Molecular and Genetic Characterization of the Sir2 Gene in Drosophila Melanogaster

by

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Prior to starting graduate school, I never would have guessed how challenging it would be nor how much sacrifice I would ask of my friends and family. I think back to all the phone calls I didn't return, all the letters I let sit idle, all the times I wasn't there for the loved ones in my life and how, in every instance, they were there for me.

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Abstract

The control of chromosome structure is important in the regulation of gene expression, recombination, DNA repair, and chromosome stability. Much work has recently been focused on the enzymes that acetylate and deacetylate histones within chromatin. Precisely how histone modification alters chromatin to promote changes in gene expression is still unclear. To better understand the role of histone modification in the regulation chromatin structure/function, I characterized the Drosophila homologue of Sir2, a yeast gene that represses transcription presumably via histone deacetylation. Sir2 is a member of a well-conserved family of histone deacetylases and has been implicated in transcriptional silencing across phylogenetic lines. In yeast, Sir2 also promotes longevity via regulation of chromatin structure. In this thesis, I tested the hypothesis that Sir2 functions as a silencing factor that regulates aging across phylogenetic lines. To accomplish this, I cloned and characterized the Drosophila *Sir2* homologue. I showed that the *dSir2* transcript and protein are dynamically regulated during development, and that dSir2 is found both at heterochromatin and euchromatic sites. I generated, and then studied the phenotype of *dSir2* mutant animals. Surprisingly, I found that *dSir2* is a non-essential gene. In genetic assays, I show that *dSir2* mutations modify chromatin structure but do not shorten lifespan. I also demonstrate an interaction between dSir2 and dCBP, a histone acetyltransferase and coactivator. In summary, my data support the hypothesis that Sir2 regulates heterochromatin across phylogenetic lines but that its role in lifespan is not conserved.

Chapter I

INTRODUCTION

Overview

Chromatin, the structural matrix of chromosomes, is no longer considered a static medium that simply serves as a scaffold for the packaging of DNA. A large body of evidence shows that chromatin regulatory complexes are maintained through successive rounds of replication and thus, can regulate genetic programs in a heritable fashion. This form of control, called epigenetic regulation, allows cells to "remember" gene expression programs to achieve proper cellular differentiation during development [1]. Epigenetic mechanisms have been shown to regulate important processes such as gene transcription, cell cycle progression, DNA replication, repair, and recombination [2]. Likewise, misregulation of chromatin formation and/or function is associated with human disease. For example, oncogenic transcription factors such as MLL-CBP and MOZ-TIF2 are believed to promote leukemia through constitutive acetylation of chromatin surrounding genes that control hematopoiesis [3]. Similarly, mutations in a DNA methyltransferase gene targeted to heterochromatin cause chromosomal abnormalities such as multiradiate chromosomes and lead to ICF syndrome[1], a devastating immunodeficiency syndrome [4]. The modulation of chromatin has also been implicated in aging pathways. Immortal cell lines and some cancers are associated with perturbations in the function of

[1] ICF syndrome (for immunodeficiency, centromere instability and facial anomalies)

chromosomal proteins such as telomerase and the centromere protein, CENP-A [5] [6]. In addition, Sir2, a gene whose product is thought to directly regulate heterochromatin structure via histone deacetylation, has also been implicated in aging across phylogenetic lines [7].

The Sir2 gene encodes one of the most well conserved chromatin remodeling proteins. A better understanding of its role in heterochromatin formation, development, and aging may help unveil some of the mystery of epigenetic regulation. This thesis describes the cloning and characterization of the Drosophila Sir2 (*dSir2*) homologue. I generated and used *dSir2* mutants to test the hypothesis that its role in regulating heterochromatin and lifespan is conserved. Specifically, I have addressed the following questions:

1) What is the role of the Drosophila homologue of Sir2 (*dSir2*), in the regulation of chromatin structure?

2) Is *dSir2* required for normal development, lifespan and/or maintenance of chromosome stability in the germ line?

3) What is the relationship between *dSir2* and *dCBP*, a well-characterized transcriptional coactivator that regulates gene expression via acetylation?

Background and Significance

Chromosomes are sub-nuclear organelles that carry genetic information. During interphase, which is the most relaxed state, the chromosomes are compacted nearly 10,000 fold (1A). Precisely how the cell manages to achieve this degree of compaction is still unclear. What is known is that DNA wraps around a histone core (5-10 fold compaction) accounting for classic "beads on a string" conformation. Further folding leads to the "30 nm fiber" (net 50 fold compaction) stabilized by H1 linker histone [8]. There is still debate about the next level of compaction although there is some evidence that the 30 nm fiber condenses along an organizing center (Figure 1B). Beyond this, however, structures are mostly speculative [9].

As the cell progresses through the cell cycle, the chromosomes continue to condense into highly compact, microscopically visible rodlets that align at

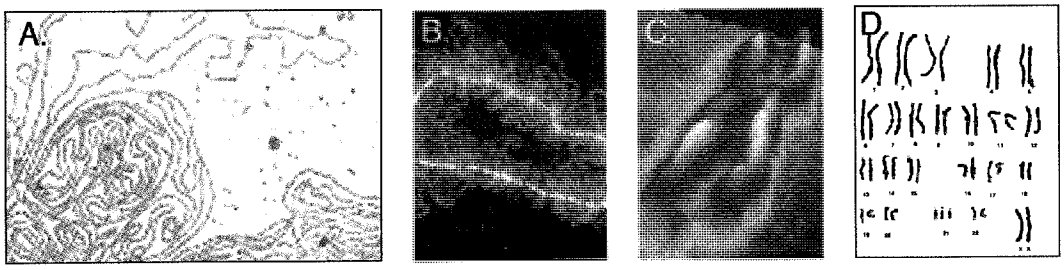


Figure 1. Chromosomes **(A)** Scanning electron micrograph showing interphase chromomsomes. **(B)** A lampbrush chromosome folded along an organizing center. **(C)** A human X chromosome imaged with scanning force microscopy. **(D)** Human karyotype [10].

the center of the nucleus (Figure 1C). The rodlets are maximally condensed at the end of metaphase and have visible substructures (Figure 1D). A constricted region, or centromere, is present at a certain point along each chromatid. It is narrower than the rest of the chromosome and contains the kinetichores, the sites of attachment by

microtubules. Flanking the centromere is a specialized domain called pericentric chromatin. Maintenance of proper centromere and pericentric structures is required for successful chromosome segregation (reviewed in [11]). A secondary constriction found on each chromosome is called the nucleolar organizer region (NOR). The NOR contains rRNA gene repeats and marks the site where the nucleolus forms. The tips of chromosomes, called telomeres, form knobby projections. Telomeres contain GT-rich sequences and are believed to be essential to prevent chromosome degradation and fusion (reviewed in [12]).

Telomeres, centromeres, NORs, and peri-centric DNA are specialized domains within chromatin. Chromatin is composed of DNA, histone proteins and non-histone proteins. Much has been learned about chromatin through the study of stained metaphase chromosomes (Figure 1D). Spreads of metaphase chromosomes, stained with quinicrine or Giemsa, show unique banding patterns along the chromosome arms [13]. These different patterns are believed to reflect differences in chromatin and/or DNA structure. When chromosomes are stained with Feulgan, a standard reagent that reacts with DNA, two classes of chromatin are identified based on the intensity of staining. Darkly stained bands, believed to be highly compacted, are called heterochromatin and lightly stained bands, euchromatin [14]. The silent human X chromosome, centromeres, pericentric chromatin, telomeres and NOR all stain darkly.

Heterochromatin is found across phylogenetic lines and is classically defined as chromatin that is visibly condensed during interphase and is resistant to DNAses [15]. Replication of heterochromatin occurs late in cell cycle and meiotic

recombination within centric heterochromatin is either low or absent [15]. Heterochromatin can be further subdivided into two classes, α and β heterochromatin. α heterochromatin, also known as satellite DNA, usually occurs as large blocks of simple repetitive sequences that are unreplicated, or underreplicated DNA. β heterochromatin consists of middle repetitive sequences, such as that found in ribosomal genes, the heterochromatin of the X and Y chromosomes, and some transposable elements [16].

Heterochromatin and euchromatin are also distinguished by differences in covalent modification of DNA. Early chromatin fractionation experiments suggested that histone proteins within heterochromatin are relatively hypoacetylated compared to histones within euchromatin [17]. Cytosine hypermethylation has also been shown to be a key feature of heterochromatin in many eukaryotes, especially in plants and mammals. In these organisms, heterochromatin is hypermethylated in both heterochromatin and silenced euchromatic domains [18]. However, in coccid insects, paternal heterochromatin is hypomethylated and Drosophila lack cytosine methylation entirely [15].

Genes that are silenced when packaged in heterochromatin are believed to be unavailable to the transcription machinery [19]. Of course, there are exceptions to this, such as the Drosophila *light* (*lt*) gene that is expressed normally in spite of its position within heterochromatin [20]. There are in fact, an estimated 40-50 genes within the pericentric heterochromatin of Drosophila [21]. The significance of their unique location remains to be determined. Regardless, it is evident that most DNA within heterochromatin is packaged in an inactive form. Many believe that

heterochromatic repression allows the cell to control harmful (repetitive DNA), foreign sequences (i.e. invading organisms) or excess genes (e.g. X chromosome in females and duplicated genes) [15], [18], [22].

The constitutive repression of genes within heterochromatin is a fascinating phenomenon that has been used as a model to understand silencing that occurs during development. Formation of silenced domains during development allow cells to define their identity by establishing stable patterns of gene expression [1]. Silencing has unique properties that are common across phylogenetic lines. First, it is determined by position (on the chromosome), not by a specific DNA sequence (e.g. promoter sequence). For example, while heterochromatin is most often composed of repetitive, gene poor sequences, the inactive X chromosome of female mammals—which assumes a heterochromatic structure and silenced state—is rich in genes and non-repetitive DNA. Furthermore, a normal gene placed near heterochromatin can also be silenced, as seen in Drosophila, when radiation-induced chromosomal translocations place euchromatic genes in or near a heterochromatic environment. In these mutants, an ordinary gene translocated near heterochromatin is expressed normally in some of the cells but is silenced in neighboring cells, resulting in a phenotype that has a mosaic or variegated appearance [16]. This heterochromatin phenomenon is called position effect variegation (PEV). Both the stochastic nature—causing the variegated phenotype—and the mechanism of domain-specific regulation is still poorly understood.

Silencing is also distinctive because it is heritable [1]. Also known as cellular memory, heterochromatic structures and the silenced state persist throughout mitosis

and meiosis. The mechanism of cellular memory has long been sought. In most eukaryotes, it has been observed that cytosine methylation is inherited in functionally significant patterns [23]. A model to account for this phenomenon, termed the "maintenance methylation model" was first described in 1975 [24]. The model was based on the observation that cytosine methylation is more likely to occur at DNA that is already hemimethylated. Thus, the pattern of methylation would be reproduced with each new round of cell division; the original hemimethylated strand would serve as the template for methylation on the newly synthesized strand. This model has fallen out of favor as it fails to explain all of the data but did set the stage for subsequent models that require that some mark of heterochromatin persist through mitosis [23].

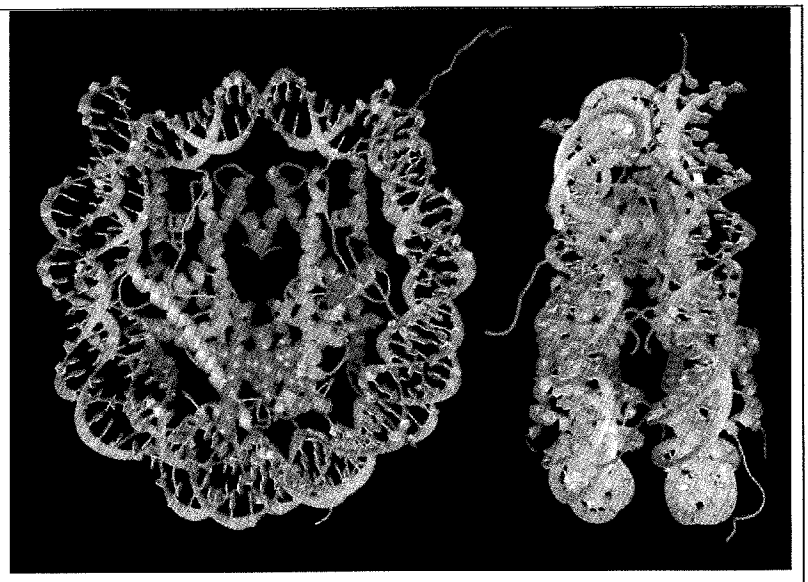
Although our understanding of heterochromatin structure and function is still primitive, an explosion of new data suggest that heterochromatin formation and function requires multiple events including nuclear sub-localization, modifications of histones, recruitment of protein complexes/enzymatic activities, alteration of DNA and sometimes, expression of certain RNAs [19], [23], and [25].

To discuss heterochromatin structure and function further, it is necessary to review the structure and function of the nucleosome, the fundamental repeating subunit of all chromatin. The core of the nucleosome is made up of well-conserved proteins known as histones. Once thought to be the heritable material in the nucleus, histones proteins form tetramers made up subunits known as H2A, H2B, H3 and H4. Long before the high-resolution crystal structure of the nucleosome particle was determined, Arthur Kornberg predicted the following properties of the nucleosome,

known as the "nucleosome hypothesis": 1) the nucleosome is composed of repeating units of an octamer, made up of two histone tetramers, 2) approximately 200 bps of DNA are associated with each octomer, 3) DNA is wrapped around the outside of the octamer, and 4) H1 is a linker molecule that binds to the outside of the nucleosome [8]. Data in support of Kornberg's model soon followed. Electron microscope images of chromatin extracted in low salt conditions assumed a "beads on a string" confirmation, consistent with the idea that DNA wrapped around regularly spaced, disk-shaped, histone octamers [26]. Biochemical studies using micrococcal nuclease digestion of chromatin showed that the nucleosome core protects approximately 146 bp of DNA [27] and the low-resolution crystal structure showed the discoid octamer wrapped in DNA [28].

A recent high-resolution structure of the core particle shows the details of 146 bp DNA wrapped around the octamer core (Figure 2). The DNA makes 1.65 turns of a flat, left-handed superhelix [29]. The inner surface of the DNA phosphodiester backbone binds to the histone octomer primarily through electrostatic interactions. There is a notable lack of contacts between the DNA bases and the histones, in keeping with the lack of sequence specific binding of histones to DNA. Each histone is made up of two domains: a well-organized central fold that lies within and constrains the DNA superhelix and an unstructured tail, protruding outside the compact core. The DNA superhelix forms channels, produced by alignment of the minor grooves, through which the H3 and H2B histone tails appear to pass. Beyond here, the tails are not visualized in the crystal and are presumed to be unstructured.

Figure 2. High-resolution structure of the nucleosome
Histone proteins fold to form the core. 146 bp of DNA in a flat superhelix wraps around the core. The histone tails protrude outside the nucleosome. (Adapted from [29])



While the structure of the nucleosome is well described, higher order chromatin structures and the interplay between those structures and the genes within are still poorly understood. More than 30 years ago, Vincent Allfrey proposed that modification of histone proteins themselves might affect the interaction between genes and chromatin [30]. Allfrey noted that histone N-termini were sprinkled with conserved lysine residues and that the lysine residues could be modified by acetylation (Figure 3). He hypothesized that acetylation would reduce electrostatic interaction between DNA and histone tails, relaxing the packing of the nucleosome, allowing activating factors to gain access to the DNA. Conversely, hypoacetylation of histones within heterochromatin would lead to tighter packing. From the crystal structure, it is now evident that the histone tails do not contact the DNA backbone. Thus, it is more likely that acetylation of histone tails affects histone: histone or histone: non-histone protein contacts, not DNA: histone interactions. Although Allfrey might have been wrong about one element, his belief that modification of the

lysines in histone tails could be an important regulatory strategy within chromatin was the foundation for the models of chromatin structure and function that followed.

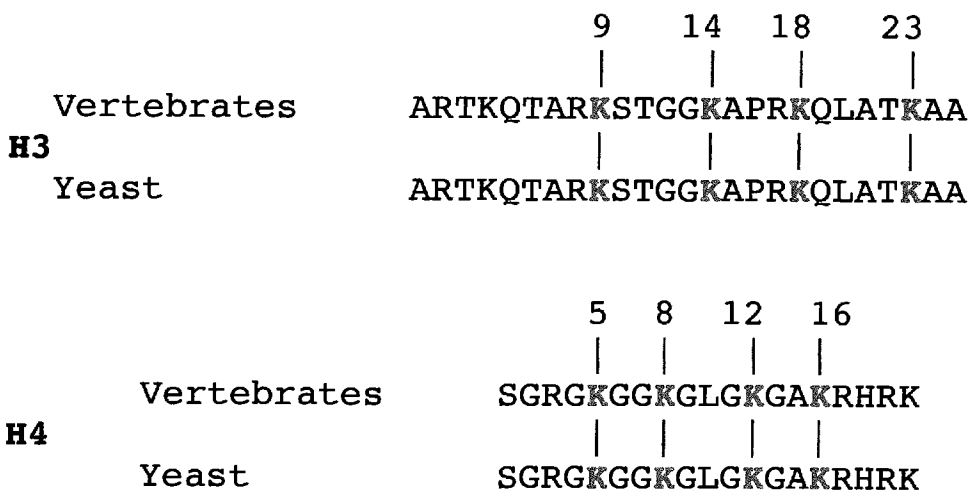


Figure 3. Histone Tails. Potential acetylation sites (shown in red) are well conserved in histone tails. Note the regular spacing of the lysine residues. Adapted from [31].

In the decades following Allfrey's pioneering observations, a positive correlation was found between gene activation and histone acetylation and vice versa. For example, using chromatin immunoprecipitation assays, workers have demonstrated that transcriptionally silent chromatin domains are relatively hypoacetylated and regions of active gene transcription are hyperacetylated [17] [32]. Evidence that this modification is required, and not just a mark of specialized domains comes from multiple organisms. For example, treatment of *S. pombe* with trichostatin A (an inhibitor of deacetylase activity) results in hyperacetylation of centric heterochromatin and loss of silencing of genes placed there [33]. On the other hand, the 2-fold active X chromosome in Drosophila—which allows for sex-linked

dosage compensation—requires hyperacetylation of H4-Lys16 [34]. Although scores of transcription factors that remodel chromatin and activate or repress genes were characterized during this productive period, the enzymes that were capable of modifying histones remained elusive for many decades.

In a technological breakthrough, the first enzyme capable of catalyzing histone acetylation was identified in 1995. Using Tetrahymena nuclei and a novel in-gel histone acetyltransferase (HAT) assay, workers purified the first nuclear HAT (type A) that acetylates histones within chromatin [35]. Surprisingly, microsequencing of this protein revealed that it was a homologue of a yeast transcriptional co-activator, GCN5 [36]. After this initial observation, HAT activity was detected in 5 different classes of proteins: p300/CBP [37, 38], GNAT super family (e.g. PCAF), MYST super family (e.g. MOF —a gene required for dosage compensation in Drosophila), basal transcription factors (e.g. TAFII250) [39], and the nuclear co-receptor co-factors (e.g. SRC1) [40]. The specificities of these enzymes differ from one another (reviewed in [31]). For example, *in vitro*, CBP/p300 can acetylate all 4 core histones, while other HATs such as Gcn5, PCAF, and SRC1 acetylate only H3 and H4. Given that many of these proteins complex with one another *in vivo* (e.g. p300/CBP binds to PCAF and SRC-1), many have hypothesized that HAT activity is fundamental to the transcriptional activity of these complexes.

CBP and p300 are two of the most well characterized HATs. They are structurally nearly identical and play similar, but not completely overlapping roles in development and tumor suppression across phylogenetic lines by way of HAT activity and transcriptional co-activation (reviewed in [41]). CBP and p300 contain

distinct domains that mediate a myriad of protein-protein interactions: CREB-binding domain, a bromodomain, and zinc fingers—known as the cys, ZZ and TAZ domains, respectively (see figure 4 below). CBP and p300 also contain a HAT domain that is required for most, but not all of the functions tested to date [41]

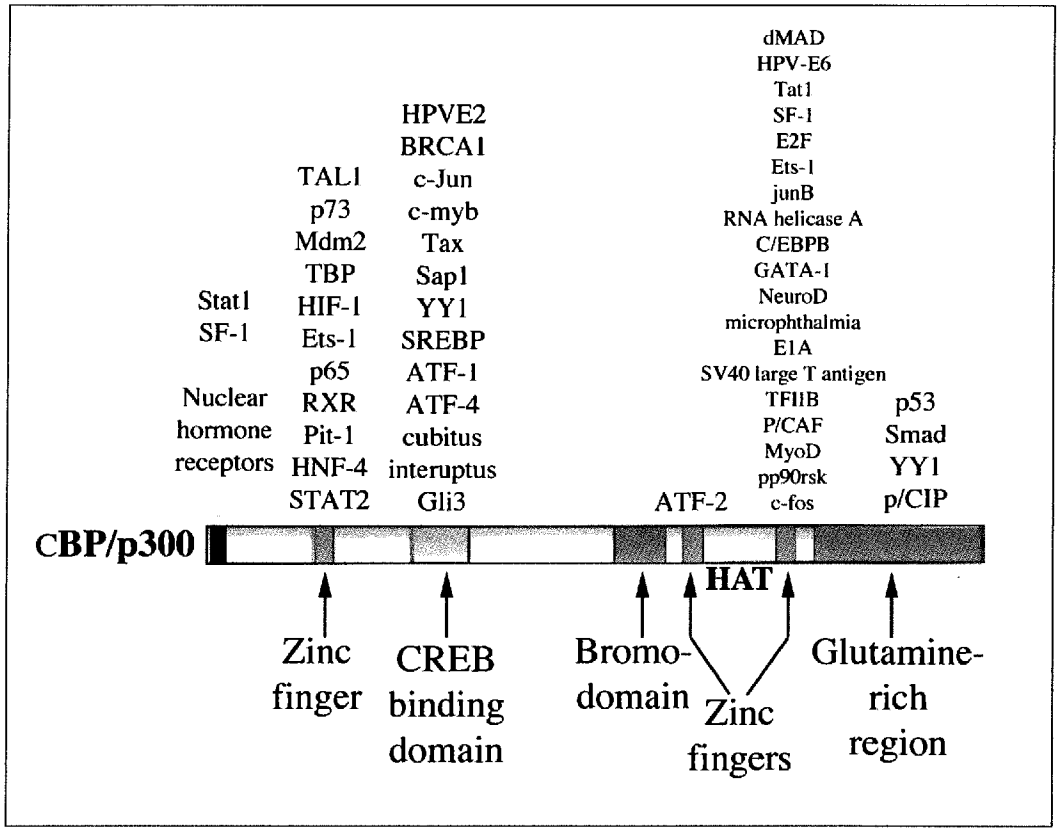


Figure 4. Functional domains of CBP/p300 Domains of CBP/p300 and selected interacting partners are shown above the domain where they bind (reproduced from [41]).

Studies of the Drosophila homologue of CBP (*dCBP*) have been particularly fruitful. Complete loss of *dCBP* causes early lethality and produces varied phenotypes—loss of thorax and head structures as well dorsal and ventral cuticular structures [42]. When maternal *dCBP* is present, the embryos progress further through embryogenesis but also show defects in the germ band —causing loss of mesoderm and ectoderm cells—and loss of naked cuticle and hairs and bristles in the denticle belts. The wide range of phenotypes suggest a role in multiple developmental pathways [41]. *dCBP* has subsequently been shown to play a role in several pathways such as determination of segment polarity (via an interaction with

cubitus interuptus, a GLI-3 homologue), gut formation (via an interaction with *dTCF*, homologue of T cell factor) and germ band formation (via an interaction with *dorsal*, a homologue of a component of NF-KB) (reviewed in [41]). Interestingly, evidence to date suggests that dCBP inactivates dTCF via direct acetylation, by way of the HAT domain[43]. This, along with a growing number of studies, has provided evidence that so-called HATs, and particularly the HAT domain of p300/CBP are capable of modifying substrates other than histones (e.g. p53, GATA1, TIIF, tubulin) (reviewed in [31]).

Given the above data, it is important to determine whether p300/CBP actually modify histones *in vivo*. In one study, mutations that affect HAT Acetyl CoA binding decrease the ability of dCBP to act on endogenous dCBP promoters in cell lines [44]. The same mutations cause a decrease in histone acetylation states *in vivo*. Using *in vitro* transcription studies, Kraus et. al. have shown that the HAT domain of p300 is required for transcriptional activation [45]. Moreover, the authors demonstrate that the HAT domain is required for H3 acetylation of chromatinized nucleosomes. These data are consistent with the model that transcriptional activation is mediated by acetylation of histones via p300.

If histone acetylases play a fundamental role in gene regulation, deacetylases would be predicted to have a role as well. The first histone deacetylase identified was isolated by affinity chromatography using trapoxin A, a small molecule inhibitor of deacetylase activity. When microsequenced, the protein was found to be homologous to the S. cerevisiae transcriptional repressor, Rpd3. Further characterization of Rpd3 revealed that it was in a complex with a second related deacetylase, Hda1 [46].

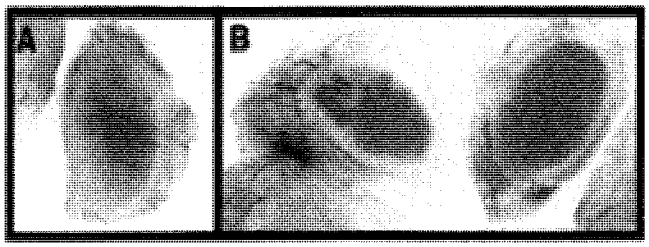
Similar deacetylases, from multiple organisms, have since been discovered and fall into two categories: those with homology to Rpd3 and those with homology to Hda1 (reviewed in [47]). Thus, Rpd3-like proteins are called class I histone deacetylases (HDAC) and Hda1-like proteins are called class II HDACs. Both deacetylases have a 390 amino acid (aa) conserved catalytic domain and are thought to require metal (a divalent zinc cation) to activate a water molecule during catalysis [48]. Three complexes, all with DNA binding and chromatin remodeling activity, have been shown to contain HDAC1 and 2: Sin3, NuRD and CoREST. Other HDAC family members (3,4,5 and 7) directly associate with the nuclear co-repressors, NCoR and SMRT (reviewed in [47]).

The discovery of HAT-mediated gene activation and HDAC-mediated gene repression further strengthened Lewey's original hypothesis that histone acetylation loosens the grip of chromatin on genes, allowing the binding of transcription factors and activation of gene transcription. However, data have begun to accumulate that suggest that histone deacetylation can also be linked with gene activation. For example, initial studies of the phenotype of yeast Rpd3 mutations demonstrated that, as expected, a number of yeast genes were activated. However, further analysis showed that transcription of a subset of Rpd3-regulated genes was actually decreased in the mutant [46]. Furthermore, mutations in the Drosophila Rpd3 homologue, known as Drosophila HDAC1 (dHDAC1) enhanced PEV in the white mottled 4 (w^{m4}) mutant [49].

The w^{m4} mutant is a radiation-induced inversion on the X chromosome that places the *white* (*w*) gene near the edge of the centromeric heterochromatin. The *w*

gene normally controls pigment deposition in the eye. A null mutation of *w* turns the normally red eyes completely white. The w^{m4} mutation positions the *w* gene near centric heterochromatin and results in white eyes with patches of red pigment [50]. It has been demonstrated that mutations in factors that maintain an open chromatin state, such as GAGA [51] and zeste, enhance the w^{m4} phenotype (less red pigment).

Figure 5. PEV in Drosophila. (**A**) The eye of the fly is usually uniformly red. In the w^{m4} mutant shown here, the red pigment appears only in patches. (**B**) w^{m4} (left) vs. w^{m4}; HP1/-(right). Mutation in the heterochromatin gene, HP1 suppress the wm4 phenotype. Adapted from [15].



On the other hand, mutations in the heterochromatin protein, HP1 [52], and in transcriptional repressors such as polycomb [53], suppress the w^{m4} phenotype (more red pigment). Thus, when the RPD3 mutant was found to enhance the w^{m4} phenotype, the hypothesis that histone deacetylation leads to gene repression was questioned.

Several new models have been proposed to account for the contradictory data. One simple explanation is that unexpected phenotype is due to an indirect effect of RPD3 [54]. In other words, RPD3 might normally repress the transcription of transcriptional activators. In the RPD3 mutants, repression would be lost and transcription of a subset of genes would be activated. The possibility of indirect effects is an important consideration when analyzing transcription factor mutants. However, one group that has carefully studied alleles of dHDAC1 has shown that different types of mutations in HDAC1 result in different phenotypes [55]. For

example, null mutations of dHDAC1 are semi-lethal and dominant enhancers of PEV. However, point mutations that are believed to destroy deacetylase activity cause a dramatic suppression of PEV and are homozygous lethal. The authors of this study propose that null mutations in transcription factors may have a less severe phenotype because similar proteins in the complex can substitute for their function. The substitute protein, however, might have subtle differences in function causing unpredictable phenotypes. Mutations that don't knock out the whole gene, on the other hand, have a different effect. Point mutations, for example, might allow the protein of interest to remain in the transcription complex, in effect poisoning the complex in a dominant negative fashion. In this model, only point mutations, unveil the "true" function of the gene in question. The authors apply this model to explain why the RPD3 knockout enhances PEV.

Strahl and Allis have recently proposed another model—the histone code hypothesis—that can also account for the RPD3 paradox [56]. This hypothesis proposes that histone tail modifications including acetylation, phosphorylation, ubiquitination, and methylation act in combination to build a "histone code" that is "read" by other proteins involved in gene regulation. In concert with its role in modifying chromatin structure, histone modifications would serve as highly regulated "receptors for protein complexes". Support for this model comes from several lines of evidence. For example, mounting evidence shows that different classes of enzymes extensively modify histones tails. The protein kinase Rsk-2, which is part of the mitogenic signaling pathway, was recently shown be an H3 kinase in vitro. Mutations in Rsk-2 are associated with Coffen-Lowry syndrome in humans, a disease

that causes severe psychomotor retardation and facial, digital and skeletal deformities [57]. Loss of Rsk-2 causes deficits in transcriptional activation in response to mitogens, suggesting that H3 phosphorylation is required for gene activation [58]. Phosphorylation of histones H1 and H3 has been implicated in chromosome condensation during mitosis [59] and dosage compensation of the male X chromosome in Drosophila [60]. Interestingly, it has long been known that histone lysines and arginines can be modified by methylation. However, there were no candidate enzymes until the recently identified nuclear receptor co-activator CARM1 was shown to have H3 methytransferase activity [61]. The second line of evidence supporting the histone code hypothesis is that some chromatin-associated proteins show a preference for modified histones. For example, bromodomains, present in many transcriptional co-activators, bind preferentially to acetylated, but not deacetylated H3 [62]. Thus, the histone code, fashioned by acetylated, methylated, ubiquinated, and phosphorylated histones could serve as a variable platform to attract different regulatory complexes to chromatin. The model can account for RPD3 paradox where presumed deacetylation results in gene activation. It also accounts for the fact that heterochromatin requires a particular pattern of acetylated and deacetylated histones [17]. In the histone code hypothesis, acetylation is not a simple switch but part of a larger pattern that promotes different chromatin structures and functions.

Using the histone code hypothesis, a new model has been proposed that provides a mechanism for inherited silencing of chromatin structure in *S. pombe* [23]. There are six genes required in trans for silencing at the mating type loci in *S. pombe*:

swi6 is an HP1 homologue, clr1 and rik1 are putative DNA binding proteins, clr3 and clr6 are HDACs and clr4 is an H3-Lys9 methytransferase. Crl3 and Rik1 are required for histone methylation and Swi6 localization depends on clr4 and rik1. HP1 contains a domain, known as the chromodomain, which binds specifically to the methylated H3 tail (K9). Disruption of this interaction causes loss of silencing. Based on the above, the following sequence of events has been proposed: histone H3 is deacetylated by clr6, favoring the methylation of H3. Swi6 binds to methylated H3 and contributes to a transcriptionally silenced chromatin conformation. If the methylation of H3 is maintained throughout DNA replication, Swi6 will continue to associate with the heterochromatin domain. Presumably, Swi6 can then recruit the complex that can deacetylate and methylate the newly arrived histone, allowing the heterochromatic state to perpetuate. This same mechanism can also account for the spreading of heterochromatin, a phenomenon observed in the PEV mutants. From this, a general principle for maintainence of chromatin states has been proposed—complexes that both "recognize a particular pattern and have the ability to achieve that pattern" can both propagate and pass on their epigenetic program [23].

While the above model works well to explain the silencing at the mating loci in *S. pombe*, a different mechanism is at work in *S. cerevisiae*. In this organism, there are three separate loci, MAT, HML and HMR, which contain α and **a** mating-type genes. At the MAT locus, the matingtype genes are expressed and specify either an α-type or **a**-type cell. At HML and HMR, however, the α and **a** genes are not transcribed, despite the fact that the cis-acting sequences of all three loci are identical [63]. As discussed above, inhibition of transcription is position-specific, heritable

and is mediated by packaging similar to heterochromatin defined in higher eukaryotes—the DNA is condensed, resistant to nucleases and the histones are primarily hypo-acetylated compared to DNA packaged in euchromatin [17]. Three genes, Sir2, 3 and 4 (Silencing Information Regulator), were identified by their requirement for maintenance of silencing at these loci [64]. The Sir genes have also been implicated in silencing at the telomeres, in association with the telomere binding protein, Rap1 [65]. Sir3 and 4 bind to histone tails and appear to repress gene expression directly by blocking transcription [66]. Interestingly, histone tails, which are not required for yeast growth, are essential for silencing [67]. A model that details the assembly of silent chromatin has been recently been proposed: Rap1, together with Ku70/80 recruits the Sir2/Sir4 complex. Sir3 is then recruited to the telomere, in what appears to be a regulated step, and stimulates the multimerization of the Sir3 and 4. This process is repeated, causing the spreading of the heterochromatin structure along the telomere (see Figure 6, below) [66].

Telomeric Sirs are also important in the maintenance of genomic integrity. After a double stranded break, Ku relocates to the damaged site, recruiting the Sir complex along with it [68]. Furthermore, mutations in Sir2, 3 and 4 confer increased sensitivity to gamma radiation [69]. Related to this, double stranded breaks cause derepression of telomeric genes, presumably because of transfer of the Sir complex to sites of repair [70]. However, the exact role of the Sir complex at the double strand break is still unclear (see Figure 6).

Interestingly, Sir2, but not Sir3 and 4, has also been shown to suppress recombination at rDNA, when complexed with Net1 and the cell cycle protein Cdc14.

Cdc14 is a phosphatase that is released from the nucleolus at the end of mitosis in order for the cell to progress through telophase [71]. Yeast rDNA loci contain between 100-200 copies of genes encoding rRNA that have an inherently high rate of recombination. In 1989, Gottlieb and Esposito showed that intrachromosomal recombination of ribosomal RNA genes increases 10-15 fold in Sir2 mutants [72]. The large array of repeated genes causes rRNA repeats to be spliced out. This leads to the formation of extra-chromosomal repeats (ERCs). Because of increased recombination in Sir2 mutants, ERC levels increase in the Sir2 mutants. rDNA is localized in the nucleolus and, as mentioned above, Sir2 is concentrated here and is found in association with Net1 and Cdc14 [71]. Subsequent studies have demonstrated that Sir2 is required for mediating a condensed, inaccessible chromatin structure at sites of rDNA (see Figure 6) [73].

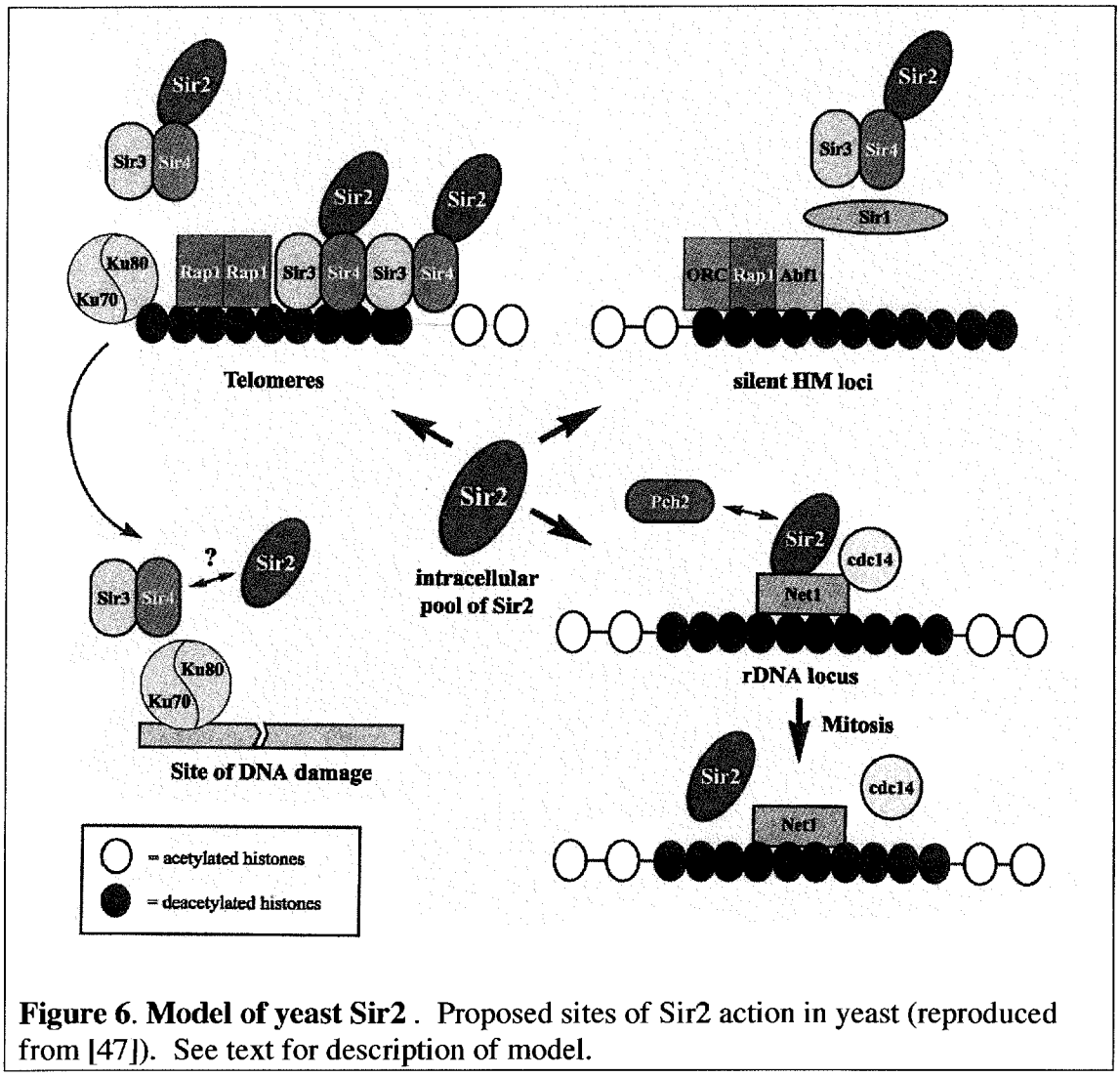


Figure 6. Model of yeast Sir2 . Proposed sites of Sir2 action in yeast (reproduced from [47]). See text for description of model.

Further investigations have revealed the existence of four other Sir2 homologues in yeast (HST1, 2,3 and 4) that are 29% to 63% identical to sir2p. A central 275 aa domain, coined the core domain, was found to be nearly identical between the five homologues. Furthermore, hst1, 3 and 4 were shown to function in silencing at the mating type loci and/or at telomeres [74]. Also of interest, a large percentage of hst3hst4 double mutants arrested at G2/M and the double mutant also had increased chromosome loss, missegregation and recombination (See Figure 6,

above). Brachmann et. al. also found that the Sir2 family extends far beyond yeast with homologues in humans, rodents and bacteria [74].

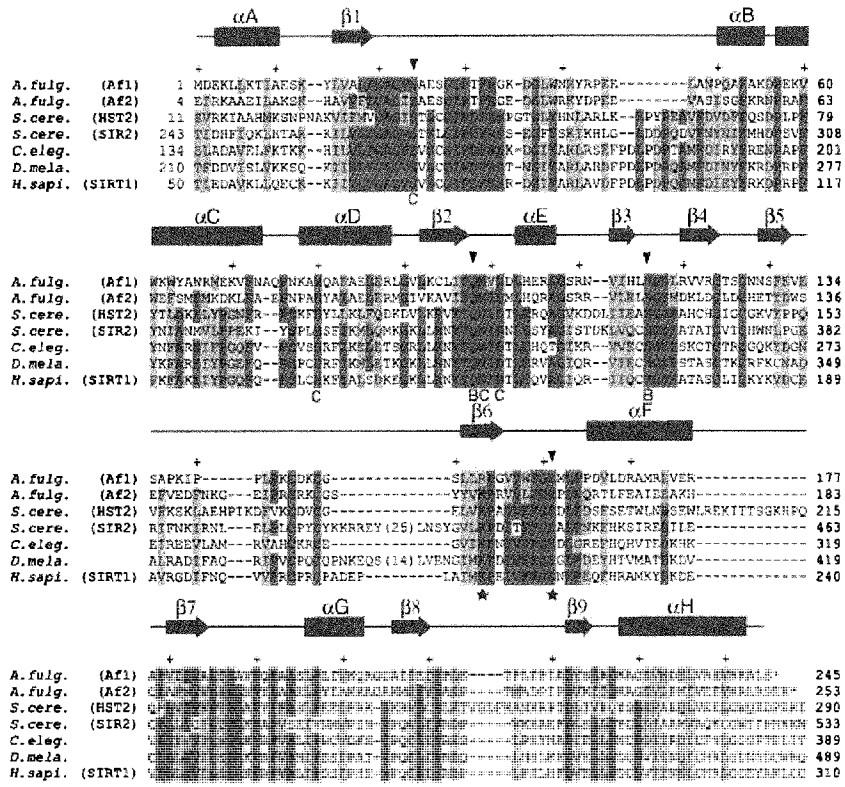


Figure 7. Alignment of sirtuin core domain
Select homologous from the sirtuin family: *Archaeoglobus fulgidus*, *S. cerevisiae*, *D. melanogaster*, and human. Amino acid residues highlighted in blue are identical in 5/6 sequences. Yellow highlights the conserved residues. The positions of the alpha and beta strands are shown above (from the crystal structure (see below)). The magenta letters correspond to sites of the NAD pocket. The magenta stars indicate the residues involved in the salt bridge. (Reproduced from [75]).

The Sir2 family, members of which have since been found in every organism, has been labeled the "sirtuin" family (see Figure 7, above). As in yeast, conservation is primarily limited to an approximately 275 amino acid core domain (See Figure 7, below) [74]. Conservation within the core domain suggests that sirtuins have an ancient and critical role in regulating the genome. But the mechanism of sirtuin

action, no doubt requiring the core domain, remained a mystery for several years to come.

One clue to sirtuin function was that overexpression of yeast Sir2 caused a global decrease in histone acetylation [76]. Likewise, in Sir2 mutants, hypoacetylation of lysine residues at the mating type loci was lost [76]. Based on the above data, Sir2 was predicted to be a histone deacetylase [76] and Sir2 deacetylase activity was pursued—but not detected (Michael Grunstein, Jef Boeke, personal communication and my own unpublished data). A breakthrough in the understanding of the role of Sir2 came from a genetic screen in Salmonella [77]. A gene called CobB was identified for its ability to compensate for mutations in another bacterial gene, CobT. CobT is a known phosphoribosyltransferase in the cobalamine biosynthesis pathway. When CobB was sequenced, it was found to be a new member of the sirtuin family. Inspired by the possibility that one sirtuin member could act as an enzyme, a second group tested the ability of the conserved core domain of both CobB and human Sir2 to transfer an ADP-ribosyl group from NAD [78], [79]. In this setting, both proteins catalyzed this reaction. Several other labs went on to demonstrate that Sir2 could actually deacetylate histone lysines if NAD was included in the reaction [80, 81]. Debate over the relevant enzymatic activity of Sir2 was resolved after a detailed enzymatic analysis showed that the ribosyltransferase reaction is a minor side reaction [82]. Sir2 from yeast and mammals has been shown to specifically deacetylate lysines 4 and 9, on H3, and lysine 16 on H4. Thus, sirtuins were determined to be bonafide histone deacetylases and define a new class of NAD-dependent histone deacetylases (class III HDACs), as predicated by genetic

experiments almost a decade earlier.

The sirtuin family is unique in that it is not structurally related to class I and II HDAC enzymes, requires NAD, and the deacetylation reaction generates a unique product, acetyl-ADP ribose. This product may have significance because it potentially links Sir2 with another family of NAD-requiring enzymes, poly (ADP-ribosyl) ation enzymes (PARPs). In response to DNA strand breaks, PARPs transfer ADP-ribose groups to proteins, forming branched polymers. PARPs are also found in association with repair, replication and transcription complexes and are thought to play a role in DNA metabolism and chromosome maintenance [83]. It has been proposed that the Sir2 reaction could inhibit PARP by 1) competing for NAD, and 2) acetyl-ADP ribose reaction product could potentially bind to PARP and block ribosyltransferase activity directly [82]. Recent data show that the Sir2 reaction by-product may have functional significance as injection of 0-acetyl-ADP-ribose into starfish oocytes causes a delay/block in blastomere cell division [84]. Further work is needed to investigate this interesting reaction product.

The crystal structure of the human and Archaeoglobus fulgidus Sir2 homologues has recently been determined at high resolution [75], [85]. Only the structure from Min et al includes NAD but both structures show nearly identical features including a large domain (called a Rossman fold) and a small domain (3-stranded zinc ribbon motif) with NAD-binding site between the two

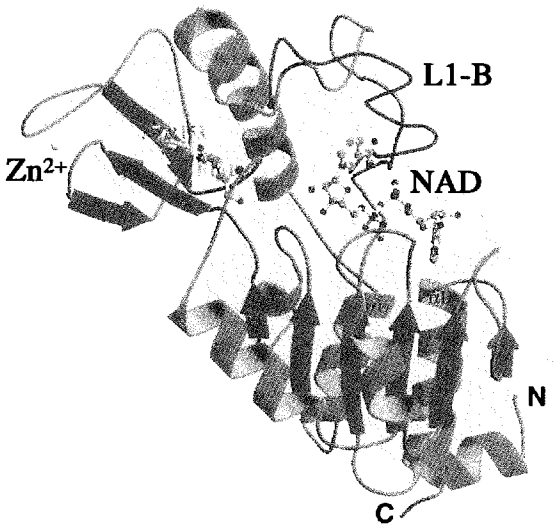


Figure 8. Sir2-Af1 crystal structure The 2.1 angstrom structure is shown in a ribbon representation. The small domain is the zinc ribbon motif, shown with a zinc molecule bound by two pairs of cystines. The NAD molecule is shown in ball and stick motif. There is a salt bridge at R153:E161 that is also represented with the ball and stick motif. The larger domain represents the Rossman fold domain. The magenta loop has a variable structure within in the crystal, probably because it is dynamic in solution. The deep surface and floor of the NAD pocket make up the active site of the enzyme (reproduced from [75].

domains (see Figure 8, above). Mutations in the invariant residues within the NAD pocket destroy Sir2 activity. Sirtuin histone deacetylation requires two steps, cleavage of the glycosidic bond between the nicotinamide group and the ADP ribose group of NAD and cleavage of the C-N bond connecting the acetyl group with lysine. Although several mechanisms of catalysis have been proposed [86], [75], the exact mechanism has yet to be elucidated [86].

The dependence of sirtuin enzymatic activity on NAD sparked interest in another observation related to Sir2 function in yeast. As mentioned above, loss of silencing at rDNA in sir2 mutants is associated with the build up of extra-chromosomal circles (ERCs). The ERCs may be toxic to yeast as cells with higher levels of ERCs reached senescence faster. Senescence in yeast has parallels to aging in higher organisms. Thus, when it was discovered that Sir2 is a NAD-dependent

enzyme, Guarente hypothesized a link between Sir2, metabolism and aging [87]. Guarente's theory is based on a series of observations, starting nearly a century ago, that animals with higher metabolic rates often have shorter life spans [88]. More recent studies have demonstrated that moderate caloric restriction in mammals can delay signs of aging and extend lifespan. These data have inspired the "rate of living hypothesis" which proposes that metabolic activity of an organism is correlated to length of life. One explanation for such a link is that free radicals generated by metabolism cause cumulative damage to proteins, nucleic acids and lipids over time, leading to progressive destruction of the organism [89]. Guarente hypothesized an alternative theory that caloric restriction would cause a shift in the "metabolic strategy" in cells, favoring longevity. Specifically, he speculated that caloric restriction would decrease consumption of NAD in glycolysis, increase available NAD, and, therefore, increase Sir2 activity. Increased Sir2 activity would enhance silencing, prevent ERC accumulation, and delay senescence (See Figure 9, below). In support of this theory, the phenotype of the null mutants of NPT1, a gene required for NAD synthesis, is similar to that of the sir2 mutant. While this theory is interesting, it is unclear how the model would translate to higher organisms, where there is no evidence of ERC production. Also, given that Guarente's model is based on the notion that lifespan is the measured by counting the number of cell divisions before cell senescence it is unclear if the model would apply to post-mitotic organisms such as C.elegans and Drosophila. Once post-mitotic organisms reach a certain developmental stage (i.e. larval stage in Drosophila), cell division stops. In these organisms, cell senescence occurs early and is not related to lifespan of the adult

animal. None-the-less, duplication of a sir2 homologue in C.elegans was recently shown to cause a doubling of lifespan and deletion of the same gene caused shortening of lifespan [90]. Thus, sirtuins are linked to regulation of lifespan across phylogenetic lines, although it is unclear exactly how.

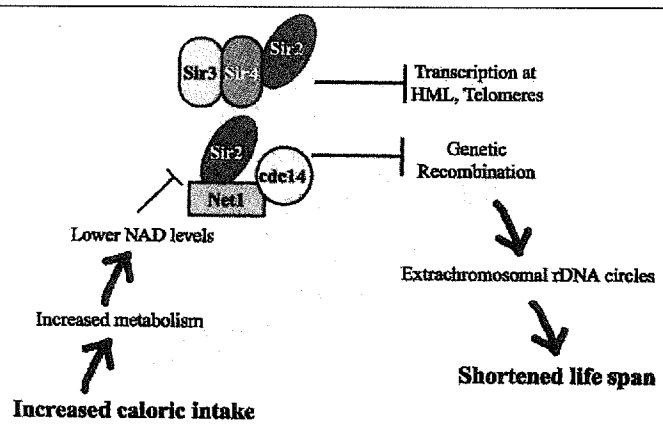


Figure 9. Link between Sir2 and lifespan (reproduced from [47]).

While the structure of the sirtuin core domain and the enzymatic activity is remarkably well conserved, new data suggest that sirtuin function is not. For example, sirtuins are present in organisms that lack histones. In fact, as mentioned above, the enzymatic activity of Sir2 was first hinted at by experiments showing that overexpression of a sirtuin in S. typhimuruim rescued a mutant strain lacking a gene encoding a phosphoribosytransferase required for cobalamin biosynthesis. It is possible that the original prokaryotic sirtuin was an NAD-dependent enzyme involved in metabolic pathways and it's role in chromosome structure evolved later. Consistent with this, several of the human sirtuins have been shown to be exclusively cytoplasmic. To date, all but one of the eukaryotic sirtuins that have been tested deacetylate histones *in vitro* but the significance of this is uncertain. For example,

two independent groups recently showed that mammalian Sir2s (human SIRT1 and mouse Sir2α) interact with and deacetylates p53 [91] [92]. Unlike the *in vitro* enzymatic studies of mammalian sirtuins using histone substrates, these papers present functional data that suggest an important interaction between mammalian Sir2 and p53. Vaziri et. al. show that expression of wild-type SIRT1 reduces p53 activity and catalytically inactive SIRT1 enhances p53-mediated apoptosis and radiosensitivity [91]. Similarly, Luo et. al. demonstrate that point mutations in Sir2 α cause an increase the sensitivity of cells to stress and that wild-type Sir2 α attenuates p53 function [92]. There are at least two important implications of this work. First, it potentially ties mammalian Sir2 into all the pathways that p53 regulates. Second, it demonstrates that sirtuins have substrates other than histones, suggesting a broader role. Given these data, it is difficult to predict which of the sirtuin functions are conserved or not. Particularly of interest is the question of whether sirtuins regulate heterochromatin across phylogenetic lines.

After decades of intense study, the interplay between chromatin structure and gene transcription is beginning to emerge. The near perfect conservation of the structure of heterochromatin has led to the hypothesis that the factors that regulate heterochromatin are also conserved. The conservation of the structure and enzymatic function within the sirtuin family provides evidence in support of this hypothesis. In this thesis, I directly test the hypothesis that Sir2's role in the regulation of heterochromatin and lifespan is conserved. To this end, I cloned and characterized the Drosophila homologue of Sir2. I examined the role of dSir2 in development, lifespan, and heterochromatin formation and demonstrated an interaction between the

histone acetyltransferase, dCBP and dSir2. These studies are described in Chapters 2 and 3.

Chapter II

MATERIALS AND METHODS

Fly strains and culture conditions.

The *TMSΔ2-3, CyO, TM2* and *SM6b, eve-LacZ* balancer chromosomes and the w^{m4}, *P (ry+t7.2=PZ} l (2) 05327* cn^1 and *P {w, LacZ} l (2) K14513* [93] chromosomes used in these studies were obtained from the Bloomington Stock Center and the Berkeley Drosophila Genome Project. They are described in Flybase [94]. The $dCBP^1$ allele used in this study has been described [42]. The $y^1w^1dCBP^1$ chromosome was generated using standard recombination techniques. Flies were cultured on standard Drosophila cornmeal-yeast source media in 8 oz plastic bottles or 28X95 mm plastic shell vials. All crosses were reared at 25°C.

Cloning and mutagenesis of *dSir2*.

Two partial *dSir2* cDNA clones were identified in a two-hybrid screen performed by Dr. Yang Chen. She used the CREB-binding domain (CBD, amino acids (aa) 825-1043) of dCBP as bait to screen a 0-6 hour Drosophila embryo library. She identified several dCBP partners, including *dSir2* [95]. I used one of the partial *dSir2* cDNA clones (base pairs (bp) 1621-3839) as a probe to isolate the full-length

cDNA using standard library screening methods at high stringency [96]. Six hybridizing phage were isolated from a Drosophila lambda gt11 head library (provided by P. Salvaterra, City of Hope, Duarte, CA). Lambda phage DNA was purified using a glycerol cushion [96] and digested with EcoRI; cDNA fragments were purified and subcloned into BlueScript KS (Stratagene). cDNA fragments were sequenced and ligated together based on homology to the known amino acid sequence of yeast Sir2 to create a contiguous cDNA clone. All sequencing was performed manually (USB 101 Sequenase Kit) or with automated sequencing (ABI Prism 310 Genetic Analyzer) using standard methods. The cDNA sequence was later confirmed using sequence from the genomic clone (see below). Five independent genomic clones were isolated from an EMBL3 Drosophila library (provided by John Tamkun, University of California, Santa Cruz). I used a 5' fragment of the cDNA (bp 270-1508) to make random-primed [^{32P}-α-dCTP]-labeled probe. Hybridizing phage were purified, subcloned into Bluescript (using SalI and EcoRI) and sequenced as above.

To define the 5' end of *dSir2*, primer extension assays were performed using an oligonucleotide 188 bp from the 5' end of the *dSir2* cDNA (bp 301-331, GAGGCGAG AGCGCAAAGCGGAGAGACCGAGG). The oligonucleotide was labeled by incubation with nucleotide kinase and [^{32}-γ-ATP] and hybridized overnight to 25 μg of total RNA isolated from Drosophila embryos. RNA was prepared using TRI Reagent (Molecular Research Center). The RNA/primer mix was ethanol precipitated, resuspended, mixed with Superscript™ enzyme (BRL), and incubated at 42°C for 90 minutes. The reaction was terminated with EDTA; RNase was added, followed by phenol/chloroform extraction and ethanol precipitation. The primer-

extended product was resuspended in TE and analyzed on a polyacrylamide gel next to a sequencing reaction that was primed with the same oligonucleotide.

Dr. Smolik mapped *dSir2* to position 34A 5,6 of chromosome 2L using standard methods [97]. Based on this chromosomal location, strains with P-elements inserted near the *dSir2* gene were obtained from the Bloomington Stock Center. I screened P-element strains with genomic Southern blotting as follows. I used the 5' EcoRI *dSir2* fragment (bp 270-1508) as a template for a random primed DNA probe (Boehringer Mannheim random primer kit). Genomic DNA was isolated from adult flies using the Genomic DNA Kit™ from Gentra Systems. Southern blotting was performed using standard techniques [96]. Plasmid rescue, which allows for direct subcloning of DNA flanking P-elements, was performed according to previously published techniques [97]. For the plasmid rescue procedures, I digested genomic DNA with XbaI and EcoRI restriction enzymes. I found and characterized two strains, *l (2) 05327, cn* ry^+ and *l (2) K14513*, w^+ that contain P-elements that insert in *dSir2*.

To generate partial deletions of *dSir2*, *l (2) 05327,cn / SM6b, eve-LacZ;* ry^{506} e^s flies were crossed to *Sp/ CyO*; *TMSΔ2-3 /TM2* flies and the *l (2) 05327,cn/ CyO;* ry^{506} e^s */ TMSΔ2-3* females were mated to ry^{506} e^s males. All of the non-*CyO*, ry^{506} e^s males that were rosy —and had therefore lost the P-element carrying the ry^+ insertion— were mated to *SM6b, eve-LacZ/ Sco;* ry^{506} e^s females. The excised *l (2) 05327,cn* chromosome was detected as l *(2) 05327,cn /SM6b, eve-LacZ;* ry^{506} e^s flies having cinnabar eye color. Such flies were saved in the balanced stock. Note that because *eve-LacZ* was transformed with ry^+ as the marker, these animals were not *ry*.

To generate the second *dSir2* mutation, w^1; *l (2) K14513,* w^+*/CyO* flies were mated to w^1; *Sp/CyO*; *TMSΔ2-3/TM2* males and the *l (2) K14523,* w^+*/CyO*; *TMSD2-3/+* male offspring were mated to w^1; *Sp/CyO* virgin females. All of the white-eyed, *CyO* males—which had lost the P element carrying the w^+ insertion—were crossed to w^1; *Sco/CyO* virgin females. The *l (2) K14513/w* deletion chromosomes were kept as a balanced stock with CyO. Forward primer BN058 (+46, TTCAATCCATATCGGTCGTAGATG) and reverse primer BN057 (+2344, CAGCGAGTAATCGAGTGTCACGGC) were used to screen genomic DNA purified using Genomic DNA Kit™ (Gentra Systems). PCRs were performed under standard conditions in 100μl reactions using 1 μl Taq DNA (BRL) 200μmols dNTPs (BRL), and 200 nmols oligonucleotide. Reactions were annealed at 60°C, extended at 72°C and 25 rounds of amplification were performed. To further define the deletion, PCRs were performed using oligos 054, 062, 040, 042 and 035. The above reaction conditions were used except the annealing temperature was 55°C and the cycle number was 30. The same strategy was applied to the *l (2) 05327* deletion strains. The deletions were sequenced by amplifying DNA using the above oligos and direct sequencing of the PCR products with ABI Prism 310 Genetic Analyzer.

Northern Blot and *In Situ* Hybridization.

RNA was prepared from Drosophila embryos using TRI REAGENT (Molecular Research Center, Inc.). For the developmental Northern analysis, mRNA was prepared using the small scale PolyAtract mRNA Isolation System (Promega Corporation). The mRNA was electrophoresed on formaldehyde agarose gels and

transferred to Hybond membranes. To make riboprobe, the C-terminal EcoRV-XhoI (1621-3839) fragment of *dSir2* was subcloned into Bluescript and *in vitro* transcribed using the Promega Riboprobe Transcription KitTM. For the Northern blot of the *dSir2* mutant, total RNA was used and the riboprobe was made from an EcoRI fragment of *dSir2* subcloned into Bluescript. The EcoRI fragment includes bp 270-1508, and encodes the N-terminal non-conserved portion of dSir2 plus most of the conserved catalytic core domain; the catalytic core domain is encoded by sequences that include bp 1098-1875. The Northern blots were hybridized with the riboprobe under high stringency conditions. For *in situ* hybridizations, the C-terminal (1621-3839) clone was used to synthesize a DIG-labeled riboprobe. The first 600 bp of the *dDnaJ-H* open reading frame was amplified by PCR using the BN058 forward primer and the more 3' BN053 reverse primer (CTGTATACTTGACGTATAA- TAG) and subcloned into Bluescript. The sequence was confirmed and the DIG-labeled riboprobe was synthesized in the same fashion as the *dSir2* riboprobes. RNA *in situ* hybridizations were performed using standard techniques [97].

Production of anti-dSir2 antibody.

The GST-*dSir2* fusion gene was constructed as follows. Plasmid CT*dSir2*: pCITE was digested with PvuII (bp 1622) and XhoI (bp 3834) and purified with Bio 101 Gene Clean Kit™. pGEX-4T3 (Pharmacia) was digested with SmaI and XhoI and purified as above. The *dSir2* fragment was ligated to the pGEX backbone, creating a SmaI/PvuII hybrid (CCC/CGT) that brings the *dSir2* coding sequence (G552-V821) in frame with the GST coding sequence. The construct was confirmed

with automatic sequencing (ABI Prism 310 Genetic Analyzer). GST-*dSir2* was transformed into DH5∝ for protein expression. 100 ml of Luria Broth (LB) plus 100 μg/ml ampicillin was inoculated with GST-*dSir2*/DH5∝ and incubated overnight at 37 °C with shaking. One-tenth of the overnight cultures was used inoculate 1 L of LB-ampicillin and was incubated until the optical density (OD) at 600 nm was 0.513. 0.4 mM IPTG was added to the culture and the culture was incubated for 3 hours. The bacteria were pelleted, washed in ice-cold PBS, pelleted again, and stored at -80°C.

GST-dSir2 protein was purified as follows. One gram of glutathione agarose beads (Sigma) was added to 50 ml PBS. The beads were incubated at 4°C over night and then washed in PBS three times. The beads were resuspended in 1 bed volume of PBS. Bacterial pellets were thawed and resuspended in ice-cold PBST (PBS plus 1% Triton, 1 mM EDTA, 10μM β-mercaptoethanol, PMSF, and 2 μg/ml leupeptin. The lysate was processed in a French press and spun at 30,000 X g for 30 minutes. The supernatant fluid was mixed with 3 ml glutathione beads and incubated on a rocking platform, overnight at 4°C. The beads were then washed three times in 15 ml ice-cold PBST and loaded onto a column. The column was washed with 3 column volumes of ice-cold PBST. GST-dSir2 was eluted from the column with 10 mM Glutathione in PBST. Five 3ml fractions were collected and 10 μl of each fraction was electrophoresed on a SDS-PAGE gel. The gel was stained with Commassie.

Gel-purification of GST-dSir2 using Promega ChromoPhor Kit™. The ChromoPhor system is designed for gel purification of proteins and is based on a dye that binds to proteins in during electrophoresis, resulting in visible bands during

electrophoresis. After electrophoresis, the protein of interest is simply cut out and eluted. The GST-dSir2 fractions were pooled, concentrated and electrophoresed in an 8% ChromoPhor SDS-PAGE gel. The putative GST-dSir2 band was excised, homogenized, and mixed with ChromoPhor elution buffer. The tubes were incubated at 37°C overnight and the protein was precipitated using acetone. The pellet was resuspended in PBS. 5 μl was electrophoresed on an SDS-PAGE gel along side a BSA standard curve and the concentration was estimated to be approximately 0.4 mg/ml. 2 mg of protein was shipped to Pocono Rabbit farm and was injected into Sprague Dawley rats along with Freund's adjuvant. Pre-immune and immune serum was collected and used at a dilution of 1:2000 (diluted in PBS/0.3% Triton/ 1% milk) on Western blots containing bacterially produced dSir2 and KC cell extracts. To decrease background, the antibody was pre-absorbed with embryos before use in Westerns or whole mount staining.

Western analyses and whole mount staining.

Whole flies (embryos, larvae and adults) were ground with a Teflon pestle in 20 μl/fly of hot 2X SDS-PAGE sample buffer[96]. The homogenate was centrifuged for 10 minutes at maximum speed and 10 μl of supernatant was separated on an 8% polyacrylamide gel. Gels were transferred to Immobilon-P membrane (Millipore), blocked with 5% BLOTTO /1%BSA, and probed with anti-dSir2 antibody using standard procedures [96] and the Immobilon P commercial protocol. Secondary anti-rat HRP conjugated antibodies were obtained from Sigma. Whole mount embryos were collected, fixed, blocked (10% horse serum in PBS plus 0.3% Triton), and

stained with anti-dSir2 antibodies (1:2000 dilution) using standard methods [97]. Secondary goat-anti rat biotinylated antibodies were obtained from Vector Scientific. Anti-acetyl histone antibodies were obtained from Upstate BioSciences and were used at a dilution of 1:100. Anti-rabbit secondary fluorescent antibodies were obtained from Vector.

Drosophila larval salivary glands were dissected, fixed in glacial acetic acid/ paraformaldehyde, squashed, and prepared for antibody staining as described ([97]). Non-fluorescing larvae from a cross between *l (2) K14513* $^{4.5}$*/CyO, Kr-GFP* females and *l (2) 053275.26/CyO, Kr-GFP* males were collected for the negative control experiment. Anti-dSir2 antibody was diluted 1:200 and anti-dCBP (described in [42]) antibody was diluted 1:1000. Donkey anti-rat Rhodamine Red™ X- labeled secondary antibody (1:100 dilution), generously donated by the Amara lab, was used with anti-dSir2 antibody. Anti-chicken Fluoresce in Isothiocyanate-labeled secondary antibody (1:100 dilution), obtained from Vector scientific, was used with the anti-dCBP antibody. Images were collected from a Zeiss fluorescent microscope using OpenLab™ software.

Deacetylase Assays.

Recombinant dSir2 was produced in E.coli BL21 (DE3) as a C-terminally (his) 6-tagged protein (provided by James Lundblad). The bacterial expression vector was constructed by inserting the full-length cDNA into pET23d (Novagen) as a PCR product using the forward primer CCAAGCTTCCATGGTGAAA-TCAAAACAAAAACATTGGCTG and the reverse primer

GGTCTAGAGTCGACCA-CTGCTGCTAACTGTCCTGGAGGC. Tagged protein was purified from cleared bacterially lysates by sequential Ni-chelate chromatography (NiNTA; Qiagen) and anion exchange chromatography (HiTrap Q-sepharose, Pharmacia). Protein was dialyzed into 25 mM Tris, pH 8.0, 1mM EDTA, 150 mM NaCl, 10 % glycerol, 1 mM DTT. Protein concentrations were estimated by BioRad protein assay using BSA as a standard. Nuclear extract from HeLa cells was prepared as previously described. Histone deacetylase assays were performed using a histone deacetylase assay kit (UBI catalogue 17-281). In this assay a peptide corresponding to the N-terminal sequence of histone H4 was chemically acetylated with 3H-acetate. Tritiated peptide (20,000 dpm; approximately 500,000 dpm/mg) was incubated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol with 0.2 mM PMSF and the indicated amount of protein, with or without 500 mM NAD+ and 50 mM sodium butyrate as indicated, for 3 hours at 37°C. Reactions were terminated by the addition of 1N HCl/0.1 N acetic acid and the released counts determined by extraction with ethyl acetate and liquid scintillation counting.

Determination of *dSir2* survival curves.

Parents for all experimental animals were 3-7 days old from uncrowded cultures. Control and experimental animals were male sibs from an outcross (*dSir2*$^{4.5}$/*Cyo* flies crossed to *dSir2*$^{5.26}$/*Cyo* flies) and thus internally controlled for humidity, parental age and crowding. Under non-stress conditions, flies were given new food every two days and individual vials were maintained at 20- 30 males per vial until populations fell below 20. Under stress conditions the flies were given new

food every four days and population densities were not optimally maintained. Standard t-test was used to determine the differences of the median and average lifespan.

Determination of the effect of *dSir2* mutations on PEV.

I used a standard protocol to assess the effect of the *dSir2* mutations on PEV [98]. Briefly, eight days post eclosion, the female progeny from a cross of w^{m4}; $dSir2^{4.5}/CyO$ virgin females to w^{m4}; $dSir2^{5.26}/Sco$ males, and the reciprocal cross, were sorted into 5 categories based on eye color, with category 1 representing almost completely white eyes and category 5 representing completely red eyes. The intermediate categories represent animals with progressively more pigment. Once scored for eye color, the *dSir2* animals were identified by the absence of *Sco*. Animals carrying the *CyO* balancer were discarded as *CyO* affects PEV [98]. At least 200 flies were scored in each blind experiment and each experiment was done twice. This protocol is at least as accurate as pigment determination protocols, which can exhibit variability due to differences in eye and animal size [98].

GST pull-down and co-immunoprecipitation assays.

GST pull-down assays were performed as described [99]. In brief, the EcoRV -XhoI fragment (bp 1621-3839) of *dSir2* was subcloned into pCITE (Novagen) and used as a template for *in vitro* translation using [^{35}S] labeled- Methionine (Amersham). The TNT reticulocyte translation kit (T7) from Promega was used, following their protocol. The radiolabeled dSir2 was mixed with either GST or

increasing amounts of GST-dCBP (CREB-binding domain) fusion protein and glutathione agarose beads (Pharmacia). The samples were incubated for 2 hours and pelleted with centrifugation at top speed in a microcentrifuge. The beads were washed, boiled in SDS-PAGE buffer and then pelleted again. The supernatant fluid was loaded onto SDS-PAGE gels and electrophoresed. The gels were dried and exposed to film. Immunoprecipitation assays were performed as follows. Two confluent 10 cm plates of Drosophila Kc cells were used for each sample. Cells were scraped into 15 ml Falcon tubes and rinsed twice in 5 ml of ice-cold PBS. The cell pellets were resuspended in 1 ml of the following lysis buffer: PBS, 0.1% NP40 and Roche "mini" EDTA free protease inhibitor tablets (1 tablet/10 ml buffer). The suspension was sonicated 4 times for 30 seconds at medium power and then transferred to microfuge tubes and centrifuged at maximum speed for 15 minutes at 4 ° C. 50 μl of a 50% slurry of Protein L agarose (Pierce) were washed in the lysis buffer and mixed with the Kc cell supernatant. 10 μl of anti-dSir2 serum (pre-immune or immune) was added to each sample and the mixtures were incubated at 4° C for an hour. The beads were washed 4 times in the lysis buffer (without protease inhibitors), pelleted as above and then boiled in 50μl 2X SDS-PAGE buffer. Samples were centrifuged at maximum speed in a microcentrifuge and the resulting supernatant fluid was analyzed on 6% SDS-PAGE gels. Westerns were performed with either anti-dSir2 or anti-dCBP antibodies as described above and as in [100].

Double-stranded RNA interference (RNAi) of dSir2 production in Drosophila Kc cells.

Drosophila Kc cells were propagated in 1X Schneider's Drosophila media (GIBCO) supplemented with 5% FBS (Hyclone) and 50 units/ml penicillin in 75-cm^2 T-flasks. The RNAi methods used were based on the paper by Clemens, et. al. [101]. A 700 bp fragment, containing *dSir2* coding sequence was amplified using PCR. Each primer contained a 5' T7 RNA polymerase binding site (GAATTAATACGACTCACTATAGGGAGA), followed by the *dSir2* sequences. The PCR products were purified (BRL DNA miniprep kit) and used as templates for Megascript T7 transcription kit (Ambion) to produce double stranded (ds) RNA fragments. The dsRNA was EtOH precipitated and annealed by incubation at 65°C for 30 minutes followed by slow cooling to room temperature. Five μg of dsRNA was analyzed on a 1% agarose gel; the RNA existed as a single band on the gel (data not shown). The RNA was stored at -20°C.

Drosophila Kc cells were diluted to 3 x 10^6 cells per/ml and one ml was used to seed each well in a 6-well culture dish using DES serum free media (Invitrogen). 15 ng of dsRNA was added directly to the medium for a final concentration of 37 nM. Cells were agitated briefly and then incubated for 30 minutes at room temperature before addition of 2 ml of 1X Schneider cell media. Cells were incubated for at least 4 days before analysis to allow for protein turnover.

Treated cells were analyzed for the presence or absence of dSir2 protein using Westerns as described previously (above). Chromosomal spreads were prepared as previously described [102]. Briefly, cells were placed in a hypertonic KCl solution, fixed in MeOH and then dropped onto slides. Slides were stained with propidium

iodide (0.3 μg/ml), cover slipped, and viewed under UV fluorescence with FITC filters (Zeiss).

Chapter III

RESULTS

Part I- Molecular Characterization of *dSir2*

Cloning of *dSir2*.

Dr. Yang Chen, a post doctoral fellow in the lab, initially identified two partial clones of *dSir2* in a two-hybrid screen using *dCBP* as bait [95]. I used one of the partial clones to isolate full-length clones from cDNA and genomic libraries. To define the transcription start site, I used primer extension assays and generated a 340 bp product that was approximately 150 bp longer at the 5' end than my longest cDNA clone (Figure 1). I also found that the most 5' *dSir2* EST fragment in Fly Base is 144 bp longer at the 5' end than my cDNA clone (GenBank Accession number AA941691). Taken together, these data suggest that *dSir2* transcription begins at the 5' end of the EST fragment. I used sequence from both sources to construct a contiguous sequence that is 3,839 bp (Figure 2). Alignment of genomic and cDNA sequences revealed a 200 bp intron that is excised after bp 1644 in the cDNA. The open reading frame of 2,463 bp begins with an ATG (bp 440) and ends with a TAA (bp 2903). The predicted protein is 821 aa, a molecular weight of 92 kD, and an

acidic pI of 4.5. The conserved "core domain" that defines the SIR2 family, and is sufficient for deacetylase activity, is highlighted in Figure 2 (Q220-S479).

Sequencing of the 5' end of the genomic clone revealed another open reading frame in the direction opposite of the *dSir2* gene (Figure 3). The predicted amino acid sequence of the open reading frame is similar to the DnaJ bacterial chaperonin protein [103]. I refer to the dDnaJ homologue as dDnaJ-H (H for Homologue).

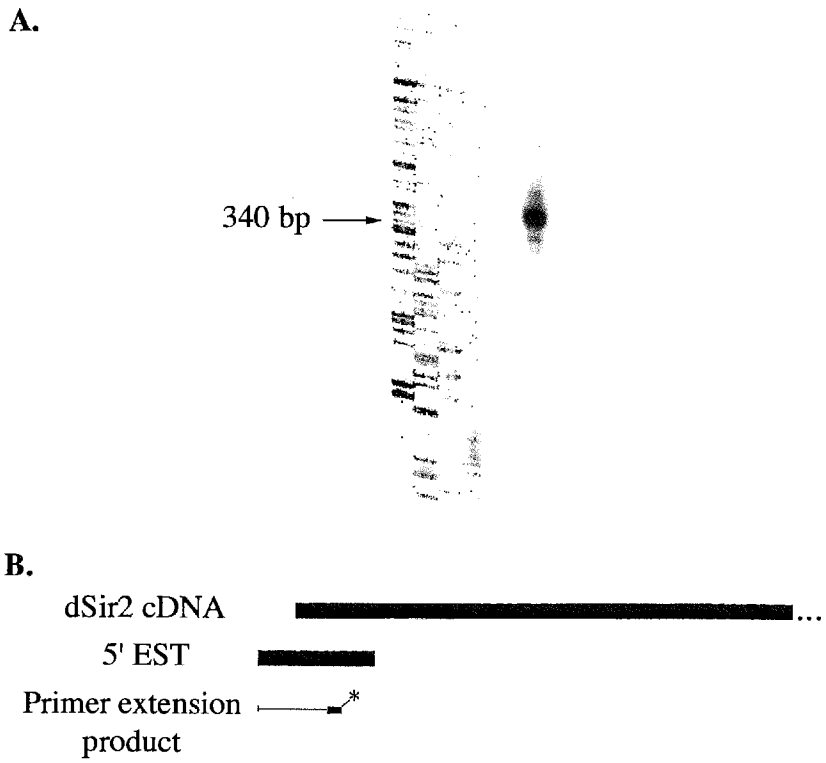
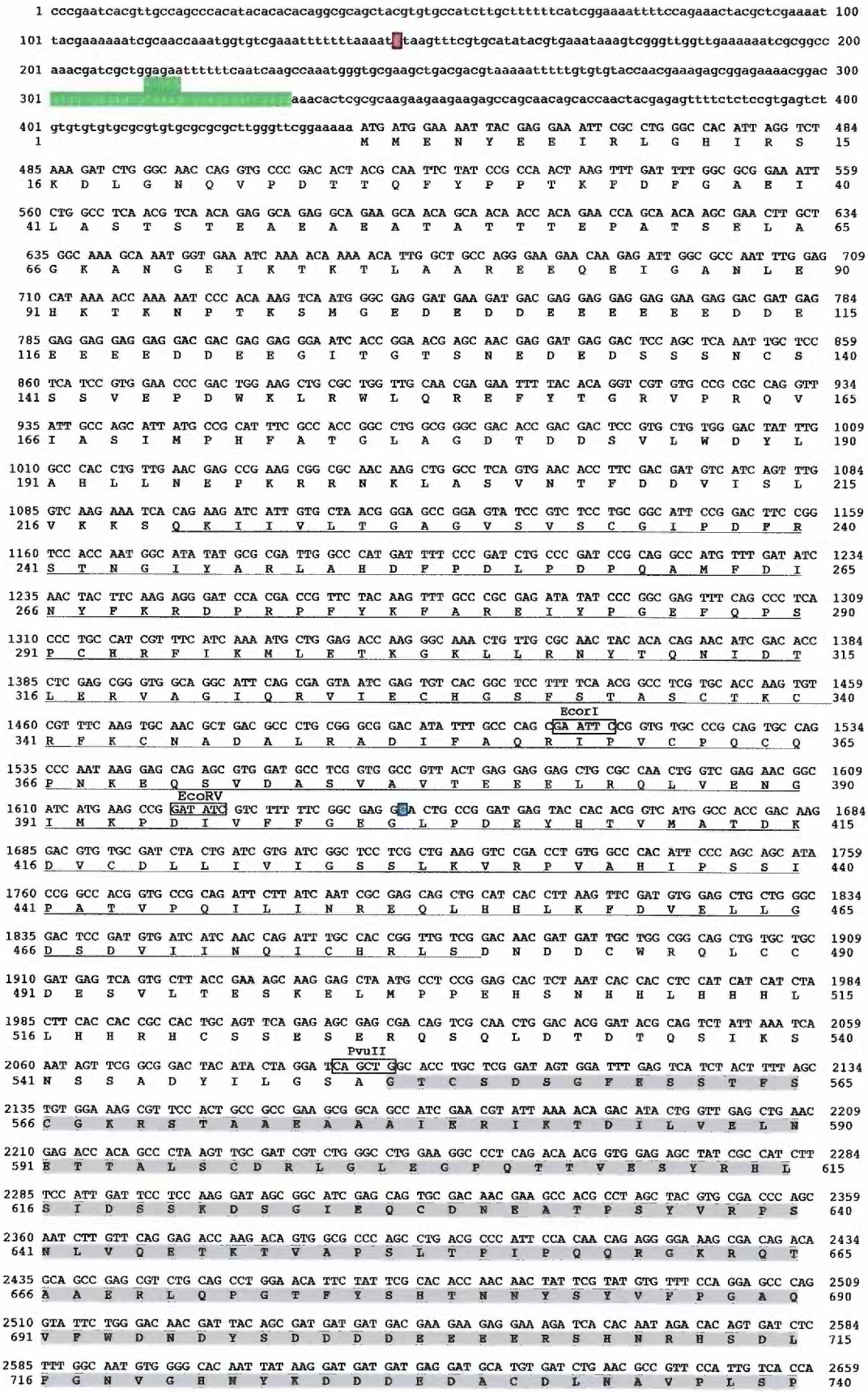


Figure 1. Primer extension defines the 5' end of dSir2. **A)** A primer extension product is shown on an acrylamide gel next to a sequencing reaction. The primer extension product was generated using a radio-labeled oligonucleotide 188 bp (oligo 065, Figure 2) from the 5' end of my longest cDNA clone. The reaction product was approximately 340 bp in length, 152 bp longer (at the 5' end) than my cDNA clone. This potential transcriptional start site corresponds to the start site identified by 5' EST fragments in GenBank. **B)** Schematic diagram showing the relationship between my cDNA clone, the 5' EST fragment and the primer extension product. The top bar represents the cDNA clone. The middle bar, representing the 5' EST fragment, extends further 5' than the cDNA clone. The bottom bar marked by the * represents the radio-labeled fragment produced in the primer extension assay. The 5' end of the EST fragment corresponds to the length of the primer extension product.


EcoRI
EcoRV
PvuII

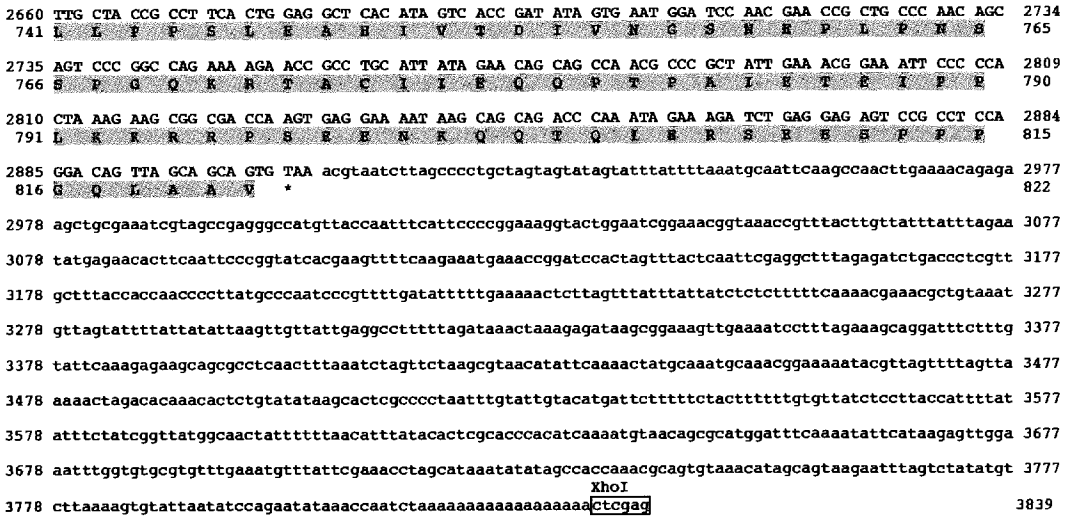
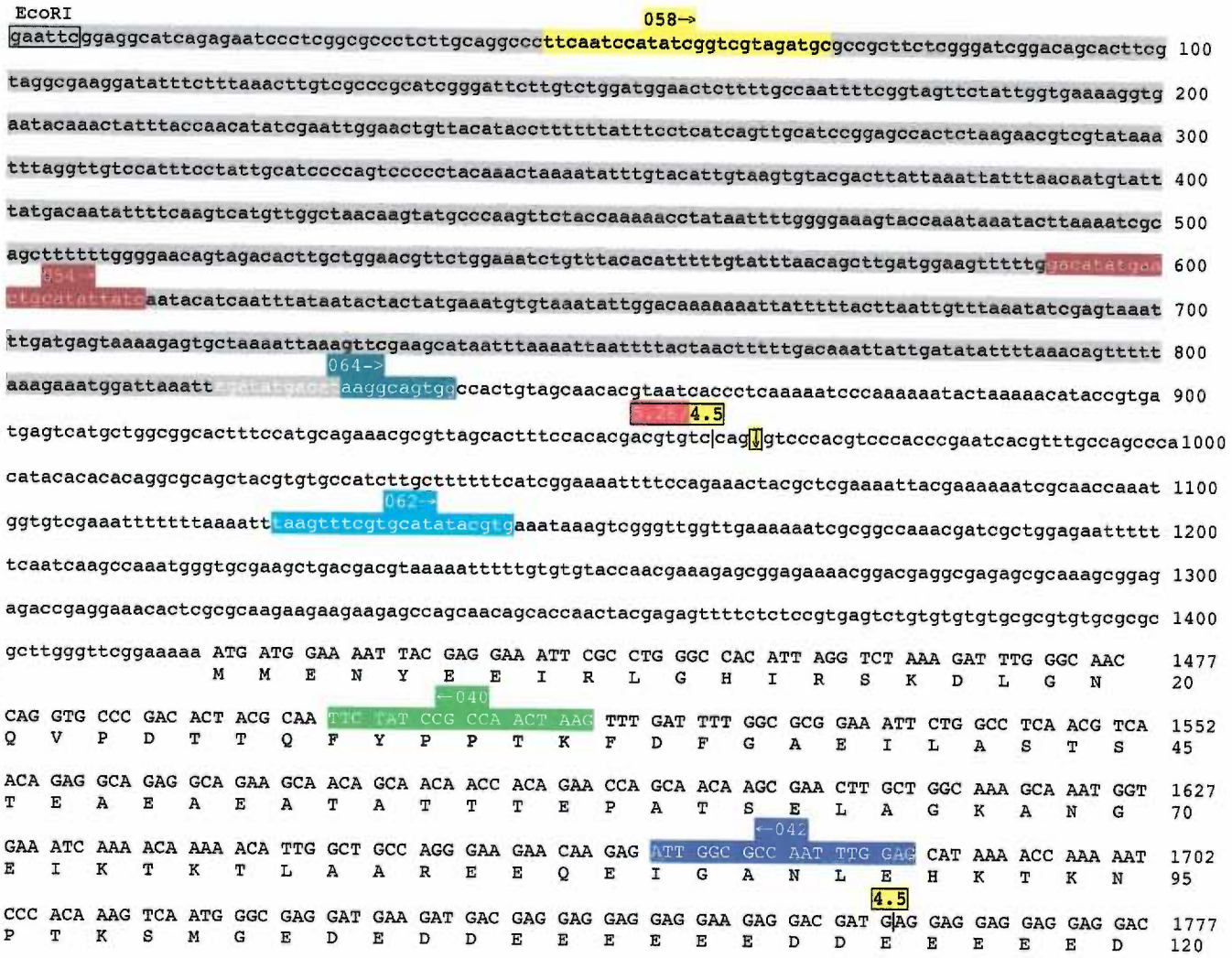


Figure 2. *dSir2* cDNA and predicted amino acid sequence. The red letter M (bp145) denotes the 5' end of the longest cDNA clone obtained from the cDNA library screen. The sequence preceeding the M was obtained from the EST sequences found in FlyBase. Oligonucleotide 065 (bp 301-331), shown in grey, was used in primer extension assays (see Figure 1). The blue letter G marks the intron-exon boundary for the 200 bp intron. The underlined sequence (Q220-S479) reveals the conserved SIR2 "core domain". The dSir2 antibody (described in Figures 8 and 9) is directed against the carboxy-terminal fragment, colored gray (G552-V821).


EcoRI
058→
064→
062→
←040
←042
4.5
4.5

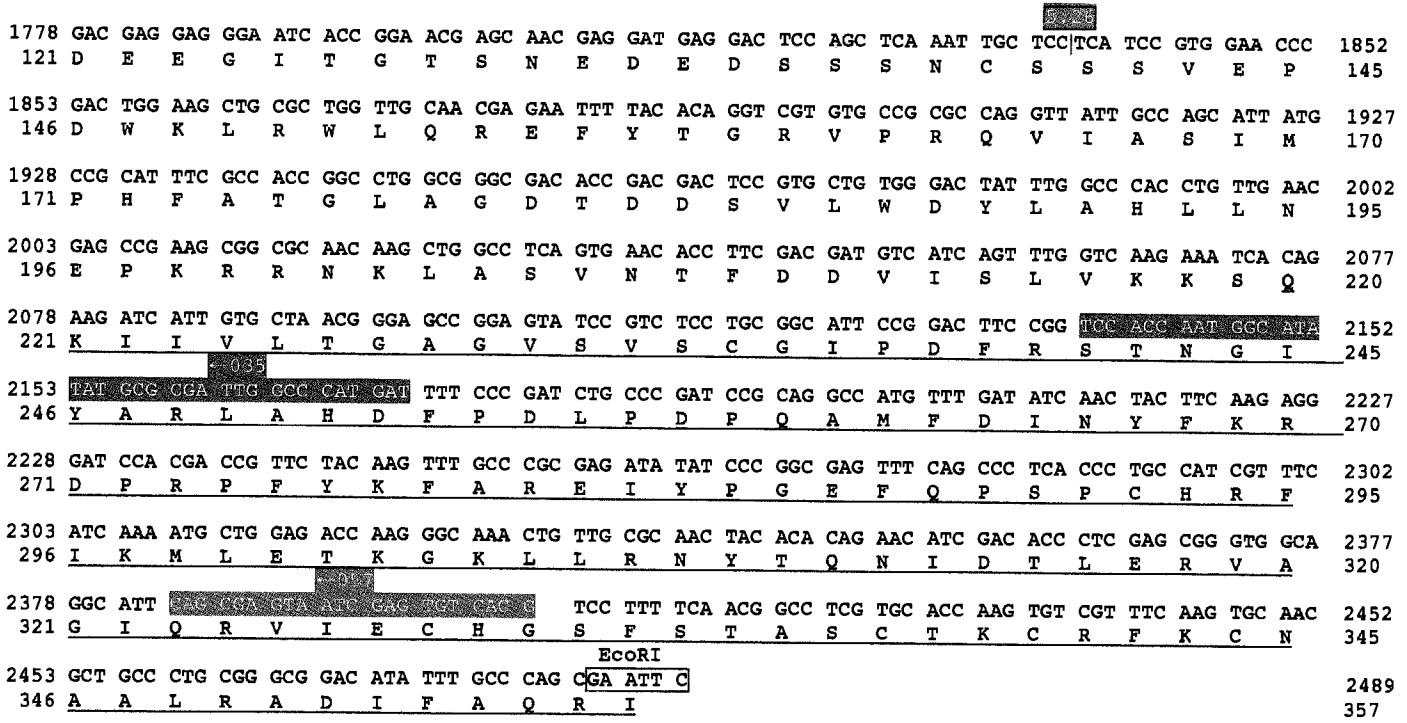


Figure 3. dSir2 genomic sequence. The nucleotide sequence of an EcoRI fragment from the *dSir2* genomic clone is shown above. The open reading frame of the neighboring gene, *DnaJ-H*, is shown in gray (ATG begins at bp 829). The *dSir2* open reading frame is marked by capital letters. The insertion site of the P-elements in strains *l(2)05327* and *l(2)K14513* is marked with an arrow (⇩). The 5.26 and 4.5 deletions share the same 5' boundary (bp 962), as indicated by the boxed text (5.26 4.5). The 3' boundaries are similarly marked at bp1760 (4.5) and bp 1839 (5.26), respectively. The oligonucleotides used to characterize 4.5 and 5.26 (see Figure 5) are in color and are numbered. The SIR2 "core domain" is underlined.

Generation of *dSir2* mutant chromosomes.

Dr. Smolik mapped the *dSir2* gene to the left arm of chromosome 2 at position 34A 5,6 by polytene chromosome *in situ* hybridization (data not shown). Based on this cytologic location, I obtained partially characterized P-element strains from the Bloomington Stock Center. Each strain reportedly contains a single P-element inserted into the fly genome and the P-element location in the genome has been roughly mapped. Using genomic Southern blotting (Figure 4) and plasmid rescue, I identified three P-element strains that have P-elements inserted in the 5' nontranscribed region of *dSir2*: *l(2)05327* (ry^+), *l(2)K14513* (w^+), and *l(2)K07223* (Figure 3). I generated partial deletions of *dSir2* from *l(2)05327* (ry^+) and *l(2)K14513* (w^+) using a standard P-element-mediated mutagenesis strategy [104]. P-elements are small transposons—mobile DNA sequences with 31 bp inverted repeats on either end. The complete transposon encodes a functional transposase and is autonomous (competent for insertion/excision). Many naturally occurring strains contain P-elements, however, not all are functional because of mutations within the transposase or because of trans- acting repressors. Engineered laboratory strains used in mutagenesis strategies contain either the inverted repeats with added functional sequences (i.e. phenotypic markers, bacterial plasmid DNA) or a functional transposase. When the engineered P-element and transposase are brought together through simple crosses, the P-elements are mobilized and "hop" around the genome, causing germ line dysgenesis. Not infrequently, errors are made during the mobilization process and wild-type sequences flanking the P-element are excised

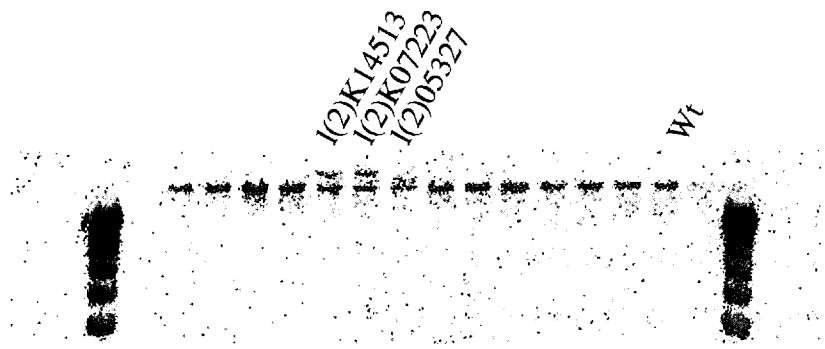


Figure 4. Southern Blot of potential dSir2 mutant P-element strains. Genomic Southern blotting reveals three strains that have a P-element inserted near the *dSir2* gene. Genomic DNA was isolated, digested with XbaI, electrophoresed on an agarose gel, and transferred to nylon. A radio-labeled *dSir2* probe hybridized to a single fragment in the sample from wild-type (Wt) DNA. However, the probe hybridized to two fragments in the samples from *l(2)K14513*, *l(2)K07223*, and *l(2)05327*, indicating insertion of the P-element near the *dSir* gene. rescued and sequenced. The P-elements were found to insert into the 5' untranslated region of *dSir2*.

along with the P-element, leaving a deletion. I took advantage of this system to generate hundreds of deletion strains, identified by loss of the P-element marker gene that controls eye color. Using oligonucleotides that flank the P-element (Figure 3), I screened approximately 100 *l(2)K14513* deletion strains by PCR, leading to the identification of one deletion mutant, $dSir2^{4.5}$, that left *DnaJ-H* sequences intact (Figure 5A). To better define the extent of the deletion, I performed PCRs using internal *dSir2* oligos (position of oligos are shown in Figure 3). When I performed PCRs using $dSir2^{4.5}$/+ genomic DNA and oligos 058, 054, 057 or 035, I found that two bands amplified—representing the mutant and wild-type versions of *dSir2* (Figure 5B). However, when I used oligos 062,040 or 042 only one band—representing the wild-type copy— amplified (Figure 5B). These results show that the region specific to oligonucleotides 062, 040, and 042 is deleted in $dSir2^{4.5}$. I sequenced the 4.5 mutant PCR product and confirmed that the deletion is 798 bp in length (bp 962-1760 of genomic sequence, Figure 3). The PCR product from the mutant DNA is only 200 bp smaller than the wild type; this apparent contradiction is resolved by the fact that approximately 600 bp of the P-element remains within the gene.

I also screened approximately 40 *l(2)05327* deletion chromosomes as above, and identified one mutant, $dSir2^{5.26}$ (Figure 5C). To define the extent of this deletion, I performed PCRs using internal oligos as above (Figure 5D). Coincidentally, the same region—specific for the oligonucleotides 062, 040, and 042—was also deleted in $dSir2^{5.26}$ mutant. I sequenced the 5.26 mutant PCR product and found that the deletion removes 877 base pairs of *dSir2* (bp 962-1939 of genomic sequence, Figure 3) and retains about 300 bp of P-element DNA.

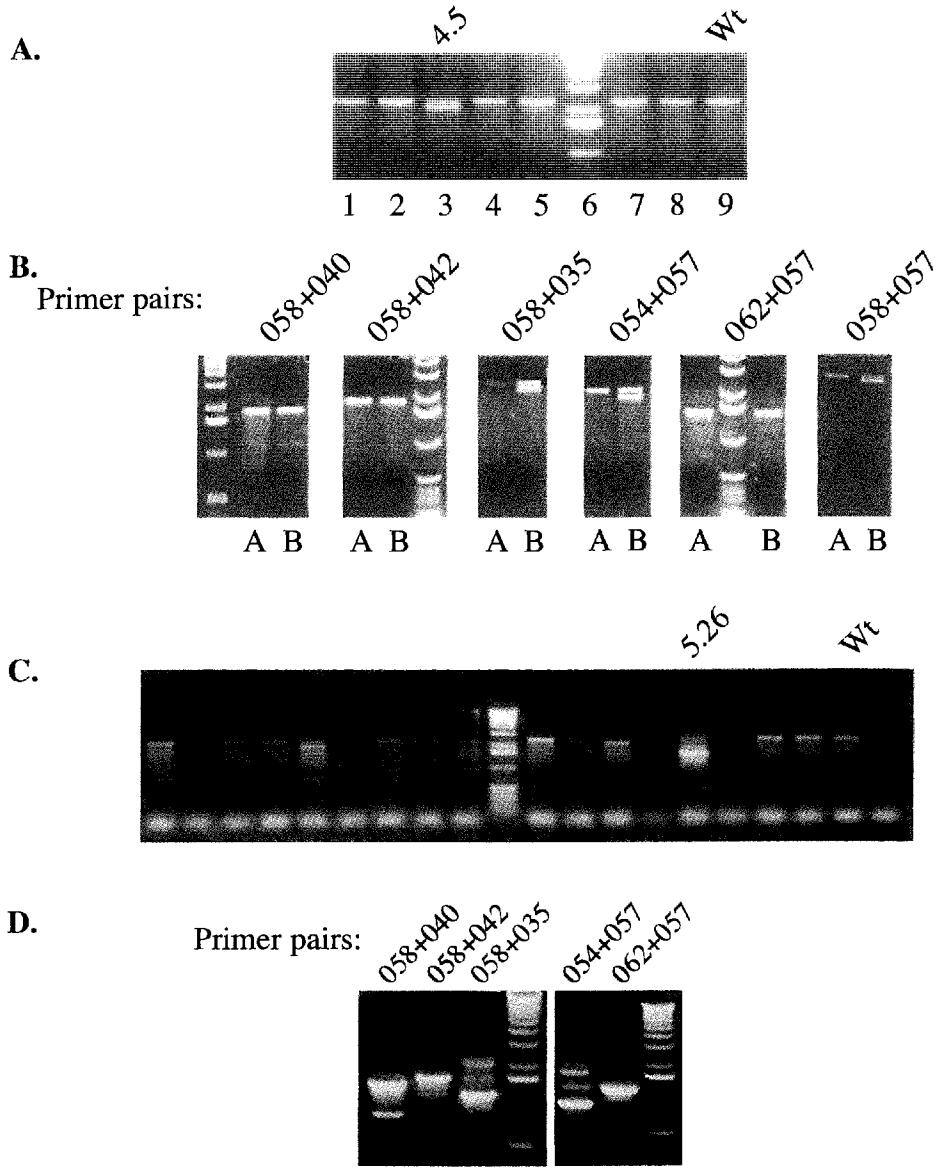


Figure 5. PCR screen to detect *dSir2* mutations **A**) Ethidium bromide-stained agarose gel showing PCR products. Genomic DNA from the *l(2)KI4513/+* hop-out strains was purified and then amplified using oligos 058 and 057 (see Figure 3). Amplification of DNA from the wild-type (Wt) chromosome yielded the expected 2.3 kb product (lane 9). Both the wild-type band and a slightly smaller band of approximately 2.2 kb were detected in DNA from hop-out strain, *4.5/+* (lane 3). Lane 6 contains 1 kb marker DNA. **B)** To better characterize the deletion from the *4.5/+* strain, different pairs of oligos (shown in Figure 3) were used to amplify DNA from either wild-type (lane A) or *4.5/+* (lane B). If the oligo sequence is not deleted, the reaction will yield both the wild-type band and the smaller, mutant band. If the oligo sequence is missing from the gene, the reaction will yield only the wild-type band, as seen when oligos 062, 040, and 042 are used. **C**) PCR screen of the *l(2)05327/+* hop-outs. A mutant band is detected in DNA from *5.26/+* (lane 15) **D**) Using oligos as discussed in B, *5.26* was characterized; oligos 062, 040, and 042 are deleted in this mutant as well.

Expression of *dSir2* transcript during development.

To determine the size, level and expression pattern of *dSir2* transcript, I analyzed *dSir2* mRNA using Northern blots and *in situ* RNA hybridization assays. Because many of the important developmental events in Drosophila occur within the first 24 hours, I collected embryos at different time points within 24 hours of fertilization. In Northern blots, the *dSir2* anti-sense riboprobe hybridized to a 4 kb transcript throughout embryogenesis (Figure 6A). The *dSir2* transcript is first detected in 0-2 hour embryos, indicating that these transcripts are maternally derived. Expression of *dSir2* decreases during cellular blastoderm and gastrulation (2-4 hour) and then increases during germ band elongation and morphogenesis (4-8 hour). RNA from $dSir2^{5.26}$/ $dSir2^{4.5}$ embryos does not hybridize to the *dSir2* probe in Northern blots (Figure 6B).

In situ RNA hybridization experiments in whole mount embryos show that *dSir2* transcript is expressed throughout the embryo in all stages of development (Figure 7A,B, C). No signal is detected in embryos probed with the sense control probe (Figure 7D). There is no *dSir2* signal in the $dSir2^{5.26}$/ $dSir2^{4.5}$ embryos (Figure 7E); wild-type embryos were included in the experiment as a positive control (Figure 7F). Both $dSir2^{5.26}$/ $dSir2^{4.5}$ and wild-type embryos probed with antisense *dDnaJ-H* show expression of the *dDnaJ-H* transcript (Figure 7G, H). This indicates that the deletion does not destroy *dDnaJ-H* expression.

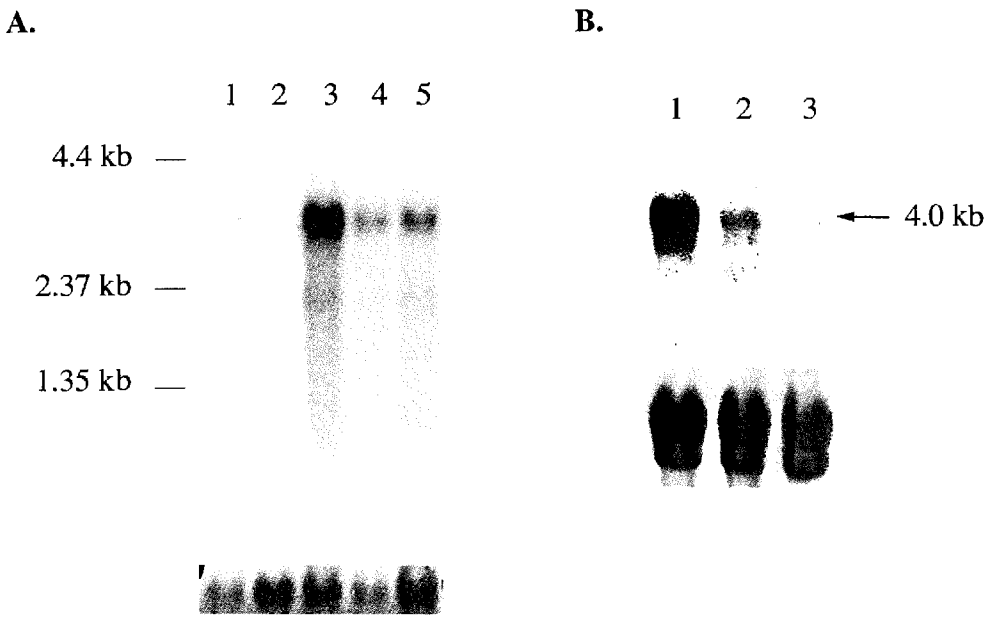


Figure 6. Northern analysis of *dSir2* transcript A) Analysis of the *dSir2* transcript during development. The top panel shows a blot containing mRNA from wild-type embryos collected at different time points in the first 24 hours: lane 1, 0-2 hours; lane 2, 2-4 hours; lane 3, 4-8 hours; lane 4, 8-12 hours; lane 5, 12-24 hours. The blot was probed with radiolabeled anti-sense *dSir2*. The lower panel shows the same blot, stained with methylene blue (highlighting rRNA), to show relative amounts of RNA in each lane. The position of the RNA size marker bands are indicated on the left. The amount of the *dSir2* transcript varies at different stages of development.
B) Analysis of *dSir2* mutants. The top panel shows a blot containing total RNA, probed with radiolabeled anti-sense *dSir2* probe. Lane 1 contains RNA from wild-type embryos, lane 2 contains total RNA from *4.5/CyO* and *5.26/CyO* embryos and lane 3 contains RNA from *4.5/5.26* embryos. The lower panel shows the same blot stained with methylene blue to show the relative amounts of RNA in each lane. Relative to wild-type, the 4.0 kb *dSir2* transcript is decreased in heterozygous mutant embryos (lane 2) and is absent in homozygous mutant embryos (lane 3).

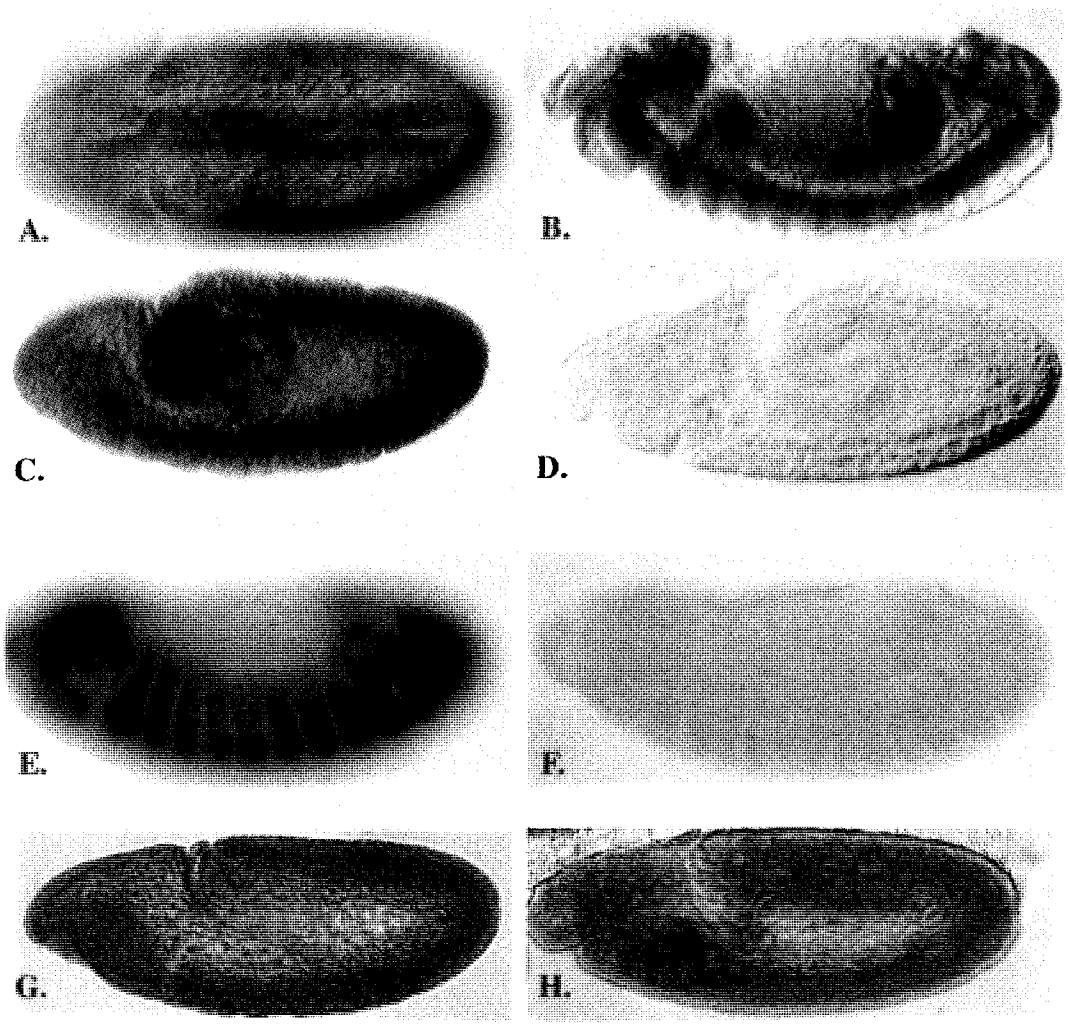


Figure 7. ***In Situ*** **analysis of** ***dSir2*** **transcript A-C)** Wild-type embryos are hybridized to DIG-labeled, anti-sense *dSir2* probe. **D)** Wild-type embryo hybridized to the *dSir2* sense control probe. **E,F)** Comparison of *dSir2* expression in wild-type **(E)** and *4.5/5.26* mutant embryos **(F)**. **G,H)** Comparison of *DnaJ-H* expresssion in wild-type **(G)** and *4.5/5.26* mutant embryos **(H)**. Embryos are stained with DIG-labeled, antisense *DnaJ-H* probe.

Characterization of dSir2 protein.

I generated a dSir2 polyclonal antibody by injecting GST-dSir2 fusion protein into rats. The fusion protein includes the C-terminal fragment of dSir2 (G552-V821), shown in Figure 2. This region shares no homology to other dSir2s, ensuring that the antibody will not cross-react with other Sir2 homologues in Drosophila. I initially purified the GST-dSir2(G552-V821) fusion protein on a GST column (Figure 8A) and then further purified it using gel purification with the aid of the ChromoPhor gel purification kit from Promega as described in the Chapter II (Figure 8B). The purified protein was sent to Pocono Rabbit farm for injection into rats.

I tested serum from dSir2-injected rats using Western blots containing Drosophila Kc cell extract and bacterially produced full-length dSir2 (Figure 9B). The predicted molecular weight of dSir2 is 92 kD, which is considerably smaller than the 125 kD band seen on immunoblots. The discrepancy between the predicted and observed size is most likely a reflection of the acidic pI rather than a post-translational modification because bacterially expressed full-length dSir2 is also 125 kD (Figure 9B). When used in Western blots of whole animal tissue, the antibody recognizes many bands (9A). However, the 125 kD band (aligned with the Kc cell extract run on the same gel) is absent in the *dSir2* mutant animal (Figure 9A).

Using Kc cells, I also show that my antibody is specific; after the antibody has been incubated with GST-dSir2, but not GST alone, it no longer recognizes the 125 kD band (Figure 9B).

In whole mount embryos, I detected dSir2 protein throughout embryogenesis. Unlike some of the mammalian Sir2 proteins, dSir2 is primarily nuclear (Figure 10A

and 10C, first row). However, at certain points during development (i.e. cellular blastoderm), dSir2 is excluded from the nucleus (Figure 10A and10C, row 2). Late in embryogenesis, dSir2 is both cytoplasmic and nuclear (Figure 10A, rows 5,6). dSir2 is present during syncitial and cellular blastoderm (Figure 10A, rows 1, 2), at high levels in the nuclei surrounding the morphogenic furrows during gastrulation (Fig. 10A, row 3) and in the germ band (Fig. 10A, row 4). In the later stages, expression is primarily detected in the CNS (Fig. 10A, row 5). Taken together, these data show that dSir2 is regulated not only in a temporal and tissue-specific fashion but also at the level of sub-cellular localization. The $dSir2^{4.5}/dSir2^{5.26}$ embryos do not express dSIR2 at any stage tested (Figure 10B).

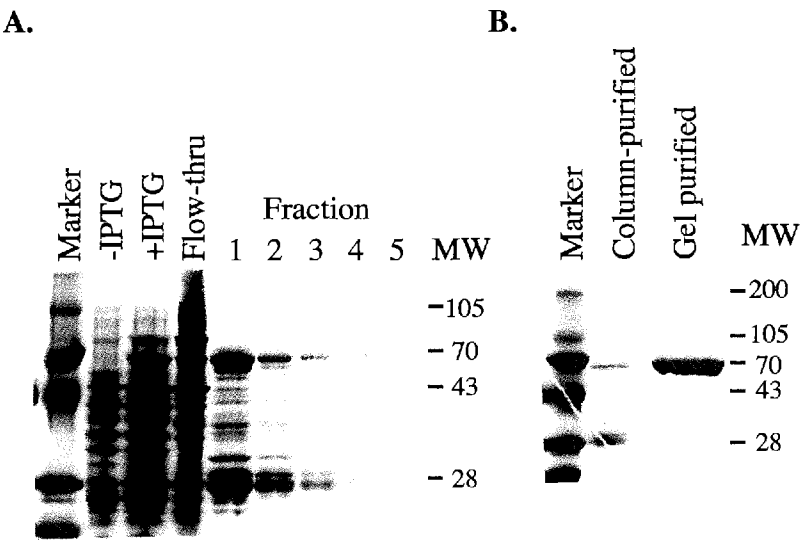


Figure 8. Purification of GST-dSir2 **A)** A Commassie-stained SDS-PAGE gel showing the purification of GST-dSir2 (G552-V821) on a Glutathione agarose column. The PvuII-XhoI fragment of *dSir2* was subcloned into pGEX-4T3 and used to produce a GST-carboxy-terminal fragment of dSir2 in bacteria. The first lane contains crude extract from uninduced bacteria. The second lane contains extract from IPTG-induced bacteria. The third lane contains the flow-through fraction and the fourth through eighth lanes contain the fractions (1-5) collected after addition of glutathione to the wash buffer. **B)** A commmassie stained SDS-PAGE gel showing gel-purified GST-dSir2. The first lane contains an aliquot of GST-dSir2 (fraction 2) before gel purificaiton. The second lane contains GST-dSir2, gel-purified using the Promega Chromophor™ system. The purified protein was sent to the Pocono Rabbit Farm for injection into Sprague-Dawley rats.

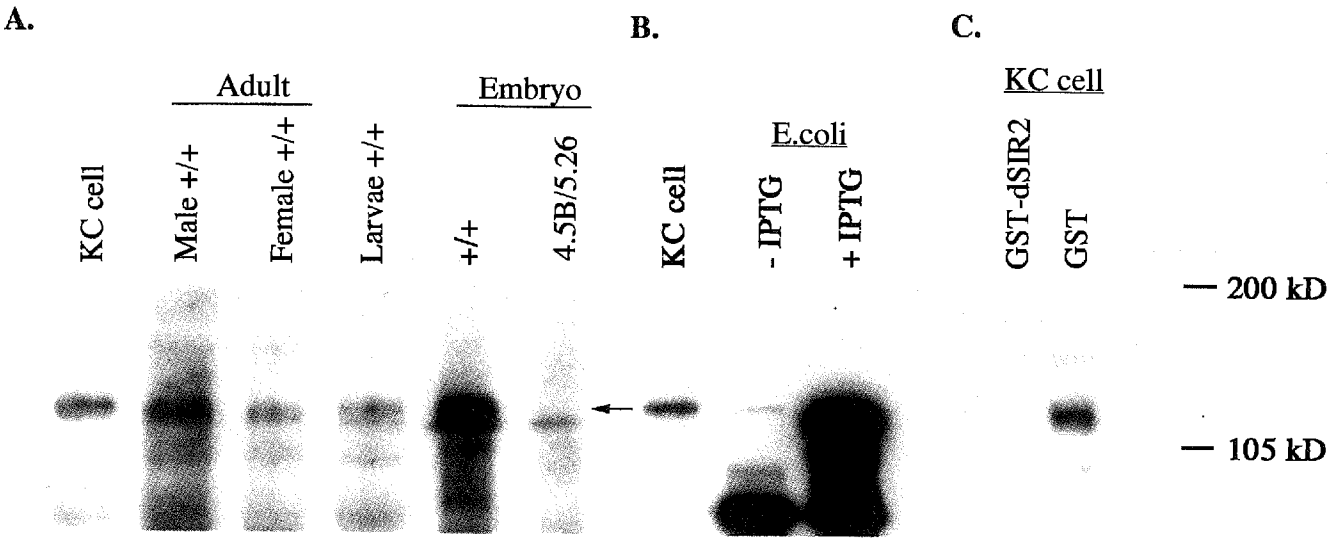


Figure 9. Western Analysis of dSir2 protein **A)** Many Drosophila tissues produce dSir2. The first lane contains Drosophila Kc cell extract. For the remaining lanes, 10 animals were homogenized in 200 μl buffer and 20μl extract was loaded per lane. A 125 kD band is present in Kc cell, wild-type adult (male and female), larval, and embryo extract. The 125 kD band is absent in the *4.5/5.26* mutant embryos . A smaller, non-specific band is present in both wild-type and mutant embryo extracts. **B)** Western blot analysis of bacterially produced, full-length dSir2. The first lane contains Kc cell extract, the second and third lanes contain extract from uninduced and induced, dSir2-transformed bacteria, respectively. dSir2 protein is present in KC cell extract, in small quantities in the uninduced sample and in excess in the induced sample. **C)** Blocking dSir2 antibody with dSir2 antigen. To show that the antibody recognizes dSir2 specifically, I mixed the antibody with an excess of either GST-dSir2 (fist lane) or GST alone (second lane) . When mixed with GST-dSir2, the antibody no longer recognizes the 125 kD band.

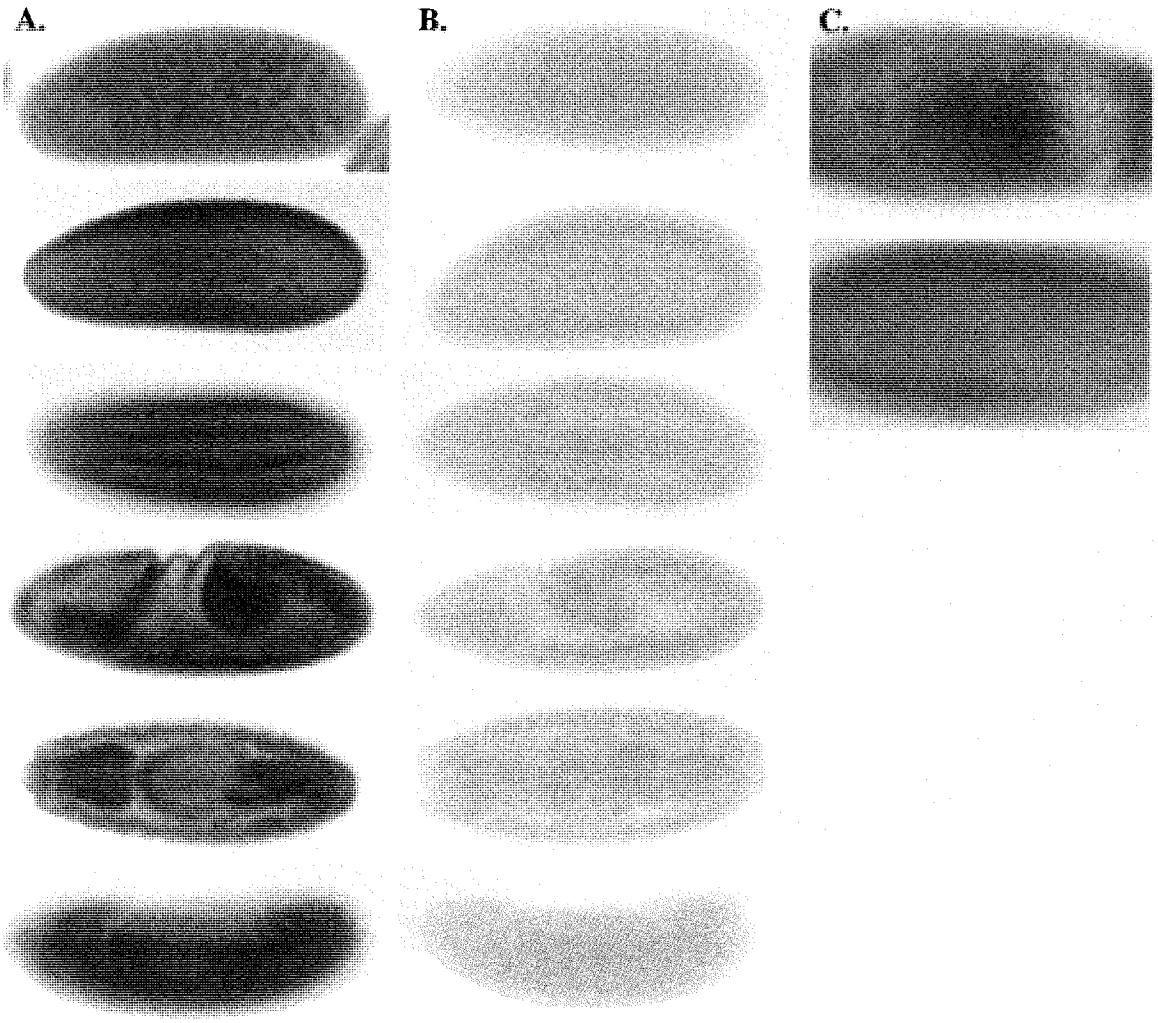


Figure 10. ***In Situ*** **Analysis of dSir2 protein** **A)** Wild-type embryos stained with anti-dSir2 antibody. The top row shows an embryo during syncitial blastoderm. The second row shows an embryo at cellular blastoderm; dSir2 is excluded from the nuclei which are at the periphery of the embryo. In the third row, the morphogenic furrow is in focus, highlighting nuclear dSir2 staining. During germ band extension stage (fourth row), dSir2 is seen in all three germ layers and is nuclear. Row five highlights the developing CNS, where dSir2 is both cytoplasmic and nuclear. The bottom row shows late expression (after segmentation). **B)** *4.5/5.26*mutant embryos are probed with the same antibody as in **A**; no specific staining is detectable. **C)** Wild-type embryos stained with anti-dSir2 are shown at higher magnification to highlight the nuclear staining (top row) and exclusion from the nucleus (bottom row).

dSir2 protein localizes to both heterochromatin and euchromatin.

Based on the affect of Sir2 mutations on PEV in yeast and the histone deacetylase activity of sirtuins, I hypothesized that *dSir2* directly modifies chromatin structure. From this, I predicted that dSir2 protein would localize to chromatin, including heterochromatin. I tested this prediction by labeling polytene chromosomes with anti-dSir2 antibody. I found that dSir2 binds to discrete bands within eukaryotic regions and is also associated with heterochromatin (Figure 11 A, B). To show the specificity of the anti-dSir2 antibody, I stained *dSir2* mutant chromosomes with the anti-dSir2 antibody. Figure 11C shows a representative nucleus stained with anti-dSir2 antibody. There is no detectable dSir2 staining. As a positive control, I labeled the *dSir2* mutant chromosome with anti-dCBP antibody (Figure 11D). Given that dCBP and dSir2 interact in the two-hybrid system, I tested whether they bind to the same sites on polytene chromosomes. I double-labeled wild type polytene chromosomes with anti-dCBP and anti-dSir2 antibodies and found that the signals do not overlap significantly (data not shown).

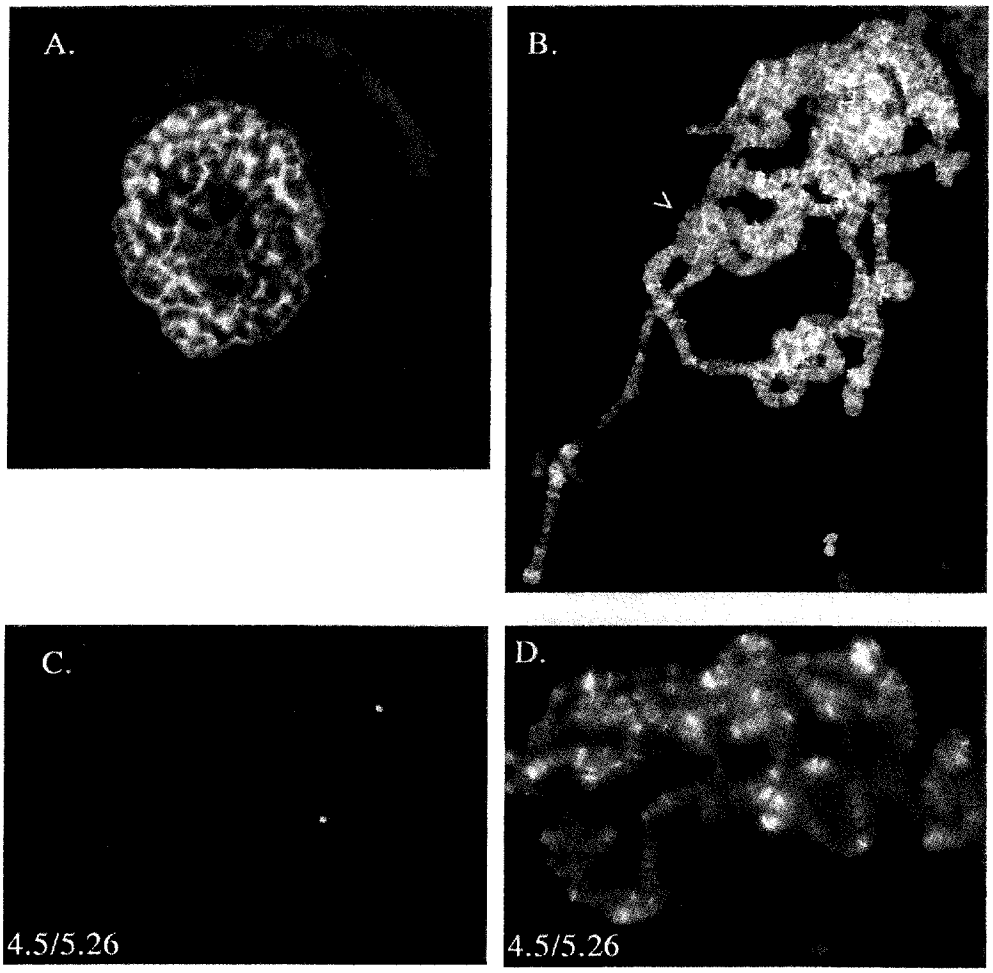


Figure 11. dSir2 protein localizes to chromatin A,B) Salivary gland tissue from wild-type larvae showing dSir2 immunostaining with rhodamine. **A)** whole mount salivary gland cell shows specific nuclear staining. **B)** dSir2 localizes to both heterochromatin (arrowhead) and to discreet bands in euchromatic regions of polytene chromosomes. **C,D)** A single salivary gland nucleus from the *4.5/5.26* mutant is double-labeled with anti-dSir2 (rhodamine) and anti-dCBP antibody (FITC). **C)** dSir2 immunostaining (rhodamine) is absent in the mutant nucleus. **D)** dCBP immunostaining (FITC) is normal in this nucleus and serves as a positive control for the experiment.

Bacterially expressed dSir2 is a NAD^+-dependent histone deacetylase.

Based on homology to other SIR2 family members, I predicted that dSir2 would have intrinsic NAD^+-dependent deacetylase activity. The Lundblad laboratory purified bacterially expressed-dSir2 and showed that dSir2 released ^{3}H-dpm from acetylated-histone H4 peptide in a NAD^+-dependent manner (Figure 12). The activity was not inhibited by sodium butyrate, consistent with idea that the sirtuins are resistant to inhibitors that the class I HDACs are sensitive to [105]. In contrast, HeLa nuclear extract — known to contain HDAC1 activity — has activity that is both NAD^+-independent and butyrate-sensitive [105]. The Sir2 data are also consistent with a recent study showing that dSir2 overexpressed in S2 cells has intrinsic NAD^+ dependent deacetylase activity [105]

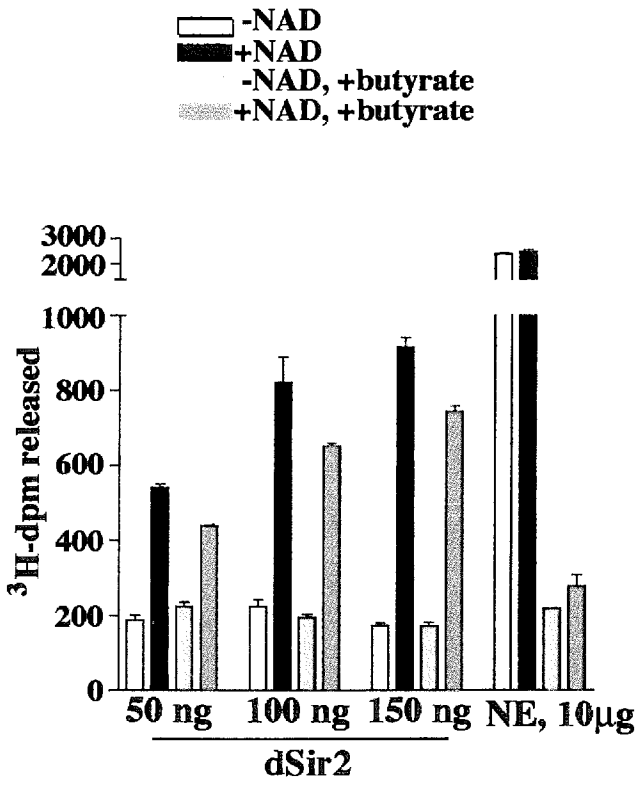


Figure 12. Drosophila Sir2 is an NAD-dependent deacetylase
Release of ethyl acetate extractable 3H-product from 3H-H4 peptide by recombinant dSir2 protein or Hela cell nuclear extract (NE), in the absence or presence of NAD+(500 μM) or sodium butyrate(50 mM). Background counts (in the absence of added protein) extracted were 200 dpm. Error bars represent standard error of the mean (n= 3).

Part II: Genetic and Biochemical Studies of *dSir2*.

dSir2 and lethality.

A recent publication [106] presented data showing that mutations in *dSir2* cause lethality. The *dSir2* mutant used in their experiments was generated from the same parental chromosome I used, *l(2)05327*. I also found that animals homozygous for the *dSir2* mutant chromosome from this parental chromosome ($dSir2^{5.26}/ dSir2^{5.26}$) do not survive beyond embryogenesis. However, it is possible that the lethality seen is not due to the *dSir2* mutation. In other words, the lethality could actually be caused from recessive lethal mutations that are carried on the dSir2 mutant chromosome. Such lethal mutations, which are not uncommon, are called second site lethal mutations. In order to demonstrate that the lethal phenotype was not due to second site lethal mutations on the parental chromosome, I generated a second *dSir2* mutant from a different parental chromosome, *l(2)K14513*. I found that animals homozygous for the second mutant chromosome ($dSir2^{4.5}/ dSir2^{4.5}$) also died early in development. Importantly, however, when I crossed $dSir2^{4.5}/CyO$ males with $dSir2^{5.26}/CyO$ females, the mutant offspring ($dSir2^{4.5}/ dSir2^{5.26}$) appeared in normal numbers (1/3 of the total number of offspring), were normal appearing, fertile and robust. This mutant animal ($(dSir2^{4.5}/ dSir2^{5.26})$ is called a transheterozygote— homozygous mutant for *dSir2* but otherwise heterozygous. By using the transheterozygote ($dSir2^{4.5}/ dSir2^{5.26}$), I was able to distinguish between the effect of second site lethal mutations and *dSir2* mutations. These experiments clearly demonstrate that *dSir2* is not required for viability and has no obvious phenotype in an otherwise wild-type animal.

***dSir2* and lifespan.**

Mutations in ySir2 decrease lifespan and overexpression of Sir2 in yeast and *C.elegans* increases lifespan. To determine whether loss of *dSir2* decreases lifespan, I compared the lifespan of flies with one or no copies of *dSir2* (Figure 13A). A total of 668 *dSir2*-/+ and 638 $dSir2^{5.26}$/ $dSir2^{4.5}$ male flies in eight simultaneous experiments were maintained under standardized environmental conditions. The average median lifespan of the $dSir2^{5.26}$/ $dSir2^{4.5}$ males is 62.53±4.98 days, slightly less than the average median lifespan of the *dSir2*- heterozygous males, which is 71.28±5.01 days (Figure 13). This difference is significant with a p=0.0035. The average mean life span of the $dSir2^{5.26}$/ $dSir2^{4.5}$ males is 85.75±6.27 days while the average mean life span of the *dSir2* heterozygous controls is 91.25±5.23 days. This difference is not significantly different (p=0.0776). Thus, there is a very slight, but significant decrease in average median lifespan, but not in the average mean lifespan in the *dSir2* transheterozygotes, compared to the heterozygotes.

Some definitions of aging describe an organism's loss of ability to handle environmental stressors [107]. To sensitize the lifespan assay, I also compared the lifespan of mutant, heterozygotic, and wild type animals under more stressful environmental conditions. To stress the animals, I delayed the time between "turning over" the vials the animals are housed in. Turning over vials is the act of transferring the flies from an "old" vial into a fresh vial. The longer the flies are kept in a vial, the wetter the food in the vial becomes and the more likely it is that the animals will get stuck in the food. Also, older food is more likely to contaminated organisms such as bacteria or fungi. In the first lifespan experiment, stocks were turned over every other

day. (Figure 13A). In the second lifespan experiment, stocks were turned over every 3-4 days (Figure 13B). The increase in the duration between turning over the vials decreased the viability of the all the animals, mutant and wildtype as shown by the left shift all the curves in the second experiment (Figure 13A and B).

In the second experiment, three genotypes were compared including 555 wild-type controls; 571 *dSir2*-/+ and 551 $dSir2^{5.26}/dSir2^{4.5}$. Four simultaneous curves were again generated. The results of the stress test show that the difference between median and mean life spans of the wild-type (41.42±5.8 and 76±5.7 days respectively) and *dSir2*-/+ (45.01±2.0 and 79±3.8 days respectively) is not significant (p=0.557 and 0.414 respectively). As seen under minimally stressful environmental conditions, the average mean life span of the $dSir2^{5.26}/dSir2^{4.5}$ transheterozygotes (80.5±4.1days) is not significantly different from the wild-type (p=0.2459) and *dSir2*/+ (p=0.6131) genotypes. However, the median life span of the $dSir2^{5.26}/dSir2^{4.5}$ flies (60.96±2.03 days) is longer than the median life spans of the controls and this difference is significant (*dSir2*/+ v. *dSir2*,p=. 0206 and wild-type v. *dSir2*,p=. 0007). Taken together, it is clear that *dSir2* mutations do not decrease lifespan.

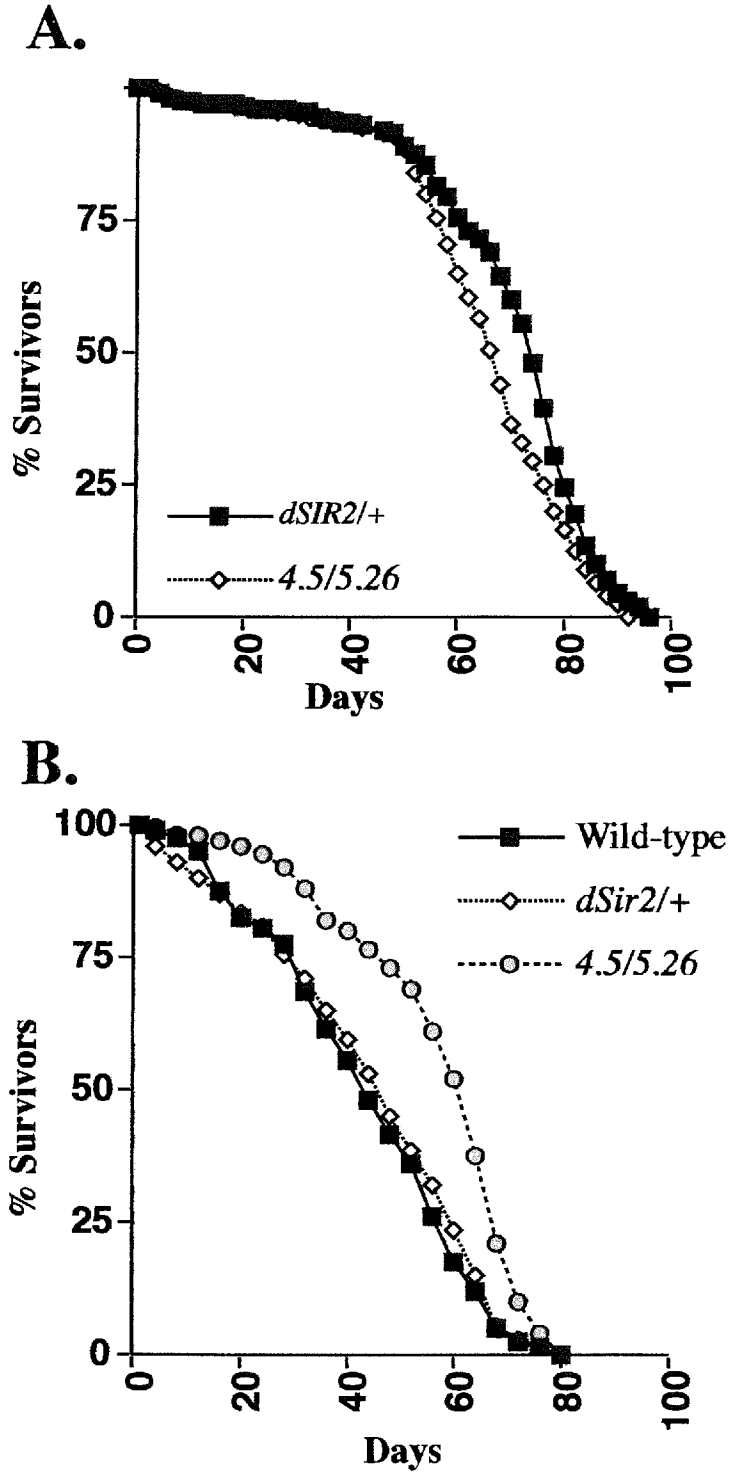
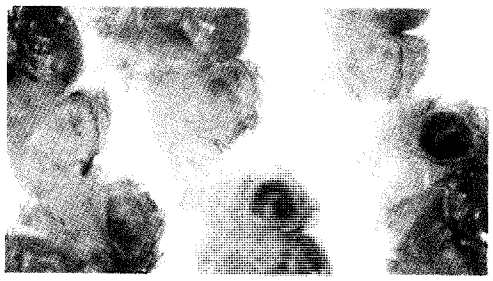


Figure 13. Effect of dSir2 mutations on life-span. **A)** Male flies maintained under minimally stressful environmental conditions (transferring the flies into fresh vials every other day). **B)** Male flies maintained under stress conditions (transferring the flies into fresh vials every third or fourth day).

***dSir2* mutations modify PEV.**

Based on the fact that *dSir2* and ySIR2 are histone deacetylases and that ySIR2 is required for heterochromatin formation, I determined whether mutations in *dSir2* modify the variegating eye color phenotype of the w^{m4} mutant. I found that *dSir2* mutations are recessive suppressors of w^{m4}, causing an increase in red pigment in the eye (Fig. 14A). To evaluate the effect of *dSIR2* mutations on PEV more quantitatively, I sorted the experimental animals into categories based on eye color. Eye pigment assays, which are commonly used to measure eye pigment, were not employed here because the assay is less sensitive and can give variable results depending on head size [98]. As per Sass and Henikoff ([98]), I established five phenotypic ranks of eye pigment; the ranks were assigned without knowledge of the genotype. I then scored the animals in each group for the presence or absence of a dominantly marked second chromosome (*Scotoid (Sco)*). I recognized the heterozygotes by the presence of the dominant marker, *Sco*, which affects the number of bristles on the thorax. Animals without the *Sco* marker are transheterozygotes ($dSir2^{4.5}/dSir2^{5.26}$). Consistent with the qualitative results, I found that the transheterozygotes were disproportionately represented in the darker eye categories compared to heterozygotes (Figure 14C). It is important to note that by using the $dSir2^{5.26}/ dSir2^{4.5}$ transheterozygotes, I am assured that the phenotypes are not due to second site mutations on the $dSir2^{5.26}$ and $dSir2^{4.5}$ chromosomes.

A.



B.

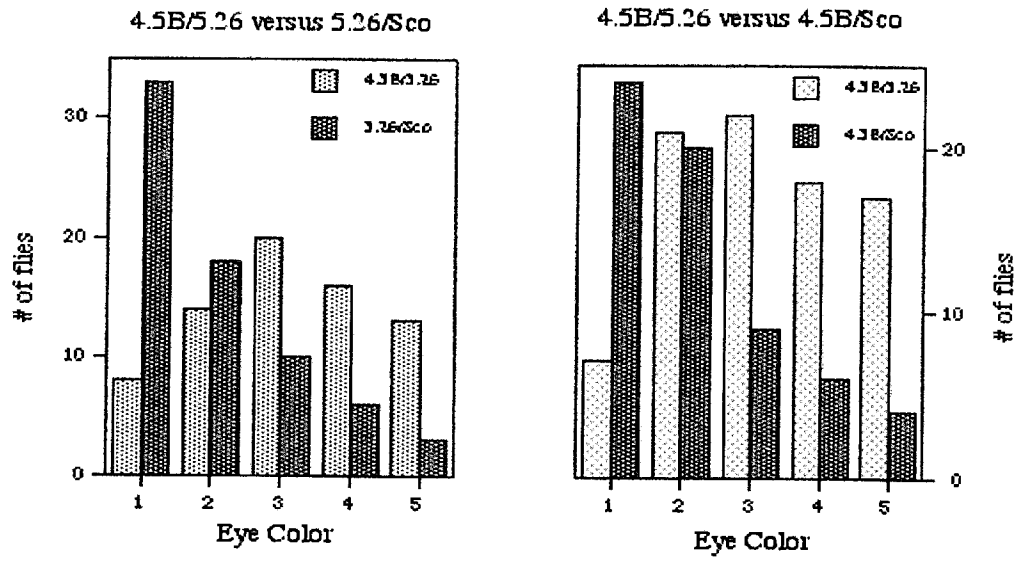


Figure 14. dSir2 mutations suppress PEV A) The animals shown in **A** are *wm4* and have either no copies (*4.5/5.26;* bottom row) or one copy (*5.26/Sco;* top row) of *dSir2*. **B)** This panel shows the effect of *dSir2* and mutations on the distribution of eye color within a population. The flies are sorted into groups based on the amount of red pigment in the eye. For example, in category 1, there is almost no red pigment in the eye; in catergory 5, the eye is nearly completely red. Loss of *dSir2* causes a shift in the distribution of the flies in each category.

dSir2 and dCBP form a complex *in vitro* and *in vivo*.

Based on the dCBP/dSir2 two-hybrid interaction, and the overlap of the distribution of dCBP and dSir2 protein and RNA (Sarah Smolik, unpublished observations), I hypothesized that dCBP and dSir2 interact physically *in vivo*. To test this, I first performed Glutathione S-transferase (GST) pull down assays and showed that GST-dCBP (aa835-1043) but not GST, precipitates radiolabeled, *in vitro* translated carboxy-terminal dSir2 (Figure 15A). To determine whether dCBP and dSir2 interact *in vivo*, I performed co-immunoprecipitation assays using Kc cell extracts. The anti-dSir2 antibody precipitates dSir2 as well as dCBP in the presence of protein L-agarose beads (Figure 15B). These data show that dCBP and dSir2 form a complex both *in vitro* and in Drosophila Kc cells.

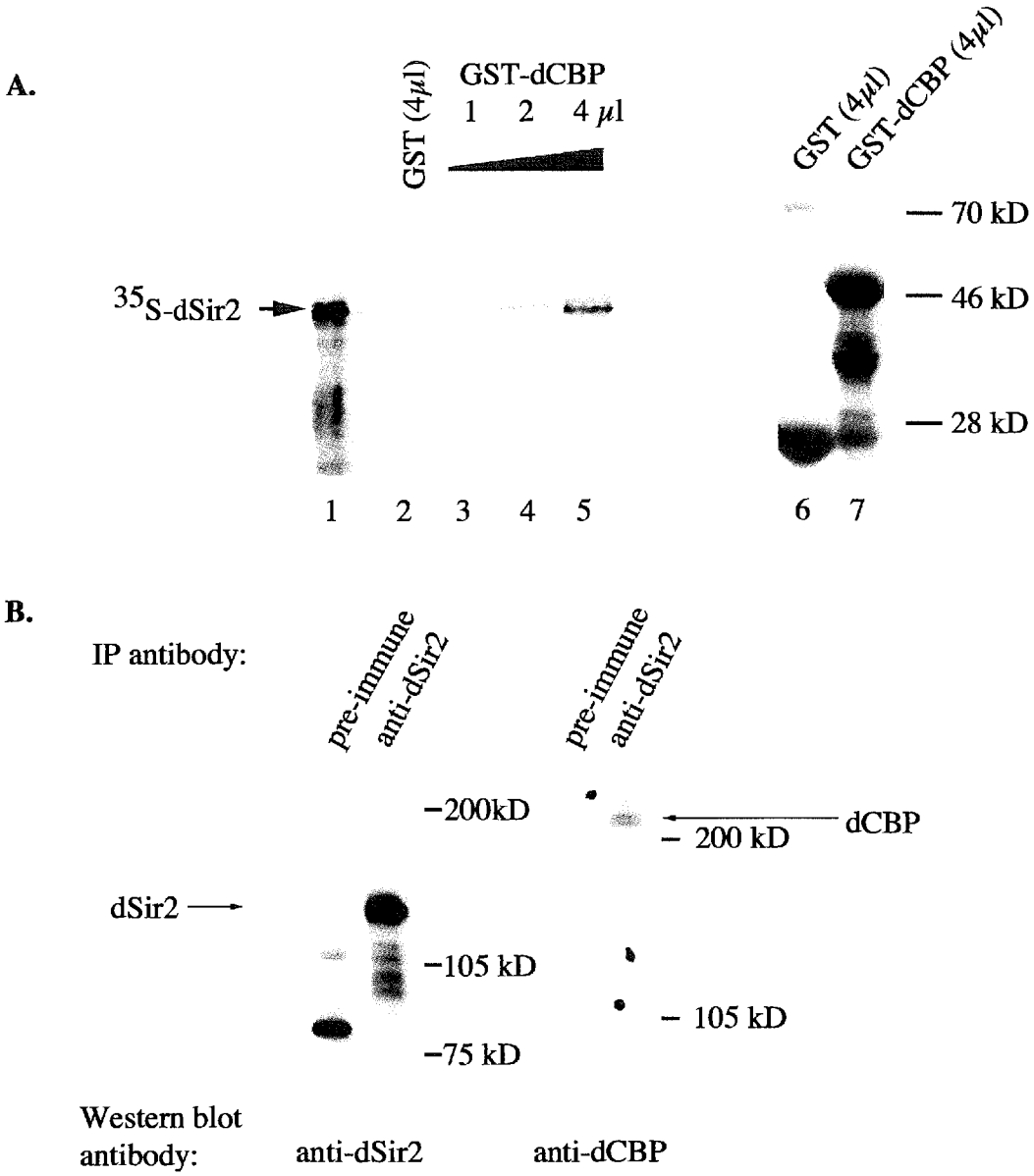


Figure 15. dSir2 and dCBP form a complex in vivo. A) Lanes 1-5 show the results from a GST pulldown assay using the CREB-binding domain of dCBP fused to GST and radiolabeled C-terminal fragment of dSir2 (aa 461-821). Glutathione beads plus GST alone did not precipitate dSir2 but increasing amounts of GST-dCBP precipitated increasing amounts of dSir2. Lanes 6,7 are a Commassie stained SDS-page gel showing relative amounts of protein used in the GST pulldown. In lane 2, 10 microliters of GST was used (as shown in lane 6). In lane 4, B) Immunoprecipitation of dCBP with the dSir2 antibody. dSir2 immune and pre-immune sera mixed with Kc cell extract was precipitated using Protein L-agarose beads, electrophoresed, transferred and probed with either anti-dSir2 or anti-dCBP antibody. Only the anti-dSir2 serum precipitated dSir2 and dCBP.

Part III: Remaining Questions

dSir2 mutations decrease histone H4 acetylation.

Based on dSir2's histone deacetylase activity and ability of *dSir2* mutations to suppress PEV, I predicted that loss of *dSir2* would cause an increase in the level of histone acetylation in embryos. To test this, I used fluorescent microscopy of whole embryos stained with antibodies that specifically recognize the hyperacetylated forms of the lysine residues on histone H4. The antibody was made using a hyperacetylated Tetrahymena N-terminal H4 histone tail peptide. The antibody recognizes tri- and tetra-acetylated histone H4 [108]. Surprisingly, I found that *dSir2* mutant animals had relatively less fluorescent signal compared to wild-type (Figure 16A,B). This suggests that H4 histone acetylation levels were decreased. The Smolik laboratory has previously shown that *dCBP* mutations also decrease the level of histone acetylation, at lysine 8 of histone H4 [44]. To test if *dCBP* and *dSir2* mutations affect the same lysine residue, I used an anti-acetyl-lysine 8 (H4) antibody to stain wild type and *dSir2* mutant embryos [32]. Similar to the results from the *dCBP* mutants, I found that the level of acetylation was decreased in the *dSir2* mutant embryos compared to wild-type embryos (Figure 16C, D).

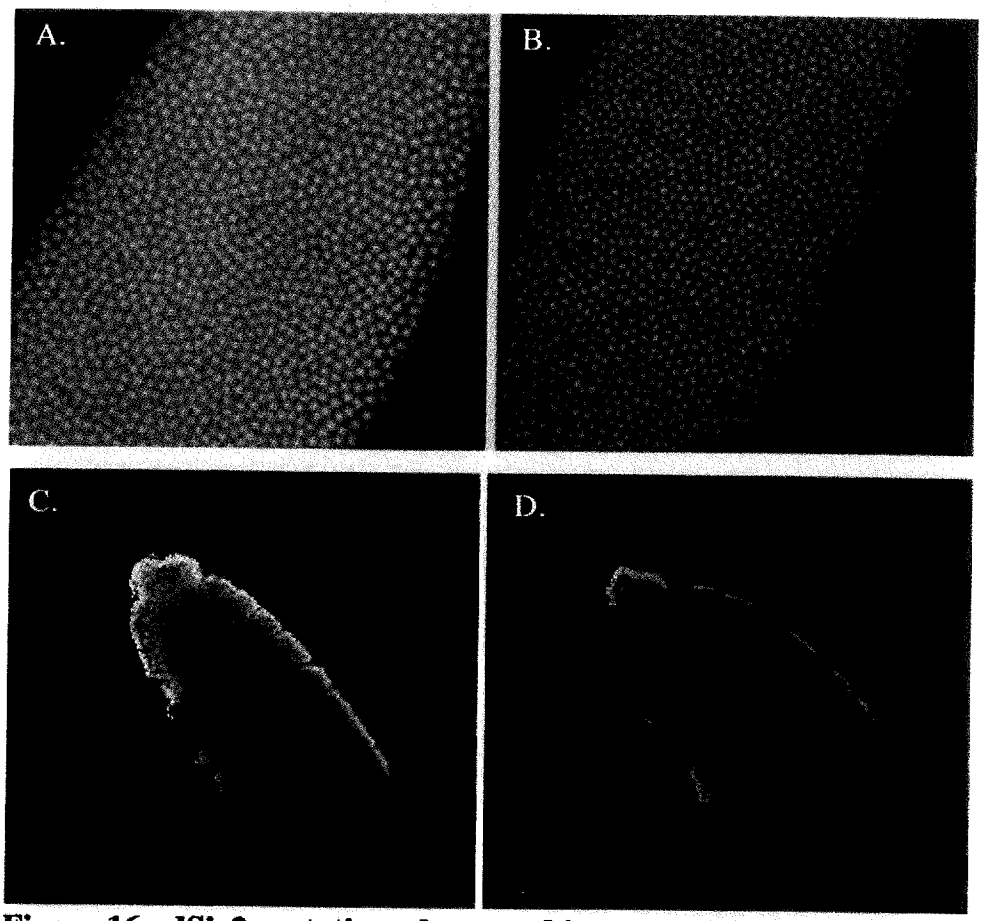


Figure 16. dSir2 mutations decrease histone acetylation levels
A)Wild-type embryo stained with anti-acetyl histone H4 antibody. **B)** *4.5/5.26* mutant embryo stained with anti-acetyl histone H4 antibody. **C)** *4.5/SM6* embryo stained with anti-acetyl H4 Lys 8 antibody. **D)** *4.5/4.5* mutant embryo stained with anti-acetyl H4 Lys 8

***dSir2* RNAi blocks dSir2 production in Kc cells, resulting in abnormal chromosome structure.**

Characterization of the ySIR2 family demonstrated an important role of the SIR2 genes in maintaining chromosomal integrity. The yeast *hst3 hst4* double mutant had a 35 fold higher rate of chromosome missegregation and non-disjunction rate twice that of baseline, suggesting a defect in sister chromatid separation [74]. Based on the data from yeast, I predicted that loss of *dSir2* would affect chromosome stability in Drosophila. Loss of chromosome stability in is readily detectable because it causes lethality, although not always in the first generation [109]. Lethality occurs as mutations accumulate in the germ line. To test whether the transheterozygote *dSir2* mutant animals continued to be viable through several generations, I followed the mutant stock for greater than 10 successive generations. The transheterozygote mutant animals remained fertile and robust. Moreover, *dSir2* mutations do not affect gross chromosome structure in larval neuroblasts (Sarah Smolik, data not shown). However, I obtained somewhat different results in my studies of *dSir2* in Drosophila Kc cells using the RNA inhibition (RNAi) technique to inhibit *dSir2* expression. I inhibited the expression of *dSir2* by treating Kc cells with double-stranded (ds) *dSir2* RNA [101]. Figure 17A shows a Western blot stained with anti-dSir2 antibody from Kc cells treated with either ds-*dSir2* RNA or mock treated. The mock-treated cells, handled in parallel with the ds-*dSir2* RNA treated cells minus the RNA, contain dSir2 after the treatment. The ds-*dSir2* treated cells, on the other hand, no longer produce dSir2 (Figure 17).

To test whether loss of *dSir2* affected chromomsomes in Kc cells, I examined gross chromosome structure in *dSir2*-treated and mock-treated cells. On the fifth day after treatment, I prepared chromosomal spreads stained with propidium iodide. Surprisingly, I found that chromosomes from the *dSir2*-treated lines had a unique morphology characterized by abnormal clumping of chromosomes, similar to those found in the *pimples* mutant in Drosophila [102]. The chromosome arms in *pimples* fail to separate at the centromere leading to chromosomes with twice the normal number of arms.

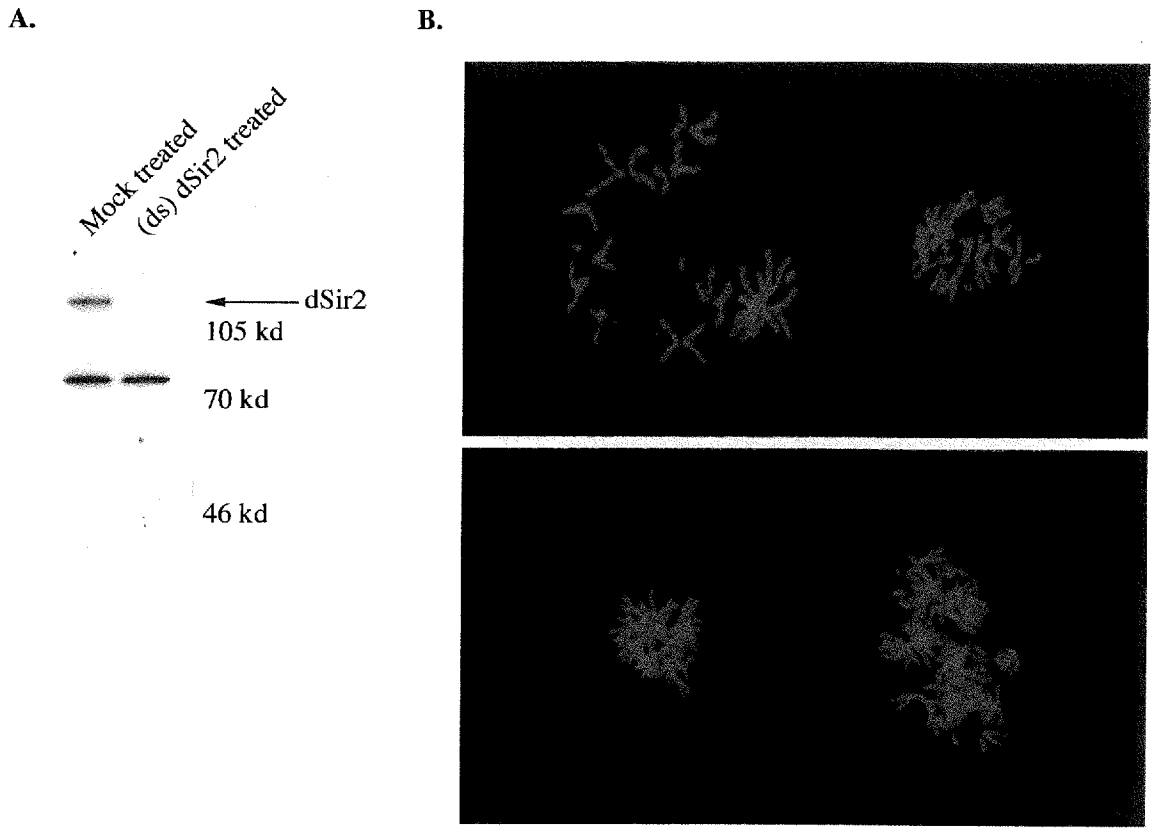


Figure 17. RNAi blocks expression of *dSir2* in Kc cells **A)** Western blot analysis of Kc cell extracts using anti-dSir2 antibody. The first lane contains extract from mock-treated cells. The second lane contains extract from *dSir2*-treated cells. Cells were treated and allowed to grow for four days before harvesting. A four hour exposure of same blot reveals a miniscule amount of dSir2 (data not shown). Sir2 production is blocked for up to eight days following RNAi transfection (data not shown). **B)** Propidium iodide-stained chromosomes from *dSir2*-treated cells have an unusual morphology compared to mock-transfected cells. The top panel shows typical chromosome spreads from mock-treated cells. The chromosomes from the *dSir2*-treated cells (lower panel) are not well spread and have a fused appearance.

Chapter IV

DISCUSSION

Overview

Epigenetics —the study of how chromatin structure regulates gene transcription in an inherited, domain specific fashion— is a rapidly growing and complex field. Simple models that propose that histone acetylation levels act as a switch to "open" and "close" chromatin for the sake of gene activation and silencing are no longer applicable. Newer models propose that histones are modified by enzymes (e.g. deacetylases, methylases, kinases) in such a way as to recruit specific complexes capable of transforming both the structure and the activity of genes in question. To better understand the regulation of chromatin structure, I chose to study the Drosophila homologue of Sir2—a member of a remarkably well-conserved gene family thought to regulate heterochromatin and lifespan via histone deacetylation across phylogenetic lines. To test the hypothesis that *dSir2's* function is conserved, I characterized the *dSir2* gene and generated *dSir2* mutants and antibodies. I used these reagents to study *dSir2* regulation and function and to demonstrate an interaction with a new partner, dCBP. My results are discussed below.

Cloning of *dSir2* I cloned and molecularly characterized the Drosophila homologue of yeast SIR2. Based on primer extension, 5' EST clones and Northern analysis, I

predict that the transcription start site is 440 bp 5' of the *dSir2* open reading frame. There are no consensus sequences (i.e. TATA, CAAT, initiator, downstream promoter element) in the sequences surrounding this site. Thus, it is likely that the transcription start site for *dSir2* is non-classical.

Approximately 600 bp upstream of the putative *dSir2* transcription start site there is an open reading frame in the direction opposite *dSir2*. This open reading frame encodes a putative homologue of the bacterial chaperonin molecule, DnaJ; I refer to this gene as *DnaJ-H*. Of note, this fact is missed in a recent publication describing the *dSir2* gene [106]. Rosenberg and Parkhurst report that the closest open reading frame, known as CG 5204, is quite remote. My own sequencing data, and that from the genome project show the presence of the *DnaJ-H* open reading frame (CG 9828) as described in the results section [94]. The close proximity of the two genes makes mutational analysis of *dSir2* more complicated. For example, a group recently created a Drosophila neurodegeneration model by overexpression of a mutated version of the human gene, Spinocerebellar ataxia type 1 (SCA1) [110]. Mutations in SCA1 (expansion of a polyglutamine tract) cause a neurodegenerative disease of the same name. Using a GMR Gal4 driver the authors overexpressed SCA-1 in the eye, leading to nuclear inclusions and cellular degradation. To identify genes that modify the SCA-1 phenotype, the authors carried out a screen looking for genes that altered SCA1-induced neurodegeneration phenotype when deleted or overexpressed. One of mutations identified that enhances their phenotype is predicted to overexpress *dSir2*. The promoter in this mutant line inserts between *dSir2* and *DnaJ-H*. It is not known if the mutation disrupts the *DnaJ-H* gene. However, given

that the authors found a suppressor line that overexpresses another DnaJ homologue (DnaJ1), it is plausible that the enhanced phenotype in the *dSir2* overexpression line is actually due to disruption of *DnaJ-H* expression, not overexpression of *dSir2*. I believe it is important to consider the effect *dSir2* mutations might have on *DnaJ-H* expression.

My *dSir2* mutagenesis scheme screened for mutations that left the *DnaJ-H* coding sequences intact and did not disrupt the pattern of *DnaJ-H* expression. As I did not use quantitative Western blots to compare *DnaJ-H* levels in the mutant and wild-type embryos, there is still a possibility that there are subtle changes in the levels of DnaJ-H in the mutant embryos. However, my *in situ* results show that the DnaJ-H transcript is present and that the level of expression of DnaJ-H RNA in the mutant and wild-type strains is similar.

Sirtuin family- Structural relationships

Yeast SIR2 is the founding member of the sirtuin family and is a non-essential gene required for silencing at the mating loci and telomeres [74], suppression of rDNA recombination [72], and repair of double stranded breaks [68]. All of SIR2's functions require the conserved "core domain", a 200 amino acid domain that defines the sirtuin family and has NAD-dependent histone deacetylase activity *in vitro* (Figure 1). There are four SIR2 homologues in yeast (HST1, 2, 3, and 4) that are also non-essential genes thought to be involved in the regulation of chromatin structure and/or integrity [74].

SIR2 homologues outside of yeast, called sirtuins, have been found in nearly every organism, from protozoa to prokaryotes to parasites to humans. This fact suggests that the sirtuin family is ancient. Interestingly, there is little homology within this large family outside the core domain [111]. Using molecular phylogenetic analysis software, 5 classes of sirtuins have been described [111]. *dSir2* is in Class I which also includes the prototypical ySIR2, yHst2, 3 and 4, several human genes, a C.elegans gene, a second Drosophila gene (D.mel2), and no prokaryotic genes. Class II is made up of a third Drosophila gene, two C.elegans genes, a human gene and several of the bacterial homologues. Most of the bacterial sirtuins and no Drosophila genes are in Class III. The last two Drosophila genes and two of the human sirtuins define Class IV. The fact that there are no Drosophila genes in Class III suggests that Drosophila may have lost this branch over time [111]. Of the five Drosophila sirtuins, *dSir2* is the most similar to ySIR2 in terms of homology in the core domain [78] (Figure 1, Chapter I). This is an important fact when comparing mutant phenotypes across phylogenetic lines.

Dynamic regulation of dSir2.

One of the strengths of the Drosophila model is the ability to study a gene in the context of a multicellular organism. To set the framework for the developmental regulation of *dSir2*, I will briefly review Drosophila embryogenesis. The fertilized egg progresses through the syncitial blastoderm stage and forms a simple cellularized embryo by about three hours. The beginning of gastrulation —after mitotic cycle 13—is marked by the formation of the ventral (ectoderm, endoderm, mesoderm) and

cephalic furrows which are invaginations that divide the embryo into 4 unequal parts. The anterior and posterior midgut invaginations (endoderm) are followed by a ventral cellular migration called germ band extension (mesoderm and ectoderm). Completion of germ band extension marks the end of gastrulation at about 4 and half hours. Embryonic segmentation accompanies germ band retraction, followed by CNS and PNS differentiation, complete by about 10 and a half hours. With the exception of the nervous system and imaginal disks, which later form the eyes, legs, and wings, cell division in the animal is complete by about 13 hours.

I examined *dSir2* mRNA during this active developmental period and found that it is present between zero and two hours and is then down-regulated between two and four hours. Interestingly, mRNA in the embryo before four hours is maternally derived (synthesized maternally and then deposited into the embryo). Previously characterized maternally derived genes have been shown to be responsible for basic patterning in the embryo and are usually required for embryonic development. It is unclear why *dSir2*, a non-essential gene, would be maternally synthesized. Perhaps in concert with the other *dSir2* homologues, *dSir2* is important, but not absolutely required for development.

Zygotic gene expression begins at about four hours and *dSir2* expression increases during this period. In general, the *dSir2* transcript is ubiquitous but levels are highest in the ventral and cephalic furrows, dorsal lateral folds, germ band, epidermis and CNS. The significance of this pattern of expression is not yet known. However, it does suggest that *dSir2* plays a general role in development, versus a specific role in a specific system.

Sub-cellular localization

Based on the histone deacetylase activity and the silencing role in yeast, sirtuins are predicted to be nuclear. However, several of the mammalian sirtuins, such as the human homologue SIRT2 [112] and two mouse sirtuins [113] have been shown be cytoplasmic. Even in S. cerevisiae, Hst2 is found only in the cytoplasm [81]. As previously discussed, these data are consistent with the idea that sirtuins have substrates other than nuclear chromatin. I found that dSir2 is primarily nuclear. However, during cellular blastoderm it appears to be excluded from the nucleus and during germ band retraction, it is detected equally in both the nucleus and cytoplasm. This finding is consistent with one of two hypotheses: dSir2 has non-nuclear substrates or the activity of dSir2 is regulated by sequestration in the cytoplasm. Interestingly, both the pattern of expression in the embryo and the sub-cellular regulation is strikingly similar to that of dCBP [44].

The distribution of dSir2 protein that I found is similar to that reported in the recent publication by Rosenberg and Parkhurst [106]. However, there is an important difference in the nuclear localization of dSir2 in the early stages of development. In my experiments, there is no dSir2 staining at the peripheral edge of the cell, which is where the nucleus sits. This suggests that dSir2 is excluded from the nucleus. Rosenberg and Parkhurst use double labeling with propidium iodide to define where dSir2 is in relation to the nucleus. In these experiments, dSir2 staining did not co-localize with propidium iodine at the syncitial blastoderm but was found to be exclusively nuclear during cellular blastoderm. Although my experiments addressed

the issue less directly, the data are still hard to reconcile. Thus, further work is needed to define the exact timing and sub-cellular localization of dSir2 during this dynamic period.

In yeast, Sir2 regulates recombination of rDNA and is concentrated in the nucleolus. Although I did not specifically look for nucleolar staining with double labeling experiments, there is no obvious concentration in, or exclusion from the nucleolus at any stage of development.

dSir2 binds to euchromatin and heterochromatin

Staining of polytene chromosomes with dSir2 antibody reveals localization to both heterochromatin and euchromatin. In yeast, dSir2 is only associated with heterochromatin [17]. Binding of dSir2 to euchromatin was also reported in a recent publication by van Steensel et. al. [114]. The authors used a new assay designed to identify *in vivo* targets of chromatin remodeling genes. They examined three Drosophila genes, HP1, GAGA and *dSir2*, and showed that *dSir2* is associated with constitutively active and activated genes such as translation factors, putative ribosomal proteins, α-tubulin, hsc4 and EIP40. However, in contrast to my results, the authors did not find that *dSir2* associates with heterochromatin. Also in contrast to the yeast data, there was no association found between *dSir2* and the ribosomal RNA genes. In a third study, workers used flag-tagged *dSir2*-transfected Drosophila SL2 cells co-labeled with anti-heterochromatin protein 1 (HP1) and anti-flag antibodies [105]. Similar to the results in van Steensel, et. al., dSir2 did not co-localize with HP1. However, my results are consistent with those obtained by Rosenberg and Parkhurst

who employed the same assay I used—staining of polytene chromosomes [106]. Rosenberg and Parkhurst showed that the dSir2 staining co-localized with a centric heterochromatin-specific antibody directed against histone H3 methyl lysine. It is unlikely that the heterochromatic staining shown here and presented by Rosenberg and Parkhurst is non-specific because a) I saw no staining of heterochromatin in the *dSir2* mutants and b) our results are the same in spite of using different primary and secondary antibodies. One possible explanation is that tissue specific variability accounts for the differences discussed above.

Mutagenesis

To study *dSir2* mutant phenotypes, I generated mutations on two unrelated chromosomes that deleted a portion *dSir2* but left *dDnaJ-H* coding sequences intact. I assessed *dDnaJ-H* expression in whole-mount embryos to ensure that the *dSir2* mutations did not disrupt *dDnaJ-H* expression. The *dSir2* deletions remove the *dSir2* transcription start site and several hundred bps of *dSir2* coding sequence. To demonstrate that no truncated versions of the gene are expressed, I examined *dSir2* expression in Northern blots and *in situ* RNA hybridization assays. I demonstrated that there is no expression of full-length or truncated versions in the *dSir2* mutant animals. I also tested for the presence of dSir2 protein in the mutants using Western blots and whole mount embryo staining. I did not detect dSir2 protein in the mutant embryos.

In genetic experiments, I used flies that were transheterozygotes so that recessive second site mutations on the parental chromosomes would not affect the

phenotype. By using transheterozygotes, I demonstrated that *dSir2* is a non-essential gene. My data directly contradicts a recent report stating that mutations in *dSir2* are lethal [106]. Both groups used the P {ry+t7.2=PZ} *l (2) 05327 cn1* chromosome to generate *dSir2* mutations. Using control experiments, I showed that this chromosome carries second site lethal mutations. Given that Rosenberg and Parkhurst used flies homozygous for this chromosome to define the *dSir2* phenotype, it is more likely that their results reflect the effect of the second site lethal mutations, not *dSir2* mutations.

Rosenberg and Parkhurst also attempt to show an interaction between *dSir2* and the Hairy/Deadpan/E (Spl) -bHLH family members. However, the strains and crosses used to define this interaction are not well described, making evaluation of their data difficult. The authors also report that *dSir2* is involved in sex-determination. First, they show that dSir2 and Deadpan, a bHLH protein, interact in GST pull down experiments. *Deadpan* (*dpn*) is known to control sex determination via negative regulation of a gene, *Sex lethal* (*sxl*). Loss of *dpn* causes overexpression of *sxl* in males and results in male-specific lethality. In the Rosenberg paper, the authors propose that *dpn* requires *dSir2* and that overexpression of *dSir2* should enhance the repression of *sxl* and vice versa. The authors test this hypothesis using their *dSir2* mutant and overexpression strains. Their assay relies on sorting embryos based on "strong" expression vs. "intermediate" expression of *sxl*. However, there is no verification of the ability of this assay to reliably distinguish between these overlapping groups. Moreover, they present data that confuses the issue further by demonstrating that the two different *dSir2* mutants have different effects: the $dSir2^{05327}$ mutant has no effect on sex ratios and the $dSir2^{ex10}$ mutant causes male lethality.

Given that both chromosomes are mutant for *dSir2* (as per the authors), the results are inconclusive. In my studies, I found no disruption in sex ratios in the transheterozygous *dSir2* mutants. In conclusion, the functional data in the Parkhurst paper do not enhance our understanding of *dSir2* function.

A recent publication by Astrom et. al. [115] presented data very similar to that presented in this thesis. The authors showed that there are second site lethal mutations on the chromosomes used to generate *dSir2* mutations in the publication by Rosenberg and Parkhurst [106]. Using chromosomes that are free of second site lethal mutations, Astrom et. al. demonstrate that deletion of *dSir2* does not cause lethality. They also showed that *dSir2* mutations do not modify sex ratios.

dSir2 mutations suppress PEV

Drosophila is a good model system in which to study chromatin structure. There are many well-studied mutants that have been used to characterize factors that control heterochromatin formation, maintenance and function. The w^{m4} mutant —caused by transposition of the white gene to the edge of centric heterochromatin — is a classic example of PEV. The variable gene expression, manifested as the mottled eye phenotype, is believed to be caused by the variable spreading of heterochromatin into the *w* gene, causing transcriptional silencing. I used the w^{m4} mutant to test whether mutations in *dSir2* alter PEV. Based on the enzymatic activity and function in yeast, I predicted that loss of *dSir2* would interfere with heterochromatin formation and suppress PEV. I found that *dSir2* mutations are recessive suppressors of PEV in the w^{m4} mutant, consistent with the model that the sirtuin family regulates

heterochromatin formation across phylogenetic lines. However, most of the known chromosomal remodeling proteins, such as HP1, act dominantly to suppress (or enhance) PEV. There are exceptions to this, such as the chromatin-remodeling gene, *zeste*. *zeste,* also a non-essential gene, is believed to stabilize an open chromatin conformation, favoring gene activation [116]. Like mutations in *dSir2*, mutations in *zeste* are recessive enhancers of PEV [116]. It is likely that genes like *dSir2* and *zeste* act to stabilize chromatin states but are not absolutely required for its formation and/or function. It would be interesting to determine how the mechanism of action of these dominant and recessive modifiers of PEV differs. One likely possibility is that the effect of *dSir2* mutations on PEV is indirect, via a gene that *dSir2* regulates. This possibility will be discussed in more detail below.

The two recent publications discussed above, characterizing *dSir2*, both showed an effect of *dSir2* mutations on PEV. Unlike my results, Rosenberg and Parkhurst reported a dominant effect of *dSir2* mutations on PEV. Of note, the authors used a control chromosome, *CyO*, which is known to skew PEV data [98]. In my experiments, I used a control chromosome, *Sco*, which has been shown to have no effect on PEV [98]. The more recent publication by Astrom et. al. [115], in agreement with my work, showed that *dSir2* mutations recessively suppress PEV.

dSir2 and dCBP

Based on Dr. Yang Chen's two-hybrid result that identified dSir2 as a dCBP interaction partner, I tested whether dCBP and dSir2 interact *in vitro* and in co-immunoprecipitation assays in Drosophila Kc cells. I found that the C-terminal half of

dSir2 and the CREB-Binding domain of dCBP interact using GST pull-down assays. Interestingly, the CREB binding domain of dCBP is also known to interact with the transcriptional activator, *Cubitus Interruptus* [95]. Thus, like in mammalian systems, the CREB binding domain of CBP appears to be a general protein-protein interaction domain. I also tested whether dCBP and dSir2 proteins interact *in vivo* using co-immunoprecipitation assays. I found that dSir2 antibody immunoprecipitated dCBP from Drosophila Kc cells, suggesting that these proteins form a complex *in vivo*. These data are consistent with the fact that *dSir2* and *dCBP* have overlapping patterns of expression during development.

dSir2 was originally isolated in a two-hybrid screen by *dCBP* as a partial clone (bp 1621-3839). The partial clone encodes the C-terminal fragment of dSir2, including the C-terminal third of the conserved core domain (aa 396-479). I also showed an interaction between one of the mammalian homologues of CBP and SIR2 (hHST1). hHST1 is an uncharacterized rat cDNA made available to the Goodman lab from Jef Boeke [74]. The only shared sequences between mammalian hHST1 and dSir2 is in the core domain, making it likely that the C-terminal third of the core domain is sufficient for binding to CBP. From the crystal structure of the human SIR2 core domain, the terminal third contains 3 three active site loops and 3 alpha helices that, in part, make up the variant of the Rossman fold, believed to be involved in NAD binding [85]. It would be interesting to test the hypothesis that dCBP binding alters the enzymatic activity of dSir2.

It is also important to discuss the fact that I did *not* find co-localization of dSir2 and dCBP on polytene chromosomes. The significance of this difference is not

known. However, there are other examples of differences in results from polytene chromosomes and Kc cells. For example, in Kc cells, HPI and a dSir2 fusion protein do not co-localize [114]. However, Rosenberg and Parkhurst showed that dSir2 and histone H3 methyl lysine co-localize on polytene chromosomes[106]. Since histone H3 lysine 9 methylation is required for HPI-mediated silencing in heterochromatin [23], it is likely that HPI and dSir2 also co-localize on polytene chromosomes. These data suggest there are differences in the regulation of chromatin complexes between Kc cells and polytene chromosomes.

Thus, the point to be taken from the data presented here is that dCBP and dSir2 *can* form a complex in KC cells. The lack of co-localization in polytene chromosomes does not change this fact. Ultimately, the more important question is whether a dCBP/dSir2 interaction has functional significance during Drosophila development.

SIR2 and the regulation of lifespan

As discussed in Chapter 1, Guarente has proposed that sirtuins provide the link between metabolism and aging across phylogenetic lines. Before discussing dSir2, however, it is worthwhile to discuss the differences between aging, lifespan and senescence. Replicative senescence is a phenomenon— seen in yeast and cells grown in culture— that limits proliferation, causing irreversible growth arrest. Replicative senescence has been proposed to protect cells from accumulating genetic damage. In at least one sense, it is a related to aging because cells are more likely to arrest as they get older. In yeast, cells divide asymmetrically, generating a larger mother cell and a smaller daughter cell. Because of the difference in cell size, the mother cell can be

followed through each generation. Previous work has demonstrated that after approximately 40 generations, the mother cell will reach senescence [117]. The number of generations it takes to reach senescence has been referred to as "lifespan" in the literature. However, this word has a different meaning when applied to multicellular organisms. For example, *C.elegans* and Drosophila are post-mitotic organisms, meaning there is no cell division after the larval stages, only cell growth. Thus, it is necessarily true that their lifespan is independent of cell senescence. Mammals, on the other hand, have both post-mitotic tissues (e.g. heart, kidneys, neurons) and actively mitotic tissues (e.g. bone marrow, colonic mucosa, skin).

The concept of aging has often been used interchangeably with the concept of lifespan. While it is clear that they are related, they are not the same. For example, through improvements in medicine and technology in the last century, humans have dramatically lengthened lifespan but have been unable to slow aging. Aging refers to specific age-related phenotypes and includes generalized organ atrophy, decreased fecundity, diminished stress tolerance, and degenerative processes such as neurodegeneration and osteoporosis. In recent years, several papers have been published showing that single gene mutations —such as Mesuthelah in Drosophila [118] and the daf mutations in C.elegans [119]— have a dramatic effect on lifespan. These studies, however, do not directly evaluate the effect of mutations on the aging process itself, except as it relates to lifespan.

How do these concepts relate to sirtuins? As discussed in Chapter 1, Guarente notes that there is a well-established link between metabolic rate and the pace of aging. He argues that cells that are calorie restricted may have an altered "metabolic

strategy" which "somehow favors longevity" and this represents the "billion dollar question". He hypothesizes that calorie restriction would increase NAD (via decreased consumption of NAD in glycolysis) and enhance sirtuin activity. How does enhanced sirtuin activity relate to aging? In yeast, sir2 mutants are defective in silencing, leading to "genome instability" characterized by the formation of ERCs causing early senescence. Guarente admits that there is no evidence that ERCs or any toxic DNA products accumulate in higher organisms during cell division. He points out, however, that silencing is important in general because it blocks "inappropriate" gene expression. Thus, in organisms with restricted caloric intake and, presumably, increased sirtuin activity, genes that are supposed to be off, stay off, protecting the organism from accidental damage over time. A specific example of this could be considered in skin, where collagenase mRNA/protein and enzyme activity increase with aging, causing loss of collagen, thinning, wrinkling and poor wound healing [120, 121]. A testable hypothesis is that collagenase transcription increases because of the inappropriate expression of genes that are normally silenced.

In support of the sirtuin/aging theory, Tissenbaum and Guarente cloned a C. elegans sirtuin, sir2.1, and showed that a mutant with two copies of sir2.1 had a significantly longer life and, conversely, that sir2.1 deletion caused shortening of lifespan [90]. In general, these data support the notion that sirtuins regulate aging across phylogenetic lines. However, it unclear if it is useful to compare the effects in yeast directly with effects in C. elegans. First, the authors show no data that suggests that dSir2 expression increases in their mutant. Second, there is no data that Sir2 plays a role in silencing in C. elegans. Finally, as mentioned earlier, it is unclear where the

link is between yeast "lifespan" and C.elegans lifespan given that C. elegans are post-mitotic. Tissenbaum and Guarente recognize this and suggest that Sir2 "may couple nutrient availability to the level of signaling through the insulin-like signaling pathway " (the insulin-like signaling pathway in C. elegans has previously been shown to regulate lifespan). Is this proposed "coupling" related to silencing or some other sir2 function? The issue remains unclear.

Based on the preceding data, it is hard to predict whether *dSir2* mutations would alter lifespan in Drosophila. I tested the effect of *dSir2* mutations on lifespan. However, I present my data with the caveat that experiments measuring lifespan are difficult to perform. Lifespan is not a simple phenotype controlled by a few genes; results can vary based on factors such as parental age, conditions of larval development, temperature, food conditions, crowding, parasites, etc [107]. An example of this is that a common cause of death is getting trapped in the food. The wetter the food, the more likely it is that flies will die this way. Thus, a small difference in the food preparation, humidity, etc. can impact the outcome of an experiment. Moreover, a fly that is simply more vigorous may appear to have a longer lifespan. There is also the challenge that there are no standard conditions for setting up lifespan experiments in Drosophila. Thus, it may be difficult to compare data from one laboratory to another.

In setting up my experiments, I attempted to use identical conditions between the experimental and control flies, especially in terms of parental age, which is a known modifier of lifespan in Drosophila. When I assessed the effect of *dSir2* mutants on lifespan I saw no significant difference between $dSir2^{5.26}$/ $dSir2^{4.5}$

transheterozygotes (no *dSir2*) and *dSir2*-/+ heterozygotes (one copy of *dSir2*). I also tested the effect of *dSir2* mutations on lifespan under more stressful environmental conditions (longer span between "turning over" the stocks). Not only did *Sir2* mutants not die faster, the $dSir2^{5.26}$/ $dSir2^{4.5}$ flies had a slightly longer median lifespan than the median lifespan of the *dSir2* heterozygotes and *Canton S* controls. However, the average mean lifespan among the genotypes was not significantly different. In conclusion, while it is possible that other sirtuins in Drosophila increase lifespan, the *dSir2* gene described here does not. Moreover, my data suggests that sirtuins do not play a universal role in the coupling of caloric intake to lifespan. It might be more valuable to focus on aging— which is the real "billion" dollar question—and the phenotypes that are associated with it. Perhaps coincidentally, a recent paper links an activated form of p53 to premature aging phenotypes in mice [122]. Given that both CBP and sirtuins have been shown to regulate p53, it would be interesting test if *dCPB/dSir2* double mutants have altered aging phenotypes.

Importantly, in contrast to my results, Astrom et. al. showed *decreased* lifespan in *dSir2* mutants. How can these opposite results be reconciled? There are several obvious differences in the methods used to perform the lifespan experiments. First, I used only males, whereas Astrom et. al. used males and females. Second, I used approximately 20 times the number of animals to generate my lifespan curves. Furthermore, it is unclear from the description of their methods if confounding variables such as crowding and parental age were controlled for. Finally, it is unfortunate that Astrom et. al. used animals in the lifespan study that were homozygous for the *dSir2* mutant chromosome. I used the transheterozygous mutant

animals in my lifespan experiments to avoid the possibility that recessive mutations contribute to the effect on lifespan.

Remaining Questions

The finding that dCBP and dSir2 form a complex *in vivo* does not fit with the model that HATs are part of gene activation complexes and HDACs are part of gene inhibition complexes. Furthermore, it is difficult to predict whether the interaction between the two proteins would be cooperative or antagonistic. To address these questions, I used anti-histone antibodies to investigate how *dSir2* mutations affect histone acetylation in whole embryos. Ludlum et al. [44] has previously shown that *dCBP* mutations (that affect acetyl CoA binding to the HAT domain of dCBP) decrease global histone acetylation levels in the embryo. I used a similar approach to test the effect of *dSir2* mutations on whole embryo histone acetylation. I first stained wild type and *dSir2* mutant embryos with a general anti-acetylated histone antibody. Surprisingly, I found that histone acetylation in *dSir2* mutants, like in the dCBP mutants, was globally decreased. These results are unexpected given that sirtuins are known histone deacetylases *in vitro*. Of note, however, these results are similar to results in yeast where histone acetylation levels are moderately reduced in the quintuple knockout hst1hst2hst3hst5sir2 strain [123]. To better define the effects of *dSir2* mutations on histones, I used a more specific antibody against acetylated histone H4, lysine 8. This is the same antibody previously used by Ludlam et. al. to show that *dCBP* mutations decrease histone acetylation. Like the results from my initial experiment, I found that the level of histone acetylation at lysine 8 of H4 was

decreased in the *dSir2* mutants. These data are consistent with the idea that the interaction between dSir2 and dCBP is cooperative.

The data that I have presented with respect to *dSir2*, *dCBP* and chromatin regulation do not fit into a simple model whereby histone acetylation causes relaxation of chromatin leading to gene activation and vice versa. While it is true that *dSir2* mutations cause the "expected " suppression of PEV, the effect is recessive. Second, the global effect of dSir2 mutations on histone acetylation is unexpected. I also show that dSir2 interacts physically with dCBP and that mutations in both genes cause decreased histone acetylation. Finally, van Steensal et. al. [114] have demonstrated that dSir2 associates with sites of active chromatin. How do these data fit with the current models of chromatin structure and function?

The first model, which I call the substitution model, proposes that like enzymes substitute for missing proteins in a complex in the setting of a null mutation. Thus, a given phenotype reflects the characteristics of the substituting gene, not the missing gene. In the case of the *dSir2* deletion, another deacetylase could substitute for *dSir2*. This could explain why loss of a gene that is well conserved, maternally deposited in the embryo and expressed in critical stages of development does not cause lethality. There are four other *Sir2* homologues in Drosophila, one of which falls into the same structural subclass as *dSir2*. Anyone of these homologues, or an entirely different deacetylase could, in theory, substitute. The substitution model could also explain why there is decreased acetylation in the *dSir2* mutant. The substituting deacetylase could inappropriately deacetylate histones during development, causing the observed decrease in histone acetylation.

The histone code hypothesis is also useful to consider in this setting. According to the histone code hypothesis, it would be appropriate to suppose that *dSir2* and *dCBP* act together to generate a specific pattern of acetylation on histone tails. For example, CBP is known to acetylate all the lysines on H3 and H4; Sir2 deacetylates only a few. A dCBP/dSir2 complex could be recruited to a promoter or domain, leaving a distinct pattern of acetylation/deacetylation required for the binding of other regulatory proteins. This model, however, cannot account for the fact that *dSir2* mutants have overall decreased histone acetylation.

Decreased histone acetylation in the *dSir2* mutants can be explained, however, if we consider the likely possibility that HDACs have targets other than histones. For example, suppose that the target of dSir2 is dCBP, not histones. Given that mammalian CBP is auto acetylated (Roland Kwok, personal communications) one could speculate that autoacetylation attenuates dCBP HAT activity. Under normal conditions, deacetylation of dCBP by dSir2 would reactivate HAT activity. In the *dSir2* mutant, however, dCBP would be less active, causing decreased histone acetylation. Based on this model, the previously paradoxical point—that *dSir2* and *dCBP* have opposite enzymatic activity but the same effect on histone acetylation—is resolved.

dSir2 can have indirect effects both by deacetylating non-histone proteins and by altering the expression of other genes. The potential for an indirect effect of dSir2 cannot be underestimated. In a recent landmark paper from the Grunstein laboratory, data are presented which explore the genome-wide effect of histone deacetylases [54]. Using modified chromatin precipitation assays, the authors studied different effects of

mutations of the yeast deacetylases, Rpd3 (and Hda1). In the first set of experiments, they examined which regions of the genome were hyperacetylated by HDAC mutations. Mutations in Rpd3 caused hyperacetylation at 531 promoters. To address the Rpd3 paradox (see Introduction for full discussion), articulated after it was demonstrated that Rpd3 mutations *repress* a subset of genes, the authors compared the 531 promoters found in their study with those known to be regulated by Rpd3 mutations [124]. They found little overlap (24/491[2]) between promoters that are hyperacetylated and genes that are repressed by the Rpd3 mutation. This is the best evidence to date that the "unexpected" effect of Rpd3 mutations, namely gene repression, is indirect. The authors also examined the association between different sites of histone acetylation and gene activity. Here, they show that acetylation at some sites (H4, K5 and 12) is strongly associated with gene activity. On the other hand, acetylation of H3, K18 and even more dramatically, H4, K16 correlates poorly with gene activity. These data highlight the importance of understanding the precise effects of HAT/HDACs, not just general trends.

Based on the above data, Robyr et. al. set a new standard for measuring the effect of HATs/HDACs: (1) enzyme binding assays to determine if the protein in question actually recognizes chromatin; (2) acetylation arrays to see where histones are de/acetylated in the presence of HAT/HDAC mutations; (3) expression arrays to determine where gene activity is altered in comparison to (2). While I have shown that dSir2 is present at heterochromatin, and that *white* gene transcription increases in w^{m4};*dSir2* mutants, I have yet to show that heterochromatin is hyperacetylated in *dSir2*

[2] The total number of genes compared is 491, not 531 because only 493 genes in the Bernstein paper overlap with the those in the Robyr paper.

mutants. In fact, in my preliminary experiments, it appears that histone acetylation globally decreases. The definitive experiment, asking whether the histones within heterochromatin are hyperacetylated in *dSir2* mutants, awaits.

It is also critical to think about indirect effects of genes like HDACs in the lifespan experiments. In yeast, Sir2 acts as a silencer of specialized domains: mating type loci, telomeres and rDNA. Thus, its role in the regulation of "lifespan" is likely quite direct, as loss of Sir2 causes loss of regulation of these important chromatin domains. In Drosophila, and other higher organisms, the issue is less clear. First, there is evidence that dSir2, unlike yeast Sir2, is not just a silencing factor, as it binds to the promoters of a number of actively transcribed, euchromatic genes [114]. Second, it is known that human Sir2 has targets other than histones (e.g. p53). Thus, the role of sirtuins in higher organisms is likely broader than in yeast. If this is true, the question of whether sirtuins control lifespan is almost irrelevant as any gene that controls hundreds of genes will probably affect a phenotype such a lifespan. An interesting question is whether there are different classes of sirtuins in higher organisms, those that control specialized chromatin domains and those that act as typical (promoter specific) transcription factors.

dSir2 and Kc cells

In yeast, the hst3hst4 double mutant suffers chromosome loss and arrests at G2/M [74]. Furthermore, several recent publications have demonstrated that a variety of genes that interfere with the formation of heterochromatin also cause defects in chromosomal segregation, presumably because of disruption of centromeres [11]. I

present preliminary data showing that inhibition of *dSir2* expression using RNAi in Drosophila Kc cells leads to gross chromosomal abnormalities. Chromosomes from cells treated with *dSir2* dsRNA are similar in appearance to the chromosomes seen in the *pimples* mutant. The *pimples* gene is required for separation of sister chromatids *in vivo* [125]. However, it is clear that there are no chromosomal abnormalities in *dSir2* mutant flies. Why do Kc cells have a different phenotype than that seen in animals? It is tempting to speculate that the mutations that lead to the transformed state in Kc cells, in combination with *dSir2* loss, lead to the phenotype as described. Given that *dSir2* is found at pericentric heterochromatin, an interesting question is whether *dSir2* plays a role in centromere structure and/or function. More work is needed to define the specific defects that cause the gross abnormalities and the role, if any, of *dSir2*.

Chapter V

SUMMARY AND CONCLUSIONS

In this thesis, I present the cloning and characterization of *dSir2*. I show that *dSir2* is developmentally regulated in a dynamic fashion, is an NAD-dependent histone deacetylase *in vitro* and a dCBP-interacting partner. In genetic assays, I show data that support the idea that *dSir2*s role in silencing at heterochromatin is conserved. In addition, in contrast to recently published work, I demonstrate that *dSir2* is non-essential. This is an important point because it is a paradox that a gene that is so well conserved and found in nearly every organism is also dispensable. An alternative hypothesis is that *dSir2* function is not dispensable but in fact duplicated in Drosophila. Deletions of *dSir2* homologues or other deacetylases, in combination with *dSir2,* may help reveal the true function of these genes. In the absence of such deletions, however, the function of *dSir2* may also be investigated using double mutants that knock out *dSir2* and potential interacting genes such as *dCBP*.

I also examined the question of whether *dSir2* targets histones *in vivo*. My preliminary experiments show that *dSir2* mutations decrease histone acetylation levels in the developing embryo. My data is in conflict with some of the genetic experiments from yeast and the biochemical properties of the sirtuin family that suggest that sirtuins act as histone deacetylases. The data that I present and review is more consistent with the hypothesis that the *in vivo* targets of sirtuins are in fact other

transcription factors (i.e. p53 and CBP). However, much work is left to further define sirtuin targets.

Several recent publications and reviews have appeared that support the hypothesis that sirtuins, via their link to NAD, provide the connection between metabolism and aging, perhaps through the protection of the genome by silencing. While my results support the idea that the sirtuin silencing function is conserved, I show that *dSir2* mutations do not decrease lifespan. However, lifespan is a complex and difficult phenotype to measure and I propose that other phenotypes of aging be used to address this interesting hypothesis.

References

1. Moazed, D., *Common themes in mechanisms of gene silencing*. Molecular Cell, 2001. 8(3): p. 489-98.
2. Jenuwein, T. and C.D. Allis, *Translating the histone code*. Science, 2001. 293(5532): p. 1074-80.
3. Cairns, B.R., *Emerging roles for chromatin remodeling in cancer biology*. Trends in Cell Biology, 2001. 11(11): p. S15-S21.
4. Xu, G.L., et al., *Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene*. Nature, 1999. 402(6758): p. 187-91.
5. Ly, D.H., et al., *Mitotic misregulation and human aging*. Science, 2000. 287(5462): p. 2486-92.
6. Blackburn, E.H., *Telomere states and cell fates*. Nature, 2000. 408(6808): p. 53-6.
7. Gems, D., *Ageing. Yeast longevity gene goes public*. Nature, 2001. 410(6825): p. 154-5.
8. Kornberg, R.D., *Chromatin structure: a repeating unit of histones and DNA*. Science, 1974. 184: p. 868-871.
9. Felsenfeld, G. and M. Groudine, *Controlling the double helix*. Nature, 2003. 421: p. 448-453.
10. Griffiths, A., et al., *An Introduction to Genetic Analysis*. fifth ed. 1993, New York: W.H. Freeman and Company.
11. Cleveland, D., Y. Mao, and K. Sullivan, *Centromeres and Kinetochores: From Epigenetics to Mitotic Checkpoint Signalling*. Cell, 2003. 112: p. 407-21.
12. Blackburn, E.H. and C. Greider, *Telomeres*. 1995, Plainview: Cold Spring Harbor Laboratory Press. 396.
13. Patil, S., S. Merrick, and H. Lubs, *Identification of each human chromosome with a modified Giemsa stain*. Science, 1971. 173: p. 821-822.
14. Heitz, E., *Das Heterochromatin der Moose*. I Jahrb Wiss Botanik, 1928. 69: p. 762-818.
15. Henikoff, S., *Heterochromatin function in complex genomes*. Biochim Biophys Acta, 2000. 1470(1): p. O1-8.
16. Henikoff, S., *Position-effect variegation after 60 years*. Trends in Genetics, 1990. 6(12): p. 422-426.
17. Braunstein, M., et al., *Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern*. Mol Cell Biol, 1996. 16(8): p. 4349-56.
18. Jones, P.A. and D. Takai, *The role of DNA methylation in mammalian epigenetics*. Science, 2001. 293(5532): p. 1068-70.
19. Wallrath, L., *Unfolding the Mysteries of Heterochromatin*. Current Opinion in Genetics and Development, 1988. 8: p. 147-153.
20. Devlin RH, et al., *Identifying a single-copy DNA sequence associated with the expression of a heterochromatic gene, the light locus of Drosophila melanogaster*. Genome, 1990. 33(3): p. 405-14.
21. Weiler, K. and B. Wakimoto, *Heterochromatin and Gene Expression in Drosophila*. Annu Rev Genetics, 1995. 29: p. 577-605.

22. Bird, A., *Gene number, noise reduction and biological complexity.* Trends in Genetics, 1995. 11: p. 94-100.
23. Richards, E. and S. Elgin, *Epigenetic Codes for Heterochromatin formation and Silencing: Rounding up the Usual Suspects.* Cell, 2002. 108: p. 489-500.
24. Holliday, R. and J. Pugh, *DNA modification mechanisms and gene activity during development.* Science, 1975. 187: p. 226-232.
25. Panning, B. and R. Jaenisch, *RNA and the Epigenetic Regulation of the X Chromosome Inactivation.* Cell, 1998. 93: p. 305-308.
26. Olins, A. and D. Olins, *Spheroid chromatin units.* Science, 1974. 183: p. 330-332.
27. Noll, M. and R.D. Kornberg, *Action of micrococcal nuclease on chromatin and the location of H1.* Journal of Molecular Biology, 1979. 109: p. 393-404.
28. Finch, J., et al., *Structure of the nucleosome core particle.* Nature, 1977. 269: p. 29-36.
29. Luger, K., et al., *Crystal structure of the nucleosome core particle at 2.8 A resolution.* Nature, 1997. 389(September 18): p. 251-260.
30. Allfrey, V., *Post-synthetic modifications of histone structure: a mechanism for the control of chromosome structure by the modulation of histone-DNA interactions,* in *Chromatin and Chromosome Structure,* H. Li and R. Eckhardt, Editors. 1977, Academic Press: New York. p. 167-191.
31. Roth, S.Y., J.M. Denu, and C.D. Allis, *Histone acetyltransferases.* Annu Rev Biochem, 2001. 70: p. 81-120.
32. Hebbes, T.R., A.W. Thorne, and C. Crane-Robinson, *A direct link between core histone acetylation and transcriptionally active chromatin.* EMBO, 1988. 7: p. 1395-1342.
33. Ekwall, K., et al., *Transient Inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres.* Cell, 1997. 91: p. 1021-1032.
34. Smith, E., et al., *The Drosophila MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation.* Mol Cell Bio, 2000. 20: p. 312-318.
35. Brownell, J.E. and C.D. Allis, *An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in Tetrahymena macronuclei.* Proceedings of the National Academy of the Sciences, 1995. 92(July): p. 6364-6368.
36. Brownell, J., et al., *Tetrahymena Histone Acetyltransferase A: A Homolog to Yeast GCN5p Linking Histone Acetylation to Gene Activation.* Cell, 1996. 84(March 22): p. 843-851.
37. Ogryzko, V., et al., *The Transcriptional Coactivators p300 and CBP Are Histone Acetyltransferases.* Cell, 1996. 87(November 29): p. 953-959.
38. Bannister, A. and T. Kouzarides, *The CBP co-activator is a histone acetyltransferase.* Nature, 1996. 384: p. 641-643.
39. Mizzen, C., et al., *The TAF(II)250 subunit of TFIID has histone acetyltransferase activity.* Cell, 1996. 87: p. 1261-70.
40. Akhtar, A. and P. Becker, *Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for doasage compensation in Drosophila.* Mol Cell, 2000. 5(2): p. 367-375.

41. Goodman, R. and S. Smolik, *CBP/p300 in cell growth, transformation, and development.* Genes and Development, 2000. 87(7): p. 1261-1270.
42. Akimaru, H., et al., *Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signalling.* Nature, 1997. 386(Apr 17): p. 735-8.
43. Waltzer, L. and M. Bienz, *Drosphila CBP represses the transcription factor TCF to antagonize Wingless signaling.* Nature, 1998. 395: p. 521-525.
44. Ludlam, W., et al., *The acetyltransferase activity of CBP is required for wingless activation and H4 acetylation in Drosophila melanogaster.* Molecular & Cellular Biology, 2002. 22(11): p. 3832-41.
45. Kraus, W., E. Manning, and J. Kadonaga, *Biochemical Analysis of Distinct Activation Functions in p300 That Enhance Transcription Initiation with Chromatin Templates.* Molecular and Celluar Biology, 1999. 19(12): p. 8123-8135.
46. Vidal, M.a.G., RF, *RPD3 encodes a second factor required to achieve maximum positive and negative transcriptional states in S. cerevisise.* Mol Cell Bio, 1991. 11(12): p. 6317-27.
47. Grozinger, C.M. and S.L. Schreiber, *Deacetylase Enzymes: Biological Functions and the Use of Small Molecule Inhibitors.* Chemistry and Biology, 2002. 9: p. 3-16.
48. Finnin, M.S., et al., *Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors.* Nature, 1999. 401: p. 188-193.
49. Rubertis, F.D., et al., *The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast.* Nature, 1996. 384(December 12): p. 589-591.
50. Mueller, H.J., *Types of Visible Variations Induced by X-rays.* Journal of Genetics, 1930. 22: p. 299-334.
51. Farakas, G., et al., *The Trithorax-like gene encodes the Drosophila GAGA factor.* Nature, 1994. 371(October 27): p. 806-808.
52. Eissenberg, J., et al., *The Heterochromatin-Associated ProteinHP-1 Is an Essential Protein in Drosophila with Dosage Dependent Effects on Position Effect Variegation.* Genetics, 1992. 131: p. 345-352.
53. Boivin, A. and J.M. Dura, *In vivo chromatin accessibility correlates with gene silencing in Drosophila.* Genetics, 1998. 150(4): p. 1539-49.
54. Robyr, D., et al., *Microarray Deacetylation Maps Determine Genome Wide Functions for Yeast Histone Deacetylases.* Cell, 2002. 109: p. 437-446.
55. Mottus, R., R.E. Sobel, and T.A. Grigliatti, *Mutational analysis of a histone deacetylase in Drosophila melanogaster: missense mutations suppress gene silencing associated with position effect variegation.* Genetics, 2000. 154(2): p. 657-68.
56. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications.* Nature, 2000. 403(6765): p. 41-5.
57. Trivier, E., et al., *Mutations in the kinase Rsk-2 associated with Coffin-Lowry Syndrome.* Nature, 1996. 384: p. 567-70.
58. Sassone-Corsi, P., et al., *Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3.* Science, 1999. 285.

59. Bradbury, E.M., *Reversible histone modifications and the chromosome cell cycle.* Bioessays, 1992. 14: p. 9-16.
60. Turner, B.M. and A. Birley, *Histone H4 Isoforms at Specific Lysine Residues Define Individual Chromosomes and Chromatin Domains in Drosophila Polytene Nuclei.* Cell, 1992. 69(April 17): p. 375-384.
61. Chen, D., et al., *Regulation of transcription by a protein methyltransferase.* Science, 1999. 284(5423): p. 2174-7.
62. Dhalluin, C., et al., *Structure and ligand of a histone acetyltransferase bromodomain.* Nature, 1999. 399: p. 491-496.
63. Laurenson, P. and J. Rine, *Silencers, Silencing and Heritable Transcription States.* Microbiologic Reviews, 1992. 56(4): p. 543-560.
64. Rivier, D. and J. Rhine, *Silencing: the Establishment and Inheritance of Stable, Repressed Transcriptional States.* Current Opinion in Genetics and Development, 1992. 2: p. 286-292.
65. Moretti, P., et al., *Evidence that a complex of SIR proteins interacts with the silencer and telomere binding protein RAP1.* Genes & Development, 1994. 8: p. 2257-2269.
66. Hecht, A., et al., *Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast.* Cell, 1995. 80: p. 583-592.
67. Kayne, P., et al., *Extremely conserved histone H4 N-terminus is dispensible for growth but essential for the silent mating loci in yeast.* Cell, 1988. 55: p. 27-39.
68. Bennett, C.B., et al., *SIR functions are required for the toleration of an unrepaired double-strand break in a dispensable yeast chromosome.* Molecular & Cellular Biology, 2001. 21(16): p. 5359-73.
69. Tsukamoto, Y., J. Kato, and H. Ikeda, *Silencing factors participate in DNA repair and recombination.* Nature, 1997. 388.
70. Martin, S.G., et al., *Relocalization of Telomeric Ku and SIR Proteins in Response to DNA Strand Breaks in Yeast.* Cell, 1999. 97: p. 621-633.
71. Shou, W., et al., *Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex.* Cell, 1999. 97(2): p. 233-44.
72. Gottleib, S. and R. Esposito, *A New Role for a Yeast Transcriptional Silencer Gene, SIR2, in Regulation of Recombination in Ribosomal DNA.* Cell, 1989. 56: p. 771-776.
73. Fritze, C., et al., *Direct evidence for SIR2 modulation of chromatin structure in yeast rDNA.* EMBO, 1997. 16(21): p. 6495-6509.
74. Brachmann, C.B., et al., *The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability.* Genes and Development, 1995. 9: p. 2888-2902.
75. Min, J., et al., *Crystal structure of a SIR2 homolog-NAD complex. [see comments].* Cell, 2001. 105(2): p. 269-79.
76. Braunstein, M., et al., *Transcriptional silencing in yeast is associated with reduced nucleosome acetylation.* Genes Dev 1993 Apr;7(4):592-604, 1993.
77. Tsang, A.W. and J.C. Escalante-Semerena, *CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of*

nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobT mutants during cobalamin biosynthesis in Salmonella typhimurium LT2. Journal of Biological Chemistry, 1998. 273(48): p. 31788-94.

78. Frye, R.A., *Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity*. Biochemical & Biophysical Research Communications, 1999. 260(1): p. 273-9.
79. Imai, S., et al., *Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase*. Nature, 2000. 403(6771): p. 795-800.
80. Tanny, J.C., et al., *An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing*. Cell, 1999. 99(7): p. 735-45.
81. Landry, J., et al., *The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases*. Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(11): p. 5807-11.
82. Tanner, K.G., et al., *Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. [see comments]*. Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(26): p. 14178-82.
83. D'Amours, D., et al., *Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions*. Biochem J, 1999. 342(Pt 2): p. 249-68.
84. Borra, M.T., et al., *Conserved enzymatic production and biological effect of O-acetyl ADP ribose by Sir2-like NAD+-dependent deacteylases*. J Biol Chem, 2002. 25: p. 25.
85. Finnin, M.S., J.R. Donigian, and N.P. Pavletich, *Structure of the histone deacetylase SIRT2*. Nature Structural Biology, 2001. 8(7): p. 621-5.
86. Denu, J.M., *Linking chromatin function with metabolic networks: Sir2 family of NAD+-depedent deacetylases*. Trends in Biochemical Sciences, 2003. 28(1): p. 41-48.
87. Guarente, L., *Sir2 links chromatin silencing, metabolism, and aging*. Genes & Development, 2000. 14(9): p. 1021-6.
88. Finkel, T. and N.J. Holbrook, *Oxidants, oxidative stress and the biology of ageing*. Nature, 2000. 408(6809): p. 239-47.
89. Crastes de Paulet, A., *[Free radicals and aging]*. Ann Biol Clin, 1990. 48(5): p. 323-30.
90. Tissenbaum, H.A. and L. Guarente, *Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans*. Nature, 2001. 410(6825): p. 227-30.
91. Vaziri, H., et al., *hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase*. Cell, 2001. 107(2): p. 149-59.
92. Luo, J., et al., *Negative control of p53 by Sir2alpha promotes cell survival under stress*. Cell, 2001. 107(2): p. 137-48.
93. Torok, T., et al., *P-lacW Insertional Mutatgenesis on the Second Chromosome of D. melanogaster: Isolation of lethals with Different Overgrowth Phenotypes*. Genetics, 1993. 135(September): p. 71-80.
94. FlyBase, *The FlyBase database of the Drosophila genome projects and community literature*. Nucleic Acids Research, 1999. 27: p. 85-88.

95. Akimara, H., et al., *nijre Encodes a Drosophila Homologue of CBP that Interacts with the Segment Polarity Gene cubitus interruptus and is required for embryogenesis.* Nature, 1997. 386: p. 735-738.
96. Sambrook, J., E. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual.* 2nd ed. 1989, Cold Spring Harbor: Cold Spring Harbor Laboratory. 1626.
97. Sullivan, W., M. Ashburner, and R. Hawley, eds. *Drosophila Protocols.* 2000, Cold Spring Harbor Laboratory Press: New York. 697.
98. Sass, G.L. and S. Henikoff, *Comparative analysis of position-effect variegation mutations in Drosophila melanogaster delineates the targets of modifiers.* Genetics, 1998. 148(2): p. 733-41.
99. Kwok, R.P.S., et al., *Nuclear Protein CBP is a co-activator for the transcription factor CREB.* Nature, 1994. 370(July 21): p. 223-226.
100. Bantignies, F., R. Goodman, and S. Smolik, *Functional Interaction between coactivator Drosophila CREB binding protein and ASH1, a member of the Trithorax group of chromatin modifiers.* Mol Cell Bio, 2000. 20: p. 9317-9330.
101. Clemens, J., et al., *Use of double-stranded RNA interferance in Drosophila cell lines to dissect signal transduction pathways.* PNAS, 2000. 97(12): p. 6499-6503.
102. Helm, S. and F. Meitelman, *Cancer Cytogenetics.* 1995, New York: Wiley, AiLiss.
103. Chuang, S.E. and F.R. Blattner, *Characterization of twenty-six new heat shock genes of Escherichia coli.* J Bacteriol, 1993. 175(16): p. 5242-52.
104. Greenspan, R., *Fly pushing: The theory and practice of Drosophila genetics.* 1997, Cold Spring Harbor: Cold Spring Harbor Press.
105. Barlow, A.L., et al., *dSIR2 and dHDAC6: two novel, inhibitor-resistant deacetylases in Drosophila melanogaster.* Experimental Cell Research, 2001. 265(1): p. 90-103.
106. Rosenberg, M. and S. Parkhurst, *Drosophila Sir2 is Required for Heterochromatic Silencing and by Euchromatic Hairy/E(Spl)bHLH Repressors in Segmentation and Sex Determination.* Cell, 2002. 109: p. 447-458.
107. Lamb, M., *Ageing*, in *The Genetics and Biology of Drosophila*, M.A.a.T.R.F. White, Editor. 1978, Academic Press Inc: New York. p. 43-104.
108. Grunstein, M., *Histone Acetylation in chromatin structure and transcription.* Nature, 1997. 389: p. 349-52.
109. Parro, R..
110. Fernandez-Funez, P., et al., *Identification of genes that modify ataxin-1-induced neurodegeneration.* Nature, 2000. 408(Nov 2): p. 101-6.
111. Frye, R.A., *Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins.* Biochemical & Biophysical Research Communications, 2000. 273(2): p. 793-8.
112. North, B., et al., *The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase.* Mol Cell, 2003. 11(2): p. 437-44.
113. Yang, Y.H., et al., *Cloning and characterization of two mouse genes with homology to the yeast Sir2 gene.* Genomics, 2000. 69(3): p. 355-69.

114. van Steensel, B., J. Delrow, and S. Henikoff, *Chromatin profiling using targeted DNA adenine methyltransferase. [see comments].* Nature Genetics, 2001. 27(3): p. 304-8.
115. Astrom, S.U., C. Thompson, and R. Jasper, *The Drosophila melanogaster sir2+ Gene is Nonessential and Has Only Minor Effects on Position-Effect Variegation.* Genetics, 2003. 163: p. 931-937.
116. Judd, B.H., *Mutations of zeste that mediate transvection are recessive enhancers of position-effect variegation in Drosophila melanogaster.* Genetics, 1995. 141(1): p. 245-53.
117. Kaeberlein, M., M. McVey, and L. Guarente, *The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms.* Genes & Development, 1999. 13(19): p. 2570-80.
118. Lin, Y., L. Seroude, and S. Benzer, *Extended life-span and stress resistance in the Drosophila mutant methuselah.* Science, 1998. 282(5390)(Oct 30): p. 943-6.
119. Dorman, J., et al., *The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans.* Genetics, 1995. 141(4)(Dec): p. 1399-406.
120. Chung, J., et al., *Modulation of skin collagen metabolism in aged and photoaged human skin in vivo.* Journal of Investigative Dermatology, 2001. 117(5): p. 1218-1224.
121. Ashcroft, G., et al., *Age-related differences in the temporal and spatial regulation of matrix metalloproteinases in normal skin and acute wounds of healthy humans.* Cell Tissue Research, 1997. 290(3): p. 581-591.
122. Tyner, S., et al., *p53 mutant mice that display early ageing-associated phenotypes.* Nature, 2002. 415: p. 45-53.
123. Smith, J.S., et al., *A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family.* Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(12): p. 6658-63.
124. Bernstein, B.E., J.K. Tong, and S.L. Schreiber, *Genomewide studies of histone deacetylase function in yeast. [erratum appears in Proc Natl Acad Sci U S A 2001 Apr 24;98(9):5368].* Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(25): p. 13708-13.
125. Stratmann, R. and C.F. Lehner, *Separation of sister chromatids in mitosis requires the Drosophila pimples product, a protein degraded after the metaphase/anaphase transition.* Cell, 1996. 84(1): p. 25-35.