**Pink1** and *parkin* demonstrate multifaceted roles when co-expressed with *Foxo*

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ABSTRACT

*Pink1* has been linked to both autosomal recessive and sporadic forms of Parkinson disease. The *Pink1* protein is thought to be involved in mitochondrial protection by interacting with *parkin* to prevent oxidative damage, maintain mitochondrial integrity and regulate mitophagy. *Pink1* 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 *Pink1/parkin*, we have performed co-expression studies to determine the effects *Pink1* and *parkin* on the *Foxo*-induced phenotype of developmental defects in the Drosophila eye. We examined directed expression of *Pink1*, *parkin*, *Pink1* or *parkin* mutants, and *Pink1* or *parkin* interfering RNAs (RNAi) with the overexpression of *Foxo* in the developing eye of Drosophila. Our findings show that reduction of *Pink1* suppresses the effects of *Foxo* overexpression, where co-overexpression with *Pink1* or *parkin* increases the severity of the phenotype. This suggests that *Pink1* and *parkin* are able to increase the pro-apoptotic effects of *Foxo*. Contrary to the view that *Pink1* and *parkin* act exclusively as protective proteins in the cell, it is likely that the *Pink1/parkin* pathway is involved in aspects of cell fate decisions other than degrading toxic proteins and maintaining mitochondrial integrity.

**Keywords:** *Pink1; Parkin; Foxo; Drosophila*

1. INTRODUCTION

*Pink1* (*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, *Pink1* is thought to be involved in mitochondrial protection by preventing oxidative damage and maintaining mitochondrial integrity, where loss of function of *Pink1*, 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 *Pink1* in mitochondrial fission/fusion events [1,9]. This involvement implicates *Pink1* 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 *Pink1* results in the ubiquitination of various mitochondrial proteins, promoting fission and mitophagy [12-15]. In contrast, studies have found that loss of *parkin* or *Pink1* 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 *Pink1/parkin* pathway in maintaining mitochondrial homeostasis.

The *Pink1/parkin* pathway has been linked to components of the insulin receptor (INR) pathway, including interaction of PTEN with *Pink1* [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 *Pink1* by *Foxo* [24,25]. It has also been suggested that *Pink1* 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 SOD2 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]. Overexpression 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 Pink1/parkin, we have performed expression studies to determine the effects of Pink1 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, Pink1 and parkin would be capable of alleviating the detrimental effects of Foxo overexpression. In contrast, our findings show that reduction of Foxo with Pink1 or parkin results in an increased severity of the Foxo-induced phenotype. These findings suggest a complex role for the Pink1/parkin pathway in cell fate decisions.

2. MATERIALS AND METHODS

2.1. Fly Stocks and Culture

The UAS-Pink1 transgenic line was created from the GH20931 Drosophila melanogaster Pink1 clone [32]. The UAS-murine Foxo1 (UAS-Foxo) and UAS-murine Foxo1AA (UAS-FoxoAA) transgenes are described in Kramer et al. [31] and the GMR-Gal4 UAS-Foxo and GMR-Gal4;UAS-FoxoAA lines were established through standard means. UAS-parkin was created previously in our laboratory [33]. The Pink1mutant line was provided by Dr. J. Chung [6]. The UAS-Pink1RNAi and UAS-parkinRNAi lines were provided by Dr. B. Lu [7,34]. The UAS-GFP control was obtained from the Bloomington stock centre. The parkinmutant 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 Pink1 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 Pink1 Decreases the Severity of the Foxo-Induced Phenotype

Overexpression of Foxo in a Pink1 mutant background (Pink1) 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 Pink1RNAi 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 Pink1 during eye development is able to alleviate the detrimental effects of Foxo. Overexpression of Foxo in a parkin mutant background (parkin) or co-overexpression with parkinRNAi resulted...
3.3. Effects of Pink1 and Parkin on the Foxo-Induced Phenotype are Independent of Akt Signalling

The constitutively active version of Foxo (Foxo) contains an alanine substitution at the T1 (T24A) and S1 (S253A) Akt phosphorylation sites [36]. Using Foxo, the severity of the Foxo-induced phenotype was seen to increase with Pink1 or parkin co-overexpression (Figure 3). Co-overexpression of Pink1 with Foxo results in significant decreases in number of ommatidia and eye area (p < 0.0001, df = 30). Co-overexpression of parkin with Foxo 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 with Pink1RNAI (Figure 2), is maintained when using the constitutively active version, Foxo (Figure 4). Co-overexpression of Foxo with Pink1RNAI results in a dramatic increase in ommatidia number and eye area (p < 0.0001, df = 30). These results indicate that the Pink1/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 is overexpressed in the Pink1 mutant background (Figure 4). As seen with Foxo overexpression, co-overexpression of Foxo with parkinRNAI 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 Pink1 by Foxo [24,25] suggests that there may be recruitment of the Pink1/parkin pathway to help maintain mitochondrial homeostasis during cell stress. Acting in this protective role, we hypothesized that Pink1 and parkin may alleviate the Foxo-induced phenotype of developmental defects in the Drosophila eye. This would presumably be
has also been shown to prevent mitochondrial dysfunction and neurodegeneration, and is proposed to function downstream of Pink1 [26]. In this protective role, Foxo is thought to act through targets including the mitochondrial superoxide dismutase SOD2, 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 Pink1 and parkin on the Foxo-induced phenotype. Co-overexpression of Pink1 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 Pink1 may also indicate a change in Foxo activity, suggesting that the relationship between Pink1 and Foxo is more complex than Pink1 indirectly activating Foxo downstream. It is also possible that Pink1 and parkin are acting outside of the INR pathway to affect cell survival. The influence of Pink1 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 Pink1 or parkin expression, making this degree of control impossible. In this instance, Pink1 and parkin may actively participate in the initiation of apoptosis, a novel role for the Pink1/parkin pathway that warrants further investigation.

Expression of the constitutively active version of Foxo (FoxoAA) with co-overexpression of Pink1, parkin or Pink1RNAi seems to indicate that the Pink1/parkin effect on the Foxo-induced phenotype is independent of Akt signalling, supporting the idea that Pink1 and parkin may be acting outside of the INR pathway. In contrast, the change in significance when expressing FoxoAA in the Pink1B9 mutant background suggests that there is Akt involvement. Interestingly, this may indicate that there is a role for Pink1 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 Pink1 expression would be partially due to the decrease in kinase activity, and partially

due to the regulation of mitochondrial fission/fusion events and through mitophagy to degrade dysfunctional mitochondrial fragments, maintaining the overall mitochondrial health of the cell [1,9]. In contrast, our results indicate that the Pink1/parkin pathway may be involved in aspects of cell fate other than protection. Our findings show that co-overexpression of Pink1 or parkin results in an increased severity of the Foxo-induced phenotype, and that a reduction in Pink1 is able to improve on the phenotype. This suggests that there may be a more complex role for the Pink1/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

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**Figure 4.** Effects of reductions in Pink1 on the Foxo-induced phenotype. Co-overexpression of FoxoAA (FoxoAA) with Pink1(RNAi) (Pink1 RNAi) results in a dramatic increase in ommatidia number and eye area. Overexpression of FoxoAA in the Pink1 mutant background (Pink1 B9) is not significantly different from the control. Co-overexpression of FoxoAA with with parkin(RNAi) (GMR-Gal4/+; UAS-FoxoAA/parkinRNAi/+), Pink1 and/or FoxoAA + GFP, GMR-Gal4/+; UAS-FoxoAA/UAS-ParkinRNAi (FoxoAA + Pink1 RNAi), Pink1RNAi, GMR-Gal4+/+; UAS-FoxoAA+/+ (FoxoAA + Pink1 B9). Error bars indicate standard error of the mean.

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due to the presence of the Pink1 protein. Future studies looking into an additional role for Pink1, apart from its kinase function, may yield new interactions and targets in the Pink1/parkin pathway.

5. CONCLUSION

In conclusion, our results show that Pink1 and parkin are able to increase the effects of Foxo in Drosophila, highlighting a possible role for the Pink1/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 Pink1 or parkin, however, suggests that there may be an additional role for Pink1 apart from its kinase function. Further studies looking at the effect of Pink1 and/or parkin on Foxo activity, and the role of the Pink1/parkin pathway in mitochondrial fission/fusion events, may uncover underlying mechanisms that mediate a shift towards apoptosis. Moreover, it is likely that the Pink1/parkin pathway is involved in various aspects of cell fate decisions, contrary to the view that Pink1 and parkin act exclusively as protective proteins.

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