The SIN3 Deacetylase Complex Represses Genes Encoding Mitochondrial Proteins

IMPLICATIONS FOR THE REGULATION OF ENERGY METABOLISM

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Deacetylation of histones by the SIN3 complex is a major mechanism utilized in eukaryotic organisms to repress transcription. Presumably, developmental and cellular phenotypes resulting from mutations in SIN3 are a consequence of altered transcription of SIN3 target genes. Therefore, to understand the molecular mechanisms underlying SIN3 mutant phenotypes in Drosophila, we used full-genome oligonucleotide microarrays to compare gene expression levels in wild type Drosophila tissue culture cells versus SIN3-deficient cells generated by RNA interference. Of the 13,137 genes tested, 364 were induced and 35 were repressed by loss of SIN3. The ~10-fold difference between the number of induced and repressed genes suggests that SIN3 plays a direct role in regulating these genes. The identified genes are distributed throughout euchromatic regions but are preferentially associated with heterochromatic regions of Drosophila chromosomes suggesting that the SIN3 complex can only access particular chromatin structures. A number of cell cycle regulators were repressed by loss of SIN3, and functional studies indicate that repression of string, encoding the Drosophila homologue of the yeast CDC25 phosphatase, contributes to the G2 cell cycle delay of SIN3-deficient cells. Unexpectedly, a substantial fraction of genes induced by loss of SIN3 is involved in cytosolic and mitochondrial energy-generating pathways and other genes encode components of the mitochondrial translation machinery. Increased expression of mitochondrial proteins in SIN3-deficient cells is manifested in an increase in mitochondrial mass. Thus, SIN3 may play an important role in regulating mitochondrial respiratory activity.

SIN3 is a component of a multiprotein complex that functions broadly in eukaryotic organisms as a transcriptional repressor of protein-coding genes (1–4). Although there is evidence that SIN3 possesses intrinsic repression activity, it primarily functions as a scaffold for assembly of the complex and interactions with other transcriptional regulatory proteins (1, 2, 5, 6). Most of the repression activity of the SIN3 complex is attributed to the histone deacetylase activity of RPD3 (1–4). The combined action of histone deacetylases and histone acetyltransferases determines the acetylation status of evolutionarily conserved lysine residues within the N-terminal tails of histones (7). In general, histone acetylation levels are correlated with transcription levels; histones are hyperacetylated near active genes and hypoacetylated near inactive genes (8). Thus, the SIN3 complex is thought to mediate transcriptional repression through the gene-specific deacetylation of histones.

Neither SIN3 nor other components of the SIN3 complex can directly bind to DNA, instead targeting of the complex to particular genes occurs through protein-protein interactions with DNA-binding repressors or corepressors that interact with DNA-binding repressors (1, 2, 5). Within the complex, the SIN3 protein is the main target of these interactions and specifically binds repressors, such as Mad family proteins, and corepressors for nuclear hormone receptors (NHRs), such as N-CoR and SMRT.

SIN3 functions in numerous biological pathways in yeast and Drosophila. It is not essential for viability in Saccharomyces cerevisiae but is required to regulate the transcription of a wide variety of genes that play roles in diverse processes such as meiosis, potassium transport, and phospholipid metabolism (9–12). In contrast, SIN3 is essential in Drosophila. SIN3 null mutants die during larval development, and elimination of SIN3 from Drosophila tissue culture cells results in delay in the G2 phase of the cell cycle (13, 14).

To identify SIN3-regulated genes involved in G2 phase cell cycle progression and to provide a more comprehensive view of the role SIN3 plays as a regulator of transcription in metazoan organisms, we have performed a genome-wide analysis of transcription changes that result from elimination of SIN3 in Drosophila tissue culture cells. We have found that SIN3 is a widely acting transcriptional regulator in Drosophila that is minimally required for repression of 364 genes and activation of 35 genes that collectively represent ~3% of the predicted Drosophila transcriptome. As anticipated, a number of these genes play roles in G2 cell cycle progression and may account for the cell cycle delay of SIN3-deficient cells. However, unex-

* This work was supported in part by startup funds from the University of Wisconsin-Madison Graduate School, Medical School, and Department of Pharmacology (to D. A. W.), by the Intramural Program in the NICHHD, National Institutes of Health (to L. A. P.), and by a grant from the Howard Hughes Medical Foundation to Gerald M. Rubin (to P. T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: NHR, nuclear hormone receptor; dsRNA, double-stranded RNA; siRNA, single-stranded RNA; RNAi, RNA interference; RMA, robust multiarray analysis; GO, Gene Ontology; HIP-1, hypoxia-inducible factor-1; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TS, thymidylate synthase; CycB, cyclin B; Trx, thioredoxin.
EXPERIMENTAL PROCEDURES

RNA Interference—To generate double-stranded RNA (dsRNA) for RNA interference (RNAi), individual fragments 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 using the TOPO TA cloning kit (Invitrogen). The following primer sets (oriented 5′→3′) were used in a standard PCR reaction: SIN3 (GAATTTGAGAACCA-CAGGCTGCTGACAC and GCTATGAAACACGACCAAC), STG (AAACC–CAGCAGTTGCGTAG and GCATAGGCTGTTGGAAGT). Sense and antisense clones were used as templates to generate single-stranded RNA (ssRNA) using a Ribomax kit (Promega). ssRNA was resuspended in annealing buffer (5 mm KCl, 10 mm NaH2PO4), and equal quantities of sense and antisense ssRNA were annealed by加热 to 95 °C for 5 min and slow cooling for 12–18 h to generate dsRNA.

dsRNA was carried out according to the protocol developed by Clemens et al. (15). Briefly, 2 × 10^6 S2 or Kc Drosophila tissue culture cells were plated into a 60-mm dish and cultured at 25°C. After 1 h, fetal bovine serum-containing media (Schneider’s Drosophila medium, Invitrogen) was removed and replaced with 2 ml of serum-free media. Approximately 40 μg of dsRNA was added and mixed by swirling. After 30 min, 4 ml of media containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin was added. RNA, to be used for probe synthesis, was extracted 3 days following addition of dsRNA. To evaluate the level of the targeted protein, Western blot analysis was routinely carried out using standard procedures, as described by Pile et al. (14). To evaluate cell cycle phenotypes resulting from RNAi treatment, fluorescence-activated cell sorting analysis was routinely carried out, as described by Pile et al. (14).

Analysis of mRNA Expression Using Oligonucleotide Arrays—Microarray analysis was performed with GeneChip Drosophila arrays (Affymetrix) using probes derived from total RNA from Drosophila S2 or Kc cells. RNA was prepared and labeled following protocols listed in Section 2 of the GeneChip Expression Analysis Technical Manual (available at www.affymetrix.com). In brief, total RNA was isolated from 0.5–1 × 10^6 cells using the RNaseasy Mini kit (Qiagen). Double-stranded cDNA was synthesized from 16–24 μg of RNA and was used as a template to synthesize biotin-labeled cRNA by in vitro transcription using the BioArray High Yield RNA Transcription Labeling kit (Enzo). Double-stranded cRNA was fragmented and hybridized to arrays according to the manufacturer’s procedures (www.affymetrix.com). Probe hybridization and data collection were performed by the Gene Expression Facility at the University of Wisconsin-Madison. For each condition (wild type S2 cells, wild type Kc cells, SIN3 RNAi S2 cells, and SIN3 RNAi Kc cells) three arrays were hybridized with independently isolated RNA.

Statistical Analysis of Microarray Expression Data—Affymetrix CEL files were used as input into log scale multivariable analysis (RMA) with an updated indexing of probe sets to genes (available at ftp://ftp.affymetrix.com). RMA was performed in both Drosophila S2 and Kc tissue culture cells with the idea that genes whose transcription is altered in both cell lines will represent general, rather than cell type-specific, targets of SIN3. For example, because G2 cell cycle genes are a common phenotype of SIN3-deficient S2 and Kc cells, this analysis should increase the probability of identifying the SIN3 target genes responsible for the cell cycle phenotype (14). S2 and Kc cell lines are derived from different Drosophila strains and are molecularly and phenotypically distinct (18). This fact is reiterated by our observation that ~1600 genes are differentially expressed between S2 and Kc cells, where ~800 genes are more highly expressed in each cell line.2

SIN3 Is a Transcriptional Repressor—High density oligonucleotide arrays representing 13,137 Drosophila genes were used to monitor gene expression differences between control Drosophila S2 or Kc cells that were mock RNAi-treated and SIN3-deficient Drosophila S2 or Kc cells in which SIN3 protein levels were drastically reduced by addition of dsRNA corresponding to an ~930-nucleotide region of the SIN3 mRNA (Fig. 1A) (14). Changes in transcript levels were measured by probing arrays with fluorescently labeled cRNA, generated from total RNA extracted from cells 3 days after RNAi treatment, at which time the maximal reduction in SIN3 protein levels was observed (data not shown).

Using analysis of variance to evaluate the expression data, 399 genes, ~3% of all predicted Drosophila genes, were identified as having altered expression levels in SIN3-deficient S2 and Kc cells compared with wild type S2 and Kc cells. At this confidence level, fewer than 10 of the 399 genes are expected to be false positives. This is likely to be a minimum number of genes regulated by SIN3, because our analysis excluded genes whose expression is altered only in S2 or Kc cells. The number of genes affected by loss of SIN3 is in line with immunofluorescence studies of Drosophila polypore chromosomes, through which it was estimated that 2–13% of Drosophila genes are directly bound by the SIN3 complex (19). The overwhelming majority of the 399 genes had altered expression levels of less than 2-fold, indicating that this group of genes would have been difficult to detect without the analysis of multiple arrays and cell types (see Supplemental data).

The 399 identified genes were further analyzed by hierarchical clustering of expression patterns (Supplemental data). The SIN3 dendrogram contains two main branches of 364 induced and 35 repressed genes. It is extremely unusual for microarray data to show such a large difference between the number of induced and repressed genes. The ~10-fold difference between the number of induced and repressed genes and the concordance between the microarray and polypore results argues that

2 L. A. Pile, P. T. Spellman, and D. A. Wassarman, unpublished observation.
SIN3 plays a direct role in transcriptional repression of the identified induced genes.

Of the 399 identified gene products, 254 have been assigned Gene Ontology (GO) terms based on their determined or predicted biochemical activity and/or role as a component of a cellular organelle or biochemical process (20). Although loss of SIN3 affected genes from a diverse number of biological processes, including signal transduction, transcription, and cell cycle regulation, a comparison of the assigned terms with the complete Drosophila GO database revealed biases for gene products involved in mitochondrial processes (Supplemental data). Proteins that localize to mitochondria are represented at a rate 4-fold higher than expected ($p = 1.43 \times 10^{-15}$), protein components of the mitochondrial matrix are represented at a rate 7-fold higher than expected ($p = 3.58 \times 10^{-11}$), and mitochondrial ribosomal proteins are represented at a rate 8-fold higher than expected ($p = 5.11 \times 10^{-12}$). Thus, SIN3 appears to play a heretofore-unknown role as a transcriptional regulator of nuclear genes that encode mitochondrial proteins.

**SIN3-regulated Genes Tend to Be Excluded from Heterochromatic Regions of the Drosophila Genome**—Analysis of the chromosomal distribution of genes mis-regulated in SIN3-deficient cells indicates that fewer genes than expected map within or adjacent to heterochromatic regions comprised by centromeres and the fourth chromosome. Only 7 of the 399 genes map within or adjacent to heterochromatic cytological positions on the X chromosome (bands 19 and 20), the second chromosome (bands 39–42), the third chromosome (bands 79–82), or the fourth chromosome (bands 101 and 102) (Fig. 2A), whereas based on the fact that 583 predicted genes are contained within these bands, 18 genes are expected to be affected (Fig. 2B). Using a chi-square test, the $p$ value for this result is $5.9 \times 10^{-3}$. In contrast, within euchromatic regions, genes affected by loss of SIN3 appear to be randomly distributed. The exception is band 55 on chromosome arm 2R, which harbors SIN3-regulated genes, including 4 glutathione transferase genes, at a rate $-2.5$-fold higher than expected.

Overall, these findings are consistent with immunofluorescence staining of Drosophila polytene chromosomes, which revealed that the SIN3 complex binds throughout euchromatic regions but is largely absent from centric heterochromatin and most of the fourth chromosome (19). Similarly, in yeast, RPD3 is excluded from telomeric heterochromatin and sub-telomeric domains (21). SIN3 may display a comparable localization pattern, because association of RPD3 with chromatin in yeast is largely dependent on SIN3 (3, 4). These findings suggest that the SIN3 complex can only access chromatin with a particular level of compaction, supporting the hypothesis that distinct transcriptional regulatory mechanisms are utilized within different chromosomal domains.

**SIN3 Regulates Genes Involved in the Oxidative Metabolism of Glucose and Fatty Acids to Acetyl-CoA**—Loss of SIN3 results in the up-regulation of numerous genes involved in glycolysis (Fig. 3A). The metabolism of glucose to pyruvate during glycolysis produces energy in the form of ATP (22). Of the 10 enzymes in the glycolytic pathway, 5 are transcriptionally up-regulated in SIN3-deficient cells, including both of the genes encoding glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, which catalyzes the final step in the pathway, the irreversible conversion of phosphoenolpyruvate to pyruvate. In contrast, the process of gluconeogenesis, in which pyruvate is converted into glucose, may be repressed in SIN3-deficient cells due to down-regulation of phosphoenolpyruvate carboxykinase. A direct role for SIN3 in repressing multiple glycolytic genes may aid in the coordinate regulation of these genes during development and in response to metabolic stress (23, 24).

Up-regulation of genes encoding components of the pyruvate dehydrogenase complex in SIN3-deficient cells may further result in the metabolism of pyruvate to acetyl-CoA. In the mitochondrial matrix, the pyruvate dehydrogenase complex oxidatively decarboxylates pyruvate to form acetyl-CoA. The pyruvate dehydrogenase complex is composed of three enzymes, two of which are transcriptionally up-regulated in SIN3-deficient cells, the E2 enzyme dihydrolipoyl transacetylase and the E3 enzyme dihydrolipoyl dehydrogenase (Fig. 3A). Up-regulation of the pyruvate dehydrogenase complex may be complemented by increased expression of the gene encoding the arsenite-transporting ATPase (CG1598). Arsenite inhibits the
pyruvate dehydrogenase complex by inactivating the E2 transacetylase and FMN adenyltransferase, which converts flavin mononucleotide into FAD, a catalytic cofactor for the pyruvate dehydrogenase complex (22). Increased expression of arsenite-transporting ATPase may decrease the local concentration of inhibitory arsenite.

Loss of SIN3 also results in the up-regulation of numerous genes involved in fatty acid oxidation (Fig. 3A). The utilization of fat as an energy source occurs by oxidation of fatty acids to acetyl-CoA in the mitochondrial matrix (22). Up-regulated mitochondrial acyl-carnitine transporter in SIN3-deficient cells initiates this process by transporting long chain acyl-CoA molecules across the inner mitochondrial membrane. Subsequently, the action of up-regulated enzymes involved in four of the six principal reactions of fatty acid oxidation yields acetyl-CoA and an acyl-CoA chain that is shortened by two carbon atoms. In contrast, the reverse reaction, fatty acid synthesis, may be inhibited by up-regulation of insulin-degrading metalloproteinase (CG5517), which catalyzes the degradation of insulin. Insulin stimulates fatty acid synthesis by activating acetyl-CoA carboxylase, the enzyme that catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, a rate-limiting step in fatty acid synthesis.

Finally, loss of SIN3 results in the up-regulation of isocitrate dehydrogenase and malate dehydrogenase, two of the seven genes encoding regulatory enzymes of the citric acid cycle (Fig. 3A) (22). Electrons from acetyl-CoA, generated by glycolysis and fatty acid oxidation, are removed in the citric acid cycle and used to form NADH and FADH$_2$. Isocitrate dehydrogenase acts as a primary control point in the citric acid cycle, functioning only when ADP and NADH levels are low. Malate dehydrogenase is also an important component of the citric acid cycle, catalyzing the only step in the cycle that has a significantly positive free energy. Thus, SIN3 is a transcriptional regulator of genes that play roles in glycolysis, fatty acid oxidation, and the citric acid cycle.

Although SIN3 has not previously been implicated in the transcriptional regulation of glycolysis or fatty acid oxidation, roles for a histone deacetylase have been established in both of these pathways. In mammalian systems, hypoxia-inducible factor 1 (HIF-1) functions as a transcriptional activator of glycolytic genes, such as glyeraldehyde-3-phosphate dehydrogenase and enolase, in response to low oxygen (hypoxic) conditions (25). Repression of HIF-1 transactivation function under nonhypoxic conditions is mediated by binding of the von Hippel-Lindau tumor suppressor protein, which in turn associates with an histone deacetylase (26). Thus, it is possible that the SIN3 complex functions as a corepressor of HIF-1 target genes, preventing inappropriate activation under nonhypoxic conditions. Similarly in mammalian systems, the NHR peroxisome proliferator-activated receptor (PPAR) directly induces the transcription of genes, such as acyl-CoA dehydrogenase and 3-hydroxy-3-methylglutaryl-CoA synthetase, to stimulate fatty acid uptake and conversion to acyl-CoA derivatives (27, 28). Because PPARα-regulated genes are induced in response to chemical inhibitors of deacetylases, and PPARs interact with the corepressors N-CoR and SMRT, it is plausible that the Drosophila SIN3 complex associates with the PPARα orthologue to repress the transcription of genes involved in fatty acid oxidation (29, 30).

**SIN3 Regulates Genes Involved in Oxidative Phosphorylation and the Response to Reactive Oxygen Species**—Genes that play roles in oxidative phosphorylation are up-regulated in response to loss of SIN3. Glycolysis, fatty acid oxidation, and the citric acid cycle form energy-rich molecules NADH and FADH$_2$ that are used to generate ATP during oxidative phosphorylation in the inner mitochondrial membrane (22). Genes encoding components of two of the four electron-transport chain complexes are transcriptionally activated in SIN3-deficient cells (Fig. 3A). A chaperone protein (CG7598) in complex I is involved in the transfer of electrons from NADH to ubiquinone. Cytochrome c$_1$ and cytochrome c-p in complex III are involved in the transfer
FIG. 3. The biological function of genes affected in SIN3-deficient cells. Genes are denoted by their common name followed in parenthesis by the CG number designated by the Berkeley Drosophila Genome Project (www.fruitfly.org). Up-regulated genes are indicated in green typeface, down-regulated genes are indicated in red typeface, and genes that do not change are indicated in black typeface. Acetyl-CoA, a key metabolite in many pathways, is indicated in pink typeface.

A, metabolic pathways involving genes affected by loss of SIN3 are organized by cellular location (cytosol, mitochondrial outer membrane, mitochondrial inner membrane, or mitochondrial matrix). Distinct pathways (glycogen metabolism, glycolysis, gluconeogenesis, inositol synthesis, amino sugar synthesis, fatty acid oxidation, citric acid cycle, and porphyrin synthesis) are denoted by colored boxes. The asterisk indicates enzymes that utilize FAD as a cofactor.

B, cytosolic and mitochondrial protein metabolic processes involving genes affected by loss of SIN3 are categorized by process. PEK-Ef2 functions as a negative regulator of translation elongation and cathepsin D, cathepsin F, and cysteine protease inhibitor function as negative regulators of protein turnover.
of electrons from the ubiquinone-cytochrome c oxidoreductase complex. Furthermore, cytochromes and catalase (discussed below) contain heme prosthetic groups that are synthesized by a pathway of enzymes up-regulated in SIN3-deficient cells.

Genes that combat reactive oxygen species (ROS) are also up-regulated in SIN3-deficient cells. In addition to generating CO₂ and H₂O, oxidative phosphorylation generates ROS that are toxic to DNA, protein, and lipids. Catalase (CG6871), glutathione peroxidases (CG12013 and CG3083), and thioredoxin peroxidase (CG1633) provide protection from hydrogen peroxide (H₂O₂) by catalyzing the formation of H₂O and O₂ from H₂O₂. Glutathione transferases also display glutathione peroxidase activity and can thus protect cells from oxidative damage. Five glutathione transferases (CG5164, CG5224, CG17530, CG17531, and CG17534) were up-regulated in SIN3-deficient cells, and two were down-regulated (CG11784 and CG1742, a microsomal glutathione transferase). Thioredoxin reductase (CG2151) transfers reducing equivalents from NADPH to thioredoxin (Trx) resulting in Trx(SH)₂, which acts as an effective intracellular antioxidant. Finally, DNA polymerase (CG1925) plays a major role in DNA replication by bypassing DNA damage caused by hydroxyl radicals (OH), the primary DNA-damaging species formed from aerobic respiration (31). In support of a role for SIN3 in the response to ROS, a deacetylase has been implicated in regulating the transcriptional response to oxidative stress mediated by the Sp1 transcriptional activator (32, 33). The SIN3 complex may provide the unidentified deacetylase activity, because it has been shown to play a role in silencing Sp1-driven transcription (34).

SIN3 Regulates Genes Involved in Mitochondrial and Cellular Protein Synthesis, Modification, Translocation, and Turnover—Nuclear genes encoding components of the mitochondrial translation machinery constitute a major class of genes up-regulated in SIN3-deficient cells (Fig. 3B). Loss of SIN3 results in the transcriptional up-regulation of 10 different aminoacyl tRNA synthetases, enzymes that catalyze the attachment of an
SIN3 Functions as a Corepressor for Max and Mad Transcription Factors and Nuclear Hormone Receptors—A priori, we expected that genes transcriptionally repressed by corepressors, such as dMax and dMnt, or NHRs, such as the ecdysone receptor ligand ecdysone have been shown to induce EIP gene transcription in Kc cells but not S2 cells (18). In contrast, genes that are indirectly regulated by ecdysone, such as catalase and pyruvate kinase are up-regulated in SIN3-deficient cells (41, 42). In addition, juvenile hormone inducible (JhI) genes (CG3298) and JhI-26 (CG3767) are up-regulated in SIN3-deficient cells. JhI-1 and JhI-26 are 2 of the 7 genes that are transcriptionally induced in S2 cells in response to treatment with juvenile hormone (JH), a steroid hormone like ecdysone that coordinates Drosophila metamorphosis (43). Finally, as discussed above, SIN3 may interact with the NHR PPAR ortholog and repress the transcription of genes encoding enzymes involved in fatty acid oxidation. Taken together, the correlation between genes identified in this study and those regulated by dMnt-dMax and NHRs provides additional evidence for a direct role for SIN3 in regulating the genes identified in this study.

Loss of SIN3 Results in an Increase in Mitochondrial Mass—The large number of genes involved in mitochondrial processes suggested that mitochondrial mass may be affected in SIN3-deficient cells. To address this hypothesis, control and SIN3-deficient cells were stained with a mitochondrion-selective amino acid to its cognate tRNA molecule. In addition, 19 of the 72 genes encoding mitochondrial ribosomal proteins, 8 from the 40S small ribosomal subunit and 11 from the 60S large ribosomal subunit, are up-regulated (20). Mitochondrial ribosomal proteins are responsible for translating the 13 mRNAs that encode proteins of the oxidative phosphorylation system, suggesting that SIN3 plays a role in regulating genes involved in the production of proteins required for mitochondrial biogenesis as well as function (35).

Translation initiation factors constitute another over-represented class of genes that are up-regulated in the absence of SIN3. Components of initiation complexes eIF2, eIF3, and eIF4E, as well as three of the four components of the eIF2 GTP exchange factor eIF2B, are up-regulated (36). However, cytosolic translation elongation factors do not appear to be affected. In fact, the rate of cytosolic polypeptide chain elongation may be inhibited in SIN3-deficient cells by inactivation of the elongation factor eEF2 through phosphorylation by up-regulated PEB-EF2 kinase. In contrast, polypeptide chain elongation on mitochondrial ribosomes may be stimulated by up-regulation of the mitochondrial-specific elongation factor EF-TuM, resulting in increased levels of the small number of proteins encoded on the mitochondrial genome, including various components of complex I, complex II, and the ATPase complex.

Protein components of the fatty acid oxidation, citric acid, and porphyrin synthesis pathways are among the 98% of mitochondrial proteins that are encoded on the nuclear genome and synthesized on cytosolic ribosomes (35). These proteins must be translocated across the outer and inner mitochondrial membranes to their site of action in the mitochondrial matrix (37). In SIN3-deficient cells transport may be facilitated by up-regulation of Tim9b (CG17767) and Tim10 (CG9878), which are components of inner membrane protein translocase machinery.

dMax heterodimers associate with SIN3 and repress transcription. A comparison of genes identified in this study to those identified in a recent study of direct gene targets for dMax and dMnt revealed considerable overlap (38). Common gene products include those involved in mitochondrial biogenesis (mitochondrial ribosomal proteins, Tim proteins, EF-TuM, and Ferrochelatase), cell cycle regulation (STG), and transcription (Jra, Bigmax, and TAF60–2). Because in mammals Myc-Max heterodimers associate with coactivators to activate transcription, Myc regulates metabolic gene transcription, and Mad/Mnt oppose Myc action through association with histone deacetylase complexes, SIN3 may function through dMnt to antagonize the action of Myc-Max (39, 40).

Up-regulation of ecdysone inducible protein (EIP) genes was not observed in SIN3-deficient cells. This is likely due to the fact that our statistical algorithm required that changes in gene expression must occur in both S2 and Kc cells, however, the ecdysone receptor ligand ecdysone has been shown to induce EIP gene transcription in Kc cells but not S2 cells (18). In contrast, genes that are indirectly regulated by ecdysone, such as catalase and pyruvate kinase are up-regulated in SIN3-deficient cells (41, 42). In addition, juvenile hormone inducible (JhI) genes (CG3298) and JhI-26 (CG3767) are up-regulated in SIN3-deficient cells. JhI-1 and JhI-26 are 2 of the 7 genes that are transcriptionally induced in S2 cells in response to treatment with juvenile hormone (JH), a steroid hormone like ecdysone that coordinates Drosophila metamorphosis (43). Finally, as discussed above, SIN3 may interact with the NHR PPAR ortholog and repress the transcription of genes encoding enzymes involved in fatty acid oxidation. Taken together, the correlation between genes identified in this study and those regulated by dMnt-dMax and NHRs provides additional evidence for a direct role for SIN3 in regulating the genes identified in this study.

L. W. M. Loo and R. N. Eisenman, personal communication.
SIN3 Regulates Mitochondrial Protein Genes

The increase in mitochondrial mass may be due to the G2 phase cell cycle delay of SIN3 RNAi cells. In a human leukemic cell line mitochondrial mass has been shown to increase as cells progress from G1 into S phase but not from S into G2/M phase (44). However, the increase in mitochondrial mass that normally occurs during the cell cycle is not as great as that observed in SIN3-deficient cells. In further support of a cell cycle-independent increase in mitochondrial mass of SIN3-deficient cells, STG-deficient cells, which are also blocked in the G2/M phase of the cell cycle, did not display a significant increase in mitochondrial mass relative to mock RNAi-treated S2 cells (Fig. 4, A, B, and D). In addition, mutation of 3-hydroxyacyl-CoA dehydrogenase, an enzyme in the SIN3-regulated fatty acid oxidation pathway, reduces the number of mitochondria in sperm and loss of ref(2)P, a gene involved in sigma rhabdovirus multiplication that is up-regulated in SIN3-deficient cells, leads to degeneration of mitochondria in spermatids of sterile male flies (Fig. 3A) (45, 46). Thus, a major physiological consequence of loss of SIN3 is an increase in the mitochondrial mass and presumably the oxidative phosphorylation capacity of cells.

Down-regulation of String Expression May Account for the G2 Phase Cell Cycle Delay of SIN3-deficient Cells—Previously we have shown that loss of SIN3 by RNAi results in G2 phase cell cycle delay (Fig. 1, B and C) (14). The cause of this delay is not known but is presumably due to altered transcription of genes involved in cell cycle control. Interestingly, several down-regulated genes in SIN3-deficient cells have been implicated in cell cycle control. Rough deal (rod, CG1569) is a regulator of mitotic chromosome segregation (47). Thymidylate synthase (TS, CG3181) is required for the synthesis of thymidylate and DNA replication, and down-regulation of TS mRNA levels results in G2/M phase cell cycle arrest (48). Cyclin B (CycB, CG3377) is a cyclin-dependent kinase regulator that acts during the G2/M transition (49). Finally, STG (CG1395), the Drosophila homolog of the yeast mitotic regulator CDC25 phosphatase, is required for G2 phase cell cycle progression (14, 50).

Our previous study demonstrated that elimination of STG by RNAi is sufficient to cause G2 phase cell cycle delay in S2 and Kc cells, indicating that transcriptional repression of STG in SIN3-deficient cells is likely to contribute to the G2 phase delay (14). In contrast, although CycB protein levels are reduced in SIN3-deficient cells, this is not sufficient to cause the G2 phase delay, because elimination of CycB by RNAi does not affect cell cycle progression. Rod and TS may contribute to the cell cycle defect of SIN3-deficient cells, but this has not been directly examined.

Finally, it is possible that the G2 phase cell cycle delay of SIN3-deficient cells is a consequence of altered expression of mitochondrial proteins (Fig. 3A). For example, in yeast, mutation of acetyl-CoA carboxylase, a regulatory enzyme in the mitochondrial fatty acid synthetic pathway, causes G2/M phase arrest of the cell cycle (51). Induction of fatty acid oxidation in SIN3-deficient cells may be equivalent to inactivation of fatty acid synthesis in acetyl-CoA carboxylase-deficient cells. If this is the case, it would reveal the existence of a G2/M phase cell cycle check point that monitors the level of certain fatty acids or fatty acid derivatives.

Summary—Analysis of the gene expression profile and physiology of cells lacking the SIN3 transcriptional repressor has provided compelling evidence that SIN3 functions as a central regulator of cytosolic and mitochondrial energy transduction pathways and mitochondrial biogenesis. Interestingly, there is substantial overlap between SIN3-regulated genes and a group of yeast genes that was shown, through microarray profiling of yeast cells treated under 300 different conditions, to be coregulated (52).

The group of yeast genes includes tRNA synthetases, 37 mitochondrial ribosomal protein genes, and 67 proteins with mitochondrial functions. Similar to genes affected by loss of SIN3, none of the yeast genes is up- or down-regulated more than 2-fold. Thus, in the absence of an activation signal, SIN3 may function to establish a chromatin structure permissive for transcription of mitochondrial protein genes but below maximal levels. The ability to eliminate SIN3 in Drosophila tissue culture cells by RNAi and examine the transcription of endogenous genes provides a tractable system to dissect the molecular mechanisms that control this complex and important regulatory system.

Acknowledgments—We thank Sandra Splinter BonDurant and Wayne Davis at the University of Wisconsin-Madison Gene Expression Center for their assistance in probing microarrays; Kathy Schell, Joan Batchelder, and Joel Puchalski at the University of Wisconsin-Madison Comprehensive Cancer Flow Cytometry Facility for their assistance in determining the mitochondrial mass of cells; Justin Haroldson and Rachel Harris for their assistance in compiling the list of SIN3 affected genes; and Rohinton Kamakaka for the Drosophila p55 antibody. We also thank Gavin Sherlock for sharing the TermFinder code prior to release.

REFERENCES

1. Ayer, D. E. (1999) Trends Cell Biol. 9, 193–198
2. Ahringer, J. (2000) Trends Genet. 16, 351–356
3. Bernstein, B. E., Tung, J. K., and Schreiber, S. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13708–13713
4. Fazzio, T. G., Koopberger, C., Goldmark, J. P., Neal, C., Bascom, R., Deldrow, J., and Tsukiyama, T. (2001) Mol. Cell. Biol. 21, 6460–6460
5. Knoepfler, P. S., and Eisenman, R. N. (1999) Cell 99, 447–450
6. Fleischer, T. C., Yun, U. J., and Ayer, D. E. (2003) Mol. Cell. Biol. 23, 3456–3467
7. Jennewein, T., and Allis, C. D. (2001) Science 293, 1074–1080
8. Fischer, W., Wang, Y., and Allis, C. D. (2003) Curr. Opin. Cell Biol. 15, 172–183
9. Sternberg, P. W., Stern, M. J., Clark, I., and Heskowitz, I. (1987) Cell 48, 567–577
10. Lamb, T. M., and Mitchell, A. P. (2001) Genetics 157, 545–556
11. Vidal, M., Buckley, A. M., Yohn, C., Hoepfner, D. J., and Gaber, R. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2370–2374
12. Hudak, K. A., Lopes, J. M., and Henry, S. A. (1994) Genetics 136, 475–483
13. Neufeld, T. P., Tang, A. H., and Robin, G. M. (1998) Genetics 148, 277–286
14. Pile, L. A., Schlag, E. M., and Wassarman, D. A. (2002) Mol. Cell. Biol. 22, 4965–4976
15. Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Machama, T., Hemmings, B. A., and Dixon, J. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6499–6503
16. Irazarry, B. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. S., Scherf, U., and Speed, T. P. (2002) Bioinformatics, in press
17. Pavlidis, P., and Noble, W. S. (2001) Genome Biol. http://www.genomebiology.com/2001/2/10/Research0042
18. Cherbas, L., and Cherbas, P. (1981) Adv. Cell Cult. 1, 91–124
19. Pile, L. A., and Wassarman, D. A. (2000) EMBO J. 21, 6131–6140
20. Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Agapitou, P. G., Bertsch, S. H., Buhrey, R. S., Galfre, G. H., George, R. A., Lewis, S. E., Richards, S. Ashburner, M., Henderson, S. N., Sutton, G. G., Wortman, J. R., Yandell, M. D., Zhang, Q., Chen, L. X., Botstein, D. C., Rogers, Y. H., Blazer, R. G., Chiam, M., Pfeiffer, B. D., Wan, K. H., Doyle, C., Baxter, E. G., Hult, G., Nelson, C. R., Borer, G. L., Abril, J. F., Abyhayan, A., An, J. H., Andrews-Pfannkuch, C., Baldwin, D., Broom, R. M., Basu, A., Bazendale, J., Rayakarturopou, L., Beasley, E. M., Beeson, K. Y., Boren, P. V., Berman, B. P., Bhandari, D., Bulskahov, S., Borkova, D., Botchan, M. R., Bouck, J., Broeckstein, P., Brottier, P., Burris, K. C., Bussam, D. A., Butler, H., Castile, E., Center, A., Chandra, I., Cherry, J. M., Cawley, S. C., Dahake, C., Davenport, L. B., Davies, P. K., de Pablo, B., Delcher, A., Deng, Z., Mays, A. D., Dew, I., Dietz, S. M., Dodson, K., Duop, L. E., Downes, M., Dugan-Rocha, S., Dunck, B. C., Dunn, P., Durbm, K. J., Evangelista, C. C., Ferrara, C., Ferriera, S., Fleischmann, W., Fod, C., Gabrielian, A. E., Garg, N. S., Gelhart, W. M., Glasser, K., Godke, A., Gong, F., Gorrell, J. H., Gu, Z., Guan, P., Harris, M., Harris, N. L., Harvey, D.

*E. M. Schlag, L. A. Pile, and D. A. Wassarman, unpublished observation.*
