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Analyzing The Regulation, Stability And Functional Differences Between Sin3 Isoforms In Drosophila

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ANALYZING THE REGULATION, STABILITY AND FUNCTIONAL DIFFERENCES BETWEEN SIN3 ISOFORMS IN *DROSOPHILA*

by

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DISSERTATION

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DEDICATION

To my parents, my brother, Pranay and the Almighty God.

ACKNOWLEDGEMENTS

A journey that started with a passion for science and brought me so far away from home is finally coming to an end. Looking back, I laugh at my naïve self who never gave a thought to the challenges that would come my way. I just knew I had a determination in me that would take me to my destination. Now when I am so close to the end, I know I had embarked on a journey that would test me every step of the way. The course of my PhD has been full of struggles and triumphs, but the struggles seemed smaller and triumphs much grander because of the fabulous people that became a part of this journey. I want to take this opportunity to say a heartfelt thank you to all those people who made it possible for me to reach where I am today.

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CHAPTER 1 INTRODUCTION

Background

Precise regulation of spatio-temporal gene expression is orchestrated by the action of many key players that govern the organization and compaction of chromatin. The SIN3 complex is one such important player that regulates several biological processes through activation or repression of a large repertoire of target genes. SIN3 was first discovered in 1987 by two independent research groups studying mating type switching in *Saccharomyces cerevisiae (Nasmyth et al., 1987; Sternberg et al., 1987).* Both groups identified SIN3 as a negative regulator of the HO endonuclease (Homothallic switching endonuclease), which is essential for mating type switching in yeast. In the decade following its discovery, SIN3 was identified in independent genetic screens under five different aliases, primarily as a negative regulator of transcription (Hudak et al., 1994; Strich et al., 1989; Vannier et al., 1996; Vidal et al., 1990; Yoshimoto et al., 1992). SIN3 itself does not possess any DNA binding or enzymatic activity and it was hypothesized that the transcriptional repression mediated by SIN3 was through its association with a histone deacetylase (Wolffe, 1996). In 1997 three separate studies, published simultaneously, showed that SIN3 associated with histone deacetylases HDAC1/2 in a multi-protein complex. (Hassig et al., 1997; Laherty et al., 1997; Zhang et al., 1997). The SIN3 complex therefore is canonically regarded as a repressor complex.

Increasing evidence over the years, however, points to a dual role in the regulation of transcription by the SIN3 complex. The transcriptional profile of a Sin3 deletion yeast strain showed upregulation of 173 transcripts confirming the role of SIN3 in gene repression (Bernstein et al., 2000). In addition, 269 transcripts were downregulated in the

absence of SIN3, suggesting a possible role in gene activation. A genome-wide study performed using a *Drosophila* cell culture system comparing wild type and *Sin3A* knockdown cells showed a similar result (Pile et al., 2003). Out of the 13,137 genes that were tested by microarray analysis, SIN3 was required for the repression of 364 genes, whereas 35 genes were activated by SIN3. Further evidence for the dual role of SIN3 came from a gene expression analysis in another important model system. Loss of SIN3 in mouse fibroblast cells resulted in differential expression of 1308 genes, out of which 977 were upregulated and 331 were downregulated (Dannenberg et al., 2005a).

Although transcriptome studies revealed several gene targets that were downregulated upon loss of SIN3, the role of SIN3 in gene activation was not well understood and was commonly attributed to indirect effects. Though activation of transcription by SIN3 could possibly be a secondary effect, several gene-specific studies suggest otherwise. In embryonic stem cells, SIN3 regulates Nanog expression either positively or negatively, in a context-dependent manner. During embryonic stem cell differentiation, phosphorylated p53 suppresses Nanog expression by recruiting mSIN3A to the Nanog promoter (Lin et al., 2005). Conversely, under proliferating conditions, the mSIN3A/HDAC complex is recruited to the Nanog promoter leading to Sox2-mediated stimulation of Nanog expression (Baltus et al., 2009). SIN3 also plays a dual role in regulation of STAT transcriptional activity (Icardi et al., 2012). STAT1 and STAT3 perform opposing functions in the regulation of cell proliferation and survival. SIN3 interacts with STAT3 and acts as a repressor of STAT3 activity. In contrast, SIN3 is required for the transcription of ISGF3 (STAT1:STAT2:IRF9) complex regulated genes. The recent study by Saha *et al.* provides a clearer picture of the role of SIN3 in gene transcription, by

integrating transcriptome data with genome-wide binding data. This study performed in *Drosophila* S2 cells reports that 92% (243/263) of genes repressed by SIN3 and 46% (162/349) of genes that are activated by SIN3 were found to be direct targets, further highlighting SIN3 as a dual regulator of transcription (Saha et al., 2016b).

The ability of SIN3 to repress or activate gene transcription is likely due to its interaction with a large repertoire of DNA binding factors. The SIN3 protein contains six highly conserved regions, four paired amphipathic alpha-helix motifs (PAH 1-4), a histone deacetylase interaction domain (HID) and a highly conserved region (HCR) (Figure 1.1) (Silverstein and Ekwall, 2005a; Wang et al., 1990). These domains, conserved from yeast to mammals, are essential for interaction with the core components of the SIN3 complex and other interacting partners that recruit the complex to its target genes. Interestingly, due to the presence of these protein-protein interaction domains, SIN3 is believed to be the scaffold that holds the complex together. Early studies describing the SIN3 complex suggest that the core complex consists of HDAC1, HDAC2, RbAp46/48, SAP30, SAP18, and SDS3 (Hassig et al., 1997; Laherty et al., 1997; Lechner et al., 2000; Zhang et al., 1997). Over the years, a multitude of proteins including SAP130 and SAP180, ING1/2, RBP1, FAM60A, BRMS1, Pf1, KDM5A/B and MRG15 has been reported to interact with the SIN3 complex, suggesting that several SIN3 sub-complexes exist (Bansal et al., 2015; Kadamb et al., 2013b; Smith et al., 2012).

Such interactions, with a variety of accessory factors and distinct enzymatic modules, contribute to the functional flexibility of the SIN3 complex (Silverstein and Ekwall, 2005a). It is also important to note that multiple isoforms of SIN3 and other complex components exist, which likely adds to the modularity of the SIN3 complex.

Intriguingly, several studies provide evidence that SIN3 isoforms perform non-redundant functions despite the presence of highly conserved protein interaction domains. This chapter focuses on understanding the structural and functional differences of SIN3 isoforms.

Isoforms of SIN3

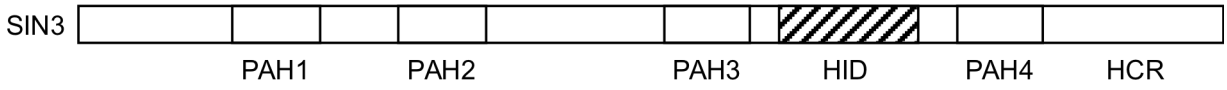


Figure 1.1. Domain structure of SIN3. The SIN3 protein contains four paired amphipathic helix domains (PAH), a histone deacetylase interaction domain (HID) and a highly conserved region (HCR).

In yeast, there is a single *Sin3* gene that gives rise to an acidic protein of approximately 170 kDa (Wang et al., 1990). This protein contains four motifs consisting of paired amphipathic helices (PAH) that are important for protein-protein interactions. When the mammalian *Sin3* gene was discovered, extensive similarity was observed with the four PAH domains in yeast (70% identity in PAH1, 56% identity in PAH2, 42% identity in PAH3 and 17% identity in PAH4) and in a large region between PAH3 and PAH4 domains (42% identity) (Halleck et al., 1995). It was later found that there are two *Sin3* genes in murine cells, *Sin3a* and *Sin3b* (Ayer et al., 1995). The SIN3A and SIN3B proteins are highly similar throughout their length, with highest homology at the PAH and histone deacetylase interaction (HID) domains. The HID is the conserved region between the PAH3 and PAH4 motifs that binds to histone deacetylases (Laherty et al., 1997). Compared to SIN3A, SIN3B has a shorter N-terminal region (Ayer et al., 1995). Multiple variant isoforms of the two mammalian *Sin3* genes have also been reported. The *Sin3a* gene can give rise to at least two alternatively spliced isoforms, SIN3A and SIN3A9. There is a nine amino acid

insert in the SIN3A9 isoform between amino acids 1205 and 1206 relative to SIN3A (Ayer et al., 1995). The *Sin3b* gene can also undergo alternative splicing. One splice form is the SIN3B protein that is 954 amino acids long and contains the conserved PAH1-4 and HID domains. The alternative form is a 293 amino acid protein, referred to as either $SIN3B_{SF}$ or SIN3B(293), which contains only the PAH1 and PAH2 domains and a unique stretch of 19 amino acids at the C-terminus (Alland et al., 1997; Yang et al., 2000). The shorter SIN3B isoform does not possess the HID region and therefore does not interact with histone deacetylases, but is still capable of repressing basal transcription (Alland et al., 1997). This leads to an intriguing possibility that the mSin3B isoforms may exercise different mechanisms of gene repression.

In the fission yeast *Schizosaccharomyces pombe*, there are three distinct *Sin3* genes, *pst1*, *pst2* and *pst3*, encoding proteins that contain PAH and HID domains exhibiting high levels of conservation with the *Saccharomyces* and mammalian SIN3 proteins (Dang et al., 1999; Silverstein and Ekwall, 2005a). Unlike fission yeast and mammalian cells, *Drosophila* has a single *Sin3A* gene, encoding a larger SIN3 protein as compared to the yeast and mammalian proteins. *Drosophila* SIN3 also possesses highly conserved PAH domains, the HID region and a conserved region beyond PAH4. Interestingly, there is a higher homology between PAH1 and PAH2 domains of *Drosophila* SIN3 and SIN3A than that between SIN3A and SIN3B (Pennetta and Pauli, 1998a). The *Drosophila Sin3A* gene produces three alternatively spliced isoforms that differ at their C-terminus (Pennetta and Pauli, 1998a; Sharma et al., 2008b). These isoforms are named SIN3 220, SIN3 190 and SIN3 187, based on their molecular weights. All three isoforms possess the protein interaction domains, PAH 1-4 and HID, but have unique stretches of

amino acids at the C-terminus. SIN3 220 has 315, SIN3 190 has 31 and SIN3 187 has 5 unique amino acids. SIN3 220 and SIN3 187 are the predominant isoforms and show differential expression during *Drosophila* embryogenesis (Sharma et al., 2008b). SIN3 190 expression is only detected in embryos and adult females. Intriguingly, there is an interplay between the predominant SIN3 isoforms, wherein, overexpression of the lower molecular weight isoform, SIN3 187, can cause a reduction in transcript and accelerated proteasomal degradation of endogenous SIN3 220 (Chaubal et al., 2016). It will be interesting to see if an inter-isoform dependent regulation of SIN3 also occurs in other species.

The mammalian SIN3 proteins, SIN3A and SIN3B exhibit distinct post-translational modifications. TOPORS is a nuclear protein that functions as a RING-dependent E3 ubiquitin ligase and as a SUMO-1 E3 ligase for p53 (Rajendra et al., 2004; Weger et al., 2005). SIN3A was identified and verified as a sumoylation substrate of TOPORS in a proteomic screen performed in Hela cells (Pungaliya et al., 2007a). Interestingly, although other SIN3 associated proteins including RbAp46, RbAp48, PSF, p54nrb and BRG1-associated factor 170, were identified as putative TOPORS substrates, SIN3B was not detected in this screen. SIN3B was instead identified as a target for the E3 ubiquitin ligase RNF220, in a yeast two-hybrid screen (Kong et al., 2010b). Further experiments conducted using HEK293 cells showed that RNF220 can ubiquitinate the N-terminal PAH1 domain as well as the C-terminus containing PAH3 and PAH4 domains of SIN3B and target it for proteasomal degradation. Since the only other proteins identified and verified in the two-hybrid screen are E2 proteins, it is probable that RNF220 specifically ubiquitinates SIN3B and not SIN3A. It is also conceivable that the SIN3A isoform may be

ubiquitinated and the SIN3B isoform may be SUMOylated in a context-dependent manner. Differential post-translational modifications of SIN3 isoforms could presumably be an active mechanism to precisely regulate the function of SIN3 isoforms in different cell types and during critical biological processes.

It is noteworthy that the different SIN3 isoforms described in various species have highly conserved protein-protein interactions domains that are responsible for the function of SIN3, and yet, as discussed below, these isoforms are non-redundant. It is possible that the evolution of SIN3 isoforms to perform unique and specialized functions has contributed to increased flexibility of SIN3 proteins in regulation of gene expression and thereby critical biological processes.

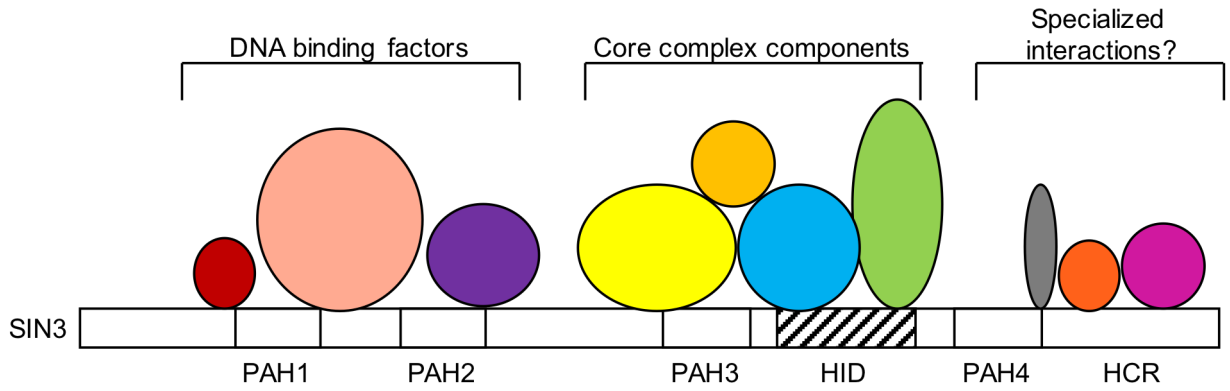


Figure 1.2. The functional domains of SIN3 are involved in several protein-protein interactions. The central region of SIN3 is involved in interactions with complex components including HDAC1/2, SDS3, SAP30, SAP 130 and SAP180, whereas the N-terminus may play a role in recruiting the SIN3 complex to its target genes by interaction with factors such as SMRTER, Opi1 and HCF-1(Grzenda et al., 2009).

Distinct protein-protein interactions exhibited by SIN3 isoforms

SIN3 proteins serve as the scaffold for a histone-modifying complex (Grzenda et al., 2009a; Kadamb et al., 2013b). The PAH and HID regions described above provide interfaces for protein-protein interactions with complex components. The central region of SIN3, which includes the PAH3 and HID domains, interacts with so named core

complex components (Figure 1.2) (Grzenda et al., 2009a). The N-terminal PAH domains, PAH1 and PAH2, bind to transcription factors that can recruit the SIN3 complex to target genes. Less is known about the interactions mediated by the PAH4 domain and the highly-conserved region (HCR) in the C-terminus. In *Drosophila*, the SIN3 220 isoform has a unique stretch of C-terminal amino acids relative to the other isoforms, which is predicted to be unstructured (Moore, 2017). It is possible that the C-terminus is involved in specialized interactions, which impact stability of the SIN3 protein and contribute to the flexibility of SIN3 function.

Interestingly, the SIN3 isoforms can form different histone modifying complexes, despite the presence of nearly identical PAH domains. In *S. pombe*, the SIN3 proteins Pst1 and Pst2 form distinct complexes that perform non-redundant functions (Nicolas et al., 2007). Pst1 is part of complex I, which contains Clr6, Prw1 and Sds3 and regulates histone acetylation at promoter regions. Pst2 is associated with complex II that includes Clr6, Prw1, Alp13, Cph1 and Cph2. Complex II primarily deacetylates histones in gene coding regions. In *Xenopus laevis*, the methyl-CpG binding protein 2 (MeCP2) forms a complex with Sin3 and a histone deacetylase (Jones et al., 1998). MeCP2 binds to methylated DNA through its methyl-CpG binding domain and recruits the SIN3 complex to promote transcriptional silencing. Co-immunoprecipitation assays performed using oocyte extracts showed that MeCP2 immunoprecipitates with the *Xenopus* Sin3A variant but not the Sin3B variant. In *Drosophila*, the predominant SIN3 isoforms SIN3 187 and SIN3 220 are part of distinct histone-modifying complexes (Figure 1.3) (Spain et al., 2010b). Both complexes contain a common set of components that include RPD3, SDS3, ING1, Pf1, Arid4B, SAP130 and BRMS1. In addition to these components, the SIN3 220

complex also contains three unique interaction partners, p55, LID and EMSY. SIN3 187 interacts with a single catalytic enzyme, RPD3, which is a histone deacetylase, whereas SIN3 220 interacts with the deacetylase and the histone demethylase LID. The *Drosophila* SIN3 isoforms thus associate with distinct histone modifying activities. It is possible that these SIN3 complexes establish distinct histone modification patterns on their target genes, which may be responsible for the non-redundant functions performed by the SIN3 isoforms.

Mammalian SIN3 isoforms also exhibit differential protein interactions. Interestingly, like the *Drosophila* SIN3 220 isoform, preferential interaction with a histone

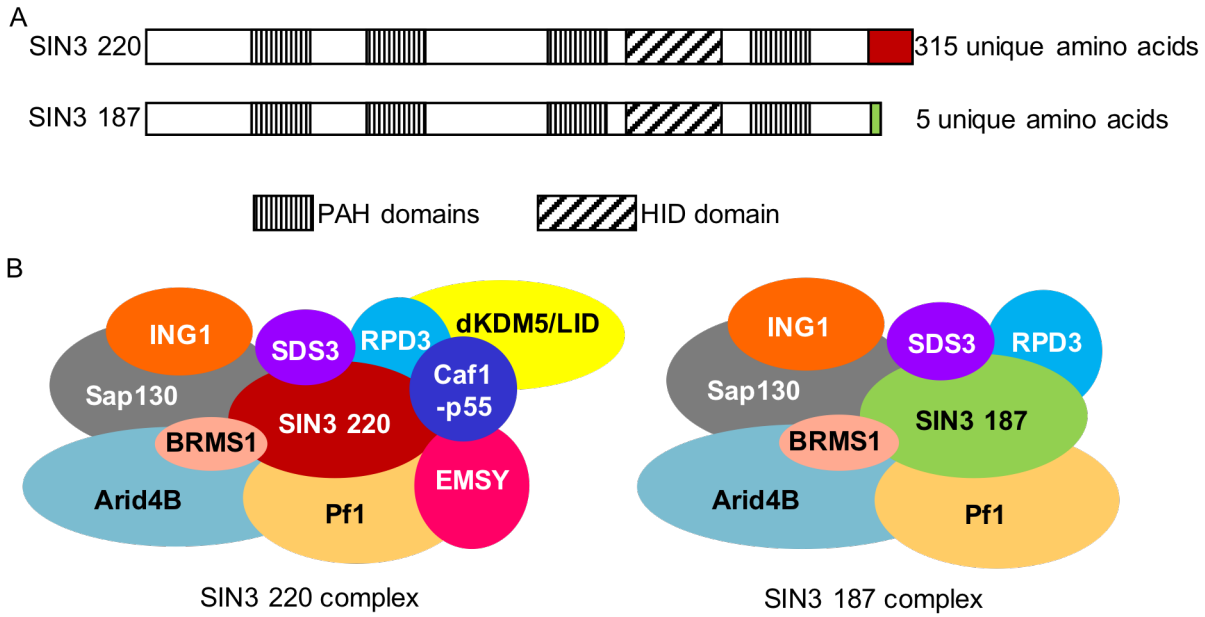


Figure 1.3. The predominant SIN3 isoforms differ at their C-terminus and form distinct histone modifying complexes. A) SIN3 220 and SIN3 187 are identical throughout the length of the protein except at the C-terminus (Pennetta and Paulli,1998; Sharma et al., 2010). B) SIN3 220 and SIN3 187 form different complexes, wherein, SIN3 220 has three unique interaction partners, LID, Caf1-p55 and EMSY (Spain et al., 2010; Moore, 2017).

demethylase is exhibited by the SIN3B protein in mammalian cells. In differentiated myotube extracts, RBP2, which is a homologue of dKDM5/LID, co-immunoprecipitated

with SIN3B but not with SIN3A (van Oevelen et al., 2008). A significant overlap in SIN3B and RBP2 binding on common target genes was also observed using high-density tilling arrays in these cells, indicating coordinated binding of these proteins. The SHMP complex, which consists of SIN3B, HDAC1, MRG15 and Pf1, is another example of distinct protein interactions exhibited by mammalian SIN3 proteins (Jelinic et al., 2011). The Pf1-SIN3B containing complex binds to constitutively transcribed genes in Hela cells and regulates their level of expression. Endogenous Pf1 preferentially interacts with SIN3B but not with SIN3A in co-immunoprecipitation assays as well as on chromatin. Loss of Pf1 and MRG15 significantly affects the recruitment of SIN3B at these genes, but the level of SIN3A is unaffected, further emphasizing on the specificity of interactions within this complex. Surprisingly, SIN3A has been reported to interact with both Pf1 and MRG15 in HEK293 cells (Yochum and Ayer, 2002). These data clearly exemplify the versatility of SIN3 proteins in forming distinct complexes in a cell-type or context-dependent manner, thereby broadening their scope of regulation of cellular processes.

Like SIN3B, SIN3A also exhibits preferential interactions with chromatin associated factors. The hormone-sensitive transcriptional corepressor SMRT physically interacts with SIN3A, but no detectable interaction was observed between SMRT and SIN3B (Nagy et al., 1997). SIN3A also interacts with ATPases involved in chromatin remodeling, BRG1 and hBRM (Sif et al., 2001). The BRG1 complex consists of SIN3A, HDAC2 and RbAp48, while the hBRM complex contains HDAC1 in addition to these proteins. It is, however, unclear whether SIN3B associates with these complexes or was not detected in these experiments as no information on SIN3B was reported in this study. Many protein-protein interaction studies have focused on single SIN3 isoforms or do not

distinguish between the different SIN3 proteins. A detailed analysis of the diverse protein interaction networks mediated by SIN3 isoforms in different species is lacking. To understand the complete picture of the fine-tuned regulation of gene expression and downstream biological processes by the master transcriptional regulator SIN3, mapping these distinct networks regulated by SIN3 isoforms is critical.

Differential regulation of biological processes by SIN3 isoforms

As discussed above, the different SIN3 isoforms interact with a variety of common as well as distinct binding partners. The presence of multiple isoforms, with evolutionarily conserved functional domains, that are capable of unique protein interactions clearly suggests that these proteins perform non-redundant biological functions. Several studies provide clear evidence for the specialized roles of SIN3 isoforms in important cellular processes. The mammalian SIN3 proteins are critical for normal embryonic development. Distinct phenotypic effects, however, are observed upon loss of SIN3A or SIN3B (Cowley et al., 2005; Dannenberg et al., 2005a; David et al., 2008). The *Sin3a* null mouse embryos survive to embryonic day 3.5 (E3.5) but cannot be detected at E6.5, indicating that SIN3A is essential in early embryo development (Cowley et al., 2005; Dannenberg et al., 2005a). Surprisingly, the presence of the highly related SIN3B protein cannot compensate for the loss of SIN3A. Instead, *Sin3b* null embryos can survive to E15.5, implying that SIN3B function is required in late gestation (David et al., 2008).

Differential roles during embryonic development can also be attributed to *Drosophila* SIN3 isoforms (Sharma et al., 2008b). In the initial stages of *Drosophila* embryogenesis, equivalent levels of the SIN3 isoforms are observed. The higher molecular weight isoform SIN3 220 gains predominance during stages 12-16 of embryo

development and is drastically reduced in stage 17, the final stage of embryogenesis. Conversely, the lower molecular weight isoforms SIN3 187 and SIN3 190 exhibit predominant expression during stage 17. This differential expression pattern of SIN3 isoforms suggests that these proteins possibly target different gene sets and play distinct roles during embryonic development. Genome-wide recruitment and transcriptome analysis performed in *Drosophila* S2 cells, identified genes that are specifically regulated by the SIN3 187 isoform (Saha et al., 2016b). Interestingly, gene ontology (GO) analysis of SIN3 187 regulated genes shows enrichment for biological processes such as post-embryonic development, metamorphosis and apoptosis, which is consistent with the observed prominant expression of SIN3 187 during later stages of embryo development.

Analysis of SIN3 isoform function in specific cell types further emphasizes the differential regulation performed by these proteins. In myoblasts and skeletal muscles, inactivation of *Sin3a* leads to a severe phenotype as compared to loss of *Sin3b* (van Oevelen et al., 2010). Mice with a *Sin3a* deletion in the myoblast compartment died within 24 hours after birth, while those with a deleted *Sin3a* in differentiated skeletal muscles did not survive beyond two weeks. Conversely, *Sin3b* deletion in myoblasts and skeletal muscles did not result in any obvious defects in development or survival as compared to control mice. Strikingly, inactivation of both *Sin3a* and *Sin3b* in skeletal muscles led to significantly shorter survival relative to the loss of each individual SIN3 protein. The mammalian SIN3 proteins also play a role in the suppression of neuronal phenotypic traits in pluripotent cells, however, SIN3A exhibits a higher degree of repression of neuronal genes as compared to SIN3B (Halder et al., 2017). Knockdown of *Sin3a* resulted in decreased expression of REST (repressor element-1 (RE-1) silencing transcription

factor) and consequent increase in the level of neuronal markers, leading to the differentiation of P19 cells into neurogenic cells. *Sin3b* silencing in these cells, however, caused a very small effect on the expression of neuronal markers and the differentiation into neuronal cells was less efficient relative to *Sin3a* knockdown. This suggests that SIN3A plays a predominant role in REST-mediated suppression of neuronal differentiation in pluripotent cells. Furthermore, SIN3A is also a player in the process of somatic cell reprogramming (Saunders et al., 2017). Knockdown of *Sin3a* significantly reduced the efficiency of Oct4, Sox2, Klf4, Myc (OSKM)-mediated MEF reprogramming. This study also showed that co-expression of SIN3A with NANOG in partially reprogrammed neural stem cells increased the efficiency of reprogramming more than three-fold as compared to NANOG alone. This reprogramming synergy with NANOG was not exhibited by SIN3B, indicating that this function is specific to SIN3A. Furthermore, SIN3A and SIN3B possibly regulate distinct pathways in hematopoietic stem cells (HSCs). *Sin3a* deletion in the bone marrow resulted in a significant loss in the number of HSCs and immediate progenitor cells (Heideman et al., 2014). In contrast, inactivation of *Sin3b* did not affect HSC viability but instead caused a defect in the differentiation of HSCs into progenitor cells (Cantor and David, 2017).

SIN3 proteins are also important players in oncogenesis (reviewed in detail by (Bansal et al., 2016)). The role of SIN3 in cancer, however, is ambiguous since different studies attribute either tumor suppressive or oncogenic functions to SIN3 proteins (Bansal et al., 2016). Interestingly, a recent study showed that the highly related SIN3 isoforms perform opposing functions in breast cancer metastasis (Lewis et al., 2016). Loss of *SIN3A* caused a significant increase in the number of invasive colonies and metastatic

potential. In contrast, *SIN3B* knockdown substantially decreased breast cancer cell invasion and resulted in reduced metastatic potential. Surprisingly, dual knockdown of *SIN3A* and *SIN3B* behaved similar to loss of *SIN3B*. In that same study, the authors performed correlation analysis investigating SIN3A and SIN3B expression levels in a number of breast cancer subtypes. When all breast cancer subtypes were considered, longer relapse-free survival of patients correlated with high expression of either SIN3A or SIN3B. However, analysis of triple-negative breast cancer samples indicated that longer relapse-free survival is correlated with either high SIN3A or with low SIN3B expression. These data suggest that there may be functional differences between the SIN3 isoforms in different molecular subtypes of cancer. This study is especially interesting in light of the current interest in SIN3 as a potential therapeutic target (Bansal et al., 2016). Future efforts should be directed toward a better understanding of the precise mechanism of regulation by individual SIN3 isoforms in different cell types and especially during cancer progression.

Conclusion

SIN3 was discovered as a transcriptional regulator three decades ago. Over the years, a plethora of studies have implicated SIN3 proteins in the regulation of several critical biological processes and revealed a large repertoire of binding partners. Despite the extensive research, we are far from understanding the complete picture of SIN3 regulation. Several pieces of the puzzle are still missing. As discussed above, the SIN3 protein consists of multiple protein interaction domains and hence is considered the scaffold that holds together the SIN3 histone modifying complex. To the best of our knowledge, however, no study has been conducted to analyze complex integrity upon

loss of SIN3. Careful biochemical analysis of SIN3 complex structure and stability will provide further insight into the scaffolding function of SIN3. Additionally, SIN3 complexes are canonically considered as repressor complexes that suppress gene expression through the activity of histone deacetylases. This model has been challenged with the acquisition of gene expression and chromatin binding data indicating that SIN3 is likely required for direct activation of a subset of targets. The gene activation function of SIN3 histone modifying complexes is not at all understood. Genome-wide analysis of histone modification patterns established by the distinct SIN3 complexes at target genes may help us better understand the role of SIN3 in both activation and repression of gene expression.

In this introduction, I have focused on isoforms of SIN3. There is a single SIN3 protein in the budding yeast, *Saccharomyces cerevisiae*, a single gene that produces multiple isoforms in *Drosophila* and two separate genes that give rise to different isoforms in mammalian cells. Despite the diversity in the number and structure of genes, the SIN3 proteins in different species contain evolutionarily conserved functional domains and form similar histone modifying complexes. It will be a worthy effort to investigate the evolution of SIN3 proteins and determine whether the presence of multiple isoforms in higher organisms contributes to the functional flexibility of SIN3. The SIN3 complexes are pleiotropic in nature and this in part contributes to their wide-range of regulation of biologically important processes. Significant efforts must be directed towards identifying the diverse common and unique interaction partners of SIN3 isoforms in different cell types. This will aid in understanding the intricate network of transcriptional regulators and in turn the critical cellular processes that may be impacted upon misregulation of SIN3.

There is also a gap in the existing knowledge regarding processes that regulate SIN3 proteins. Understanding the mechanisms that regulate the global transcriptional regulator SIN3 are crucial, especially since altered levels of SIN3 have been detected in several types of cancer. Furthermore, the SIN3 isoforms may regulate distinct biological pathways in different cell types. It is imperative to carefully dissect the functional differences between SIN3 isoforms and identify gene targets that are differentially regulated. This will prove to be particularly important in designing therapeutics that are targeted for specific cancer subtypes.

In summary, I have listed a few exciting avenues to further our understanding of epigenetic regulation of gene expression by SIN3 complexes. Although a great deal is known about the interactions of SIN3 proteins and the biological processes regulated by them, the current need is to delve deeper into the intricacies of this network. Ascertaining the overlapping and specialized functions of individual SIN3 isoforms will not only unravel novel strategies of gene regulation but will also expand the current repertoire of therapeutic targets.

Project outline

SIN3 is a widely studied global transcriptional regulator. A large repertoire of genes is regulated by SIN3. Transcriptome analyses have revealed that more than 3% of the annotated genes in the *Drosophila* genome are regulated by SIN3 (Gajan et al., 2016; Pile et al., 2003). Surprisingly, although the role of SIN3 in gene regulation has been well documented, the mechanisms regulating SIN3 itself are still not well known. In my PhD thesis research, I have studied factors affecting the regulation and stability of SIN3

isoforms in *Drosophila*. I have also analyzed the functional differences between the histone modifying complexes formed by the SIN3 isoforms.

To understand the regulation of SIN3, I explored if there was a feedback between the predominant SIN3 isoforms that controlled the level of SIN3 in the cell. In Chapter 2, I demonstrated that there is an interplay between the SIN3 isoforms, wherein, overexpression of SIN3 187 reduces the level of the endogenous SIN3 220 transcript and affects the stability of the protein by targeting it to the proteasome. This is a novel mechanism of regulation, which might ensure that a specific level of SIN3 is maintained in the cells.

Further, I investigated factors affecting the stability of SIN3 220 protein. I attempted to identify post-translationally modified species of SIN3 in *Drosophila.* I have also determined whether the N- or the C-terminus is important for SIN3 220 stability. This research is described in Chapter 3.

In Chapter 4, I have addressed the functional differences between the SIN3 isoform specific histone-modifying complexes that may be responsible for the non-redundant functions of SIN3 isoforms. I have shown that the SIN3 187 and SIN3 220 complexes establish distinct histone modification at SIN3 target genes and differentially regulate the expression of a subset of SIN3 target genes.

Taken together, these findings make a significant contribution in understanding the regulation and function of the predominant SIN3 isoforms. The experiments performed in this study also provide preliminary data for future research. I have discussed key questions that may be addressed in the future in Chapter 5.

CHAPTER 2 INTER-ISOFORM-DEPENDENT REGULATION OF THE *DROSOPHILA* MASTER TRANSCRIPTIONAL REGULATOR SIN3

A version of this work has been published:

Ashlesha Chaubal, Sokol V. Todi, and Lori A. Pile. Inter-isoform-dependent regulation of the *Drosophila* master transcriptional regulator SIN3. J Biol Chem (2017), 6:22.

Introduction

Normal cell function requires precise and coordinated regulation of abundance, localization and interaction of numerous proteins and associated factors. This systematic regulation is brought about by several synchronized processes that govern the production, subcellular location and timely degradation of proteins. Key among these processes is the ubiquitin-proteasome system, which eliminates specific proteins at determined time points (Komander and Rape, 2012). Disturbance of the ubiquitin-proteasome system has serious consequences in cellular function that can directly cause cell death (Ciechanover, 1998). This is especially true for controlling the steady-state levels of master regulatory proteins that regulate diverse transcriptional networks. Specific examples include the histone modifying enzymes, which govern chromatin organization and thus regulate gene networks. Dysregulation of histone modifying enzymes can be disastrous for the cell, since it not only leads to aberrant gene expression, but also affects genome stability (Bannister and Kouzarides, 2011).

The SIN3 HDAC complex, evolutionarily conserved from yeast to mammals, is one such important histone modifying complex (Grzenda et al., 2009b; Silverstein and Ekwall, 2005b). The protein SIN3 serves as a scaffold for the assembly of this complex (Grzenda et al., 2009b). SIN3 is a master transcriptional regulator, which, when deleted or mutated, causes embryonic lethality in *Drosophila* and mice (Cowley et al., 2005; Dannenberg et

al., 2005b; Neufeld et al., 1998; Pennetta and Pauli, 1998b). Previous work from our laboratory showed that depletion of *Drosophila* SIN3 affects several biological processes resulting in severe developmental defects, increased sensitivity to oxidative stress and reduced life span (Barnes et al., 2014; Sharma et al., 2008a; Swaminathan and Pile, 2010). Although many of the gene networks and biological processes regulated by SIN3 are known, the regulation of the SIN3 protein itself is poorly understood.

In *Drosophila,* a single *Sin3A* gene gives rise to multiple SIN3 isoforms, SIN3 187, SIN3 190 and SIN3 220. These isoforms vary only at the C-terminus due to the presence of unique C-terminal exons, form distinct HDAC complexes, are functionally non-redundant, and are differentially expressed during development (timeline summarized in Fig. 1A; (Sharma et al., 2008a; Spain et al., 2010a)). SIN3 220 is the predominant isoform expressed in proliferating cells whereas SIN3 187 expression is comparatively higher in differentiated tissue (Sharma et al., 2008a). This distinct pattern of expression led us to wonder what regulates the isoforms so that they function at different stages during development and in adults. We found a highly interdependent relationship between SIN3 187 and SIN3 220 proteins. SIN3 187 expression causes increased proteasomal degradation of SIN3 220 while also reducing its mRNA levels. To the best of our knowledge, this type of multi-level, inter-isoform regulation that dictates the abundance of a master regulatory protein has not been reported previously.

Materials and methods

Cell culture

Drosophila Schneider cell line 2 (S2) cells were cultured in Schneider's *Drosophila* medium (1X) + L-glutamine (Gibco) with 10% heat-inactivated fetal bovine serum (Gibco)

and 50 mg/ml gentamicin (Gibco) and incubated at 27°C. For S2 cells expressing a transgene, SIN3 187 with an HA tag (SIN3 187HA cells), 0.1 mg/ml penicillin/streptomycin (Gibco) and 0.1 mg/ml Geneticin (Gibco) was added for selection. For S2 cells carrying an HA-tagged *lid* (*little imaginal discs*) transgene, 300 µg/ml Hygromycin B (Invitrogen) was added for selection.

***Drosophila* stocks**

Drosophila melanogaster stocks were maintained and crosses were performed according to standard laboratory procedures. The fly stocks used were as follows: *en*-Gal4 (#8828) and EGFP (#6658) obtained from the Bloomington Stock Center and UAS-SIN3 187HA (described in reference (Sharma et al., 2008a)).

Immunostaining

Wing imaginal discs were dissected from wandering third instar larvae in 1 X phosphate buffered saline (PBS). 20-30 discs were fixed in 4% formaldehyde in 1 X PBS and blocked with 5% normal goat serum, 0.3% Triton X in 1 X PBS. The discs were stained as described previously (Sharma et al., 2008a) using the following antibodies: rabbit anti-SIN3 220 (1:500) (Sharma et al., 2008a), mouse anti-HA-FITC (1:200; Sigma) and Alexa 594 donkey anti-rabbit secondary antibody (1:1000; Invitrogen). The discs were stained with 2 µg/ml DAPI solution and mounted in Vectashield (Vector laboratories). Photographs were taken using an Olympus IX81 or BX53 microscope. All images were taken using the 10X objective lens (Numerical aperture: 0.25) at room temperature. 5% deconvolution was applied to the images using the Microsuite Basic edition software.

Cycloheximide and MG132 assay

3 x 10^6 cells were treated with 0.7 M $CuSO_4$ for 16 h prior to cycloheximide treatment. Cycloheximide (A.G. Scientific) was added to the cells to a final concentration of 100 µg/ml for 10 h. A second set of cells were simultaneously treated with 50 µM MG132 (Sigma) to inhibit the proteasome. Cycloheximide, dissolved in water, and MG132, dissolved in DMSO, were replenished after 6 h.

Time course assay

4 x 10^6 cells were treated with 0.7 M $CuSO_4$ for 48 h. Protein and RNA extracts were made as described below at the indicated time points.

Real-time quantitative reverse transcription PCR assay

Using the RNeasy mini kit (Qiagen), total RNA was extracted from 1 x 10^7 SIN3 187HA cells treated with $CuSO_4$ for different amounts of time. cDNA was generated from the total RNA with random hexamers using the ImProm-II Reverse Transcription System (Promega). The cDNA was used as template in a real-time quantitative PCR assay carried out in a Stratagene Mx3005P real-time thermocycler. Primers (5'-3') used in the PCR reaction were as follows:

SIN3 220: (TTAAAGGCGTATTGCTCGGC and TTGCGCTACAGAGAAGGTGG)

SIN3187HA: (AAATCGATTGCCGTGTAACC and GCGTAATCTGGAACATCGTATGGG)

SIN3 PAN: (AAATCGATTGCCGTGTAACC and GAGCGCAGGATTCGCCAACC)

Taf1: (CTGGTCCTGGTGAGGTGA and CCGGATTCTGGGATTTGA)

Western blot

Protein was extracted by pelleting 10^6 cells through centrifugation, followed by lysis using Laemmli sample buffer (Bio-Rad). Protein concentrations were determined using the Bio-Rad Dc protein assay reagent in accordance with the manufacturer's protocol. Western blot analysis was performed according to standard protocols (Sambrook and Russell, 2001) and as described previously (Gajan et al., 2016). Primary antibodies used were as follows: HA-HRP (1:6000; Sigma), SIN3 PAN (1:2000; (Pile and Wassarman, 2000)), SIN3 220 (1:2000; (Sharma et al., 2008a). Donkey anti-rabbit HRP-conjugated IgG (1:3000; GE Healthcare) was used as the secondary antibody. The antibody signals were detected using the ECL Prime western blot detection agent (GE Healthcare). The blots were photographed using the FOTO/Analyst Investigator (FOTODYNE) or Versa Doc imaging system (Bio-Rad) and quantitated using the TotalLab TL 100 software (Nonlinear Dynamics) and Quantity one software (Bio-Rad), respectively.

Statistical analyses

Significance values were determined by the student t-test using Graphpad. (http://www.graphpad.com/quickcalcs/index.cfm)

Results

During *Drosophila* development, SIN3 isoforms exhibit differential levels of protein expression (Summarized in Fig. 2.1A, based on data previously published by our laboratory (Sharma et al., 2008a)). SIN3 220 is predominantly expressed during stages 12-16 of embryogenesis and markedly reduced during stage 17, the final stage of embryogenesis (Sharma et al., 2008a). Conversely, the lower molecular weight isoforms, SIN3 187 and SIN3 190, exhibit a gradual increase in expression toward the later stages

of embryogenesis, peaking at stage 17 (Sharma et al., 2008a). SIN3 190 expression is limited to embryos and adult females, and so will not be further considered. Additionally, we have observed that cultured *Drosophila* S2 cells expressing HA-tagged SIN3 187 (SIN3 187HA cells), show a significant reduction in the level of endogenous SIN3 220 protein upon induction of SIN3 187HA (Saha et al., 2016a). Collectively, these earlier observations led us to investigate whether the SIN3 187 isoform controls SIN3 220 protein.

We utilized the UAS-Gal4 system (Brand and Perrimon, 1993) to analyze the impact of SIN3 187 on SIN3 220 in developing *Drosophila* tissue. *Drosophila* larval wing imaginal discs predominantly express SIN3 220 (Sharma et al., 2008a). We mated virgin females carrying a HA-tagged SIN3 187 transgene (UAS-SIN3 187HA) to *engrailed*-Gal4 driver males. Progeny of this cross exogenously express SIN3 187HA specifically in the posterior half of wing imaginal discs of wandering third instar larvae. Cells of the anterior half of the wing disc do not express the SIN3 187HA transgene and therefore serve as an internal control for endogenous SIN3 220 protein levels (Fig. 2.1B). We observed that the posterior half of the wing discs, which expressed the SIN3 187HA transgene, had reduced SIN3 220 staining as compared to the anterior half (Fig. 2.1B). The wing imaginal discs obtained from UAS-SIN3 187HA flies, which do not carry a Gal4 driver, and those obtained from a control cross between virgin females carrying the UAS-EGFP transgene and *engrailed*-Gal4 driver males showed uniform SIN3 220 staining throughout the wing disc, indicating that the reduction in SIN3 220 is a specific effect of SIN3 187HA expression (Fig. 2.1B). These data indicate that altering the amount of the SIN3 187 isoform impacts SIN3 220 protein levels *in vivo*.

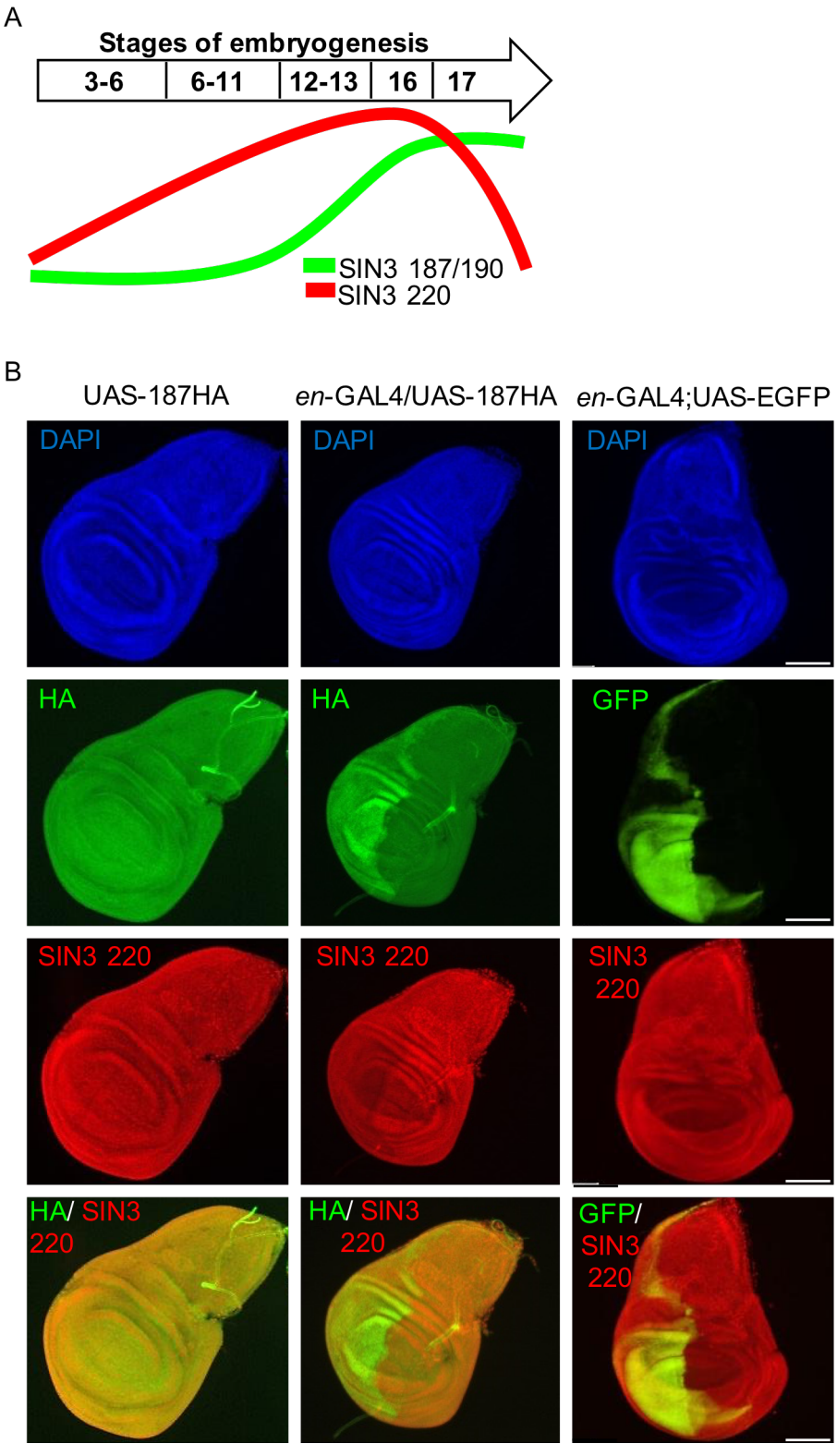


Figure continued on next page

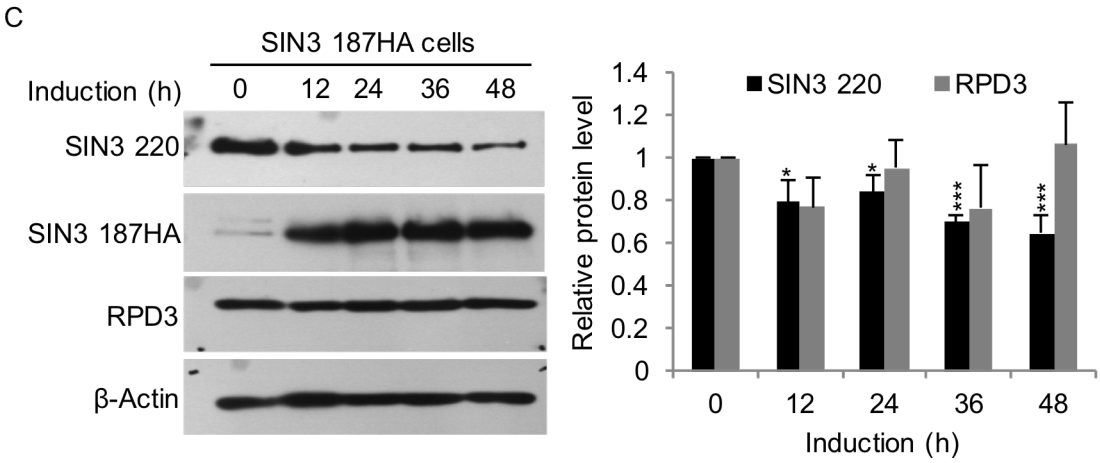


FIGURE 2.1. Ectopic expression of SIN3 187 causes a reduction in endogenous SIN3 220 protein. A) Representation of differential expression of SIN3 isoforms during development, based on data shown in (11). Numbers within the arrow indicate stages of embryogenesis. B) Wing imaginal discs were isolated from wandering third instar larvae of the indicated genotype. Wing discs were immunostained with α-HA to detect SIN3 187HA and α-SIN3 220 to detect endogenous SIN3 220. The green fluorescence observed in the UAS-187HA control (left panels) is due to background signal. Wing discs obtained from the UAS-EGFP X *en*-GAL4 cross were immunostained with SIN3 220 antibody. The green fluorescence observed in this wing disc is due to EGFP expression driven by *engrailed*-Gal4. Scale bars represent 100 μm. C) SIN3 187HA cells were treated with $CuSO_4$ for the indicated times to induce expression of the SIN3 187HA transgene. Protein extracts were probed with HA, RPD3 and SIN3 220 antibodies. β-Actin levels are shown as the loading control. The amount of SIN3 220 and RPD3 protein relative to actin is quantitated in the graph below. The results are the average of three independent biological replicates. Error bars represent standard error of the mean. *, $p < 0.05$, ***, $p < 0.005$.

To further examine this relationship between SIN3 isoforms, we turned to cultured S2 cells that, like wing imaginal disc cells, are proliferative and predominantly express SIN3 220 (Sharma et al., 2008a). We performed a time-course experiment using SIN3 187HA cells. At distinct times following induction of SIN3 187HA, whole cell protein extracts were prepared and the expression of SIN3 isoforms monitored by western blot. Compared to time 0 h, the endogenous SIN3 220 protein gradually decreased upon induced SIN3 187 expression (Fig. 2.1C). This effect was particularly noticeable at 36

and 48 h of induction, compared with non-induced cells (Fig. 2.1C). These data are consistent with the observation made using developing fly tissue (Fig. 2.1B) that SIN3 187 expression significantly impacts the amount of its 220 counterpart. To determine whether the effect of SIN3 187HA was specific for SIN3 220, we analyzed the protein level of another SIN3 complex component, RPD3. No significant change was observed on the RPD3 protein level upon SIN3 187 induction (Fig. 2.1C). Together, the results obtained from the larval wing imaginal discs and S2 cells argue that SIN3 187 specifically regulates SIN3 220 protein levels.

To gain more mechanistic insight into this interplay, we examined the stability of endogenous SIN3 220 upon exogenous expression of SIN3 187 by conducting cycloheximide-based pulse-chase experiments. Cycloheximide halts the translation of new protein, thus allowing us to monitor the turnover of existing SIN3 220 protein over time. SIN3 187HA cells were induced to express SIN3 187. Non-transfected S2 cells treated in the same way were used as the control. Both sets of cells were then treated with cycloheximide and the stability of endogenous SIN3 220 protein was monitored by western blot. In S2 cells, which express little SIN3 187, SIN3 220 is highly stable (Fig. 2.2A). When SIN3 187HA expression is induced, however, the turnover of endogenous SIN3 220 is significantly accelerated (Fig. 2.2B). The SIN3 220 protein level was markedly diminished upon induction of SIN3 187 expression, within 10 h of cycloheximide treatment, as compared to the level in S2 cells.

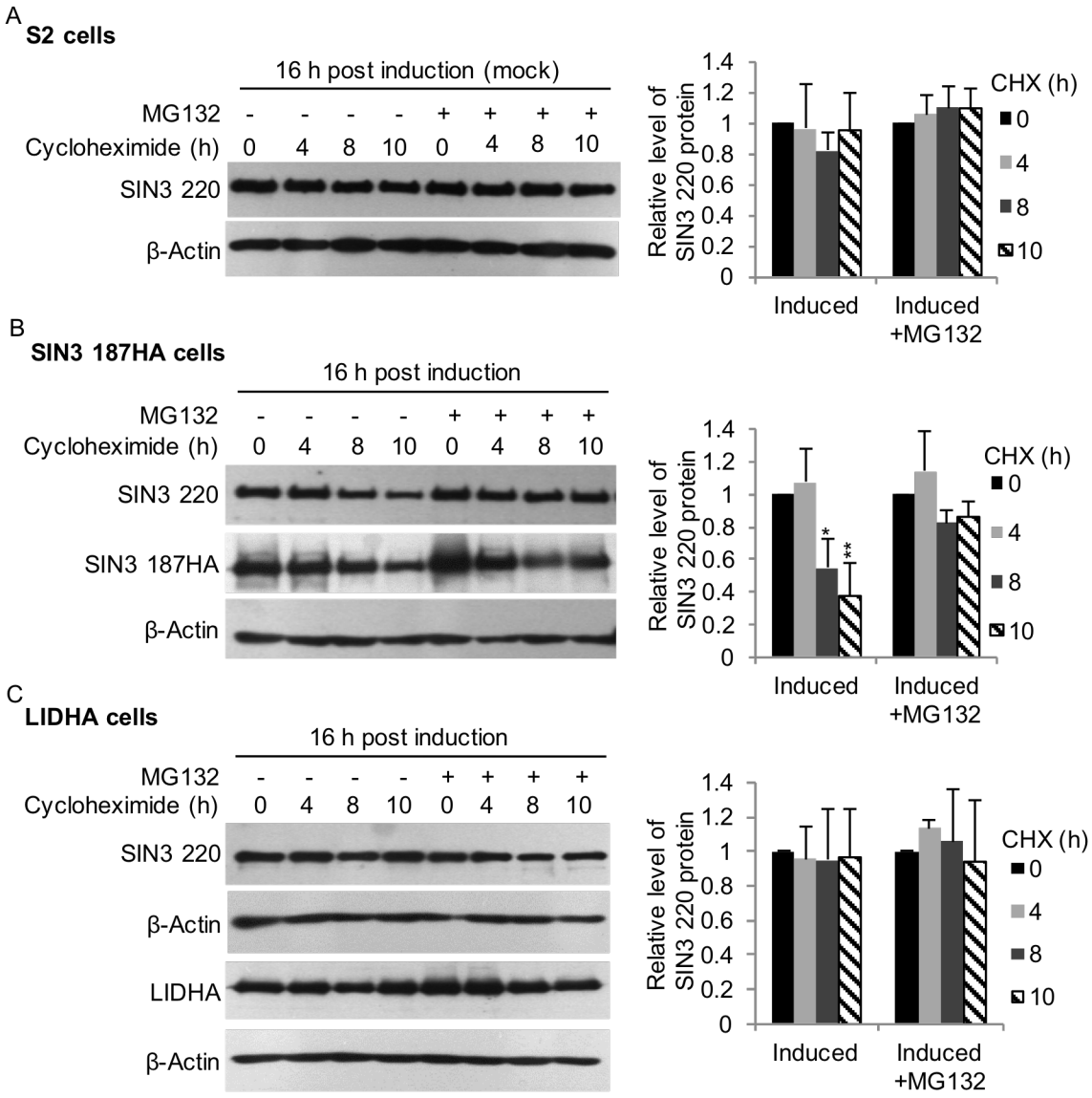


FIGURE 2.2 SIN3 187 increases SIN3 220 protein turnover by targeting it for proteasome-dependent degradation. Cycloheximide treatment was performed for (*A*) S2 (*B*) SIN3 187HA and (*C*) LIDHA cells. Protein extracts isolated at 0, 4, 8 and 10 h were analyzed by western blot. SIN3 220 levels are analyzed for two sets of cells: cells treated with $CuSO_4$ for 16 h (Induced) and cells treated with $CuSO_4$ for 16 h and then treated with the proteasome inhibitor MG132 for indicated time (Induced + MG132). Protein extracts obtained from SIN3 187HA cells were probed with HA and SIN3 220 antibodies. Protein extracts obtained from S2 and LIDHA cells were probed with pan-SIN3 antibody. LIDHA protein extracts were also probed with HA antibody. β-Actin levels were used as the loading control. Relative level of SIN3 220 protein is quantitated in the adjoining graphs. The results are the average of three biological independent replicates. Error bars represent standard error of the mean.*, $p < 0.05$, **, $p < 0.01$.

Next, we examined whether increased turnover of SIN3 220 in the presence of SIN3 187 was proteasome-dependent. To this end, we performed the cycloheximide-based pulse-chase in the presence of the proteasome inhibitor MG132. Treatment of cells with MG132 significantly slowed the degradation of SIN3 220 (Fig. 2.2B). As an additional control, we determined whether expression of a component of the SIN3 complex has a similar effect on SIN3 220 turnover. We used an S2 cell line that carries a transgene for expression of HA-tagged dKDM5/LID (Little imaginal discs), referred to as LID-HA cells. Overexpression of dKDM5/LID in the presence of cycloheximide did not alter the turnover rate of SIN3 220 protein (Fig. 2.2C). We conclude that SIN3 187 specifically leads to increased proteasomal degradation of SIN3 220, a protein that is normally quite stable.

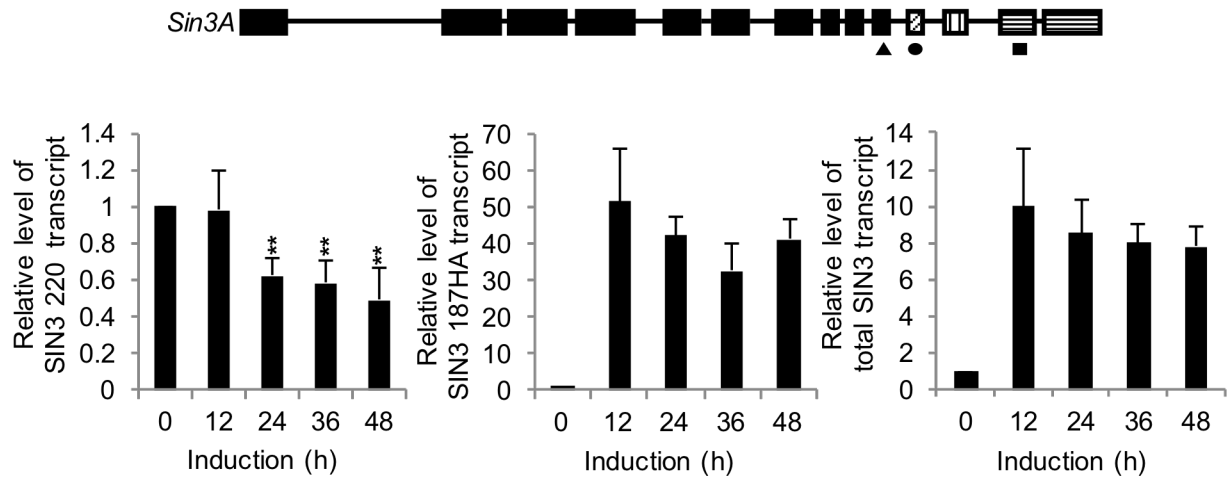


FIGURE 2.3. Presence of SIN3 187 causes a reduction in the SIN3 220 transcript. RT-qPCR analysis using SIN3 isoform-specific primers. In the schematic representing the *Sin3A* gene, filled squares indicate common exons, squares with diagonal, vertical and horizontal lines indicate unique SIN3 187, SIN3 190 and SIN3 220 exons, respectively. Small triangle, circle and square indicate the positions of the primers. *Taf1* was used as a control for normalizing transcript levels. The results are the average of three biological independent replicates. Error bars represent standard error of the mean. **, $p < 0.01$.

The impact of SIN3 187 on SIN3 220 protein turnover led us to wonder whether the interplay between these isoforms occurs at multiple levels. To explore this possibility, we analyzed the effect of SIN3 187 expression on SIN3 220 transcript. Total RNA was

extracted from SIN3 187HA cells that had been induced for different amounts of time to express the SIN3 187 transgene. Real-time quantitative reverse transcription PCR (RT-qPCR) analysis was performed using isoform-specific primers to quantify the level of the different *Sin3A* transcripts (Fig. 2.3). We observed a reduction in the amount of SIN3 220 transcript upon induction of SIN3 187HA compared with non-induced cells (Fig. 2.3). The data in Figures 2.2 and 2.3 provide evidence of multiple levels of control exerted by SIN3 187 on to SIN3 220, which collectively result in decreased SIN3 220 protein levels.

Discussion

SIN3 is well studied as a master transcriptional regulator that governs several important cellular pathways, including cell proliferation and energy metabolism (Kadamb et al., 2013a). While SIN3 functions continue to be explored, the processes that regulate SIN3 itself remain poorly understood. In this study, we report an interplay between the predominant isoforms of SIN3. SIN3 187 expression caused a substantial reduction in the level of SIN3 220 protein in developing flies and in cultured cells. Expression of SIN3 187 impacted SIN3 220 at both transcript and protein levels. The 187 isoform led to reduced 220 mRNA, while also increasing the proteasomal turnover of its protein. Collectively, our data suggest the presence of an active regulatory signal that is triggered by SIN3 187 to reduce the amount of SIN3 220. Control of SIN3 220 at multiple levels likely ensures efficient removal of this isoform during specific developmental stages and highlights the possibility that regulation of SIN3 isoform expression is critically important.

We showed the involvement of the proteasome in maintaining the level of SIN3 220. It is likely that post-translational modifications play a role in targeting SIN3 220 to this degradative machinery. Mammalian SIN3 is SUMOylated and ubiquitinated (Kong et

al., 2010a; Pungaliya et al., 2007b). It remains to be determined whether a similar situation exists in *Drosophila*. In initial attempts to examine post-translational modifications of *Drosophila* SIN3, we performed stringent immunoprecipitation experiments using either antibodies for endogenous and HA-tagged SIN3, or a high-affinity ubiquitin binding resin to detect ubiquitinated SIN3 species. No distinct higher molecular weight bands indicative of ubiquitinated SIN3 were observed in our preliminary experiments (data not shown). As an alternative approach, we selected three lysine residues in the fly ortholog that are reportedly ubiquitinated in the human counterpart (Kim et al., 2011; Mertins et al., 2013). Mutating these residues into the similar but non-ubiquitinatable amino acid arginine, alone or in combination, did not impact SIN3 220 cellular protein levels (data not shown). It is possible that other lysine residues in *Drosophila* SIN3 are ubiquitinated, or that its proteasomal degradation might be ubiquitin-independent. There is a growing number of proteins that do not require ubiquitination to be degraded by the proteasome (Blount et al., 2014). Our initial studies, however, do not definitively rule out the possibility that ubiquitination of SIN3 220 is involved at some point to regulate its turnover.

Drosophila SIN3 isoforms differ only at the C-terminal region. 187 and 220 isoforms both interact with a core group of HDAC complex components that are conserved across species (Spain et al., 2010a). Perhaps the SIN3 protein needs to be amidst this core complex to be stable. One possibility to account for SIN3 220 protein reduction by SIN3 187HA expression is that excess SIN3 187 sequesters the common complex components and exposes SIN3 220 for proteasomal degradation.

SIN3 220 transcript is also reduced upon exogenous expression of SIN3 187. The molecular mechanism governing this effect remains to be elucidated. Perhaps some regulatory factor detects the presence of SIN3 187 transcript and alters splicing at the *Sin3A* gene, resulting in reduced SIN3 220 transcript. Another possibility could be transgene-induced post-transcriptional gene silencing (Cogoni and Macino, 2000). When the SIN3 187HA transgene is expressed, the overall level of *Sin3A* mRNA is very high. This may trigger degradation of the *Sin3A* transcript. Further investigation will help us better understand which mechanism is responsible for regulating the amount of SIN3 220 transcript.

It will be interesting to determine whether mechanisms akin to the ones that we reported exist to regulate SIN3 in organisms other than *Drosophila*. Our finding of inter-isoform-dependent regulation of SIN3 expands the overall understanding of avenues through which master switches are controlled during development. It also suggests that similar processes may apply to other key proteins with isoform-specific properties.

CHAPTER 3 INVESTIGATING THE STABILITY AND POST-TRANSLATIONAL MODIFICATION OF *DROSOPHILA* SIN3 220

Introduction

SIN3 is a global transcriptional regulator involved in the regulation of diverse processes such as development, energy metabolism, cell proliferation and cellular senescence (Kadamb et al., 2013b). Several studies have also implicated SIN3 proteins in the process of oncogenic transformation (Bansal et al., 2016). Since SIN3 governs the expression of a vast number of gene targets, understanding factors that control the stability and thereby function of SIN3 proteins is critical, both for normal biology and disease.

The level of proteins in a cell can be accurately maintained by controlling their rate of synthesis and degradation. Post-translational modifications, in particular ubiquitination and SUMOylation, are key players that can modulate stability of proteins and target them for degradation. The process of ubiquitination involves linking a conserved protein, ubiquitin, to a target substrate through three enzymatic steps catalyzed by E1 activating enzymes, E2 conjugating enzymes and E3 ligases (Hershko and Ciechanover, 1992). The target protein, which can be mono-ubiquitinated or poly-ubiquitinated, is then targeted to the proteasome for degradation and the ubiquitin moieties are recycled. Several key proteins, such as cell cycle regulators, transcription factors, tumor suppressors and oncoproteins are regulated by the ubiquitin system (Hershko and Ciechanover, 1998). The small ubiquitin-related modifier (SUMO) protein also post-translationally modifies a large repertoire of proteins in the cell. The process of SUMOylation is similar to that described for ubiquitin (Kim et al., 2002). In addition to targeting proteins for degradation, SUMOylation also plays a role in protein translocation,

subnuclear structure formation and as an antagonist to ubiquitination. SUMOylation and ubiquitination may both be involved in regulation of mammalian SIN3 proteins. RNF220, an E3 ubiquitin ligase, ubiquitinates the mammalian SIN3B protein (Kong et al., 2010b). The SIN3A protein is SUMOylated by the E3 ligase, TOPORS (Pungaliya et al., 2007a). Very little is known however, about other key players and mechanistic details regarding post-translational modification of SIN3 proteins.

Although progress has been made in understanding the regulation of the global transcriptional regulator SIN3, a great deal remains to be elucidated. The *Saccharomyces cerevisiae* SIN3 protein has two regions with significant PEST scores (Wang et al., 1990). PEST regions are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) amino acid residues and are considered indicators of rapid protein degradation (Rogers et al., 1986). The role of PEST regions in SIN3 degradation has not been extensively addressed. SIN3 itself has been implicated in influencing the stability of several proteins such as p53, SMRTER, Mad4 and Myc, but the factors governing SIN3 stability are not yet understood (Kadamb et al., 2013b). Since SIN3 plays an important role in regulating several critical biological processes, it is essential to maintain an accurate level of SIN3 in the cells. SIN3 187 and SIN3 220 are the predominant SIN3 isoforms in *Drosophila* (Sharma et al., 2008b). Unpublished work from our laboratory has shown that ubiquitous expression of a N-terminal TAP-tagged (N-TAP) SIN3 220 protein in *Drosophila* results in embryonic lethality. The same result, however, was not obtained upon ubiquitous expression of SIN3 220 with a C-terminal HA (C-HA) tag (Spain et al., 2010b). It had also been determined that when these tagged-SIN3 proteins were expressed in S2 cells, the level of N-TAP SIN3 220 protein was higher compared to C-HA SIN3 220. We therefore

hypothesized that N-TAP SIN3 220 is not efficiently degraded, leading to an increased total level of SIN3 220 protein, which in turn causes embryonic lethality when expressed during fly development.

In this research, we aimed to identify factors that influence the stability of SIN3 protein in *Drosophila*. We demonstrate that both the N and C-terminus of SIN3 proteins are important for targeting SIN3 for degradation. We also investigated whether loss of specific lysine residues in potential ubiquitination sites impacted the level of SIN3 220 protein in the cell.

Materials and methods

Cell culture

S2 and SIN3 187HA cells were cultured as described in Chapter 1. S2 cells expressing a transgene, SIN3 220 with an HA tag (SIN3 220HA cells), were cultured in Schneider's *Drosophila* medium (1X) + L-glutamine (Gibco) with 10% heat-inactivated fetal bovine serum (Gibco). 0.1 mg/ml penicillin/streptomycin (Gibco) and 0.1 mg/ml Geneticin (Gibco) was added for selection. 1 μl/ml of 0.7M $CuSO_4$ was added to the relevant cell lines for induction of tagged proteins.

Generation of lysine to arginine mutants

To generate lysine to arginine mutants at putative ubiquitination sites we used a plasmid containing the SIN3 220 gene with a sequence encoding a C-terminal HA tag (C-HA SIN3 220) in the pRMHa4 vector. Using site-directed mutagenesis, we replaced the codon for lysine at amino acid position 71, 1209 and 1229 individually with that for arginine. The mutations at these specific positions were verified by DNA sequencing (Eton Biosciences). After verification, the single mutants (SIN3 220 K71R, SIN3 220

K1209R and SIN3 220 K1229R) were used for a second round of site-directed mutagenesis to create double lysine mutants (SIN3 220 K71 1209R, SIN3 220 K71 1229R, and SIN3 220 K1209 1229R), which were verified by DNA sequencing. To generate the triple lysine mutant, SIN3 220 K71 1209R was used to perform site-directed mutagenesis and the mutation was confirmed by DNA sequencing. The primers used for site-directed mutagenesis are listed in Table 3.1.

Table 3.1: Primers used for site-directed mutagenesis

Primer Name	Sequence
Ubq sdm acK71R forward	CACGCCACGCTTACGAGTGGAGGATGCG
Ubq sdm acK71R reverse	CGCATCCTCCACTCGAAGCGTGGCGTG
Ubq sdm acK1209R forward	GAAGTACTACCTCCGGTCTCTCGATCAC
Ubq sdm acK1209R reverse	GTGATCGAGAGACCGGAGGTAGTACTTC
Ubq sdm acK1229R forward	GCCCTGCGCTCACGGAGTCTGTTTAAC
Ubq sdm acK1229R reverse	GTTAAACAGACTCCGTGAGCGCAGGGC

Nuclear fraction extraction and immunoprecipitation

Nuclear fraction extraction and immunoprecipitation was carried out from SIN3 220HA cells as previously described (Spain et al., 2010). In brief, 150 μl of interaction buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 0.5 mM EDTA,1% Triton X-100) was added to 850 μl of nuclear extract prepared from 1 x 10^8 cells along with 40 μl of anti-HA agarose beads (Sigma) and incubated at 4°C overnight. The beads were then washed with IP wash buffer 1 (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), IP wash buffer 2 (20 mM HEPES (pH 7.4), 500 mM NaCl,

0.5 mM EDTA,1.5% Triton X-100, 0.1% sodium deoxycholate and 10% glycerol) and IP wash buffer 3 (20 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and 1.5%Triton X-100) for 5 minutes each at 4°C. The immunoprecipitated proteins were eluted using Laemmilli buffer (Bio-Rad) for 20 minutes at room temperature. The eluted proteins were analyzed by western blotting.

Transient transfection

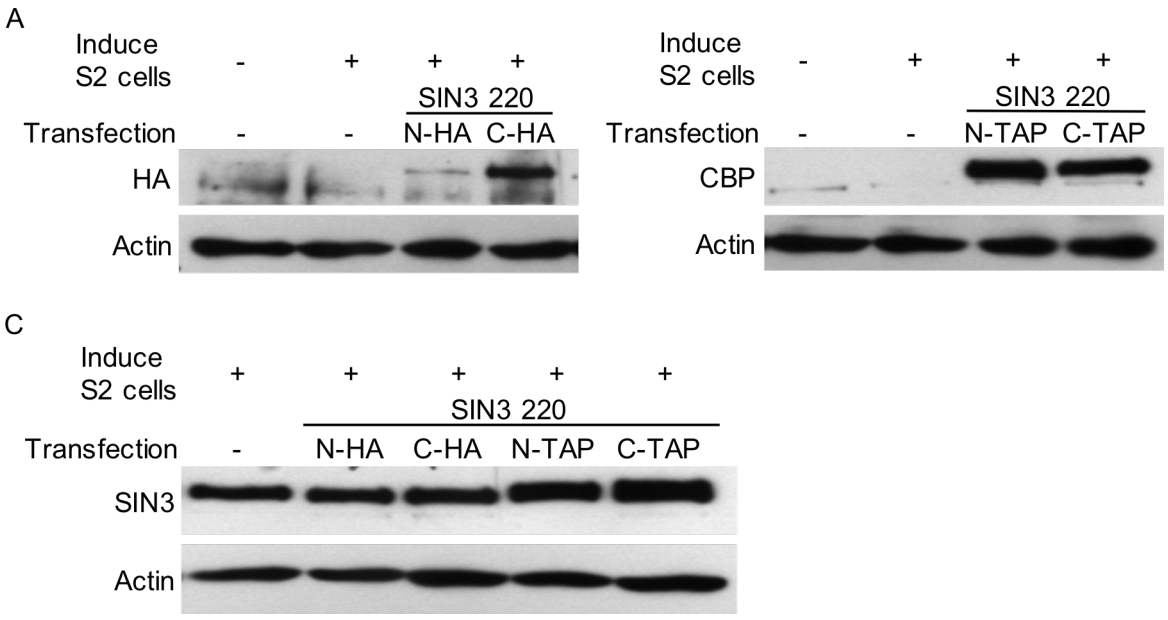
SIN3 220 tagged constructs and lysine mutants were transiently transfected into S2 cells and SIN3 187HA cells using the Effectene transfection kit (Qiagen) as per the manufacturer's protocol. Briefly, 1.0 μg of plasmid DNA was mixed with 8 μl Enhancer and incubated for 5 minutes followed by addition of 10 μl Effectene reagent and a 15 minute incubation at room temperature. The solution was then added to 2 x 10^6 cells after mixing with 1 ml of appropriate cell culture media. 1 ml of cell culture media was also added to mock transfected cells. The cells were induced with 1 μl/ml of 0.7M $CuSO_4$ after 24 hours. Protein extracts were made after 24 hour induction and analyzed by western blotting.

Western blotting

Western blot was performed as described in Chapter 1. Primary antibodies used were as follows: HA-HRP (1:6000; Sigma), SIN3 PAN (1:2000; Pile and Wasserman, 2000), CBP (1:3000, Upstate Cell Signaling Solutions), RPD3 (1:3000, Pile and Wasserman, 2000), Actin (1:1000, Cell Signaling) and Tubulin (1:1000, Cell Signaling), SUMO-2/3 (kindly provided by Dr. Xiang-Dong Zhang, Subramonian et al., 2014). Secondary antibodies used are donkey anti-rabbit HRP-conjugated IgG (1:3000; GE Healthcare) and sheep anti-mouse HRP-conjugated IgG (1:3000; GE Healthcare).

Results and discussion

As described in Chapter 2, overexpression of SIN3 187 leads to the proteasomal degradation of SIN3 220. This interplay is not only a possibly unique regulatory mechanism but also a tool to analyze factors that affect the stability of SIN3 220. We have previously observed that exogenous expression of un-tagged SIN3 220 protein or C-HA SIN3 220 in S2 cells does not lead to an increase the overall level of SIN3. We hypothesize that the level of SIN3 220 is critically controlled in S2 cells and that excess SIN3 220 protein is targeted for degradation. Therefore, any factor that interferes with the degradation of SIN3 220, thereby stabilizing the protein, should result in a higher total level of SIN3 220 relative to control in S2 and SIN3 187HA cells.



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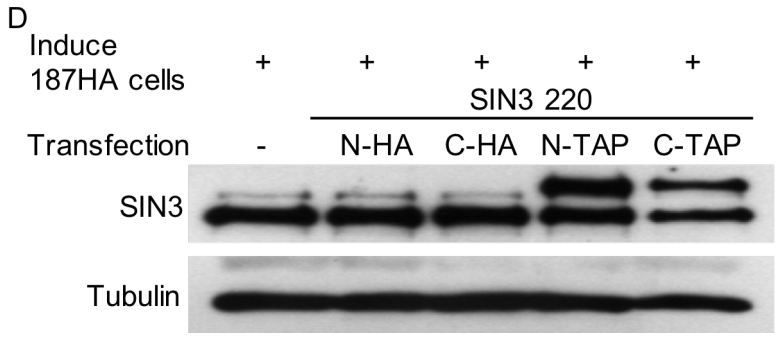


Figure 3.1. Presence of a TAP tag at either N or C-terminus interferes with the degradation of SIN3 220 protein. SIN3 220 HA and TAP-tagged constructs were transiently transfected in S2 cells (A), (B) and (C) and 187HA cells (D). Protein extracts isolated after 24 h $CuSO_4$ induction were analyzed by Western blotting. (A) Protein extracts from mock transfected and SIN3 220 N-HA and SIN3 220 C-HA transfected cells were probed with HA antibody to verify expression of these constructs. (B) Protein extracts from mock transfected and SIN3 220 N-TAP and SIN3 220 C-TAP transfected cells were probed with CBP antibody to verify expression of constructs. (C) Protein extracts from mock transfected and SIN3 220 N-HA, SIN3 220 C-HA, SIN3 220 N-TAP and SIN3 220 C-TAP transfected cells were probed with SIN3 antibody to determine the total level of SIN3 protein upon transfection. Beta-actin levels were used as loading control. (D) Protein extracts from mock transfected and SIN3 220 N-HA, SIN3 220 C-HA, SIN3 220 N-TAP and SIN3 220 C-TAP transfected cells were probed with SIN3 antibody to determine the stability of SIN3 220 tagged constructs upon SIN3 187 overexpression. Tubulin levels were used as loading control.

Unlike C-HA SIN3 220, N-TAP SIN3 220 can be expressed at a high level in S2 cells. This led us to ask whether the position or the nature of a tag affects the stability of SIN3 220 protein. To address this question, we generated SIN3 220 constructs to express SIN3 220 with an HA or a TAP tag on either the N terminus (N-HA or N-TAP) or C the terminus (C-HA or C-TAP). Details of construction of these plasmids is provided in the undergraduate honors thesis of Michael Sobolic. We transiently transfected N-HA SIN3 220, C-HA SIN3 220, N-TAP SIN3 220 and C-TAP SIN3 220 encoding constructs into S2 cells and SIN3 187HA cells. The stability of these tagged proteins was analyzed by western blotting. The expression of the TAP-tagged and HA-tagged SIN3 protein was verified by probing the blots with tag-specific antibodies (Figure 3.1A, B). In S2 cells, the

TAP-tagged SIN3 proteins exhibit a higher protein level as compared to the HA-tagged proteins (Figure 3.1C). Similarly, in SIN3 187HA cells, the level of N-HA SIN3 220 and C-HA SIN3 220 is reduced upon SIN3 187 expression like endogenous SIN3 220 (Figure 3.1D). Conversely, the level of N-TAP SIN3 220 and C-TAP SIN3 220 remains high in the presence of SIN3 187 and is comparable to that of the exogenously expressed SIN3 187 (Figure 3.1D). This suggests that both the N-terminus and the C-terminus may be important for SIN3 220 stability. Since the TAP tag (~ 21kDa) is a larger tag as compared to HA (~ 1.1kDa), it is possible that presence of a TAP tag protects the SIN3 220 protein from being targeted for degradation. Interestingly, in mammalian cells, both the N-terminal and the C-terminal domains of SIN3B are ubiquitinated by RNF220 (Kong et al., 2010b). It is therefore possible, that presence of a large tag at the N or C-terminus interferes with the post-translation modification of SIN3 220, thereby affecting its degradation.

Although there is evidence for post-translational modification of SIN3 in mammalian cells, ubiquitination or SUMOylation of SIN3 has not been reported in *Drosophila* or any other model system. We attempted to identify ubiquitinated SIN3 species by either immunoprecipitating endogenous SIN3 or by enriching ubiquitinated proteins and analyzing the immunoprecipitates by western blotting using specific antibodies. No conclusive evidence, however, was obtained from these reciprocal immunoprecipitation experiments (data not shown). We therefore addressed this question using an alternative approach. The database of post-translational modifications (dbPTM) (http://dbptm.mbc.nctu.edu.tw/index.php) is a comprehensive database for protein post-translational modifications (PTMs) that integrates information for experimentally validated and putative PTMs generated from several predictive tools (Lee et al., 2006). For our

study, we identified specific lysine residues in the mammalian SIN3 protein predicted by dbPTM as potential ubiquitination sites. Using the sequence alignment tool Clustal W, we observed that these specific lysine residues are conserved in the *Drosophila* SIN3 protein (Figure 3.2A). We hypothesized that if these lysine residues are putative ubiquitination sites, mutating them to arginine should stabilize the SIN3 220 protein

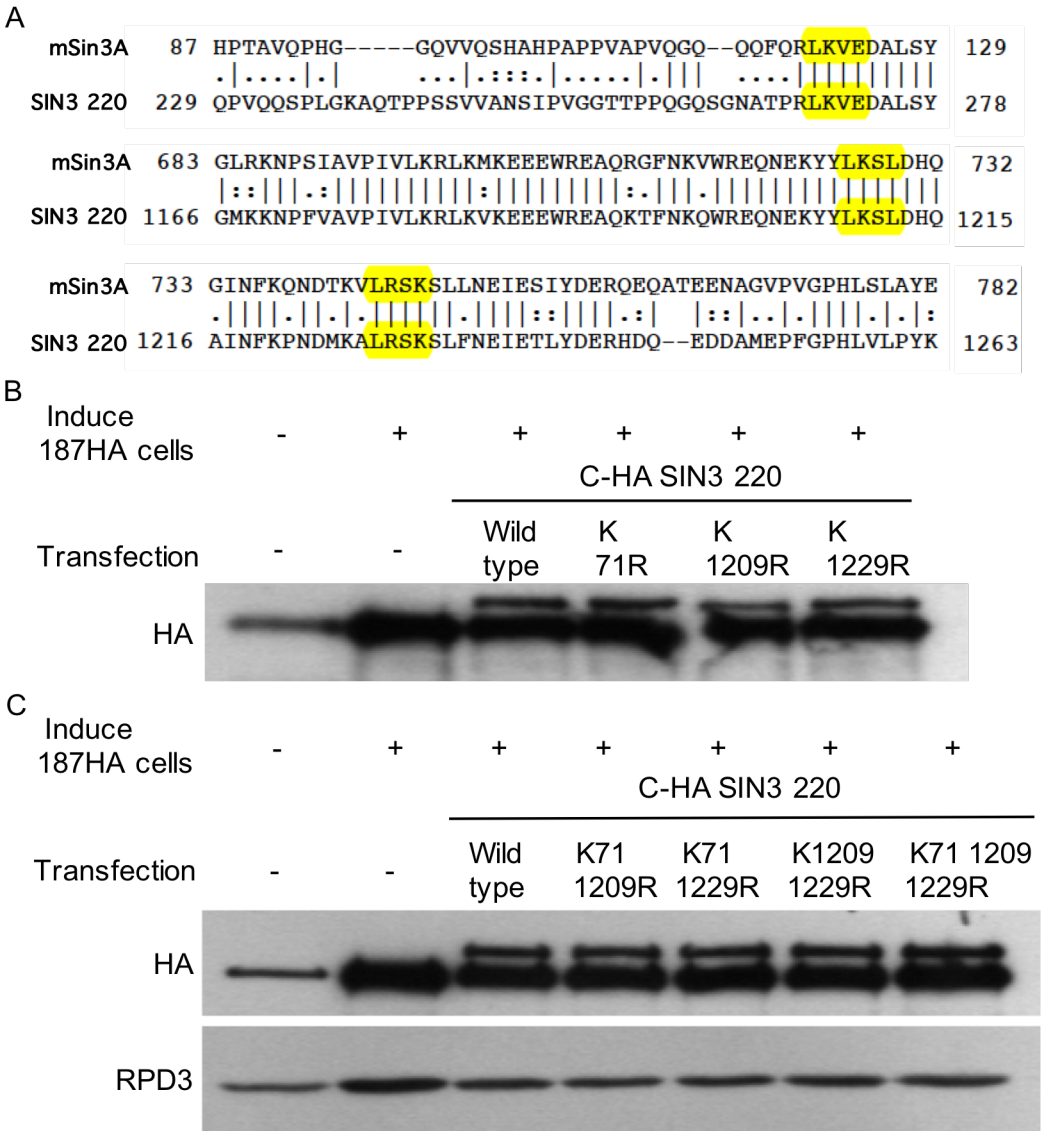


Figure 3.2. Lysine to arginine mutations at putative ubiquitination sites do not significantly alter the stability of SIN3 220. (A) Sequence alignment of specific regions in mammalian Sin3A and *Drosophila* SIN3 220 proteins. Putative ubiquitination sites conserved between these proteins are highlighted. The lysine (K) residues in these

putative sites were mutated to arginine (R) residues using site-directed mutagenesis. SIN3 187HA cells were transiently transfected with SIN3 220 3'HA constructs containing specific single (B), double and triple (C) K to R mutations. Protein extracts isolated after 24 h $CuSO_4$ induction were analyzed by Western blotting. (B) Protein extracts from mock transfected and wild type C-HA SIN3 220, SIN3 220 K71R, SIN3 220 K1209R and SIN3 220 K1229R transfected cells were probed with HA antibody to determine the stability of constructs upon SIN3 187 overexpression. (C) Protein extracts from mock transfected and wild type SIN3 220 3'HA, SIN3 220 K71 1209R, SIN3 220 K71 1229R, SIN3 220 K 1209 1229R and SIN3 220 K71 1209 1229R transfected cells were probed with HA antibody to determine the stability of these constructs upon SIN3 187 overexpression. RPD3 levels were used as loading control.

Using site-directed mutagenesis we created single (K71R, K1209K, K1229R), double (K71 1209R, K71 1229R, K1209 1229R) and triple (K71 1209 1229R) lysine to arginine mutations in C-HA SIN3 220 at potential ubiquitination sites. These SIN3 220 encoding constructs were transiently transfected in SIN3 187HA cells and the stability of the mutated proteins was analyzed by western blotting. C-HA SIN3 220, which was used to create these mutants, was used as a control. Converting lysine to arginine at putative ubiquitination sites individually or in combination did not significantly alter the stability of SIN3 220 (Figure 3.2 B, C). This suggests that these specific lysine residues may not play a role in targeting SIN3 220 for degradation. It is conceivable that other lysine residues may be important for ubiquitination of SIN3 220 or that SIN3 220 may be targeted for degradation by other post-translational modifications.

As mentioned, the mammalian SIN3A isoform, which is a paralog of SIN3B, is SUMOylated by TOPORS (Pungaliya et al., 2007a). Since SIN3A exhibits higher similarity to *Drosophila* proteins relative to SIN3B, we asked whether *Drosophila* SIN3 proteins undergo SUMOylation. To detect SUMOylated SIN3 species we performed immunoprecipitation experiments in S2 cells expressing a transgene encoding HA-tagged SIN3 220 (SIN3 220HA cells) under the control of a metallothionien promoter. Similar to S2 cells, the total level of SIN3 is tightly regulated in SIN3 220HA cells and does not

increase even upon induction of the SIN3 220 transgene (Saha et al., 2016b). We predict that upon induction and subsequent expression of SIN3 220HA, the excess SIN3 220 protein is targeted for degradation, thus maintaining a specific level of SIN3 220 in the cell. We attempted to enrich SUMOylated SIN3 species by immunoprecipitating HA-tagged SIN3 220 using anti-HA agarose beads. The immunoprecipitates were analyzed by western blotting using SIN3 and SUMO-2/3 antibodies. No distinct signal was observed for SIN3 in the blot probed with anti-SUMO-2/3 indicating that SUMOylated SIN3 species could not be detected in our experiment (Figure 3.3).

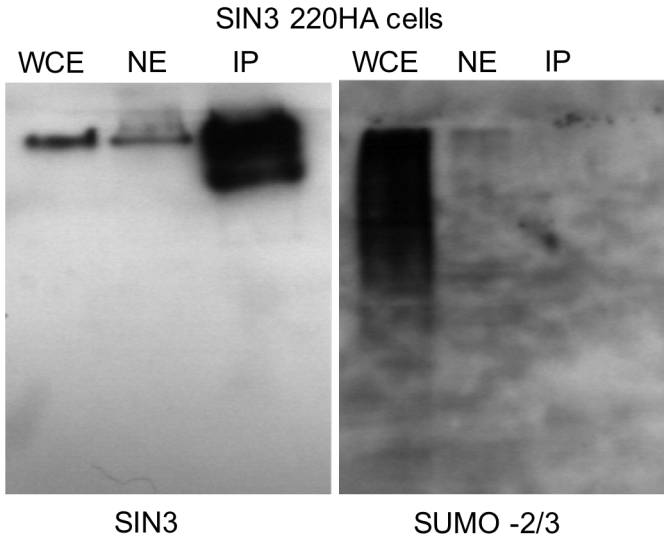


Figure 3.3. SUMOylated species of SIN3 could not be detected upon immunoprecipitation of tagged SIN3 220. Co-immunoprecipitation assays were performed with extracts prepared from SIN3 220HA cells using anti-HA agarose beads. Protein extracts were analyzed by Western blotting. Whole cell extracts (WCE), nuclear extracts (NE) and immunoprecipitate (IP) were probed with SIN3 and SUMO-2/3 antibodies to detect SUOMylated SIN3 species.

In conclusion, we have demonstrated that the N and C-terminus play an important role in SIN3 220 stability and that the presence of a large protein tag at either terminus possibly interferes with the degradation of SIN3 220. No post-translationally modified species of SIN3 could be detected in our studies. It is possible that ubiquitinated or

SUMOylated species of SIN3 220 exist but are very transient and hence were not identified in our immunoprecipitation experiments (Andreou and Tavernarakis, 2009). We tested specific lysine residues at potential ubiquitination sites for their ability to influence SIN3 220 stability. Although loss of these lysine residues did not affect the stability of SIN3 220 protein it is possible that there may be other lysine residues that are critical for targeting SIN3 for proteasomal degradation.

CHAPTER 4 UNDERSTANDING THE FUNCTIONAL DIFFERENCES BETWEEN SIN3 ISOFORM-SPECIFIC COMPLEXES IN *DROSOPHILA*

Introduction

In *Drosophila*, the predominant SIN3 isoforms, SIN3 187 and SIN3 220, are differentially expressed during embryonic development (Sharma et al., 2008b). This suggests that the SIN3 isoforms perform distinct functions during the different developmental stages. Additionally, we demonstrated through experiments described in Chapter 2 that there is a possible mechanism in place that can ensure that the correct isoform is present during the appropriate stage of embryogenesis (Chaubal et al., 2016). The SIN3 isoforms also differ in their ability to rescue lethality in *Sin3A* null flies, further emphasizing the differential functions performed by these isoforms (Spain et al., 2010b).

SIN3 187 and SIN3 220 arise from alternative splicing of a single *Sin3A* gene and possess identical PAH 1-4 (paired amphipathic helices) and HID (histone deacetylase interaction domain) regions (Pennetta and Pauli, 1998a; Sharma et al., 2008b). These functional domains are involved in complex formation and interactions with DNA binding factors that target the SIN3 complex to its target genes. Despite the presence of these identical protein interaction domains, SIN3 187 and SIN3 220 form distinct histone modifying complexes and perform non-redundant functions (Spain et al., 2010b). Both of the SIN3 isoform specific complexes contain a common core of complex components that includes SDS3, ING1, BRMS1, ARID4B, SAP130, Pf1 and the histone deacetylase RPD3. In addition to these components, the SIN3 220 complex also associates with EMSY, Caf1-p55 and the histone demethylase dKDM5/LID. It is likely that the unique stretch of 315 amino acids at the SIN3 220 C-terminus is involved in interactions with these specific binding partners. Previous work in our laboratory has shown that Caf1-p55

is capable of directly interacting with the SIN3 220 unique C-terminus (Uni-C), whereas EMSY does not interact with the Uni-C. (Saha, 2017; Moore, 2017). Interaction of dKDM5/LID with the Uni-C has not yet been characterized. Furthermore, it is possible that the presence of EMSY, Caf1-p55 and dKDM5/LID is responsible for the non-redundant functions performed by the SIN3 220 complex. The interaction of EMSY with the SIN3 220 complex was identified only in S2 cells but not in *Drosophila* embryo nuclear extracts (Spain et al., 2010). For this research therefore, we have focused on the possible role of dKDM5/LID and Caf1-p55 in the SIN3 220 complex.

Little imaginal discs (dKDM5/LID) is a JmjC-domain containing histone demethylase that specifically removes the H3K4me3 histone modification (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007). Like SIN3, dKDM5/LID is essential for survival in *Drosophila* and a dual regulator of gene expression, in that it is involved in both activation and in repression (Gajan et al., 2016; Gildea et al., 2000). Additionally, dKDM5/LID functions coordinately with SIN3 in regulation of cell proliferation and wing development. Interestingly, dKDM5/LID inhibits the activity of the histone deacetylase RPD3 in a complex isolated from *Drosophila* embryos that includes dPf1, MRG15 and CG13367 (Lee et al., 2009). It is possible that presence of dKDM5/LID in the SIN3 220 complex impacts the histone deacetylase activity of RPD3. In support of this hypothesis, the SIN3 220 complex exhibits a decreased deacetylase activity as compared to SIN3 187 *in vitro* (Spain et al., 2010). Those authors also determined that SIN3 187 and SIN3 220 complexes differentially impact global H3K9Ac and H3K14Ac levels. Overexpression of SIN3 187 caused a noticeable decrease in H3K9Ac and H3K14Ac, whereas overexpression of SIN3 220 reduced only the H3K9Ac mark. Whether SIN3 isoform

specific complexes establish distinct histone modification patterns at target genes and thereby differentially regulate gene expression has not been elucidated.

Transcriptome analysis in *Drosophila* S2 cells has shown that SIN3 and dKDM5/LID regulate several common gene targets (Gajan et al., 2016). Another unique binding partner of SIN3 220, Caf1-p55 also affects the expression of a subset of SIN3 target genes (Saha, 2017). Caf1-p55 is a WD-40 repeat containing protein that can directly bind to histone proteins (Henning et al., 2005). Caf1-p55 is present in several chromatin-modifying complexes including the SIN3 complex, the NURF complex and Polycomb repressive group complex (Czermin et al., 2002; Hassig et al., 1997; Laherty et al., 1997; Martinez-Balbas et al., 1998; Muller et al., 2002). Since the SIN3 187HA complex does not include dKDM5/LID and Caf1-p55, there is an intriguing possibility that binding of the SIN3 187 complex to target genes instead of the SIN3 220 complex may result in gene expression changes that mimic those caused by loss of Caf1-p55 and dKDM5/LID. These changes in gene expression can in turn, be responsible for the functional differences between the SIN3 isoform specific complexes.

In this chapter, we show that dKDM5/LID does not interact with the unique C-terminus of SIN3 220. Loss of *lid* by RNAi impacts histone acetylation patterns at a subset of targets genes suggesting that presence of dKDM5/LID in the SIN3 220 complex may influence the activity of RPD3. We also demonstrate that recruitment of the SIN3 187 complex to SIN3 targets genes in place of the SIN3 220 complex alters the expression of a subset of genes. Furthermore, we analyze whether these gene expression changes are similar to those caused due to the double knockdown of *Caf1-55* and *lid*. We also demonstrate that distinct histone modifications patterns are established by the SIN3 187

and SIN3 220 complexes. This research is a significant contribution towards the understanding of key functional differences between the predominant SIN3 isoforms in *Drosophila*.

Materials and Methods

Cell culture

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

Paraquat treatment

4 x 10^6 S2 and SIN3 187HA cells in 4 ml appropriate culture medium were induced with $CuSO_4$ for transgene activation. After 24 hour induction, cells were treated with 10 mM paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride (Sigma Aldrich)). After 24 hour paraquat treatment, cells were stained with Trypan blue and cell counts were determined as per hemocytometer standards.

Transient transfection

Transient transfection was performed in LID-HA cells as described in Chapter 3.

RNA interference

RNAi in S2 cells was performed as described previously (Gajan et al., 2016). Briefly, 4 x10^6 cells were plated in 60-mm dishes in 4 ml Schneider's *Drosophila* medium containing gentamycin. Cells were left undisturbed for 3 hours to facilitate adhering to the dish. After 3 hours, the culture medium was replaced with serum-free medium. 50 μg of appropriate dsRNA was added to each dish. Schneider's *Drosophila* medium containing gentamycin was added after 30 minutes incubation with dsRNA. Construction of dsRNA targeting *lid, Caf1-55* and the *GFP* gene is described previously (Gajan et al., 2016, Saha

et al., 2016). Knockdown was verified by standard PCR using gene specific primers. *Taf-1* was used as a loading control.

Chromatin immunoprecipitation and qPCR

Chromatin preparation and immunoprecipitation was performed as previously described (Saha et al., 2016). Briefly, 75 μg of prepared chromatin was immunoprecipitated using: anti-HA agarose beads (40 μl), anti-H3C (4 μl), anti-H3K4me3 (3 μl), anti-H3K9Ac (3 μl), anti-H3K14Ac (3 μl), anti-H3K27Ac (3 μl) and pre-immune IgG (10 μl). Immunoprecipitated samples after reverse-crosslinking were analyzed by quantitative PCR. Primer pairs used are listed in Table 4.1.

Table 4.1: Primers used for ChIP-qPCR analysis

Gene	Forward primer	Reverse Primer
Ahcy13	CGAAGCCCAGCTACAAAGTC	AATAGATGCAATTCACCCGC
MS (CG10623)	CGGAAAACGTACAGCAGTGA	GCATTTGACCAGAATTGGCT
Sam-S	CCACACCTCCACCGTCTACT	CCTCTGTTCAAGTCGTGCAA
Pyk	GACGACGCTTTCAGCGAT	TTTGAAGCTCGGGTCTGC
Jumu	GCGACTTCGAATACGAGACC	GCCGTGATCTCTGCACTTTT
Lea	TGAATTTCGCTTTCGTTGGT	CAATTAAGGAGGCGAAACGA
Sli	AAACACCGCTAATCCAATCG	AGAAAAGCGCAAAAGTCGAA
Ds	TGCCAACCATCCTAACGG	CTGTGGAGGACACAGGGG

Gene expression analysis by RT-PCR

Total RNA was isolated from 1 x 10^7 cells using the RNAeasy kit (Qiagen). cDNA was prepared from the isolated RNA using random hexamers and the ImProm-II Reverse

Transcription Kit (Promega). cDNA was analyzed by quantitative PCR using gene specific primers listed in Table 4.2. Relative fold change in gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *Dmn* was used as a normalizer.

Table 4.2: Primers used for RT-qPCR analysis

Gene	Forward primer	Reverse primer
Ahcy13	AGACCTTGGTCTTCCCCG	GACACCGGTGGTCGTCTC
MS (CG10623)	TCCAAAGTCGGAAGGCTG	GGCCACTTTGGTAAGCGA
Sam-S	AAACTTTGACCTCAGGCCC	CGCTGGTATATCGGCTGG
Pyk	GGCTCCGGCTTCACAA	TTCCTGAGCGGCAGAATTTATT
Jumu	AGGAGATGCTGAACGTGGAC	TCGCGGATATAGCTTCCAGT
Lea	TGAATTTCGCTTTCGTTGGT	CAATTAAGGAGGCGAAACGA
Sli	AAACACCGCTAATCCAATCG	AGAAAAGCGCAAAAGTCGAA
Reph	CTGATGGTGGAGAACCGC	TTTGGCTTGAATGCCTCC
Dmn	GACAAGTTGAGCCGCCGCCTTAC	CTTGGTGCTTAGATGACGCA

Nuclear fraction extraction and immunoprecipitation

Nuclear fraction extraction was performed as described in Chapter 3. Immunoprecipitation was carried out using anti-HA agarose beads (40 μl) and anti-Flag resin (40 μl). The protein extracts were analyzed by western blotting.

Western blotting

Western blotting was performed as described in Chapter 2. Primary antibodies used for analysis are as follows: anti-HA (1:6000), anti-SIN3 PAN (1:2000), anti-RPD3 (1:3000), anti-Caf1-p55 (1:3000, Abcam).

Statistical Analyses

All significance values were calculated by the unpaired two sample Student's t test using GraphPad (https://www.graphpad.com/quickcalcs/ttest1/?Format=SEM) as described in Chapter 2.

Results and Discussion

To understand the functional differences between the SIN3 isoform specific complexes, we first analyzed the role of dKDM5/LID and Caf1-p55 in the SIN3 220 complex. Co-immunoprecipitation (Co-IP) assays in S2 cells and immunoprecipitation (IP) following bacterial expression has shown that Caf1-p55 can directly interact with the Uni-C (Saha, 2017). To determine whether dKDM5/LID can interact with the Uni-C, we transiently transfected a construct encoding HA-tagged Uni-C (Uni-C HA) into S2 cells carrying a HA/Flag-tag *lid* encoding transgene (LID-HA cells). Mock transfected LID-HA cells were used as a control. The expression of the transgenes was induced by addition of $CuSO_4$ and Co-IP was performed using anti-Flag resin. The immunoprecipitates were analyzed by western blotting using HA, SIN3 and RPD3 antibodies. A clear signal for Uni-C HA in whole cell extracts obtained from transfected cells, but not from mock transfected cells, indicated successful transient transfection (Figure 4.1). No signal, however, was obtained for the immunoprecipitate suggesting that dKDM5/LID does not interact with the unique C-terminus of SIN3 220. Consistent with published data, endogenous SIN3 and

RPD3 immunoprecipitated with dKDM5/LID and served as a positive control for the Co-IP assay (Gajan et al., 2016). Although we did not observe an interaction of dKDM5/LID with the Uni-C it is possible that dKDM5/LID interacts with this region in the context of the full-length SIN3 220 protein.

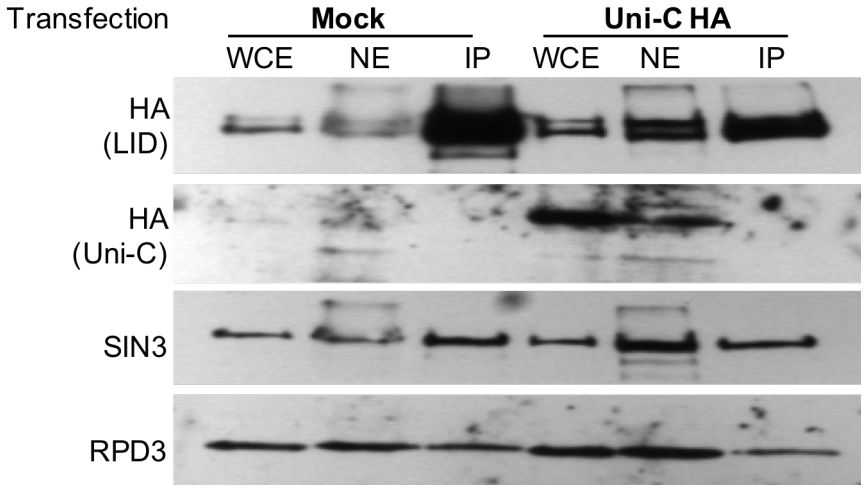


Figure 4.1. dKDM5/LID does not interact with the unique C-terminus of SIN3 220. Co-immunoprecipitation assays were performed using extracts prepared from LID-Flag/HA cells that were transiently transfected with Uni-C HA or mock transfected. The extracts were incubated with anti-Flag resin. Samples were analyzed by Western blotting. Whole cell extract (WCE), nuclear extract (NE) and immunoprecipitate (IP) were probed with HA, SIN3 and RPD3 antibodies.

Another possibility is that dKDM5/LID interacts with SIN3 220 through Caf1-p55. The dKDM5/LID protein contains multiple conserved domains, namely, Jumonji C (JmjC), JmjN, ARID (A/T rich interaction domain), C_5HC_2 zinc finger and three PHD motifs (plant homeobox domain). The third PHD domain (PHD3) of dKDM5/LID is essential for development in *Drosophila* (Li et al., 2010). Additionally, it recognizes and binds to di- and trimethylated lysine 4 of histone H3 and possibly aids in the recruitment of dMyc to these methylated regions. We hypothesized that the dKDM5/LID PHD3 domain may also be important for its interaction with SIN3 and Caf1-p55. To address this hypothesis, we

used a stable cell line expressing a transgene encoding a HA-tagged LID protein that lacks the PHD3 domain (LID-HA∆PHD3). We performed Co-IP assays in LID-HA and LID-HA∆PHD3 cells using anti-HA resin. The immunoprecipitates were analyzed by western blotting using antibodies against HA, SIN3 and Caf1-p55. The loss of the PHD3 domain did not significantly alter co-immunoprecipitation of either endogenous SIN3 or Caf1-p55 with dKDM5/LID, suggesting that this domain is not important for interaction with these proteins (Figure 4.2). Further analysis can be conducted by eliminating other conserved protein interaction domains of dKDM5/LID to assess their requirement for interaction with SIN3 or Caf1-p55.

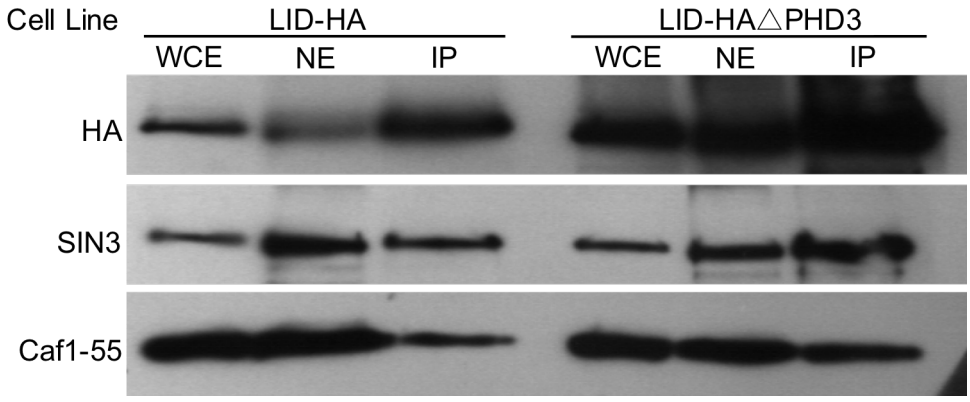


Figure 4.2. PHD3 domain of dKDM5/LID is not required for interaction with SIN3 220 and Caf1-p55. Co-immunoprecipitation assays were performed using extracts prepared from LID-HA and LID-HA△PHD3 cells. The extracts were incubated anti-HA agarose beads. Samples were analyzed by Western blotting. Whole cell extract (WCE), nuclear extract (NE) and immunoprecipitate (IP) were probed with HA, SIN3 and Caf1-p55 antibodies.

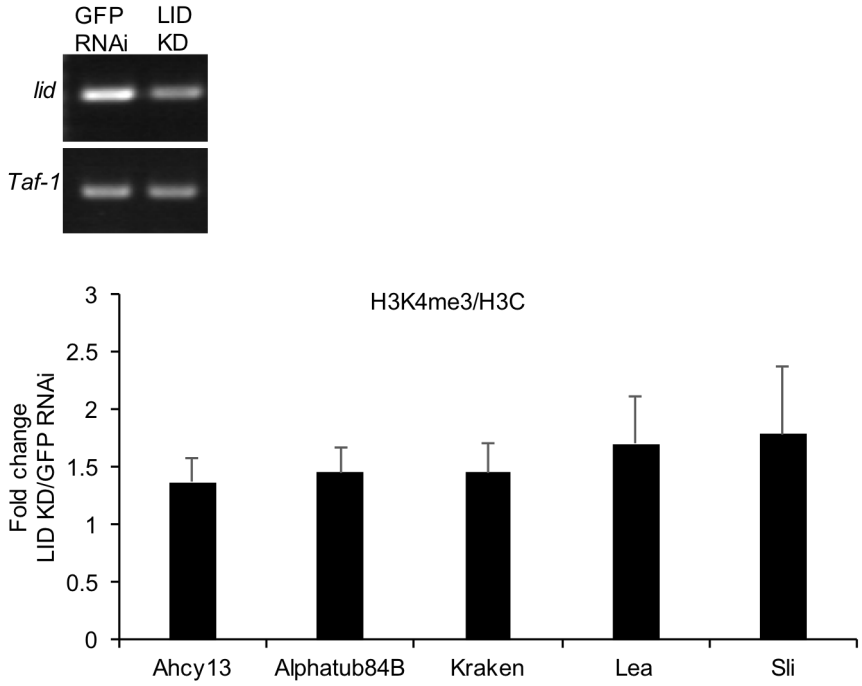


Figure 4.3. Loss of dKDM5/LID increases the level of H3K4me3 mark. A) RT-PCR analysis was performed to confirm *lid* knockdown in S2 cells. *Taf-1* was used as a loading control B) ChIP-qPCR analysis was performed post *lid* knockdown using anti-H3K4me3. Percent input signal obtained was normalized to H3C. Fold change relative GFP RNAi was plotted. The results are the average of three biological independent replicates. Error bars represent standard error of the mean.

Next, we analyzed the role of dKDM5/LID in the histone modification activity of the SIN3 complex. dKDM5/LID is a histone demethylase and does not possess any catalytic activity towards the histone acetylation mark (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007). An alteration in H3K9Ac however, was observed upon *lid* knockdown at two LID regulated genes, *Sesn* and *ssdp*, although the change was not statistically significant (Gajan et al., 2016). To further examine the effect of *lid* knockdown on histone acetylation marks regulated by RPD3, we performed ChIP-qPCR using antibodies against H3K9Ac, H3K14Ac and H3K27Ac in S2 cells treated with dsRNA against *lid* (Kurdistani et al., Tie et al., 2009; 2002; Spain et al., 2010). Efficient knockdown

of *lid* was verified by assessing the transcript level using standard RT-PCR (Figure 4.3A). S2 cells treated with dsRNA against GFP were used as a control. Since LID is a histone demethylase, ChIP-qPCR was also performed using anti-H3K4me3 as a positive control. We expect that *lid* RNAi should result in an increase in the level of H3K4me3 at target genes. IgG was used as a non-specific control. We tested five genes namely, *Ahcy13* (*Adenosylhomocysteinase*), *AlphaTub84B* (*α-Tubulin at 84B)*, *Kraken*, *Lea*/*Robo2* (*Roundabout 2*) and *Sli* (*Slit)* that were previously determined to be regulated by both SIN3 and dKDM5/LID (Gajan et al., 2016). Knockdown of *lid* lead to a noticeable although statistically insignificant increase in the level of H3K4me3, which is consistent with published data (Figure 4.3B) (Gajan et al., 2016). Interestingly, *lid* RNAi caused a modest yet statistically significant increase in the H3K9Ac mark at four of the five genes tested (Figure 4.4A). Alteration in the pattern of H3K14Ac and H3K27Ac was also observed upon *lid* knockdown (Figure 4.4B, C). These data suggest that dKDM5/LID impacts the level of histone acetylation at target genes possibly by influencing either the recruitment or activity of RPD3. Further analysis is required to provide evidence in support of this hypothesis.

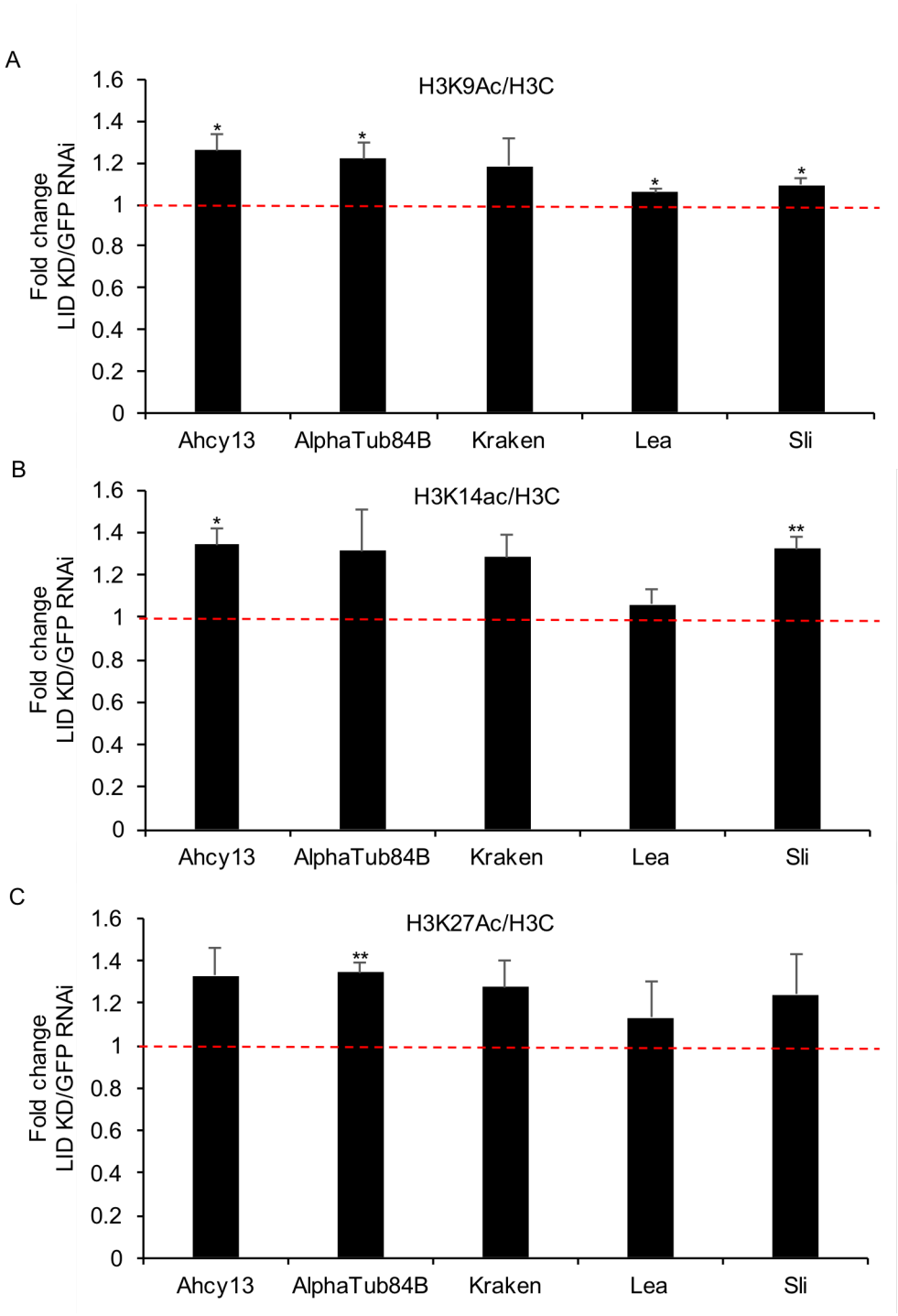
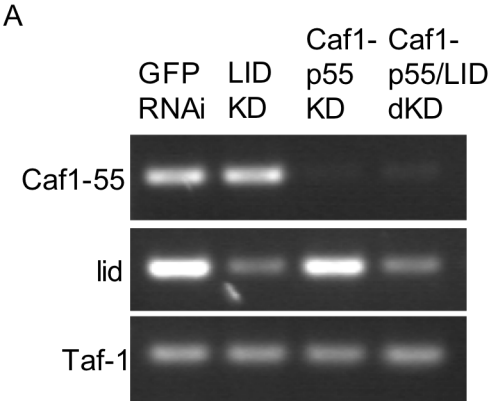
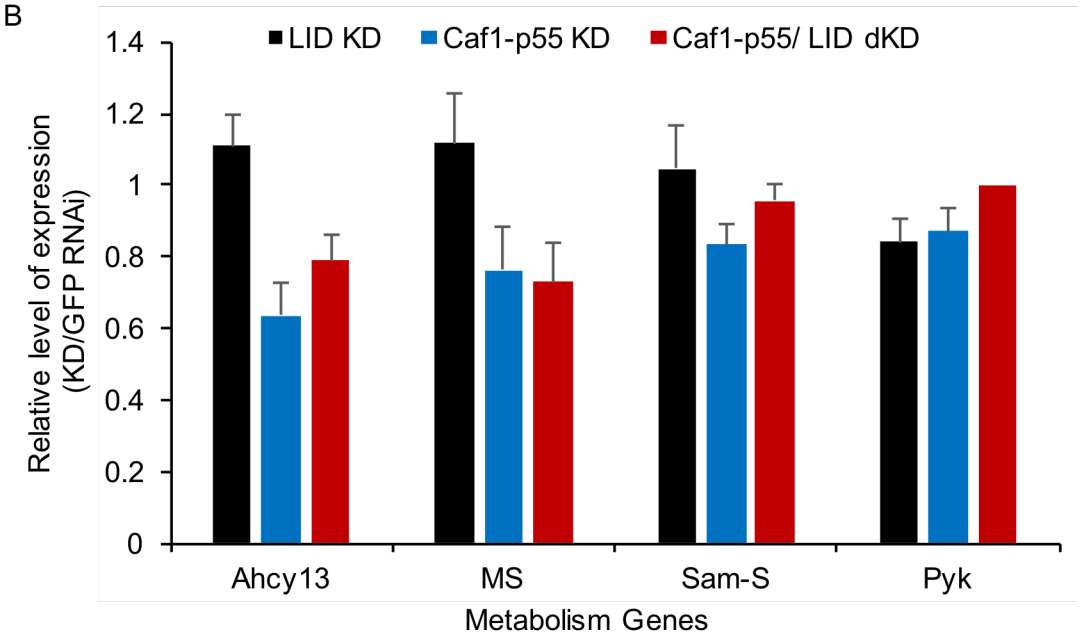


Figure 4.4. Loss of dKDM5/LID impacts histone acetylation patterns. ChIP-qPCR analysis was performed post *lid* knockdown using anti-H3K9Ac (A), anti-H3K14Ac (B) and anti-H3K27Ac (C). Percent input signal obtained was normalized to H3C. Fold change relative to GFP RNAi was plotted. The results are the average of three biological independent replicates. Error bars represent standard error of the mean. .*, $p < 0.05$, **, $p < 0.01$.

A


GFP RNAi
LID KD
Caf1-p55 KD
Caf1-p55/LID dKD
Caf1-55
lid
Taf-1

B


LID KD
Caf1-p55 KD
Caf1-p55/ LID dKD
Relative level of expression (KD/GFP RNAi)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ahcy13
MS
Sam-S
Pyk
Metabolism Genes

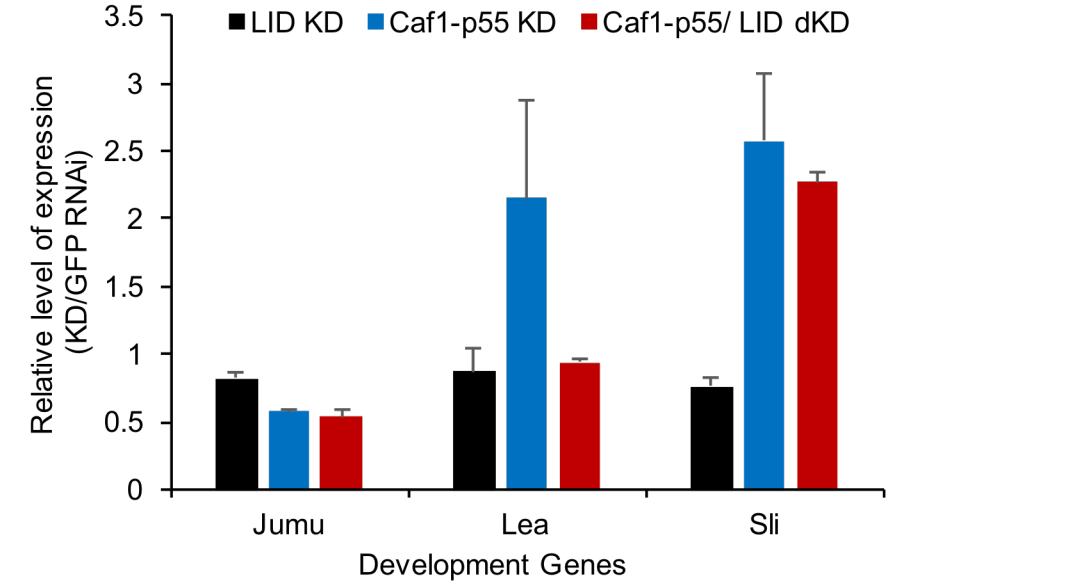

LID KD
Caf1-p55 KD
Caf1-p55/ LID dKD
Relative level of expression (KD/GFP RNAi)
3.5
3
2.5
2
1.5
1
0.5
0
Jumu
Lea
Sli
Development Genes

Figure 4.5. dKDM5/LID and Caf1-p55 may act through separate pathways to regulate SIN3 gene expression. RT-qPCR analysis was performed in S2 cells treated with dsRNA against *lid* and *Caf1-55,* individually and in combination. GFP RNAi was used as a non-specific control. *Dmn* was used as a control for normalizing transcript levels. The results are the average of three biological independent replicates. Error bars represent standard error of the mean.

dKDM5/LID and Caf1-p55 regulate expression of a subset of SIN3 target genes (Gajan et al., 2016; Saha, 2017). We therefore asked whether dKDM5/LID and Caf1-p55 function coordinately in the SIN3 complex. For this study, we chose gene targets from two GO categories, metabolism and neuron development, that are enriched in genes regulated by both SIN3 isoforms (Saha et al., 2016). According to Flybase, *Sam-S (*S-adenosylmethionine (SAM) synthetase*)*, *Ahcy13* and CG10623 ((*MS*) Methionine synthase) are *Drosophila* genes likely involved in methionine metabolism (http://flybase.org/). SIN3 regulates the level of H3K9Ac and H3K4me3 at the promoters of these methionine metabolic genes and consequently their level of expression (Liu et al., 2016). *Pyk* (*Pyruvate kinase*) encodes a key enzyme in glucose metabolism and its expression is increased upon loss of SIN3 (Pile et al., 2003). *Jumu* (*Jumeau*) is a transcriptional regulator that regulates dendrite morphogenesis in *Drosophila* (Parrish et al., 2006). *Sli* and *Lea/Robo2* are key players in the process of axon guidance (Kidd et al., 1999; Rajgopalan et al., 2000; Simpson et al., 2000). *Jumu*, *Sli* and *Lea* are direct SIN3 targets and their transcription is activated by SIN3 (Saha et al., 2016). To analyze the effect of dKDM5/LID and Caf1-p55 on the expression of these SIN3 targets, we performed individual and double knockdown of *lid* and *Caf1-55* in S2 cells. GFP RNAi was used as a non-specific control. Total RNA was isolated from these cells, converted to cDNA and analyzed by qPCR using gene specific primers. Efficient knockdown of *lid* and *Caf1-55*, individual as well as double knockdown was confirmed by standard RT-

PCR (Figure 4.5A). The reduction of dKDM5/LID and Caf1-p55 individually, resulted in the downregulation of *Pyk* and *Jumu* (Figure 4.5B). Double knockdown of *lid* and *Caf1-55* at these genes did not exhibit an additive effect. Conversely, the individual loss of dKDM5/LID and Caf1-p55 exhibits opposing effects on the expression of *Ahcy13*, *Sam-S*, *MS*, *Lea* and *Sli* (Figure 4.5B). Reduction in the level of both dKDM5/LID and Caf1-p55 by RNAi results in either an intermediate effect relative to the individual knockdown (*Ahcy13*, *Sam-S*) or follows the trend of the individual knockdowns (*MS*, *Lea*, *Sli*). This suggests that dKDM5/LID and Caf1-p55 may regulate the expression of a subset of SIN3 gene targets by distinct mechanisms.

The SIN3 187 complex does not contain dKDM5/LID or Caf1-p55. It is therefore possible that when the SIN3 187 complex is recruited to SIN3 target genes instead of the SIN3 220 complex, it results in gene expression changes that are similar to those observed upon double knockdown of *lid* and *Caf1-55*. To address this possibility, we took advantage of the interplay between SIN3 isoforms that was described in Chapter 2. *Drosophila* S2 cells predominantly express the SIN3 220 isoform, and therefore SIN3 target genes are bound by the SIN3 220 complex in S2 cells (Saha et al., 2016). As demonstrated in Chapter 2, when SIN3 187 is overexpressed, the endogenous SIN3 220 protein is rapidly degraded through the proteasome. Since the level of endogenous SIN3 220 is drastically reduced, we hypothesized that the SIN3 187 complex replaces the SIN3 220 complex at SIN3 regulated genes. This provided us with an experimental system wherein the two cell types S2 and SIN3 187HA exhibit the presence of distinct SIN3 isoforms, enabling us to analyze the differences in gene transcription in the presence of different SIN3 isoform specific complexes. To analyze changes in gene expression, we

studied the same metabolic and developmental genes as mentioned above. To verify localization of SIN3 187 at these genes, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) in SIN3 187HA cells. As demonstrated in Chapter 2, induction of SIN3 187HA in these cells substantially decreased the level of endogenous SIN3 as compared to S2 cells (Figure 4.6A). Chromatin was prepared from S2 cells and SIN3 187HA cells and immunoprecipitated using anti-HA agarose beads. S2 cells serve as a non-specific control since they do not express any HA-tagged protein. qPCR analysis of immunoprecipitated DNA using gene specific primers confirms that SIN3 187HA binds to all the gene targets mentioned above and does not bind to the intronic region of *ds,* which serves as the negative control (Figure 4.6B).

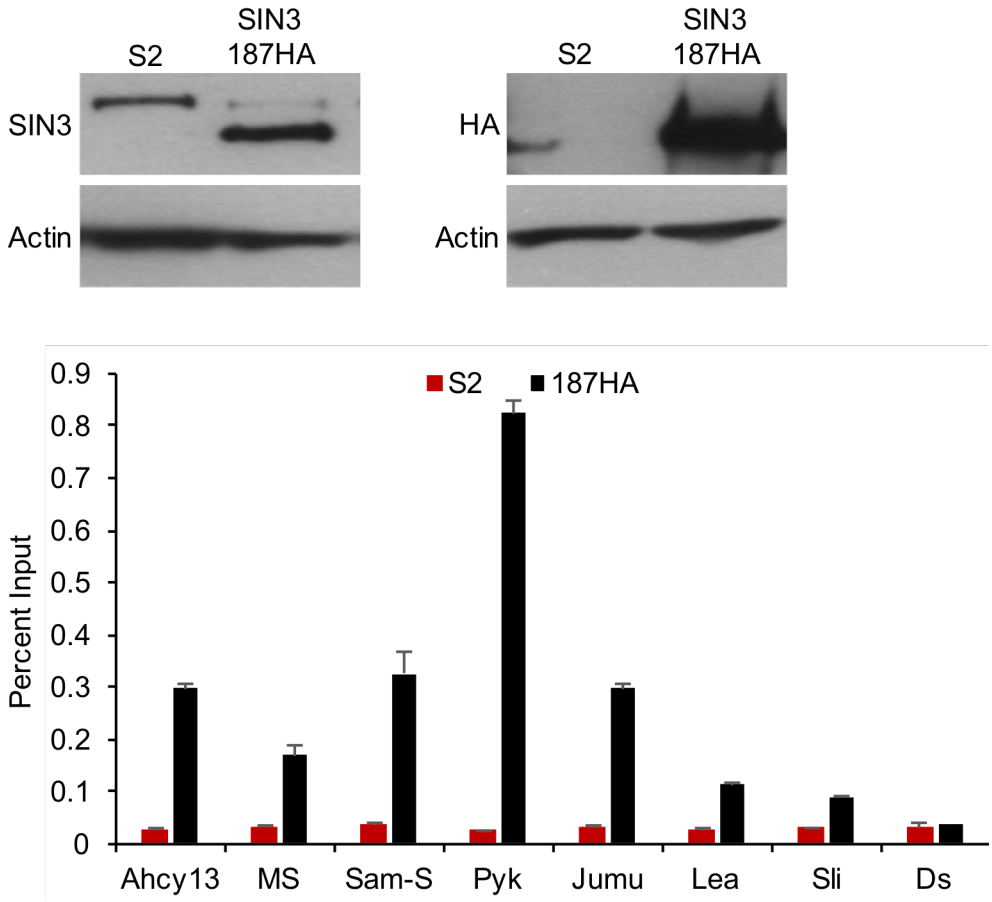


Figure 4.6. Verification of SIN3 187HA binding to SIN3 gene targets. A) Western blotting analysis of proteins isolated from S2 and SIN3 187HA cells after 48 hour induction with $CuSO_4$. Protein extracts were probed with SIN3 and HA antibodies. Actin was used as a loading control. B) ChIP-qPCR analysis was performed in S2 and SIN3 187HA cells using anti-HA agarose beads. S2 cells serve as a non-specific control. The results are the average of three biological independent replicates. Error bars represent standard error of mean.

After verification of SIN3 187HA binding to specific gene targets, we analyzed the expression pattern of these genes in S2 and SIN3 187HA cells. RNA was isolated from S2 and SIN3 187HA cells after 48 hour induction by $CuSO_4$. RNA obtained from these cells was then converted to cDNA and analyzed by qPCR using gene specific primers. All the metabolic genes tested, *Ahcy13*, *Sam-S*, and *Pyk*, with the exception of *MS* were significantly upregulated in SIN3 187HA cells as compared to S2 cells (Figure 4.7). Among the genes important for neuronal development, *Lea* and *Sli* exhibit several fold

higher expression in SIN3 187HA cells relative to S2 cells (Figure 4.7). This suggests that recruitment of the SIN3 187 complex instead of the SIN3 220 complex can alter the gene expression pattern of a subset of SIN3 target genes. Further, comparison of gene expression changes in SIN3 187HA cells to those observed upon loss of LID and Caf1-p55 did not exhibit similar patterns, with the exception of *Sli*. (Figure 4.5, Figure 4.7). This suggests that differential gene expression in SIN3 187HA cells relative to S2 cells is not merely due to the absence of dKDM5/LID and Caf1-p55 in the SIN3 187 complex. It is possible that dKDM5/LID and Caf1-p55 play a role in the regulation of a subset of SIN3 target genes, such as *Sli*. Analyzing a larger number of genes involved in different biological pathways may help us identify the cellular context wherein binding of the SIN3 187HA complex mimics the loss of dKDM5/LID and Caf1-p55.

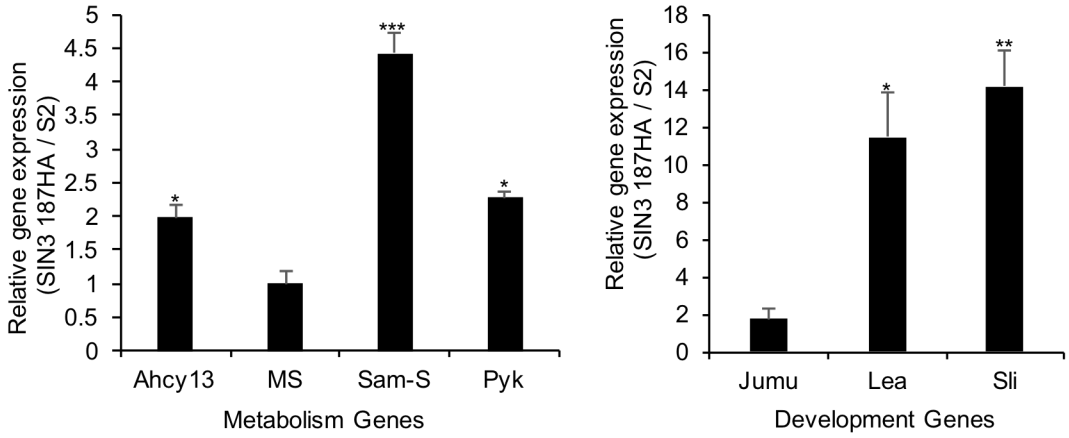


Figure 4.7. Binding of SIN3 187 complex to target genes in place of the SIN3 220 complex results in differential gene expression. RT-qPCR analysis was performed in S2 and SIN3 187HA cells using gene specific primers. *Dmn* was used as a control for normalizing transcript levels. The results are the average of three biological independent replicates. Error bars represent standard error of the mean. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Additionally, as demonstrated above, loss of dKDM5/LID impacts the pattern of histone acetylation marks at targeted genomic loci. To further analyze the functional differences between the SIN3 isoform specific complexes, we asked whether the absence dKDM5/LID in the SIN3 187 complex results in the establishment of differential histone modification patterns at target genes by this complex. Chromatin was prepared from S2 and SIN3 187HA cells and ChIP was performed using antibodies against H3K4me3, H3K9Ac and H3K14Ac histone marks. These histone modifications were analyzed because dKDM5/LID specifically demethylates the H3K4me3 mark and the SIN3 187 complex alters global levels of H3K9Ac and H3K14Ac (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007; Spain et al., 2010). The percent input signal for each histone modification was normalized to H3C. IgG was used as a non-specific ChIP control. The SIN3 187 complex establishes a distinct histone modification pattern at a subset of SIN3 target genes (Figure 4.8).

Four out of the seven genes tested in this study, namely, *Sam-S*, *Pyk*, *Lea* and *Sli*, show an increase in the level of H3K4me3 following induction of SIN3 187HA (Figure 4.8A). An increase in the H3K4me3 mark is consistent with the loss of the histone demethylase dKDM5/LID, which is predicted if dKDM5 is recruited by the SIN3 220 complex. The gene *MS*, however, exhibits a significant decrease in H3K4me3, suggesting that the differences in the level of histone methylation at SIN3 target genes upon SIN3 187 binding cannot solely be attributed to the absence of dKDM5/LID in the complex. Analysis of histone acetylation at H3K9 and H3K14 positions in SIN3 187HA cells reveals alteration in the level of these marks relative to S2 cells at a subset of gene targets (Figure 4.8B, C). *Ahcy13*, *Sam-S*, *Pyk* and *Jum*u do not exhibit any significant

changes in the level of H3K9Ac at their promoters. Like the H3K4me3 mark, there is a significant decrease in the level of H3K9Ac at the *MS* gene promoter. Reduction in the level of two active histone marks, H3K4me3 and H3K9Ac, however, does not cause a substantial decrease in the expression of *MS*, indicating that other factors may play a role in maintaining the appropriate expression of *MS* in the cell (Figure 4.7). The change in gene expression for *Lea* and *Sli*, on the other hand, is consistent with the histone modification changes. There is a highly significant increase in the level of H3K4me3 and H3K9Ac at *Lea* and *Sli*, which correlates with the several fold upregulation of transcription of these genes (Figure 4.7). Although not statistically significant, there is also an increase in level of H3K14Ac that may contribute to the activation of expression of *Lea* and *Sli*. This is interesting because Lea and Sli are key players in neuron development. It is possible that the switch in SIN3 isoforms from SIN3 220 to SIN3 187 during embryogenesis occurs to alter the pattern of histone modification at specific developmental genes ensuring appropriate level of expression of these genes. More genes important for embryonic and post-embryonic development need to be tested to provide further evidence to support this hypothesis. The change in the level of H3K14Ac at the genes tested upon SIN3 187 binding is not statistically significant, except at *Jumu*. It is, however, possible that this change may be biologically significant by recruiting chromatin associated factors that can recognize and bind this histone mark.

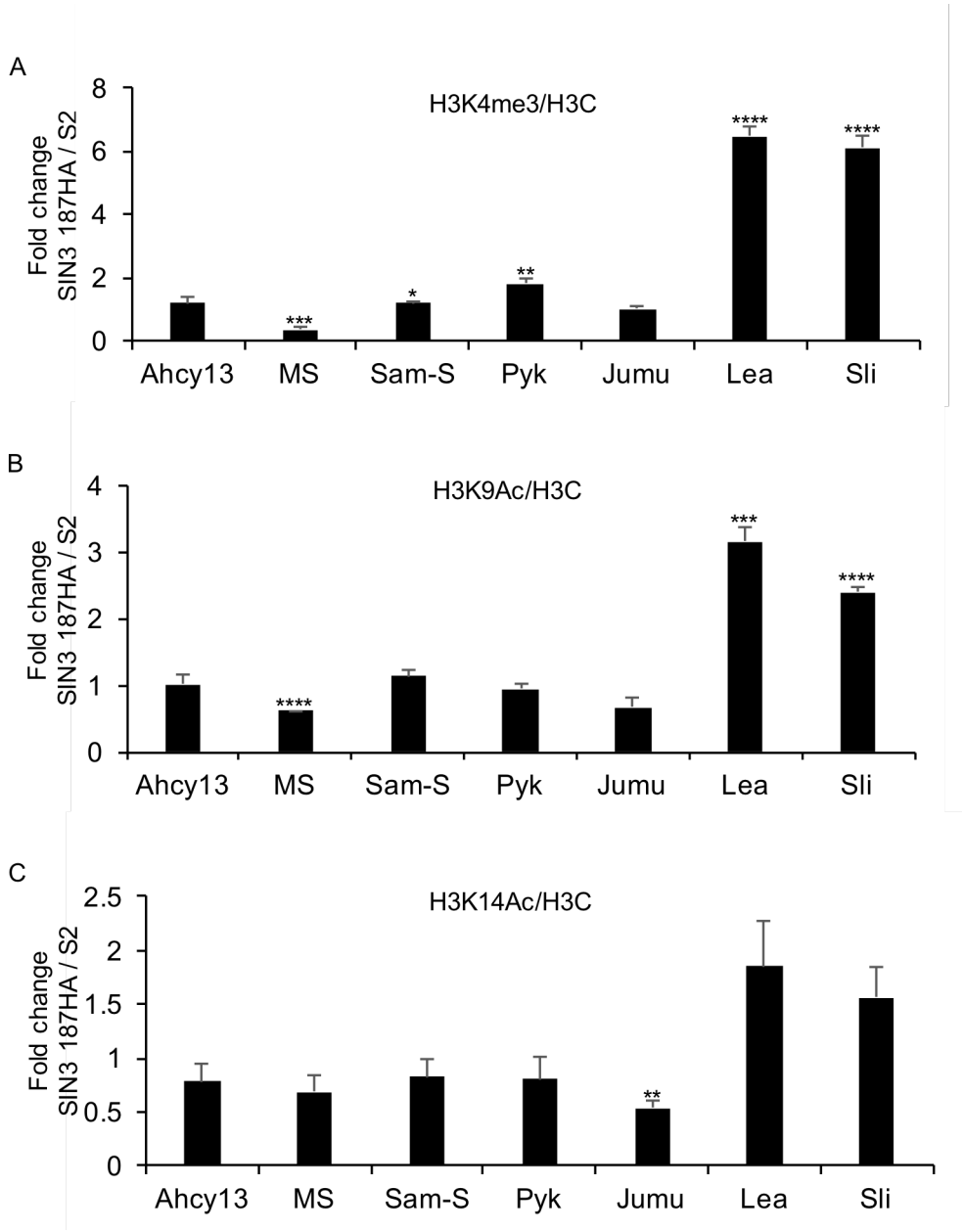


Figure 4.8. Binding of SIN3 187 complex to target genes in place of the SIN3 220 complex results in differential histone modification patterns. ChIP-qPCR analysis was performed in S2 and SIN3 187HA cells using anti-H3K4me3 (A) anti-H3K9Ac (B) and anti-H3K14Ac (C). Percent input signal obtained was normalized to H3C. Fold change relative to S2 was plotted. The results are the average of three biological independent replicates. Error bars represent standard error of the mean. .*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

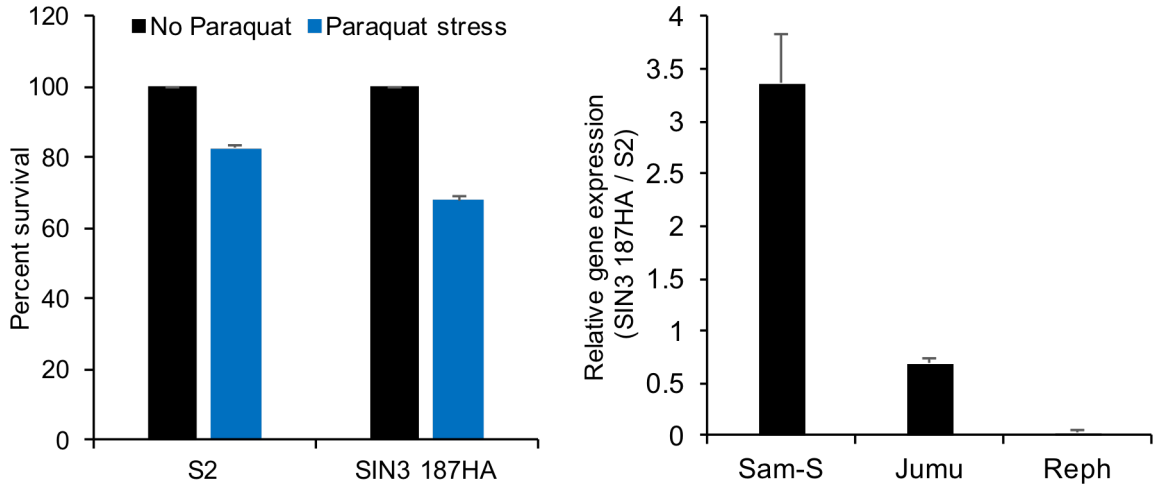


Figure 4.9. SIN3 187HA cells are more sensitive to oxidative stress relative to S2 cells and exhibit differential gene expression. A) S2 and SIN3 187HA cells were treated with paraquat for 24 hours post induction by $CuSO_4$. Percent cell survival relative to untreated cells was plotted. The results are the average of three biological independent replicates. Error bars represent standard error of the mean. B) RT-qPCR analysis was performed in S2 and SIN3 187HA cells treated with paraquat for 24 hours post induction by $CuSO_4$. *Dmn* was used as a control for normalizing transcript levels. The results are the average of two biological independent replicates. Error bars represent standard deviation.

SIN3 and dKDM5/LID play an important role in the regulation of oxidative stress response (Gajan et al., 2016). Since the SIN3 187 complex does not contain dKDM5/LID, we predicted that the SIN3 187 HA cells are more sensitive to oxidative stress as compared to S2 cells. We treated S2 and SIN3 187HA cells with paraquat to induce oxidative stress. Cell survival was determined after 24 hour paraquat treatment. SIN3 187HA cells exhibit lower survival as compared to S2 cells (Figure 4.9A). We also analyzed the expression pattern of three genes, *Sam-S*, *Jumu* and *Reph* (Regulator of eph expression) that are regulated by SIN3 and dKDM5/LID under oxidative stress conditions (Gajan et al., 2016). *Sam-S* is upregulated, whereas *Jumu* and *Reph* are downregulated upon *Sin3A* and *lid* double knockdown in S2 cells under oxidative stress

conditions (Gajan et al., 2016). Interestingly, when SIN3 187HA cells are subjected to oxidative stress, we also observe an upregulation of *Sam-S* and downregulation of *Jumu* and *Reph* (Figure 4.9B), suggesting that the SIN3 220 complex may play a crucial role during stress response due to the presence of dKDM5/LID in the complex. We have, however, tested a limited number of genes. More stress response genes need to be analyzed to better understand the role of SIN3 187 and SIN3 220 complexes in stress tolerance.

In summary, we have demonstrated that dKDM5/LID does not interact with the unique C-terminus of SIN3 220 and that the PHD3 domain of dKDM5/LID is not required for interaction with either SIN3 220 or Caf1-p55. Loss of dKDM5/LID alters the level of histone acetylation at gene targets. Furthermore, dKDM5/LID and Caf1-p55 may act independently to regulate expression of a subset of SIN3 target genes. We have also shown that there is differential gene expression of specific target genes between S2 and SIN3 187HA cells which, predominantly express SIN3 220 and SIN3 187 isoforms, respectively. These differences in expression of target genes are not solely because of the absence of specific complex components in the SIN3 187 complex. Interestingly, the SIN3 187 and SIN3 220 complexes establish distinct histone modification patterns that may contribute to differences in gene expression and thereby differences in function during critical biological processes.

CHAPTER 5 FUTURE DIRECTIONS

In my PhD thesis research, I have studied the regulation, stability and functional differences of the SIN3 isoforms in *Drosophila*. SIN3 is a global transcriptional regulator that plays a key role in several important biological processes such as cell proliferation, development, metabolism and cancer progression. Although SIN3 has been well studied for over three decades, critical questions regarding the regulation of expression and stability of SIN3 proteins, evolution and functional differences between SIN3 isoforms and mechanisms underlying the role of SIN3 in gene activation are yet to be answered. In my study, I have demonstrated that a feedback mechanism between the predominant SIN3 isoforms in *Drosophila* may regulate the level of SIN3. Additionally, both the N and C-terminus are important for stability and proteasomal degradation of SIN3 proteins. Furthermore, the SIN3 187 and SIN3 220 complexes establish distinct histone modification patterns at target genes that may be responsible for change in the level of expression of these genes and thereby the non-redundant functions performed by the SIN3 isoforms. Based on published literature and the current research, some important unanswered questions are discussed below.

What factors are involved in alternative splicing of *Sin3A* gene in *Drosophila*?

In this research, we have discussed the regulation of the predominant SIN3 isoforms, SIN3 187 and SIN3 220. These isoforms are produced by alternative splicing of the *Sin3A* gene in *Drosophila*. The key players that are responsible for splicing the *Sin3* gene in *Drosophila* and other species are not known. An RNAi screen can be performed in *Drosophila* S2 cells using dsRNA against known and putative splicing factors. S2 cells predominantly express the SIN3 220 isoform (Sharma et al., 2008). If loss of a splicing

factor impacts the splicing of *Sin3A* gene, we may observe an accumulation of unspliced SIN3 transcript. Putative factors identified in this study can then be studied further by conducting biochemical and bioinformatic analysis. Binding of these proteins to *Sin3A* at specific locations near exon-intron boundaries using ChIP-qPCR can be analyzed. Additionally, the effect of loss of these factors on *Drosophila* embryogenesis can be studied. Furthermore, using bioinformatics tools homologues of these factors in other species and their expression pattern in distinct tissues, can be identified which will help us understand the splicing and thereby regulation of the SIN3 isoforms in other model systems.

Which complex components influence the stability of SIN3 proteins?

In this study, we have demonstrated that overexpression of SIN3 187 can target the existing SIN3 220 protein for proteasomal degradation. We hypothesize that the excess SIN3 187 protein sequesters common core complex components from the SIN3 220 complex, thereby destabilizing SIN3 220 and targeting it for degradation. To identify complex components that may influence SIN3 220 stability, we first have to determine which components directly interact with SIN3. Based on experiments performed in our lab, we know that Caf1-p55 and ING1 directly interact with the unique C-terminal region of SIN3 220, whereas EMSY, SAP130, SDS3 and RPD3 do not (Saha, 2017; Moore 2017). These studies were, however, performed using only the unique SIN3 220 C-terminus, and the full length SIN3 protein may have additional interactions with these complex components.

To identify all the protein-protein interactions within the SIN3 complex, the endogenous SIN3 complex could be immunopurified, followed by quantitative mass

spectrometry (MS) analysis after chemical cross-linking (Sharon et al., 2006). SIN3 isoform specific complexes can be purified from SIN3 187HA and SIN3 220HA cells by affinity purification using anti-HA beads. S2 cells can be used as a control for immunoprecipitation to detect non-specific interactions. Comparison between the protein complex samples before and after cross-linking may reveal new protein bands due to cross-linking between complex interaction partners. New bands appearing after cross-linking will be excised and subjected to peptide digestion and analyzed by MS/MS. This will capture all the protein-protein interactions within the SIN3 complex and provide potential candidates that may influence SIN3 stability. Additionally, since the SIN3 complex is conserved from yeast to mammals, our study will also add to the existing knowledge about SIN3 complex structure in mammalian systems. This is especially important since several studies are targeting interaction of SIN3 with its complex components for therapeutic use (Hurst et al., 2008; Smith et al., 2010, Farias et al., 2010; Bansal et al., 2015).

How is SIN3 targeted to the proteasome for degradation?

We found that inhibiting the proteasome using the inhibitor MG132 stabilizes the SIN3 220 protein. Our attempts to detect SIN3 species that are post-translationally modified were without success. It is possible that post-translationally modified SIN3 is very unstable and gets rapidly degraded escaping detection. In an alternative approach, the proteins that may be involved in targeting SIN3 to the proteasome can be analyzed. In SIN3 187HA cells, overexpression of SIN3 187 targets SIN3 220 for degradation. Similarly, in SIN3 220HA cells, upon induction of the SIN3 220 transgene, excess SIN3 protein is degraded to maintain a specific level of SIN3 in the cell. SIN3 187HA and SIN3

220HA cells could be treated with $CuSO_4$ to induce the expression of the transgenes followed by treatment with MG132. This will result in accumulation of SIN3 proteins that are targeted for degradation. The endogenous SIN3 protein can then be immunoprecipitated along with its binding partners and subjected to quantitative mass spectrometry. Mass spectrometric analysis will help us identify proteins such as SUMO/ubiquitin E3 ligases that may post-translationally target SIN3 and target it to the proteasome. This study will help identify important players that may regulate the stability and function of SIN3.

What is the genome-wide pattern of histone modifications established by SIN3 187 and SIN3 220 complexes?

We demonstrated that the SIN3 isoform specific complexes can establish different patterns of histone modifications at certain target genes. We have, however, tested a limited number of genes. To understand the complete picture of differential regulation of histone modifications by the SIN3 isoform specific complexes, a genome-wide analysis of histone post-translational modifications at all SIN3 target genes can be performed using ChIP-seq. Chromatin will be isolated from S2 and SIN3 187HA cells and ChIP will be performed using antibodies against specific histone marks namely, H3K9Ac, H3K14Ac, H3K27Ac and H3K4me3. H3C will be used as a normalizer and IgG as a non-specific control. This analysis will primarily provide a detailed map of key histone modifications at SIN3 target genes. It will help identify the differences in the pattern of histone modification upon SIN3 187 recruitment in place of SIN3 220. Additionally, we can determine whether this differential pattern of histone marks is localized to gene promoters or is present throughout the gene. By combining histone modification ChIP-

seq data with the existing RNA-seq data for SIN3 regulated genes, we can correlate differences in histone marks with changes in gene expression. This study will help us determine the true functional differences between SIN3 187 and SIN3 220 complexes.

There are multiple SIN3 isoforms in different species that perform non-redundant functions. Although the role of SIN3 in regulation of several biological processes has been well-studied, the exact roles of different SIN3 isoforms are not well characterized. Recently, researchers are focusing on understanding how SIN3 isoforms may perform distinct functions in important processes such as stem cell maintenance and oncogenic transformation (Lewis et al., 2016; Halder et al., 2017). This study and the experiments described in this chapter make a significant contribution towards dissecting the role of SIN3 isoforms in transcriptional regulation and will aid in further advancing the field of epigenetic regulation of cellular processes.

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ABSTRACT

ANALYZING THE REGULATION, STABILITY AND FUNCTIONAL DIFFERENCES BETWEEN THE SIN3 ISOFORMS IN DROSOPHILA

by

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SIN3 is a global transcriptional regulator, conserved from yeast to mammals, that acts as a scaffold protein for a histone modifying complex. In *Drosophila*, a single *Sin3A* gene is alternatively spliced to produce distinct SIN3 isoforms; SIN3 220, SIN3 190 and SIN3 187, that differ only at their C-terminus. These isoforms are differentially expressed during development. We have shown that there is an interplay between the predominant isoforms of SIN3, SIN3 220 and SIN3 187, that possibly regulates the overall level of SIN3 in the cell. Exogenous expression of SIN3 187 reduces the level of transcript and accelerates the proteasomal degradation of endogenous SIN3 220. This feedback can possibly ensure that the appropriate isoform is present during the correct developmental stage during embryogenesis. Differential expression of the SIN3 isoforms during embryo development suggests that they perform unique and specialized functions. The SIN3 proteins form distinct isoform specific complexes. SIN3 187 interacts with a single catalytic enzyme, the HDAC RPD3, while SIN3 220 interacts with two enzymes, RPD3 and the HDM dKDM5/LID. This differential interaction of SIN3 isoforms with distinct

histone modifying activities may play a role in the non-redundant functions performed by SIN3. Using previously published transcriptome data, we have identified common and unique gene targets of SIN3 and LID. In *Drosophila* S2 cells, knockdown of LID results in an increase in the level of H3K9ac, H3K14ac and H3K27ac at genes commonly regulated by SIN3 and LID. Since LID preferentially interacts with the SIN3 220 complex, we have investigated the histone modification patterns established by the SIN3 isoform specific complexes. We utilized *Drosophila* cultured cells that express either the SIN3 187 or the SIN3 220 complex. The SIN3 187 and SIN3 220 complexes establish distinct histone modification patterns at target genes and differentially regulate the expression of these genes. It is possible that the differential histone modification patterns and the consequent alteration of target gene expression contributes to the functional differences between the SIN3 isoforms. This work enhances our understanding of SIN3 isoform function and provides further insight into the molecular mechanisms of epigenetic control of gene expression by histone modifying complexes

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