Drosophila SIN3 Is Required at Multiple Stages of Development

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SIN3 is a component of a histone deacetylase complex known to be important for transcription repression. While multiple isoforms of SIN3 have been reported, little is known about their relative expression or role in development. Using a combination of techniques, we have determined that SIN3 is expressed throughout the Drosophila life cycle. The pattern of expression for each individual isoform, however, is distinct. Knockdown of all SIN3 expression reveals a requirement for this protein in embryonic and larval periods. Taken together, the data suggest that SIN3 is required for multiple developmental events during the Drosophila life cycle. Developmental Dynamics 237:3040–3050, 2008. © 2008 Wiley-Liss, Inc.

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INTRODUCTION

Development of multicellular organisms requires coordinate spatial and temporal regulation of gene expression. Levels of gene expression have long been correlated with the amount of histone acetylation (Allfrey et al., 1964). Overall acetylation levels are regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kuo and Allis, 1998). Mutations in HATs and HDACs lead to developmental defects and/or lethality in a variety of metazoans, likely due to altered expression of genes encoding key developmental regulators (Lin and Dent, 2006). For example, Drosophila melanogaster reared on food containing the HDAC inhibitor trichostatin A (TSA) exhibited a developmental delay at low concentrations and lethality at the highest concentrations tested (Pile et al., 2001).

Multiple HDACs are present in Drosophila. SIN3 is a component of one multisubunit HDAC complex conserved from yeast to human (Silverstein and Ekwall, 2005). SIN3 is believed to serve as a scaffold protein for assembly of the complex and has been shown to be the major subunit that targets the complex to specific promoters (Silverstein and Ekwall, 2005). Null mutations in Drosophila Sin3A result in embryonic lethality with only a few animals surviving to the first larval instar stage (Neufeld et al., 1998; Pennetta and Pauli, 1998). For this reason, investigating the role of SIN3 in the regulation of specific developmental pathways has proven difficult. SIN3 is believed to be involved in various biological processes linked to development and cell cycle progression. For instance, SIN3 has been implicated in eye development as Drosophila Sin3A was isolated in a screen to identify factors involved in modulation of the rough eye phenotype caused by ectopic expression of seven in abstensia (Neufeld et al., 1998). SIN3 has also been linked to developmental regulation through hormone signaling. SIN3 has been shown to associate with SMRTER, a 20-hydroxy-ecdysone (ecdysone) steroid hormone corepressor (Tsai et al., 1999). SIN3 colocalizes with SMRTER on polytene chromosomes isolated from Drosophila third instar larvae salivary glands (Pile and Wassarman, 2000). SIN3 binding to ecdysone steroid hormone regulated genes was shown to be dynamic and coincident with a developmental expression pattern of these genes.
genes in response to hormone signal. SIN3 has also been shown to be important for cell proliferation. Knockdown of SIN3 in *Drosophila* tissue culture cells by RNA interference (RNAi) resulted in a G2 phase delay in cell cycle progression (Pile et al., 2002). Furthermore, comparison of gene expression profiles from wild-type and RNAi-induced SIN3-deficient cells revealed differences in expression of genes encoding proteins that control multiple cellular processes, including cell cycle progression, transcription, and signal transduction (Pile et al., 2003). Taken together, the phenotypes of the *Drosophila* mutants and the tissue culture knock down cells, along with the links to hormone signaling, suggest that SIN3 is a critical regulator of development and cell cycle progression.

*Drosophila* Sin3A is represented by a single gene. Multiple cDNAs that correspond to alternatively spliced transcripts, however, have been isolated (Neufeld et al., 1998; Pennetta and Pauli, 1998). These transcripts are predicted to produce distinct protein isoforms that differ in amino acid sequence only at the carboxy (C)-terminus of each protein (Fig. 1). An antibody raised against a region of the predicted proteins of splice sites, but also are similar at the protein level to SIN3 187 and SIN3 220 (Supplementary File 1, which can be viewed online). The Sin3A orthologs in more distantly related species, including beetle, honeybee and wasp, are all similar to SIN3 220. A search of expressed sequence tag (EST) databases in these species failed to recover other isoforms. The conservation of multiple Sin3 isoforms that differ at the C-terminus supports our hypothesis that these are functionally important distinct proteins.

### RESULTS AND DISCUSSION

#### Alternative Splicing of SIN3 Is Conserved

To investigate if production of multiple isoform transcripts by alternative splicing of the Sin3A gene is conserved, a sequence comparison of *Drosophila* Sin3A orthologs to other insect species was carried out. Similar to *Drosophila*, we find evidence of multiple Sin3 isoforms in three species of mosquito (Fig. 1B). These isoforms not only have conservation of splice sites, but also are similar at the protein level to SIN3 187 and SIN3 220 (Supplementary File 1, which can be viewed online). The Sin3A orthologs in more distantly related species, including beetle, honeybee and wasp, are all similar to SIN3 220. A search of expressed sequence tag (EST) databases in these species failed to recover other isoforms. The conservation of multiple Sin3 isoforms that differ at the C-terminus supports our hypothesis that these are functionally important distinct proteins.

#### SIN3 Isoforms Are Differentially Expressed During Fly Development

To investigate expression of the Sin3 isoforms throughout the *Drosophila* life cycle, we carried out a reverse transcription-polymerase chain reaction (RT-PCR) analysis using isoform-specific primer sets (Fig. 1). SIN3 187- and SIN3 220-specific mRNA was detected in embryos, larvae, and adults (Fig. 2). In contrast, SIN3 190-specific mRNA was detected only in embryos and adult females. It was not detected in either larvae or adult males. We hypothesize that the SIN3 190 isoform may serve an important gene regulatory function during early fly development or in the adult female germ line. It is possible that only adult females produce the SIN3 190 isoform. The 190 isoform may be maternally deposited during oogenesis, providing a possible explanation for its presence in embryos.

To further our analysis of SIN3 isoform expression, we analyzed protein expression in multiple tissues during distinct time points of fly development. To determine the relative levels of the different isoforms during embryogenesis, protein extracts prepared from embryos collected at different stages were analyzed by Western blot using the SIN3 pan antibody (Fig. 3A,B). This antibody was raised against recombinant protein containing sequence common to all reported isoforms (Fig. 1; Pile and Wasserman, 2000). Two protein bands were detected at all time points tested. One corresponded to SIN3 220, and the other corresponded to SIN3 187, SIN3 190, or a mixture of the two. The difference in molecular weight between SIN3 187 and SIN3 190 is too small to be resolved on an eight percent polyacrylamide gel.

SIN3 220 expression was highest in extracts prepared from embryos at stages 12–16. Germ band retraction, dorsal closure, and ventral nerve shortening occur during these stages (Campos-Ortega and Hartenstein, 1985). SIN3 220 expression dropped at stage 17, the final stage of embryogenesis when the ventral nerve cord continues to retract and the majority of morphogenesis is complete (Campos-Ortega and Hartenstein, 1985). Expression of the lower molecular weight isoforms steadily increased as embryogenesis progressed, and remained high at the final stage 17 (Fig. 3A,B). The relative levels of SIN3 220 and SIN3 187/190 thus change during embryogenesis. This finding that the SIN3 isoforms have a differential temporal expression pattern during embryonic development suggests that they may have different functions. It is possible that the different isoforms regulate distinct sets of genes that are required for specific developmental events.

To analyze expression in different tissues and developmental stages of the fly, protein extracts prepared from various larval and adult tissues were...
Fig. 1.
analyzed by Western blot using the SIN3 pan antibody (Fig. 3C,D). Larval imaginal discs are comprised of committed cells that undergo differentiation resulting in the adult body structures. SIN3 220 is expressed at a higher level relative to the lower molecular weight isoforms in the wing and leg imaginal discs of third instar larvae. The isoforms are expressed at similar levels to each other in eye and antennal discs and larval brain tissue. SIN3 220 expression is much lower compared with the lower molecular weight isoforms in adult brain and total adult tissue. Of interest, in embryogenesis, larogenesis, and adulthood, the ratio of SIN3 220 to the lower molecular weight isoforms is generally reflective of the ratio of undifferentiated to differentiated cells. SIN3 220 appears to be expressed equivalently to, or more than, the lower molecular weight isoforms in tissues that have numerous mitotic cells and that are less differentiated. In the most differentiated tissue, such as that of the final stage of embryogenesis, and in adults, SIN3 220 expression is very low. In support of this hypothesis, continuous Drosophila tissue culture cell lines primarily express SIN3 220 (Supplementary Figure S1).

Fig. 1. Genomic organization of the Drosophila Sin3A locus. A: The intron-exon map of the SIN3 isoforms is shown. Exons are indicated by boxes and introns by thin lines. Untranslated regions (UTRs) are indicated in grey and protein-coding regions in black. The transcript structures are based on cDNAs described by Neufeld et al. (1998) (SIN3 187, GenBank AF024604; SIN3 190, GenBank AF024603) and Penneta and Pauli (1998) (SIN3 220, GenBank AJ007518). PA, PB, PD represent the protein-coding regions of SIN3 190, SIN3 220, and SIN3 187, respectively. B: The transcript structures of Sin3A isoform orthologs are shown. Gene names are given by orthology relationship to Drosophila Sin3A isoforms. Two ancient introns are marked due to their conservation in all investigated insect species. Tree on the left drawn based on Flybase (http://flybase.bio.indiana.edu/blast/). Scale bar shows the divergence time between species. Species name abbreviations: Aaeg, Aedes aegypti; Agam, Anopheles gambiae; Amel, Aiptasia mellifera; Cpip, Culex pipiens; Dmel, Drosophila melanogaster; Nvit, Nasonia vitripennis; Tcas, Tribolium castaneum.

SIN3 Isoforms Are Expressed in a Similar Spatial Pattern During Fly Embryogenesis

Given that all isoforms were detected in the embryo, we wished to investigate whether the expression of individual isoforms was restricted to specific cell types or localized to particular regions of the embryo. To determine the spatial expression patterns of individual SIN3 isoforms, we used either in situ hybridization or immunohistochemistry, depending on available reagents. To determine RNA expression patterns, we performed in situ hybridization in fly embryos of different stages using the SIN3 220 antibody. Antibodies specific for the SIN3 220 isoform were generated using recombinant protein consisting of the unique C-terminal region of SIN3 220 (amino acids 1748-2062). We tested the specificity of the antibody by Western blot analysis. The antibody recognizes a protein of approximately 220 kDa in tissue culture and embryonic extracts (Supplementary Figure S1). Reduced signal was detected in extracts prepared from SIN3-deficient tissue culture cells (Supplementary Figure S1). In addition to the SIN3 220 antibody, we performed immunohistochemistry using the SIN3 pan antibody that recognizes SIN3 187, 190 and 220. A pool of 0–18 hr embryos was immunostained for SIN3 using either the SIN3 pan or the SIN3 220 antibody. The expression pattern observed using the SIN3 220 antibody is very similar to the pattern observed using the SIN3 pan antibody and to that observed for the SIN3 RNA transcripts (Fig. 5). The SIN3 220 protein is ubiquitously expressed, and exhibits its strong expression in the germ band, ventral nerve cord and brain during later development. Confocal microscopy images revealed that SIN3 is localized to the nucleus (Fig. 5C,D). The results generated from the in situ hybridization and immunohistochemistry experiments suggest that SIN3 187, SIN3 190, and SIN3 220 are expressed in similar spatial patterns during fly embryonic development. SIN3 is thus ubiquitously expressed during embryogenesis, consistent with a previous descriptive report of
SIN3 embryonic expression (Pennetta and Pauli, 1998).

**Generation of SIN3 Conditional Mutants**

The expression pattern analysis indicates that the SIN3 187 and 220 isoforms are expressed throughout *Drosophila* development. Because SIN3 is required for embryogenesis, characterization of the role of SIN3 in larval and pupal development has not been possible (Neufeld et al., 1998; Pennetta and Pauli, 1998). Therefore, to determine whether SIN3 is also required for postembryonic development, we designed a conditional knockdown transgenic fly. In *Drosophila*, conditional knockdown can be achieved by developmental stage-specific induction of RNA interference (RNAi) using the GAL4-UAS system (Duffy, 2002; Lee and Carthew, 2003). We constructed a transgene (UAS-SIN3RNAi) designed to target the degradation of all SIN3 isoforms. Tubulin, actin, and heat shock GAL4 driver lines were used to knock down SIN3 expression in all tissues. Progeny resulting from the cross of UAS-SIN3RNAi lines to GAL4 driver lines are referred to as SIN3-deficient flies.

**SIN3 Is Required for Postembryonic Development**

To induce ubiquitous loss of SIN3, we crossed heterozygous tub-GAL4 or Act-GAL4 driver males to homozygous UAS-SIN3RNAi females (test crosses; Fig. 6). Half of the progeny are expected to be SIN3-deficient. Nine independent UAS-SIN3RNAi lines were tested. As a control, both UAS-SIN3RNAi females and GAL4 driver males were crossed to *w*^1118^ males and females respectively (Fig. 6, and data not shown). Progeny of all crosses were allowed to develop to adulthood. Three independent test and control crosses were set up for each UAS-SIN3RNAi line. The minimum number of adults that were scored was 54. In eight of nine test crosses, no SIN3-deficient flies survived to adulthood. Flies from the single test cross that survived to adulthood showed no obvious phenotype. Western blot analysis revealed that these adult flies expressed SIN3 at levels comparable to control animals, suggesting that SIN3 is not being effectively knocked down.
in that single viable test cross (data not shown). The finding that ubiquitous loss of SIN3 resulting from RNAi leads to lethality is consistent with previous reports demonstrating that SIN3 is essential during early stages of development (Neufeld et al., 1998; Pennetta and Pauli, 1998).

To determine the stage of development during which the SIN3-deficient flies die, we followed the development of embryos from the control and test crosses (Fig. 6). In the control crosses, 100% of embryos survived to adulthood. In the test cross, 72% of embryos hatched into first instar larvae, but only 51% developed into wandering third instar larvae (consistent with the predicted ratio of progeny that will be SIN3-deficient). All surviving wandering third instar larvae developed into adults. All surviving adults in the test cross had stubble bristles, indicating that they were not SIN3-deficient flies. These results indicate that SIN3-deficient embryos die during embryonic and first, second, or early third instar larval development.

To verify the knock down of SIN3, and to analyze the SIN3-deficient embryos, embryos from the test cross were immunostained for SIN3 using the SIN3 pan antibody, and DNA using DAPI. Initially we analyzed a pool of 0–20 hr embryos. Loss of SIN3 upon induction of RNAi is inferred by the decrease in SIN3 staining intensity in 37% of these embryos (n = 94). This number is smaller than the predicted 50%. RNAi-induced loss of SIN3 expression is a consequence of degradation of transcribed RNA by the introduction of double stranded RNA (dsRNA) and of degradation of existing protein by normal cellular turnover. Depending on the stability of SIN3, the RNAi-induced effect may be delayed long after induction of the dsRNA from the UAS-SIN3RNAi transgene. To allow for protein turnover, we collected embryos for 2 hr, and allowed these embryos to age 18 hr. This pool of 18- to 20-hr embryos was immunostained with the SIN3 pan antibody. In this aged population, 48% of the embryos (n = 75) had little to no SIN3 staining (Supplementary Fig. 2). DNA staining of the 0- to 20-hr collection with DAPI revealed that the SIN3-deficient embryos fell into different phenotypic categories. Some of the SIN3-deficient embryos had wild-type morphology and the stage of development could thus be determined. 29% of the SIN3-deficient embryos were in stages 9–11, 26% in stages 12–14, and 11% in stage 15. The remaining 34% of the SIN3-deficient embryos had poor DNA staining by DAPI (4’,6-diamidine-2-phenylindole-dihydrochloride), and in some, the cells of the embryo appeared to pull away from the periphery, suggesting embryo degeneration. Due to loss of recognizable cellular structure, a stage of development for these embryos could not be assigned. As no SIN3-deficient embryos at stage 16 or later were identified, ubiquitous loss of SIN3 by RNAi appears to allow development for approximately 13 hr, to stage 15. The finding that some embryos develop to stage 15 is likely due to the presence of maternally deposited SIN3 that is not targeted by the RNAi pathway (Pennetta and Pauli, 1998). That some SIN3-deficient animals survive to larvae is possibly due to the presence of a low level of SIN3 that allows development to that stage. Eventually SIN3 is reduced to lethal levels in all animals having both the GAL4 driver and the UAS-SIN3RNAi transgenes. The SIN3-deficient larvae appeared phenotypically normal, but failed to continue to develop into wandering third instar larvae. Loss of SIN3 by RNAi in Drosophila tissue culture cells resulted in loss of cell proliferation, likely due to a G2 cell cycle block (Pile et al., 2002). Homozygous null SIN3 clones in the developing eye resulted in scars across the eye consistent with a role for SIN3 in cell survival or proliferation (Neufeld et al., 1998). Given these previous findings, we hypothesize that lethality following
RNAi induced loss of SIN3 in developing Drosophila results either from loss of cell proliferation or cell viability. To determine the effect of loss of SIN3 on postembryonic development, we induced SIN3 RNAi at different stages of larval development by crossing hsp70-GAL4 males to UAS-SIN3RNAi females (Fig. 7). Control crosses were set up as mentioned above. Embryos were collected and subjected to initial heat shock at different stages of development to induce SIN3 RNAi. The developing larvae were subjected to heat shock by incubating at 37°C for 1 hr. The larvae were subjected to heat shock each day, with a 24-hr recovery period at room temperature between heat shock treatments, until the larvae either died or developed into adults. The number of animals that survived to wandering third instar, pupae, and adulthood was determined. Induction of SIN3 RNAi in first or second instar larvae caused lethality before the wandering third instar, while inducing loss of SIN3 in wandering third instar larvae or pupae had no detectable effect on fly viability (Fig. 7, and Supplementary Figure S3). Inspection of the SIN3-deficient dead larvae revealed no gross phenotypic abnormalities, and the surviving adults appeared phenotypically normal (data not shown).

The transitions from larva to prepupa, and from prepupa to pupa, are each driven by pulses of the steroid hormone ecdysone (Riddiford, 1993). Induction of loss of SIN3 in first, second, or early third instar, before the ecdysone pulse at the end of the third instar larval stage, results in lethality, while induction during or after the time frame of this pulse has no effect on fly viability. SIN3 has been found to bind to ecdysone-regulated genes on polytene chromosomes isolated from third instar larval salivary glands (Pile and Wassarman, 2000). It is possible that the larval lethality is due to aberrant expression of ecdysone responsive genes. Loss of SIN3 might lead to premature activation or lack of down-regulation of ecdysone target genes, resulting in altered expression of genes required for morphogenesis.

Through the use of a conditional knock down transgenic fly system, we have established that SIN3 is required for both embryonic and early larval development. Results from our experiments have not detected a role for SIN3 in late larval, pupal, or adult development. We do not, however, rule out the possibility that SIN3

Fig. 5. SIN3 proteins are localized to the nucleus and show a similar spatial expression pattern. A–D: The expression pattern of SIN3 was determined by immunostaining with the SIN3 pan antibody (A, red; C, green) or SIN3 220 antibody (B, red; D, green). Embryos were counterstained with DAPI (4',6-diamidino-2-phenylidole-dihydrochloride; A,B, blue) or propidium iodide (PI; C,D). Numbers at the bottom left corner of each picture denote the approximate stage of the embryo. C,D: Confocal images of the surface of stage 5 embryos. The punctate staining signifies that the staining is in the nucleus. Embryos are oriented with anterior to the left.

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functions in these late stages of development, when, as in the early stages, expression is detected. Again, depending on the stability of SIN3, the RNAi-induced effect may occur after induction of the dsRNA from the UAS-SIN3 RNAi transgene. Thus, even though we induced RNAi during the late larval and pupal stages, SIN3 protein levels may have remained at levels sufficient to allow development to the adult stage. It is also possible that in the latter stages of Drosophila development, other proteins are able to compensate for SIN3-deficiency.

To confirm that the embryonic and larval lethality is a consequence of SIN3-deficiency and not due to an RNAi off target effect, we introduced a UAS-187 transgene (UAS driven expression of cDNA for the SIN3 187 isoform) into flies that are SIN3-deficient. Interestingly, while SIN3 220 is also able to rescue SIN3 RNAi-induced lethality, the amount of rescue is not the same as that of SIN3 187. Expression of SIN3 220 from UAS-220 increased survival of SIN3-deficient flies from 0 to 53% in males and 0 to 55% in females. The difference in the ability of the individual isoforms to suppress the lethal phenotype is unlikely due to differences in the amount of expression from the two transgenes as the protein level of SIN3 187 and SIN3 220 in extracts prepared from adult females were similar (Supplementary Figure S4). In contrast, the level of SIN3 220 in males compared with females was quite dissimilar. Like the SIN3 187 transgenic flies, surviving SIN3 220 transgenic adults appeared phenotypically normal. The finding that SIN3 187 or 220 can individually rescue the lethal phenotype due to knock down of expression of all SIN3 proteins suggests that SIN3 187 or 220 can partially substitute for the other isoforms. That the extent of rescue is different in males and females suggests the possibility that there may be sex-specific roles of the isoforms that cannot be compensated for by another isoform.

**SUMMARY**

It is well established that regulation and maintenance of histone acetylation levels are important for normal development (Lin and Dent, 2006). In this study, we have investigated the expression and postembryonic requirement of SIN3, one component of a multisubunit HDAC complex. We find that SIN3 isoforms are expressed throughout development.
**Experimental Procedures**

**Collection of Insect Sin3A Gene Orthologs**

The *Drosophila melanogaster* Sin3A sequence (Flybase FBgn0022764) was retrieved from Flybase and used as query in searches against the genome databases of yellow fever mosquito (*Aedes aegypti* genome database version 1.0), African malaria mosquito (*Anopheles gambiae* genome database version 2.2), Northern house mosquito (*Culex pipiens* genome database version 1.0), red flour beetle (*Tribolium castaneum* (Georgia GA2 genome database version 2.1)), honeybee (*Apis mellifera* DH4 genome database version 4.0), and parasitic wasp (*Nasonia vitripennis* genome assembly 1.1) with BLASTP or TBLASTX (Altschul et al., 1997). Multiple protein sequence alignments were generated with T-Coffee (Notredame et al., 2000) and inspected manually (Supplementary File S1). The predicted gene structure of Sin3A isoform orthologs was retrieved from the NCBI database.

The C-terminal regions were confirmed by cDNA clones present in the EST database of each species except for honeybee and wasp (Supplementary Table S1). The intron position in the C-terminal region of two uncharacterized Sin3A 187 (PD) isoform orthologs, Cpip_PD and Agam_PD, were manually located based on the conserved dipteran Sin3A PD splice acceptor site (GTGCCAG@GTCAAAC) within intron 7 of Cpip_PB, and *A. gambiae* EST clone (GenBank BM58707), respectively.

** Antibody Production**

Rabbit polyclonal antibodies were generated against recombinant protein containing the unique region of SIN3 220, amino acids 1748-2062. The IgG fraction was prepared using the Econo-Pac serum IgG purification kit (Bio-Rad).

**Western Blot Analysis**

Western blot analysis was performed in accordance with standard protocols (Sambrook and Russell, 2001). To prepare embryo extracts, approximately 25 μl of *Canton S* embryos from different embryonic developmental stages were homogenized in 100 μl of Lae-mml sample buffer (Bio-Rad). To prepare whole tissue extracts, dissected tissues were homogenized in Laemmli sample buffer. Protein concentration was determined using the DC protein assay reagent (Bio-Rad) according to the manufacturer’s protocol. Protein extract (15 to 20 μg) was fractionated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membrane, PVDF (Pall), and probed with IgG purified polyclonal rabbit antibodies against SIN3 pan (1:2,000) and RPBS (1:1,000; as a loading control), followed by donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G, IgG, (1:3,000; GE Healthcare) and detected with enhanced chemiluminescence reagents (GE Healthcare).

**Whole-Mount In Situ Hybridization**

Whole-mount in situ hybridization on fly embryos was performed using a
modified version of the protocol by Lehmann and Tautz (Goldstein and Fyrberg, 1994). To generate riboprobes for in situ hybridization, DNA fragments containing sequences to be targeted were amplified by PCR from Canton S genomic DNA and cloned in both orientations into the pCR-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen). The following primer sets (oriented 5′–3′) were used a standard PCR reaction: SIN3 3′ (TAAATCTAGAGTGCTCTCGATACAGGCTTG and ATTGTCTAGATGATGGCCAGATATGCCGGCA), SIN3 187 (TATTCTAGAGTGCTCATACCAAGGCTTG and GGGTCTAGATGATATCCTTCTTGGC), and SIN3 190 (ATGTCTAGACACAACATCCACA and ATGTCTAGACTTTCGTTAAG). Linearized plasmids were used as templates in an in vitro transcription reaction (RiboMax, Promega) with digoxigenin-labeled UTP (Roche) to synthesize labeled RNA probes. Alkaline phosphatase substrate, NBT-BCIP (Roche), was used for color development. The time allowed for color development (15 min to 3 hr) was comparable for the pan and SIN3 187 riboprobes. The time for color development (24 hr) for the SIN3 190 probe was considerably longer. Individual experiments were repeated a minimum of three times. Visualization and photography was done with a Zeiss Axioscope 2 fitted with an Axio-phot photography system.

**Immunostaining**

Immunostaining of Canton S, w^{1118}, and SIN3-deficient embryos was performed according to the protocol of Patel (Goldstein and Fyrberg, 1994). Embryos were fixed in 4% formaldehyde solution then blocked with 0.1% bovine serum album, 0.1% Triton X-100, 5% normal goat serum in 1× phosphate buffered saline. The embryos were incubated with IgG purified polyclonal rabbit antibodies against SIN3 pan (1:50) or SIN3 220 (1:50), followed by Alexa 594 or Alexa 488 goat anti-rabbit IgG (1:100) secondary (Invitrogen). DAPI (100 ng/µl) or propidium iodide was used to stain the DNA. Embryos were mounted in 70% glycerol. Images were captured on a Zeiss Axioscope 2 fitted with an Axio-photography system. Confocal microscopy was performed using Leica Confocal Software on a Leica TCS SP2 microscope.

**Reverse Transcription PCR Assay**

Total RNA was extracted from different morphogenetic forms of the fly using Trizol reagent (Invitrogen). cDNA was generated from total RNA extracted from different morphogenetic forms (0- to 18-hr embryo, wandering third instar larva, adult male and adult female) using the ImProm-II Reverse Transcription System (Promega) with random hexamers. The cDNA was used as template in a standard PCR assay. The forward primer was common in all SIN3 reactions. The following primers (oriented 5′–3′) were used: SIN3 F (AAACT-GATTGCGGTGTAAAC), SIN3 187 R (TATGTATTTTCAAGCTTG), SIN3 190 R (TCTGTTGIGCAATTCTG), SIN3 220 R (CTCTTGGCCAGCAGCAGCTG); TAF1 F (GTGGAG-CAGGCAAGGGACC) and TAF1 R (TCCCGCTCCTTGTGCGAATG). A 730-bp region of the third exon of Sin3 was generated by PCR using genomic DNA isolated from w^{1118}; Act-GAL4 (Bloomington #4414), Hsp70-GAL4 (Bloomington #1799) and tub-GAL4 (Bloomington #5138).

**Drosophila Stocks**

Drosophila melanogaster stocks were maintained and crossed were performed according to standard laboratory procedures. For embryo collection, embryos were collected on apple juice/agar plates supplemented with yeast paste and then allowed to age according to the procedure. The following stocks were used: Canton S, w^{1118}, Act-GAL4 (Bloomington #4414), Hsp70-GAL4 (Bloomington #1799) and tub-GAL4 (Bloomington #5138).

**Cloning of the UAS-SIN3RNAi Construct**

A 730-bp region of the third exon of Sin3 was generated by PCR using genomic DNA isolated from Drosophila Kc167 tissue culture cells as template with the following primers (oriented 5′–3′) TA- AATCTAGAGTGCTCTCGATACAGGCTTG and ATTGTCTAGATGATGGCCAGATATGCCGGCA. The SIN3 UAS-SIN3RNAi P-element was constructed by inserting this 730-bp PCR product into the pWiz vector (obtained from the Drosophila Genomics Resource Center) at each of the AaeII and NheI restriction sites, in opposite orientations (Lee and Carthew, 2003). The clone was confirmed by sequencing.

**Generation of Transgenic Flies Carrying the UAS-SIN3RNAi Construct**

w^{1118} embryos were injected with the UAS-SIN3RNAi construct at Model System Genomics, Duke University, according to standard protocol (Rubin and Spradling, 1982). Homozygous lines for each single site transgene insertion were generated.

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