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The SIN3/RPD3 Deacetylase Complex Is Essential for G_2 Phase Cell Cycle Progression and Regulation of SMRTER Corepressor Levels

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The SIN3 corepressor and RPD3 histone deacetylase are components of the evolutionarily conserved SIN3/RPD3 transcriptional repression complex. Here we show that the SIN3/RPD3 complex and the corepressor SMRTER are required for *Drosophila* G_2 phase cell cycle progression. Loss of the SIN3, but not the p55, SAP18, or SAP30, component of the SIN3/RPD3 complex by RNA interference (RNAi) causes a cell cycle delay prior to initiation of mitosis. Loss of RPD3 reduces the growth rate of cells but does not cause a distinct cell cycle defect, suggesting that cells are delayed in multiple phases of the cell cycle, including G_2. Thus, the role of the SIN3/RPD3 complex in G_2 phase progression appears to be independent of p55, SAP18, and SAP30. SMRTER protein levels are reduced in SIN3 and RPD3 RNAi cells, and loss of SMRTER by RNAi is sufficient to cause a G_2 phase delay, demonstrating that regulation of SMRTER protein levels by the SIN3/RPD3 complex is a vital component of the transcriptional repression mechanism. Loss of SIN3 does not affect global acetylation of histones H3 and H4, suggesting that the G_2 phase delay is due not to global changes in genome integrity but rather to derepression of SIN3 target genes.

Posttranslational acetylation of evolutionarily conserved lysine residues within the N-terminal tails of histones has been implicated in the regulation of transcription (33). In general, histone acetylation levels are correlated with transcription levels; nucleosomes located near active genes contain hyperacetylated histones, while those located near inactive genes contain hypoacetylated histones (5, 20). Histone acetylation levels are determined by the relative activities of various histone acetyltransferases (HATs) and histone deacetylases (HDACs) that display specificity for particular lysine residues (33). Thus, targeting of an HDAC to a given promoter provides a mechanism for transcriptional repression (29, 55). Histone deacetylation may repress transcription by strengthening histone tail-DNA interactions and thereby blocking access of transcriptional regulators to the DNA template or by removing acetyl moieties on histone tails that are important for the interaction of transcriptional regulators with chromatin (17, 25, 37, 63, 67).

SIN3 and the RPD3 deacetylase are components of a multiprotein complex that represses the transcription of many eukaryotic genes (3). The SIN3/RPD3 complex does not directly bind DNA but is targeted to specific genes through protein-protein interactions between SIN3 and DNA-binding proteins or corepressors that interact with DNA-binding proteins. The mammalian SIN3/RPD3 complex (which we refer to as the SIN3/HDAC1 complex and which contains SIN3A and/or SIN3B and HDAC1 and/or HDAC2) is involved in the regulation of transcription by nuclear hormone receptors (NHRs), the Myc/Mad/Max family of transcription factors, and a variety of other transcription factors (12, 18, 21, 28, 35, 44). NHRs and Myc/Mad/Max proteins participate in both activation and repression of genes. In the absence of hormone, type II NHRs, including the thyroid hormone receptor and the retinoic acid receptor, bind their cognate DNA sequences and repress transcription (15, 47). Early studies indicated that repression is mediated by targeting of the SIN3/HDAC1 complex through association of SIN3 with the corepressors SMRT and N-CoR, which, in turn, bind unliganded NHRs (1, 21, 44, 72). The preponderance of evidence suggested a model in which conversion of NHRs from repressors to activators involved reversal of repression, by ligand-dependent dissociation of the SIN3/RPD3 complex, and recruitment of coactivator complexes that possess intrinsic HAT activity (15, 47). However, involvement of the SIN3/HDAC1 complex in transcriptional repression by unliganded NHRs has recently come into question (65). While a *Xenopus* N-CoR/SIN3/RPD3 complex has been purified, mammalian SIN3 and HDAC1 do not purify with endogenous SMRT-containing complexes (27). Other HDACs, including HDAC3, associate with SMRT and N-CoR complexes and have been implicated in repression by NHRs (24, 38).

Aspects of the corepressor-to-coactivator conversion model have been addressed by using a *Drosophila* system. Ecdysteroid hormones, such as ecdysone, control *Drosophila* metamorphosis by activating transcription through the Ecdysone receptor (EcR), a member of the type II NHR family (51). *Drosophila* SMRTER, the functional homologue of SMRT and N-CoR, binds EcR and SIN3 to mediate repression in the absence of a hormone (62). Heterozygous *EcR* and *SIN3* mutant flies show synthetic lethality and developmental phenotypes, providing in vivo evidence for a functional link between EcR and SIN3. Furthermore, SIN3, RPD3, and SMRTER colocalize at numerous loci in *Drosophila* salivary gland polytene chromosomes and the level of binding of SIN3 and RPD3 to ecdysone-regulated loci decreases upon ecdysone-induced transcrip-

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tional activation and increases coincident with a reduction in transcription (48). Taken together, these findings suggest that repression of EcR-regulated genes is relieved by dissociation of the SIN3/RPD3 complex upon ecdysone binding.

Studies suggest that, in addition to affecting histone acetylation levels, dissociation of the SIN3/RPD3 complex upon gene activation affects the stability of SIN3-interacting proteins. For example, the interaction between SIN3 and the p53 tumor suppressor protein is not only important for the ability of p53 to repress transcription but is also important for protection of p53 from proteasome-mediated degradation (43, 83).

Histone acetylation has also been implicated in regulation of progression through the cell cycle (39). In yeast, proper acetylation of histones H3 and H4 is essential for progression through the G_2/M phase of the cell cycle. Loss of certain HATs that preferentially acetylate histones H3 or H4 or mutation or deletion of conserved lysine residues in the N-terminal tail of histone H4 leads to arrest in the G_2/M phase (22, 41, 42, 71, 79). Similarly, chemical inhibitors of HDACs have been reported to have antiproliferative effects on mammalian cells, including arrest of the cell cycle in the G_1 and/or G_2 phases (14, 30, 31, 36, 45, 49, 52, 58, 74, 75, 76). These observations highlight the importance of the balance of histone acetylation-deacetylation during the cell cycle. The SIN3/RPD3 deacetylase complex may participate in regulation of G_2 cell cycle progression, as ecdysone treatment of *Drosophila* tissue culture cells causes arrest in the G_2 phase of the cell cycle (4, 9, 11, 19).

Thus, in this study, we examined the cell cycle requirement for individual components of the SIN3/RPD3 complex and the corepressor SMRTER. In addition to SIN3 and RPD3, the SIN3/RPD3 complex contains p55 (also known as chromatin assembly factor 1 [CAF-1] and RbAp46/48) and SIN3-associated polypeptides 18 (SAP18) and 30 (SAP30) (3, 18, 34, 35, 80, 82). p55 is a component of numerous complexes involved in histone metabolism, including CAF-1, nucleosome-remodeling HDAC (Mi-2/NuRD), and nucleosome remodeling factor, and is thought to target these complexes to histone H4 (40, 61, 64, 66, 68, 73, 81). SAP30 directly interacts with SIN3 and RPD3, and in yeast, *SAP30* mutants display many, but not all, of the phenotypes observed in *SIN3* and *RPD3* mutants (35, 82). Finally, in mammalian cells, SAP18 binds to SIN3 and enhances SIN3/RPD3-mediated transcriptional repression (80).

By using an RNA interference (RNAi) approach to eliminate specific proteins in *Drosophila* tissue culture cells, we show that progression through the G_2 phase of the cell cycle requires SIN3 and SMRTER but not p55, SAP18, and SAP30, suggesting that SIN3/RPD3 complex components play distinct roles in vivo. RPD3 RNAi cells do not display a distinct cell cycle phenotype but are growth impaired, possibly reflecting roles for RPD3 at multiple points in the cell cycle, including G_2 phase progression. Global histone acetylation levels are increased in RPD3 RNAi cells, but this is likely due to the activity of RPD3-containing complexes other than SIN3/RPD3, as global acetylation levels are not affected in SIN3 RNAi cells. Surprisingly, the G_2 phase delay caused by loss of SIN3 or SMRTER is independent of EcR, as the delay occurs in SIN3/EcR and SMRTER/EcR double-RNAi cells. The SIN3/RPD3 complex appears to act through SMRTER to control cell cycle progression, as loss of SIN3 or RPD3 leads to a reduction in the level of SMRTER protein. This is consistent with a role for the SIN3/RPD3 complex in protecting corepressors from proteolysis.

MATERIALS AND METHODS

Cell culture. *Drosophila* Schneider cell line 2 (S2) cells were cultured at 22°C in Schneider's *Drosophila* medium (Life Technologies) containing 10% fetal bovine serum (FBS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For ecdysone treatment, 20-hydroxyecdysone (Sigma) was dissolved in dimethyl sulfoxide and added to cells in culture medium at a concentration of 10^{-6} M.

dsRNA production. Individual DNA fragments, approximately 700 to 1,200 bp in length and containing sequences encoding the protein to be targeted by RNAi, were amplified by PCR from *Drosophila melanogaster* genomic DNA and cloned in both orientations into the pCRII-TOPO cloning vector by using the TOPO TA cloning kit (Invitrogen). The following primer sets (oriented 5′–3′) were used in a standard PCR: SIN3 (GAATTTGAAGACCACAACCTCG and GATGGCG ATATGTCCGGCAC), RPD3 (GACCGGCACCAAAGTAAACC and CTTG GTCATCTCATCGGCAG), SMRTER (TGAACTACCTGCCACCACAC and AATGGCAACCATGGTCTGCC), SAP30 (ACATCGCGCTGTCGAAAGAA and CAGGTGGTGTCGTTGCCAAG), SAP18 (TTGATATAGTTATCGAAA AGAGC and AGTTCGTGTTACTTGTATTCCAC), p55 (TCACACCATCTG CTTGTGGG and AGATTGTACAATCTGCTGAC), STG (AACACCAGCA GTTCGAGTAG and GCATAGGCTTTGCTGAAGTC), PP1-87B (AGTACT TGGACTCGTATGG and GAGGACAGCAATCTGTCGAAG), and EcR (CT ATGACCACAGCTCGGAC and TCGGTTGGGGGCGCCATTAC). Sense and antisense clones were used as templates to generate single-stranded RNA (ssRNA) with a Ribomax kit (Promega). ssRNA was resuspended in annealing buffer (5 mM KCl, 10 mM NaH_2PO_4), and equal quantities of sense and antisense ssRNAs were annealed by heating to 95°C for 5 min and slow cooling for 12 to 18 h to generate double-stranded RNA (dsRNA). dsRNA was stored at −80°C.

RNAi. RNAi was carried out on the basis of the protocol of Clemens et al. (10). Briefly, 2×10^6 cells were plated into a 60-mm-diameter dish. After 1 h, FBS-containing medium was removed and replaced with 2 ml of serum-free medium. Approximately 40 μg of dsRNA (as little as 10 μg has been tested and found to be effective) was added per dish and mixed by swirling. After 30 min, 4 ml of medium containing 10% FBS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml was added. Cells were assayed at a specified day following addition of dsRNA. To determine the growth curves of RNAi cells, cells were mixed and counted each day following the addition of dsRNA for a total of 4 days.

Western blotting or reverse transcription (RT)-PCR analysis was routinely carried out for both single- and double-RNAi-treated cells to evaluate the level of the targeted protein or mRNA, respectively. Representative examples are shown in Fig. 1. Western blot assays were performed as described below, and RT reactions were carried out in accordance with standard procedures by using total RNA extracted from cells with Trizol reagent (Life Technologies) (57).

Western blot analysis. Western blot analysis was performed in accordance with standard protocols (57). To prepare whole-cell extracts, cells were pelleted by centrifugation and then lysed in Laemmli sample buffer (Bio-Rad) at a concentration of 1.5×10^4 cells/μl of buffer. Protein concentration was determined with the Bio-Rad Dc protein assay reagent in accordance with the manufacturer's protocol. Extract (5 to 20 μg) was fractionated by sodium dodecyl sulfate (SDS)–8 or 10% polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon P polyvinylidene difluoride membrane (Millipore), and probed with various rabbit primary antibodies, followed by donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG; 1:3,000; Amersham Pharmacia Biotech), and detected with ECL reagents (Amersham Pharmacia Biotech). Primary antibodies included IgG-purified anti-SIN3 (1:500) and anti-RPD3 (1:500), anti-SMRTER (1:2,000; kindly provided by R. Evans), anti-SAP18 (1:2,000), and anti-p55 (1:6,000; kindly provided by R. Kamakaka) (48, 62, 64). The SAP18 polyclonal antibody was generated in rabbits by using a full-length recombinant *Drosophila* SAP18 protein as the antigen.

For anti-histone Western blot assays, crude whole-cell acid-soluble protein extracts were prepared as follows. Cells (2×10^7 to 5×10^7) were pelleted by centrifugation and resuspended in 1 ml of 1× phosphate-buffered saline (PBS)–10 mM sodium butyrate. Sulfuric acid was added to a final concentration of 0.4 N. Cells were incubated on ice for 30 min and then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was dialyzed against 0.1 N acetic acid for 1 to 3 h; this was followed by two changes of distilled water for 1 to 3 h and then

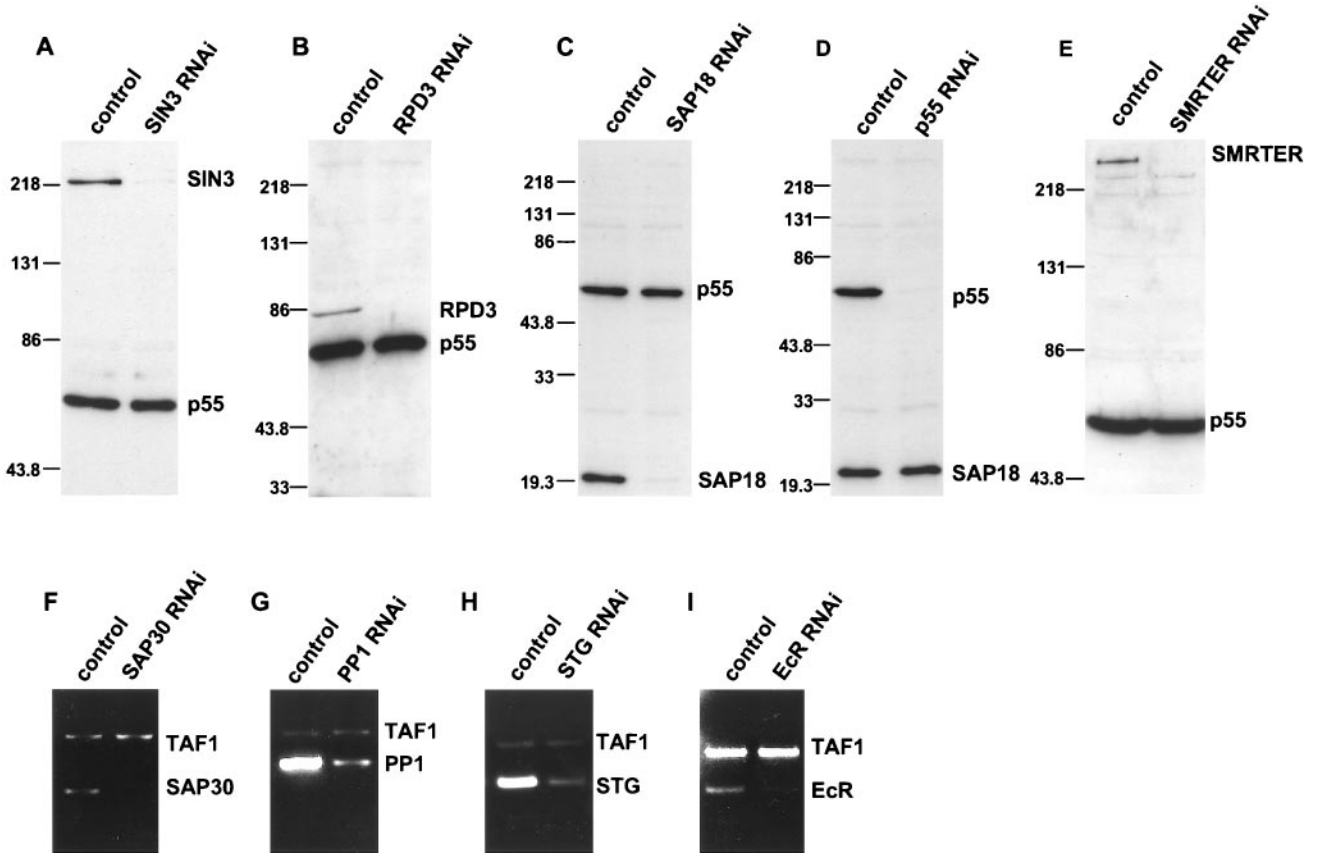

FIG. 1. Specific genes can be targeted by RNAi. (A to E) Western blot assays of whole-cell extracts from control or RNAi cells. Each blot was probed with two antibodies, one specific for the protein targeted by RNAi, i.e., SIN3 (A), RPD3 (B), SAP18 (C), p55 (D), or SMRTER (E), and a second specific for a protein that was not targeted by RNAi, i.e., p55 (A to C and E) or SAP18 (D). Protein molecular weight markers are indicated in thousands to the left of each panel. (F to I) Ethidium bromide-stained agarose gels of RT-PCR products. Total RNA was isolated from control and RNAi cells and subjected to RT-PCR analysis. Two primer sets were used in each reaction mixture, one set for the mRNA targeted by RNAi, *SAP30* (F), *PP1* (G), *STG* (H), and *EcR* (I), and a second set specific for the *TAF1* (formally designated $TAF_{II}250$) mRNA that was not targeted by RNAi.

overnight incubation at 4°C. Dialyzed supernatant was subjected to trichloroacetic acid (TCA) precipitation to isolate acid-soluble proteins. A 200-μl volume of 100% TCA was added to approximately 1 ml of dialyzed supernatant, and the solution was mixed and placed on ice for 30 min. Precipitated proteins were isolated by centrifugation at 12,000 × *g* for 15 min. All but 10 μl of the liquid was removed, 1 ml of ice-cold acetone was added, and the pellet in acetone was centrifuged at 12,000 × *g* for 2 min. The acetone was removed, and the pellet was allowed to briefly air dry. The TCA pellet was resuspended in 100 to 200 μl of Laemmli sample buffer (Bio-Rad). Protein concentration was determined by using the Bio-Rad Dc protein assay reagent. A 12-μg extract sample was separated by SDS–15% PAGE, transferred to Immobilon P polyvinylidene difluoride membrane (Millipore), probed with various rabbit primary antibodies, followed by donkey anti-rabbit horseradish peroxidase-conjugated IgG (1:3,000; Amersham Pharmacia Biotech), and detected with ECL reagents (Amersham Pharmacia Biotech). All histone antibodies were obtained from Upstate Biotechnology, including anti-phos H3 (1:2,000), anti-H3Ac9/14 (1:10,000), anti-H4Ac8 (1:600), anti-H4Ac12 (1:1,000), and anti-H4Ac5/8/12/16 (1:2,000).

FACS analysis. To prepare cells for fluorescence-activated cell sorter (FACS) analysis, 10^6 cells were washed twice with 1× PBS and resuspended in 2 ml of 1× PBS–0.25% Triton X-100–4 μg of propidium iodide per ml and 10 μl of RNase A (10 mg/ml) was added. Stained cells were analyzed with a Becton Dickinson FACScan machine, and the data were analyzed with CELLQuest software.

Immunofluorescence assay. Cells were seeded onto coverslips at a density of 5×10^5/ml and fixed in 2% formaldehyde in 1× PBS for 10 min. After a brief wash with 1× PBS, cells were blocked with 1% bovine serum albumin–0.1% Triton X-100 in 1× PBS for 30 min at 25°C, incubated with rhodamine-conjugated phalloidin antibody (1:100; Molecular Probes) in 1× PBS–0.1% Triton X-100–1% normal goat serum for 1 h at 25°C, washed three times for 10 min each time with 1× PBS, and mounted onto slides with Vectashield mounting medium (Vector Laboratories, Inc.).

RESULTS

SIN3 is required for progression through the G_2 phase of the cell cycle. To determine the physiological requirements for SIN3 in *Drosophila* cells, we have used RNAi methodology to reduce the level of SIN3 protein in S2 tissue culture cells (10). RNAi causes degradation of a specific mRNA, which, in turn, causes a reduction in the level of the encoded protein. In brief, RNAi was carried out by adding dsRNA, corresponding to an ~930-nucleotide region of the SIN3 mRNA, to S2 cells and culturing the cells for 3 days prior to analysis. We refer to these cells as SIN3 RNAi cells. Western blot analysis of whole-cell extracts with an antibody directed against SIN3 showed that RNAi treatment resulted in near elimination of the SIN3 protein (Fig. 1A). In accord with an earlier report, the RNAi effect in *Drosophila* tissue culture cells appears to be all or none (16). Immunofluorescence staining of SIN3 RNAi cells with SIN3 antibody showed that ~95% of the cells had nearly complete

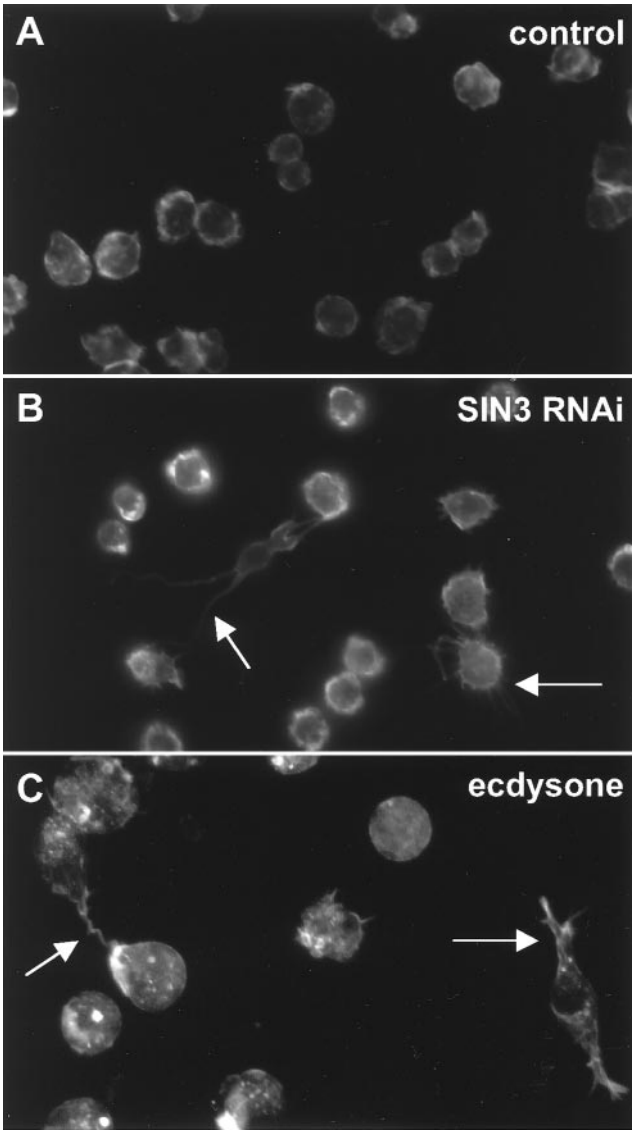

FIG. 2. Loss of SIN3 results in altered cellular morphology. On the third day following the addition of dsRNA, RNAi cells were fixed and stained with a phalloidin antibody to visualize the cell surface. Panels: A, control; B, SIN3 RNAi; C, ecdysone treated. The arrows in panels B and C indicate abnormal cells that contain long, thin projections.

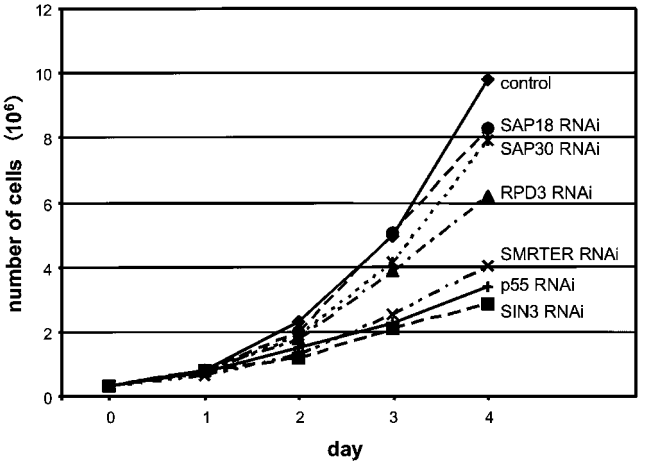

FIG. 3. Loss of some SIN3/RPD3 complex subunits and SMRTER affects cell growth. Growth curves for control and RNAi cells are indicated by different symbols and labeled at the day 4 time point. Cell numbers were determined each day following addition of dsRNA for a total of 4 days. Results of a single experiment are shown, but identical relative growth rates for the seven samples were observed in multiple independent experiments.

loss of expression, while ~5% of the cells appeared to be unaffected and expressed SIN3 at or near wild-type levels. Furthermore, the RNAi effect was specific for SIN3, as Western blot analysis revealed that the level of other components of the SIN3/RPD3 complex in SIN3 RNAi cells was not reduced (Fig. 1A; see also Fig. 8; data not shown).

To monitor the physiological effect of the loss of SIN3, we analyzed SIN3 RNAi cells for morphology (Fig. 2), growth rate (Fig. 3), and cell cycle progression (Fig. 4). Loss of SIN3 caused morphological changes in *Drosophila* S2 cells. Control cells were round and had a fairly smooth surface, whereas many SIN3 RNAi cells were flattened and had long, thin projections (Fig. 2A and B). Counting of control and SIN3 RNAi cells for 4 days following addition of dsRNA revealed that loss of SIN3 increased the doubling time of S2 cells from ~1 day to ~2 days (Fig. 3). To examine this phenotype in more detail, the cell cycle distribution of SIN3 RNAi cells was determined by FACS analysis. By comparison with mock RNAi-treated cells, fewer SIN3 RNAi cells had a DNA content of 2N and more SIN3 RNAi cells had a DNA content of 4N, suggesting that SIN3 is required for progression through the G_2/M phase of the cell cycle (Fig. 4A and B). SIN3 RNAi-treated *Drosophila* Kc167 tissue culture cells were also delayed in the G_2/M phase, indicating that SIN3 plays a role in cell cycle progression in multiple cell types (data not shown). Note that we refer to the cell cycle phenotype of SIN3 RNAi cells as a G_2 phase delay and not a G_2 phase arrest because it is unclear whether SIN3 RNAi cells ever progress through G_2 phase and initiate mitosis. The distinction between cell cycle delay and arrest is difficult to assess since the RNAi effect is transient and SIN3 protein levels begin to increase 5 to 6 days after addition of dsRNA (10; data not shown).

To determine if SIN3 RNAi cells were delayed in G_2 or M phase, we examined the phosphorylation state of histone H3. One hallmark of initiation of mitosis is phosphorylation of serine 10 of histone H3 (23, 70). Western blot analysis of whole-cell acid-soluble protein extracts with an antibody directed against phosphorylated serine 10 of histone H3 (anti-phos H3) indicated that SIN3 RNAi cells have extremely low levels of phosphorylated histone H3 relative to asynchronously dividing control cells (Fig. 5A). We also fluorescently stained SIN3 RNAi cells with the phos H3 antibody and determined that 5.5% of the control cells (n = 868) and 1.5% of the SIN3 RNAi cells (n = 1,465) were stained with the phos H3 antibody. Thus, SIN3 RNAi cells do not initiate mitosis, indicating that SIN3 is required for G_2 phase cell cycle progression.

Positioning of SIN3 upstream of protein phosphatase 1 (PP1) also supports a role for SIN3 prior to mitosis. In *Caenorhabditis elegans*, PP1/Glc7 was shown to be responsible for dephosphorylation of serine 10 of histone H3, allowing for

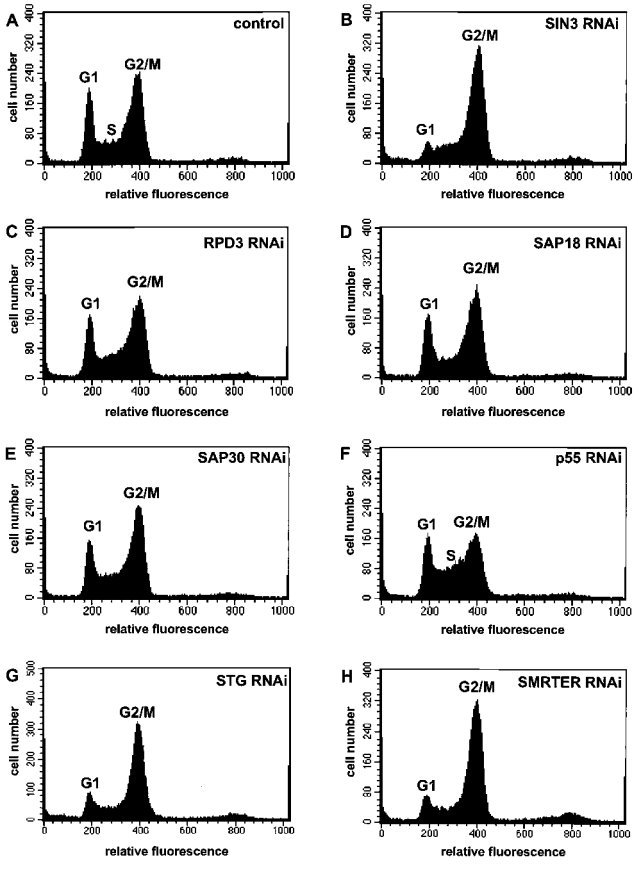

FIG. 4. Loss of some SIN3/RPD3 complex subunits, SMRTER, and STG affects cell cycle progression. Control and RNAi cells were analyzed by FACS on the third day following addition of dsRNA (A to H). G_1 phase (2N DNA content, relative fluorescence of 200) and G_2/M phase (4N DNA content, relative fluorescence of 400) peaks are indicated. S phase (2N to 4N DNA content, relative fluorescence of 200 to 400) is indicated in panels A and F. The gene targeted by RNAi is indicated in the upper right corner of each panel.

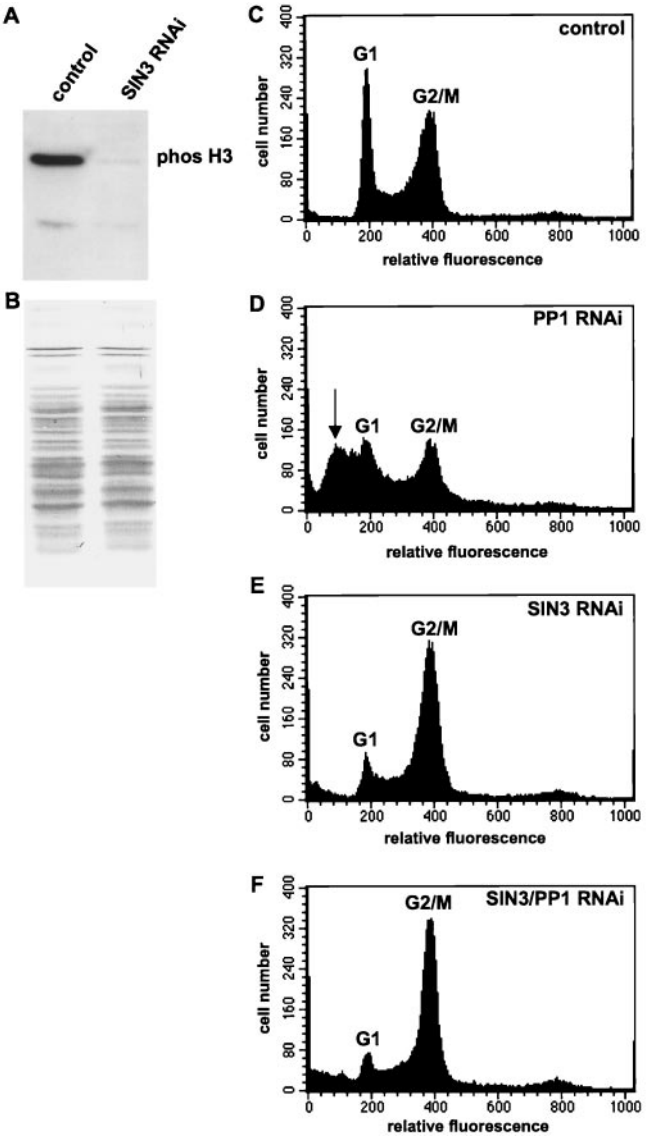

FIG. 5. SIN3 RNAi cells are delayed in the G_2 phase of the cell cycle prior to initiation of mitosis. Whole-cell acid-soluble protein extracts from control and SIN3 RNAi cells were subjected to Western blot analysis with an antibody specific for histone H3 phosphorylated at serine 10 (A). India ink was used to visualize proteins bound to the membrane probed in panel A, demonstrating that equal quantities of protein were contained in each sample (B). Control and RNAi cells were analyzed by FACS on the third day following addition of dsRNA (C to F). G_1 phase (2N DNA content, relative fluorescence of 200) and G_2/M phase (4N DNA content, relative fluorescence of 400) peaks are indicated. The arrow in panel D indicates the sub-G_1 peak representing cells with a DNA content of less than 2N. The gene(s) targeted by RNAi is indicated in the upper right corner of each panel.

chromatin decondensation at the end of mitosis (23). Null mutants of *Drosophila* PP1-87B die at the larval stage, and their cells fail to exit mitosis and exhibit overcondensed chromatin (2, 13). Loss of PP1 in S2 cells by PP1-87B RNAi strongly disrupted the cell cycle (Fig. 1G and 5D). FACS analysis revealed a reduction in both the G_1 and G_2/M phase peaks and a new sub-G_1 phase peak, which may be due to progression through mitosis prior to completion of DNA replication. Interestingly, the FACS profile of SIN3/PP1-87B double-RNAi cells was similar to the SIN3 RNAi profile, indicating that SIN3 acts upstream of PP1 in cell cycle progression (Fig. 5E and F).

Not all components of the SIN3/RPD3 complex are required for progression through the G_2 phase of the cell cycle. To address the mechanism underlying the SIN3 requirement for G_2 cell cycle progression, we asked whether other components of the SIN3/RPD3 complex are also required for this process. RNAi was carried out for each of the other subunits of the complex, RPD3, SAP18, SAP30, and p55. Western blot analysis, using antibodies directed against RPD3, SAP18, or p55 showed, in each case, that addition of dsRNA drastically reduced protein expression (Fig. 1B, C, and D). Similarly, RT-PCR analysis of SAP30 RNAi cells showed that the level of *SAP30* mRNA was drastically reduced (Fig. 1F).

As with SIN3, we examined the morphology, growth rate, and cell cycle profile of RPD3, SAP18, SAP30, and p55 RNAi cells. Unlike loss of SIN3, loss of other SIN3/RPD3 complex subunits did not cause drastic changes in cell morphology (data not shown). Some RPD3 RNAi cells had small projections, but none were as prominent as those observed in SIN3 RNAi cells.

Loss of some subunits of the SIN3/RPD3 complex slowed the rate of growth relative to that of control cells (Fig. 3). SAP18 and SAP30 RNAi cells exhibited growth rates similar to that of control cells. RPD3 RNAi cells had a moderate reduction in growth rate, doubling in ~1.5 days, compared to ~1 day for control cells. Finally, p55 RNAi cells showed a strong reduction in growth rate, equivalent to that seen in SIN3 RNAi cells.

Similar to and most likely a reflection of the growth rate, loss of individual components of the SIN3/RPD3 complex resulted in distinct cell cycle phenotypes (Fig. 4C, D, E, and F). p55 RNAi cells (which exhibited the slowest growth) showed the strongest deviation from the control cell cycle profile (Fig. 4F). The number of cells in both the G_1 and G_2/M phases was reduced, with a concomitant increase in the number of cells in S phase, possibly reflecting the role of p55 in chromatin assembly during DNA replication (64). More subtle effects were observed in RPD3, SAP18, and SAP30 RNAi cells, each having a slight reduction in the number of cells in the G_1 phase (Fig. 4C, D, and E). While the cell cycle distribution of RPD3 RNAi cells was similar to that of control cells, the reduced growth rate of these cells suggests a delay at multiple points in the cell cycle, presumably including both the G_1 and G_2/M phases. Chemical inhibitors of deacetylases cause both G_1 and G_2 phase arrests, supporting a role for RPD3 at these points in the cell cycle (14, 30, 74, 75). Taken together, the growth rate and cell cycle distribution data indicate that SIN3, RPD3, and p55 play regulatory roles during the cell cycle but SAP18 and SAP30 do not.

Loss of RPD3, but not SIN3, affects global histone acetylation. Since the SIN3/RPD3 complex possesses HDAC activity, we examined whether global effects on histone acetylation contribute to the cell cycle defect of SIN3 or RPD3 RNAi cells. Yeast *RPD3*-null mutants exhibit a global increase in acetylation at lysine 5 (K5) and K12 of histone H4 and K9/18 and K14 of histone H3 (54). Furthermore, treatment of mammalian tissue culture cells with HDAC inhibitors, including sodium butyrate, trichostatin A, and trapoxin, leads to a global increase in histone acetylation levels and arrest in the G_1 and G_2 phases of the cell cycle (14, 30, 31, 36, 45, 49, 52, 58, 74, 75, 76).

Histone acetylation levels in SIN3 and RPD3 RNAi cells were monitored by Western blot analysis of whole-cell acid-soluble protein extracts with antibodies against specific acetylated histone lysine residues. As predicted by studies with yeast, loss of RPD3 caused an increase in the acetylation of K8 and K12 of histone H4 and K9/14 of histone H3 (Fig. 6, compare lanes 1 and 3). Surprisingly, histone acetylation levels in SIN3 RNAi cells were equivalent or even slightly reduced relative to levels in control asynchronously dividing cells (Fig. 6, compare lanes 1 and 2).

In plants, global histone acetylation levels are not constant throughout the cell cycle; rather, they vary depending on the cell cycle phase (26). Since SIN3 RNAi cells are delayed in the G_2 phase, the overall level of acetylated histones may be decreased relative to the level in control asynchronous cells. Therefore, we compared the histone acetylation level of SIN3 RNAi cells with that of cells independently blocked in the G_2 phase by String (STG) RNAi. STG is the *Drosophila* homologue of the yeast mitotic regulator Cdc25 phosphatase that is required for G_2 phase cell cycle progression (50). *STG* mRNA levels were drastically reduced by RNAi, and FACS analysis

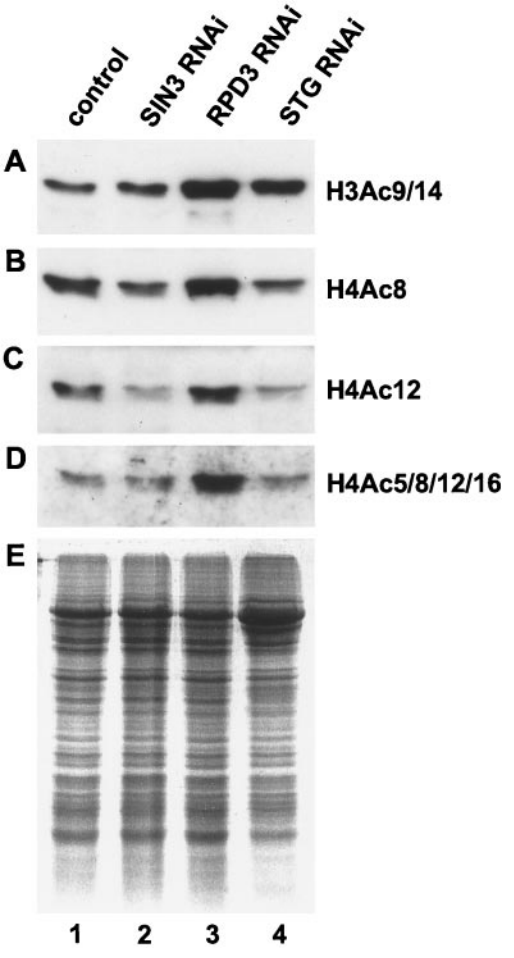

FIG. 6. Loss of RPD3, but not SIN3, results in a global increase in histone acetylation. (A to D) Western blot analysis of whole-cell acid-soluble protein extracts from control and RNAi cells with antibodies specific for acetylated histones, H3Ac9/14 (A), H4Ac8 (B), H4Ac12 (C), and H4Ac5/8/12/16 (D). (E) Samples identical to those probed in panels A to D were subjected to SDS-PAGE analysis, and proteins were visualized by staining with Gelcode Blue Stain Reagent (Pierce), demonstrating that the same quantity of protein was contained in each sample.

revealed that STG RNAi cells were delayed in the G_2 phase of the cell cycle (Fig. 1H and 4G). STG RNAi cells showed a small reduction in the acetylation of K8 and K12 of histone H4, indicating that G_2 cells probably have lower overall histone H4 acetylation levels than do control cells and demonstrating that in *Drosophila* cells, acetylation levels vary through the cell cycle (Fig. 6, compare lanes 1 and 4). The histone acetylation levels of SIN3 and STG RNAi cells were very similar. Thus, unlike loss of RPD3, loss of SIN3 does not cause a global increase in histone acetylation. Therefore, morphological and cell cycle phenotypes associated with loss of SIN3 may result from gene-specific changes in histone acetylation levels.

The corepressor SMRTER is required for progression through the G_2 phase of the cell cycle. Genetic and biochemical studies functionally link the SMRTER corepressor and the SIN3/RPD3 complex (48, 62). Therefore, SMRTER RNAi was carried out to determine whether SMRTER is involved in cell

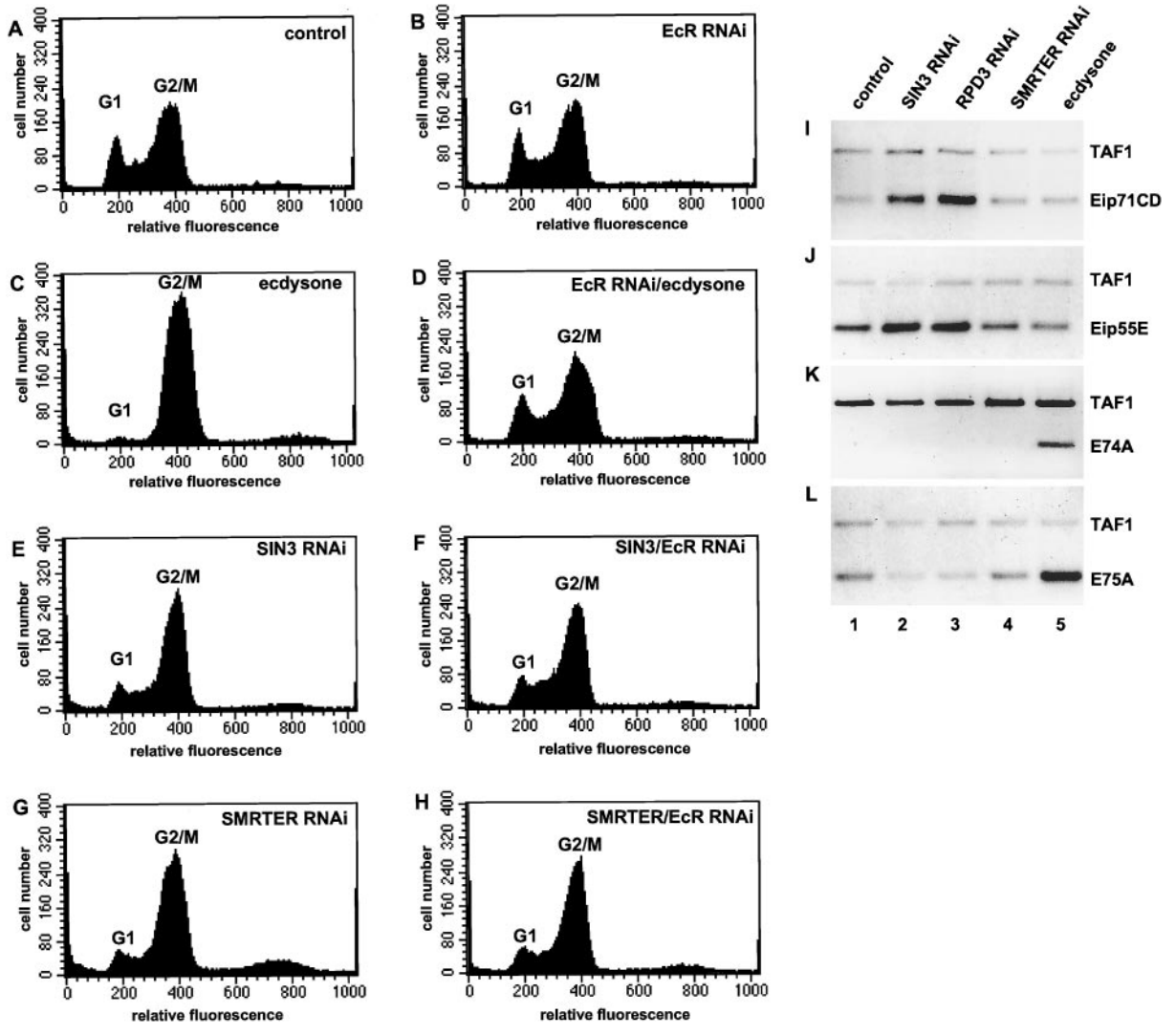

FIG. 7. The G_2 phase cell cycle delay resulting from loss of SIN3 or SMRTER is unaffected by loss of EcR. Control, RNAi, and ecdysone-treated cells were analyzed by FACS on the third day following addition of dsRNA (A, B, and D to H). Cells in panel C were analyzed by FACS 1 day following addition of ecdysone. For cells analyzed in panel D, ecdysone was added 2 days following the addition of EcR dsRNA, 1 day prior to FACS analysis. This provided sufficient time for the EcR RNAi effect to occur prior to ecdysone treatment. G_1 phase (2N DNA content, relative fluorescence of 200) and G_2/M phase (4N DNA content, relative fluorescence of 400) peaks are indicated. The gene(s) targeted by RNAi is indicated in the upper right corner of each panel. Ethidium bromide staining of agarose gels of RT-PCR products (I to L) was used to examine the expression of ecdysone-regulated genes, *Eip71CD* (I), *Eip55E* (J), *E74A* (K), and *E75A* (L), in control asynchronously dividing cells (lane1), SIN3 RNAi cells (lane 2), RPD3 RNAi cells (lane 3), SMRTER RNAi cells (lane 4), and ecdysone-treated cells (lane 5). As a loading control, *TAF1* mRNA levels were determined for each sample. RT-PCR products are labeled on the right of each panel.

cycle progression (Fig. 1E). Similar to that of SIN3 RNAi cells, the growth rate of SMRTER RNAi cells was strongly reduced (Fig. 3). In addition, FACS analysis of SMRTER RNAi cells revealed that SMRTER is required for G_2/M phase progression of the cell cycle (Fig. 4H). Thus, SMRTER most likely recruits the SIN3/RPD3 complex to genes that need to be repressed for progression through the G_2 phase of the cell cycle.

SIN3 and SMRTER function independently of EcR during the cell cycle. In accord with published observations, we found that treatment of S2 cells with ecdysone caused a rapid and severe G_2/M phase cell cycle block (Fig. 7C) (4, 8, 11, 19, 53). Thus, the SIN3/RPD3 complex, SMRTER, and EcR are required at similar points in the cell cycle. In addition, ecdysone treatment of S2 cells caused changes in cell morphology, including the generation of long, thin projections (Fig. 2C). Phenotypic similarities between SIN3 RNAi cells, SMRTER RNAi cells, and ecdysone-treated cells and biochemical and genetic links between the SIN3/RPD3 complex and EcR suggested that the SIN3/RPD3 complex represses EcR-regulated genes to allow progression through the G_2 phase of the cell cycle. In other words, loss of the SIN3/RPD3 complex or SMRTER by RNAi allows activation of EcR-regulated genes, which mimics activation of EcR-regulated genes by ecdysone.

To address this proposition, we examined whether EcR is required for the ecdysone-induced G_2 phase cell cycle block.

EcR RNAi reduced the level of *EcR* mRNA but did not cause a cell cycle defect, suggesting that EcR is not required for cell cycle progression in the absence of a steroid (Fig. 1I and 7B). In contrast, the G_2 phase block caused by ecdysone treatment was suppressed by EcR RNAi, indicating that EcR is required for the cell cycle phenotype of ecdysone-treated cells (Fig. 7D).

However, two lines of evidence suggest that the SIN3/RPD3 complex functions independently of EcR during progression through the G_2 phase of the cell cycle. First, FACS analysis revealed that SIN3/EcR and SMRTER/EcR double-RNAi cells were delayed in the G_2 phase of the cell cycle (Fig. 7F and H). Thus, while EcR is required for the ecdysone-induced G_2 phase block, EcR is not required for the G_2 phase delay caused by loss of SIN3 or SMRTER. Second, SIN3, RPD3, and SMRTER RNAi had different effects on gene expression than ecdysone treatment (Fig. 7I to L). The steady-state transcription levels of four genes (*Eip71CD*, *Eip55E*, *E74A*, and *E75A*) that are regulated by ecdysone during *Drosophila* development were examined by RT-PCR (8, 59). Relative to that in control asynchronously dividing cells, *Eip71CD* and *Eip55E* transcription was upregulated in SIN3 and RPD3 RNAi cells but not in ecdysone-treated cells while *E74A* and *E75A* transcription was upregulated in ecdysone-treated cells but not in SIN3 and RPD3 RNAi cells. Substantial changes in transcription were not observed in SMRTER RNAi cells. At least in the case of these four genes in S2 cells, these results suggest that loss of SIN3 or RPD3 is not sufficient to derepress transcription of genes that are responsive to ecdysone. In summary, it appears that the SIN3/RPD3 complex and EcR do not work in concert to repress transcription of ecdysone-inducible genes to allow progression through the G_2 phase of the cell cycle.

SIN3- and RPD3-dependent regulation of SMRTER protein levels is required for progression through the G_2 phase of the cell cycle. SIN3 has been directly and indirectly implicated in regulation of the stability of transcription factors (46, 78, 83). Thus, we were interested in determining if SMRTER levels are affected by loss of the SIN3/RPD3 complex. To test this possibility, we examined SMRTER expression levels in SIN3 RNAi cells. Western blot analysis of extracts prepared from control and SIN3 RNAi cells revealed that SMRTER protein levels were reduced in SIN3 RNAi cells (Fig. 8A, lanes 1 and 2). This reduction occurred posttranscriptionally, as SMRTER mRNA levels were not reduced in SIN3 RNAi cells (Fig. 8C, lanes 1 and 2). SMRTER protein levels, but not mRNA levels, were reduced in RPD3 RNAi cells (Fig. 8A, lane 3, and C, lane 3), and were unchanged in p55, SAP18, SAP30, and STG RNAi cells (Fig. 8A, lanes 8, 7, 6, and 5, respectively). STG RNAi cells were included as a control for cells blocked in the G_2 phase independently of SIN3/RPD3 complex activity.

In contrast to SMRTER protein levels, SIN3 protein levels were not affected by loss of any other SIN3/RPD3 complex subunit, by loss of SMRTER or STG, or by ecdysone treatment (Fig. 8B). Levels of RPD3, p55, SAP18, and SAP30 were also unchanged in SIN3 RNAi cells relative to those in control cells (Fig. 1; data not shown). In addition, transcriptional activation of EcR-regulated genes by ecdysone treatment did not lead to a reduction in SMRTER levels, indicating that SMRTER levels are not linked to ecdysone gene activation in this cell type (Fig. 8A, lane 4). Thus, SMRTER levels appear to be regulated by a posttranscriptional mechanism in response to loss of SIN3 or RPD3. The SIN3/RPD3 complex may regulate translation of the SMRTER mRNA, but given the documented role of SIN3 in the protection of transcription factors from proteosomal degradation, it is likely that loss of SIN3 or RPD3 leads

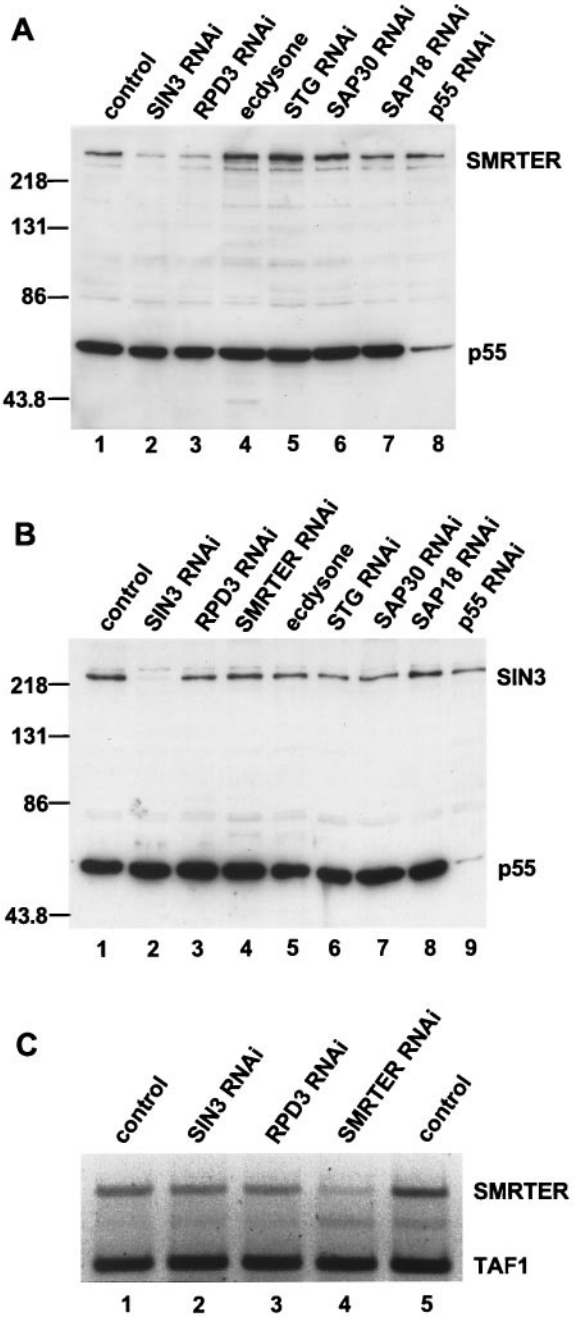

FIG. 8. SMRTER protein, but not mRNA, levels are reduced in cells lacking SIN3 or RPD3. Treatments are indicated above the lanes. (A and B) Western blot analysis of whole-cell extracts from control and RNAi cells. (A) Western blot probed with antibodies to SMRTER and p55. (B) Western blot probed with antibodies to SIN3 and p55. Protein molecular weight markers are indicated in thousands to the left of each blot. (C) Ethidium bromide staining of agarose gels of RT-PCR products to examine *SMRTER* mRNA levels. As a loading control, *TAF1* mRNA levels were determined for each sample. RT-PCR products are labeled on the right of the panel.

to degradation of SMRTER and that stabilization of SMRTER by the SIN3/RPD3 complex is required for progression through the G_2 phase of the cell cycle.

DISCUSSION

This study demonstrates that the SIN3/RPD3 complex is essential for G_2 phase cell cycle progression. Elimination of SIN3 causes cells to be delayed in the cell cycle with a 4N DNA content prior to initiation of mitosis. Loss of SIN3 does not cause a global change in the acetylation level of histones H3 and H4, suggesting that the G_2 phase delay is due to local, not global, changes in chromatin structure that presumably alter the expression of a limited number of genes. The SMRTER corepressor probably recruits the SIN3/RPD3 complex to these genes, as loss of SMRTER causes a G_2 phase delay and SMRTER protein levels are reduced in response to loss of SIN3 or RPD3. Hormone-bound EcR blocks G_2 phase progression but does so independently of the SIN3/RPD3 complex and SMRTER, leaving open the identity of the DNA-binding protein that recruits the SIN3/RPD3/SMRTER assemblage. In addition, this establishes at least two transcriptional repression mechanisms that are essential for progression through the G_2 phase. SAP18 and SAP30 are not required for the cell cycle regulatory activity of the SIN3/RPD3 complex, indicating that they are not essential for stability of the complex, recruitment of the complex to some promoters, or the deacetylase activity of the complex. These findings provide in vivo evidence for (i) distinct roles for individual components of the SIN3/RPD3 complex, (ii) a functional link between the SIN3/RPD3 complex and the SMRTER corepressor, and (iii) a role for the SIN3/RPD3 complex, not just SIN3, in regulation of the level of corepressor proteins. Furthermore, these findings point to the SIN3/RPD3 complex as an essential regulator of progression through the G_2 phase of the cell cycle and suggest that the SIN3/RPD3 complex is a target of deacetylase inhibitors that cause G_2 phase arrest of cancer cells (31, 36, 45, 58).

RNAi reveals distinct roles for components of the SIN3/RPD3 complex. We have found that loss of individual components of the SIN3/RPD3 complex leads to distinct cellular phenotypes. Elimination of SIN3 or RPD3 by RNAi reduces the rate of cell growth, causes cells to delay in the G_2 phase of the cell cycle, and results in reduced levels of the corepressor SMRTER. However, G_1 phase cell cycle progression is not affected in SIN3 RNAi cells but may be affected in RPD3 RNAi cells; cell morphology is affected in SIN3, but not RPD3, RNAi cells; and global histone acetylation levels are not affected in SIN3 RNAi cells but are affected in RPD3 RNAi cells. Phenotypic differences between SIN3 and RPD3 RNAi cells are presumably due to inactivation of other RPD3-containing complexes. RPD3 is a component of the Mi-2/NuRD complex and has been shown to interact with a number of repressors in the absence of SIN3 (7, 32, 61, 68, 73, 81). Thus, a strong G_2 phase delay in RPD3 RNAi cells may be masked by cell cycle defects resulting from inactivation of RPD3 activities that are independent of SIN3. SIN3-independent activities of RPD3 are clearly demonstrated by the global change in histone acetylation observed in RPD3 RNAi cells but not in SIN3 RNAi cells (Fig. 6). On the other hand, it is possible that differences are due to loss of SIN3 activities that are independent of RPD3. Mutations in SIN3 that prohibit RPD3 binding diminish, but do not abolish, transcriptional repression in mammalian cells, and SIN3 binds sites on *Drosophila* polytene chromosomes that are not bound by RPD3, suggesting that SIN3 can function independently of RPD3 (18, 34, 48, 72). Thus, elimination of SIN3 and RPD3 by RNAi has uncovered multiple roles for these proteins. Many of these roles are independent of one another, with the exception of roles in the regulation of SMRTER protein levels and in G_2 cell cycle progression, which appear to require both proteins, presumably in the context of the biochemically defined SIN3/RPD3 complex.

Similar to loss of SIN3 and RPD3, loss of p55 causes a growth rate reduction. However, p55 RNAi cells are delayed in the S phase of the cell cycle and SMRTER protein levels are not affected. Phenotypes observed in p55 RNAi cells may be due to the functioning of p55 as a component of the Mi-2/NuRD, CAF-1, or nucleosome remodeling factor complex (40, 61, 64, 68, 81). Finally, in contrast to loss of SIN3, RPD3, and p55, loss of SAP18 and SAP30 does not cause cell morphology changes, cell growth defects, cell cycle phenotypes, or loss of SMRTER protein. Thus, in these cellular processes, SAP18 and SAP30 are not essential for recruitment of the SIN3/RPD3 complex to promoters, deacetylation of substrates by the SIN3/RPD3 complex, or stability of the SIN3/RPD3 complex. The lack of a requirement for SAP30 for some SIN3/RPD3 activities is consistent with the observation that inactivation of SAP30 by antibody microinjection does not affect repression mediated by artificial tethering of SIN3 to a promoter (35). Our data suggest that if SAP18 and SAP30 are important for SIN3/RPD3 complex function, then they play gene-specific roles that do not involve regulation of progression through the G_2 phase of the cell cycle.

Progression through the G_2 phase of the cell cycle requires the SIN3/RPD3 complex. Global changes in the histone acetylation level cause cell cycle phenotypes, including arrest in the G_2 phase. Mutation of N-terminal lysine residues that are normally acetylated in histone H4 results in a G_2/M phase delay (41, 42). HDAC inhibitors such as trichostatin A, trapoxin, and sodium butyrate have been reported to cause a cell cycle arrest in the G_1 and/or G_2 phase (14, 30, 31, 36, 45, 49, 52, 58, 74, 75, 76). Mutation of HATs such as GCN5, ELP1, and SAS3 results in arrest in the G_2/M phase of the cell cycle (22, 71, 79). In each of these cases, the cell cycle phenotype has been attributed to global changes in histone H3 or H4 acetylation. In one case, the global change in genome integrity has been shown to activate the RAD9-dependent DNA damage checkpoint pathway that induces a delay in the G_2 phase (41). Furthermore, the mammalian homologue of RPD3, HDAC1, exists in a complex with RAD9 (6). We found that loss of SIN3 causes a G_2 phase delay but does not cause global changes in histone acetylation, raising the question of the mechanism underlying the cell cycle delay.

The RAD9-dependent DNA damage checkpoint pathway does not appear to be activated by loss of SIN3. The checkpoint signaling mechanism sequentially involves sensing of DNA damage by RAD proteins, activation of the CHK1 protein kinase (known as Grapes in *Drosophila*) by phosphorylation, inactivation of the Cdc25 phosphatase (known as STG in *Drosophila*) by CHK1-dependent phosphorylation, and inhibi-

tion of the Cdc2-cyclin B complex by phosphorylation of Cdc2 (50, 56, 69, 77). Disruption of RAD9 suppresses the G_2/M phase delay caused by mutating the four conserved lysines that are acetylated in the N-terminal tail of histone H4 (41). However, we found that SIN3/RAD9 double-RNAi cells were delayed in the G_2 phase, suggesting that the delay in SIN3 RNAi cells is independent of RAD9 (data not shown). In addition, we have examined the steady-state levels of mRNAs encoding components of the RAD9 pathway, as well as the p53 pathway, that cause growth arrest in the G_2/M phase (60). *Grapes*, *String*, *p53*, and *Gadd45* mRNA levels were not affected in SIN3 RNAi cells (data not shown). The cyclin B protein levels in SIN3 RNAi cells were reduced, but this is not sufficient to cause the G_2 phase delay, as cyclin B RNAi cells were not delayed in the G_2 phase (data not shown). Thus, other than a reduction in the level of SMRTER protein, events that occur downstream of loss of the SIN3/RPD3 complex that lead to the G_2 phase delay are unclear, but they do not appear to be caused by global changes in genome integrity.

A role for the SIN3/RPD3 complex in regulating corepressor levels. In addition to a role in transcriptional repression, SIN3 has recently been shown to play a role in regulating protein stability. SIN3 interacts with the p53 tumor suppressor protein, and SIN3 and p53 are localized, along with deacetylated histones, to a p53-regulated promoter (43). A recent report has shown that SIN3 stabilizes p53 and protects it from proteasome-mediated degradation in mammalian cells (83). Therefore, SIN3 appears to be required for deacetylase activity at a promoter, as well as stability of the repressor involved in recruitment of the SIN3/RPD3 complex to a promoter. In addition, the corepressor N-CoR, but not SMRT, appears to be subject to regulated proteolysis by mSiah2, which binds ubiquitin-conjugating enzymes (78). Interestingly, *Drosophila SIN3* mutants were isolated in a genetic screen as dominant enhancers of a phenotype caused by ectopic expression of Sina, the *Drosophila* homologue of mSiah2, suggesting that SIN3 counteracts the activity of Sina (46). Thus, SIN3 has been directly and indirectly implicated in regulation of the protein stability of transcription factors that are targeted for degradation.

We found that SMRTER protein levels are regulated by SIN3 and RPD3. This regulation occurs posttranscriptionally, as SMRTER message levels are not affected in SIN3 or RPD3 RNAi cells. The SIN3/RPD3 complex may regulate SMRTER translation or stability. Given the previously described role for SIN3 in regulation of the proteosomal degradation of transcriptional regulators, it is most likely that the SIN3/RPD3 complex regulates SMRTER stability. This would provide the first evidence that the protein stabilization function of SIN3 is carried out as a component of the SIN3/RPD3 complex and that the deacetylase activity of the SIN3/RPD3 complex is important for this function. Sina does not appear to play a role in the regulation of SMRTER protein levels, as SMRTER levels are reduced in SIN3/Sina double-RNAi cells and the cells were delayed in the G_2 phase of the cell cycle (data not shown).

This study demonstrates that RNAi is a powerful approach to investigate of the cellular requirements for individual proteins and to determine the epistatic relationship between proteins that function in a given cellular process. The RNAi approach can now be used to identify factors that function along with the SIN3/RPD3 complex to regulate progression through the G_2 phase of the cell cycle, including DNA-binding factors that recruit the SIN3/RPD3 complex.

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