A complex interplay between SAM synthetase and the epigenetic regulator SIN3 controls metabolism and transcription

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The SIN3 histone-modifying complex regulates the expression of multiple methionine catabolic genes, including SAM synthetase (Sam-S), as well as SAM levels. To further dissect the relationship between methionine catabolism and epigenetic regulation by SIN3, we sought to identify genes and metabolic pathways controlled by SIN3 and SAM synthetase (SAM-S) in Drosophila melanogaster. Using several approaches, including RNAi-mediated gene silencing, RNA-Seq— and quantitative RT–PCR—based transcriptomics, and ultra-high-performance LC-MS/MS— and GC/MS—based metabolomics, we found that, as a global transcriptional regulator, SIN3 impacted a wide range of genes and pathways. In contrast, SAM-S affected only a narrow range of genes and pathways. The expression and levels of additional genes and metabolites, however, were altered in Sin3A and Sam-S dual knockdown cells. This analysis revealed that SIN3 and SAM-S regulate overlapping pathways, many of which involve one-carbon and central carbon metabolisms. In some cases, the factors acted independently; in some others, redundantly; and for a third set, in opposition. Together, these results, obtained from experiments with the chromatin regulator SIN3 and the metabolic enzyme SAM-S, uncover a complex relationship between metabolism and epigenetic regulation.

Control of cellular metabolism is critical for cell homeostasis and, ultimately, for cell and organismal viability. The cellular response to changes in metabolite levels often involves activation of signaling pathways that leads to posttranslational protein modifications and a change in gene expression patterns (1). There is a direct link between metabolism and control of gene expression. DNA is packaged into chromatin, of which histones make up the bulk of the packaging proteins. Histones are subject to posttranslational modifications. The type and extent of histone modification affect chromatin structure and binding of transcription factors, which, in turn, control gene activity (2). Methylation and acetylation are two histone modifications that impact transcription (3). The methyl and acetyl groups are derived from SAM and acetyl-CoA, respectively. The levels of these two key cellular metabolites are directly influenced by central metabolic pathways, including folate and methionine metabolism, for SAM, whereas glycolysis and fatty acid catabolism are important for acetyl-CoA (1).

The feedback between epigenetic control and metabolism is complex. Global histone modification levels respond to changes in metabolic flux. In humans, inhibition of glycolysis leads to a decrease in acetyl-CoA that, in turn is reflected in a decrease in the global levels of histone H3 and histone H4 lysine acetylation (4). In yeast, alteration of either glucose or serine levels affects histone H3K4 methylation and H3T11 phosphorylation (5). In plants, Caenorhabditis elegans, and Drosophila melanogaster, reduction of SAM synthetase (SAM-S),2 the key enzyme necessary to generate SAM as well as the rate-limiting enzyme in methionine catabolism (6), results in decreased global histone methylation relative to controls (7–9). Alternatively, mutation or reduction of histone-modifying enzymes can impact the expression of enzymes that control metabolism as well as cellular metabolite concentration. For example, in rats or mice, knockdown or knockout of genes encoding two enzymes involved in histone deacetylation, HDAC1 and SIRT6, or the histone H3K9 demethylase JHDM2A, leads to changes in the expression of multiple metabolic genes and altered metabolic profiles (10–12). Our understanding of the full range of mechanisms that control the cross-talk between metabolism and epigenetics remains incomplete.

The SIN3 complex is one of the major multisubunit histone-modifying complexes present in cells. Distinct SIN3 complexes

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2 The abbreviations used are: SAM-S, SAM synthetase; TCA, tricarboxylic acid; qRT–PCR, quantitative RT–PCR; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization, and Integrated Discovery; PLS-DA, partial least squares discriminant analysis; ChiP-Seq, ChiP sequencing; ssRNA, single-stranded RNA; KD, knockdown; ANOVA, analysis of variance; H3K4me3, tri-methylation at the 4th lysine residue of the histone H3 protein; SESAME, Serine-responsive SAM-containing Metabolic Enzyme; H3K9ac, acetylation at the 9th lysine residue of the histone H3 protein.
have been identified and described in organisms ranging from yeast to humans (13). The very large majority contain the deacetylase HDAC1, whereas a subset additionally contain a histone H3K4me3 demethylase (14). Sin3A, as an essential gene in Drosophila and mammals, plays an important role in regulating transcription, cell proliferation, and development (15). Genome-wide transcriptome analyses performed in different model organisms reveal that SIN3 regulates genes involved in stress response, the cell cycle, development, and metabolism (16–19). In addition to affecting the expression of genes encoding metabolic enzymes, we recently demonstrated that SIN3 links epigenetic regulation and metabolism, specifically as related to methionine catabolism and global histone methylation (20). Knockdown of Sin3A in Drosophila S2 cultured cells leads to an increase in the expression of Sam-S, with a concomitant increase in the methyl donor SAM and global levels of H3K4me3. In contrast, knockdown of Sam-S results in a decrease in global H3K4me3 levels. Cultured cells in which both SAM-S and SIN3 are reduced have nearly WT levels of global H3K4me3; thus, SAM-S and SIN3 act in opposition to affect histone methylation.

In Drosophila, there is a single known Sam-S gene, and it is essential (9, 21). Three types of mammalian SAM-S, called MATI, MATII, and MATIII, are encoded by three methionine adenosyltransferase genes: MAT1A, MAT2A, and MAT2B (22). Although it is a metabolic enzyme, yeast and mammalian SAM-S can be found in nuclei and can localize to chromatin (5, 23, 24). Furthermore, SAM-S has been demonstrated to affect transcription. Genes involved in cell proliferation, cell differentiation, signaling pathways, and the immune response are misregulated when Mat1a is depleted in mice (25). In yeast, SAM-S associates with other metabolic enzymes to form the SESAME complex, which regulates gene expression through affecting histone modifications by sensing glycolysis and glucose-derived serine metabolism (5).

In this work, to determine the gene- and metabolism-regulatory networks controlled by SIN3 and SAM-S, we performed a genome-wide transcriptome analysis and generated a metabolomic profile in cells with altered SIN3 and SAM-S levels. We found that, although SAM-S influences the levels of metabolites found in multiple pathways, the number of genes affected was few. Previous studies indicate that SIN3-regulated metabolic genes include those involved in glycolysis and gluconeogenesis, the citric acid cycle (TCA cycle), as well as fatty acid, GSH, and pyrimidine metabolism (16, 17, 26). Consistent with these studies, we found that metabolites involved in many pathways of central carbon metabolism are impacted by reduction of SIN3. Comparing the metabolic pathways controlled by SIN3 and SAM-S, we found a complex interaction between these factors. For some metabolic pathways, SIN3 and SAM-S act in a redundant fashion. For others, however, reduction of SAM-S counteracts some of the effects of Sin3A knockdown, similar to the effect observed for global histone H3K4me3 levels. Multiple carbon-processing pathways are thus subject to control by both metabolic sensing and epigenetic regulation.

Results

RNA-Seq analysis identifies common and distinct genes regulated by SIN3 and SAM-S

To investigate the underlying transcriptional network regulated by SIN3 and SAM-S, we performed an RNA-Seq experiment to identify genome-wide changes in gene expression upon RNAi-mediated reduction of Sin3A or Sam-S or both in Drosophila S2 cells. We selected to perform our studies using the Drosophila model, as Sin3A and Sam-S are single genes in Drosophila, unlike mammals, which have multiple genes encoding different isoforms of these proteins. Additionally, Drosophila has been demonstrated to be useful for studies of metabolic homeostasis and disease (27). Use of the S2 cell line allows the study to be conducted in a homogenous cell system. Cells treated with dsRNA targeting GFP acted as the control. Knockdown of Sin3A and Sam-S was verified by Western blot analysis and RT-PCR or real-time qRT-PCR, respectively (Fig. S1). The RNA-Seq data obtained from Sin3A RNAi–treated and control S2 cells have been published previously (17, 28). The reproducibility of the Sam-S and Sin3A + Sam-S knockdown data were confirmed by performing a Pearson’s correlation analysis of the three biological replicates (Fig. S2). To verify the RNA-Seq data, we utilized the RNAi knockdown samples prepared for the metabolomics study, described below, to analyze mRNA levels by real-time qRT-PCR. All tested genes showed similar expression trends between the real-time qRT-PCR results and RNA-Seq data (Fig. S3).

Differential expression analysis was performed by comparing the knockdown samples with the control. To identify significantly affected genes, we selected genes having more than or equal to a 1.5-fold change in expression with a p value cutoff of 0.05 and the false discovery rate (FDR) cutoff of 0.05 (Fig. 1 and Table S1). Because reduced SAM-S leads to decreased global H3K4me3 levels (9, 20), a histone mark associated with active genes (29, 30), we predicted that the expression of a large number of genes would change upon knockdown of Sam-S. Contrary to our expectations, only 18 genes were affected (Fig. 1A). Of these, 11 were up-regulated and 7 were down-regulated in Sam-S knockdown cells compared with the control (Fig. 1A). In contrast to the limited transcriptional effects observed following Sam-S knockdown, Sin3A regulates a large percentage of the genome, as described previously (17, 28). In Sin3A single knockdown cells, 263 genes were up-regulated and 349 were down-regulated (Fig. 1B). Of note, as reported, Sam-S is a direct target of SIN3 regulatory control (20). Interestingly, when Sam-S and Sin3A were reduced simultaneously by RNAi, the number of impacted gene targets was significantly higher than that of Sin3A knockdown alone. Of the 734 genes misregulated upon dual knockdown of Sin3A and Sam-S, 258 genes (35%) were up-regulated, and 476 genes (65%) were down-regulated (Fig. 1C). This finding suggests that the repertoire of SAM-S target genes is larger than that revealed in the Sam-S single knockdown sample. Additionally, the data indicate that there are a number of common genes regulated by SIN3 and SAM-S in which the presence of either factor facilitates control levels of expression and that a measurable change in expression occurs only when both factors are reduced by RNAi.


Cross-talk of SAM-S and SIN3 on metabolism and transcription

Evaluation of the specific gene targets indicates that there are shared and unique misregulated genes upon individual knockdown of Sin3A or Sam-S (Fig. 2). Of the 18 targets of SAM-S, 13 were also targets of SIN3 (Fig. 2). The data suggest that, for these 13 genes, SIN3 and SAM-S function independently to regulate their expression levels, as loss of either factor is sufficient to impact gene activity. Additionally, of the 13 targets, 12 genes were dysregulated in all three knockdown samples (Fig. 2). Interestingly, with the exception of the single gene Sam-S, which increased following Sin3A knockdown and decreased in Sam-S and Sin3A + Sam-S knockdown compared with the control, the rest of the 11 shared targets were misregulated in the same direction when comparing all three tested conditions (Fig. 2). These findings indicate that SIN3 and SAM-S function to regulate these 11 genes in a similar way, acting as either a corepressor or coactivator at these sites. Of the shared 13 targets, E(spl)mbeta-HLH was the only one misregulated in each of the Sin3A and Sam-S knockdown samples, but in the opposite direction, suggesting that the regulatory role of SIN3 and SAM-S on this gene is different.

As single knockdown of Sam-S impacted the expression of only a limited number of genes, we focused our analysis on Sin3A knockdown targets compared with Sin3A + Sam-S knockdown targets. 612 genes were targets of SIN3, and this number increased to 734 in the dual knockdown sample (Fig. 1, A and C). Of these, 403 genes were common, with 12 genes dysregulated in all three knockdown samples (Fig. 2). The degree of expression changes for the overlapping 391 genes (category I) was not significantly altered between Sin3A knockdown and Sin3A + Sam-S knockdown (Table S1). These results strongly suggest that these 391 genes are regulated by SIN3 but not by SAM-S, as their expression was impacted following Sin3A knockdown, and no obvious effect was observed following additional knockdown of Sam-S. The nonoverlapping genes identified when comparing those affected in the two conditions, Sin3A knockdown and Sin3A + Sam-S knockdown, are interesting targets for this study. 208 genes (category II) were misregulated in Sin3A knockdown but not changed in Sin3A + Sam-S knockdown relative to the control (Fig. 2). The data suggest that these genes are regulated by SIN3 and also by SAM-S but that the role of SAM-S on these targets is in opposition to the role of SIN3. Additionally, SAM-S impacts these genes only in the absence of SIN3, as the expression of these genes was not altered in Sam-S knockdown sample relative to the control. In contrast, 328 genes (category III) were misregulated only upon dual reduction of SIN3 and Sam-S (Fig. 2), indicating that these genes are regulated redundantly by SIN3 and SAM-S.

Next we sought to determine the biological processes and pathways regulated by SIN3 and SAM-S. We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses on the significantly regulated genes identified by RNA-Seq using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (31, 32). Although the RNA-Seq data of the Sin3A knockdown sample were analyzed in two previous publications, the authors used a different -fold change cutoff to determine differentially expressed genes and selected a subgroup of genes for GO and KEGG pathway analyses (17, 28). Therefore, we reperformed GO and KEGG pathway analyses for SIN3 targets in this study.

First we determined the pathways and processes associated with each of the three samples: Sam-S knockdown, Sin3A knockdown, and Sin3A + Sam-S knockdown. Because only 18 genes were misregulated in Sam-S knockdown compared with the control, protein folding and determination of adult lifespan were the only significantly enriched biological processes, and no significantly enriched KEGG pathway was identified for this sample (Fig. S4A and Table S2). As expected, comparison of the processes and pathways that were significantly enriched in

Figure 1. Reduction of SIN3 and SAM-S affects cellular gene expression profiles. A–C, scatterplots indicating the changes in gene expression in Sam-S knockdown cells (A), Sin3A knockdown cells (B) as reported previously (28), and Sin3A + Sam-S knockdown cells (C) compared with GFP RNAi control cells. The results are the average of three biological replicates. Cuffdiff was used to identify statistically significant genes, p < 0.05 and FDR < 0.05 were considered statistically significant. Red spots, significantly changed genes having more than or equal to 1.5-fold change expression; purple spots, significantly changed genes having less than 1.5-fold change expression; gray spots, nonsignificantly changed genes.
Sin3A knockdown and dual knockdown cells indicated that both shared and distinct categories were affected under these different conditions (Fig. S4, B and C, and Table S2). Next, to focus on the gene-regulatory effects of these two factors acting independently, in opposition or redundantly, we performed the analysis using the shared and unique gene sets from categories I, II, and III from Sin3A knockdown and Sin3A + Sam-S knockdown cells. For the 391 SIN3-only–regulated genes of category I, transport, sleep, determination of adult lifespan, as well as fatty acid degradation, other glycan degradation, nicotinate, and nicotinamide metabolism were specific (Fig. 3A and Table S2). For the 208 genes of category II, for which reduction of Sam-S appeared to counter the effect of reduction of Sin3A, autophagy as well as sphingolipid metabolism, starch and sucrose metabolism, biosynthesis of antibiotics, and amino sugar and nucleotide sugar metabolism were unique (Fig. 3B and Table S2). Interestingly, for categories I and II, the processes and pathways with the largest number of genes included metabolism (Fig. 3A, A and B, and Table S2). For the 328 genes of category III, which were regulated redundantly by SIN3 and SAM-S, RNA metabolic process, RNA polymerase, regulation of chromatin silencing, cell cycle, DNA replication, nucleotide excision repair, and mismatch repair were specific (Fig. 3C and Table S2). The results indicate that SIN3, a cofactor associated with histone modification, and SAM-S, a metabolic enzyme, each affect the expression of genes encoding proteins that control a number of critical metabolic processes in the cell. Independently, SAM-S controls significantly fewer pathways compared with SIN3. In the absence of SIN3, however, additional roles of this metabolic enzyme are revealed. These data indicate that there is a complex interplay between epigenetic and metabolic control of gene expression.

**Figure 2. Venn diagram showing shared and unique genes misregulated upon knockdown of Sin3A or Sam-S or both.** Categories I, II, and III are described in the text. For the 391 genes of category I, \( p < 1.2 \times 10^{-11} \). The significance was calculated by hypergeometric test.

A diverse set of metabolites is regulated by SIN3 and SAM-S

The finding that SIN3 and SAM-S control the expression of genes enriched in different metabolic pathways suggests that these factors may impact the levels of numerous cellular metabolites. To test this prediction, we performed an untargeted metabolomics analysis on cells with Sin3A, Sam-S, or Sin3A + Sam-S knockdown and compared these results with GFP RNAi control cells. RNAi efficiency was routinely validated by Western blot analysis and RT-PCR (Fig. S1).

Partial least squares discriminant analysis (PLS-DA) indicates that each sample has a unique metabolic profile (Fig. 4A). To identify significantly regulated metabolites, we used a \( p \) value cutoff of 0.05 (Table S3). Of the 53 metabolites misregulated in the Sam-S knockdown sample relative to the control, 27 metabolites (51%) were up-regulated, and 26 metabolites (49%) were down-regulated (Fig. 4B). Consistent with the gene expression profiles, more metabolites were affected in Sin3A knockdown and Sin3A + Sam-S knockdown cells compared with Sam-S knockdown cells. Of the 248 metabolites dysregulated following Sin3A knockdown, 45 metabolites (18%) were up-regulated, and 203 metabolites (82%) were down-regulated (Fig. 4B). Of the 207 metabolites misregulated upon dual knockdown of Sin3A and Sam-S, 46 metabolites (22%) were up-regulated, and 161 metabolites (78%) were down-regulated (Fig. 4B). These metabolic data clearly indicate that altering the level of SIN3 or SAM-S leads to a change in the cellular metabolome. The finding that knockdown of Sin3A results in a more profound change to the cellular metabolome compared with the Sam-S knockdown sample strongly suggests that SIN3 regulates multiple metabolic pathways, not only methionine catabolism, which has been described previously (20).
Individual knockdown of Sin3A or Sam-S affects shared and unique metabolites (Fig. 5). Of the 53 metabolic targets of SAM-S, 25 were also targets of SIN3 (Fig. 5). The data suggest that, for these 25 metabolites, SIN3 and SAM-S function independently to regulate their amount, as loss of either factor alone is sufficient to impact metabolite concentration. 14 of the 25 shared metabolites were dysregulated in all three knockdown samples (Fig. 5). The levels of the other 11 metabolites of the 25 shared targets as well as 4 of the 14 common metabolites were changed in the opposite direction when comparing the two tested conditions (Fig. 5). These findings indicate that SIN3 and SAM-S function to regulate these 15 metabolites in opposite ways. For the other 10 metabolites of the 14 common targets, the factors function in a similar regulatory manner.

More dysregulated metabolites were observed in Sin3A knockdown and Sin3A/H11001 Sam-S knockdown cells compared with Sam-S knockdown cells. Of the 154 metabolites altered in both Sin3A and Sin3A/H11001 Sam-S knockdown samples, 14 targets were dysregulated under all three knockdown conditions (Fig. 5). Interestingly, except for a single metabolite, SAM, which increased following Sin3A knockdown and decreased upon Sam-S and Sin3A/H11001 Sam-S knockdown compared with the control, the rest of the 153 shared metabolites, comparing the Sin3A knockdown and Sin3A/H11001 Sam-S knockdown samples,
were misregulated in the same direction (Fig. 5). Of the overlapping 140 targets (category I), the concentration of only seven metabolites was significantly altered when comparing Sin3A knockdown and Sin3A/H11001 Sam-S knockdown samples (Table S3). These results indicate that the majority of the 140 shared metabolites are largely affected by SIN3 only, as their levels were altered following Sin3A knockdown, and no obvious effect was observed following additional knockdown of Sam-S. 83 metabolites (category II) were misregulated in Sin3A knockdown but not changed in Sin3A + Sam-S knockdown (Fig. 5). The data suggest that these metabolites are regulated by both SIN3 and SAM-S. For these metabolites, SAM-S and SIN3 act...
in opposition because SAM-S reduction negates the effect caused by loss of SIN3. Additionally, SAM-S impacts these metabolites only following reduction of SIN3, as the concentration of these metabolites was not altered in the Sam-S knockdown sample relative to the control. In contrast, 35 metabolites (category III) were misregulated only upon dual reduction of SIN3 and SAM-S (Fig. 5), indicating that these metabolites are regulated redundantly by SIN3 and SAM-S.

To further investigate the function of SIN3 and SAM-S as related to metabolism, we performed a pathway analysis with the significantly regulated metabolites using MetaboAnalyst module 4.0 (33). This tool takes into account both the enrichment analysis as well as pathway topology analysis, which is an indication of the impact of the metabolites in the pathway. The large majority of the pathways identified in this analysis were found in all three samples (Fig. 6 and Table S4). For example,
consistent with work published previously (20), methionine catabolism was impacted under all three conditions (Fig. 6 and Table S4). A limited number of pathways, including phenylalanine metabolism, fructose and mannose metabolism, galactose metabolism, and nicotinate and nicotinamide metabolism, were uniquely associated with the Sin3A knockdown sample (Fig. 6, A and C, and Table S4). No pathways were exclusively affected in either Sam-S knockdown or Sin3A + Sam-S knockdown cells (Fig. 6, A, B, and D, and Table S4). Next, to focus on the metabolite-regulatory effects of these two factors acting independently or redundantly, we performed the analysis using the shared and unique metabolite sets from categories I, II, and III between Sin3A knockdown and Sin3A + Sam-S knockdown cells, similar to the analysis we used for the transcriptome data-sets. For the 140 metabolites of category I, which were largely regulated by Sin3 only, GSH metabolism, histidine metabolism, pyruvate metabolism, riboflavin metabolism, and sulfur metabolism were specific (Fig. S5, A and B, and Table S4). The finding that a number of pathways were controlled specifically by Sin3 is likely a reflection of the fact that Sin3 is considered a global transcriptional regulator that affects the expression of many genes with varied functions. For the 83 metabolites of category II, for which reduction of Sam-S appeared to counter the effect of reduction of Sin3A, phenylalanine metabolism, valine, leucine and isoleucine biosynthesis, fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, starch and sucrose metabolism, and nicotinate and nicotinamide metabolism were unique (Fig. S5, A and C, and Table S4). For the 35 metabolites of category III, which only changed in the dual knockdown sample, no pathways unique to this category were found (Fig. S5, A and D, and Table S4). Taken together, this analysis indicates that Sin3 and Sam-S regulate a number of overlapping metabolic pathways, many of which are involved in one-carbon and central carbon metabolism. For some metabolites in specific pathways, the factors act independently; for others, they have redundant function; for a third set, they act in opposition.

**The impact of Sin3 and Sam-S on one-carbon and central carbon metabolism**

For the next step in our study, we investigated the gene expression effects that were possibly linked to the altered metabolic pathways. Previous work from our group and others has found that Sin3 regulates genes that control cellular energy metabolism and mitochondrial function (16, 18, 34). Additionally, we reported that reduction of Sin3 affects the expression of genes involved in methionine catabolism, the level of the methyl donor Sam-S, and the amount of global H3K4me3 (20). In that work, we also found that Sin3 and Sam-S function in opposition to control global H3K4me3 levels. The current metabolomics study showed that the superpathways of amino acid, carbohydrate, and energy metabolism as well as the KEGG subpathways of cysteine and methionine metabolism, glycolysis or gluconeogenesis, and citrate cycle (TCA cycle) were enriched under all three conditions (Fig. 6 and Table S4). Therefore, we focused on methionine catabolism (Fig. 7A), glycolysis/gluconeogenesis (Fig. 8A), as well as the TCA cycle (Fig. 9A) for an integrative analysis of the transcriptome and metabolome data.

**Sam-S and SAM were the only significantly changed gene and metabolite involved in methionine catabolism when Sam-S was knocked down (Fig. 7, B and C).** Our previous work revealed that reduced Sam-S leads to decreased global H3K4me3 levels (9, 20). These data indicate that Sam-S affects Sam-S levels, which then likely influences global H3K4me3 levels. Our previous study also found that Sam-S is a direct target of Sin3 (20). Comparing Sin3A knockdown and Sin3A + Sam-S knockdown cells, the expression of methionine metabolic genes, including Adenosylhomocysteinase (Acy13), CG10623, and Cystathionine B-synthase (Cbs), showed similar trends of gene expression changes relative to control cells (Fig. 7B). These data suggest that these genes are largely regulated by Sin3. As for metabolites, Sam-S was low in the dual knockdown sample, as the cells were missing the key synthesis enzyme Sam-S (Fig. 7C). Compared with levels in control cells, homocysteine, cystathionine, and cysteine were also lower when both Sin3A and Sam-S were reduced, whereas methionine and S-adenosylhomocysteine were not altered in any significant way (Fig. 7C). Consideration of the transcriptome and metabolic data indicates that, although both Sin3A and Sam-S play important roles in control of methionine catabolism, the mechanisms of control are quite distinct. Sin3 controls gene expression to likely influence protein levels of multiple enzymes and then the concentration of metabolites in the pathway, whereas Sam-S activity appears to be limited to controlling the level of Sam-S.

The expression of Phosphofructokinase (Pfk), which encodes the key rate-limiting enzyme of glycolysis (35), was significantly changed in Sin3A knockdown cells (Fig. 8B), consistent with our previous reports (16, 34). Analysis of the ChIP-seq dataset (28) indicates that Sin3 binds to the promoter of Pfk (Fig. 8C). Reduction of Sin3 led to altered levels of multiple metabolites involved in glycolysis/gluconeogenesis (Fig. 8D). Metabolites from glycolysis I, including glucose, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-biphosphosphate, were significantly lower in Sin3A knockdown cells compared with control cells (Fig. 8D). Metabolites from glycolysis II, including 3-phosphoglycerate and pyruvate, were significantly higher in Sin3A knockdown cells (Fig. 8D). Taken together, the gene expression, binding profile and metabolomic data suggest that Sin3 may directly regulate Pfk expression to affect glycolysis/gluco- neogenesis. Although pyruvate was the only significantly changed metabolite in both Sam-S and Sin3A + Sam-S knockdown cells relative to control cells, we noted that the cellular concentration of most observed intermediates involved in glycolysis/gluconeogenesis in the dual knockdown sample was intermediate between the two single-knockdown samples (Fig. 8D). These data strongly suggest that Sin3 and Sam-S regulate glycolysis/gluconeogenesis in opposite ways.

Compared with the control sample, reduction of Sin3 led to increased expression of Isocitrate dehydrogenase (Idh) and Isocitrate dehydrogenase 3b (Idh3b) (Fig. 9B), which control the key rate-limiting step of the TCA cycle (36). Our previously published ChIP-seq data (28) indicate that Sin3 binds to the promoters of Idh and Idh3b (Fig. 9C). Consistent with the observation that reduction of Sin3 results in aberrant mito-
Cross-talk of SAM-S and SIN3 on metabolism and transcription

In this study, we identified genes and metabolites regulated by SIN3 and SAM-S through transcriptomics and metabolomics analyses. We found that reduction of SAM-S did not affect the expression of many genes but did impact the cellular concentration of numerous metabolites. Furthermore, for some genes and metabolites, SIN3 and SAM-S acted independently; for others, redundantly; and for a third set, in opposition. Methionine catabolism and glycolysis/gluconeogenesis were controlled by both SIN3 and SAM-S, whereas the TCA cycle was largely affected by SIN3 only. Together, these data indicate a complex relationship between SIN3 and SAM-S in the regulation of transcription and metabolism.

Consistent with the role of a global transcriptional regulator, many genes were independently regulated by SIN3. Given that many SIN3 gene targets encode multiple metabolic enzymes, it is likely that SIN3 impacts gene expression, which, in turn, influences the protein levels of metabolic enzymes and then the corresponding cellular metabolite concentration. Data obtained in our previous ChIP-seq study indicate that many SIN3 gene targets are bound by SIN3. Our previous work...
also reported that SIN3 alters H3K4me3 and H3K9ac levels at the promoters of SIN3-bound genes (20). Therefore, it is likely that SIN3 affects histone modifications to directly control the expression of SIN3-bound gene targets.

SAM-S activity directly influences the concentration of SAM, which, in turn, affects global H3K4me3 levels (37). We found here that reduction of SAM-S affects not only the concentration of SAM but also metabolites in pathways other than one-carbon metabolism. Although the levels of multiple metabolic intermediates were affected in Sam-S knockdown cells relative to control cells, the expression of only a few genes responded to a decrease in SAM-S. There are several possible reasons to account for the minimal gene expression impact following SAM-S reduction. First, it may be due to incomplete depletion of SAM-S. Given that Sam-S is a highly expressed gene (17), it is possible that the remaining SAM-S following RNAi knockdown is enough to maintain function to a certain degree. Second, although the global levels of H3K4me3 are decreased in Sam-S knockdown cells relative to the control (9, 20), this change may not affect the H3K4me3 peak width at many genes to affect their expression. In this respect, it has been reported that H3K4me3 peak width, not the height and area of H3K4me3 peaks, is associated with gene transcription (38).

There was also a set of genes and metabolites (category II) regulated by SIN3 and also regulated by SAM-S, but only when SIN3 was reduced. SAM-S has been demonstrated to localize on chromatin and recruit regulatory proteins to affect gene expression (5, 23, 24). The mechanism of how SAM-S is
recruited to chromatin, however, is not fully understood. It is possible that reduction of SIN3 alters chromatin structure by changing histone modifications, which, in turn, affects the binding of SAM-S on chromatin and results in a change of gene expression. When these gene targets encode metabolic enzymes, the metabolite concentration in the specific pathway may be impacted.

Another group of genes and metabolites (category III) was redundantly influenced by SIN3 and SAM-S so that the gene expression level or metabolite concentration was only affected when both factors were reduced. Interestingly, in the Sin3A/Sam-S knockdown sample, the global H3K4me3 levels were similar to that found in the control, which indicates that the effects of SIN3 and SAM-S for these genes and metabolites are likely not due to a change in global histone methylation. Whether the genome-wide pattern of H3K4me3 is affected under these conditions, however, is unknown. It is possible that SAM-S reduction leads to a change in histone methylation peak width at some genes. Additionally, Sam-S knockdown could affect methylation of certain transcription factors. Nonhistone protein lysine methylation has been demonstrated to impact protein activity and protein–protein interactions (39). Effects on genes and metabolites may be negligible when SAM-S alone is reduced. In combination with a decrease in SIN3, however, this influence is likely amplified, leading to the observed changes. It has been reported that, in C. elegans, distinct methyltransferases and stress genes have differential responses to low SAM levels under a variety of stress conditions (40). For methyltransferases or/and demethylases required for the expression of category III genes, their response to decreased SAM-S may be dependent on the presence of SIN3, which has been found to influence the stress response (26).

There is a discrepancy between the cellular SAM concentration and global histone H3K4me3 levels under the different conditions. Our previous work suggested that SIN3 directly regulates the expression of methionine metabolic genes to...
increase SAM, which, in turn, leads to increased global H3K4me3 (20). Although the concentration of SAM was low in both Sam-S and Sin3A+Sam-S knockdown cells (Fig. 7C), global H3K4me3 levels were only impacted in the Sam-S knockdown sample relative to the control (20). This could be because the sensitivity of histone methylation to the concentration of SAM may be influenced by other SAM-consuming pathways, such as methylation at nonhistone proteins, lipids, and other metabolites (41). For example, Tu and co-workers (42) recently determined that, in yeast, SAM depletion leads to demethylation of protein phosphatase 2A (PP2A), which, in turn, increases phosphorylation and chromatin binding activity of the histone demethylase Rph1, resulting in altered global histone methylation levels. In Sam-S single-knockdown and dual-knockdown cells, reduction of SAM-S leads to a low concentration of SAM. The global gene expression pattern resulting from a decrease in SIN3 and SAM-S in the dual knockdown sample, however, is widely different from the control or Sam-S single-knockdown cells. Expression of genes encoding proteins in multiple metabolic pathways is altered in the dual-knockdown sample. The altered gene expression and metabolic status of these cells could influence other SAM-consuming pathways, which, in turn, impacts the cellular pool of SAM and then histone methylation.

Methionine catabolism generates the major methyl donor SAM. Glycolysis and the TCA cycle produce the key acetyl source acetyl-CoA and the critical energy fuel ATP, respectively. Given the importance of these pathways, we investigated whether and how SIN3 and SAM-S regulate one-carbon and central carbon metabolism. Both factors have an effect on methionine catabolism and glycolysis, but the TCA cycle is impacted by SIN3 only. SIN3 regulates the expression of genes encoding rate-limiting enzymes such as Sam-S, Pfk, Idh, and Idh3b to affect these pathways. In contrast, SAM-S controls the concentration of SAM to influence methionine catabolism. Interestingly, we observed an impact of SAM-S on glycolysis. The mechanism by which the enzyme that catalyzes the synthesis of SAM influences the glycolytic pathway, however, is not understood. It has been reported that, in yeast, Sam-S, as part of the SESAME complex, can be recruited to the promoter region of Pyk1 to affect its expression when glucose is present (5). In our experiments with Drosophila cells, expression of Pyk was not affected upon Sam-S knockdown relative to the control (Table S1), which suggests that SAM-S can affect glycolysis through additional unknown mechanisms.

It is well established that cancer cells have altered metabolism (43). There are overlapping metabolic pathways, comparing those disrupted in human cancer cells and the SIN3 and SAM-S regulated pathways we discovered in Drosophila cells. The phenomenon known as the Warburg effect, in which highly proliferative cells generate biomass through an increase in glycolytic flux (44), occurs in cancer cells (45). Genes that encode glycolytic enzymes and TCA cycle enzymes are overexpressed in a range of cancer cell types (46). In addition to reprogramming of glycolysis and the TCA cycle, enzymes and metabolites involved in one-carbon metabolism are also often misregulated in cancer cells (47). A recent study demonstrated that control of dietary methionine affects cancer progression in a mouse soft tissue sarcoma model as well as in a patient-derived xenograft model for colorectal cancer (48). Therefore, these metabolic pathways are promising targets for anti-cancer therapeutic agents (49).

The expression and/or function of SIN3 and SAM-S are also under consideration as new therapeutic targets. Work from multiple independent groups has indicated that disruption of a SIN3 complex is one possible cancer treatment (50). Dependent on cell type, SIN3 has been found to have either oncogenic or tumor-suppressive activity (50). The expression level of SIN3 isoforms has been correlated with survival outcomes in triple-negative breast cancer samples (51). Waxman and co-workers (52–54) used small-molecule inhibitors and peptide decoys to dissociate SIN3 from its interacting partners, which then lessens the oncogenic potential of cancer cell lines to form tumors when injected into mice. It is known that SAM-S misregulation is linked to liver disease, including liver cancer (55). A number of independent studies have reported that MAT1A, one of three mammalian Sam-S genes, is decreased, whereas MAT2A and MAT2B, the other two mammalian Sam-S genes, are increased in liver cancer (55). Additionally, it was recently reported that SAM-S inhibition suppresses primary tumor and lung metastasis in a triple-negative breast cancer mouse model (56).

In this study, we observed a complex relationship between the histone-modifying complex SIN3 and the metabolic enzyme SAM-S in the regulation of transcription and metabolism. The data generated here suggest that the function of SIN3 and SAM-S in the regulation of one-carbon and central carbon metabolisms may contribute to their roles in cancer progression. Future investigations will be directed toward the study of the intersection between epigenetic gene regulation and metabolic flux by SIN3 and SAM-S and the impact on the specific link between control of metabolism and cancer cell proliferation.

**Experimental procedures**

**Cell culture**

*Drosophila* Schneider cell line 2 (S2) cells were cultured at 27 °C in Schneider’s *Drosophila* medium (1×) with l-glutamine (Life Technologies), 10% heat-inactivated FBS (Invitrogen), and 50 mg/ml gentamycin (Thermo Fisher Scientific, Waltham, MA).

**dsRNA production**

The constructs containing targeting sequences in the pCRII-Topo vector were generated previously (9, 57). dsRNA against GFP was used as a control. dsRNA was produced based on modification of a protocol described previously (57). In brief, sense- and antisense-containing DNA constructs were digested to produce linearized DNA templates that were purified further using phenol–chloroform. Single-stranded RNA (ssRNA) was generated from the DNA templates with a RiboMAX kit in accordance with the manufacturer’s protocol (Promega, Madison, WI) and cleaned up by phenol–chloroform. Purified ssRNA was resuspended in 100 mM NaCl. Equal amounts of sense and antisense ssRNA were combined, heated at 95 °C for 5 min, and then slowly cooled for 12–18 h to generate dsRNA.
RNAi

RNAi was produced based on modification of a protocol described previously (57). Briefly, 4 × 10^6 Drosophila S2 cells were seeded in a 60-mm Petri dish with 4 ml of Schneider’s medium containing FBS. After 3 h, FBS-containing medium was removed and replaced with 2 ml of serum-free medium. 50 μg of dsRNA per gene was added, and the sample was mixed well by swirling. 4 ml of Schneider's medium containing FBS was added after 30 min. Cells were assayed 4 days following addition of dsRNA. Western blot and RT-PCR analyses were routinely carried out for both single- and double-RNAi–treated cells to verify efficient knockdown of Sin3A and Sam-S, respectively.

Western blot analysis

The Western blot analysis was performed based on modification of a protocol described previously (9, 57). For whole-cell protein extraction, 1.5 × 10^6 RNAi-treated Drosophila S2 cells were subjected to centrifugation at 845 relative centrifugal force (rcf) for 5 min. The cell pellet was resuspended in 100 μl of Laemml buffer (Bio-Rad), and the sample was heated at 95 °C for 5 min. The total protein concentration of the samples was determined by conducting a Protein DC assay in accordance with the manufacturer’s protocol (Bio-Rad). 20 μg of whole-cell protein extracts was separated on an 8% SDS-PAGE gel and transferred to a PVDF membrane (Thermo Fisher Scientific). The membranes were blocked with 5% milk for 1 h, washed with PBS buffer containing Tween 20 (PBST), incubated with the primary antibody for 2 h, washed, and incubated with the secondary antibody for 1 h. The antibody signals were then detected with the ECL Prime Western blot detection system (GE Healthcare). The membranes were reblocked overnight, washed, reprobed with the primary antibody targeting the loading control protein for 2 h, washed, and incubated with the secondary antibody for 1 h, and then the signal was detected. Primary antibodies included SIN3 (1:2000 (58)) and α-tubulin (1:1000, Cell Signaling, Danvers, MA). Donkey anti-rabbit HRP-conjugated IgG (1:3000, GE Healthcare) was used as the secondary antibody.

RT-PCR and real-time qRT-PCR

Total RNA was extracted from RNAi-treated Drosophila S2 cells using the RNaseasy Mini Kit in accordance with the manufacturer’s protocol (Qiagen, Hilden, Germany). Complementary DNA was generated from total RNA using the ImProm-II reverse transcription system with random hexamers following the manufacturer’s protocol (Promega). DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) was used for RT-PCR. For RT-PCR, primer sets (5’ to 3’) TTC CAA AAC ACA AGT AAC CTG C (forward) and TTG TGA CTT GTG AGA AGT TCC G (reverse) were used to verify the knockdown of Sam-S. TBP-associated factor 1 (Taf1), using primer set (5’ to 3’) GTG GAG GCA ACA AGG GAG CC (forward) and TTC CGG TCC TTG TGC GAA TG (reverse), was used as the loading control for RT-PCR. The primers used in real-time qRT-PCR have been described previously (9). Real-time qRT-PCR was performed using ABSolute Blue SYBR Green ROX Master Mix (Fisher Scientific, Hampton, NH) and carried out in a Stratagene Mx3005P real-time thermocycler. Taf1 was used to normalize RNA levels. The gene expression changes are represented as the mean (± S.E.) of the -fold changes observed in knockdown samples compared with GFP RNAi control cells. Real-time qRT-PCR results are the average of three biological replicates.

Gene expression analysis by RNA-Seq

Three biological replicates of RNAi-treated Drosophila S2 cells were harvested, flash-frozen, and sent to the Applied Genomics Technology Center at Wayne State University for RNA isolation and next-generation sequencing. The RNA-Seq experiment and bioinformatics analysis were performed as described previously (17). In brief, total RNA was extracted with an RNA Universal Tissue Kit in accordance with the manufacturer’s protocol (Qiagen). RNA was used to generate adapter-ligated PCR fragments by using a TruSeq RNA Sample Preparation Kit, following the manufacturer’s protocol (Illumina, San Diego, CA). The PCR fragments were sequenced on an Illumina HiSeq 2500 sequencing system. The significantly differentially expressed genes are listed in Table S1. GO and KEGG pathway analyses were performed using DAVID version 6.8 (31, 32). We pooled related GO categories with p < 0.05 into a single broader category, as described previously (28). Detailed information regarding GO and KEGG pathway analyses is shown in Table S2.

Metabolomics

Five biological replicates of RNAi-treated Drosophila S2 cells were harvested, flash-frozen, and sent to Metabolon Inc. for the metabolomics study. Sample preparation and metabolomic analysis were conducted at Metabolon Inc. as described previously (59). In brief, samples were prepared using the automated MicroLab STAR system (Hamilton Co., Reno, NV). Proteins were precipitated with methanol under vigorous shaking for 2 min, followed by centrifugation. The resulting extracts were analyzed with ultra-performance LC-MS/MS and GC-MS. The raw data were normalized to total protein concentration based on a Bradford assay. The normalized data of each compound was then rescaled to obtain the relative intensity by setting the median level equal to 1. The PLS-DA was performed using SIMCA software version 15.0. The metabolomics profile is provided in Table S3. The pathway analysis was performed from MetaboAnalyst 4.0 (33) by uploading the data with KEGG ID, and the detailed results are shown in Table S4. The pathway analysis was designed for two groups. The relative intensity of each compound was used for all three conditions and category II and III pathway analysis. The -fold change of each compound between Sin3A KD/GFP RNAi and Sin3A + Sam-S KD/GFP RNAi was used for category I pathway analysis.

Statistical analyses

Cuffdiff was used to identify statistically significant genes in RNA-Seq. One-way ANOVA post hoc analysis was used to compare gene expression of category I genes between Sin3A knockdown and Sin3A + Sam-S knockdown cells. All significance values in real-time qRT-PCR were calculated by unpaired two-sample Student’s t test from GraphPad. The p values for the intersection of Venn diagrams were calculated by hypergeo-
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metric test in R. The p values for the GO and KEGG pathway analyses were calculated by modified Fisher’s exact test from DAVID. One-way ANOVA with contrasts was used to determine statistically significant metabolites. The pathway analysis in the metabolomics study was performed from MetaboAnalyst using a hypergeometric test for enrichment analysis and relative betweenness centrality for the pathway topology analysis.

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