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# *Pink*1 and *parkin* demonstrate multifaceted roles when co-expressed with *Foxo*

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## ABSTRACT

***Pink*1 has been linked to both autosomal recessive and sporadic forms of Parkinson disease. The *Pink*1 protein is thought to be involved in mitochondrial protection by interacting with *parkin* to prevent oxidative damage, maintain mitochondrial integrity and regulate mitophagy. *Pink*1 and *parkin* have been linked to components of the insulin receptor (INR) pathway, including PTEN, Akt and *Foxo*, but their effects in the INR pathway have been largely overlooked. To further investigate the roles of *Pink*1/parkin, we have performed co-expression studies to determine the effects *Pink*1 and *parkin* on the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. We examined directed expression of *Pink*1, *parkin*, *Pink*1 or *parkin* mutants, and *Pink*1 or *parkin* interfering RNAs (RNAi) with the overexpression of *Foxo* in the developing eye of Drosophila. Our findings show that reduction of *Pink*1 suppresses the effects of *Foxo* overexpression, where co-overexpression with *Pink*1 or *parkin* increases the severity of the phenotype. This suggests that *Pink*1 and *parkin* are able to increase the pro-apoptotic effects of *Foxo*. Contrary to the view that *Pink*1 and *parkin* act exclusively as protective proteins in the cell, it is likely that the *Pink*1/*parkin* pathway is involved in aspects of cell fate decisions other than degrading toxic proteins and maintaining mitochondrial integrity.**

**Keywords:** *Pink*1; *Parkin*; *Foxo*; Drosophila

## 1. INTRODUCTION

*Pink*1 (*PTEN induced putative kinase* 1) encodes a serine-threonine kinase which has been linked to autosomal recessive and some sporadic forms of Parkinson disease [1-3]. Targeted to the mitochondria, *Pink*1 is thought to be involved in mitochondrial protection by preventing oxidative damage and maintaining mitochondrial integrity, where loss of function of *Pink*1, in humans and in *Drosophila melanogaster*, show substantial mitochondrial defects in sensitive tissues [4-8]. It is becoming increasingly apparent that protection during cell stress is due to the involvement of *Pink*1 in mitochondrial fission/fusion events [1,9]. This involvement implicates *Pink*1 as a key regulator of fission/fusion, acting upstream of the E3 ubiquitin ligase, parkin, to maintain proper mitochondrial integrity and function [4,6,10,11]. In this role, recruitment of *parkin* to the mitochondria by *Pink*1 results in the ubiquitination of various mitochondrial proteins, promoting fission and mitophagy [12-15]. In contrast, studies have found that loss of *parkin* or *Pink*1 function can also result in increased fission, promoting mitophagy [16,17]. Although the fission/fusion decision is not fully understood, results do highlight the importance of the *Pink*1/*parkin* pathway in maintaining mitochondrial homeostasis.

The *Pink*1/*parkin* pathway has been linked to components of the insulin receptor (INR) pathway, including: interaction of PTEN with *Pink*1 [18] and DJ-1 [19,20], an indirect interaction with Akt through *parkin* [21], an interaction with Akt through DJ-1 [22,23], and transactivation of *Pink*1 by *Foxo* [24,25]. It has also been suggested that *Pink*1 may activate *Foxo* indirectly through Sir2 [26]. This is thought to be a protective mechanism, where *Foxo* activation results in the transcription of genes such as *SOD*2 and *Thor*. In addition to genes that promote stress resistance, under conditions of oxidative stress or starvation, *Foxo* transcription factors may also target genes that promote cell cycle arrest and apoptosis [27,28]. Overexprssion of *Foxo* has been linked to neurotoxicity [29,30] and overexpression in the developing Drosophila eye results in a characteristic phenotype with reductions in cell number and area [31]. Genetic expression studies using the fly eye have been enormously successful in the study of neurodegeneration. This is due to the conservation of key signaling pathways between humans and Drosophila, and the ease of quantifying degeneration of photoreceptor neurons associated with each

Drosophila ommatidium. To further investigate the roles of *Pink*1/parkin, we have performed expression studies to determine the effects of *Pink*1 and *parkin* on the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. We hypothesized that through an interaction with the INR pathway, or through mitochondrial protective effects, *Pink*1 and *parkin* would be capable of alleviating the detrimental effects of *Foxo* overexpression. In contrast, our findings show that reduction of *Pink*1 is able to suppress the effects of *Foxo* overexpression, where co-overexpression of *Foxo* with *Pink*1 or *parkin* results in an increased severity of the *Foxo*-induced phenotype. These findings suggest a complex role for the *Pink*1/*parkin* pathway in cell fate decisions.

## 2. MATERIALS AND METHODS

### 2.1. Fly Stocks and Culture

The *UAS-Pink*1 transgenic line was created from the GH20931 *Drosophila melanogaster Pink*1 clone [32]. The *UAS-murine Foxo*1 (*UAS-Foxo*) and *UAS-murine Foxo*1$^{AA}$ (*UAS-Foxo*$^{AA}$) transgenes are described in Kramer *et al.* [31] and the *GMR-Gal4 UAS-Foxo* and *GMR-Gal*4;*UAS-Foxo*$^{AA}$ lines were established through standard means. *UAS-parkin* was created previously in our laboratory [33]. The *Pink*1$^{B9}$ mutant line was provided by Dr. J. Chung [6]. The *UAS-Pink*1$^{RNAi}$ and *UAS- parkin*$^{RNAi}$ lines were provided by Dr. B. Lu [7,34]. The *UAS-GFP* control was obtained from the Bloomington stock centre. The *parkin*$^{45}$ mutant line was provided by Dr. L. Pallanck [35]. All crosses were performed using standard techniques. All flies were cultured on standard cornmeal/yeast/molasses/agar media at 25˚C.

### 2.2. Scanning Electron Microscopy of the Drosophila Eye

Flies were aged three days past eclosion on standard cornmeal/yeast/molasses/agar media at 25˚C. Flies were then frozen at −80˚C and examined under dissecting microscope. Flies were mounted, desiccated overnight and coated in gold before photography at 170 times magnification with a Hitachi S-570 SEM. Area of the eye was measured as per the ocular area, regardless of the presence of ommatidia. This was determined by outlining the ocular margin and/or ridge bristles indicating the postocular area. Eye areas and ommatidial counts were compared using GraphPad Prism 5, using unpaired *t*-test with a significance level of 0.05.

## 3. RESULTS

### 3.1. *Parkin* Increases the Severity of the *Foxo*-Induced Phenotype

Overexpression of *Foxo* in the developing Drosophila eye results in a characteristic phenotype with reductions in cell number and area [31]. When co-overexpressed with *parkin*, there is a significant increase in the severity of the *Foxo*-induced phenotype (**Figure 1**), including a significant reduction in number of ommatidia and overall area of the eye ($p < 0.0001$, $df = 31$). This suggests that the addition of *parkin* further reduces the number of viable cells available during eye development. Co-overexpression with *Pink*1 shows no significant increase in the *Foxo*-induced reduction of ommatidia ($p = 0.1150$, $df = 29$) and area ($p = 0.2335$, $df = 29$) (**Figure 1**).

### 3.2. Reduction in *Pink*1 Decreases the Severity of the *Foxo*-Induced Phenotype

Overexpression of *Foxo* in a *Pink*1 mutant background (*Pink*1$^{B9}$) results in a significant increase in ommatidia number ($p = 0.0008$, $df = 21$) and eye area ($p = 0.0015$, $df = 21$) (**Figure 2**). In addition, co-overexpression of *Foxo* with *Pink*1$^{RNAi}$ shows an even greater effect, with significant increases in ommatidia number and area ($p < 0.0001$, $df = 30$) (**Figure 2**). These results suggest that the absence or depletion of *Pink*1 during eye development is able to alleviate the detrimental effects of *Foxo.* Overexpression of *Foxo* in a *parkin* mutant background (*parkin*$^{45}$) or co-overexpression with *parkin*$^{RNAi}$ resulted

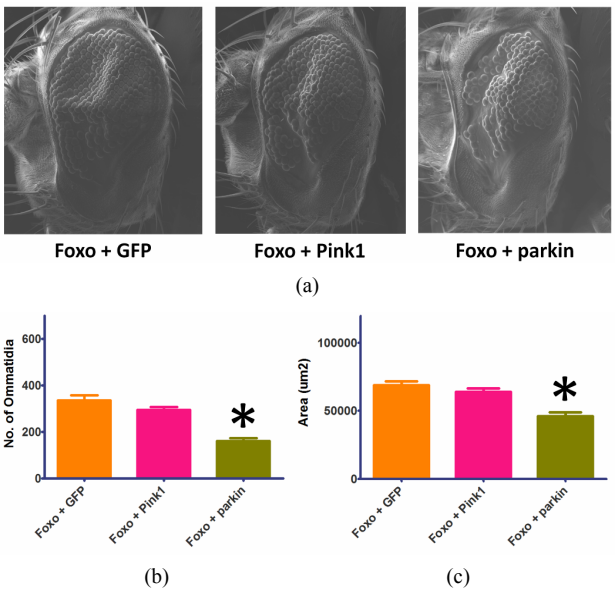


**Figure 1.** *Parkin* increases the severity of the *Foxo*-induced phenotype. Co-overexpression of *Foxo* with *parkin* shows a significant reduction in number of ommatidia and overall area of the eye. Co-overexpression of *Foxo* with *Pink1* shows no significant increase in the *Foxo*-induced reduction of ommatidia and area. Genotypes shown include *GMR-Gal*4 *UAS-Foxo/UAS-GFP* (*Foxo* + GFP), *GMR-Gal*4 *UAS-Foxo/+*; *UAS-Pink*1/+ (*Foxo* + *Pink*1), *GMR-Gal*4 *UAS-Foxo/+*; *UAS-parkin/+* (*Foxo* + parkin). Error bars indicate standard error of the mean.

 

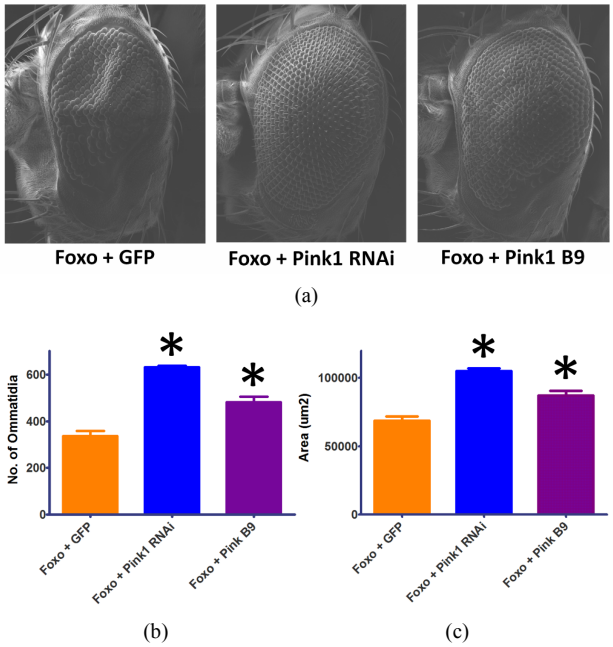


**Figure 2.** Reduction in *Pink*1 decreases the severity of the *Foxo*-induced phenotype. Overexpression of *Foxo* in the *Pink*1 mutant background (*Pink*1 B9) results in a significant increase in ommatidia number and eye area. Co-overexpression of *Foxo* with *Pink*1$^{RNAi}$ (*Pink*1 RNAi) shows significant increases in ommatidia number and area. Overexpression of *Foxo* in a *parkin* mutant background (*GMR-Gal*4 *UAS-Foxo*/+; *parkin*$^{45}$/*parkin*$^{45}$) or co-overexpression with *parkin*$^{RNAi}$ (*GMR-Gal*4 *UAS-Foxo*/+; *parkin*$^{RNAi}$/+) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal*4 *UAS-Foxo/UAS-GFP* (*Foxo* + GFP), *GMR-Gal*4 *UAS-Foxo*/+; *UAS-Pink1*$^{RNAi}$/+ (*Foxo* + *Pink*1 RNAi), *Pink1*$^{B9}$/*y*;*GMR-Gal*4 *UAS-Foxo*/+ (*Foxo* + *Pink*1 B9). Error bars indicate standard error of the mean.

in apparent synthetic lethality with no surviving progeny. This implies that the broad protective functions of *parkin* are necessary to maintain a viable organism during this development.

### 3.3. Effects of *Pink*1 and *Parkin* on the *Foxo*-Induced Phenotype are Independent of Akt Signalling

The constitutively active version of *Foxo* (*Foxo*$^{AA}$) contains an alanine substitution at the T1 (T24A) and S1 (S253A) Akt phosphorylation sites [36]. Using *Foxo*$^{AA}$, the severity of the *Foxo*-induced phenotype was seen to increase with *Pink*1 or *parkin* co-overexpression (**Figure 3**). Co-overexpression of *Pink*1 with *Foxo*$^{AA}$ results in significant decreases in number of ommatidia and eye area ($p < 0.0001$, $df = 30$). Co-overexpression of *parkin* with *Foxo*$^{AA}$ also results in significant decreases in number of ommatidia ($p = 0.0090$, $df = 29$) and eye area ($p = 0.0190$, $df = 29$). The apparent rescue of the *Foxo*-induced phenotype, seen when co-overexpressing *Foxo*

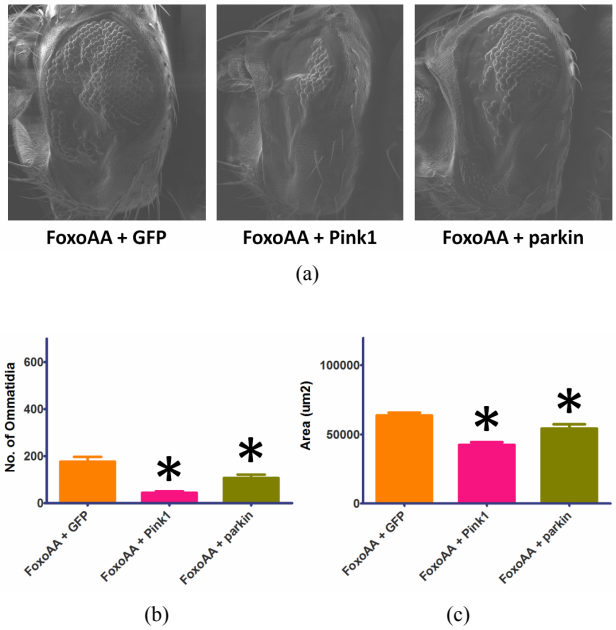


**Figure 3.** Effects of *Pink*1 and *parkin* on the *Foxo*-induced phenotype, independent of Akt signalling. Co-overexpression of *Foxo*$^{AA}$ (*Foxo*AA) with *Pink*1 results in significant decreases in number of ommatidia and eye area. Co-overexpression of *Foxo*$^{AA}$ with *parkin* results in significant decreases in number of ommatidia and eye area. Genotypes shown include *GMR-Gal*4/*UAS-GFP*;*UAS-Foxo*$^{AA}$/+ (*Foxo*AA + GFP), *GMR-Gal*4/+; *UAS-Foxo*$^{AA}$/*UAS-Pink*1 (*Foxo*AA + *Pink*1), *GMR-Gal*4/+; *UAS-Foxo*$^{AA}$/*UAS-parkin* (*Foxo*AA + parkin). Error bars indicate standard error of the mean.

with *Pink*1$^{RNAi}$ (**Figure 2**), is maintained when using the constitutively active version, *Foxo*$^{AA}$ (**Figure 4**). Co-overexpression of *Foxo*$^{AA}$ with *Pink*1$^{RNAi}$ results in a dramatic increase in ommatidia number and eye area ($p < 0.0001$, $df = 30$). These results indicate that the *Pink*1/*parkin* interaction with *Foxo* is independent of Akt signalling. In contrast, there is no significant difference in ommatidia number ($p = 0.2131$, $df = 29$) or eye area ($p = 0.8027$, $df = 29$) when *Foxo*$^{AA}$ is overexpressed in the *Pink*1$^{B9}$ mutant background (**Figure 4**). As seen with *Foxo* overexpression, co-overexpression of *Foxo*$^{AA}$ with *parkin*$^{RNAi}$ resulted in apparent synthetic lethality with no surviving progeny.

## 4. DISCUSSION

Under cell stress conditions, *Foxo* transcription factors are activated and target genes that promote cell survival and/or apoptosis [27,28]. The transactivation of *Pink*1 by *Foxo* [24,25] suggests that there may be recruitment of the *Pink*1/*parkin* pathway to help maintain mitochondrial homeostasis during cell stress. Acting in this protective role, we hypothesized that *Pink*1 and *parkin* may alleviate the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. This would presumably be

 

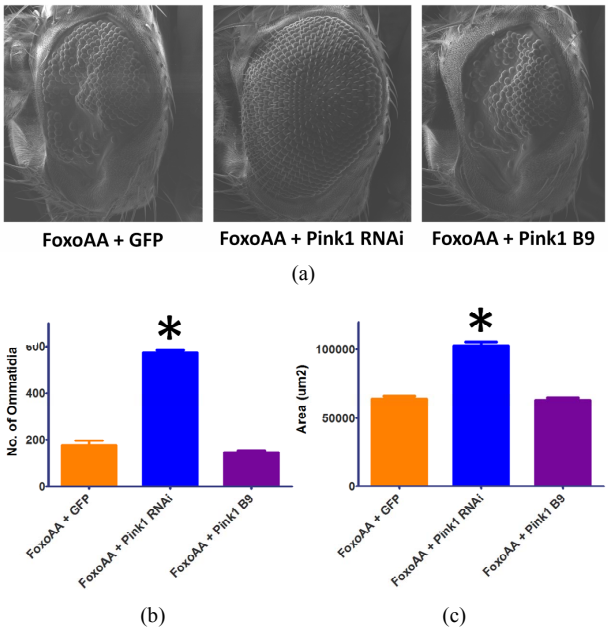


**Figure 4.** Effects of reductions in *Pink*1 on the *Foxo*-induced phenotype, independent of Akt signalling. Co-overexpression of $Foxo^{AA}$ (*Foxo*AA) with $Pink1^{RNAi}$ (*Pink*1 RNAi) results in a dramatic increase in ommatidia number and eye area. Overexpression of $Foxo^{AA}$ in the *Pink*1 mutant background (*Pink*1 B9) is not significantly different from the control. Co-overexpression of $Foxo^{AA}$ with with $parkin^{RNAi}$ (*GMR-Gal4*/+; *UAS-*$Foxo^{AA}$/$parkin^{RNAi}$/+) resulted in apparent synthetic lethality. Genotypes shown include *GMR-Gal4/UAS-GFP*; *UAS-*$Foxo^{AA}$/+ (*Foxo*AA + GFP), *GMR-Gal4*/+; *UAS-*$Foxo^{AA}$*/UAS-*$Pink1^{RNAi}$ (*Foxo*AA + *Pink*1 RNAi), $Pink1^{B9}$/*y*; *GMR-Gal4*/+; *UAS-*$Foxo^{AA}$/ + (*Foxo*AA + *Pink*1 B9). Error bars indicate standard error of the mean.

due to the regulation of mitochondrial fission/fusion events and through mitophagy to degrade dysfunctional mitochondrial fragments, maintaining the overall mitochondria health of the cell [1,9]. In contrast, our results indicate that the *Pink*1/*parkin* pathway may be involved in aspects of cell fate other than protection. Our findings show that co-overexpression of *Pink*1 or *parkin* results in an increased severity of the *Foxo*-induced phenotype, and that a reduction in *Pink*1 is able to improve on the phenotype. This suggests that there may be a more complex role for the *Pink*1/*parkin* pathway under cell stress conditions.

Many transcriptional targets of *Foxo* have been identified, including molecules involved in metabolism, oxidative stress resistance, cell cycle arrest and apoptosis [27,28]. The *Foxo*-induced phenotype of developmental defects in the Drosophila eye is likely due to the transcription of pro-apoptotic gene targets. Drosophila studies link phosphorylation of *Foxo* to neurodegeneration, and have identified the pro-apoptotic *Hid* gene as one responsible target, where overexpression of *Hid* causes dramatic eye degeneration [29,30,37]. In contrast, *Foxo* has also been shown to prevent mitochondrial dysfunction and neurodegeneration, and is proposed to function downstream of *Pink*1 [26]. In this protective role, *Foxo* is thought to act through targets including the mitochondrial superoxide dismutase *SOD*2, a gene involved in stress resistance. With the ability to promote cell survival or apoptosis, changes in *Foxo* activity may be the mechanism behind the effects of *Pink*1 and *parkin* on the *Foxo*-induced phenotype. Co-overexpression of *Pink*1 or *parkin* may affect *Foxo* activity to increase the transcription of pro-apoptotic targets, thus increasing the severity of the phenotype. Improvement of the *Foxo*-induced phenotype seen with reductions in *Pink*1 may also indicate a change in *Foxo* activity, suggesting that the relationship between *Pink*1 and *Foxo* is more complex than *Pink*1 indirectly activating *Foxo* downstream. It is also possible that *Pink*1 and *parkin* are acting outside of the INR pathway to affect cell survival. The influence of *Pink*1 and *parkin* on mitofission/mitofusion events is not fully understood, and some mechanisms involved are closely tied to apoptosis. It may be that under certain conditions these mechanisms are utilized to promote apoptosis instead of cell protection. One example would be the ubiquitination of VDAC by *parkin* [14]. VDAC is a major component of the permeability transition pore (PTP), and is involved with mitochondrial outer membrane permeabilization (MOMP) through interactions with pro-apoptotic Bcl-2 proteins [38]. With both the PTP and MOMP implicated as initiators of apoptosis, mitofission events triggered by ubiquitination of VDAC must be controlled so to prevent release of apoptotic factors from the mitochondria. Compounding factors, such as the effects of Hid in the *Foxo*-induced phenotype, may result in overwhelming instability during increases in *Pink*1 or *parkin* expression, making this degree of control impossible. In this instance, *Pink*1 and *parkin* may actively participate in the initiation of apoptosis, a novel role for the *Pink*1/*parkin* pathway that warrants further investigation.

Expression of the constitutively active version of *Foxo* ($Foxo^{AA}$) with co-overexpression of *Pink*1, *parkin* or $Pink1^{RNAi}$ seems to indicate that the *Pink*1/*parkin* effect on the *Foxo*-induced phenotype is independent of Akt signalling, supporting the idea that *Pink*1 and *parkin* may be acting outside of the INR pathway. In contrast, the change in significance when expressing $Foxo^{AA}$ in the $Pink1^{B9}$ mutant background suggests that there is Akt involvement. Interestingly, this may indicate that there is a role for *Pink*1 in the cell that is independent of its kinase function, and that this additional role is somehow involved in the Akt signalling pathway. In this respect, the apparent rescuing effect of the *Foxo*-induced phenotype during decreases in *Pink*1 expression would be partially due to the decrease in kinase activity, and partially

 

due to the presence of the *Pink*1 protein. Future studies looking into an additional role for *Pink*1, apart from its kinase function, may yield new interactions and targets in the *Pink*1/*parkin* pathway.

## 5. CONCLUSION

In conclusion, our results show that *Pink*1 and *parkin* are able to increase the effects of *Foxo* in Drosophila, highlighting a possible role for the *Pink*1/*parkin* pathway in cell death. In addition, the constitutively active version of *Foxo* allows us to exclude a general requirement for Akt during increased expression of *Pink*1 or *parkin,* however, suggests that there may be an additional role for *Pink*1 apart from its kinase function. Further studies looking at the effect of *Pink*1 and/or *parkin* on *Foxo* activity, and the role of the *Pink*1/*parkin* pathway in mitochondrial fission/fusion events, may uncover underlying mechanisms that mediate a shift towards apoptosis. Moreover, it is likely that the *Pink*1/*parkin* pathway is involved in various aspects of cell fate decisions, contrary to the view that *Pink*1 and *parkin* act exclusively as protective proteins.

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