SIN3 gene regulatory activity is linked to RNA polymerase II pausing

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Summary statement: SIN3 cofactors can activate or repress genes. Using bioinformatic analysis, we find that histone methylation and RNA polymerase II binding profiles differ at SIN3-regulated genes with distinct transcriptional outcomes.

ABSTRACT

The chromatin environment has a significant impact on gene expression. Chromatin structure is highly regulated by histone modifications and RNA polymerase II binding dynamics. The SIN3 histone modifying complex regulates the chromatin environment leading to changes in gene expression. In *Drosophila melanogaster*, the *Sin3A* gene is alternatively spliced to produce different protein isoforms, two of which include SIN3 220 and SIN3 187. Both SIN3 isoforms are scaffolding proteins that interact with several other factors to regulate the chromatin landscape. The mechanism through which the SIN3 isoforms regulate chromatin is not well understood. Here, we analyze publicly available data sets to allow us to ask specific questions on how SIN3 isoforms regulate chromatin and gene activity. We determined that genes repressed by the SIN3 isoforms exhibited enrichment in histone H3K4me2 and H3K4me3 near the transcription start site. We observed an increase in the amount of paused RNA polymerase II on the promoter of genes repressed by the isoforms as compared to genes that require SIN3 for maximum activation. Furthermore, we analyzed a subset of genes regulated by SIN3 187 that suggest a mechanism in which SIN3 187 might exhibit hard regulation as well

as soft regulation. Data presented here expand our knowledge of how the SIN3 isoforms regulate the chromatin environment and RNA polymerase II binding dynamics.

INTRODUCTION

Nucleosomes are composed of DNA strands that wrap around histone proteins. This interaction facilitates DNA compaction into the nucleus of the cell. Histone proteins are subject to post-translational modifications such as acetylation and methylation. The presence or absence of specific histone modifications can impact gene activity (Tse et al., 1998). The regulation of gene activity by histone modifications occurs through two major mechanisms. First, the modification of histone N-terminal amino acids (histone tails), such as the acetylation of lysine residues, brings about the nullification of the ionic interaction between the positively charged lysine and the negatively charged DNA. The loss of such interaction leads to a less compact nucleosome, making the DNA more accessible for RNA polymerase binding (Nightingale et al., 1998). The second mechanism is through signaling and transcription regulator recruitment. Histone tail modifications can act as a signal that recruits transcription factors and chromatin effectors to a gene locus, conferring an effect on gene regulation (Cheung et al., 2000).

One such chromatin effector complex is the SIN3 histone deacetylase (HDAC) complex, which regulates the acetylation and methylation of histone tails (Silverstein and Ekwall, 2005). The SIN3 complex is composed of the SIN3 scaffolding protein, which recruits other proteins, including histone deacetylase 1 (HDAC1), and the histone demethylase dKDM5A/LID (Moshkin et al., 2009; Spain et al., 2010). *Sin3A* is an essential gene in metazoans. The knockout of *Sin3A* leads to loss of viability in both Drosophila and mouse models (Dannenberg et al., 2005; Neufeld et al., 1998; Pennetta and Pauli, 1998). Drosophila SIN3 regulates genes encoding proteins in many important pathways including cell cycle, one carbon, and central carbon metabolism (Pile et al., 2003). Consistent with the changes in gene expression, RNA interference-mediated reduction of *Sin3A* in Drosophila S2 cultured cells leads to a G2 arrest in the cell cycle (Pile et al., 2002). Furthermore, reduction of *Sin3A* in S2 cells leads to the deregulation of many one carbon and central carbon metabolites, indicating a role for the SIN3 complex in metabolic regulation of the cell (Liu and Pile, 2017; Liu et al., 2020).

58 The Drosophila *Sin3A* gene encodes multiple isoforms of SIN3 through differential
59 splicing (Pennetta and Pauli, 1998). Two of the most prevalent isoforms are SIN3 220
60 and SIN3 187, named based on the predicted molecular weight. The isoforms have
61 differential expression patterns and distinct abilities to rescue a genetic mutation in
62 *Sin3A*. SIN3 isoforms are expressed at similar levels in the initial stages of Drosophila
63 embryogenesis (Sharma et al., 2008). SIN3 220 levels increase in stages 12-16 but fall
64 at the final stage of embryogenesis, at which time, the expression of SIN3 187 becomes
65 predominant (Sharma et al., 2008). The lethality due to genetic disruption of the *Sin3A*
66 gene can be rescued by a transgene designed to express SIN3 220, while a transgene
67 encoding SIN3 187 is unable to suppress the lethal phenotype (Spain et al., 2010).
68 Additionally, while both isoforms interact with HDAC1 along with other core SIN3
69 complex components, our group has shown that these isoforms bind unique proteins as
70 well. For example, dKDM5A/LID is found in the SIN3 220 complex and not the SIN3 187
71 complex (Spain et al., 2010).

72 The SIN3 isoforms are recruited to thousands of genes throughout the Drosophila
73 genome, many of which are common targets between the two isoforms, suggesting an
74 overlapping mechanism of regulation by the isoforms (Saha et al., 2016). Specific gene
75 targets that are differentially regulated by the isoforms and belong to distinct gene
76 ontology (GO) categories, however, have also been identified. The differential
77 interaction of specific proteins with the isoforms could lead to the differential regulation
78 of the observed GO pathways (Saha et al., 2016). Both of the SIN3 isoforms have been
79 implicated in gene repression and gene activation, yet the chromatin context in which
80 SIN3 complexes can activate or repress genes is not well understood. RNA polymerase
81 II (RNA Pol II) transcribes eukaryotic genes in three general steps. First, RNA Poll II is
82 recruited to gene promoters to form the pre-initiation complex (PIC) where the RNA
83 polymerase interacts with DNA at the transcription start site (TSS). Next, the C-terminal
84 domain (CTD) of the polymerase is phosphorylated at serine (Ser) 5, transcription is
85 initiated and then RNA Pol II pauses 20-60 base pairs downstream of the TSS. This
86 promoter proximal pausing is dependent on pausing factors such as NELF-A and the
87 DSIF complex containing SPT5 (Adelman and Lis, 2012). To release the polymerase
88 from its paused state, the transcription elongation factor TEFb phosphorylates RNA Pol

II, NELF-A and the DSIF complex, leading to the disassociation of NELF-A. Once transcription is terminated, RNA Pol II is released and recycled for another round of transcription. It has been long suggested that histone acetylation affects RNA Pol II dynamics, and many publications support this idea (Nightingale et al., 1998; Vaid et al., 2020). Studies from Nightingale et al. (1998) showed that histone acetylation positively affects RNA Pol II initiation. Furthermore, results of a recent study suggest a link between histone deacetylation activity and RNA Pol II pausing on a subset of developmental and signaling genes (Vaid et al., 2020). The authors found that HDAC inhibition leads to the release of RNA Poll II from the promoter paused state, leading to the elongation of select gene transcripts.

SIN3 is well-studied in its role as a gene regulator and scaffolding protein. However, the way in which SIN3 isoforms repress or activate genes has not been fully elucidated. We recently explored a potential mechanism of gene regulation by SIN3 (Mitra et al., 2021). We predicted that histone modifying complexes such as the SIN3 complex regulate housekeeping genes in a soft repression manner rather than an on/off switch. Soft repression is a mechanism whereby gene regulators affect the expression of genes by a small fold change to fine-tune the transcriptional output (<2 log2 fold change). For that study, we focused on genes repressed by the SIN3 220 isoform. Here, we investigate the mechanism of differential regulatory activity of the SIN3 isoforms by metagene analysis of SIN3-regulated genes. Our results support the idea that histone modifications regulated by SIN3 impact RNA Pol II dynamics. Additionally, our findings are consistent with our prediction that SIN3 acts as a soft repressor. Interestingly, we also obtain data to suggest that the SIN3 187 isoform acts as a hard regulator on some genes. In summary, we provide evidence to suggest that SIN3 fine tunes the expression of genes by regulating histone methylation and elongation dynamics of RNA Pol II.

RESULTS

SIN3 220 and SIN3 187 binding is enriched at the TSS of repressed and activated gene targets

To understand how SIN3 isoforms can lead to either gene repression or gene activation, we sought to uncover possible differences in protein binding and the chromatin

119 landscape comparing SIN3 repressed and SIN3 activated direct gene targets. We did
120 this by analyzing SIN3 isoform binding to genomic loci and comparing the common and
121 unique targets. In this study, we expanded on our published analysis of the binding of
122 the SIN3 isoforms by including targets unique to one isoform. In our previous study, we
123 determined that the majority of SIN3 220 targets are also regulated by SIN3 187, while
124 SIN3 187 regulates several genes that are specific to that isoform (Saha et al., 2016).
125 To analyze the binding profile of the SIN3 isoforms, published genomic data were
126 downloaded from NCBI's GEO database (Fig. 1A) and analyzed using the Galaxy
127 platform (Afgan et al., 2018). To determine the list of genes directly regulated by the
128 SIN3 isoforms, we integrated SIN3 RNA-seq data (Gajan et al., 2016) with ChIP-seq
129 data (Saha et al., 2016). SIN3 220 directly regulates 405 genes; 60% (242/405) of those
130 genes are repressed by SIN3 220, while 40% (163/405) are activated. Furthermore, 859
131 genes are directly regulated by SIN3 187, 55% (469/859) are repressed, while 45%
132 (390/859) are activated. Of the 405 genes directly regulated by SIN3 220, 83%
133 (335/405) are also regulated by SIN3 187 with only 17% (70/405) of genes uniquely
134 regulated by SIN3 220 (Fig. 1B). For SIN3 187 targets, only 39% (335/859) of genes are
135 also regulated by SIN3 200, with the majority of genes, 61% (524/859), uniquely
136 regulated by SIN3 187.

137 Our previous binding analysis showed that SIN3 isoforms are enriched at the TSS of
138 genes (Saha et al., 2016). To follow up from that study, we asked, what are the
139 differences between the enrichment of SIN3 at genes regulated by both isoforms, and
140 genes regulated by SIN3 187 alone? Consistent with our previous analysis, SIN3 220
141 binding is enriched at the TSS of repressed genes and activated genes (Fig. 2A, B)
142 compared to the gene body. Additionally, SIN3 220 binding is more enriched at
143 repressed genes compared to activated genes (Fig. 2B). Likewise, SIN3 187 binding is
144 more enriched at the TSS for both repressed and activated genes (Fig. 2A, 2B)
145 compared to the gene body. To a lesser extent but similar to the SIN3 220 binding
146 profile, genes repressed by SIN3 187 exhibited more binding at the TSS compared to
147 activated genes (Fig. 2B). Next, we looked at genes exclusively regulated by SIN3 187
148 and not by SIN3 220. These genes are bound by SIN3 187 and change in expression
149 only when SIN3 187 levels are perturbed and not when SIN3 220 levels are reduced

150 (Saha et al. 2016). Interestingly, at this set of targets we do not observe a notable
151 difference in SIN3 187 binding comparing the levels at repressed to activated genes
152 (Fig. 2A, 2B). These data indicate that for the common repressed SIN3 gene targets,
153 SIN3 complex activity might be impacting the PIC or other factors at the TSS. At
154 activated targets and at the genes specific to SIN3 187, the location of gene regulatory
155 activity may be more variable as complex binding is less TSS directed.

156 **SIN3 repressed genes have more enrichment of H3K4me2 and H3K4me3**
157 **compared to activated genes**

158 SIN3 is a scaffold protein for assembly of a histone modifying complex that binds to
159 gene promoters and can affect the neighboring chromatin environment (Gajan et al.,
160 2016; Liu and Pile, 2017). SIN3 is associated with two proteins shown to regulate the
161 post-translational modification profile of histone proteins, histone deacetylase HDAC1
162 and histone demethylase dKDM5/LID. Here, we wished to analyze the promoter histone
163 modification profiles at SIN3-regulated genes. Due to lack of histone acetylation data
164 generated from S2 cells, we were not able to examine the acetylation profile of SIN3
165 gene targets. We previously determined that the histone demethylase dKDM5/LID is a
166 part of the SIN3 220 complex but not SIN3 187 complex (Spain et al., 2010). This
167 finding led us to ask if genes regulated by the SIN3 isoforms have differential
168 enrichment of methylation at target genes. To address this question, we determined the
169 level of histone methylation at the direct SIN3 isoform targets. We chose H3K4me1,
170 H3K4me2, and H3K4me3 to gain a full understanding of the H3K4 methylation pattern
171 of SIN3-regulated genes. We expected genes to have low to no H3K4me1 levels, since
172 this mark has been associated with gene enhancers rather than at the TSS (Heintzman
173 et al., 2007). Additionally, we expected genes to have higher enrichment of H3K4me2
174 and H3K4me3 when compared to H3K4me1 levels. Our reasoning is that both of these
175 marks are associated with actively expressed genes (Bernstein et al., 2005), and SIN3
176 has been shown to fine-tune the expression of expressed genes (Mitra et al., 2021). We
177 downloaded H3K4 methylation data from NCBI GEO (Fig. 1A) and overlapped it with
178 genes bound by SIN3 to generate heat maps and binding profiles. We found no
179 difference in H3K4me1 enrichment between activated and repressed genes (Fig. 3A).

180 On the other hand, we found higher enrichment of H3K4me2 and H3K4me3 at genes
181 repressed by SIN3 220 compared to those genes that are activated (Fig. 3A). This
182 enrichment was seen ~700 bp downstream of the TSS. For SIN3 187, we observe a
183 similar trend wherein H3K4me1 enrichment was similar at repressed and activated
184 targets, while SIN3 187 repressed genes showed higher enrichment of H3K4me2 and
185 H3K4me3, compared to genes activated by SIN3 187 (Fig. 3A). To further dissect the
186 histone methylation profiles of SIN3-regulated genes, we looked at genes exclusively
187 regulated by SIN3 187 and not SIN3 220. To our surprise, we saw a similar but smaller
188 trend in which H3K4me2 and H3K4me3 were enriched at repressed genes and not
189 activated genes (Fig. 3B), though the level of these marks at the unique targets was
190 lower in comparison to the common targets. Because SIN3 187 was not found to
191 interact with dKDM5/LID, these results strongly suggest that dKDM5/LID is not the sole
192 factor that affects the histone methylation profile of SIN3-regulated genes.

193 **RNA Pol II and negative elongation factor (NELF) are paused at the TSS of SIN3**
194 **220 and SIN3 187 repressed genes and not at activated genes**

195 Transcription elongation by RNA pol II is regulated in the cell by pausing factors such as
196 negative elongation factor-A (NELF-A) and DRB sensitivity-inducing factor (DSIF)
197 (Adelman and Lis, 2012). HDAC1 influences the release of RNA pol II from the TSS of a
198 subset of development and signaling genes (Vaid et al., 2020). Since HDAC1 is one of
199 the core components of both SIN3 220 and SIN3 187 complexes, we asked if RNA pol II
200 is paused at SIN3-regulated genes. To do this, previously published RNA Pol II and
201 NELF-A genome-wide binding data (Mazina et al., 2021) was downloaded and
202 overlapped with the SIN3 binding data (Fig. 1A). We observed an enrichment of RNA
203 Pol II 50 bp downstream of the TSS of genes repressed by SIN3 220 (Fig. 4A). RNA Pol
204 II was also enriched 50 bp downstream of the TSS of genes repressed by SIN3 187
205 (Fig. 4A). To quantify the difference in enrichment of RNA Pol II at SIN3 220 and SIN3
206 187 regulated genes, we determined the pausing index using a method modified from
207 Vaid et al. (2020). To calculate the pausing index, the binding enrichment value at
208 promoter region (-50 bp to +50 bp) was divided by the enrichment value along the genic
209 region (+300 bp to -100 TES). SIN3 220 and SIN3 187 repressed genes exhibited

210 higher RNA Pol II pausing when compared to genes that were activated (Fig. 4B).
211 These data suggest that SIN3 repression of genes involves the regulation of RNA Pol II
212 elongation dynamics.

213 To further investigate whether SIN3 repressed genes exhibit high RNA Pol II pausing,
214 we asked if the binding of the pausing factor NELF-A is also different between
215 repressed and activated genes. NELF-A is a pausing factor that plays an important role
216 in RNA Pol II promoter proximal pausing (Yamaguchi et al., 2013). In line with our
217 expectations, the NELF-A binding pattern was similar to that seen with RNA Pol II.
218 NELF-A had a higher enrichment near the TSS of SIN3 220 and SIN3 187 repressed
219 genes compared activated genes (Fig. 4A). These data support the idea that SIN3 220
220 and SIN3 187 repress some genes through a similar mechanism. Additionally, these
221 findings suggest a mechanism in which SIN3 represses genes by regulating RNA Pol II
222 pausing, possibly involving NELF-A.

223 **SIN3 isoforms share common DNA binding motifs**

224 Neither of the SIN3 isoforms have DNA binding capabilities, but likely are recruited to
225 DNA targets through their association with transcription factors (Kasten et al., 1996).
226 Thus, we conducted a motif analysis to identify enriched motifs at the binding sites of
227 SIN3 isoforms. Furthermore, we parsed out repressed and activated genes to determine
228 if gene regulation outcome is correlated with the binding motif. To do this, we used the
229 MEME software (Buske et al., 2010) through the Galaxy platform. Our analysis found
230 that many binding motifs were different, only 1 out of the top 6 motifs overlapped
231 between the isoforms (Fig. 5, 6). Next, we asked if the SIN3 binding motifs are shared
232 with any transcription factor binding motifs. To this end, we used Tomtom to compare
233 the DNA binding motifs of the SIN3 isoforms with known transcription factor motifs
234 (Gupta et al., 2007). Transcription factors daughterless (Da) and tinman (Tin) binding
235 motifs overlapped with SIN3 220 binding motifs on both activated and repressed genes
236 (Fig. 5A,B). Da is a broadly expressed transcription factor (Cronmiller and Cummings,
237 1993) with a wide range of regulatory functions, including proliferation (Smith et al.,
238 2002) and development (Cummings and Cronmiller, 1994). *Tin* is a homeobox gene that
239 codes for a transcription factor involved in differentiation and development (Liu et al.,

240 2009; Ranganayakulu et al., 1998; Zaffran et al., 2006). Additionally, the transcription
241 factor longitudinals lacking (Lola) binding motif overlapped with SIN3 187 activated and
242 repressed binding motifs as well as SIN3 220 repressed binding motifs (Fig. 5A, 6). Lola
243 is a transcription factor that plays a role in cell fate determination by antagonizing notch
244 (Zheng and Carthew, 2008). Lola has also been shown to regulate genes involved in
245 programmed cell death (Bass et al., 2007). The finding that multiple transcription factor
246 motifs are enriched at promoters of SIN3-regulated genes are consistent previous
247 results demonstrating that SIN3 recruitment is widespread and that direct gene targets
248 fall into multiple GO categories (Pile and Wassarman, 2000; Saha et al., 2016).

249 **SIN3 isoforms function as soft repressors**

250 We previously determined that SIN3 220 functions as a soft repressor (Mitra et al.,
251 2021).This term signifies that SIN3 is capable of fine-tuning the expression of target
252 genes rather than switching them on and off. This action results in small but
253 physiologically and statistically significant changes in gene expression. To expand on
254 our previous study in which we investigated SIN3 220 repressed targets, here we
255 looked at the levels of regulatory action at SIN3 220 activated genes and at genes
256 regulated by SIN3 187. Using direct targets of SIN3 220, we analyzed the log2 fold
257 changes (log2FC) in gene expression of SIN3 220 active and repressed genes. 242
258 direct repressed targets of SIN3 220 were identified, of which 99.6% (241/242) of genes
259 demonstrated less than 2 log2FC (Fig. 7). A total of 163 directly active targets of SIN3
260 220 were identified. Of these 163 genes, 98% (160/163) of them showed less than 2
261 log2FC and only 2% (3/163) of genes fell in the range of 2 to 3 log2FC when levels of
262 SIN3 220 were perturbed (Fig. 7). These data indicate that SIN3 220 indeed acts as a
263 soft regulator on both active as well as repressed gene targets. Next, we asked whether
264 SIN3 187 also functions as a soft regulator to fine-tune gene expression. We
265 hypothesized that since both isoforms have many overlapping targets, they might have
266 similar effects on gene expression. 469 directly repressed targets of SIN3 187 were
267 identified, of which 89.8% (421/469) of genes showed less than 2 log2FC with the
268 overexpression of SIN3 187. 6.4% (30/469) were in the 2 to 3 log2FC category and
269 3.8% (18/469) of genes showed greater than 3 log2FC in gene expression (Fig. 7). A

270 similar pattern of soft regulatory activity was observed for the direct gene targets
271 activated by SIN3 187. Of the total 390 genes identified, 69.5% (271/390) of genes
272 changed less than 2 log2FC in expression. 13.3% (52/390) showed 2 to 3 log2FC while
273 17.2% (67/390) of genes had greater than 3 log2FC in expression levels (Fig. 7). These
274 results show that a majority of direct gene targets of both isoforms exhibited small
275 changes in gene expression, demonstrating they function as soft repressors and soft
276 activators. As this mechanism of regulation is seen for repressed as well as activated
277 genes, this modulating activity appears to be the preferred mode of action of this global
278 transcriptional regulator.

279 **SIN3 fine-tunes the expression of moderately expressed genes**

280 To further investigate whether SIN3 regulates in an on/off or soft regulation manner, we
281 asked if SIN3 regulated gene targets are active or silenced in cells. We hypothesized
282 that if SIN3 is acting as a soft regulator, gene targets should exhibit moderate
283 expression. On the other hand, if SIN3 is acting in an/off manner, gene targets should
284 exhibit either high or silent/low expression, depending on the action of SIN3. To test our
285 hypothesis, we analyzed the expression level of all of the expressed genes in
286 Drosophila S2 cells, as determined by RNA-seq reads reported as fragments per
287 kilobase of exon per million mapped fragments (FPKM). We divided the genes into four
288 quadrants (Fig. 8) using a slightly modified classification as in Kalashnikova and
289 colleagues (Kalashnikova et al., 2021). We considered genes with FPKM >1000 as
290 strongly expressed, genes with FPKM 10-1000 as moderately expressed and genes
291 with FPKM < 1 as silenced (Kalashnikova et al., 2021). We saw that 87% (211/242) of
292 genes repressed by SIN3 220 and 70% (114/163) of genes activated by SIN3 220 were
293 moderately expressed (Fig. 8A). Similarly, to a lesser extent, 87% (406/469) of genes
294 repressed by SIN3 187 and 55% (215/390) of genes activated by SIN3 187 were
295 moderately expressed (Fig. 8A). These data support our hypothesis that SIN3 regulates
296 moderately expressed genes by modulation of activity and not by turning off genes.
297 Interestingly, 14% (53/390) of genes activated following the ectopic expression of SIN3
298 187 had an FPKM value of 1 or less in control cells, while only 2% of the other gene
299 sets had such a low FPKM value (Fig. 8A). This finding suggests that SIN3 187 may act

300 as a hard regulator on a subset of genes. To further investigate, we looked closely at
301 the 53 genes predicted to be regulated in an on/off manner by SIN3 187. We saw that
302 96% (51/53) of the genes were activated by more than 3 log2FC following ectopic
303 expression of SIN3 187. Additionally, we asked if these 53 genes activated by SIN3 187
304 are also regulated by SIN3 220. We found that while 74% (39/53) are bound by SIN3
305 220, only 6% (3/53) change in expression when SIN3 220 levels are perturbed. These
306 data indicate that SIN3 187 acts as a hard regulator on a subset of genes. To further
307 corroborate our findings, we asked if SIN3 187 binding on those 53 genes is distinct
308 from the binding of SIN3 187 on all SIN3 187 directly activated genes. Comparing the
309 binding of SIN3 187 on the 53 genes with all SIN3 187 directly regulated genes, we saw
310 that SIN3 187 binding on all directly activated genes is localized at the TSS, while SIN3
311 187 binding on the 53 genes is not strictly localized to the TSS (Fig. 8B). Data
312 presented here provide evidence to suggest that SIN3 187 might have two modes of
313 regulation, soft and hard regulation. We predict that soft regulation occurs due to
314 binding at the promoter of genes, while hard regulation does not.

315 **DISCUSSION**

316 Here, we analyzed the binding patterns of the SIN3 isoforms in the context of the
317 chromatin environment. Metagene analysis revealed that the binding of the SIN3
318 isoforms occurs mostly at the TSS of genes. Interestingly, both SIN3 isoforms exhibited
319 higher binding intensity on genes repressed compared to genes activated (Fig. 2). In
320 contrast, genes that are uniquely repressed by SIN3 187 exhibited a similar binding
321 profile to those genes uniquely activated by SIN3 187. One possible reason for this
322 difference is that SIN3 187 lacks some SIN3 220 complex interactors, some which may
323 help in the recruitment of SIN3 to target genes. Additionally, the difference in binding at
324 the TSS between SIN3 220 and SIN3 187 unique targets leads to the prediction that
325 SIN3 187 can act as a hard regulator while SIN3 220 does not. Soft regulation is
326 predicted to depend on transcriptional regulators binding promoter proximally where
327 they lead to fine-tuning of gene expression (Mitra et al., 2021). Consistent with our
328 hypothesis, soft regulation occurs when SIN3 isoforms are localized to the promoter
329 proximal regions, while hard regulation of genes occurs when SIN3 187 binding is

330 localized away from the TSS. Genes repressed by the SIN3 isoforms exhibited enriched
331 H3K4me3 levels immediately after the TSS (Fig. 3). Given that SIN3 binds at expressed
332 genes, this finding was not surprising since H3K4me3 is enriched on actively expressed
333 genes (Bernstein et al., 2005). The finding that both isoforms are located at targets with
334 similar levels of H3K4me3 enrichment suggests that SIN3 220 and SIN3 187 dampen
335 gene expression through a similar soft regulation mechanism. On the other hand, it is
336 intriguing that both 220 and 187 regulated genes exhibited similar methylation patterns,
337 since the SIN3 220 complex but not the SIN3 187 complex, contains dKDM5/LID (Spain
338 et al., 2010)), a histone demethylase that targets H3K4me3. The H3K4me3 enrichment
339 patterns suggest that H3K4me3 levels at the promoters are not regulated by
340 dKDM5/LID alone.

341 Furthermore, genes repressed by the SIN3 isoforms exhibited higher RNA pausing at
342 the TSS when compared to genes activated by the isoforms (Fig. 4B). This finding
343 indicates that the mechanism of SIN3 repression is similar between the isoforms and
344 likely involves regulation of RNA Pol II pausing. In line with this prediction, genes
345 repressed by the isoforms showed enrichment of NELF-A, a pausing factor, at the TSS
346 (Fig. 4A). One enzyme that could play a role in the regulation of RNA Pol II binding
347 dynamics is HDAC1. Indeed, both SIN3 isoform complexes contain HDAC1 (Spain et
348 al., 2010). Previously published findings indicate that HDAC activity could lead to RNA
349 Pol II pausing, and the inhibition of HDAC activity leads to the release of the paused
350 polymerase (Vaid et al., 2020). Consistent with the proposed mechanism of regulation,
351 while not statistically significant, SIN3 187 regulated genes had higher average RNA Pol
352 II pausing when compared to SIN3 220 (Fig. 4B). We previously determined that the
353 Vmax of the HDAC activity of the SIN3 187 complex is higher when compared to the
354 SIN3 220 complex (Spain et al., 2010). We predict that the higher HDAC activity in the
355 SIN3 187 complex is responsible for a higher pausing index when compared to the
356 SIN3 220 complex. Overall, data presented here along with published data, allow us to
357 propose a model in which SIN3 repression activity is in part dependent on the role of
358 HDAC1 in regulating RNA Pol II pausing.

359 Soft regulation is predicted to affect gene expression in a less dramatic but biologically
360 significant manner. Our findings support the role of both SIN3 220 and SIN3 187 in soft
361 regulation. The perturbation of either isoform leads to small but significant changes in
362 the level of expression of target genes (Fig. 7). Furthermore, both isoforms
363 predominantly bind at the TSS of genes, a feature predicted of soft regulation (Mitra et
364 al., 2021). Through analysis of the measured expression level of genes regulated by the
365 SIN3 isoforms, we observed that most of the gene targets are expressed at moderate
366 levels in S2 cells. This finding is consistent with our idea of soft regulation, wherein
367 genes expressed at moderate levels are attenuated and not turned on/off. Interestingly,
368 a subset of genes activated by SIN3 187 exhibited the opposite effect (Fig. 8B). These
369 genes were not expressed in wild-type cells and are turned on by SIN3 187 to more
370 than 3 log2 fold change. These data indicate that the SIN3 187 complex might regulate
371 genes by two mechanisms, soft and hard regulation. Future studies will be aimed at
372 further testing the model that the SIN3 complexes impact histone modifications at
373 housekeeping gene targets to modulate expression levels in response to cellular
374 demands.

375 **MATERIALS AND METHODS**

376 All of the software packages were used through the public servers at the Galaxy web
377 platform (Afgan et al., 2018)) with the exception of the RNA Pol II pausing index
378 calculation and Tomtom.

379 **ChIP-Seq analysis**

380 Data were downloaded from the Sequence Read Archive (SRA), using NCBI SRA
381 toolkit (Leinonen et al., 2011). To determine the binding profiles, we first mapped all
382 reads to the *Drosophila melanogaster* reference genome (dm3) using the software
383 package Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). Uniquely mapped
384 reads were extracted using the filter SAM option in SAMtools (Li et al., 2009).

385 **Heat maps and binding profiles**

386 Using the UCSC table browser (Karolchik et al., 2004), we created a genome assembly
387 using a list of genes of interest such as SIN3 220 activated genes, SIN3 220 repressed

genes, and so on. ChIP-seq data was then mapped to the assembled genome and a matrix was created using deepTools2: computeMatrix (Ramírez et al., 2016). This matrix, centered around the TSS and +/- 3 kb, was then used to generate heat maps and graphs plotting the binding profiles of the protein or histone mark of interest.

Pausing index

Rpb3 enrichment on SIN3-regulated genes was calculated and placed into a matrix using deepTools2: computeMatrix (Ramírez et al., 2016), through the Galaxy web platform. This matrix was then downloaded from Galaxy and analyzed through R Studio. Rpb3 enrichment was then calculated at the promoter region (-50 bp to +50 bp) and divided by the genic region (+300 bp to -100 bp TES). Statistical analysis using the Mann-Whitney test was done.

MEME and Tomtom analysis

MACS2 (version 2.1.1) (Zhang et al., 2008) was used to call peaks using the default parameters. Genomic locations were extracted and binding motifs were determined using MEME suite (Bailey and Elkan, 1994). To compare the binding motifs of SIN3 isoforms to transcription factor binding motifs, Tomtom package using default settings was used (Tanaka et al., 2011).

FPKM levels of genes in S2 cells

RNA-seq data from control cells previously published by our group (Gajan et al., 2016) was downloaded and divided into five categories based on the FPKM as previously published (Kalashnikova et al., 2021).

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AUTHOR CONTRIBUTIONS

I.S and L.A.P conceived the project. I.S and A.M performed the analysis. I.S and L.A.P wrote and edited the manuscript.

COMPETING INTERESTS

The author(s) declare no competing interests.

DATA AVAILABILITY

All data used in the study are available through the GEO database. GEO accession numbers are listed in Figure 1A.

424 **Figure Legends**

425 Fig. 1. Datasets used in this study. A. Data sets used in this study are shown with the
426 associated NCBI GEO number. B. Genes bound and regulated by SIN3 220 and SIN3
427 187 are plotted in a Venn diagram.

428

429 Fig. 2. SIN3 isoform binding is different between activated and repressed genes. A.
430 SIN3 220 and SIN3 187 binding on genes that change in expression when SIN3 220
431 and SIN3 187 levels are perturbed, visualized using heat maps. Uniquely regulated
432 genes are those that are bound by SIN3 187 and only change in expression when SIN3
433 187 levels are changed. B. Average binding intensity of SIN3 isoforms on regulated
434 genes plotted from -3 kb to 3 kb. R = repressed, A = activated, UR = uniquely
435 repressed, UA = uniquely activated, TSS = transcription start site.

436

437 Fig. 3. H3K4 methylation at genes regulated by the SIN3 isoforms. A. H3K4me1,
438 H3K4me2, and H3K4me3 enrichment was mapped to SIN3 220 and SIN3 187 regulated
439 genes. Heat maps and binding profiles were generated spanning genes from -3 kb to 3
440 kb. B. H3K4me1, H3K4me2, and H3K4me3 enrichment was mapped to genes bound
441 by SIN3 187 and that only change in expression when SIN3 187 levels are changed.
442 Heat maps and binding profiles were generated spanning genes from -3 kb to 3 kb. R =
443 repressed, A = activated, UR = uniquely repressed, UA = uniquely activated, TSS =
444 transcription start site.

445

446 Fig. 4. RNA pol II pausing at SIN3 regulated genes. A. RNA Pol II subunit Rpb3 and
447 pausing factor NELF-A binding were mapped against genes regulated by both SIN3
448 isoforms. B. Pausing index of genes regulated by both isoforms. Rpb3 binding
449 enrichment value at the promoter (-50 bp to +50 bp) was divided by Rpb3 enrichment
450 value at the gene body (+300 bp to -100 bp TES). R = repressed, A = activated, TSS =

451 transcription start site, TES = transcription end site. Significance was determined by
452 Mann Whitney U Test. *** indicates p value <0.001.

453

454 Fig. 5. SIN3 220 binding motifs and alignment. A. SIN3 220 activated and B. repressed
455 genes binding motifs were generated using MEME software and top three motifs are
456 shown. Motifs were then compared to known Drosophila transcription factor (TF) motifs
457 using TOMTOM software and the three most statistically significant TFs are shown.
458 Pearson correlation coefficient statistical analysis was utilized (Gupta et al., 2007;
459 Pietrokovski, 1996).

460

461 Fig. 6. SIN3 187 binding motifs and alignments. Binding motifs of genes A. activated
462 and B. repressed by SIN3 187 were generated using MEME software and top three
463 Motifs are shown. Motifs were then compared to known Drosophila transcription factor
464 (TF) motifs using TOMTOM software and the three most statistically significant TFs are
465 shown. Pearson correlation coefficient statistical analysis was utilized (Gupta et al.,
466 2007; Pietrokovski, 1996).

467

468 Fig. 7. SIN3 isoforms act as soft regulators. Genes regulated and bound by the SIN3
469 isoforms were parsed based on the expression level change when SIN3 levels were
470 perturbed. R = repressed, A = activated.

471

472 Fig. 8. SIN3 187 acts as a hard regulator on a subset of genes. A. FPKM values of
473 genes in Drosophila S2 cells were parsed based on the expression level of those genes
474 in wild-type cells and the regulation of those genes by the SIN3 isoforms. Genes with
475 FPKM >1000 are considered strongly expressed, genes with FPKM 10-1000 as
476 moderately expressed and genes with FPKM < 1 as silenced. B. SIN3 187 binding (from
477 -3 kb to +3 kb from the TSS) on the 53 genes activated by ectopic expression of SIN3
478 187 and are silent in wild-type S2 cells (black line). This binding profile was compared to

479 the profile of all genes activated by ectopic expression of SIN3 187 (red line). TSS =
480 transcription start site.

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624

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SIN3 187	GSE72173
H3K4me1	GSE50366
H3K4me2	GSM2756643
H3K4me3	GSE50369
Rpb3	GSM4745377
NELF-A	GSM4745369

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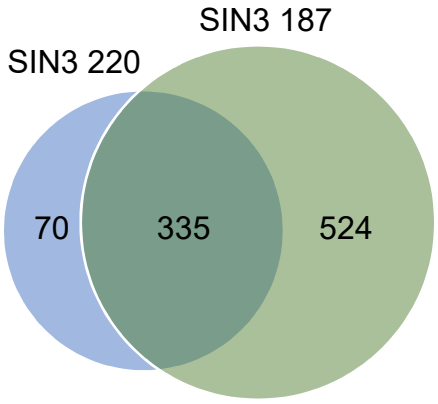

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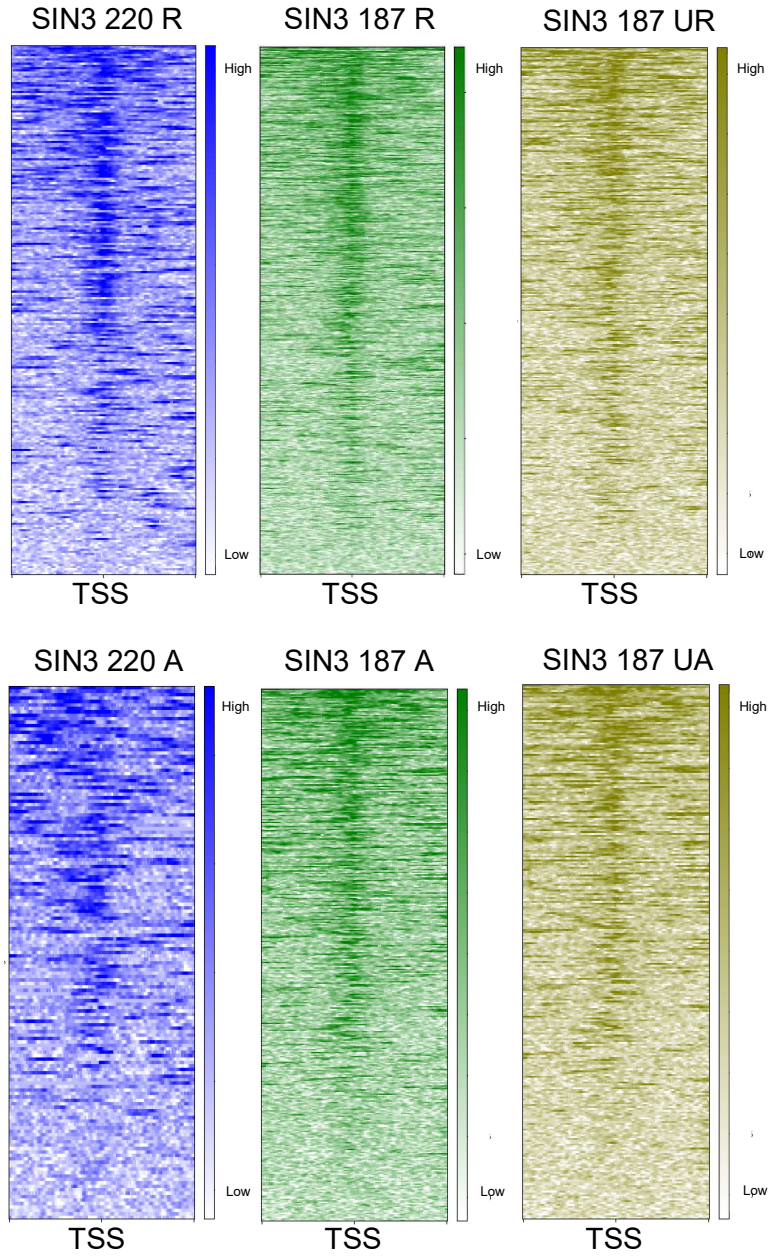
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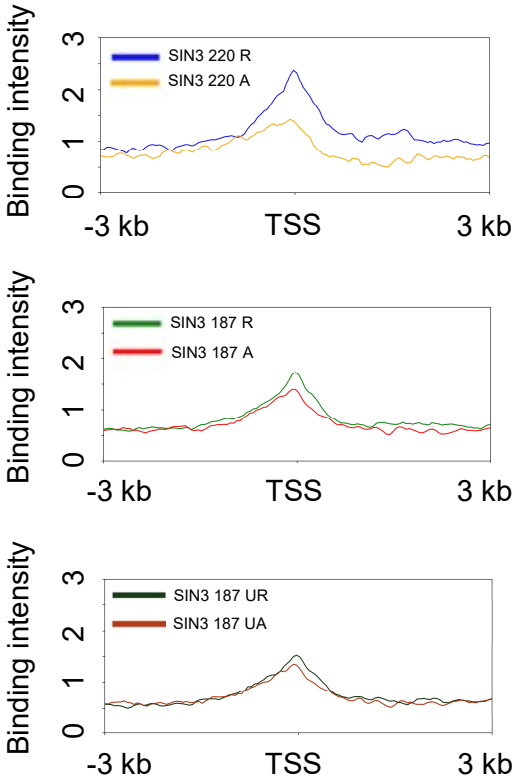
High
Low
TSS

B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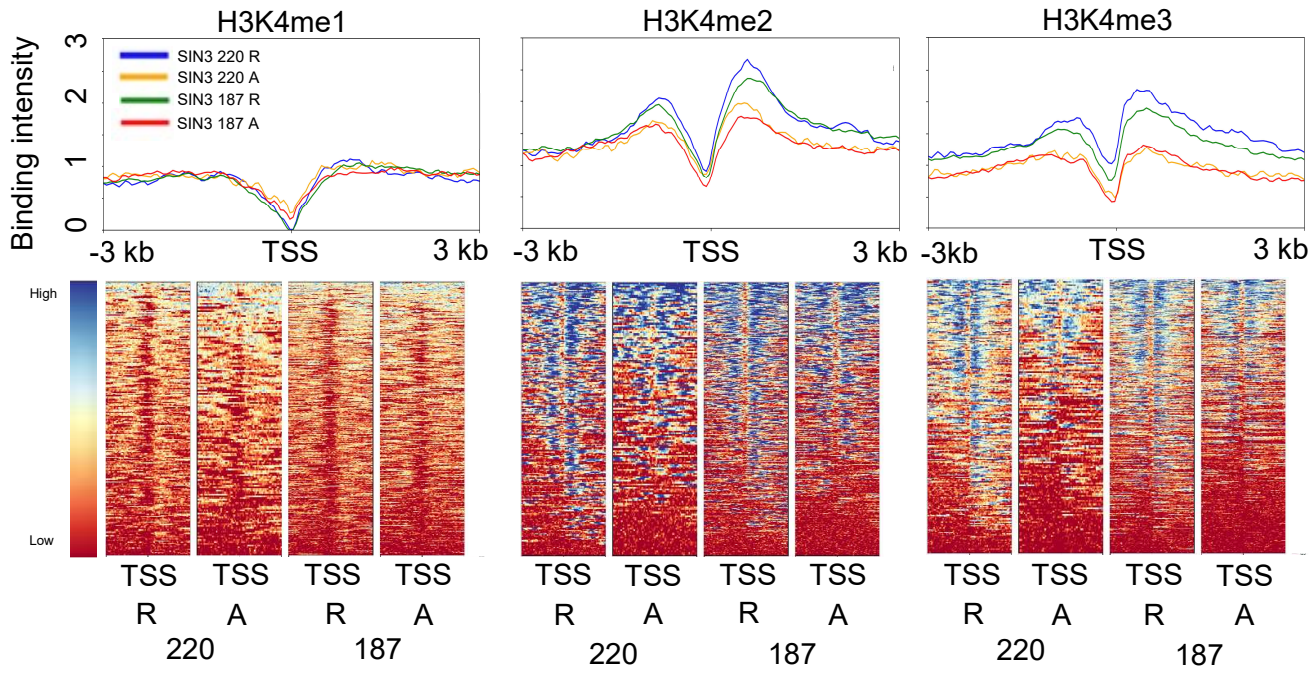
-3 kb
TSS
3 kb

A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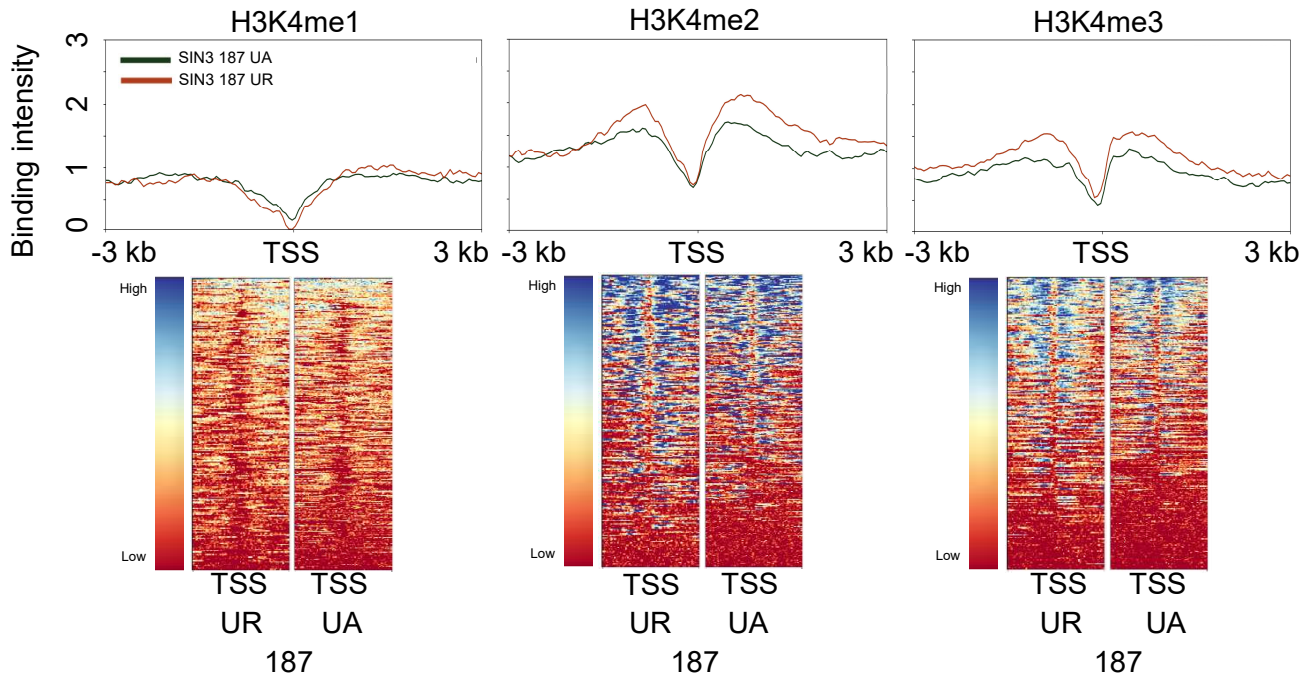
SIN3 187 A
-3 kb
TSS
3 kb
-3kb
High
Low
TSS R 220
TSS A 220
TSS R 187
TSS A 187

B

E

F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TSS UR 187
TSS UA 187

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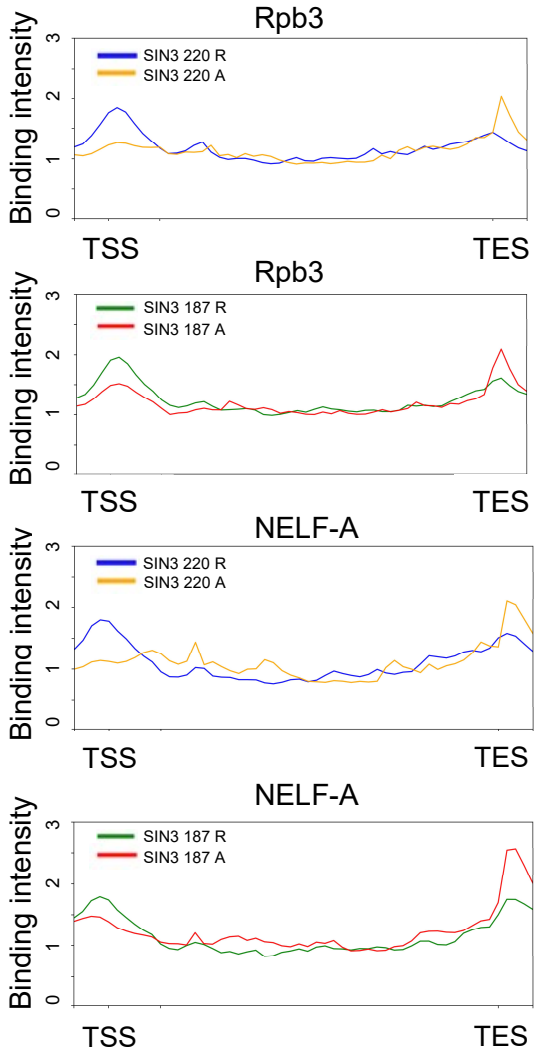
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Binding intensity
0
1
2
3
TSS
TES
NELF-A
SIN3 220 R
SIN3 220 A
Binding intensity
0
1
2
3
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TES
NELF-A
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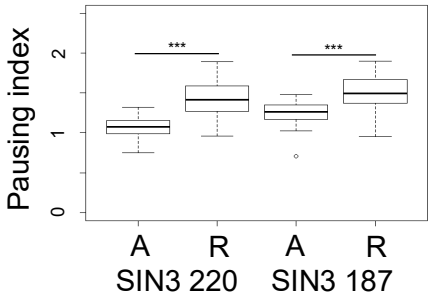
SIN3 187 A
Binding intensity
0
1
2
3
TSS
TES

B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2
***
***
A
R
A
R
SIN3 220
SIN3 187

A

SIN3 220 activated genes

SIN3 binding motif	TF motif	TF	P value
		Da	0.0037
		Sna	0.0057
		Btd	0.0050
		Luna	0.0008
		CG3065	0.0006
		Tin	0.0014
		Hand	0.0006
		Phol	0.0021
		Peb	0.0034

B

SIN3 220 repressed genes

SIN3 binding motif	TF motif	TF	P value
		Lola	0.0010
		Ase	0.0019
		Br	0.0048
		Ci	0.0021
		Da	0.0037
		Tin	0.0056
		Ttk	0.0035
		Gl	0.0038
		CG12155	0.0049

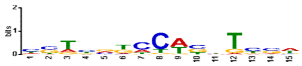
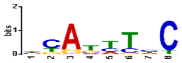
A

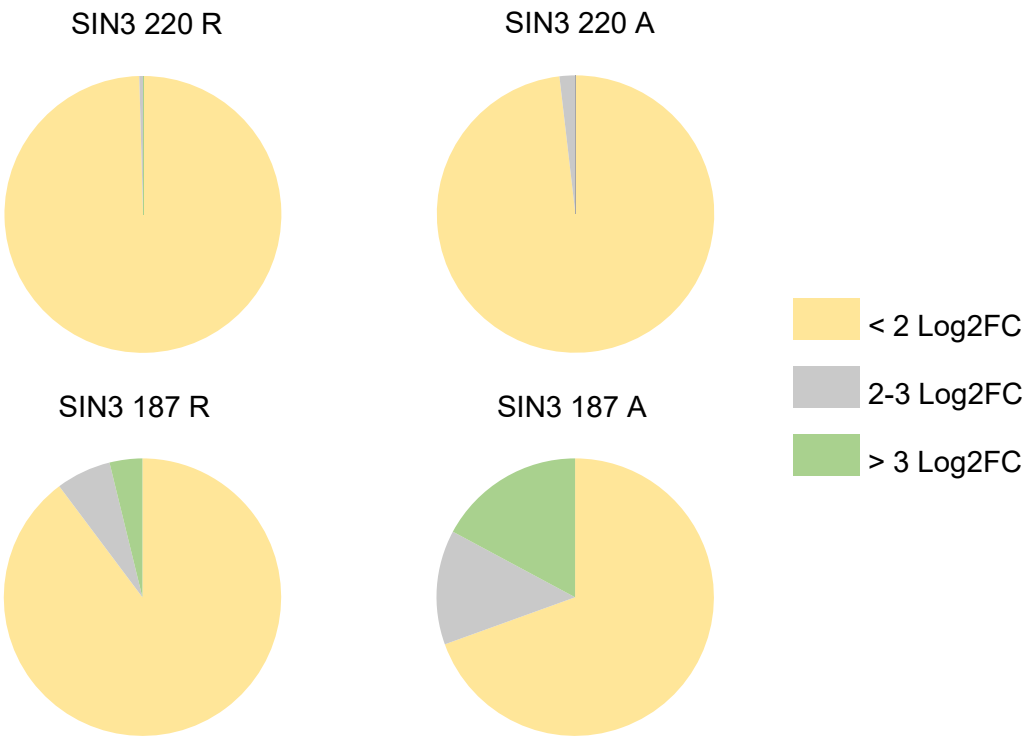
SIN3 187 activated genes

SIN3 binding motif	TF motif	TF	P value
		Lola	0.0018
		Wor	0.00571
		CG4360	0.0001
		Lola	0.0013
		CG3407	0.0022
		D18A	0.0005
		Disco-r	0.0008
		Dichaete	0.0028

B

SIN3 187 repressed genes

SIN3 binding motif	TF motif	TF	P value
		Pho	0.00361
		CG13897	0.0009
		Srp	0.00286
		Fer1	0.00317
		Ets96B	0.00430
		D19A	0.00471
		Ets65A	0.00516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SIN3 220 A
SIN3 187 R
SIN3 187 A
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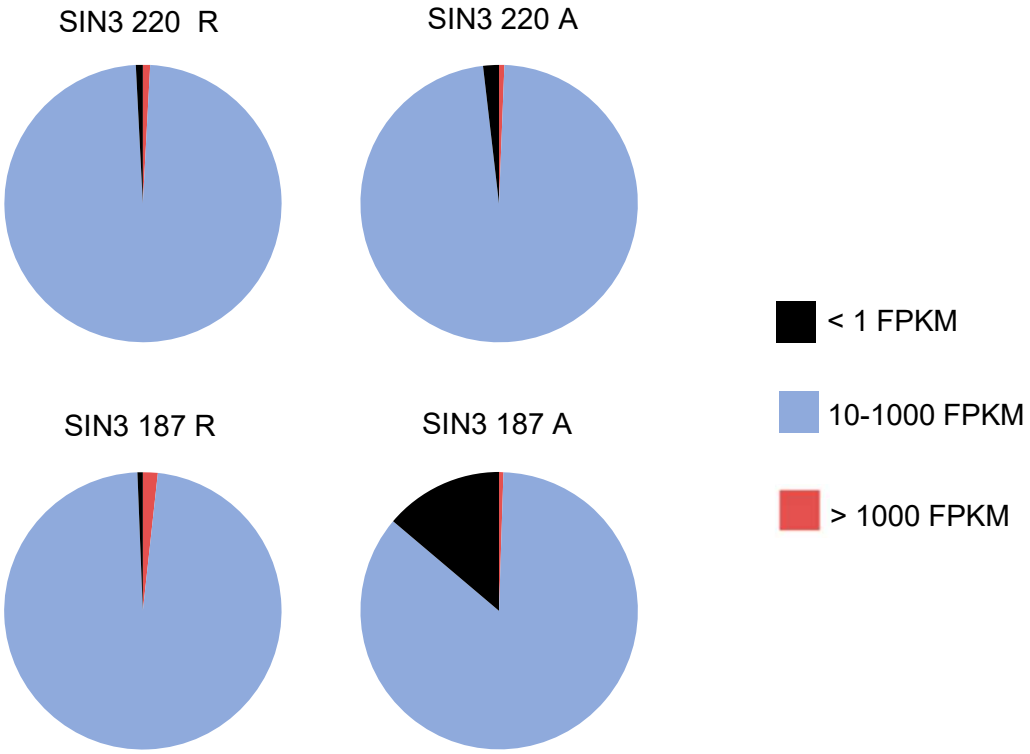
2-3 Log2FC
> 3 Log2FC

A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SIN3 220 A
SIN3 187 R
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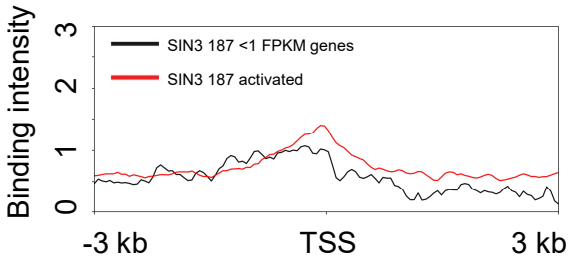
10-1000 FPKM
> 1000 FPKM

B


Binding intensity
0
1
2
3
SIN3 187 <1 FPKM genes
SIN3 187 activated
-3 kb
TSS
3 kb