**Drosophila** SIN3 Isoforms Interact with Distinct Proteins and Have Unique Biological Functions*

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The SIN3 corepressor serves as a scaffold for the assembly of histone deacetylase (HDAC) complexes. SIN3 and its associated HDAC have been shown to have critical roles in both development and the regulation of cell cycle progression. Although multiple SIN3 isoforms have been reported in simple to complex eukaryotic organisms, the mechanisms by which such isoforms regulate specific biological processes are still largely characterized. To gain insight into how SIN3 isoform-specific function contributes to the growth and development of a metazoan organism, we have affinity-purified two SIN3 isoform-specific complexes, SIN3 187 and 220, from *Drosophila* S2 cells and embryos. We have identified a number of proteins common to the complexes, including the HDAC RPD3, as well as orthologs of several proteins known to have roles in regulating cell proliferation in other organisms. We additionally identified factors, including the histone demethylase little imaginal discs and histone-interacting protein p55, that exhibited a preferential interaction with the largest SIN3 isoform. Our experiments indicate that the isoforms are associated with distinct HDAC activity and are recruited to unique and shared sites along polytene chromosome arms. Furthermore, although expression of SIN3 220 can substitute for genetic loss of other isoforms, expression of SIN3 187 does not support *Drosophila* viability. Together our findings suggest that SIN3 isoforms serve distinct roles in transcriptional regulation by partnering with different histone-modifying enzymes.

Transcriptional regulation by SIN3 histone deacetylase (HDAC) complexes is essential for a number of important biological processes. For instance, SIN3 complexes are required for viability, as demonstrated by the finding that mutations in SIN3 result in embryonic lethality in both *Drosophila* and mouse (1–4). Furthermore, genome-wide localization and gene expression studies have mapped the SIN3 regulatory network to include nuclear genes involved in mitochondrial biogenesis and function (5), genes involved in DNA replication and repair (6), and genes involved in development (2, 5, 6). Additionally, functional studies in both *Drosophila* and mammalian systems have shown that SIN3 is an important factor in the regulation of cell cycle progression and exit (2, 3, 7–10). Together, these studies highlight the importance of SIN3 in both growth and development.

The SIN3 corepressor serves as a scaffold for the assembly of HDAC complexes. These complexes are recruited to chromatin, where the catalytic subunit, RPD3 in yeast and *Drosophila* and HDAC1 and -2 in mammals, deacetylates histones to repress transcription (11, 12). Compositionaly similar SIN3 complexes from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and mammals have been isolated and characterized, illustrating the conservation of SIN3 complex proteins among eukaryotes (11, 13). This similarity further suggests that the essential functions of these complexes may be conserved as well.

In yeast, two distinct mechanisms of SIN3-mediated repression of gene transcription have been characterized. First, SIN3 complexes, such as the Rpd3L (large) complex, can be recruited to the promoter of target genes by DNA-binding factors or other corepressors to inhibit transcription (14–17). Second, SIN3 complexes, such as Rpd3S (small), can be recruited by chromatin-associated proteins to the coding region of a target gene to repress transcription from internal cryptic promoters (14, 15, 18). In mammals, a third mechanism has been demonstrated in which SIN3 is recruited by the transcription factor E2F4 to downstream regions of cell cycle-regulated genes (6). After recruitment, SIN3 spreads farther downstream to permanently silence these genes during differentiation (6). Despite these recent advances, the molecular mechanisms by which SIN3 complexes regulate transcription at specific subsets of target genes to affect the growth and development of multicellular organisms are still not well understood.

SIN3 isoforms have been shown to form distinct complexes that function differently. For example, in *S. pombe*, three SIN3 proteins (PST1, -2, and -3) are encoded by separate genes (11, 19). The PST1-containing complex, similar to Rpd3L of *S. cerevisiae*, is recruited to promoters (14, 15, 18). The complex containing PST2, similar to the Rpd3S complex of *S. cerevisiae*, is recruited to coding regions (14, 15, 18). Mammalian Sin3A and -B, also encoded by separate genes, are known to perform different functions as well. For example, mSin3A has been shown to promote cell proliferation during embryonic development (1, 2), whereas mSin3B is up-regulated in response to oncogenic
stress and is required for cellular senescence (9). Although these studies establish SIN3 isoforms as distinct proteins that serve separate essential roles in a developing organism, a systematic analysis of metazoan isoform-specific complexes is still lacking.

The *Drosophila* Sin3A gene produces multiple alternatively spliced isoforms that differ only at the C terminus (3, 4). These isoforms are differentially expressed and are hypothesized to perform different functions during *Drosophila* development (20). SIN3 220, the isoform of 220 kDa, is the predominantly expressed isoform in highly proliferative cells, such as immortalized cell lines and larval imaginal discs, whereas SIN3 187 is the predominant isoform in differentiated tissues, such as those found in late stage embryos and adult flies (20). A third isoform, SIN3 190, which is not conserved in other insect species, is detected only in adult females and embryos.

Although expression patterns of the *Drosophila* SIN3 isoforms suggest that they perform unique roles during development, the nature of these roles has not been characterized. To understand how SIN3 isoforms contribute to the growth and development of a metazoan organism, we have affinity-purified SIN3 isoform-specific complexes from both *Drosophila* S2 cells and embryos. We report here that *Drosophila* SIN3 187 and 220 are found in distinct HDAC complexes containing both shared and unique proteins. We further show that these complexes exhibit different HDAC activity and that SIN3 187 and 220 are localized to discrete regions on polytene chromosomes. Additionally, isoform-specific rescue experiments indicate that expression of SIN3 187 is insufficient to support fly viability. Overall, these results clearly demonstrate that SIN3 187 and 220 are functionally distinct. The results further suggest that isoform-specific proteins likely contribute to the unique regulatory functions of distinct SIN3 complexes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—S2 cells were grown in Schneider’s *Drosophila* medium (1×) + 1-glutamine with 10% heat-inactivated fetal bovine serum (Invitrogen). 50 mg/ml gentamycin was added to the medium for S2 control cells, whereas 0.1 mg/ml penicillin/streptomycin and 0.1 mg/ml Geneticin were added to stably transfected cell lines. Lines carrying expression constructs for HA-tagged SIN3 187 or 220 or FLAG-tagged p55 were made by transfecting S2 cells with a pMT vector containing cDNA for tagged SIN3 187, SIN3 220, or p55. The cells were simultaneously transfected with the pV9 vector that carries the neomycin gene to allow for selection of the transformed cells. Cells that carried chromosomal insertions of both transgenes were then selected for by growth in Geneticin. TAP-tagged SIN3 187 and 220 cell lines were constructed in the same manner, except that the TAP vector carries the Hygromycin B marker. Cells were thus grown in medium with 50 mg/ml gentamycin and 300 μg/ml Hygromycin B. Details regarding construction of the expression plasmids are available upon request.

**Drosophila Stocks**—*Drosophila melanogaster* stocks were maintained, and crosses were performed according to standard laboratory procedures. The following stocks were used: UAS-p55 RNA interference (RNAi) (26455GD), obtained from the Vienna *Drosophila* RNAi Center; Act-GAL4 (4414), tub-GAL4 (5138), en-GAL4 (8828), and Sin3A08268 (12350), all obtained from the Bloomington Stock Center; UAS-SIN3RNAi, as described (20); and w1118 and Sin3Ae376 (gift from Dr. David Wassarman). The UAS-190,220RNAi lines were generated according to the protocol given in Ref. 20. The targeting sequence that was cloned into the pWiz vector was generated using the following primers (oriented 5’ to 3’): CAGTTCTAGAGCGTAACTCAGGCAGAATAC and CAGTTCTAGACGTCGAGAAGTGTATCAC. To generate lines with constitutive ubiquitous expression of either SIN3 187HA or 220HA, the UAS-187HA or UAS-220HA transgenes were recombined onto chromosomes containing tub-GAL4 or Act-GAL4, respectively. For the rescue of lethality experiments, the UAS-187HA and UAS-220HA transgenes were recombined onto the Sin3A08268 and Sin3Ae376 mutant chromosomes. A stock containing this recombinant chromosome and tub-GAL4 transgene was created and maintained over CyO and Sb balancer chromosomes.

**Cell Culture Nuclear Extract Preparation and Co-immunoprecipitation**—Nuclear extracts were prepared from both S2 control and SIN3HA-transformed cells. 1 × 10⁶ cells were pelleted at 1250 × g for 5 min. Cells were washed with 10 ml of phosphate-buffered saline (PBS) and centrifuged for 5 min. Cells were lysed in 5 ml of cell lysis buffer (50 mM Hepes (pH 7.4), 1 mM MgCl₂, 0.1% Triton X-100, 0.5 mM EDTA, 150 mM NaCl, 1 mM DTT, 1 Complete protease inhibitor minitabit (Roche Applied Science) per 10 ml of buffer. Lysis was monitored by trypsin blue staining. Isolated nuclei were pelleted at 2000 × g for 5 min through 1 ml of nuclear lysis buffer plus sucrose (15 ml of 1 M sucrose was added to 50 ml nuclear lysis buffer) and resuspended in 1 ml of nuclear lysis buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 Complete Protease Inhibitor minitabit (Roche Applied Science) per 10 ml of buffer and incubated on ice for 1–2 h. Debris was cleared by centrifugation at 16,000 × g for 10 min. Approximately 550 μl of nuclear extract was incubated with 40 μl of anti-HA beads (HA-7 monoclonal antibody conjugated to Sepharose beads (Sigma). The total volume was increased to 650 μl with interaction buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 10% glycerol) in a 1.5 ml microcentrifuge tube. Extracts were incubated with the antibody beads overnight. The beads were washed with each of the following three buffers for the indicated times: radioimmune precipitation buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfide, 0.1% sodium deoxycholate) one time for 5 min; Wash 2 (20 mM Hepes (pH 7.4), 500 mM NaCl, 0.5 mM EDTA, 1.5% Triton X-100, 0.1% sodium deoxycholate, 10% glycerol) two times for 5 min; Wash 3 (10 mM Tris-HCl (pH 8), 300 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) one time for 5 min. Bound proteins were eluted by incubation with 25 μl of Laemmli buffer (Bio-Rad) for 5 min.

**Embryo Whole Cell Extract Preparation and Co-immunoprecipitation**—Whole cell extracts were prepared from 0–24 h 187HA, 220HA, and w1118 embryos as described previously (21) with minor modifications. 300–400 μl of extract was incubated with 40 μl of anti-HA beads for 2–6 h at 4 °C. Bound proteins were eluted with 25 μl of Laemmli buffer.
Histone Extract Preparation—$5 \times 10^7$ cells were pelleted at 1250 $\times g$ for 5 min. Histones were acid-extracted by resuspending the cells in 500 $\mu$L of 0.4 N sulfuric acid in PBS, incubating for 30 min on ice, and centrifuged at 12,000 $\times g$ for 10 min. The extracts were dialyzed overnight at 4 °C against 0.1 N glacial acetic acid in distilled H$_2$O and for 4 h in distilled H$_2$O the next day. After TCA precipitation, protein concentrations were determined using the DC Protein Assay (Bio-Rad).

Embryo Nuclear Extract Preparation—Nuclear extracts were prepared from 0–24 h 187HA, 220HA, and w$^{118}$ embryos as described previously (22) with the following minor modifications. 10–15 g of washed, dechorionated embryos were first homogenized in Buffer I (1 ml per 1 g of embryos) using a mortar and pestle. The homogenate was then transferred to a Dounce homogenizer and disrupted with 20 strokes of a loose pestle. The soluble nuclear extract was cleared by centrifugation at 10,000 $\times g$ for 50 min.

Affinity Purification and Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) Analysis—4 ml of nuclear extract prepared from 4 $\times 10^6$ S2 cells or 10–15 g of embryos, as described above, was incubated with 150–200 $\mu$L of anti-HA beads overnight at 4 °C. To control for nonspecific interactions, immunoprecipitations were also performed using extracts prepared from non-transfected S2 cells and non-transgenic wild type w$^{118}$ Drosophila embryos. Bound proteins were eluted with 3 $\times$ 200 $\mu$L of 500 $\mu$L/ml HA peptide, and samples were concentrated using 10 kDa molecular mass cut-off Amicon Ultra 4 spin columns (Millipore).

The LC/MS/MS analysis was performed at the Proteomics Facility Core of the Institute of Environmental Health Sciences, Wayne State University. Eluted proteins were separated by PAGE, and the resulting lanes were cut into gel slices. Proteins were reduced, alkylated, and digested with trypsin in gel. Peptides were separated by reverse phase chromatography before introduction into a linear ion trap mass spectrometer (LTQ- XL, Thermo Scientific). Peptide identification of MS2 spectra was scored using Proteome Discoverer 1.1 (Thermo) and Mascot (Matrix Science) software using the latest FlyBase D. melanogaster protein data base (r5.24). A MudPIT-type strategy was employed (all LC/MS/MS runs for a lane were grouped into one file), and proteins were positively identified if minimally two unique peptides per protein scored above the 5% false discovery rate cut-off. Nonspecific proteins identified in the control immunoprecipitations were removed from the list. Protein abundance was estimated by spectral counting. Results were normalized to the number of SIN3 peptides in each group and further adjusted to account for protein size. Fold difference protein binding in SIN3 187 and SIN3 220 immunofinity pull-down experiments was determined by dividing normalized spectral counts of the 220 experiment by those of 187 for each protein identified.

Gel Staining—Gels were stained using the SilverSnap Silver Stain kit from Pierce or the Silver Stain kit from Owl Scientific. Gels from which bands were excised for LC/MS/MS analysis were stained with SYPRO Ruby (Sigma).

Western Blotting—Western blot analysis was performed in accordance with standard protocols (23). Proteins were separated on an 8% SDS-polyacrylamide gel, 15% for histone extracts, and transferred to a polyvinylidine difluoride (PVDF) membrane (Pall). Membranes were incubated with the primary antibodies HA-HRP (1:6000; Sigma), FLAG (1:5000; Sigma), SIN3 187 (1:2000 (24)), RP3D (1:1000 (24), p55 (1:500; kindly provided by Dr. Carl Wu), CBP (1:5000; Millipore), H3 (1:30000; Abcam), H3K9/K14Ac (1:5000; Millipore), H3K9Ac (1:6000; Millipore), H3K14Ac (1:5000; Millipore), H4K8Ac (1:600; Millipore), or H4K12Ac (1:1500; Millipore), followed by incubation in donkey anti-rabbit HRP-conjugated IgG (1:3000; GE Healthcare) secondary antibody where applicable. The antibody signals were detected using the ECL + Western blot detection system (GE Healthcare).

Histone Deacetylase Activity Assays—Histone deacetylase activity was monitored using the HDAC fluorimetric assay/drug discovery kit AK-500 (Enzo Life Sciences). SIN3 187 and 220 complexes were affinity-purified as described above. 18 $\mu$L of the eluted complexes was incubated with 25–500 $\mu$L Mu Fluor de Lys substrate at 30 °C for 0–20 min. After the addition of the developer, the fluorescence was read using a Gemini XPS Microplate Spectrofluorometer (MDS Analytical Technologies) with excitation at 360 nm and fluorescence at 460 nm. Counts were normalized to the levels of RPD3 as determined by Western blot analysis. Kinetic data were analyzed by calculating the change in arbitrary fluorescent units (AFU)/min between the 10 and 20 min time points and plotting this data versus substrate concentration. The $K_m$ and $V_{max}$ were determined using Prism curve-fitting software (GraphPad Software). At least three independent experiments were performed at each substrate concentration.

Polytene Chromosome Preparation and Staining—Polytene chromosome preparation and staining was performed as described previously (24) using the following antibodies: mouse anti-HA-FITC (1:100; Sigma), rabbit anti-SIN3 220 (1:500 (20)), and secondary antibody Alexa 594 donkey anti-rabbit (1:400; Invitrogen).

Statistical Analyses—An unpaired two-tailed Student’s t test was used to determine significance for the p55 genetic rescue experiments, whereas a paired t test was used to determine significance for the differences in histone acetylation levels among S2, 187HA, and 220HA cells. For the kinetic HDAC analyses, the statistical function present in the Prism curve-fitting software (GraphPad Software) was used to compare the $K_m$ and $V_{max}$ for 187HA and 220HA.

RESULTS

SIN3 Isoforms Serve as the Scaffold for Unique Histone Deacetylase Complexes—To identify isoform-specific SIN3-interacting factors, we generated stably transfected S2 cell lines or transgenic Drosophila that express either the SIN3 187 or SIN3 220 isoform with a C-terminal HA tag. The transgenes used for S2 cell expression were under the control of the inducible metallothionein promoter, whereas transgene expression in flies was controlled by the GAL4-UAS system. To ensure that the SIN3 187 and 220 proteins were expressed in cultured cells, we prepared whole cell extracts from 187HA, 220HA, and S2 control cells and probed a Western blot with antibody to the HA tag. Western blot analysis confirmed that SIN3 187HA and 220HA are expressed (Fig. 1A). The transgenic fly lines that...
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were generated have a single chromosome carrying both a ubiquitous GAL4 and a UAS-SIN3 187HA or 220HA transgene. To verify that these flies express SIN3 187HA and 220HA, we generated whole cell extracts from adult SIN3 187HA, 220HA, and \( w^{1118} \) control flies. Western blot analysis with antibody to the HA tag confirmed that the transgenic flies express SIN3 187HA and 220HA (Fig. 1A).

To identify proteins that interact with SIN3 187 and 220, we affinity-purified SIN3HA complexes from the S2 cells and flies described above. Nuclear extracts prepared from SIN3 187HA and 220HA S2 cells and transgenic \( Drosophila \) were incubated with anti-HA-agarose affinity gel, and bound proteins were eluted with HA peptide. Three independent purifications from both S2 cells and embryos were performed. To determine whether the SIN3 187 and 220 isoforms interact with different sets of proteins, the co-immunoprecipitated proteins were separated by SDS-PAGE and visualized by silver staining (Fig. 1B). The co-immunoprecipitated proteins were then identified by LC/MS/MS. Through this analysis, we identified a number of proteins common to both isoforms as well as some proteins unique to one SIN3 protein or the other (Fig. 1C and supplemental Tables 1 and 2). Although obvious differences in interacting proteins are not apparent in the silver stain of the embryo samples, distinct proteins were definitely identified by the more sensitive LC/MS/MS analysis. The sets of interacting factors, which were very similar between S2 cells and embryos, included a number of proteins previously found to be part of either a yeast or mammalian SIN3 complex (supplemental Table 3). These findings clearly demonstrate that SIN3 complexes are conserved from yeast to \( Drosophila \) to mammals.

Analysis of the SIN3 purifications from the cultured cells identified seven proteins in addition to SIN3 that were isolated as part of SIN3 complexes from other organisms (Fig. 1C). RPD3, SDS3, and ARID4B were found in similar levels in both \( Drosophila \) complexes, whereas BRMS1L, SAP130, ING1, and p55 were present at higher levels in the SIN3 220 complex. Orthologs of six of these proteins were purified as components of an mSin3A complex from human erythroleukemia cells (25) (supplemental Table 3). Additionally, both \( Drosophila \) complexes appear to be more similar to the yeast Rpd3L/Complex I as opposed to Rpd3S/Complex II (supplemental Table 3). The identification of two \( Drosophila \) SIN3 complexes similar to Rpd3L/Complex I does not necessarily imply that an Rpd3S/Complex II type complex is absent from \( Drosophila \). The purification approach used in this study may have precluded recovery of such a complex. Instead, the presence of two isoform-specific SIN3 complexes similar to Rpd3L/Complex I suggests that there are likely additional mechanisms for SIN3-mediated regulation in \( Drosophila \) that may not be present in yeast.

A particularly interesting attribute of the \( Drosophila \) complexes is that p55 was present almost exclusively in the SIN3 187 complex. Prw1, the ortholog of p55 in \( S. pombe \), is present in both Complex I and Complex II. Also, a similar but not orthologous protein, Umel1, is present in both Rpd3L and S complexes. In addition, p55 orthologs are thought to be part of a catalytic core, important for the HDAC activity of SIN3 complexes (26–28). As such, we expected that p55 would interact strongly with both SIN3 187 and 220. The absence of p55 from the SIN3 187 complex suggests that p55 is not essential for the HDAC activity of all SIN3 complexes. It is possible, instead, that through an ability to bind histone H4 (29, 30), p55 could be important for recruiting or stabilizing the SIN3 220 complex to a specific subset of target genes.

In addition to the proteins described above, two factors, LID (little imaginal discs) and ESMY (CG15356), not previously identified as SIN3 core complex components, were found to interact with SIN3 220. LID is a histone H3K4 demethylase that has recently been shown to interact with RPD3 in \( Drosophila \) (31, 32). A LID complex containing RPD3, Pf1, MRG15, and CG13367, but not SIN3, was previously isolated from \( Drosophila \).
illa embryo nuclear extracts (31). Drosophila SIN3 was co-immunoprecipitated with LID, however, as part of the RLAF complex, of which RPD3, MRG15, Pf1, and EMSY are also members (32). The LAF complex is composed of the same proteins as RLAF except that RPD3 is not present (32). The mass spectroscopic analysis of S2 cell culture purifications in the current study identified RPD3, LID, and EMSY but not MRG15 or Pf1 as SIN3 220-interacting factors. This result, together with the LID, LAF, and RLAF purifications, illuminates the existence of multiple complexes of varying composition with different combinations of histone-modifying activity. These data further suggest that the specific interaction of LID and EMSY with SIN3 220 likely contributes to the functional differences between SIN3 187 and 220, thus adding to the complexity of SIN3-mediated regulation of transcription.

Mass spectroscopic analyses of the embryo purifications were similar to that of S2 cells with a few notable differences. Overall, the analyses yielded a list of 59 proteins common to both isoforms, an additional 19 proteins enriched in the SIN3 220 immunoprecipitation, and 11 proteins enriched in the SIN3 187 immunoprecipitation (Fig. 1C and supplemental Table 2). In addition to p55, BRMS1L exhibited a preferential interaction with the SIN3 220 isoform. EMSY and ARID4B were not found in either complex, whereas Pf1, which is present in multiple mSin3 complexes (33–35), was found to interact with both embryonic SIN3 187 and 220. The heterogeneous cell population present in embryos likely contributed to the identification of a larger number of proteins in embryos than in S2 cells.

Two proteins, SAP18 and SAP30, predicted to be part of the Drosophila SIN3 complex (28, 36, 37) were absent from both the S2 cell and embryo purifications. Consistent with these data, a Western blot of cell culture and embryo purifications probed with antibody to SAP18 did not detect co-immunoprecipitation of SAP18 with either SIN3 187 or 220 (data not shown). Furthermore, RNAi knockdown of SIN3, RPD3, and p55 arrested S2 cell proliferation, whereas knockdown of SAP18 or SAP30 had no effect (7). Together, these results suggest that SAP18 and SAP30 are not components of the Drosophila SIN3 complexes.

RPD3 Interacts with Both SIN3 187 and 220—The histone deacetylase RPD3 is the catalytic subunit responsible for the majority of SIN3 complex-mediated repression (16, 38). Drosophila RPD3 co-localizes with SIN3 on polytenic chromosomes, and binding of SIN3 and RPD3 to ecdysone-regulated loci correlates with a decrease in transcription from those loci (24). As described above, LC/MS/MS analysis of SIN3 187HA and 220HA purifications revealed an interaction of RPD3 with both isoforms, as expected. Similarly, Western blot analysis of HA purifications from both cultured cells and embryos probed with antibodies to the HA tag and to RPD3 showed that RPD3 co-immunoprecipitated with both SIN3 187 and 220 (Fig. 2, A and C). No signal for RPD3 appeared in the lane containing the bound fraction of the S2 control cell extract (Fig. 2A). These results confirm the interaction of RPD3 with SIN3 obtained by mass spectroscopic analysis and suggest that both the SIN3 187 and 220 complexes have enzymatic activity.

p55 Exhibits a Preferential Interaction with SIN3 220—Based on the literature, we predicted that p55, the smallest subunit of CAF-1 (chromatin assembly factor 1), would co-immunoprecipitate with Drosophila SIN3 187 and 220 (34, 39–42). LC/MS/MS analysis detected p55 in the triple complex SIN3 220 affinity purifications in both S2 cells and embryos. Unexpectedly, it was not detected in any of the embryo SIN3 187 purifications and was detected in only one of the S2 cell replicates. Additionally, Western blot analysis of HA purifications from both cultured cells and embryos showed a strong signal for p55 in the SIN3 220 bound fraction, and a weak signal in the SIN3 187 bound fraction (Fig. 2, A and C). Together, these results identified a preferential interaction of p55 with SIN3 220.

To confirm the interaction of p55 with SIN3 220, we affinity-purified p55 from a stably transfected cell line and a transgenic fly line that express the cDNA for p55 with a 2× N-terminal FLAG tag. Endogenous SIN3 220 co-immunoprecipitated with FLAGp55 in cultured cells as shown by Western blot analysis with an antibody recognizing all isoforms of SIN3 (Fig. 2B). As mentioned earlier, S2 cells predominantly express SIN3 220 (20). As such, we affinity-purified FLAGp55 from embryo whole cell extracts to confirm the preferential interaction of p55 with SIN3 220 in a tissue that endogenously expresses both isoforms in detectable amounts. Western blot analysis showed a strong signal for SIN3 220 but a relatively weak signal for SIN3 187 in the bound fraction (Fig. 2D), confirming the preferential interaction of p55 with SIN3 220. Furthermore, HA and FLAG purifications from SIN3 187HA or 220HA cells transiently transfected with the FLAGp55 expression vector yielded similar results (data not shown). We also generated stable cell lines carrying transgenes that can be induced to express N-terminally TAP-tagged SIN3 187 and 220 isoforms. When SIN3 187NTAP or 220NTAP were affinity-purified from these cells, Western blot analysis using antibody to the TAP tag confirmed the preferential interaction of p55 with SIN3 220 (supplemental Fig. 1), suggesting that none of the affinity tags are interfering with the interactions. Together, these results...
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FIGURE 3. Overexpression of SIN3 220 rescues lethality caused by p55 knockdown. Flies carrying an RNAi construct for p55 were crossed to the tub-GAL4 driver line or to recombinant flies expressing SIN3 187 under the control of GAL4 driven by the tubulin enhancer (SIN3 187HA) as well as to an Act-GAL4 driver line and flies expressing SIN3 220 under the control of GAL4 driven by the actin enhancer. Crosses were scored for the number of viable flies carrying the p55 RNAi and the GAL4 driver constructs, as compared with the flies with the p55 RNAi, GAL4 driver, and SIN3HA constructs. 187HA-2, 220HA-2, and 220HA-1, 2 represent distinct recombinant lines. Error bars, S.E.; n = 3 except for Act-GAL4 (n = 5); p55 RNAi/tub-GAL4 flies compared with p55 RNAi/187HA-2, p = 0.07 and RNAi/187HA-3, p = 0.09; p55 RNAi/Act-GAL4 flies compared with p55 RNAi/220HA-1, p = 9 \times 10^{-4} (***) and RNAi/220HA-2, p = 0.01 (**).

support an isoform-specific interaction of p55 with SIN3 220 and suggest that p55 is particularly important for the activity of SIN3 220.

p55 Genetically Interacts with SIN3 220—to explore the possibility of a functional interaction between p55 and SIN3 220, we performed genetic experiments using the GAL4-UAS system (43) in combination with RNAi (44). To determine whether we could detect biologically relevant isoform-specific interactions with p55, we crossed flies that ubiquitously express SIN3 187HA or 220HA with flies carrying an RNAi construct to induce p55 knockdown. The RNAi construct consists of a UAS enhancer that drives expression of an inverted repeat of p55 RNAi, which causes a reduction in protein expression. In this way, we generated flies that are knocked down for p55 but overexpress either SIN3 187 or 220.

p55 RNAi driven by ubiquitous GAL4 expression resulted in an extremely complete lethality (Fig. 3). Similarly, when SIN3 187 was overexpressed along with the p55 double-stranded RNA, virtually no flies survived to adulthood. When SIN3 220 was overexpressed, however, 58–70% of the flies were able to survive. The ability of SIN3 220 but not SIN3 187 to suppress the lethal phenotype is not due to the presence of higher levels of the 220 protein as compared with 187. SIN3 187HA levels are similar to or higher than that of 220HA, as shown by Western blot analysis of extracts prepared from embryos or adults of the parent lines (Fig. 1A) (data not shown). Furthermore, lethality caused by knockdown of SAP130, a protein that biochemically interacts with both isoforms, could not be rescued by overexpression of either SIN3 187 or 220 (data not shown). This result suggests that the rescue is specific to the SIN3 220-p55 interaction. We hypothesize that rescue by SIN3 220 occurs because the RNAi knockdown of p55 is incomplete. It is possible that the exogenous SIN3 220HA sequesters the remaining p55 into the complex to support its essential functions. A second possibility is that the overexpression of SIN3 220 allows the cell to bypass the requirement for p55. These genetic results support the biochemical data indicating that p55 preferentially interacts with SIN3 220. Furthermore, these findings demonstrate that there are functional differences between the 187 and 220 isoforms and suggest that the distinct activities of the complexes are likely directed by the action of isoform-specific proteins.

SIN3 187 and 220 Exhibit Distinct HDAC Activity in Vitro—The ability of overexpression of SIN3 220 but not SIN3 187 to suppress lethality due to p55 RNAi knockdown establishes a functional difference between these two isoforms. This functional difference is potentially due to a distinction in HDAC activity between the complexes. It is possible that unique components of the SIN3 220 complex, such as p55, could be important for its HDAC activity. p55 as well as the histone demethylase LID have been shown to affect HDAC activity (26, 31). To determine whether the presence of unique proteins, such as p55 and LID, influence the HDAC activity of the SIN3 220 complex, we compared the activity of affinity-purified SIN3 187HA and 220HA using an in vitro HDAC fluorimetric assay. S2 nuclear extracts were incubated with HA beads as a control. The samples were incubated with 50 μM substrate for either 0 or 20 min with or without the HDAC inhibitor trichostatin A, and the HDAC activity was detected by measuring the fluorescence of each sample (Fig. 4A). The 187 and 220 samples each exhibited detectable HDAC activity, consistent with the presence of RPD3 in the complexes. The activity of both samples was inhibited by trichostatin A, as expected. The SIN3 220 complex demonstrated a consistently lower, although not statistically significant, level of activity at 20 min as compared with the SIN3 187 complex (Fig. 4A).

To investigate this difference further, we analyzed the kinetics of the SIN3 187 and 220 complexes. Affinity-purified SIN3 187 and 220 were incubated with increasing concentrations of substrate for 0–20 min, and the K_{m} and V_{max} for each complex were determined (Fig. 4B). The values for K_{m} were similar for SIN3 187 and 220. The values for V_{max} differed significantly, with SIN3 220 exhibiting a lower V_{max} (Fig. 4B). These results suggest that the enzymes have similar affinity for the substrate but that the 220 complex has a slower rate than the 187 complex. It is possible that the activity of RPD3 in the SIN3 220 complex is inhibited to some extent by the presence of SIN3 220-specific proteins, such as LID.

Overexpression of SIN3 187 and 220 Differentially Affect Global Histone Acetylation Levels—The decreased activity rate exhibited by the SIN3 220 complex suggests that RPD3 is a less effective enzyme when complexed with SIN3 220 as opposed to SIN3 187. To further distinguish the HDAC activity of the SIN3 isoforms, we analyzed the effect of overexpressing SIN3 187 and 220 on global histone acetylation levels. Histones were extracted from S2 control, SIN3 187HA, and SIN3 220HA cells. The extracts were analyzed by SDS-PAGE followed by Western blotting with site-specific antibodies to acetylated H3K9 and H3K14, H4K8 and H4K12, and H3 (Fig. 5A). The Western blot signals were quantified, and histone acetylation signals were normalized to the H3 signals. These values were then used to determine relative acetylation levels. SIN3 187 overexpression resulted in a significant decrease in H3K9/14 acetylation (p = 0.03), as shown by antibody recognizing the dual H3K9/14Ac
antibody to H3K14Ac alone, 187 overexpression exhibited a noticeable although statistically insignificant decrease, whereas 220 overexpression showed no change. Together these results suggest that the SIN3 187 complex affects both global H3K9 and K14 acetylation, whereas SIN3 220 affects H3K9 alone. Additionally, small reductions in histone H4 acetylation upon overexpression of SIN3 187 were observed, although the changes were not statistically significant. Western blot analysis of whole cell protein extracts showed that the level of RPD3 remained constant upon overexpression of either SIN3 187 or 220 despite SIN3 187 having been expressed at higher levels than SIN3 220 (Fig. 5A). Overall, these data are consistent with the in vitro analysis indicating that SIN3 187 exhibits more robust HDAC activity and confirm that the isoforms are functionally distinct.

**SIN3 187 and 220 Localize to Distinct Regions on Polytene Chromosomes**—SIN3-mediated repression is accomplished by the recruitment of SIN3 complexes to chromatin (11, 16). It is possible that the *Drosophila* SIN3 isoforms are targeted to different subsets of genes or that they target different genomic regions as in yeast. For this reason, we sought to determine whether SIN3 187 and 220 are recruited to distinct locations on polytene chromosomes. Polytene spreads were prepared from the recombinant flies expressing SIN3 187HA or 220HA. The polytene chromosomes from the 187HA flies were stained with antibody to the HA tag to visualize SIN3 187 and with antibody specific to SIN3 220 to visualize endogenous SIN3 220 (Fig. 6). The staining showed that SIN3 187 and 220 co-localized at many euchromatic regions. There are several distinct loci, however, that were bound by one isoform or the other, suggesting that the isoforms regulate different genes or that they act at different regions of the same genes (Fig. 6). Polytene chromosomes from the 220HA flies were also stained with antibodies to the HA tag and to endogenous SIN3 220 as a control (Fig. 6). The complete co-localization of the two antibodies suggests that the HA tag is not interfering with SIN3 localization. These results are consistent with genome-wide localization by chromatin immunoprecipitation of mammalian Sin3A and -B, indicating that these isoforms are recruited to many similar promoters as well as to some distinct genes (6). Additionally, these results suggest that the unique functional roles of the isoforms are probably mediated by the observed differences in gene localization as well as the distinct HDAC activities.

**SIN3 Isoforms Are Non-redundant**—Mutations in the SIN3A gene, presumably resulting in loss of all SIN3 isoform expression, lead to lethality during the embryonic stage of *Drosophila* development (3, 4). Results of the affinity purifications, p53 genetic assays, and polytene co-localization analysis suggest that individual SIN3 isoforms perform distinct functions during *Drosophila* development. To determine whether expression of a single isoform could support viability, we performed two experiments, both taking advantage of the GAL4-UAS expression system (43). In the first experiment, we generated flies that carry the SIN3 187HA or 220HA transgene on the same chromosome as one of two SIN3 mutants, *Sin3A*08268, an EMS allele, or *Sin3A*02834, a p-element insertion allele. We then crossed these flies with the tub-GAL4 driver for ubiquitous expression of the tagged isoform, creating flies that are *Sin3A*08268−/−,
TABLE 1
SIN3 isoforms vary in their ability to rescue lethality of genetic Sin3A loss of function alleles

| Sin3A allele | Percentage survival | Sin3A−/− | Sin3A+/−, 187HA+ | Sin3A−/−, 187HA+ |
|--------------|---------------------|----------|-----------------|-----------------|
| P-element    | %                   | 0        | 100             | 6 ± 0.5         |
| EMS          | %                   | 0        | 100             | 6 ± 1.3         |
| Sin3A−/−     | %                   | Sin3A+/− | Sin3A+/−, 220HA+ | Sin3A−/−, 220HA+ |
| P-element    | %                   | 0        | 100             | 74 ± 1.5        |
| EMS          | %                   | 0        | 100             | 66 ± 6.3        |

TABLE 2
Simultaneous knockdown of SIN3 190 and SIN3 220 results in lethality

Multiple independent UAS-190,220RNAi/UAS-190,220RNAi fly lines were crossed with the tub-GAL4/Sb driver line, and the progeny were analyzed and counted. All adult progeny had stubble bristles, indicating that they did not express the GAL4 activator required for SIN3 knockdown. The number of flies reported represents the total number from two independent parental crosses for each line.

| RNAi line | Number of adults observed |
|-----------|---------------------------|
| UAS-190,220RNAi | UAS-190,220RNAi/tub-GAL4 | UAS-190,220RNAi/Sb |
| UAS-190,220RNAi 4  | 0                        | 362               |
| UAS-190,220RNAi 16 | 0                        | 447               |
| UAS-190,220RNAi 17a | 0                        | 574               |
| UAS-190,220RNAi 18  | 0                        | 575               |

These data clearly show that the isoforms have non-redundant activity.

To confirm that SIN3 187 alone does not support fly viability, we performed RNAi knockdown in transgenic flies to eliminate expression of two of the three SIN3 isoforms. The transgene UAS-SIN3 190,220RNAi drives expression of an inverted repeat of the SIN3 transcript designed to target both SIN3 190 and 220. To verify that the expressed double-stranded RNA resulted in knockdown of SIN3 190 and 220, we used a GAL4 driver specific for eye imaginal disc expression. Western blot analysis of whole cell extracts prepared from SIN3 190,220 knockdown larval eye discs indicated a decrease in SIN3 220 and a small increase in SIN3 187 expression, demonstrating the specificity of the double-stranded RNA (supplemental Fig. 3). The lower molecular weight signal is specific to SIN3 187, because we have previously shown that SIN3 190 expression is not detectable in larvae (20). Next, the Act-GAL4 driver line was used to knock down SIN3 190 and 220 expression in all tissues. We did not observe any viable SIN3 190, 220 knockdown adult flies (Table 2). The RNAi knockdown results as well as the results of the rescue experiments demonstrate that expression of SIN3 187 cannot compensate for loss of the other isoforms. In contrast, expression of SIN3 220 on its own supports viability. These data, in addition to the biochemical data, strongly indicate that SIN3 isoforms have distinct non-redundant functions.

**DISCUSSION**

In this study, we have shown that *Drosophila* SIN3 isoforms interact with distinct proteins and perform unique biological functions. The difference in histone deacetylase activity observed between affinity-purified SIN3 187 and 220 suggests that the isoforms assemble into functionally distinct complexes. The differences in global histone acetylation levels.
observed upon overexpression of SIN3 187 and SIN3 220 further support this distinction. SIN3 187 and 220 localize to many similar but also some distinct loci on polytene chromosomes, suggesting that they act uniquely to regulate certain subsets of genes or genomic regions. The specific association of SIN3 220 with proteins that function in histone modification and chromatin recruitment likely contributes to the observed differences in HDAC activity and localization between SIN3 187 and 220 complexes. Furthermore, the ability of SIN3 220, but not SIN3 187, to suppress lethality due to p55 RNAi knockdown and to support viability on its own indicates that the isoforms serve different biological functions. Taken together, our data support a model in which SIN3 isoforms partner with different chromatin-interacting factors to regulate transcription in a gene-specific manner to affect different biological processes.

SIN3 Isoforms Interact with a Common Set of Proteins—From mass spectroscopic analysis, we identified eight proteins that have been isolated as SIN3 core complex components in other systems (supplemental Table 3). Seven of these proteins, including RPD3, SDS3, ING1, SAP130, PFI (embryo), ARID4B (S2 cells), and BRMS1L (S2 cells), were common to both SIN3 187 and 220. Five of the common proteins are considered to be established SIN3 core components (11, 13), and BRMS1L and PFI have been identified as SIN3-interacting factors in multiple studies (35, 45–47). Orthologs of the histone deacetylase, RPD3, serve as the catalytic subunit of SIN3 complexes in virtually all organisms studied to date (34, 48), so its interaction with both isoforms was expected. Likewise, SDS3 is an established SIN3 core complex protein in both yeast and mammals (14, 18, 38), and ING family proteins have been purified as part of SIN3 complexes in S. cerevisiae (49, 50), S. pombe (15), and mammals (51). SAP130 and ARID4B were initially purified as part of an mSin3A complex from human cultured cells (25) and have since been established as core SIN3 components in that system (34). BRMS1 and BRMS1-like have not been purified with every SIN3 complex but are considered to assemble into mammalian SIN3 complexes of varying composition along with ING1 (45, 46). In summary, five of the six core components identified are common to both isoforms. This result is consistent with previously observed conservation of SIN3 core complex composition among eukaryotic organisms and suggests that the function of the Drosophila SIN3 complexes is conserved as well.

p55 Is an Isoform-specific Factor—Unexpectedly, p55 did not interact with both isoforms, despite being considered an important core complex component in multiple organisms. p55 and its mammalian ortholog, RBP4/7, constitute part of a catalytic HDAC core present in both SIN3 and NuRD complexes (39–42). The isoform-specific complexes Complex I and II in S. pombe each possess a p55 ortholog, Prw1 (15), and a non-orthologous WD40 repeat protein is present in both the Rpd3L and S complexes in S. cerevisiae (14, 18). For this reason, we expected that Drosophila p55 would interact strongly with both SIN3 187 and 220. To our surprise, peptides for p55 were identified in none of the SIN3 187 purifications from embryos and in only a single S2 cell purification at substoichiometric amounts. Equally surprising was the strong HDAC activity exhibited by the affinity-purified SIN3 187 complex, despite the absence of p55. These results show that a functional SIN3 HDAC complex without p55 is present and that p55, consistent with its ability to directly bind histones (30), may therefore be more important for SIN3 220 complex activity at the level of recruitment or stability on chromatin.

LID and EMSY Show a Preferential Interaction with SIN3 220—Two proteins not established as SIN3 complex proteins exhibited a preferential interaction with SIN3 220. The histone demethylase LID interacted with SIN3 220 in both S2 cells and embryos, and EMSY was identified as a SIN3 220-specific factor in S2 cells. Recent evidence has linked Drosophila LID and EMSY to SIN3 regulatory and HDAC activity. When in a complex with RPD3, PFI, MRG15, and CG13367, LID inhibited the HDAC activity of RPD3 without the requirement of its demethylase activity (31). Recruitment of the RALF complex, composed of SIN3, RPD3, LID, PFI, MRG15, and EMSY, to Notch target genes by the H2A/H2B histone chaperone, NAP1, resulted in both demethylation of H3K4me3 and deacetylation of H3 (32). These studies suggest that LID is capable of inhibiting the HDAC activity of RPD3 but that the presence of other proteins can affect this ability. In the current study, LID exhibited a preferential interaction with the SIN3 220 isoform. Interestingly, the SIN3 220 complex demonstrated weaker HDAC activity than the 187 complex in our in vitro studies. Furthermore, overexpression of SIN3 187 resulted in a global decrease in H3K9/14Ac and a small decrease in H4K8 and K12Ac levels, whereas overexpression of SIN3 220 affected H3K9Ac levels alone. It is possible that the presence of LID in the SIN3 220 complex is responsible for the observed differences in HDAC activity. Alternatively, the LID-containing SIN3 220 complex could be important for HDAC activity at a specific subset of target genes, and interactions of proteins such as LID and EMSY with specific histone chaperones or transcription factors could facilitate recruitment to those target genes. In support of the latter, Sin3 HDAC complexes containing the mammalian ortholog of LID, RBP2, were found to be recruited by E2F4 to permanently silence a subset of genes in differentiated muscle cells (6).

SAP18 and SAP30 Do Not Interact with SIN3 187 or 220—Two additional proteins, SAP18 and SAP30, predicted to be part of the Drosophila SIN3 complex were absent from our purifications. SAP18 was purified as part of a mammalian SIN3 complex (28). It has also been shown to interact with Drosophila bicoid, which interacts with the PAH3 and -4 domains of SIN3 (37, 52). Based on these results, Singh et al. (37) hypothesized that SAP18 would be a component of the Drosophila SIN3 complex. SAP18 was not identified in any of our affinity purifications, suggesting that SAP18 is not part of the complex. It is possible that the stringency of the washing conditions affected the binding of SAP18 to SIN3 or another member of the complex. It is also possible that SAP18 interacts with Drosophila SIN3 in a gene-specific manner or in response to a particular stimulus. Although SAP18 was isolated as part of a mammalian SIN3 complex (28), there is evidence to suggest that SAP18 is important for histone deacetylation at a specific subset of genes (53, 54). In this way, it is possible that SAP18 is present in a
subset of SIN3 complexes that were not abundant under our experimental conditions.

SAP30, a core complex component in *S. cerevisiae* and mammals, is thought to be important for bridging interactions between mSin3 and ING1, ARID4A, and ARID4B (55–58). The presence of ING1 and ARID4B in the *Drosophila* SIN3 complexes despite the absence of a SAP30 ortholog suggests that the interactions by which SIN3 complexes assemble in *Drosophila* differ somewhat from those of mammals. SIN3 complex function in *S. pombe* is conserved despite the absence of SAP18 and SAP30 from that genome (59, 60). Likewise, SIN3 complex function in *Drosophila* does not appear to require SAP18 or SAP30.

**SIN3-interacting Proteins Are Linked to Cellular Growth or Differentiation**—The majority of the identified SIN3-interacting proteins, including SIN3, have been implicated in the regulation of cell cycle progression, cellular growth, or differentiation. For instance, SD3 is important for Rpd3L complex regulation of the G1/S transition in *S. cerevisiae* (61) and for the G2/M phase transition in mouse embryonic fibroblasts (62). ING family proteins are tumor suppressors important for the regulation of cell growth in mammalian systems (51, 55). In fact, human ING2 was recently found to recruit mSin3A complexes to the p21 promoter, a gene encoding a tumor suppressor protein (63). Mammalian ARID4A and -B interact with SAP30 to recruit mSin3-HDAC complexes to E2F promoters to inhibit cellular proliferation and induce senescence (58), and mammalian BRMS1 and BRMS1L have both been shown to inhibit cell growth (46).

In addition to the factors common to both isoforms, p55, EMSY, and LID have also been implicated in cell proliferation. p55 has been shown to be involved in repressing E2F/RBF target genes in *Drosophila* (64), and knockdown of p55 in S2 cells results in an S phase arrest (7). EMSY has been linked to differentiation and quiescence (65). In addition, EMSY binds the BS69 corepressor, which is important for cellular senescence (66, 67). *Drosophila* LID was shown to interact with dMyc to promote cell growth (68). In contrast, mammalian RBP2 was shown to interact with Mad1 to repress the c-Myc target gene, hTERT (69), and to be recruited along with mSin3 complexes to permanently silence cell cycle-regulated genes (6).

Although many of the common factors are involved in regulating proliferation and differentiation, the factors specific to SIN3 220 potentially allow that complex to regulate these processes in a gene-specific manner, independently of SIN3 187. SIN3 187 and 220 are recruited to many similar but also some unique sites on polytene chromosomes. Furthermore, simultaneous knockdown of SIN3 190 and 220 isoforms resulted in complete lethality, and SIN3 187 was unable to rescue viability of *Sin3A−/−* embryos. Interestingly, expression of SIN3 220, but not 187, was able to suppress lethality due to RNAi-induced p55 knockdown. These results suggest that the 220-specific factor p55, along with EMSY and LID, could be important for recruiting SIN3 220 complexes to particular subsets of target genes important for regulating proliferation and differentiation during early development.

In summary, our data demonstrate that SIN3 187 and 220 are functionally distinct proteins with unique biological roles in the developing organism. We are currently working to examine the contributions of both common and distinct factors to the regulatory activity of SIN3 187 and 220 and thus to their roles in metazoan development. It will be of great interest to link the SIN3 220-specific factors to particular gene-regulatory functions that affect developmental processes, such as cell proliferation and differentiation.

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