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Ret rescues mitochondrial morphology and muscle degeneration of *Drosophila Pink1* mutants

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Abstract

Parkinson's disease (PD)-associated Pink1 and Parkin proteins are believed to function in a common pathway controlling mitochondrial clearance and trafficking. Glial cell line-derived neurotrophic factor (GDNF) and its signaling receptor Ret are neuroprotective in toxin-based animal models of PD. However, the mechanism by which GDNF/Ret protects cells from degenerating remains unclear. We investigated whether the *Drosophila* homolog of *Ret* can rescue *Pink1* and *park* mutant phenotypes. We report that a signaling active version of *Ret* (*Ret*^{MEN2B}) rescues muscle degeneration, disintegration of mitochondria and ATP content of *Pink1* mutants. Interestingly, corresponding phenotypes of *park* mutants were not rescued, suggesting that the phenotypes of *Pink1* and *park* mutants have partially different origins. In human neuroblastoma cells, GDNF treatment rescues morphological defects of PINK1 knockdown, without inducing mitophagy or Parkin recruitment. GDNF also rescues bioenergetic deficits of PINK knockdown cells. Furthermore, overexpression of *Ret*^{MEN2B} significantly improves electron transport chain complex I function in *Pink1* mutant *Drosophila*. These results provide a novel mechanism underlying Ret-mediated cell protection in a situation relevant for human PD.

Keywords Drosophila; neurodegeneration; neurotrophic factors; OXPHOS; Parkinson's disease

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

The etiology of Parkinson's Disease (PD) is highly complex and largely unknown, involving both environmental and genetic risk factors. Mitochondrial dysfunction, oxidative stress and protein aggregation are believed to be central events in the pathological process, but their interconnection remains unclear (Schapira & Jenner, 2011; Exner *et al*, 2012; McCoy & Cookson, 2012). The first indications of a role for mitochondria came with the discovery that the toxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) causes Parkinsonism in humans and animal models (Burns *et al*, 1983; Langston *et al*, 1983). Its active metabolite, 1-methyl-4-phenyl-pyridinium ion (MPP^+), is selectively imported into dopaminergic neurons via the dopamine transporter, and inhibits complex I of the electron transport chain (ETC). Several other mitochondrial toxins, including paraquat and rotenone, generating either mitochondrial reactive oxygen species (ROS) or specifically inhibiting complex I, have been linked to PD in epidemiological studies and animal models (de Lau & Breteler, 2006). Furthermore, patients with sporadic PD can have decreased activity of complex I in brain and other tissues (Schapira *et al*, 1989; Parker & Swerdlow, 1998), or less complex I proteins in the substantia nigra (Mizuno *et al*, 1989).

Autosomal recessive PD-associated proteins Parkin, PINK1 and DJ-1 (OMIM #600116, 605909, 606324) have been shown to have functions related to mitochondrial integrity, (reviewed in Exner *et al*, 2012; Martin *et al*, 2011). In three seminal studies, *Pink1* mutant *Drosophila* displayed mitochondrial abnormalities and muscle degeneration in a manner highly similar to *park* mutants, and Parkin overexpression largely rescued the phenotypes of *Pink1* mutants, but not *vice versa*, suggesting that the two proteins act in a common linear pathway (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006). Manipulation of the mitochondrial remodeling machinery rescues some *Pink1* and *park* mutant phenotypes in *Drosophila* and in mammalian cell lines. However, while increasing fission rescues the *Drosophila* phenotypes, shifting the fusion/fission balance in the opposite direction rescues mammalian cell lines, but the underlying mechanisms are not fully understood (Deng *et al*, 2008; Poole *et al*, 2008; Lutz *et al*, 2009). PINK1, a mitochondrial Ser/Thr kinase, and Parkin, an E3 Ubiquitin ligase, were found to

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regulate clearance of damaged mitochondria via mitophagy (Geisler *et al*, 2010; Narendra *et al*, 2010; Vives-Bauza *et al*, 2010), and microtubular transport (Weihofen *et al*, 2009; Wang *et al*, 2011). However, other studies have reported additional functions of Parkin in the regulation of stress response proteins and mitochondrial biogenesis (Bouman *et al*, 2011; Shin *et al*, 2011), in promoting NF-κB signaling (Henn *et al*, 2007; Muller-Rischart *et al*, 2013), and in controlling cytochrome-c release (Berger *et al*, 2009). PINK1 also has additional functions, unrelated to recruiting Parkin, such as regulating mitochondrial calcium buffering (Gandhi *et al*, 2009; Sandebring *et al*, 2009; Heeman *et al*, 2011). Furthermore, *PINK1* mutant mitochondria have decreased activity of complex I of the ETC (Morais *et al*, 2009), and overexpression of a yeast substitute for complex I rescued many of the functional impairments of *Pink1* mutant flies (Vilain *et al*, 2012). Additional studies are required to elucidate which of the functions reported for Parkin and PINK1 are critical for causing Parkinson pathology.

The neurotrophic factor Glial cell line-derived neurotrophic factor (GDNF) promotes the survival of dopamine neurons (Lin *et al*, 1993) and protects nigral dopamine neurons from cell death in rodent and primate toxin-models of PD such as 6-hydroxydopamine (6-OHDA) and MPTP (Kearns & Gash, 1995; Sauer *et al*, 1995; Tomac *et al*, 1995; Gash *et al*, 1996). Several clinical trials have been performed with mixed outcomes, but ongoing research and development aims at improving delivery methods of GDNF (Deierborg *et al*, 2008). GDNF signals via the GPI-anchored co-receptor GFR-α1 and the receptor tyrosine kinase Ret (Airaksinen & Saarma, 2002). Endogenous Ret expression is required for long-term survival of a fraction of nigral dopamine neurons in aged mice (Kramer *et al*, 2007). Conversely, mice that express a constitutively active Ret receptor in dopamine neurons (Ret^{MEN2B}) show increased numbers of dopamine neurons (Mijatovic *et al*, 2007). The mechanism by which GDNF/Ret protects dopamine neurons from cell death is not fully elucidated. We hypothesized that Ret-activated signaling pathways converge with functions of proteins associated with familial PD. We recently reported that *Ret* and *DJ-1* double loss-of-function in aged mice exacerbates the neuron loss observed in *Ret* single mutants (Aron *et al*, 2010). Here, we investigated whether *Ret* interacts genetically with *park* and *Pink1* in *Drosophila*. We found that constitutively active Ret^{MEN2B} specifically rescues phenotypes of *Pink1* mutants, including muscle degeneration, mitochondrial morphology and function, whereas *park* mutants remained unaffected. Moreover, Ret signaling rescued mitochondrial morphological and functional defects of PINK1-deficient human SH-SY5Y cells, without activating mitophagy. Mechanistically, Ret signaling restored the activity of complex I of the ETC, which is reduced in *Pink1*, but not *park* mutant flies. Thus our study indicates that Ret signaling can specifically ameliorate Pink1 loss-of-function deficiencies that are relevant to human Parkinson's disease.

Results

Active Ret rescues *Pink1* but not *park* mutant muscle degeneration

To study whether *Ret* can modify *Pink1* and *park* phenotypes, we utilized the *Drosophila* indirect flight muscles (IFMs) as a model system. Here, *Pink1* and *park* mutants undergo significant muscle degeneration, likely because of the high energy consumption of the IFMs, and display enlarged mitochondria with broken cristae. Late stage pupae display normal muscle morphology, but soon after eclosion, the muscle tissue degenerates (Greene *et al*, 2003; Clark *et al*, 2006; Park *et al*, 2006). In 3- to 5-day-old *Pink1* and *park* mutant animals housed at 18°C, interrupted muscles were found, and one or several of the six muscles displayed degenerated, highly irregular myofibrils with abnormal sarcomere structure, hereafter referred to as "degenerated" (Fig 1I and K) in approximately 65% of the animals as compared to controls, which never displayed this phenotype (Fig 1A, B, E, F, L). To investigate whether Ret signaling could modify muscle degeneration, we utilized the constitutively active version, Ret^{MEN2B}, which has an activating point mutation in the kinase domain (M955T) (Read *et al*, 2005). In an expression analysis of endogenous *Ret* by reverse transcriptase PCR (RT-PCR), we detected high levels of *Ret* mRNA in larvae and pupae, and lower levels in the adult thorax and IFMs (Supplementary Fig S1). To achieve robust overexpression of activated Ret specifically in muscles, we used the *UAS-GAL4* system and the *Myocyte enhancer factor-2* (*Mef2*) *GAL4* driver, which is active in all muscle tissues from the early embryo throughout larval and pupal stages and in the adult fly. *Mef2* > Ret^{MEN2B} overexpression caused lethality at 25°C, but at 18°C, viable progeny eclosed with lower frequency. Surviving transgenic flies displayed mild muscle abnormalities, including deposits of actin dispersed over the muscle tissue, and some abnormally thick and irregular myofibrils (Fig 1C, G, J). A recent RNAi screen for modifiers of muscle development (Schnorrer *et al*, 2010) identified a large number of lines with a highly reminiscent phenotypic class and designated this "actin blobs", we therefore refer to this by the same term. When Ret^{MEN2B} was overexpressed in the background of *Pink1* mutants, the majority of flies showed significantly improved muscle morphology, with only 12% of flies displaying degenerated myofibrils (Fig 1D and L). The frequency of flies with actin blobs also decreased markedly compared to Ret^{MEN2B} expressing controls, suggesting that Pink1 function may be required for this phenotype. However, in contrast to *Pink1* mutants, *park* mutants overexpressing Ret^{MEN2B} showed no improvement as the frequency of degenerated myofibrils remained unchanged (Fig 1H and L). Expression of the Ret^{MEN2B} protein was examined by Western Blot of thorax homogenates and levels were similar between the *Pink1* and *park* mutants, indicating that differences in transgene expression were not a likely cause of the differential response (Fig 1M). To determine if Ret protein expression or Ret signaling was required for the phenotypic rescue, we overexpressed wild-type (WT) Ret using the same *GAL4* driver. We found that Ret^{WT} was unable to modify the phenotype probably because the putative *Ret* ligand was not present in the IFMs at significant levels at this stage (Supplementary Fig S2). Moreover, the effects of Ret on IFM morphology appeared rather specific, since overexpression of a constitutively active fibroblast growth factor receptor (FGFR), $UAS\text{-}htl^{\lambda}$, caused a dramatic change in IFM fate (data not shown).

Rescue of *Pink1* mutants is not developmental

The partial embryonic lethality and appearance of actin blobs by *Mef2* > Ret^{MEN2B} overexpression indicated that high levels of Ret signaling interfered with normal muscle development. Other receptor

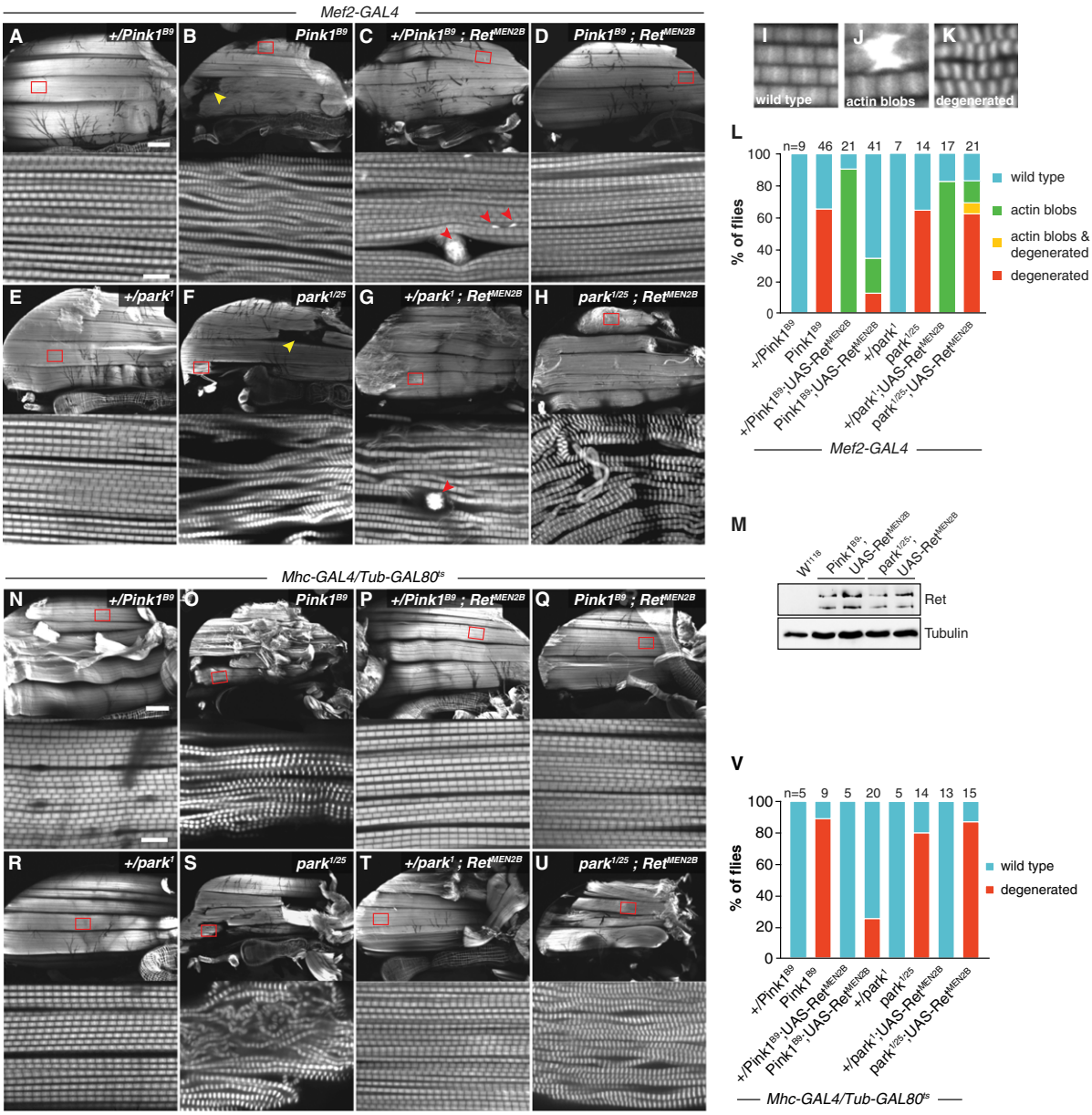

Figure 1. Ret^{MEN2B} overexpression rescues *Pink1* but not *park* mutant muscle degeneration.

A–K *Drosophila* hemi-thoraces stained with phalloidin at low magnification (upper panels) showing overall indirect flight muscle (IFM) morphology, and at higher magnification (lower panels). High-magnification images of WT sarcomeres (I), sarcomeres with 'actin blobs' (J), and degenerated sarcomeres (K). Heterozygous controls (A, E) display normal IFM layout (upper panels), myofibril morphology (lower panels) and sarcomeres (I). *Pink1* (B) and *park* mutants (F) display abnormal morphologies with truncated muscles (yellow arrow heads, upper panels) and disorganized myofibrils (lower panels) with degenerated sarcomere structure (K). Animals overexpressing *Ret^{MEN2B}* (C, G) display normal IFM layout (upper panels), fairly normal myofibril morphology with occasional deposits of mislocated actin filaments, and actin blobs, (red arrow heads, lower panels and J). *Ret^{MEN2B}* overexpression in *Pink1* mutants largely rescues the mutant phenotypes, as the majority of animals display normal IFM morphology (D), while *park* mutants are not rescued (H).

L Percentage of flies with phenotype "wild type" (blue), "actin blobs" (green), "degenerated" (red) or "actin blobs and degenerated" (yellow).

M Western blot analysis of Ret expression in thorax homogenates from *w^{1118}* controls, and *Pink1*, or *park* mutants overexpressing *Ret^{MEN2B}*, indicating similar levels of Ret overexpression between the two mutant backgrounds. Tissue from three animals per sample. Tubulin was used as a loading control.

N–U Overexpression of *UAS-Ret^{MEN2B}* under control of *Mhc-GAL4* and *Tub-GAL80^{ts}*, pupae were shifted from 18 to 30°C at pupal stage 11, activating expression after muscle formation is completed. Heterozygous controls (N, R) and *Ret^{MEN2B}* late overexpressing animals display normal muscle and myofibril morphologies (N, P, R, S, T). *Pink1* (O) and *park* mutants (S) display abnormal morphologies with truncated muscles and disorganized myofibrils with degenerated sarcomere structure (lower panels). Late *Ret^{MEN2B}* overexpression in *Pink1* mutants (Q) largely rescues the mutant phenotypes, while *park* mutants (U) are not rescued.

V Percentage of flies with phenotype "wild type" (blue) or "degenerated" (red). Number of animals per genotype as depicted in figure.

Data information: Scale bars: upper panels, 100 μm; lower panels, 10 μm.
Source data are available online for this figure

tyrosine kinases such as epidermal growth factor receptor (EGFR) and FGFR are known to regulate embryonic myoblast specification via Ras/Erk signaling (Carmena *et al*, 1998; Halfon *et al*, 2000), and the insulin receptor controls muscle size (Demontis & Perrimon, 2009). Therefore, it is plausible that active Ret^{MEN2B} affects these, or similar developmental processes. To verify that the rescue of the *Pink1* mutants is not a developmental interaction, we utilized the $GAL80^{ts}$ system which permits transgene expression in a defined time window regulated by temperature. To drive Ret^{MEN2B} expression, we chose the *GAL4* driver, *Myosin heavy chain (Mhc) GAL4*, which expresses only in differentiated muscles, not in myoblasts, in difference to *Mef-GAL4* and generates higher expression. Unlike *Mef2-GAL4*, it causes complete lethality when driving Ret^{MEN2B} from embryonic stages. Flies were crossed at 18°C (non-permissive temperature), after which pupae were shifted to 30°C (permissive temperature) at pharate adult stage P11 ± 3 h (equivalent of 75 h APF at 25°C) (Flybase FBdv:00005349), a time well after completion of IFM development, but before the onset of apoptotic degeneration in *Pink1* and *park* mutants (Greene *et al*, 2003; Clark *et al*, 2006). Analyses were again performed at 3–5 days post-eclosion. Using this protocol, *Pink1* and *park* mutants showed degenerated myofibrils with a frequency of approximately 90% and 80% respectively as compared to controls (Fig 1N, O, R, S, V), the higher penetrance being likely due to the increased temperature. Ret^{MEN2B}-overexpressing flies eclosed with Mendelian frequencies and displayed fully normal muscle morphology, without the presence of actin blobs, confirming the hypothesis that the lethality and actin blob phenotypes have developmental origins (Fig 1P, T, V). When Ret^{MEN2B} was expressed in *Pink1* mutants from this late pupal stage and onwards, it again largely rescued muscle degeneration, indicating that the rescue is not due to a developmental interaction, but a direct protective effect of Ret signaling on degenerating tissue (Fig 1Q and V). Interestingly, *park* mutants were again not rescued using this expression protocol (Fig 1U and V).

Ret signaling rescues mitochondrial morphology in flight muscles

One possibility is that Ret^{MEN2B} inhibits muscle degeneration without directly targeting the primary cause of the *Pink1* phenotype: mitochondrial impairments (Clark *et al*, 2006). To test this possibility, we analyzed the ultrastructure of mitochondria using transmission electron microscopy. IFMs from control flies showed regular organization of myofibrils and densely packed mitochondria with intact cristae (Fig 2A, E, L, M). *Pink1* and *park* mutants displayed a heterogeneous population of mitochondria with the majority having significantly enlarged sizes and mild or severe disruption of their cristae structure, when compared to control mitochondria (Fig 2B, F, I-M). *Mef2* > Ret^{MEN2B} overexpression in control flies did not alter normal mitochondria morphology (Fig 2C, G, L, M). However, in *Pink1* mutants, Ret^{MEN2B} overexpression significantly reduced the fraction of severely impaired mitochondria and increased the fraction of mitochondria with WT-like cristae structure (Fig 2D and L). In contrast, *park* mutants showed no improvement of structural impairments when Ret^{MEN2B} was overexpressed (Fig 2H and M).

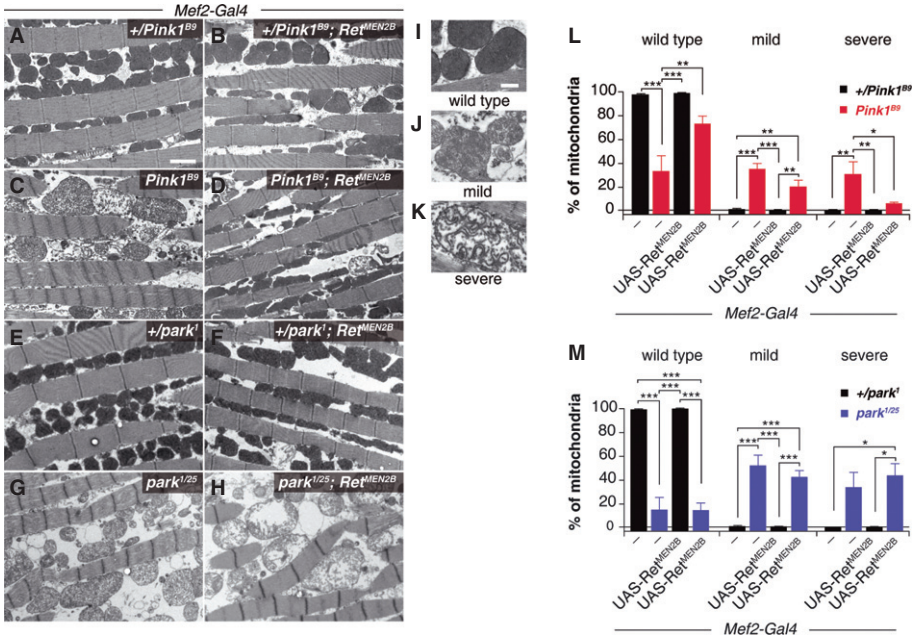

Figure 2. Ret^{MEN2B} rescues mitochondrial cristae structure of *Pink1* mutants.

A–K Transmission electron microscopy images of indirect flight muscles. Heterozygous controls (A, E) and animals overexpressing Ret^{MEN2B} (B, F) display normal mitochondria of similar size with highly dense cristae structure. *Pink1* and *park* mutants have enlarged mitochondria with broken cristae (C, G). Phenotype can vary from mild to severe. High-power images of mitochondria are shown for the categories wild type (I), mild (J), severe phenotype (K). Ret^{MEN2B} overexpression partially restores mitochondrial size and cristae structure in *Pink1* (D), but not *park* mutants (H). Scale bar, 2 μm.

L, M Percentages of mitochondria of the indicated categories, 500–800 mitochondria per animal, averages of 6 animals per genotype.

These results demonstrate that Ret^{MEN2B} can rescue mitochondrial impairments of *pink1* but not *park* mutants, suggesting that the mitochondrial deficiencies of the two mutant strains have partially different origins.

Ret rescues mitochondrial morphology in dopaminergic neurons

To address whether Ret^{MEN2B} also rescues the morphology of mitochondria in dopaminergic neurons, we overexpressed Ret^{MEN2B} using TH-GAL4 together with the mitochondrial marker mitoGFP (Pilling *et al*, 2006). *Pink1* and *park* mutants displayed severely enlarged mitochondria as compared to controls (Fig 3A, B, E, F, I, J). Ret^{MEN2B} overexpression in a control background did not significantly alter the normal mitochondrial background (Fig 3C, G, I, J). However, when overexpressed in *Pink1* mutants, mitochondrial size was significantly rescued (Fig 3D and I). Quantification of mitochondrial volumes revealed that in the presence of Ret^{MEN2B} the abundance of normal mitochondria was increased, while the fraction of enlarged mitochondria decreased to levels similar to those of control flies. Merely, the 4% largest mitochondria were not rescued. In line with the analysis of mitochondria in muscle, mitochondrial morphology in neurons of *park* mutants was not rescued by Ret^{MEN2B} (Fig 3H and J).

GDNF/Ret signaling rescues mitochondrial defects in mammalian cells

In order to assess whether signaling from endogenous Ret can also rescue mitochondrial impairments caused by loss of PINK1 function, we used the human dopaminergic neuroblastoma cell line SH-SY5Y, which expresses endogenous Ret. Acute knock-down of PINK1 in this cell line was previously shown to cause fragmentation of the mitochondrial network (Lutz *et al*, 2009) (Fig 4A, B, D). Stimulation of Ret by GDNF and soluble GFRα-1 rescued mitochondrial fragmentation, demonstrating that endogenous mammalian Ret can rescue mitochondrial impairments (Fig 4C and D). A semi-quantitative RT-PCR analysis of PINK1 mRNA controlled that GDNF/GFRα-1 stimulation did not upregulate PINK1 levels (Fig 4E).

Ret rescues mitochondrial morphology independently of Parkin-induced mitophagy

Although the data so far suggested that Ret rescues Pink1-deficient mitochondria independently of Parkin, we cannot exclude that Ret signaling activates Parkin translocation to mitochondria, thus promoting their clearance through mitophagy. To test this hypothesis, we treated SH-SY5Y cells overexpressing Parkin with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to depolarize mitochondria. CCCP treatment induced recruitment of Parkin to mitochondria (detected 2 h after adding CCCP) followed by the removal of depolarized mitochondria in about 50% of Parkin-expressing SH-SY5Y cells (monitored 24 h later) (Fig 4G and N). Parkin-induced mitophagy required the presence of PINK1, as described previously (Geisler *et al*, 2010; Narendra *et al*, 2010; Vives-Bauza *et al*, 2010), but was not impaired in cells silenced for Ret expression (Fig 4H, I, J, N, O). Moreover, the overexpression of constitutively active Ret^{MEN2A} did not induce Parkin translocation or mitophagy under any condition, including PINK1 knock-down with or without Parkin overexpression (Fig 4K, L, M, N). Similar results were obtained when GDNF and soluble GFRα-1 was used to activate signaling via endogenous Ret (Fig 4N). Furthermore, GDNF/GFRα-1 treatment also rescued mitochondrial fragmentation induced by PINK1 silencing HeLa cells, a cell type which does not express endogenous Parkin (Denison *et al*, 2003; Pawlyk *et al*, 2003), further indicating that Ret signaling rescues PINK1 loss-of-function phenotypes independently of Parkin (Supplementary Fig S3).

Ret signaling rescues impaired bioenergetics of Pink1-deficient cells

It has been reported previously that PINK1 deficiency impairs mitochondrial respiration (Gautier *et al*, 2008, 2012; Gandhi *et al*, 2009; Lutz *et al*, 2009; Morais *et al*, 2009). We therefore investigated whether activation of Ret signaling via GDNF/GFRα-1 treatment could influence this phenotype. We measured mitochondrial function under basal and stress conditions in SH-SY5Y cells silenced for PINK1 expression by using an extracellular oxygen flux analyzer. In comparison to control siRNA-treated cells, PINK1-deficient cells were characterized by a decreased oxygen consumption rate even under basal conditions (Fig 5A). Moreover, the spare respiratory capacity (difference between maximal and basal respiration) was markedly reduced, indicating that the ability of PINK1-deficient cells to respond to an increased energy demand under stress conditions is severely impaired. Remarkably, GDNF/GFRα-1 treatment fully rescued basal respiration and increased maximal respiration in PINK1-deficient cells, indicating that the beneficial effect of increased Ret signaling in PINK1-deficient models can be explained by influencing the bioenergetic capacity of mitochondria rather than mitophagy.

Complex I deficiency of *Pink1* mutants rescued by Ret signaling

To investigate whether Ret signaling also rescued mitochondrial functionality in *Drosophila*, we measured ATP content of thoracic homogenates. As previously shown (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006; Vos *et al*, 2012), *Pink1* and *park* mutants showed reduced ATP content in the thorax to approximately 40% of controls, including flies carrying the Mef2-GAL4 driver (Fig 5B and C). *Mef2 > Ret^{MEN2B}* overexpression in control flies caused a slight reduction in ATP as compared to controls, possibly as a result of their mild muscle phenotype. In line with the rescue of myofibril and mitochondrial structures, *Ret^{MEN2B}* overexpression largely rescued ATP levels in *Pink1* mutants, while ATP levels of *park* mutants did not significantly improve (Fig 5B and C). To unravel the underlying mechanism of the improved mitochondrial respiration, we turned our attention to complex I of the ETC. Recent reports had found that *Pink1*, in contrast to *park* mutants had decreased activity of the ETC, and specifically of complex I function (Morais *et al*, 2009; Vilain *et al*, 2012). For these reasons, we measured complex I activity in *Ret^{MEN2B}*-overexpressing *Pink1* mutants, by monitoring rotenone-sensitive NADH oxidation by spectrophotometry, normalized to the activity of citrate synthase. As previously observed, *Pink1* mutants displayed markedly reduced complex I activity (Fig 5D). Interestingly, *Ret^{MEN2B}* significantly increased complex I activity to levels similar to controls (Fig 5D). In accordance with previously reported data, *park* mutants showed no decreased complex I

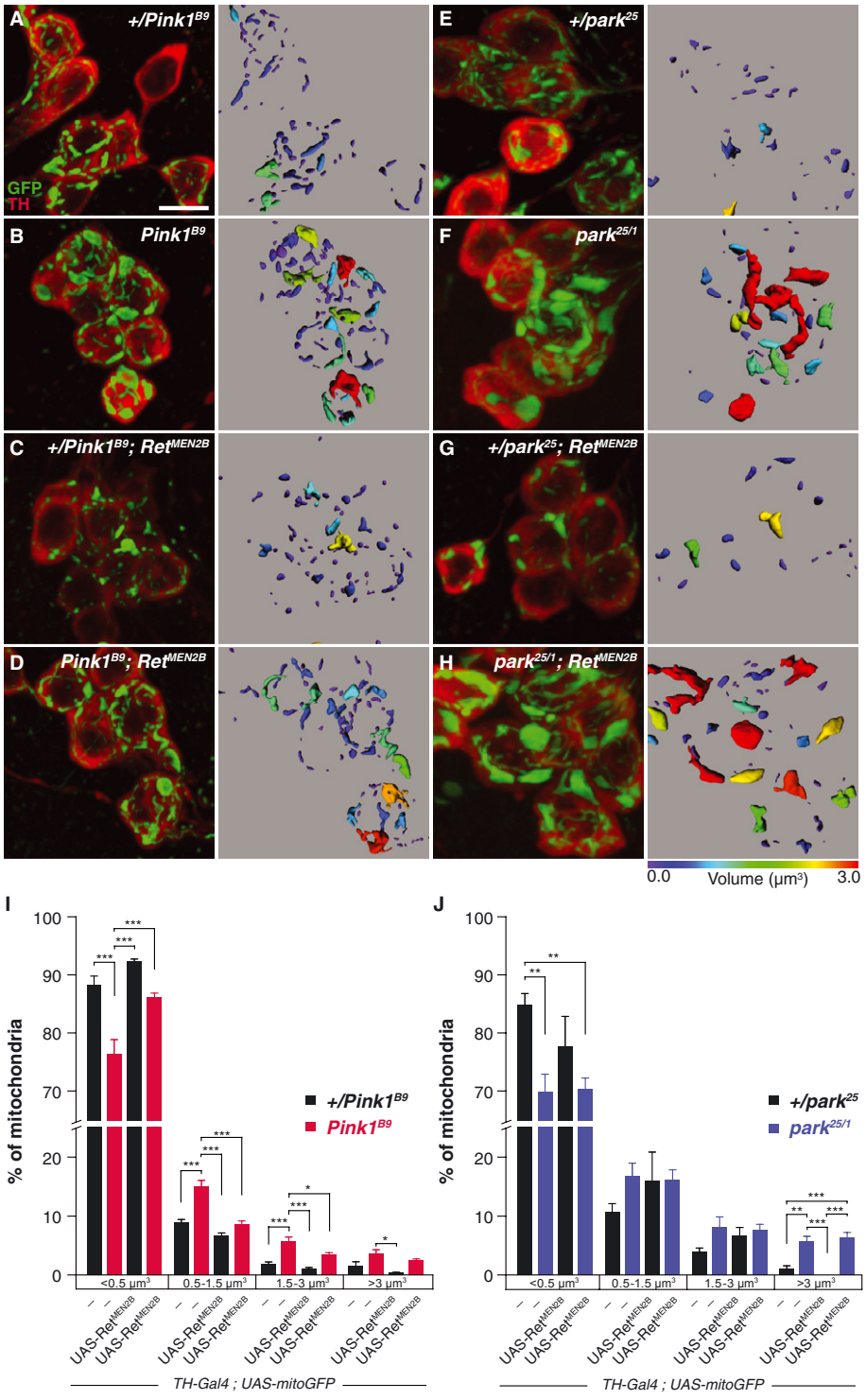

Figure 3. Rescue of Pink1 mutant dopamine neuron mitochondria by Ret^{MEN2B}.

A–H Confocal maximum projections (left panels) and isosurface renderings (right panels) of dopamine neuron mitochondria in the PPL1 cluster of dopaminergic neurons, visualized by mitoGFP and immunostainings against GFP and TH. Genotypes: All flies contain *TH-GAL4* and *UAS-mitoGFP* and *Pink1*, *park* mutant alleles, as well as *UAS-Ret^{MEN2B}* as indicated. Isosurface renderings are color-coded according to volume from 0 to 3 µm^3. Ret^{MEN2B}-overexpressing control animals (C, G) display normal mitochondrial morphology as compared to non-transgenic controls (A, E). *Pink1* mutants (B) and *park* mutants (F) display severely enlarged mitochondria, and Ret^{MEN2B} partially rescues mitochondrial size in *Pink1* mutants (D), but not in *park* mutants (H). Scale bar, 5 µm.

I, J Mitochondrial volume distributions of (A–D) and (E–H) in categories as indicated. Due to differences in staining and imaging conditions, data between the *Pink1* and *park* datasets cannot be directly compared. *n* = 8–20 animals per genotype.

activity as compared to controls (Fig 5E). Depleting the complex I subunit (CG11455) from muscles by RNAi abrogated most complex I activity (Fig 5F), and Ret^{MEN2B} overexpression was not able to rescue this defect (Fig 5F), suggesting that Ret signaling does not activate alternative means of NADH oxidation as previously shown for the yeast protein Ndi1p (Vilain *et al*, 2012). The mechanism by which Pink1 controls complex I function is still unknown. *Drosophila* complex I contains 48 subunits, six of which are mitochondrially encoded, the rest being nuclear. The supply of commercially available antibodies for *Drosophila* complex I is limited to the subunit NDUFS3, which has recently been shown to be reduced in *Pink1* mutants (Liu *et al*, 2011). By Western blot, we could confirm the reduction of NDUFS3, but did not observe an upregulation by Ret^{MEN2B} (Supplementary Fig S4A and B). We performed a semi-quantitative RT-PCR screen of other complex I subunits in *Pink1* mutants compared to Ret^{MEN2B}-overexpressing *Pink1* mutants. Of 45 subunits analyzed, most were unchanged, but the transcript of CG6485, orthologous to human NDUFV2, was moderately elevated in Ret^{MEN2B}-overexpressing *Pink1* mutants (Supplementary Fig S4C). Interestingly, when compared to controls, CG6485 mRNA was reduced by 46% in *Pink1* mutants, and significantly increased to 117% of controls by Ret^{MEN2B} overexpression (Fig 5G and H). This effect may at least in part be responsible for the Ret-mediated rescue of Pink1 deficiency.

Discussion

The receptor tyrosine kinase Ret is already known to be required for long-term survival of nigral dopamine neurons in mice, and stimulation with its ligand GDNF protects dopamine neurons from cell death in a variety of toxin-based rodent and primate models of PD. In the present work, we found that a signaling-active version of the *Drosophila* homolog of Ret suppresses degeneration of muscle tissue and mitochondrial abnormalities in *Pink1* mutants. Interestingly, *park* mutants were not rescued. In human SH-SY5Y cells, stimulation of endogenous Ret by GDNF rescued both morphological and bioenergetic defects of mitochondria in PINK1-depleted cells. Pink1 and Parkin were previously shown to interact genetically in *Drosophila* in what was proposed to be a linear pathway, and a significant body of work has described how Pink1 and Parkin function to initiate mitophagy of impaired mitochondria, and arrest of mitochondrial trafficking. However, in our cell culture model, Ret signaling did not induce mitophagy or Parkin recruitment, arguing that Ret rescues PINK1 deficits independently of Parkin. A recent study demonstrated that *Pink1* mutants in contrast to *park* mutants have decreased function of complex I of the electron transport chain, suggesting that Pink1 is required for maintaining efficient complex I enzymatic activity and that this function is upstream of mitochondrial remodeling. We found that Ret rescued both the impairment of complex I activity, and partially the mitochondrial morphology in *Pink1* mutants, suggesting that complex I is a target of Ret signaling. Previous studies of complex I inhibition or genetic depletion have shown mild morphological impairments in *Drosophila* muscle, contrary to the stronger phenotype of *Pink1* mutants. Therefore, it was somewhat unexpected that restoring complex I activity would be sufficient to rescue also morphological defects. One interpretation is that the *Pink1* mutant morphological phenotype is more severe due to a synergistic effect of deficits in remodeling/mitophagy and complex I activity, which in this study was partially rescued. Another possibility is that Ret signaling not only targets complex I, but also morphology in a Parkin-independent manner.

Extrapolated to mammalian models, our results suggest a novel mechanism by which the GDNF family of neurotrophic factors may promote survival of dopamine neurons in PD. Several of the mammalian models where the neuroprotective effects of GDNF treatment were initially discovered, were in fact models of mitochondrial dysfunction, either directly via complex I inhibition by MPTP treatment (Tomac *et al*, 1995; Gash *et al*, 1996), or the more general ROS toxicity of 6-OHDA (Kearns & Gash, 1995; Sauer *et al*, 1995), which also includes complex I impairments (Glinka *et al*, 1997). In light of our findings, it would be interesting to investigate whether or not GDNF improves complex I activity in these model systems. GDNF has been tested in models of alpha-synuclein overexpression, a pathology that is not known to cause complex I deficiency, but did not show any neuroprotective effects, fitting with our hypothesis (Lo Bianco *et al*, 2004; Decressac *et al*, 2011).

The current findings support recent evidence showing that Pink1 has an important function related to complex I activity, which is independent of its function in recruiting Parkin to the outer mitochondrial membrane upon loss of membrane potential. This model is consistent with a partial rescue of Pink1 deficiencies, e.g. by either overexpressing Parkin or the yeast complex I equivalent NADH dehydrogenase, or, in the current work, Ret^{MEN2B} (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006; Vilain *et al*, 2012). In addition, our findings are consistent with a recent study showing that Pink1-deficient flies but not Parkin-deficient flies can be rescued by TRAP1, which also seems to have beneficial effects on complex I activity (Zhang *et al*, 2013).

The pathways by which Ret signaling targets complex I and rescues *Pink1* mutants requires further investigation. Also, the mechanism by which Pink1 regulates complex I remains elusive, it may regulate for example gene expression, phosphorylation status or assembly (Salvi *et al*, 2005; Pagliarini & Dixon, 2006) (Fig 6). Our gene expression analysis showed that most subunits are unchanged by Ret^{MEN2B}, but interestingly one subunit was moderately downregulated in *Pink1* mutants and upregulated by Ret^{MEN2B}, which may improve function. However, we do not exclude the possibility that Ret signaling targets complex I, and perhaps other metabolic components, by different means.

Brain-derived neurotrophic factor (BDNF) protects mouse cortical neurons against drug-induced excitotoxicity, an effect that was blocked by the complex I inhibitor Rotenone and a MEK1/2 inhibitor, suggesting that BDNF signaling via the Ras/Erk pathway can regulate complex I function (Markham *et al*, 2012). The signaling properties and functions of *Drosophila* Ret are not characterized in great detail, but it is structurally homologous to mammalian Ret and can, to some extent, activate the same signaling pathways (Abrescia *et al*, 2005). Mammalian Ret on the other hand, has been extensively characterized and is known to activate a number of downstream signaling pathways including Ras/ERK, phosphoinositol-3 kinase (PI3K)/Akt, phospholipase C-gamma (PLCγ), Janus kinase (JAK)/STAT, and ERK5, several of which have pro-survival effects, most notably the PI3K/Akt pathway (Sariola & Saarma, 2003; Pascual *et al*, 2011). Recent studies of *Pink1* and *park* mutant *Drosophila* have indicated that PI3K/Akt signaling or components downstream of this pathway rather exacerbates *Pink1* and *park*

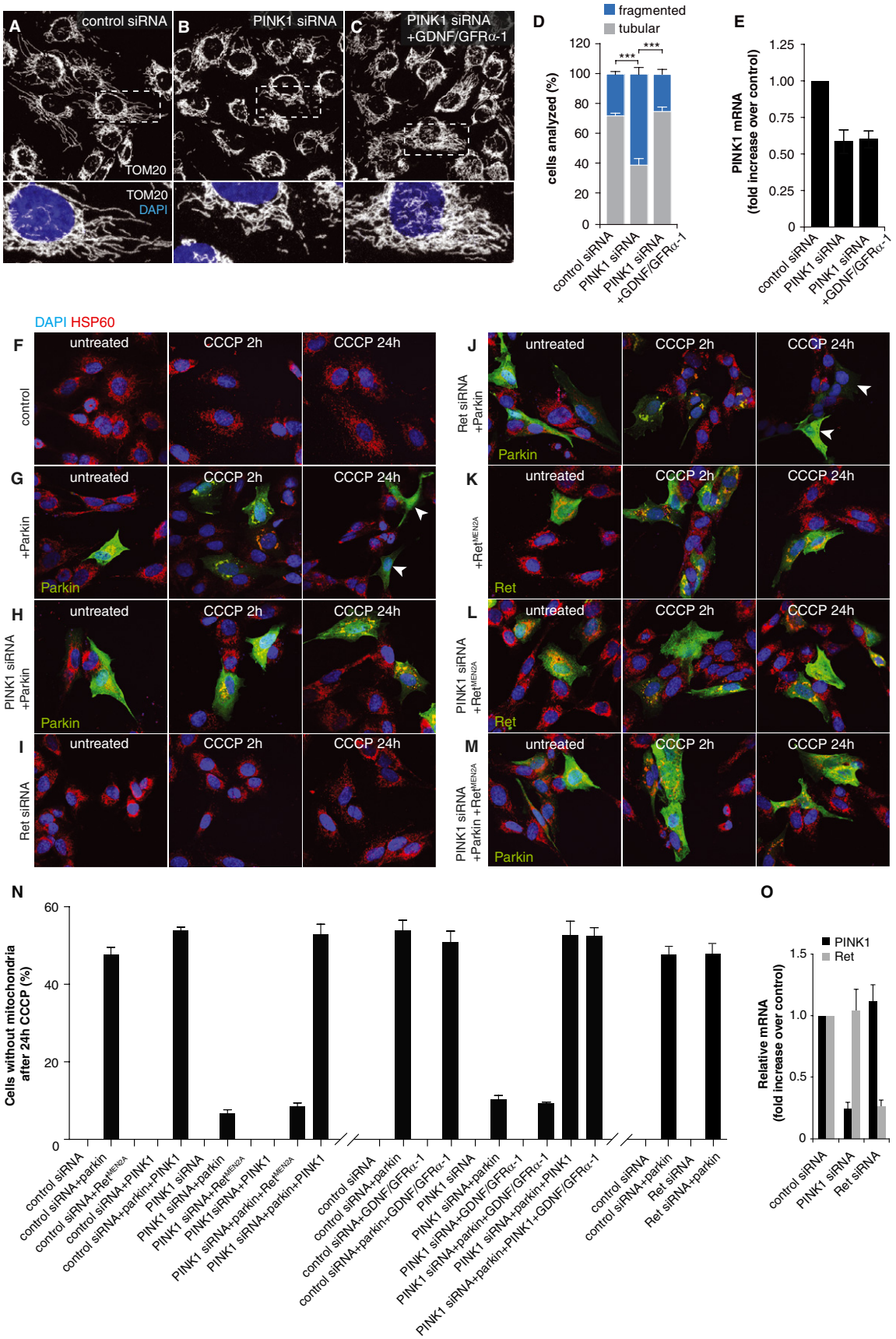

Figure 4. Activation of Ret signaling mammalian cells rescues PINK1 deficiency, but has no effect on mitophagy.

A–C SH-SY5Y cells expressing endogenous Ret, transfected with scrambled control siRNA (A) display normal tubular mitochondrial morphology, visualized by immunostaining for TOM20 (white); DAPI (blue) indicates nuclei. Cells silenced for PINK1 expression display increased mitochondrial fragmentation (B). Stimulation of Ret signaling by treatment of cells with GDNF together with soluble GFRα-1 rescues mitochondrial fragmentation after PINK1 knockdown (C).

D Quantification of cells with either tubular (gray) or fragmented (blue) mitochondria.

E Quantification of PINK1 mRNA by quantitative RT-PCR indicates that GDNF/GFRα-1 treatment has no effect on PINK1 expression.

F–M SH-SY5Y cells were treated with CCCP for 2 or 24 h to depolarize mitochondria, and then stained for HSP60 (red), DAPI (blue) and Parkin or Ret (green) as indicated. Cells with endogenous Parkin expression display low levels of mitophagy (F) and no cells fully cleared of mitochondria were detected 24 h after CCCP treatment. Cells overexpressing Parkin display translocation of Parkin to mitochondria 2 h after CCCP treatment and complete clearance of mitochondria by 24 h after adding CCCP (G). White arrowheads indicate cells without detectable mitochondria. Silencing of PINK1 by siRNA largely inhibits Parkin translocation and mitophagy (H), whereas silencing of Ret has no effect on mitophagy alone (I) or in cells overexpressing Parkin (J). Overexpression of constitutively active Ret^{MEN2A} does not activate mitophagy in control or PINK1-silenced cells (K, L), and does not modulate Parkin translocation or mitophagy in Parkin-overexpressing cells (M).

N Quantification of the experiments described in (F–M).

O Quantification of mRNA after PINK1 or Ret silencing by quantitative RT-PCR.

mutant phenotypes (Tain *et al*, 2009; Liu & Lu, 2010), making it an unlikely candidate for rescue.

Additional studies are required to elucidate the details by which Pink1 and Ret regulate complex I activity, and whether this finding is transferrable to mammalian models. In summary, this work shows that Ret signaling can rescue phenotypes of *Pink1* mutants by restoring mitochondrial respiration and specifically complex I function, and thereby suggests a potential novel mechanism underlying GDNF-mediated protection in mammalian PD models. In the future, screening of PD patients for complex I deficiencies and subjecting specifically those individuals to GDNF treatment may provide a new therapeutic strategy.

Materials and Methods

Fly strains and procedures

Mef2-GAL4;UAS-Ret^{MEN2B} is lethal at 25°C, therefore all crosses were performed at 18°C. All analyses were performed with 2- to 5-day-old flies. In experiments with Mhc-GAL4;Tub-$GAL80^{ts}$, pupae were shifted from 18 to 30°C at pharate adult stages P11-P12 (Flybase FBdv:00005349) and analyzed at 3–4 days post eclosion. $park^{25}$ (Greene *et al*, 2003) was provided by Leo Pallanck, $park^{1}$ (Cha *et al*, 2005) and $Pink1^{B9}$ (Park *et al*, 2006) were provided by Jongkyeong Chung, $Pink1^{B9}$*::Mef2-GAL4* (Tain *et al*, 2009) was provided by Alex Whitworth, *UAS-*Ret^{MEN2B} (Read *et al*, 2005) was provided by Ross Cagan, *TH-GAL4* (Friggi-Grelin *et al*, 2003), was provided by Hiromu Tanimoto, *Mef2-GAL4 (Ranganayakulu et al, 1996)*, *Tub-*$GAL80^{ts}$ (McGuire *et al*, 2003), and UAS-mitoGFP (Pilling *et al*, 2006) were obtained from the Bloomington stock center, *UAS-*$CG11455^{RNAi}$ (#12838) was obtained from Vienna Drosophila RNAi Center. "+" controls depict *Pink1* and *park* WT alleles from w^{1118} (Bloomington stock #5905). In For all histology experiments, flies were genotyped by PCR to assure correct genotypes and control for X-chromosome non-disjunction, for list of primers see Supplementary information.

Myosin heavy chain – GAL4 flies

A 2.5 kb Mhc enhancer was amplified from genomic DNA using primers FS124 (5′-tcaggtaccGGCCGCTCTAGAAATGATATGTG-3′) and FS125 (5′-tcacgcggccgcATTATCCTTGCTTAAATTTCGTTTAG-3′) and cloned with Asp718/NotI into a GAL4-containing Casper-based P-element transformation vector. Transgenic flies were generated using standard procedures. In contrast to the formerly published GAL4 line (Schuster *et al*, 1996), which shows a rather weak activity in embryos, larvae and adults, this new *Mhc-GAL4* line is very strong and very specifically expressed in differentiated muscles from embryonic stages onwards (FS, unpublished).

Histology, transmission electron microscopy and analysis of mitochondrial morphology

Hemi-thoraces were prepared as described previously (Schnorrer *et al*, 2010), stained with Phalloidin-Alexa Fluor-568 (Molecular Probes), and single plane images were acquired on an Olympus FV1000 confocal scanning microscope. For transmission electron microscopy, hemi-thoraces were fixed in 2.5% Glutaraldehyde, from which semithin sections were prepared and stained with toluidine-blue, subsequently ultrathin serial sections were prepared using a Leica EM UC6 Ultramicrotome. Images at 5,000× magnification were acquired using a JEOL JEM-1230 transmission electron microscope at 80 kV, equipped with a Gatan Orius SC1000 digital Camera. Six TEM Images per animal were acquired from randomly selected regions of the indirect flight muscles. All mitochondria in these images (500–800 per animal) were grouped into three categories, based on the integrity of the cristae structure, with genotypes blinded to the experimenter, using the ImageJ software (NIH). Whole mount immunostaining of fly brains was performed according to standard procedures. The following antibodies were used: rabbit anti-tyrosine hydroxylase (ab152, Millipore; 1:200) and chick anti-GFP (Abcam ab13970; 1:500). The PPL1 cluster was imaged using an Olympus FV1000 confocal microscope with a 60× NA 1.3 objective with 4× zoom. 52 z-sections of 0.3 μm spacing were acquired and deconvolved by the nearest neighbor algorithm using Metamorph 7.5 (Molecular Devices). A volume corresponding to 26 × 26 × 15 μm was cropped, subjected to linear rescaling and analyzed in Imaris x64 6.4.2 (Bitplane Scientific Software). Mitochondrial volume was measured by 3D isosurface rendering using a fixed threshold.

Immunoblot analysis

Thoraces from three animals per sample were homogenized in Triton-lysis buffer, protein concentration was determined using the BCA method (BioRad), equal amounts of protein were separated using SDS-PAGE and blotted according to standard procedures.

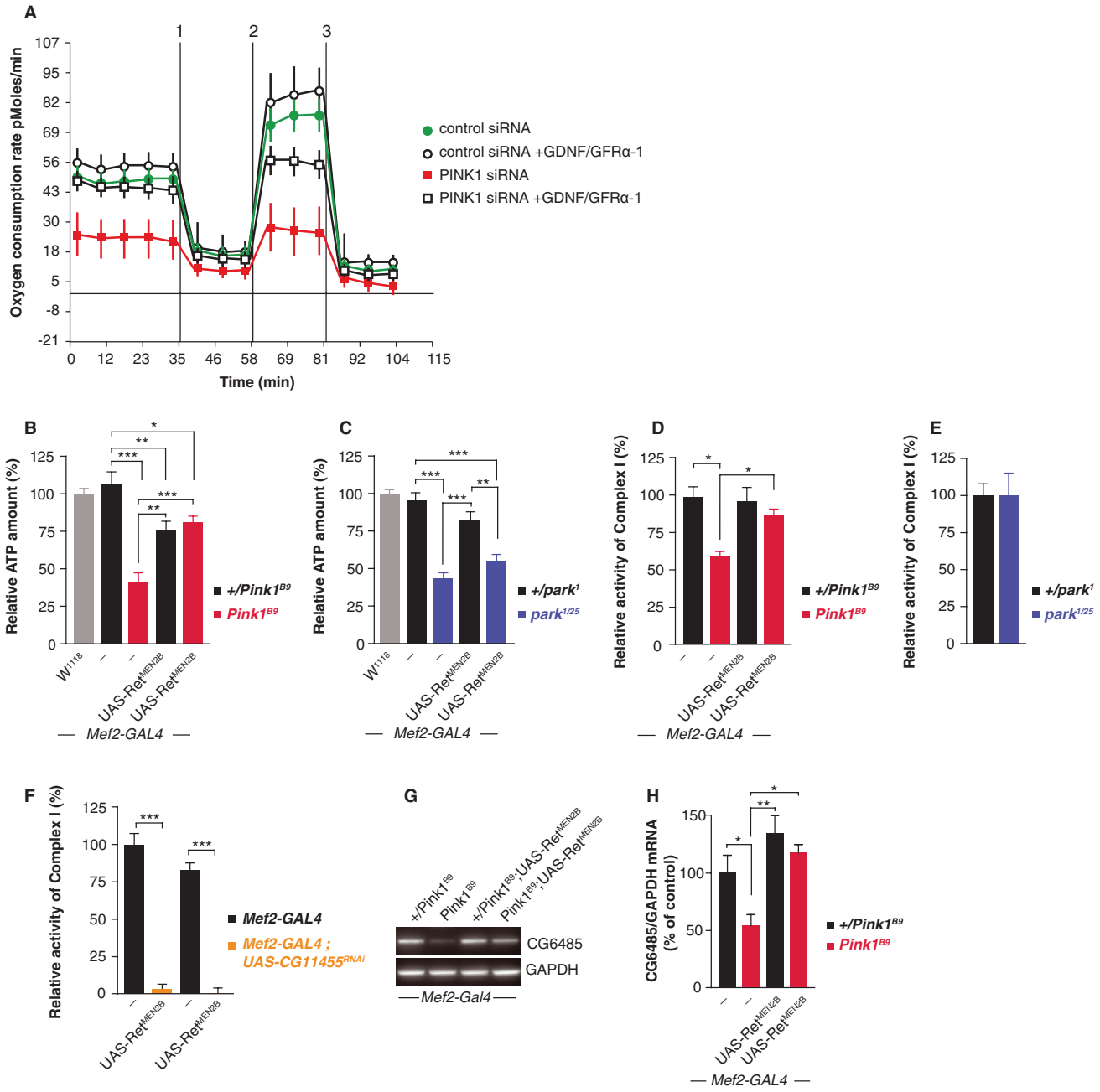

Figure 5. Ret signaling rescues mitochondrial respiration and complex I function in PINK1-deficient cells.

A Oxygen consumption rate in SH-SY5Y cells determined by an extracellular flux analyzer. 1: Injection of the F_1F_O–ATPase inhibitor oligomycin; 2: injection of the uncoupler FCCP; 3: injection of the complex I inhibitor rotenone and the and complex III inhibitor antimycin A. Under basal conditions, as well as FCCP-evoked maximum respiration, PINK1 knockdown cells (red squares) displayed markedly reduced oxygen consumption as compared to controls (green circles). Treatment of PINK1 knockdown cells with GDNF/GFRα-1 rescued basal respiration and increased FCCP-evoked respiration (open squares).

B, C Relative ATP content in the thorax, normalized by total protein, expressed as percentage of w^{1118} controls. *Pink1* and *park* mutants have reduced ATP amounts. Ret^{MEN2B} overexpression partially rescues ATP deficiency in *Pink1* (B), but not *park* mutants (C). Averages of 6–12 animals per genotype.

D–F Activity of Complex I (rotenone sensitive), normalized to citrate synthase activity, percentage of heterozygous controls. *Pink1* mutants have impaired complex I function, which is rescued by Ret^{MEN2B} overexpression (D). *park* mutants have normal complex I activity as compared to controls (E). Inactivation of the complex I subunit CG11455 by RNAi driven by Mef2-GAL4 causes dramatically reduced complex I activity as compared to controls (F) and this was not rescued by Ret^{MEN2B} overexpression.

G Semi quantitative RT-PCR analysis of complex I subunit CG6485 indicates upregulation by Ret^{MEN2B} overexpression in *Pink1* mutants, GAPDH was used as a loading control.

H Quantification of CG6485 mRNA normalized to GAPDH, averages of 3 experiments, RNA from 3 thoraces per sample.

Antibodies used were: panRet (provided by C. Ibanez) and alpha-Tubulin (clone DM1A, Sigma).

Cell culture, treatments and RNA Interference

SH-SY5Y (DSMZ number ACC 209) cells were cultivated as described previously (Henn *et al*, 2005; Schlehe *et al*, 2008). For acute stimulation of Ret, cells were incubated for 3–4 h with recombinant hGDNF (Shenandoah Biotechnology Inc.) and hGFRα-1 (R&D Systems) at a final concentration of 100 ng/mL. PINK1 and Ret gene silencing was performed with the following stealth siRNA oligos (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen): PINK1 human HSS127945 (SH-SY5Y), Ret human HSS109181.

Assessment of mitochondrial morphology

SH-SY5Y: Cells grown on 15-mm glass coverslips were fixed with 3.7% PFA in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 5% BSA in PBS at room temperature. Fixed cells were sequentially incubated with primary antibody diluted in blocking solution (TOM20 pAb, overnight at 4°C) and secondary antibody diluted in blocking buffer (goat anti rabbit Alexa555- coniugated, 2 h at room temperature). Nuclei were counterstained with DAPI. Coverslips were mounted on glass slides and images were acquired with a Zeiss LSM710 confocal microscope equipped with a 63× oil objective (NA 1.4). Cells displaying an intact network of tubular mitochondria were classified as tubular. When this network was disrupted and mitochondria appeared either globular or rod-like they were classified as fragmented. The mitochondrial morphology of the cells was determined in a blinded manner. Quantifications were based on 150 cells from at least 3 independent experiments.

Assessment of mitophagy

SH-SY5Y cells were plated on glass coverslips and reversely transfected with siRNA and 24 h later with the indicated DNA plasmid. Human GDNF and GFRalpha were added to the cells 24 h after siRNA transfection and 3 h before CCCP treatment. The next day, cells were treated with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) for 2 or 24 h. Recruitment of parkin to mitochondria (after 2 h CCCP) and removal of mitochondria (after 24 h CCCP) was detected by indirect immunofluorescence using a monoclonal anti-Parkin antibody (PRK8, Santa Cruz Biotechnology) and a polyclonal antibody against HSP60 (Santa Cruz Biotechnology). Nuclei were stained by DAPI. Cells

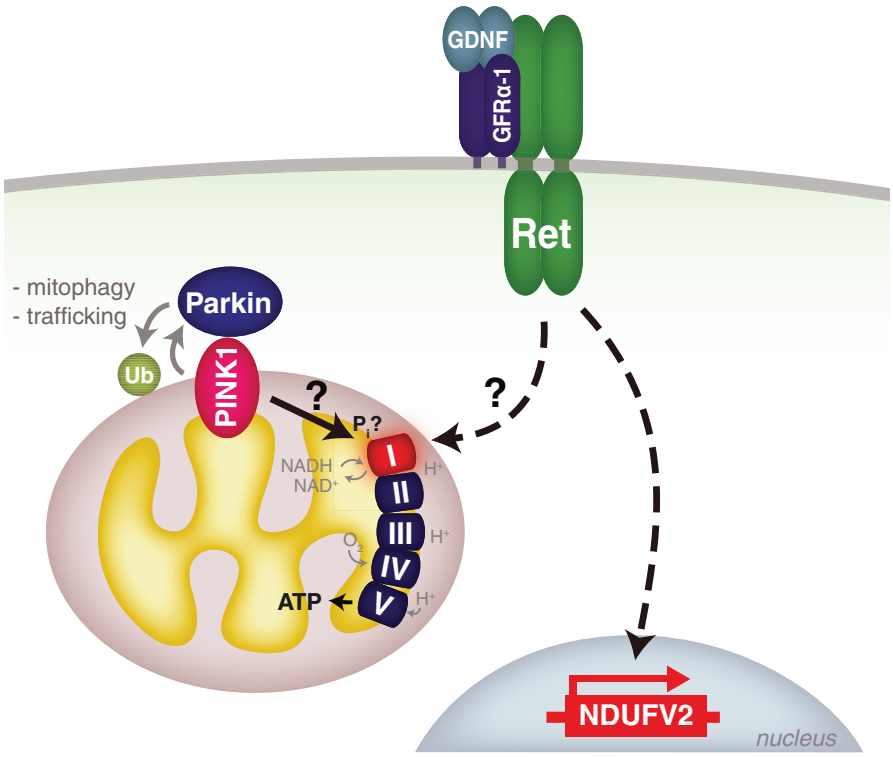

Figure 6. Model of Pink1 and Ret^{MEN2B} functions.
Our results suggest a dual role for Pink1: One in recruiting Parkin to the mitochondria and initiating mitochondrial clearance or regulating mitochondrial trafficking, a second in regulating the activity of complex I via an as yet unclear pathway. This could be mediated, for example, via phosphorylation of the protein complex or by regulating expression of complex I components. Loss of Pink1 decreases complex I activity and respiratory function. Ret rescues specifically *Pink1* mutants, by restoring complex I activity, respiration and ATP production, in part by upregulating the mRNA levels of the complex I subunit NDUFV2 (CG6485).

were analysed by fluorescence microscopy using a Leica DMRB microscope and confocal images were taken using a Zeiss LSM710 confocal microscope equipped with a 63× oil objective (NA 1.4). Quantifications are based on three independent experiments. At least 1,500 cells were analysed for each condition.

Real-time RT-PCR, cultured cells

Knock-down efficiency of PINK1 and Ret was evaluated by real-time RT-PCR with the 7500 Fast Real Time System (Applied Biosystems) as previosly described (Bouman *et al*, 2011). Statistical analysis of RT-PCR data is based on at least four independent experiments with triplicate samples. For list of primers, see Supplementary information.

Measurement of mitochondrial oxygen consumption

The oxygen consumption rate was determined using a Seahorse XF 96 analyzer (Seahorse Biosciences). SH-SY5Y cells were reversely transfected and plated in a XF 96 cell culture microplate. The next day, fresh medium containing human GDNF/GFRα-1 was added to the cells where indicated. The cells were incubated with low-glucose (1 mM) medium overnight and the sensor cartridge was hydrated overnight according to the manufacturers' instructions. Measurements were performed 48 h after transfection. The measured values were normalized to protein levels. PINK1 knockdown did not induce apoptosis under these conditions. The cells were washed using the XF Prep Station three times with Seahorse Medium containing 10 mM galactose and 1 mM pyruvate. Mitochondrial function was analyzed using the XF Cell Mito Stress Test Kit (Seahorse Biosciences) and all measurements were carried out at 37°C. The following drugs were diluted in Seahorse Medium and loaded on the sensor cartridge: oligomycin (injection port A), carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP; injection port B), rotenone and antimycin A (both injection port C). The drugs were diluted in Seahorse Medium and loaded on the sensor cartridge. Measured values were normalized to protein levels.

ATP measurement

Measurements of thoracic ATP were performed using a luciferase assay as described previously (Park *et al*, 2006) with some modifications: Briefly, single thoraces from 3-day-old flies with heads and wings removed were homogenized in 50 μl of extraction buffer (100 mM Tris-HCl, 4 mM EDTA pH 7.8) with 6 M Guanidine-HCl using a teflon-on-glass dounce homogenizer. The lysate was boiled for 3 min and cleared by centrifugation at 20,000 *g* for 1 min. The samples were diluted 1:100 in extraction buffer before analyzing using the ATP determination kit (Invitrogen), according to the manufacturer's instruction. Values were normalized to total protein content, measured by absorbance at 280 nm using a NanoDrop spectrophotometer. All measurements were performed in triplicate.

Enzymatic measurements

Activity of complex I (NADH: ubiquinone oxidoreductase) was assessed by monitoring the oxidation of NADH as previously described (Fischer *et al*, 1986). Briefly, thoraces from 20 animals were homogenized in 250 mM sucrose, 10 mM Tris pH 7.4, 0.15 mM $MgCl_2$, after which mitochondria were isolated as described previously (Walker *et al*, 2006). Enzymatic activity of complex I was assessed by NADH oxidation, monitored at A340 nm as described (Bugiani *et al*, 2004), and rotenone insensitive activity was subtracted. The activity of complex I was normalized to Citrate Synthase activity, which was measured indirectly by AcCoA-SH formation, as described (Ferguson & Williams, 1966).

RT-PCR, *Drosophila* complex I subunits

Thoraces were dissected and snap-frozen, homogenized in RLT buffer (Qiagen) using a rotor-stator homogenizer. Total RNA was prepared using the RNeasy mini kit according to instructions. Samples were treated with DNase1 on-column for 15 min (RNase-free DNase set, Qiagen). RT-PCR analysis was performed using the OneStep RT-PCR kit (Qiagen) using 20 ng of template RNA and 35–40 cycles of PCR amplification depending on signal strength of the primer pair. Primers were designed using the primerBLAST tool (NCBI), and when possible exon-junction spanning primers were used, for list of primers, see Supplementary information. As some of the analyzed transcripts are single-exon, control reactions omitting the reverse transcriptase amplification step were performed to assure that samples were free of contaminating genomic DNA, despite DNase1 treatment.

Statistical analysis

Data represent mean ± SEM. Statistical analysis was carried out using analysis of variance (ANOVA) or Student's *t*-test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

PK designed, performed and analyzed the majority of the experiments. CS and FS contributed to the design of the fly genetics and analysis of muscle morphology, and FS generated the Mhc-GAL4 line. EM and AKM-R designed, performed and analyzed the SH-SY5Y experiments. KFW supervised the cell culture work and contributed to the analysis of the fly data. RK supervised the project, designed experiments and co-wrote the manuscript with PK.

Conflict of interest

The authors declare that they have no conflict of interest.

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