar metabolism (*P*-value: 0.079) and drug metabolism (*P*-value: 0.0865).

Another enriched category of genes regulated by SIN3 is the heat shock response genes. This category of genes is also enriched upon *lid* knockdown (Fig. 3.5B). In fact, GO analysis of dKDM5/LID regulated genes determined that the top enriched biological processes are related to heat shock response. Nine heat shock genes (*Hsp22*/*Hsp67Bb*, *Hsp23*, *Hsp26*, *Hsp27*, *Hsp68*, *Hsp70Ba*, *Hsp70Bb*, *Hsp70Bbb* and *Hsp70Bc*) are regulated by both SIN3 and dKDM5/LID. Two additional heat shocks genes (*Hsp67Ba* and *Hsp67Bc*) are also misregulated in the dual knockdown (Fig. 3.5C).

The heat shock response is typically induced through the heat shock transcription factor (HSF) which activates heat shock proteins (Hsps) in response to multiple cellular stresses such as heat, oxidative stress, toxins and bacterial infections (Akerfelt et al., 2010). Hsps bind denatured proteins produced during cellular stress and mediate refolding or degradation of these proteins counteracting proteotoxicity and can thereby influence organismal life span (Tower, 2011). Some heat shock proteins are also induced by the JNK signaling pathway and the transcription factor Foxo, which are also implicated in life span determination (Wang et al., 2005). SIN3 and dKDM5/LID have both been associated with life span determination (Barnes et al., 2014; Li et al., 2010; Liu et al., 2014). Interestingly, an RNAi screen genetically links SIN3 to the JNK signaling pathway, where SIN3 acts as an enhancer of JNK phosphorylation (Bond and

Foley, 2009). Recently, dKDM5/LID was reported to interact with Foxo and help recruit Foxo to genes coregulated by dKDM5/LID and Foxo (Liu et al., 2014). Understanding the coordinate function of these stress response regulators would provide further insight to the mechanisms of life span extension.

KEGG pathway analysis of LID regulated genes identified glutathione metabolism (*P*-value: 0.0032), metabolism of xenobiotics by cytochrome P450 (*P*-value: 0.0257) and drug metabolism, all involving the *Glutathione S transferase* genes, as enriched pathways. Another enriched pathway is endocytosis (*P*-value: 0.0456), which includes the heat shock genes. Glutathione metabolism is the top enriched pathway identified based on both SIN3 regulated and dKDM5/LID regulated genes. The Glutathione S transferase genes *GstD3*, *GstD7*, *GstE2* and *GstT4* are regulated by SIN3, while dKDM5/LID regulates *GstD1*/*GstD9*, *GstD7*, *GstE2*, *GstE9* and *GstT4*. These genes are also misregulated in the double knockdown of *Sin3A* and *lid* with the exception of *GstE2*, which is regulated in opposing directions by SIN3 and dKDM5/LID. In addition to the above genes, *GstO1* also shows significant downregulation upon dual knockdown.

Glutathione is an antioxidant, which plays a key role in defense against oxidative stress. The catalytic activity of glutathione peroxidases reduces hydrogen and lipid peroxides while oxidizing glutathione (Lu, 2013). Both glutathione peroxidases and glutathione S transferases can reduce organic peroxides. Another important role of glutathione involves detoxification of xenobiotics including drugs, where glutathione S transferases conjugate glutathione to a wide range of xenobiotics leading to their elimination from the cell (Ramsay and Dilda, 2014). Glutathione is also implicated in the

modulation of cell proliferation and cell death (Pallardo et al., 2009). In *Drosophila*, glutathione supplementation results in increased survival of flies treated with the oxidative stressor paraquat (Barnes et al., 2014; Bonilla et al., 2006). Thus the glutathione pathway may provide a critical link to the observed phenotypes associating SIN3 and dKDM5/LID to stress tolerance, lifespan extension and cell proliferation.

Differing from the single knockdowns, GO analysis of genes regulated by *Sin3A* and *lid* dual knockdown identified cell cycle related biological processes as the most enriched (Fig. 3.5C). A large number of distinct and overlapping genes fall under each of these biological processes. While no significant enrichment of cell cycle related processes were identified upon GO analysis of dKDM5/LID regulated genes, a few cell cycle related processes were enriched upon GO analysis of SIN3 regulated genes (Supplementary Data 2). These processes are, however, less enriched in the single knockdown due to the small number of genes being regulated. As described above, double knockdown of *Sin3A* and *lid* resulted in an increase in the number of genes that exhibit a significant change in gene expression (Fig. 3.3). In addition, a general increase in the level of expression change was also seen at most genes (Fig. 3.4 and Supplementary Data 1). Taken together these results suggest that SIN3 and dKDM5/LID can regulate a large number cell cycle related genes in a coordinated manner. The additive regulatory effects of both SIN3 and dKDM5/LID seems important at these genes to bring about significant changes in gene expression.

KEGG pathway analysis of genes regulated by double knockdown of *Sin3A* and *lid* identified DNA replication (*P*-value: 6.00-E-05), mismatch repair (*P*-value: 0.0036) and endocytosis (*P*-value: 0.0337) to be the most enriched pathways. In addition,

similar to single knockdown of *Sin3A*, multiple metabolic pathways, namely, pyrimidine metabolism (*P*-value: 0.0337), glutathione metabolism (*P*-value: 0.0360), glycolysis or gluconeogenesis (*P*-value: 0.0675), cysteine and methionine metabolism (*P*-value: 0.0825) and fatty acid metabolism (*P*-value: 0.0870) were enriched upon double knockdown.

Expression profiles upon knockdown of *Sin3A* or *lid* and induction of oxidative stress share similarities

Based on the literature linking both SIN3 and dKDM5/LID to oxidative stress response, we wished to identify genes regulated by these proteins under conditions of oxidative stress. To test for a transcriptional response upon activation of oxidative stress, we treated cells subjected to RNAi knockdown with 8.3 mM paraquat (1,1′-Dimethyl-4,4′-bipyridinium dichloride (Sigma Aldrich)) for 24 hr. Paraquat treatment generates the reactive oxygen species (ROS), superoxide anion, where accumulation of ROS results in oxidative stress in the cell. Isolated mRNA from S2 cells treated with paraquat in addition to dsRNA against GFP as control, *Sin3A*, *lid* or both were subjected to RNAseq analysis. Induction of oxidative stress in control cells resulted in expression changes of 212 genes (Table 3.1 and Fig. 3.6C).

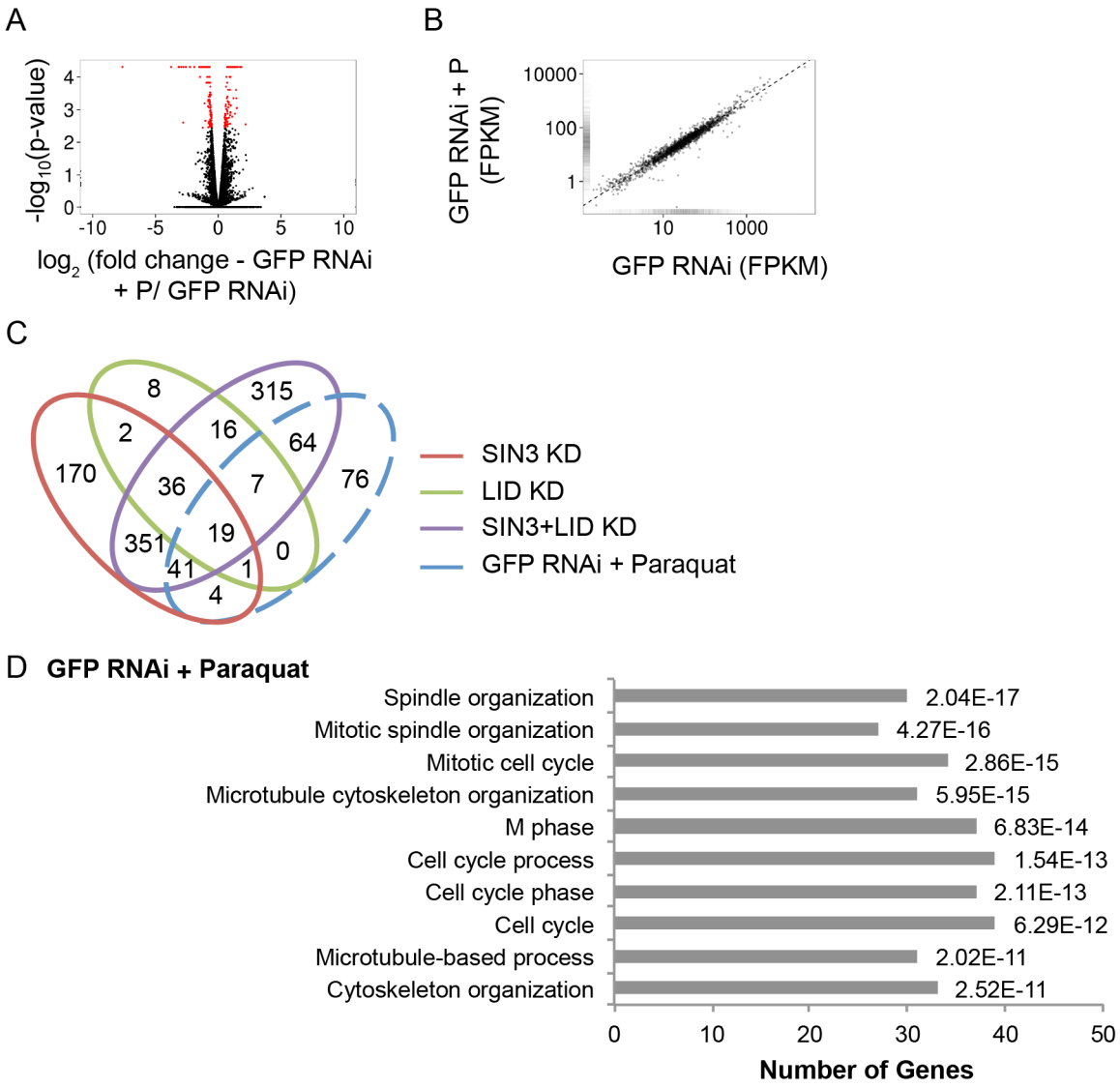


Figure 3.6: RNAseq analysis identifies genes regulated by induction of paraquat mediated oxidative stress in S2 cells. (A) Volcano plot depicting log fold change in expression. Red – significant change in expression, Black – no significant change in expression. (B) Scatter plot depicting expression trends of all significantly regulated genes. (C) Venn Diagram indicating significant overlap of genes regulated by SIN3, dKDM5/LID, both and stress. KD – knockdown (D) Gene Ontology (GO) analysis of genes regulated upon induction of paraquat mediated oxidative stress. *P*-value for each enriched category is displayed on the right of the bars. KD – knockdown, P - paraquat.

Upon comparing SIN3 or dKDM5/LID regulated genes to genes misexpressed upon induction of oxidative stress considerable overlap was noted. 65 genes regulated

by SIN3 and 27 genes regulated by dKDM5/LID showed regulation upon paraquat treatment. 131 differentially expressed genes showed an overlap between double knockdown of SIN3 and dKDM5/LID and paraquat treatment.

Furthermore, GO analysis of regulated genes upon paraquat treatment showed a high enrichment for cell cycle related processes, similar to what was observed for dual knockdown of *Sin3A* and *lid* (Fig. 3.5C and 3.6D). In addition, the heat shock response genes misregulated in both *Sin3A* or *lid* knockdowns were also misregulated upon paraquat treatment. KEGG pathway analysis of genes regulated upon paraquat treatment identified ribosome (*P*-value: 1.45E-07), DNA replication (*P*-value: 1.35E-05), mismatch repair (*P*-value: 2.85E-05), glycolysis or gluconeogenesis (*P*-value: 3.36E-04), fructose and mannose metabolism (*P*-value: 0.0213) and nucleotide excision repair (*P*-value: 0.0319) pathways to be enriched. Considerable overlap is thus seen between enriched pathways for genes regulated by paraquat treatment and dual knockdown of *Sin3A* and *lid*. These results implicate a critical role for SIN3 and dKDM5/LID in stress tolerance and suggest that the loss of these proteins may partially mimic oxidative stress conditions in the cell.

RNAseq identifies a transcriptional profile associated with SIN3 and dKDM5/LID upon oxidative stress

Analysis of differential gene expression upon reduced levels of SIN3 or dKDM5/LID under oxidative stress conditions identified a large number of regulated genes. As above, an FDR cutoff of 0.05 was used to determine significantly regulated genes. A minimum of 1.4 fold change in expression was observed for all genes

identified as significantly regulated. 1136 genes changed in expression upon *Sin3A* knockdown compared to non stressed controls (Table 3.1). 514 (45%) genes were upregulated while 622 (55%) were downregulated. The equal distribution of genes that were repressed or activated by SIN3 under oxidative stress is similar to the trend observed under non stressed conditions. *lid* knockdown upon paraquat mediated oxidative stress conditions resulted in the misregulation of 357 genes where 141 (39%) genes were upregulated while 216 (61%) were downregulated. Observed gene expression changes under both normal and conditions of oxidative stress indicate that dKDM5/LID predominantly acts as an activator of gene transcription. In oxidative stress conditions, however, a near 10% increase in the proportion of genes repressed is observed. 1308 genes changed in expression upon double knockdown of *Sin3A* and *lid* compared to non stressed controls. Of these 622 (48%) were upregulated while 686 (52%) were downregulated.

Of the 1136 genes regulated by SIN3 in conditions of oxidative stress, 588 were genes that did not significantly change upon *Sin3A* knockdown under non stressed conditions or upon induction of stress alone (Fig. 3.7G). Of the 358 genes regulated by dKDM5/LID under oxidative stress conditions, 185 did not alter in expression in non stressed conditions or under stress alone (Fig. 3.7H). Upon double knockdown of *Sin3A* and *lid* in oxidative stress conditions, 594 of 1308 genes did not significantly change under non stressed conditions or upon induction of stress alone (Fig. 3.7I). Overall a large number of additional genes were regulated upon knockdown of *Sin3A*, *lid* or both in an environment of oxidative stress. In addition to a larger number of genes being regulated under paraquat mediated oxidative stress conditions, an increase in the level

of gene expression changes was also noted at many genes (Fig. 3.4 and Supplementary Data 1).

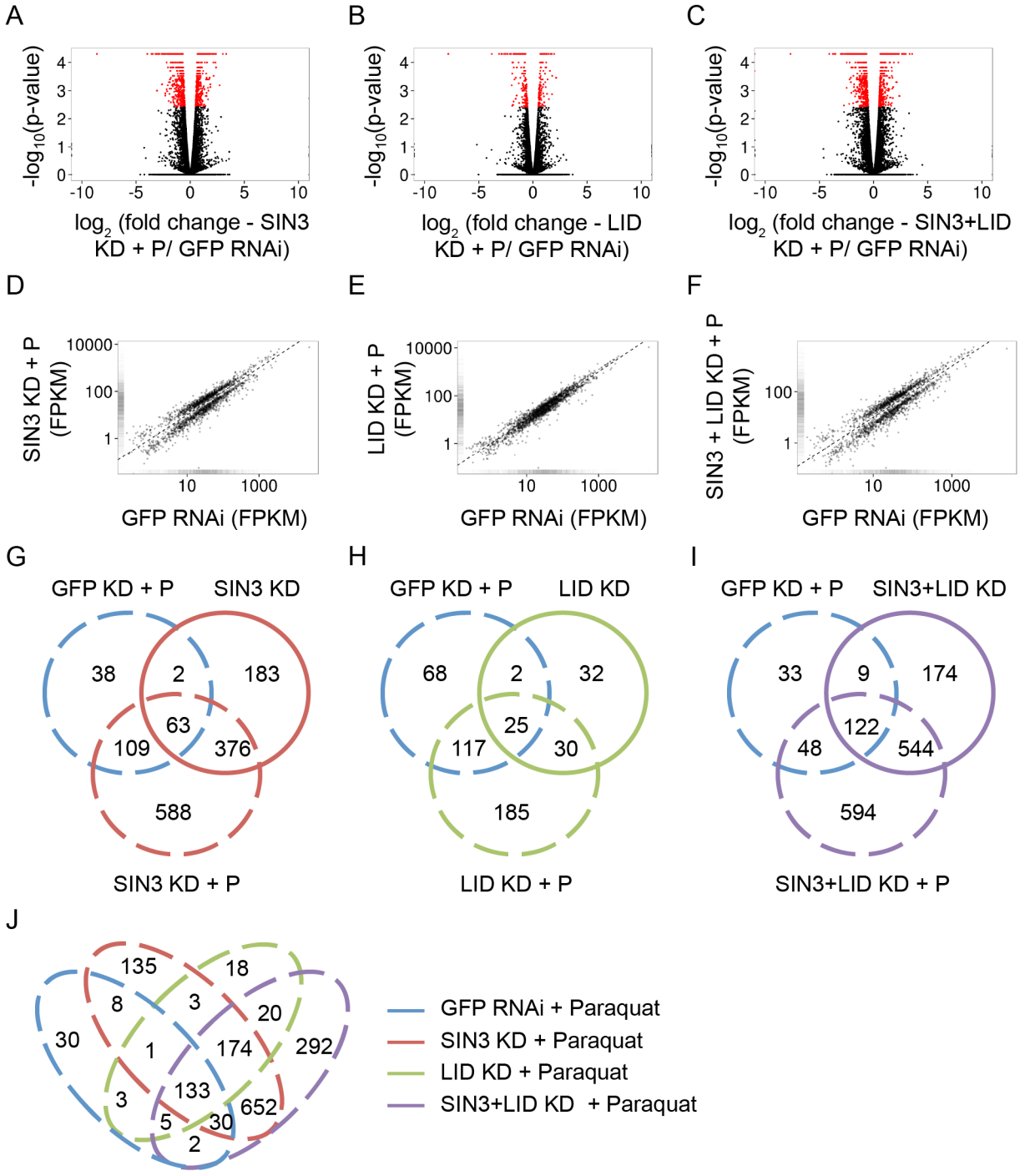


Figure 3.7: RNAseq analysis identifies genes regulated by SIN3, dKDM5/LID or both under paraquat mediated oxidative stress conditions in S2 cells. (A-C) Volcano plots depicting log fold change in expression of indicated samples. Red – significant change in expression, Black – no significant change in expression. (D-F) Scatter plots depicting expression trends of all significantly regulated genes of indicated samples. (G-J) Venn Diagrams indicating significant overlap of genes regulated upon indicated conditions. KD – knockdown, P – paraquat.

GO analysis of genes regulated by SIN3, dKDM5/LID or both under conditions of oxidative stress identified similar processes related to cell cycle regulation to be enriched under all knockdown conditions (Fig. 3.8). This was in contrast to normal conditions, where cell cycle related processes were most enriched only upon double knockdown of *Sin3A* and *lid*. The enrichment of genes involved cell cycle related processes, however, is a common trend observed under stress environments as induction of paraquat mediated stress alone lead to similar results.

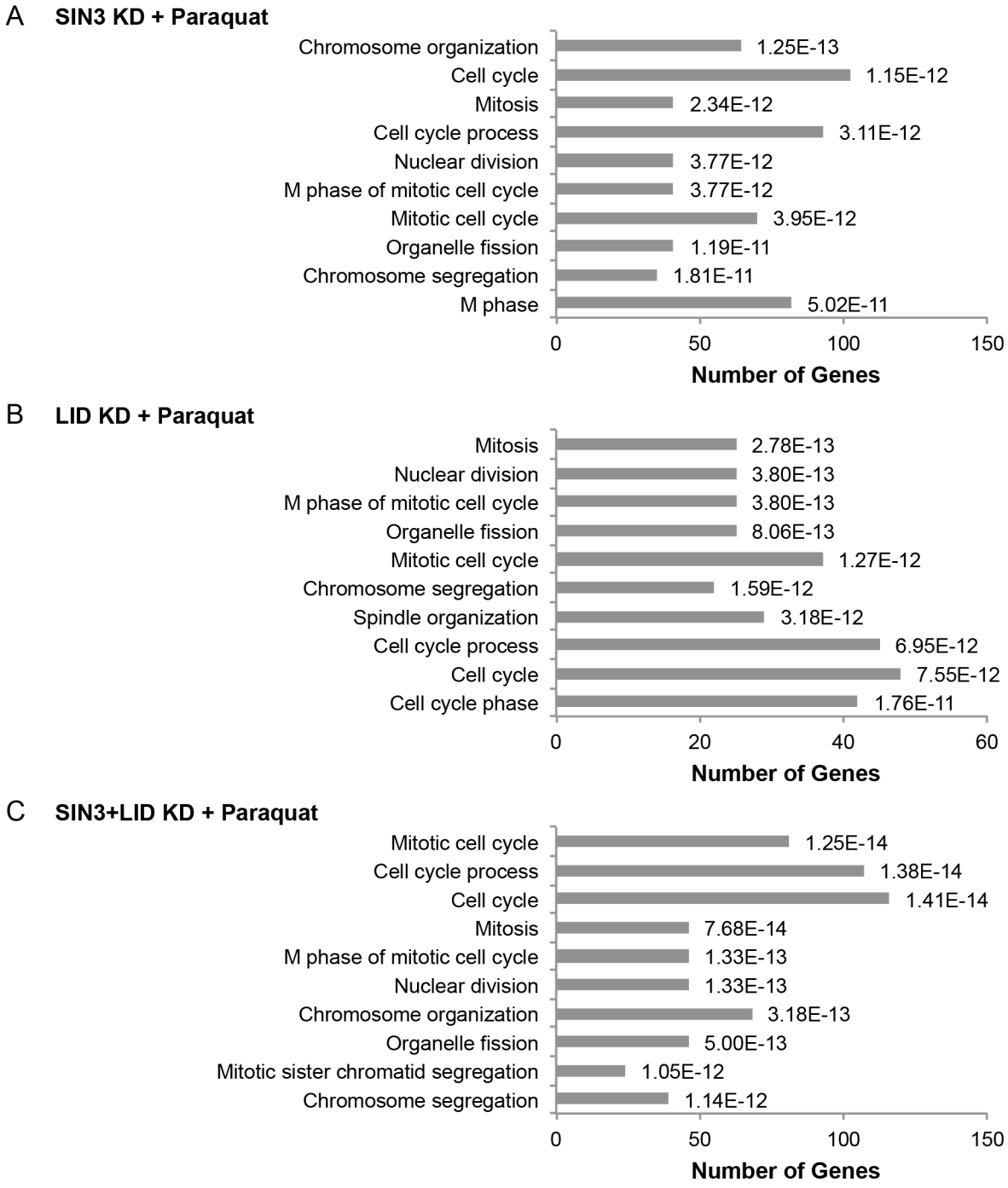


Figure 3.8: Genes regulated by SIN3, dKDM5/LID or both under paraquat mediated stress conditions are enriched in cell cycle related processes. (A-C) Gene Ontology (GO) analysis of genes regulated by SIN3, LID or both as indicated. *P*-value for each enriched category is displayed on the right of the bars. KD – knockdown.

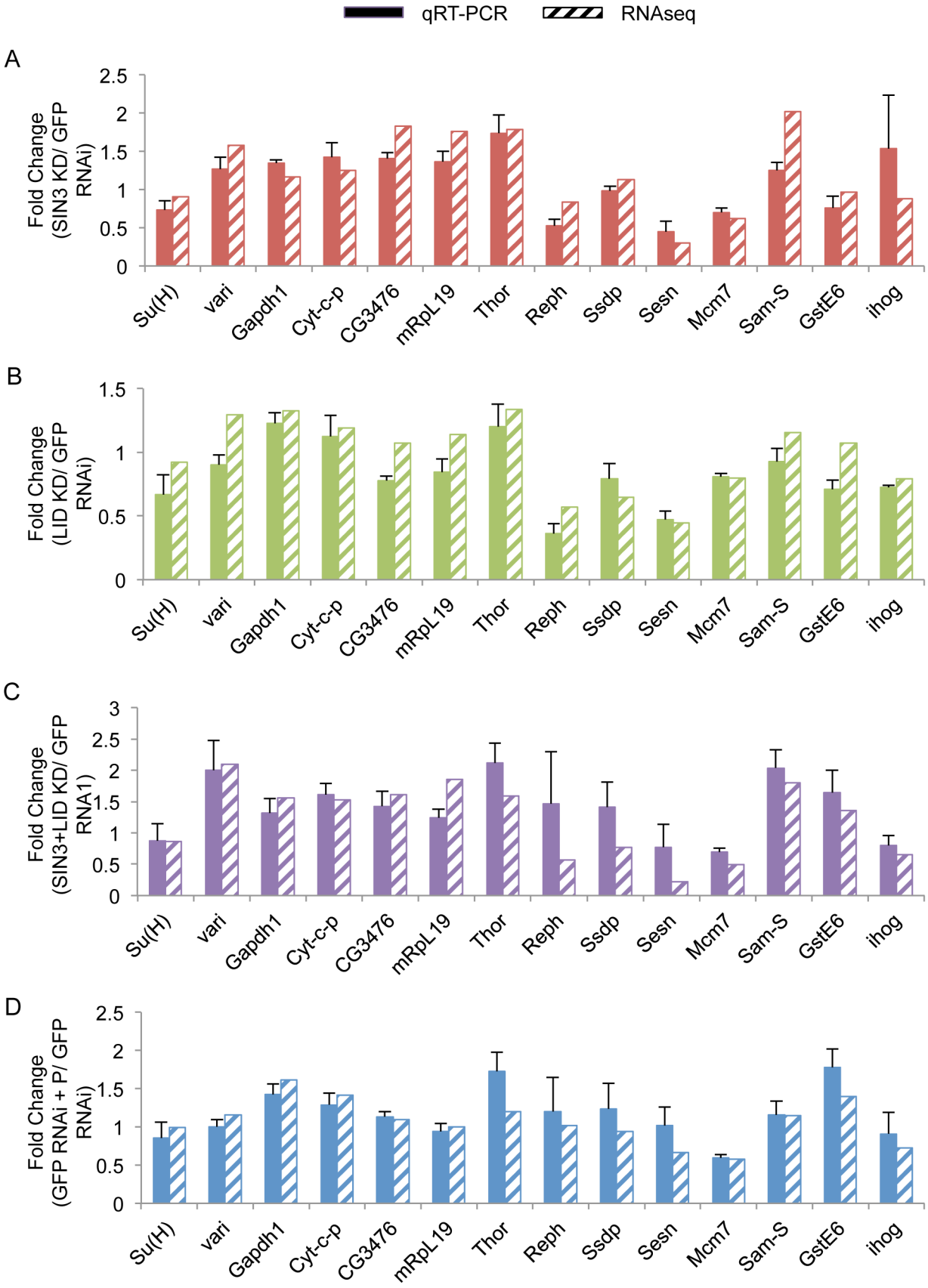
As knockdown of *Sin3A* and *lid* both affect cell proliferation in S2 cells and wing imaginal disc tissues as described in Chapter 2, the observed enrichment of genes involved in cell cycle related processes that are regulated by SIN3 and LID are of specific interest. In *Drosophila*, RNAi mediated knockdown of *Sin3A* leads to cell cycle arrest at the G2/M phase (Pile et al., 2002). Similarly mSin3A affects the G2/M transition of the cell cycle (Dannenberg et al., 2005). Our RNAseq data identified several genes implicated in the G2/M transition of the cell cycle. In *Drosophila*, proper levels of Cyclins A and B (CycA and CycB) and phosphorylated cyclin dependent kinase 1 (cdk1) are important for the G2/M phase transition (Lee and Orr-Weaver, 2003). String (STG) is a phosphatase that can remove an inhibitory phosphate on cdk1 and thereby promote G2/M transition (Chen et al., 2007; Edgar and O'Farrell, 1990). Our RNAseq results show that *CycA* is significantly downregulated upon dual knockdown of *Sin3A* and *lid* under induction of oxidative stress. *CycB* is downregulated upon knockdown of *Sin3A* or double knockdown of *Sin3A* and *lid* under oxidative stress conditions. Further, *Cdk1* is significantly downregulated upon double knockdown of *Sin3A* and *lid* as well as upon induction of oxidative stress. *Stg* is downregulated upon double knockdown of *Sin3A* and *lid* under oxidative stress conditions. Only small (statistically non significant) decreases in expression of these genes were noted upon knockdown of Sin3A or lid under normal conditions. These changes in expression, however, could be biologically relevant considering the observed cell proliferation defects described in Chapter 2. Surprisingly, *grapes* (*grp*), encoding a checkpoint kinase that can inhibit STG function when activated, was also downregulated upon dual knockdown of *Sin3A* and *lid* and upon induction of oxidative stress.

In mammals KDM5 proteins have been linked to cell cycle regulation through the E2F/RB pathway, which affects G1/S transition (Benevolenskaya, 2007). In *Drosophila*, Cyclin E (CycE) and Cdk2 promote the G1/S transition (Lee and Orr-Weaver, 2003). Cdk4 can also promote progression to S phase by phosphorylating the tumor suppressor Rb, which in turn disrupts is association with E2F (Coqueret, 2002). We find that *CycE* is significantly downregulated upon dual knockdown of *Sin3A* or double knockdown of *Sin3A* and *lid* under oxidative stress conditions. *Cdk2* is significantly downregulated upon knockdown of *Sin3A* or double knockdown of *Sin3A* and *lid* under oxidative stress conditions. *Cdk4* is significantly downregulated upon double knockdown of *Sin3A* and *lid* as well as upon induction of oxidative stress. Interestingly, *Dacapo* (*dap*), encoding an inhibitor of the CycE/Cdk2 kinase activity, is also downregulated upon double knockdown of *Sin3A* and *lid* under oxidative stress conditions. Down regulation of both activators and inhibitors of cell cycle progression upon knockdown of *Sin3A* and or *lid* suggests that these proteins are involved in activating many cell cycle regulatory genes. The combinatorial expression levels of these regulators in turn determine proper progression of the cell cycle.

Real time qRT-PCR validates RNAseq results

To verify RNAseq data we repeated the RNAi knockdown experiments under normal or paraquat induced oxidative stress conditions. Isolated RNA was analyzed by real-time RT-PCR to determine if expression trends observed by RNAseq analysis were reproducible by real time qRT-PCR. For this purpose, we tested 15 candidate genes that were identified as regulated by SIN3 alone, dKDM5/LID alone or both, or showed

increased expression levels upon paraquat treatment (Fig. 3.9). Taf1, a component of the basal transcription machinery, which did not show significant changes in any of the tested conditions based on RNAseq data and previous microarray expression data for knockdown of *Sin3A*, was used to normalize expression across samples. Real time qRT-PCR results validated the expression trends observed by RNAseq.


qRT-PCR
RNAseq
A
Fold Change (SIN3 KD/ GFP RNAi)
B
Fold Change (LID KD/ GFP RNAi)
C
Fold Change (SIN3+LID KD/ GFP RNA1)
D
Fold Change (GFP RNAi + P/ GFP RNAi)
Su(H)
vari
Gapdh1
Cyt-c-p
CG3476
mRpL19
Thor
Reph
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Mcm7
Sam-S
GstE6
ihog

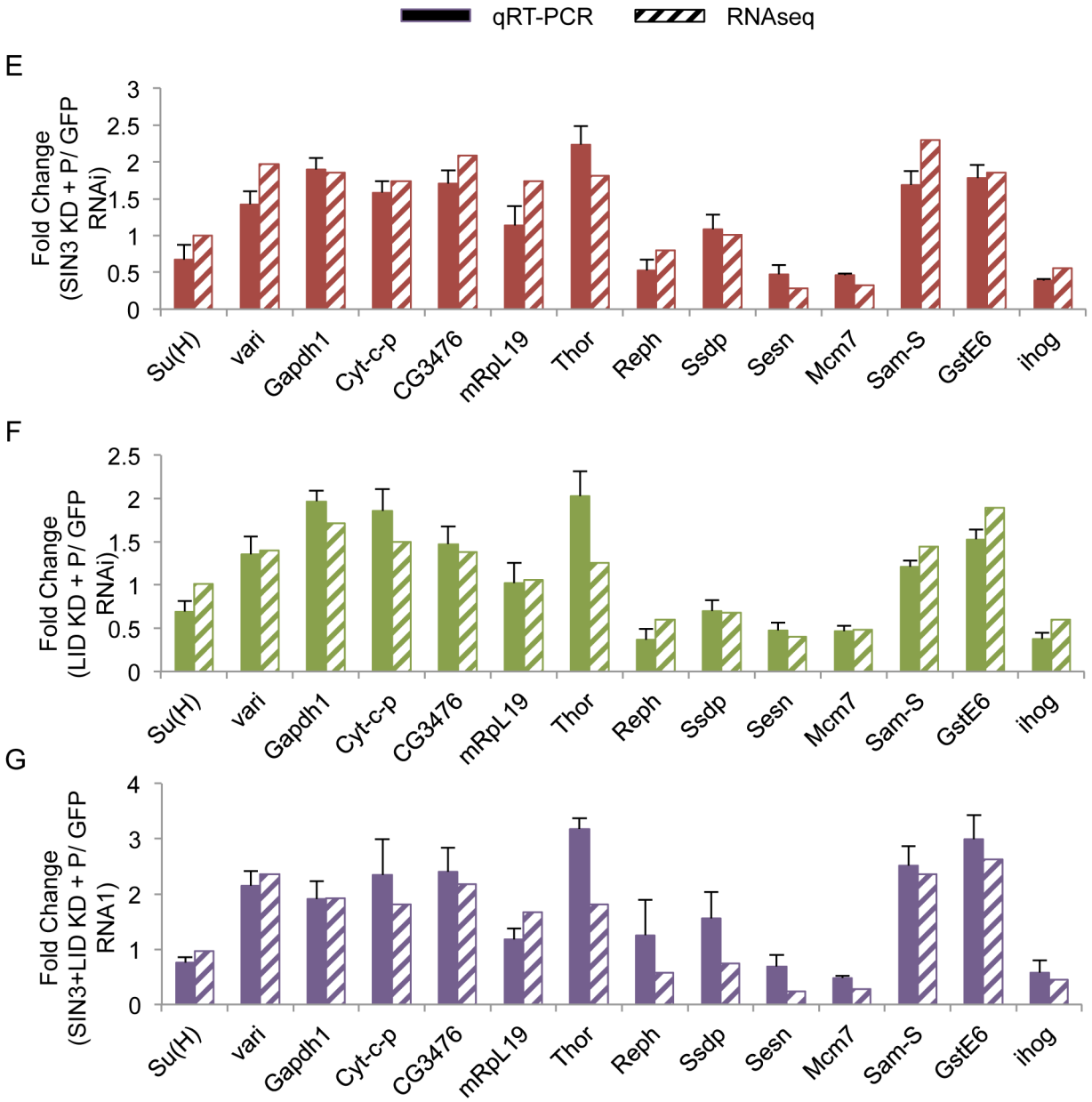

Figure 3.9: Real time qRT-PCR validates RNAseq data. Real time quantitative RT-PCR analysis of total RNA extracts from S2 cells treated with dsRNA targeting indicated genes to induce knockdown (KD) under non stressed conditions (A) or paraquat induced oxidative stress conditions (B). All treated samples (Exp, experimental) were compared to non stressed GFP dsRNA treated controls. Primers targeting indicated genes were used for PCR amplification. The results are the average of five biological replicates. Error bars represent standard error of the mean. KD – knockdown.

The RNAseq data has enabled us to identify a large number of genes regulated by SIN3 and dKDM5/LID. We identified multiple regulated genes involved in key processes affected by SIN3 and dKDM5/LID such as cell cycle regulation and tolerance to stress. The identified SIN3 and dKDM5/LID target genes can greatly aid in dissecting the roles of SIN3 and dKDM5/LID in said cellular pathways. Our findings therefore, provide a great framework for analyzing the transcriptional network through, which SIN3 and dKDM5/LID affect diverse functions in the cell.

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CHAPTER 4

REGULATION OF HISTONE MODIFICATIONS AT SIN3 AND/OR dKDM5/LID TARGET GENES IN *DROSOPHILA* S2 CULTURED CELLS

INTRODUCTION

DNA is compacted and packaged within the nucleus with the aid of histone proteins forming chromatin, a nucleoprotein complex. The basic repeating unit of chromatin is formed by 147 bp of DNA that wraps around a histone octamer composed of two copies each of histones H2A, H2B, H3 and H4 (Kornberg, 1974; Luger et al., 1997). The linker histone H1 binds linker DNA present between adjacent nucleosomes and helps in higher order chromatin folding (Szerlong and Hansen, 2011). While enabling packaging, the formation of nucleosomes, however, hinders DNA dependent processes, such as transcription, DNA replication and repair by preventing accessibility to the DNA template (Luger et al., 2012). To retain chromatin structure while allowing DNA accessibility, chromatin is dynamically regulated. The role of histone post-translational modifications (PTMs) brought about by histone modifying enzymes in regulating chromatin structure has been under study for the past few decades (Zentner and Henikoff, 2013). Over a hundred different histone modifications have been identified and novel histone PTMs are still being discovered.

Most histone modifications discovered occur on amino acid residues of the more accessible N-terminal tails of histones that protrude from the nucleosome core (Kouzarides, 2007). Acetylation, methylation, phosphorylation, ubiquitylation and sumoylation are some such histone modifications. Multiple writer proteins can put on these modifications. A large number of reader proteins can recognize these

modifications and recruit multiple other proteins, thereby controlling downstream DNA dependent processes such as transcription (Bannister and Kouzarides, 2011). Histone PTMs, based on the type of modification and the amino acid residue modified, have been linked to different transcriptional outcomes. Marks such as histone acetylation and methylation of H3K4, H3K36 and H3K79 are associated with euchromatin while methylation of H3K9, H3K27 and H4K20 is associated with heterochromatin (Kouzarides, 2007). Genome wide studies in *Drosophila* identified multiple chromatin modifications along different regions of actively transcribed genes (Filion et al., 2010; Kharchenko et al., 2011). Promoter proximal regions are enriched for H3K4me2/me3 and H3K9ac, while exons are enriched for the transcriptional elongation mark H3K36me3. H3K27ac, H3K4me1 and H3K18ac mark intronic regions. The X chromosome is enriched for H4K16ac along with the elongation mark H3K36me3.

SIN3 is the scaffold protein of a histone modifying complex and co-purifies with the catalytic components RPD3 and dKDM5/LID in *Drosophila* (Moshkin et al., 2009; Spain et al., 2010). Studies in yeast highlight a lack of substrate specificity by RPD3 for its deacetylase function (Kadosh and Struhl, 1998; Rundlett et al., 1996). RPD3 deacetylates lysine residues of histone H3 and H4 with greater effect at H3K9, H4K5 and H4K12. dKDM5/LID, in contrast specifically removes H3K4me3 (Eissenberg et al., 2007; Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007). The mammalian homologs of dKDM5/LID, however, demethylate both H3K4me2 and me3 (Christensen et al., 2007; Hayakawa et al., 2007; Klose et al., 2007).

In mammals, two distinct genes code for Sin3A and Sin3B proteins (Ayer et al., 1995). KDM5A, the mammalian homolog of dKDM5/LID, associates with Sin3B

(Hayakawa et al., 2007). Early work in mammals suggested that SIN3 is recruited to gene promoters by sequence specific DNA binding proteins (Ayer et al., 1995; Silverstein and Ekwall, 2005). Genome wide ChIP-chip analysis indicates that both Sin3A and Sin3B bind predominantly to regions downstream of the TSS (van Oevelen et al., 2008). Genes repressed by SIN3 were bound immediately downstream of the TSS, while SIN3 spread further downstream at non repressed genes. KDM5A was also found to colocalize at a large proportion of Sin3 target genes (van Oevelen et al., 2008). SIN3 and KDM5A bind at overlapping positions immediately downstream of the TSS. Genome wide binding analysis of SIN3 in *Drosophila* embryos (0-12 hr) and cultured cells (Kc167) also indicates binding near the TSS at a large proportion of gene targets (Celniker et al., 2009; Filion et al., 2010). ChIPseq analysis of *Drosophila* wing imaginal disc tissue revealed a large number of binding sites for dKDM5/LID where majority mapped to the TSS of gene targets (Lloret-Llinares et al., 2012). Roughly 80% of dKDM5/LID targets were also enriched for H3K4me3. Depletion of dKDM5/LID by RNAi resulted in an increase in abundance of H3K4me3 at target genes.

In Chapter 3, we described the identification of a large number of genes that are regulated by SIN3, dKDM5/LID or both. We wished to determine if these genes are directly bound by SIN3 or dKDM5/LID as well as investigate associated changes in histone acetylation and methylation patterns at gene targets. While genome wide binding patterns of *Drosophila* SIN3 and dKDM5/LID has been analyzed previously, these studies were not conducted using S2 cultured cells, which were used in our gene expression analysis (Celniker et al., 2009; Filion et al., 2010; Lloret-Llinares et al., 2012). As different tissue and cell types can show variation in gene expression

regulation, we wished to investigate SIN3 and dKDM5/LID binding at candidate genes utilizing ChIP-qPCR in S2 cells. Furthermore, while genome wide changes in H3K4me3 levels upon *lid* knockdown were mapped in wing imaginal discs, changes in histone modification patterns at specific gene promoters upon depletion of *Sin3A* have not been tested. We utilized ChIP-qPCR to identify changes in acetylation and methylation of H3K4 at target genes upon depletion of SIN3 and dKDM5/LID proteins. We find that both SIN3 and dKDM5/LID bind around the TSS of several gene targets. Depletion of SIN3 results in increased histone acetylation at some genes while no significant changes in histone marks are observed upon *lid* knockdown.

MATERIALS AND METHODS

Cell Culture

Cell lines utilized and culture conditions are described in Chapter 2.

RNA interference

RNAi methodology and dsRNA constructs are described in Chapter 2.

Western Blotting

Whole cell protein extract preparation and western blotting methodology is described in Chapter 2. For protein extracts enriched for histones, 5 x 10^7 cells were pelleted by centrifugation at 1250xg for 5 min and resuspended in 500 μl of 0.4N sulfuric acid. Resuspended cells were incubated on ice for 30 min and subjected to centrifugation at 12000xg for 10 min. The pellet was resuspended in 1.9 ml glacial acetic acid and dialyzed overnight in 1 L of dH_2O at 4^0C. Samples were further dialyzed

2 x 2 hr in 2 L of dH_2O and precipitated in 100 μl TCA per 500 μl of protein sample and incubated overnight at 4^0C. Precipitated samples were spun at 16000xg for 10 min and the pellet was resuspended in 100 μl of Laemmli sample buffer (Bio-Rad). 1 μl of unbuffered Tris base was added to neutralize the acid. 5 – 10 μg of histone enriched protein samples were separated on a 15% SDS-polyacrylamide gel and subjected to western blot analysis. Antibodies utilized are listed below.

Primary antibodies: HA-HRP (1:6000; Roche, 2013819), SIN3 (1:2000; (Pile and Wassarman, 2000)), alpha-tubulin (1:1000; Cell Signaling, 2144), H3 (1:30,000; Abcam, ab1791), H4 (1:30,000; Abcam, ab10158) H3K9/K14ac (1:5000; Millipore, 06-599), H3K9ac (1:6000; Millipore, 07-352), H3K4me2 (1:2000; Millipore, 07-030), H4K4me3 (1:2000; Active Motif, 39159)

Secondary antibody where applicable: donkey anti-rabbit HRP-conjugated IgG (1:3000; GE Healthcare, NA9340)

Drosophila Stocks

Drosophila melanogaster stocks were maintained, and crosses were performed according to standard laboratory procedures. The following stocks were used: En-GAL4 (8828) obtained from the Bloomington Stock Center and UAS-LID$^{RNAi\text{-}KK}$ (103830) obtained from Vienna *Drosophila* RNAi Center.

Immunostaining

Methodology is described in Chapter 2. Antibodies against histone H3 lysine 4 trimethylation (1:500; Active Motif, 39159) followed by donkey anti-rabbit Alexa 594

(1:1000; Life Technologies, A21207) were used for staining.

Chromatin immunoprecipitation and real-time quantitative PCR

4 x 10^7 cells were crosslinked with 1% formaldehyde for 10 min and quenched with 125 mM glycine. The cells were then pelleted and washed 3 times with 1 X PBS, with centrifugation each time at 1250xg at 4^0C for 5 min. The obtained pellet was resuspended in 15 ml of resuspension buffer (10 mM Tris (pH 8), 10 mM KCl, 3 mM $CaCl_2$, 0.34 M Sucrose, 1 mM DTT, 0.1% Triton X-100, 0.2 mM EGTA, 1 Roche complete protease inhibitor tablet) and incubated on ice for 15 min. The resuspended cells were then homogenized by a dounce homogenizer using a loose pestle 10 times and a tight pestle 15 times. The homogenized cells were pelleted at 170xg at 4^0C for 10 min. The pellet was then resuspended in 200 µl of 10X MNase digest buffer (15 mM Tris (pH 8), 60 mM KCl, 15 mM NaCl, 1mM $CaCl_2$, 0.25 M sucrose, 1 mM DTT) and subjected to MNase digestion using 20 units of MNase for 10 min at room temperature. 10 mM EDTA was added to stop the reaction. Samples were diluted with NaCl buffer (140 mM NaCl, 10 mM Tris (pH 7.6), 2 mM EDTA) to a final volume of 1.2 ml and subjected to sonication for seven 30 sec pulses with 1 min intervals at 20% amplitude using an Ultrasonic dismembrator (Model 500 (Fisher Scientific)) sonicator. Sonicated samples were subjected to centrifugation at 15,000xg for 15 min at 4^0C and the pellet was discarded. 75 µg of prepared chromatin was diluted to a final volume of 500 µl with NaCl buffer and used for immunoprecipitation. For immunoprecipitation of HA tagged proteins 30 µl of anti-HA beads (monoclonal anti-HA agarose conjugate clone HA-7 (Sigma, A2095)) were added to 500 µl of prepared chromatin samples and placed on a

nutator at 4^0C for 4 hr. For immunoprecipitation of native protein or modified histones, antibodies specific to SIN3 ((Pile and Wassarman, 2000) (5 µg)), H3 C-terminus (Abcam, ab1791 (4 µg)), H3K9Ac (Millipore, 07-352 (3 µl)), H3K4Me2 (Millipore, 07-030 (3 µl)), H3K4Me3 (Active Motif, 39159 (3 µl)) or pre-immune IgG (2.5 µg) as control was added to 500 µl of prepared chromatin samples and placed on a nutator at 4^0C overnight. 30 µl of anti-IgG beads (Protein A agarose (Pierce)) were then added to antibody treated chromatin samples and the tubes were placed on a nutator at 4^0C for 4 hr. Anti-HA or anti-IgG beads were then washed with Wash 1 buffer (50 mM Tris (pH 7.6), 280 mM NaCl, 2 mM EDTA, 0.3% sodium dodecyl sulfide), Wash 2 buffer (25 mM Tris (pH 7.6), 500 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100) and Wash 3 buffer (10 mM Tris (pH 7.6), 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% Triton X-100) for 10 min each at 4^0C. Finally beads were rinsed with Tris-EDTA (pH 8.0) and eluted with 500 µl of elution buffer (1% sodium dodecyl sulfide, 0.1 M $NaHCO_3$) at 65^0C for 1 hr. Eluted samples were treated with 0.05 µg/µl RNase A at 37^0C for 15 min and DNA:protein crosslinks were reversed by overnight incubation at 65^0C after addition of 200 mM NaCl. Samples were Proteinase K treated (0.04 µM Proteinase K, 10 µM EDTA, 20 µM Tris (pH 7.5)) at 45^0C for 1.5 hr and subjected to phenol chloroform extraction and ethanol precipitation. Precipitated DNA was resuspended in 50 µl of ddH_20. Input DNA was prepared from 75 µg of chromatin samples directly after RNase treatment and reversal of crosslinks as described above. Input DNA (diluted 1:100) and immunoprecipitated samples (diluted 1:4) were subjected to real-time quantitative PCR with Absolute SYBR Green ROX master mix (Fisher Scientific) using a Stratagene Mx3005P real-time thermocycler. The following primer

sets (5' to 3' – forward and reverse) were used in the PCR reactions:

Su(H) (CCA CTG CCA TCC AAA TCC and GCG GCT GGC TTC TGT TTA)

vari (CCC AAC AAA GAA GTG GCG and TCC AAC AGC GCA AAA ACA)

Cyt-c-p (GCA AAT TTT CCA GAG GCT TTC and GCC GAT TTT TCA CGA ATG AC)

CG3476 (CTG CAA TCG ATA GCT GAA TGT and GCG CGG TAT TAT AAT TTC CAT)

mRpL19 (TGG CAG TAC CCT TCC AAT TAT and ACA CAC TGC TGT GTC AAC CTA TT)

Thor (CGA GAG AGC AGG CGA AAG and TGT GTT CAC CGT TGG CTG)

Ssdp (CAC TGA AAA TGG CGT GCT T and AAG TTG CGT CGT CGT CGT)

Sesn (TCG TTG CGA TTC GTT TCA and CGC TTT TCT AGC CGG ACA)

Hsp27 (CCT GGT TGC CAT GCA CTA and TGC TTC AAC GTT TGC CTT C)

Mcm7 (ACA CCT TTG GCA AGC AGC and ATT CCG CCA GAT CAT CCA)

Sam-S (TTG AAC GCA GGT TGA GCA and CGC TCC GGA GTG AAC TGT)

GstE6 (TTT TCT CTT TCA TTG ATC CCA A and GAC TGG GGT CCA AAC CGT)

ihog (TCC ACT GTA CCG CGA TGA and GGG ATG CTG GAA CTG GAA)

CG31819 (AGC GCT GCC AGA AGA AGA and GGT CAA AGT CTC CCA ATT TTC A)

Statistical analyses

All significance values were calculated by the two sample Student's *t*-test using GraphPad. http://www.graphpad.com/quickcalcs/index.cfm.

RESULTS AND DISCUSSION

In Chapter 2, we described biochemical and genetic interactions of SIN3 and

dKDM5/LID. We further showed that reduction of these proteins result in similar phenotypes during cell proliferation and wing development. In Chapter 3, we described the identification of multiple gene targets that are regulated by both proteins. Based on these observations, we asked if there are underlying changes at the chromatin template upon misexpression of these proteins.

dKDM5/LID specifically decreases global levels of H3K4Me3

The SIN3 complex acts as a global transcriptional repressor (Silverstein and Ekwall, 2005). We and others have shown that *Drosophila* SIN3 interacts with both an HDAC, RPD3, and a KDM, dKDM5/LID (Moshkin et al., 2009; Spain et al., 2010). The link for histone acetylation in transcription activation is well established (Verdin and Ott, 2014). The HDAC activity of RPD3 has been well characterized, where RPD3 contributes to the formation of a repressive environment at target genes (Kadosh and Struhl, 1998; Yang and Seto, 2008). The role of methylation in transcription, however, is determined by the amino acid residue modified. In *Drosophila*, work by several groups indicates that dKDM5/LID, a JmjC domain containing KDM, specifically removes the histone H3K4 trimethyl mark, which is associated with active transcription (Eissenberg et al., 2007; Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007). In addition, homozygous *lid* mutant larvae have decreased enrichment of histone H3 acetylation along polytene chromosomes (Lloret-Llinares et al., 2008). While dKDM5/LID is not thought to have HDAC activity itself, this result suggests that methylation and acetylation might be coordinately regulated. That possibility, however, has not been universally supported by experiments performed in other cell types. In

contrast to the observation in polytene chromosomes, alterations in global histone acetylation levels were not observed in extracts prepared from *lid* knockdown flies (Eissenberg et al., 2007).

Based on the association of dKDM5/LID with an HDAC complex, we sought to test the possible role of dKDM5/LID in regulation of histone acetylation in *Drosophila* S2 cells as well as to verify the role in regulating histone methylation. For this purpose, we performed western blot analysis of protein extracts from control S2 cells, *lid* knockdown or dKDM5/LID FLAG-HA overexpressing cells. *lid* knockdown was achieved by RNAi and verified by real time qRT-PCR (Fig. 3.1 and data not shown). Global levels of histone H3K4me3 increased while H3K4me2, H3K9ac and H3K14ac remained unchanged upon decreased expression of dKDM5/LID relative to mock treated samples (Fig. 4.1A and B). Consistent with these data, increased expression of dKDM5/LID results in lowered H3K4me3, with no changes in other modifications tested (Fig. 4.1A and B). Based on these results, we conclude that dKDM5/LID is a histone H3K4me3 specific demethylase that does not affect global levels of acetylation in S2 cells. To demonstrate the histone H3K4 demethylase activity of dKDM5/LID in the developing fly, we performed H3K4me3 immunostaining of wing imaginal discs in *Drosophila* larvae with altered levels of dKDM5/LID (Fig. 4.1C). For this analysis, we again utilized the GAL4-UAS system in flies, described in Chapter 2, to knock down *lid* in the posterior region of the wing imaginal discs using an En-GAL4 driver. As expected, upon knock down of *lid,* we saw increased staining of H3K4me3 in the posterior region of the wing imaginal discs compared to the anterior region, which expressed wild type levels of dKDM5/LID.

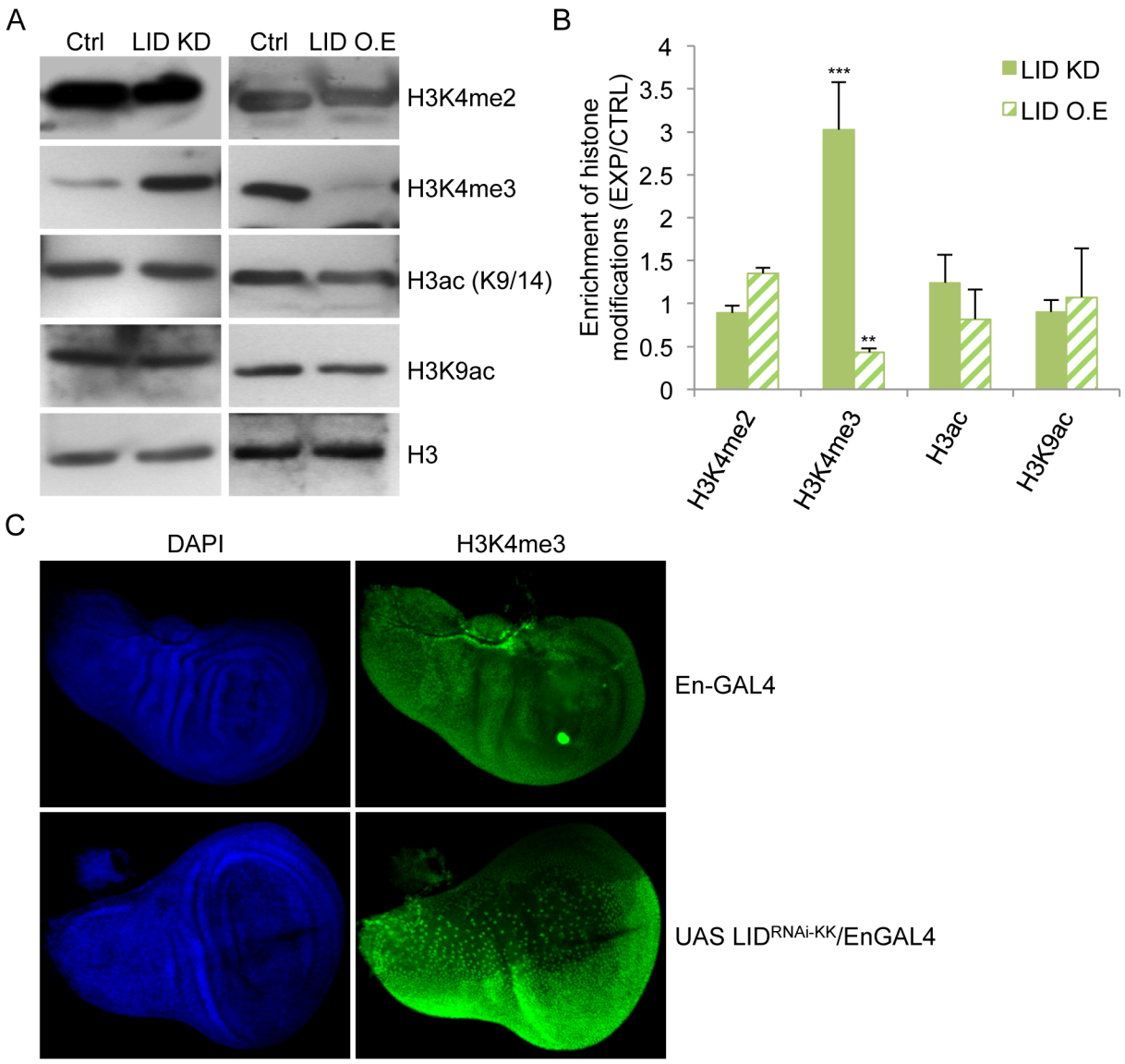

Figure 4.1: dKDM5/LID acts as an H3K4me3 specific demethylase in *Drosophila*. (A) Western blot analysis of histone extracts from S2 cells mock treated (CTRL) or treated with dsRNA targeting *lid* (LID KD) (left panel) or whole cell protein extracts from control S2 cells (CTRL) or S2 cells overexpressing dKDM5/LID FLAG-HA (LID O.E) (right panel) probed with antibodies to the histone modification marks indicated on the right or histone H3 as a loading control. (B) Quantification of western blot signals from (A) normalized to the signal from H4 and or H3; n = 2-6. Error bars represent standard error of the mean. (C) Immunostaining of control (En-GAL4) or *lid* knockdown (UAS-LID$^{RNAi-KK}$/En-GAL4) wing imaginal discs stained with DAPI (left panel) or antibody to H3K4me3 (right panel). KK – RNAi line from Vienna *Drosophila* RNAi Center, ** – $P < 0.01$, *** – $P < 0.001$.

A second demethylase active against H3K4me3 has been identified in flies, namely dKDM2 (Kavi and Birchler, 2009). dKDM5/LID and dKDM2 genetically interact (Li et al., 2010). Lethality observed in double mutants of dKDM5/LID and dKDM2 could be rescued by wild type *lid* but not a demethylase mutant *lid* transgene, implicating functional redundancy in the demethylase function of these proteins. Our data indicating significant changes in global levels of H3K4me3 upon misregulation of dKDM5/LID suggest that it is not completely redundant in demethylase function with dKDM2 and highlight an important role for this enzyme in the cell. Further, while reduced expression of dKDM5/LID results in a similar curved wing phenotype as *Sin3A* knockdown (Fig. 2.5A), reduced expression of dKDM2 suppresses the *Sin3A* knockdown phenotype (Swaminathan et al., 2012). This highlights possibly opposing roles for these two demethylases of H3K4me3 in wing development.

SIN3 and dKDM5/LID bind to TSS proximal regions of target genes

Having established that dKDM5/LID can alter global levels of H3K4Me3, we tested if dKDM5/LID directly bound to target genes along with SIN3 by ChIP-qPCR. We analyzed binding of SIN3 and dKDM5/LID at several genes identified by RNAseq as regulated by SIN3, dKDM5/LID or both under normal or oxidative stress conditions. Flybase gene ontology (GO) terms were also taken into consideration in the selection of genes for testing (St Pierre et al., 2014). Due to the roles of SIN3 and dKDM5/LID in cell proliferation and wing development (described in Chapter 2), we selected several genes involved in these processes. We tested *Mcm7*, a DNA helicase that is part of the DNA replication preinitiation complex, which is also implicated in G2/M DNA damage

checkpoint regulation (Kondo and Perrimon, 2011). It is of interest that *Sin3A* RNAi in *Drosophila* S2 cells results in a G2/M phase cell cycle arrest (Pile et al., 2002). Tested genes *Thor*, the eukaryotic intiation factor 4E (eIF-4E) binding protein and *Sesn*, are both implicated in negative regulation of cell growth through the TOR signaling pathway (Cully et al., 2010; Hernandez et al., 2005; Jacinto and Hall, 2003; Lee et al., 2010; Miron et al., 2001). The regulation of cell size by these genes lead to corresponding changes in wing size thereby implicating a role in wing development (Lee et al., 2010; Miron et al., 2001). The genes, *Sequence-specific single-stranded DNA-binding protein* (*Ssdp*), S-adenosylmethionine Synthetase (*Sam-S*), *Heat shock protein 27* (*Hsp27*) and *interference hedgehog* (*ihog*) were selected for their implication in wing development. The protein product of *Ssdp* is part of the CHIP-Apterous complex, which triggers a signaling cascade that regulates wing development (Bronstein et al., 2010). *Sam-S* expression is regulated by SSDP and is implicated in wing development through its genetic interaction with *Ssdp*. *Hsp27* has been implicated in the determination of wing shape (Carreira et al., 2011). *ihog* regulates wing development mediated by the Hedgehog signaling pathway, where Ihog binds Hedgehog activating the signaling cascade (Camp et al., 2010; Yao et al., 2006). Gene ontology analysis of RNAseq data, described in Chapter 3, identified septate junction assembly genes as highly enriched among SIN3 regulated genes. We selected *varicose* (*vari*) involved in open tracheal system development (Beitel and Krasnow, 2000). Metabolic genes were also highly enriched among SIN3 regulated genes. We tested several genes involved in multiple metabolic processes. *Sam-S*, implicated in wing development also synthesizes the major methyl donor S-adenosylmethionine (Larsson et al., 1996). The regulation of

Sam-S by SIN3 is of interest as this could affect histone methylation, a mark modified by dKDM5/LID. We tested *Glutathione S transferase E6* (*GstE6*), implicated in glutathione metabolism, a pathway that was highly enriched among both SIN3 and dKDM5/LID regulated genes (Saisawang et al., 2012). *Cyt-c-p* is involved in mitochondrial energy production through oxidative phosphorylation (Limbach and Wu, 1985). CG3476 is predicted to encode a mitochondrial membrane transporter involved in acyl carnitine transport (FlyBase-Curators et al., 2004-). Due to the enrichment of genes functioning in the mitochondria among genes regulated by SIN3, we further selected *mitochondrial ribosomal protein L19* (*mRpL19*), a constituent of the mitochondrial large ribosomal subunit implicated in translation. Further, both SIN3 and dKDM5/LID regulated genes were implicated in stress response and life span determination processes. *Thor* and *Hsp27*, implicated in cell cycle and wing development pathways, respectively, are also involved in stress response and lifespan determination pathways (Hao et al., 2007; Wang et al., 2004a; Zid et al., 2009). In addition, we analyzed binding at *Su(H)*, which did not show any significant regulation upon loss of either SIN3 or dKDM5/LID. *Su(H)* encodes a component of the Su(H)/Hairless DNA binding complex, which can physically interact with dKDM5/LID, and is important for tethering a SIN3 and dKDM5/LID containing complex to Notch target genes. (Liefke et al., 2010; Moshkin et al., 2009). Further, as a transcriptional effector of Notch signaling, Su(H) is implicated in wing imaginal disc dorsal / ventral pattern formation (Koelzer and Klein, 2006; Nagel et al., 2005). CG31819, a gene located in a region devoid of SIN3 or dKDM5/LID binding based on published genome wide binding data, was used as a negative control (Celniker et al., 2009; Filion et al.,

2010; Lloret-Llinares et al., 2012).

Drosophila S2 cells predominantly express SIN3 220, the larger isoform. We utilized *Drosophila* S2 cells that express either HA tagged SIN3 220 or FLAG-HA tagged dKDM5/LID. S2 cells not carrying any transgene were used as a control. We prepared chromatin from control cells or cells expressing the HA tagged proteins and immunoprecipitated using anti-HA beads. Quantitative real-time PCR was used to determine enrichment of SIN3 and dKDM5/LID at gene targets selected based on the RNAseq results described in Chapter 3. Published genome wide binding data indicate that both SIN3 and dKDM5/LID bind predominantly at the 5' end of transcribed genes (Celniker et al., 2009; Filion et al., 2010; Lloret-Llinares et al., 2012). We designed qPCR primers for amplification of 5' regions that spanned the TSS of selected genes.

ChIP-qPCR results showed a greater than three fold enrichment of SIN3 220 compared to control at most of the tested genes with the exception of *vari* and *GstE6*, where no significant enrichment was observed (Fig. 4.2A). No enrichment was noted at the negative control gene *CG31819*. The highest enrichment of ~ 11 fold was seen at CG3476. We further validated binding of SIN3 to target genes by immunoprecipitating SIN3 using an antibody targeting endogenous SIN3 protein. For this purpose we prepared chromatin from *Drosophila* S2 cells and immunoprecipitated using anti-SIN3 antibody. Immunoprecipitation was performed with pre-immune IgG as a control. Quantitative real-time PCR was used to determine enrichment as previously. Similar enrichment at gene targets was observed for both endogenous SIN3 protein and HA tagged SIN3 protein (Fig. 4.2A and 4.3B, blue bars). No significant enrichment was observed in the control IP (Fig. 4.3A).

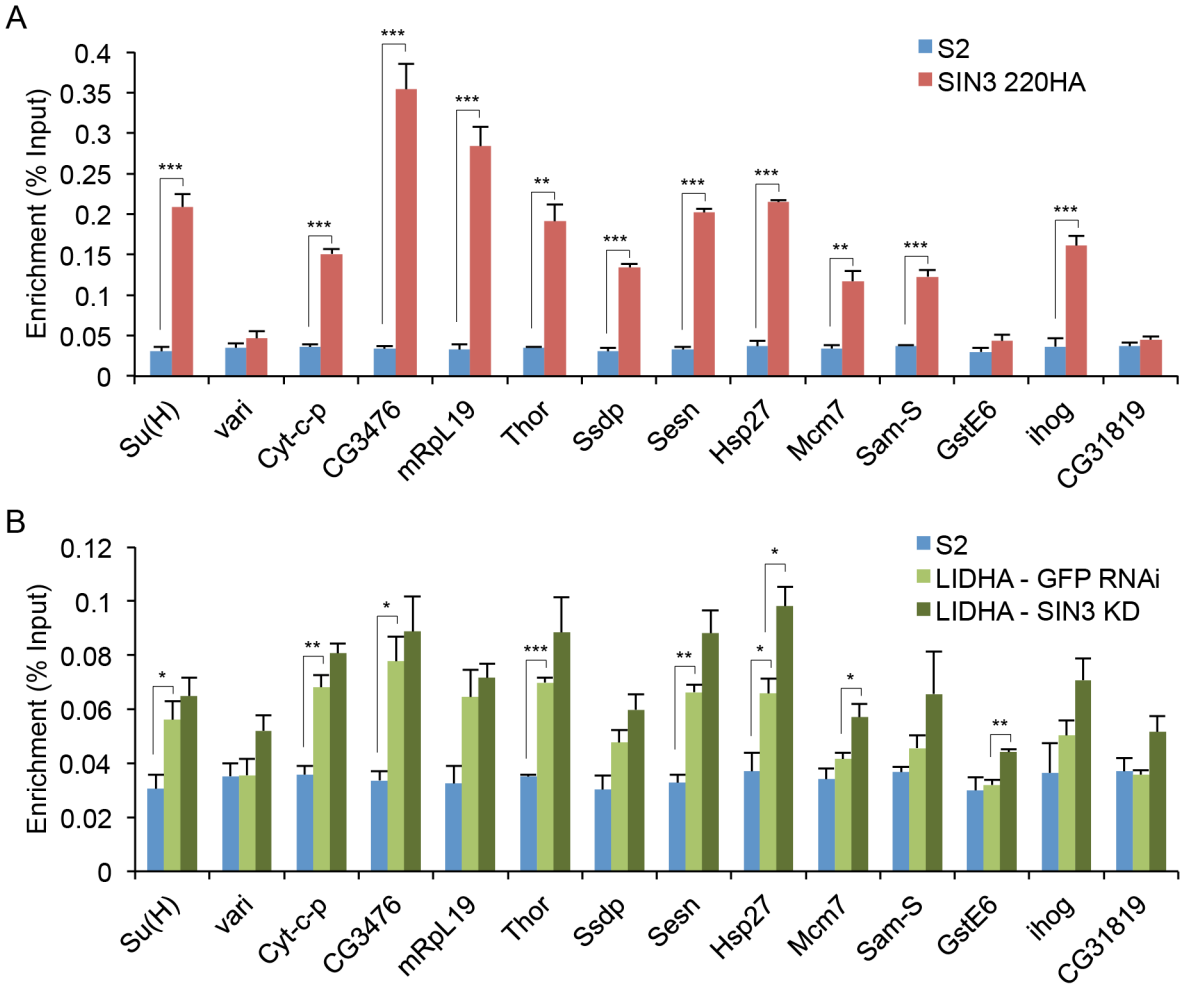

Figure 4.2: SIN3 and dKDM5/LID bind to common gene targets. Real-time quantitative PCR analysis of chromatin prepared from indicated cell lines immunoprecipitated with beads conjugated to antibody to the HA tag. (A) Enrichment of SIN3 220 at predicted target genes. (B) Enrichment of dKDM5/LID at target genes upon GFP RNAi as control or *Sin3A* knockdown. Primers used in the PCR amplification target regions spanning the TSS of indicated genes. *CG31819* acts as a negative control. The results are the average of three biological replicates. Error bars represent standard error of the mean. KD – knockdown, * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.01$.

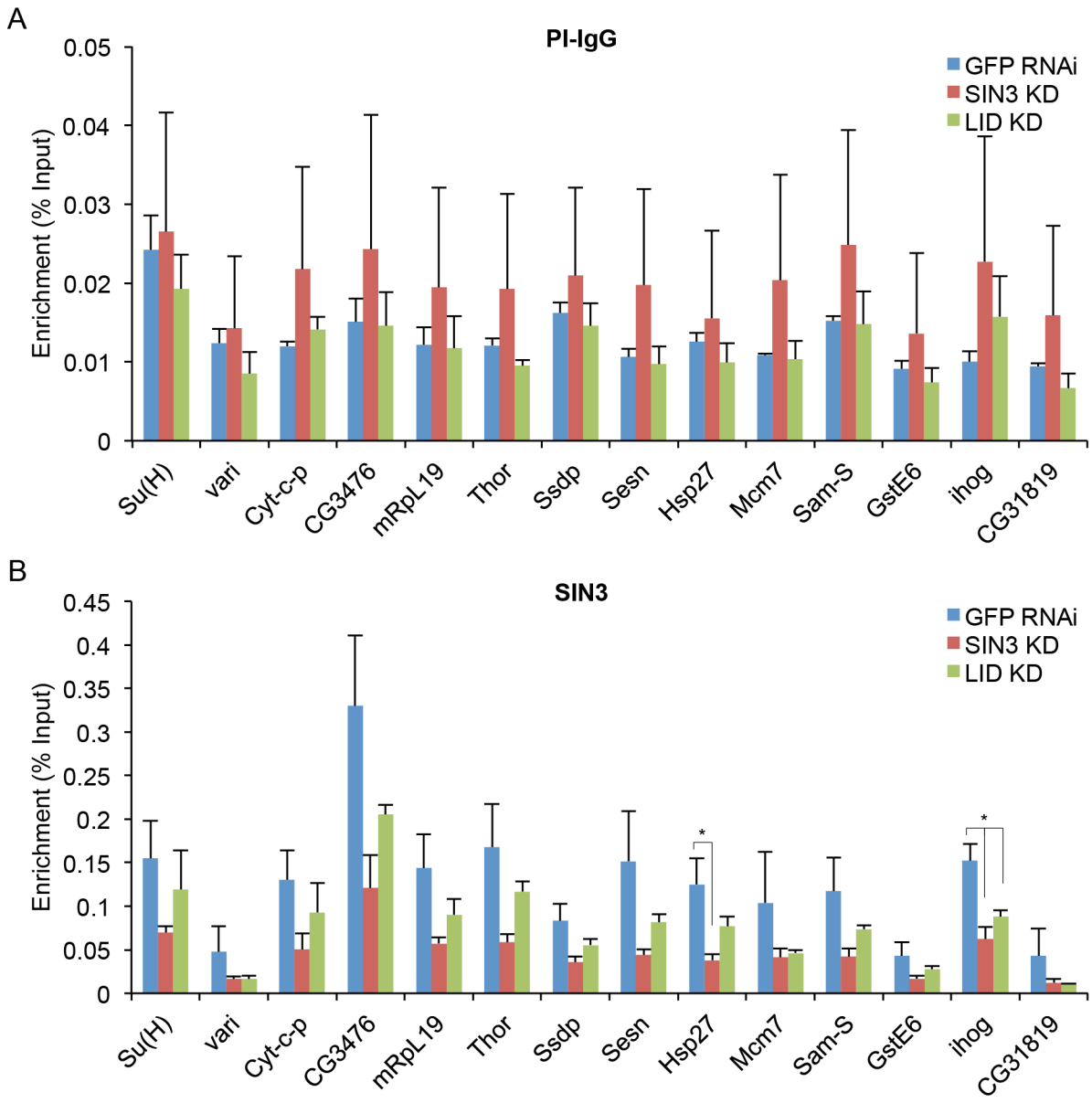

Figure 4.3: *lid* knockdown affects recruitment of SIN3 to gene targets. Real-time quantitative PCR analysis of chromatin prepared from indicated cell lines immunoprecipitated with preimmune IgG (PI-IgG) as control (A) or antibody to SIN3 (B). Primers used in the PCR amplification target regions spanning the TSS of indicated genes. The results are the average of three biological replicates. Error bars represent standard error of the mean. KD – Knockdown, * – $P < 0.05$.

Of the two tested genes with no significant enrichment of SIN3 at the TSS, *vari* expression is upregulated upon depletion of *Sin3A* under both normal and oxidative

stress conditions (Chapter 2). Thus the binding data suggests that this gene maybe an indirect target of SIN3. Upregulation of *GstE6* expression by SIN3 was only observed under paraquat mediated oxidative stress conditions. Thus it is possible that SIN3 is recruited to this gene only under such conditions. Of the genes with significant enrichment of SIN3, *CG3476*, *mRpL19*, *Thor* and *Sam-S* are negatively regulated by SIN3. *Sesn*, *Hsp27* and *Mcm7* are positively regulated by SIN3. *Cyt-c-p* and *ihog* are only significantly upregulated upon knockdown of *Sin3A* under induction of oxidative stress. Knockdown of *Sin3A* did not result in any significant changes in expression of *Su(H)* and *Ssdp*, yet the TSS of these genes are bound by SIN3. The varying transcriptional outcomes of genes bound by SIN3 suggest a complex transcriptional program involving SIN3.

The histone modifying functions of SIN3 associated proteins RPD3 and dKDM5/LID remove marks associated with active transcription suggesting a role in gene repression. We, however, identified a large number of genes to be positively regulated by SIN3. We hypothesized that genes that are activated by SIN3 may not show direct binding but be regulated indirectly. Our results contradict this hypothesis, where both genes positively and negatively regulated by SIN3 are bound by SIN3 at TSS proximal regions. These data suggest additional functions for SIN3 in gene regulation that are distinct from its affect on histone PTMs.

Compared to SIN3, dKDM5/LID was only modestly enriched at tested genes, where all genes showed less than 2.5 fold enrichment compared to control (Fig. 4.2B, light green bars). Only *Su(H)*, *Cyt-c-p*, *CG3476*, *Thor*, *Sesn*, *Hsp27* and *iHog* had statistically significant enrichment compared to control. All genes bound by SIN3,

however, showed above background levels of enrichment for dKDM5/LID. Similar to SIN3, highest enrichment of dKDM5/LID was seen at *CG3476*. These observations suggest that both SIN3 and dKDM5/LID may be recruited together as a complex to gene targets. The low enrichment of dKDM5/LID may indicate a lower stoichiometry of the protein at gene targets compared to SIN3. It is, however, not possible to exclude technical differences. Therefore, the observed differences may arise due to varying DNA:protein crosslinking efficiencies for SIN3 and dKDM5/LID.

Of genes showing significant enrichment of dKDM5/LID at the TSS, significant transcriptional regulation is seen only for *Sesn* and *Hsp27*, where both genes are downregulated upon *lid* knockdown. *Cyt-c-p* and *CG3476* are upregulated upon *lid* knockdown under conditions of oxidative stress. All genes with dKDM5/LID enrichment are also enriched for SIN3. It is likely that due to interaction with the SIN3 complex, dKDM5/LID too binds to gene targets bound by SIN3, while its effect on transcriptional regulation is restricted to a subset of these genes. Surprisingly, SIN3, but not dKDM5/LID, is significantly enriched at *Ssdp,* a gene with significant transcriptional regulation by dKDM5/LID but not SIN3. While not statistically significant, dKDM5/LID was enriched above control levels at *Ssdp*. The overall lower enrichment of dKDM5/LID at all genes may have contributed to the observed low enrichment at this gene. Similar to SIN3, dKDM5/LID too bound to *Su(H)*, the protein product of which is thought to facilitate binding of a SIN3 and dKDM5/LID complex to Notch target genes (Moshkin et al., 2009). As neither SIN3 or dKDM5/LID affect *Su(H)* expression levels under normal or oxidative stress conditions, other protein associations may influence the regulation of this gene.

Overall, significant overlap is seen in the binding patterns of SIN3 and dKDM5/LID at target genes suggesting that dKDM5/LID maybe recruited to SIN3 targets as part of the SIN3 complex. The gene regulatory effects of these two proteins, however, vary among genes. Common and unique protein interactions of SIN3 and dKDM5/LID may contribute to the similar and distinct transcriptional outcomes at different gene targets.

Reduced levels of dKDM5 affect recruitment of SIN3 to gene targets

Next we wished to test if loss of SIN3 or dKDM5/LID could affect binding of the other to gene targets. To test for the effect of SIN3 on dKDM5/LID binding we treated FLAG-HA tagged dKDM5/LID expressing cells with dsRNA targeting *Sin3A* and performed ChIP-qPCR. We further subjected S2 cells to RNAi targeting *Sin3A*, *lid* or both and tested for enrichment of SIN3 using antibody to SIN3. Based on the biochemical interaction of SIN3 with dKDM5/LID we predicted that dKDM5/LID could bind to target genes as part of the SIN3 complex. Thus we would expect the depletion of the scaffold protein of the complex, SIN3, to affect binding of associated proteins to gene targets. ChIP-qPCR results show that *Sin3A* knockdown does not decrease the moderate enrichment for dKDM5/LID observed at target genes (Fig. 4.2B, dark green bars). In fact, an increase in enrichment was noted at all genes tested including the negative control with statistically significant increases at *Hsp27*, *Mcm7* and *GstE6*. It is not clear if the observed increases in dKDM5/LID binding upon *Sin3A* knockdown are biologically relevant. The increased enrichment seen at all genes is possibly due to an experimental artifact due to sample variability rather than of any biological significance.

It is, however, possible that reduced enrichment of SIN3 results in an increased stabilization of dKDM5/LID at the chromatin template.

Sin3A knockdown results in a decrease in the enrichment of SIN3 at gene targets as would be expected (Fig. 4.3B). No significant changes were observed in the control IP samples (Fig. 4.3A). Thus, the level of *Sin3A* knockdown achieved by RNAi was sufficient to decrease the enrichment of SIN3 at target genes. Such decrease of SIN3, however, did not reduce dKDM5/LID binding to targets (Fig. 4.2B), suggesting that dKDM5/LID maybe recruited independently of SIN3. This leads to the hypothesis that dKDM5/LID transiently interacts with the SIN3 complex or occurs as part of a subcomplex and associates with the SIN3 core complex at the chromatin template. It is, however, not possible to exclude the idea that the amount of SIN3 still present upon RNAi mediated depletion of SIN3 is sufficient to recruit dKDM5/LID to targets.

Knockdown of *lid* on the other hand resulted in a moderate reduction of SIN3 enrichment at target genes (Fig. 4.3B). While less than two fold reduction of SIN3 binding at targets was observed, the data imply that dKDM5/LID could affect binding or recruitment of SIN3 to gene targets. How dKDM5/LID affects the binding of SIN3 to gene targets is, however, unclear. Further investigation of dKDM5/LID and SIN3 associated proteins are needed to understand the potential role of dKDM5/LID in regulating recruitment of SIN3 to chromatin.

Decrease in SIN3 or dKDM5/LID levels do not alter histone density at the TSS of target genes

Having established that both SIN3 and dKDM5/LID directly bind to several target

genes, we wished to analyze possible changes in histone modifications regulated by SIN3 complex proteins. First, we tested if SIN3 or dKDM5/LID can affect overall histone density at target genes. For this purpose, we isolated chromatin from S2 cells treated with dsRNA against *Sin3A*, *lid* or GFP as a control and immunoprecipitated with antibody against the histone H3 C-terminus. Pre-immune IgG was used as a control for immunoprecipitation. ChIP-qPCR was performed as before to investigate possible changes in histone density. Obtained results indicate that knockdown of neither *Sin3A* nor *lid* affect histone density at regions spanning the TSS of gene targets (Fig. 4.4).

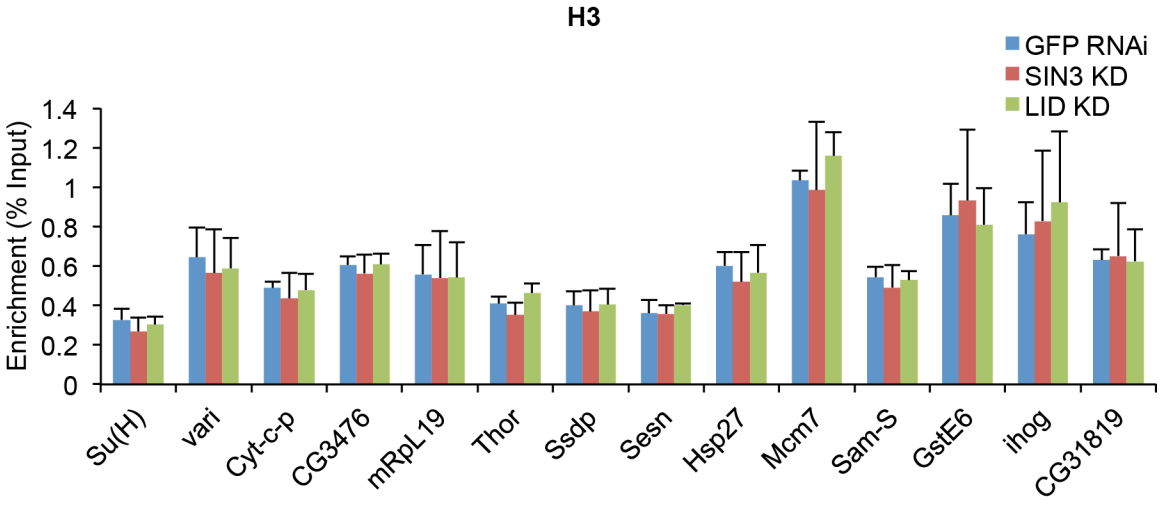

Figure 4.4: *Sin3A* or *lid* knockdown does not significantly alter histone density. Real-time quantitative PCR analysis of chromatin prepared from indicated cell lines immunoprecipitated with antibody to the histone H3 C-terminus (H3). Primers used in the PCR amplification target regions spanning the TSS of indicated genes. The results are the average of three biological replicates. Error bars represent standard error of the mean. KD – Knockdown.

Decrease in SIN3 results in increased levels of histone acetylation at the TSS of some target genes

Due to the association of the HDAC RPD3 with SIN3 we wanted to test if

depletion of SIN3 can affect histone acetylation levels at target genes. Published work analyzing global changes in acetylation levels in *Drosophila* S2 cells found that depletion of RPD3, but not SIN3, by RNAi resulted in increased acetylation of H4K8 and K12 as well as H3K9/14 (Pile et al., 2002). Overexpression of SIN3, however, resulted in decreased global levels of acetylation at H3K9 and K9/14 (Spain et al., 2010). To understand the effect of SIN3 and dKDM5/LID on histone acetylation at gene targets, we performed ChIP-qPCR with antibody specific to H3K9ac.

The level of enrichment of H3K9ac at the TSS of tested target genes varied across genes (Fig. 4.5). Comparatively low enrichment of H3K9ac was observed at several gene targets. Of those genes with significant enrichment of H3K9ac, an increase in acetylation was observed only at *CG3476*, *mRpL19* and *ihog* upon depletion of *Sin3A*. *CG3476* and *mRpL19* are both genes upregulated upon knockdown of *Sin3A*. Thus the increase in acetylation levels upon *Sin3A* knockdown at these genes correlates with gene expression trends. On the other hand *ihog* is activated by SIN3 under conditions of oxidative stress but not in normal conditions. While only some genes showed changes in acetylation levels at the TSS upon depletion of SIN3, it is possible that changes in acetylation levels may occur further upstream or downstream at other SIN3 target genes.

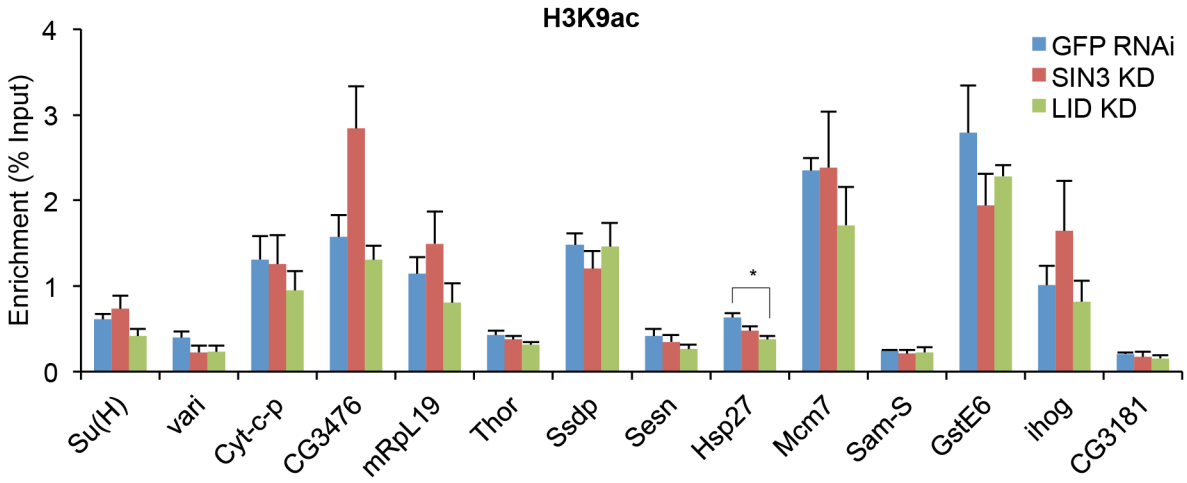

Figure 4.5: *Sin3A* knockdown leads to increased H3K9ac levels only at some target genes. Real-time quantitative PCR analysis of chromatin prepared from indicated cell lines immunoprecipitated with antibody to histone H3K9ac. Primers used in the PCR amplification target regions spanning the TSS of indicated genes. The results are the average of three biological replicates. Error bars represent standard error of the mean. KD – knockdown, * – $P < 0.05$.

lid knockdown resulted in small decreases in the level of acetylation at many of the tested genes. Due to the small changes observed it is not possible to make any conclusions regarding the role of dKDM5/LID on histone acetylation levels. In published work, however, a decreased enrichment of histone H3 acetylation along polytene chromosomes in homozygous *lid* mutant larvae has been observed (Lloret-Llinares et al., 2008).

Decrease in SIN3 and dKDM5/LID do not significantly alter levels of H3K4 methylation at the TSS of gene targets

Due to the demethylase activity of dKDM5/LID resulting in global changes of H3K4me3, we tested for possible changes in H3K4me2 and H3K4me3 levels at target genes using specific antibodies by ChIP-qPCR as described above. Similar to the

enrichment of H3K9ac, several genes had comparatively low levels of enrichment of both H3K4me2 and me3 (Fig. 4.5 and 4.6). Interestingly, all genes with comparatively high enrichment for H3K9ac also had high enrichment of H3K4me2 and me3. Consistent with published genome wide data, our results imply that these marks are enriched at overlapping regions (Filion et al., 2010; Kharchenko et al., 2011). Surprisingly, no significant changes were observed for H3K4me2 or me3 levels upon loss of *Sin3A* or *lid* (Fig. 4.6). It is thus unclear what the role of the demethylase is at target genes. As we have only tested for enrichment of histone marks at regions spanning the TSS of target genes, we cannot exclude the possibility that other regions of the gene may be significantly affected. Recent work, however, calls into question the general positive effect on transcription attributed to H3K4 methylation and H3K36 methylation (Lenstra et al., 2011). Deletion of enzymes that add these marks result in altered expression of only about 1% of budding yeast genes. Further, in budding yeast cells, dramatic transcriptional changes occur upon reentry into the cell cycle from quiescence. Genome wide ChIPseq analysis during this transition detected no significant changes in patterns of histone methylation at growth and stress genes while significant changes were observed in the patterns of histone acetylation (Mews et al., 2014).

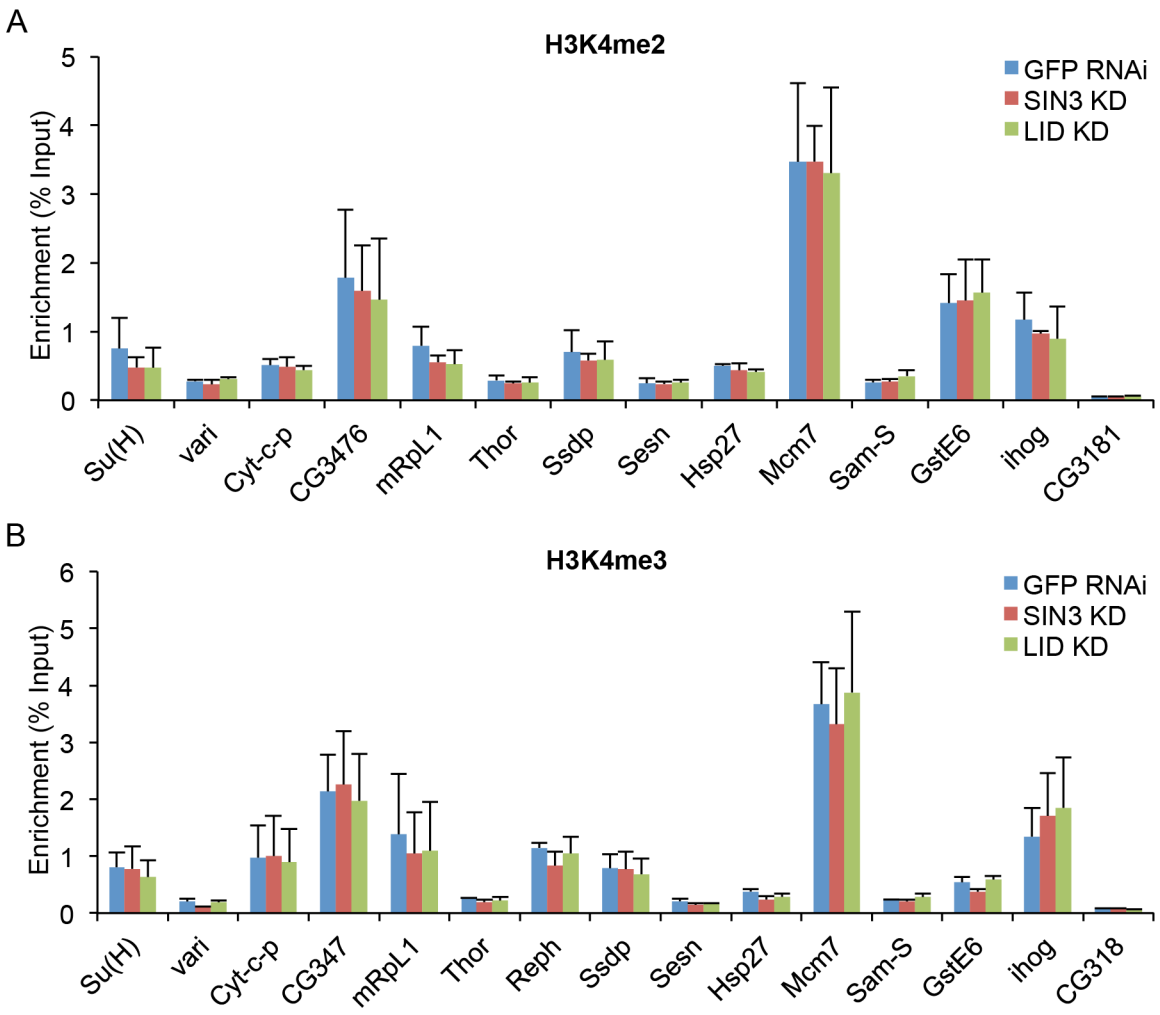

Figure 4.6: *Sin3A* or *lid* knockdown does not significantly alter H3K4 methylation patterns at target genes. Real-time quantitative PCR analysis of chromatin prepared from indicated cell lines immunoprecipitated with antibody to histone H3K4me2 (A) or H3K4me3 (B). Primers used in the PCR amplification target regions spanning the TSS of indicated genes. The results are the average of three biological replicates. Error bars represent standard error of the mean. KD – knockdown.

Overall, our data indicate that SIN3 and dKDM5/LID bind at overlapping sites of many gene targets. The regulation of transcriptional outcome at these genes, however, differs. Much work is required to elucidate how these proteins are recruited to gene targets and how they regulate gene transcription. While histone modifications may be a

means of gene regulation by these proteins, other protein interactions possibly make important contributions to underlying gene regulation.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Epigenetic regulation through histone modifications is among the multiple mechanisms that control transcriptional outcome. The enzymes that bring about these histone modifications thus are important players in transcriptional regulation. As multiple histone modifications can coordinately affect gene expression, it is no surprise that histone modifying enzymes too can coexist in multi subunit complexes. SIN3, NuRD, CoREST and NCoR are such complexes that incorporate both histone deacetylase and demethylase modules (Hayakawa and Nakayama, 2011). Our work has focused on the SIN3 complex in the *Drosophila* model system.

Work from our laboratory and others determined that *Drosophila* SIN3, the scaffold protein of the SIN3 complex, associates with both the HDAC RPD3 and the KDM dKDM5/LID (Moshkin et al., 2009; Spain et al., 2010). The current work has focused on validating the interaction of dKDM5/LID with SIN3 and characterizing the functions of dKDM5/LID with relevance to the SIN3 complex. We utilized co-immunoprecipitation and reciprocal co-immunoprecipitation methods to validate the interaction of dKDM5/LID with SIN3 and the associated HDAC RPD3 (Fig. 2.2).

Having established the interaction of dKDM5/LID with SIN3, we went on to determine the potential roles of dKDM5/LID in SIN3 regulated processes. Previous work in *Drosophila* cultured cells identified that RNAi mediated depletion of SIN3 hindered progression through G2 to M phase of the cell cycle (Pile et al., 2002). Furthermore, depletion of SIN3 in wing imaginal discs affected clonal cell growth (Swaminathan and

Pile, 2010). We conducted similar experiments knocking down *Sin3A* and *lid* by RNAi in both cell culture and wing imaginal discs. Our results indicate that similar to SIN3, dKDM5/LID affects cell proliferation both in cultured cells and developing flies (Fig. 2.4). The effect of dKDM5/LID, however, is less pronounced than of SIN3, suggesting that other components of the SIN3 complex may also contribute to the observed cell proliferation defect.

Depletion of SIN3 in wing imaginal discs is also known to affect wing development resulting in a curved wing phenotype (Swaminathan and Pile, 2010). Similarly we identified that knockdown of *lid* in wing imaginal discs resulted in adult flies with curved wings (Fig. 2.5). Apart from *lid* knockdown, overexpression of *lid* resulted in additional wing defects implicating a role for dKDM5/LID in wing development. Furthermore, overexpression of *lid* in wing imaginal discs in a *Sin3A* knockdown background resulted in rescue of the curved wing phenotype (Table 2.2). These observations suggest overlapping functions for SIN3 and dKDM5/LID in their affect on wing morphology.

Having established that both SIN3 and dKDM5/LID function in similar processes we next determined the underlying changes in gene expression. We conducted RNAseq analysis upon knockdown of *Sin3A*, *lid* or both in S2 cultured cells. We identified 624 and 90 genes to be regulated by SIN3 and dKDM5/LID respectively (Fig. 3.2). 850 genes were misregulated upon double knockdown of both *Sin3A* and *lid*. SIN3 has generally been associated with transcription repression (Silverstein and Ekwall, 2005). Our data indicate that nearly equal number of genes are activated and repressed by SIN3. dKDM5/LID, however, predominantly acts as an activator. We further find that a

significant portion of genes regulated by dKDM5/LID is also regulated by SIN3. GO analysis of regulated genes highlights that a large portion of SIN3 regulated genes is involved in metabolic processes, corroborating published findings (Fig. 3.3) (Pile et al., 2003). The most enriched category of genes regulated by dKDM5/LID are involved in stress response, which are also regulated by SIN3. Interestingly, the most enriched categories of genes misregulated upon dual knockdown of *Sin3A* and *lid* are related to cell cycle processes. In addition multiple genes implicated in wing development were also found to be regulated by SIN3 and or dKDM5/LID. Further investigation into the regulation of these genes could lead to a better understanding of the role of SIN3 and dKDM5/LID in cell cycle progression and wing development.

Published work indicates that depletion of both SIN3 or dKDM5/LID lead to increased sensitivity to oxidative stress in flies (Barnes et al., 2014; Li et al., 2010). We further tested gene expression changes upon knockdown of *Sin3A, lid* or both under paraquat induced oxidative stress conditions. We find a significant overlap between genes that are regulated by SIN3 or dKDM5/LID and genes regulated upon induction of oxidative stress (Fig. 3.4). This suggests that loss of SIN3 or dKDM5/LID can partly mimic oxidative stress induced conditions in the cell. In addition, depletion of SIN3 and dKDM5/LID under oxidative stress conditions resulted in the regulation of a large number of additional genes (Fig. 3.6). Interestingly, genes involved in cell cycle processes were the most enriched among regulated genes under stress induced conditions (Fig. 3.7). This potentially highlights a link between the roles of SIN3 and dKDM5/LID in stress tolerance and cell cycle progression.

The histone modifying functions of SIN3 associated proteins RPD3 and

dKDM5/LID involve removal of marks associated with active transcription. We, however, identified a large proportion of genes to be activated by SIN3 and dKDM5/LID. Therefore, to understand the role of these histone modifying activities in SIN3 and dKDM5/LID dependent regulation of transcription, we studied the changes in histone modification patterns upon knockdown of *Sin3A* or *lid* at regulated genes.

First we performed ChIP-qPCR analysis to determine direct binding of SIN3 or dKDM5/LID to regions spanning the TSS of selected genes. Genes bound by SIN3 were also bound by dKDM5/LID suggesting that these proteins maybe recruited together to chromatin (Fig. 4.2). While knockdown of *Sin3A* did not affect binding of dKDM5/LID to targets, knockdown of *lid* resulted in some decrease in the enrichment of SIN3 at targets (Fig. 4.2 and 4.3). This suggests that dKDM5/LID may help recruit SIN3 to chromatin. Although both SIN3 and dKDM5/LID bound similar gene targets, the effect of these proteins on transcriptional outcome was varied. In addition, while depletion of SIN3 resulted in an increase of histone acetylation at some target genes, the loss of dKDM5/LID did not result in significant changes in histone methylation patterns (Fig. 4.5 and 4.6). These results suggest that gene regulation by the SIN3 complex may not solely depend on the histone modifying ability of the complex.

This work has contributed to our understanding of the role of dKDM5/LID in SIN3 related regulation of transcription under normal and oxidative stress induced conditions, cell proliferation and wing developmental processes. The molecular mechanisms and gene regulatory pathways by which these processes are regulated is yet unclear. A schematic of regulation by SIN3 and dKDM5/LID is depicted in Fig 5.1. Some of the questions that remain unanswered with regard to the association of SIN3 and

dKDM5/LID and their function in the cell are discussed below.

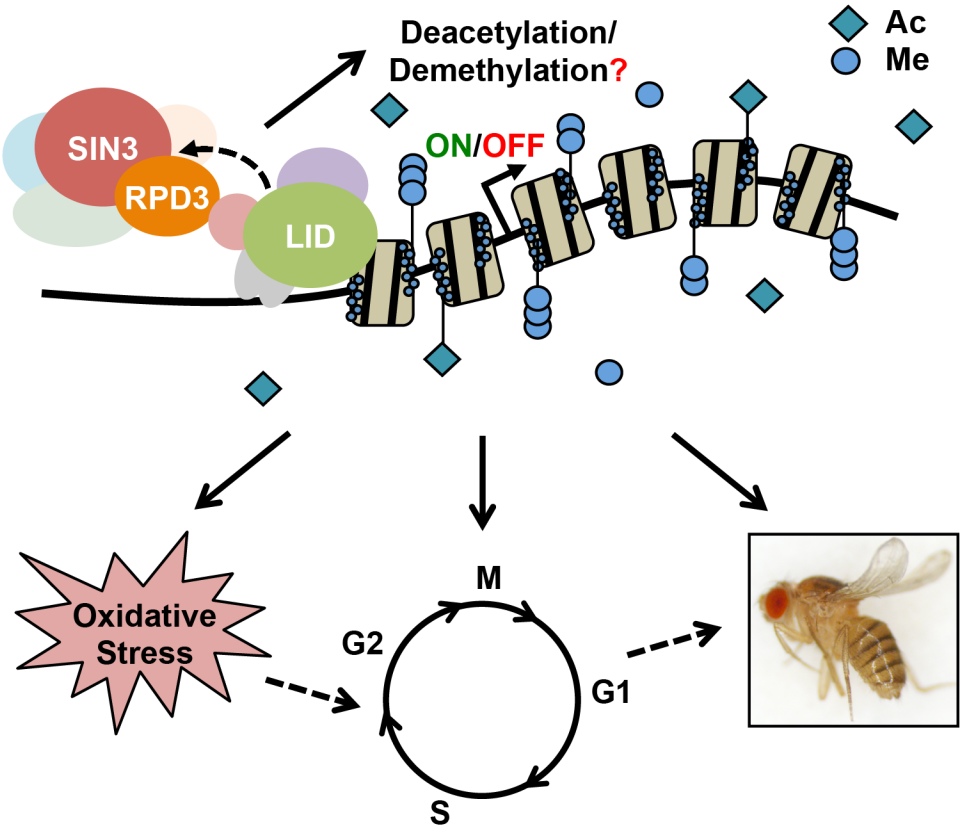

Figure 5.1: Model for regulation by SIN3 and dKDM5/LID. In *Drosophila*, SIN3 associates with two histone modifying enzymes RPD3, an HDAC and dKDM5/LID, a KDM. These proteins are recruited together to TSS proximal regions of gene targets. dKDM5/LID could potentially affect recruitment of SIN3 to targets. The activity of RPD3 and LID can lead to the removal of H3 and H4 acetylation and H3K4me3 respectively. Through their histone modifying ability or by other means these proteins regulate gene transcription of common or distinct genes. This in turn can affect regulation of cellular stress, cell cycle progression and wing development.

Is dKDM5/LID a stable component of the SIN3 HDAC core complex?

We and others have determined that dKDM5/LID interacts with SIN3 220 and RPD3 (Moshkin et al., 2009; Spain et al., 2010). However, based on a dKDM5/LID purification another group reported interaction with RPD3 but not SIN3 (Lee et al.,

2009). In our studies we find that knockdown of *Sin3A* and *lid* results in common misregulated genes and phenotypes. Further, both SIN3 and dKDM5/LID bind common gene targets. These observations strengthen the idea that these proteins occur together as a complex and are recruited to chromatin together. Due to the direct binding of many core complex proteins to SIN3 via its PAH and HID domains, SIN3 is considered the scaffold protein of the complex (Grzenda et al., 2009; Silverstein and Ekwall, 2005). Thus it would be expected that the depletion of the scaffold protein would lead to depletion of SIN3 associated proteins at chromatin target sites. We, however, observed no significant reduction of dKDM5/LID upon knockdown of *Sin3A* at gene targets. This implies that dKDM5/LID may only transiently interact with SIN3 or may be part of a subcomplex that associates with SIN3 at the chromatin template. To address this possibility, dKDM5/LID can be immunoprecipitated and subjected to glycerol gradient fractionation. Obtained fractions can be tested for the presence of SIN3 and/or dKDM5/LID with specific antibodies or subjected to mass spectrometric analysis. This experiment would help determine if dKDM5/LID is part of a subcomplex that associates with SIN3. If dKDM5/LID occurs as a subcomplex, the determination of interacting proteins would aid in understanding how dKDM5/LID interacts with SIN3 and is recruited to chromatin.

What components of the SIN3 complex directly interact with dKDM5/LID?

While we have validated the interaction of SIN3 with dKDM5/LID, we do not know if these proteins directly interact. Mass spectrometric analysis of both SIN3 and dKDM5/LID purifications have determined RPD3, Pf1 and EMSY proteins as interactors

(Moshkin et al., 2009; Spain et al., 2010). It is possible that dKDM5/LID associates with SIN3 through its interaction with these proteins. Tagged versions of the above proteins and other SIN3 complex proteins can be expressed in bacterial systems and subjected to pull down assays to determine direct interaction with dKDM5/LID and or SIN3. Alternatively, chemical crosslinking coupled to mass spectrometry methods can be utilized to identify the multiple interactions between SIN3 complex components.

How are SIN3 and dKDM5/LID recruited to chromatin?

We found that while RNAi mediated knockdown of *Sin3A* did not affect binding of dKDM5/LID to chromatin, knockdown of *lid* resulted in a slightly reduced enrichment of SIN3 at target sites. This suggests the possibility that dKDM5/LID is recruited first to chromatin, which in turn recruits SIN3 and associated proteins to target genes. KDM5 proteins contain an ARID domain, which is implicated in sequence specific or non specific binding to DNA (Kortschak et al., 2000; Scibetta et al., 2007; Tu et al., 2008). Moreover, KDM5 proteins also contain multiple PHD domains. PHD domains have been implicated in binding to methylated lysines of histones (Iwase et al., 2007; Shi et al., 2006; Wysocka et al., 2006b). Cells expressing dKDM5/LID mutants of these domains can be used in ChIP-qPCR experiments to test for binding of dKDM5/LID to chromatin and recruitment of SIN3 to chromatin. However, other proteins may help target dKDM5/LID and or SIN3 to target genes. As described above if we determine that dKDM5/LID occurs as a subcomplex of the SIN3 220 complex, proteins that are part of such a subcomplex would be ideal candidates to be tested for a role in targeting dKDM5/LID or SIN3 to chromatin. ChIP-qPCR experiments upon RNAi mediated

depletion of such proteins would help elucidate their role in recruitment.

What are genome wide binding patterns of dKDM5/LID and changes in H3K4me3 patterns in Drosophila S2 cells?

We performed genome wide expression analysis to identify genes regulated by SIN3 and dKDM5/LID in *Drosophila* S2 cells. Targeted ChIP-qPCR analysis of selected genes suggests that while these proteins bind near the TSS of many genes, the transcriptional response at these genes are varied. In addition, while dKDM5/LID affected global levels of H3K4me3 (Fig. 4.1), no significant changes in H3K4me3 were observed at the specific genes tested (Fig. 4.6). A caveat in our selective ChIP-qPCR analysis is that we have only tested for binding and histone modification changes at regions spanning the TSS of putative target genes. It is possible that other regions upstream or downstream of the TSS may display binding or changes in histone modifications at target genes. The analysis of genome wide binding patterns of SIN3 and dKDM5/LID and histone modification changes upon depletion of these proteins would provide comprehensive insight into gene regulation by these proteins. Currently, work in our laboratory has generated genome wide binding data of SIN3 in S2 cells by ChIPseq (Saha, unpublished). Expanding this work to dKDM5/LID binding and histone modification changes, such as H3K9ac and H3K4me3, upon RNAi mediated depletion of SIN3 and dKDM5/LID, together with our data from RNAseq analysis, would enhance our understanding of transcriptional regulation by these proteins.

What are the gene regulatory pathways through which SIN3 and dKDM5/LID affect cell proliferation and wing development?

In Chapter 2 we described cell proliferation and wing developmental phenotypes associated with loss of SIN3 and dKDM5/LID. As described in Chapter 3, we have performed RNAseq expression analysis upon RNAi mediated knockdown of *Sin3A*, *lid* or both under normal or oxidative stress conditions and found a large number of genes to be regulated. GO analysis of regulated genes implicates multiple regulated genes in cell cycle and wing developmental processes. Targeted genetic screens that knock down *Sin3A* or *lid* and their regulated genes, determined by RNAseq, can reveal enhancers or suppressors of the observed cell cycle and wing developmental phenotypes. These data would help to determine underlying gene regulatory pathways involved in these processes.

What is the role of dKDM5/LID in modulating stress response and lifespan in adult flies?

Our laboratory recently highlighted a critical role for SIN3 in the regulation of stress response, which in turn can affect adult lifespan (Barnes et al., 2014). In the current work, we found that genes involved in stress response and determination of adult lifespan, such as the heat shock genes and glutathione S transferase genes, are enriched among genes regulated by both SIN3 and dKDM5/LID. Survival assays of flies knocked down for *Sin3A* or *lid* and genes implicated in above processes under normal or stress induced conditions can elucidate the signaling pathways involved and determine a role for dKDM5/LID in the process.

We have currently determined overlapping roles for SIN3 and dKDM5/LID in cell cycle and wing developmental processes. Further, we determined a large number of genes to be regulated by SIN3, dKDM5/LID or both. We have, therefore, generated the tools necessary to dissect the molecular mechanisms by which these proteins affect the above cellular processes.

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ABSTRACT

ANALYZING THE INTERACTIONS OF KDM5/LID AND SIN3 IN *DROSOPHILA MELANOGASTER*

by

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SIN3, the scaffold protein of a histone modifying complex is conserved from yeast to mammals. *Drosophila* SIN3 associates with both a histone deacetylase RPD3 and a histone demethylase dKDM5/LID. Immunopurification of dKDM5/LID verifies a previously observed interaction with SIN3 and RPD3. Furthermore, deficiency of dKDM5/LID phenocopies deficiency of SIN3 in many cellular and developmental processes. Knockdown of both *Sin3A* and *lid* hinder cell proliferation in *Drosophila* cultured cells and developing flies. Knockdown of these genes also results in a curved wing phenotype implicating a role in wing development. Analysis of underlying gene expression changes upon decreased expression of SIN3, dKDM5/LID or both at a genome wide level determined multiple genes that are commonly regulated by SIN3 and dKDM5/LID. Common gene targets of SIN3 and dKDM5/LID are implicated in processes related to stress tolerance. Additive roles of these proteins seem important in the regulation of cell cycle associated genes. Induction of paraquat mediated oxidative stress found a higher number of genes to be regulated by both SIN3 and dKDM5/LID,

with an enrichment of genes involved in cell cycle regulation. Moreover, SIN3 and dKDM5/LID were found to bind the TSS proximal regions of several regulated genes suggesting direct regulation of these targets. Determination of histone modification changes at the transcriptional start sites of target genes upon knockdown of *Sin3A* or *lid* reveal changes in histone acetylation levels at some genes with no significant changes in histone methylation levels. This suggests an important role for the histone deacetylase activity of the complex in affecting gene regulation, while, the contribution of the demethylase activity appears to be minimal. It is, however, possible that the role of demethylation is highly context specific and allows for fine tuning of gene regulation under specific conditions or during specific developmental time points. This work emphasizes the important contributions of the histone demethylase dKDM5/LID to regulation of cellular events by the SIN3 complex.

Supplementary files included are as follows:

- Supplementary Data 1_AG – Excel spreadsheet containing RNAseq differential expression analysis outputs
- Supplementary Data 2_AG – Excel spreadsheet containing Gene Ontology analysis data

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