Altered expression of *CG5961*, a putative *Drosophila melanogaster* homologue of *FBXO9*, provides a new model of Parkinson disease

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Received February 24, 2016
Accepted March 8, 2016
Published May 6, 2016
DOI http://dx.doi.org/10.4238/gmr.15028579

ABSTRACT. F-box proteins act as the protein recognition component of the Skp-Cul-F-box class of ubiquitin ligases. Two members of a gene sub-family encoding these proteins, *FBXO7* and *FBXO32*, have been implicated in the onset and progression of degenerative disease. *FBXO7* is responsible for rare genetic forms of Parkinson disease, while *FBXO32* has been implicated in muscle wasting. The third gene in this family, *FBXO9*, is related to growth signaling, but the role of this gene in degenerative disease pathways has not been thoroughly investigated. Characterizing the putative *Drosophila melanogaster* homologue of this gene, *CG5961*, enables modeling and analysis of the consequence of targeted alteration of gene function and the effects on the overall health of the organism. Comparison of the protein domains of *Homo sapiens* *FBXO9* and the putative *D. melanogaster* homologue *CG5961* revealed a high degree of conservation between the protein domains. Directed expression of *CG5961* (via *CG5961* EP) and inhibition of *CG5961* (through a stable RNAi transgene) in the developing *D. melanogaster* eye caused abnormalities in adult structures (ommatidia and inter-ommatidial bristles). Directed expression of either *CG5961*...
or CG5961-RNAi in the dopaminergic neurons led to a reduced lifespan compared to that in lacZ controls. We showed that protein structures of CG5961 and FBXO9 are highly similar and studied the effects of altered expression of CG5961 in neuron-rich tissues. Our results suggest that CG5961 activity is necessary for the proper formation of neuronal tissue and that targeted alteration of gene expression in dopaminergic neurons leads to a reduced lifespan.

**Key words:** CG5961; Drosophila melanogaster; E3 ubiquitin ligase; FBXO9; Neurodegeneration; Parkinson disease

**INTRODUCTION**

Progressive and continuous loss of function of neurons in specific areas of the brain can lead to neurodegenerative disorders such as Parkinson disease (PD) and Alzheimer disease (AD) (Xie et al., 2014). Neuronal dysfunction can be caused by environmental factors, such as exposure to pesticides and neurotoxic metals, or by the inheritance of disease-causing alleles in specific genes (Johnson, 1991; Chin-Chan et al., 2015). Although either environmental or genetic influences can independently lead to these diseases, in many cases a combination of genetic risk factors and toxin exposure determines the disease onset (Kalia and Lang, 2015). The identification and characterization of genes responsible for inherited forms of neurodegenerative disease may lead to new therapeutic approaches and novel treatments to slow and potentially prevent disease progression, regardless of the underlying cause.

PD and AD, the two most prevalent human neurodegenerative disorders, may result from protein accumulation. A hallmark of PD is the presence of Lewy bodies, which are intracellular cytoplasmic inclusions composed of clusters of α-synuclein (α-SYN) produced by altered expression of SNCA (Olanow and McNaught, 2011; Todd and Staveley, 2012). In the conventional model of AD, the presence of Tau inclusions, which are hyper-phosphorylated clusters of the microtubule stabilizing protein TAU, and an over-abundance of the amyloid precursor protein cause pre-mature neuronal death and disease symptoms (Wright et al., 1991; Goedert et al., 1995). Although these proteins were originally implicated in the pathogenesis of either PD or AD, aberrant activity of α-SYN and Tau products has been observed in both conditions. α-SYN has been implicated in the pathogenesis of AD; it is thought to play a role in abnormal synapse formation, while high levels of TAU in cerebral spinal fluid have been used as a powerful diagnostic tool for pre-screening of PD (Kim et al., 2004; Kang et al., 2013). This suggests that the mechanism of neuronal destruction and disease may be somewhat conserved between PD and AD. Aberrant protein clusters generated by mutant forms of α-SYN and TAU prevent normal cellular function and increase the oxidative stress level in the cell, eventually leading to pre-mature mortality (Atkin and Paulson, 2014). Although the cell is equipped with mechanisms to prevent permanent damage associated with aberrant protein function, defects in intracellular protein turnover have been associated with neurodegenerative disease.

The ubiquitin proteasome system is one of the basic cellular mechanisms devoted to the degradation of intracellular components in a tightly regulated and highly specific manner (Glickman and Ciechanover, 2002). This complex selectively targets proteins within the cell and adds a single or multiple ubiquitin moieties, which in turn either alters the function of the protein or marks it for future destruction by the 26S proteasome (Chau et al., 1989; Zeng et
This system functions through the activity of three enzymes: 1) the E1, activating enzyme; 2) the E2 conjugating enzyme; and 3) the E3 ligating enzyme (Ciechanover et al., 2000). Mutation of E3 enzymes has been suggested as a cause of disease pathology (Bielskiene et al., 2015). A sub-class of E3 enzymes contains unique proteins known as F-box proteins. These proteins contain a conserved F-box domain used to bind to the Skp-Cullin complex, creating the Skp-Cullin-F-box (SCF) ubiquitin ligase complex (Cardozo and Pagano, 2004). The SCF ubiquitin ligase complex is integral to proper cellular health and protein turnover with impaired function, leading to premature cell death and disease phenotypes (Genschik et al., 2013). Current research into new therapeutics has focused on emulating or mimicking the activity of the E3 components of these complexes to stimulate the removal of harmful proteins from the cell (Bulatov and Ciulli, 2015). The targeting function of the F-box proteins is an essential aspect of the SCF ubiquitin ligase complex.

In particular, the FBXO group of proteins is of interest in the study of disease, as a number of these are involved in essential biological processes and have been implicated as causative factors in disease onset and progression. Two of these FBXO genes have been directly implicated in degenerative disorders: FBXO7, also known as PARK15, has been linked to a severe early onset form of PD known as Parkinson-Pyramidal syndrome (Di Fonzo et al., 2009), while FBXO32, or atrogin, has been linked to muscle wasting phenotypes (de Palma et al., 2008). Both FBXO7 and FBXO32 belong to the same evolutionary sub-family along with another gene, FBXO9, which has not been well-characterized (Cenciarelli et al., 1999). Understanding the role of FBXO9 in the ubiquitination pathway may lead to a better understanding of various disease phenotypes.

The Drosophila melanogaster homologue of FBXO7/nutcracker (ntc) was originally identified as a factor involved in the terminal differentiation of sperm with ntc mutations causing male sterility (Bader et al., 2010). The highly conserved F-box binding domain in FBXO7 is conserved in ntc, and altered expression of ntc in both the neuron-rich D. melanogaster eye and dopaminergic neurons has been found to lead to degenerative, PD-like phenotypes, mimicking the symptoms observed with altered FBXO7 function in Homo sapiens (MerzettiEM, Dolomount LA and Staveley BE, unpublished results). The high degree of functional conservation between FBXO7 in humans and Drosophila suggests that FBXO9 may also have a functional homologue in D. melanogaster. We have attempted to identify and characterize the putative fly homologue of FBXO9 and found that altered gene expression can influence eye development and reduce longevity when expressed in the dopaminergic neurons.

**MATERIAL AND METHODS**

**Media**

The standard cornmeal-yeast-molasses-agar medium was composed of 65 g/L cornmeal, 10 g/L nutritional yeast, and 5.5 g/L agar supplemented with 50 mL/L Crosby’s fancy-grade molasses and 5 mL 0.1 g/mL methyl 4-hydroxybenzoate in 95% ethanol and 2.5 mL propionic acid in standard plastic vials. Media was stored at 4°C but warmed to room temperature before use.

**Transgenic lines**

The Ddc-Gal4^HL4.3D (Ddc-Gal4) line was a generous gift from Dr. Jay Hirsh (University
of Virginia) (Lin et al., 2000). The following lines were obtained from the Bloomington Drosophila Stock Center at Indiana University-Bloomington: 1) to drive expression behind the morphogenetic furrow in the developing eye disc Glass Multiple Reporter-Gal4 (GMR-Gal4; Freeman, 1996), stock number 1104; 2) to act as a control for the ectopic expression of transgenes UAS-lacZ (UAS-lacZ; (Brand and Perrimon, 1993)), stock number 1776; 3) to alter the expression of CG5961, P{CG5961G4347} (CG5961EP) stock number 30076 and P{TRiP.JF01332}attP2 [CG5961-JF01332]CG5961-RNAi stock number 27390.

Scanning electron microscopy of D. melanogaster eyes

Female virgins of GMR-Gal4 were mated with UAS-lacZ, CG5961EP, and CG5961-RNAi males. Male progeny of each cross were collected, aged for 3-5 days, and frozen at -80°C. Flies were mounted to metal stubs and desiccated for 24 h pre-imaging. The eyes of mounted flies were imaged via scanning electron microscopy at 130X magnification with a Mineral Liberation Analyzer 650F scanning electron microscope (FEI, Hillsboro, OR, USA). Total bristle count and ommatidia count were obtained using ImageJ software (Schneider et al., 2012) (NIH, Bethesda, MD, USA).

Ageing analysis

Female virgins of the Ddc-Gal4 line were mated with UAS-lacZ, CG5961EP, and CG5961-RNAi males. Male progeny of each cross were collected each day and placed into separate vials containing no more than 20 flies. Vials were scored for survival every 2 days and the media was changed after each death event or every 3 days. Survival curves were compared by the log-rank (Mantel Cox) test and graphs were created using GraphPad Prism software (La Jolla, CA, USA).

RESULTS

FBXO9 belongs to a sub-family of genes responsible for target specificity in ubiquitin ligase complexes along with the well-established Parkinson gene FBXO7/PARK15. Phylogenetic analysis showed that evolution between the FBXO orthologues is consistent among species (Figure 1A). Although divergent evolution has occurred, the functional domains and conserved residues were well-conserved between both FBXO9 and FBXO7, suggesting that both genes are important in organism survival. A bioinformatics search (tBLASTn) using human FBXO9 as a query identified a single putative D. melanogaster homologue, CG5961. The overall similarity between the two proteins was 33%; however, the level of conservation within the protein functional domains was greater. The human FBXO9 protein is 437 amino acids in length, while the putative Drosophila protein is 442 amino acids. Each of the two proteins shares 4 distinct domains: a microtubule interacting and trafficking domain (MIT), a tetratricopeptide repeat sequence, an F-box domain, and a carboxyl terminal nuclear localization sequence (Figure 1B). Of these domains, the highest degree of similarity was observed in the F-box domain, which is 51 amino acids in length with 33 absolutely conserved amino acid residues and 12 well-conserved functionally similar amino acid residues (Figure 1C). However, human FBXO9 contains an HNH nuclease family domain that we were unable to identify in the D. melanogaster protein (Jin et al., 2004). The high degree of
similarity between the conserved active domains suggests that CG5961 is the D. melanogaster homologue of FBXO9.

The D. melanogaster eye has been used to determine the neurodevelopmental effects of gene alteration, as both ommatidia and bristles, the two main tissues composing the eye, are of neuronal origin (Cook et al., 2011). To determine the broad-scale neuronal implications of altered CG5961 activity, we directed the expression of CG5961 (via CG5961	extsuperscript{EP}) and the
inhibition of CG5961 (via a CG5961-RNAi transgene) using the GMR-Gal4 transgene in the developing D. melanogaster eye. These experiments were conducted at both at 25° and 29°C, with the former acting as the standard physiological condition and the latter used to elevate the activity of Gal4-UAS system expression. Tissue-specific expression of CG5961 caused a significant decrease in both ommatidia and bristle number compared to lacZ controls at 25°C (Figure 2). Directed inhibition achieved by expressing the CG5961-RNAi construct caused a similar loss of ommatidia and bristle number compared to lacZ controls at 25°C. Thus, CG5961 is tightly regulated in the neurons composing the D. melanogaster eye and changes in expression have a significant negative effect.

To determine whether altered CG5961 expression in neuronal cells is directly linked to neurodegenerative disease, both the CG5961EP and a CG5961-RNAi transgenes were expressed in dopaminergic neurons under control of the Ddc-Gal4 transgene. Since dopaminergic neurons form dopamine, which is a neurotransmitter present in low abundance in cases of PD, a gene that negatively impacts overall organism survival when expressed solely in dopaminergic neurons would likely lead to PD-like phenotypes. Dopaminergic-specific expression of CG5961EP significantly decreased the mean lifespan compared to lacZ controls (Figure 3). Fifty percent of the CG5961EP flies died by day 48, while the control lacZ flies did not reach 50% survival until day 60. This is a significant decrease in lifespan of approximately

Figure 2. Altered tissue specific expression of CG5961 in the Drosophila melanogaster eye causes a decrease in ommatidia and bristle number. A. Scanning electron micrographs of D. melanogaster eyes taken at a horizontal field width of 500 µm. Genotypes are as follows: I) GMR-Gal4 / UAS-lacZ 25°C; II) GMR-Gal4 / UAS-lacZ 29°C; III) GMR-Gal4 / CG5961EP 25°C; IV) GMR-Gal4 / CG5961EP 29°C; V) GMR-Gal4 / CG5961-RNAi 25°C; VI) GMR-Gal4 / CG5961-RNAi 29°C. Images were taken with a FEI MLA 650. B. D. melanogaster eye shows a decrease in number of ommatidia and bristles when either CG5961EP or a CG5961-RNAi transgene is under the control of GMR-Gal4. This decrease in tissue formation is more severe at the elevated temperature of 29°C likely due to the increased efficiency of the Gal4-UAS system. Comparisons were measured using a one-way ANOVA and significance was tested using a Tukey post-hoc test, N = 10. *P < 0.05, **P < 0.01, ***P < 0.001.
Expression of CG5961-RNAi in dopaminergic neurons caused an even greater decrease in longevity (44 days) compared to lacZ controls of approximately 25% (Figure 3). This decrease was slightly more severe than that observed with the expression of CG5961EP and indicates that in dopaminergic neurons, much like in the D. melanogaster eye, CG5961 is tightly regulated. Altered expression of this gene has severe negative consequences.

DISCUSSION

The four protein domains conserved between human FBXO9 and the putative D. melanogaster homologue provide insight into the function of this gene. The highly conserved F-box domain is present in each of the D. melanogaster homologues of the FBXO family of F-box proteins including FBXO7 and FBXO32 and has been found to be responsible for mediating the protein-protein interaction between the F-box protein and the SCF ubiquitin ligase complex (Cardozo and Pagano, 2004). The microtubule interacting and trafficking domain has been implicated in endosomal trafficking and microtubule movement (Ciccarelli et al., 2003). Current knowledge of MIT domains focuses on the endosomal trafficking ATPase Vps4, which is activated by the direct binding of an additional protein known as the MIT-interacting motif (Hurley and Yang, 2008). The tetratricopeptide repeat domain is a 34-amino acid sequence motif found in a number of diverse proteins responsible for scaffold formation for protein-protein interactions (Blatch and Lässle, 1999). The structure of TPR closely resembles that of MIT, suggesting that both play a role in protein-protein interactions within the cell, a role suited to the targeting function associated with other FBXO proteins. The nuclear localization sequence tags a protein for import into the nucleus, which suggests that FBXO9 activity may be localized to this location (Marfori et al., 2011). Taken together, the presence of these conserved domains indicates that human FBXO9 and the putative homologue
CG5961 function as components of a multi-component SCF ubiquitin ligase complex.

The ommatidia and bristles in the D. melanogaster eye are formed from neuronal precursors and allow for highly sensitive characterization of mutations that alter neural developmental function (Sang and Jackson, 2005). Specific altered expression of CG5961 in the neuron-rich D. melanogaster eye leads to the disruption of tissue formation and decreased ommatidia and bristle numbers. Expression of CG5961 appears to be highly regulated in the neurons of the eye, and deviation of this expression leads to severe phenotypes. Although there have been no previous studies evaluating the effects of altered CG5961 expression in the D. melanogaster eye, CG5961 appears to be necessary for proper neuronal cell development.

Dopaminergic neurons are essential in the production of dopamine, a neurotransmitter critical for motor control. Impaired dopamine production resulting from improper neuron functioning leads to severe defects and premature mortality. To determine whether the observed negative phenotype associated with driven expression of CG5961 in the eye was conserved in disease-associated neuronal tissues, we evaluated the effects of altered expression in dopaminergic neurons to determine the consequences on the overall lifespan of D. melanogaster. Directed dopaminergic expression of either CG5961 or CG5961-RNAi leads to a significant decrease in overall lifespan compared to lacZ controls. This decrease in lifespan indicates that CG5961 is essential for the proper function of dopaminergic neurons. Furthermore, CG5961 is regulated in a highly controlled manner. Mutations in both FBXO7 and FBXO32 have been linked to human disease phenotypes, with altered expression of FBXO7 causing a PD-like phenotype in D. melanogaster (MerzettiEM, Dolomount LA and Staveley BE, unpublished results). A decrease in longevity in response to altered expression of the D. melanogaster homologue of FBXO9 produced in dopaminergic neurons suggests a link between the human FBXO9 gene and human degenerative disease.

The FBXO family of genes has been implicated in a number of inherited genetic disorders. Although FBXO7 and FBXO32 were found to participate in the processes of neurodegeneration and muscular degeneration, respectively, a closely related gene, FBXO9, has not been well-characterized. We show that D. melanogaster CG5961 is highly similar to Homo sapiens FBXO9 and that altered expression of this gene leads to degenerative phenotypes in both the eye and dopaminergic neurons of flies. This is the first study demonstrating a potential link between FBXO9 and degenerative disease phenotypes and shows that the putative D. melanogaster homologue of FBXO9 may be important in neuronal development and function. Further research into the pathways and interacting proteins involved in FBXO9-mediated proteolytic activity may lead to new targets for disease prevention and therapeutic strategies.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

E.M. Merzetti was partially funded by Department of Biology Teaching Assistantships and a School of Graduate Studies Fellowship from Memorial University of Newfoundland. BES received research support from the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant.
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