C. elegans Model Identifies Genetic Modifiers of α-Synuclein Inclusion Formation During Aging

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Abstract

Inclusions in the brain containing α-synuclein are the pathological hallmark of Parkinson’s disease, but how these inclusions are formed and how this links to disease is poorly understood. We have developed a C. elegans model that makes it possible to monitor, in living animals, the formation of α-synuclein inclusions. In worms of old age, inclusions contain aggregated α-synuclein, resembling a critical pathological feature. We used genome-wide RNA interference to identify processes involved in inclusion formation, and identified 80 genes that, when knocked down, resulted in a premature increase in the number of inclusions. Quality control and vesicle-trafficking genes expressed in the ER/Golgi complex and vesicular compartments were overrepresented, indicating a specific role for these processes in α-synuclein inclusion formation. Suppressors include aging-associated genes, such as sir-2.1/SIRT1 and laq-1/LASS2. Altogether, our data suggest a link between α-synuclein inclusion formation and cellular aging, likely through an endomembrane-related mechanism. The processes and genes identified here present a framework for further study of the disease mechanism and provide candidate susceptibility genes and drug targets for Parkinson’s disease and other α-synuclein related disorders.

Introduction

Sporadic as well as familial Parkinson’s disease are characterized by protein inclusions in the brain containing α-synuclein [1]. Similar inclusions are also present in other neurodegenerative diseases, including dementia with Lewy bodies [2]. The α-synuclein gene is causatively related to Parkinson’s disease, since mutations in the gene, and duplication or triplication of the α-synuclein locus cause familial forms of Parkinson’s disease in humans [3–5]. Sporadic Parkinson’s disease, seen in 1–4% of the population over 65 years of age, appears to be unrelated to mutations or multiplications of the α-synuclein locus. How α-synuclein inclusions are produced is unknown, but identifying cellular factors and processes involved in the formation of these inclusions may provide some understanding of the molecular cause of Parkinson’s disease and of the link between aging and the sporadic form of the disease. To study pathological α-synuclein accumulation, we used a genetic model organism, the nematode Caenorhabditis elegans. We chose C. elegans for its thoroughly characterized aging properties, its amenability to genome-wide RNAi screening, and its transparency throughout its lifetime, which allows visualization of inclusions in living animals during aging. We expressed human α-synuclein fused to yellow fluorescent protein (YFP) in C. elegans under control of the unc-54 promoter, which drives expression to the body wall muscle cells. Muscle expression rather than neuronal expression was chosen for several reasons. The unc-54 promoter is strong and muscle cells are large, allowing for visual detection of α-synuclein expression and its subcellular localization. Furthermore, RNAi by feeding seems to work more efficiently in muscles than in neurons, which better allows for genome-wide RNAi screening. Finally, muscle expression has been used successfully to model protein-misfolding diseases and to identify modifier genes in previous studies [6–8]. The α-synuclein-YFP chimaeric protein is recognized by an antibody specific for human α-synuclein and an antibody for YFP (Figure 1B). YFP fusion to human α-synuclein relocates to inclusions (Figure 1A), which are visible as early as day 2 after hatching and increase in number and size during the animals’ aging up to late adulthood. As YFP alone remains diffusely localized throughout aging, this indicates that relocation of α-synuclein-YFP into foci is caused by intrinsic properties of the α-synuclein protein.

Results/Discussion

To visually trace expression of α-synuclein, we expressed human α-synuclein fused to yellow fluorescent protein (YFP) in C. elegans under control of the unc-54 promoter, which drives expression to the body wall muscle cells. Muscle expression rather than neuronal expression was chosen for several reasons. The unc-54 promoter is strong and muscle cells are large, allowing for visual detection of α-synuclein expression and its subcellular localization. Furthermore, RNAi by feeding seems to work more efficiently in muscles than in neurons, which better allows for genome-wide RNAi screening. Finally, muscle expression has been used successfully to model protein-misfolding diseases and to identify modifier genes in previous studies [6–8]. The α-synuclein-YFP chimaeric protein is recognized by an antibody specific for human α-synuclein and an antibody for YFP (Figure 1B). YFP fusion to human α-synuclein relocates to inclusions (Figure 1A), which are visible as early as day 2 after hatching and increase in number and size during the animals’ aging up to late adulthood. As YFP alone remains diffusely localized throughout aging, this indicates that relocation of α-synuclein-YFP into foci is caused by intrinsic properties of the α-synuclein protein.

One of the characteristics of late inclusions in the brains of Parkinson’s patients is the presence of electron-dense filamentous and granular protein material, which is typical for aggregated protein [9]. To address whether α-synuclein was aggregated within the inclusions in our C. elegans model, we measured the mobility of the α-synuclein-YFP chimaera by fluorescence recovery after
We observed two types of inclusions. One type contained mostly mobile material (Figure 2P–2T, 2W; 80% fluorescence recovery), whereas the other type contained immobilized material (Figure 2K-2O, 2X; 40% fluorescence recovery), similar to Q40-YFP aggregates (Figure 2F-2J, V; 30% fluorescence recovery), indicating aggregated protein, a characteristic of α-synuclein deposits in Parkinson’s disease.

Notably, there was an increase in the number of “immobile” inclusions relative to “mobile” inclusions during aging (Figure 2Y and 2Z), which appeared unrelated to the expression level of α-synuclein-YFP (Figure 1C). Interestingly, immobile inclusions were not observed before late adulthood, which is consistent with age-related aggregation in Parkinson’s disease patients [11]. We noted that the motility of the mobile material contained in inclusions was similar at all ages, suggesting that aggregation was not a gradual process but caused by a sharp transition from mobile to immobile material (data not shown). Taken together, the time-dependent accumulation and immobilization of α-synuclein in inclusions verify the suitability of our α-synuclein-YFP C. elegans system as a model for human Parkinson’s disease.

To identify processes involved in inclusion formation we searched for genes that, when inactivated, increased the amount of inclusions using a genome-wide RNAi screen. Worms were screened by visual inspection using fluorescence microscopy at days 4 and 5 after transfer of synchronized, first stage (L1) larvae to RNAi clones. Clones were scored positive when at least five out of the first ten worms screened showed an increase in the amount of inclusions (Figure 3). Genes for which RNAi results were confirmed in duplicate, in liquid culture and on agar plates, were considered suppressors of inclusion formation.

In this screen we found 80 suppressors of inclusion formation (Table 1). Forty-nine of these genes have an established human ortholog (Blast E-value ≤2.5e-5), indicating involvement of these genes in molecular pathways conserved between humans and nematodes (Table S1 online). The effect of RNAi was confirmed in genetic deletion strains for three genes: sir-2.1, an NAD+-dependent protein deacetylase, lagr-1, a sphingolipid synthase, and ymel-1, a mitochondrial protease, which is an ortholog of the human presenilin associated metalloprotease (PAMP) (Figure 4).

The modifier genes function in a variety of biological processes, some of which have previously been suggested to be involved in Parkinson’s disease, such as vesicular transport and lipid...
metabolism. Lipid metabolism, lipid membranes, and vesicle-mediated transport have previously been linked to α-synuclein pathology in a yeast model [12,13]. In Parkinson’s patients, lipids and membrane material have been found to be directly associated with α-synuclein in lipid droplets and Lewy bodies [11,14–16].

Although we did identify a C. elegans ortholog of the recently discovered modifier of neuronal alpha-synuclein toxicity SIRT2, we did not pick up two other modifiers of neuronal alpha-synuclein pathology: the G-protein coupled receptor kinase 2 and the molecular chaperone Hsp70 [17–19]. In Drosophila, overexpression of G-protein coupled receptor kinase 2 (Gprk2) increases neuronal toxicity of α-synuclein [18]. In addition, expression of an S129A mutant of α-synuclein, which cannot be phosphorylated by Gprk2, is less toxic, while forming more aggregates [18]. Based on these observations, one might expect that knockdown of Gprk2 would result in an increase in the

Figure 2. Fluorescent Recovery after Photo Bleaching Reveals α-Synuclein Inclusions Contain Mobile as well as Immobilized Protein Material. (A,F,K,P) Images of YFP, Q40-YFP and α-synuclein-YFP transgenic animals. (B-E,G-J, L-O,Q-T) High magnification images of the area indicated (red box) before photo bleaching and during recovery. (U-X) Graphical representation of fluorescence recovery after photo bleaching in (B-E, G-J, L-O, Q-T). Relative fluorescence intensity (RFI) value on y-axis represents percentage fluorescence corrected for background bleaching. (Y) Average number of inclusions larger than ~2 μm2 per animal between tip of the nose and pharyngeal bulb during aging (n = 9 for day 11, n = 10 for days 9, 13, 15 and 17). (Z) Percentage of foci containing immobile material during aging. Bar in d-g represents 50 μm (overview) and 5 μm in higher magnification images. Error bars in (Y) indicate standard deviation.

doi:10.1371/journal.pgen.1000027.g002
amount of inclusions as well. Two orthologs of the Gprk2 gene are present in C. elegans, grk-1 and grk-2. We tested the effect of knockdown of these two genes by RNAi on the formation of inclusions. Unexpectedly, RNAi knockdown of grk-1 or grk-2, and not RNAi of random tyrosine or serine kinases, resulted in a decrease in the amount of inclusions (Figure 5A and data not shown), indicating that we could not have recovered these genes in our screen for more inclusions. There was no obvious divergence in the level of α-synuclein-YFP expression at day 5 after synchronization, indicating that formation of the inclusions themselves is affected (Figure 5D). The effect of knockdown of grk-1 and grk-2 by RNAi was confirmed by crossing in deletion alleles of both genes, grk-1(ok1239) and grk-2(gk268), into the α-synuclein-YFP strain (Figure 5B). Note that an RNAi screen for a reduction in the amount of inclusions yielded only one other kinase, which supports the idea that GRKs act specifically (data not shown). In all, C. elegans orthologs of Gprk2 act as modifiers of α-synuclein inclusion formation. Knockdown of Gprk2 in Drosophila or overexpression of grk-1 or grk-2 in C. elegans will be required to establish whether their specific role in alpha-synuclein pathology is comparable between the two species.

While Gprk2 overexpression increases toxicity of α-synuclein in Drosophila, Hsp70 overexpression has been shown to decrease its toxicity [19]. Because we did not recover Hsp70 as a modifier in our screen, we tested RNAi for the Hsp70 (C12C8.1) gene independently. Knockdown by RNAi of Hsp70 did not obviously increase the amount of inclusions, whereas the same RNAi clone did increase the amount of inclusions in a polyglutamine worm (Figure 6). This result is consistent with the findings in Drosophila—that the decreased toxicity in the presence of Hsp70 is independent of a change in the amount of alpha-synuclein inclusions [19]. Apart from a direct role in the detoxification of misfolded monomers or oligomers of alpha-synuclein, Hsp70 in Drosophila neurons may also act indirectly, for example as an inhibitor of apoptosis, which may be independent of aggregation [20].

To further characterize the inclusion mechanism, we classified the complete set of identified modifiers according to seven subcellular locations as annotated by the UniProt entries of the human orthologs (see Table S1) and compared this classification with that of sets of random genes (Figure 7A). This analysis revealed that the number of genes that function in the ER-Golgi and vacuolar compartment are more than 2-fold higher (p<0.005) and more than 3-fold higher (p<0.001), respectively, than would be expected by chance (Figure 7A).

A number of these endomembrane-localized genes are involved in vesicle-mediated trafficking, protein quality control, and detoxification of the ER (Table 2). Of particular interest is the cytochrome P450 gene (cyt34A7), whose closest human ortholog inactivates neurotoxic compounds that are known to increase the risk of developing Parkinson’s disease in humans, such as organophosphate insecticides and paraquat [16,21–23].

We also identified several molecular regulators of lifespan as modifiers of inclusion formation, such as lagr-1, a sphingolipid synthase, and sir-2.1, the C. elegans homolog of Sirtuin1, both of which we confirmed by crossing the deletion strain for both genes, lagr-1(gk331) and sir-2.1(ok434), into the α-synuclein strain (Figure 4 A–4D) [24]. Deletion of sir-2.1 or lagr-1 both result in an increase of approximately 35% in the number of α-synuclein inclusions in nine-day old adult worms (Figure 4 A–D). The homolog of lagr-1 in yeast, LAG1, regulates its lifespan and is homologous to LASS2 in humans. Sirtuin-1 is an NAD+-dependent protein deacetylase, which regulates lifespan in yeast, fruit fly and C. elegans [25–28]. In C. elegans, overexpression of sir-2.1, the homolog of human sirtuins 1 to 3, extends its lifespan by up to 50%, probably by inhibiting expression of ER-stress genes [25,26]. These findings suggest a link between the molecular mechanism of aging and α-synuclein pathology.

In all, our screen recovered genes and processes that have previously been identified in other cell or animal models as modifiers of neuronal α-synuclein toxicity, such as sir-2.1 and genes involved in vesicle trafficking [12,17,29]. Based on these findings, we expect other genes from our screen to provide insight into neuronal α-synuclein pathology as well. Future studies in neurons will resolve which of these gene processes are implicated in neuronal α-synuclein pathology.

In a screen performed previously for modifiers of polyglutamine aggregation in C. elegans, we found a large variety of proteasomal genes and chaperones [6]. These genes are typically found to be involved in protein misfolding and aggregation and are suggested to be generally involved in protein-misfolding diseases. Surprisingly, very few of such genes were found in the α-synuclein screen.
Table 1. Suppressors of α-Synuclein Inclusion Formation.

| Function | Cosmid no. | Gene | Description | Strength |
|----------|------------|------|-------------|----------|
| CS       | R05H10.6   | cdh-7 | FAT tumor suppressor homolog 3 | +        |
| CS       | Y54E2A.2   |      | Similar to Ca2+-binding actin-bundling protein (spectrin) | +        |
| DR       | E03A3.2    | rcp-5 | DEAD/DEAH box helicase | +        |
| DR       | F32D1.10   | mcm-7 | DNA-replication licensing factor | +        |
| DR       | W02D9.1    | pri-2 | Eukaryotic-type DNA primase, large subunit | ++       |
| ECM      | F41F3.4    | col-139 | Structural component of the cuticle, phosphate transport | +        |
| EM       | C07A9.8    |      | Bestrophin, anion channel | ++       |
| EM       | C28H8.11   |      | Tryptophan 2,3-dioxygenase | ++       |
| EM       | R03E1.2    |      | Renin-precursor, lysosomal ATPase H+ transporter | ++       |
| EM       | T14F9.1    | vha-15 (phi-52) | ATPase coupled proton transport, Vacuolar ATP synthase sub | +        |
| EM       | Y37H9.6    | ndx-4 | NUDIX hydrolase | +        |
| ET       | B0213.12   | cyp-3A47 | Cytochrome P450 2B6 | +        |
| ET       | W01B11.2   | sulp-6 | STAS domain, Sulfate transporter family | ++       |
| ET       | W01B11.6   |      | Thioredoxin, Yeast: required for ER-Golgi transport, stress protection | ++       |
| GM       | F29F11.12  |      | UDP-glucuronosyltransferase 1–8 precursor | ++       |
| GM       | K07A3.1    | fbp-1(N07A3.b) | Fructose-1,6-bisphosphatase | +        |
| LM       | C28C12.7   | spp-10 | sphingolipid metabolism/lysosomal | ++       |
| LM       | F28H1.4    |      | Membrane-associating domain, Plasmodulin | ++       |
| LM       | F54F11.1   |      | Lipolytic enzyme, putative phospholipase | ++       |
| LM       | K09H9.6    | lpd-6 | RNA-binding protein required for 60S ribosomal subunit biogenesis | +        |
| LM       | T08H10.1   |      | Aldo-keto reductase family 1 member B10, oxido-reductase | +        |
| LM       | W03G9.6    | paf-1 | acetylhydrolase/phospholipase A2 | ++       |
| LM       | Y48G9.10   |      | Carnitine O-palmitoyltransferase I | ++       |
| LM       | Y63B11.10  | lasg-1 | LAG1, (dihydro)ceramide synthase, sphingolipid synthesis, ER | ++       |
| PD       | C46F9.3    | math-24 | MATH domain, Ubiquitin carboxyl-terminal hydrolase 7 | ++       |
| PD       | C47B2.1    |      | F-box domain | +        |
| PD       | F54B2.6    |      | Peptidase family M1 | +        |
| PD       | M03C11.5   | ymel-1 | Peptidase M41, FtsH, metallo protease: unfolded mito-proteins | ++       |
| PD       | R09B3.4    | ubc-12 | Ub-conj. enzyme (E2), NEDD8-conj. enzyme NCE2 | +        |
| PD       | R151.6     |      | Human Derlin-2, protein degradation in ER | +        |
| PD       | T06A4.1    |      | Peptidase M14, carboxypeptidase A, Zinc-metalloprotease | +        |
| PD       | Y63D3A.9   | fbxb-93 | F-box | +        |
| PF       | F52C12.2   |      | Uncharacterized conserved protein | +        |
| PF       | R151.7     |      | Hsp90 protein | +        |
| PS       | F42A10.4   | efk-1 | Ca/calmodulin-dep. kinase, Elongation factor 2 kinase | +        |
| Function | Cosmid no. | Gene | Description | Strength |
|----------|------------|------|-------------|----------|
| PS       | F50H6.9    |      | Protein of unknown function, DUF288 | +        |
| PS       | Y67D8A.370.a | pdf-4 | Translational repression, Splice Isoform 2 of Pumilio homolog 2 | ++       |
| RSP      | C04F5.5    | srb-2 | 7TM chemoreceptor, GPCR activity, DNA binding/Zn-finger | +        |
| RSP      | C28H8.5    |      | ShK domain | ++       |
| RSP      | D2089.1    | rfp-7 | Splicing factor, arginine/serine-rich | ++       |
| RSP      | F26H11.4   |      | Neurofilament triplet H protein | ++       |
| RSP      | T12A2.3    |      | Protein AF-9 | ++       |
| RSP      | Y113G7B.18 | mdt-17 | Transcriptional cofactor | +        |
| RSP      | Y116A8C.35 | udf-2 | Splicing factor U2AF 35 kDa subunit | +        |
| RSP      | Y48G1A.6   | Y48G1A.53.a | Polycomb group protein SCM/13M8T | ++       |
| SG       | C24B9.8    | str-13 | 7TM chemoreceptor, GPCR activity | ++       |
| SG       | F10E9.3    |      | Splice Isoform 1 of Death domain-associated protein 6 | ++       |
| SG       | F12A10.6   |      | Serine/threonine kinase (haspin family) | ++       |
| SG       | F19H8.2    |      | ~ to Rho kinase | +        |
| SG       | F21F3.3    |      | Isoprenylcysteine carboxyl methyltransferase family, yeast: ER | ++       |
| SG       | R09E12.1   | srbc-59 | 7TM chemoreceptor, srbc family | ++       |
| SG       | R24.4      |      | Chemoreceptor | +        |
| SG       | T28B9.2    |      | Protein phosphatase 1, catalytic subunit, alpha isoform 3 | ++       |
| SG       | T28F2.2    |      | Splice Isoform 1 of COMM domain-containing protein 4 | ++       |
| SG       | W05B5.2    |      | GPCR, membrane | ++       |
| SG       | Y171G12B20 | mab-20 | Semaphorin-4G precursor | ++       |
| SG       | ZK355.1    | ZK355.h | Tyr-kinase Receptor | ++       |
| U        | B0238.11   |      | HMG box-containing protein, transcriptional | +        |
| U        | C02E7.6    |      | Splice Isoform 1 of AMME syndrome candidate gene 1 protein | ++       |
| U        | C29F9.1    |      | Unknown | ++       |
| U        | D1022.5    |      | Dienelactone hydrolase family | +        |
| U        | F01G12.5a  | let-2 | Unknown | ++       |
| U        | F47F6.5    |      | Similarity to C-type lectin | ++       |
| U        | F54E7.6    |      | Unknown | ++       |
| U        | F56C4.1    |      | Unknown | ++       |
| U        | H23L24.e   |      | Unknown | ++       |
| U        | K01G12.3   |      | HIV TAT specific factor 1 | +        |
| U        | R09H3.3    |      | Unknown | ++       |
| U        | R09H3.3    |      | Unknown | ++       |
| U        | R10H10.2   | spe-26 | kelch-like 20 | ++       |
| U        | R11A8.4    | sir-2.1 | NAD-dependent deacetylase sirtuin-1 | ++       |
| U        | T05A10.4   |      | Similarity to cysteine-rich secretory protein 2 precursor | +        |
| U        | T06G6.8    |      | Unknown | ++       |
Table 1. cont.

| Function       | Cosmid no.   | Gene                  | Description                                                                 | Strength |
|----------------|--------------|-----------------------|----------------------------------------------------------------------------|----------|
| Chondroitin 6-sulfotransferase | U XE249     | Chondroitin 6-sulfotransferase | +                                                            | ++       |
| C-type lectin  | U Y19D10A.j | C-type lectin         | +                                                            |          |
| Insulin-like peptide | U ZC334.9  | ins-28                | ++                                                           |          |
| C-type lectin  | U ZK1290.11 | Unknown               | ++                                                           |          |
| VT C07G1.5     | VT C34C12.2  | Role in preribosome assembly or transport/t-snare domain | +                                                            | ++       |
| VT M151.3      | VT T05G5.9  | Ran-binding protein 2-like 4/yeast: ER-Golgi/SNARE assembly | +                                                            | ++       |
| VT W02A11.2    |              | ESCRT-II complex subunit, Yeast: vacuolar protein sorting protein 25 | +                                                            |          |

U: Unknown, RSP: RNA synthesis and processing, PT: Protein Transport, PS: Protein synthesis, PF: Protein Folding, DR: DNA replication, ECM: Extracellular matrix, CS: Cytoskeleton, EM: Energy metabolism, GM: Glucose Metabolism, SG: Signaling, VT: vesicle transport, ET: electron transport.

Only a single gene found to increase α-synuclein inclusion overlapped with the screen for polyglutamine aggregation we performed in C. elegans. Perhaps even more strikingly, the modifiers of polyglutamine aggregation that we had identified previously located primarily to cytosol and nucleus (Figure 7B) [6]. This suggests that different cellular processes are involved in preventing the development of inclusions in these protein-misfolding disorders. In conclusion, we have developed a transgenic C. elegans model showing α-synuclein inclusions that share important characteristics of inclusions in Parkinson’s disease. The formation of inclusions shows a strong age-dependency and clearly distinct, consecutive phases of progressive aggregation. Our transgenic worms therefore provide a versatile model for the characterization of the molecular steps preceding pathologic inclusion formation. In a global RNAi screen we identified modulators of α-synuclein aggregation that are distinct from those in other protein-aggregation disorders. Most importantly, our list of suppressors of age-dependent inclusion formation reveals a clear link between α-synuclein inclusion formation and cellular aging, most probably via an endomembrane-related mechanism. Our results provide an important guide for further elucidation of the pathogenic mechanisms and genetic susceptibility factors in human Parkinson’s disease and other α-synuclein related disorders.

Materials and Methods

Constructs

To create P(unc-54)/α-synuclein: YFP (pRP2386) α-synuclein was amplified from cDNA (provided by S. Lindquist, MIT, MA) by PCR using primers adding a KpnI and an AgeI site, respectively (3’-agctagatccggaagctactgaaaggg-5’, 3’- gactacgaacctgagcttccaggctgccacctc-5’), to the α-synuclein gene, which was then cloned into the pPD30.38-YFP expression vector.

Media and Strains

Standard conditions were used for C. elegans propagation at 20°C. Worms were synchronized by hypochlorite bleaching, hatched overnight and were subsequently cultured on NGM plates containing 5- Fluoro-2-deoxy-uridine (FUDR) to prevent eggs from hatching. The following strains were used: NL5901 (pkl2386 [α-synuclein::YFP unc-119(+)]), VC199(ok434), OW8 (sir-2.1(ok434)IV; pkl2386[α-synuclein::YFP unc-119(+)]), VC765(gk331), OW13 (gk-1(ok1239)), pkl2386[α-synuclein::YFP unc-119(+)], OW15 (gk-1(ok1239)X; pkl2386[α-synuclein::YFP unc-119(+)]), OW15 (gk-2(gk268)III; pkl2386[α-synuclein::YFP unc-119(+)]), OW6 (ame-1(m1920)III; pkl2386[α-synuclein::YFP unc-119(+)]), OW101 (lagr-1(k331); pkl2386[α-synuclein::YFP unc-119(+)]), AM134 (rmls126[P(unc-54)Q0::YFP]), and AM141 (rmls135-P(unc-54)Q0::YFP) [7]. The α-synuclein YFP fusion is driven by an unc-54, muscle specific, promoter to obtain expression in the body-wall muscle cells. Muscle expression was chosen to allow for efficient RNAi and for visual detection of inclusions [6–8].

Creation of Transgenic Strains

Transgenic C. elegans were generated using standard ballistic transformation by co-bombarding unc-119 rescue vector (pRP2512) and the α-synuclein-YFP expression construct into unc-119(ed3) nematodes [30].

Immunoblotting Analysis

Nematode protein extracts were prepared from whole animal frozen pellets in PBS containing proteinase inhibitors (Roche, Indianapolis, USA) by using Fastprep24 (MP, Solon, USA).
Figure 4. Sir-2.1, ymel-1 and lagr-1 Are Suppressors of α-Synuclein Inclusion Formation. (A,B) Confocal images showing α-synuclein-YFP transgenic animals and the transgenic strains containing a deletion in the sir-2.1 gene (sir-2.1(ok434)) on day 9. (C) Average number of inclusions between tip of the head and pharyngeal bulb of the worm (n = 8), *P<0.025 (Student’s t test). (D, E, F) Confocal images showing α-synuclein-YFP transgenic worms and the transgenic strains containing a deletion in the ymel-1 and lagr-1 gene (ymel-1(tm1920) and lagr-1(gk331)), on day 9. (G) Average number of inclusions between tip and pharyngeal bulb of the worm (n = 8 (wt and ymel-1), n = 7 (lagr-1)). *P<0.05, **P<0.05 (Student’s t test).

doi:10.1371/journal.pgen.1000027.g004

Figure 5. RNAi and Deletion of grk-1 and grk-2 Decreases Inclusion Formation. (A) Confocal images showing head region of α-synuclein-YFP transgenic animals fed on OP50 bacteria, bacteria containing empty RNAi vector (L4440), and expressing double stranded RNA targeting grk-1 and grk-2. (B) Confocal images of α-synuclein-YFP C. elegans, in wild type background, grk-1(ok1239) and grk-2(gk268) background. Scale bar represents 25 μm. (C) Quantification of the number of worms within the population (n = 20) with the same amount (wt), fewer (<wt) or no inclusion (none). (D) Western blot analysis of protein extracts from 20 staged transgenic C. elegans with a grk-1 or grk-2 genetic deletion showing similar levels of fusion protein expressed and actin (loading control).

doi:10.1371/journal.pgen.1000027.g005
Samples were boiled 5 min with sample buffer containing SDS and DTT, and separated on 10% SDS/PAGE. Proteins were transferred to PVDF membranes. Antibody binding was visualized by binding of horse-radish peroxidase-coupled secondary antibody and chemiluminescence (ECL Lumi-Light, Roche, Germany). Antibodies used were α-synuclein (1:3,000; LB509, Zymed, San Francisco, USA), anti-actin (1:8,000; Clone C4, ICN, Aurora, OH, USA) and anti-GFP polyclonal serum [31]. Goat-anti-mouse and goat-anti-rabbit IgG-HRP (1:5,000; BioRad, Hercules, CA, USA) were used as secondary antibodies.

CLSM and Fluorescence Recovery after Photo Bleaching (FRAP)

Transgenic worms were mounted on 2% agarose pads containing 40 mM NaN₃ as anaesthetic on glass microscope slides. Images were captured using a Leica DMIRE2 confocal microscope with an x40 oil immersion lens (HCX PL APO CS). Images shown are 2D maximal projections of zseries. Immobilized animals were subjected to FRAP analysis with minor modifications as described [20]. An area of ~4 μm² was bleached for 5 s (five iterations at 100% intensity), after which images were collected (at low intensity). Relative fluorescence intensities (RFI) were calculated as described by using the RFI = (Net/Y1)/(Net/Y0) equation [10]. For quantification of the number and motility of inclusions (Figure 1 y and z) all foci larger than 2 μm² between the nose and pharyngeal bulb were analyzed by FRAP. Measurements on inclusions were performed using ImageJ software [26]. Statistical significance was determined using t tests.

RNAi Screen

Synchronized larvae were grown in liquid culture in 96-well plates, on bacterial strains from the Ahringer bacterial RNAi library as described [6,32]. Worms grown on the different RNAi clones were visually selected for increased inclusion formation four and five days after bleaching. Bacterial clones, that exhibited increased inclusion formation, were confirmed in an independent experiment in duplicate in liquid culture. RNAi clones confirmed to increase inclusion formation were then fed to α-synuclein-YFP transgenic C. elegans on NGM plates for reconfirmation. All gene targets of the positive-RNAi foods were verified by sequencing of the insert of the RNAi plasmids.

Bioinformatics and Statistical Analysis

Subcellular location data were tested for overrepresentation using expected relative frequencies based on a Poisson distribution (e−μY/Y!), where Y is the observed percentage of genes observed for a given class (Y) and μ is the expected (mean) percentage of this class among all annotated genes. The observed results were compared to a random list of 55 genes (n = 20) created from...
random UniProt entries with annotated subcellular locations. Genes functioning in more than one location were assigned to both locations.

Supporting Information

Table S1  Human orthologs. Found at: doi:10.1371/journal.pgen.1000027.s001 (0.08 MB XLS)

Acknowledgments

We thank I. Braakman and C. Wijmenga for critically reading the manuscript. We thank the Caenorhabditis Genetics Center, funded by the NIH National Center for Research Resources, and the Mitani lab for C. elegans strains, J. Ahiringer for the RNAi library, and K. Spillemia from the UMCG Microscopy and Imaging Center (UMIC) for advice on microscopy, and J. Senior for editing the manuscript.

Author Contributions

Conceived and designed the experiments: TvH RP EN. Performed the experiments: TvH KT. Analyzed the data: TvH KT RB. Contributed reagents/materials/analysis tools: RB RH RP. Wrote the paper: TvH EN. Advice: RH.

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