

Drosophila as a Model to Study Mitochondrial Dysfunction in Parkinson's Disease

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Identification of single gene mutations that lead to inherited forms of Parkinson's disease (PD) has provided strong impetus for the use of animal models to study normal functions of these "PD genes" and the cellular defects that occur in the presence of pathogenic PD mutations. *Drosophila* has emerged as an effective model in PD-related gene studies. Important insights into the cellular basis of PD pathogenesis include the demonstration that two PD genes, *PINK1* and *parkin*, function in a common pathway, with *PINK1* positively regulating *parkin*, to control mitochondrial integrity and maintenance. This is accomplished through regulation of mitochondrial fission/fusion dynamics. Subsequent observations in both fly and mammalian systems showed that these proteins are important for sensing mitochondrial damage and recruiting damaged mitochondria to the quality-control machinery for subsequent removal. Here, I begin by reviewing the opportunities and challenges to understanding PD pathogenesis and developing new therapies. I then review the unique tools and technologies available in *Drosophila* for studying PD genes. Subsequently, I review lessons that we have learned from studies in *Drosophila*, emphasizing the *PINK1/parkin* pathway, as well as studies of *DJ-1* and *Omi/HtrA2*, two additional genes associated with PD implicated in regulation of mitochondrial function. I end by discussing how *Drosophila* can be used to further probe the functions of *PINK1* and *parkin*, and the regulation of mitochondrial quality more generally. In addition to PD, defects in mitochondrial function are associated with normal aging and with many diseases of aging. Thus, insights gained from the studies of mitochondrial dynamics and quality control in *Drosophila* are likely to be of general significance.

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with a prevalence second only to that of Alzheimer's disease. There is no cure or treatment that can halt disease progression. A primary pathological hallmark of the disease is degeneration of multiple neuronal types including, most notably, dopaminergic neurons in the substantia nigra

of the midbrain (Dauer and Przedborski 2003; Shulman et al. 2011). However, pathology of many non-dopaminergic neurons including olfactory and brain stem neurons predates that of DA neurons (Braak et al. 2003). Patients with PD present with characteristic "motor symptoms," such as resting tremor, slowness of movement, rigidity, postural instability, and gait difficulty.

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Although the mainstay of current medical treatment for PD is dopamine replacement, this is not very satisfying. First, the dopamine replacement only alleviates some of the motor symptoms (does not help gait problems), becomes less effective over time, and is often associated with intolerable side effects. Second, PD patients also present with a combination of non-motor symptoms (Simuni and Sethi 2008) including dementia, which occurs in more than one-third of patients; psychiatric symptoms such as depression, anxiety, and obsession; autonomic dysfunction (involving cardiac and digestive systems); sleep disruption; skin lesions; and musculoskeletal abnormalities (Meissner et al. 2011b). Some of these non-motor symptoms may be more debilitating than the motor impairment, but they do not usually respond to dopamine replacement. Third, because of the progressive, debilitating nature of the disease, there is an urgent need to develop disease-modifying therapies that can prevent progression of the illness and perhaps even revert some of the pathology. In summary, PD is a multisystem disease affecting more than DA neurons. Therefore, although therapies targeted to dopaminergic neurons or their targets (such as dopamine replacement, cell transplantation, deep brain stimulation) can provide some therapeutic benefit to patients, particularly with respect to the motor symptoms, we still need to develop therapies that target the underlying cellular defects so as to treat both motor and non-motor symptoms and to prevent disease progression in the first place. A prerequisite for developing these new therapies is that we understand the pathogenesis of PD at the cellular and molecular level in tissues beyond dopaminergic neurons.

IDENTIFICATION OF PD GENES PROVIDES STRONG IMPETUS FOR STUDIES USING ANIMAL MODELS

Vertebrate models have been used in the PD field for many years. Once believed to be solely an environmental disease, much early work focused on toxin models of PD to study disease pathogenesis and develop therapies. Following the landmark discovery that exposure to the mi-

tochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to loss of dopaminergic neurons and motor-related PD-like symptoms in humans (Langston et al. 1983; Bove et al. 2005), rodent and primate models were developed that used chemical depletion of dopamine using 6-hydroxy-dopamine, or feeding with mitochondrial toxins such as MPTP, rotenone, or paraquat (Bove et al. 2005). The results of these studies led to the important conclusion that dopaminergic neuronal degeneration results from oxidative stress and mitochondrial dysfunction (Abou-Sleiman et al. 2006).

Although the above toxin models show dopaminergic neuronal degeneration, new anti-PD drugs and neuroprotective agents that work well to alleviate symptoms in these animal models have largely failed to yield positive effects in human trials (Linazasoro 2004; Meissner et al. 2011b). These results suggest that the disease mechanisms underlying pharmacological models of PD may be different from those acting in the vast majority of sporadic PD patients.

The identification of genes that mediate familial PD has transformed our understanding of the genetic contribution to PD and is providing insight into the cellular mechanisms underlying PD pathogenesis (Hardy et al. 2009; Martin et al. 2011). Over the past 15 years, mutations in several genes have been definitively shown to mediate familial PD. Mutations in SNCA (encoding α -synuclein to *PARK1* [Polymeropoulos et al. 1997] and *PARK4* [Singleton et al. 2003], *Leucine-rich repeat kinase 2* (*LRRK2*)/*Dardarin* (*PARK8*) [Paisan-Ruiz et al. 2004; Zimprich et al. 2004]) were identified several years ago as being associated with autosomal-dominant forms of the disease. More recently, mutations in *VPS35* (Vilarino-Guell et al. 2011; Zimprich et al. 2011) and *EIF4G1* (Chartier-Harlin et al. 2011) have been reported to cause autosomal-dominant forms of PD. Mutations in *PARKIN* (*PARK2*) (Kitada et al. 1998), *DJ-1* (*PARK7*) (Bonifati et al. 2003), and *PTEN-induced kinase 1* (*PINK1*, *PARK6*) (Valente et al. 2004) are associated with autosomal-recessive forms of PD. Mutations in *ATP13A2* (*PARK9*), which encodes a lysosomal ATPase, have also been found in an atypical, autosomal-recessive parkinsonism



(Ramirez et al. 2006) with distinct clinical manifestations (Schneider et al. 2010). These single-gene, Mendelian forms represent ~10%–15% of all PD cases. Some patients with familial forms of PD show clinical symptoms that are similar to those present in patients with sporadic cases. In addition, genome-wide association studies have provided evidence that polymorphic variants in some of the genes associated with familial PD contribute to sporadic PD (Satake et al. 2009; Simon-Sanchez et al. 2009). Thus, the distinction between sporadic and familial forms of PD has become increasingly blurred, and the knowledge gained from studies of genes associated with inherited forms of PD will likely provide insight into mechanisms underlying sporadic forms of the disease as well.

Although human genetic studies have allowed the identification of PD genes from patient-based studies, ethical and technical considerations constrain the utilization of human subjects as experimental systems. Human cell-based studies are limited by tissue availability and provide only limited information when studying processes at the tissue, organ, or whole-animal levels. Postmortem studies are also limited by tissue availability and seldom allow identification of the initial pathological events during presymptomatic stages. Overall, it is difficult to determine the causality of mechanisms from human studies. Thus, animal models are indispensable tools for the analysis of PD genes.

Why do we need invertebrate models if vertebrate models can be easily generated? Vertebrate models include non-human primates, rodents (mice and rats), zebrafish, frogs, and chickens. Mouse models predominate, because of the large number of genetic technologies available and the long history of cell biology and analysis of behaviors in this organism. It might be assumed that mouse models are superior to invertebrate models, because mice are evolutionarily closer to humans, and we can perhaps more easily relate observations of mouse pathology and behaviors such as locomotion, learning, and memory to their counterparts in humans. However, work from many laboratories has shown that rodent models do not always re-

capitulate human disease pathology (Dawson et al. 2010). *Drosophila* models, however, surprisingly to some, have replicated many pathological processes related to human neurological disorders (Zoghbi and Warren 2010). Next, I outline the advantages that *Drosophila melanogaster* offers as a model organism, and insights that studies in *Drosophila* have provided for PD research.

THE ADVANTAGES OF USING *DROSOPHILA* AS A MODEL SYSTEM TO STUDY PD

The identification of PD genes provides an unprecedented opportunity to understand PD pathogenesis through in vivo studies. The key questions in the field are: What are the normal functions of these PD genes? How do PD pathogenic mutations affect PD gene function? Do these PD genes function in a common pathway, or in parallel pathways?

Among the various model organisms, *Drosophila melanogaster* has emerged as an especially effective tool to study PD genes. *Drosophila* has been used extensively for investigating fundamental biological processes, such as cell death (Hay et al. 2004; Hay and Guo 2006), as well as cell proliferation, growth, and migration (for review, see Bellen et al. 2010). In addition, flies display complex behaviors such as circadian rhythms, learning and memory, sleep, and aggression (Bellen et al. 2010). Some of these were thought to be exclusive to humans, and studies now suggest that the genetic bases of these behaviors are conserved in humans. Accumulated studies from more than a century have left the modern fly field with powerful molecular genetic tools (Adams and Sekelsky 2002; St Johnston 2002; Venken and Bellen 2005). *Drosophila* also has a compact genome size (1/30th of the human genome), limited genetic redundancy, and a short generation time (10 days) and life span (60–80 days). The complete sequence of the *Drosophila* genome has revealed that 77% of human disease genes are conserved in the fly (Rubin 2000; Bier 2005). These features make flies an excellent model system in which to study the function of disease genes including those involved in neurodegenerative diseases (Lessing and Bonini 2009; Lu and Vogel 2009), and in

which to dissect genetic pathways related to these disease genes.

Drosophila has emerged as an effective tool for studying PD pathogenesis (for review, see Dodson and Guo 2007; Lu and Vogel 2009; Guo 2010; Whitworth 2011). The *Drosophila* genome encodes homologs of *DJ-1*, *PINK1*, *PARKIN*, *LRRK2*, and *VPS35*. The adult brain of *Drosophila* contains clusters of dopaminergic neurons (Nassel and Elekes 1992), and these neurons degenerate when flies are fed rotenone (Coulom and Birman 2004), a complex I inhibitor that also triggers dopaminergic neuronal degeneration in mammals (Sherer et al. 2003; Bove et al. 2005). Among the genes that mediate familial PD, only *α-synuclein* does not have a homolog in *Drosophila*. Nevertheless, expression of human wild-type and PD-causing mutant forms of *SNCA* in *Drosophila* results in dopaminergic neuronal loss (Feany and Bender 2000; Trinh et al. 2008). In this review, we mainly focus on *Drosophila* homologs of genes associated with recessive forms of PD: *PINK1*, *PARKIN*, and *DJ-1*. We also emphasize the implications that these studies in *Drosophila* have for advancing our understanding of PD in clinical settings. It is important to note that although dopaminergic neurons do show slight degeneration in some *Drosophila* models of PD (Whitworth et al. 2005; Trinh et al. 2008), the significance of *Drosophila* models of PD involves much more than dopaminergic neuron pathology. Flies show defects in multiple systems, reminiscent of the multiple system involvement in PD patients. It is the understanding of the cellular basis of these defects that will provide insight into the pathogenesis of PD.

VARIOUS TOOLS IN *DROSOPHILA*

It would be a chapter in itself to review fully the tools available in *Drosophila*, but here I summarize those that are particularly relevant to building disease models.

Mutagenesis- and Loss-of-Function-Based Studies

Transposons including *P*-element, *piggyBac*, and *Minos* can insert in either coding sequence or

regulatory regions of a gene resulting in loss of function of the gene. The advantage of using *P*-elements, and possibly *Minos*, is that one can generate a null mutation via imprecise excision of *P*-elements located near or in a gene (Adams and Sekelsky 2002). The fly community has generated a large number of transposon insertion lines, and their precise insertion sites in the genome have been determined (Bellen et al. 2004; Thibault et al. 2004). It is estimated that in the next several years, collectively, transposon insertions will be available to disrupt >90% of all *Drosophila* genes. The availability of these transposons has largely replaced the need for chemical mutagenesis, which requires lengthy and tedious mapping strategies. In genomic areas in which transposable elements are lacking, one can perform homologous recombination to generate deletions or disease-related knockin alleles (Rong and Golic 2000; Wesolowska and Rong 2010).

Tissue-Specific Overexpression and Knockdown Can Be Easily Accomplished In Vivo

Temporally controlled, tissue-specific overexpression can be easily accomplished in vivo using the UAS-GAL4 system, a bipartite transcription activation system (Brand and Perrimon 1993). In this system, one transgene consists of a yeast transcription factor (GAL4), driven by a ubiquitous promoter such as tubulin or a tissue-specific promoter such as the dopaminergic neuron-specific TH. The second transgene consists of a gene of interest that is under the control of UAS (upstream activating sequences), which drives transcription in response to GAL4 binding. Therefore, for example, transgenic flies carrying a version of Gal4 driven by the tyrosine hydroxylase promoter (TH-Gal4) and UAS-*PINK1* will express *PINK1* in a dopaminergic neuron-specific pattern. This versatile system allows expression of genes both during development and adulthood, which is very useful for modeling diseases. More recently, several variants on this two-component expression system have been generated. (These are reviewed in detail elsewhere.) Suffice it to say that they allow one to turn gene expression on (or off; see below)

in almost any desired tissue or cell type, in an inducible manner (del Valle Rodriguez et al. 2012). Transposon mutagenesis has also resulted in the creation of a large number of *P*-element-bearing lines that carry a UAS element near one end. These lines, known as EP lines, allow for GAL4-dependent expression of nearby genes, if the UAS element is 5' to the gene, and pointing so as to drive expression of the gene (Rorth 1996).

Tissue-specific and inducible gene silencing can be accomplished through the use of the GAL4 system as well. In brief, flies can be generated that carry UAS-transgenes expressing long double-stranded RNAs (dsRNAs) or microRNAs. When crossed to specific GAL4 drivers or the many variants referenced above, gene silencing is brought about in cells expressing GAL4. Lines of flies carrying UAS-dsRNA or UAS-microRNA transgenes have been, or are being, generated for most *Drosophila* genes (Dietzl et al. 2007; Ni et al. 2011). These lines are publicly available.

Site-Specific Transgenesis Allows Precise Controls over Genetic Background and Expression Levels

Site-specific gene integration can also be brought about using donor plasmids carrying target sites for a site-specific integrase, and fly lines carrying a source of integrase and a “landing pad” target site inserted at specific positions in the genome. Multiple versions of site-specific integration technology and landing pads scattered throughout the genome are available for the fly (Venken and Bellen 2007; del Valle Rodriguez et al. 2012). This technology is important because it allows one to test the activity of a series of mutant proteins, for example, having confidence that all other genomic variables have been kept constant.

Genetics and Compound Screens Provide Unbiased Methods for Identifying Genes and Pathways Important for PD Pathogenesis and Its Suppression

One of the most important tools in the *Drosophila* toolbox lies in the ability to perform large-scale screens for new mutations or conditions of

gene overexpression or silencing that enhance or suppress phenotypes associated with disease models. A number of screen strategies are available (for review, see St Johnston 2002). The goal of unbiased genetic screens is to identify new components that either function in the same genetic pathway of the disease model or in parallel pathways to regulate a common process (the disease state). The power of these screens cannot be understated, because they do not require any existing knowledge related to the function of the disease gene.

In parallel, *Drosophila* has also become a valuable *in vivo* model in which to perform compound screens for suppressors of disease states (Gladstone and Su 2011; Pandey and Nichols 2011). *In vivo* systems like the fly are advantageous for several reasons. First, flies are cheap as compared with mammalian disease models such as mice and show disease phenotypes during development or as young adults. Second, many disease states cannot be easily modeled in cell culture. Third, and perhaps most importantly, *in vivo* screens are unbiased in that they focus on the bottom line—suppression of the disease state. This stands in contrast to many cell-based compound screens, which focus on identifying inhibitors or activators of a particular protein. Although obviously a valuable approach, focus on modifying the activity of a particular protein commits the researcher to that molecule, to the exclusion of all other pathways and molecules, many of which may not be known as regulators of the disease state under study. It may be particularly challenging to implement specific target-based drug screens in cell culture for diseases that result from loss of gene function, as with several of the familial forms of PD, because in these cases one must know the identity of a pharmacologically relevant target in another gene ahead of time.

WHAT LESSONS HAVE WE LEARNED FROM STUDIES IN *DROSOPHILA*?

PINK1 and *Parkin* Function in a Common Pathway to Regulate Mitochondrial Integrity

PARKIN encodes a protein with RING-finger motifs and has E3 ubiquitin ligase activity in



in vitro assays. The initial hypothesis related to *PARKIN* function was that loss of *parkin* resulted in the aberrant accumulation of toxic proteins, perhaps as a result of a failure of the ubiquitin-proteasome system to degrade substrates of Parkin ubiquitination. Consistent with this hypothesis, Parkin catalyzes K48-mediated polyubiquitination, which targets substrates for proteasomal degradation, and multiple proteins have been shown to interact with Parkin and are ubiquitinated in a Parkin-dependent manner in vitro. However, few of these substrates have been shown to accumulate in vivo, in *PARK2* patients, or *parkin* knockout mice, leaving their significance unclear (for review, see West et al. 2007; Dawson and Dawson 2010). Subsequent studies indicated that Parkin also catalyzes other forms of ubiquitination (for review, see West et al. 2007), which can influence cellular processes such as signal transduction, transcriptional regulation, and protein and membrane trafficking, without promoting substrate degradation (Mukhopadhyay and Riezman 2007). However, the exact role of Parkin remained unclear.

The first in vivo indication that *parkin* regulates mitochondrial integrity came from studies of *Drosophila parkin* mutants. Flies lacking *parkin* show dramatic mitochondrial defects in several energy-intensive tissues (Greene et al. 2003; Pesah et al. 2004), and a subset of dopaminergic neurons undergoes degeneration (Whitworth et al. 2005). Subsequent studies in *parkin* knockout mice indicated that although severe defects in mitochondrial morphology are not observed, these animals do display defects in mitochondrial respiration (Palacino et al. 2004).

Key studies that implicate mitochondrial dysfunction as a central mechanism for PD pathogenesis come from studies of *Drosophila PINK1* and its interaction with the fly *parkin*. *PINK1* encodes a protein with a mitochondrial targeting sequence and a serine-threonine kinase domain. We and others reported that a large fraction of *PINK1* is localized to mitochondria (Clark et al. 2006), and *Drosophila* lacking *PINK1* show phenotypes very similar to those of flies lacking *parkin*. *PINK1* and *parkin* mutants are viable but show striking defects in mitochondrial integrity—swollen mitochondria

that have severely broken cristae—in energy-intensive tissues. The males are sterile, adult muscle undergoes degeneration and cell death, and a subset of dopaminergic neurons undergoes degeneration (Clark et al. 2006; Park et al. 2006; Yang et al. 2006). *PINK1* mutants also show reduced ATP levels and mitochondrial DNA (mtDNA) content, and increased stress sensitivity (Clark et al. 2006; Park et al. 2006; Yang et al. 2006). Flies lacking endogenous *PINK1* function but expressing PD-associated mutant forms of *PINK1*, either by overexpression (Yang et al. 2006) or under the control of the endogenous *PINK1* promoter (Yun et al. 2008), show phenotypes similar to those of *PINK1*-null mutants, consistent with *PINK1*-associated disease being the result of loss of function.

What is the relationship between *PINK1* and *parkin*? Stringent genetic studies in *Drosophila* allow one to construct a genetic pathway. *parkin* overexpression in flies suppresses all *PINK1* mutant phenotypes tested (Clark et al. 2006; Park et al. 2006; Yang et al. 2006), whereas *PINK1* overexpression does not compensate for loss of *parkin* function (Clark et al. 2006; Park et al. 2006). Furthermore, double mutants lacking both *PINK1* and *parkin* have phenotypes identical to, rather than stronger than, either single mutant (Clark et al. 2006; Park et al. 2006; Yang et al. 2006). In addition, several groups have reported that Parkin and *PINK1* can physically interact in at least some contexts (Kim et al. 2008; Xiong et al. 2009; Sha et al. 2010). Together, these observations provide compelling in vivo evidence that *PINK1* and *parkin* act in a linear pathway to regulate mitochondrial integrity and maintenance, with *parkin* functioning downstream from *PINK1*.

Several lines of evidence suggest that these observations on *PINK1* and *parkin* function in flies are relevant to humans. First, PD patients who harbor mutations in *PINK1* or *PARKIN* are clinically indistinguishable (Ibanez et al. 2006), and mice lacking both *PINK1* and *parkin* show phenotypes no worse than those of the single mutants (Kitada et al. 2009), consistent with the hypothesis that these genes function in a common genetic pathway. Second, expression of human *PINK1* (Clark et al. 2006; Yang et al. 2006)



or *PARKIN* in *Drosophila* suppresses phenotypes caused by loss of function of *PINK1* or *parkin*, respectively, suggesting that the human and fly proteins are functionally conserved. Third, cells from patients and/or mouse knock-out models of *PINK1* or *parkin* also show defects in mitochondrial morphology and/or mitochondrial respiration, particularly in complex I activity in a variety of cell types (Greene et al. 2003; Muftuoglu et al. 2004; Palacino et al. 2004; Exner et al. 2007; Hoepken et al. 2007; Stichel et al. 2007; Gautier et al. 2008; Poole et al. 2008; Wood-Kaczmar et al. 2008; Dagda et al. 2009; Gegg et al. 2009; Morais et al. 2009; Sandebring et al. 2009; Grünewald et al. 2010; Billia et al. 2011; Schmidt et al. 2011; Shim et al. 2011). Fourth, in neurons derived from pluripotent stem cells from PD patients harboring *PINK1* mutations, recruitment of Parkin to mitochondria is impaired (Seibler et al. 2011).

It is important to note that, in contrast to prior toxin models of PD in which dopaminergic neurons are selectively killed, *PINK1* or *parkin* genetic models lead to defects in multiple tissues in addition to those in dopaminergic neurons, which are largely degenerative in nature. Therefore, these genetic models have the potential to uncover new therapies for neuroprotection, as well as targeting the cellular defects that cause cell loss in both dopaminergic neurons and non-dopaminergic tissues.

The *PINK1*/*Parkin* Pathway Promotes Mitochondrial Fission and/or Inhibits Fusion

How do *PINK1* and *parkin* regulate mitochondrial function? Examination of *PINK1* and *parkin* mutant phenotypes provided an important clue. During *Drosophila* spermatogenesis, mitochondria in spermatids undergo mitochondrial fusion, creating a spherical structure composed of two intertwined mitochondria. Subsequently, the structure unfurls, yielding two mitochondrial derivatives. In both *PINK1* and *parkin* mutants, however, only one mitochondrial derivative is seen, suggesting a defect in mitochondrial fission or an overabundance

of fusion (Riparbelli and Callaini 2006; Deng et al. 2008).

Mitochondria are dynamic and continually undergoing cycles of fission and fusion. Mitochondrial fusion is promoted by *mitofusin* (*mfn*), which is required for outer membrane fusion, and *Optic atrophy 1* (*Opa-1*), which is essential for inner membrane fusion. Mitochondrial fission is promoted by Dynamin-related Protein 1 (*Drp1*), a predominantly cytoplasmic protein recruited to mitochondria during fission (for review, see Chen and Chan 2009).

Several key findings support the hypothesis that the *PINK1*/*parkin* pathway regulates mitochondrial dynamics. First, mitochondria in *PINK1* or *parkin* mutants are clumped in both dopaminergic neurons and flight muscle. They are also swollen and have disrupted cristae. Second, these cellular defects in mitochondrial morphology, as well as other defects such as the degeneration of flight muscle, cell death, locomotion defects, and a decrease in dopamine levels in fly heads, can be suppressed by increasing the expression of the pro-fission molecules *drp1*, and/or decreasing levels of the pro-fusion molecules *mitofusin* or *opa1* (Deng et al. 2008; Poole et al. 2008; Yang et al. 2008; Park et al. 2009). Third, loss of *PINK1* function leads to defects in assembly of the electron transport chain, which can be rescued by overexpression of *drp1* (Liu et al. 2011). Fourth, heterozygosity for *drp1* is lethal in a *PINK1* mutant background, consistent with the idea that *PINK1* and *drp1* work in the same direction to promote fission (Deng et al. 2008; Poole et al. 2008). That said, it is important to note that the phenotypes associated with loss of *PINK1* or *parkin*, and loss of *drp1*, are distinct (Deng et al. 2008), indicating that *PINK1* and *Parkin* are not core components of the fission machinery, but instead regulators of the process. Molecular mechanisms of how this is accomplished are suggested in part by studies showing that *Parkin* can ubiquitinate and degrade *Mfn* (Poole et al. 2010; Ziviani et al. 2010). Because both cellular defects and organismal defects can be rescued by manipulating mitochondrial dynamics, manipulation of mitochondrial dynamics provides a novel therapeutic strategy.



Observations that point to roles of *PINK1* and *parkin* in regulating mitochondrial morphology have also been obtained in mammalian systems. However, in contrast to the story in *Drosophila*, which is consistent across cell types and laboratories, in mammalian systems various effects have been observed. Some studies suggest that loss of *PINK1* results in fission, with decreased levels of *drp1* resulting in suppression (Exner et al. 2007; Dagda et al. 2009; Lutz et al. 2009; Sandebring et al. 2009). In other studies, however, enlarged mitochondria have been observed in *PINK1* striatal neurons (Gautier et al. 2008), and in COS7 cells mitochondrial phenotypes associated with *PINK1* knockdown are suppressed by *fis1* or *drp1* overexpression, as in *Drosophila* (Yang et al. 2008). Moreover, the *PINK1/parkin* pathway promotes *drp1*-dependent mitochondrial fission and/or inhibits *opa1*-dependent fusion in primary neurons, mammalian hippocampal, and dopaminergic neurons (Yu et al. 2011). In addition, in fibroblasts from patients with *PINK1* or *PARKIN* mutations, Mfn ubiquitination is affected (Rakovic et al. 2011). These studies, in conjunction with those suggesting that Mfn is degraded by Parkin in mammalian cells (Chan et al. 2011; Tanaka et al. 2011), suggest that the *PINK1/parkin* pathway is likely to play a conserved role in mammals. The reasons for the differences in how loss of *PINK1/parkin* results in defects in mitochondrial fusion and fission are not entirely clear. But it is likely that the final mitochondrial morphology phenotype observed in any particular cell type, particularly with respect to the presence or absence of *PINK1/parkin*, will depend on many variables. In any case, what is most important is not the specific morphology observed, but the functional state of the mitochondrial population and the ways in which this is influenced by *PINK1* and *parkin*. Recent observations in mammals and flies detailed below have provided several important insights.

***PINK1* and *Parkin* Promote Mitophagy**

In a series of elegant studies, Youle and colleagues showed that in mammalian cells, Parkin is recruited to mitochondria whose inner mem-

brane has been depolarized (an outcome common to multiple forms of mitochondrial damage), or that have been treated with the herbicide Paraquat, an inducer of complex-1-dependent reactive oxygen species (Narendra et al. 2008). Recruitment of Parkin was followed by removal of these damaged mitochondria through a specialized form of autophagy known as mitophagy, in which mitochondria are specifically degraded following engulfment by autophagosomes (for review, see Goldman et al. 2010). Recent findings suggest that mitophagy is intimately linked with changes in mitochondrial size and shape brought about through fission and fusion (for review, see Hyde et al. 2010). Mitophagy requires both the loss of fusion and the presence of fission. Importantly, decreased mitophagy results in the accumulation of oxidized proteins and decreased cellular respiration, strongly suggesting that the end result of this process is the selective removal of damaged mitochondria (Twig et al. 2008). Mitophagy, but not recruitment of Parkin to depolarized mitochondria, requires Drp1, indicating that Parkin-dependent mitophagy requires fission (Narendra et al. 2008).

Work in flies and mammals further shows that recruitment of Parkin to mitochondria depends on PINK1 (Narendra et al. 2009; Geisler et al. 2010; Vives-Bauza et al. 2010; Ziviani et al. 2010). Parkin fails to be recruited to depolarized mitochondria in cells lacking *PINK1*. PINK1's ability to recruit Parkin requires PINK1 kinase activity. In flies, early work suggested a role for PINK1-dependent phosphorylation of Parkin as important for recruitment (Kim et al. 2008). Follow-up studies in mammalian cells have yielded conflicting results. In one study, PINK1 was shown to phosphorylate Parkin, but in a second study, no evidence was found for binding of PINK1 to Parkin, phosphorylation of Parkin by PINK1, or ubiquitination of PINK1 by Parkin (Vives-Bauza et al. 2010).

How does PINK1 promote the recruitment of Parkin specifically to damaged mitochondria? PINK1 protein levels are specifically up-regulated on damaged mitochondria (Narendra et al. 2009; Vives-Bauza et al. 2010; Ziviani et al. 2010). PINK1 is a membrane protein with its

carboxyl terminus facing the cytoplasm (Zhou et al. 2008). In healthy mitochondria, PINK1 is constitutively cleaved, releasing its carboxy-terminal kinase domain into the cytoplasm, where it is degraded in a proteasome-dependent manner. In damaged mitochondria that have lost their membrane potential, cleavage decreases and full-length PINK1 remains anchored to the membrane (Narendra et al. 2009). Mitochondrial anchorage is all that is required, because tethering of PINK1 to the mitochondrial membrane through other methods is sufficient to recruit Parkin (Narendra et al. 2009). As expected based on these observations, overexpression of *PINK1* in a wild-type background, but not a *parkin* mutant background, is also sufficient to promote Parkin recruitment (Vives-Bauza et al. 2010; Ziviani et al. 2010). In cultured *Drosophila* cells, recruitment of Parkin results in the ubiquitination and removal of Mfn, whereas loss of *PINK1* and *parkin* results in accumulation of Mfn (Poole et al. 2010; Ziviani et al. 2010). Ubiquitination of Mfn may function to prevent outer mitochondrial membrane fusion, thus facilitating the segregation and isolation of damaged mitochondria. Ubiquitinated Mfn may also serve as a signal for mitophagy, because loss of *mitofusin* in *Drosophila* results in decreased recruitment of Parkin to mitochondria (Ziviani et al. 2010), suggesting that these proteins may be involved in recruitment as well.

Once recruited and activated, Parkin has several effects on the mitochondria-associated proteome. In addition to Mfns, many mitochondrial proteins are ubiquitinated and/or degraded in a Parkin-dependent manner. These include Milton and Miro (Chan et al. 2011), Bcl-2 (Chen et al. 2010), components of the TOM mitochondrial protein import complex (Chan et al. 2011; Yoshii et al. 2011), VDAC (Geisler et al. 2010; Narendra et al. 2010a), and Drp-1 (Wang et al. 2011a).

How is PINK1 cleavage regulated? Early work in *Drosophila* showed that PINK1 cleavage required the inner mitochondrial membrane protease rhomboid 7 (Whitworth et al. 2008). This, coupled with an earlier observation that the *rhomboid-7* mutant phenotype includes a loss of mitochondrial fusion (McQuibban et al.

2006), suggested a model in which PINK1's pro-fission activity is negatively regulated through cleavage by rhomboid 7. Early experiments in mammalian systems excluded the rhomboid protease PARL as the protease cleaving PINK1 based on the results of RNAi knockdown (Narendra et al. 2010b). However, more recent observation by the same group (Jin et al. 2010) and others (Deas et al. 2011; Meissner et al. 2011a; Shi et al. 2011) clearly show that PINK1 is cleaved by PARL and that this activity is regulated by mitochondrial membrane potential.

Although these findings are intriguing, it is important to note that the recruitment of Parkin to mitochondria in *Drosophila* and mammalian studies has been performed in cell lines (Ziviani et al. 2010). It remains to be shown that recruitment of Parkin occurs in vivo in tissues that show phenotypes when *PINK1/parkin* are removed, and that this is associated with mitophagy.

The findings that the *PINK1/parkin* pathway regulates mitochondrial dynamics and mitophagy suggest an exciting model in which a failure of mitochondrial quality control lies at the heart of PD pathogenesis. In this model, when mitochondria undergo damage, PINK1 senses the damage, becomes stabilized, and recruits Parkin specifically to the damaged mitochondria. Parkin then promotes degradation of Mitofusin, which prevents these mitochondria from fusing with the network. Parkin-dependent ubiquitination of other substrates then results in mitophagy. In *PINK1/parkin*-mediated PD, damaged mitochondria fail to be cleared, allowing damaged mitochondria, which may carry mutant genomes, to accumulate in cells.

The *PINK1/Parkin* Pathway and Mitochondrial Transport

Observations in *Drosophila* have also led the way in identifying links between *PINK1*, *parkin*, and mitochondrial motility. Mitochondria are not located randomly in cells. They are actively trafficked to regions of the cell where ATP or Ca^{2+} buffering demands are high using a microtubule-based system. Two proteins that mediate microtubule-based transport of mitochondria,

Miro and Milton, were first identified in *Drosophila*, in screens for mutants with defects in synaptic transmission and locomotion (Stowers et al. 2002; Guo et al. 2005). Miro sits in the mitochondrial outer membrane (Fransson et al. 2006), and Milton links Miro-bound mitochondria to Kinesin-1-heavy chain, which promotes mitochondrial movement on microtubules (Glater et al. 2006). Thus, in mutants for these genes, mitochondria accumulate in the soma and fail to be transported to axons and dendrites. Miro also functions as a Ca^{2+} sensor, promoting the release of mitochondria from microtubules in areas of the cell with high levels of intracellular Ca^{2+} (Cai and Sheng 2009).

Recent observations from *Drosophila* and mammalian laboratories show that mitochondrial PINK1 binds Miro (Weihofen et al. 2009; Wang et al. 2011b; Liu et al. 2012). This leads, either through phosphorylation (Wang et al. 2011b) or through a phosphorylation-independent mechanism (Liu et al. 2012), to Parkin-dependent ubiquitination and degradation of Miro, resulting in a loss of mitochondrial movement. Other work has shown that Miro is lost from depolarized mitochondria (Chan et al. 2011), stationary mitochondria are less likely than motile mitochondria to undergo fusion (Twig and Shirihai 2011), and that loss of Miro promotes mitophagy (Liu et al. 2012). Together these observations suggest that PINK1 and Parkin are important regulators of mitochondrial motility, and that one of their functions in mitochondrial quality control is to sequester damaged mitochondria by severing their connections to the microtubule network, preparing them for autophagy. Such an activity is likely to be particularly important in neurons with long processes.

Links between the PINK1/Parkin Pathway and Other PARK Loci

Omi/HtrA2 (*PARK13*) encodes a serine protease (Vande Walle et al. 2008) that is localized to the mitochondrial matrix. *Omi/HtrA2* has been suggested to function downstream from PINK1 in a common pathway (Plun-Favreau et al. 2007), based on the findings that mammalian

Omi/HtrA2 binds PINK1 and that the phosphorylation of *Omi/HtrA2* is dependent on PINK1 in mammalian cells (Plun-Favreau et al. 2007). One group has also reported the presence of mutations/polymorphisms in *Omi/HtrA2* in sporadic PD patients (Strauss et al. 2005). Loss of *Omi/HtrA2* leads to loss of non-dopaminergic neurons in the striatum (Rathke-Hartlieb et al. 2002; Jones et al. 2003; Martins et al. 2004).

Does *Omi/HtrA2* function as a downstream target of PINK1 in vivo? An overexpression-based genetic interaction is observed between PINK1 and *omi/HtrA2* (Whitworth et al. 2008; Yun et al. 2008). However, in contrast to PINK1 mutants, *omi/HtrA2*-null mutants have normal mitochondrial morphology in both muscle and testes, and a normal number of dopaminergic neurons (Yun et al. 2008). In addition, extensive loss-of-function-based genetic interaction studies fail to provide any in vivo evidence supporting the hypothesis that *Omi/HtrA2* functions in the same pathway, either upstream or downstream of PINK1, to either positively or negatively regulate PINK1. They also do not provide any clear evidence that *Omi/HtrA2* acts in a parallel manner to regulate mitochondrial morphology (Yun et al. 2008). These loss-of-function-based analyses are more relevant to PD than are *Omi/HtrA2* overexpression-based analyses, because reported *Omi/HtrA2* mutations associated with PD are proposed to represent loss-of-function or dominant-negative mutations (Strauss et al. 2005). In addition, mutant forms of the *Drosophila* *Omi/HtrA2* analogous to the reported disease form in sporadic PD patients, and a mutant lacking a serine thought to be a target for PINK1-dependent phosphorylation (Plun-Favreau et al. 2007), retain significant, if not full, *Omi/HtrA2* function in vivo (Yun et al. 2008). Thus, loss-of-function studies strongly suggest that *Omi/HtrA2* does not play an essential role in regulating mitochondrial integrity in the PINK1/*parkin* pathway in *Drosophila* (Yun et al. 2008). Recent studies in mice show that neurodegeneration in the *Omi/HtrA2* mutant is not rescued by overexpression of *parkin* in mice (Yoshida et al. 2010). The implication of these studies is that *Omi/HtrA2* may not



function in the *PINK1/parkin* pathway. This conclusion is also supported by studies that found no association between mutations in *Omi/HtrA2* and PD, and work showing that the PD-associated mutation in *Omi/HtrA2* occurs with comparable frequency in unaffected populations, suggesting they represent simple polymorphisms (Ross et al. 2008; Simon-Sanchez and Singleton 2008).

Mutations in *DJ-1* (*PARK7*) cause very rare autosomal-recessive forms of PD (Bonifati et al. 2003). Many diverse activities have been proposed for *DJ-1*, including functions as a cysteine protease, a redox-regulated chaperone, a transcription coactivator and RNA-binding protein, and a regulator of survival signaling through interactions with Daxx or the kinase ASK1 (for review, see Wilson 2011). What is clear is that *DJ-1* is cytoprotective and that this activity requires a conserved cysteine. Although *DJ-1* is predominantly cytoplasmic, oxidative stress enhances its association with mitochondria (Canet-Aviles et al. 2004; Blackinton et al. 2005, 2009; Ooe et al. 2005; Lev et al. 2008). In addition, versions of *DJ-1* targeted specifically to mitochondria by fusing a mitochondrial localization sequence to its amino terminus show enhanced cytoprotective functions, suggesting that the mitochondria is one (of perhaps many) important site of *DJ-1* action (Junn et al. 2009). Finally, it is worth noting that triple knockout mice lacking *PINK1*, *parkin*, and *DJ-1* fail to show nigral degeneration, even with aging (Kitada et al. 2009). This somewhat surprising result could reflect action in a common pathway as with *PINK1* and *parkin*. Alternatively, it could reflect developmental compensation, the presence of which is strongly hinted at in experiments using conditional, adult-specific *parkin* knockout mice (Shin et al. 2011; cf. Goldberg et al. 2003; Itier et al. 2003; Von Coelln et al. 2004; Perez and Palmiter 2005).

In *Drosophila*, flies lacking *DJ-1* (deletions) are viable, with their most prominent phenotype being increased sensitivity to oxidative stress as assayed by reduced survival upon Paraquat or rotenone feeding (Menziez et al. 2005; Meulener et al. 2005, 2006). In some studies, overexpression of *DJ-1* fails to rescue *PINK1* mutant muscle phenotypes (Yang et al. 2006)

or male sterility due to lack of *PINK1* or *parkin* (M Guo, unpubl.). In other studies, *Drosophila DJ-1* mutants show mitochondrial defects, but these are in many ways distinct from those associated with loss of *PINK1* or *parkin* (Hao et al. 2010). In addition, although expression of *DJ-1* is capable of rescuing muscle defects due to loss of *PINK1*, it cannot rescue identical-looking muscle defects due to loss of *parkin*. Expression of either *PINK1* or *Parkin* in a *DJ-1* mutant also has the surprising effect of causing organismal lethality. All of these results suggest complex, not linear, interactions between *DJ-1* and the *PINK1/parkin* pathway.

CONTRIBUTIONS OF *DROSOPHILA* AND FUTURE DIRECTIONS

In summary, work in *Drosophila* has provided several key insights in understanding PD. Flies provided the first in vivo evidence that *PINK1* and *parkin* regulate mitochondrial integrity. The stringent epistatic studies provided compelling evidence that *PINK1* and *parkin* function in a common genetic pathway, with *PINK1* positively regulating *parkin*. In contrast, studies have not provided in vivo support for *Omi/HtrA2* as a component of the *PINK1/parkin* pathway. Studies in *Drosophila* also suggest that the *PINK1/parkin* pathway promotes mitochondrial fission and/or inhibits fusion. These studies provide the first demonstration that manipulation of mitochondrial dynamics can suppress *PINK1/parkin* phenotypes both at the cellular level (mitochondrial integrity) and organismal level (muscle degeneration, dopamine levels, and locomotion), thereby providing novel therapeutic targets. More recent work in cultured cells shows that *PINK1* and *parkin* regulate mitophagy in *Drosophila*, paralleling work in mammals and suggesting the exciting possibility that failure of mitophagy, and thus mitochondrial quality control, underlies the pathogenesis of *PINK1/parkin*-mediated PD. The unique attributes of *Drosophila* (robust phenotypes, straightforward genetics providing opportunities for genome-wide genetic screens and drug screens) suggest that *Drosophila* studies will continue to move the field forward and help identify

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novel therapies for PD, and potentially other aging-related neurodegenerative disorders. In support of this contention, compounds and a number of genes (either over- or underexpressed, or exogenous) have been identified as suppressors of *PINK1* and/or *parkin* phenotypes in the fly (Greene et al. 2005; Whitworth et al. 2005; Deng et al. 2008; Poole et al. 2008; Yang et al. 2008; Park et al. 2009; Tain et al. 2009; Hao et al. 2010; Imai et al. 2010; Liu and Lu 2010; Fernandes and Rao 2011; Saini et al. 2011; Koh et al. 2012; Vilain et al. 2012). Given that defects in mitochondria accumulate in normal aging as well as in PD, identifying multiple ways of activating mitophagy may be generally useful therapeutically in many diseases of aging. In short, *Drosophila* provides an indispensable and unique opportunity to contribute to the PD field.

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