Characterization of the Sirt6 Knockout in Drosophila Melanogaster

Characterization of the *Sirt6* knockout in *Drosophila melanogaster*

by

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I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

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Thesis Advisor

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Abstract

Parkinson's disease (PD) is a movement disorder that is characterized by the loss of dopaminergic neurons in the midbrain. It is known that midbrain dopaminergic neurons demonstrate differential vulnerability to Parkinson's disease pathology and aging. Here, I took a novel approach to elucidate the molecular mechanisms of this differential vulnerability to PD-induced pathology. Specifically, I carried out a meta-analysis of published human-derived microarray datasets to identify gene candidates that show a similar expression signature in populations of resistant neurons, such as the medial substantia nigra and the ventral tegmental area (VTA) neurons, and an altered signature in vulnerable neurons, such as those of the lateral substantia nigra. I identified *Sirt6*, which codes for a component of the DNA repair complex, as a candidate neuroprotective gene as it has high levels of expression in the VTA and the medial substantia nigra and low expression levels in the lateral substantia nigra. I used immunohistochemical analysis to confirm this differential expression pattern of *Sirt6* in the midbrain of wild type mice. To further investigate the effects of altered *Sirt6* expression on the nervous system, I used CRISPR genome editing tools to create a *Sirt6* knockout *Drosophila melanogaster* model. I use histological analyses to show that the recessive mutation leads to early-onset neurodegeneration, as evidenced by a doubling of neuropil vacuolization in 30-day-old flies. I use behavioral studies to demonstrate that mutant flies have significant climbing activity loss compared to age-matched control flies. In addition, I demonstrate that overexpression of *Sirt6* significantly attenuates loss of locomotor function and neurodegeneration in an alpha-synuclein transgenic fly model. Lastly, I discuss the

promise of our findings with regards to gaining a deeper understanding of the aging brain and Parkinson's disease pathology.

Introduction

Parkinson's Disease

Parkinson Disease is the second most common progressive neurodegenerative disorder affecting nearly 5% of the population by age 85 (de Lau and Breteler, 2006). The English surgeon, James Parkinson, first defined the disease nearly two centuries ago (Lang & Lozano, 1998). As Parkinson describes it in his 1817 essay, patients often present with "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards; and to pass from a walking to a running pace; the senses and intellects being uninjured" (Parkinson, 1817). The celebrated French neurologist Jean-Martin Charcot later coined the term Parkinson's Disease (PD) and further characterized bradykinesia and/or akinesia, rigidity, postural instability, and tremor-at-rest as the cardinal symptoms of the disease (Lang & Lozano, 1998 and Goetz, 2011). However, the first pathological studies on PD patients came nearly half a century later when Brissaud, Foix, and Nicolesco identified pathological changes in the substantia nigra of PD patients' post-mortem brain tissues (Goetz, 2011). Interestingly, a decade later, Rolf Hassler demonstrated that neuronal loss is not homogenous in the substantia nigra and that a group of neurons in this region survive in PD patients (Saper, 1999; Duke, 2007; and McNaught, 2010). As I will discuss later, this important pathological finding has paved the way for numerous investigations, including

this project, into the molecular basis of PD's pathology. Throughout the past decades, it has been shown that while substantia nigra pathology is the hallmark of the PD, there is also evidence of neuronal loss in the locus coeruleus, dorsal nucleus of vagus, hypothalamus, nucleus basalis of Meynert, olfactory bulb, and the enteric nervous system (Forno, 1996). Together, these pathological changes in the brain lead to a constellation of motor and non-motor symptoms, such as tremor, bradykinesia, gait instability, olfactory loss, intestinal dysmotility, and sleep abnormalities (Chaudhuri and Schapira, 2009).

Differential vulnerability of Dopaminergic neurons to PD pathology

Numerous studies have demonstrated that the pathology in the substantia nigra's pars compacta is responsible for the disease's motor symptoms (Rodriguez-Oroz, 2009). Substantia nigra pars compacta houses the cell bodies of a group of dopaminergic neurons that are a component of two neuronal pathways, termed the direct and indirect pathway, responsible for facilitating motor function. In the direct pathway, the dopaminergic neurons function to release the tonic inhibition of the thalamus on the cortex, thereby facilitating motor function. In the indirect pathway, the dopaminergic neurons enhance the thalamus' tonic inhibition of the cortex, thereby repressing movement. Together, substantia nigra's dopaminergic projections fine-tune cortex's motor commands (kandel, 2000). As a result, many investigators have focused on elucidating the pathological mechanisms that lead to loss of dopaminergic neurons in the substantia nigra pars compacta. Interestingly, it has been long known that there is an anatomical pattern to neurodegeneration amongst these dopaminergic neurons. In 1938,

Rolf Hassler provided pathological evidence that showed that the ventrolateral region of the substantia nigra pars compacta was most severely affected in the post-mortem brains of PD patients (González-Hernández, 2010). Since then, numerous studies have confirmed that neuronal death is more abundant in the ventral pars compacta compared to both the dorsal pars compacta and the medial neurons of the ventral tegmental area, a neighboring area that houses dopaminergic neurons involved in the reward circuit (Duke, 2007 and McNaught, 2010).

Identifying neuroprotective genes

Over the past two decades, most PD research groups have been focused on using transgenic or neurotoxic-induced animal models to study the cellular pathways that are negatively affected in SN's vulnerable cells. Their analyses of degenerating neurons suggest that PD deregulates the following downstream pathways: energy metabolism, proteostasis, inflammatory responses, vesicle trafficking, and DNA damage (McNaught, 2010; Zheng, 2010; and Lim, 2013). Nevertheless, it is unlikely that this strategy would be suitable for identifying potential therapeutic targets, as many of these pathways are concurrently deregulated in the degenerating neuron. Moreover, it is highly possible that dysregulation of these pathways merely correlate with PD pathology and that they do not play a causative role in cell death. Simply put, modulation of these downstream pathways would be unlikely to rescue pathology. Here, and in contrast to the field's current strategy, I proposed a novel approach to elucidate the molecular distinctions that render groups of midbrain neurons resistant to PD-induced neurodegeneration. Specifically, I

aimed to identify the genes that show a similar expression signature in populations of resistant neurons, such as the medial SN and the VTA neurons, and an altered signature in vulnerable neurons, such as those of the lateral SN. To this end, I have carried out a meta-analysis of microarray datasets that carried the gene expression profile of the VTA, medial SN, and lateral SN in the post-mortem human tissue of four healthy human subjects (Roth, 2006 and Duke, 2007). My analysis revealed *Sirt6*, a member of the Sirtuin family of genes, as a potential neuroprotective gene.

Sirtuins

Sirt6 is a member of the sirtuin family, a group of highly conserved NAD+-dependent enzymes, which have played a major role in metabolic and chromatin regulation throughout evolution (Vassilopoulos, 2011). Silent Information Regulator 2 (SIR2), the first member of the sirtuin family, was discovered in 1981 by researchers at the Cold Spring Harbor Laboratories studying yeast genetics. Klar et al. described the discovery of a protein responsible for transcriptional silencing at the silent mating loci and telomeres in the Saccharomyces cervesiae (Klar, 1981). However, sirtuins only came into the spotlight in 1999 following a string of three major findings by Leonard Guarente's research group at MIT. In 1999, the group demonstrated that upregulation of SIR2 is sufficient to promote longevity in yeast (Kaeberlein and McVey, 1999). A year later, the researchers established that SIR2 is a histone deacetylase and that its deacetylase activity is responsible for its role in transcriptional silencing and longevity (Imai and Armstrong, 2000). Importantly, the group further demonstrated that SIR2 overexpression extends

lifespan in Caenorhabditis elegans, which has a post-mitotic adult life (Tissenbaum, 2001). Since the early 2000's, numerous SIR2-like proteins have been discovered. These proteins are grouped into group classes I-IV (Huang, 2007) (Figure 1). Prokaryotic and eukaryotic organisms express different members of each class. Humans express members of all four classes: SIRT1-7 (Frye, 2000). SIRT1-3 are class I, SIRT4 is class II, SIRT5 is class III, and SIRT6 and SIRT7 are class IV (Frye, 2000). While a highly conserved NAD+-binding catalytic domain unites these seven proteins, they each have unique expression patterns and subcellular localization.

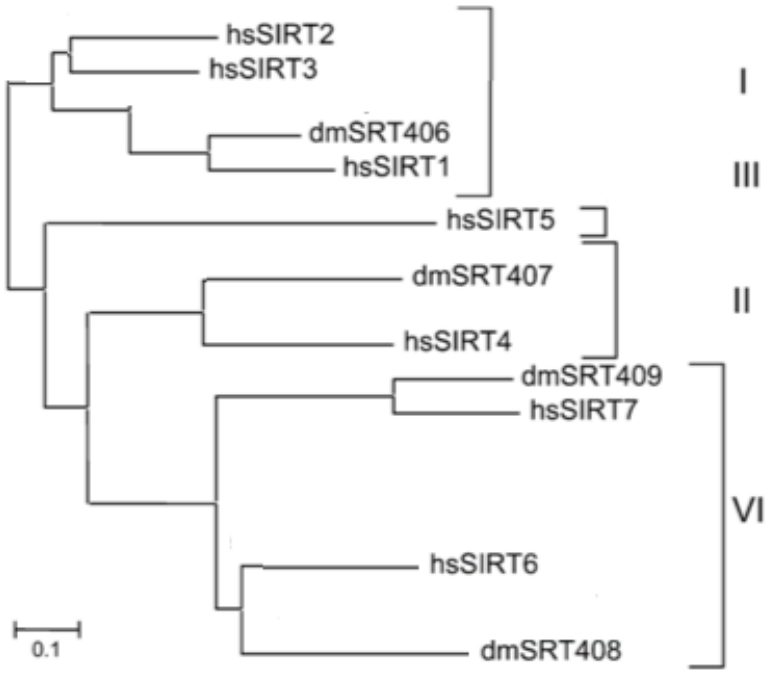
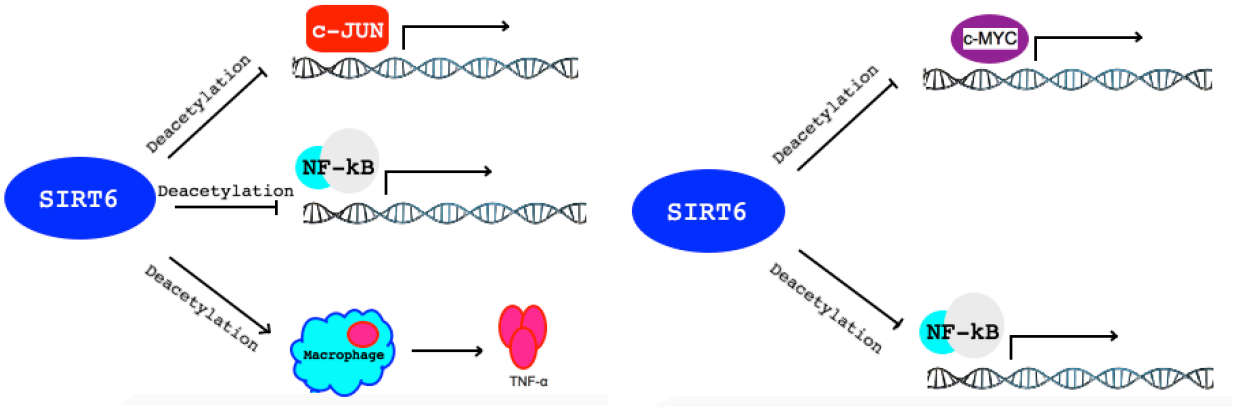


Figure 1: The molecular phylogeny of eukaryotic sirtuins. Dm and hs represent *Drosophila* melanogaster and Homo sapiens, respectively. Figure modified from Huang et al. 2007.

Sirt6

Human *Sirt6* was first identified and mapped to chromosome 19q13.3 in the year 2000 (Frye, 2000). Initial characterization of the protein came five years later when Fredrick Alt's group demonstrated that the protein was intranuclear and tightly bound to chromatin. The group also created the first knockout mouse model of *Sirt6* and found that these mice were small, had a progeroid phenotype, only lived about four weeks, and had a constellation of severe abnormalities, such as profound lymphopenia, lordokyphosis, lipodystrophy, low levels of serum insulin growth factor receptor-1 (IGF-1), and severe hypoglycemia. These observations were in line with *Sirt6*'s wide expression pattern in the body, with the highest expression levels seen in the brain, muscle, and thymus (Mostoslavsky, 2006). Over the past few years, numerous groups have begun to shed light on the underlying mechanisms of *Sirt6*'s pleiotropic effects in different tissues. Currently, we know that *Sirt6* is a NAD+-histone deacetylase and a weak ADP-ribosylase (Michishita, 2008 and Mao, 2011). Specifically, *Sirt6* deacetylates histone H3 at lysine 9 (H3K9) and lysine 56 (H3K56). Since histone deacetylation promotes the chromatin to acquire a closed conformation, it was speculated that *Sirt6* might play a role in regulating gene expression, DNA repair, and telomere maintenance (Grewal, 2003 and Kugel, 2014). Figure 2 illustrates our current understanding of *Sirt6*'s role as a negative regulator of cellular pathways.

(a and b)



(c) (d)

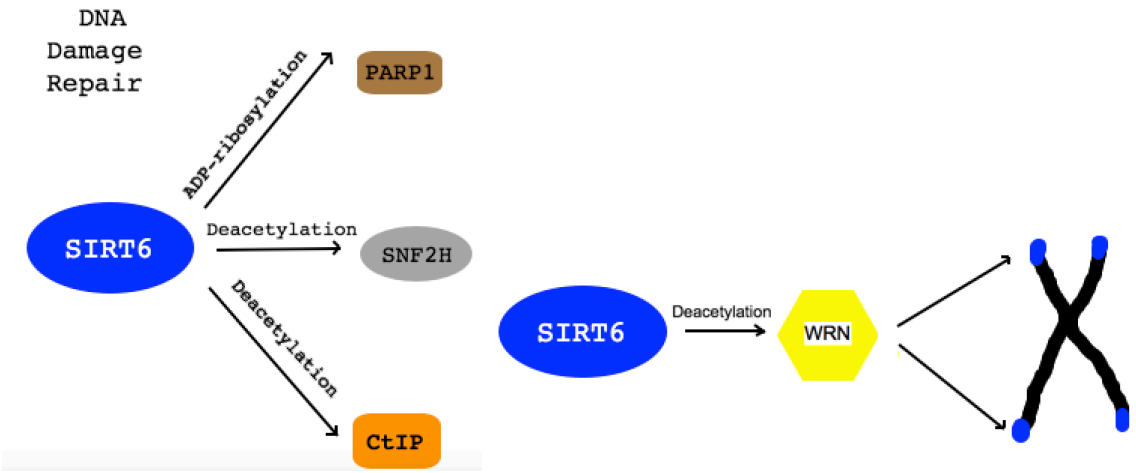


Figure 2| SIRT6 modulates multiple cellular pathways. (a and b) SIRT6's histone deacetylation at H3K9 and H3K56 decrease the access of transcription factors (c-JUN, NF-kB, c-MYC) to their target genomic regions and thereby, downregulates the expression of their target genes. In macrophages, SIRT6's deacetylase activity promotes the release tumor necrosis factor alpha (TNF-alpha). (c) SIRT6 ADP-ribosylates poly-(ADP-ribose) polymerase 1 (PARP1), enhances the recruitment of SNF2H to sites of DNA double strand breaks, and directly deacetylates C-terminal binding protein (CtBP) interacting protein(CtIP). (d) SIRT6 deacetylates H3K9 and H3K56 that promotes stabilization of Werner syndrome protein (WRN), which in turn, enhances to telomere maintenance.

***Sirt6* regulates aging-related cellular pathways**

Since Fred Alt's group first demonstrated *Sirt6* knockout mice's progeroid phenotype, numerous investigators have elucidated multiple pathways in which *Sirt6*'s absence might contribute to an early aging phenotype. Sirt6's regulatory role in these pathways is described below.

Inflammatory pathways: *Sirt6* promotes the secretion of tumor necrosis factor-alpha (TNF-alpha), a key pro-inflammatory cytokine, from macrophages. At the same time, *Sirt6* inhibits the inflammatory response by attenuating nuclear factor kB (NF-kB) signaling. *Sirt6* deacetylates H3K9 at the promoter of NF-kB target genes and decreases inflammatory-dependent apoptosis and senescence (Kawahara, 2009). In addition, *Sirt6* inhibits the expression of c-JUN-dependent pro-inflammatory genes, such as Interleukin-6 and TNF-alpha in macrophages (Xiao, 2012).

Cellular senescence: *Sirt6* overexpression prevents premature senescence in porcine fetal fibroblasts that were incubated with cytotoxic agents, such as tert-butylhydroperoxide, by reducing DNA damage build up in the nucleus (Xie, 2012). *Sirt6* is thought to postpone aging as it has been shown to decelerate cellular senescence, DNA damage build up, and telomere dysfunction in endothelial cells (Cardus, 2013).

Cancer metabolism: Raul Mostoslavsky's group at MGH first unraveled a major role for *Sirt6* in cancer metabolism. Specifically, the group has shown that *Sirt6* is a potent tumor suppressor and that its expression level is a predictor of tumor-free survival in patients suffering from pancreatic cancer or colorectal carcinoma (Sebastián, 2012). It is also

known that *Sirt6* overexpression, through the protein's ADP-ribosylase activity, triggers apoptosis in cancer cell lines (Van Meter, 2011).

DNA damage repair: *Sirt6* is a key member of the DNA repair complex and is involved in base excision repair (BER), telomere maintenance, and double-strand break (DSB) repair (Mostoslavsky, 2006). Katrin Chua's laboratory at Stanford was the first group to identify a physiological function for *Sirt6*. They demonstrated that *Sirt6* is bound to telomeres where its H3K9 deactylation function is required to prevent replication-associated telomere defects and premature ageing (Michishita, 2008). Specifically, *Sirt6* deacetylates H3K9 and H3K56 residues during DNA replication and this stabilizes the Werner syndrome (WRN) protein, which is a component of a protein complex that caps telomeres. Mutations in either *Sirt6* or WRN lead to genomic instability (Michishita, 2008 and 2009).

***Sirt6* regulates the repair of single and double strand DNA break**

SIRT6 is also involved in both Base Excision Repair (BER) and DNA double strand break (DSB) repair. In their 2006 landmark publication, Fred Alt's group demonstrated that deletion of *Sirt6* in both Mouse embryonic fibroblasts (MEFs) and Embryonic Stem (ES) cells leads to increased sensitivity to oxidative agents, which cause DNA breaks that are mainly repaired by BER (Mostoslavsky, 2006). Vera Gorbunova's group at University of Rochester later found that *Sirt6* physically interacts with PARP1, a protein involved in both BER and the repair of double Strand DNA breaks (Mao, 2011). More recently, the group has shown that *Sirt6* deletion in Mouse embryonic fibroblasts (MEFs) leads to a 40% decline in BER's efficacy and that an overexpression of *Sirt6* reverses the decline of

BER in aging foreskin fibroblasts. They have also demonstrated that both of *Sirt6*'s enzymatic activities, deacetylase and mono ADP-ribosyl transferase, are required for efficient BER (Xu, 2015). However, the molecular mechanisms with which *Sirt6* regulates BER remain to be elucidated. Currently, the molecular mechanisms of *Sirt6*'s involvement in DSB repair are better understood than its role in BER. Specifically, *Sirt6* is recruited to the site of DSBs in the genome where it interacts with and mono-ADP-ribosylates PARP1 and enhances its activation at the DNA break site (Mao, 2011). PARP1 recruits and modifies a number of the DNA damage repair complex proteins, such as XRCC1 and ATM, by poly ADP-ribosylating these proteins (Michèle Rouleau, 2010). Overexpression of *Sirt6* in mammalian cells significantly enhances the clearance of gamma-H2AX foci, a marker for DSB, and reduces DNA fragmentation, as seen on comet assay, following oxidative stress (Mao, 2011).

***Sirt6* maintains genomic stability**

It has been long known that transposable elements (also known as 'jumping genes') are a major source of genomic instability in mammalian cells. Transposons, which have given rise to nearly half of the human genome, promote DNA double-strand breaks, insertion-mediated deletions, genomic conversion and rearrangement, ectopic recombination, and the formation of mutation-prone microsatellite islands. Retrotransposons are a group of transposons that replicate through reverse transcription of RNA intermediates and insert themselves into new locations in the genome (Lander, 2001; Cordaux and Batzer, 2009). Transposable elements are packaged in heterochromatin regions where they remain transcriptionally repressed. However, aging leads to heterochromatin relaxation and this,

in turn, leads to transposon's loss of repression (Li, 2012). In fact, it is known that reactivation of retrotransposons play an active role in neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) (Li, 2015; Laska, 2012). Long interspersed nucleotide element-1 (LINE-1) is a subgroup of retrotransposons that comprise 17% of the entire human genome and is responsible for most of retroransposition in the human population (Brouha, 2002). Interestingly, new findings by Vera Gorbunova's group have shown that *Sirt6* plays a key role in repressing the transcription of LINE-1. *Sirt6* binds to the LINE-1 promoter and suppresses its transcription by modulating its heterochromatin status. *Sirt6* deletion in mice leads to a seven-fold increase in LINE-1 transcription in the brain. Overexpression of *Sirt6* in MEF cells reduces LINE-1 retrotransposition by 71%. Interestingly, *Sirt6* leaves its position at the LINE-1 promoter in response to stress and normal aging leading to the de-repression of dormant LINE-1 (Van Meter, 2014). Based on these findings, Van Meter el al. have proposed that in addition to DNA damage repair, *Sirt6* also protects cells from genomic instability by keeping the most active subgroup of transposons in an epigenetically silent state. In summary, there is ample evidence to support a crucial role for *Sirt6* in protecting cells from damage accumulation in the telomeres and the genome.

Alpha-synuclein is involved in PD's pathology

For almost two hundred years, our understanding of PD was limited to our observations in the neurology clinic and at the microscope. This all changed in 1997 when a group of European scientists identified a mutation, an alanine to threonine mutation at residue 53 of alpha-synuclein, in one Italian kindred and three unrelated Greek families that showed

an autosomal dominant pattern of PD inheritance (Polymeropoulos, 1997). Within a few months, Virginia Lee and John Trojanowski's group at University of Pennsylvania demonstrated strong alpha-synuclein staining in the aggregates accumulated in the substantia nigra of six idiopathic PD patients (Spillantini, 1997). Since then, two more additional SNCA missense mutations, A30P and E46K, and a wild type triplication have been identified in PD patients (Zarranz, 2004; Kruger, 1998; and Singleton, 2003). SNCA codes for alpha-synuclein, a presynaptic protein that promotes the assembly of protein complexes required for neurotransmitter packaging in and release from vesicles at the presynaptic nerve terminal. Specifically, alpha-synuclein directly associates with vesicle-associated membrane protein 2 (VAMP2) and promotes soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex assembly at the presynaptic terminal (Burré, 2010). While a clear molecular connection between alpha-synuclein and PD pathology remains to be elucidated, it is thought that mutations and/or overexpression of SNCA ultimately leads to the protein's misfolding, abnormal post-translational processing, and aggregation (Breydo, 2012). Numerous studies have showed that increased alpha-synuclein expression levels correlates with PD pathology. First, transgenic mice that express human SNCA in their nervous system show signs of progressive motor dysfunction, dopaminergic synapse loss in the basal ganglia, and alpha-synuclein positive protein aggregation in the SN pars compacta (Masliah, 2000). Second, alpha-synuclein overexpression in rodents and monkeys have been shown to induce parkinsonian motor symptoms, loss of dopaminergic neurons in the SN pars compacta, and alpha-synuclein positive inclusion bodies in neurons (Kirik, 2003). Third, patients with

SNCA duplication have a late age-of-onset, slow progressing, and a milder cognitive decline course, while patients with SNCA triplication have an early age-of-onset, prominent dementia, and accelerated cognitive decline (Chartier-Harlin, 2004). Together, these findings suggest that overexpression of alpha-synuclein is a representative model of PD pathology.

Drosophila **model of PD**

Our *Drosophila* alpha-synuclein model is based on the QF2-QUAS expression system developed by Christopher Potter's group at Stanford University. In this system, the QF2 transcription factor is downstream of the neuronal-specific Synaptobrevin promoter and the human alpha-synuclein is downstream of a QUAS promoter (Figure 3). As a result, human alpha-synuclein's expression is only driven in neurons. Our laboratory has demonstrated that in this model, neuronal expression of human alpha-synuclein leads to significant and accelerated vacuole formation throughout the brain, accelerated apoptosis, significant neuronal loss in the medulla, and early-onset locomotor function loss in flies (Ordonez, 2018).

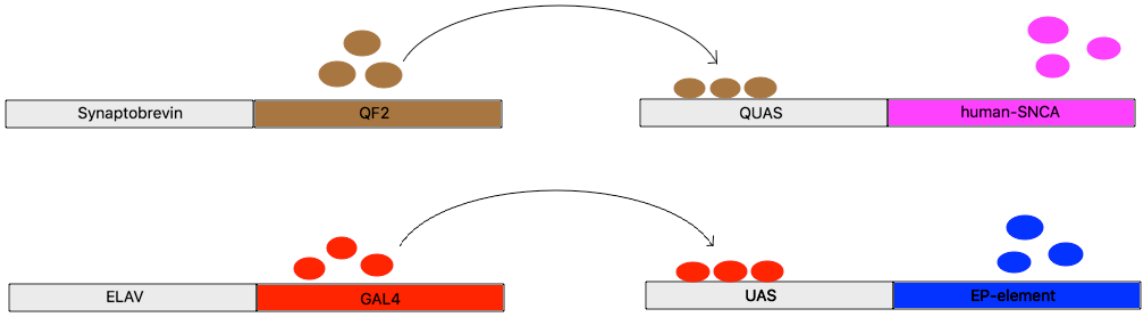


Figure 3| Driver lines expressing QF2 and GAL4 in neurons are crossed to QUAS- and UAS- reporter lines, respectively. The human-*SNCA* and *Sirt6 EP-element* are respectively fused to QF-2 and GAL4 bindings sites. The activators (QF2 and GAL4) drive the expression of *SNCA* and *Sirt6* in the *Drosophila* nervous system.

Methods

Construction of the CRISPR SIRT6 $^{-/-}$ line

To create *Sirt6* knockout flies, two gRNA sequences that serve to guide the Cas9 nuclease to specific genomic locations were designed and inserted into pl100-pBFv-U6 (Perrimon laboratory). The sgRNA sequences are as follows: AACCATGAGCTGCAACTACGcgg (at ATG site) and GCAATTACTTCATTACTTTGagg (after the stop codon). The plasmid (150 ng/µl) was injected into approximately 200 Cas9-expressing flies (*yw; attP40{nos-Cas9}/CyO)* by BestGene. There were 42 surviving adults, which were then crossed to *Tm3/Tm6* balancer flies in a series of crosses. The F1 of these crosses were screened by PCR to identify flies that potentially harbored a third chromosome that lacked the *Sirt6* gene. Only one line was found to carry this deletion. Two flies from this line were crossed to *Tm3/Tm6* balancers and to each other, in order to maintain a stock and create SIRT6 $^{-/-}$ flies, respectively (Figure 4). Whole *Sirt6* deletion in SIRT6$^{-/-}$ line was verified by PCR.

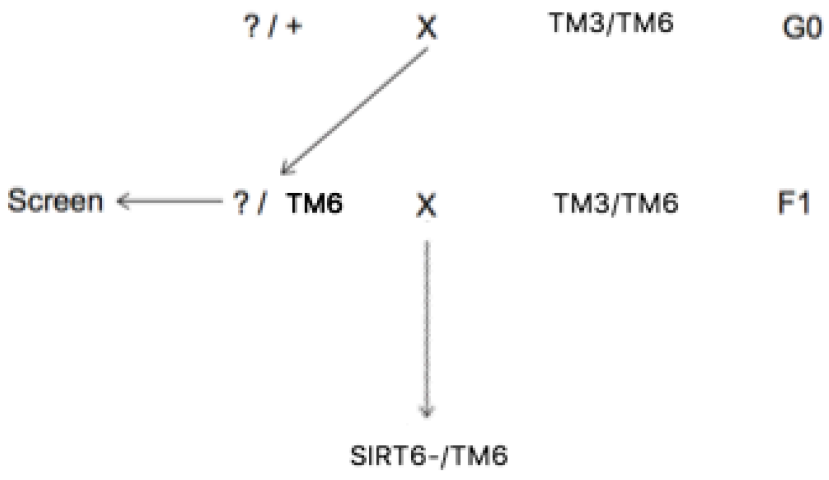


Figure 4| Crossing outline to generate balanced SIRT6^{-}/TM6 *Drosophila* stock. Following simultaneous injection of two sgRNAs into embryos (yw; attP40{nos-Cas9}/CyO), the modified chromosome is detected by first crossing the injected G0 flies to TM3/TM6 stock and isolating individual F1 flies for screening. Screening is done by genotyping the wings of F1 flies to identify deletion of SIRT6.

PCR verification of the CRISPR transgenic line

PCR primers to verify the deletion of *Sirt6* from the *Drosophila* genome via CRISPR were designed in the following manner. The 5' primer was targeted against a genomic region 400bp upstream of *Sirt6*'s start codon while the 3' primer was targeted against a genomic region 300bp downstream of *Sirt6*'s stop codon. Hence, when *Sirt6* is entirely deleted, a 710bp PCR product is produced.

Sirt6 Deletion reporter-F: CAGCCGTTGTTTACAGTGTGACC

Sirt6 Deletion reporter-R: CAGATGCCAAGATCTGGCCC

Sirt6 inside Reporter-F: GGCTGAATTGATCAAGAAATCGGG

Sirt6 inside Reporter-R: GTGGTTCCAAGCGCAATATTTAGG

Histology and Immunostaining

Adult fly heads were fixed in formalin and embedded in paraffin. They were subsequently sectioned in a frontal orientation. Serial 2- or 4-µm sections spanning the entire *Drosophila* brain were obtained and positioned on a glass slide. Brain histology was evaluated by staining paraffin sections with H&E according to standard protocols. Prior to immunostaining, Sodium citrate–based antigen retrieval (boiling for15 minutes, 10 mM sodium citrate, pH 6.6) was carried out. Immunostaining was performed using an avidin-biotin-peroxidase complex method (VECTASTAIN ABC Kit; Vector Laboratories) or

secondary antibodies coupled to AlexaFluor-488 or AlexaFluor-555 (Invitrogen). Primary antibodies included anti-SIRT6 (1:1000; NB100-2522), anti-Tyrosine Hydroxylase (1:500; ImmunoStar).

Quantitative PCR. RNA was extracted from eight *Drosophila* heads by surgically removing the heads and immediately freezing them on dry ice. Frozen tissues were thawed in QIAZOL (Qiagen) for isolation of total RNA. RT-PCR was performed in a two-step procedure. A Nanodrop ND-1000 Spectrophotometer was used to measure total RNA concentration. For each sample, 1 μg of total RNA was reverse transcribed using a cDNA Reverse Transcription Kit (Applied Biosystems), and the resulting cDNA was used for a qPCR reaction. PCR reactions were set up in a 16-μl-reaction volume using SYBR Green PCR Master Mix, and *RpL32* was used as the internal control. Each data point is the result of three biological and three technical replicates.

RT-PCR primers

Sirt6-RNAi forward: GTCATGTTTTGTGGGCTGCA;

Sirt6-RNAi reverse: TTGCGCTTGGAACCACTTTG;

Locomotion assay. At least 20 flies per genotype were collected on the day of eclosion and each fly was individually placed in a fresh vial without anesthesia. Locomotion was measured on the afternoon of days 2, 4, 8 and 11. Each fly was tapped gently to the bottom of the vial and the vial was placed on a gridded surface. Locomotion was measured by counting the number of centimeters a fly walked in 30 seconds. This measurement was carried out four times for each fly, with at least one-minute interval

between each measurement. A total of 18-20 flies were assayed per genotype.

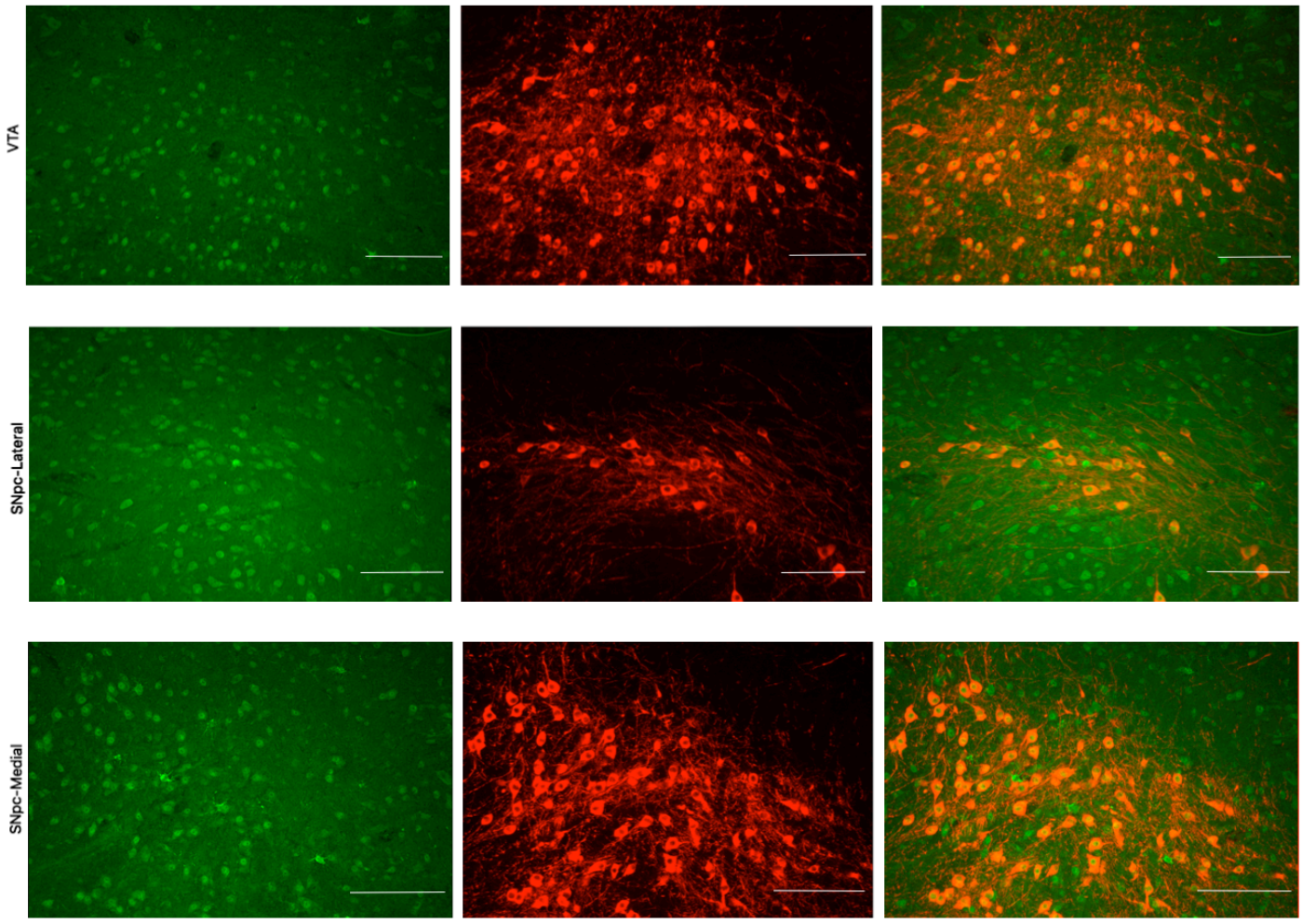
Results

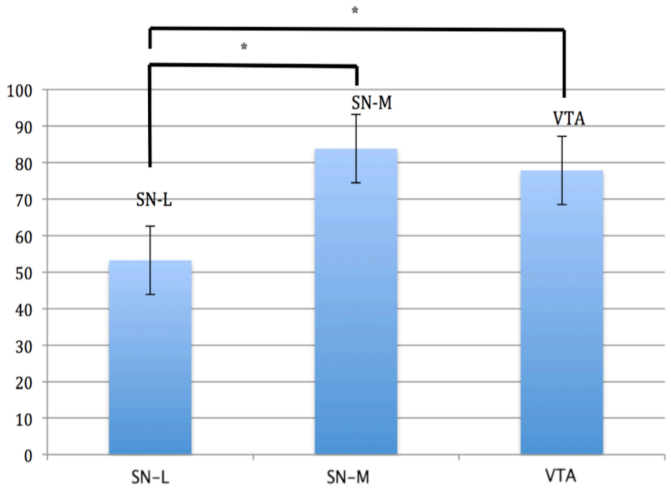
***Sirt6* is upregulated in resistant dopaminergic neurons compared to vulnerable neurons**. To identify the genes that show a similar expression signature in populations of resistant neurons, such as the medial SNpc and the VTA neurons, and an altered signature in vulnerable neurons, such as those of the lateral SNpc, I carried out a meta-analysis of two publicly available raw microarray datasets from Roth et al. 2006 and Duke et al. 2007. Specifically, I extracted the raw micro-array data of four human controls with no history of a neurological disease from each study (Roth et al. 2006 and Duke et al. 2007). Gene expression profiles for the lateral and medial SNpc were extracted from the 2007 publication of Duke et al. while the gene expression profile of the VTA was obtained from the 2006 findings of Roth et al. The brains were fresh-frozen upon autopsy and the post-mortem delay was less than 21.7 hours for all subjects. As both studies had used two comparable versions of Affymetrix HG_U133 chips for their transcriptional analysis, I was able to normalize each dataset to the same housekeeping gene and combine them to create one data set that contained transcriptional profiles of the media and lateral SNpc and the VTA. I used Pearson coefficient of correlation to identify the top ten gene transcripts that were significantly upregulated in both the medial substantia nigra and the ventral tegmental area, compared to the lateral substantia nigra (Table 1). Among these candidate gene transcripts, I decided to focus on *Sirt6* as it is highly expressed in the brain and has been shown to regulate genome stability, DNA damage repair, and telomere

integrity (Kugel & Mostoslavsky 2014). Specifically, SIRT6 $^{-/-}$ mice have severe degenerative and aging-like symptoms and reduced lifespan while brain-specific *Sirt6* knock out mice have a smaller brain size and significant growth-defects (Schwer, 2010). At the cellular level, Toiber et al. have reported that apoptosis and DNA damage markers are upregulated in *Sirt6* deficient neurons (Toiber, 2013). To validate our meta-analysis findings, with regards to *Sirt6*'s differential transcription pattern in the midbrain, I used immunohistochemical analysis to assess *Sirt6* expression levels in midbrain sections of five wild type mice. Quantification of *Sirt6* signal in the nucleus of dopaminergic neurons of the SNpc and the VTA confirmed that the protein has a significantly higher expression levels in the resistant dopaminergic neurons, VTA and medial SNpc, compared to the neurons of the lateral SNpc (Figure 5). These preliminary findings prompted us to generate a *Sirt6* knockout fly model to further study *Sirt6*'s potential role as a neuroprotective gene.

Gene	Function
ASMTL_AS1	RNA Gene, affiliated with the non-coding RNA class
SIRT6	NAD-dependent protein deacetylase
EFCAB13	EF-Hand Calcium Binding Domain 13
PELI2	Involved in the TLR and IL-1 signaling pathways
XXRA1	
ARID3A	important for normal embryogenesis
Myo15B	Unconventional Myosin
MALAT1	RNA Gene, affiliated with the non-coding RNA class
TSPAN10	
ST6GALNA	intracellular transport of apical glycoproteins

Table 1| Genes significantly upregulated in both the medial substantia nigra (SN-M) and the ventral tegmental area (VTA), compared to the lateral substantia nigra (SN- L). Our Meta-analysis of microarray datasets obtained from Roth et al. and 2006 and Duke et al. 2007 identified Sirt6 as a neuroprotective candidate gene.


VTA
SNpc-Lateral
SNpc-Medial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VTA

Figure 5| *Sirt6* is differentially expressed in the mouse midbrain. In non-transgenic mice, neurons of the lateral SN have lower expression levels of Sirt6 compared to the VTA and the medial SN as assessed by immunohistochemistry (*p <0.05; one-way ANOVA). The left panel sections stained with the anti-SIRT6 antibody. The middle panel sections stained with the anti-tyrosine hydroxylase (TH) antibody. The right panel is the merged image. Scale bar is 50um.

Creating SIRT6$^{-/-}$ flies using sgRNAs

To characterize *Sirt6*, I generated a null allele using CRIPSR/Cas9 with dual sgRNAs. I designed two sgRNAs to target the sequences that flank the *Sirt6*'s entire gene (Figure 6). The two sgRNAs were sent to BESTGENE where they were co-injected into approximately 200 *Drosophila* embryos with the genotype: yw; attP40{nos-Cas9}/CyO. From the 200 injected embryos, 42 survived, eclosed, and gave rise to fertile flies. These flies were crossed to the TM3/TM6 balancer to give rise to F1 flies. F1 flies were further crossed to TM3/TM6 and the progeny F2 from each cross was collected and maintained at 25'c incubator (Figure 4). I designed two pair of reporter primers to screen for a potential *Sirt6* deletion in the wings of F1 flies. Only one F1 fly was found to harbor the *Sirt6* deletion (Figure 7.a). The F2 progeny of this fly's cross to the TM3/TM6 were used to establish a stock. The stock was further characterized by PCR analysis, sequencing, and quantitative PCR analysis. PCR amplification confirms the absence of any *Sirt6* allele in SIRT6$^{-/-}$ flies (Figure 7.b). Sequencing results confirm the deletion of 6441 bases of genomic sequence, reflecting a simultaneous Cas9 excision directed by the two sgRNAs (Figure 7.c). My quantitative PCR analysis did not produce any detectable *Sirt6*

transcripts in SIRT6 $^{-/-}$ (Figure 8). Together, the PCR amplification and the sequencing data confirm that our balanced fly stock carries a *Sirt6* deletion allele.

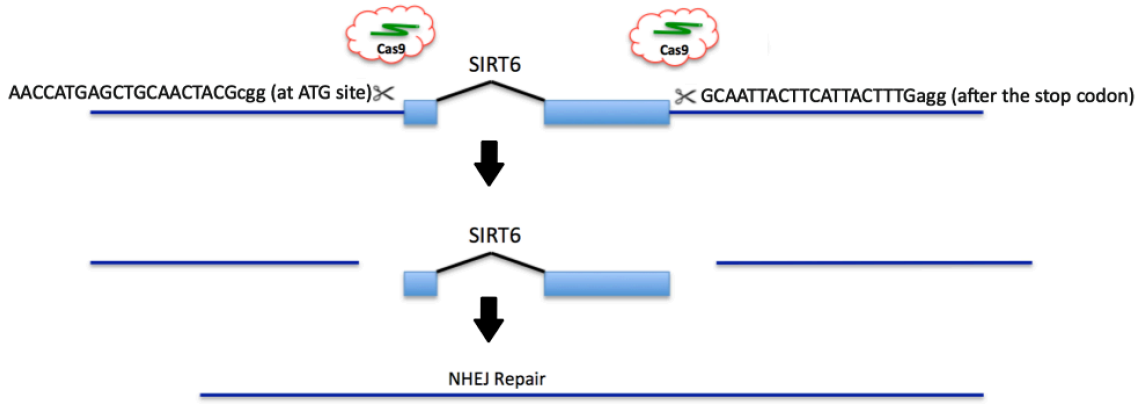
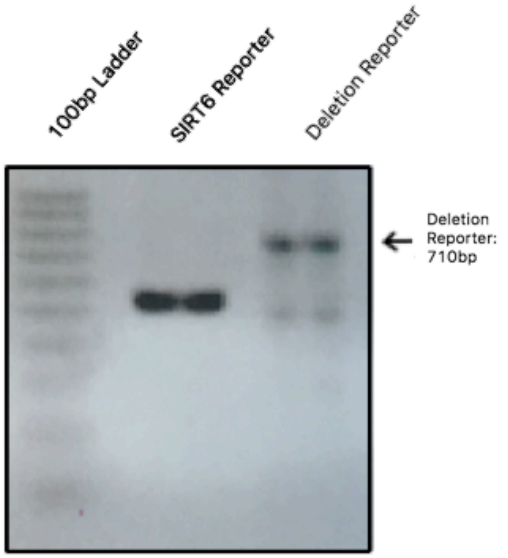
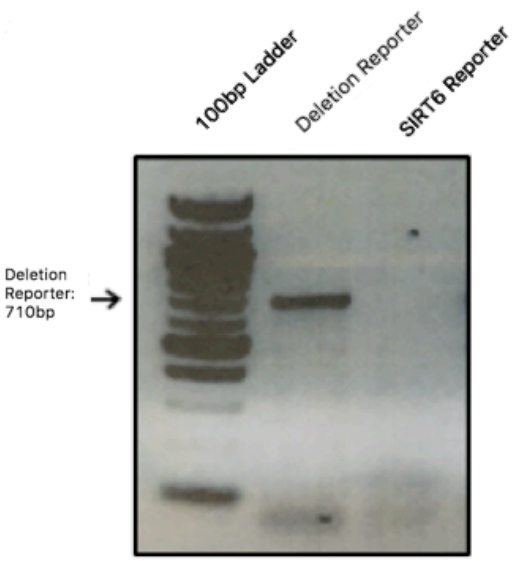


Figure 6 | Schematic of two sgRNAs targeting the Sirt6 locus to create a Sirt6 deletion using the *Drosophila* nos-Cas9/sgRNA system. Blue boxes represent exons. gRNA_1 targets the start codon and gRNA_2 targets downstream of the stop codon.

(a)


100bp Ladder
SIRT6 Reporter
Deletion Reporter
Deletion Reporter: 710bp

(b)


100bp Ladder
Deletion Reporter
SIRT6 Reporter
Deletion Reporter: 710bp

(c)

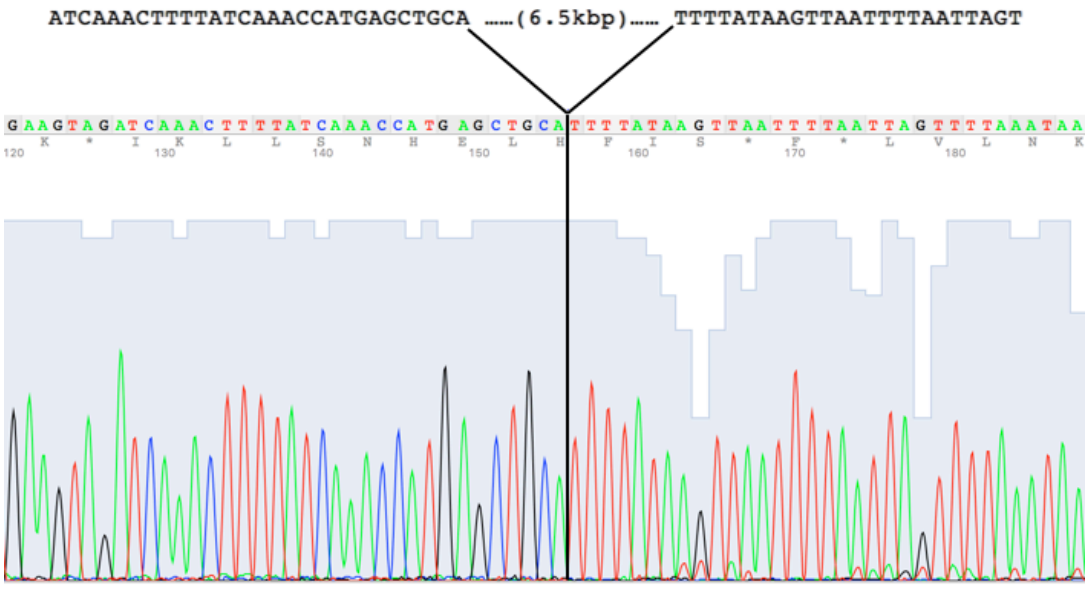

ATCAAACTTTTATCAAACCATGAGCTGCA ……(6.5kbp)…… TTTTATAAGTTAATTTTAATTAGT
GAAGTAGATCAAACTTTTATCAAACCATGAGCTGCATTTTTATAAGTTAATTTTAATTAGTTTTTAAATAA
120 130 140 150 160 170 180

Figure 7| (a) PCR analysis confirms deletion in one allele of *Sirt6 in* SIRT6^{-}/TM6 flies. The second lane is the PCR product that is produced by the *Sirt6* reporter and confirms the presence of a wild type *Sirt6* allele. The third lane carries a 710 bp PCR product that is produced by the *Sirt6* deletion reporter primer pair. (b) PCR analysis confirms deletion of both *Sirt6* copies *in* SIRT6$^{-/-}$ flies. The second lane carries a 710 bp PCR product that is produced by the Sirt6 deletion reporter primer pair. The third lane is the PCR product that is produced by the Sirt6 reporter. In the presence of Sirt6 allele, a ~500bp product would have been produced. (b) DNA sequencing confirms a ~6.5 kb deletion of Sirt6 from the third chromosome in SIRT6$^{-/-}$ flies.

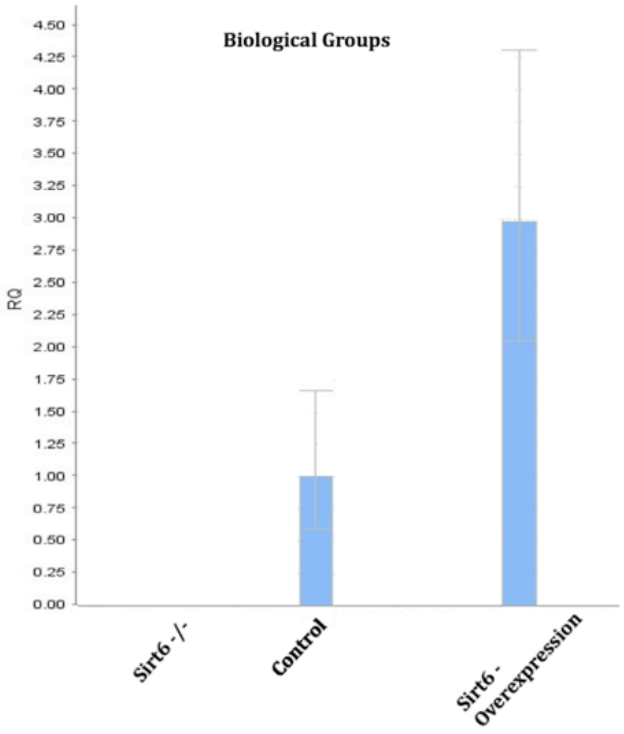


Figure 8| Quantitative RT-PCR analysis of cDNA generated from 1-day-old fly mRNA with control, Sirt6 deletion, and overexpressing Sirt6 using the UAS/GAL4 expression system and the neuronal elav-GAL4 driver. Sirt6 mRNA levels are plotted for control, Sirt6 deletion, and Sirt6 overexpression. mRNA levels were normalized to RpL32 mRNA. Control is elav-GAL4/+.

Loss of *Sirt6* causes progressive, age-dependent neurodegeneration

Histological examination of SIRT6$^{-/-}$ flies' brain sections incubated for 20 and 30 days at 25°C demonstrated a mild but accelerated and progressive vacuole formation in the neuropil compared to wild type (W^{1118}) flies ($p<0.05$)(Figure 9). By day 20 post-eclosion, the number of vacuoles, smaller than 5 um in diameter, doubles in SIRT6$^{-/-}$ flies compared to control flies. Vacuoles could be found throughout the neuropil, especially in the medulla and lateral to the ellipsoid body.

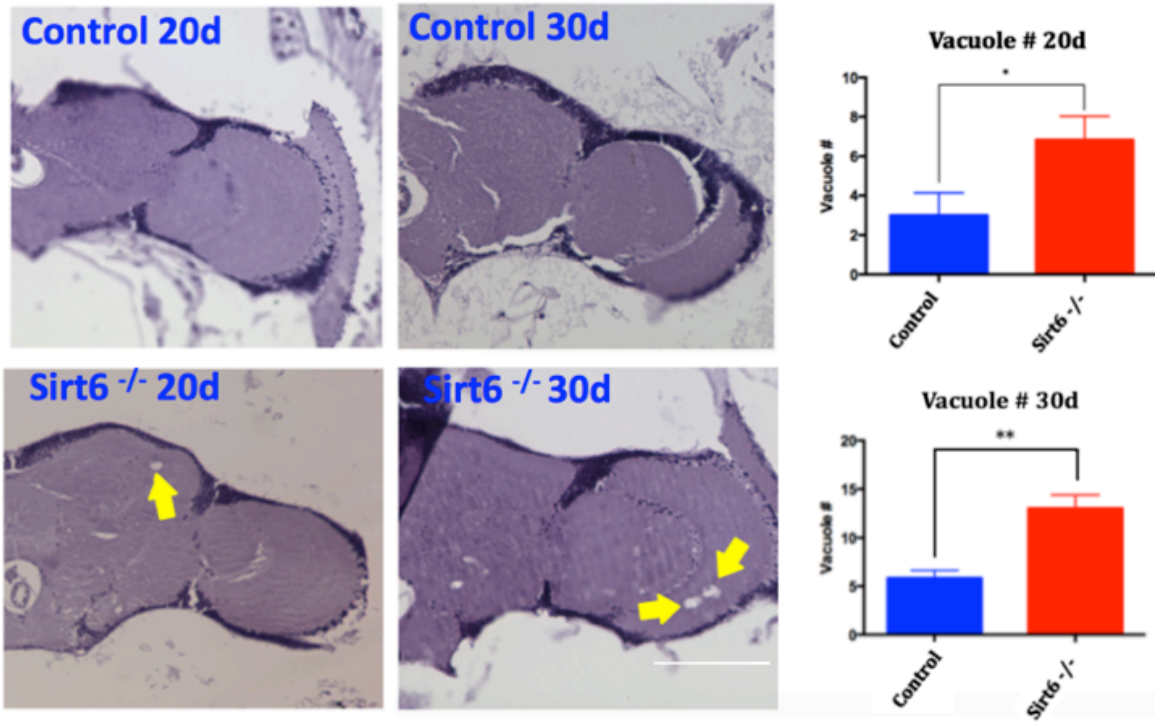


Figure 9| Sirt6 deletion causes mild neurodegeneration. Frontal sections from control and Sirt6 $^{-/-}$ flies at 20 and 30 days of age. The esophagus is visible as the hole at the bottom left of each panel. Results are from 6 flies per genotype and time point (*$p<0.05$, ** $p<0.01$; unpaired t-test). Values shown are mean +/- SEM. Control is w^{1118}. Scale bar is 20 um.

***Sirt6* homozygous flies exhibit premature loss of climbing activity and no clear lifespan shortening.** The mild neurodegeneration in SIRT6 $^{-/-}$ flies prompted us to look for a potential behavioral phenotype. Wild type flies demonstrate a robust negative geotaxis as they rapidly climb up when tapped to the bottom of the vial (Feany, 2000). This negative geotaxis response is progressively lost with normal aging and in established *Drosophila* models of neurodegenerative diseases (Feany, 2000; Marsh, 2006; Diaper, 2013). SIRT6 $^{-/-}$ flies show a robust premature loss of climbing activity from an early age ($p<0.05$) (Figure 10). When tapped to the bottom of vials, these flies climb a shorter distance compared to wild type (W^{1118}) flies. The motor deficit becomes progressively worse as the flies age. In contrast to the robust climbing activity phenotype, SIRT6$^{-/-}$ deletion does not significantly shorten the lifespan of flies (Figure 11).

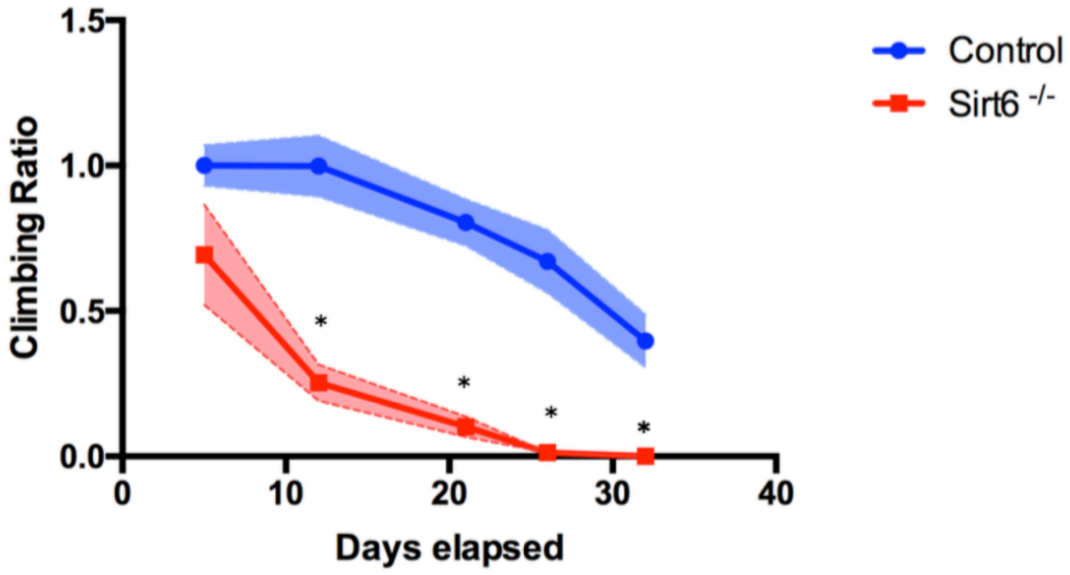


Figure 10 | Sirt6 $^{-/-}$ flies show premature loss of climbing activity. Mutant flies are significantly different in their climbing activity compared to age-matched control flies (*$p<0.05$; one-way ANOVA). Flies were raised and aged at 25°C, collected upon eclosion, and aged at 25°C. Shading indicates +/- SEM. Control is w 1118.

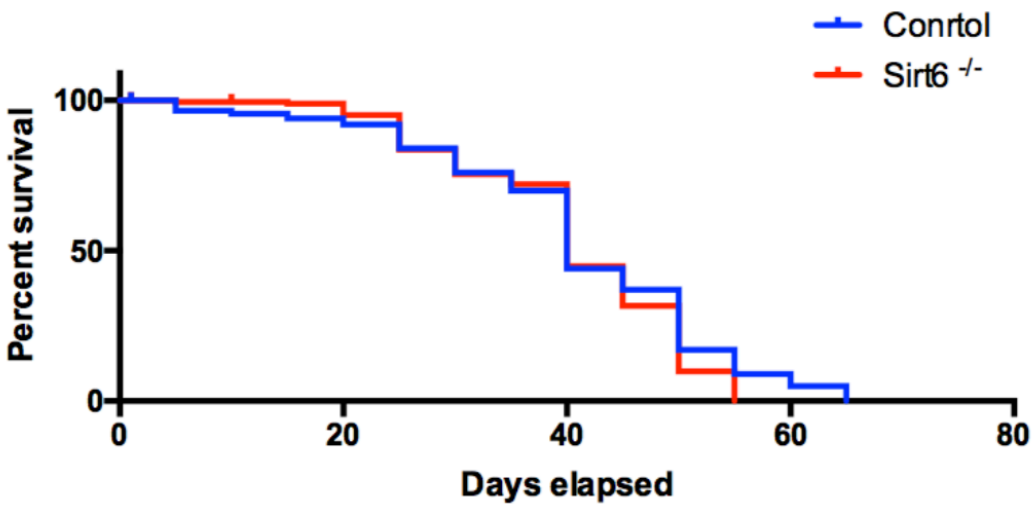


Figure 11 | Sirt6 deletion does not clearly reduce lifespan. Each point represents the mean from 200 flies. Control is w^{1118}.

Sirt6 expression modifies alpha-synuclein toxicity

Here, I sought to determine whether *Sirt6* could modulate alpha-synuclein induced neurotoxicity. To this end, I crossed our alpha-synuclein fly model to the *Sirt6* overexpresser line to upregulate SIRT6 expression in the nervous system. I also crossed the alpha-synuclein line to the SIRT6 $^{-/-}$ flies, screened for recombinant flies, and generated alpha-synuclein expressing flies that did not carry the SIRT6$^{-/-}$ gene. In addition, I crossed alpha-synuclein line and elav-gal4 flies to W^{1118} to generate our control groups. I collected, aged, and carried out the following two studies on the F1 of the above four crosses. First, I measured the locomotor activity of F1 flies at 2, 4, 8, 11 days post-eclosion. In accordance with our group's previous findings, the alpha-synuclein line showed significant reduction in locomotor activity compared to control flies as early as

day 4 post-eclosion ($p<0.05$)(Figure 12). This loss of locomotor function was partially rescued by overexpression of *Sirt6* in the alpha-synuclein flies ($p<0.05$). Interestingly, alpha-synuclein flies harboring total *Sirt6* deletion did not show a worsened locomotor activity compared to alpha-synuclein flies expressing wild type *Sirt6* (Figure 12). Next, I assessed the degree of neurodegeneration at day 11 post-eclosion in the four fly groups. I carried out hematoxylin staining on measured cell density in the frontal medulla region. As per our group's previous findings, alpha-synuclein expressing flies had significant, approximately 40%, neuronal loss in the medulla ($p<0.05$) (Figure 13). Overexpression of *Sirt6* attenuated this alpha-synuclein-mediated neuronal loss in the frontal medulla as cell density was in this region was comparable to control flies (Figure 13). However, loss of *Sirt6* expression in alpha-synuclein expressing flies did not further worsen neurodegeneration (Figure 13). This finding was in line with the results of the locomotion assay, which demonstrated that *Sirt6* loss does not exacerbate the phenotype of alpha-synuclein flies. Taken together, these findings suggest that *Sirt6* overexpression has neuroprotective effects in our fly model of neurodegeneration.

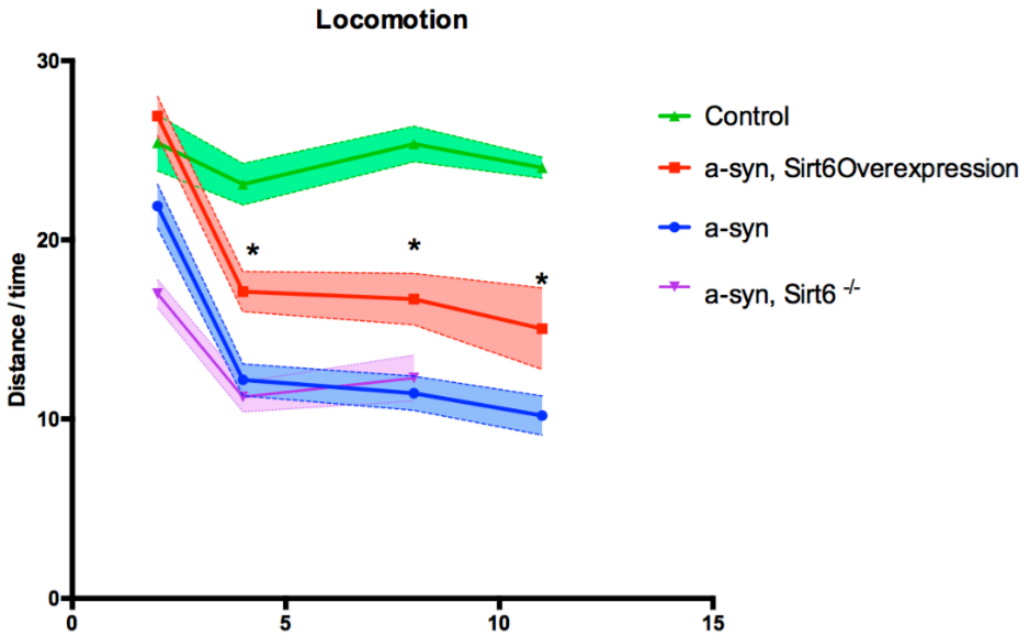


Figure 12| Overexpression of Sirt6 significantly attenuates loss of locomotor function in an alpha-synuclein transgenic fly model relevant to Parkinson's disease. Locomotor activity was assessed by measuring the distance that the fly walked in 30 seconds. Results are from 20 flies per genotype. (*p<0.05; one-way ANOVA). Values shown are mean +/- SEM. Alpha-synuclein is expressed using the QF system and the Syb-QF2 driver. Control is elav-GAL4/+.

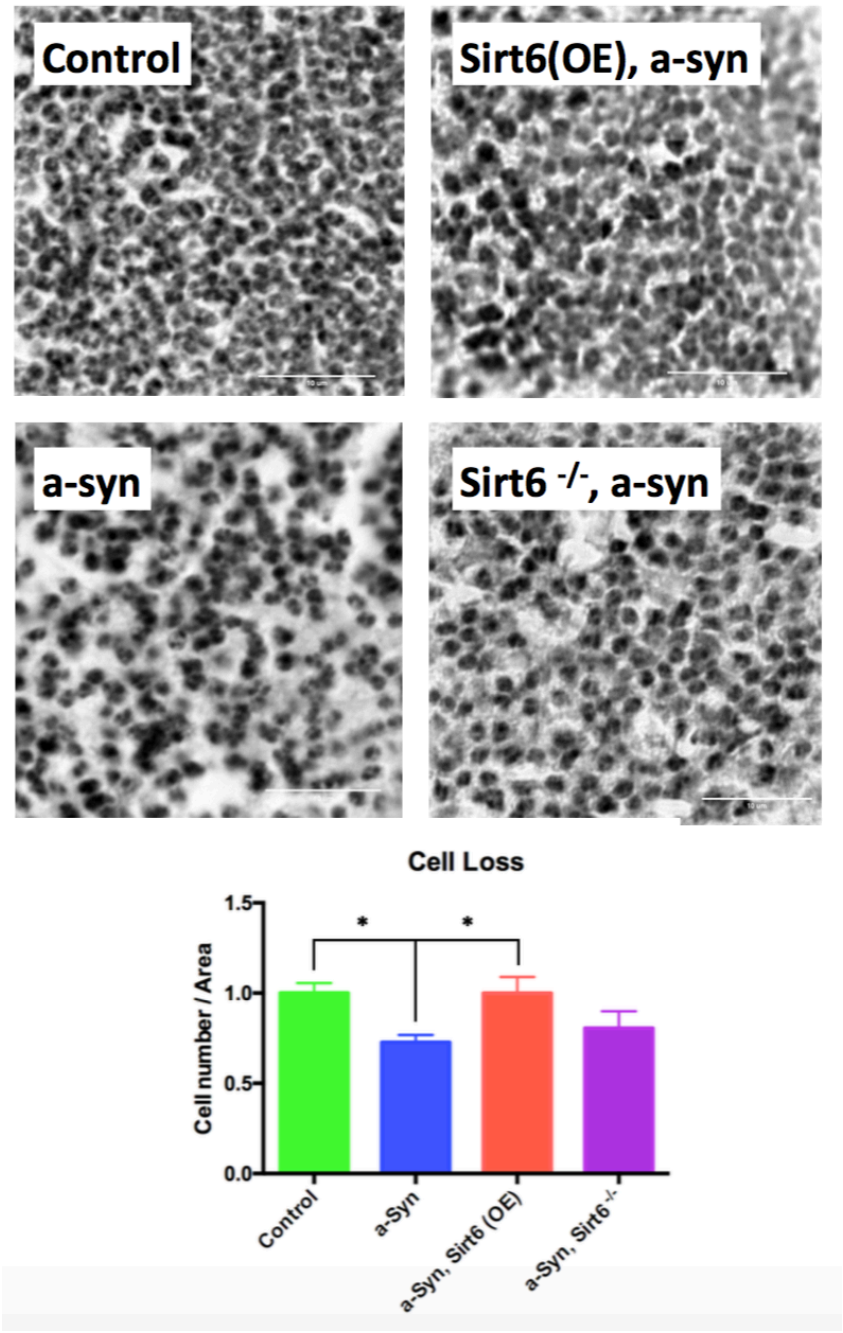


Figure 13| Overexpression of Sirt6 significantly attenuates neurodegeneration in alpha-synuclein transgenic flies. Sections from the medullary cortex of 10-day-old adults. 6 flies were used per genotype (*p<0.05; one-way ANOVA). Values shown are mean +/- SEM. Scale bars are 10 µm. Control is elav- GAL4/+ .

Discussion

***Sirt6* differential expression correlates with neuronal vulnerability in PD-induced pathology.** The differential vulnerability of midbrain's dopaminergic neurons in PD has long been a robust but unexplained neuropathological finding in post-mortem PD brain tissues. Here, I proposed a novel approach to identify the genes that show a similar expression signature in populations of resistant neurons, such as the medial SN and the VTA neurons, and an altered signature in vulnerable neurons, such as those of the lateral SN. I carried out a meta-analysis of microarray datasets that carried the gene expression profile of the VTA, medial SNpc, and lateral SNpc in the post-mortem human tissues (Roth, 2006 and Duke, 2007). I identified *Sirt6* as gene that is significantly upregulated in both the medial SNpc and the VTA (more resistant region), compared to the lateral SN (more vulnerable region). *Sirt6* is a member of the sirtuin gene family that has been heavily indicated in a host of neurodegenerative diseases and has been shown to regulate energy metabolism and DNA damage in non-neuronal tissues. Therefore, I hypothesized that constitutive expression of *Sirt6* will have neuroprotective effects in the context of a neurodegenerative insult. To assess this hypothesis, I needed to establish that the differential *Sirt6* transcription levels identified in our meta-analysis corresponded to differential *Sirt6* translation levels. Recent advances in RNA sequencing and proteomics technology has demonstrated that a gene's mRNA level frequently does not correlate with its protein level as mRNA post-transcriptional processes heavily influence protein levels (Vogel, 2012). As a result, I carried out immunohistochemical analysis to establish that *Sirt6*'s differential expression pattern in the mouse midbrain matched our human-

derived microarray analysis. It is important to note that due to a lack of any previous studies of *Sirt6* expression in the nervous system, I was unable to reliably assess our antibody's specificity in neuronal tissues.

***Sirt6* deletion causes behavioral and neuronal deficits in Flies.** I used the CRISPR-Cas9 technology to remove the entire *Sirt6* gene in *Drosophila*. I observed that SIRT6$^{-/-}$ flies had clear climbing activity deficits. Since Cas9 had removed *Sirt6* throughout the entire organism, it is possible that the flies' robust behavioral deficits were secondary to non-CNS abnormalities, such as metabolic defects. To determine whether *Sirt6* deletion lead to any CNS abnormalities, I examined CNS pathology and found that SIRT6$^{-/-}$ flies showed mild and progressive vacuolization. In accordance with this mild phenotype, I did not observe a clear shortening of life span in SIRT6$^{-/-}$ flies. These findings are surprising as *Sirt6* deletion leads to a significant shortening of lifespan in mice, albeit likely secondary to severe metabolic derangement (Mostoslavsky, 2006). There are a number of possible explanations for this mild neuronal loss and normal life span. First, it is possible that other histone deacetylase proteins might compensate for *Sirt6*'s absence. In addition, neurons have *Sirt6* -independent pathways, such as nucleotide excision and mismatch repair. At the chromatin level, cells rely on a number of different chromatin modifiers to regulate their heterochromatin levels. Thus, in the absence of *Sirt6*, other histone deacetylase proteins can potentially play a larger role in these regulatory pathways. Lastly, heterochromatin loss and DNA damage buildup are age-dependent processes; therefore, it is possible that their dysregulation will only lead to a severe phenotype in the late stages of life or in the presence of an extrinsic cellular insult/stress.

***Sirt6* overexpression modifies alpha-synuclein toxicity.** To study the potential effect of *Sirt6* modulation on neuronal survival in the context of PD-disease, I generated alpha-synuclein expressing fruit flies that either overexpressed *Sirt6* or carried a whole deletion of *Sirt6*. I demonstrated that upregulation of *Sirt6* significantly attenuated premature loss of locomotor function and reversed cell loss in the nervous system. There are a number of possible explanations for *Sirt6*'s neuroprotective effects. First, higher levels of *Sirt6* expression may lead to enhanced repair of PD-induced DNA damage in neurons and decelerate neuronal loss. Second, PD-related neuronal insults likely causes DNA breaks that attract the DNA damage repair complex, which includes *Sirt6*, to the site of damage. This pulls *Sirt6* away from its site of action, where it maintains parts of the chromatin in a transcriptionally silent heterochromatin state, and lead to dysregulation of the cell's transcriptional pathways. This explanation is in line with recent findings that suggest that recruiting *Sirt6* to DNA break sites and away from the heterochromatin regions of chromosomes leads to derepression of transposons, which in turn, leads to genomic instability. A number of research groups have recently demonstrated that derepression of a subgroup of transposons (Human Endogenous Retrovirus-K) likely contributes to neurodegeneration and disease pathogenesis in sporadic amyotrophic lateral sclerosis (ALS). As it has been demonstrated that *Sirt6* regulates multiple cellular pathways, it is likely that *Sirt6* neuroprotective effects stems from its simultaneous regulation of multiple pathways. A third possible explanation is that the neuroprotective effects of *Sirt6* overexpression is an indirect effect of high *Sirt6* levels, which are not physiologically normal, perturbing other cellular pathways. This perturbation, in turn, attenuates

neuronal death. Nevertheless, the molecular mechanisms of *Sirt6*'s neuroprotective effect remains to be determined. Surprisingly, PD flies with a total *Sirt6* knockout did not show any exacerbation of their behavioral and pathological phenotype, compared to PD flies expressing wild type *Sirt6*. It is likely that human alpha-synuclein expression in fruit flies is so toxic that a second insult, from the lack of *Sirt6* expression, is unable to further induce neuronal pathology. It is possible that a more sensitive assay might unravel worsening of neuropathology in these flies. Lastly, it is important to note that the expression levels of human alpha-synuclein in the fruit flies are an important determinant of neurotoxicity and its resultant behavioral and cellular phenotype. It is possible that overexpression of *Sirt6* could interfere with the expression of human alpha-synuclein. To minimize this probability of this interference, I utilized two independent expression systems, QF2-QUAS and GAL4-UAS, to express human alpha-synuclein and overexpress *Sirt6.* Nevertheless, future experiments should establish that alpha-synuclein levels are comparable among PD flies that have varying levels of *Sirt6* expression.

Conclusion

It has been long known that neuronal degeneration in the substantia nigra is the pathological hallmark of PD (Saper, 1999). Nearly a century ago, it was discovered that the dopaminergic neurons of the substantia nigra show differential vulnerability to PD (Saper, 1999; Duke, 2007; and McNaught, 2010). The aim of this thesis was to identify the molecular correlates of this differential vulnerability to PD-induced pathology in the midbrain. To this end, I analyzed transcription profiles of human dopaminergic neurons and identified the histone deacetylase *Sirt6* as a potential neuroprotective factor in the substantia nigra. I created *Sirt6* knockout flies and carried out histological and behavioral assays to study the effect of *Sirt6* modulation on the central nervous system. I observed that *Sirt6* deletion leads to early-onset neurodegeneration on histology and significant climbing activity loss on behavioral assays in flies. In addition, I demonstrated that *Sirt6* overexpression significantly attenuates loss of locomotor function and neurodegeneration in an alpha-synuclein transgenic fly model of Parkinson's disease. It is hoped that the findings presented in this thesis can promote further investigation into the molecular mechanisms underlying *Sirt6*'s neuroprotective role in the nervous system. An understanding of this molecular cascade will provide us with potential modulation targets to tackle neurotoxicity in normal aging, Parkinson's disease, and other neurodegenerative diseases.

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