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Functional Analysis Of Sin3 Isoforms In Drosophila

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by

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DEDICATION

To my parents, my wife, my son and Bhomvola:

Thanks for your support through this long journey!

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CHAPTER 1 INTRODUCTION

The term chromatin biology describes a myriad of processes that ranges from chromatin dynamics to gene regulation, which in turn impacts several cellular activities. A multitude of proteins and protein modifications are involved in translating the information from DNA to gene expression that ultimately determine the fate of life.

Chromosome organization

Nucleosomes are macromolecular structures that are comprised of a histone octamer core wrapped by 147 base pairs of DNA and histone H1. The histone octamer is formed by two copies of each of the core histones - H2A, H2B, H3 and H4 (Finch et al., 1977; Kornberg and Thomas, 1974; Luger et al., 1997; Noll, 1974; Van Holde et al., 1974). Histone H1 is associated with approximately 20 base pairs of DNA and acts a linker protein connecting the repeating nucleosome units (Noll and Kornberg, 1977). The linear array of nucleosomes further condenses to form higher order chromatin structures (Hayes and Hansen, 2001; Woodcock and Dimitrov, 2001) and thus can be packaged inside the small nucleus of eukaryotic cells (Figure 1.1).

The carboxy terminal domains of histones are highly organized to form the core of the octamer (Arents et al., 1991; Richmond et al., 1984), whereas the amino termini are very flexible and protrude out of the core (Weintraub and Van Lente, 1974; Whitlock and Simpson, 1977). The N-termini of core histones are extremely basic due to the presence of a high proportion of lysine and arginine residues (Luger and Richmond, 1998). Several

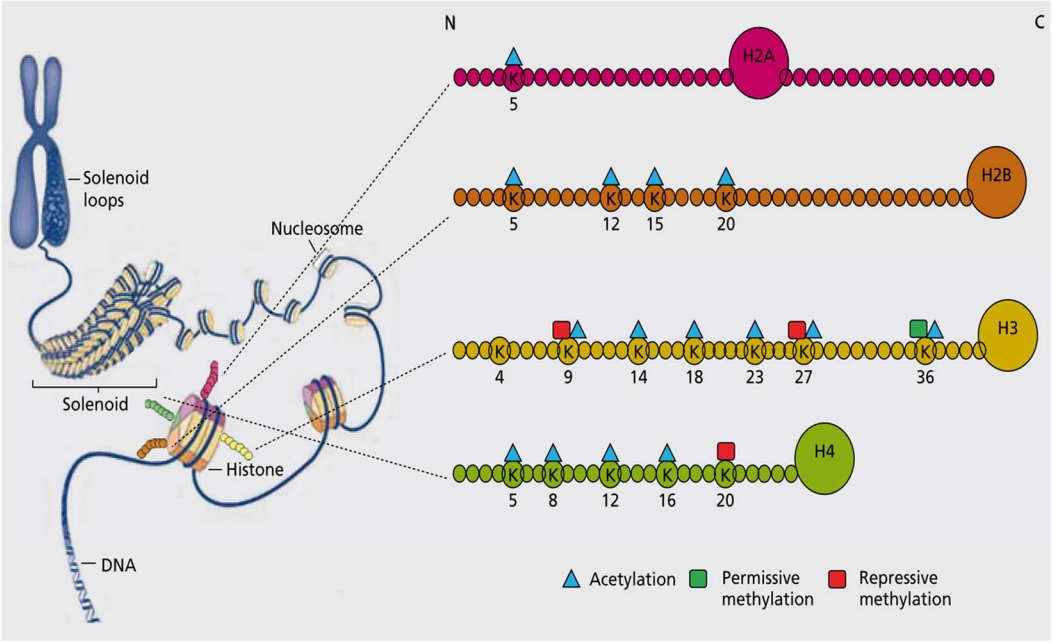


Figure 1.1: Eukaryotic chromosomal organization and histone modifications. Double stranded DNA is wrapped around histones to form nucleosomes. Nucleosomes are further compacted and packaged into chromosomes. Histone tails are post-translationally modified by different covalent marks. These modifications play important roles in the regulation of gene transcription.

of these amino acid residues are subject to covalent post translational modifications (PTMs) (Davie, 1998) (Figure 1.1), which are implicated in determining the dynamic nature of the chromatin structure and influencing gene regulation.

Histone modifications and their role in transcription

A large number of histone PTMs such as acetylation, methylation, phosphorylation, ubiquitination and sumolylation have been identified (Tan et al., 2011). Acetylation of the histone tails was one of the first modifications by a chemical moiety to be discovered (Allfrey et al., 1964; Phillips, 1963). Work by Allfrey et al., also suggested that the addition of acetyl groups on histone tails results in an increase of RNA synthesis (Allfrey et al.,

1964). Subsequently, several histone acetyl transferase enzymes, or histone acetylases, were discovered (Struhl, 1998). These enzymes were demonstrated to play a role in gene activation. For example, p300/CBP, a histone acetyl transferase, was shown to function as a transcriptional coactivator (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). In general, acetylation of histones is correlated to gene activation (Kuo and Allis, 1998). Unlike acetylation, site specific lysine methylation of histones has varied functions and has been shown to be enriched at both active and repressed genes (Martin and Zhang, 2005). For example, histone H3 lysine 4 trimethylation (H3K4me3) was demonstrated to be enriched over promoter regions of active genes, histone H3 lysine 4 monomethylation (H3K4me1) marks are associated with enhancer elements and histone H3 lysine 36 trimethylation (H3K36me3) is correlated with transcription elongation (Bannister et al., 2005; Barski et al., 2007; Heintzman et al., 2007; Santos-Rosa et al., 2002). On the other hand, repressed genes are marked by histone H3 lysine 27 trimethylation (H3K27me3) and histone H3 lysine 9 dimethylation (H3K9me2) (Boyer et al., 2006; Bracken et al., 2006; Lehnertz et al., 2003). An overview of different PTMs is demonstrated in Figure 1.1.

Chromatin modifying complexes, which act upon different histone PTMs, affect the chromatin structure and subsequent transcriptional outcomes. To date, several chromatin modifying proteins that regulate gene expression have been identified. These factors form multiprotein complexes to modify histones, alter nucleosome position or recruit other *trans* factors to switch gene transcription on or off. The role of histone acetyl transferases (HATs) to activate gene transcription is well documented (Sterner and Berger, 2000). The earliest identified histone acetyl transferase, Gcn5, was shown to acetylate both free and nucleosomal histones (Wang et al., 1997; Xu et al., 1998b; Yang et al., 1996) and was

shown to be important for transcriptional activation of specific genes (Brownell et al., 1996; Georgakopoulos and Thireos, 1992). The requirement of the HAT activity of PCAF to induce gene transcription was demonstrated in several molecular processes such nuclear receptor function, muscle differentiation, development and homeostasis (Blanco et al., 1998; Korzus et al., 1998; Puri et al., 1997; Xu et al., 1998a). Histone methyl transferases have also been shown to affect gene activity (Zhang and Reinberg, 2001). For example, SUV39H1, a protein that has intrinsic methyl transferase activity, leads to heterochromatic gene silencing (Melcher et al., 2000; Rea et al., 2000; Tschiersch et al., 1994).

Another class of chromatin modifying complexes act as ATP-dependent chromatin remodelers. These enzymes facilitate nucleosome positioning to control chromatin architecture and gene transcription (Henikoff, 2008; Schnitzler, 2008). In *Drosophila melanogaster*, NURF was discovered as an ATP-dependent chromatin remodeler involved in activation of genes *in vitro* and *in vivo* (Badenhorst et al., 2002; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995). Conversely, in *Saccharomyces cerevisiae*, the ATP-dependent nucleosome remodeler Isw2 can position nucleosomes to hinder transcription (Whitehouse and Tsukiyama, 2006).

Sequential recruitment of protein complexes to chromatin affects transcriptional outcome. SWI/SNF is recruited by Swi5p at the *HO* gene promoter (Cosma et al., 1999). SWI/SNF in turn is required for recruitment of the HAT Gcn5 complex to the *HO* gene promoter (Cosma et al., 1999; Krebs et al., 1999). Subsequently, another activator, SBF, is recruited to the promoter that ultimately leads to RNA polymerase II-mediated transcription of the *HO* gene (Fry and Peterson, 2002). Recruitment of the MLL complex

by Menin leads to robust increase in the expression of HOX genes in leukemogenesis (Caslini et al., 2007; Chen et al., 2006; Hughes et al., 2004; Yokoyama et al., 2005). Transcriptional silencing has also been demonstrated through recruitment of specific enzymes facilitated by other factors (Thiel et al., 2010). For example, in *Xenopus laevis*, methylated DNA tethers to MeCP2, which in turn recruits histone deacetylases to silence gene transcription (Jones et al., 1998).

Chromatin states

The actions of chromatin modifying machinery and the presence or absence of histone PTMs determine whether the underlying chromatin is favorable for gene transcription to occur. Classically, chromatin states are broadly referred to as euchromatin and heterochromatin, based on transcriptional activity (Allis and Jenuwein, 2016). Euchromatin is considered to be transcriptionally active. Transcriptionally active genes were shown to be targeted by histone acetyl transferases and histones at these sites are hyperacetylated (Schubeler et al., 2004; Wang et al., 2009). In addition, transcriptionally active domains are sensitive to DNase1 mediated-cleavage, which indicates an open structural conformation of euchromatic regions (Cockerill, 2011). Heterochromatin domains on the other hand, are associated with transcriptional silencing and condensed chromatin structure (Grewal and Jia, 2007). Previous work demonstrated silencing of reporter transgenes inserted in these chromatin regions (Handler and Harrell, 1999; Markstein et al., 2008). These domains are enriched in features such as SUV39H1, HP1 and histone H3 lysine 9 methylation (H3K9me) (Grewal and Jia, 2007). Paradoxically, there are reports that indicate an opposing role of some of these features, which were used to define heterochromatin (Hediger and Gasser, 2006). Both HP1 and H3K9me

were demonstrated to be enriched at a subset of transcriptionally active sites, contradicting their role in heterochromatin formation (Cryderman et al., 2005; Greil et al., 2003; Piacentini et al., 2003). At these transcriptionally active sites, HP1 very likely recruits distinct group of effector proteins to induce gene transcription (Grewal and Jia, 2007). Thus, heterochromatin state needs to be redefined by integrating additional features that are specific to repressive chromatin domains.

With the development and advancement of next generation sequencing technology, researchers have been successful to classify the chromatin states in finer detail by integrating distinct chromatin binding proteins, histone modifications, accessibility to chromatin and global transcriptomic analysis. Nine chromatin states were defined upon combinatorial patterning of differentially enriched histone modifications (Kharchenko et al., 2011). Chromatin state 1 is enriched with H3K4me3/me2 and histone H3 lysine 9 acetylation (H3K9ac) marks, which are both associated with active transcription (Kharchenko et al., 2011). Approximately half of the DNase1 hypersensitive sites were found to be present at active transcription start sites and to contain features of active chromatin state 1 as predicted based on the decondensed nature of euchromatin (Kharchenko et al., 2011). Pericentromeric silent domains were marked with high levels of H3K9me2/me3 and HP1 protein (Kharchenko et al., 2011).

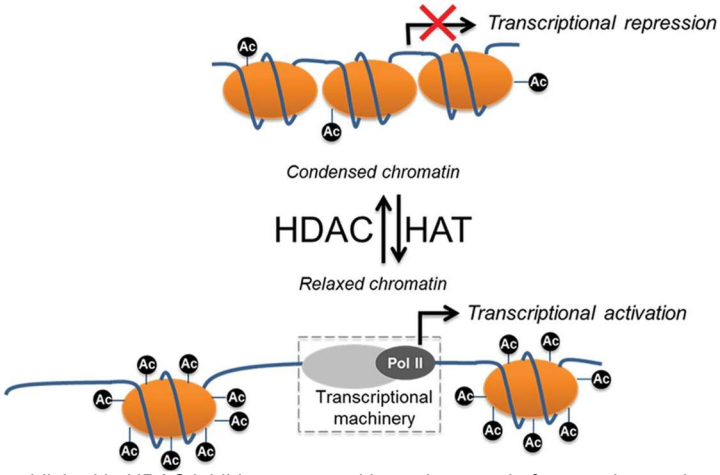
In another study, chromatin was classified into five states based on the localization of 53 chromatin modifying proteins (Filion et al., 2010). In this study, red and yellow chromatin states were determined as transcriptionally active regions, while blue, green and black chromatin states were considered as different forms of silent and heterochromatic regions (Filion et al., 2010). One conspicuous difference between red

and yellow chromatin domains was enrichment of H3K36me3 in the yellow chromatin state (Filion et al., 2010). The black chromatin domain that accounted for majority of the fly genome contains silenced genes and is devoid of classically defined heterochromatin features like H3K9me2/me3, SUV39H1 and HP1 (Filion et al., 2010). Blue chromatin corresponds with polycomb group complex mediated silenced domains and exhibits an enrichment of H3K27me3 and polycomb group complex proteins (Filion et al., 2010). The green chromatin state forms the classically defined heterochromatin, showing enrichment of H3K9me2/3 and HP1 (Filion et al., 2010). Determination of these different chromatin states significantly advances our knowledge of how chromatin structure, in concert with components that determine chromatin states, functions in the epigenetic regulation of biological processes.

Histone deacetylation and deacetylases

Histone deacetylation is the opposing process of histone acetylation and leads to the removal of acetyl marks. Histone deacetylation results in the removal of the positively charged acetyl marks on the histones, therefore impeding transcription due to the resulting compaction of chromatin (Ayer, 1999). Histone acetylation had been demonstrated to favor transcription by allowing transcription factor binding by restricting the interaction between histone tails and underlying DNA (Lee et al., 1993; Vettese-Dadey et al., 1996). Therefore, histone deacetylation very likely enables interaction of histone tails with DNA, inhibits transcription factor binding and represses transcription (Ayer, 1999). Additionally, histone deacetylation favors formation of higher order chromatin as acetylation of histones had been demonstrated to facilitate transcription by decreasing

the compact nature of chromatin structure (Annunziato et al., 1988; Ayer, 1999; Garcia-Ramirez et al., 1995; Tse et al., 1998; Ura et al., 1997) (Figure 1.2).



This figure was originally published in HDAC inhibitors as cognitive enhancers in fear, anxiety and trauma therapy: where do we stand? Nigel Whittle, Nicolas Singewald. Biochemical Society Transactions. Apr 01, 2014.

Figure 1.2: Histone acetylation and transcriptional regulation. Histone acetylation carried out by histone acetyl transferases (HAT) leads to an open chromatin structure and transcription activation. Conversely, histone deacetylase (HDAC) removes acetyl marks resulting in compact chromatin structure and transcriptional silencing.

Several enzymes responsible for deacetylation of histones have been reported. Rpd3 was one of the earliest factors discovered to play a role in transcriptional repression (Vidal and Gaber, 1991). Rpd3 was shown to be important for transcriptional repression via histone deacetylase activity (Kadosh and Struhl, 1998). Subsequently, the mammalian orthologues of Rpd3, HDAC1 and HDAC2, were discovered using a deacetylase inhibitor based assay (Taunton et al., 1996).

Histone deacetylases are generally associated with large multimeric protein complexes. The Mi-2/NURD complex is highly conserved in the animal kingdom (Brehm et al., 2000; Tong et al., 1998; Wang et al., 1998; Xue et al., 1998). In addition to HDAC1, this repressive complex has been demonstrated to possess an ATPase-dependent

remodeling activity (Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998). HDAC1/2 in the NURD complex are required for deacetylation of histones (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998), while CHD3/CHD4 have been shown to have helicase-like ATPase activity (Woodage et al., 1997). In addition to being a core component of the NURD complex, HDACs are found in the CoREST complex. (Humphrey et al., 2001; Tontsch et al., 2001; You et al., 2001). The CoREST complex consists of CoREST, HDAC1/HDAC2, Sox-like protein, p80, ZNF 217 and LSD1 (You et al., 2001). The CoREST protein contains two SANT domains, which are essential for interacting with HDAC1/2 (You et al., 2001). The CoREST complex possesses deacetylase activity (You et al., 2001). In addition to the deacetylase activity, the CoREST complex has demethylase activity and this activity is mediated by LSD1, the histone demethylase component of the complex (Shi et al., 2004; Shi et al., 2005). HDAC1 is found in another major large multiprotein complex referred to as the SIN3 histone modifying complex, which is described in detail in the next section (Hassig et al., 1997; Nagy et al., 1997; Zhang et al., 1997). In summary, histone deacetylases are important enzymes involved in the maintenance of homeostasis between gene activation and repression, thereby controlling transcriptional output.

The SIN3 histone modifying complex

SIN3 was initially discovered in a genetic screen studying the process of mating type switching in budding yeast (Nasmyth et al., 1987; Sternberg et al., 1987). SIN3 is conserved across eukaryotes (Grzenda et al., 2009; Kadamb et al., 2013; Silverstein and Ekwall, 2005). SIN3, in general, has been implicated in the negative regulation of gene transcription, though several pieces of evidence suggest a role of the protein in

transcription activation (Grzenda et al., 2009; Kadamb et al., 2013). In mouse, mSIN3A is required for p53-mediated repression of gene targets (Murphy et al., 1999). In another example, yeast Sin3 and Rpd3 interact with Ume6 to repress gene transcription (Washburn and Esposito, 2001). In *Drosophila*, *Sin3A* knockdown led to alteration of expression of many genes of which the large majority was upregulated indicating that SIN3 acts as a repressor (Pile et al., 2003). Alternatively, SIN3 has also been shown to act as an activator of transcription. In yeast, Sin3 is required for inducing the expression of Gam2 and Sta1 (Yoshimoto et al., 1992). The *Nanog* gene promoter has been shown to be bound and activated by mSin3A-HDAC via Sox2 (Baltus et al., 2009). In *Drosophila*, SIN3 acts an activator of genes involved in cell migration (Das et al., 2013).

Sin3A is an essential gene higher eukaryotes (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008; Neufeld et al., 1998; Pennetta and Pauli, 1998) and is implicated in several biological processes (Grzenda et al., 2009; Silverstein and Ekwall, 2005). SIN3 has been shown to play a role in protein stability, metabolism, oncogenic transformations, cell survival and cellular senescence (Bansal et al., 2016; Kadamb et al., 2013). In *Drosophila* cultured cells, SIN3 was shown to regulate approximately 3% of genes (Pile et al., 2003). Consistent with the role in these important cellular processes, loss of SIN3 leads to a cell proliferation defect in yeast, mammals and *Drosophila* (Cowley et al., 2005; Dannenberg et al., 2005; Pile et al., 2002; White et al., 2009). SIN3 has also been shown to play a critical role in oxidative stress response and regulating mitochondrial activity (Barnes et al., 2014; Gajan et al., 2016). Structurally, SIN3 has four paired amphiphatic helix domains (PAH) domains, which have been demonstrated to interact with accessory proteins. A histone deacetylase interacting domain (HID) is

present between the third and the fourth PAH domains of SIN3. The HID is required for interacting with histone deacetylase, the main catalytically active component of the SIN3 complex (Laherty et al., 1997). A DNA binding domain is absent in SIN3 (Wang and Stillman, 1993), therefore, it is recruited to chromatin via DNA binding factors or other corepressor proteins (Ayer et al., 1996; Kadosh and Struhl, 1997; Varier et al., 2016; Xu et al., 2008) (Figure 1.3).

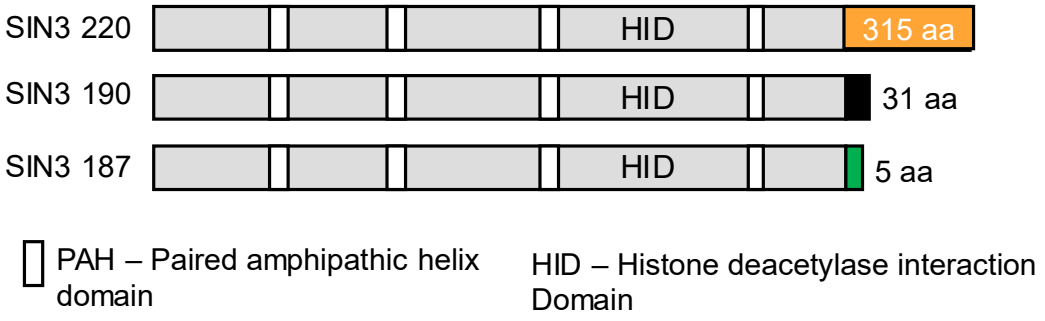


Figure 1.3: *Drosophila* SIN3 isoforms. A single *Sin3A* gene encodes for multiple isoforms of SIN3. Structurally, all SIN3 isoforms have four paired amphiphatic helix (PAH) domains shown as white boxes and a histone deacetylase interaction domain (HID). SIN3 isoforms differ at the C-terminal region. The unique C-terminal domains of SIN3 isoforms are represented as following; SIN3 220 - orange, SIN3 190 - black and SIN3 187- green.

A large number of proteins, including HDAC1/2, SDS3, Sap30, Rbbp4/7, ING1/2, Sap180 and BRMS1, form the core of a large SIN3 complex (Doyon et al., 2006; Fleischer et al., 2003; Hassig et al., 1997; Kasten et al., 1997; Kuzmichev et al., 2002b; Meehan et al., 2004; Skowyra et al., 2001; Vidal and Gaber, 1991; Zhang et al., 1997). Some of these components such as SDS3 and BRMS1 are thought to be important for maintaining the structural integrity of the complex and required for catalytic activity (Lechner et al., 2000; Meehan et al., 2004). In yeast, *Sds3* knockout results in the abolishment of the Sin3 and Rpd3 interaction and loss of HDAC activity (Lechner et al., 2000). In a human

metastatic cell line, BRMS1 was demonstrated to be required for the catalytic activity of HDAC complexes (Meehan et al., 2004). In this study, immunoprecipitation was carried out against full length and truncated forms of BRMS1. HDAC activity assay performed on immunoprecipitated samples showed a loss of HDAC activity associated with the truncated forms of BRMS1 compared to the full length (Meehan et al., 2004). Rbbp4 and Rbbp7 are predicted to stabilize the SIN3 complex on chromatin (Grzenda et al., 2009; Kadamb et al., 2013; Silverstein and Ekwall, 2005).

From yeast to mammals, SIN3 forms various protein complexes suggesting a diverse role of these complexes in animal development (Kadamb et al., 2013). In mammals, three isoforms of Sin3 from two *mSin3* genes have been reported. mSin3A and mSin3B are the major isoforms, while a third, $mSin3B_{SF}$ has also been reported (Ayer et al., 1995; Yang et al., 2000). mSin3A and mSin3B proteins form discrete complexes and function in mammalian development. In mice, mSin3A and mSin3B function in cell proliferation and cell cycle exit, respectively (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008; van Oevelen et al., 2008). Recently, mSin3A and mSin3B have been shown to play opposing roles in breast cancer metastasis (Lewis et al., 2016). mSin3A was found to be a suppressor of metastasis, while mSin3B has been demonstrated to promote cell invasion (Lewis et al., 2016). In budding yeast, a single Sin3 protein forms two distinct complexes, Rpd3L and Rpd3S. These complexes were shown to repress transcription by distinct mechanisms (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Li et al., 2007a; Li et al., 2007b). In fission yeast, three isoforms of SIN3 (i.e Pst1, Pst2 and Pst3), encoded by three different genes, have been reported (Dang et al., 1999; Silverstein and Ekwall, 2005). The heterogeneous population of SIN3

interacting proteins other than the core complex components very likely imparts specificity to these distinct SIN3 complexes.

In *Drosophila*, a single *Sin3A* is alternatively spliced to produce distinct SIN3 isoforms (Neufeld et al., 1998; Pennetta and Pauli, 1998). SIN3 220, SIN3 190 and SIN3 187 are the major transcripts identified in flies (Pennetta and Pauli, 1998) (Figure 1.3). As in yeast and mammals, these *Drosophila* SIN3 isoforms have four PAH domains and a HID between the third and the fourth PAH domains, indicating that SIN3 is structurally conserved (Neufeld et al., 1998; Pennetta and Pauli, 1998). *Drosophila* SIN3 isoforms differ at their C-terminal regions. The isoforms are expressed differentially during development (Sharma et al., 2008). SIN3 220 is predominant in proliferative cells, while SIN3 187 is expressed in differentiated tissues (Dobi et al., 2014; Sharma et al., 2008). Expression of SIN3 190 is limited to adult females and ovaries (Sharma et al., 2008). SIN3 220, but not SIN3 187, is essential for fly viability (Spain et al., 2010). SIN3 220 and SIN3 187 isoforms form distinct SIN3 complexes, with the same core components present in both of the complexes (Spain et al., 2010). Caf1-55, dKDM5/LID and EMSY proteins

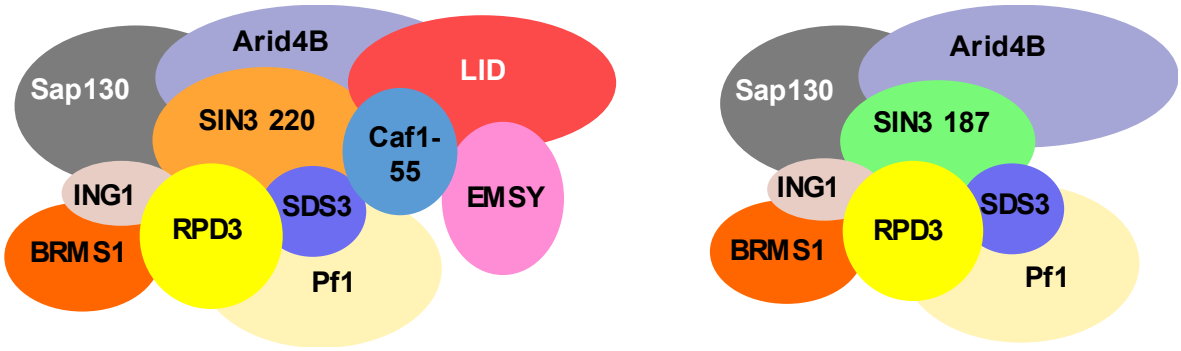


Figure 1.4: *Drosophila* SIN3 isoform specific complexes. SIN3 220 and SIN3 187 form distinct complexes. These complexes share core protein components. The SIN3 220 complex specifically contains dKDM5/LID, Caf1-55 and EMSY.

preferentially interact with the SIN3 220 complex (Spain et al., 2010) (Figure 1.4), suggesting a differential function of the SIN3 220 complex compared to the SIN3 187 complex. These data suggested that SIN3 isoforms have non-redundant functions during fly development and warranted further investigation.

Role of Caf1-55 in the SIN3 220 complex

In *Drosophila*, Caf1-55 was first identified as a component of the Caf1 complex (Tyler et al., 1996). The Caf1 complex acts as a histone chaperone required for the deposition of histones on newly synthesized strands of DNA in both mammals and *Drosophila* (Kamakaka et al., 1996; Kaufman et al., 1995; Smith and Stillman, 1989, 1991; Stillman, 1986). Comparison of the amino acid sequence of Caf1-55 shows that it is homologous to mammalian Rbbp4/7 proteins (Tyler et al., 1996). Caf1-55 has WD40 repeats that give it a β-barrel structure (Song et al., 2008). The WD40 repeats are approximately 40 amino acid long structures often terminating with a tryptophan and aspartic acid dipeptide sequence (Neer et al., 1994). The WD40 repeat is an excellent interface for protein-protein interactions (Suganuma et al., 2008). X-Ray crystallographic analysis demonstrated that Caf1-55 interacts with histones H3 and H4 and, interestingly, this interaction is dependent on post-translational modifications on histone tails (Nowak et al., 2011; Schmitges et al., 2011; Song et al., 2008). Based on the structural properties, Caf1-55 is thought to act as a platform for the assembly of chromatin modifying complexes.

In addition to the SIN3 complex, Caf1-55 has been found to be an interacting partner of several other chromatin modifying complexes. Caf1-55 is a component of the ATP-dependent remodeling complexes including the nucleosome remodeling factor

(NURF) complex and the nucleosome remodeling deacetylase (NuRD) complex (Smith and Stillman, 1989; Tyler et al., 1996; Verreault et al., 1996; Wade et al., 1998; Zhang et al., 1998). Caf1-55 has also been shown to play a role in a cytoplasmic acetyl transferase Hat1p complex and the Polycomb Group Repressive complex (Czermin et al., 2002; Hassig et al., 1997; Kuzmichev et al., 2002a; Muller et al., 2002; Parthun et al., 1996; Zhang et al., 1997).

Among the SIN3 isoform specific complexes in *Drosophila*, Caf1-55 preferentially interacts with the SIN3 220 complex (Spain et al., 2010). Reduction of Caf1-55 by RNA interference results lethality in *Drosophila* (Spain et al., 2010). Interestingly, overexpression of the SIN3 220 isoform, but not SIN3 187, can rescue lethality due to Caf1-55 knockdown (Spain et al., 2010). Together, these data suggest that the interaction between SIN3 220 and Caf1-55 is essential for development.

Project outline

In *Drosophila*, how SIN3 isoforms function in regulating gene expression throughout fly development is poorly understood. In addition, the function of unique protein components in the SIN3 220 complex is not well characterized. In my PhD thesis research, the major goal was to study the biological processes regulated by SIN3 isoforms. Furthermore, I investigated the function of Caf1-55 in modulating the activity of the SIN3 220 complex.

We took a functional genomics approach to study the biological processes regulated by SIN3 isoforms. In the process of performing the genomic analysis, I developed a protocol for chromatin immunoprecipitation (ChIP). ChIP is a standard technique to identify genomic loci bound by a particular chromatin or transcription factor.

The technique I developed is based on preparation of chromatin using micrococcal nuclease based chromatin fragmentation. The details of the optimized ChIP protocol are described in Chapter 2. Next, I carried out genome-wide binding and expression analysis to study the role of SIN3 isoforms in regulating distinct biological processes. This part of the research is described in Chapter 3.

Caf1-55 preferentially interacts with the SIN3 220 isoform specific complex. The function of Caf1-55 in the SIN3 220 complex is not well understood. I used a biochemical approach to investigate the role of Caf1-55 in the SIN3 220 complex. This part of the research is described in Chapter 4.

Overall, these studies have enhanced our understanding of gene regulation by the SIN3 isoforms. The role of SIN3 187 and SIN3 220 complexes in the fly development, however, still remains unclear. In Chapter 5, I briefly describe potential projects that would facilitate our understanding of the requirement of distinct SIN3 complexes during the *Drosophila* life cycle.

CHAPTER 2 A MICROCOCCAL NUCLEASE DIGESTION-MEDIATED CHROMATIN SHEARING PROTOCOL FOR EFFICIENT CHROMATIN IMMUNOPRECIPITATION

Introduction

The chromatin immunoprecipitation (ChIP) assay is an invaluable method to study protein-DNA interaction *in vivo*. In this method, a protein-DNA complex is immunoprecipitated using antibody against the protein under study. The level of enrichment of the protein at chromatin is then quantified by PCR-based methods or next generation sequencing technologies. X-ChIP and N-ChIP are two broad strategies to perform ChIP (O'Neill and Turner, 2003; Orlando, 2000). The major difference between these two strategies is that X-ChIP is performed by crosslinking the protein-DNA complex, while N-ChIP is performed on native chromatin. X-ChIP has been suggested to be more suitable to study protein-DNA interaction and N-ChIP has been shown to be optimal to investigate histone modifications (Turner, 2001). For X-ChIP, protein is crosslinked to DNA by crosslinking reagents such as formaldehyde, dimethyl adipimidate and dimethyl 3,3'-dithiobispropionimidate (Zeng et al., 2006). Chromatin is then fragmented to a desired size by sonication or enzymatic digestion. The sheared protein-DNA complex is immunoprecipitated using antibody against the protein of interest.

Chromatin preparation is one of the critical steps necessary to perform successful chromatin immunoprecipitation. Sonication, which is the most extensively used method to fragment DNA, is fraught with several technical challenges. Chromatin shearing by sonicators like Diagenode bioruptors (https://www.diagenode.com/applications/chromatin-shearing) or Covaris (http://covarisinc.com/applications/chromatin-shearing/) is very efficient, however these instruments are expensive. More economical sonicators, like the Sonic Dismembrator

(Thermo Fisher Scientific), give highly variable results making optimization of chromatin preparation both labor intensive and time consuming. In addition, sonication leads to frothing of samples affecting the efficiency of the process. Enzyme-mediated fragmentation of chromatin is an alternative method to overcome these issues. A protocol describing chromatin preparation from a mammalian cell line using micrococcal nuclease (MNase) has been developed and described by Cell Signaling (http://www.cellsignal.com/contents/resources-protocols/simplechip-chromatin-immunoprecipitation-protocol-(agarose-beads)/chip-agarose?N=102288+4294956305&fromPage=plp). A recent advancement of the technique described the use of MNase to fragment chromatin followed by bursts of sonic waves to extract chromatin from *Drosophila* cultured cells (Skene and Henikoff, 2015). The sonication buffer used in that study contains the detergent sodium dodecyl sulfate (SDS) that raises a concern regarding the efficacy of the chromatin immunoprecipitation assay. The presence of SDS in the sonication buffer often induces foaming of samples. Foaming leads to an increase in surface tension, which in turn can disrupt protein conformation, affecting the overall experiment (Clarkson et al., 1999). In addition, foaming introduces variability across samples.

I initially performed ChIP following a protocol that utilized sonication and brief MNase digestion as the mode of chromatin fragmentation. Briefly, following formaldehyde crosslinking, S2 cells were lysed in buffer containing the detergent SDS. Lysed cells were sonicated using the Sonic Dismembranator (Thermo Fisher Scientific) I further digested the sonicated chromatin with MNase. I next treated the chromatin to reverse the DNA-protein crosslinks and verified chromatin fragmentation using a protocol described in

detail below. This methodology of chromatin preparation did not produce desired chromatin fragment size as shown in Figure 2.1A.

In this report, I present an optimized MNase digestion followed by sonication based chromatin fragmentation protocol to perform ChIP on sample from *Drosophila* cultured cells using a sonication buffer free of SDS. This protocol is optimized in a way such that every step can be controlled, producing highly reproducible results. In addition, this protocol can be efficiently applied to map chromatin bound transcription factors and histone modifications, both locally and globally.

Materials and Methods

Formaldehyde crosslinking of *Drosophila* cells

I started with 4 x 10^7 S2 cells in 10 ml of serum free media. Using this number of cells, a final concentration of 300 ng/µl to 500 ng/µl of chromatin extract could be obtained. Cells were subjected to formaldehyde crosslinking. Formaldehyde was added to cells to a final concentration of 1% and the cells were incubated on a shaker for 10 min at room temperature. Excess formaldehyde was quenched by adding freshly prepared glycine solution to a final concentration of 125 mM followed by shaking at room temperature for 10 min. Following formaldehyde crosslinking and quenching, the cells were subjected to centrifugation at 800 x g at 4^{o}C for 5 min in a clinical centrifuge. The cell pellet was resuspended in ice cold 1 X PBS to remove any trace of media and crosslinking reagents. This cell pellet wash process was repeated three times. Formaldehyde treated cells were then processed to prepare chromatin as described below.

Cell Lysis and MNase Digestion

Douncing was used to lyse the cells. Washed cells were resuspended in 15 ml of lysis buffer (10 mM Tris (pH 8), 10 mM KCl, 3 mM $CaCl_2$, 0.34 M Sucrose, 1 mM DTT, 0.1% Triton X-100 and 0.2 mM EGTA) (Li and Arnosti, 2010). One protease inhibitor pellet (Roche) was added to the lysis buffer to prevent protein degradation upon cell lysis. A single protease inhibitor pellet is sufficient for chromatin preparation from 4 x 10^7 cells. The cell suspension was then incubated on ice for 15 min. Cells were then dounced 10 times using a loose pestle and 15 times by a tight pestle, keeping the douncer on ice. Douncing leads to cell lysis, while the nuclei are left intact. The nuclei were recovered by centrifugation of the sample at 170 x g, 4^{o}C for 10 min in a clinical centrifuge. The supernatant was removed leaving the pellet undisturbed.

Intact nuclei were then resuspended in 200 µl of MNase digest buffer (15 mM Tris (pH 8), 60 mM KCl, 15 mM NaCl, 1 mM DTT, 0.25 M Sucrose and 1 mM $CaCl_2$) and transferred to a 2 ml microfuge tube. The sample was then subjected to MNase digestion by addition of 20 units of MNase (Worthington Biochemicals) followed by incubation on a nutator for 30 min at room temperature. This is a very critical step in the preparation of chromatin as over digestion may lead to formation of mononucleosomes, which are not optimal for efficient immunoprecipitation. The enzymatic activity of MNase varies between different manufacturers and can also be variable among different lots from the same manufacturer. Care should be taken to make sure that incubation with MNase produces the desired DNA fragment size. A more detailed description of optimizing this step is outlined in the Appendix. The MNase reaction was quenched by addition of EDTA to final concentration of 10 mM followed by incubation on ice for 5 min.

Extraction of chromatin from MNase-digested nuclei

A few cycles of brief sonication lead to efficient extraction of chromatin from nuclei. 1 ml of resuspension buffer (140 mM NaCl, 10 mM Tris (pH 7.6) and 2 mM EDTA) was added to the MNase digested nuclei to bring the total volume to 1.2 ml. Sonication was carried out using the Sonic Dismembrator (Thermo Fisher Scientific) for 3 min and 30 sec with 30 sec pulse on and 1 min pulse off at 20% amplitude. Frothing of samples was not observed as this buffer does not contain SDS. Measures should be taken to keep the sample cold during the sonication process. The sonicated sample was subjected to centrifugation at 21,000 x g for 15 min at 4^{o}C to separate the soluble chromatin fraction from nuclear debris. After centrifugation, the chromatin fraction was transferred to a new 1.7 ml microfuge tube. The chromatin can be quantified using a spectrophotometer. At this point, the chromatin can be stored at -80^{o}C after flash freezing in liquid nitrogen or can be directly used to reverse crosslinks to prepare input chromatin and assess the quality of the chromatin prepared. Assessing of chromatin quality using visualization by agarose gel electrophoresis is recommended for each chromatin preparation.

To prepare DNA to use as “input” for the experiment, approximately 75 µg of chromatin was subjected to a reverse crosslink reaction. The total volume of chromatin was made up to 500 µl using resuspension buffer and treated with 0.05 µg/µl of RNase for 20 min at 37^{o}C. The sample was then incubated overnight at 65^{o}C with 200 mM NaCl. Next, the mixture was incubated at 45^{o}C for 1 hr 30 min with 0.04 µM Proteinase K (Thermo Fisher Scientific), 10 µM EDTA and 20 µM Tris (pH 8). After incubation, one volume of buffered phenol:chloroform was added to the sample, which was then vortexed. The mixture was subjected to centrifugation for 5 min at 21,000 x g and 500 µl of the top layer was distributed equally to two new 1.7 ml tubes. Phase lock tubes (5 Prime) may be

used to increase the efficiency of sample recovery at this step. The DNA was precipitated by adding 3 volumes of chilled 100% ethanol, 3 M sodium acetate to a final concentration of 0.03 M and 0.5 µl of pellet paint (Novagen). After mixing, the tubes were incubated at room temperature for 10 min. The sample was subjected to centrifugation at 21,000 x g and 4°C for 15 min to pellet the DNA. After washing once with 70% ethanol, the pellet was air dried and resuspended in 25 µl of water. For long-term storage purpose, the pellet may be dissolved in 10 mM Tris. The protocol and recipe of buffers are provided in detail in the Appendix.

Chromatin immunoprecipitation

I chose Caf1-55 to show the efficiency of the ChIP protocol. I performed ChIP followed by quantitative PCR (qPCR). Caf1-55 is a component of several chromatin remodeling complexes and binds to histones H3 and H4 (Martinez-Balbas et al., 1998; Nowak et al., 2011; Smith and Stillman, 1989; Song et al., 2008; Spain et al., 2010; Tyler et al., 1996; Verreault et al., 1996). I generated a stable cell line carrying a transgene for expression of Caf1-55 tagged with HA, under the control of a metallothionein promoter (Figure 4.1A). The DNA plasmid containing the transgene was acquired from the Berkeley *Drosophila* Genome project (BDGP) ORFeome collection (Yu et al., 2011). I prepared chromatin from this cell line and performed ChIP using anti-HA agarose beads. Chromatin prepared from S2 cells that do not express any HA-tagged protein was used as a non-specific ChIP control. Approximately 75 µg of chromatin was incubated with 30 µl of anti-HA beads. The sample was placed on a nutator at 4°C for 4 hr. Chromatin bound HA beads were washed once with a modified 2 X RIPA buffer (50 mM Tris (pH 7.6), 280 mM NaCl, 2 mM EDTA, 0.3% SDS), once with IP1 buffer (25 mM Tris (pH 7.6), 500 mM NaCl,

1 mM EDTA, 0.1% SDS and 1% Triton X-100) once with IP2 buffer (10 mM Tris (pH 7.6), 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate and 0.5% Triton X-100) and once with 1 X TE buffer. The chromatin was eluted by addition of elution buffer (1% SDS, 0.1 M $NaHCO_3$) followed by incubation at 65^oC for 30 min. The elution step was performed twice yielding a total volume of 500 µl. The DNA-protein crosslinks of the immunoprecipitated samples were reversed as described above for input DNA preparation. Details of this protocol and buffer preparation are outlined in the Appendix.

Results and Conclusion

The extent of DNA fragmentation was visualized by performing gel electrophoresis. The process of chromatin preparation described above resulted in the formation of predominantly mono-, di- and tri-nucleosomes (Figure 2.1B).

Previously, we have described a SIN3 220 histone deacetylase complex that contains Caf1-55 (Spain et al., 2010). We thus predicted that Caf1-55 would localize to the same genes that are targeted by SIN3 220. We performed qPCR to assay the binding of Caf1-55 over gene promoters targeted by SIN3 220. We selected promoter regions of *MME, Sam-S, Ahcy13, CG10623, Cyt-c1* and *Pyk* as putative binding sites of Caf1-55 and the intronic region of the *ds* gene as a negative control. The primers used for qPCR are listed in the Table 4.2. The enrichment was calculated using $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) and is represented as a percentage of input. The results of the ChIP-qPCR demonstrate that Caf1-55 localized to putative gene promoters, while very little enrichment was observed at the negative control region (Figure 4.1B).

In conclusion, we describe a very efficient, highly reproducible protocol to perform ChIP using a *Drosophila* cell line. This protocol has also been successfully used to assay

histone modification enrichment and map corepressor recruitment sites by next generation sequencing (Gajan et al., 2016; Liu et al., 2015; Saha et al., 2016).

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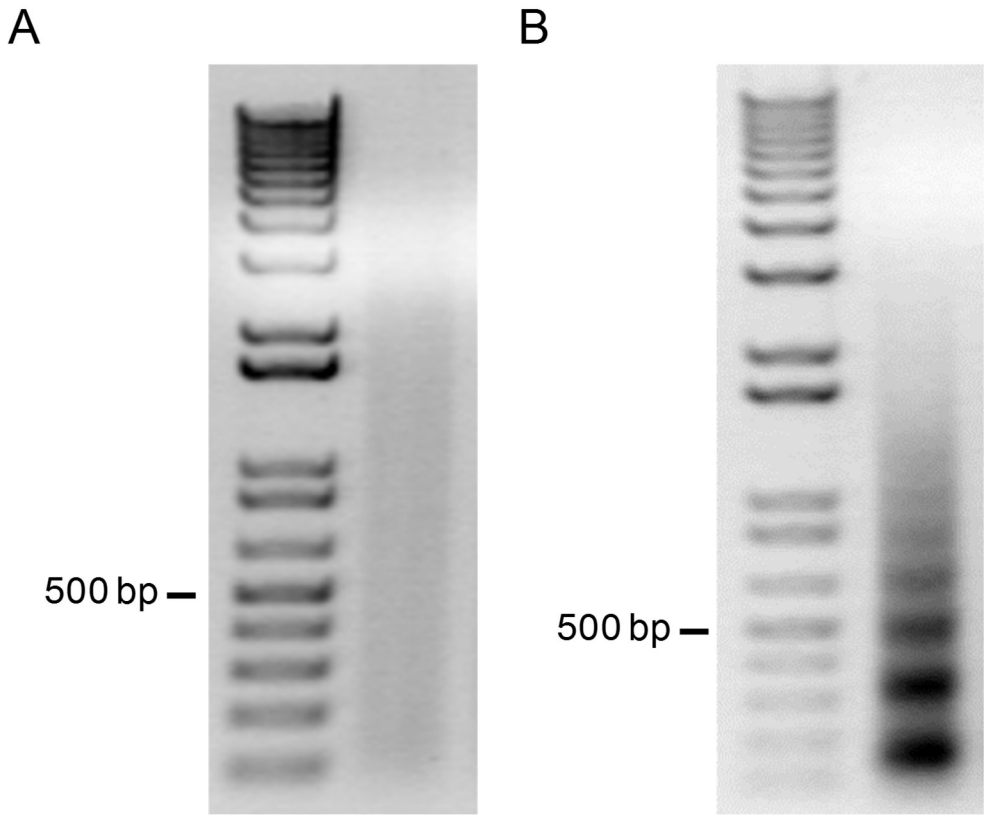

Figure 2.1: Preparation of chromatin. (A) Chromatin preparation from S2 cells showing the DNA fragmentation pattern using sonication mediated chromatin fragmentation. (B) Chromatin preparation from S2 cells showing the DNA fragmentation using micrococcal nuclease (MNase) digestion. The purified DNA sample (right lane) was subjected to electrophoresis on a 1% agarose gel. DNA ladder was loaded in the left lane.

CHAPTER 3 GENOME-WIDE STUDIES REVEAL NOVEL AND DISTINCT BIOLOGICAL PATHWAYS REGULATED BY SIN3 ISOFORMS

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Introduction

Nucleosomes are comprised of DNA wrapped around histone proteins to form a stable chromatin structure. Post translational modifications (PTMs) of histones influence chromatin structure and the transcriptional state of genes (Zentner and Henikoff, 2013). Histone acetylation, which is one of the earliest discovered histone PTMs (Allfrey et al., 1964; Phillips, 1963), has been well established as a transcriptional activation mark (Kuo and Allis, 1998; Struhl, 1998). The reverse, histone deacetylation, is correlated with transcription repression (Kuo and Allis, 1998). Histone lysine acetyltransferases (KATs) and histone deacetylases (HDACs) are key effectors conferring histone acetylation and deacetylation, respectively. These enzymes are often found in complexes where they associate with a scaffold protein and accessory factors. The accessory proteins are thought to finely tune the enzymatic activity of complexes (Lalonde et al., 2014). One such multiprotein complex is the SIN3/RPD3 HDAC complex, in which SIN3 acts as the master transcriptional adapter protein that interacts with the deacetylase RPD3 and other accessory proteins (Grzenda et al., 2009; Silverstein and Ekwall, 2005). The primary role of the SIN3 complex is to mediate gene repression, however, evidence of transcriptional activation promoted by SIN3 has been documented (Bernstein et al., 2000; Dannenberg et al., 2005; Das et al., 2013; Fazzio et al., 2001; Nawaz et al., 1994; Pile et al., 2003).

SIN3 activity is linked to several cellular processes throughout metazoan development. SIN3 has been shown to be involved in protein stability, oncogenic transformations, senescence and cell survival (Kadamb et al., 2013). SIN3 is also implicated in cell cycle regulation. Reduction of SIN3 in *Drosophila* S2 cells compromises the G2/M phase transition during cell cycle progression (Pile et al., 2002). Additionally, RNA interference (RNAi) mediated knockdown of *Sin3A* causes curved wings in adult flies (Swaminathan and Pile, 2010). Rescue of the curved wing phenotype by overexpression of String, a G2/M regulator, highlights the importance of SIN3 in cell cycle progression (Swaminathan and Pile, 2010). In mouse, knockdown of *mSin3A* impacts both G1 and G2/M phases of the cell cycle (Cowley et al., 2005; Dannenberg et al., 2005). Null mutants of *sin3* in budding yeast show an accumulation of cells in G2 phase (White et al., 2009). SIN3 has also been linked to metabolism. Several metabolic processes including glucose metabolism, oxidative metabolism, oxidative phosphorylation, mitochondrial biogenesis, fatty acid oxidation as well as mitochondrial and cellular protein synthesis have been previously reported to be regulated by SIN3 as determined by expression profiling of S2 and Kc *Drosophila* cell lines in which *Sin3A* was knocked down (Pile et al., 2003). In addition, studies in yeast and fly models indicate that SIN3 plays a critical role in regulating mitochondrial activity and oxidative stress (Barnes et al., 2014; Barnes et al., 2010). In mouse, two genes encode highly related Sin3 proteins, mSin3A and mSin3B (Ayer et al., 1995; Silverstein and Ekwall, 2005). Several investigators have shown that the two highly similar mouse Sin3 proteins play distinct roles during development. For example, *mSin3A* is essential for early embryonic development, survival and growth of cultured cells (Cowley et al., 2005; Dannenberg et al., 2005), while

mSin3B plays a regulatory role during the late gestation period of embryogenesis, suggesting the functional non-redundancy of the *mSIN3* genes (David et al., 2008). Despite being an area of active research, the complete array of functions and complexity of SIN3 regulatory networks remains largely unknown.

The intricate mechanism of gene regulation by SIN3 is augmented by the presence of distinct SIN3 isoforms or discrete SIN3 HDAC complexes in multiple eukaryotic model systems (Silverstein and Ekwall, 2005). In *Drosophila,* splicing of a single gene results in the expression of multiple isoforms of SIN3 (Pennetta and Pauli, 1998). Additionally, subcomplexes of SIN3 with and without RPD3 have been isolated (Moshkin et al., 2009). Unlike *Drosophila*, *Saccharomyces cerevisiae* express a single Sin3 protein (Wang et al., 1990). Although a single protein is present, multiple complexes containing Sin3 and Rpd3 have been identified, suggesting a functional diversity of yeast SIN3 HDAC corepressor complexes. In budding yeast, Rpd3 predominantly forms a large complex (Rpd3L) and a small complex (Rpd3S) (Carrozza et al., 2005). The core components of these complexes are Sin3, Rpd3 and Ume1. Interestingly, Rpd3L acts as a corepressor at promoters of transcribed genes, while Rpd3S suppresses cryptic transcription by recognizing Set2 methylated histones in the gene body (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005; Li et al., 2007a; Li et al., 2007b). Furthermore, several subcomplexes of mSin3A and mSin3B have been reported (Jelinic et al., 2011; Yang and Seto, 2008). The distinct activities of the different SIN3 complexes are an area of active research. Taken together, these data confirm the presence of functional variations among SIN3 HDAC complexes in different model organisms.

Previously, we demonstrated the existence of distinct SIN3 HDAC complexes in *Drosophila* (Spain et al., 2010). SIN3 187 and SIN3 220 are the most prevalent isoforms of SIN3 expressed during fly development (Sharma et al., 2008). SIN3 220 is the predominantly expressed isoform in proliferative cells such as cultured *Drosophila* S2 cells and larval imaginal disc cells, whereas SIN3 187 is the prevalent isoform during the latter stages of embryonic development and in adults (Sharma et al., 2008). Additionally, expression of the SIN3 220 isoform was found to rescue lethality due to a genetic null allele, while SIN3 187 was essentially unable to suppress the lethal phenotype (Spain et al., 2010). Differential spatial and temporal patterns of expression of SIN3 isoforms support the idea that they have distinct functions. Recently, SIN3 187, but not SIN3 220, was found to play an active regulatory role in the mesoderm (Dobi et al., 2014), which is in accord with the expression of SIN3 187 in differentiated cells. Taken together, results from our laboratory and others strongly suggest non-overlapping activity of SIN3 isoforms in *Drosophila*.

Genome-wide binding sites of SIN3 have been previously published by several groups (Das et al., 2013; Filion et al., 2010; Negre et al., 2011), however, no distinction between isoform specific genomic localization was indicated in those studies. To explore the functional differences of SIN3 isoforms in modulating biological processes, we carried out genome-wide assays in *Drosophila* cultured cells that predominantly express one or the other major isoform. To our knowledge, it is the first time the binding sites of SIN3 isoforms across the *Drosophila* genome have been mapped. Interestingly, we found that overexpression of SIN3 187 led to replacement of SIN3 220 at a majority of the genomic sites, indicating that the binding sites of the SIN3 isoforms overlap with each other. RNA-

seq analysis, however, demonstrate that SIN3 187 plays unique gene regulatory roles, in addition to having some functions in common with SIN3 220.

Materials and Methods

Cell culture

The *Drosophila* S2 cells were cultured in *Drosophila* Schneider's media (1X) + L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). Gentamycin (50 mg/ml) was added to S2 cells. S2 cell lines stably transfected with expression constructs of HA-tagged SIN3 187 and SIN3 220 were grown in the media with 0.1 mg/ml of penicillin/streptomycin and 0.1 mg/ml Geneticin. Cells were maintained at 27^{o}C.

Western blot

Western blot was carried out following standard protocols (Sambrook J, 2001). To prepare whole cell extract, approximately 1.5x10^{6} cells were harvested by centrifugation and lysed in Laemmli Sample buffer (Bio-Rad). Proteins (15 to 20 µg) were resolved on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride (PDVF) membrane (Pall). Membranes were incubated with primary antibody: HA-HRP (1:6000; Sigma), SIN3 PAN (1:2000 (Pile and Wassarman, 2000)), SIN3 220 (1:2000 (Sharma et al., 2008)), α-Tubulin (1:1000, Cell signaling) or β-Actin (1:1000, Cell signaling). Following incubation with primary antibody, membranes were incubated with donkey anti-rabbit HRP-conjugated IgG (1:3000, GE healthcare) secondary antibody wherever applicable. The antibody signal was detected using the ECL prime western blot detection system (GE Healthcare).

Chromatin preparation by MNase digestion

Approximately $4x10^7$ exponentially growing S2 cells were subject to cross-linking by the addition of formaldehyde to a final concentration of 1% for 10 min at room temperature. Excess formaldehyde was quenched by addition of glycine to a final concentration of 125 mM. The cross-linked cells were washed with 1X phosphate buffered saline and resuspended in lysis buffer (10 mM Tris-HCl pH 8, 10 mM KCl, 3 mM $CaCl_2$, 0.34 M sucrose, 1 mM DTT, 0.1% Triton X-100), 0.2 mM EGTA, proteinase inhibitor (Roche), followed by incubation on ice for 15 min. These cells were disrupted by dounce homogenizer (10 times by loose pestle, 15 times by tight pestle) followed by low speed centrifugation (170 x g for 10 min). The pellet was resuspended in 200 µl MNase digest buffer (15 mM Tris-HCl pH 8, 60 mM KCl, 15 mM NaCl, 1 mM DTT, 0.25 M sucrose, 1 mM $CaCl_2$) and subjected to micrococcal nuclease digestion by addition of 20 units of MNase (Worthington Biochemicals) for 10 min at room temperature. 10 mM EDTA was added to quench the MNase activity. Following MNase digestion, samples were made up to 1.2 ml with low salt buffer (140 mM NaCl, 10 mM Tris-HCl pH 7.6, 2 mM EDTA) and sonicated using a probe sonicator (Thermo Fisher Scientific) to extract cross-linked chromatin in the solution.

Approximately 75 µg of crude chromatin was used to prepare input for the ChIP experiment. Briefly, samples were treated with 0.05 µg/µl of RNase A (Sigma) and incubated overnight with 200 mM NaCl at 65^{o}C. Samples were treated with 0.04 µM Proteinase K (Thermo Fisher Scientific), 10 µM EDTA and 20 µM Tris (pH 8) and incubated at 45^{o}C for 1.5 hr. DNA was purified by standard phenol chloroform extraction. This method leads to the formation of mono, di, tri-nucleosomal DNA fragments as determined by agarose gel electrophoresis analysis of purified DNA.

Chromatin immunoprecipitation

For HA-tag ChIP, we used monoclonal anti-HA-agarose antibody (Sigma). Anti-HA-agarose bead slurry was washed with RIPA buffer. 75 µg of chromatin was incubated with 30 µl washed anti-HA-agarose for 4 hr at 4^{o}C. Chromatin prepared from wild type S2 cells was used as control. Chromatin bound HA-agarose was then washed, once with 1 ml of modified 2X RIPA buffer (50 mM Tris pH 7.6, 280 mM NaCl, 2 mM EDTA, 0.3% SDS) for 8 min at room temperature, once with 1 ml of IP1 buffer (25 mM Tris pH 7.6, 500 mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100) at 4^{o}C for 10 min, once with 1 ml of IP2 buffer (10 mM Tris pH 7.6, 250 mM LiCl, 1mM EDTA, 0.5% sodium deoxycholate, 0.5% Triton X-100) at 4^{o}C for 10 min and once with 1 ml of Tris-EDTA (pH 8). To perform SIN3 PAN ChIP, we incubated chromatin with 10 µl polyclonal SIN3 antibody (Pile and Wassarman, 2000) overnight at 4^{o}C. Chromatin incubated with pre-immune IgG was used as the non-specific ChIP control. We added 30 µl of washed Protein-A-agarose beads to the anti-SIN3 or pre-immune IgG incubated chromatin followed by incubation for 4 hr at 4^{o}C. Bound beads were washed once with 1 ml of 1x RIPA at 4^{o}C for 10 min, once with 1 ml of high salt buffer at 4^{o}C for 10 min, once with 1 ml of low salt buffer at 4^{o}C for 10 min and once with 1 ml of TE buffer. For both the HA-tag and the anti-SIN3 ChIP, cross-linked protein-DNA was eluted in elution buffer (1% SDS, 0.1 M $NaHCO_3$) at 65^{o}C for 30 min. Elution was done twice in a total volume of 500 µl. Cross-links were reversed in the eluted samples as for the ChIP input DNA preparation. Purified DNA samples from HA-tag ChIP were quantified by real-time PCR using $2^{-\Delta\Delta CT}$ method of analysis (Livak and Schmittgen, 2001). All genes tested by ChIP-qPCR validate the ChIP-seq results (Figure 3.1). All validation experiments

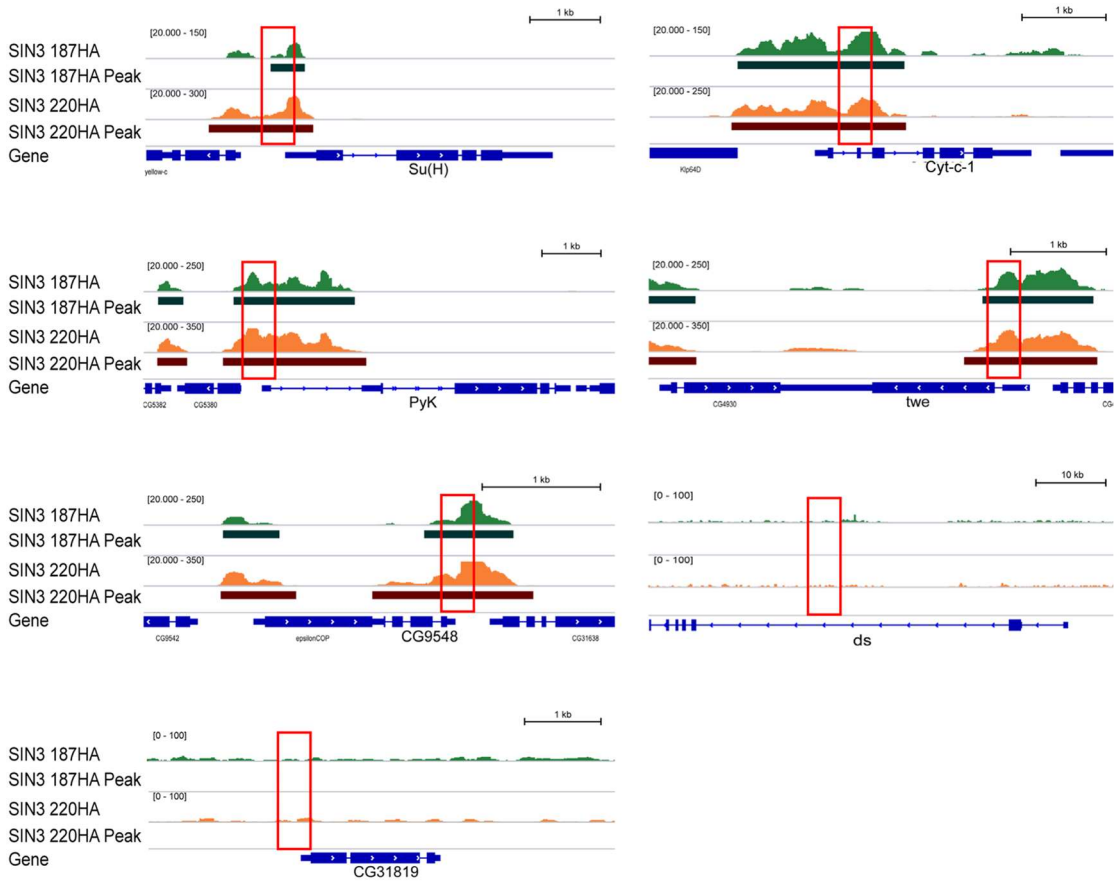

Figure 3.1: ChIP-seq tracks. ChIP-seq gene tracks showing the enrichment of SIN3 isoforms over the genes shown in Fig 3.4C and 3.4D. Red boxes mark the approximate region amplified by qPCR following ChIP as shown in Fig 3.4C and 3.4D.

were performed at least three times and data are represented as the mean of percentage of input. Information about the primers used for ChIP-qPCR assay is reported in Table 3.1. HA-tag ChIP was used for the ChIP-seq experiment.

ChIP-seq library preparation

We prepared sequencing libraries using the method previously described by Ford et al., 2014 (Ford et al., 2014). In brief, we used approximately 10 ng of ChIP or input samples to prepare sequencing libraries. ChIP or input DNA was end repaired,

Table 3.1: Primers used for ChIP-qPCR analysis

Gene	Gene ID	Forward Primer	Reverse Primer
Su(H)	*CG3497*	CCACTGCCATCCAAATCC	GCGGCTGGCTTCTGTTTA
Cyt-c1	*CG4769*	TTGCAGGCGAAGAACCTC	GGTTAGGTCCGATCGGGTA
PyK	*CG7070*	GACGACGCTTTCAGCGAT	TTTGAAGCTCGGGTCTGC
CG9548	*CG9548*	CGGGTCCTCCACAGATGA	CATTGGACGCCTGTGTGA
twe	*CG4965*	ATCAGCCGTGCAGGAGAC	GGCTGGGATTCTCTGGGT
ds	*CG17941*	TGCCAACCATCCTAACGG	CTGTGGAGGACACAGGGG
CG31819	*CG31819*	AGCGCTGCCAGAAGAAGA	GGTCAAAGTCTCCCAATT

adenylated and ligated to indexed adapters. Gel extraction of ligated adapter DNA fragments was carried out on a 2% low-melting agarose gel (Lonza). 5 cycles of PCR was conducted to convert all Y-shaped structure adapter-DNA fragments to double-stranded DNA structure as previously described (Ford et al., 2014). This step was followed by size selection of DNA samples by gel extraction. Finally, size selected DNA fragments were amplified for 12 cycles by PCR. Together, we used 17 cycles of PCR to amplify adapter ligated ChIP DNA. In all steps, DNA was purified by Beckman Coulter Ampure XP resin. The prepared sequencing libraries were sent to the University of Wisconsin Biotechnology Center, where all sequencing samples were multiplexed and sequenced on an Illumina HiSeq2500 platform sequencing system in single end mode with a read length of 100 bp. Two independent biological replicates of the ChIP-seq experiment were performed. ChIP-seq data are available through GEO under GSE72173.

ChIP-seq analysis

To map the binding sites of SIN3 isoforms, we used Bowtie2 (Langmead and Salzberg, 2012) to align all reads to the *Drosophila* genome. Reference *Drosophila melanogaster* sequence (dm3) was downloaded from the UCSC table browser and raw

reads were aligned to the reference genome. Only uniquely mapped tags were subsequently used for further analysis. Input DNA tags were used to normalize ChIP DNA tags. Protein bound regions were called using Model-based analysis of ChIP-seq (Macs 2.1.0) (Zhang et al., 2008) using the default parameters and -- keep-dup = all. The correlation of the two biological replicates was performed through analysis of the Irreproducible Discovery Rate (IDR) (Kundaje A, 2012; Landt et al., 2012; Li Q, 2011) (Figure 3.2 and Supplemental Data 1). Final peaks were called using an IDR of 0.1%.

Peaks were assigned to UCSC Refseq genes (dm3). Using the bedtools (Quinlan and Hall, 2010) windows function, we assigned peaks within –1 Kb of transcription start sites (promoter) to +100 bp of transcription end sites to genes. We applied the ngsplot tool (Shen et al., 2014) to perform the metagene analysis and represented the data as the average ChIP signal from -2 Kb of the TSS to + 2 Kb of the TES. Venn diagrams were plotted in R (version: 3.1.1).

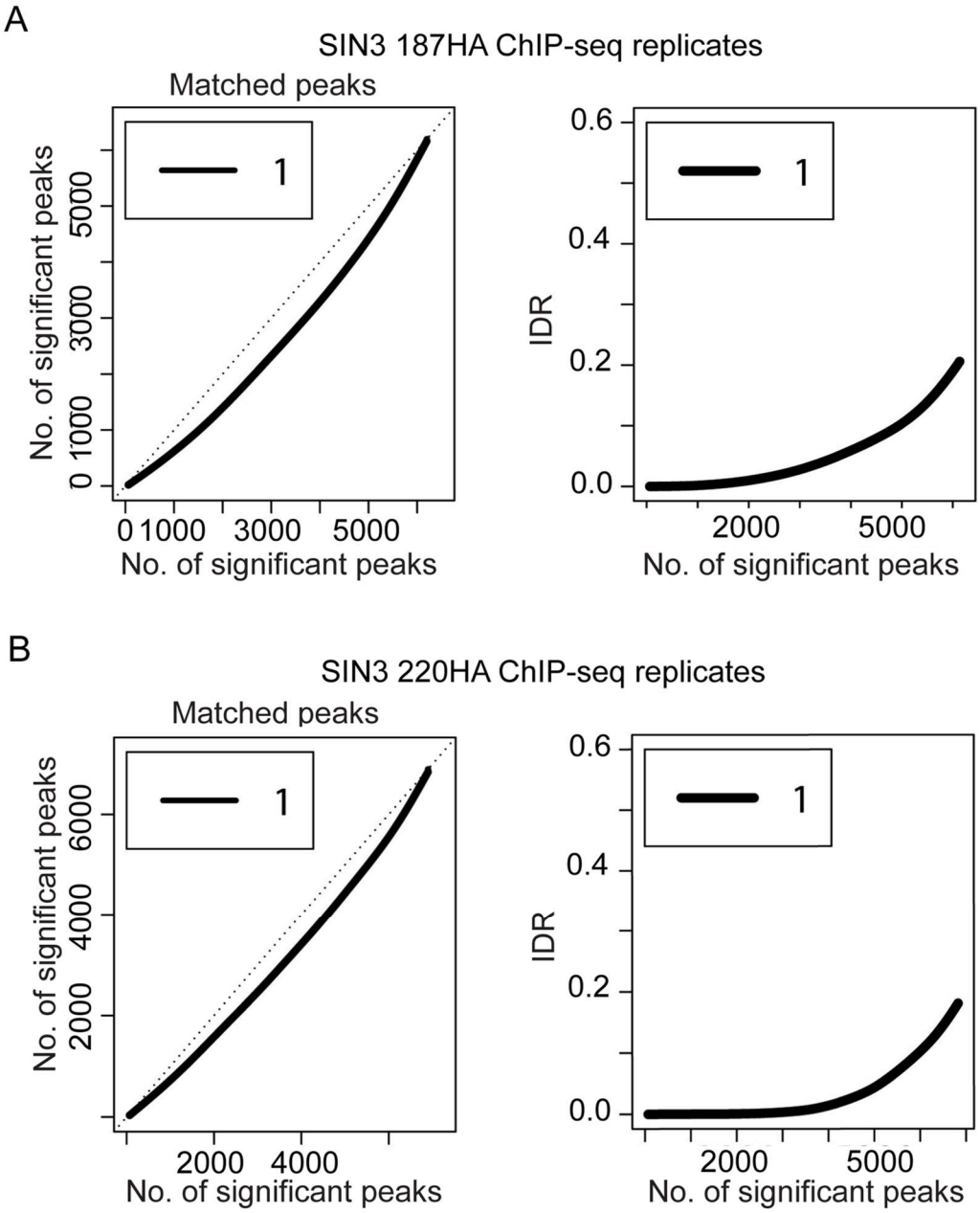

Figure 3.2: Irreproducible discovery rate analysis. IDR analysis showing the correlation between the two biological replicates of ChIP-seq analysis for SIN3 187HA samples (A) and SIN3 220HA samples (B).

RNA interference

RNAi mediated *Sin3A* knockdown was carried out based on a published protocol (Pile et al., 2002) with slight modifications. In brief, approximately 4 x 10^6 cells were plated in 4 ml of *Drosophila* Schneider's medium. After 3 hr, FBS-containing media was carefully removed and supplemented with 2 ml of serum free media. 50 µg of dsRNA was added to plates and mixed by swirling. After 30 min, 4 ml of complete *Drosophila* media was added. Cells were incubated for 4 days. Construction of dsRNA targeting *Sin3A* was previously described (Pile et al., 2002). dsRNA against GFP was used as a control. dsRNA targeting GFP was generated using T7 polymerase mediated *in vitro* transcription of a PCR DNA template (kindly provided by Dr. Russell L. Finley, Jr.) coding *GFP* sequence. The primer pairs to amplify *GFP* containing sequence are GAA TTA ATA CGA CTC ACT ATA GGG AGA TGC CAT CTT CCT TGA AGT CA (forward primer) and GAA TTA ATA CGA CTC ACT ATA GGG AGA TGA TGT TAA CGG CCA CAA GTT (reverse primer). Efficient knockdown of *Sin3A* compared to GFP RNAi was verified by western blot analysis.

Overexpression of SIN3 187HA

S2 cells carrying stably transfected HA-tagged SIN3 187 transgene under the metallothionein promoter was induced by addition of 0.07 M $CuSO_4$. Ectopic expression of SIN3 187HA was verified by western blotting.

Gene expression analysis by RT-qPCR

Gene expression analysis by RT-qPCR was carried out as previously described (Barnes et al., 2010). The change in gene expression due to SIN3 187HA overexpression was compared to wild type S2 cells, while alteration of gene expression due to knockdown

of *Sin3A* was compared to GFP RNAi S2 cells. The primers used for RT-qPCR are listed in Table 3.2. The *Taf1* gene was used to normalize the mRNA level. At least three independent biological replicates were performed. Results are represented as the mean of the fold difference between the experimental and control samples.

Table 3.2: Primers used for gene expression analysis

Gene	Gene ID	Forward Primer	Reverse Primer
Stat92E	*CG4257*	TGAAGACCAACACACGCTTC	GATGATTGACCGGATAGGCT
Sam-S	*CG2674*	AAACTTTGACCTCAGGCCC	CGCTGGTATATCGGCTGG
Gclm	*CG4919*	CATTCCGTCCATTCTCCG	GCGTGGTGGTTGAAAAGG
tum	*CG13345*	CAGCACCGTCCACTGATG	CCGACATCTCAGCCCAAC
CG1969	*CG1969*	GGAAGCAACTGGGAAAACTG	TGATCAGCTTGTCCTTGCAG
Ance	*CG8827*	GGCTACACGCCACTCAAAAT	TCAATGATCGACTTGTCCCA
Taf-1	*CG17603*	CTGGTCCTGGTGAGGTGA	CCGGATTCTGGGATTTGA

RNA-seq and data analysis

Procedures from RNA isolation to next generation sequencing were performed at the Applied Genomics Technology Center, Wayne State University. Briefly, total RNA was extracted using the EZ1® RNA Universal Tissue Kit (Qiagen). Cell lysis and homogenization was carried out by bead-milling on the TissueLyser® II (Qiagen) in 750 µl QIAzol™ lysis reagent. Extracted RNA was purified on the EZ1® Advanced (Qiagen). Additional DNase treatment was done to remove any residual DNA. Quantification and quality assessment of purified total RNA was done using the DropSense96® Microplate Spectrophotometer (Trinean) and microfluidics using the RNA R6K assay for the Agilent 2200 TapeStation, respectively. cDNA was generated by reverse transcription of the purified mRNA. The TruSeq RNA Sample Preparation Kit (Illumina) was used to prepare sequencing libraries following the manufacturer's protocol. Single indexed samples were

multiplexed and sequenced on an Illumina HiSeq 2500 sequencing system in paired end mode with a read length of 2 x 50 bp. A total of three independent biological replicates were performed. In the current study, we report the RNA-seq analysis done on total RNA isolated from cells overexpressing SIN3 187HA. Wild type S2 cells treated in the same way as the SIN3 187HA cell line was used as control. Correlation among replicates is shown in Figure 3.3. SIN3 187HA RNA-seq data is available through GEO under GSE72173. The RNA-seq dataset for *Sin3A* knockdown versus GFP RNAi was retrieved through GEO under GSE68775 (Gajan et al., 2016).

The Tophat pipeline was utilized for the analysis (Trapnell et al., 2012). Reads obtained from RNA sequencing were aligned to the UCSC reference genome (dm3) using Bowtie2/Tophat (Trapnell et al., 2012). Cufflinks was used to estimate transcripts levels of a total of 14,540 Refseq genes. Differentially expressed genes were identified using cuffdiff (Trapnell et al., 2012) with default parameters at FDR ≤ 0.05 for the *Sin3A* knockdown sample and FDR ≤ 0.001 for the SIN3 187HA overexpression sample. The R statistics environment was used to visualize the data. The volcano and scatter plots were generated using the ggplot2 package.

GO analysis

Genes bound by SIN3 isoforms and that also showed change in expression upon alteration in the level of SIN3 isoforms were included in the GO analysis. GO analysis, performed separately on genes repressed or activated by SIN3 isoforms, was done utilizing the online tool DAVID (Huang da et al., 2009). Utilizing visual inspection, we pooled related gene ontology categories into a single broader category. For example, determination of adult lifespan (GO:0008340), multicellular organismal aging

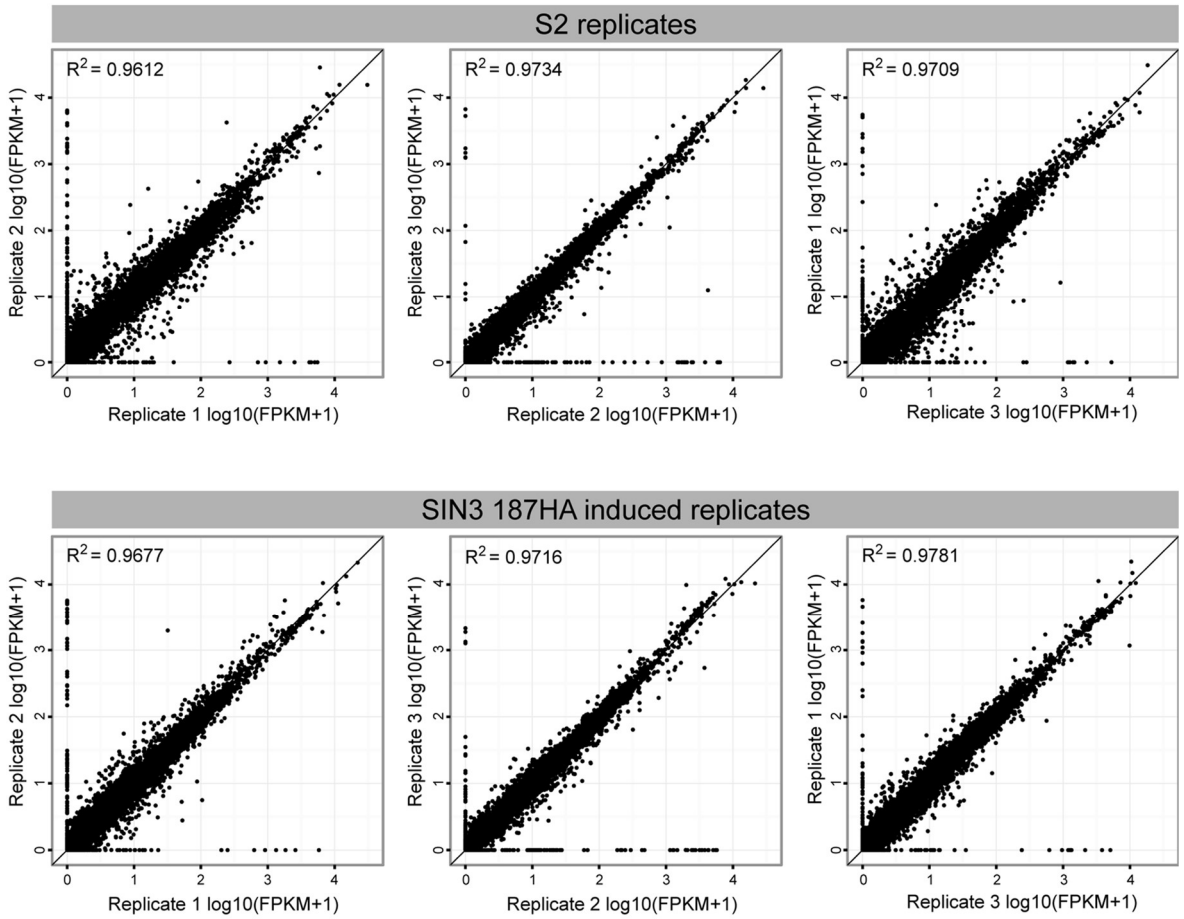

Figure 3.3: RNAseq correlation analysis. Scatter plots showing the correlation between the replicates of gene expression analysis by RNA-seq using total RNA extracted from S2 cells (top) or the SIN3 187HA stably transfected cell line (bottom). Data representing the correlation between three independent biological replicates.

(GO:0010259) and aging (GO:0007568) categories found to be enriched for genes activated by SIN3 187, were pooled into one broader category Aging. Detailed information regarding the GO analysis is shown in Supplemental Data 2. GO categories with *P*-value < 0.05 were pooled.

Statistical Analysis

Statistical calculations were done using the online statistical tool Graphpad and significant values were calculated using the unpaired Student's *t*-test.

Availability of supporting data

ChIP-seq and RNA-seq data can be accessed from the GEO database (www.ncbi.nlm.nih.gov/geo/). ChIP-seq datasets are deposited under accession number GSE72171 and RNA-seq data for SIN3 187 overexpression are deposited under accession number GSE72172. These two datasets are included in a superseries GSE72173. RNA-seq datasets for *Sin3A* knockdown samples can be retrieved from GSE68775.

Results

To analyze gene regulatory activity of SIN3 isoforms, we established two S2 cultured cell lines in which cells express either of the two major SIN3 isoforms with a tag for immunoprecipitation (Figure 3.4A). The predominantly expressed isoform of SIN3 in S2 cells is SIN3 220 (Figures 3.4A and 3.4B). To express SIN3 220 with an HA tag, a stable S2 cell line carrying a transgene with SIN3 220HA cDNA was generated (Spain et al., 2010). (Figure 3.4B, left panel). In addition to the stable SIN3 220HA cell line, we also generated a stable cell line for expression of SIN3 187HA (Figure 3.4B, right panel) (Spain et al., 2010). Interestingly, overexpression of SIN3 187HA resulted in an almost complete reduction of SIN3 220, when compared to S2 cells (Figure 3.4B, right panel). These results indicate that the SIN3 187 isoform modulates the expression of SIN3 220. Important for this study, we have established a homogeneous cell culture system in which cells express almost exclusively one isoform or the other.

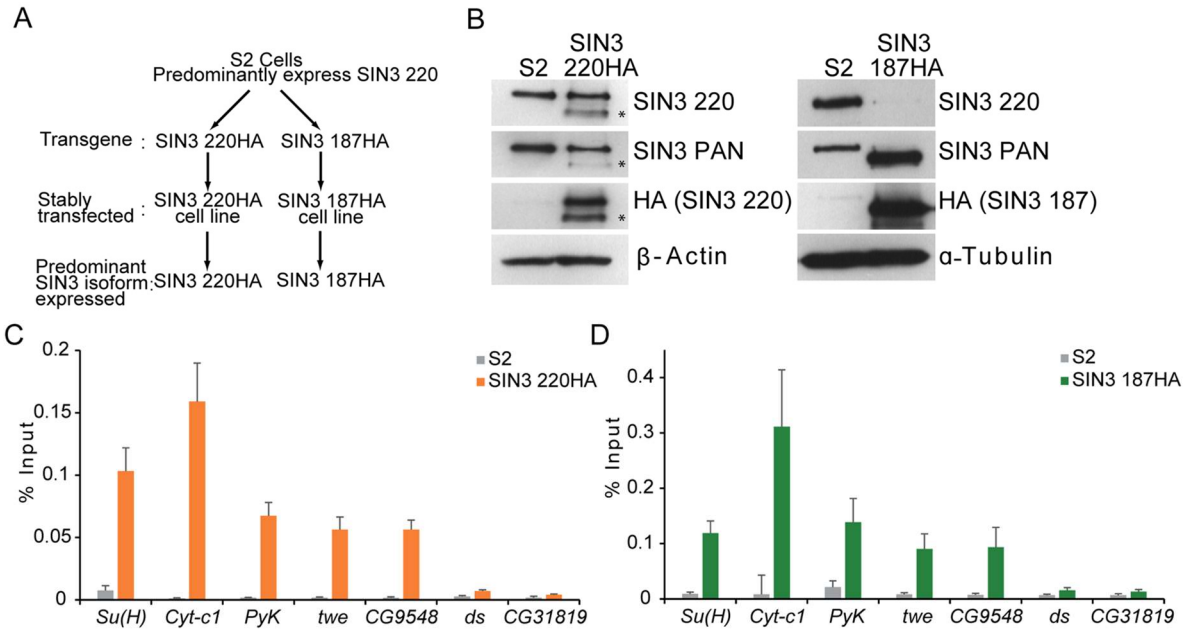

Figure 3.4: Expression of SIN3 187 affects levels of SIN3 220. (A) Schematic representing the different cell lines used in this study as well as the SIN3 isoform expressed in that line. (B) Western blot analysis of whole cell extract prepared from S2 cells, SIN3 220HA (left) and SIN3 187HA (right) cell lines. The expression of SIN3 220 with a C-terminal HA tag was driven by an inducible metallothionein promoter. Due to leakiness of the metallothionein promoter, the maximum level of expression of SIN3 220HA was achieved without induction. The SIN3 187HA cell line was treated with 0.07 M of $CuSO_4$ to induce the transgene. Blots were probed with antibodies listed at the right. SIN3 PAN antibody recognizes all SIN3 isoforms.

Utilizing this cell culture system, we first set out to study the binding of SIN3 isoforms at putative gene targets, predicted from previous studies (Filion et al., 2010; Negre et al., 2011; Pile et al., 2003). To confirm the localization of SIN3 220 to specific targets, we prepared chromatin from the SIN3 220HA cell line and performed chromatin immunoprecipitation (ChIP) using antibody against the HA tag followed by quantitative PCR (qPCR). As predicted, ChIP-qPCR data showed the enrichment of SIN3 220 at putative gene targets *Supressor of hairless* (*Su(H)), Cytochrome c1 (Cyt-c1), Pyruvate*

kinase (Pyk), twine (twe), CG9548 but not at control regions *dachsous (ds)* or *CG31819* (Figure 3.4C). Next, we sought to measure the binding of SIN3 187 to the predicted targets. We performed ChIP on chromatin prepared from the SIN3 187HA cell line using antibody against the HA tag. Following induction of SIN3 187HA for 48 hr, we observed considerable enrichment of SIN3 187 at the same targets bound by SIN3 220 (Figure 3.4D), suggesting that SIN3 187 is recruited to the same genes as those bound by the SIN3 220 isoform. Chromatin from the non-transfected S2 cell line was used as a non-specific ChIP control. As expected, little SIN3 enrichment at any of the tested targets was observed in these control cells (Figures 3.4C and 3.4D). We performed an additional ChIP-qPCR experiment using antibody that recognizes both SIN3 isoforms. The results confirm the recruitment of SIN3 187 to SIN3 220 targeted genomic sites (Figure 3.5). Based on the western blot analysis, we conclude that expression of SIN3 187 impacts the level of expression of SIN3 220. Additionally, following ectopic expression of SIN3 187, SIN3 220 is replaced by SIN3 187 at the tested genes. Most importantly, the ChIP-qPCR results demonstrate that we were able to effectively immunoprecipitate chromatin fragments bound by SIN3 isoforms. Next, using ChIP followed by deep sequencing, we set out to map the binding sites of SIN3 isoforms across the entire *Drosophila* genome at a high resolution.

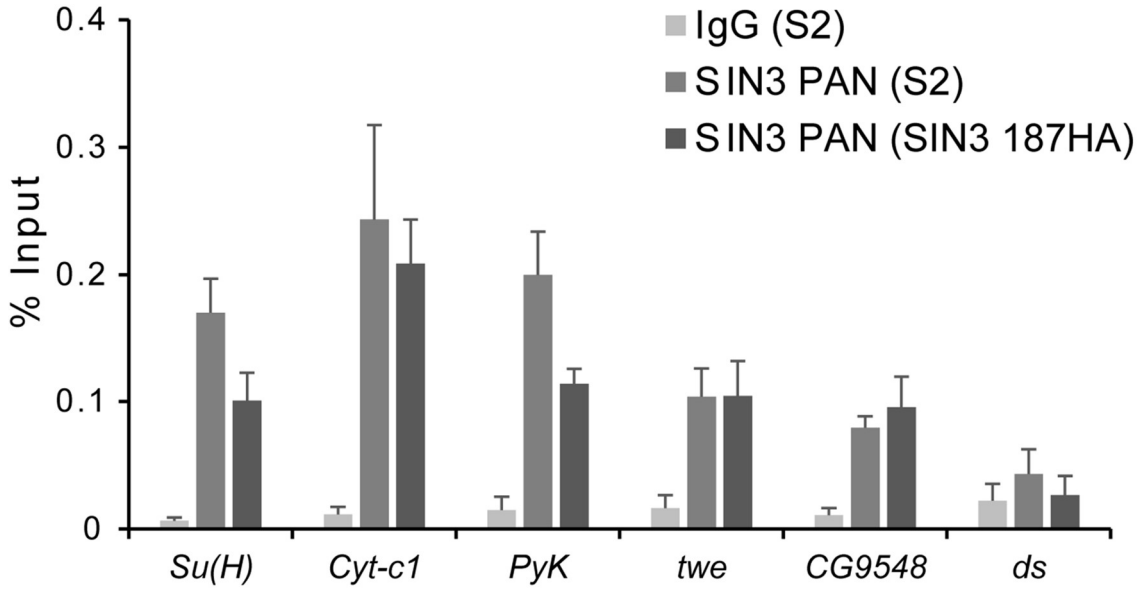

Figure 3.5: SIN3 ChIP. ChIP-qPCR assay was performed using antibody against SIN3 on chromatin prepared from S2 cells or cells that were induced to overexpress SIN3 187HA.

SIN3 isoforms bind to overlapping genomic targets

Although genome-wide SIN3 occupancy has been previously mapped (Das et al., 2013; Filion et al., 2010; Negre et al., 2011), the genes that are differentially bound by SIN3 isoforms have not been determined. In the current study, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) from chromatin prepared from the stable cell lines that express either of the SIN3 isoforms tagged with HA (Figure 3.4). We performed two independent biological replicates of the ChIP-seq experiment, for which we prepared separate sequencing libraries for SIN3 187HA and SIN3 220HA ChIP DNA samples. Additionally, we prepared separate libraries using input DNA samples for each replicate. Following sequencing of the ChIP samples, we used MACS2 to call peaks at an irreproducible discovery rate (IDR) of 0.1 (Figure 3.2 and

Supplemental Data 1). Further, we retained only those peaks that were three fold or more enriched over the input sample. 4903 and 5810 peaks were called for the SIN3 187 and the SIN3 220 libraries, respectively. A comparison of SIN3 isoform bound peaks in S2 cells with those of the binding sites of SIN3 mapped previously in the *Drosophila* embryos (Negre et al., 2011) showed a substantial level of correlation (more than 50% overlap) (Figure 3.6). This overlap between the occupancy of the SIN3 isoforms in S2 cells in this study and published SIN3 binding in *Drosophila* embryos suggest that in the whole organism, SIN3 isoforms are likely recruited to many genomic targets by a mechanism similar to that in S2 cells. Additionally, the differences in the binding patterns suggest that there are tissue-specific binding sites for the isoforms.

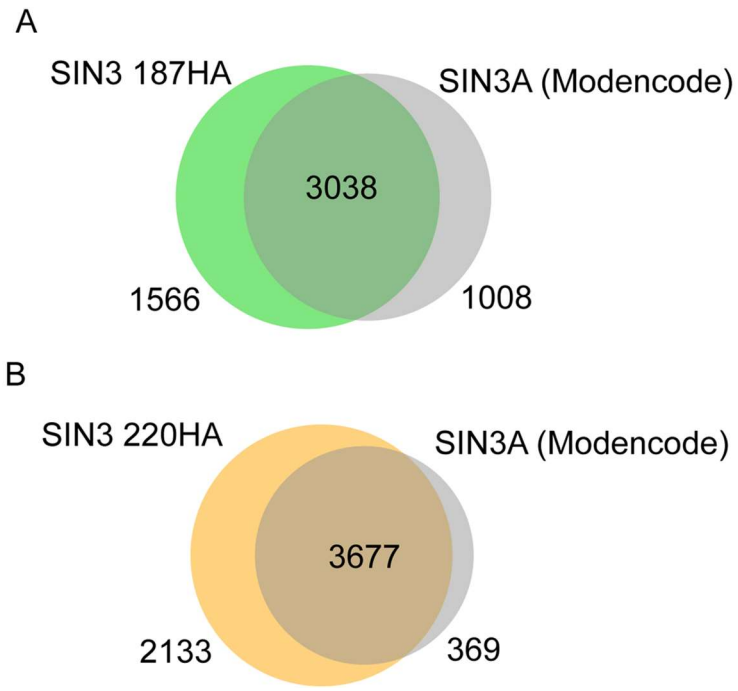

Figure 3.6: Comparison between Modencode SIN3 ChIP-seq and SIN3 isoforms ChIP-seq data. Venn diagrams showing the comparison of the number of peaks identified in this study for SIN3 187HA (A) or SIN3 220HA (B) to the number of peaks identified previously (Modencode) (Negre et al., 2011).

Through investigation of the chromosomal distribution of SIN3 isoforms, we found that they are enriched over the euchromatic regions of the *Drosophila* genome as determined by the cis-regulatory enrichment annotation system (CEAS) analysis (Shin et al., 2009). Specifically, we found that 99% of the peaks identified for SIN3 isoforms were represented on the euchromatic arms of chromosome 2L, 2R, 3L, 3R and X (Figure 3.7A and Supplemental Data 3). This result is in accord with SIN3 binding at euchromatic regions in polytene chromosomes (Pile and Wassarman, 2000). We found that a large majority of peaks of the SIN3 isoforms localized around the transcription start sites (TSS) of genes, which suggests that both SIN3 isoforms bind the promoter regions of genes to regulate transcriptional activity (Figure 3.7B). We also note a slight enrichment of the SIN3 isoforms around the transcription end sites (TES) of genes. We do not rule out the possibility that some of these peaks may overlap with the promoter region of other closely localized genes. The peaks of SIN3 binding were assigned to genomic features and grouped into eight categories (Figure 3.7C). More than 50% of the peaks were located within 1 kb upstream of the TSS and 5'UTR region. Enrichment of SIN3 isoforms beyond 1 kb upstream of the TSS was observed but at a low level. We also note that SIN3 isoforms were preferentially located over introns versus coding exons. Very little or negligible binding of SIN3 isoforms was observed at the 3'UTR or distal intergenic regions.

The enrichment patterns of SIN3 187 and SIN3 220 at genomic features (promoter, 5'UTR, intron, gene body and 3'UTR) are similar to each other (Figure 3.7C). Additionally, a comparison of ChIP-seq profiles of SIN3 isoforms revealed that SIN3 isoforms localize to overlapping genomic loci (Figure 3.8A). We next directly compared the peaks identified

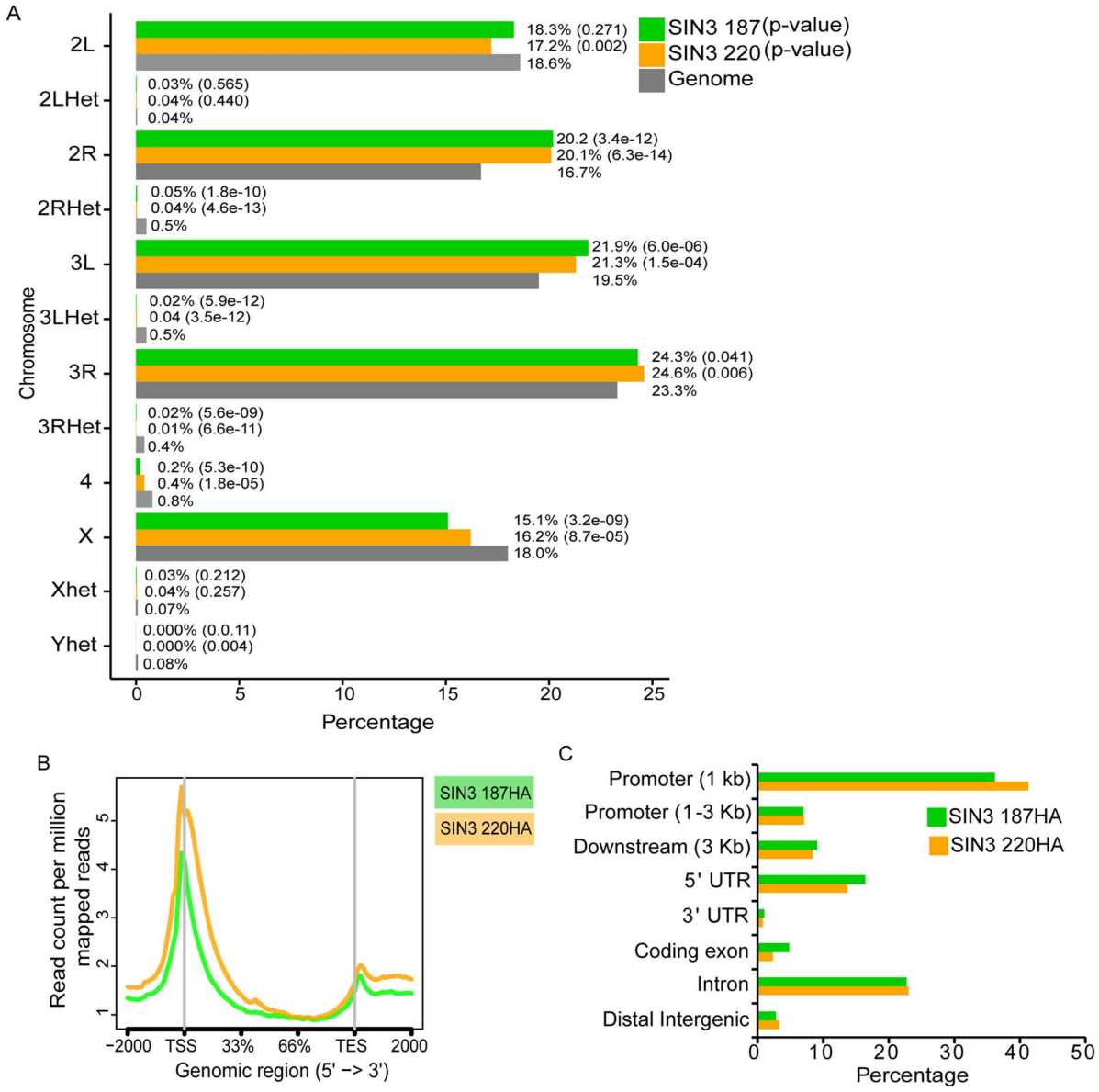

Fig 3.7: Genome-wide occupancy profile of SIN3 isoforms. (A) The chromosomal distribution of SIN3 isoforms across the *Drosophila melanogaster* genome as determined by the cis-regulatory element annotation system (CEAS). The *P*-value represents the significance of relative enrichment over genome. It was calculated using a one-sided binomial test. (B) The metagene analysis of all peaks showing the enrichment of SIN3 isoforms around the transcription start site (TSS) and the transcription end site (TES). (C) Bar plot representing the enrichment of SIN3 isoforms over genomic features.

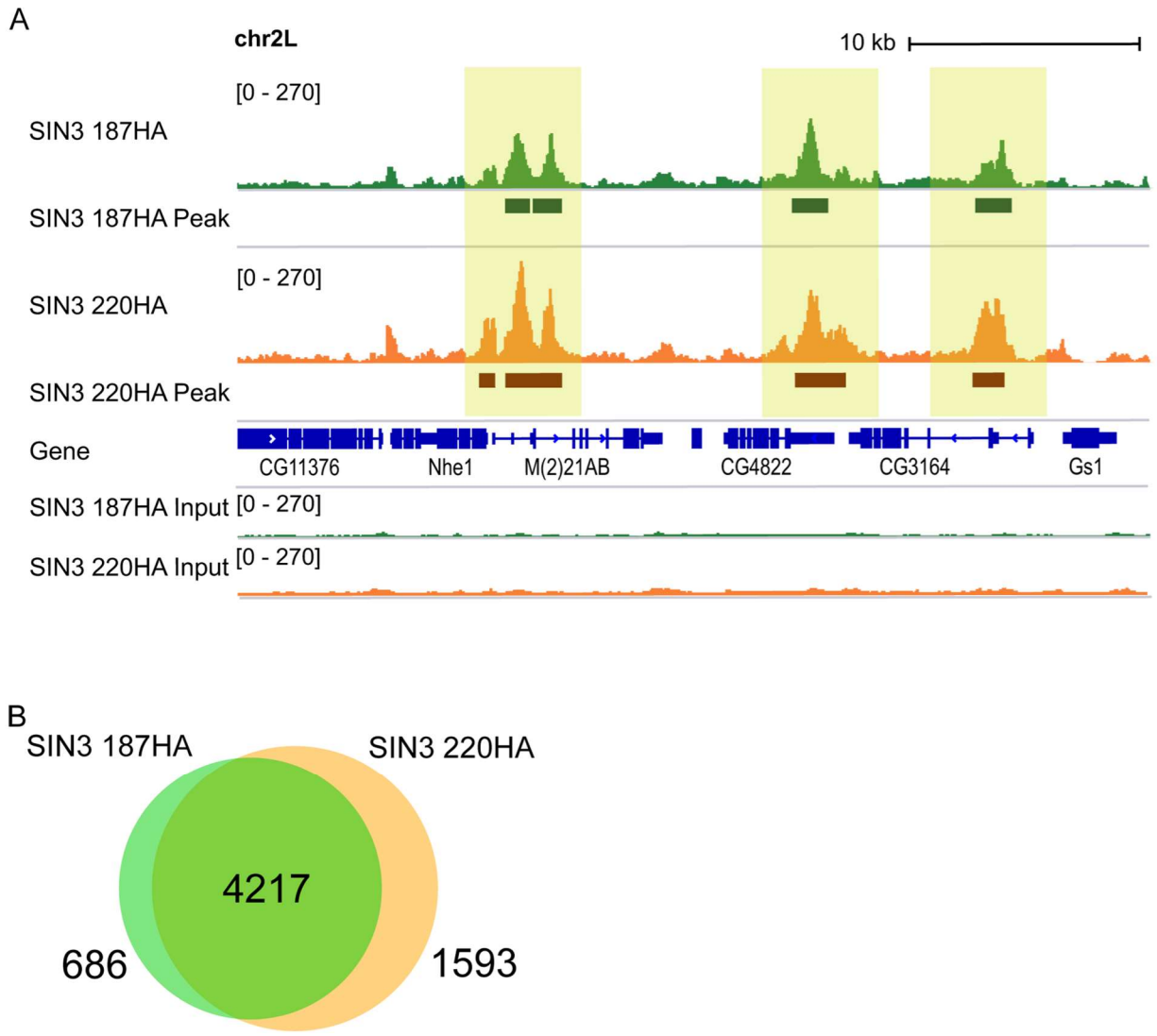

Figure 3.8: SIN3 isoform binding sites largely overlap. (A) Binding of SIN3 187HA and SIN3 220HA are depicted as standard genomic tracks on the integrated genomic viewer. Peaks were aligned to the built-in *Drosophila melanogaster* gene annotation track, shown in blue. Exons are shown in solid blue, introns as blue lines and arrows indicate the directionality of genes. SIN3 187HA and SIN3 220HA peaks as called by MACS2 are displayed in dark green and dark orange bars, respectively, below their enrichment tracks. Input tracks are shown in green for SIN3 187HA and orange for SIN3 220HA. All peaks shown are highly significant, *P*-value < 1e-20. (B) Venn diagram showing the overlap of peaks between the SIN3 187HA and the SIN3 220HA ChIP-seq data. A minimum of 50% overlap between peaks of the two SIN3 isoforms was considered.

for SIN3 187 and SIN3 220. We considered the peaks to be bound by SIN3 isoforms as similar only if the sequence of overlap between the peaks was at least 50%. Using this

criterion, we found 86% of SIN3 187 peaks called overlapped with 73% of SIN3 220 peaks, indicating that the majority of genomic sites targeted by SIN3 isoforms are common (Figure 3.8B). These data demonstrate that expression of SIN3 187 leads to its enrichment at the genomic targets that were previously bound by the SIN3 220 isoform.

Our next goal was to identify genes that are possibly bound by SIN3 isoforms. For this objective we assigned peaks to genes. If a peak under investigation mapped within 1 kb upstream of the TSS and 100 bp downstream of the TES, it was assigned to that gene. Therefore, a sample peak could be assigned to one or more genes, depending on the directionality and the distance between genes. Utilizing this method, we extensively mapped the genes that are bound by SIN3 isoforms. We identified 5903 (approximately 34% of the *Drosophila* genome) and 6905 (approximately 40% of the *Drosophila* genome) genes bound by SIN3 187 and SIN3 220, respectively, supporting the idea of a global transcriptional role of SIN3 isoforms. Additionally, the ChIP-qPCR data showing localization of the SIN3 isoforms at putative gene targets (Figures 3.4C and 3.4D) were validated by the ChIP-seq results (Figure 3.1). Taken together, our data demonstrate that SIN3 isoforms are targeted to many overlapping genomic loci and a substantial enrichment of SIN3 is observed around the TSS. Overall, these data are in accord with previous findings showing that SIN3 localizes to euchromatic regions of the genome and the extent of SIN3 binding to the *Drosophila* genome supports previous results indicating that SIN3 is a global transcriptional regulator (Filion et al., 2010; Negre et al., 2011; Pile and Wassarman, 2000).

SIN3 220 directly regulates genes involved in metabolism and cell cycle progression

The high resolution ChIP-seq profile identified genomic targets of the SIN3 220 isoform. To investigate the genes regulated by the SIN3 220 isoform, we performed transcriptome analysis by RNA-seq on total mRNA isolated from S2 cells treated with *Sin3A* dsRNA or GFP dsRNA as a control. As SIN3 220 is the predominant isoform in S2 cells, we considered genes misregulated upon *Sin3A* knockdown in S2 cells to be regulated by the SIN3 220 isoform and we refer to these genes as SIN3 220 targets. Efficient knockdown of *Sin3A* was verified by western blot (Figure 3.9A). For the expression profile we selected genes that showed a change in expression of ≥1.5 fold (FDR ≤ 0.05) for *Sin3A* knockdown samples compared to GFP RNAi samples. We identified 602 genes as misregulated upon reduction of *Sin3A* in S2 cells (Figure 3.10A). 263 were upregulated and 349 genes were downregulated upon *Sin3A* knockdown. Based on this RNA-seq analysis, the SIN3 220 isoform acts as both a corepressor, for those genes upregulated upon *Sin3A* knockdown, and as a co-activator, for the genes demonstrating a decrease in expression following *Sin3A* knockdown.

To identify the genes regulated directly by SIN3 220, we integrated the SIN3 220 ChIP-seq and the *Sin3A* knockdown RNA-seq data. We retained only those genes that responded to reduced SIN3 expression and were bound by SIN3 220. Approximately 92% (243/263) and 46% (162/349) of the genes repressed and activated by SIN3 220, respectively, are direct targets (Figure 3.9B, red and black dots). This finding is particularly interesting because the data suggest that genes repressed by SIN3 220 are more likely to be direct targets in comparison to the genes activated by SIN3 220. We next performed metagene analysis on genes directly regulated by SIN3 220 to study the

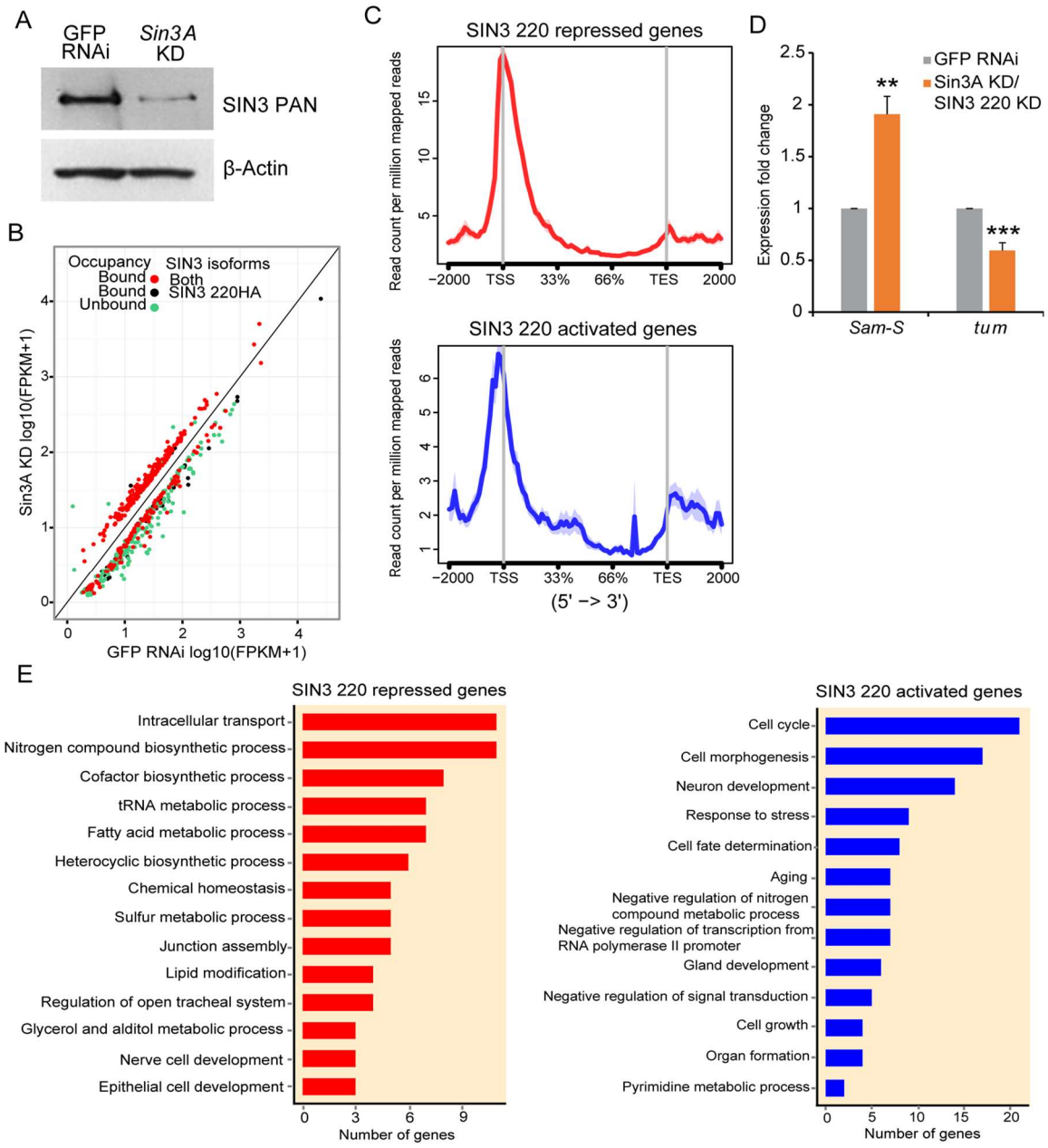

Figure 3.9: ChIP-seq and transcriptome analysis reveal the genes directly regulated by SIN3 220. (A) Western blot for SIN3 of whole cell extracts prepared from GFP RNAi or *Sin3A* knockdown (KD) cells. β-Actin was used as a loading control. (B) Scatter plot showing the correlation between genes bound by SIN3 220HA and those that change in expression following *Sin3A* knockdown. Green dots denote genes regulated by SIN3 220 but not bound. Red dots indicate genes that are regulated by SIN3 220 and bound by both SIN3 isoforms. Black dots refer to genes uniquely bound and

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regulated by the SIN3 220 isoform. (C) Average ChIP signal of SIN3 220HA over the genes as identified in (B). (D) qRT-PCR verification of genes identified by RNA-seq to change in expression upon *Sin3A* knockdown versus GFP RNAi. *P*-value ** < 0.01, *** < 0.0001. (E) Gene-ontology analysis of the genes identified in (B) are represented as bar plots. The Y-axis lists the broad GO categories. Red: categories of genes that are directly repressed. Blue: categories of genes directly activated. *P*-value (gene ontology categories) < 0.05.

distribution across gene features. A large majority of SIN3 220 peaks were found at the transcription start sites of the repressed and activated genes (Figure 3.9C). We also observed some enrichment around the transcription end site, exclusively at genes activated by SIN3 220. We note that the majority of the genes directly regulated by SIN3 220 are also bound by SIN3 187, as indicated by the SIN3 187 ChIP-seq data (Figure 3.9B, red dots). Regulation of these genes by SIN3 187 is explained in more detail below.

The genomic experiments identified several genes directly regulated by SIN3 220. To verify the RNA-seq data, we selected genes that showed a change in expression upon *Sin3A* knockdown and were bound by SIN3 220 as determined by the ChIP-seq analysis. We utilized reverse transcription followed by quantitative PCR (RT-qPCR) to verify gene expression changes resulting from *Sin3A* knockdown. We selected *S-adenosyl methionine synthetase* (*Sam-S*) and *tumbleweed* (*tum*), which were found to be altered upon *Sin3A* knockdown, and bound by SIN3 220 as well (Figure 3.11). Consistent with the RNA-seq data, we found that *Sam-S* was upregulated upon *Sin3A* knockdown, while expression of *tum* was reduced (Figure 3.9D)**.** These data indicate that in S2 cells, SIN3 220 acts as a corepressor for *Sam-S* and co-activator for *tum.*

Next, we sought to investigate the biological processes that are regulated by SIN3 220. We performed gene ontology (GO) analysis on the genes that were directly repressed and activated by SIN3 220 using the DAVID gene annotation module (Figure 3.9E and

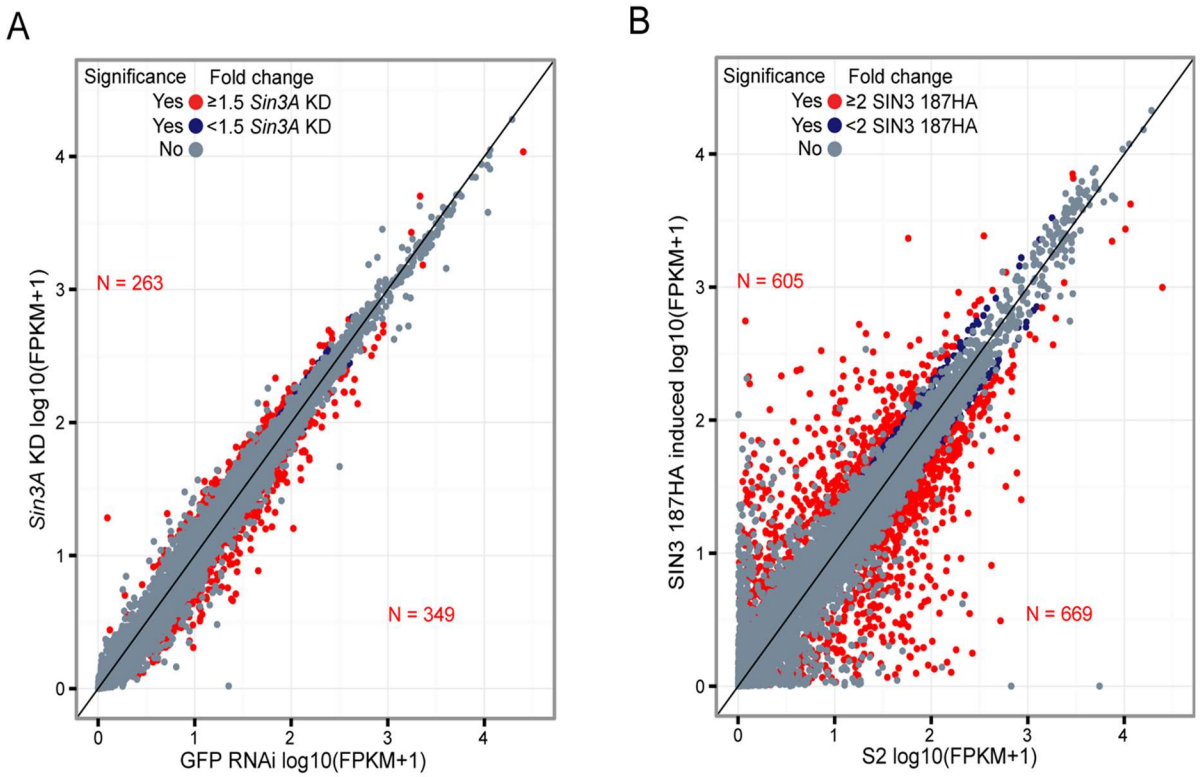

Figure 3.10: Differential gene expression analysis. Scatter plots showing the gene expression change due to *Sin3A* knockdown (KD) compared to GFP RNAi (A) and SIN3 187HA overexpression versus S2 mock induced cells (B).

Supplemental Data 2).52% (126/243) of the repressed genes and 57% (92/162) of the activated genes were enriched for GO terms. Metabolic pathways associated with perturbed levels of SIN3 have been characterized in S2 and Kc cell lines (Pile et al., 2003). Consistent with the published data, the category of metabolism was overrepresented in the genes repressed by SIN3 220. In addition to the metabolic and biosynthetic processes, SIN3 220 repressed genes are linked to intracellular transport and junction assembly processes (Figure 3.9E). Additionally, GO analysis revealed several well studied and novel biological processes overrepresented by genes activated by SIN3 220 (Figure 3.9E). SIN3 220 was found to be important for activation of several genes

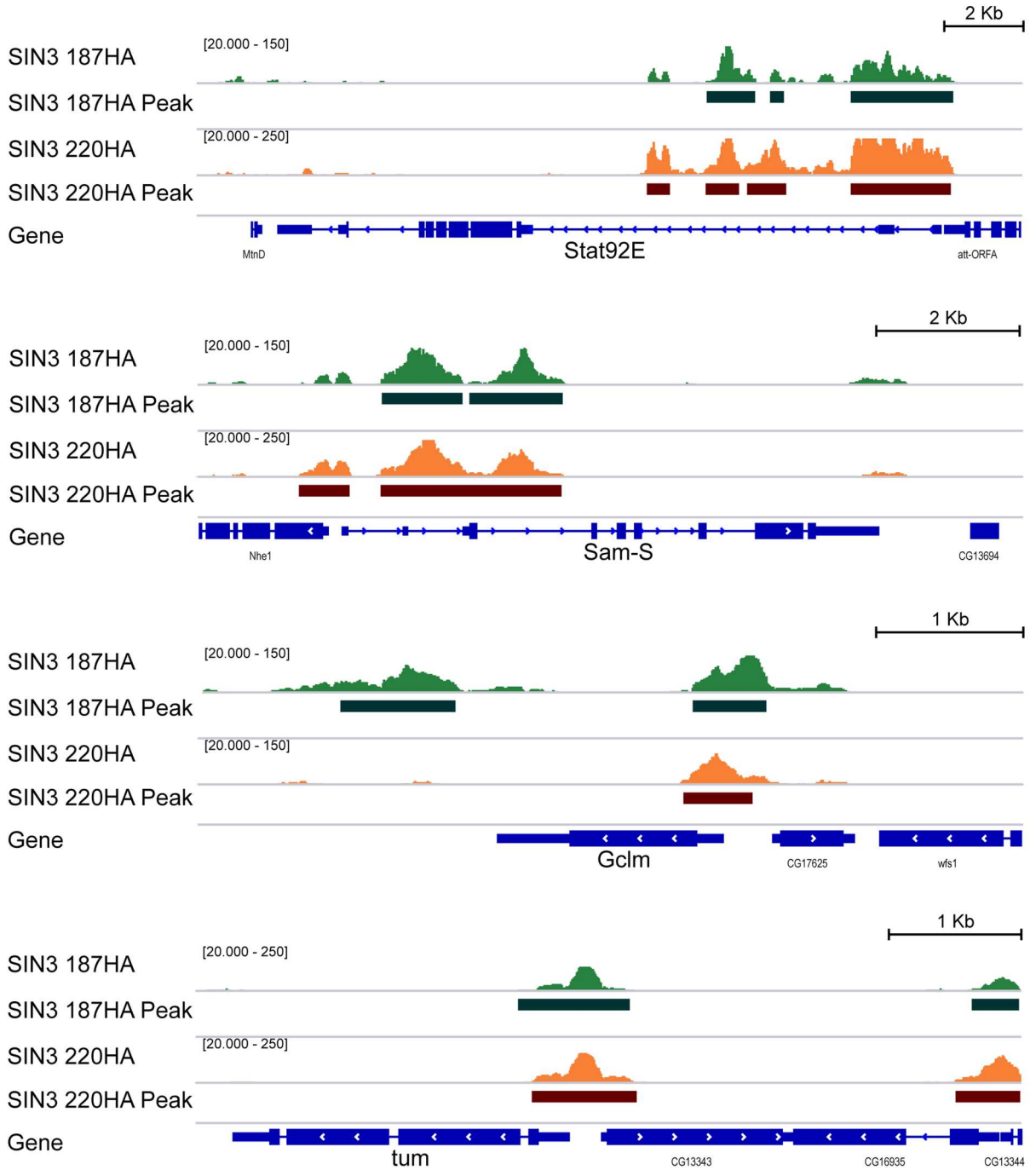

Figure 3.11: ChIP-seq gene tracks showing enrichment of SIN3 isoforms over *Stat92E, Sam-S, Gclm* and *tum*.

involved in cell cycle. This is particularly interesting because SIN3 was previously implicated in cell cycle regulation (Pile et al., 2003). Data from this current study indicate that the SIN3 220 isoform directly modulated expression of genes involved in cell cycle progression. The GO analysis indicated that SIN3 220 activated genes include those that regulate stress response and aging, which is in accord with our previous findings (Barnes et al., 2014). Furthermore, these data demonstrate that SIN3 220 regulates genes involved in novel pathways such as cell morphogenesis, cell fate determination and cell growth. Taken together, our findings in S2 cells demonstrate that SIN3 220 plays a critical role in mediating expression of genes encoding proteins involved in a wide array of biological processes. Consistent with the widespread role of histone modifications in modulating gene expression, the gene regulatory transcriptional network controlled by SIN3 220 is quite diverse.

SIN3 187 regulates many similar genes as those regulated by SIN3 220

Our findings demonstrate that the ectopic expression of SIN3 187 in S2 cells led to a reduction in the level of SIN3 220 (Figure 3.4B). In addition, we found that SIN3 220 was replaced by SIN3 187 at genomic targets (Figures 3.4 and 3.8A). During early *Drosophila* embryogenesis, the levels of SIN3 isoforms are comparable (Sharma et al., 2008). During the final stage, however, the relative level of the SIN3 220 isoform is reduced with an increase in expression of SIN3 187 (Sharma et al., 2008). Based on these data, we hypothesized that SIN3 187 likely regulates a different set of genes from those of SIN3 220. To test our hypothesis, we utilized RNA-seq to perform differential gene expression analysis comparing the expression of genes in cells that express SIN3 187HA to the gene expression of non-transfected S2 cells (Figure 3.4B, right panel).

Overexpression of SIN3 187 leads to the loss of SIN3 220 isoform in S2 cells, thus making this system ideal to investigate the regulatory roles of SIN3 187. Since SIN3 187HA was overexpressed in S2 cells, we used a stringent threshold of ≥2 fold gene expression (FDR ≤ 0.001) alteration between the wild type S2 cells and cells that express SIN3 187HA. Using this criterion, we identified 1274 genes to be differentially regulated due ectopic expression of SIN3 187. The number of genes repressed and activated by SIN3 187 is 669 and 605, respectively (Figure 3.10B and Supplemental Data 4). To identify the genes directly regulated by SIN3 187, we combined the binding profile of SIN3 187 with the gene expression changes due to SIN3 187 overexpression. By combining the recruitment data and gene expression changes, we found SIN3 187 bound approximately 42% (282/669) of repressed genes and 56% (338/605) of activated genes (Figure 3.12A), indicating direct regulation of these genes by SIN3 187.

So far in our analysis, we have identified direct targets of SIN3 187 and SIN3 220 in S2 cells. We next made four comparisons (Figure 3.12B): 1. genes directly repressed by SIN3 220 to those directly repressed by SIN3 187, 2. genes directly activated by SIN3 220 to those directly activated by SIN3 187, 3 and 4. genes directly regulated by SIN3 187 and SIN3 220 in opposing directions. Little overlap between gene sets was observed. These data imply that the isoforms are regulating very different sets of genes. Careful consideration of the model system, however, suggests in fact, that this is not the case. These comparisons yielded four classes of genes: A. Genes directly repressed (A_r) or activated (A_a) by SIN3 220, B. Genes directly repressed (B_r) or activated (B_a) by SIN3 220 as well as SIN3 187, C. Genes directly repressed (C_r) or activated (C_a) by SIN3 187 alone and D. Genes that are directly regulated in opposing directions by SIN3 187 and

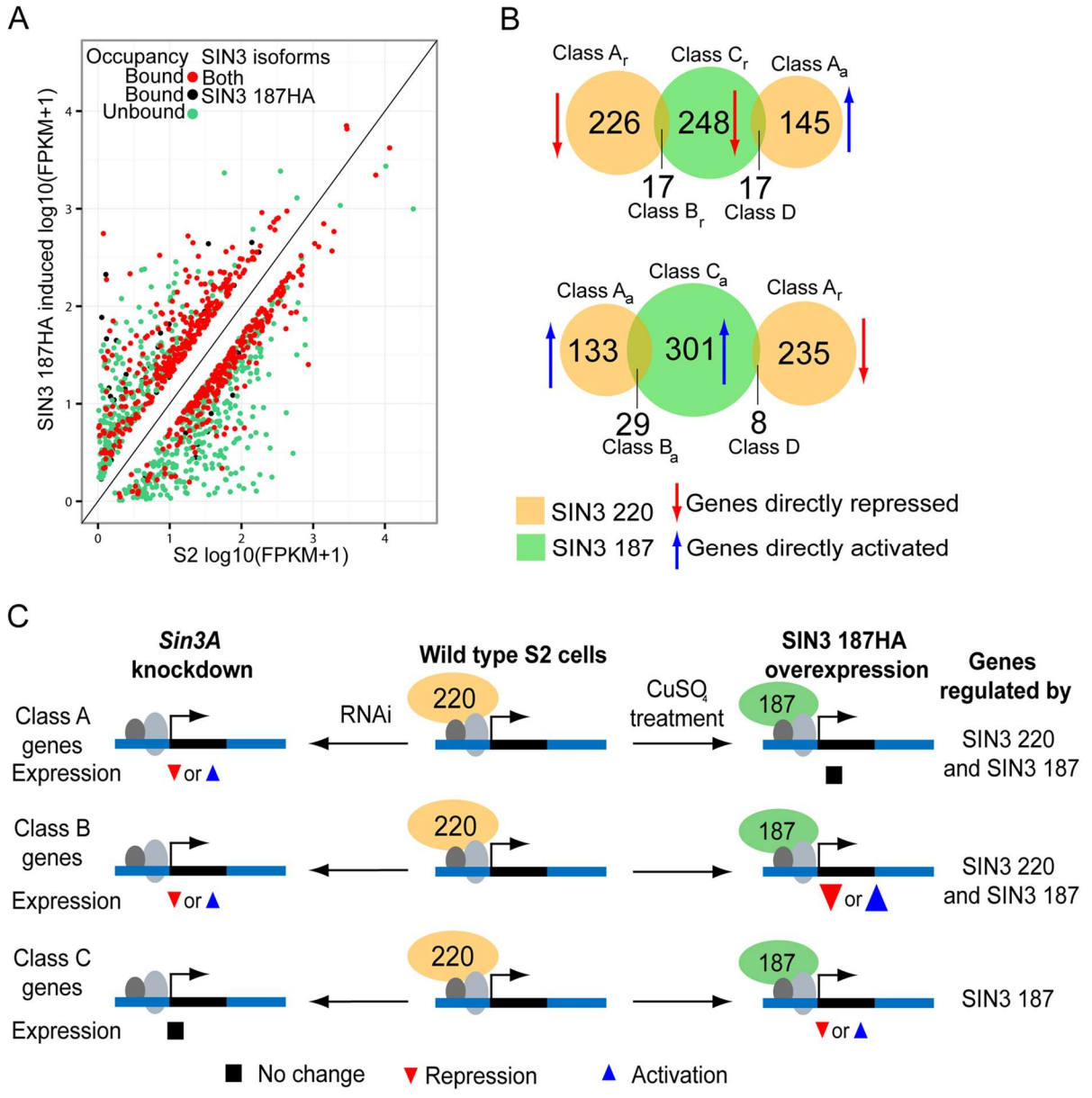

Figure 3.12: SIN3 187 regulates distinct as well as common genes as that of SIN3 220. (A) Scatter plots showing the correlation between genes bound and that change in expression upon overexpression of SIN3 187. Green dots denote genes regulated by SIN3 187 but not bound. Red dots indicate genes that are regulated by SIN3 187 and are bound by both SIN3 isoforms. Black dots refer to genes uniquely bound and regulated by SIN3 187. Continued on the next page.

(B) Venn diagrams showing the comparison of genes directly regulated by SIN3 isoforms. Different classes of genes are labelled as A, B, C and D. r, repression. a, activation. (C) Schematic showing the regulation of Class A, B and C genes by the SIN3 isoforms. The middle column shows the genes bound by SIN3 220 in wild type cells. Gene expression upon *Sin3A* (SIN3 220) knockdown is depicted in the left column and gene expression upon ectopic expression of SIN3 187HA is represented in the right column.

SIN3 220 (Figures 3.12B and 3.12C). For Class A genes, we did not observe gene expression changes between wild type S2 cells and cells that overexpress SIN3 187. This, however, does not exclude a role for SIN3 187 at Class A genes. Ectopic expression of SIN3 187 replaces SIN3 220 and thus may perform the same role as SIN3 220 without additional expression changes at gene targets compared to wild type cells. 95% (214/226) and 62% (83/133) of the directly repressed and directly activated Class A genes, respectively, are bound by SIN3 187, suggesting that majority of these genes are likely regulated by both SIN3 isoforms (Figure 3.12C). Class A genes bound only by SIN3 220 are likely to be unique targets of SIN3 220 (Figure 3.9B, black dots). Surprisingly, the majority of the genes specifically bound by SIN3 220 were activated. Of note, we cannot rule out the possibility that SIN3 187 might also be recruited to these potential SIN3 220 unique genes, as SIN3 187 peaks might not have been identified in the ChIP-seq assay due to statistical thresholds. A small number of genes were either repressed or activated by both SIN3 187 and SIN3 220 (Class B). All Class B genes are directly regulated by both SIN3 isoforms, suggesting that SIN3 220 and SIN3 187 regulate Class B genes in a similar way. SIN3 187, however, led to increased changes in expression of these genes relative to the wild type cells, implying that SIN3 187 has a stronger effect in regulating these genes compared to SIN3 220 (Figure 3.12C). Of high interest are the Class C

genes, which are genes uniquely regulated by SIN3 187. Class C genes will be discussed in detail below. Another set of genes falls into Class D, which includes genes that are regulated in opposing directions by SIN3 187 and SIN3 220 (Figure 3.12B). Using RT-qPCR, we validated the differential effects of the isoforms on two representative genes of this class (Figure 3.13). This class also includes genes that are potentially regulated only by SIN3 220. This gene set may show a change in expression upon overexpression of SIN3 187 due to the loss of SIN3 220 rather than enrichment of SIN3 187. Taken together,

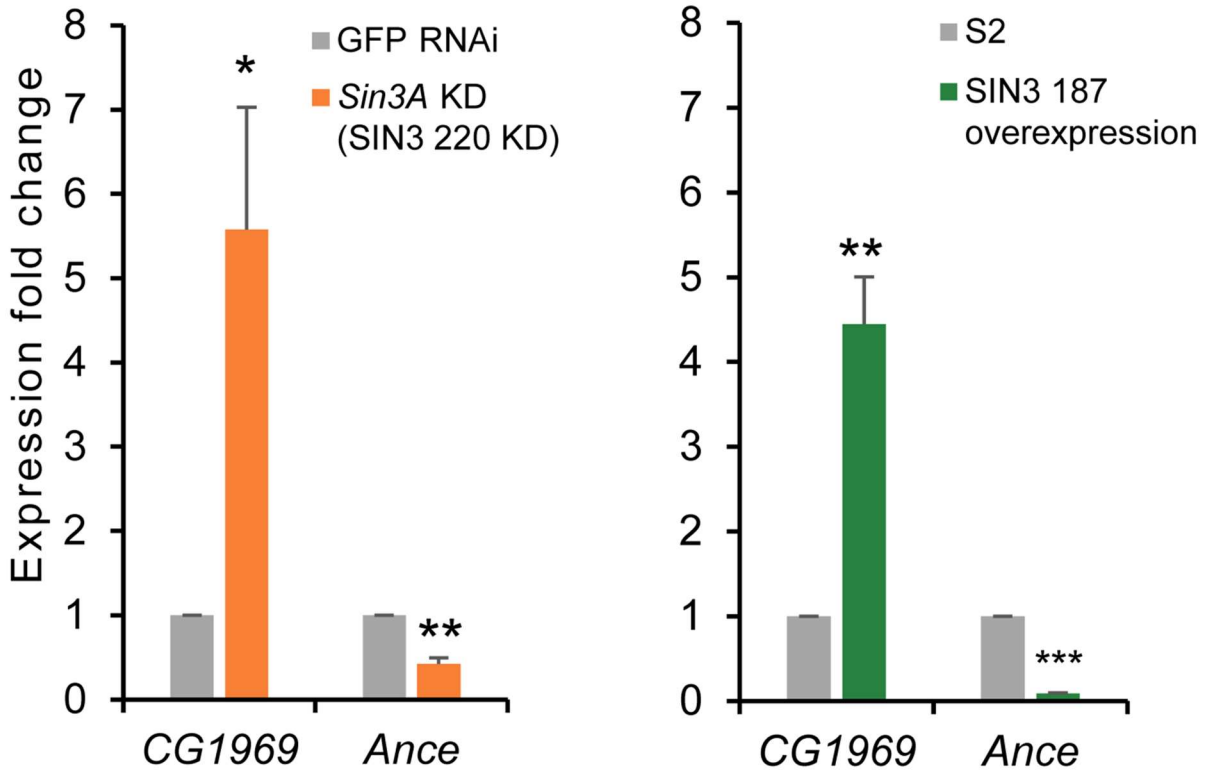

Figure 3.13: RT-qPCR verification of Class D genes. The level of CG1969 mRNA is upregulated upon Sin3A (SIN3 220) knockdown or SIN3 187 overexpression indicating that this gene is repressed by SIN3 220 and activated by SIN3 187.Conversely, expression of Ance is downregulated due to alteration in the level of SIN3 isoforms demonstrating that SIN3 220 and SIN3 187 act as a co-activator or as a co-repressor, respectively. P-value * < 0.05, ** < 0.01, *** < 0.0001.

we conclude that majority of the genes that are directly regulated by SIN3 220 are also regulated by SIN3 187 in a similar fashion. In addition, we identified several genes to be distinctly regulated by SIN3 187.

SIN3 187 plays distinct roles to that of SIN3 220 in regulating gene expression

We performed metagene analysis on genes directly repressed and activated by SIN3 187. This analysis revealed that the preferential binding sites of SIN3 187 are the transcriptional start sites of genes (Figure 3.14A). Similar to the observation for SIN3 220 binding, we found substantial enrichment of SIN3 187 around the transcriptional end sites of genes that are activated upon SIN3 187HA overexpression compared to those that are repressed (Figure 3.14A). Interestingly, 95% of the genes directly repressed and 90% of the genes directly activated by SIN3 187 were genes also bound by the SIN3 220 isoform in the SIN3 220HA cell line (Figure 3.12A, red dots). These findings indicate that upon expression of SIN3 187HA, the SIN3 187 isoform occupied the same sites previously bound by SIN3 220. Interestingly, a number of these commonly bound genes demonstrated a change in expression upon ectopic SIN3 187 expression, but not upon *Sin3A,* essentially SIN3 220, knockdown. Thus, SIN3 187 specifically modulates expression of this set of genes (Figure 3.12C, Class C). We identified 248 genes repressed and 301 activated uniquely by SIN3 187. Next, we selected two genes to verify the gene regulatory functions of SIN3 187. Based on the RNA-seq data, we chose *Signal-transducer and activator of transcription protein at 92E* (*Stat92E*) and *Glutamate-cysteine ligase modifier subunit* (*Gclm*), which are shown to be repressed and activated specifically by the SIN3 187 isoform, respectively. In addition, these genes are bound by SIN3 187 at the promoter region as determined by the ChIP-seq data (Figure 3.11). Using RT-

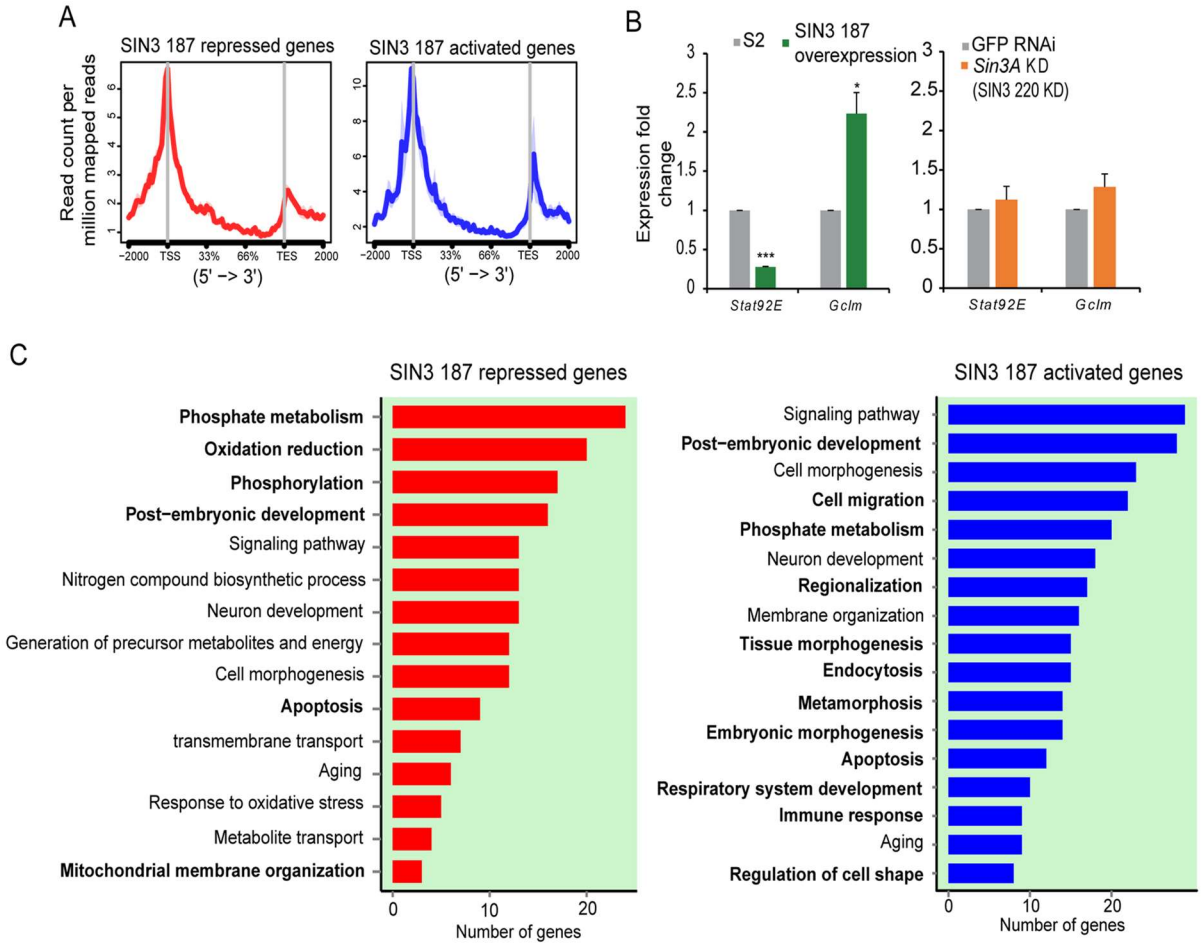

Figure 3.14: SIN3 187 distinctly regulates differentiation related genes. (A) Average ChIP signal of SIN3 187HA over the genes directly regulated by SIN3 187. (B) RT-qPCR verification of genes identified by RNA-seq to change in expression after overexpression of SIN3 187 versus control (left) but not affected upon *Sin3A* knockdown versus GFP RNAi (right). *P*-value * < 0.05, *** < 0.0001. (C) Gene ontology analysis of the Class C genes identified in Fig 3.12 are represented as bar plots. The Y-axis lists the broad GO categories. Red: categories of genes that are directly repressed. Blue: categories of genes that are directly activated. *P*-value (gene ontology categories) < 0.05. Biological processes labelled in bold indicate the unique processes that are overrepresented for genes distinctly regulated by SIN3 187.

qPCR, we demonstrate that overexpression of SIN3 187 led to repression of *Stat92E* and activation of *Gclm*, thus validating the RNA-seq experiment (Figure 3.14B). Furthermore, we analyzed the expression of these genes upon knockdown of *Sin3A* (SIN3 220). Our results demonstrate that reduction of *Sin3A* (SIN3 220) did not impact the expression of these genes (Figure 3.14B), although SIN3 220 was bound to those genes in wild type cells (Figure 3.11). These results indicate that *Stat92E* and *Gclm* genes are specific targets of SIN3 187 activity.

Our previous research demonstrated that during the late stages of *Drosophila* embryogenesis, expression of SIN3 187 increases and the relative level of the SIN3 220 isoform is reduced (Sharma et al., 2008). These results led to the hypothesis that SIN3 187 might play a specific role during the latter stages of embryo development. In the current study, we identified the genes specifically regulated by SIN3 187 and bound by SIN3 187 in cultured cells (Figure 3.12, Class C). Next, we sought to examine the biological processes that are represented by these genes. We performed GO analysis on the unique direct SIN3 187 targets (Supplemental Data 2). Approximately, 63% (157/248) of the genes repressed and 56% (170/301) of the genes activated by SIN3 187 were enriched for GO terms (Figure 3.14C). Strikingly, the GO analysis demonstrates that SIN3 187 regulated genes are involved in post-embryonic development, metamorphosis and apoptosis. Interestingly, these categories of biological processes are largely specific to SIN3 187 regulated genes when compared to the genes regulated upon *Sin3A* (SIN3 220) knockdown (compare Figures 3.14C and 3.9E). Additionally, we found that genes involved in phosphate metabolism, phosphorylation, endocytosis, embryonic morphogenesis and development of respiratory system were regulated specifically by the

SIN3 187 isoform. Further, we identified a few biological processes regulated by SIN3 187 that were also impacted upon *Sin3A* (SIN3 220) knockdown. For example, biological processes such as neuron development, cell morphogenesis and aging are overrepresented by both SIN3 187 regulated genes and genes that show a change in expression due to reduction of *Sin3A* (SIN3 220). Collectively, these data indicate that SIN3 187 specifically modulates biological processes involved in differentiation and development, in addition to regulating processes in common with the SIN3 220 isoform.

Discussion

In this research, we have described the differential role of the SIN3 isoforms in regulating gene activity, summarized in Figure 3.15. We have shown that the level of the largest isoform, SIN3 220, is regulated by ectopic expression of SIN3 187. In addition, upon overexpression of SIN3 187, SIN3 187 replaces SIN3 220 at genomic targets. Based on the ChIP-seq data, we determined that SIN3 isoforms localize to overlapping genomic regions, the large majority of which occupy the promoter region of genes. Additionally, global transcriptome analysis revealed that SIN3 isoforms play overlapping roles in regulating processes involved in metabolism, cell cycle and aging. Interestingly, we identified unique genes modulated by SIN3 187. These genes are overrepresented for biological processes that link the activity of SIN3 187 to the control of genes encoding proteins that function in post-embryonic development, endocytosis, phosphate metabolism and embryonic morphogenesis. Together, the current study demonstrates functional overlap as well as non-redundancy of SIN3 isoforms.

In cultured *Drosophila* cells, ectopic expression of SIN3 187 led to a reduced level of SIN3 220 (Figure 3.4). This switch in expression mimics the expression pattern of SIN3

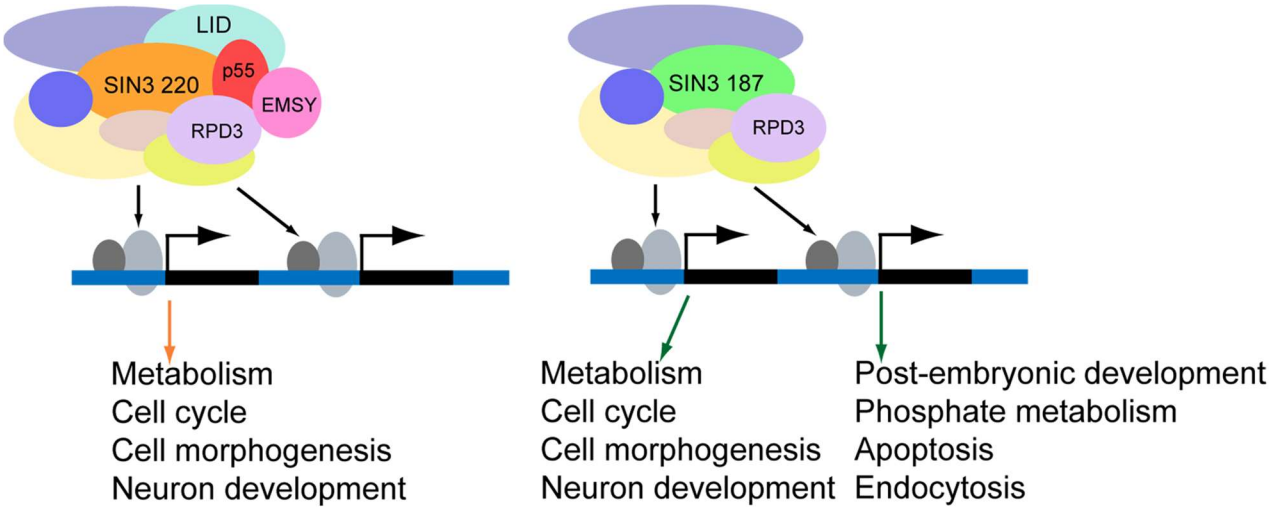

Figure 3.15: A model depicting the role of SIN3 isoforms in the regulation of gene expression.

isoforms observed during *Drosophila* embryonic development (Sharma et al., 2008). Together, the fly and S2 cell culture data suggest that SIN3 187 regulates the expression and/or stability of the SIN3 220 isoform. The mechanism through which ectopic expression of SIN3 187 may affect the amount of SIN3 220, however, remains unknown. Preliminary studies from our laboratory suggest that both transcriptional and post-transcriptional regulation is occurring in the cells (data not shown). The ChIP data indicate that ectopically expressed SIN3 187 replaces SIN3 220 at genomic targets. We also determined that the enrichment of SIN3 187 at genomic loci was dependent on the level of SIN3 220 present (data not shown). Therefore, it is plausible that a critical ratio of SIN3 220 to SIN3 187 is essential to determine which of the two isoforms will occupy a gene target, thereby modulating the transcriptional outcome. Additionally, the stage specific expression of SIN3 isoforms during the life cycle of *Drosophila melanogaster* (Sharma et al., 2008) favors the idea that maintenance of a critical ratio of SIN3 isoforms is important.

The gene expression analysis demonstrates that SIN3 187 might play similar roles as that of SIN3 220 as well as carry out unique functions. It is therefore curious that, unlike SIN3 220, expression of SIN3 187 alone does not rescue fly lethality caused by a null mutation in the *Sin3A* gene (Spain et al., 2010). One possibility is that SIN3 187 plays a critical gene regulatory role only in the presence of SIN3 220. This scenario is supported by the finding that expression of both SIN3 187 and SIN3 220 in a *Sin3A* null background led to a greater percentage of surviving flies compared to expression of SIN3 220 alone (Spain et al., 2010). Additionally, we determined that SIN3 187 is recruited to similar gene targets as that of SIN3 220. We speculate that localization of SIN3 187 to chromatin is dependent or partially dependent on SIN3 220. The preferential interaction of SIN3 220 with p55, dKDM5/LID and EMSY (Spain et al., 2010) suggests a differential genomic binding capacity of the SIN3 220 isoform that might facilitate the subsequent recruitment of SIN3 187. p55, a WD-40 repeat containing protein, has been demonstrated to provide an interaction framework for histones and other proteins to form multimeric complexes (Hennig et al., 2005; Nowak et al., 2011; Song et al., 2008). Therefore, it is likely that p55 plays an important role in the chromatin recruitment of SIN3 220. dKDM5/LID facilitates the removal of the histone H3K4 trimethyl mark (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007). A recent study using a mouse cell line revealed that the level of histone H3K4me3 influences the recruitment of mSin3A to target sites (Cheng et al., 2014). Therefore, the LID-containing SIN3 220 complex might alter the chromatin composition in a way that enables subsequent recruitment of SIN3 187.

One of the most surprising findings from our study is that despite the extensive amount of overlapping localization of SIN3 isoforms, the SIN3 187 isoform regulates the

level of expression of a distinct set of genes (Figure 3.14). One possibility for this result is that binding of SIN3 187 leads to differential chromatin modification. We have previously shown that ectopic expression of SIN3 187 significantly affects both global histone H3K9 and H3K14 acetylation (Spain et al., 2010). On the other hand, global changes of only histone H3K9 acetylation were observed upon overexpression of SIN3 220 (Spain et al., 2010). In addition, the SIN3 187 isoform specific complex might recruit other *trans*-factors to chromatin, thereby altering transcription in distinct ways. To identify the mechanisms by which SIN3 isoforms regulate gene expression, more extensive evaluation of the interplay of *cis*-elements and *trans*-factors in relation to the SIN3 complexes is required.

Although we mapped the occupancy of SIN3 isoforms to approximately 6000 genes, the transcriptome analysis in S2 cells showed that expression of approximately 1000 genes is affected due to alteration in the level of SIN3 isoforms. We question why many binding events of the SIN3 isoforms do not affect gene expression (Figures 3.8, 3.9 and 3.14). One explanation is that S2 cells do not represent the complexity of gene regulation in the whole organism. SIN3 isoforms might contribute to gene expression changes depending on their interaction with stage specific transcription factors during the developmental stages of the whole organism. In addition, we do not rule out the possibility that some fortuitous associations of SIN3 isoforms occur, which do not contribute to gene expression alterations, a possibility that has been previously suggested (Georlette et al., 2007; Li et al., 2008). A recent publication reported a list of possible false positive binding peaks referred to as phantom peaks (Jain et al., 2015). The authors suggested that these regions are occupied by any transcription factor irrespective of their biological relevance.

Approximately 40% of peaks identified for SIN3 isoforms by our ChIP-seq assay overlapped with the phantom peaks (data not shown). Approximately 10% of these peaks, however, can be assigned to genes that demonstrated expression changes due to alterations in the level of SIN3 isoforms, indicating that enrichment of SIN3 isoforms at these loci have functional outcomes.

The large majority of peaks identified for the SIN3 isoforms, mapping around the 5' end of genes (Figures 3.7 and 3.14), is consistent with observations in other model systems. Interestingly, we observed a substantial level of enrichment of the SIN3 isoforms around the transcription end sites of the genes activated by SIN3 isoforms (Figures 3.9 and 3.14). Alteration of gene expression due to changes in higher order chromatin structure has been reported and reviewed (Dean, 2011). Therefore, we wonder if there is any possibility that gene looping influences the function of SIN3 isoforms as co-activators of genes. More extensive studies, however, are required to understand the possibility of gene looping and its role in gene regulation by SIN3 isoforms.

Conclusion

In summary, our results indicate complex mechanisms through which genes are regulated by the SIN3 187 and 220 isoforms. This result suggests that the highly similar SIN3 isoforms likely work in concert with other factors that differentially affect transcript levels. These data suggest a novel mechanism of gene regulation by SIN3 isoforms, by which the SIN3 isoforms localize to overlapping genomic sites and modulate some similar and many distinct biological processes. Collectively, these findings set a solid framework for further understanding of the network of biological processes regulated by SIN3 histone modifying complexes.

Acknowledgements

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CHAPTER 4 CAF1-55 IMPACTS RECRUITMENT OF SIN3 220 TO CHROMATIN AND AFFECTS EXPRESSION OF A SUBSET OF SIN3 220 GENE TARGETS

Introduction

In eukaryotes, double stranded DNA is wrapped around a histone octamer to form the basic chromatin structure known as a nucleosome. Chromatin modifying complexes and the modification of histones play a major role in regulating gene expression, which in turn ultimately determines cell fate (Dawson and Kouzarides, 2012).

Caf1-55 is a subunit of the Caf1 complex, which mediates DNA-replication based chromatin assembly (Verreault et al., 1996). Caf1-55 is an evolutionarily conserved WD40 repeat-containing protein that associates with several chromatin modifying complexes (Hennig et al., 2005). Caf1-55 is required for ATP-dependent chromatin remodeling by the NURF complex (Martinez-Balbas et al., 1998). Caf1-55 has been shown to interact with the polycomb group repressive complex (PRC) (Czermin et al., 2002; Muller et al., 2002), and the mammalian homologues Rbbp4/7 have been demonstrated to interact with Retinoblastoma (Rb) protein (Qian et al., 1993). Furthermore, association of Caf1-55 with HDAC complexes have been reported (Ayer, 1999; Hassig et al., 1997; Laherty et al., 1997; Taunton et al., 1996).

Caf1-55 functions in several biological processes, such as the regulation of the cell cycle and development of anatomical structures in metazoans. For example, loss of Caf1-55 in *Drosophila* S2 cells leads to cell cycle arrest during the S phase (Pile et al., 2002). In addition, three mutant alleles of *Caf1-55* were found to suppress the *Senseless* overexpression phenotype in *Drosophila* eyes (Anderson et al., 2011). These *Caf1-55* mutants are linked to the disruption of H3K27me regulation and lead to homeotic defects

in eyes, antenna and wings in flies, which can be rescued by overexpression of HOX genes. (Anderson et al., 2011).

Previously, our lab has demonstrated a genetic and a biochemical interaction between *Caf1-55* and *Sin3A* (Spain et al., 2010). Therefore, we are interested in understanding the mechanism by which Caf1-55 modulates the activity of the SIN3 histone modifying complex.

SIN3 is a master transcriptional adapter protein historically understood to act as a corepressor complex (Grzenda et al., 2009; Silverstein and Ekwall, 2005). The role of SIN3 in different biological processes is well studied (Kadamb et al., 2013). In mammals, two SIN3 isoforms, mSin3A and mSin3B, were shown to play distinct gene regulatory roles (Cowley et al., 2005; Dannenberg et al., 2005; Grandinetti and David, 2008). For example, in mice, mSin3A and mSin3B play a role in cell proliferation and cell cycle exit, respectively (Cowley et al., 2005; Dannenberg et al., 2005; David et al., 2008; Grandinetti and David, 2008; van Oevelen et al., 2008). In yeast, the large Rpd3/Sin3 complex represses transcription from gene promoters and the small complex silences transcripts arising from cryptic promoters within genes (Keogh et al., 2005). In *Drosophila*, multiple isoforms of SIN3 are expressed from a single gene (Pennetta and Pauli, 1998). These isoforms have been demonstrated to play distinct roles during development (Sharma et al., 2008; Spain et al., 2010). *Drosophila* SIN3 complexes possess core and unique protein components, and it is therefore likely that the unique components impart specificity thereby impacting the function of distinct SIN3 complexes.

In *Drosophila*, SIN3 187 and SIN3 220 are the predominantly expressed alternatively spliced isoforms (Pennetta and Pauli, 1998). Previous studies showed that

these isoforms are differentially expressed during developmental stages and form distinct complexes (Sharma et al., 2008; Spain et al., 2010). The SIN3 isoform specific complexes share several protein components such as RPD3, ING1, PF1, Arid4B and BRMS1. The SIN3 220 complex, however, has additional unique components. Caf1-55, dKDM5/LID and EMSY preferentially interact with the SIN3 220 complex (Spain et al., 2010). Overexpression of SIN3 220, but not SIN3 187, can rescue lethality in flies caused by knockdown of *Caf1-55* (Spain et al., 2010). These data suggested that the interaction between the SIN3 220 complex and Caf1-55 is critical for proper fly development.

In this research, we demonstrate that Caf1-55 is partially required for localization of SIN3 220 at specific gene targets. We also demonstrate that Caf1-55 regulates a subset of SIN3 220 targets. We found that Caf1-55 physically interacts with the unique C-terminal domain of SIN3 220 and mutations in the histone H4 binding domain of Caf1-55 affect this interaction. Surprisingly, we found that overexpression of the histone H4 binding mutant of Caf1-55 does not affect transcription of SIN3 220 regulated genes. These data indicate that the interaction of SIN3 220 with the histone H4 binding pocket of Caf1-55 is likely not essential for recruitment of the SIN3 220 complex to chromatin.

Materials and Methods

Generation of protein expression plasmids

To generate a HA-tagged SIN3 220 unique C-terminal clone (UniC) for expression in *Drosophila* S2 cells, we used a previously available SIN3 220HA construct in pHRMA-4 vector (Spain et al., 2010). The first exon of UniC starts at 6061 base pair of the *Sin3A* cDNA sequence (NCBI accession number: AJ007518). Using site-directed mutagenesis, an EcoRI site was inserted prior to the 5’ end of the UniC exon of *Sin3A* cDNA sequence

carried on the vector. In addition to the EcoRI site, an ATG start sequence was incorporated at the start of the *UniC* cDNA sequence. A second EcoRI site was already present on the vector at the 3' end of the gene. Restriction digestion was carried out by EcoRI and the excised fragment was sub-cloned into an empty pHRMA-4 vector. To express UniC in bacterial cells, cDNA of *UniC* was sub-cloned into an empty pMAL-c2X vector. We carried out PCR using the above mentioned pHRMA-4 clone carrying *UniC* cDNA as a template. We inserted EcoRI and XbaI sites at 5' and 3' end of the *UniC* coding sequence, respectively. PCR amplified *UniC* was digested by EcoRI and XbaI and ligated into the pMAL-c2X vector. Both the UniC clones for expression in *Drosophila* S2 cells and bacterial cells have a HA tag incorporated at the 3' end of the *UniC* cDNA. Primer pairs used to generate these clones are listed in Table 4.1.

To express Caf1-55 in bacterial cells, we carried out site-directed mutagenesis to insert BamHI and XhoI sites at the 5' and 3' end *Caf1-55* coding sequence, respectively. Site-directed mutagenesis was performed on a pHRMA-4 vector containing *Caf1-55* cDNA sequence. Restriction enzyme mediated digested *Caf1-55* cDNA fragment was excised and inserted into an empty pET-28a vector. Sequences of primers used are listed in Table 4.1.

We purchased the construct of Caf1-55 in the pMK33 vector (FMO025549) from the Berkeley *Drosophila* Genome project (BDGP), ORFeome collection (Yu et al., 2011). To create mutant forms of Caf1-55, site-directed mutagenesis was performed on the wild type FLAG-HA tagged Caf1-55 plasmid. Table 4.1 has the primer sequences used to create these clones. All clones were sequenced to verify that the mutation was present.

Table 4.1: Primers used to generate expression clones

Plasmid	Sequence (5' -> 3')	Expression system
UniC	CCACCTTCTCTGTAGCGCAACATCTCGGCAGTATCTTCGGAATTCGCCTCCGTCTTG	S2 cells
	CAAGACGGAGGCGAATTCCGAAGATACTGCCGAGATGTTGCGCTACAGAGAAGGTGG	
UniC	ACGTCGGAATTCTTGCGCTACAGAGAAGG	E.coli
	GGCCGCGTCTAGATTAAGCGTAATCTGG	
Caf1-55	GTATTTTCAGGGCGCCGGATCCATGGTGGATCGCAGC	E.coli
	GCTGCGATCCACCATGGATCCGGCGCCCTGAAAATAC	
Caf1-55	CCAATACCGCTTGACTCGAGGGTACCAAGCTTGTC	E.coli
	GACAAGCTTGGTACCCTCGAGTCAAGCGGTATTGG	
Caf1-55 H4M	GAGGAGCAGAGTACGGCGGCTGCTGCGGCCGGACCACCCGAGCTGC	S2 cells
	GCAGCTCGGGTGGTCCGGCCGCAGCAGCCGCCGTACTCTGCTCCTC	
Caf1-55 H3M	CACACCGCCGTTGTTCAGGACGTGGCTTGG	S2 cells
	GCCAAGCCACGTCCTGAACAACGGCGGTGTG	
	CGGATCGGTGGCTAAAGACCAGAAGCTGATG	
	CATCAGCTTCTGGTCTTTAGCCACCGATCCG	
	CGCTCACACAGCGCAAGTCAATTGCTTG	
	CAAGCAATTGACTTGCGCTGTGTGAGCG	

Cell culture

S2 cells were cultured as described in Chapter 3. Cell lines expressing wild type or mutant forms of Caf1-55 were generated as described previously (Gajan, 2015).

Chromatin immunoprecipitation and qPCR

Chromatin was prepared as described in Chapter 2. The immunoprecipitation assay, which was performed using HA-conjugated resin, is described in Chapter 2. Immunoprecipitation for SIN3 and histone modifications were performed by adding 10 µl of anti-SIN3, 3 µl of anti-histone H3K4me3 (Active motif), 3 µl of anti-histone H3K9ac (Millipore) and 4 µl anti-histone H3 (Abcam) and 10 µl of pre-immune IgG to approximately 75 µg of prepared chromatin. Chromatin was incubated with antibody on a nutator at 4^{o}C overnight. Samples were then incubated with 30 µl of Protein A Agarose resin (Thermo Fisher Scientific) for 4 hr at 4^{o}C. After incubation, resins were washed with 1X RIPA buffer (25 mM Tris (pH 7.4), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS), IP1 buffer (25 mM Tris (pH 7.6), 500 mM NaCl, 1mM EDTA, 0.5% SDS and 1% Triton X-100)

and IP2 buffer (10 mM Tris (pH 7.6), 250 mM LiCl, 1 mM EDTA, 0.5% sodium deoxycholate and 0.5% Triton X-100) at 4^{o}C for 5 min each. Elution of immunoprecipitated DNA was done by addition of elution buffer (1% SDS, 0.1 M $NaHCO_3$). These samples were then subjected to reverse crosslinking as described in Chapter 2. qPCR was performed to quantitate the enrichment of Caf1-55, SIN3 and histone modifications at specific genomic locations. All primer pairs used in this study are listed in Table 4.2.

Table 4.2: Primers used for ChIP-qPCR analysis

Gene	Gene ID	Forward primer	Reverse primer
Sam-S	*CG2674*	CCACACCTCCACCGTCTACT	CCTCTGTTCAAGTCGTGCAA
MME1	CG3476	CTGCAATCGATAGCTGAATGT	GCGCGGTATTATAATTTCCAT
PyK	*CG7070*	GACGACGCTTTCAGCGAT	TTTGAAGCTCGGGTCTGC
Cyt-c1	CG4769	TTGCAGGCGAAGAACCTC	GGTTAGGTCCGATCGGGT
Ahcy13	CG11654	CGAAGCCCAGCTACAAAGTC	AATAGATGCAATTCACCCGC
CG10623	CG10623	CGGAAAACGTACAGCAGTGA	GCATTTGACCAGAATTGGCT
Cbs	*CG1753*	CCCTTCCTGTTTCCATCTGA	TGCGAAATTGCGTGAGATTA
ds	CG17941	TGCCAACCATCCTAACGG	CTGTGGAGGACACAGGGG

Gene expression analysis by RT-qPCR

Gene expression analysis was performed as previously described (Barnes et al., 2010). In brief, we utilized the RNAeasy purification kit (Qiagen) to extract total RNA from *Drosophila* cultured cells. Total RNA was converted into cDNA using the ImProm-II Reverse Transcription System (Promega) with random hexamers. qPCR was carried out

using cDNA as template in a Stratagene Mx3005P real-time PCR. Primer sets used for the RT-qPCR analysis are listed in Table 4.3.

Table 4.3: Primers used for gene expression analysis

Gene	Gene ID	Forward primer	Reverser primer
CG1969	CG1969	GGAAGCAACTGGGAAAACTG	TGATCAGCTTGTCCTTGCAG
Sam-S	CG2674	AAACTT TGACCTCAGGCC C	CGC TGG TAT ATC GGC TGG
MME1	CG3476	GCCATCCATTGGACACAATA	GGCGGCACAATCTATGACT
PyK	CG7070	GGCTCCGGCTTCACCAA	TTCCTGAGCGGCAGAATTTATT
Cyt-c1	CG4769	GATTCCATGGCTTGAGATTG	GGGAGGAGAGGTTCTTCG
Ahcy13	CG11654	AGACCTTGGTCTTCCCCG	GACACCGGTGGTCGTCTC
CG10623	CG10623	TCCAAAGTCGGAAGGCTG	GGCCACTTTGGTAAGCGA
Cbs	CG1753	TGCAACTGTTGGTGAGGC	CATCCTGATCCACGACGG
SIN3 220	CG8815	TTAAAGGCGTATTGCTCGGC	TTGCGCTACAGAGAAGGTGG
Taf1	CG17603	CTGGTCCTGGTGAGGTGA	CCGGATTCTGGGATTTGA

Nuclear fraction extraction and immunoprecipitation

Preparation of nuclear extracts from S2 cells or stable cells expressing wild type or mutant forms of Caf1-55 were carried out as previously described (Spain et al., 2010). Briefly, 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 and incubated with 35 µl of anti-FLAG resin (Sigma) overnight at 4^{o}C. Resins were then washed with wash buffer 1 (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), 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 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 min each at 4^{o}C. Elution of immunoprecipitated protein was carried out by incubating the resin with 35 µl of Laemmlli Buffer (Bio-Rad) for 15 min at room temperature. Eluted samples were then subjected to western blot analysis.

The immunoprecipitation assay on nuclear extract prepared from transiently transfected S2 cells was performed using the protocol described above. In brief, S2 cells were transiently transfected by adding pHRMA-4 vector carrying an HA-tagged UniC cDNA sequence using the Effectene transfection kit (Qiagen). Immunoprecipitation was carried out using anti-HA beads and eluted samples were analyzed by western blot.

Direct protein-protein interaction assay

The bacterial expression system was used to express both the unique C-terminal region of SIN3 220 and Caf1-55. We co-transformed *E. coli* BL21 (DE3) cells by adding a mixture of pET-28a and pMAL-c2X vectors carrying *Caf1-55* and *UniC* cDNA, respectively. The expression vectors pET-28a possess a kanamycin resistance gene and pMAL-c2X contain an ampicillin resistance gene. Colonies transformed with both of these plasmids were selected by their ability to grow on media containing 100 mg/ml of ampicillin and 50 mg/ml of kanamycin. For control experiments, BL-21 cells were transformed by only pET-28a vector carrying *Caf1-55* cDNA. Expression of proteins in bacterial cells were carried out using an autoinduction method described previously (Studier, 2005). In brief, starter culture of bacteria containing the desired expression plasmids were inoculated in the autoinduction media (lysogeny broth with added supplements; 50 µM $(NH4)_2SO_4$, 100 µM KH_2PO_4, 100 µM Na_2HPO_4, 1 mM $MgSO_4$, 4% glycerol, 14 µM glucose and 29 µM lactose) and incubated in a shaker overnight at 20^oC. Bacterial cells were harvested by centrifugation at a speed of 3220 x g. The bacterial pellet was resuspended in lysis buffer (500 mM NaCl, 10 mg lysozyme (MP Biopharmaceuticals), 0.001% of universal nuclease (Pierce), 90% 1 X PBS and protease inhibitor tablet (Roche)) and incubated on ice for 1 hr. These cells were then subjected to

sonication at amplitude of 30% with 2 sec pulse on and 2 sec pulse off for a total time of 2 min. Sonicated samples were then centrifuged at 600 x g for 30 min and the supernatant was stored for downstream immunoprecipitation assay as described below.

Caf1-55 was tagged with a 6X histidine at the N-terminus of the protein. On the other hand, UniC has HA and MBP tags at its end. To perform the immunoprecipitation, we added 50 µl anti-HA resin to bacterial lysate containing recombinant UniC and Caf1-55 and incubated on a nutator at 4^{o}C for 1 hr. The anti-HA beads were washed with wash buffer 1 (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate), 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 wash buffer 3 (20 mM HEPES (pH 7.4), 300 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA, 10% glycerol and 1.5% Triton X-100) for 5 min each at 4^{o}C. Elution of immunoprecipitated protein was performed by incubating resins in 50 µl of Laemmli buffer (Bio-Rad) for 15 min at room temperature. These samples were subjected to western blot analysis.

Western blot

Western blot analysis was performed as described in Chapter 3. Primary antibodies used to probe western blots are as following, HA-HRP (1:6000; Sigma), SIN3 (1:2000 (Pile and Wassarman, 2000)), RPD3 (1:3000, (Pile and Wassarman, 2000)), HIS (1:1000; Qiagen), β-Actin (1:1000; Cell signaling). 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).

RNA Interference

RNAi-mediated knockdown (RNAi) of *Caf1-55* was carried out using a protocol described in Chapter 3. The primer sequences used to generate *Caf1-55* dsRNA are (oriented 5' to 3') AGGAGCATAGGGTTATTGATGC and CTCGTCGTTGTAAACATTCTCG. To design the *Caf1-55* dsRNA primer pair, sequences were obtained from *Drosophila* RNAi Library 1.0 on Open Biosystems. These primers were used to amplify S2 genomic DNA as a template. The amplicon was inserted into the pCRII-Topo vector using the TOPO TA Cloning kit (Thermo Fisher Scientific). Generation of GFP dsRNA is described in Chapter 3. Western blotting was routinely performed for all experiments to evaluate the efficiency of RNAi-mediated knockdown of *Caf1-55* in S2 cells.

Statistics

Significance values were calculated by two samples unpaired Student's *t*-test using the GraphPad software.

Results and Discussion

Caf1-55 is recruited to SIN3 220 gene targets

In *Drosophila melanogaster*, SIN3 220 and SIN3 187 are the most prevalent isoforms of SIN3 (Neufeld et al., 1998; Pennetta and Pauli, 1998). SIN3 220 is the predominant SIN3 isoform expressed in S2 cells (Spain et al., 2010). Previously, we demonstrated that Caf1-55 interacts with the SIN3 220 specific complex (Spain et al., 2010). Based on these findings, we questioned whether Caf1-55 is localized to genomic

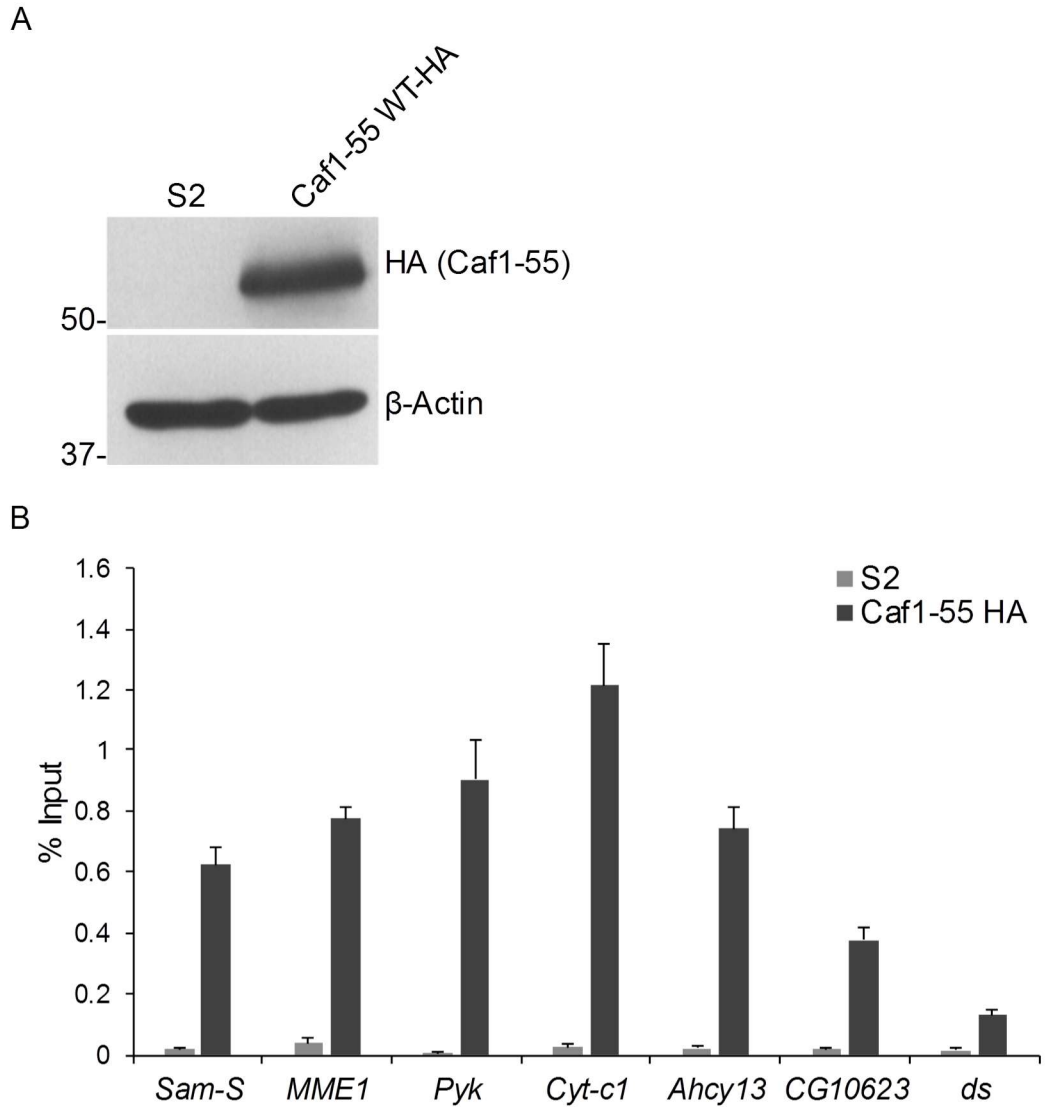

Figure 4.1: Caf1-55 localizes to SIN3 220 gene targets. (A) Western blot analysis of whole cell extract prepared from S2 or Caf1-55 WT-HA cells induced to express the tagged Caf1-55 protein. The blot was probed with antibodies as listed. β-Actin was used as a loading control. (B) ChIP-qPCR analysis for Caf1-55 HA. ChIP-qPCR was performed using HA-antibody conjugated resin. ChIP-qPCR on chromatin prepared from S2 cells was used as a non-specific ChIP control. *ds* was used as a predicted negative control for Caf1-55 binding. Error bars represent standard error of mean (n=3).

loci targeted by SIN3 220. We made a stable S2 cell line by transfecting a cDNA construct for expression of Caf1-55 tagged with HA and FLAG peptides at the C-terminus. Western

blot analysis of the whole cell extract prepared from Caf1-55 stable cells confirmed expression of Caf1-55. Extract from S2 cells was used as a control (Figure 4.1A).

To test whether Caf1-55 is localized to genes bound by SIN3 220, we performed chromatin immunoprecipitation followed by qPCR (ChIP-qPCR). ChIP was performed on chromatin prepared from cells that express HA-tagged Caf1-55. Chromatin from S2 cells not expressing any HA-tagged protein was used as a non-specific ChIP control. Specific gene targets that were previously demonstrated to be bound by SIN3 220 were chosen for this analysis (Saha et al., 2016). We analyzed the localization of Caf1-55 at the promoter region of *Sam-S*, *MME1*, *Pyk*, *Cyt-c-1*, *Ahcy13*, *CG10623* and at an intronic region of *ds*. We previously demonstrated that the intronic region of *ds* is not bound by SIN3 220 and thus was used as a negative control (Saha et al., 2016). ChIP-qPCR analysis indicates that Caf1-55 was localized to the genomic targets of SIN3 220 (Figure 4.1B). These data suggest that Caf1-55 interacts with SIN3 220 at genomic loci and therefore could potentially regulate the activity of the SIN3 220 complex.

Caf1-55 impacts recruitment of SIN3 220 at gene targets

Previously, we demonstrated that SIN3 220 rescued lethality in flies due to knockdown of *Caf1-55* (Spain et al., 2010). Since Caf1-55 is bound to the same promoters as SIN3 220, we asked whether Caf1-55 is required for the recruitment of SIN3 220 to gene targets. We used RNAi to knock down *Caf1-55* in S2 cells. S2 cells were treated with double-stranded RNA (dsRNA) targeting either *Caf1-55* or GFP, which was used as the mock control. Approximately 75% of Caf1-55 was knocked down post RNAi treatment as demonstrated by the western blot analysis Figure 4.2A.

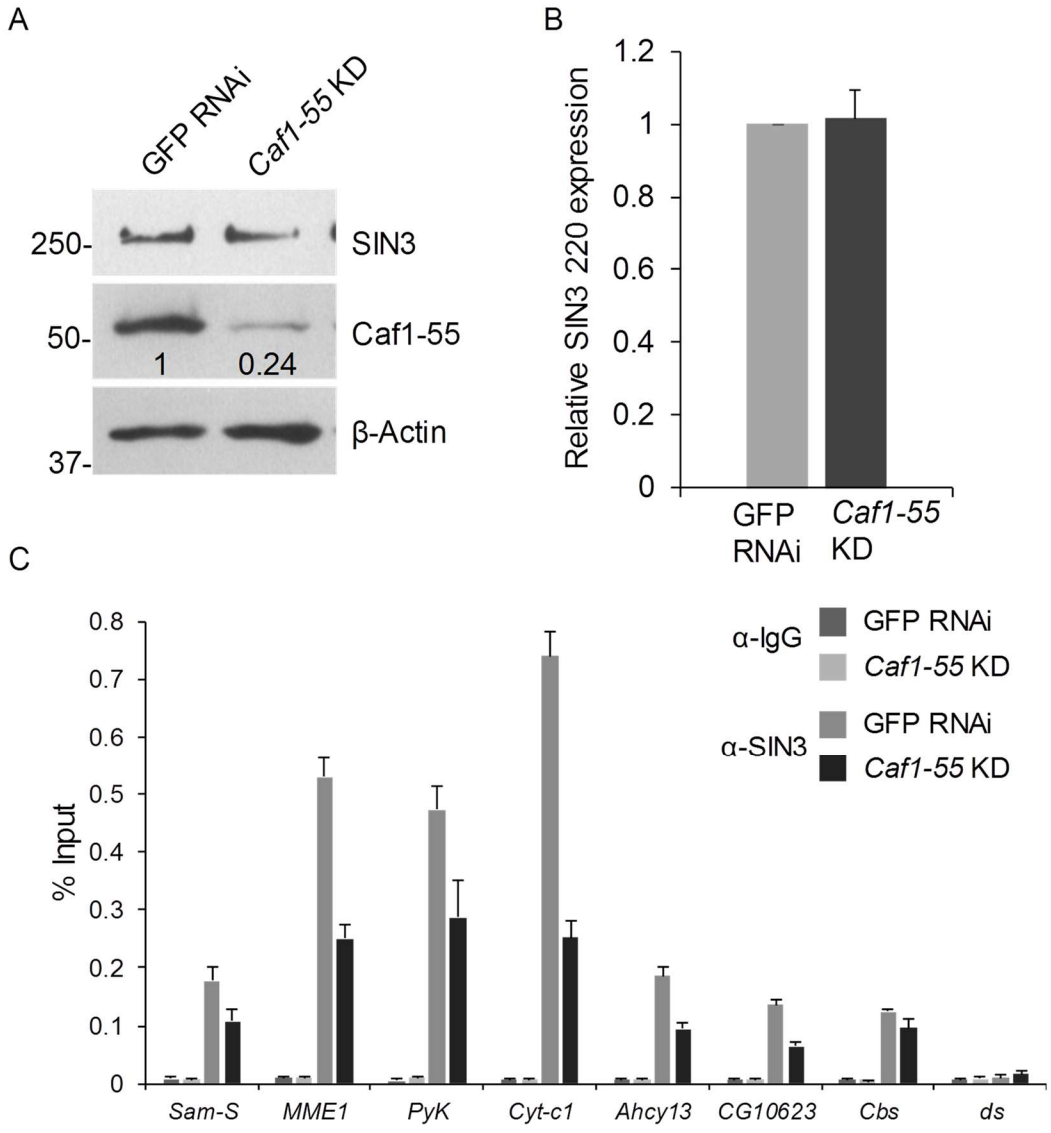

Figure 4.2: Loss of Caf1-55 negatively impacts SIN3 220 localization to chromatin. (A) Western blot analysis of whole cell extract prepared from cells post RNAi treatment against *Caf1-55*. dsRNA against *GFP* was used as a control. The blot was probed with antibodies as listed. β-Actin was used as a loading control. Densities were normalized to β-Actin and represented as relative level of Caf1-55 remaining after RNAi treatment. (B) RT-qPCR analysis measuring the level of SIN3 220 expression upon reduction of Caf1-55. The primer pair was designed to amplify a unique exon of SIN3 220 (n=3). The *Taf1* gene was used to normalize the expression of SIN3 220. *p*-value > 0.05 (C) ChIP-qPCR analysis for SIN3 post *Caf1-55* knockdown. ChIP-qPCR was performed using SIN3 antibody. Chromatin was prepared from S2 cells treated with either GFP or *Caf1-55* dsRNA. ChIP using pre-immune IgG was used as a non-specific ChIP control. *ds* was used as a negative control. Error bars represent standard error of mean (n=3).

To rule out the possibility that knockdown of *Caf1-55* affects the stability of SIN3 220, we asked whether loss of Caf1-55 has any effect on the expression of SIN3 220. Knockdown of *Caf1-55* had a negligible effect on the protein level of SIN3 (Figure 4.2A). To test whether reduction of Caf1-55 has any effect on the gene expression of *Sin3A*, we performed RT-qPCR analysis using primers targeting an exon specific to the SIN3 220 isoform. RNA was prepared from cells treated with GFP or Caf1-55 dsRNA. The reduction of Caf1-55 had no effect on the transcript level of SIN3 220 (Figure 4.2B). Our data demonstrate that expression of SIN3 220 is insensitive to a reduction in the level of Caf1-55.

To test the effect of *Caf1-55* knockdown on the recruitment of SIN3 220 to chromatin, we performed ChIP-qPCR using an antibody against SIN3. ChIP was performed on chromatin prepared from cells treated with dsRNA targeting *Caf1-55* or GFP. ChIP using pre-immune IgG antibody was used as a non-specific control. We tested localization of SIN3 220 at *Sam-S, MME1, PyK, Cyt-c1, Ahcy13, CG10623* and *Cbs. ds* was used as a negative control gene. The transcript level of each these genes, except Cbs, is upregulated upon reduction of *Sin3A* (Gajan et al., 2016; Saha et al., 2016). We observed negligible enrichment of specific target regions from pre-immune IgG ChIP. As expected, we found that SIN3 220 was highly enriched at genes that were previously demonstrated to be SIN3 220 targets and was not enriched to any appreciable level at the negative control gene (Figure 4.2C). Upon *Caf1-55* knockdown, the localization of SIN3 220 at target sites was reduced (Figure 4.2C). Interestingly, the reduced level of Caf1-55 did not affect localization of SIN3 220 at the promoter of *Cbs*, a gene whose

transcripts levels are downregulated by *Sin3A* knockdown (Gajan et al., 2016; Saha et al., 2016), suggesting that SIN3 220 is recruited at this gene via distinct mechanism.

The level of SIN3 220 reduction at genomic targets ranged from 1.5 to 2 fold. Although approximately 75% of Caf1-55 was lost upon RNAi treatment, the remaining Caf1-55 might be able to recruit SIN3 at gene targets, which could explain why there is not a more robust loss of SIN3 220 at genomic loci. Alternatively, it is also possible that other SIN3 complex components play a synergistic role with Caf1-55 to recruit SIN3 to chromatin. Previously, we have demonstrated that *lid* knockdown affects SIN3 220 localization at gene targets (Gajan, 2015). Therefore, it will be interesting to study the effect of double knockdown of *Caf1-55* and *lid* on the recruitment of SIN3 220 to chromatin. Our data indicate that Caf1-55 facilitates the recruitment of SIN3 220, albeit partially.

Loss of *Caf1-55* affects expression of a subset of SIN3 220 regulated genes

To test whether Caf1-55 is required for the SIN3 220 complex gene regulatory activity, we measured gene expression changes upon *Caf1-55* RNAi. Total RNA was prepared from S2 cells treated with *Caf1-55* or GFP dsRNA as a control. cDNA was prepared from total RNA using a reverse transcriptase reaction. We carried out real-time PCR (RT-qPCR) to measure the relative expression of genes that have been previously demonstrated to be regulated by SIN3 220 (Barnes et al., 2010; Gajan et al., 2016; Pile et al., 2003; Saha et al., 2016). We analyzed the expression of *CG1969, Sam-S, MME1, PyK, Cyt-c1, Ahcy13, CG10623* and *Cbs*. All of these genes except *Cbs* are upregulated when the level of SIN3 is reduced compared to wild type (Gajan et al., 2016; Pile et al., 2003; Saha et al., 2016). Reduction of Caf1-55 led to a subtle upregulation of a subset of

genes tested (Figure 4.3). These data are consistent with the recruitment data. Interestingly, reduction of SIN3 220 leads to downregulation of *Cbs* expression (Gajan et al., 2016; Pile et al., 2003; Saha et al., 2016)**,** while reduction of Caf1-55 led to a slight upregulation of *Cbs.* These results suggest that SIN3 220 and Caf1-55 regulate this gene via differing mechanisms compared to the genes upregulated upon reduction of SIN3.

Caf1-55 knockdown did not result in a robust change in gene expression level. In fact, for several of the genes tested, the level of mRNA transcripts did not show a statistically significant alteration upon reduction of Caf1-55 compared to the control. We speculate that this moderate level of expression change is due to an incomplete loss of SIN3 220 binding from the gene targets when the level of Caf1-55 was reduced (Figure 4.2C)

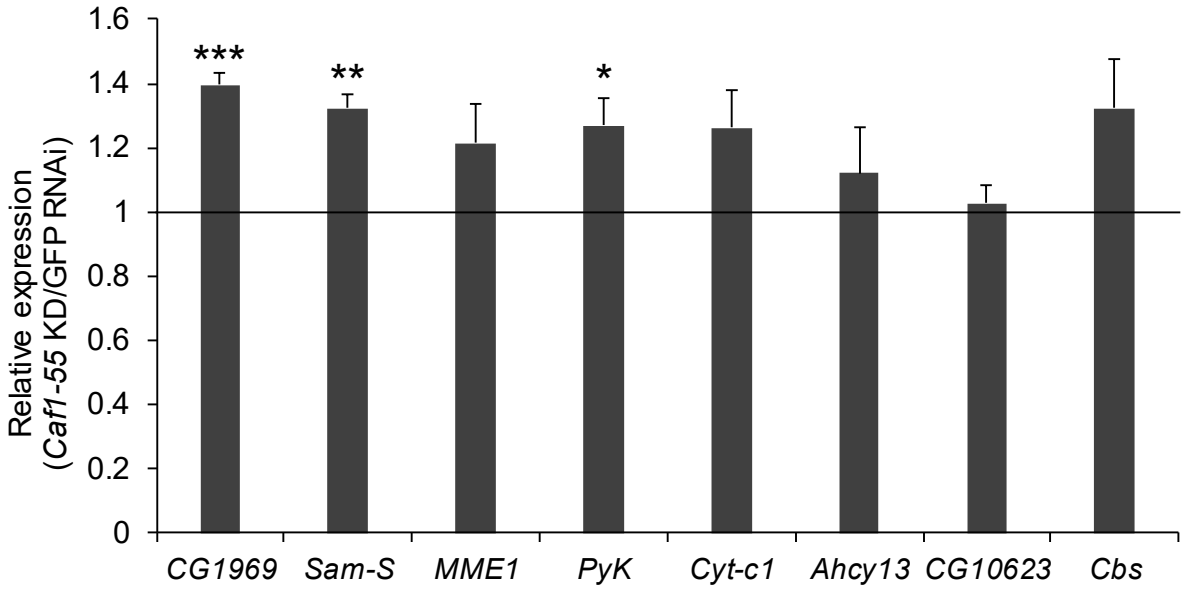

Figure 4.3: Knockdown of *Caf1-55* affects expression of a subset of SIN3 220 gene targets. RT-qPCR analysis measuring expression of genes upon *Caf1-55* knockdown compared to cells treated with GFP dsRNA. The *Taf1* gene was used normalize mRNA level. Error bars represent standard error of mean (n=3). * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

Caf1-55 moderately affects the histone H3K4me3 mark at SIN3 220 target genes

As loss of Caf1-55 impacts recruitment of SIN3 to target genes, we asked whether *Caf1-55* knockdown affects histone modifications. The SIN3 220 complex contains the HDAC RPD3 and the H3K4me3 HDM dKDM5/LID (Spain et al., 2010). We predicted that reduction in the enrichment of SIN3 220 at target sites would lead to an increase in transcriptionally active histone marks. To investigate the effect of *Caf1-55* RNAi on the histone marks H3K9ac and H3K4me3, we performed a ChIP-qPCR assay. S2 cells were treated with *Caf1-55* dsRNA and the knockdown efficiency was verified by western blot analysis. As a control, we performed ChIP on chromatin prepared from cells treated with GFP dsRNA. We measured the enrichment of histone H3 and histone marks H3K9ac and H3K4me3 at the promoter region of two genes, *Sam-S* and *MME1*. *ds* was used as a negative control. We did not observe a difference in the enrichment of histone H3 upon *Caf1-55* knockdown compared to control (Figure 4.4A). Unexpectedly, reduction of *Caf1-55* did not affect enrichment of the histone mark H3K9ac (Figure 4.4B). The histone mark H3K4me3, however, was affected as predicted. Loss of *Caf1-55* led to a slight increase in the level of the histone mark H3K4me3 at the promoters of *Sam-S* and *MME1* (Figure 4.4C). Although SIN3 binding was impacted upon reduction of Caf1-55, we did not observe a robust alteration in the level of histone marks. We speculate that the loss of Caf1-55 does not completely abolish SIN3 localization to chromatin and therefore has a very mild effect on histone modifications.

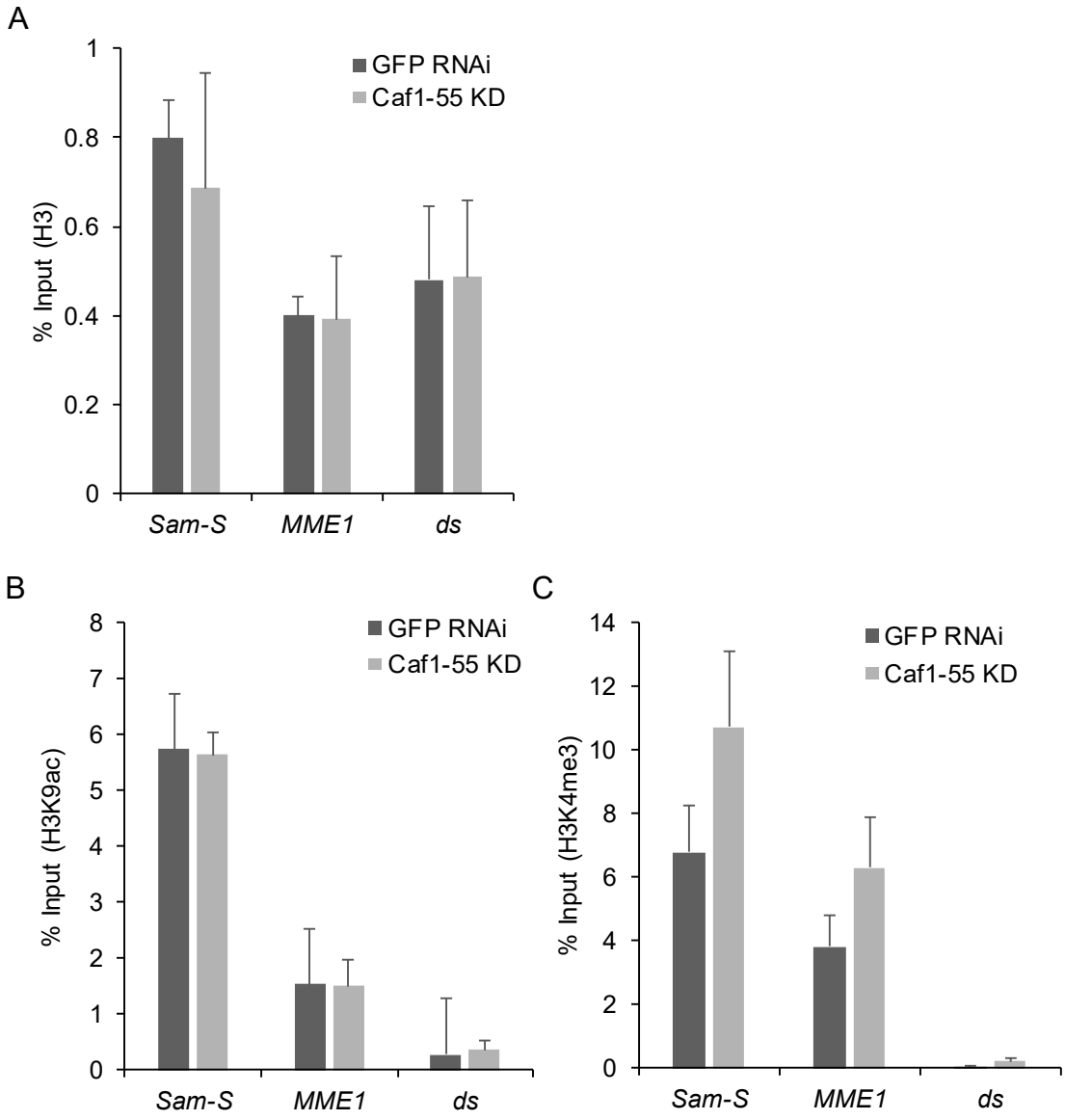

Figure 4.4: Loss of Caf1-55 has a subtle effect on histone H3K9ac and H3K4me3. ChIP-qPCR analysis was carried out to measure the relative enrichment of histone H3 (A) or histone H3K9ac (B) or histone H3K4me3 post *Caf1-55* knockdown at SIN3 220 target genes. *ds* was used as a negative control. Error bars represent standard error of mean from three independent biological experiments.

Caf1-55 physically associates with the unique C-terminal region of SIN3 220.

Previously, we demonstrated that Caf1-55 preferentially interacts with the SIN3 220 complex, one of the two SIN3 complexes in *Drosophila (Spain et al., 2010)*. Structurally, SIN3 220 and SIN3 187 differ only at their C-terminal regions. SIN3 220 has 315 amino acids at the C-terminal that are encoded by two unique exons (Pennetta and Pauli, 1998). These amino acids are absent from the C-terminus of the SIN3 187 isoform (Figure 4.5A). Therefore, we hypothesized that the unique C-terminal region (UniC) of SIN3 220 interacts with Caf1-55. We cloned a cDNA encoding *UniC* with an HA tag into the pRHMA-4 vector. We transiently transfected Caf1-55-HA/FLAG stable S2 cells with the UniC construct and carried out immunoprecipitation experiments using antibody-conjugated resin targeting the FLAG tag. Immunoprecipitated protein samples were analyzed by western blot. The western blot was probed with antibody targeting the HA tag allowing the detection of both UniC and Caf1-55. As predicted, we found that Caf1-55 could efficiently pull down UniC (Figure 4.5B). This interaction between UniC and Caf1-55 was specific as we did not detect any interaction between dKDM5/LID and UniC (data not shown). In addition, we performed a reverse co-immunoprecipitation assay to demonstrate the interaction between UniC and Caf1-55. We transiently transfected S2 cells with the UniC-HA expression construct and performed an immunoprecipation using HA antibody-conjugated resin. The western blot was probed with antibodies against Caf1-55 and the HA tag. Our data clearly demonstrate that that UniC efficiently immunoprecipitated Caf1-55 in S2 cells (Figure 4.5C). Together, these data indicate that Caf1-55 interacts with the C-terminal region of SIN3 220.

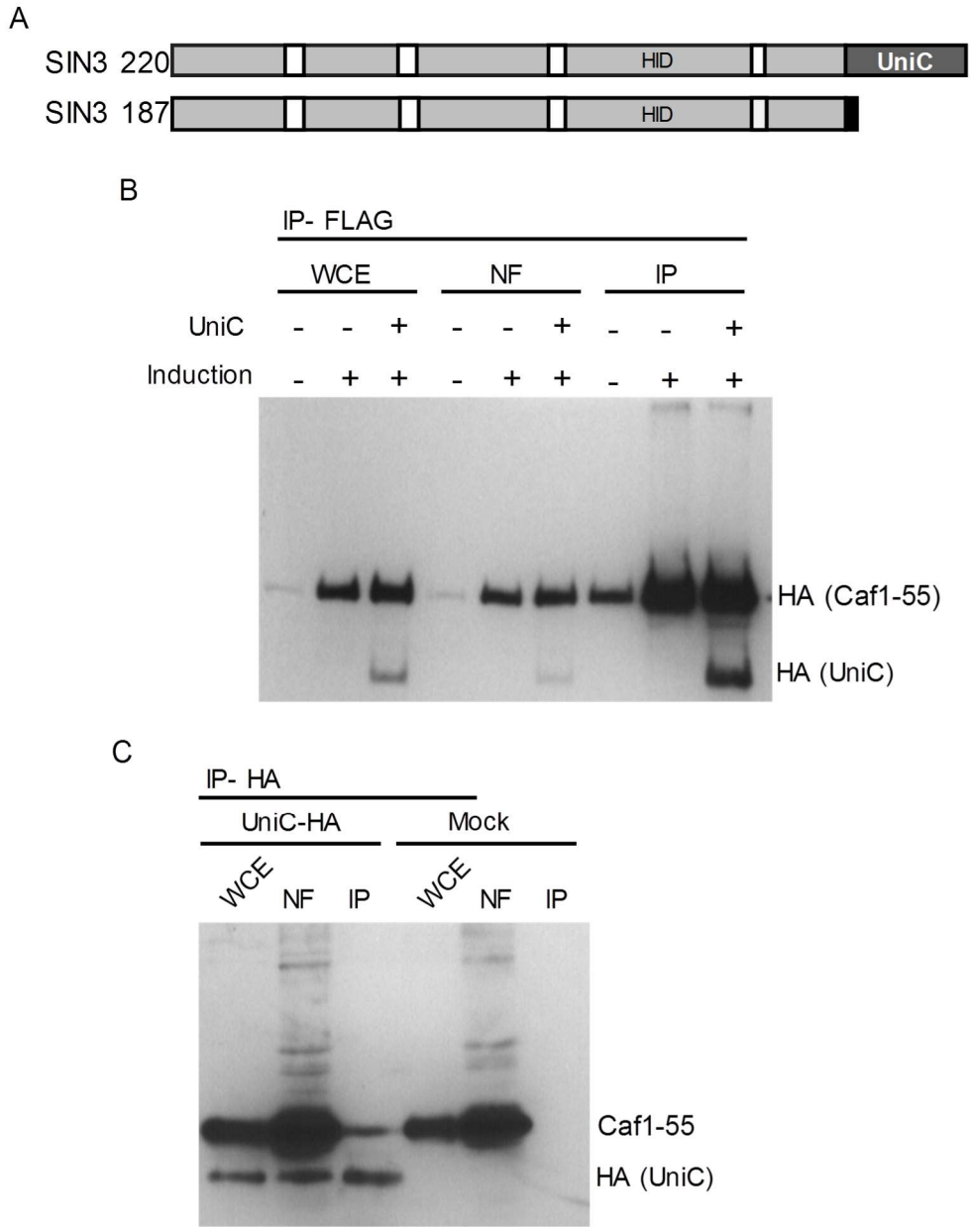

Figure 4.5: Unique C-terminal domain of SIN3 220 interacts with Caf1-55. (A) Illustration of the structure of two SIN3 isoforms. White boxes indicate paired amphiphatic helix domains, light grey indicates exons shared by SIN3 isoforms, black indicates SIN3 187 specific exon, dark grey indicates SIN3 220 specific exons (UniC). HID, histone deacetylase interaction domain. (B), (C) Immunoprecipitation assays were performed using antibody as labelled. Caf1-55 FLAG/HA (B) or S2 (C) cells were transfected with plasmids as indicated. Western blots were probed with antibodies as listed. These experiments have been performed a minimum of three times. WCE - whole cell extract, NF - Nuclear fraction, IP - Immunoprecipitation.

To investigate whether Caf1-55 directly interacts with UniC biochemically, we utilized a bacterial expression system to express recombinant Caf1-55 and UniC, followed by immunoprecipitation assays. cDNA encoding *Caf1-55* and *UniC* were cloned into the pET28-a and pMAL-c2X vectors, respectively. While encoded Caf1-55 has 6X histidine tag, UniC has a MBP/HA tag at its terminus. We co-transformed *Escherichia coli* BL-21(DE3) cells with a mixture of Caf1-55 and UniC expression plasmids. Resulting colonies were screened based on the two different antibiotic selectable markers present on the two expression vectors. We used auto-induction method to express the recombinant proteins (Studier, 2005). Soluble fraction was prepared from bacterial cells, which contained the recombinant protein. We carried out an immunoprecipitation assay by incubating the soluble fraction with resin conjugated with HA antibody. As a negative

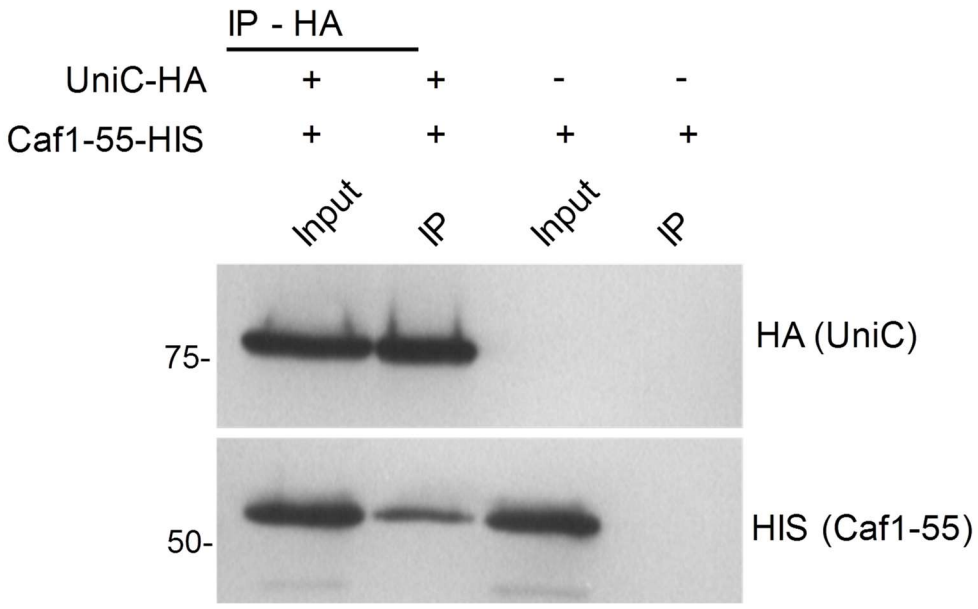

Figure 4.6: Caf1-55 physically associates with the unique C-terminal domain of SIN3 220. Immunoprecipitation assay was performed using HA antibody. *E.coli* BL21 (DE3) cells were transformed with plasmids as listed. Western blot was probed with antibody as indicated. These experiments have been performed for a minimum of three times. IP – Immunoprecipitation.

control, we performed immunoprecipitation assay using the soluble fraction containing HIS tagged Caf1-55 only. Western blot analysis of the immunoprecipitated fractions showed that UniC efficiently interacted with Caf1-55 (Figure 4.6). On the other hand, we did not observe Caf1-55 in the control pull down experiment (Figure 4.6). These data establish that the unique C-terminal region of SIN3 220 acts as a surface that facilitates interaction with Caf1-55.

The histone H4 binding pocket of Caf1-55 is partially required to interact with SIN3 220

An *in vitro* structural analysis identified domains of Caf1-55 critical for interaction with histone H3 and H4 (Nowak et al., 2011). Amino acids Asp-362, Asp-365 and Glu-235, Asp-252, Glu-279 in the Caf1-55 sequence are essential for interacting with histones H4 and H3, respectively (Nowak et al., 2011). Interestingly, the histone H4 binding pocket of Caf1-55 also interacts with Su(z)12, a component of the polycomb group repressive complex (Nowak et al., 2011; Schmitges et al., 2011), suggesting that the histone H4 binding domain of Caf1-55 might be a domain that interacts with several distinct proteins. Therefore, we hypothesized that the histone H4 binding region of Caf1-55 interacts with SIN3 220. To generate histone H4 binding mutant of Caf1-55 (Caf1-55 H4M), we performed site-directed mutagenesis to change Glu-361, Asp-362A, Glu-364 and Asp-365 to alanine. Using a similar approach, we mutated Glu-235 to glutamine, Asp-252 to lysine and Glu-279 to glutamine to generate a cDNA construct for a histone H3 binding mutant of Caf1-55 (Caf1-55 H3M). Site-directed mutagenesis was performed on the Caf1-55 WT plasmid obtained from BDGP. By transfecting S2 with these mutated plasmids, we generated two inducible stable cell lines to ectopically express HA-FLAG tagged Caf1-

55 H4M or Caf1-55 H3M. Western blot analysis of whole cell extract prepared from S2 cells that express wild type or mutant forms of Caf1-55, indicated a robust expression of these proteins compared to the endogenous levels of Caf1-55 (Figure 4.7).

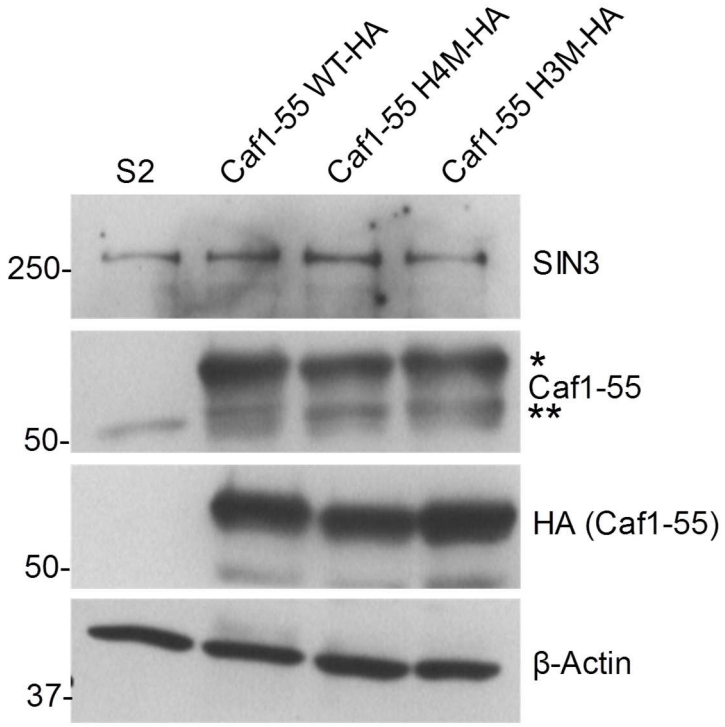

Figure 4.7: Expression of wild type and mutant forms of Caf1-55. Western blot analysis of whole cell extract prepared from stable cell lines expressing Caf1-55 WT-HA, Caf1-55 H4M-HA or Caf1-55 H3M-HA. Extract prepared from S2 cells was used as a control. Antibodies used to probe the blot are listed on the side. * FLAG/HA tagged Caf1-55, ** Endogenous Caf1-55.

We carried out immunoprecipitation assays using an antibody against the FLAG tag to pull down wild type Caf1-55 (Caf1-55 WT), Caf1-55 H4M or Caf1-55 H3M. Immunoprecipitation of proteins in nuclear extract prepared from S2 cells that lack a FLAG-tagged protein was used as a negative control. Bound fractions were analyzed by western blot. Wild type and mutant forms of Caf1-55 were efficiently pulled down by as indicated by HA antibody probe (Figure 4.8A, B). We did not detect any HA-tagged protein

in the IP fraction from S2 cells that lacked HA-tagged proteins (Figure 4.8A, B). As previously demonstrated (Spain et al., 2010), we observed a robust interaction of SIN3 and RPD3 with Caf1-55 WT (Figure 4.8A, B). Interestingly, the interactions between SIN3

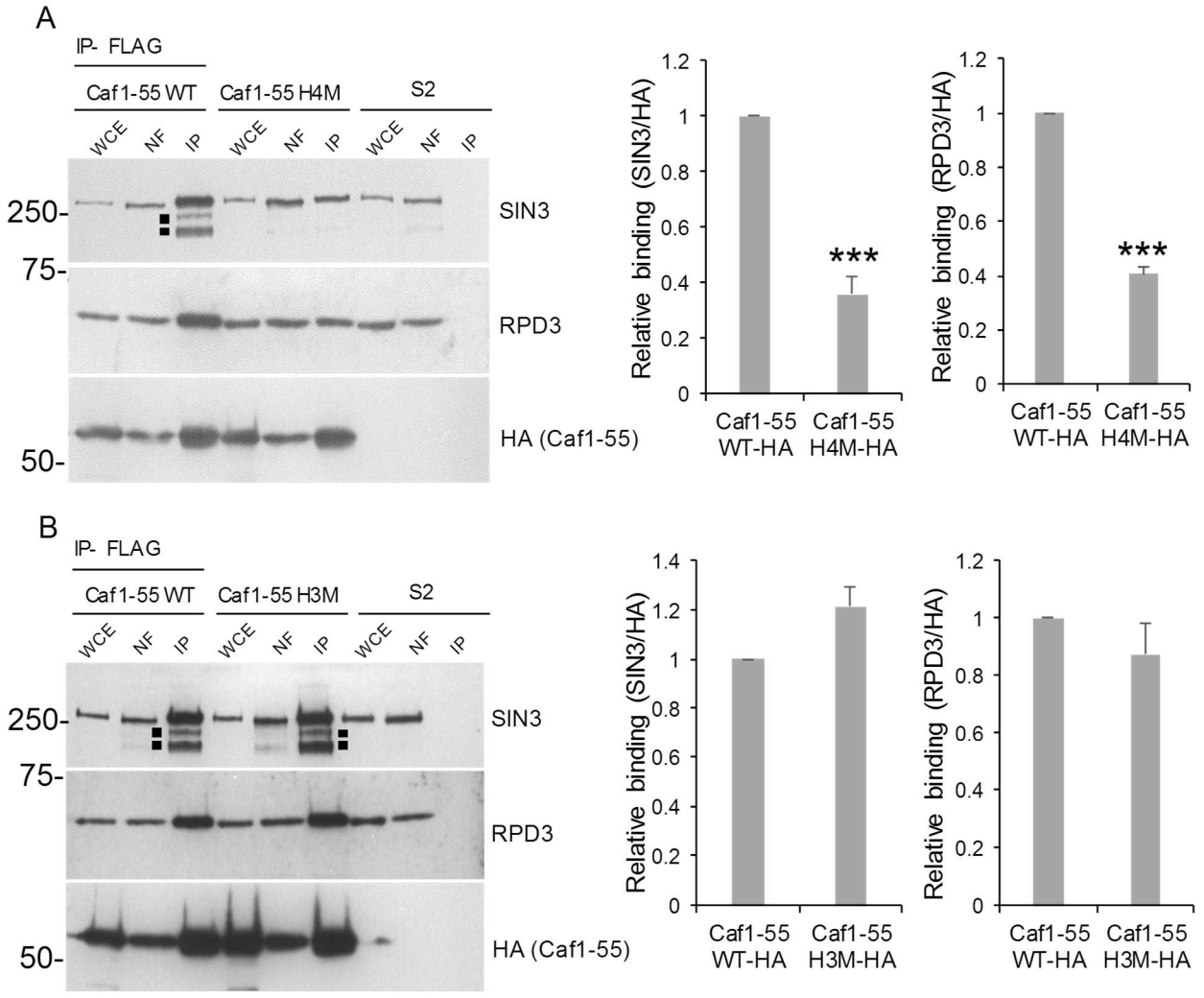

Figure 4.8: The histone H4 binding pocket of Caf1-55 interacts with SIN3 220. Investigation of the interaction between Caf1-55 H4M (A) or Caf1-55 H3M (B) with SIN3 or RPD3 using immunoprecipitation assay (IP). The immunoprecipitation was performed using antibody against FLAG. Stable cells expressing HA/FLAG tagged wild type or mutant forms of Caf1-55 are indicated. S2 cells were used as a control. Western blots were probed with antibodies as listed. These experiments were performed at least three times. WCE - whole cell extract, NF - Nuclear fraction, IP - Immunoprecipitation. The relative level of the reduction of interaction between mutants of Caf1-55 and SIN3 or RPD3 is shown in the right panel. Black squares indicate degradation products of SIN3 220 and therefore not used for quantification. *** P-Value < 0.001.

and RPD3 with Caf1-55 H4M were reduced drastically (Figure 4.8A). Mutation of the histone H3 binding domain of Caf1-55, however, did not impact the association of SIN3 and RPD3 with Caf1-55 (Figure 4.8B). Mutation of the histone H4 binding domain of Caf1-55 led to approximately 2.5-fold reduction in the level of interaction with both SIN3 and RPD3 (Figure 4.8B). We did not observe a complete loss of the interaction between Caf1-55 H4M and SIN3 and RPD3. This result strongly suggests that other amino acids are involved in mediating these interactions. The results of this assay show that the amino acids of Caf1-55 known to interact with the histone H4 are important but not essential for interaction between Caf1-55 and SIN3 and RPD3.

Expression of SIN3 220 gene targets is not affected upon induction of Caf1-55 H4M

The interaction between Caf1-55 and SIN3 220 was significantly reduced upon mutating the histone H4 binding domain of Caf1-55. Based on this finding, we asked whether overexpression of Caf1-55 H4M will affect expression of genes regulated by SIN3 220, which in turn would indicate an effect on SIN3 220 recruitment to gene targets. Firstly, we analyzed the effect on Caf1-55 localization to chromatin upon mutating the histone H4 binding pocket. We prepared chromatin from stable cell lines expressing HA-tagged Caf1-55 WT and Caf1-55 H4M proteins. ChIP was performed using HA antibody conjugated resin. ChIP on chromatin extracted from S2 cells, which does not express any HA-tagged protein, was used as the non-specific ChIP control. We analyzed binding of Caf1-55 at *MME1, Sam-S, Ahcy13, CG10623*, *Cbs* and *ds.* No significant difference comparing recruitment of wild type to mutant Caf1-55 was observed. This analysis indicates that the amino acids of Caf1-55, previously shown to be important for the interaction to H4 (Nowak et al. 2011), were not essential for recruitment of Caf1-55 to

chromatin (Figure 4.9). Since this mutant form of Caf1-55 still localizes to chromatin, we wondered whether the recruitment of SIN3 220 to chromatin is mediated via interaction with the histone H4 binding pocket of Caf1-55.

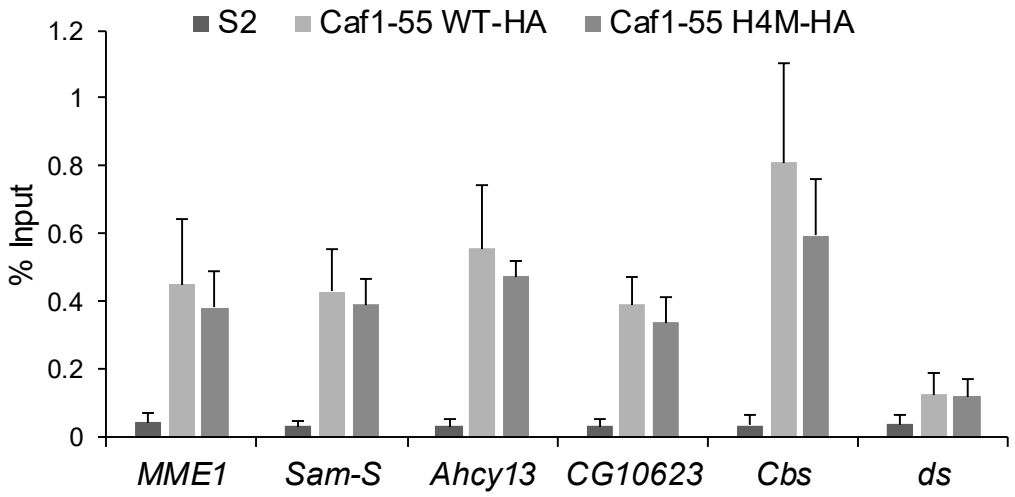

Figure 4.9: Localization of Caf1-55 to chromatin does not require histones H4. HA antibody-conjugated resin was used to perform ChIP on chromatin prepared from cells expressing Caf1-55 H4M-HA or wild type Caf1-55 (Caf1-55 WT-HA). Chromatin from S2 cells was used as a non-specific ChIP control. Error bars represent standard error of mean from three independent biological experiments.

We investigated whether ectopic expression of Caf1-55 H4M impacts expression of genes known to be regulated by SIN3 220. We performed RT-qPCR on cells that express Caf1-55 WT or Caf1-55 H4M. Total RNA extracted from S2 cells, which do no express any tagged protein, was used as a control. qPCR was performed using primes to target four genes, *Sam-S, Ahcy13, CG10623* and *Cbs*. To our surprise, we found that overexpression of Caf1-55 H4M did not alter the expression of the genes compared to the expression in cells with wild type Caf1-55 (Figure 4.10). These data suggest that expression of Caf1-55 H4M does not impact the recruitment of SIN3 220 to gene targets.

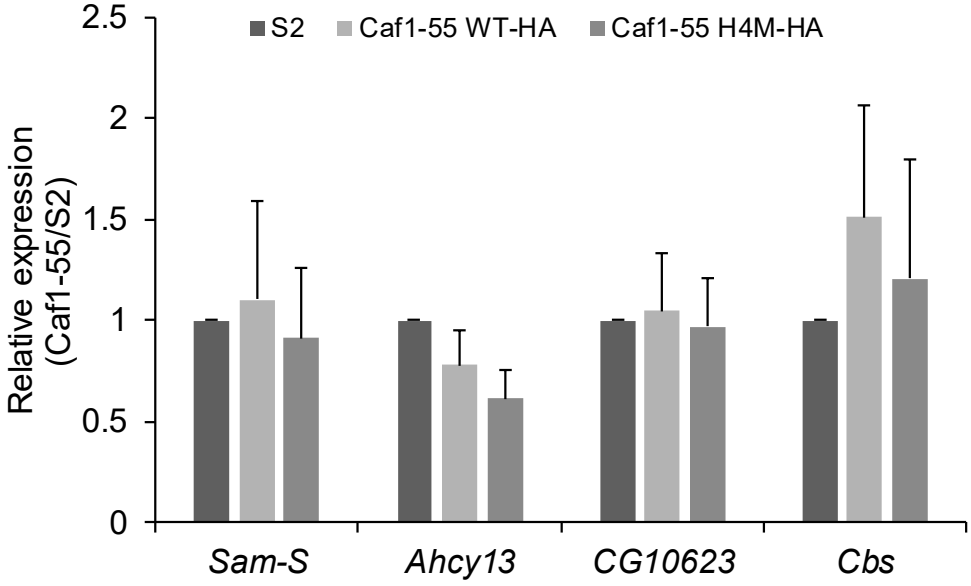

Figure 4.10: Mutations of Caf1-55 at the histone H4 binding pocket has no effect on expression of SIN3 220 gene targets. RT-qPCR analysis was carried out on total RNA prepared from cells expressing Caf1-55 WT-HA or Caf1-55 H4M-HA. Total RNA from S2 cells was used as a control. Error bars represent standard error of mean from at least three independent biological replicates. The *Taf1* gene was used to normalize mRNA level. P-value > 0.05.

To test this, we performed SIN3 ChIP upon ectopic expression of Caf1-55 H4M and compared to Caf1-55 WT data. Our preliminary data (not shown) suggest that the recruitment of SIN3 220 to chromatin was not affected due to ectopic expression Caf1-55 H4M. It is possible that endogenous Caf1-55 present in these cell lines facilitates SIN3 220 localization on chromatin. Ectopic expression of Caf1-55 WT or Caf1-55 H4M, however, is extremely robust and very likely replaces endogenous protein (Figure 4.7). We speculate that the partial loss of interaction between Caf1-55 H4M and SIN3 220 is not sufficient to hinder recruitment of SIN3 220 at gene targets. Together, these data indicate that the domain of Caf1-55 that we predicted to interact with SIN3 220 may not be essential for localization of SIN3 220 at chromatin.

Histone H3 binding domain is not required for localization of Caf1-55 to chromatin

We showed that the interaction between Caf1-55 and SIN3 220 was not affected upon the mutation of the histone H3 binding motif of Caf1-55. The *in vitro* data demonstrated that this mutation impacts association of Caf1-55 with histone H3 (Nowak et al., 2011; Schmitges et al., 2011). We therefore hypothesized that Caf1-55 might be anchored to chromatin via the histone H3 binding motif to facilitate recruitment of SIN3 220 to target genes. Firstly, we investigated the effect on the localization of Caf1-55 to chromatin upon mutating the histone H3 binding motif. We performed ChIP using HA antibody conjugated resin on chromatin prepared from stable cell lines expressing either

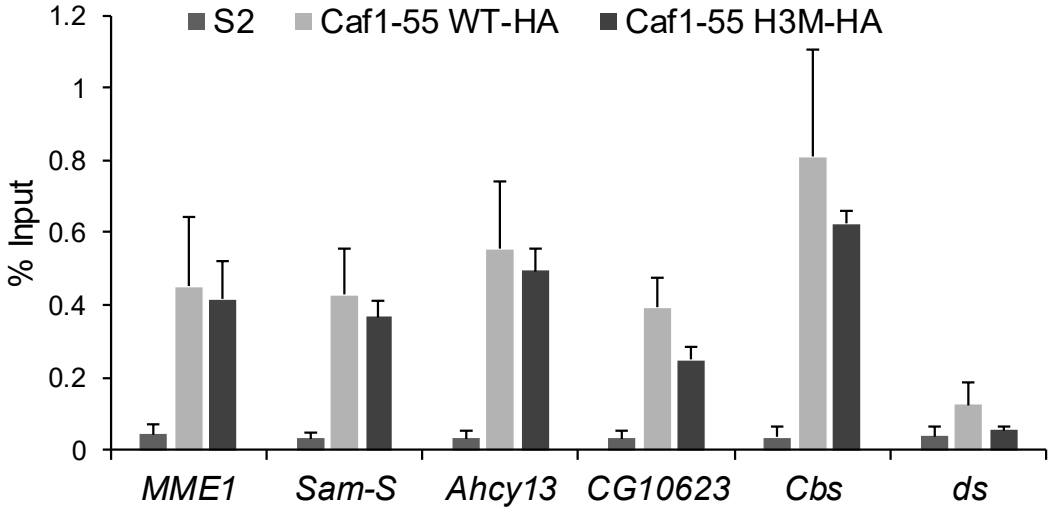

Figure 4.11: Mutation of the histone H3 binding motif has no effect on localization of Caf1-55 to chromatin. ChIP-qPCR was performed using HA antibody-conjugated resin to study the recruitment of Caf1-55 upon mutating the histone H3 binding motif. Chromatin was prepared from cells expressing Caf1-55 H3M-HA and Caf1-55 WT-HA. S2 cells was used as a control. Error bars represent standard error of mean from three independent biological experiments.

HA-tagged Caf1-55 WT or Caf1-55 H3M. ChIP that was carried out on chromatin prepared from S2 cells was used a nonspecific ChIP control. Surprisingly, we did not observe any effect on the localization of mutant Caf1-55 at target genes as predicted from the *in vitro* studies (Figure 4.11). These data suggest that Caf1-55 localizes to chromatin via another domain other than the histone H3 binding motif. These data also suggested that expression of Caf1-55 H3M would not affect recruitment of SIN3 220 to target genes as well as the expression of those genes. We measured the changes in expression of SIN3 220 target genes in the presence of Caf1-55 H3M by RT-qPCR. As expected, we found that induction of Caf1-55 H3M did not affect the expression of known targets of

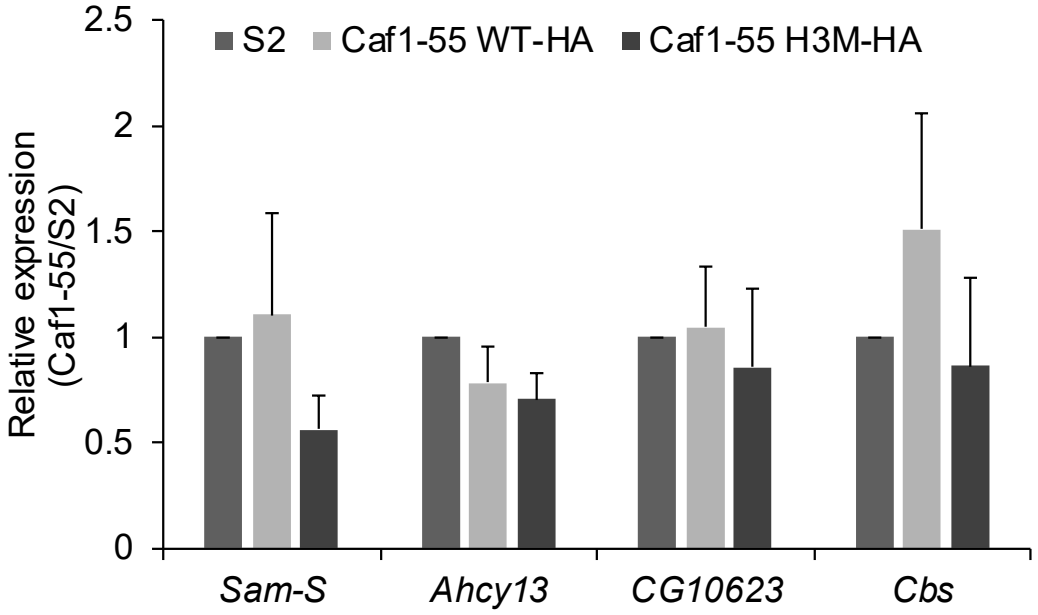

Figure 4.12: Effect of Caf1-55 H3M on expression of SIN3 220 gene targets. RT-qPCR analysis on cells expressing Caf1-55 WT-HA or Caf1-55 H3M-HA. RT-qPCR on total RNA extracted from S2 cells was used as a control. Error bars represent standard error of mean from at least three independent biological replicates. The *Taf1* gene was used to normalize mRNA level. P-value > 0.05

SIN3 220 (Figure 4.12) and we speculate that it is due to maintenance of SIN3 220 at those sites.

Conclusion

In summary, we have demonstrated that Caf1-55 localizes to the gene targets of SIN3 220. Knockdown of Caf1-55 impacts localization of SIN3 220 at target genes and affects expression of some of these genes. Using both the *Drosophila* cell culture system and the bacterial expression system we found that the unique C-terminal region of SIN3 220 physically interacts with Caf1-55. Interestingly, we found that mutation at the histone H4 binding pocket of Caf1-55 negatively affects the interaction between SIN3 220 and Caf1-55. Su(z)12 has also been demonstrated to interact with Caf1-55 at the region that binds histone H4 (Nowak et al., 2011; Schmitges et al., 2011), indicating that the domain previously considered to associate with histone H4 is a multi-protein associating region. In addition, our data demonstrate that the histone H3 interacting domain does not interact with SIN3 220. To our surprise, we found that mutations of either of the histone binding domains does not affect Caf1-55 localization to chromatin. Although mutation of the histone H4 binding pocket of Caf1-55 reduces the interaction with SIN3 220 significantly, it does not affect expression of genes regulated by SIN3 220. These data suggest that another domain of Caf1-55, which interacts with SIN3 220 facilitates the recruitment of SIN3 220 to chromatin. Together, our data demonstrate an important but not essential function of Caf1-55 in modulating the gene regulatory activity of the SIN3 220 complex.

CHAPTER 5 FUTURE DIRECTIONS

In my PhD thesis research, I have identified binding sites of the SIN3 isoforms across the *Drosophila* genome. The data demonstrate that a large majority of the gene targets of SIN3 220 are also targeted by SIN3 187 and expression of SIN3 187 leads to a reduction in the level of SIN3 220. Together, these data suggest that SIN3 220 occupancy is lost upon induction of SIN3 187 and genomic targets, which were initially occupied by the SIN3 220 isoform, become SIN3 187 targets. Furthermore, we performed global transcriptome analysis to identify the genes regulated by the SIN3 isoforms. Gene ontology analysis indicates that SIN3 220 and SIN3 187 regulate similar categories of genes such as cell cycle and metabolism. Interestingly, SIN3 187 additionally regulates genes involved in post-embryonic morphogenesis and apoptosis. These results are intriguing as SIN3 187 alone cannot rescue lethality in flies caused by the loss of *Sin3A* (Spain et al., 2010). However, the percent of survival is increased when both the 187 and 220 isoforms are used in rescue compared to SIN3 220 alone (Spain et al., 2010). Therefore, a major objective in the future is to understand the role of the SIN3 isoforms in regulating biological processes during the course of development in *Drosophila*. I briefly describe several approaches to answer some outstanding questions.

Generation of fly lines to express either the SIN3 187 isoform or the SIN3 220 isoform.

To understand how the SIN3 isoforms function during fly development, we will generate fly lines that can solely express either SIN3 187 or SIN3 220. Here, I propose the utilization of the CRISPR/Cas9 system to conditionally or ubiquitously express either SIN3 187 or SIN3 220. Using this system, we can knockout multiple SIN3 isoforms and

leave the gene intact to express only one isoform, which is the subject of our study. The donor plasmid will be designed to insert of an epitope tag at the C-terminal end of either SIN3 187 or SIN3 220. In addition, we will design the constructs to insert two FRT sequences, flanking unique exons of the SIN3 190 isoform and the epitope tagged SIN3 isoforms (Figure 5.1). These flies will be crossed to flies expressing Flippase enzyme (FLP). Expression of FLP will result in the homologous recombination of the two FRT sites. This will result in the loss of expression of SIN3 190 and the tagged isoform while leaving the endogenous isoform unaltered. The procedure is illustrated in Figure 5.1. Using heatshock-mediated FLP expression, we will be able to conditionally express only SIN3 187 or SIN3 220, which will provide a system to study its role.

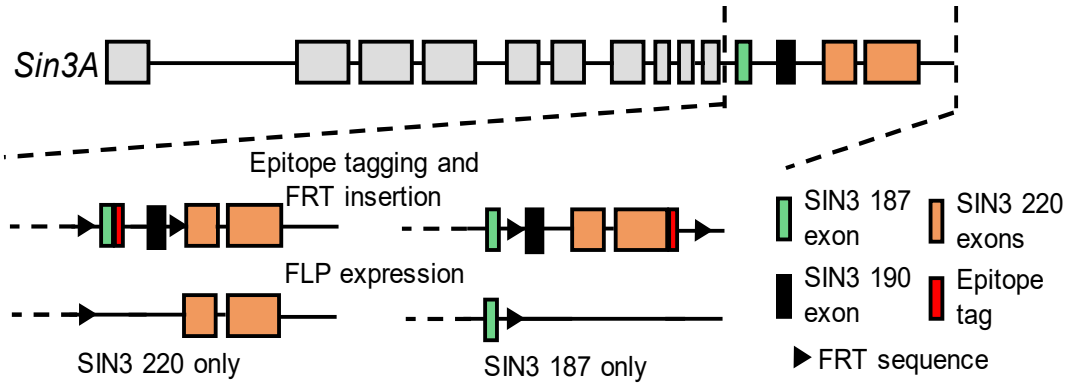

Figure 5.1: Schematic illustration for generation of fly lines to express either SIN3 187 or SIN3 220 by CRISPR/Cas9 mediated gene engineering. FLP; Flippase.

Does loss of SIN3 isoforms affect embryonic to larval morphogenesis?

In this research, we have demonstrated that SIN3 187 and not SIN3 220 regulates genes that are involved in post-embryonic development. Based on these results, we hypothesize that SIN3 187 controls the process of transition from the embryonic stage to the larval stage of development. Using CRISPR/Cas9 to conditionally knockout SIN3

isoforms, we will monitor whether expression of only SIN3 187 or SIN3 220 can support the transition from embryo to larva. In wild type organisms, the process of embryogenesis takes approximately 22 hr and can be divided into 17 stages. Heatshock of embryos post egg laying will be performed to knock out SIN3 isoforms and the time of transition from the embryonic stage to the larval stage will be measured. These experiments will indicate whether SIN3 187 affects post-embryonic development in flies. In addition, we can measure gene expression changes upon loss of either of the SIN3 isoforms during the last stage of embryogenesis. We will perform RNAseq on the total RNA extracted from the stage 17 embryo, which has had a SIN3 isoform knocked out with the wild type condition serving as a control.

Role of SIN3 isoforms in cell proliferation

SIN3 has been demonstrated to regulate cell proliferation (Cowley et al., 2005; Dannenberg et al., 2005; Pile et al., 2002; Swaminathan and Pile, 2010; White et al., 2009). In *Drosophila*, whether all of the SIN3 isoforms regulate cell cycle processes is unclear. Several pieces of evidence suggest that SIN3 220 plays the essential role in cell proliferation. Wing imaginal discs, which consist of proliferative cells, predominantly express SIN3 220 (Sharma et al., 2008). Additionally, in S2 cultured cells SIN3 220 is the most prevalent isoform expressed. Knockdown of *Sin3A* results in a cell proliferation defect and arrest of cells at the G2/M phase of the cell cycle (Pile et al., 2002). Clonal analysis in wing imaginal discs demonstrated that *Sin3A* knockdown results in cell cycle defect (Swaminathan and Pile, 2010). Consistent with the cell cycle defect phenotype, we demonstrated that SIN3 220 regulates genes involved in cell cycle both in this study as well as in a previous study (Gajan et al., 2016). In addition to the role of SIN3 220 in the

cell cycle, our transcriptome data indicates that SIN3 187 also regulates genes involved in cell cycle. Therefore, we want to test whether the processes of the cell cycle are subjected to regulation by the SIN3 220 isoform and/or the SIN3 187 isoform. We can answer this question by integrating the CRISPR/Cas9 system with the fly FUCCI system. Fly FUCCI is a novel approach to study cell cycle regulation (Zielke et al., 2014). In this system, different fluorochromes can be assigned to cells depending on specific stages of cell cycle. To investigate the role of SIN3 isoforms in cell proliferation, we can use the CRISPR/Cas9 system to express either of the SIN3 isoforms as described in the previous section, and use the FUCCI system to monitor cell cycle progression. This experiment is predicted to provide a definitive answer as to the isoforms responsible for cell cycle regulation.

What are the mechanisms of gene regulation by the SIN3 isoforms?

To understand the mechanism of gene regulation by the SIN3 isoforms, we plan to identify enriched DNA sequence motifs in the ChIP-seq dataset for both SIN3 isoforms. Motif analysis will be performed using the MEME software suite. Based on the motif analysis, we will be able to use the modENCODE transcription factor ChIP-seq datasets to identify transcription factors that have overlapping binding sites with the SIN3 isoforms. We can then use a candidate factor approach to test how the identified transcription factors may modulate the activity of the SIN3 isoform specific complexes. We would be able to use tools such as RNAi, ChIP-qPCR to study the role of different transcription factors influencing the function of the SIN3 isoforms.

A previous study from our laboratory showed by western blot analysis that the SIN3 isoforms differentially affect global histone modifications. It is still not well studied,

however, how the SIN3 isoforms influence different histone PTMs at chromatin sites, which may contribute to the distinct method of gene regulation by the SIN3 isoforms. More interestingly, while the SIN3 220 isoform specific complex contains both a histone deacetylase (RPD3) and demethylase (dKDM5/LID), the SIN3 187 complex has only the deacetylase component (Spain et al., 2010). Thus we predict that the SIN3 isoforms regulate gene expression by modulating distinct histone PTMs. To test this hypothesis, we can to perform ChIP-seq analysis of the histone marks H3K9ac, H3K14ac, H3K27ac and H3K4me3 upon altering the levels of SIN3 isoforms using the S2 cell line system described in Chapter 3.

Interestingly, our study demonstrates that several genes are activated by the SIN3 isoforms. These findings are unexpected as SIN3 histone modifying complexes are generally considered as corepressor complexes. To investigate the mechanism by which genes are activated by SIN3, we will map the binding of RPD3 upon altering the levels of the SIN3 isoforms. Since RPD3 has the deacetylase activity, we predict an effect by RPD3 at genes that are induced by the SIN3 isoforms. In addition, identification of the chromatin factors and mapping of histone PTMs as discussed above will potentially contribute to our understanding of the mechanism by which SIN3 acts as a transcriptional activator.

How does SIN3 187 expression effect Caf1-55 localization?

In this research, we demonstrate that expression of SIN3 187 leads to reduction of the level of SIN3 220 protein. In addition, SIN3 187 replaces SIN3 220 at genomic targets. The fate of the unique components of the SIN3 220 complex at chromatin, however, is unclear. To test if SIN3 187 affects localization of Caf1-55, a unique component of the SIN3 220 complex, we will carry out ChIP-qPCR using an antibody

targeting Caf1-55 upon expression of the SIN3 187 isoform and subsequent decrease of SIN3 220. Chromatin from cells that do not express SIN3 187 will be used as a control. This study can be further extended to test the localization of dKDM5/LID and EMSY upon expression of SIN3 187 at chromatin, as these are also unique components of the 220 complex. If the enrichment of Caf1-55, dKDM5/LID or EMSY is reduced at genomic loci, it will indicate that SIN3 187 affects localization of the SIN3 220 specific complex components. These results will contribute to our understanding of the mechanism by which the SIN3 isoforms modulate gene expression in distinct ways.

I investigated the role of Caf1-55 in modulating the binding and gene regulatory activity of the SIN3 220 complex (Chapter 4). I found that Caf1-55 is partly required for the recruitment of the SIN3 220 complex to some of the gene targets. In addition, my data demonstrate that SIN3 220 directly interacts with Caf1-55. It is not clear, however, whether recruitment of the SIN3 220 complex is the main function of Caf1-55. Below, I briefly describe several ongoing experiments that will allow further understanding of the regulatory activity of Caf1-55 in the SIN3 220 complex.

Generation of a Caf1-55 knock out cell line.

As described in Chapter 4, we observed an approximately 1.5 to 2.5 fold reduction of SIN3 220 binding from gene targets due to loss of Caf1-55, which is indicative of the importance, but not absolute requirement of Caf1-55, for SIN3 220 recruitment to chromatin. There are two possibilities to explain this observation. Firstly, Caf1-55 is a highly expressed protein in *Drosophila* S2 cells. The average knockdown efficiency of Caf1-55 upon RNAi treatment is approximately 75%. Thus, the remaining 25% of Caf1-55 may be sufficient to facilitate localization of SIN3 220. Alternatively, other complex

components along with Caf1-55 may be required for the process of recruiting SIN3 220. To test which of these possibilities is true, we can use CRISPR/Cas9 system to knockout Caf1-55 in S2 cells. To create a *Caf1-55* knockout cell line, we will follow the protocol described previously (Bassett et al., 2014). We can use this *Caf1-55* knockout cell line to interrogate how Caf1-55 modulates the gene regulatory activity of the SIN3 220 complex.

Does Caf1-55 affect SIN3 220 activity globally?

I have shown that the level of SIN3 220 is reduced at gene targets upon reduction of Caf1-55. ChIP-seq can be used to test if loss of *Caf1-55* affects SIN3 220 localization to chromatin globally. ChIP will be carried out using an antibody targeting SIN3 on chromatin prepared from *Caf1-55* knockout cells followed by deep sequencing. My preliminary ChIP-qPCR data imply that the level of SIN3 220 reduction varies between individual genes. Results of ChIP-seq experiments will provide information on the level of reduction of SIN3 220 at all sites, which can be used to ask whether Caf1-55 is of high importance for a subset of SIN3 220 target genes.

Our preliminary data indicate that Caf1-55 modulates expression of a subset of SIN3 220 target genes. We will perform global transcriptome analysis upon *Caf1-55* knockout and compare results to that of the known SIN3 220 regulated gene targets. This study will determine whether Caf1-55 is required for SIN3 220 mediated gene regulation.

Identification of the motif on Caf1-55 that is required for interaction with SIN3 220.

This study has shown that a mutation in the histone H4 binding pocket of Caf1-55 significantly reduces, but does not abolish, the interaction with SIN3 220. These data suggest that other amino acids are required for mediating the interaction between Caf1-55 and SIN3 220. In addition, the mutation of the histone H4 binding pocket of Caf1-55

has no effect on genes regulated by SIN3 220 as indicated by gene expression analysis. To identify the domain of Caf1-55 that interacts with SIN3 220, we can express truncated Caf1-55 and perform immunoprecipitation assay to test the interaction between Caf1-55 and SIN3 220. We will perform ChIP and expression analysis to investigate whether expression of the truncated Caf1-55 mutants will influence the gene regulatory activity of SIN3 220 at target genes. We can perform ChIP using antibody against SIN3 upon expression of the truncated forms of Caf1-55. Using RT-qPCR, we can test whether truncated forms of Caf1-55 affect expression of SIN3 220 regulated genes. Furthermore, we plan to use X-Ray crystallography to solve the structure of the Caf1-55 and SIN3 220 interaction. These results will contribute to our understanding of the way in which SIN3 220 is recruited to chromatin via Caf1-55.

Does Caf1-55 regulate the activity of the SIN3 220 complex by mediating interaction with dKDM5/LID and EMSY?

Caf1-55, dKDM5/LID and EMSY preferentially interact with the SIN3 220 complex between the known SIN3 isoform specific complexes in *Drosophila*. Caf1-55 has an ideal structure for interactions with other proteins. I hypothesize that Caf1-55 acts as sub-scaffold to mediate the interaction of dKDM5/LID and EMSY with the SIN3 220 complex. To test this hypothesis, we will immuno-purify the SIN3 220 complex post knockdown or knockout of *Caf1-55* and investigate the presence of dKDM5/LID and EMSY in the complex using western blotting. I predict that Caf1-55 plays a role in integrating dKDM5/LID and EMSY to the SIN3 220 complex.

The SIN3 complex is essential for the development of *Drosophila melanogaster*. Identifying the functions of the distinct SIN3 isoform complexes is key to understand how

these complexes regulate different stages of fly development. In addition, dissecting the role of individual complex components in the SIN3 complexes is pivotal to study the mechanism by which the SIN3 complexes modulate gene regulation. The experiments described in this chapter are anticipated to elucidate the role and the underlying molecular mechanism, whereby the SIN3 isoform specific complexes regulate gene expression essential for cell fate decisions and development.

APPENDIX

Chromatin preparation from S2 cells

Unless noted, all steps are performed at room temperature

1. Count cells. Transfer 4 X 10^7 cells to 10 ml of serum free media. Use 50 ml falcon tube.
2. To crosslink DNA-protein, add formaldehyde to a final concentration of 1% (625 µl of 16% formaldehyde). Place tube on a shaker for 10 min.
3. Quench reaction by adding freshly prepared glycine solution to a final concentration 125 mM for 10 min under shaking conditions.

   Note: Prepare 1 M glycine (0.375 gm of glycine in 5 ml of sterile water). Add 1.25 ml of 1 M glycine to 10 ml of crosslinked cell sample.
4. Wash cells 3 times by addition of cold 1X PBS. After each wash, spin the cells at 4^{o}C at 800 x g for 5 min.
5. After the final wash, resuspend cells in 15 ml of lysis buffer. Add 1 tablet of protease inhibitor tablet (Roche or Pierce) to 15 ml of cell suspension. Completely dissolve the protease inhibitor and incubate on ice for 15 min.
6. Use dounce homogenizer to lyse the cells. Dounce 10 times using a loose pestle and 15 times using a tight pestle. Transfer homogenized cell suspension to a new 15 ml tube.

   Note: To keep the cells cold during this step, place douncer on ice.
7. Centrifuge at 170 x g at 4^{o}C for 10 min.
8. Resuspend pellet in 200 µl of MNase digest buffer.
9. Add 20 units of MNase. Incubate for 30 min.

Note: Add 1.3 µl of 15 units/µl stock MNase (Worthington Biochemicals). This step needs optimization depending on the effectiveness of MNase. In general, incubation of chromatin for 10 to 30 min with MNase is sufficient.

10. Add EDTA to a final concentration of 10 mM to stop the reaction. Incubate for 5 min.
11. Use resuspension buffer to make the volume up to 1.2 ml.
12. Sonicate the sample for 3 min 30 sec and 20% amplitude.

    Note: Sonication: 30 sec pulse on and 60 sec pulse off. Use ice-ethanol as a coolant. Sonication should be performed in 2 ml tubes with the probe inserted to the 0.5 ml mark of the 2 ml tube.
13. Centrifuge at 21,000 x g at 4^{o}C for 15 min.
14. Transfer supernatant to a new tube for input DNA preparation and chromatin immunoprecipitation.

    Note: At this step, the chromatin can be stored in -80^{o}C by flash freezing in liquid nitrogen.
15. Measure concentration of prepared chromatin.
16. For input DNA preparation, use approximately 75 µg of the chromatin sample.

    Note: Depending on the experimental requirement, lower amounts of chromatin can be used for reversal of crosslinks for input DNA preparation.

Reverse Crosslink DNA:Protein

1. Depending on the concentration of chromatin, make the volume up to 500 µl by adding resuspension buffer.
2. To 500 µl of chromatin, add 4.0 µl of 10 µg/µl RNase A. Incubate at 37^{o}C for 15 min.
3. Add 20 µl of 5 M NaCl and incubate at 65^{o}C overnight.

4. Add 2 µl of 10 µg/µl Proteinase K, 10 µl of 0.5 M EDTA, 10 µl of 1M Tris (pH 7.6).
5. Incubate at 45ºC for 1 hr.
6. Prepare phase lock tube (5 Prime) by centrifugation at 21,000 x g for 30 sec.
7. Add equal volume phenol/chloroform to the treated chromatin sample. Vortex the mixture and transfer to the pre-spun phase lock tube.
8. Centrifuge for 5 min at 21,000 x g.
9. Transfer aqueous (top) phase to a new tube.

   Note: Distribute 500 µl equally to two tubes for ease of processing downstream steps.
10. Add 50 µl of 3 M NaOAc (pH 5.2).
11. Add 3 volumes of chilled 100% ethanol.
12. Add 0.5 µl of pellet paint (Novagen).
13. Invert well to mix and let the mixture stand for 10 min.
14. Centrifuge tubes for 20 min at 4ºC at 21,000 x g.
15. Wash pellet in 1 ml of 70% ethanol and centrifuge for 15 min at 4ºC at 21,000 x g.
16. Discard supernatant carefully and allow the pellet to air dry.
17. For long-term storage dissolve the pellet in 50 µl 10 mM Tris (pH 8). Store at -20ºC.
18. Perform gel electrophoresis to verify chromatin fragmentation.

Buffers

Lysis buffer

10 mM Tris (pH 8)

10 mM KCl

3 mM $CaCl_2$

0.34 M sucrose

1 mM DTT

0.1% Triton X-100

0.2 mM EGTA

MNase digest buffer

15 mM Tris (pH 8)

60 mM KCl

15 mM NaCl

1 mM DTT

0.25 M sucrose

1 mM $CaCl_2$

Resuspension buffer

140 mM NaCl

10 mM Tris (pH 7.6)

2 mM EDTA

Chromatin Immunoprecipitation using anti-HA beads

1. Aliquot 75 µg of chromatin to a new tube. Adjust the volume to 500 µl with resuspension buffer.
2. Add 30 µl of prepared HA agarose beads (Sigma).

   Note: HA agarose beads preparation: Wash beads 5 times by adding 1 ml 1 X RIPA buffer.
3. Place the tube on nutator and incubate for 4 hr at 4^{o}C.
4. IP washes

   Note: For each washing step transfer bead slurry into a new tube. All IP washes are done at 4^{o}C and centrifugation steps are performed at 3,400 x g for 1 min at 4^{o}C.

A) 2 X RIPA (modified) buffer - 1 time incubate for 8 min. Spin

B) IP1 - 1 time incubate for 10 min. Spin

C) IP2 - 1 time incubate for 10 min. Spin

D) TE buffer- 1 ml quick rinse. Spin

5. Add 250 µl of elution buffer and incubate at 65°C for 30 min. Repeat. Reverse crosslink to purify DNA.

Buffers

1 X RIPA buffer

25 mM Tris (pH 7.6)

140 mM NaCl

1 mM EDTA

1% Triton X-100

0.1% SDS

2 X RIPA (modified) buffer

50 mM Tris (pH 7.6)

280 mM NaCl

2 mM EDTA

0.3% SDS

IP1 buffer

25 mM Tris (pH 7.6)

500 mM NaCl

1 mM EDTA

0.1% SDS

1% Triton X-100

IP2 buffer

10 mM Tris (pH 7.6)

250 mM LiCl

1 mM EDTA

0.5% Sodium deoxycholate

0.5% Triton X-100

Elution buffer

1% SDS, 0.1M $NaHCO_3$

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ABSTRACT

FUNCTIONAL ANALYSIS OF SIN3 ISOFORMS IN *DROSOPHILA*

by

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The multisubunit SIN3 complex is a global transcriptional regulator. In *Drosophila*, a single *Sin3A* gene encodes different isoforms of SIN3, of which SIN3 187 and SIN3 220 are the major isoforms. Previous studies have demonstrated functional non-redundancy of SIN3 isoforms. The role of SIN3 isoforms in regulating distinct biological processes, however, is not well characterized. In addition, how the components of the SIN3 complex modulate the gene regulatory activity of the complex is not well understood. In this study, I identified the biological processes regulated by the SIN3 isoforms. Additionally, I explored how Caf1-55 impacts the gene regulatory activity of the SIN3 220 complex.

For the purpose of the study, I developed a highly reproducible ChIP protocol using micrococcal nuclease (MNase)-mediated chromatin preparation from *Drosophila* cultured cells. This protocol can be used to perform ChIP to map both histones and non-histone chromatin binding proteins locally and globally across the genome.

Next, we identified the biological processes regulated by the SIN3 isoforms. We established a *Drosophila* S2 cell culture model system in which cells predominantly express either SIN3 187 or SIN3 220. To identify genomic targets of SIN3 isoforms, we performed chromatin immunoprecipitation followed by deep sequencing. Our data

demonstrate that upon overexpression of SIN3 187, the level of SIN3 220 decreased and the large majority of genomic sites bound by SIN3 220 were instead bound by SIN3 187. We used RNA-seq to identify genes regulated by the expression of one isoform or the other. In S2 cells, which predominantly express SIN3 220, we found that SIN3 220 directly regulates genes involved in metabolism and cell proliferation. We also determined that SIN3 187 regulates a unique set of genes and likely modulates expression of many genes also regulated by SIN3 220. Interestingly, biological pathways enriched for genes specifically regulated by SIN3 187 strongly suggest that this isoform plays an important role during the transition from the embryonic to the larval stage of development.

Finally, I investigated the function of Caf1-55 in the SIN3 220 complex. Our data demonstrate that Caf1-55 localizes to SIN3 220 gene targets and is partly required for recruiting SIN3 220 to chromatin. In addition, we show that the C-terminal domain of SIN3 220 physically interacts with Caf1-55. We found that the interaction between SIN3 and Caf1-55 is significantly reduced upon mutating the histone H4 binding pocket of Caf1-55. Surprisingly, the reduced interaction between the histone H4 binding mutant of Caf1-55 and SIN3 220 is not sufficient to cause a change in the expression of SIN3 220 regulated genes. Together, these data provide evidence of a novel role of Caf1-55 in impacting recruitment of a component of a chromatin modifying complex to genomic loci.

In summary, our research reveals important insights of how the SIN3 isoform specific complexes might function during the course of fly development.

Supplementary files are included:

- Supplementary Data 1 – Excel spreadsheet containing detailed information on peaks identified by ChIP-seq analysis.

- Supplementary Data 2 – Excel spreadsheet containing detailed information of the GO analysis performed on genes directly regulated by SIN3 isoforms.
- Supplementary Data 3 – Excel spreadsheet containing detailed annotation of ChIP-seq peaks for SIN3 187HA (SIN3 187HA ceas) or SIN3 220HA (SIN3 220HA ceas) as determined by the cis-regulatory enrichment annotation (CEAS) system.
- Supplemental Data 4 – Excel spreadsheet containing detailed information about differential gene expression analysis upon overexpression of SIN3 187HA as determined by Cuffdiff

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