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Caenorhabditis elegans as an Experimental Model Organism to Study Parkinson's Disease-Related Genes

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Functional Analysis of Parkin and α-Synuclein

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Erklärung

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Ehrenwörtliche Versicherung

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Meinen Eltern und Großeltern

Thus spake Zarathustra:

“Ye have made your way from the worm to man, and much within you is still worm…"

(Friedrich Nietzsche, *Zarathustra’s Prologue*, 1.3)

1 Summary

Parkinson's disease (PD) is the second most common neurodegenerative disorder, mainly characterized by motor dysfunctions resulting from massive and selective loss of dopaminergic neurons. Mutations in the human *parkin* gene, which encodes an E3 ubiquitin ligase, are the most frequent causes of hereditary PD, leading to autosomal-recessive juvenile Parkinsonism. However, the cell biological role of Parkin and the molecular pathogenic mechanisms by which mutations cause PD are unclear.

In this study, the *Caenorhabditis elegans parkin* ortholog, *pdr-1*, was identified and characterized in detail. PDR-1 is functionally conserved, since it physically associates and cooperates with enzymes of the ubiquitylation/degradation system to mediate ubiquitin conjugation. Strikingly, in contrast to *pdr-1* loss-of-function mutants, the in-frame deletion mutant protein PDR-1(Δaa24-247) still interacts with its co-enzymes, and moreover, the corresponding mutant *pdr-1(lg103)* is hypersensitive towards misfolded protein conditions. In this mutant, both cytosolic stress conferred by overexpression of mutant human α-synuclein, a gene linked to autosomal-dominant forms of PD, as well as endoplasmatic reticulum (ER)-derived folding stress result in severe developmental defects and lethality. Although expression of *pdr-1* is regulated by all three activators of the unfolded protein response (UPR), IRE-1, PEK-1, and ATF-6, genetic analyses established a function of PDR-1 in parallel to IRE-1 signalling.

Therefore, PDR-1/Parkin plays an essential role in the regulation of different proteotoxic stress pathways: it contributes to the ER-specific UPR, but also participates in the cytosolic detoxification of protein aggregates, including α-synuclein. The truncated protein PDR-1(Δaa24-247) seems to mediate a toxic misfunction by sequestering critical components of the protein folding/degradation machinery, which is related to the stress hypersensitivity in the *pdr-1(lg103)* mutant. In this study, an experimental animal system was established which is well suited to identify modifiers of toxicity and relevant compounds. Such studies might allow to dissect the molecular and cellular pathways involved in the pathogenesis of PD and to identify potential therapeutic drug targets.

2 Introduction

2.1 Clinical Characteristics and Pathology of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting about 1-2% of the population over the age of 65 (Riess et al., 2003). The clinical manifestations of this disease, originally described in 1817 by James Parkinson in his "Essay on the Shaking Palsy", are mainly motor dysfunctions. This encompasses a spectrum of core clinical features, from which today over one million patients suffer: rigidity (stiffness), bradykinesia (slowness of movement), tremor at rest (rhythmic shaking and involuntary movement) and postural instability (disturbance of balance) (Lang and Lozano, 1998a; Lang and Lozano, 1998b). However, PD is a heterogeneous disorder, as many patients also develop cognitive dysfunctions, including anxiety, depression and dementia (Dawson and Dawson, 2002), or abnormalities in olfactory and visual perception (Chung et al., 2003). This chronic progressive disease proceeds relentlessly until the patient dies.

Pathologically, PD is characterized by the specific and massive loss of dopamine (DA) containing neurons in the Substantia Nigra pars compacta (SNpc) and the Locus Coeruleus (Figure 1).

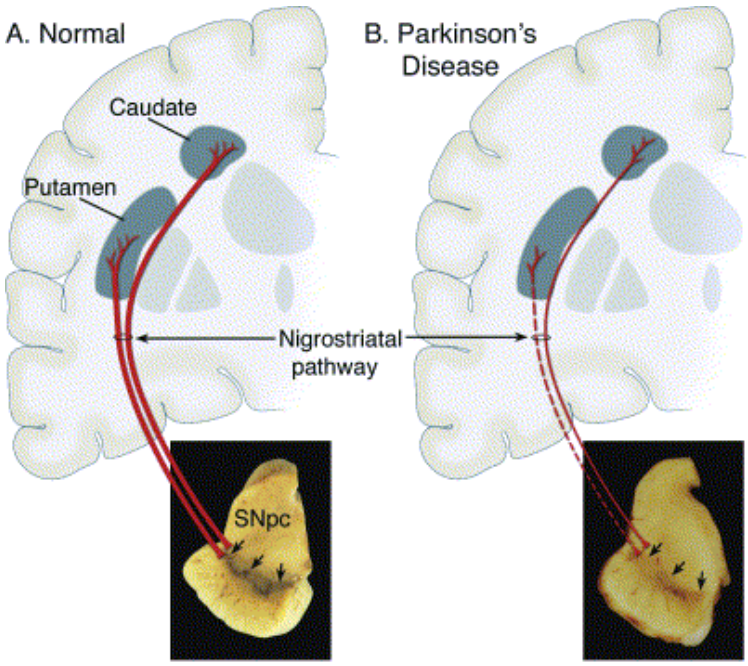


Figure 1. Pathology of Parkinson's Disease.

Schematic representation of (**A**) the normal and (**B**) the diseased nigrostriatal pathway (in red). DA neurons (arrows) of the SNpc project into the striatum (i.e., putamen and caudate). The photographs demonstrate (**A**) the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons, as well as (**B**) an obvious depigmentation (i.e., loss of dark-brown pigment neuro-melanin) of the SNpc due to the marked loss of dopaminergic neurons (Taken from Dauer and Przedborski, 2003).

Loss of greater than 80% of these SNpc neurons leads to nigrostriatal DA deficiency, which is responsible for the major symptoms of PD. Although DA is synthesized only in a small subset of neurons, it accomplishes an exceedingly important role in the nervous system, as it not only controls movement, but also regulates emotional behaviour, cognitive functions, and memory.

The main neuropathological hallmark of idiopathic PD are small (5-25 µm) and spherical cytoplasmic, and more abundantly neuritic inclusions (Lewy bodies and neurites) (Goedert, 2001), originally identified by Friedrich Lewy in 1912 (Figure 2).

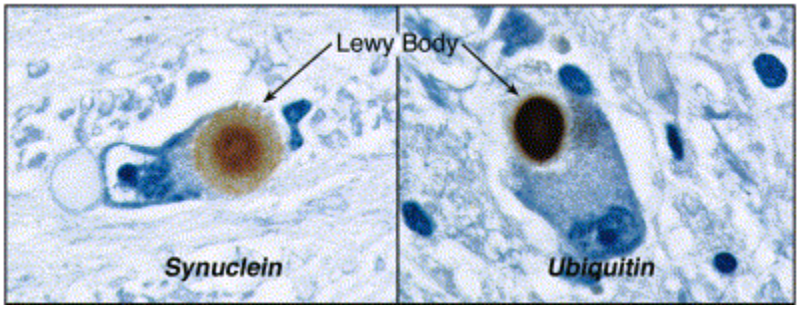


Figure 2. Lewy Body Pathology of PD Brain.

Immunohistochemical staining of Lewy bodies in dopaminergic neurons. **Left:** antibody against α-synuclein. **Right:** antibody against ubiquitin (Taken from Dauer and Przedborski, 2003).

These eosinophilic, hyaline aggregates contain a variety of different aggregation-prone proteins, as well as enzymes involved in protein folding, degradation, and detoxification. The major components of Lewy bodies (LBs) are α-synuclein and its interaction partner synphilin-1, as well as ubiquitin, Parkin, UCH-L1, proteasomal subunits and chaperones (Dev et al., 2003a). Interestingly, some of these constituents are directly associated with familial forms of PD.

The mechanism of LB biogenesis and their contribution to neurodegenerative processes are unknown. However, formation of aggresomes which are proteinaceous inclusions that facilitate and increase the degradation of excess amounts of unwanted and possibly cytotoxic proteins, is a cellular defence mechanism against unfolded protein stress (Garcia-Mata et al., 2002; Kopito, 2000; Sherman and Goldberg, 2001). Similarities between aggresomes and LBs with respect to structural organization, protein content, and intracellular localization have already been identified, and suggest that these inclusions are related and could be formed in similar ways (Olanow et al., 2004).

The major medical approaches to treating this disease are DA replacement therapies by the precursor L-DOPA (levodopa) or different agonists, as well as medications interfering with different enzymatic steps of DA metabolism, nerve terminal release and re-uptake (Kitamura et al., 2002). Although, current PD medications alleviate most of the symptoms, efficiency over the time is limited, and none halt or retard degeneration of DA neurons. Thus, neuro-protective (to prevent cell death) or neuro-restorative (to repair neurons) therapies must be developed (Dawson and Dawson, 2002), but these have to be based on understanding its molecular and biochemical pathogenesis of PD.

2.2 Pathogenesis of Parkinson's Disease

Although the mechanisms underlying pathogenesis of PD are unknown, mainly two cellular dysfunctions are implicated: mitochondrial respiration defects and the resulting oxidative stress, as well as dysfunctions in protein folding/degradation pathways and the consequent abnormal protein aggregation.

2.2.1 Mitochondrial Dysfunction and Oxidative Stress

Originally, mitochondrial impairment and consequent oxidative damage have been detected in pathological analyses of PD brains (Beal, 2003; Jenner and Olanow, 1998). Inhibition of complex I and IV of the mitochondrial respiratory chain have been revealed in affected DA neurons which seem particularly vulnerable due to their high basal rate of oxidation (Betarbet et al., 2002; Goedert, 2001). Thereby, DA itself might act as an endogenous neurotoxin, as its metabolism can generate harmful reactive oxygen species (ROS), and some of its adducts confer cytotoxicity to nerve cells (Lotharius and Brundin, 2002; Lotharius and O'Malley, 2001; Nass and Blakely, 2003). This is further supported by data from intoxication and genetic experimental models. Exposure to specific drugs causes a syndrome that mimics the core neurological symptoms and selective dopaminergic neurodegeneration, with or without Lewy body formation (Dauer and Przedborski, 2003). These PD mimetics, mostly mitochondrial complex I inhibitors, have been extensively studied in cell culture systems and in a variety of animal models (Reviewed by Betarbet et al., 2002;

Dauer and Przedborski, 2003; Shimohama et al., 2003) including primates, rodents, and nematodes (Braungart, 2004; Nass et al., 2002). In addition, the discovery of some PD-associated genes, and their analysis in cell culture systems or gene-knockout models in mice and flies further implicated mitochondrial defects and oxidative stress in the pathogenesis of PD (Shen and Cookson, 2004).

2.2.2 Proteasomal Dysfunction

Studies of toxin-based PD models and the function of genes implicated in inherited forms of PD strongly suggested that impairment of protein degradation along with an age-related tendency to accumulate damaged proteins is crucial and may play a major role in the pathophysiology of PD (McNaught et al., 2003; McNaught and Olanow, 2003). The ubiquitin-proteasome dependent degradation pathway regulates protein turnover in the cytosol and in the nucleus of all eukaryotic cells (Figure 3).

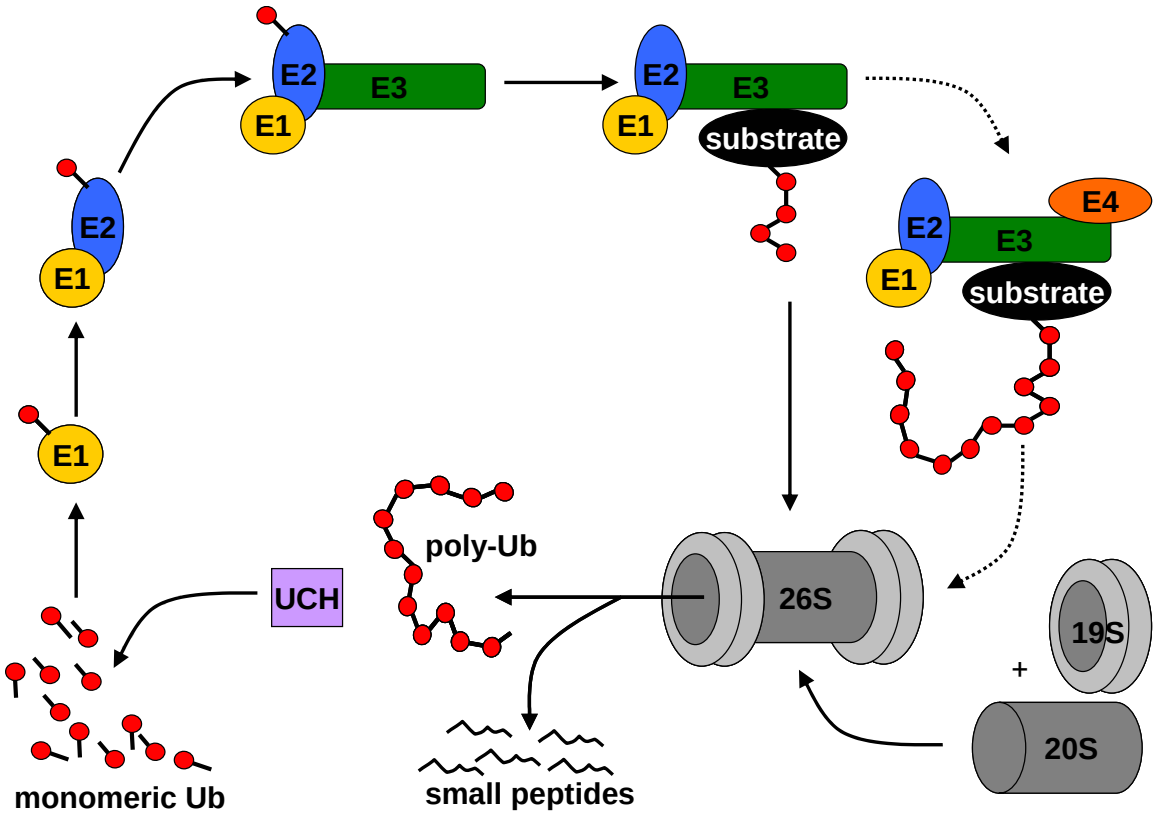


Figure 3. The Ubiquitin-Proteasome System.

Abbreviations: Ub, ubiquitin; **E1**, ubiquitin-activating enzyme; **E2**, ubiquitin-conjugating enzyme; **E3**, ubiquitin protein ligase; **E4**, multiubiquitin-chain assembly factor; **UCH**, ubiquitin C-terminal hydrolase. For a description of the ubiquitin-proteasome system, see further text.

Thereby, short-lived as well as damaged or misfolded proteins are targeted for destruction by conjugation to ubiquitin, mediated by a concerted action of different enzymes in a sequential cascade (Pickart, 2001). First, free ubiquitin gets activated by an ubiquitin-activating enzyme (E1) through an ATP-dependent mechanism, and is transferred to an ubiquitin-conjugating enzyme (E2 or UBC). Next, an ubiquitin-protein ligase (E3), which confers substrate specificity, mediates attachment of ubiquitin to an acceptor protein. Sequential rounds of ubiquitylation, in which each following ubiquitin moiety is linked to the previous, leads to formation of poly-ubiquitylated substrates. Sometimes, this is mediated in conjunction with an additional multichain assembly factor (E4), to ensure efficient substrate multi-ubiquitylation. Poly-ubiquitylated proteins are recognized by the associated 26S proteasome, and subsequently degraded (Hochstrasser, 1996). The 26S proteasome is a multicatalytic proteinase complex, composed of a barrel-shaped 20S core particle, with a proteolytic active cavity, and a 19S regulatory complex, which is attached at either or both ends. The 19S complex promotes recognition and binding of ubiquitin chains, as well as substrate unfolding and translocation into the 20S core proteinase (Baumeister et al., 1998; Verma and Deshaies, 2000). Thereby, substrates are cleaved into short peptides and poly-ubiquitin chains, which are then disassembled by ubiquitin C-terminal hydrolases (UCH), de-ubiquitylating enzymes, to regenerate free monomeric ubiquitin (Chung and Baek, 1999; Kim et al., 2003).

It is noteworthy that several PD associated genes are either directly involved in, or are turned over by the ubiquitin-proteasome dependent degradation system (Giasson and Lee, 2003). In addition, proteasome inhibition causes formation of aggresome/Lewy body-like structures and cytotoxicity in DA neurons in culture (McNaught et al., 2002). Moreover, it has already been shown that proteasomal activity is impaired in substantia nigra of sporadic PD patients. Low steady-state levels of proteasome activators in specifically DA neurons may render these cells more susceptible to proteolytic stress than other brain cells (Ciechanover and Brundin, 2003).

Furthermore, a clear link between protein folding and degradation pathways has already been established. Dysfunction in the ER leads to accumulation and consequent aggregation of misfolded proteins, thereby impairing proteasomal activity (Bence et al., 2001) which in turn aggravates ER stress (Paschen, 2003).

2.2.3 Dysfunction of the Endoplasmatic Reticulum

The accumulation of misfolded and/or aggregated proteins in the lumen of the endoplasmatic reticulum (ER) activates an intracellular signalling pathway, the unfolded protein response (UPR) (Figure 4). This adaptive homeostatic pathway augments ER folding capacity by transcriptional induction of ER-resident chaperones, folding catalysts and protein degradation complexes, and, in addition, limits further accumulation of unfolded proteins in the ER by translational attenuation (Rutkowski and Kaufman, 2004).

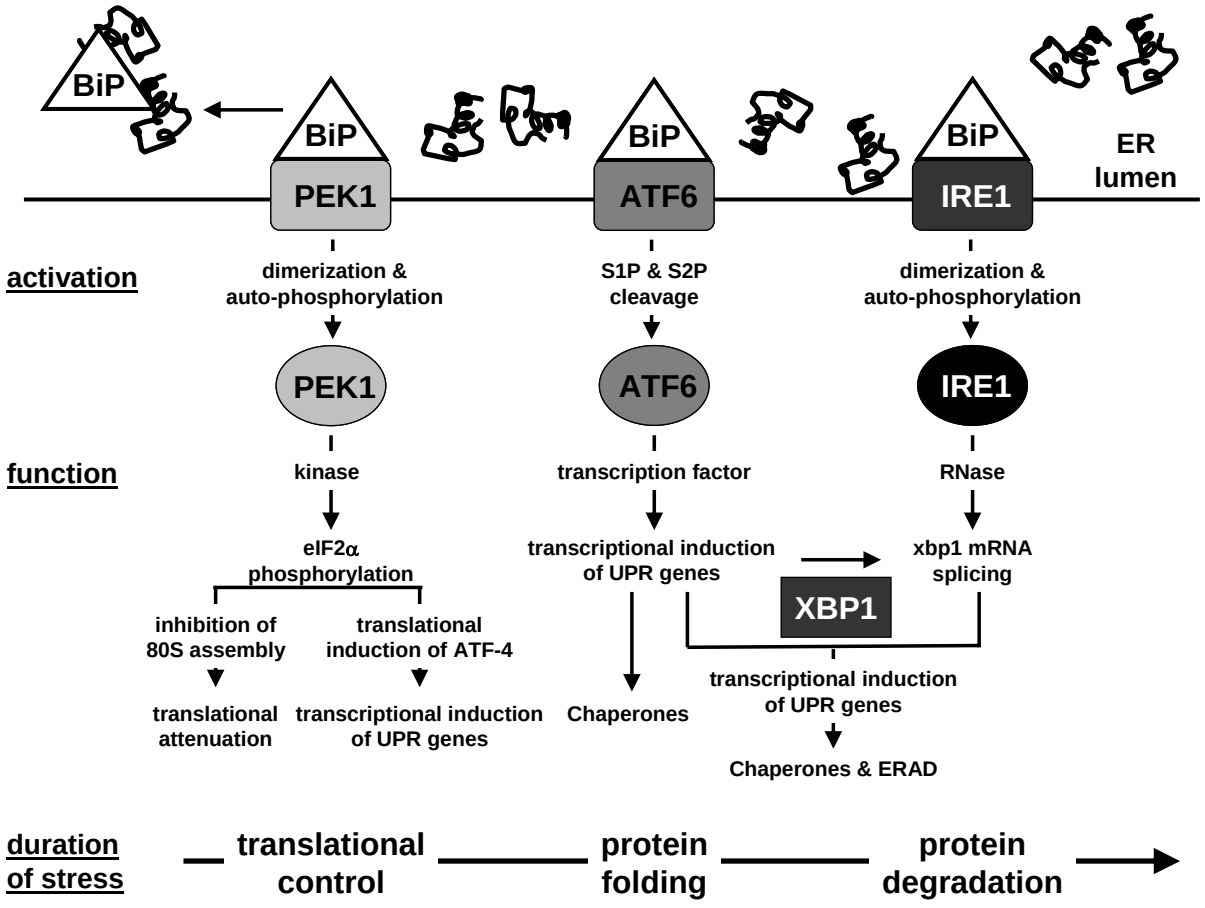


Figure 4. The Unfolded Protein Response.

The different phases of the UPR are executed by a time-dependent shift, according to duration of stress signals (Yoshida et al., 2003). First, the kinase PERK/PEK1 mediates translational attenuation by direct phosphorylation of the initiation factor eIF2α, to limit further protein synthesis. On the other hand, this selectively promotes translation of specific mRNAs (e.g. ATF4). Next, the transcription factor ATF6, activated through Golgi transport and cleavage, regulates a group of genes encoding ER-resident molecular chaperones, in an attempt to correct the defects by refolding. Finally, IRE1 initiates a transcriptional activation through splicing of *xbp1* mRNA. The transcription factor XBP1 itself directly targets a set of genes encoding ER-resident chaperones, folding catalysts, and protein degradation complexes, to promote degradation of misfolded proteins.

Under normal physiological conditions, this signal transduction pathway is repressed by binding of the ER chaperone BiP to the luminal domains of three ER-membrane resident effectors: the protein-kinase and site-specific endoribonuclease IRE1; the eukaryotic translation initiation factor 2 kinase PERK/PEK; and the transcriptional activator ATF6 (Zhang and Kaufman, 2004). Upon accumulation of unfolded proteins BiP is released to encompass its chaperone function, permitting the activation of the UPR (Zhang and Kaufman, 2004). When the UPR is overwhelmed or fails, affected cells can be set on a pathological trajectory that culminates in their dysfunction and death (Forman et al., 2003).

Unfolded proteins in the ER are retro-translocated to the cytosol and turned over by the ER-associated degradation pathway (ERAD) (Bonifacino and Weissman, 1998; Kostova and Wolf, 2003; Plemper and Wolf, 1999), a process regulated by the UPR. Both pathways are required for the coordinated disposal of misfolded proteins even in the absence of acute stress (Friedlander et al., 2000; Travers et al., 2000). The involvement of ER stress pathways in the pathogenesis of PD was further substantiated, as a variety of PD mimetics, like 6-OHDA (6-hydroxydopamine) and MPTP/MPP^+ (1-methyl-4-phenylpyridinium), specifically induce ER stress and activate the UPR (Ghribi et al., 2003; Holtz and O'Malley, 2003; Kheradpezhouh et al., 2003; Ryu et al., 2002). Furthermore, impairment of the UPR pathway increases sensitivity to parkinsonism-inducing toxins (Ryu et al., 2002).

Thus, proteolytic stress defined as a state in which levels of unwanted proteins (mutant, misfolded, denatured or damaged) exceed the capacity for clearance due to increased protein production and/or inadequate folding/proteolysis, is strongly implicated as a major key event in the pathogenesis of sporadic and the various familial forms of PD (Forman et al., 2003; McNaught and Olanow, 2003; Sherman and Goldberg, 2001).

2.3 Aetiology of Parkinson's Disease

While the causes of PD are largely unknown, considerable evidence suggests a multifactorial aetiology as a result of cumulative effects of environmental and complex genetic factors, with toxins and advancing age as the main risk factors (Shastry, 2001). Most cases of idiopathic PD appear sporadically, whereas familial cases are rare with a monogenic inheritance less than 5% (Cordato and Chan, 2004). Recent studies revealed several susceptibility loci of which six certain monogenic forms of familial PD have already been identified (Table 1). This has provided some explanation for the clinical heterogeneity in this disorder: different age of onset from juvenile to late, transmission dominant and recessive, progression from very slow to rapid, differences in the clinical features, with or without LB pathology (Warner and Schapira, 2003).

locus PARK	position	gene	onset	TM	LB	reference
1/4	4q21-22	**α-synuclein**	middle-late	AD	+	(Polymeropoulos et al., 1997; Singleton et al., 2003)
2	6q25-27	***parkin***	early-juvenile	AR	-*	(Kitada et al., 1998)
3	2p13	unknown	late	AD	+	(Gasser et al., 1998)
5	4p14	**UCH-L1**	late	AD	n.d.	(Leroy et al., 1998)
6	1p35-36	**PINK1**	early	AR	n.d.	(Valente et al., 2004)
7	1p36	**DJ-1**	early	AR	n.d.	(Bonifati et al., 2003)
8	12p11.2-q13.1	**Dardarin/ LRRK2**	late	AD	+	(Paisan-Ruiz et al., 2004; Zimprich et al., 2004)
9	1p36	unknown	juvenile	AR	n.d.	(Hampshire et al., 2001)

Table 1. Loci and Genes Linked to Hereditary PD.

Abbreviations: **TM**, transmission; **LB**, Lewy body; **AD**, autosomal dominant; **AR**, autosomal recessive; **n.d.**, not determined; *****, except one case (Farrer et al., 2001).

2.3.1 α-synuclein

The first rare monogenic form of autosomal-dominant PD was linked to mutations in the gene encoding α-synuclein. This highly abundant presynaptic protein is implicated in many biological processes (Lykkebo and Jensen, 2002), but the mechanism of mediating toxicity remains elusive (Dev et al., 2003a). α-synuclein, the major component of LBs, normally is a soluble monomeric protein, but displays a concentration-dependent tendency to polymerize into misfolded aggregates acquiring fibrillar structures (Goedert, 2001). Although α-synuclein aggregates have been shown to impair proteasomal activity (Lindersson et al., 2004; Snyder et al., 2003) a cytoprotective function of α-synuclein aggregation or fibril formation, by sequestering potentially toxic soluble forms (Xu et al., 2002), has been suggested (Tanaka et al., 2004). However, which conformational state of α-synuclein may confer toxic properties remains uncertain (Dev et al., 2003a).

So far, three autosomal-dominant point mutations (Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004) as well as genomic multiplications (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Singleton et al., 2003) of the α-synuclein locus cause PD. Thus, there is a clear dosage effect according to the number of supernumerary copies of α-synuclein since even elevated protein levels mediate pathology. This has already been confirmed by transgenic animal models, including primates, rodents, as well as flies and nematodes (Feany and Bender, 2000; Giasson et al., 2002; Lakso et al., 2003; Lee et al., 2002; Masliah et al., 2000; Neumann et al., 2002; Yamada et al., 2004). Overexpression of human α-synuclein caused motoric dysfunctions, formation of LB-like structures, and neurotoxicity, sometimes even associated with death of DA neurons (Reviewed by Maries et al., 2003). On the other hand, gene knock-outs of α-synuclein in mice resulted in resistance to MPTP intoxication (Dauer et al., 2002) but also in functional deficits of the dopaminergic system (Abeliovich et al., 2000). Although many insights have been gained by α-synuclein studies over the past few years, the exact molecular and cellular processes underlying neurotoxicity and pathogenesis of PD are still unknown and have to be elucidated.

2.3.2 Parkin

In contrast to rare mutations in the majority of PD associated genes, most of familial cases are linked to mutations in the *parkin* gene, causing autosomal-recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). A great variety of about 100 different mutations have been identified so far, including exon rearrangements, small deletions or insertions, as well as single base pair substitutions (Reviewed by Hedrich et al., 2004; Mata et al., 2004). Although it appears that most mutations are recessive and so complete loss-of-function results in the pathogenesis, rare heterozygous alleles have been identified, suggesting dominant negative or toxic gain-of-misfunction mutations in some cases (Reviewed by Ciechanover and Brundin, 2003; West et al., 2002). Furthermore, missense mutations or small deletions seem to be associated with an earlier onset and a more pronounced phenotype than complete loss-of-functions by large deletions (Lohmann et al., 2003).

Parkin has been suggested to function as an E3 ubiquitin ligase (Shimura et al., 2000) for aggregation-prone proteins (Tsai et al., 2003). A variety of un-related substrate proteins have been identified, including α-Sp22, a rare modified species of α-synuclein (Shimura et al., 2001). Parkin-mediated ubiquitylation enhances proteasomal degradation of substrate proteins at least in cell culture (Reviewed by Kahle and Haass, 2004). Human Parkin consists of several domains (Figure 5) which all seem to be essential for its functional integrity as missense mutations cluster in each. Some familial *parkin* mutations interfere with its ubiquitylation activity and therefore inhibit its protective function (Shimura et al., 2000).

The extreme N-terminus of Parkin is homologous and structurally related to ubiquitin (Sakata et al., 2003). This ubiquitin-like domain (UBL) is thought to be involved in coupling to the proteasome (Sakata et al., 2003; Tsai et al., 2003), substrate recognition (Shimura et al., 2001), and, regulation of Parkin stability (Finney et al., 2003). The following unique Parkin domain (UPD) is of yet unknown function, although auto-ubiquitylation clusters among others in this region (Finney et al., 2003). Consistently, Parkin was shown to be rapidly degraded by the 26S proteasome (Choi et al., 2000), due to its auto-ubiquitylation activity.

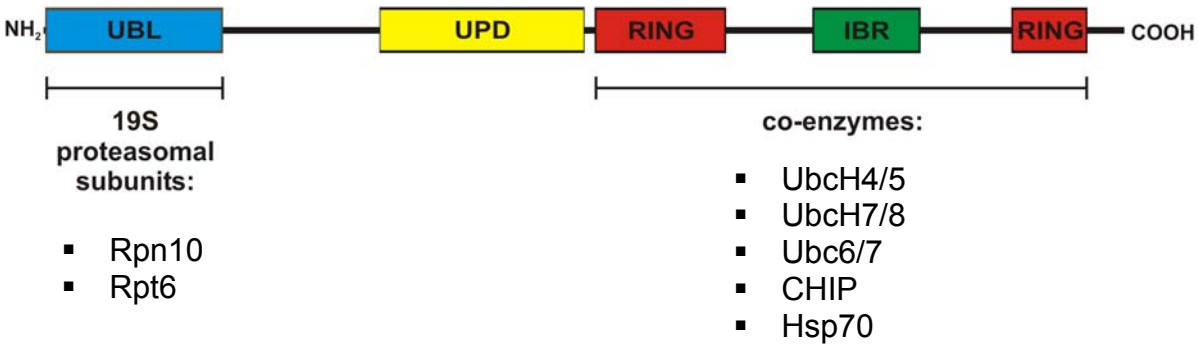


Figure 5. Human Parkin and Associated Proteins.

Schematic view of human Parkin protein architecture. The different domains are colour-boxed: ***blue*****:** ubiquitin-like domain (UBL); ***yellow*****:** unique parkin domain (UPD); ***red*****:** C_3HC_4 ring-finger domains (RING); ***green*****:** C_6HC in-between ring-finger domain (IBR). Parkin associates with the proteasomal subunits listed via the UBL domain, and with the co-enzymes listed via the RING box structure. With the exception of α-Sp22, all other so far identified substrates bind to the RING box of Parkin.

The C-terminus consists of two C_3HC_4 zinc-finger domains (RING) separated by a C_6HC in-between RING finger domain (IBR). This particular arrangement, the RING box, defines a protein superfamily, which includes dorfin and ariadne ubiquitin ligases (Marin and Ferrus, 2002). It was shown that this configuration mediates selective target recognition and/or binding, as well as association with specific co-enzymes of the ubiquitylation machinery. Human Parkin cooperates with E2 and E4 enzymes involved in cytosolic protein stress response and the ERAD pathway (Imai et al., 2002; Shimura et al., 2001; Zhang et al., 2000), thereby enabling and facilitating its E3 ubiquitin ligase activity.

LBs were conspicuously absent in brains of patients with homozygous *parkin* mutations conferring most likely complete loss-of function (Kitada et al., 1998). However, examination of a compound heterozygous patient carrying an in-frame deletion and a missense mutations with retained biochemical activity, which has been demonstrated to confer a toxic gain-of-function (Cookson et al., 2003), displayed LB pathology (Farrer et al., 2001). Interestingly, some mutations, particularly those located within the UBL and RING domains, cause altered protein localization and aggregation into large cytoplasmic and nuclear aggresome-like structures (Cookson et al., 2003; Gu et al., 2003). Moreover, inhibition of the proteasome as well as co-expression of *parkin* and some of its substrates resulted in formation of LB-like ubiquitin-positive cytosolic inclusions (Ardley et al., 2003; Chung et al., 2001b; Junn et al., 2002; Muqit et al., 2004; Zhao et al., 2003). Therefore, Parkin has been

suggested to be a prerequisite for LB formation (Chung et al., 2001b; Tanaka et al., 2004). Thus, the early and severe form of cell death that occurs in AR-JP may be related to the absence of LB formation and their potential protective effect (Olanow et al., 2004).

A variety of *parkin* knock-out models have already been established in flies (Greene et al., 2003; Pesah et al., 2004) and mice (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004; Von Coelln et al., 2004). Parkin-deficient animals are viable and display only subtle behavioural defects, mitochondrial dysfunctions, as well as alterations of DA metabolism. However, none of the knock-outs showed impairment or loss of dopaminergic neurons of the nigrostriatal pathway (Reviewed by Kahle and Haass, 2004). Surprisingly, none of the known Parkin substrates were found to accumulate in the brains of *parkin* deficient mice (Goldberg et al., 2003; Lorenzetti et al., 2004; Palacino et al., 2004). These data suggest either that redundant ubiquitylation pathways for Parkin substrates may exist, Parkin-mediated ubiquitylation does not target substrates for proteasomal degradation, or Parkin substrates identified so far are erroneous.

However, human Parkin has been suggested to be involved in the UPR pathway, although the molecular mechanism is poorly understood, especially *in vivo*. Upon increased accumulation of misfolded polypeptides, *parkin* expression seems to be induced (Imai et al., 2000). Furthermore, a specific stimulation of Parkin's E3 ligase activity through de-phosphorylation in response to ER stress has been demonstrated (Yamamoto et al., 2004). In addition, Parkin mediates the ubiquitylation of its cell death inducing unfolded target protein, Pael-R, an ER transmembrane protein (Imai et al., 2001), thereby directly suppressing cytotoxicity. The human E4 enzyme CHIP which links protein folding and degradation (Cyr et al., 2002; Murata et al., 2003), is able to augment Parkin E3 ubiquitin ligase activity (Imai et al., 2002). Knock-down of *parkin* in cell culture systems combined with unfolded protein stress either caused by expression of the Parkin substrates α-synuclein or Pael-R, or by proteasome inhibition resulted in neurotoxicity and selective neurodegeneration. Co-expression of *parkin* ameliorated loss of DA neurons (Petrucelli et al., 2002; Yang et al., 2003).

Nevertheless, until now, functional studies on *parkin* have not provided a direct explanation for the pathogenic mechanism of mutations, suggesting that additional animal models may be required to elucidate its biological role.

2.3.3 Other PD-Associated Genes

Besides *parkin*, two other genes have been linked to autosomal recessive forms of PD. Mutations in the DJ-1 gene are the second most frequent cause of recessive PD after *parkin* mutations. DJ-1 seems to be a multifuntional protein, which exerts chaperone activity, senses oxidative stress, acts as an antioxidant, and, mediates multiple protein-protein interactions. Furthermore, it is suggested to be involved in the cellular response to a variety of other stresses (Bonifati et al., 2004). Only few mutations have been found in the PINK1 gene which encodes a putative mitochondrial protein kinase that has been suggested to be involved in cellular stress response (Shen and Cookson, 2004). This suggests that loss of either of those two proteins renders DA neurons more vulnerable to injury, as both have been shown to protect cells against proteasomal dysfunction.

In addition to α-synuclein, two other genes associated with autosomal dominant forms of familial PD have been cloned. A single mutation has been identified in the gene encoding the C-terminal ubiquitin-hydrolase UCH-L1, a neuron-specific de-ubiquitylating enzyme that is necessary for recycling of free ubiquitin. Such a mutation could possibly impair the overall efficiency of proteasomal protein degradation (Chung et al., 2001a). An opposing ubiquitin ligase activity of UCH-L1 created a link to aggregation of α-synuclein (Liu et al., 2002). However, a mouse UCH-L1 deletion model developed neurodegeneration, though distinct from PD pathology (Saigoh et al., 1999). The latest identified PD-associated gene is Dardarin which encodes a leucin-rich kinase (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). However, so far nothing is known about its function or the pathogenic mechanism of the identified mutations.

To date, a number of other candidate genes have been implicated in sporadic and familial cases and may play a minor role in the aetiology of PD. These include genes responsible for DA neuron differentiation and survival, genes involved in DA synthesis, metabolism and function, as well as detoxification enzymes. Although there seems to be some convergence at the cellular level between the genes/proteins associated with familial PD, their exact cellular and physiological roles, as well as the pathogenic mechanisms of mutations are still unknown.

2.4 The Model Organism *Caenorhabditis elegans*

Originally, the invertebrate *Caenorhabditis elegans* (*C. elegans*) was selected by Sydney Brenner because of its rapid life cycle, fecundity, genetic tractability, and simple cellular complexity as a favourable, experimental model to study fundamental aspects of developmental and neuronal biology (Brenner, 1974).

C. elegans, a member of the smooth-skinned, unsegmented roundworms, is a small (~ 1.3 mm; Ø 80 µm), non-parasitic and free-living soil nematode, found abundantly in many parts of the world. At 20° C, *C. elegans* has a generation time of about 3.5 days, developing from an egg through four larval stages (L1 to L4) to the reproductive adult animal, with each stage separated by a molt (Figure 6).

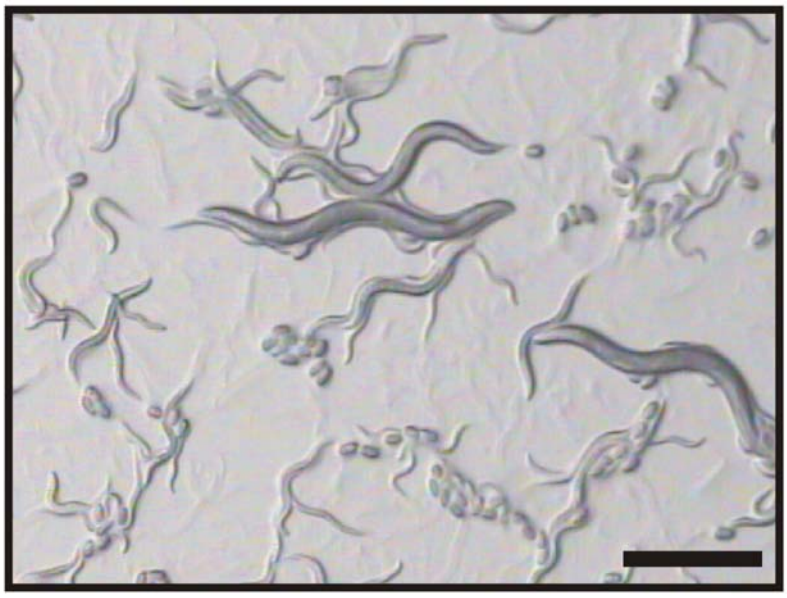


Figure 6. The Nematode *Caenorhabditis elegans*.

Shown are all developmental stages of the N2 wild type strain. *C. elegans* develops from an egg through four larval stages (L1 to L4) to the reproductive adult animal. Scale bar 0.5 mm

C. elegans usually has a short life-span of about 2-3 weeks (Byerly et al., 1976), but, at unfavourable conditions, animals can go through an alternative developmental stage in which a resistant dauer larval form is produced, surviving extreme conditions (desiccation and lack of food) for several months. In the laboratory, *C. elegans* can be easily cultured and maintained with *Escherichia coli* (*E. coli*) as a food source, on an agar substrate in Petri dishes, in liquid culture, or even in microtiter plates, making it amenable to high-throughput approaches. Moreover, stocks can be frozen at -80° C or in liquid nitrogen for indefinite storage.

C. elegans is diploid and has five pairs of autosomal chromosomes (LG I-V) and one pair of sex chromosomes (LG X). Two sexes exist (Figure 7): a self-fertilizing hermaphrodite (XX) which produces both sperms and oocytes; and a male (X0) which occasionally appears at a frequency of ~ 0.2%, as a result of spontaneous X chromosome loss. This hermaphroditism facilitates genetic analysis as the strains are normally propagated asexually, giving rise to a large number of self-progeny (>300), forming clones. However, males, which can be generated experimentally by heat-shock, are capable of mating with hermaphrodites, producing mainly cross-progeny. *C. elegans* has a simple body structure and a small invariant number of 959 somatic cells (1031 in the male) from which the complete cell lineage, from fertilized egg to adult, is known.

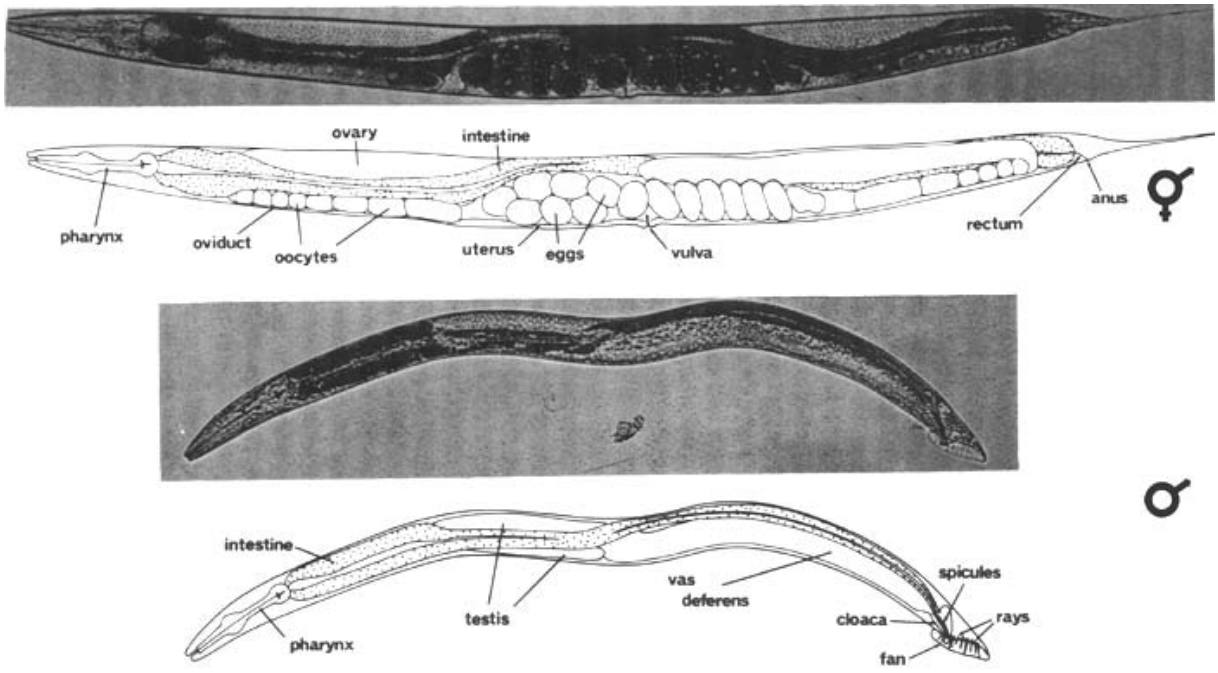


Figure 7. Transmission Light Microscopic Images and Schematic Body Plan of *C. elegans*.
Lateral views of adult wild type (**top**) hermaphrodite and (**bottom**) male animals (Sulston and Horvitz, 1977).

The nervous system, in comparison to the complexity of the human brain that contains over 100 billion neurons, is extremely simple. However, the 302 neurons of the adult hermaphrodite, which on the basis of morphology and connectivity can be assigned to 118 different neuronal classes, represent most of the nerve cells identified in other organisms (White, 1988). Nerve cells in *C. elegans* are small (Ø < 5 µm) and are largely concentrated in a nerve ring, a ventral and a smaller dorsal nerve cord, and a complex head sensory system. Furthermore, the chemical complexity of the nervous system is highly conserved, as *C. elegans* contains many of the known signalling components and neurotransmitter systems (Brownlee and Fairweather, 1999) found in the mammalian nervous system.

A broad set of tools and methodologies for anatomical, behavioural, genetic, transgenic, biochemical, and pharmacological manipulations have been developed. Due to its transparency *C. elegans* is perfectly suited for the analysis of *in vivo* expression patterns of genes, as well as determination of the subcellular localization of their products, as it can easily be transformed with reporter constructs, e.g. green fluorescence protein (GFP) or β-galactosidase, by microinjection or bombardment. *C. elegans* is amenable to both forward (phenotype to genotype) and reverse (genotype to phenotype) genetic approaches, as well as to pharmacological screens involving large numbers of animals. A selection of gene inactivation and deletion strategies are available, ranging from random, chemical mutagenesis, over targeted transposon insertion, to transient loss of function by double stranded RNA (dsRNA)-dependent gene silencing (RNAi). A large collection of more than 2.500 mutants is available of which many have already been experimentally characterized, resulting in a wide spectrum of analyzed phenotypes and behavioural patterns. The 100 megabase (Mb) genome which has been completely sequenced in 1998, encodes a total of about 20.000 predicted genes of which 43% have human homologs, including numerous disease genes (Culetto and Sattelle, 2000).

Given the simplicity and genetic tractability, *C. elegans* is a favourable model organism for the detailed study of gene/protein functions, and has already successfully been used to elucidate the molecular mechanisms involved in different human diseases, including various neurodegenerative disorders (Reviewed by Driscoll and Gerstbrein, 2003).

2.5 Aim of the Work

Many experimental PD animal models, including gene- (knock-out and transgenic) as well as toxin-based systems, have already been established. So far, these have provided some useful insights into the pathogenesis of PD, but the molecular and cellular mechanisms of DA cell death remain enigmatic. None of the current models fully recapitulates all key features, which clinically and pathologically characterize PD. Moreover, fly and mouse *parkin* knockout models generated so far, show only subtle phenotypes, and therefore could not provide a direct explanation for the pathogenic mechanism. Thus, additional model systems might be required to elucidate the biological role of Parkin *in vivo* and to dissect the mechanisms/pathways involved in the pathophysiology of PD.

Most of the known molecular components involved in DA signalling in mammals are also present in the nematode, including biosynthesis, metabolism, transport and re-uptake (Nass et al., 2001; Wintle and Van Tol, 2001). Indeed, DA has already been shown to be used as a neurotransmitter system in *C. elegans*, which is responsible for a variety of different behaviours, including locomotion and egg-laying. In contrast to the 100 thousands of DA neurons in mammals which are rather inaccessible, *C. elegans* has only eight putative mechanosensory neurons (Figure 8) containing DA (Nass and Blakely, 2003).

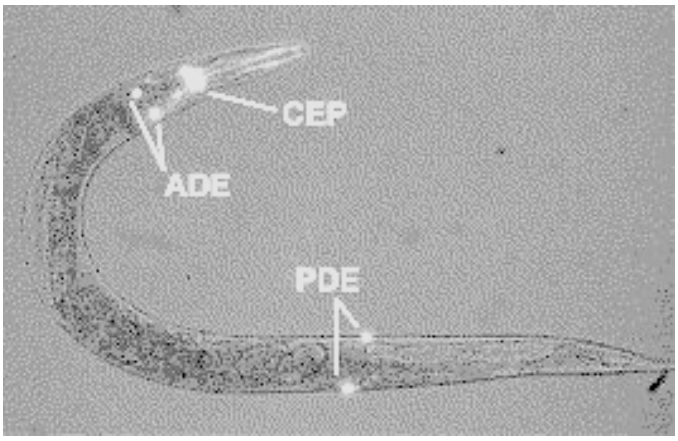


Figure 8. Dopaminergic Neurons in *C. elegans*.

Hermaphrodites contain four symmetrically arranged cephalic cells (CEPs) and two bilateral anterior deirids (ADEs) in the head, as well as two bilateral posterior deirids (PDEs) in the tail. In addition, the male contains another three pairs of DA neurons in the tail, as well as four DA containing male-specific spicule socket cells. Cells were visualized by GFP expression under the promoter of the *C. elegans* DA transporter *dat-1* (Modified from Suo et al., 2004).

Furthermore, with the exception of α-synuclein, which seems to be unique to vertebrates since flies also lack an obvious counterpart, all PD-associated genes are present in the *C. elegans* genome. The nematode genes are well conserved concerning protein sequence and architectural structure of the encoded proteins. Most of the PD-associated genes have single homologs, while others have multiple (Table 2). Moreover, genes involved in the pathways implicated in the pathogenesis of PD, like protein ubiquitylation/degradation (Jones et al., 2002) or the unfolded protein response (Ma and Hendershot, 2001) are also highly conserved from worms to humans.

Homo sapiens	***Caenorhabditis elegans***
α-synuclein	no homolog
parkin	*pdr-1* (K08E3.7)
UCH-L1	F46E10.8, Y40G12A.1, Y40G12A.2
PINK1	EEED8.9
DJ-1	B0432.2, C49G7.11
Dardarin/LRRK2	*lrk-1* (T27C10.7)

Table 2. PD-Associated Genes are Conserved in *C. elegans*.

The table depicts known human PD-associated genes and their homologous *C. elegans* genes (predicted open reading frames).

First attempts have already been made to model degeneration of DA neurons in *C. elegans*. Overexpression of human wild type (WT) or alanine53->threonine mutation (A53T) from a pan-neuronal promoter (*aex-3*) resulted in motor deficits as well as in neuronal and dendritic loss, also of dopaminergic neurons (Lakso et al., 2003). In addition, PD mimetics have already been administered to *C. elegans*. Brief exposure to 6-OHDA caused membrane blebbing of axons and dendrites, as well as selective DA neuron degeneration (Nass et al., 2002). Furthermore, susceptibility of *C. elegans* to MPP^+ treatment and the amelioration of neurotoxicity by anti-PD drugs has also been demonstrated (Braungart, 2004). However, gene knockout studies of PD-associated genes in *C. elegans* have not been performed so far.

Aim of this thesis was to establish an experimental *C. elegans* model for PD, with the main focus on *parkin*, to study its functions as well as the mechanisms of pathogenesis on a molecular and cellular level *in vivo*.

3 Results

3.1 *C. elegans pdr-1* is the Homolog of Human *parkin*

3.1.1 Analysis of PDR-1/Parkin Proteins

By BLAST search analysis, a single *C. elegans* open reading frame (ORF) K08E3.7 with high similarity to human *parkin* was identified, and named *pdr-1* (**P**arkinson's **D**isease **R**elated gene-1). Cloning of the corresponding cDNA by reverse transcriptase coupled polymerase chain reaction (RT-PCR) revealed an additional coding exon (exon IV) not recognized by gene predictions. It encodes a 386 amino acid protein, PDR-1, sharing the same characteristic domain structure with human Parkin, along with 29%/41% overall amino acid sequence identity/similarity, and up to 50%/69% in highly conserved domains, respectively (Figure 9).

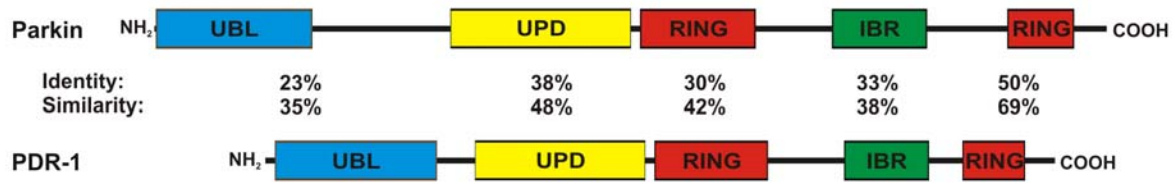


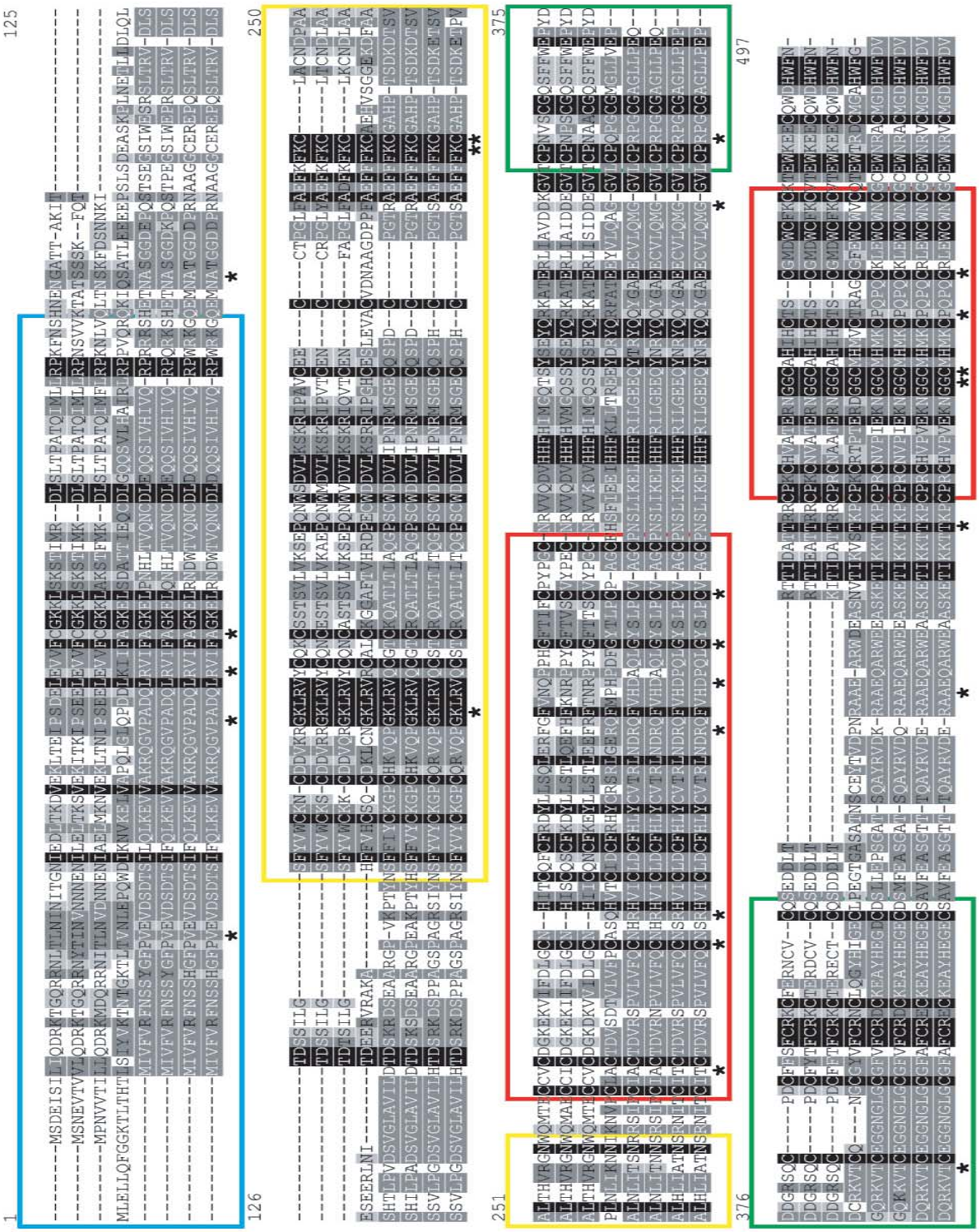
Figure 9. Domain Structure of Human Parkin and *C. elegans* PDR-1 Protein.

The respective domains are colour-boxed: **blue:** ubiquitin-like domain (UBL); **yellow:** unique parkin domain (UPD); **red:** C_3HC_4 ring-finger domains (RING); **green:** C_6HC in-between ring-finger domain (IBR). Identity and similarity values of amino acid sequences for each domain are shown.

For sequence comparison, human and fly *parkin* cDNAs were amplified from the respective libraries, the homologous *pdr-1* genes from two related nematodes of the genus *Caenorhabditis sp*. were identified, and the corresponding cDNAs were cloned by RT-PCR. Alignment of the sequences revealed high conservation of Parkin proteins, and suggests an important function across all species (Figure 10).

Figure 10 (shown on next page). PDR-1/Parkin Protein Sequence Alignment.

Length of PDR-1/Parkin proteins in amino acids (aa): ***Caenorhabditis elegans*:** 386 aa; ***Caenorhabditis briggsae*:** 385 aa; ***Caenorhabditis remanei*:** 387 aa; ***Drosophila melanogaster*:** 468 aa; ***Mus musculus*:** 464 aa; ***Rattus norvegicus*:** 465 aa; ***Bos taurus*:** 465 aa; ***Homo sapiens*:** 465 aa. Black shading indicates sequence identity, grey shading sequence similarity. The different domains are colour-boxed as described in Figure 9. Asterisks indicate positions of familial PD missense mutations in human *parkin* (Mata et al., 2004).



C. elegans, *C. briggsae* and *C. remanei* PDR-1 show the same degree of sequence conservation to human Parkin, and about 80% sequence identity among each other. However, despite the already mentioned domains, bioinformatic analyses predicted a eukaryotic thiol (cysteine) protease active site at the extreme C-terminus only for vertebrate Parkin proteins. Why fly and worm Parkin proteins lack this domain and whether vertebrate Parkin indeed has additional function(s) is unclear. Nevertheless, many of the identified human familial Parkin mutations are highly conserved among all species, underlining their importance. Some of these affect important cysteine residues of the RING box, and might therefore interfere with folding. Others affect putative modification sites, and might therefore abrogate ubiquitin-linkage or phosphorylation.

3.1.2 *pdr-1* Gene Structure

C. elegans pdr-1 is located on the extreme 3` end of the right arm of chromosome III (LGIII), in close vicinity to its adjacent genes (Figure 11). The upstream ORF *cyk-4* (K08E3.6) is located in a head-to-head orientation, whereas *pdr-1* and its downstream gene K08E3.8 form an operon, and thus are co-transcribed from a single promoter (Blumenthal et al., 2002).

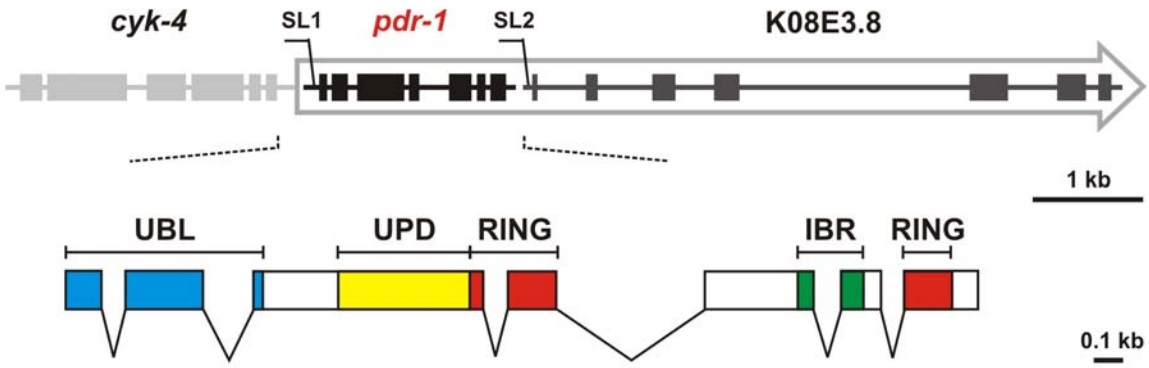


Figure 11. Genomic Organization and Gene Structure of *C. elegans pdr-1*.

Top: The gene structure and relative positions of *pdr-1* and its neighbouring genes are shown. Coding exons are depicted as boxes, introns as lines. An arrow indicates the *pdr-1*/K08E3.8 operon, whereas the upstream gene *cyk-4* (K08E3.6) is orientated into the opposite direction. *pdr-1* is trans-spliced to splice leader SL1, the downstream gene K08E3.8 is trans-spliced to SL2. Scale bar: 1 kb. **Bottom:** Annotated view of the *pdr-1* gene structure. Coding exons are indicated as boxes, introns as lines. Parts encoding the different domains are colour-boxed, as described in Figure 9. Scale bar: 0.1 kb.

Such transcriptional units are frequently used in *C. elegans* (~20% of all genes are organized within an operon) as a mechanism to co-regulate genes involved in fundamental cellular processes. By contrast, genes that encode tissue- or cell-type specific proteins are never found in operons. *C. elegans* operons are clusters (2-8 genes per operon) of closely spaced genes (~100bp), which are transcribed on a single polycistronic precursor mRNA from a promoter at the 5' end of the gene cluster. This pre-mRNA is processed co-transcriptionally by 3' end formation to generate monocistronic mRNAs, which are spliced in *trans* to short diverse splice leaders (SL). The first gene in an operon is always spliced to SL1, whereas the following ORFs are spliced to SL2 or its variants, as an unique feature of downstream genes (Blumenthal and Gleason, 2003). RT-PCR analyses of the trans-splicing mechanism confirmed the association of *pdr-1* cDNA with SL1, whereas the downstream genes K08E3.8 showed SL2 specific trans-splicing. As both genes are indeed co-transcriptionally regulated by a single promoter, the downstream gene K08E3.8 served as an internal control in this study.

3.1.3 Comparative Genomics of the *pdr-1* Operon

Comparative genomics of homologs from closely related organisms often allow the identification of important regulatory promoter and enhancer elements as well as the unambiguous assignment of coding sequences. *C. elegans*, *C. briggsae*, and *C. remanei* are estimated to have diverged about 80-100 million years ago (Stein et al., 2003). Even though non-coding sequences have usually fully diverged within this period of time, exon/intron structures and control elements driving expression of homologous genes are often conserved.

The genomic region spanning the entire *pdr-1*/K08E3.8 operon was subcloned from the related nematode species by a 'PCR Walking' strategy, using single circularized genomic DNA fragments as a template and a combination of designed species specific and degenerated primers. Comparison of the deduced genomic sequences of both genes revealed conservation of *pdr-1* and K08E3.8 genes and the operon structure (Figure 12).

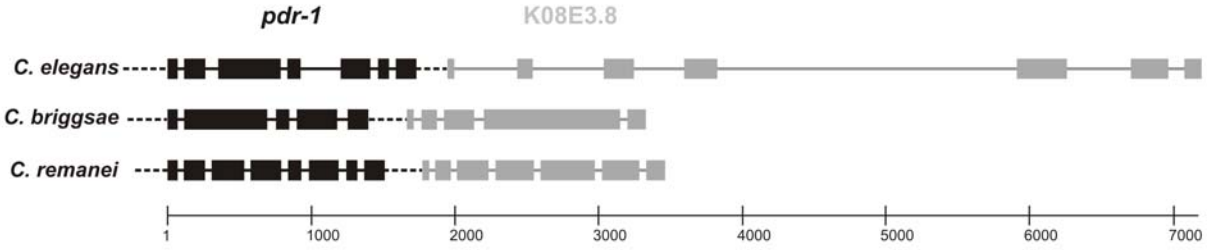


Figure 12. Comparison of the *pdr-1*/K08E3.8 Gene Structure from the Genus *Caenorhabditis sp*.
Exons are depicted as boxes, intron as lines, intergenic regions as dotted lines. **Black:** *pdr-1*; **grey:** K08E3.8.

However, *C. elegans* introns of both genes, and in particular of K08E3.8, are much larger compared to the corresponding introns in the related nematodes. In particular, intron IV of *C. elegans* K08E3.8 contains two 768 bp inverted repeats, and is therefore unusually large (2031 bp) compared to a mean *C. elegans* intron size (67 bp) (Stein et al., 2003). In addition, *C. briggsae* contains fewer introns in both genes, whereas *C. remanei pdr-1* encloses more introns than in *C. elegans*, in line with data from other analyzed genes (Stein et al., 2003).

The identified genomic sequences were aligned using Dialign software (http://bibiserv.techfak.uni-bielefeld.de/dialign/). Then, sequences were further analyzed with MatInspector (http://genomatix.gsf.de/cgi-bin/matinspector.pl) and TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCHJ.html) software. Sequences from single introns that are not present in the analyzed nematodes, were neglected, and therefore excluded from the search for important regulatory elements. *In silico* analyses of *pdr-1* promoter sequences, about 650 bp 5' of the translational start could not reveal apparent TATA or CCAAT boxes in any of the analyzed nematode species. In contrast to human *parkin* promoter, which also lacks these boxes, but is driven by CpG islands, the promoter of *pdr-1* is not very GC-rich in sequence (Asakawa et al., 2001; West et al., 2001). However, search for binding sites of transcriptional activators and repressors in the *pdr-1* promoter revealed some promising candidates, for example members of the cAMP-responsive element binding (CREB) protein family, including ATF6. Furthermore, binding sites for *C. elegans* cell-death specification protein CES-2 and the maternal gene product SKN-1 were detected. A complete list of all predicted transcription factor binding sites can be found in Tables 14-16 (see Appendix Section).

Although these putative binding sites are not always fully conserved among all three nematodes regarding their orientation and position, there is at least some partial overlap. However, these data have to be carefully interpreted, and, thus, physiological relevance could not be proposed for any. In addition, it is likely that additional regulatory elements exist in the nematode further upstream, or probably even downstream of the operon. Nevertheless, these data might give first insights into the transcriptional regulation of *pdr-1* and should further help to identify key enhancer or repressor elements.

3.1.4 The Downstream Gene K08E3.8

Although functional correlation between genes of the same operon it not necessarily presupposed, relationship between single genes from *C. elegans* operons have already been described (Blumenthal and Gleason, 2003). In order to test for a functional correlation of *pdr-1* and its downstream gene, K08E3.8 was subjected to further analyses. Cloning of the K08E3.8 cDNA by RT-PCR confirmed that the exon-intron structure of was correctly predicted by genefinder. K08E3.8, previously named *mdt-29* as a member of the **M**e**D**ia**T**or gene class, shares only moderate homology with yet identified genes from other organisms. However, it shows similarity to human Q96RN5 positive cofactor 2 glutamine/Q-rich-associated protein (PCQAP), implicated in schizophrenia and DiGeorge/velocardiofacial syndrome (DGS/VCFS), as well as to *S. cerevisiae* CYC8, which both act as parts of transcriptional co-repressor/co-activator complexes (Berti et al., 2001; Conlan et al., 1999).

K08E3.8 encodes a 441 aa protein with proline (P)- and glutamine/asparagine (Q/N)-rich ('prion') domains. Proteins bearing ‘prion’ domains can sometimes exist in at least two different physical states, mediated by conformational changes in the Q/N-rich domain. Prion domains are both modular and transferable to other proteins, on which they can confer a heritable epigenetic alteration of function. It is important to note that this is distinct from, though mechanistically analogous to, disease states associated with prion propagation and amyloidogenesis (http://www.wormbase.org/). In addition, K08E3.8 carries a mitochondrial energy transfer protein-signature for inner membrane transport. To identify conserved amino acids and important domains of K08E3.8, the corresponding cDNAs from the related nematode species were cloned by RT-PCR and analyzed (Figure 13).

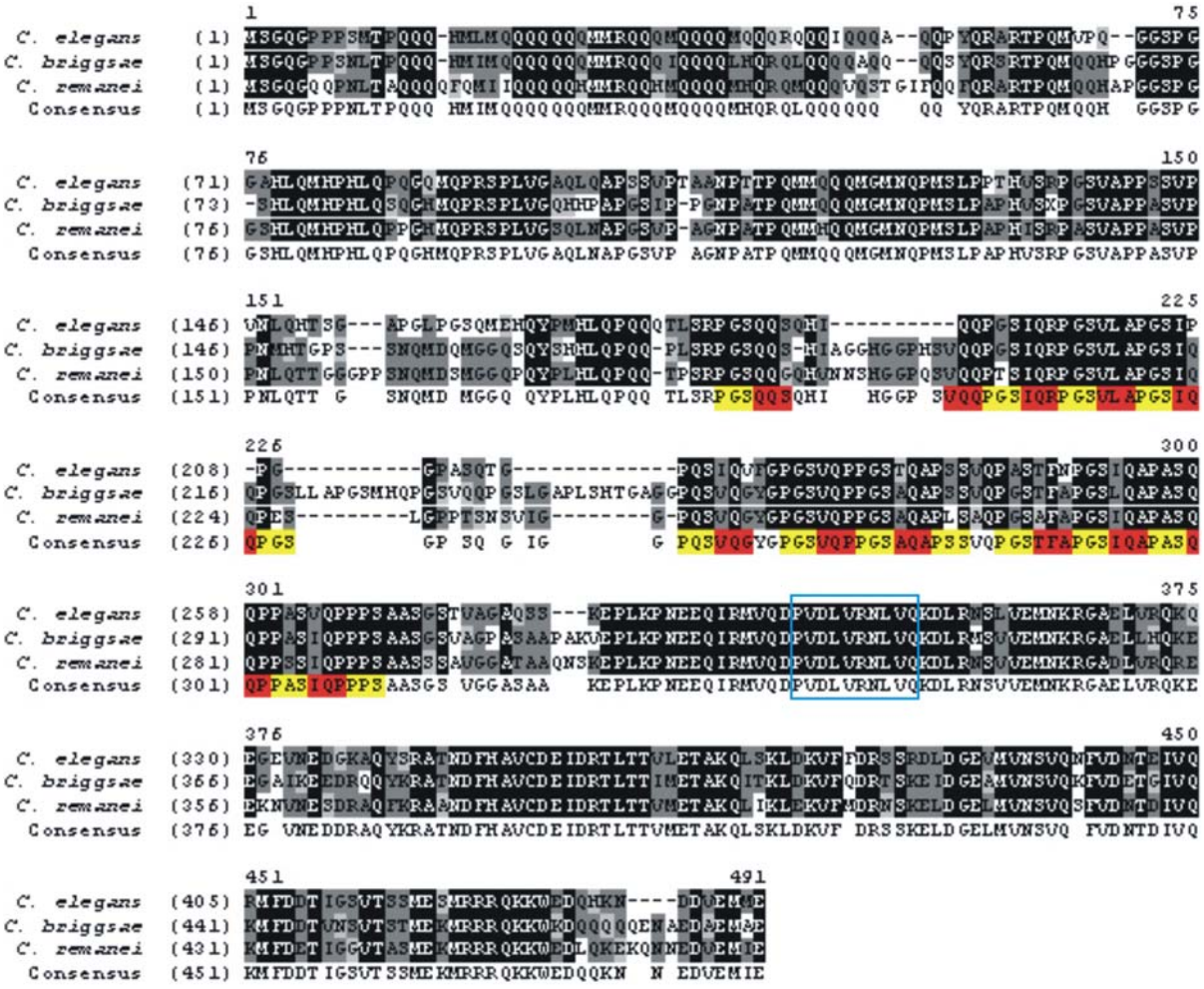


Figure 13. K08E3.8 Protein Sequence Alignment.

Length of K08E3.8 proteins in amino acids (aa): ***Caenorhabditis elegans*****:** 442 aa; ***Caenorhabditis briggsae*****:** 482 aa; ***Caenorhabditis remanei*****:** 472 aa. Black shading indicates sequence identity, grey shading sequence similarity. The N-termini consist of glutamine- and proline-rich domains. The mitochondrial substrate carrier motif is marked by a blue box. Internal repeats are colour highlighted.

The mean percent identity between orthologs from *C. elegans* and *C. briggsae* at the amino acid level is 75%, similar to the divergence between mouse and human protein pairs (Stein et al., 2003). In contrast to PDR-1, K08E3.8 protein is less conserved among the nematode species (~ 69% average sequence identity on amino acid level). Since in other organisms no definite K08E3.8 homologs have been identified, the biological role of K08E3.8 remains unclear. Nevertheless, its similarity to transcriptional co-repressor/co-activator subunits, and in addition, its association with the homeobox transcription factor CEH-40 in a yeast-two-hybrid system (Li et al., 2004) and with SEL-7 in *vitro* (http://www.wormbase.org/), might suggest a nuclear role. However, the functional significance of these interactions is unknown.

3.2 Expression Analysis of the *pdr-1* Gene

3.2.1 Alternative Splicing of *pdr-1*

Alternative splicing has an important role in expanding protein diversity. Differential splicing of the *parkin* gene has been observed not only in different organisms, but also in different human tissues or cell types, and is suggested to be physiologically relevant (Dagata and Cavallaro, 2004; Kitada et al., 2000; Sunada et al., 1998).

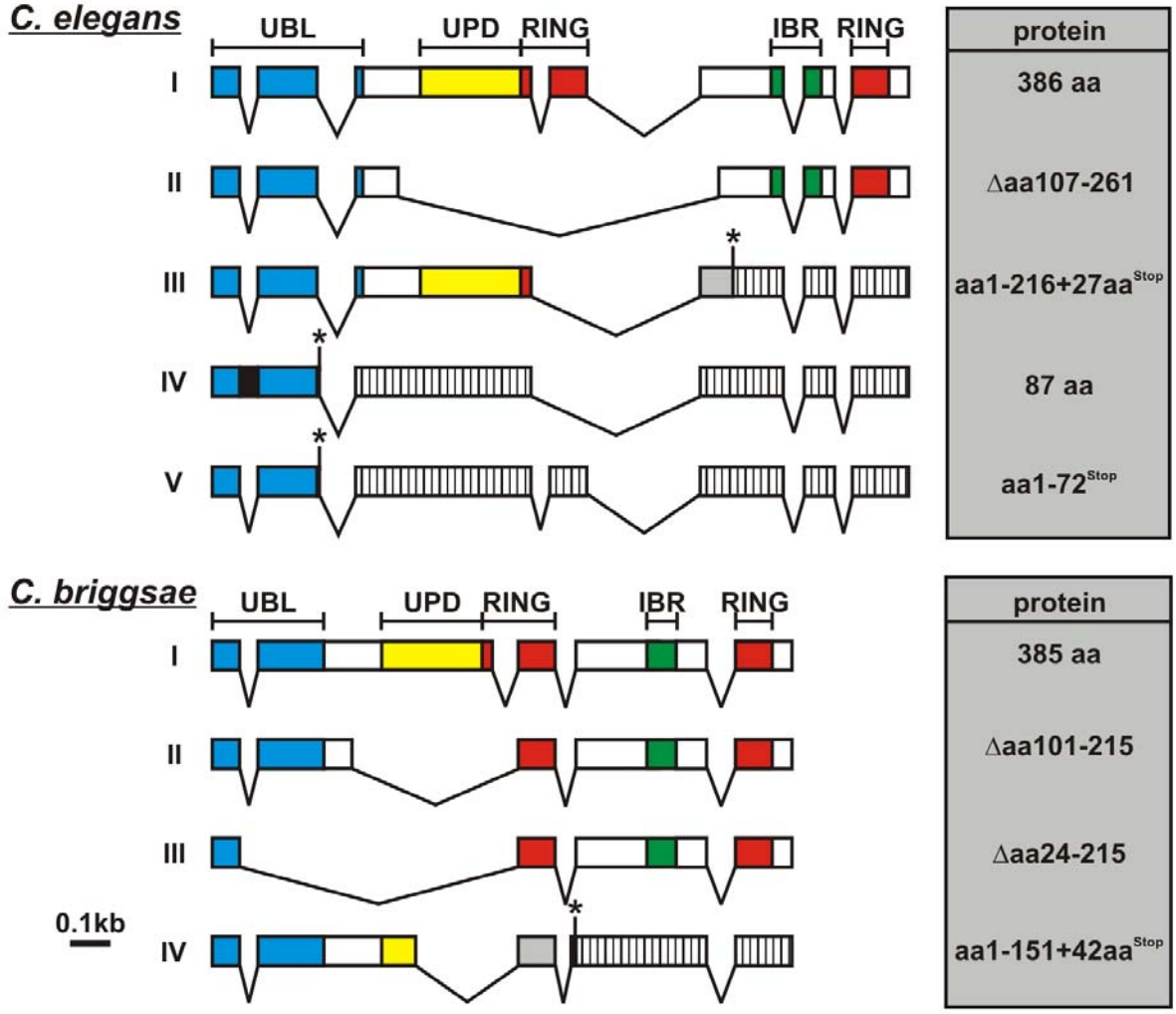


Figure 14. *pdr-1* Splicing Variants and Protein Isoforms.

Top: *C. elegans* splice variants. **I:** full-length *pdr-1* (1158 bp); **II:** in-frame (Δ bp 321-784); **III:** out-of-frame (Δ bp 649-740); **IV:** out-of-frame (45 bp insertion after position 69, 4 bp insertion after position 215, and Δ bp 649-740); **V:** out-of-frame (4 bp insertion after position 215). **Bottom:** *C. briggsae* splice variants. **I:** full-length *pdr-1* (1155 bp); **II:** in-frame (Δ bp 301-645); **III:** in-frame (Δ bp 70-645); **IV:** out-of-frame (Δ bp 454-645, insertion of 8 bp after position 736). Asterisks indicate premature stops, followed by un-translated regions (hatch boxes). Black shading depicts newly spliced coding regions, grey shading shift into another reading frame. Scale bar 0.1kb.

By RT-PCR on total RNA, five different *pdr-1* splice variants were identified from *C. elegans*, and another four of the *C. briggsae* homolog (Figure 14). The encoded PDR-1 isoforms have different amino acid compositions, and most important, different molecular architectures. Some of the nematode *pdr-1* splice variants do not perfectly resemble the already identified *parkin* transcripts, others however are well conserved, even in humans. Minor variations between nematode and mammalian *parkin* splice variants, certainly arise from different gene structures and splice sites, which are highly conserved among rat, mouse and human, but distinct in nematodes. However, alternative splice variants detected so far, might only represent a subset and the total number might be far greater, but one cannot exclude different evolutionary conservation for some *parkin* splice variants, as well.

Alternative splicing of *parkin* could potentially generate a large number of protein isoforms that might impart different properties on the cells displaying them. Furthermore, the expression of the different isoforms could be differentially affected by mutations of the *parkin* gene. This might provide an explanation for the broad spectrum of phenotypic abnormalities observed in AR-JP patients.

3.2.2 *pdr-1* Transcription is Developmentally Regulated

To analyze the temporal expression pattern of *pdr-1*, Northern blot analyses were performed using total RNA from each developmental stage of *C. elegans* wild type animals. *pdr-1* transcription becomes active in embryogenesis and is maintained throughout all developmental stages until adulthood (Figure 15).

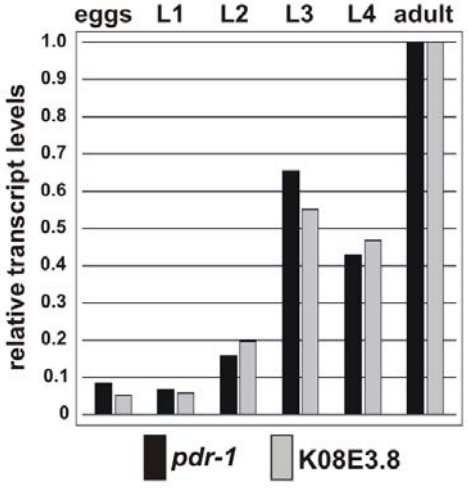


Figure 15. *pdr-1* is Developmentally Regulated.

Northern blot analyses show co-transcriptional regulation of *pdr-1* and K08E3.8 during all developmental stages, from embryogenesis (eggs) throughout larval stages (L1-L4) until adulthood (adult). *pdr-1* and K08E3.8 transcript levels are specifically up-regulated beginning in L2 and strongly increasing in L3. All transcript levels indicated are relative to young adult levels and were adjusted for equal loading with the corresponding *ama-1* level.

Notably, *pdr-1* as well as K08E3.8 transcript levels are specifically up-regulated beginning at the larval L2 stage and strongly increasing at the L3 stage, reaching a maximum in the adult. These data corroborate the proposed transcriptional co-regulation of PDR-1 and K08E3.8, and additionally suggest development-specific function(s).

3.2.3 *pdr-1 in vivo* Expression Pattern

The *parkin* gene has been shown to display a widespread expression, not only in humans, but also in a variety of other vertebrates and invertebrates (Horowitz et al., 2001; Huynh et al., 2001; Solano et al., 2000; Stichel et al., 2000).

To determine the expression pattern of *C. elegans pdr-1 in vivo* different green fluorescent protein (*gfp*) reporter constructs were generated. Two variants of a promoter *gfp* construct (P_{pdr-1}*::gfp* long and short) were generated, containing either 4.0 kb or at least 650 bp of upstream sequence, fusing the *pdr-1* start codon to the *gfp* coding region (plasmids pBY1013 and pBY1909, respectively) (Figure 16). In addition, to identify the subcellular localization of PDR-1, a translational fusion construct P_{pdr-1}*::gfp::pdr-1* (plasmid pBY1794) was generated. To ensure proper expression of the reporter gene, this constructs retains the complete genomic context of the operon, including both genes of the transcriptional unit (Figure 16).

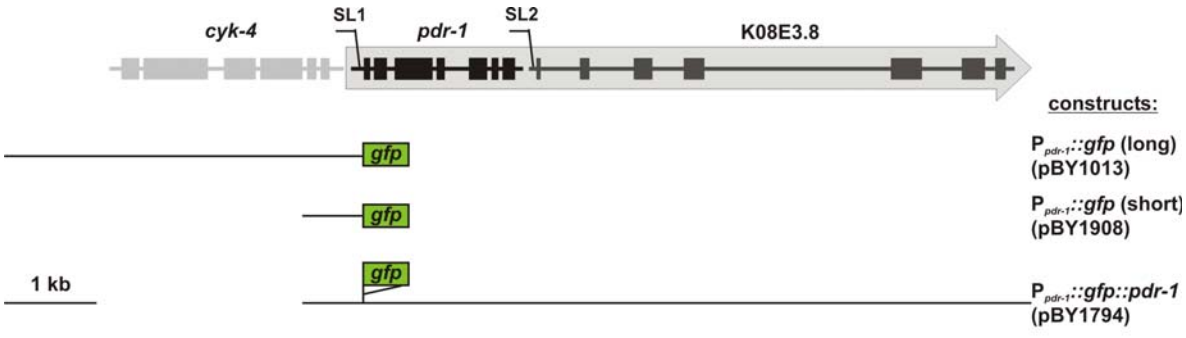


Figure 16. *pdr-1* Reporter Constructs.

Position and extent of the *gfp* reporter constructs (P_{pdr-1}*::gfp* and P_{pdr-1}*::gfp::pdr-1*) relative to *pdr-1* are shown. Lines represent genomic sequences contained in the different reporter constructs. Promoter *gfp* fusions (P_{pdr-1}*::gfp*) contain either ~4.0 kb or 650 bp upstream sequence (long and short, respectively). In P_{pdr-1}*::gfp::pdr-1* the *gfp* coding sequence is fused in-frame to the *pdr-1* translational start to yield a N-terminal tagged GFP::PDR-1 protein.

The engineered *pdr-1* reporter constructs were microinjected into N2 wild type worms to obtain stable lines of transgenic animals, expressing the *gfp* fusions from extrachromosomal arrays (Figure 17).

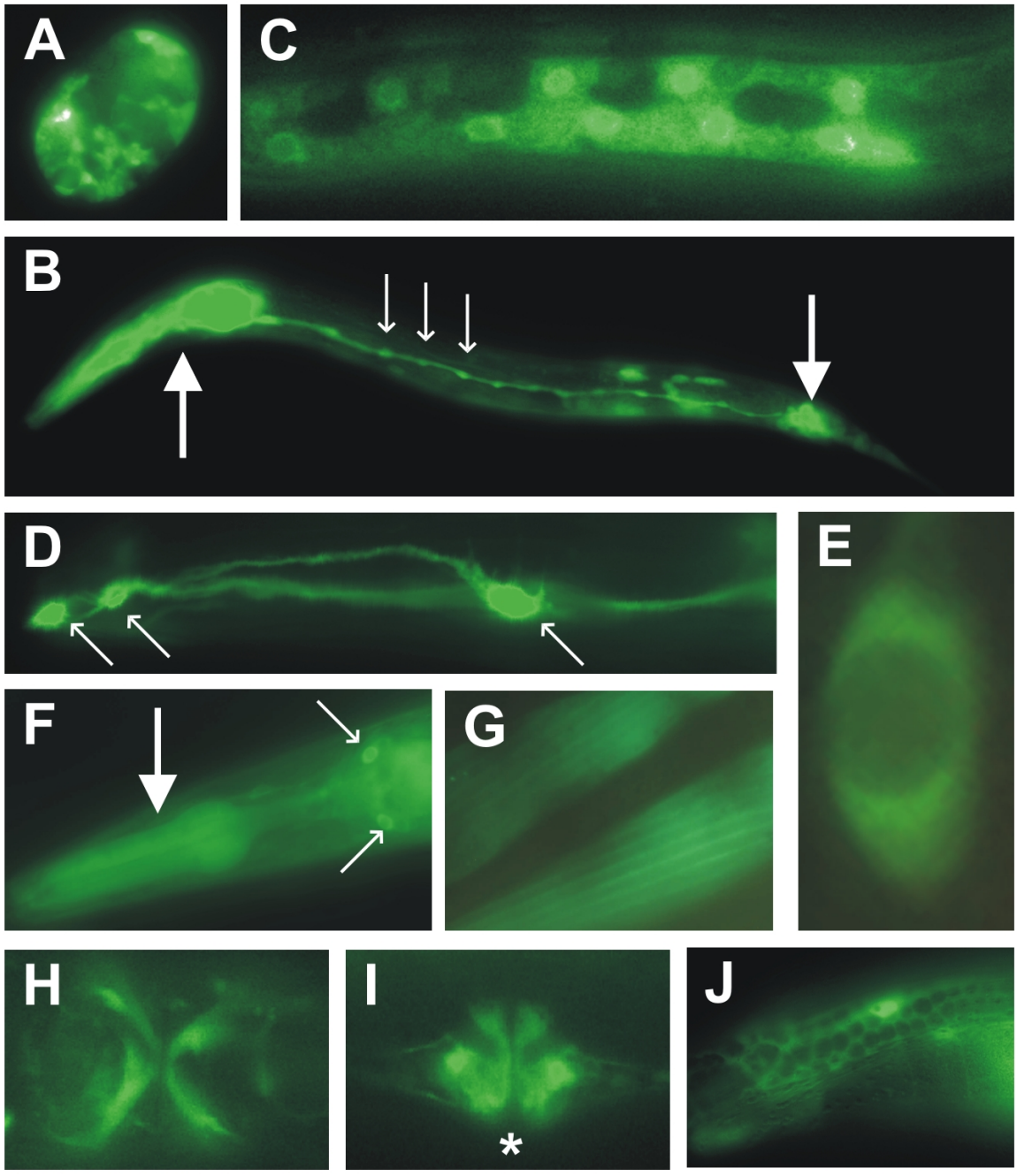


Figure 17. *C. elegans pdr-1* is Ubiquitously Expressed.

Transgenic expression of different *pdr-1::gfp* reporter constructs in N2 wild type animals. (**A**) Embryo expressing *gfp* in almost all cells. (**B**) L2 larval *gfp* expression in pharyngeal and anal muscles (closed arrows) as well as in neurons of the ventral nerve cord (open arrows). (**C**) L3 larval *gfp* expression in hypodermal cells. (**D**-**J**) *gfp* expression in almost all tissues of adult worms. (**D**) Cell bodies (open arrows) and processes of head neurons. (**E**) Cytoplasmatic localization of GFP::PDR-1 in a neuron. (**F**) Pharyngeal muscles (closed arrow) and neurons of the head (open arrows). (**G**) Body-wall

muscles. (**H**) Vulval muscles (ventral view). (**I**) Vulval muscles (lateral view). The vulva opening is marked by an asterisk. (**J**) Gonadal *gfp* expression.

Although mosaic in individual worms, *gfp* expression patterns were almost identical in all 12 independent transgenic strains examined In total, 12 independent transgenic lines were analyzed, six for the promoter constructs pBY1013 and pBY1909 (strains BR1948 and BR3187-91, respectively), as well as another six for the translational fusion construct pBY1794 (strains BR3045-50).

In vivo analysis of the *gfp* reporter constructs confirmed the temporal expression pattern of *pdr-1* observed in transcriptional analyses. GFP signals were detected from embryogenesis (Figure 17A) throughout all developmental stages (Figure 17B and C) until adulthood (Figure 17D-J). GFP::PDR-1 is highly expressed in most neurons of the head, the tail and the nerve cords, localizing to cell bodies as well as to processes (Figure 17B, 17E-F). GFP staining is mostly cytoplasmic and mainly excluded from the nucleus (Figure 17E). Furthermore, GFP signal was observed in all muscle cells (Figure 17B and 17F-I), as well as in a variety of other tissues, like hypodermal cells (Figure 17C) and gonads (Figure 17J), as well as spermatheca and intestine.

Noteworthy, it appeared that both promoter constructs, although injected at the same concentration as the translational fusion construct, showed slightly stronger GFP signal, suggesting a regulation of PDR-1 at the protein level, most likely by degradation. However, PDR-1 is enriched in neurons and muscles, but present in almost all tissues of the animal, and so conceivably plays an important role in all cells.

3.3 Biochemical Analysis of PDR-1 Protein

3.3.1 Yeast-Two-Hybrid Protein Interaction Studies

To investigate the role of PDR-1, the yeast-two-hybrid system was used to screen for interaction partners. Full-length *pdr-1* cDNA fused to the Gal4p DNA-binding domain (DB) was used as a bait to screen two different *C. elegans* Gal4p activation domain (AD) cDNA libraries. To reduce false positives, high stringency screens were performed, selecting on expression of four different reporter gene markers. However, low-affinity proteins, which mediate only weak interaction, may have been missed by this approach.

In summary, three independent screens were performed, evaluating about 150.000 transformants in total. 57 of 70 putative interactors were classified as true positives, which showed a significant reproducible phenotype after plasmid isolation and retransformation of yeast. These true positives represent 40 different genes in total, since for some of them 2-8 independent clones have been isolated. The identified PDR-1 interactors are involved in a variety of biological processes. Interestingly, many of the identified genes could be assigned to protein degradation pathways (Figure 18).

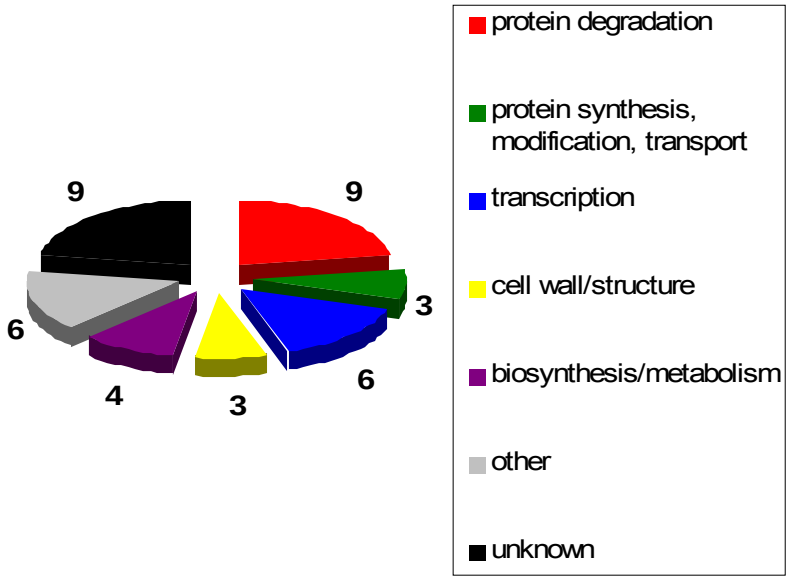


Figure 18. Biological Functions of Identified PDR-1 Interactors.

Distribution of PDR-1 interactors among various biological functions. Shown are the numbers of PDR-1 interactors identified in the yeast-two-hybrid screen that are implicated in the respective biological functions. Other biological functions include for example mitochondrial transport and RNA-binding. A complete and annotated list of all identified PDR-1 interacting clones can be found in Table 17 (see Appendix Section).

The remaining genes identified in the PDR-1 interaction screen, were dispersed among numerous and diverse functional categories, like gene transcription, cell structure, different metabolism pathways, transport, signalling pathways, or were determined to be of complete unknown function.

Most prominently represented were members of four distinct classes of the papain superfamily of cysteine (thiol) proteases (Figure 19): CPL-1, a homolog of human cathepsin L (ORF T03E6.7, eight clones); CPR-4, CPR-6 and F57F5.1, homologs of human cathepsin B (ORF F44C4.3, two clones; ORF C25B8.3, one clone; F57F5.1, two clones, respectively); ASP-3, a homolog of human cathepsin D (ORF H22K11.1, one clone). Cathepsins have long been thought to function exclusively in the terminal degradation of proteins in the lysosomes, but recent findings suggested physiological functions in other compartments (Goulet et al., 2004; Reinheckel et al., 2001). Even more interesting, age-related lysosomal changes and spillage of hydrolytic enzymes from lysosomes into the cytoplasm has been shown to correlate with necrotic cell death in neurodegeneration (Syntichaki and Tavernarakis, 2003).

Figure 19. PDR-1 Interacts with Several Cathepsins.

Yeast Two-Hybrid studies of PDR-1 interactions. Growth analysis of cells expressing the indicated combinations of control (-), PDR-1 and its associated proteins, spotted in doublets on selective medium plates (lacking leucine, tryptophan, histidine, and adenine). Strength of PDR-1/cathepsin interactions judged by growth on increasing concentrations: T03E6.7 > H22K11.1 > C25B8.3 > F44C4.3 > F57F5.1.

Many other identified PDR-1 interactors are related to the ubiquitin/proteasome-dependent protein degradation pathway. Two independent clones were isolated for RPT-2, a 19S regulatory subunit of the *C. elegans* proteasome (Figure 20A). The UBL domain of human Parkin has already been shown to mediate coupling to the proteasome by direct interaction with the subunits Rpn10 and Rpt6 (Sakata et al., 2003; Tsai et al., 2003). Furthermore, PDR-1 interacts with F52C6.2, an ubiquitin-like protein that might represent a new modifier homologous to ubiquitin (Reviewed by Jentsch and Pyrowolakis, 2000; Schwartz and

Hochstrasser, 2003). In addition, PDR-1 associates with F49C12.9 that contains an ubiquitin-associated (UBA) domain, capable of binding UBL domains as well as multi-ubiquitin chains (Buchberger, 2002). Interestingly, CHN-1 (Hoppe et al., 2004), the *C. elegans* homolog of human E4 enzyme CHIP which regulates Parkin E3 ligase activity (Imai et al., 2002), was identified as a co-enzyme of PDR-1 (Figure 20B). Moreover, PDR-1 specifically associates with *C. elegans* E2 enzymes UBC-2, UBC-18 and UBC-15 (Figure 20C), homologs of the human E2 enzymes UbcH4/5, UbcH7/8, and Ubc6, which cooperate with Parkin (Imai et al., 2002; Shimura et al., 2001; Zhang et al., 2000). However, PDR-1 does not interact with other *C. elegans* E2 enzymes UBC-14, UBC-6, or UBC-7, homologs of Ubc7 (Jones et al., 2002).

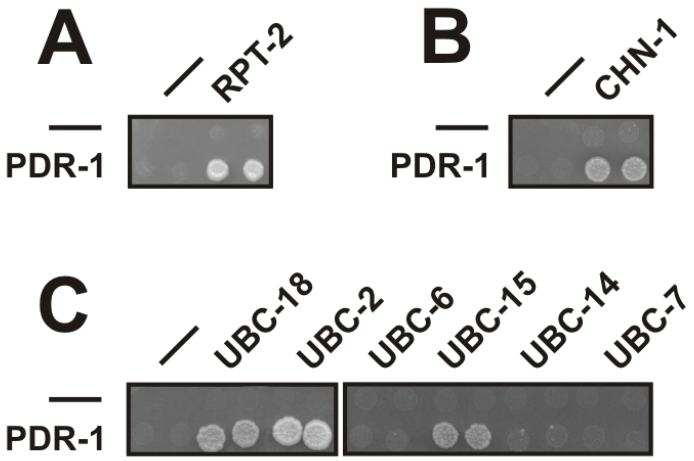


Figure 20. PDR-1 Interacts with a Highly Conserved *C. elegans* Protein Degradation Machinery.

Yeast-Two-Hybrid analysis as described in Figure 19. (**A**) PDR-1 associates with the *C. elegans* proteasome via the 19S regulatory subunit RPT-2. (**B**) PDR-1 binds to CHN-1, homolog of the human E4 enzyme CHIP. (**C**) PDR-1 specifically associates with *C. elegans* E2 enzymes UBC-18, UBC-2 and UBC-15, but not with UBC-14, UBC-6 or UBC-7.

As PDR-1 was found associated with the RING finger protein, T24D1.3, the yeast-two-hybrid technique was used to further analyze its dimerization ability. PDR-1 is indeed able to homo-dimerize with itself, as well as to hetero-dimerize with T12E12.1 (Figure 21A), the *C. elegans* homolog of fly ariadne-2 another member of the RING-box superfamily. Consistently, T12E12.1 itself is able to homo-dimerize and partially binds the same *C. elegans* E2 enzymes like PDR-1. The homolog of *C. elegans* UBC-18, human UbcH7/8 has already been already shown to bind to the human homolog of Ariadne (HHARI) (Ardley et al., 2001).

Since co-transcriptional regulation of *pdr-1* and its downstream gene K08E3.8 have been confirmed, a functional relationship of both gene products was tested. Interestingly, PDR-1 indeed physically associates with K08E3.8 (Figure 21B). It is noteworthy, that also other Q/N-rich ('prion') domain containing proteins were

identified as PDR-1 interactors, PQN-32 and PQN-38 (this study), as well as PQN-95 from an independent screen (Li et al., 2004).

Figure 21. PDR-1 Dimerizes and Interacts with K08E3.8.

Yeast-Two-Hybrid analysis as described in Figure 19. (**A**) Homo- and hetero-dimerization of PDR-1. PDR-1 binds to itself and to another RING box protein T12E12.1, the *C. elegans* homolog of human Ariadne-2. (**B**) PDR-1 physically interacts with K08E3.8, suggesting a functional relationship between both genes from the operon.

Both, homo-dimerization of PDR-1 as well as interaction of PDR-1 and K08E3.8 have been confirmed by an alternative method, using the split-ubiquitin system (D. Dirnberger, personal communication). However, why PDR-1 and K08E3.8 interact with each other remains elusive. Nevertheless, the co-transcriptional regulation of both genes from the operon as well as the physical interaction of their products indicate an interesting functional connection between PDR-1 and K08E3.8.

In addition to new PDR-1 partners identified in this study, many of the reported protein interactions of human Parkin were shown to be highly conserved in *C. elegans*. However, some interactions of human Parkin could not be demonstrated for *C. elegans* PDR-1, at least not using the yeast two-hybrid system. In yeast, PDR-1 does not interact e.g. with HSP-1, a Hsp-70 chaperone homolog, or with UNC-59 and UNC-61, homologs of the human Parkin substrate CDCrel-1.

It is worth mentioning that at least one of the newly identified PDR-1 interactors (ORF Y39B6A.1) has also been recognized in an independent screen (Li et al., 2004), supporting the functional relevance of these studies. From this *C. elegans* genome-wide protein-interaction study, great data is available, including information about further binding partners of the PDR-1 interactors identified here. By integration of the already published data (Li et al., 2004) into the novel PDR-1 interaction data described in this study, an extended PDR-1 protein interaction map was established using i-View software (http://vidal.dfci.harvard.edu/). This '2nd level' interaction map enabled to detect distinct connections between different PDR-1 interactors, resulting in a complex network.

So, in a first step, PDR-1 interactors were identified that share other binding partners, in addition to PDR-1. Interestingly, more than half of all PDR-1 interactors associate with at least one other PDR-1 interactor. In particular, e.g. the gene product of Y39B6A.1 binds to twelve other proteins identified as PDR-1 interactors. Y39B6A.1 shows some similarity to human Hornerin, a protein rich in glycine, serine, and glutamine residues. A second gene product C39D10.7, binds to ten other PDR-1 interactors. Two independent clones encoding this protein that is implicated in chitin metabolism and shares homology to the Mucin 2 precursor have been identified as PDR-1 interactors in this study. Even more interesting, the already mentioned ubiquitin-like modifier F52C6.2 associates with eight other PDR-1 interacting proteins. From this finding certainly, an important question arises: Does PDR-1 simply bind to F52C6.2 protein, or additionally, does PDR-1 catalyze the conjugation of F52C6.2 protein to substrate(s) as an E3 ligase, in a manner analogous to other ubiquitin-like modifiers? Other interesting candidates, like the nuclear hormone receptor NHR-111 (ORF F44G3.9), identified from the genome-wide screen as a PDR-1 interactor (Li et al., 2004), or the gene product of F29G6.3 that shows some homology to Y39B6A.1, associate with seven other proteins identified as binding partners of PDR-1. Twelve other candidate proteins not further mentioned here bind to at least 2-4 other known PDR-1 interactors.

Furthermore, in a second step, binding partners that are shared among all PDR-1 interactors, but were not found to directly interact with PDR-1, were identified. Apparently, among these are some additional ‘prion’ domain proteins, e.g. PQN-54 and PQN-5 (ORFs R09B5.5 and C03A7.4, respectively) as well as ABU-10 and ABU-11 (ORFs F35A5.3 and T01D1.6, respectively) that associate with at least 1-6 different PDR-1 interactors. The latter are members of the **a**ctivated in **b**locked **U**PR protein family that comprises eleven ER transmembrane proteins (Urano et al., 2002). Interestingly, these proteins are induced in response to ER stress and protect, as a back-up mechanism, worms with a defective UPR against ER stress. In addition, other protein that bind to at least 2-5 different PDR-1 interactors are implicated in UPR-related biological functions, i.e. protein synthesis or ER-Golgi protein transport.

From this evaluation, at least it is noteworthy that many PDR-1 interactors also interact with each other, and distinct candidates often associate with the same set of proteins, like the above mentioned (for the PDR-1 interaction map see Figure 22).

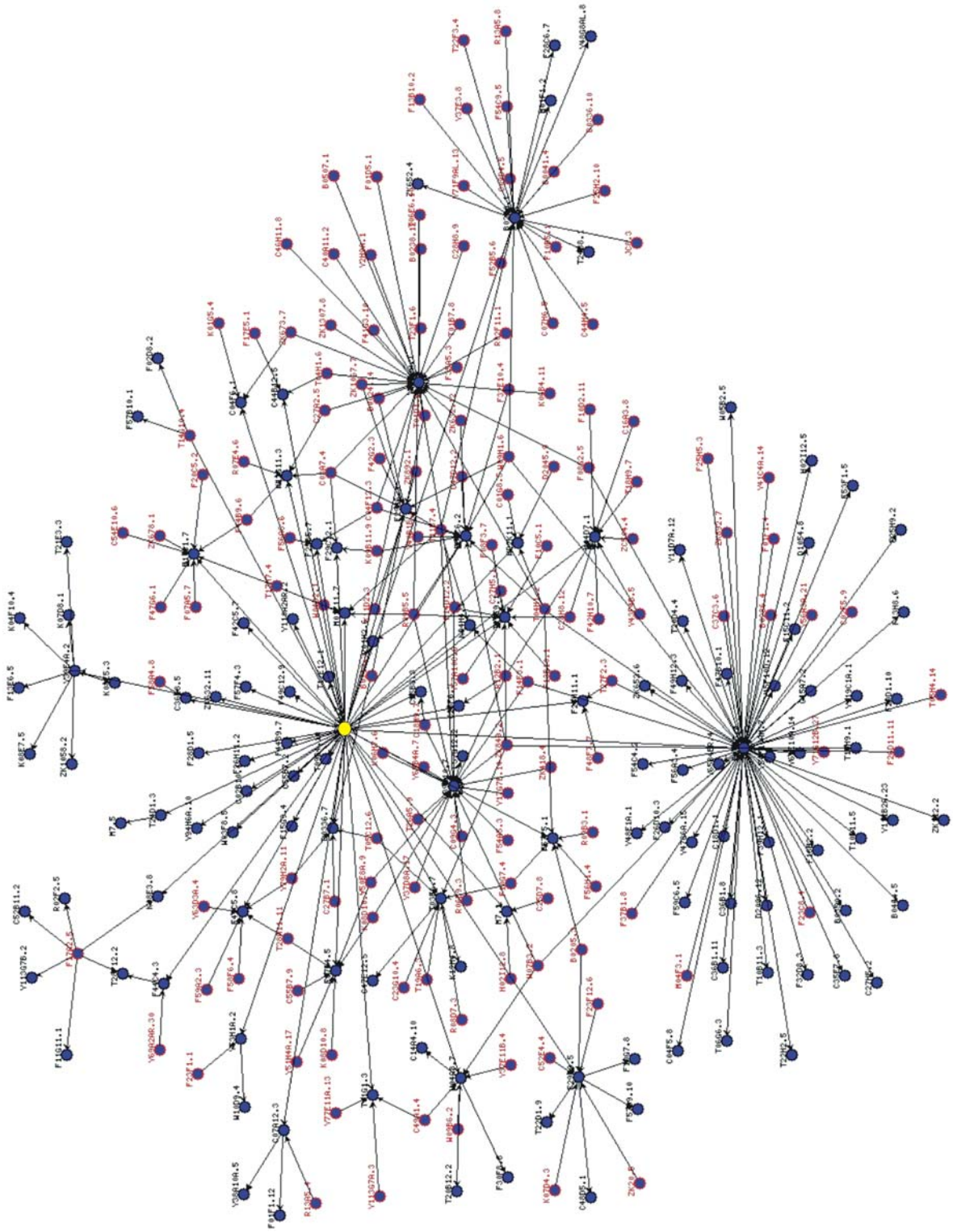


Figure 22. Extended Protein Interaction Map of PDR-1.

To generate this protein interaction map, all known PDR-1 interactors as well as all other binding partners of these proteins have been integrated (Li et al., 2004, and this study). **Nodes:** PDR-1 (yellow), bait (blue) and prey (red) proteins. **Lines:** protein interactions. The collective protein interaction data was visualized using i-View software (http://vidal.dfci.harvard.edu/).

However, these protein interactions have to be confirmed by alternative methods. Nevertheless, first evaluation already revealed quite promising putative connections between PDR-1 and single biological functions of protein metabolism, in general. Such studies might indeed give important hints in order to identify multiple components of novel and biologically relevant PDR-1/Parkin protein complexes.

3.3.2 GST-Pull Down Experiments

To confirm the specificity of PDR-1 interactions with the *C. elegans* ubiquitylation machinery by an alternative method, glutathione S-transferase (GST)-pull down assays were used. GST-tagged and immobilized ubiquitylation enzymes purified from *E. coli* were incubated with *in vitro* translated PDR-1 labelled with ^{35}S methionine/cysteine. GST-tagged E2 enzymes UBC-18 and UBC-2, as well as the E4 enzyme CHN-1, respectively, were able to bind and pull down radioactive PDR-1 (Figure 23).

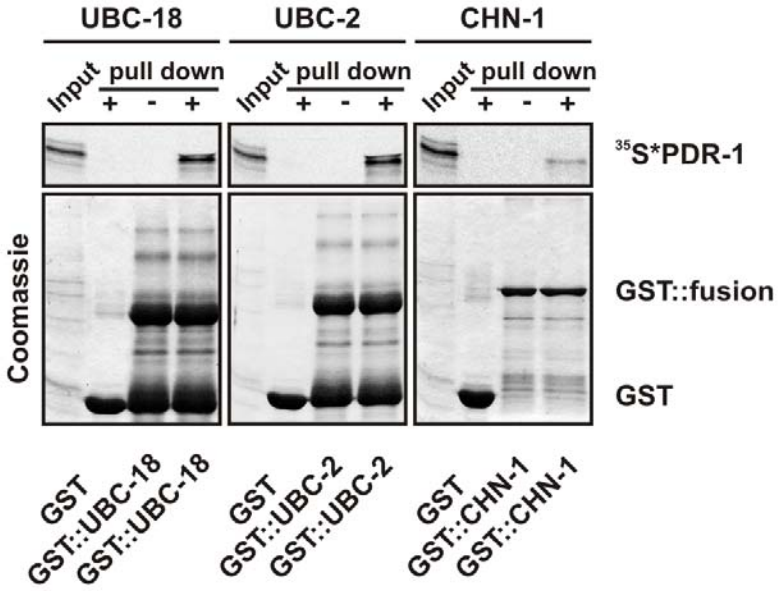


Figure 23. Confirmation of PDR-1 Interactions by GST-Pull Down Experiments.

Recombinant proteins purified from *E. coli* were bound to glutathione-Sepharose beads and incubated with in vitro translated PDR-1 After extensive washing under physiological conditions, the reaction was analyzed by SDS-PAGE followed by coomassie blue staining and autoradiography. GST alone was used as a negative control. PDR-1 binds the *C. elegans* E2 enzymes UBC-18 and UBC-2, as well as the E4 enzyme CHN-1.

Taken together, PDR-1 specifically physically associates with a conserved *C. elegans* ubiquitylation machinery, as demonstrated by two alternative methods in this study.

3.3.3 Expression and Purification of Recombinant PDR-1

To purify recombinant full-length PDR-1 expressed in *E. coli*, several approaches were performed. PDR-1 produced from bacteria tended to aggregate into inclusion bodies under a variety of different expression methods. PDR-1 protein fused to 6xHIS-tag, either at the N-terminus (pBY1118) or at both ends (pBY1119), could only be purified under denaturing conditions. Unfortunately, fusion of PDR-1 with a GST-tag did not enhance solubility to an extent that natively folded PDR-1 could have been purified in larger amounts.

In order to purify native PDR-1, a eukaryotic expression system was chosen since specific post-translational modification might be essential for its proper folding. SF9 insect cells were transfected with a recombinant Baculovirus to produce GST::myc::PDR-1 fusion protein. Using this expression system, small amounts of soluble full-length PDR-1 have been purified under native conditions.

3.3.4 PDR-1 Mediates E3 Ubiquitin Ligase Activity

Self-ubiquitylation is characteristic for RING-type E3 ligases that belong to the ubiquitin system (Lorick et al., 1999). To study the enzymatic activities of PDR-1, an assay in which self-ubiquitylation in the absence of a specific substrate occurs on the ubiquitin ligase itself, was performed. Therefore, the entire poly-ubiquitylation system was reconstituted with purified ubiquitin, E1 enzyme, *C. elegans* E2 enzyme UBC-2 or human E2 UbcH7, as well as *C. elegans* E4 enzyme CHN-1 (Figure 24).

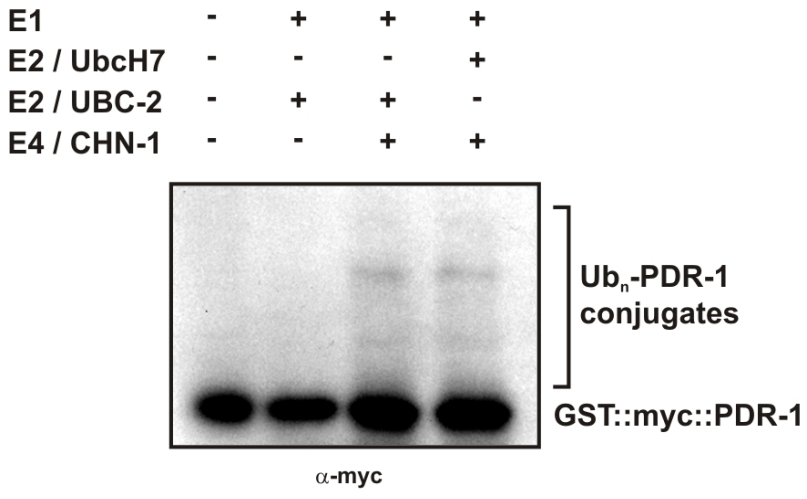


Figure 24. *In vitro* Self-Ubiquitylation of PDR-1.

GST::myc::PDR-1, expressed and purified from insect cells, was incubated with the combination of ubiquitylation enzymes purified from *E. coli*. Reactions were analyzed by SDS-PAGE and subsequent western blotting probed with anti-myc antibody. Efficient self-ubiquitylation of PDR-1 requires E1, E2 (*C. elegans* UBC-2 or human UbcH7) and the E4 enzyme CHN-1.

In conjunction with the complete set of enzymes and even in concert with human E2 enzyme UbcH7, a known binding partner of human Parkin (Shimura et al., 2001), PDR-1 shows self-ubiquitylation (Figure 24). Although, human Parkin is able to poly-ubiquitylate certain substrate proteins efficiently, self-ubiquitylation activity seems to be weaker and was demonstrated by a more sensitive approach, using I^{125}-labelled ubiquitin (Shimura et al., 2000). Therefore, it is not surprising that only a minor fraction of ubiquitylated PDR-1 is detectable in this assay.

In summary, these biochemical data demonstrate that PDR-1 acts as an E3 enzyme in a highly conserved ubiquitylation complex and thus represents the functional *C. elegans* ortholog of human Parkin.

3.3.5 Antibody Generation and Purification

To generate a PDR-1 specific antibody, two rabbits each were immunized with one of two synthesized peptides: peptide I (MSDEISILIQDRKTG) represents the first 15 aa and is located within the UBL domain; peptide II (QTSYSEYQRKATER) comprises aa 260-273 and resides between the first RING and the IBR domain.

Sera were tested after each bleeding in western blots on purified recombinant PDR-1 protein or on whole cell lysates from SF9 cells. As expected, affinity and specificity of the antibodies improved upon consecutive immunizations. However, first preliminary affinity purification on western blots using recombinant PDR-1, did not further advance the ability to detect PDR-1 to a satisfying extent, since ~10-100 ng purified protein was the minimum amount recognized. Unfortunately, endogenous PDR-1 was never detected in western blots on whole worm lysates, even not after preceding treatment with the proteasome inhibitor MG132 (100µM for 6h) which stabilizes short-lived proteins. This is consistent with data from *in vivo* analyses of the GFP::PDR-1 translational fusion, which already suggested extremely low cellular PDR-1 protein levels. In addition, proteins that are present only at low levels are often masked by the highly abundant collagens of the nematode cuticle that constitute a significant percentage of the total protein content. However, endogenous PDR-1 could also not be detected *in vivo* by immunostaining of worms.

Thus, larger scale affinity purification accompanied by enrichment of the antibodies is recommended to be able to detect endogenous PDR-1 on western blots and perhaps *in vivo*.

3.4 Analyses of *pdr-1* Deletion Mutants

3.4.1 Identification of Different *pdr-1* Deletion Mutants

To further investigate the *in vivo* function of *pdr-1*, deletion mutants were isolated by PCR-screening of UV/Trimethylpsoralen (TMP)-mutagenized *C. elegans* libraries with *pdr-1* specific primers (Figure 25). The mutants *lg103* and *lg101* were obtained from EleGene (Munich, Germany), whereas the alleles *tm598* and *tm395* were provided by Dr. Shohei Mitani (National Bioresource Project for the nematode, Japan).

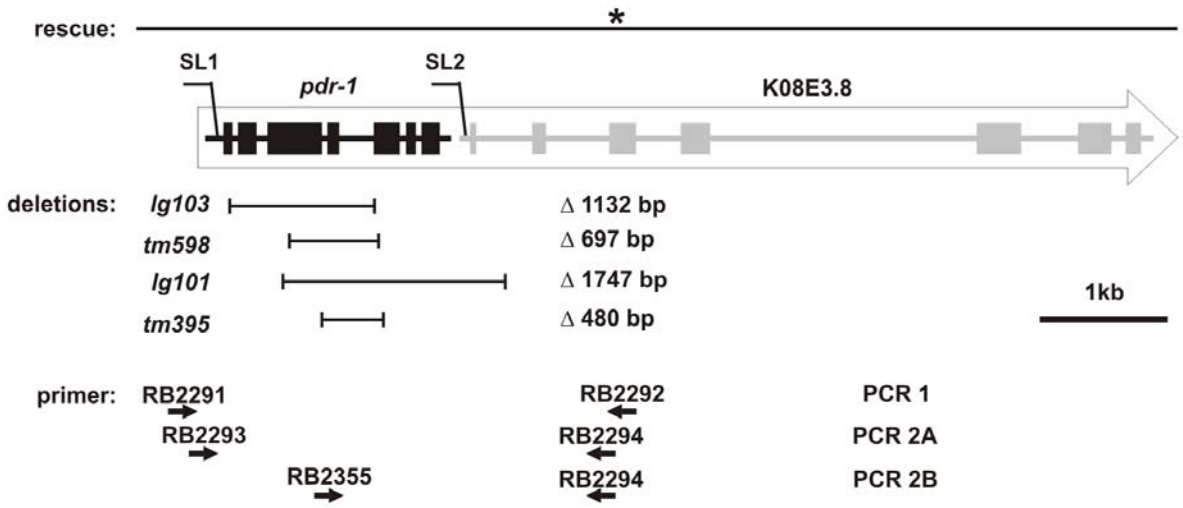


Figure 25. Schematic View of *pdr-1* Deletion Alleles.

The gene structure and relative positions of *pdr-1* and its neighbouring genes is shown. Coding exons are depicted as boxes, introns as lines. An arrow indicates the *pdr-1*/K08E3.8 operon. Position and extent of the rescuing construct (rescue) relative to *pdr-1* is shown. The asterisk indicates the position of an engineered frame-shift mutation in the downstream gene of the rescuing clone. The position and extent of the four analyzed *pdr-1* deletions, depicted by lines, is shown. Small arrows indicate the position of the primer pairs used to identify the *pdr-1* deletions.

To identify these mutants, 'nested PCR' was performed using two pairs of primers (PCR 1: RB2291/RB2292, external; PCR 2A: RB2293/RB2294, internal) annealing outside the *pdr-1* coding region. Mutants bearing a *pdr-1* deletion give rise to an accordingly shorter PCR-product compared to wild type animals (Figure 26). To distinguish homo- from heterozygous mutants, a second 'nested PCR' round was performed using an additional primer that is located within the deleted regions (PCR 2B: RB2355/RB2294, in-deletion).

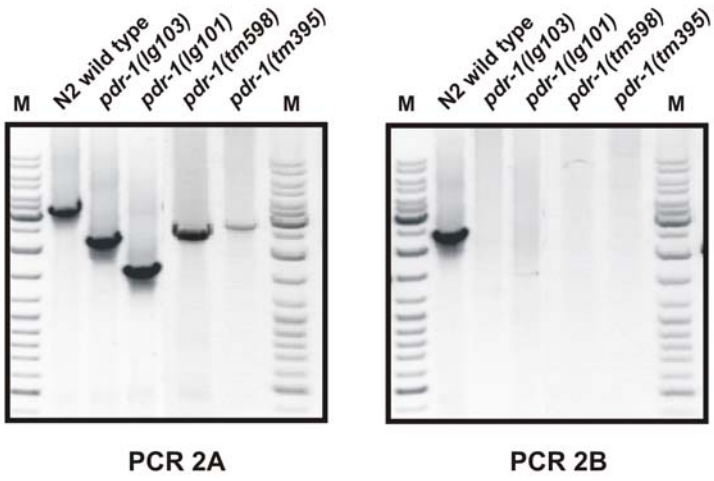


Figure 26. Identification of *pdr-1* Deletion Mutants.

SW-PCR confirmation of the *pdr-1* deletions by analysis of genomic DNA. For primers used see Figure 24. **Left:** PCR 2A: *pdr-1* mutants show accordingly shorter PCR products than wild type (wild type: 3.2 kb; *lg103*: 2.1 kb; *lg101*: 1.5 kb; *tm598*: 2.5 kb; *tm395*: 2.7 kb). **Right:** PCR 2B: The absence of a product in the PCR reaction with *pdr-1* mutants confirms their homozygosity (wild type 2.4 kb). M: Marker (GeneRuler™ DNA Ladder Mix, MBI Fermentas, Vilnius, Litauen)

This PCR reaction results in the amplification of a wild type fragment from heterozygous mutants, whereas homozygous mutants do not give rise to a product (Figure 26). To eliminate background mutations in the *pdr-1* deletion mutants, strains were backcrossed with N2 wild type animals several times. After each crossing, homozygous mutants were verified by Single-Worm-PCR (SW-PCR) again.

Sequencing of these genomic PCR products from *pdr-1* mutants revealed the exact deletion breakpoint of the alleles. The deletions *lg103*, *tm598* and *tm395* remove only parts of the *pdr-1* ORF, and therefore represent 'clean' *pdr-1* alleles. In contrast, the deletion of allele *lg101* in addition eliminates the complete first exon including the translational start of the downstream gene K08E3.8, and therefore affects both genes from the operon (for details of deletion breakpoints and backcrossing of the single *pdr-1* alleles see 5.5.4 in the Experimental Procedures Section).

3.4.2 Transcriptional Analysis of *pdr-1* Deletion Mutants

To confirm that the *pdr-1* single gene deletions do not affect transcription of the downstream gene K08E3.8, northern blot analyses of total RNA from the respective mutants was performed. These experiments demonstrated that the *pdr-1* gene in the mutant allele *lg103* is indeed transcribed on a truncated mRNA at amounts comparable to wild type level (Figure 27).

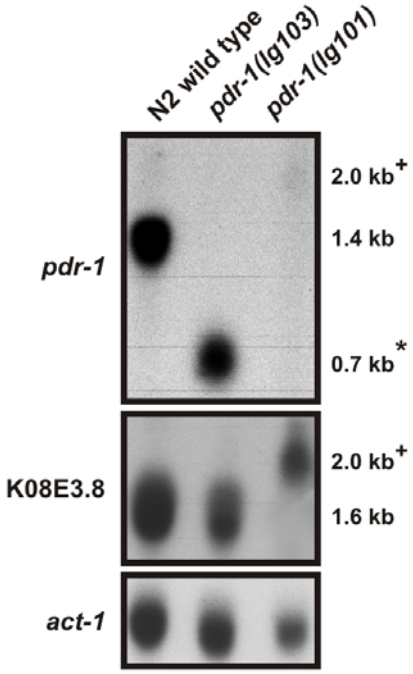


Figure 27. Northern Blot Analysis of Mutant *pdr-1* Transcripts.

pdr-1(lg103) produces a truncated transcript marked with an asterisk (0.7 kb) at levels comparable to wt *pdr-1* (1.4 kb). The deletion does not affect transcription of the downstream gene K08E3.8 (1.6 kb). In contrast, the deletion *pdr-1(lg101)* produces a hybrid transcript from the remaining parts of both genes, *pdr-1* and K08E3.8 marked with a plus (2.0 kb). This band was detectable with both, *pdr-1* and K08E3.8 specific probes. An *act-1* specific probe shows slightly unequal loading (also see Figure 34 for normalized data).

Importantly, transcription of the downstream gene K08E3.8 is unaffected by this deletion. In contrast, the allele *lg101*, which affects both genes of the operon, showed compared to wild type a longer hybrid transcript, which was detectable with both *pdr-1* and K08E3.8 specific probes. Reverse transcription followed by polymerase chain reaction (RT-PCR) on total RNA of the *pdr-1* mutants, resulted in the isolation of the respective truncated cDNAs (Figure 28).

The allele *lg103* bears an 1132 bp deletion, fusing exon 1 of *pdr-1* in-frame to exon 5. The corresponding mutant protein PDR-1(∆aa24-247) is internally truncated and only contains the intact IBR and second RING-finger domains. In *tm598*, 697 bp of the genomic *pdr-1* ORF are deleted fusing parts of exons 3 and 5, also resulting in an in-frame deletion. In contrast to *lg103*, this allele produces an internally truncated protein PDR-1(∆aa140-263) that still bears the intact UBL domain.

However, the alleles *lg101* and *tm395* remove most of the *pdr-1* ORF and result in out of-frame mutations, followed by premature stops. The allele *lg101*, eliminates 1747 bp of the *pdr-1*/K08E3.8 ORFs, and gives rise to a protein that is terminated after aa 121 of PDR-1, only encoding the intact UBL domain. In *tm395*, 480 bp of the genomic *pdr-1* ORF are deleted, resulting in a frame-shift causing a premature stop. The translated polypeptide is truncated after aa 199, only representing the intact UBL and UPD domains.

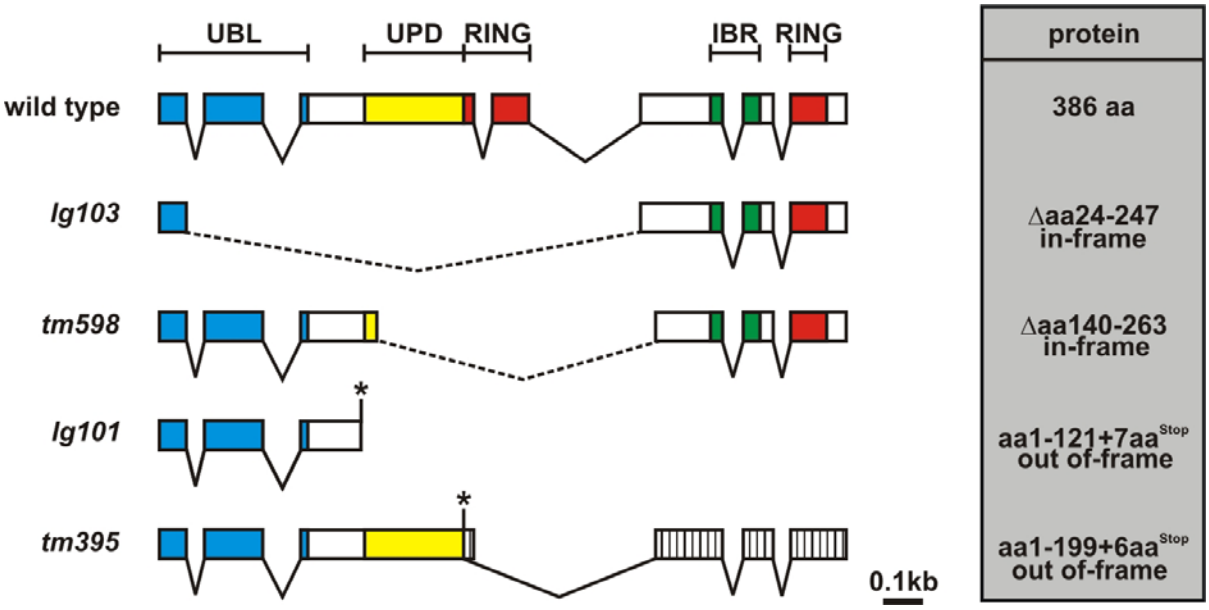


Figure 28. A Set of Different *pdr-1* Deletion Mutants.

Detailed view of the *pdr-1* deletions, their splicing, and their mutant gene products. Colour-coding as described in Figure 9. The translated mutant proteins are listed. In-frame deletions are indicated by dotted lines, splicing of the out-of-frame deletion *tm395* by lines. The out-of-frame deletion of the allele *lg101* extends into the neighbouring gene K08E3.8. Early translational stops generated by the out of-frame deletions are marked with asterisks. Un-translated exons are depicted by hatched boxes.

3.4.3 Biochemical Analysis of Mutant PDR-1 Gene Product

To test for residual biochemical activity of the internally truncated PDR-1(Δaa24-247) protein, interaction studies similar to that of full-length PDR-1 were performed using both methods the yeast two-hybrid system (compare Figures 20 and 29) and GST-pull down assays (compare Figures 23 and 30). Truncated PDR-1(Δaa24-247) binds the same set of E2 and E4 enzymes like full-length PDR-1 (Figure 29A and 30) since it bears the intact IBR and second RING finger domains. Evidently, association of PDR-1(Δaa24-247) with RPT-2 is disrupted (Figure 29B), as *pdr-1(lg103)* mutants lack an intact UBL domain that is essential for coupling to the proteasome (Sakata et al., 2003). However, truncated PDR-1(Δaa24-247) is still capable of dimerizing with its wild type form (Figure 29C). This interaction has been confirmed using the split-ubiquitin system (D. Dirnberger, personal communication).

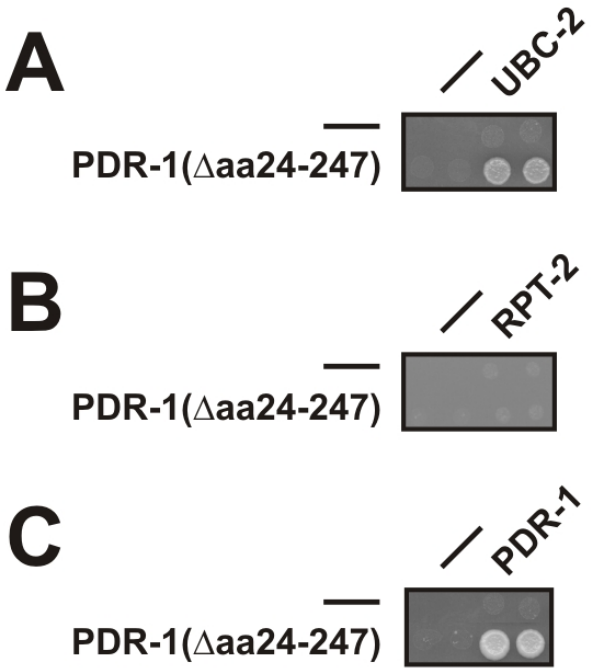


Figure 29. Truncated PDR-1(∆aa24-247) Retains Residual Binding Activity.

Growth analysis of yeast cells was performed as described for wild type PDR-1 in Figure 20. (**A**) UBC-2.Truncated PDR-1(∆aa24-247) still binds the E2 enzyme UBC-2. (**B**) Association of truncated PDR-1(∆aa24-247) with the proteasomal subunit RPT-2 is abrogated. (**C**) Truncated PDR-1(∆aa24-247) is still able to dimerize with its full-length version.

In addition, the yeast two-hybrid interactions of truncated PDR-1(∆aa24-247) were confirmed by GST-pull down experiments (Figure 30).

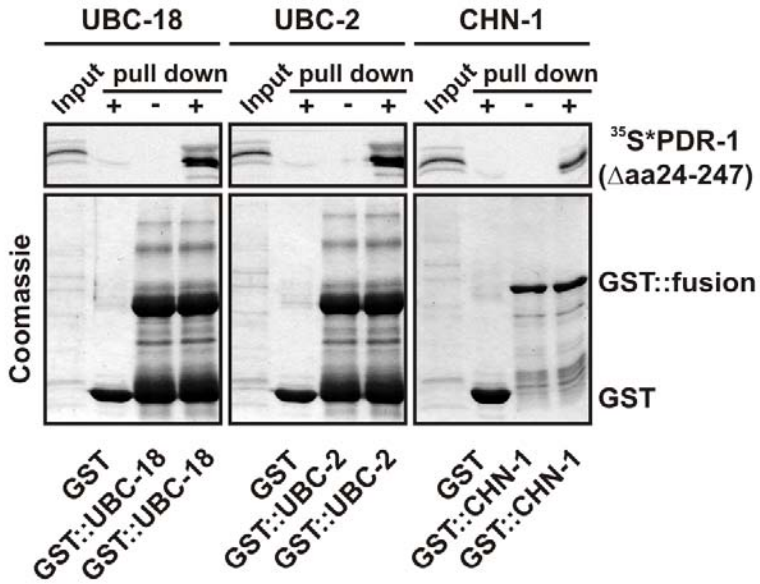


Figure 30. Confirmation of Truncated PDR-1(∆aa24-247) Protein Interactions.

Experiments were performed as described for wild type PDR-1 in Figure 23. Immobilized GST-tagged ubiquitylation enzymes bind and pull down truncated PDR-1(∆aa24-247), labelled with ^{35}S methionine/ cysteine. Truncated PDR-1(∆aa24-247) interacts with the *C. elegans* E2 enzymes UBC-18 and UBC-2, as well as the E4 enzyme CHN-1 as its wild type form.

Taken together, these data demonstrate that the RING-box structure, in particular the IBR and 2nd RING domain, mediates not only specific E2 and E4 binding, but also homo- as well as hetero-dimerization of RING box proteins. Furthermore, in-frame deleted *pdr-1(lg103)* animals give rise to a mutant protein PDR-1(∆aa24-247) which retains association with the ubiquitylation machinery but lacks essential coupling to the proteasome.

3.4.4 Phenotypical Analysis of *pdr-1(lg101)*

Prior to phenotypical analyses, the allele *lg101* was backcrossed nine times to the N2 wild type strain, in order to eliminate putative second-site mutations. Notably, this deletion affects *pdr-1* and K08E3.8, the second gene of the operon.

Homozygous *pdr-1(lg101)* mutants displayed a variety of different phenotypes, including morphological and developmental alterations as well as defects in different behaviours. First, *pdr-1(lg101)* mutants show a dumpy (Dpy) phenotype, appearing shorter and thicker than wild type animals. Second, possibly due to their altered morphology, *pdr-1(lg101)* mutants are uncoordinated (Unc) and perform 30% less body-bends/min compared to wild type (wt: 47.6 ± 1.5; *pdr-1(lg101)*: 33.0 ± 2.3; n = 25; $p < 0.0001$). Third, *pdr-1(lg101)* mutants show an egg-laying defective (Egl-D) phenotype, which was further characterized in detail. About half of all *pdr-1(lg101)* mutants died earlier because of a 'bag-of-worm' phenotype (wt: 0%; *pdr-1(lg101)*: 48% ± 5%; n = 100; $p < 0.0001$). Due to the inability to lay eggs, the progeny hatches and develops inside the mother, resulting in their deterioration. In line with these data, *pdr-1(lg101)* mutants showed a ~12h delayed start of egg-laying, a slower egg-laying rate/h (wt: 6.8 ± 0.3; *pdr-1(lg101)*: 3.7 ± 0.3; n = 15; $p < 0.0001$), and more eggs in the uterus at later stages of the egg-laying phase (wt: 1st day: 23.5 ± 0.7, 2nd day: 30.9 ± 1.0, 3rd day: 1.8 ± 0.3; *pdr-1(lg101)*: 1st day: 11.7 ± 1.0; 2nd day: 22.6 ± 0.6; 3rd day: 13.2 ± 1.0; n = 25; all $p < 0.0001$). Finally, *pdr-1(lg101)* mutants produced less progeny and thus showed ~ 40% reduced brood size compared to wild type (see later Table 3). First preliminary pharmacological analyses using serotonin (5-HT), the 5-HT re-uptake inhibitor imipramine, as well as the DA antagonist chlorpromazine that all induce egg-laying in *C. elegans*, suggested neuronal, rather than muscular defects underlying the Egl-D phenotype of *pdr-1(lg101)* mutants.

However, none of these phenotypes observed in homozygous *pdr-1(lg101)* mutants has been detected in heterozygous *pdr-1(lg101)/+*, trans-heterozygous *pdr-1(lg101)/pdr-1(lg103)* or other homozygous *pdr-1* deletion alleles. Therefore, all characterized abnormalities of *pdr-1(lg101)* mutants can be attributed to a recessive loss-of-function of the downstream gene K08E3.8. Moreover, an effect of any of the four *pdr-1* deletions on the closely linked upstream gene *cyk-4* can almost be excluded, since a variety of different mutations in this gene cause an embryonic lethal phenotype (http://www.wormbase.org/).

3.5 Analyses of *pdr-1* Mutants under ER Stress Conditions

Homozygous *pdr-1* single gene mutants are viable and display no alterations of morphology, development, fertility, or life-span. In addition, they show no behavioural defects in motility, egg-laying, defecation, chemotaxis, or mechanosensation. In addition, *pdr-1* deletions do not affect dopaminergic neuron survival as judged by an integrated DA transporter *gfp* reporter gene construct $P_{dat\text{-}1}$*::gfp* (Nass et al., 2002). Since *pdr-1* single gene mutants behaved like N2 wild type animals in all performed experiments, at least under normal growth conditions, *pdr-1* mutants were subjected to further pharmacological analyses using different compounds.

3.5.1 The *pdr-1(lg103)* Mutant is Sensitized to ER Stress

Worms were treated with the reducing agents dithiothreitol (DTT) and β-mercaptoethanol (β-ME), or with tunicamycin, a specific inhibitor of *N*-linked glycosylation, leading to accumulation of unfolded proteins in the ER (Gething and Sambrook, 1992). It appeared that particularly *pdr-1(lg103)* mutants are hypersensitive to ER stress conditions, resulting in severe developmental defects and lethality at early larval stages (Figure 31). In contrast, N2 wild type animals are able to cope with moderate ER stress and were unaffected.

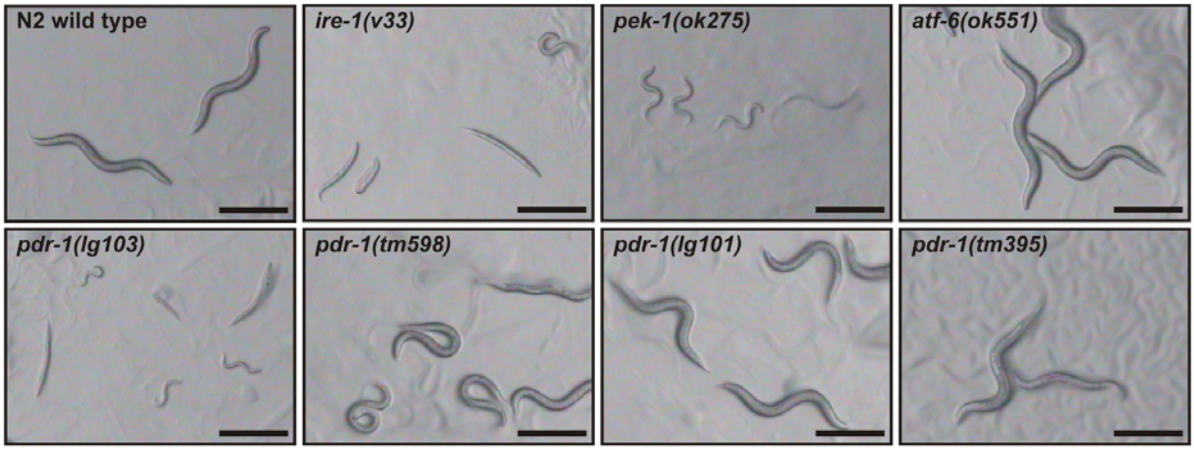


Figure 31. Particular *pdr-1(lg103)* Mutants are Hypersensitive to ER Stress.

Photos show ER stressed worms, treated with 1.5 µg/ml tunicamycin, after three days growth from synchronized eggs at 20°C. Most of the N2 wild type animals reach adulthood, whereas the majority of *ire-1(v33)* and *pek-1(ok275)* mutant animals either arrest or die at early larval stages. *pdr-1(lg103)* animals, in contrast to other *pdr-1* deletion alleles, show the same characteristic ER stress hypersensitivity as mutants of the UPR. Scale bar: 0.5 mm.

Surprisingly, none of the other three analyzed *pdr-1* deletion alleles showed a comparably strong phenotype than *pdr-1(lg103)* (Figure 31), even not at higher doses of exogenous ER stress. However, this phenotype is similar to the one observed with mutants defective in the proper execution of the UPR pathway (Shen et al., 2001).

C. elegans encodes single homologs of each stress sensor, *ire-1*, *pek-1* and *atf-6,* and mutants are sensitive to elevated ER stress. An intact UPR is absolutely required for normal development as *ire-1(v33)* loss-of-function mutants suffer from developmental defects and a reduced brood size, whereas *pek-1(ok275)* and *atf-6(RNAi)* loss-of-function mutants are indistinguishable from wild type animals. In *C. elegans*, IRE-1 is the central regulator of the UPR, whereas PEK-1 and ATF-6 provide redundant protection against ER stress (Calfon et al., 2002; Shen et al., 2001; Urano et al., 2002). However, mutants are hypersensitive to elevated ER stress, as both, tunicamycin treated single mutants (Figure 31) as well as non-stressed *ire-1;pek-1*, *xbp-1;pek-1*, or *xbp-1;atf-6(RNAi)* double mutants, arrest at or prior to larval stage L3 (Shen et al., 2001; Urano et al., 2002).

To quantify the effects of ER stress on *pdr-1* mutants, low concentrations of tunicamycin [1.5 µg/ml] were chosen, as DTT and β-ME also affected growth of the *E. coli* food source. A stress inducible P_{hsp-4}::*gfp* transcriptional reporter (Calfon et al., 2002) was used in all experiments to monitor efficient induction of the UPR. *C. elegans hsp-4* is a homolog of the mammalian ER chaperone BiP, which gets up-regulated upon ER stress. At this concentration of tunicamycin, the overwhelming majority of wild type animals were resistant to elevated ER stress and matured to fertile adults. In contrast, almost 90% of *ire-1(v33)* and *pek-1(ok275)* mutant animals arrested during development and died, indicating increased stress sensitivity of mutants in the UPR. Notably, almost 70% of homozygous *pdr-1(lg103)* animals showed ER stress hypersensitivity, similar to *ire-1* and *pek*-1 loss-of-function mutants (Figure 32). Since *pdr-1(lg103)* responded differently to tunicamycin treatment than the other *C. elegans parkin* alleles, it is possible that this mutation confers a dominant negative or gain-of-misfunction phenotype. In agreement with such a model is the retained protein interaction capability of the encoded mutant protein. To test this, *pdr-1* gene doses were altered by analyzing hetero- and trans-heterozygous mutants (Figure 32). All heterozygous *pdr-1* alleles showed resistance to increased ER stress, comparable to wild type.

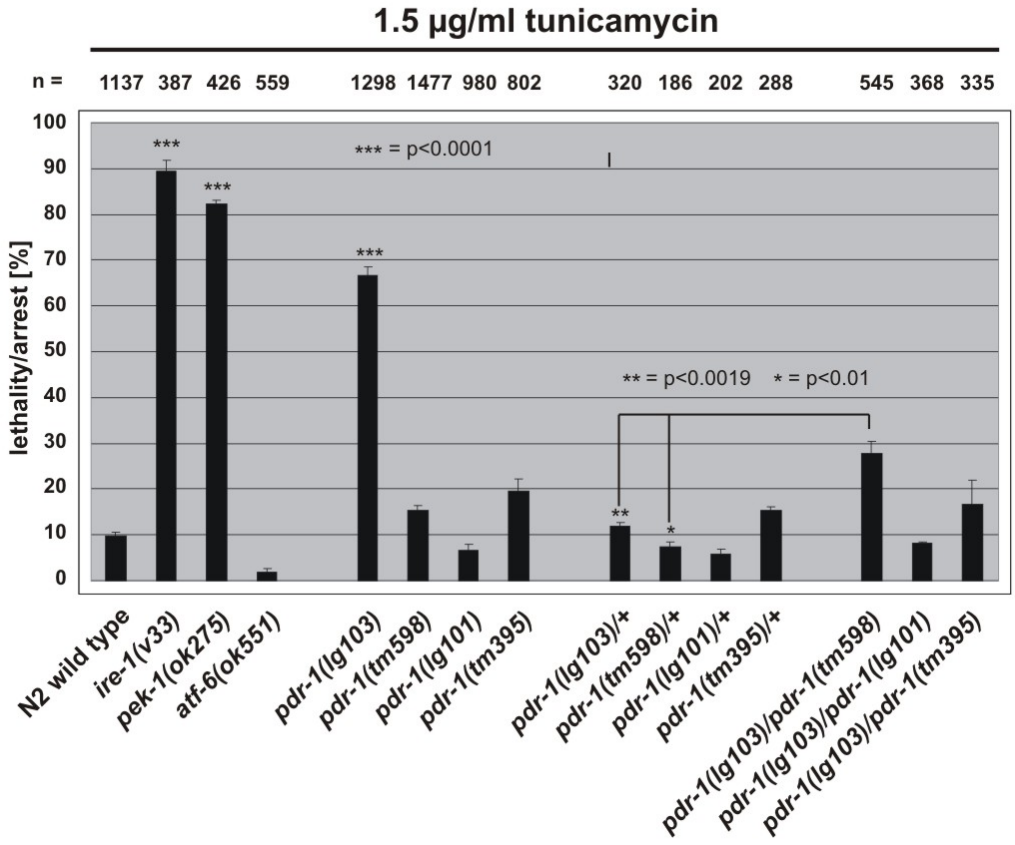


Figure 32. Quantitative Analysis of the *pdr-1(lg103)* ER Stress Hypersensitivity.

Each strain was allowed to lay eggs for three hours on 1.5 µg/ml tunicamycin. Progeny determined to be either dead or arrested at larval stages was counted after three days growth at 20° C. Only homozygous *pdr-1(lg103)* mutants are hypersensitive to tunicamycin, but transheterozygous *pdr-1*(*lg103)/pdr-1(tm598)* animals also showed a significantly increased sensitivity. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

Whereas homozygous *pdr-1(lg103)* mutants showed the highest sensitivity upon ER stress, the combination of the two in-frame-deletion alleles in the transheterozygous mutant *pdr-1(lg103)/pdr-1(tm598)* also resulted in significantly increased sensitivity. Notably, none of the other transheterozygous combinations, like *pdr-1(lg103)/pdr-1(lg101)* or *pdr-1(lg103)/pdr-1(tm395),* showed a significant increase in sensitivity, compared to respective heterozygous alleles (Figure 32).

3.5.2 Rescue of the Tunicamycin Hypersensitivity

The tunicamycin hypersensitivity of homozygous *pdr-1(lg103)* can be restored to wild type behaviour by transgenic expression of *pdr-1*. This rescue was achieved by microinjection of the cosmid K08E3, a genomic subclone containing the complete *pdr-1* operon (pBY1500), or an engineered rescue construct (pBY1908), which

carries a frame-shift mutation in exon III of the downstream gene K08E3.8 (for a schematic view see Figure 25).

In total, 14 independent lines were analyzed: five were transgenic for the cosmid K08E3 (BR2726-2730: *pdr-1(lg103);byEx429-433*), three were transgenic for pBY1500 (BR2768-2770: *pdr-1(lg103);byEx434-436*), and six were transgenic for pBY1908 (BR3136-3141: *pdr-1(lg103);byEx417-422*). At higher concentrations of tunicamycin [2.5 µg/ml], almost 90% of *pdr-1(lg103)* showed severe developmental defects and lethality. Wild type animals were much more resistant to ER stress, even at higher doses, as only 32% died or arrested during at early larval stages. In all analyzed lines expressing the rescuing transgene, lethality/arrest was significantly reduced down to 40%, as compared to non-rescued control animals (Figure 33).

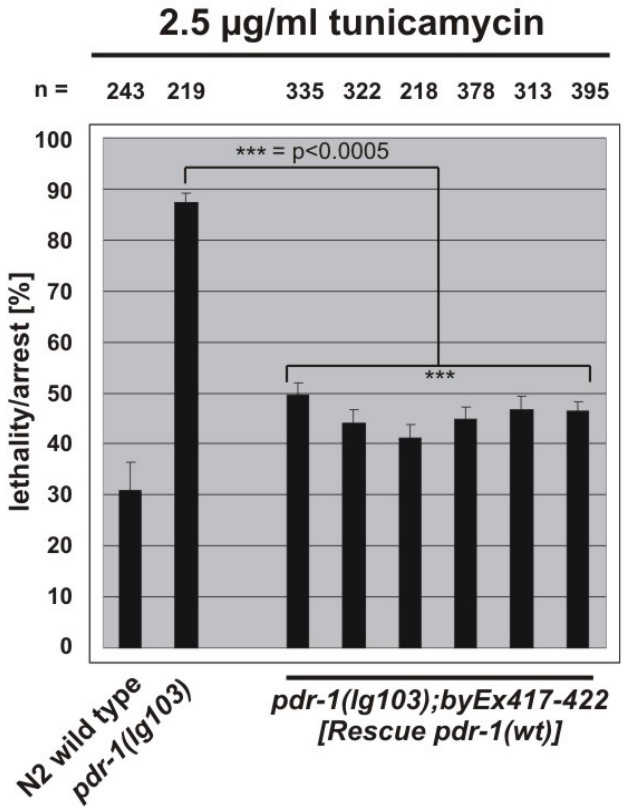


Figure 33. Rescue of the *pdr-1(lg103)* ER Stress Hypersensitivity by a Wild Type Transgene.

Wild type copies of *pdr-1*, expressed from independent transgenic arrays in the mutant background (*pdr-1(lg103); byEx417-422[pdr-1]*) significantly restored survival at 2.5 µg/ml tunicamycin. Experiments were performed as described in (A). Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

Notably, the presence of one wild type copy of *pdr-1*, either genomic in a *pdr-1(lg103)*/+ heterozygote or from a transgenic array, was sufficient to restore viability and fertility (Figures 32 and 33). Furthermore, in contrast to *pdr-1* loss-of-function alleles, in-frame deletions bearing the intact IBR and second RING finger domains, either were hypersensitive to ER stress or at least served as a sensitized background

in compound heterozygotes. These results clearly rule out a dominant negative function of *pdr-1(lg103)*, but rather suggest a semi-dominant role as a gain-of-misfunction allele. The severe developmental defects and lethality observed in ER stressed *pdr-1(lg103)* mutants further suggest a role of PDR-1 in the UPR.

3.5.3 *pdr-1* is Involved in the UPR

To further elucidate the function of *pdr-1* in the UPR pathway, genetic interactions between different *pdr-1* alleles and the UPR-mutants *ire-1(v33)*, *pek-1(ok275)* and *atf-6(ok551)* were tested. Therefore, double mutants were generated and the brood sizes of the respective strains in the absence of exogenous ER stress were counted.

First, the brood sizes of *pdr-1* mutants were analyzed in detail. Homozygous *pdr-1(lg101)* mutants produced significantly less progeny (62%) than N2 wild type (100%). Nonetheless, all other homozygous *pdr-1* alleles *lg103*, *tm598* and *tm395*, as well as the transheterozygous *pdr-1(lg101)/pdr-1(lg103)* animals showed normal brood sizes (~90%) compared to wild type (Table 3). Thus, the reduced brood size observed in homozygous *pdr-1(lg101)* animals can be assigned to a recessive loss of K08E3.8 function in this allele. Conclusively, 'clean' deletions of the *pdr-1* gene do not significantly influence fertility, offspring production or egg-laying behaviour.

Like previously published (Shen et al., 2001), *ire-1(v33)* single mutants defective in the proper execution of the UPR produced a significantly smaller brood size than wild type (57%; $P < 0.0001$). In contrast, *pek-1(ok275)* and *atf-6(ok551)* single mutants showed normal numbers of progeny (86%; $P = 0.0002$ and 90%; $P = 0.1032$, respectively), compared to wild type (Table 3).

Strikingly, *ire-1(v33);pdr-1(lg103)* and *ire-1(v33);pdr-1(tm598)* double mutants showed a dramatically reduced brood size with respect to each single mutant (Table 3). Specifically the tunicamycin-sensitive *pdr-1* in-frame deletion alleles exacerbated the phenotype of *ire-1* loss-of-function mutants. While the *pdr-1* allele *lg103* reduced the brood size by half (47% reduced), the allele *tm598* had milder effects (29% reduced) on the brood size of *ire-1(v33)* single mutants (100%). Notably, complete loss of *pdr-1* function in the *ire-1* mutant background, i.e. in the double mutant *ire-1(v33);pdr-1(tm395)*, had no effect on the brood size. In contrast, the brood size of *ire-1(v33);pdr-1(lg101)* double mutants was strongly diminished (60% reduced).

genotype	n	progeny mean ± SEM	% relative brood size	*P* value
N2 wild type	**63**	**319 ± 4**	**100**	**-**
pdr-1(lg103)	**62**	**294 ± 6**	**92 ± 2**	**= 0.0002**
pdr-1(tm598)	**18**	**301 ± 6**	**94 ± 2**	**= 0.8961**
pdr-1(tm395)	**17**	**284 ± 6**	**89 ± 2**	**= 0.2731**
pdr-1(lg101)	**82**	**197 ± 9**	**62 ± 3**	**< 0.0001**
pdr-1(lg101)/pdr-1(lg103)	**22**	**285 ± 7**	**89 ± 2**	**= 0.1646**
ire-1(v33)	**57**	**181 ± 8**	**100**	**-**
ire-1(v33);pdr-1(lg103)	**60**	**94 ± 7**	**53 ± 4**	**< 0.0001**
ire-1(v33);pdr-1(tm598)	**18**	**127 ± 10**	**71 ± 6**	**< 0.0001**
ire-1(v33);pdr-1(tm395)	**18**	**178 ± 10**	**100 ± 6**	**= 0.1811**
ire-1(v33);pdr-1(lg101)	**36**	**71 ± 9**	**40 ± 5**	**< 0.0001**
pek-1(ok275)	**35**	**275 ± 6**	**100**	**-**
pek-1(ok275);pdr-1(lg103)	**34**	**280 ± 7**	**102 ± 3**	**= 0.7200**
pek-1(ok275);pdr-1(lg101)	**28**	**208 ± 6**	**76 ± 2**	**< 0.0001**
atf-6(ok551)	**30**	**287 ± 7**	**100**	**-**
atf-6(ok551);pdr-1(lg103)	**30**	**311 ± 5**	**108 ± 2**	**= 0.0063**

Table 3. Genetic Interaction of *pdr-1* in-frame Deletions And *ire-1(v33)* Loss-Of-Function.

The table depicts mean and relative brood sizes (progeny) of all mutant *pdr-1* alleles, as well as of *ire-1(v33)*, *pek-1(ok275)*, and *atf-6(ok551)* single and double mutants at 20° C. The total numbers of brood sizes scored are listed (n). The brood size of each strain is listed as mean ± SEM, and in percent. The brood sizes of the *pdr-1* single mutants were calculated relative to wild type level; brood sizes of the different *pdr-1* double mutants were calculated relative to the level of the respective UPR-single mutant *ire-1(v33)*, *pek-1(ok275)* or *atf-6(ok551)*. *P* values were calculated by *t* test analysis.

However, compared to wild type (100%), both single mutants *pdr-1(lg101)* and *ire-1(v33)* caused a strong reduction of the brood size (38% and 43% reduced, respectively). Therefore, it is likely that the decreased brood size of *ire-1(v33);pdr-1(lg101)* double mutants results from additive effects of both single mutants, independent of *pdr-1*. On the contrary, the strongly reduced brood size caused by the alleles *lg103* and *tm598* in the *ire-1* loss-of-function background results from a synergistic effect, and is dependent on a *pdr-1* in-frame deletion.

Nevertheless, the strong *pdr-1* in-frame deletion allele *lg103* had no effect on the brood sizes of other UPR-mutants, e.g. the double mutants *pek-1(ok275);pdr-1(lg103)* and *atf-6(ok551);pdr-1(lg103)* behaved like the respective single mutants. Not surprising, the brood size of *pek-1(ok275);pdr-1(lg101)* double mutants was decreased independent of *pdr-1*, behaving like *pdr-1(lg101)* single mutants. Previous

experiments suggested that *ire-1(v33)* represents a complete null allele (Shen et al., 2001, and this study). Therefore, the most obvious explanation for the strong synergistic effects of specifically *pdr-1* in-frame deletions and *ire-1* loss-of-function, is that both genes act in parallel pathways of the UPR. In contrast, *pdr-1* deletion alleles had no effect on *pek-1* or *atf-6* mutations. Since there is currently no indication of a fourth branch of the UPR, these results suggest that *pdr-1* may be assigned either to the *pek-1* or *atf-6* signalling pathway. *ire-1;pdr-1* double mutants show a milder phenotype compared to *ire-1;pek-1*, *xbp-1;pek-1*, or *xbp-1;atf-6(RNAi)* double mutants, which all arrest during development (Shen et al., 2001; Urano et al., 2002). Such analysis further indicates that *pdr-1* has to be positioned downstream of the three signal transducers IRE-1, PEK-1, and ATF-6.

Taken together, in contrast to *pdr-1* loss-of-function alleles, *pdr-1* in-frame deletions specifically aggravated the phenotype of an *ire-1* loss-of-function. These results are completely in line with data from the tunicamycin-induced stress tests. The in-frame deletion allele *lg103* confers a stronger phenotype than *tm598* in ire-1 mutant background, while *pdr-1* out-of-frame deletions *tm395* and *lg101* had no effect, at least not *pdr-1* dependent. These data support the proposed gain-of-misfunction of *pdr-1* in-frame deletions.

3.5.4 *pdr-1* is Regulated by the UPR

To further elucidate the position of *pdr-1* in, and its regulation by the UPR, *pdr-1* transcript levels in the UPR mutant backgrounds were analyzed by Northern blots. Transcript level of both genes, *pdr-1* and K08E3.8, were significantly reduced in *ire-1(v33)* and *pek-1(ok275)* loss-of-function alleles to about 50% of wild type levels. Interestingly, both *pdr-1* and K08E3.8 mRNA levels were significantly up-regulated in *atf-6(ok551)* mutants by about 50%, compared to wild type background (Figure 34).

Closer examination of the *atf-6(ok551)* deletion breakpoints, suggested that this mutation most likely represents a hypermorphic allele. The resulting mutant protein resembles the cleaved and activated form of the ATF-6 transcription factor (Yoshida et al., 2000), and might therefore be constitutively active, even under non-stress conditions. Consistently, transcript levels of the control genes *hsp-4* and *xbp-1* were reduced in *ire-1(v33)* as well as in *pek-1(ok275)* mutant background, but were unaffected in *atf-6(ok551)* mutant background (Figure 34). *C. elegans* ATF-6, in

contrast to mammalian, is not involved in induction of known UPR targets (Shen et al., 2001)

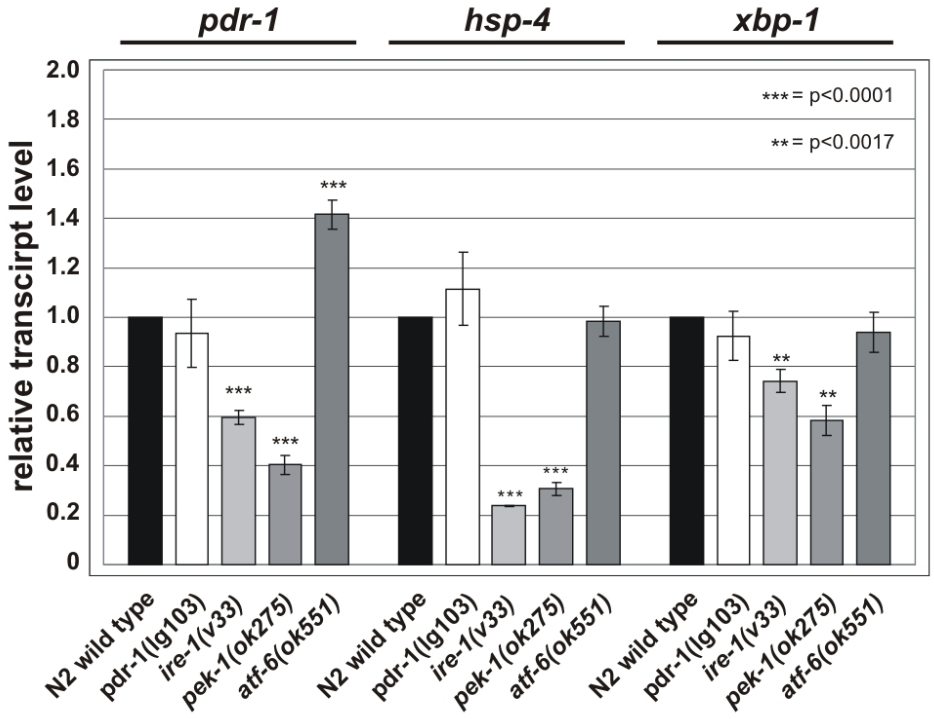


Figure 34. *pdr-1* Transcription is Regulated by the UPR.

Northern blot analyses of total RNA from mutants of the UPR showed reduced transcript levels of *pdr-1* and K08E3.8 in *ire-1(v33)* and *pek-1(ok275)* mutants, but elevated levels in *atf-6(ok551)* mutants, relative to wild type. Control genes *hsp-4* and *xbp-1* showed a reduction of transcription rate in *ire-1(v33)* and *pek-1(ok275)* mutants, whereas levels in *atf-6(ok551)* mutants were equal to wild type. Wild type levels of each transcript were set to 1.0 fold induction. An *act-1* specific probe was used to adjust for equal loading. Shown are mean values of relative transcript level +/- SEM of 3-14 independent quantifications.

To test if *pdr-1(lg103)* confers endogenous ER stress and so induces the UPR itself, chaperone expression *in vivo* and in Northern blot analyses was monitored. In all four *pdr-1* mutant backgrounds, the UPR remained un-induced but was still inducible by exogenous ER stress, judged by P_{hsp-4}::*gfp* reporter expression. Consistently, transcript levels of both C*. elegans* BiP homologs, *hsp-4* and *hsp-3*, were unaffected in all *pdr-1* mutants as measured by Northern blot analyses (Figure 34). Taken together, *pdr-1* transcript levels of *pdr-1* were reduced in *ire-1(v33)* and *pek-1(ok275)* loss-of-function alleles, and were strongly upregulated in *atf-6(ok551)* mutants. Therefore one can conclude that *C. elegans parkin* expression is controlled by all three regulators of the unfolded protein response, IRE-1, PEK-1, and ATF-6, and may therefore be positioned downstream of the UPR.

3.6 Ectopic Expression of α-synuclein in *C. elegans*

Accumulation of α-synuclein in Lewy bodies is one of the hallmarks of sporadic PD and of its hereditary forms caused by mutations in the α-synuclein gene. Since the *C. elegans* genome does not encode an obvious homolog, human α-synuclein WT as well as pathogenic mutations A53T and A30P were ectopically expressed in *C.* elegans from different endogenous promoters. First, *C. elegans* wild type animals transgenic for α-synuclein variants expressed from the pan-neuronal *unc-119* promoter were analyzed by immunofluorescence (Figure 35).

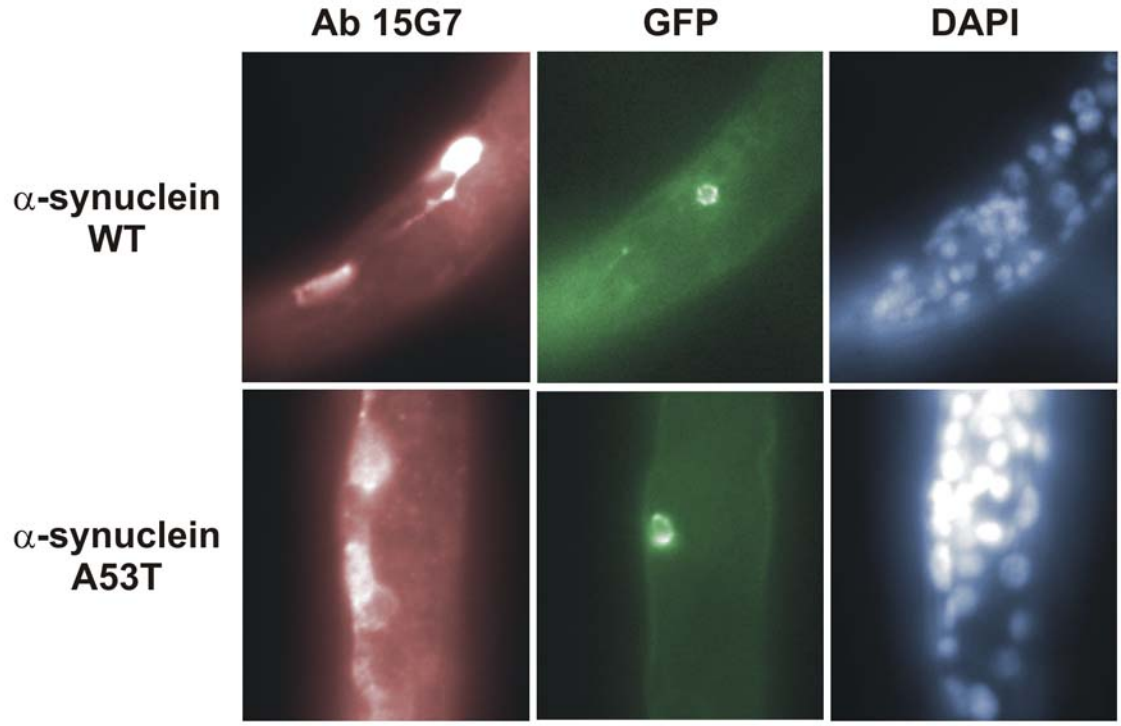


Figure 35. Ectopic Expression of Human α-synuclein in *C. elegans*.

α-synuclein variants were expressed from the pan-neuronal *unc-119* promoter. Photographs display representative examples of the specific staining patterns. **Upper row:** α-synuclein WT; **Lower row:** α-synuclein A53T; **Left:** antibody α-synuclein (15G7); **Middle:** GFP signal in a dopaminergic neuron; **Right:** DAPI counter-staining.

Upon mosaic expression of α-synuclein WT and A53T mutation massive accumulation of α-synuclein was seen in a variety of neuronal cell bodies and processes. The neurites appeared significantly swollen, reaching diameters that exceed that of their somata. This might be caused by aggregation of α-synuclein into large cytoplasmic and dendritic inclusions. Whether these depositions indeed resemble aggresome/LB-like structures and result in cell death has not been investigated so far.

However, to further analyze the effects of human α-synuclein ectopic expression in *C. elegans* on specific cell types and to test for dosage-dependency, a variety of other endogenous promoters were chosen. To examine the impact of human α-synuclein on particularly DA neuron integrity and survival, α-synuclein variants were expressed from the *dat-1* promoter. Because LB formation has to be considered a long-lasting age-dependent phenomenon and the short life-span of *C. elegans* may vastly preclude LB formation, α-synuclein levels may have to be increased in order to exacerbate aggregation. Therefore, the *sel-12* promoter was used to ensure strong and ubiquitous expression of human α-synuclein during all stages of the worm. Thus, a set of transgenic lines was generated by micro-injection of the respective plasmids into *C. elegans* wild type. These strains offer great potential to investigate the specific effects of different α-synuclein overexpression conditions.

3.6.1 Mutant α-Synuclein Expression Leads to Developmental Arrest and Lethality of *pdr-1(lg103)*

To test whether there exists a genetic link between *pdr-1* dysfunction and α-synuclein aggregation in *C. elegans*, α-synuclein was ectopically expressed in *pdr-1* mutant background. This time, genomically integrated copies of human wild type α-synuclein and A53T mutation were expressed from the pan-neuronal *aex-3* promoter ($P_{aex\text{-}3}$*::α-synuclein*) (Lakso et al., 2003). Whereas neuronal expression of wild type α-synuclein resulted in no observable phenotype, the α-synuclein A53T mutation caused developmental defects and a temperature-sensitive lethal phenotype in *pdr-1(lg103)* (Figure 36). Although α-synuclein WT and A53T mutation have not been cloned in exactly the same way (Lakso et al., 2003), it is unlikely that this contributes to the observed phenotypical differences between both variants. α-synuclein WT is N-terminally fused to codons 1-49 of the *aex-3* gene, whereas the A53T mutation is directly fused to the *aex-3* promoter. This results, as observed in Western blots, in a slight difference of the molecular weights of the expressed α-synuclein proteins (Figure 47, see Discussion Section).

Noticeably, ectopic expression of human α-synuclein in any of the different mutant *pdr-1* backgrounds did not enhance loss of dopaminergic neurons as judged by the *gfp* expression pattern of an integrated $P_{dat\text{-}1::gfp}$ marker (Figure 36).

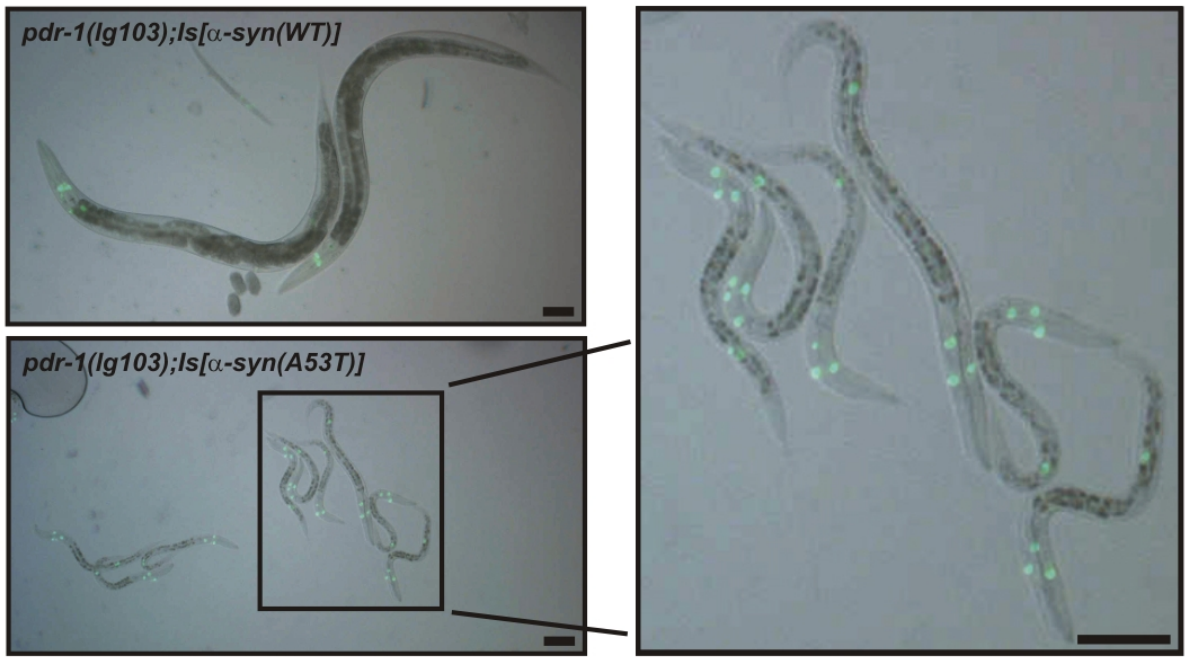


Figure 36. Ectopic Expression of Human α-synuclein A53T Mutation in *pdr-1(lg103)* Mutants Leads to Developmental Arrest and Lethality.

Pictures show *pdr-1(lg103)* mutant animals ectopically expressing α-synuclein WT or A53T mutation from a pan-neuronal promoter ($P::_{aex\text{-}3}$) from integrated arrays. Worms were grown from synchronized eggs for three days at 20° C. Ectopic expression of α-synuclein WT in *pdr-1(lg103)* mutant background showed no effect on survival/development of the animals, as almost all worms developed to fertile adults. In contrast, Ectopic expression of α-synuclein A53T in *pdr-1(lg103)* mutant background resulted in a dramatic lethality/arrest, as almost all animals arrested and/or died during development. The enlarged sector shows a three fold magnified view on *pdr-1(lg103);ls[α-syn(A53T)]* arrested at early larval stages. Expression of α-synuclein in *pdr-1* mutant background did not accelerate or enhance loss of dopaminergic neurons as judged by integrated co-injection marker $P_{dat\text{-}1}$*::gfp*. Scale bar: 0.1 mm.

At 15° C, 15% of *pdr-1(lg103)* mutants expressing α-synuclein A53T, arrested and died at early larval stages (Figure 37A). This lethal phenotype of *pdr-1(lg103)* mutants became fully penetrant by increasing temperature to 20° C (Figure 37B). In contrast, expression of α-synuclein A53T mutation did not cause a phenotype in other *pdr-1* alleles, even not at 25° C (Figure 37C). Moreover, expression of wild type α-synuclein was inconspicuous in any *pdr-1* mutant background (Figure 37). Expression of any α-synuclein variant in wild type animals had no effect on development or viability of worms.

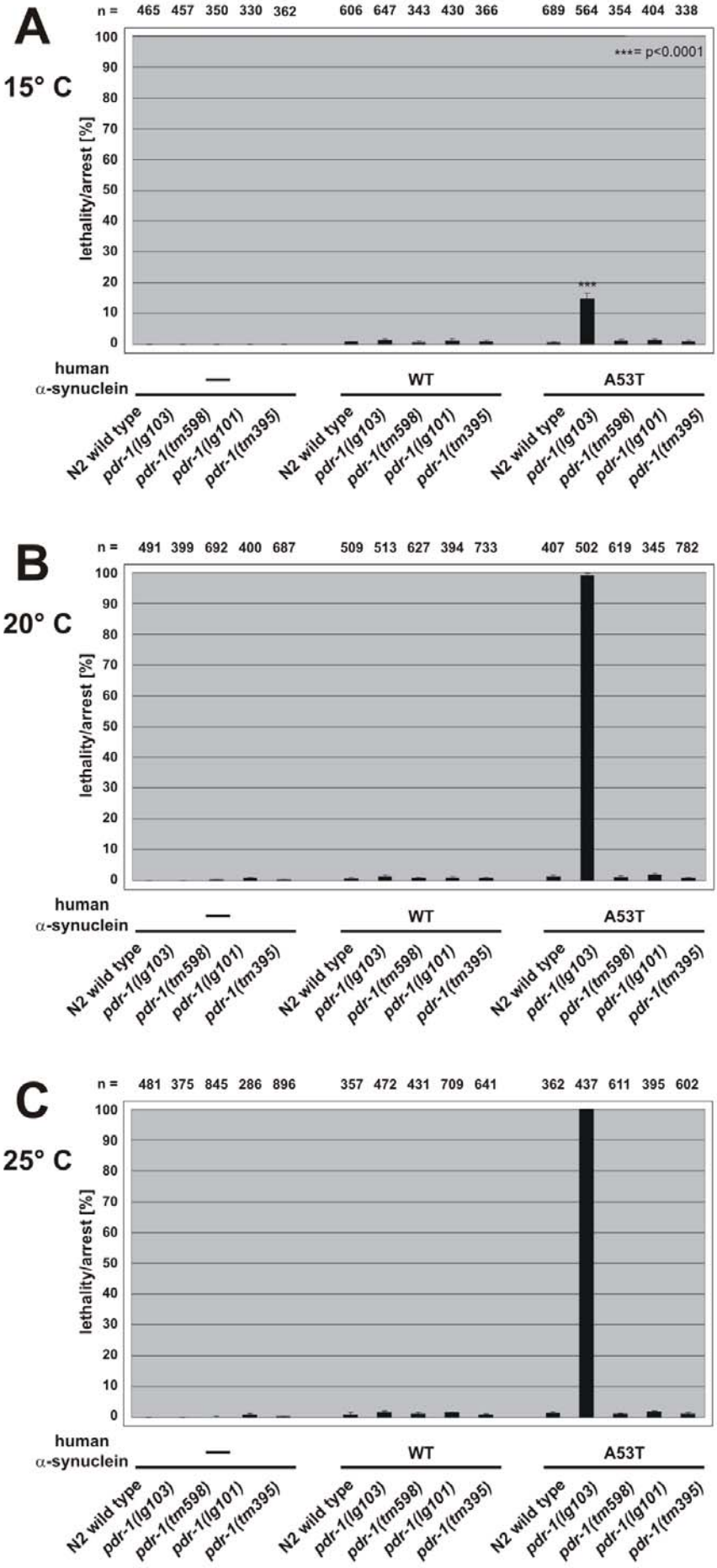


Figure 37. Survival Analyses of *pdr-1* Mutants Ectopically Expressing α-synuclein WT and A53T Mutation.

The Y-axis depicts the percentage of dead/arrested animals after 3-4 days grown from synchronized eggs at different temperatures. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n). (**A**) Only α-synuclein A53T ectopically expressed in *pdr-1(lg103)* animals, results in weak but significant lethality/arrest at 15° C. (**B**) α-synuclein A53T induced lethality/arrest in *pdr-1(lg103)*, but not in other *pdr-1* mutant animals, is dramatically enhanced at 20° C. (**C**) Ectopic expression of α-synuclein at 25° C.

3.6.2 Cytotoxicity Is Dependent on Levels of both Mutant Proteins

The incidence of PD and formation of Lewy bodies generally increases with the age of the patients. In order to generate a PD model in a short-lived animal like *C. elegans* (lifespan generally lower than 20 days) obviously the expression levels of α-synuclein have to be increased compared to the situation in humans.

Genetic tools were used to monitor the consequences of different levels of a-synuclein accumulation in various *pdr-1* mutant backgrounds (Table 4).

genotype	expression of α-synuclein A53T	n	mean ± SEM % lethality/arrest
N2 wild type	**+/+**	**407**	**1.6 ± 0.7**
pdr-1(lg103)	**+/+**	**502**	**99.0 ± 0.6 *****
pdr-1(tm598)	**+/+**	**619**	**1.1 ± 0.4**
pdr-1(lg101)	**+/+**	**345**	**1.9 ± 0.7**
pdr-1(tm398)	**+/+**	**782**	**0.8 ± 0.3**
pdr-1(lg103)	**+/-**	**384**	**0.3 ± 0.3**
pdr-1(lg103)/+	**+/-**	**306**	**0.3 ± 0.3**
pdr-1(lg103)/+	**+/+**	**258**	**0.7 ± 0.4**
pdr-1(lg103)/pdr-1(tm598)	**+/+**	**229**	**4.4 ± 0.5 **/***
pdr-1(lg103)/pdr-1(lg101)	**+/+**	**198**	**0.8 ± 0.5**
pdr-1(lg103)/pdr-1(tm598)	**+/+**	**202**	**1.3 ± 0.6**

******* *P* value against *α-synuclein A53T(+/+)* < 0.0001
****** *P* value against *pdr-1(lg103)/+;α-synuclein A53T(+/+)* = 0.0010
***** *P* value against *pdr-1(tm598);α-synuclein A53T(+/+)* = 0.0308

Table 4. Survival of Heterozygous *pdr-1* Mutants Ectopically Expressing α-synuclein A53T.

Analyses were performed at 20° C and as described in Figure 37. Only α-synuclein A53T expressed from both integrated copies, confers cytotoxicity in specifically homozygous *pdr-1(lg103)* mutant background, and, although milder, in transheterozygous mutants carrying both in-frame deletions *pdr-1(lg103)/pdr-1(tm598)*. Shown are mean values of lethality/arrest in percent +/- SEM, the total number of animals analyzed is listed (n).

Several results from these experiments are noteworthy: Firstly, only strong expression of α-synuclein A53T (two copies of the transgene) resulted in a strong phenotype in *pdr-1(lg103)* mutants. Secondly, similar to the results obtained after exposing the animals to ER stress, only a homozygous *pdr-1(lg103)* mutant background or, to some extent, a transheterozygous *pdr-1(lg103)/pdr-1(tm598)*

genetic background resulted in a phenotype in α-synuclein A53T expressing worms. Thirdly, a single copy of the wild type *pdr-1* or of the other *pdr-1* alleles was sufficient to prevent a phenotype, even in animals that expressed two copies of α-synuclein A53T.

Previous experiments already suggested that *pdr-1(lg103)* might cause a gain-of-misfunction phenotype. If this is true, then further increasing the expression level of this mutant should result in an aggravation of the α-synuclein A53T lethal phenotype. In order to test this hypothesis, the consequences of α-synuclein A53T expression in the *pdr-1(lg103);atf-6(ok551)* double mutant background was analyzed. Since the mutant allele *atf-6(ok551)* enhances the transcriptional rate of *pdr-1*, increased PDR-1 protein levels can be proposed in this genetic background. Lethality/arrest was strongly enhanced already at 15°C with respect to the *pdr-1(lg103)* single mutant (Figure 38).

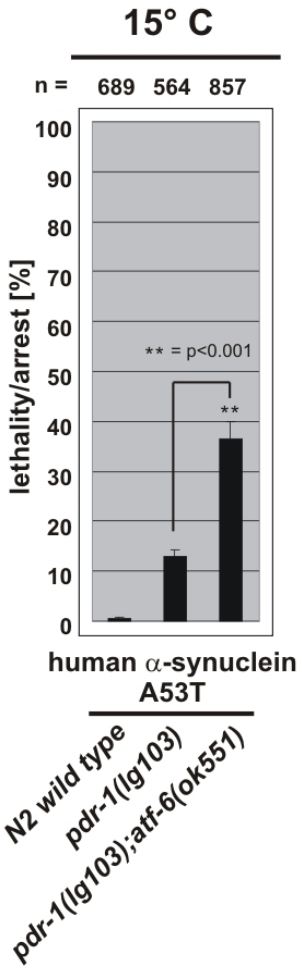


Figure 38. Transcriptional Enhancement of *pdr-1(lg103)* Aggravates Toxicity.

pdr-1(lg103);atf-6(ok551) double mutants expressing α-synuclein A53T mutation show a significantly enhanced lethality/developmental arrest at 15° C. The Y-axis depicts the percentage of dead/arrested animals after four days survival of synchronized eggs at 15° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

In contrast, knock-down of either α-synuclein A53T or *pdr-1(lg103)* gain-of-misfunction, should ameliorate the lethal phenotype of *pdr-1(lg103)* mutants ectopically expressing α-synuclein A53T mutation. Therefore, animals were subjected to dsRNA-mediated interference (RNAi) studies by feeding (Figure 39). Only weak, but not significant reduction of lethality/arrest was observed in *pdr-1(lg103)*;α-synuclein A53T animals subjected to α-synuclein RNAi when compared to control animals.

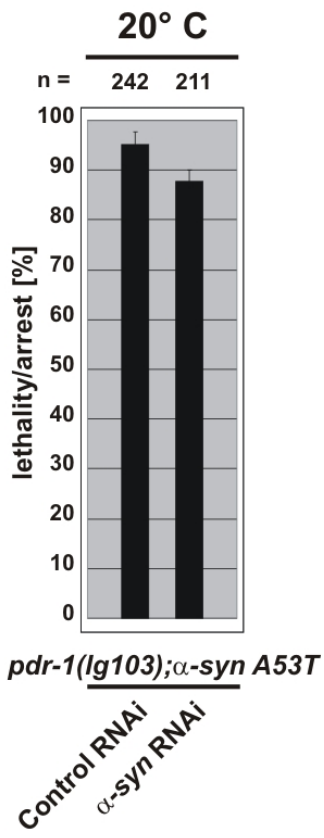


Figure 39. α-synuclein RNAi Studies.

pdr-1(lg103) mutants expressing α-synuclein A53T mutation were subjected to α-synuclein RNAi, in order to modulate cellular levels of α-synuclein protein, and thus, cytotoxicity. Therefore, animals were fed with engineered *E. coli* producing dsRNA of either control or α-synuclein. The Y-axis depicts the percentage of dead/arrested animals after 3-4 days grown from synchronized eggs at 20° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

The most likely explanation for this is, that RNAi mediated knock-down was simply incomplete, and cellular levels of mutant proteins could not be reduced below a certain toxic threshold. This is in line with data from other preliminary RNAi knock-down studies of *pdr-1*, mutant *pdr-1(lg103)* or K08E3.8. Although RNAi is a great tool to reduce/eliminate expression of distinct genes, some *C. elegans* tissues as well as genes are less sensitive to dsRNA-mediated knock-down, at least by feeding methods (Simmer et al., 2002). However, to be sure, one has to perform control experiments like quantitative RT-PCR, in order to compare endogenous mRNA levels of the respective gene.

In summary, a strong neurotoxic phenotype results from the expression of mutant α-synuclein in the sensitized background of recessive gain-of-misfunction alleles of *C. elegans parkin*. Cytotoxicity is dependent on temperature and on protein levels of both, human α-synuclein A53T mutation and PDR-1(Δaa24-247).

3.6.3 Blockage of the UPR is Not Sufficient for α-Synuclein A53T Mediated Cytotoxicity

The aggravation of a mutant α-synuclein phenotype by *pdr-1(lg103)* could either be caused by blocking the ER stress response or by an effect of the mutant protein that does not involve its role in the UPR. In order to distinguish between these two possibilities, animals expressing α-synuclein in a defective UPR background or treated with exogenous ER stress by tunicamycin, were analyzed. Expression of neither wild type α-synuclein nor the A53T variant caused a detectable phenotype in the *ire-1(v33)* mutants, not even at higher temperatures (Figure 40). Not surprising, *ire-1(v33)* single mutants themselves showed a temperature-dependent effect as

lethality/arrest was increased at 25°C. This is consistent with temperature being a critical determinant of protein folding/degradation pathways (Figure 40).

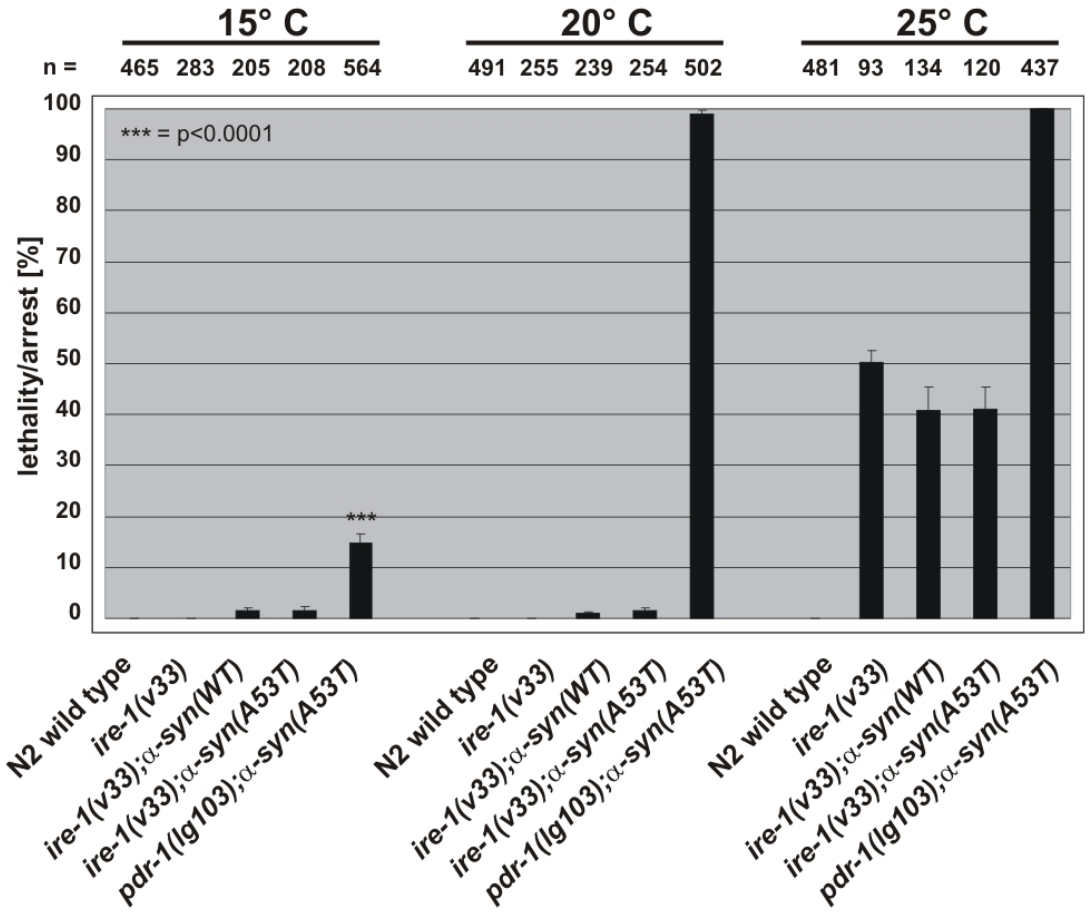


Figure 40. Cytotoxicity of α-synuclein A53T Is Independent of UPR Dysfunction.

Ectopic expression of α-synuclein WT and A53T in *ire-1(v33)* mutant background did not affect survival, even not at elevated temperatures. The Y-axis depicts the percentage of dead/arrested animals after three days survival of synchronized eggs at 15, 20 and 25° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

To test the effects of exogenous ER stress on cytotoxicity of α-synuclein, worms overexpressing WT or A53T mutation were treated with tunicamycin. Consistent with data from *ire-1* mutants, animals expressing α-synuclein are not more sensitive to exogenous ER stress when treated with tunicamycin compared to wild type (Figure 41). Complete in line with the latter findings, α-synuclein expressing strains did not induce the UPR, as monitored by *hsp-4* and *xbp-1* transcript levels in Northern blot analyses. Consistently, the *hsp-4::gfp* reporter gene remained un-induced in animals ectopically expressing human α-synuclein in wild type or in the *pdr-1(lg103)* mutant background.

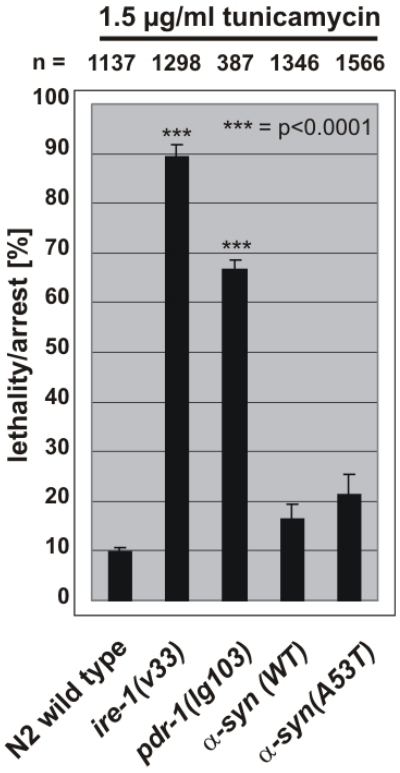


Figure 41. α-synuclein Expressing Worms are Not Sensitive to Exogenous ER Stressors.

The Y-axis depicts the percentage of dead/arrested animals after three days survival of synchronized eggs at 20° C plates containing 1.5 µg/ml tunicamycin. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

To test if disruption of *chn-1* function would aggravate α-synuclein A53T mediated cytotoxicity in *pdr-1(lg103)* at low temperatures, animal were subjected to *chn-1* RNAi (feeding). Knock-down of *chn-1* in *pdr-1(lg103)* mutants ectopically expressing α-synuclein A53T mutation at 15° C, enhanced toxicity weakly, but not significantly (Figure 42).

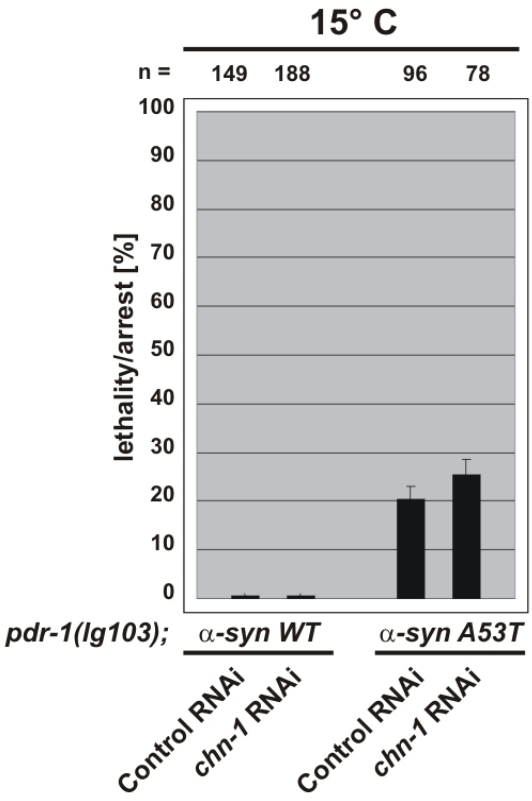


Figure 42. *chn-1* RNAi Studies.

pdr-1(lg103) mutants expressing either α-synuclein WT or A53T mutation were subjected to *chn-1* RNAi, in order to enhance cytotoxicity. Therefore, animals were fed with engineered *E. coli* producing dsRNA of either control or *chn-1*. The Y-axis depicts the percentage of dead/arrested animals after 3-4 days grown from synchronized eggs at 15° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

This either suggests that *chn-1* might indeed function in the affected pathway, or simply that RNAi, at least by feeding methods as in the case of α-synuclein and *pdr-1(lg103)*, does not function properly. As a follow up strategy, *pdr-1(lg103);chn-1(-/-)* double mutants overexpressing α-synuclein should be constructed and analysed. However, so far a genetic interaction can neither be established nor excluded, though *chn-1* loss-of-function mutants are not sensitive to tunicamycin treatment (T. Hoppe, personal communication). Taken together, these results demonstrate that the phenotype caused by the combination of α-synuclein A53T expression and *pdr-1* gain-of-misfunction is not mediated by impairment of an ER stress pathway.

3.6.4 *pdr-1(lg103)* and α-synuclein A53T Mediated Toxicity is Independent of Oxidative and Heat Stress Pathways

To further assign cytotoxicity mediated by overexpression of α-synuclein A53T in *pdr-1(lg103)* mutant animals to a certain pathway, worms were tested under oxidative and heat stress conditions (Figures 43 and 44, respectively).

Paraquat (methylviologen) was used as an endogenous inducer of oxidative stress. *mev-1(kn-1*) control mutants carry a missense mutation in a cytochrome *b* subunit of mitochondrial respiratory complex II.

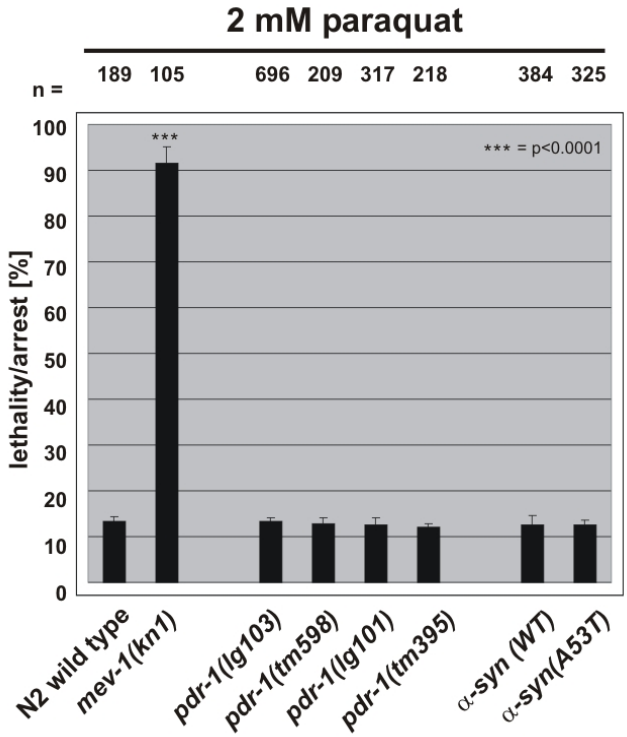


Figure 43. *pdr-1* Mutants and Worms Ectopically Expressing α-synuclein are Not Hypersensitive to Oxidative Stress.

Oxidative stress had no effect on viability and development of *pdr-1* mutants or worms overexpressing human α-synuclein WT or A53T mutation. In contrast, *mev-1(kn1)* sensitive control mutants arrested early in their development. The Y-axis depicts the percentage of dead/arrested animals treated with 2mM paraquat after three days exposure of synchronized L1 larvae at 20° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

These mutants are hypersensitive towards increased oxidative stress, and thus arrest early in their development (Ishii et al., 1998). However, all *pdr-1* mutants, as well as animals overexpressing human α-synuclein, were resistant towards oxidative stress, indistinguishably from wild type (Figure 43).

In addition, the development and survival of *pdr-1* mutants and animals overexpressing human α-synuclein after short heat stress treatments was analyzed. *ire-1(v33)* mutants showed a significantly increased lethality/arrest by 2h heat shock at 35°C. In contrast, all *pdr-1* mutants and animals overexpressing α-synuclein were resistant to short heat stress treatments and showed no discernable phenotype, like wild type (Figure 44).

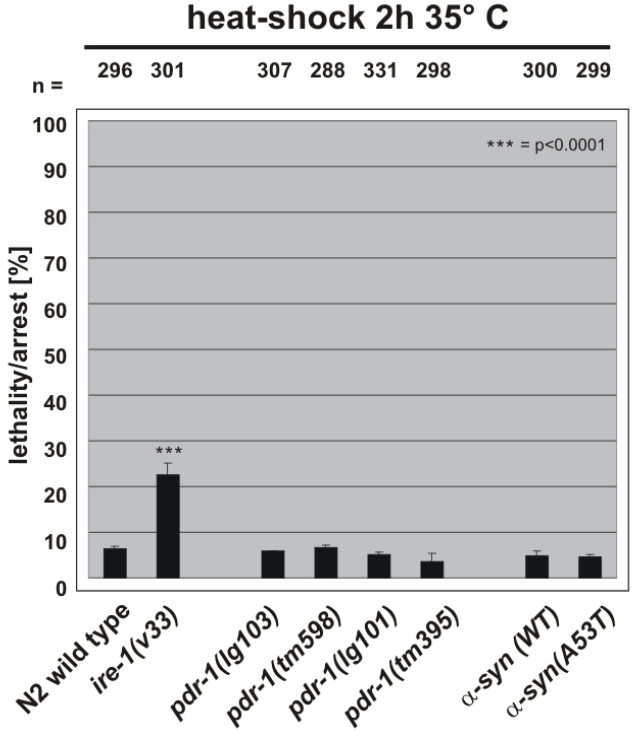


Figure 44. *pdr-1* Mutants and Worms Ectopically Expressing α-synuclein are Not Hypersensitive to Heat Stress.

The total number of animals scored is listed above each column (n). Synchronized L2/L3 larvae were heat-stressed for 2h at 35° C. The Y-axis depicts the percentage of arrested/dead animals after two days recovery at 20° C. Shown are mean values +/- SEM, the total number of animals analyzed is listed above each column (n).

In summary, ectopic expression of human α-synuclein A53T, but not WT, in specifically *pdr-1(lg103)* mutant background causes temperature-dependent cytotoxicity, resulting in developmental defects and lethality. The exerted cytotoxicity is independent of ER stress or the UPR and independent of oxidative or heat stress pathways. Thus, these results most likely suggest a cytosolic mechanism for α-synuclein A53T mediated toxicity, corroborating a misfunction of *pdr-1(lg103)* in two distinct protein stress pathways.

4 Discussion

The underlying molecular and cellular pathways that mediate PD are largely unknown. Mutations in human *parkin* have been associated with particularly severe recessive forms of PD. Human Parkin is involved in several cellular processes, and like other RING-finger containing proteins acts as an E3 ubiquitin ligase that targets several substrates for degradation. Until now, functional studies on *parkin* have not provided a direct explanation for the disease mechanism(s), suggesting that additional animal models may be required. In the present study, a nematode model was developed to investigate the biological role of the *parkin* gene *in vivo*, in order to gain insights into the pathogenesis of PD.

4.1 *C. elegans* PDR-1 Is the Functional Equivalent of Human Parkin

First, the *Caenorhabditis elegans* protein PDR-1 was identified as the ortholog of human Parkin. Expression analyses revealed that PDR-1 is present in all tissues but enriched in neurons and muscles, and mainly localizes to the cytoplasm (see Figure 17). PDR-1 was shown in this study to interact and cooperate with co-enzymes of the ubiquitin/proteasome pathway, in order to mediate E3 ubiquitin ligase activity (see Figure 24). Like human Parkin, PDR-1 specifically associates with E2 and E4 enzymes (see Figures 20 and 23) involved in the cytosolic protein stress response and the ERAD pathway (Imai et al., 2002; Shimura et al., 2001; Zhang et al., 2000). Noteworthy, ubiquitylation and degradation machineries have already been reported to be highly conserved from nematodes to humans (Davy et al., 2001; Jones et al., 2002).

The identification of PDR-1 protein interactions with the product of the downstream gene K08E3.8 (see Figure 21B), as well as with several other ‘prion’ domain proteins identified in this and in an independent study (Li et al., 2004), suggest a high affinity of PDR-1/Parkin and interactors for such Q/N-rich domains (see Figure 22 for the PDR-1 interaction map). The *C. elegans* genome encodes about 100 predicted ‘prion’ domain proteins (PQN-), dispersed among numerous cellular functions. However, these proteins may represent due to the modulatory

capacity of the 'prion' domain that can acquire conformational changes, aggregation-prone proteins, which are targeted by PDR-1. This further substantiates the implication of human Parkin in the removal of aggregation-prone polypeptides, including polyQ proteins (Tsai et al., 2003). Although the cellular role of K08E3.8 is unknown, the phenotypes caused by a K08E3.8 mutation suggest an important function. The deletion *pdr-1(lg101)* eliminates codons 1-63 of K08E3.8, and therefore the translational start. The described phenotypes of *pdr-1(lg101)* mutants have been linked to a presumably recessive K08E3.8 loss-of-function. However, the arising question for the functional correlation between PDR-1 and its downstream gene K08E3.8, and the biological role of their interaction, cannot be answered, yet. Nevertheless, the developmentally regulated co-expression of *pdr-1* and K08E3.8 as well as the physical interaction of both gene products, suggest an interesting new field of research that needs to be further investigated.

Although a variety of different proteins with diverse and un-related functions were identified, many novel PDR-1 interaction partners are related to protein degradation pathways. However, whether these proteins are co-enzymes that are associated with a specific function of PDR-1/Parkin or serve as potential substrates for PDR-1/Parkin mediated ubiquitylation still has to be elucidated. Each of the *C. elegans* homologs of human cathepsins, which were most prominently found in the PDR-1 interaction screen, might represent a candidate substrate for PDR-1 (see Figure 19). Age-related lysosomal damage and subsequent release of destructive enzymes into the cytoplasm has been linked to necrotic cell death (Syntichaki and Tavernarakis, 2003). Nevertheless, some of these *C. elegans* cathepsins were also found associated with 19S regulatory subunits and E1 enzymes in an independent screen (Li et al., 2004). Although cathepsins have long been implicated only in end-stage protein degradation in lysosomes, potential change for substrate specificity and localization has been reported (Goulet et al., 2004; Reinheckel et al., 2001). Thus, these results might provide a possible direct functional link between lysosomal damage and the pathophysiology of PD.

Substrates of human Parkin identified so far are diverse and associated with numerous un-related cellular functions. Candidate Parkin substrates that are aggregation-prone proteins range from the ER transmembrane protein Pael-R (Imai et al., 2001) to cytosolic modified α-synuclein forms (Shimura et al., 2001) and polyQ proteins (Tsai et al., 2003). Parkin substrates that have direct toxic effects include

free, monomeric forms of α/β-tubulins (Ren et al., 2003) and cyclin E, which promotes apoptosis (Staropoli et al., 2003). In addition, specific synaptic proteins that are targeted by Parkin have been identified: the septins CDCrel-1/2 (Choi et al., 2003; Zhang et al., 2000), synaptotagmin XI (Huynh et al., 2003), and, most recently identified, the dopamine transporter (Jiang et al., 2004). The aminoacyl-tRNA synthetase subunit p38 (Corti et al., 2003) completes the list of the diverse and un-related Parkin substrates identified so far.

Why PDR-1/Parkin carry two RING fingers, while, in principle, a single one is sufficient for E2 binding and ubiquitin ligase activity of many E3 proteins, is unclear (Joazeiro and Weissman, 2000; Moynihan et al., 1999). The identified homo-dimerization ability of PDR-1, and the binding to other RING finger proteins (see Figures 21A and 28C), may suggest a possible function of PDR-1/Parkin as a molecular scaffold for the assembly of a multisubunit complex (Dev et al., 2003b). Furthermore, this also provides a mechanistic explanation for the wide-ranged affinity of PDR-1/Parkin for proteins with diverse and un-related functions. Therefore, the simply extended interaction surface might allow association with other proteins to acquire possibly changes of specificities and functions. A second possibility is that RING box proteins may ligate also other ubiquitin-like modifiers in addition to ubiquitin. Since PDR-1 was found to interact with the ubiquitin-like protein F52C6.2, the question arises whether PDR-1 simply binds this protein or indeed catalyzes the conjugation of this putative UBL modifier to itself and/or to certain substrate proteins. Although speculative, such a linkage may also imply new un-revealed functions other than proteasome-dependent degradation, as it is the case for the UBLs Nedd8 (neural precursor cell-expressed and developmentally down-regulated gene) or SUMO (small ubiquitin-related modifier). However, interaction of PDR-1 with either the SUMO- or Nedd8-specifc E2 enzymes, UBC-9 or UBC-12 respectively, could not be demonstrated, at least not using the yeast-two-hybrid system.

Nevertheless, the newly identified PDR-1 interactions have to be further validated and characterized. In order to analyze the interactions of PDR-1 with several candidate proteins, appropriate GST-fusion constructs have been generated for some of them (see Table 12), and first attempts to purify the corresponding recombinant proteins have been made. Next, protein interactions have to be confirmed in GST-pull down experiments, and PDR-1/Parkin mediated ubiquitylation has to be tested.

4.2 PDR-1 Is Part of the UPR Pathway

The UPR is an intracellular signalling pathway that mediates an adaptation to ER stress at both transcriptional and translational levels. It augments folding and degradation capacity and also acts by reducing the protein load in the ER (Rutkowski and Kaufman, 2004). Treatment with tunicamycin, an inhibitor of *N*-linked glycosylation, results in high amounts of unfolded proteins in the ER lumen, and thus, tunicamycin is a potent inducer of the UPR.

Particularly one *C. elegans parkin* allele, *pdr-1(lg103)*, an in-frame deletion resulting in a PDR-1 protein without functional UBL and RING1 domains, was specifically sensitive towards ER stress conditions. These animals suffered from severe developmental defects and lethality (see Figures 31 and 32), but were, at the same time, not sensitive to oxidative or heat stress (see Figures 43 and 44). The hypersensitivity of *pdr-1(lg103)* animals towards ER stress is similar to the phenotype of mutants defective in the proper execution of the UPR, which is required for normal development in *C. elegans*. Tunicamycin treated UPR mutants, or non-treated double mutants of the pathway, including *ire-1;pek-1*, *xbp-1;pek-1*, or *xbp-1;atf-6(RNAi)* animals, typically arrest at the L2/L3 larval stages due to the degeneration of the intestine (Shen et al., 2001; Urano et al., 2002). At L2, the *C. elegans* intestine induces high-level synthesis of secretory proteins which in the absence of proper UPR function make the animals more susceptible towards ER stress (Shen et al., 2001). PDR-1 contributes to this anti-stress response, and is consequently up-regulated in the L2/L3 larval stages (see Figure 16).

In mammals, the UPR is mediated by two branches, one specifically induced by ER stress (ATF-6 and IRE-1) and one shared by different cellular stresses (PERK/PEK), the so called integrated stress responses (Harding et al., 2002). These pathways converge on the level of translational attenuation through induction of different kinases that phosphorylate eIF2α, like PERK/PEK. To date, cross-talk and feedback mechanisms have been reported within the two branches, as well as between them (Rutkowski and Kaufman, 2004). Because the translation attenuation is usually transient, the transcriptional aspect of the UPR mediated by IRE1 and ATF6 becomes more important in adapting cellular processes with the accumulation of unfolded proteins (Shen et al., 2004). Although conserved, the coordination of the

three pathways and their specific contribution to the metazoan ER stress response is unclear.

Since *pdr-1(lg103)* mutants shared the ER stress hypersensitivity of UPR mutants, genetic interactions were tested. *pdr-1(lg103)* and *pdr-1(tm598)* specifically cooperate with *ire-1* loss-of-function but not with *pek-1* or *atf-6* mutants, as shown by synthetic effects of *ire-1(v33)* and *pdr-1(lg103)* or *pdr-1(tm598)* in the respective double mutant (see Table 3). This suggests that *pdr-1* may act to some extent in parallel to *ire-1* signalling, perhaps by directly contributing to either the *pek-1* or *atf-6* pathway. In *C. elegans*, IRE-1 exerts nearly complete control over the induction of well characterized components of ER client protein processing machinery, and PEK-1 provides redundant protection against ER stress (Shen et al., 2001). While *pek-1(ok275)* represents a complete loss-of-function mutant, the deletion *atf-6(ok551)* most likely functions as a hypermorphic allele, which is constitutively active, even under non-stressed conditions. This is supported by the fact that *atf-6(ok551)* mutants are not sensitive to tunicamycin treatment like *ire-1* or *pek-1* loss-of-function mutants (see Figures 31 and 32). To be able to further assign PDR-1 function to either the PEK-1 or the ATF-6 pathway, genetic interaction of *pdr-1(lg103)* with a clear *atf-6* loss-of-function has to be studied. However, un-stressed *ire-1;pek-1*, *xbp-1;pek-1*, or *xbp-1;atf-6(RNAi)* double mutants arrest at early larval stages and therefore show an earlier/stronger phenotype than *ire-1;pdr-1* double mutants, suggesting a position for PDR-1 downstream of these UPR transducers.

Unfolded proteins in the ER are retro-translocated to the cytosol (Tsai et al., 2002) and turned over by the ERAD pathway (Ahner and Brodsky, 2004) which function is increased by the UPR (Kostova and Wolf, 2003). Genes active in the ERAD pathway are not essential under normal growth conditions, but become indispensable under stress or when the UPR is blocked. Similar to *pdr-1(lg103)*, mutations in ERAD genes and in the recently identified *C. elegans abu* gene family (**a**ctivated in **b**locked **U**PR) are synthetic lethal with a defective UPR, too, but activate the UPR themselves (Friedlander et al., 2000; Travers et al., 2000; Urano et al., 2002). In contrast to *pdr-1* in-frame deletions, which exacerbated the phenotype of un-stressed *ire-1* mutants, the effects of *ire-1* or *xbp-1* mutants are enhanced by *abu-1(RNAi)* only under stress conditions. Moreover, the UPR reporter *hsp-4* is not induced in any of the *pdr-1* mutant backgrounds, even not in *pdr-1(lg103)* mutants (see Figure 33). This rules out a susceptibility of worms to unfolded protein stress

conferred by increased steady state level of endogenous ER stress in the *pdr-1* mutants. As it is known for ERAD genes, expression of the *pdr-1* gene is also induced by the UPR. Loss of IRE-1 or PEK-1 function resulted in about half-fold reduction of *pdr-1* transcript levels (see Figure 33). In contrast, *pdr-1* transcript levels are elevated half-fold by the *atf-6(ok551)* mutation. However, as already mentioned this special allele most likely represents a hypermorphic allele, from which an otherwise only stress-induced, activated form of the transcription factor ATF-6 might be generated constitutively, even in the absence of stress. Consequently, this suggests that *pdr-1* gene expression is controlled by all three pathways IRE-1, PEK-1, and ATF-6. Nonetheless, though very unlikely, it cannot be excluded that *atf-6(ok551)* mutants confer a loss-of-function. This would disprove the direct regulation of *pdr-1* gene expression by ATF-6 signalling. If so, then, most likely IRE-1 and PEK-1 dependent back-up mechanisms may be responsible for the enhanced *pdr-1* expression in *atf-6(ok551)* mutants, since cross-talk between the three UPR pathways is known. However, *hsp-4* and *xbp-1* gene expression is not enhanced in *atf-6(ok551)* mutants (see Figure 34).

Nevertheless, a similar back-up mechanisms has been identified for members of the *abu* gene family, although transcriptional induction was only seen in additionally ER stressed (tunicamycin-treated) mutants defective in the IRE-1/XBP-1 pathway. Whether induction of *abu* gene expression is dependent on PEK-1 or ATF-6, is unknown (Urano et al., 2002). ABU proteins are type I ER-transmembrane proteins similar to mammalian cell surface scavenger receptors of endothelial cells that bind chemically modified extracellular proteins and direct their lysosomal degradation. *abu* genes play an important role in protecting animals with a defective UPR against ER stress, as a back-up mechanism. Therefore, a function within the endomembrane system by binding to altered ER client proteins and modulating their intracellular fate was suggested, similar to one of the distantly related mammalian receptors (Urano et al., 2002). The *abu* gene family encodes nine relatively homologous proteins and two more distantly related members. Interestingly, at least these two (ABU-10 and ABU-11) have been detected to associate with distinct PDR-1 interactors (Li et al., 2004), although the significance of these interactions is unclear.

Earlier studies indicated an specific up-regulation of human Parkin expression in response to ER stress in cell culture (Imai et al., 2000), though differences

between astrocytic and neuronal cells have been found as well (Ledesma et al., 2002). However, the involved pathways have not been investigated. Interestingly, a very recent report demonstrated the specific stimulation of Parkin E3 ubiquitin ligase activity through de-phosphorylation in response to ER stress (Yamamoto et al., 2004). In summary, these collective data suggest a general role for PDR-1/Parkin in the UPR as well as a complex mode of regulation and modulation of PDR-1/Parkin function during the execution of this pathway (Figure 45).

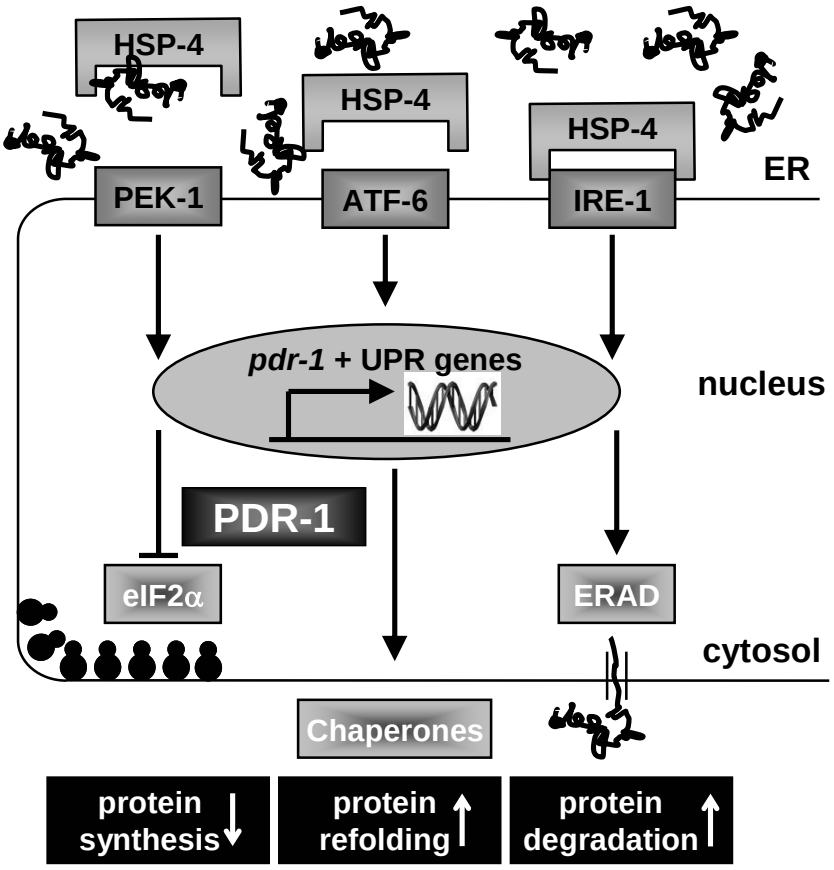


Figure 45. Regulation of PDR-1 By And Involvement In the UPR.

Accumulating unfolded proteins in the ER are bound by HSP-4/BiP. The release of HSP-4 from PEK-1/PERK, ATF-6 and IRE-1 activates these signal transducers. By translational and transcriptional responses, a program to sustain ER function is set in motion. So, *pdr-1* gene expression is controlled by all three UPR signalling pathways PEK-1, ATF-6, and IRE-1. Furthermore, PDR-1 protein acts in parallel to IRE-1 signalling, most likely downstream in the PEK-1 and/or ATF-6 pathway.

The data presented here fully correlate with the proposed model for a widespread involvement of ER stress and the UPR in the pathophysiology of PD (Forman et al., 2003; Sherman and Goldberg, 2001). For example, it was shown that some PD mimetics like 6-OHDA, MPP^+, and rotenone specifically induce ER stress and activate the UPR in cultured neuronal cells. Furthermore, impairment of the UPR pathway increases sensitivity to parkinsonism-inducing toxins (Ryu et al., 2002). The biochemistry of stress induction is most likely very similar in *C. elegans*, as recently the susceptibility of worms to MPP^+ treatment and the amelioration of neurotoxicity by anti-PD drugs was demonstrated (Braungart et al., 2004).

4.3 PDR-1 Is Involved in the Cytosolic Stress Response

Accumulation of the cytosolic protein α-synuclein in Lewy bodies is a hallmark of PD and mutations result in autosomal dominant familial PD. The A53T mutation enhances aggregation of α-synuclein by accelerated fibril formation (Conway et al., 2000). This in turn impairs the proteolytic system (Stefanis et al., 2001) and increases the sensitivity of cells to proteasome inhibition (Petrucelli et al., 2002; Tanaka et al., 2001). However, not only missense mutations are causative for PD, but also multiplications of the gene locus, and therefore enhanced expression levels of wild type α-synuclein have been associated with PD (Chartier-Harlin et al., 2004; Ibanez et al., 2004; Singleton et al., 2003). This has already been proven in transgenic animal models (Reviewed by Maries et al., 2003).

The heat shock protein Hsp70 provides a link between α-synuclein toxicity and the cytosolic protein folding machinery (Auluck et al., 2002). Co-expression of Hsp70 prevented loss of DA neurons associated with α-synuclein in flies. In fact, several heat shock proteins and co-chaperones have been detected in LBs of human post-mortem tissue, suggesting a key role of molecular chaperones in PD progression (Reviewed by Muchowski and Wacker, 2005). Thereby, chaperones like Hsp70 are important in both refolding misfolded proteins and directing proteins towards proteasomal degradation (Cyr et al., 2002; Young et al., 2004).

Furthermore, several lines of evidence suggest a relationship between Parkin and α-synuclein: a rare modified species of α-synuclein was identified as a substrate of Parkin E3 ligase activity (Shimura et al., 2001) and overexpression of Parkin ameliorated DA neuron loss in an α-synuclein transgenic fly model (Yang et al., 2003). However, a physiological role and a direct explanation for the pathogenic mechanism have not been determined so far. In *C. elegans*, overexpression of human α-synuclein wild type or A53T mutation from a pan-neuronal promoter (*aex-3*) resulted in motor deficits as well as in neuronal and dendritic loss, also of DA neurons (Lakso et al., 2003).

In the present study, it was shown that human α-synuclein accumulates in the *C. elegans* neurons both in the cytoplasm and in axonal processes (see Figure 35). Moreover, mutant α-synuclein A53T, but not its wild type form, exerts a cytotoxic effect specifically in *pdr-1(lg103)* mutant animals, resulting in severe developmental defects and lethality at early larval stages (see Figure 36).

Similar to the behaviour after tunicamycin treatment (see Figure 32), other homozygous *pdr-1* mutants were unaffected by ectopic expression of either α-synuclein wild type or A53T mutation (see Figure 37). It is noteworthy that the allele *pdr-1(tm598)* served also in this experiment as a sensitized background in *pdr-1(lg103)/pdr-1(tm598)* compound mutants (see Table 4). Interestingly, cytotoxicity is dependent on temperature (see Figure 37) and on genes doses of both α-synuclein A53T mutation and *pdr-1(lg103)* mutant (see Figure 38). The fact that this phenotype in *C. elegans* arises already at larval stage L2 (~2-3 days after fertilization) is remarkable, given that α-synuclein aggregation in mice is only toxic after months (Giasson et al., 2002). It is conceivable that this toxicity was only observed as a synthetic effect in animals harbouring both the in-frame deletion allele *pdr-1(lg103)* and ectopically expressed mutant α-synuclein.

The phenotype caused by ectopic expression of α-synuclein A53T mutation in *pdr-1(lg103)* mutant background is similar to the one observed under ER stress conditions (compare Figures 31 and 35). In both cases, animals arrest at the same stage of development, in line with the temporal up-regulation of *pdr-1* expression in specifically larval stages L2/L3 (see Figure 15). However, cytotoxic effects of mutant α-synuclein are not seen in tunicamycin-treated animals or in the background of mutants in the UPR pathway. Expression of α-synuclein in wild type worms treated with tunicamycin or in *ire-1* deficient worms did not impair development or viability (see Figures 40 and 41). Consistently, the ER stress marker *hsp-4::gfp* was not up-regulated by transgenic expression of α-synuclein in either wild type worms or *pdr-1(lg103)* mutants. Thus, *pdr-1(lg103)* exacerbates mutant α-synuclein-induced toxicity in an UPR independent fashion. In addition, other cellular stress pathways were found to be un-affected in α-synuclein transgenic worms, too, as animals were not sensitive to oxidative or heat stress conditions (Figures 43 and 44).

Taken together, α-synuclein A53T mutation, but not WT, confers cytotoxicity specifically in *pdr-1(lg103)* mutant background that is dependent on temperature and gene doses of both mutant proteins. This suggests that *pdr-1(lg103)* can tolerate a certain threshold of aggregation-prone α-synuclein A53T mutation, but high cellular levels cause developmental defects and lethality. However, this toxicity is independent of ER, oxidative or heat stress pathways, and therefore is most likely mediated by a cytosolic mechanism.

4.4 PDR-1/Parkin Loss-Of-Function Vs. Gain-Of-Misfunction

Mutations in the *parkin* gene are the most frequent cause of familial PD, and are related to a very early-onset: before 40 years of age, average 26.1 years (Hattori and Mizuno, 2004). So far, about 100 various *parkin* mutations have been identified which seem to contribute to the clinical and pathological heterogeneity of the disease (Hedrich et al., 2004). In contrast to larger deletions, which result in complete loss of Parkin function, it has been proposed that missense mutations or small deletions are associated with a more pronounced phenotype, since patients exhibited earlier onset as well as expansion of the clinical features (Lohmann et al., 2003). Although it appears that most *parkin* mutations are recessive, rare heterozygous alleles have already been identified, suggesting dominant negative or toxic gain-of-misfunction mutations in some cases (Lohmann et al., 2003). In contrast to the absence of LBs in most *parkin*-proven cases, the brain of a human compound heterozygote carrying an in-frame deletion and a missense mutation with retained E3 ubiquitin ligase activity displayed LB pathology (Farrer et al., 2001). Moreover, there have been several recent reports of aggresome formation elicited by specific *parkin* mutations residing in the RING finger domains (Reviewed by Kahle and Haass, 2004). Thus, it would be very interesting to study the effects of different mutations (e.g. truncations and large deletions vs. specific missense mutations) on the molecular properties of Parkin *in vivo*, in order to shed light on whether different mutations indeed result in distinct pathological and clinical features.

In contrast to the severeness of AR-JP in human patients, *parkin*-deficient animals showed only subtle phenotypes and no loss of dopaminergic neurons. Moreover, none of the proposed Parkin substrates was stabilized in *parkin* knock-out mice, implying that either the bona-fide substrates have not been identified yet, or that the protection/detoxification mechanism to which Parkin contributes does not depend on the degradation of toxic substrates. Alternatively, redundant pathways for the *parkin*-mediated ubiquitylation may exist or the loss of Parkin activity requires another insult, such as a toxic stimulus or cellular stressor to induce a PD-like syndrome (Reviewed by Kahle and Haass, 2004). In the present study, a set of four different *C. elegans pdr-1* mutants (two loss-of-function alleles and two in-frame deletions) was characterized in both genetic and pharmacological analyses. Similar to complete loss of fly (Greene et al., 2003; Pesah et al., 2004) or mouse (Goldberg

et al., 2003; Itier et al., 2003; Palacino et al., 2004; Von Coelln et al., 2004) *parkin*, simple knock-out mutants of the *C. elegans* ortholog *pdr-1* are viable and not significant sensitive to various cellular stress conditions (see Figures 32, 37, 43 and 44). However, homozygous *pdr-1(lg103)* in-frame deletion mutants are particularly sensitive to ER-derived and cytosolic protein folding stress, resulting in severe developmental defects and lethality (see Figures 32 and 37). Although the second in-frame deletion mutant *pdr-1(tm598)* did not show increased protein stress sensitivity per se, it at least served as a sensitive allelic background for *pdr-1(lg103)*. *pdr-1(lg103)/pdr-1(tm598)* compound heterozygotes showed an augmented susceptibility to tunicamycin treatment and α-synuclein A53T expression, compared to the respective heterozygous alleles or to other transheterozygous combinations with *pdr-1* loss-of-function alleles (see Figure 32 and Table 4). The differences between *pdr-1* in-frame deletions and loss-of-function mutants become clearer by data from genetic interactions of *pdr-1* and *ire-1* mutants. Both *pdr-1* in-frame deletion alleles exacerbated the phenotype of *ire-1* loss-of-function mutants, while *pdr-1* loss-of-function had no effect on the brood size of *ire-1* mutants (see Table 3). Nevertheless, the allele *pdr-1(lg103)* exhibited a stronger phenotype than *pdr-1(tm598)*. These data suggest that in-frame deletions of *pdr-1* result in PDR-1 misfunction, in contrast to complete loss-of-function alleles that were inconspicuous compared to wild type. The observation that the increased transcription of *pdr-1(lg103)* in the *atf-6(ok551)* hypermorphic background exacerbates the α-synuclein A53T conferred cytotoxicity (see Figure 38) even at low temperatures supports a gain-of-misfunction model for the genetics of *pdr-1(lg103)*.

Both in-frame deletions *pdr-1(lg103)* and *pdr-1(tm598)* lack the UPD and the first RING domain (see Figure 25). The resulting truncated proteins PDR-1(Δaa24-247), and perhaps also PDR-1(Δaa140-263) still bind specific E2 enzymes and CHN-1, like wild type PDR-1 (compare Figures 20 to 29 and 23 to 30). However, the UBL domain is missing in *pdr-1(lg103)*, whereas it remained intact in *pdr-1(tm598)* (see Figure 25). Mutant PDR-1(Δaa24-247) retained the capability to associate with its co-enzymes of the ubiquitylation machinery, but did no longer bind to the proteasomal subunit RPT-2 (see Figure 29B). Interestingly, it was recently shown that the UBL domain regulates the stability of the Parkin (Finney et al., 2003). In line with these data, higher amounts of the corresponding truncated PDR-1(Δaa24-247) protein from

recombinant expression in SF9 insect cells compared to full-length PDR-1 was obtained. Therefore, an increased intracellular concentration of PDR-1(Δaa24-247) may confer a stronger phenotype than mutant PDR-1(Δaa140-263), encoded by *pdr-1(tm598)*. In contrast to complete elimination of the UBL domain, some pathogenic missense mutations located within the UBL decrease the stability of Parkin protein, leading to its rapid degradation (Henn et al., 2005). Although various pathogenic C-terminal mutations of Parkin were found to be inactivated by misfolding and aggregation, N-terminal deletions of, or pathogenic missense mutations within the UBL domain, however, did not affect solubility (Henn et al., 2005; Winklhofer et al., 2003). Nevertheless, it has been reported that interfering with the UBL domain, can also impair Parkin E3 ubiquitin ligase activity (Corti et al., 2003; Huynh et al., 2003). Moreover, expression of a smaller Parkin variant (Δaa1-79) that lacks the UBL domain has been observed to reduce overall amount of ubiquitylated proteins, compared to cells expressing of full-length Parkin (Henn et al., 2005).

This suggests that such in-frame deletions of *pdr-1* (and possibly other PD-related *parkin* variants with similar capacities) may confer a toxic misfunction by their residual binding activity and/or altered regulation since essential components of the protein folding/degradation machinery may be sequestered and inactivated by this mutant. Such a blockade of the ubiquitylation and probably of the chaperone-mediated refolding machinery renders cells sensitive towards proteotoxic stress, whereas mutations that eliminate or reduce expression do not confer such an effect (for a model see Figure 46).

The co-chaperone and E4 enzyme CHIP provides the physical and functional link between chaperones and protein degradation machinery (Murata et al., 2003). Recently, it was shown that CHN-1 is expressed in the cytosol and binds to chaperones (Hoppe et al., 2004), similar to its human ortholog CHIP, which is involved in the chaperone/parkin mediated quality control of the ER protein Pael-R (Imai et al., 2002). Thus, CHN-1 might assist in regulating the cellular balance between folding and degradation and its titration could lead to a dramatic change in the folding capacity of the cytosol. Moreover, chaperones and co-chaperones have already been implicated as modulators of disease pathology in the neurodegenerative disorders by their ability to modify protein aggregates (Reviewed by Muchowski and Wacker, 2005; Slavotinek and Biesecker, 2001).

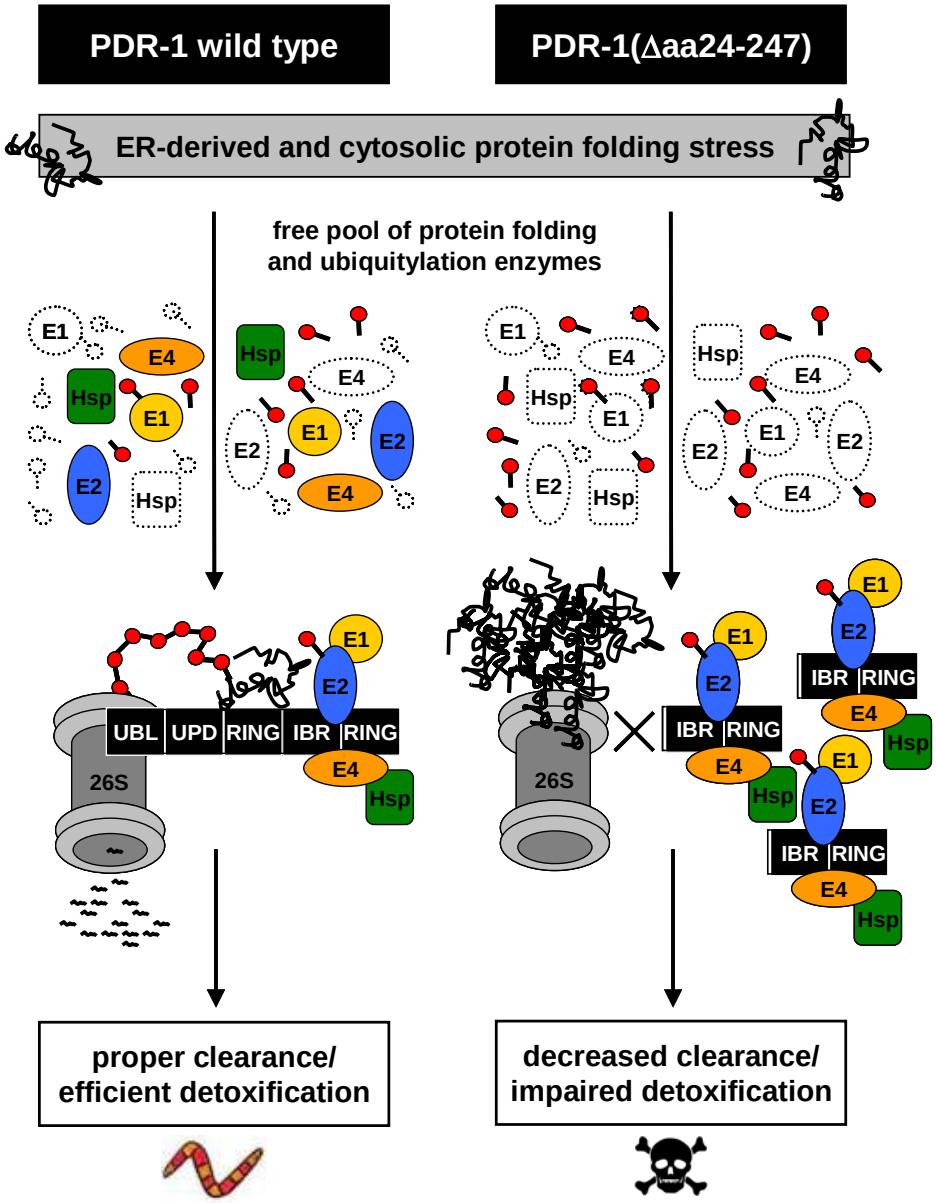


Figure 46. Working Model of *pdr-1(lg103)* Exerted Hypersensitivity Towards Proteotoxic Stress.

Left: Wild type animals are able to deal with ER-derived and/or cytosolic protein stress conditions through efficient detoxification and proper clearance. **Right:** *pdr-1(lg103)* gain-of-misfunction mutants are hypersensitive to proteotoxic stress, resulting in developmental arrest and lethality. The more stable mutant protein PDR-1(Δaa24-247) most likely sequesters important components of the cellular protein folding and ubiquitylation machinery. This impairs detoxification and decreases clearance of misfolded proteins, and thereby, promotes their accumulation and aggregation (Abbreviations and colour coding as described in Figure 3).

Taken together, these results suggest that studying gain-of-misfunction or dominant negative *parkin* alleles may provide further insights into the biological role of Parkin in addition to existing knock-out models.

4.5 The Biological Role of PDR-1/Parkin

Morphologically visible damage of dopaminergic neurons in *pdr-(lg103)* animals treated with tunicamycin or expressing mutant α-synuclein were not observed (see Figure 36). Instead, the early lethality which is detrimental for the animals is consistent with death by intestinal degeneration, as seen in mutants with an impaired UPR (Kaufman et al., 2002). Unlike in vertebrates, in *C. elegans* it is the intestine that is the tissue most vulnerable for protein stress (Kaufman et al., 2002). There is evidence from other disease models that, despite different organs being affected by the mutant phenotype, the underlying mechanism and its biochemistry is highly related in *C. elegans* and human cells (Eimer et al., 2003). However, death of dopaminergic neurons in *pdr-1(lg103)* mutants in later stages or probably aged worms cannot be excluded. Nevertheless, one can speculate that the underlying molecular mechanisms that are responsible for toxicity in these different tissues might be highly conserved and derived from a common origin of ER dysfunction.

In pathological states of the brain where levels of unfolded proteins exceed proteasomal degradation capacity and/or when the ubiquitin/proteasome pathway is impaired (Chung et al., 2001a) protein aggregates are formed. When the UPR cannot be activated sufficiently to restore ER functioning, a self-aggravating process is initiated. Protein aggregates are formed, which block proteasomal function in the cytosol, and proteasomal inactivation then aggravates ER stress. The connection between proteotoxic stress in the ER and the cytosol is compatible with an important role of PDR-1/Parkin in both pathways. PDR-1/Parkin was shown to physically associate and cooperate with E2 enzymes of the cytosolic stress response as well as those involved in the ERAD pathway (see Figures 20 and 23) (Imai et al., 2002; Shimura et al., 2001; Zhang et al., 2000). This is further substantiated by the severe developmental defects and early larval lethality of *pdr-1(lg103)* mutants observed in response to both ER dysfunction and cytosolic protein stress. The results presented in this study suggest that a fundamental role of PDR-1/Parkin in the UPR and the cytosolic stress response might be the ancient and conserved function from invertebrates to vertebrates. This might be specialized during evolution to maintain dopaminergic neuronal integrity.

Despite defects in protein folding/degradation pathways and the consequent abnormal protein aggregation, mitochondrial damage and the consequent oxidative

stress have also been implicated in the pathophysiology of PD. Although, many reciprocal influences between these two essential cellular functions are known, primary events have to be dissected from secondary downstream effects to better understand the underlying mechanism that provoke neuronal cell loss. Mitochondrial impairment and oxidative damage have been detected in PD patients, and mitochondrial deficiencies were constantly observed in *parkin* knock-out animals. However, *pdr-1* mutants and animals overexpressing α-synuclein are not sensitive to oxidative stress. Although mitochondrial damage or dysfunction in those worms was not analyzed in detail using functional assays or vital dyes, resistance to paraquat treatment suggested that mitochondria are unaffected.

Several reports already suggested communication between ER and mitochondria at least under apoptotic conditions via Ca^{2+} (Hacki et al., 2000; Nakamura et al., 2000). However, a growing body of evidence suggests that mitochondrial dysfunction might be a downstream event of proteotoxic stress and signalling from the ER but at least is involved in the propagation of cellular injury that ultimately leads to neuropathology (Reviewed by Paschen, 2003). It was already shown that the UPR up-regulates cellular functions that are beyond the scope of protein folding, secretion or degradation (Shen et al., 2004). A recent report demonstrated a novel signalling pathway by transmission of cell stress from the ER to mitochondria (Hori et al., 2002). Suppression of cytosolic protein synthesis under ER stress had a complex effect to sustain mitochondrial properties, while impairment of the UPR can produce mitochondrial dysfunction. If severe ER stress is sustained and not alleviated, prolonged activation of the UPR induces mitochondrial stress and causes subsequent accumulation of ROS, in the end resulting in cell death (Haynes et al., 2004). Furthermore, inhibition of proteasomal function decreases mitochondrial protein synthesis and activity, and increases the production of reactive oxygen species (Sullivan et al., 2004).

This study supports the model for a widespread involvement of ER dysfunction and the UPR, as well as of cytosolic protein misfolding/aggregation in the pathophysiology of PD and suggests that PDR-1/Parkin functions as a central regulator of both proteotoxic stress pathways.

4.6 Outlook

The present study provides a promising tool to investigate the biological role(s) of Parkin and the pathophysiology of PD *in vivo* on a molecular and cellular level. Given the simplicity and genetic tractability of *C. elegans*, this model system might now be used to uncover important aspect of PDR-1/Parkin function by a combination of biochemical, genetic and pharmacological methods.

The established model can now be used to dissect the role of PDR-1/Parkin in and its regulation by the UPR with the genetic tools available. Since many *C. elegans* mutants deficient in known UPR/ERAD regulators or targets have been described, further genetic interactions can be studied directly. Double mutants of *pdr-1(lg103)* with *xbp-1* and *atf-6* loss-of-function mutants might help to further restrict PDR-1/Parkin function to either PEK-1 and/or ATF-6 signalling. Additionally, double mutants of *pdr-1(lg103)* with mutants deficient in downstream genes of the UPR, like ERAD components and chaperones, or later induced mediators of the cell death machinery, might help to identify the level of genetic interaction between IRE-1 and PDR-1/Parkin. Moreover, the system can be used to identify novel important genetic modifiers of toxicity and potential therapeutic drug targets. The severe phenotype of *pdr-1(lg103)* mutants expressing α-synuclein A53T and its temperature- and dose-dependency is perfectly suited to explore specific enhancers as well as suppressors of the PDR-1 ER-stress/α-synuclein induced proteotoxicity. Such screens could be rapidly performed in a genome-wide approach, using RNAi-feeding libraries in order to knock-down each single *C. elegans* gene in combination with tunicamycin treatment or α-synuclein overexpression in the *pdr-1(lg103)* mutant background. For this purpose, an appropriate strain has already been constructed into which the RNAi-hypersensitive *rrf-3(pk1426)* (Simmer et al., 2002) mutation has been introduced by crossing (strain BR3205). Such experimental approaches would certainly reveal new genetic interactions and might help to gain more insights into the affected cellular stress pathways. In addition, this might reveal further interactions and feedback-mechanism between the UPR and the cytosolic stress response that are important to completely understand the central role of PDR-1/Parkin.

Since knock-down of *pdr-1(lg103)* by RNAi could not sufficiently suppress the hypersensitivity towards proteotoxic stress conditions, an alternative strategy was chosen in order to confirm *in vivo* the toxic gain-of-misfunction mediated by *pdr-*

1(lg103). Some attempts to ectopically express the corresponding in-frame deleted ORF in *pdr-1* loss-of-function background, this time in order to induce proteotoxic stress hypersensitivity, have been made. However, micro-injection of the *pdr-1(lg103)* mutant genomic ORF (construct pBY1569) into *pdr-1(lg101)* mutant background did not result in increased hypersensitivity against tunicamycin treatment or ectopic expression of mutant α-synuclein in the generated strains (BR2789-2792 and BR3236-3239, respectively). Nevertheless, this is most likely caused by mosaic expression along with too low cellular levels of the corresponding protein PDR-1(Δaa24-247), as judged by the expression of the co-injection marker *sel-12::gfp*. To circumvent these experimental problems, a follow-up strategy was already initiated by generation of the constructs pBY1792 and pBY1793 (*pdr-1* wild type and *pdr-1(lg103)*, respectively) which can be used to transform *C. elegans* by micro-particle bombardment. This method should result in the isolation of strains carrying genomically integrated copies of the transgenes, thus preventing mosaic expression and ensuring sufficient cellular protein levels. Alternatively, this could also be done using a GFP-tagged mutant PDR-1 variant, in order to be able to compare intracellular localization of WT and mutant protein *in vivo*.

In addition, the respective *pdr-1* wild type and mutant cDNAs have been cloned into appropriate cell culture vectors in order to test hypersensitivity/resistance towards proteotoxic stress conditions. Since all *pdr-1* variants are expressed in human cells with the expected molecular weights, now, the stability of the different mutant PDR-1 proteins should be studied. Furthermore, the expressed proteins should also be used to address the question of residual E3 ligase activity of *pdr-1* in-frame deletion mutants.

In analogy, this nematode model can also be used for rapid transgenic analyses of human *parkin* variants *in vivo*. Wild type *parkin* and different AR-JP causing mutations can be introduced into different *pdr-1* mutant backgrounds to study the function of human Parkin and how mutations interfere with this. Differences in specific *parkin* mutations concerning their functional consequence (loss-of-function or toxic gain-of-function) as well as their ability to form aggresomes have already been identified. This model might help to explore the pathogenic mechanisms conferred by specific *parkin* mutations and their contribution to the pathophysiology of AR-JP. In addition, this might also help to understand the mechanisms of Parkin-mediated detoxification and its function in LB formation.

In order to elucidate the mechanism of PDR-1(Δaa24-247)/α-synuclein A53T mediated toxicity several experiments can be performed using the presented model. To analyze protein levels and folding of α-synuclein variant in *pdr-1* mutant background, first preliminary biochemical analyses have already been performed. However, total amounts of α-synuclein proteins variants have not been found altered in *pdr-1(lg103)* mutant background, compared to *C. elegans* wild type background. Noticeably, separation of detergent-soluble (supernatant) and -insoluble (pellet) protein fractions showed lower levels of only α-synuclein A53T, but not WT, in the pellet fraction of *pdr-1(lg103)* mutants compared to N2 wild type background (Figure 47).

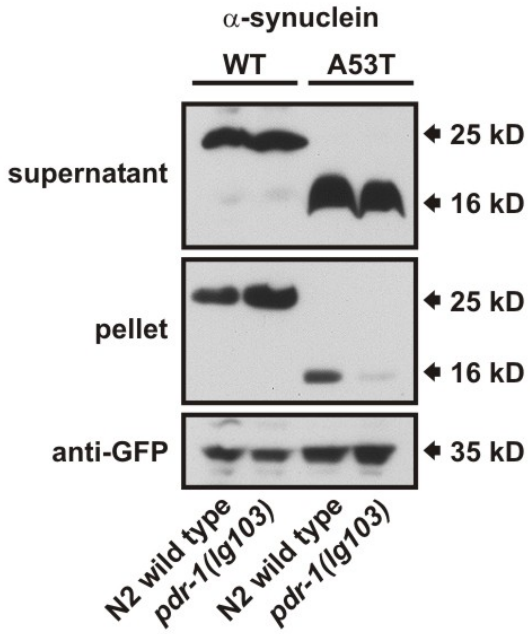


Figure 47. Analysis of α-synuclein Protein Expressed in *pdr-1(lg103)* Mutants.

Shown are Western blots of total worm lysates separated by centrifugation into detergent-soluble (supernatant) and -insoluble (pellet) fractions. Pellet fractions were solubilized using urea containing buffer. Western blots were stained with α-synuclein antibody 15G7, and GFP antibody as a loading control. The different molecular weights observed for α-synuclein WT and A53T variants, arise from different cloning strategies. Although lower protein levels for α-synuclein A53T in the pellet fraction of *pdr-1(lg103)* mutants can be seen, a coincident increase of α-synuclein A53T protein in the supernatant fraction cannot be determined due to protein overload.

Although these results have to be considered preliminary and thus have to be verified, they support the model of a Parkin-mediated detoxification mechanism through cytoprotective aggresome/LB formation. Additionally, these results are complete in line with the suggested neurotoxic role of specifically soluble α-synuclein species. However, aggregate formation of α-synuclein in *pdr-1* mutant background has to be studied *in vivo,* too. In order to characterize the nature and localization of potential inclusions on a sub-cellular and molecular level, immuno-histological and biochemical studies should be performed. In addition, electron microscopy should be considered, to ultimately characterize aggregates.

A variety of other remaining question should be addressed using the presented model. Does co-expression of specific chaperones (e.g. Hsp70) suppress hypersensitivity of *pdr-1(lg103)* mutants towards proteotoxic stress? Are *pdr-1(lg103)* mutants equally hypersensitive to expression of other known substrates of human Parkin? Are *pdr-1* mutants or animals ectopically expressing α-synuclein affected by specific PD-mimetics and inhibitors of protein turnover? Are mitochondria affected in *pdr-1* mutants or in animals overexpressing α-synuclein, and if so, due to a primary or a secondary event? Are the interaction partners of PDR-1, identified from the yeast-two hybrid screen, physiologically relevant, and if so, what are their functions?

Although many questions are beyond the scope of this study and remain un-answered, the compelling model presented here will certainly help to shed light onto the molecular and cellular pathways involved into the pathophysiology of PD.

5 Experimental Procedures

5.1 Microbiology Techniques

All *E. coli* and *S. cerevisiae* strains used in this study are listed in Tables 5 and 6, respectively (see Materials Section). General methods were used for handling, cultivation, storage, etc. of *E. coli* and *S. cerevisiae* as described (Ausubel, 1987; Sambrook et al., 1989) and according to the manufacturer's instructions. Preparation and transformation of $CaCl_2$- or electro-competent *E. coli* cells, was done following standard procedures as described (Maniatis et al., 1982; Sambrook et al., 1989). Preparation and transformation of competent *S. cerevisiae* cells by PEG/LiAc methods was performed as described (Gietz and Woods, 2002; Sherman, 1991).

5.2 DNA Techniques

Sequencing of DNA was performed by Toplab (Martinsried) or GATC (Konstanz). DNA and RNA concentrations were measured by photometry or estimated from an appropriate gel. Agarose-gel electrophoresis was performed using standard methods. Restriction digestion, dephosphorylation, and ligation of DNA were performed following standard cloning methods (Maniatis et al., 1982; Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed using Taq- or Pfu-polymerase, or a mixture of both, according to the manufacturer's instructions. Single parameters were adjusted for each separate reaction. All vectors used and all constructs generated in this study are listed in Tables 11 and 12, respectively (see Materials Section). All primers used in this study are listed in Table 13 (see Materials Section).

5.2.1 DNA Preparation and Purification

Extraction and purification of DNA fragments from agarose-gels or enzymatic reactions was done using extraction spin-columns following instructions of the manufacturer (QIAGEN, Hilden). Plasmid and cosmid preparation from *E. coli* was performed by alkaline lysis method after a modified protocol (Birnboim and Doly,

1979) and purified using DNA prep-columns according to the manufacturer's instructions (QIAGEN, Hilden).

5.2.2 Plasmid Isolation from *S. cerevisiae*

Cells from 3ml over night cultures were harvested and disrupted by adding 200µl yeast-miniprep-solution (2% TritonX-100; 1% SDS; 100mM NaCl; 10mM Tris/HCl pH8.0; 1mM EDTA) and an equal volume of glass beads (200-300 µm diameter) and subsequent vortexing for 1 min. DNA was extracted from the hydrous phase after addition of 200µl Phenol::$CHCl_3$::Isoamyl (25:24:1), 2 min vortexing and centrifugation (5 min, 14.000 rpm). 1µl of extracted DNA was used for transformation of *E. coli* by electroporation.

5.2.3 Plasmid Excision from Phages

In vivo excision of plasmids from *C. elegans* cDNA clones, supplied as λZAPII phages, was carried out using the following protocol: 200µl *E. coli* XL-1 blue MRF' (grown o/n at 30° C in LB medium + 0.2% maltose + 10mM $MgSO_4$; OD_{600} = 1) were co-infected with 2µl phages and 2µl helper phages for 15 min at 37° C. 3 ml LB medium was added for further incubation at 37° C for 2-3 h. Cells were lysed at 65-70° C for 20 min, and pelleted by centrifugation (1000g, 15 min). 10µl of supernatant (stored at 4° C) was mixed with 100µl SOLR (OD_{600} = 1), incubated for 15 min at 37° C, and spread on LB plates containing ampicillin o/n at 37° C.

5.2.4 Preparation of Genomic DNA from *C. elegans*

Four to five plates (Ø 9 cm) with worms were washed 2-3 times with M9 buffer, frozen in liquid nitrogen and stored at -80° C until the DNA was prepared. Frozen worm pellets worms were lysed in 500 µl of lysisbuffer (0.2M NaCl; 0.1M Tris-HCl pH8.5; 50mM EDTA; 0.5% SDS; + 10µg proteinase K) for 30 min at 65° C. 5 µg RNAse A were added and slurry was incubated for further 30 min at 37° C. Genomic DNA was prepared using standard phenol/chloroform extraction methods.

5.3 RNA Techniques

5.3.1 *In vitro* Transcription

To produce dsRNA *in vitro* transcription was performed using T7 Megascript Kit according to the manufacturer's instructions (Ambion). Reactions were carried out on 1µg DNA template (for antisense RNA: PCR-product of primer RB1152/T7 on NcoI-digested pBY1248; for sense RNA: PCR-product of primer T7/RB1153 on SalI-digested pBY1248). After removal of the DNA template, RNA was precipitated using LiCl and suspended in H_20. Sense and antisense RNA were mixed at same amounts, heated for 10 min at 70°C, and allowed to anneal for 30 min at 37°C. dsRNA was used for RNAi by micro-injection and soaking methods.

5.3.2 Preparation of RNA from *C. elegans*

Four to five plates (Ø 9 cm) with well fed worms were washed 2-3 times with M9 buffer, frozen in liquid nitrogen and stored at -80° C until the RNA was prepared. Frozen worm pellets worms were transferred into a sterile mortar and homogenized with a sterile pestle. 600 µl of lysisbuffer were added to the homogenized worms and the resulting extract was transferred to a cold 1.5 ml Eppendorf tube. The extract was drawn five to seven times into a 2 ml syringe carrying a needle with 0.9 mm diameter. To remove the worm debris the extract was centrifuged at 16000 g for ten minutes at 4° C. The supernatant was transferred to a new cold 1.5 ml Eppendorf tube and mixed with an equal volume of 70% ethanol. The RNA was purified with the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN).

5.3.3 RT-PCR

RT-PCR was used to clone full-length cDNAs of various *C. elegans* genes. First strand cDNA synthesis was carried out using oligo-dT primer and reverse transcriptase with total RNA samples of the respective strains. All cDNAs were isolated by PCR on first strand cDNA, using combinations of oligo-dT and gene specific primers. Designed species-specific oligonucleotides were then used to amplify the respective *pdr-1* ORFs from related species of the genus *Caenorhabditis*.

5.3.4 Northern Blot Analyses

5 µg of total RNA was loaded per lane on 1.0% agarose RNA gels and blots onto Hybond N+ membranes were performed following standard procedures (Sambrook et al., 1989). DNA fragments (25 ng) were labelled with α^{32}P dCTP (~50 µCi) using the Megaprime labelling kit according to the manufacturer's instructions (Amersham). None-incorporated radioactive nucleotides were removed using sephadex columns (Boehringer). *ama-1* and *act-1* specific probes were used as controls to adjust for equal loading (Johnstone and Barry, 1996; Shen et al., 2001). Blots were hybridized and washed at 65° C according to standard procedures (Church and Gilbert, 1984). For quantification of relative transcript levels, blots were exposed on a phosphor imager screen (Molecular Dynamics) and read with a phosphor scanner. The intensity of bands was determined using ImageQuant software.

5.4 Protein Techniques

All antibodies used in this study are listed in Table 10 (see Materials Section). Following general protein biochemistry methods were done as described (Ausubel, 1987; Sambrook et al., 1989) and according to the manufacturer's instructions. Protein concentrations were determined by the Bradford method according to manufacturer's instructions (BIO-RAD Laboratories GmbH). Proteins were separated by sodium-dodecyl-sulfate (SDS) or Tricin polyacrylamid-gel electrophoresis (PAGE), depending on the expected size of the corresponding protein. Proteins from gels were visualized by standard coomassie staining methods. Gels were dried under vacuum. Western blot were performed according to standard methods, using PVDF membranes. Proteins were detected by ponceau S staining methods and or by immunoblotting.

5.4.1 Yeast-Two-Hybrid Screen

Protein interaction studies were performed using the MATCHMAKER GAL4 Two-Hybrid System 3 according to the manufacturer's instructions (Clontech). As a bait, full-length PDR-1 was fused to the GAL4 DNA-binding domain (pGBKT7) and

transformed into yeast AH109. *C. elegans* GAL4 activation domain libraries (gift of Robert Barstead) were used as prey. Protein interaction studies were carried out under high stringency conditions using selective plates lacking LEU (leucine), TRP (tryptophan), HIS (histidine), and ADE (adenine), and supplemented with 1mM 3-aminotriazol (3-AT) and 20 µg/ml x-α-Gal (5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside), according to the manufacturer's instructions. Transformation efficiency was monitored by growth of different dilutions on plates lacking the aa required for plasmid selection, only. To evaluate identified clones, plasmids were isolated from yeast. These were used to re-transform yeast together with either the bait construct or the empty vector control. To confirm interactions 3µl yeast cells (OD_{600} = 0.25) were retested by spotting onto appropriate selective plates again. True positive clones were subjected to sequencing and identified by blast search analyses. For further yeast-two-hybrid analyses, full-length cDNAs of *pdr-1, pdr-1(lg103), ubc-2, ubc-6, ubc-7, ubc-14, ubc-15, ubc-18*, *chn-1*, *rpt-2*, *cpl-1*, *cpr-4*, *cpr-6*, F57F5.1, H22K11.1, T12E12.1 and K08E3.8 were cloned into vectors pGBKT7 and pGADT7, to test interactions in both combinations. Correct expression of the constructs were monitored by protein extraction, SDS-PAGE and western blotting with anti-myc and anti-HA antibodies.

5.4.2 Expression and Purification of Proteins from *E. coli*

To generate recombinant 6xHIS-tagged PDR-1 protein, full-length cDNA was cloned into vector pET28b(+) (Novagen), tagging at either (N-terminus: pBY1230; C-terminus: pBY1229), or both termini (pBY1118). pET21a-UbcH7 (gift of Martin Scheffner) was used to produce human E2 enzyme UbcH7 in *E. coli*. For *in vitro* ubiquitylation assays *chn-1* ORF was cloned into vector pET21a(+) (Novagen).To generate GST-fusion proteins, *ubc-2* and *ubc-18* ORFs were cloned into the pGSTparallel-3 (Sheffield et al., 1999) vector (pBY1457 and pBY1456, respectively). Recombinant GST::CHN-1 was expressed from a pGEX4T1 (Pharmacia) based construct (Hoppe et al., 2004).

After induction of 500 ml BL21(pRIL) culture with 1mM IPTG for 4h at 37° C, cells were frozen and lysed in 40 ml lysis-buffer [10% glycerin; 500mM NaCl; 10mM Tris/HCl, pH8.0; + complete protease inhibitor (Boehringer)], sonified, and

centrifuged (16.000 rpm, 4° C, 20 min). Some proteins could be purified under native conditions from the supernatant. For purification of GST-fusion proteins (GST::UBC-2 / GST::UBC-18 / GST::CHN-1 / GST-myc::PDR-1) cleared lysates were allowed to bind to 500 µg of glutathione-Sepharose beads (Pharmacia). After extensive washing in lysis buffer + 0.1% Triton X-100, GST-fusion proteins were eluted with 10mM glutathione.

For purification of aggregated proteins from inclusion bodies under denaturing conditions, the pellet fraction was resuspended in buffer A [6M GuHCl; 100mM NaH_2PO_4; 10mM Tris/HCl; pH8.0], and incubated 1 h at room temperature. The supernatant was incubated 2 h at room temperature with pre-equilibrated (3x wash with buffer A + 5mM imidazole + 100 mM NaCl) Ni^{2+} NTA-Agarose (QIAGEN). After extensive washing procedure (each wash with 10 ml for 10 min, separated by centrifugation for 3 min at 750g: 2x buffer A + 5mM imidazole + 100 mM NaCl; 3x buffer B [8M urea; 100mM NaH_2PO_4; 10mM Tris/HCl; 100mM NaCl; pH8.0]; 2x buffer C (buffer B + 10 mM imidazole); 2x buffer D (buffer B + 20 mM imidazole); 1x buffer F (buffer B + 30 mM imidazole)), matrix was loaded in gravitation columns (Biorad) and bound proteins were eluted with 10 ml buffer E (buffer B + 500 mM imidazole). Single fractions taken contained protein samples of estimated 99% purity, as judged from coomassie stained gels. Prior to further analyses, some aliquots had to be dialyzed for 30 min against buffer B, using 0.0025 µm filters (Millipore).

5.4.3 Preparation of Yeast Protein Extracts

To prepare protein extracts for immunoblotting, *S. cerevisiae* was grown in the respective drop-out media to an OD_{600} of 2-5 and harvested by centrifugation. The cell pellet was resuspended in 1 ml cold deionised water and after addition of 150µl 1.85M NaOH; 7.5% β-ME, lysed on ice for 15 min. To precipitate proteins 150µl 55% trichloracetic acid (TCA) were added and incubated on ice for 10 min. Precipitated proteins were pelleted by centrifugation (10 min; 14.000 rpm; 4° C), and pellet was resuspended in 50-100µl HU-buffer (8 M urea; 5% SDS; 200mM Tris, pH6.8; 1mM EDTA; BPB; 1.5% DTT). Proteins were analyzed by SDS-PAGE, western blotting and detected using anti-myc or anti-HA antibodies.

5.4.4 Expression and Purification of Proteins from SF9 Cells

pdr-1 and *pdr-1(lg103*) ORFs were cloned into a modified Baculovirus vector pAcUW51 (Pharmingen). The resulting constructs (pBY1898 and pBY1644, respectively) were co-transformed along with linearized BaculoGold DNA into SF9 insect cells to generate recombinant viruses, according to the instruction manual of the Baculo Gold System (Pharmingen). For protein production, 20 ml of infected SF9 cells ($3x10^6$ cells/ml) were grown for 2 days. Cells were lysed in twice the volume of the cell pellet in lysis-buffer [10mM Tris pH 8; 10mM DTT or 10mM β-ME + complete protease inhibitors (Boehringer)] using a dounce homogenizer.

5.4.5 Protein Extraction from *C. elegans*

For quick analyses, whole animal lysates were prepared by resuspending washed worms in five volumes of SDS-PAGE sample buffer followed by 5 min boiling (to enhance solubilization of worms, glass-beads were added, and slurry was vortexed). To prepare native proteins from *C. elegans*, washed worm pellets were resuspended in native lysis buffer and cracked open by the liquid nitrogen grinding method followed by sonification. The soluble SDS extracted fraction was separated by centrifugation at 55.000 rpm for 1h at 4° C. Proteins from the insoluble pellet fraction were extracted using 8 M Urea buffer (HU-buffer see protein preparation from *S. cerevisiae*).

5.4.6 *In vitro* Translation

pdr-1 and *pdr-1(lg103)* ORFs were cloned into vector pCite-4a(+) (Novagen) (pBY1494 and pBY1645, respectively) to produce radioactively labelled proteins PDR-1 and PDR-1(Δaa24-247). *In vitro* transcription and translation was performed using ^{35}S methionine/cysteine together with the rabbit TNT Coupled Reticulocyte Lysate System according to the manufacturer's instructions (Promega). Successful *in vitro* translation was monitored by SDS-PAGE and autoradiography.

5.4.7 GST-Pull Down

Lysates of radioactively labelled PDR-1 full-length and PDR-1(∆aa24-247) mutant version were incubated over night at 4°C on glutathione-Sepharose beads (Amersham) loaded with the respective GST-fusion proteins and washed at least five times in lysis-buffer + 0.1% Triton X-100 and 150 mM NaCl. Reactions were separated by SDS-PAGE and visualized by coomassie blue staining and autoradiography.

5.4.8 *In vitro* Ubiquitylation

Reactions were done as previously described (Koegl et al., 1999). Purified rabbit E1 (Affiniti), purified GST-UBC-2 as well as UbcH7 and CHN-1 crude *E. coli* cell extracts were used for self-ubiquitylation reactions of purified GST::myc::PDR-1. Reactions were separated by SDS-PAGE followed by western blotting using 9E10 anti-myc antibody.

5.4.9 Production of Antiserum

To generate PDR-1 specific antibodies, two rabbits each were immunized by Eurogentec with two different synthetic peptides as follows. Immunization DE01648 [antigen code EP012277-KLH-MBS (aa 1-15: MSDEISILIQDRKTG); animal codes: SA1109 (antibody RB12) and SA1110 (antibody RB13)] and immunization DE01649 [antigen code: EP012278-KLH-MBS (aa 260-273: QTSYSEYQRKATER); animal code: SA1111 (antibody RB14) und SA1112 (antibody RB15). Sera were tested after each bleeding in western blots on purified recombinant PDR-1 protein, or on whole cell lysates from SF9 cells or *C. elegans*. Affinity and specificity of the sera increased during ongoing immunizations. Using antibodies in 1/500 dilution 10-100ng of purified recombinant protein was the lowest concentration detectable on western blots with any of the four antibodies, as judged by distinct dilutions.

5.4.10 Affinity Purification of Antibodies

Affinity purification of antibodies was performed as described (Burke et al., 1982). About 100µg recombinant purified PDR-1 protein was loaded on a SDS-PAGE gel, western blotted and visualized by ponceau S staining. The identified band was cut out and incubated in 500µl sera. Antibodies were eluted from the nitrocellulose membrane after several washes by pH. After neutralization, antibodies were used undiluted on western blots.

5.5 *C. elegans* Methods

5.5.1 Breeding of *C. elegans*

All strains used in this study are listed in Tables 7, 8 and 9 (see Materials Section). The animals were maintained on NGM plates seeded with *E. coli* OP50 like previously described (Brenner, 1974; Wood, 1988). Petri dishes with the diameters 3.5cm, 5cm and 9cm were used in this work. Animals were kept in air permeable cardboard boxes at 15, 20 or 25°C. The basic culture methods (handling of *C. elegans*, freezing, etc.) were done like previously described (Lewis and Fleming, 1995; Stiernagel, 1999).

For decontamination or synchronization of *C. elegans* cultures, worms were subjected to alkaline hypochlorite treatment. Synchronized L1 larvae were spotted onto 9 cm plates seeded with OP50 and allowed to grow for 6 hours, 18h, 30h, 42h and 54h for L1, L2, L3, L4 and young adult stages, respectively. Worms were inspected visually before harvesting to confirm that the worms were at the correct stage.

5.5.2 Genetic Crosses

All strains constructed by crossings in this work are listed in Table 9 (see Materials Section). L4 hermaphrodites were mated with males at a ratio of 1:3 on small NGM agar plates. Worms were transferred to a fresh plate every 24h for four consecutive days, and finally removed. Progeny laid within the first 24 hours was discarded, due to high percentage of self progeny vs. cross progeny. The success of the crosses

was monitored by the amount of males in the F1 generation. About ten of the following F1 animals were singled and their progeny (F2) was further analyzed. The double mutant of interest, identified by SW-PCR and/or visual markers, was isolated in the F2 generation, and confirmed from the F3 generation.

5.5.3 Worm Lysis for Single Worm PCR (SW-PCR)

All primer used for the identification of mutants are listed in Table 13 (see Materials Section). Single worms were transferred into PCR tubes containing 10µl Worm Lysis Buffer [50mM KCl; 1mM Tris/HCl pH 8.2; 2.5 mM $MgCl_2$; 0.45% NP-40; 0.45% Tween 20; 0.01% gelatine) + 0.5mg/ml proteinase K] and frozen at -80°C for at least 30 minutes. 0.5 µl lysate, produced by incubation at 65°C for one hour and at 95°C for 10 minutes, was used as a template for the following PCR. For the identification of deletion mutants from populations or single worms two rounds of PCR were performed, using nested primer pairs. First PCR was done with external primer pairs and served as a template for the following PCR. Internal primer pairs were used for the identification of a deletion band, and for confirming homozygosity, used in combination with a primer annealing inside the deletion.

5.5.4 Generation and Isolation of *C. elegans pdr-1* Deletion Mutants

pdr-1(lg101) and *pdr-1(lg103)* mutant strains analyzed in this work were obtained from Claudia Rudolph (EleGene). *pdr-1(tm598)* and *pdr-1(tm395)* mutants were provided by Shohei Mitani (NBP-Japan). All mutants were generated by screening of deletion libraries constructed by UV/TMP (Trimethylpsoralen) treatment (Gengyo-Ando and Mitani, 2000; Yandell et al., 1994). Size and nature of the *pdr-1* deletion alleles (deletion breakpoints / cosmid K08E3 coordinates): *lg103*: 1132 bp in-frame deletion (30885/30886-32017/32018); *lg101*: 1747 bp out of-frame deletion (31312/31313-33059/33060); *tm598*: 697 bp in-frame deletion (31365/31366-32062/32063); *tm395*: 480 bp out of-frame deletion (31601/31602-32081/32082). Prior to analysis, the mutants *lg103* and *lg101* were backcrossed with N2 wild type animals 7-9 times, respectively, the alleles *tm598* and *tm395* at least twice.

5.5.5 Transformation of *C. elegans*

Transgenic animals were constructed by micro-injection as previously described (Mello et al., 1991). All strains constructed by micro-injection in this work are listed in Table 8 (see Materials Section). The injected constructs are described in Tables 11 and 12 (see Materials Section).

Expression Analyses: The promoter *gfp* constructs, $P_{pdr\text{-}1}$*::gfp* long and short (pBY1013 and pBY1909), were generated by insertion of either 4.0 kb or 650 bp fragments, immediately 5' of the predicted initiation ATG codon of *pdr-1*, and ligated in-frame with the *gfp* coding sequence into *C. elegans* expression vectors pPD95.75 or pPD117.01, respectively (ftp://www.ciwemb.edu/pub/FireLabInfo/FireLabVectors/). The translational fusion construct, $P_{pdr\text{-}1}$*::gfp::pdr-1* (pBY1794), was engineered by in-frame ligation of an 8.5 kb fragment, containing the complete genomic region of *pdr-1*, into the promoter construct $P_{pdr\text{-}1}$*::gfp*. 25ng/µl of the *gfp* reporter constructs were co-injected with 75ng/µl pRF4, a dominant *rol-6* injection marker, into N2 wild type animals.

Rescue of *pdr-1(lg103)*: Rescue plasmids were constructed by subcloning a 13707 bp EcoRV fragment of cosmid K08E3, containing the complete operon (pBY1500). The engineered rescuing construct (pBY1908) was generated by insertion of 4 bp (GTAC) into the Acc65I restriction site and re-ligation. This created a frame-shift in exon3 of K08E3.8 after bp 191 of the coding sequence, generating a protein truncated after aa 63. For rescue, 5ng/µl of cosmid K08E3 or 25ng/µl of the plasmids pBY1500 or pBY1908 were co-injected with 25ng/µl pBY1153 (*sel-12*::*gfp*) into *pdr-1(lg103)* mutant animals. The amount of DNA was adjusted to a total concentration of 100ng/µl using plasmid pBluescriptSK.

Overexpression of *pdr-1(lg103)*: In order to ectopically express the ORF of the toxic gain-of-misfunction allele *pdr-1(lg103)* in *pdr-1* loss-of-function mutant background, the construct pBY1569 was generated. This plasmid contains the complete operon of the *pdr-1(lg103)* in-frame deletion mutant, similar to pBY1500. 25ng/µl of pBY1569 were micro-injected together with 25ng/µl pBY1153 marker into *pdr-1(lg101)* mutants. As a follow-up strategy the inserts of pBY1500 and pBY1569 were cloned into pBY232, which contains an *unc-119* rescuing cassette. The resulting constructs pBY1792 and pBY1793, respectively, can be used to transform *C. elegans unc-119*

mutants by micro-particle bombardment, in order to generate integrated transgenic lines.

Overexpression of α-synuclein: To generate constructs for human α-synuclein overexpression the ORF of the respective variant (WT, A53T and A30P) was cloned under were under the control of the *sel-12* promoter (pBY1158, pBY1159 and pBY1160, respectively) as well as under control of the *dat-1* promoter (pBY1168, pBY1169 and pBY1170, respectively). 20ng/µl of each α-synuclein construct together with 35ng/µl of the corresponding co-injection marker, which drives expression of *gfp* from either the *sel-12* or *dat-1* promoter (pBY1153 and pBY266) was used. The total DNA concentration was adjusted was pBluescript vector to 100ng/µl and injected into N2 wild type animals to generate stable transgenic lines.

5.5.6 Immunohistochemistry

Fixation: *C. elegans* were harvested from plates and washed five times in 1x PBS at RT. Last washing step was performed in H_2O, and worms were placed on ice. An equal volume of 2x MRWB buffer (160mM KCl; 40mM NaCl; 14 mM Na_2EDTA; 1mM spermidin HCl; 0.4mM spermin; 30mM Na PIPES, pH7.4; 0.2% β-ME; 50% methanol) was added. 10% fresh prepared paraformaldehyde solution was added to a final concentration of 1% (dry paraformaldehyde was dissolved in 2 drops NaOH and heated in 65° C water bath for 15 min). Samples were immediately mixed and frozen in liquid nitrogen. 2-3 times samples were defrosted under warm water and quick frozen again. After last defrosting, samples were incubated on ice for 30 min.

Reducing disulfides to -SH: Samples were washed twice in Tris Triton buffer (100mM Tris-HCl, pH7.4; 1% Triton X-100; 1mM EDTA), and incubated for 2h at 37°C in Tris triton + 1% β-ME with agitation. Worms were washed once in 10x volumes of 1X BO_3 buffer (10X BO_3 buffer: 1M H_3BO_3; 0.5M NaOH; pH9.5) + 0.01% Triton X-100, and incubated in 1X BO_3 buffer + 10mM DTT for 15 min at RT with agitation.

Oxidation of -SH groups to $-SO_3$: Samples were incubated in 1X BO_3 buffer + 0.01% Triton X-100 + 0.3% H_2O_2 for 15 min at RT with agitation, and washed afterwards in 10fold volume with 1X BO_3 buffer + 0.01% Triton X-100. Next, worms were washed with Antibody buffer B (1x PBS; 0.1% BSA; 0.1% Triton X-100; 0.2% Na azide; 1mM EDTA) at least for 15 min.

Antibody incubation: Antibody incubation was performed in Antibody buffer A (identical to Antibody buffer B, except 1% BSA), washing steps in Antibody buffer B. 20µl worm suspension was incubated with the appropriate primary antibody dilution in buffer A o/n at 4° C. Worms were washed 5 times in buffer B, and incubated o/n in buffer B. Incubation with secondary antibody was performed o/n in buffer A. Worms were washed 5 times in buffer B, and incubated o/n in buffer B.

Microscopy: 3µl stained worms were placed on 2% agarose pads, and mixed with 3µl solution NPG + DAPI (20mg n-propyl gallate dissolved in 0.7 ml glycerine and 0.3 ml H_20; 100 µl solution + 10µl Tris pH9.5 + 1µl 1mg/ml DAPI (4',6'-diamidino-2-phenylindole hydrochloride).

5.5.7 RNA Interference (RNAi)

For RNAi the respective cDNAs were cloned into vector pPD129.36 (gift of Andrew Fire), flanked by two T7 promoters. For RNAi by feeding HT115DE *E. coli* cells were transformed with the constructs and experiments were performed as previously described (Kamath et al., 2001). dsRNA production was induced with 1mM IPTG on plates seeded with the respective bacteria strains. L4-stage worms were placed on RNAi-producing plates and were allowed to produce progeny. Adults were removed or transferred to new RNAi-producing plates. First and third generation progeny grown on RNAi plates were scored a phenotype. Injection and soaking of dsRNA was performed as described (Fire et al., 1998).

5.5.8 Assays for Developmental and Behavioural Phenotypes

Most of the phenotypical analyses were performed according to previously described methods (Summarized by Hope, 1999).

Bag-of-worms: For estimation of bagging worms, 100 L4 animals (10 per plate) were analyzed at 20° C. Worms were transferred to new plates every day to prevent overcrowding. A bag-of-worms was defined when the progeny hatched inside their mother. Each day bagging worms were recorded and discarded. The remaining worms were transferred every day to new plates and progeny was discarded. The experiment was stopped as soon as no further progeny was laid.

Body-bends: Body-bends per min were measured as previously described (Mendel et al., 1995). Worms were maintained and analyzed at 20°C. The wave frequency of the sinusoidal movement was measured over time. 25 worms were analyzed for three consecutive minutes.

Brood size: Individual L4 hermaphrodites were placed onto fresh plates and incubated at 20°C (for analysis of cross-progeny, 3-5 males of the respective *C. elegans* strain were used in addition). To prevent overcrowding, worms were transferred daily onto fresh plates for three consecutive days. The progeny was counted two to three days after removal of the P0.

Chemical avoidance: A 2 cm ring of a noxious solution (8M glycerin or NaCl, stained with xylencyanol) was printed onto an agar plate free of food. Animals were picked into the centre and the fraction of worms crossing the noxious ring after few minutes was determined.

Chemotaxis: Chemotaxis assays were performed as previously described (Bargmann et al., 1993; Bargmann and Horvitz, 1991). Petri dishes were prepared by spotting 1 µl of either 100% ETOH (-, control spot) and 1µl of odorant (e.g. diacetyl) diluted in EtOH (+, odorant spot). Additionally 1µl azide was spotted onto both spots, to immobilize animals once they reach these spots. Staged adult worms were washed several times and spotted in a thin lane in the middle of the Petri dish between both spots. After 60-90 min the distribution of worms was analyzed, and the chemotaxis index calculated (number of worms at odorant spot – number of worms at control spot/ total number of animals).

Defecation: Analysis of defecation was done as previously described (Thomas, 1990). Worms were maintained and analyzed at 20°C. The length of one defecation cycle and the time between these cycles were measured. Length of one defecation cycle was defined as the duration between the posterior muscular contraction (pBoc) and the expulsion (Exp). The time between was two defecation cycles was defined as the duration between two consecutive expulsions. Ten consecutive defecation cycles were measured for each animal.

Drug treatments: Adult worms were allowed to lay eggs for three hours at 20°C on NGM agar plates containing varying concentrations of DTT, ß-mercaptoethanol or tunicamycin (Calbiochem). Eggs were counted and progenies were studied three

days later (Shen et al., 2001). Synchronized L1 worms were treated with 2mM paraquat (Sigma) solution and survival at 20° C was studied three days later (Ishii et al., 1998). Mean values were calculated from different experimental groups of 3-10 independent assays, each.

Eggs-in-uterus: For measuring the number of eggs inside the uterus, individual worms were analyzed at three consecutive days of the egg-laying period. Therefore, staged adults were dissolved by hypochlorite treatment on the respective day in microtiter plates (25 worms/day). By this procedure, eggs were released from uterus of the worms and could easily be counted in the individual wells.

Eggs per hour: Measurement of the egg-laying rate per hour was done as previously described (Trent et al., 1983). Worms were maintained and analyzed at 20°C. Single hermaphrodites that had reached adulthood one day before were allowed to lay eggs for five hours. Eggs were counted after each hour.

Egg-laying pharmacology: Assays were performed as previously described (Desai et al., 1988; Trent et al., 1983; Waggoner et al., 1998; Weinshenker et al., 1995). Worms were treated for 60-90 min with distinct dilutions of different compounds: serotonin (5-HT), imipramine, or chlorpromazine.

Heat-shock treatments: Synchronized L2 larvae grown at 20° C were heat stressed for 2h at 35° C and afterwards further maintained at 20° C. Development and survival was scored 2 days later. Mean values were calculated from different experimental groups of six independent assays.

Lifespan: For the lifespan analysis, 5 to 10 adult hermaphrodites were transferred onto fresh plates for egg laying and removed after 3-4 hours. Animals were cultured at 20°C or 25° C and examined every day until death. They were scored death when they did no longer move in response to prodding them with a platinum pick. Each day, dead worms were recorded and removed from the plates. Experiments were started with 100 worms per genotype (10 per plate) and the wild type (N2) was always included as a control.

Mechanosensation: Mechanosensation of worms was assayed as previously described (Chalfie and Sulston, 1981; Chiba and Rankin, 1990; Way and Chalfie, 1989). Response of animals to the following stimuli was analyzed: simple tapping of the plate, eyelash-touch on the side of the body or prodding of worms with a pick at either the head or the tail.

5.6 Software and Microscopy

Quantitative evaluation of Northern blots was performed using ImageQuant 5.0 software (Molecular Dynamics). Sequence alignments were generated using Vector NTI version 6.0 (InforMax). Pictures of GFP were taken with an AxioPlan 2 Microscope (Zeiss) using the AxioVision 3.0 software. Different software tools used in this study can be found at http://www.expasy.org/tools/. i-View software can be found at http://vidal.dfci.harvard.edu/), Dialign software at http://bibiserv.techfak.uni-bielefeld.de/dialign/, MatInspector at http://genomatix.gsf.de/cgi-bin/matinspector.pl, and TFSEARCH at http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCHJ.html. Links and general information about *C. elegans* can be found at http://elegans.swmed.edu/ and http://www.wormbase.org/. Information about *C. elegans* mutants can be found at http://biosci.umn.edu/CGC/CGChomepage.htm, http://shigen.lab.nig.ac.jp/c.elegans/index.jsp, http://celeganskoconsortium.omrf.org/ and http://www.wormbase.org/. For informations about the used *C. elegans gfp* reporter constructs see ftp://www.ciwemb.edu/pub/FireLabInfo/FireLabVectors/).

6 Materials

If not stated otherwise, chemicals and reagents (analytical grade) were purchased from Merck, Sigma, Roth, Calbiochem, Fluka and Biorad. Reagents for molecular biology, restriction enzymes and other enzymes were purchased from MBI Fermentas, New England Biolabs (NEB), QIAGEN, Promega, Pharmacia, Molecular labs, La Roche and Boehringer Ingelheim. Media for cultivation of bacteria, yeast and worms were obtained from Serva, Difco and Gibco BRL. Radiolabelled chemicals were purchased from Amersham Pharmacia or Perkin Elmer. Secondary POD-coupled antibodies were purchased from Dianova.

6.1 Strains

6.1.1 *E. coli* Strains

Strains used in this study

Strain	Genotype	Reference
OP50	*ura*$^-$	(Brenner, 1974)
DH5α	*end*A1, *hsd*R17(r_κ^-,m_κ^+), *sup*E44, *thi, rec*A1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F) U169, Φ80d*lac*ZΔM15	(Hanahan, 1985; Woodcock et al., 1989)
HT115 (DE3)	*F*$^-$*, mcr*A*, mcr*B, IN*(rrnD-rrnE)1, lambda*$^-$*, rnc14::Tn10(DE3 lysogen:*lacUV5 promoter-T7 polymerase, RNAse III minus	(Takiff et al., 1989)
BL 21 DE3 (pRIL)	B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal*λ(DE3) *endA* Hte [*argU ileY leuW* Cam^r]	Stratagene
XL1-Blue MRF'	Δ(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*^{q} [F´ *proAB lacI*^{q}*Z* Δ*M15* Tn*10* (Tet^r)]	Stratagene
SOLR	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ uvrC umuC::*Tn*5* (Kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R [F´ *proAB lacI*^{q}*Z* Δ*M15*] Su$^-$ (nonsuppressing)	Stratagene

Table 5. List of Used *E. coli* Strains.

6.1.2 *S. cerevisiae* Strain

Strains used in this study

Strain	Genotype	Reference
AH109	*MATa, trp1-901, leu2-3, 112, ura3-53, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::GAL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	(James et al., 1996)

Table 6. List of Used *S. cerevisiae* Strain.

6.1.3 *C. elegans* Strains

Some of the strains were provided by the "*C. elegans* Genetic Center" (CGC) at the University of Minnesota, USA (http://biosci.umn.edu/CGC/CGChomepage.htm), others from Elegene (Munich), Shohei Mitani (NBP-Japan), David Ron, Randal Kaufman, and Garry Wong.

Strains used in this study

Strain	Genotype	Reference
N2	*C. elegans* wild type *var. Bristol*	CGC
EM464	*C. remanei* wild type	CGC
VT847	*C. briggsae* wild type	CGC
NL2099	*rrf-3(pk1426)II*	(Simmer et al., 2002)
	pdr-1(lg101)III	EleGene, Munich
	pdr-1(lg103)III	EleGene, Munich
BR3224	*pdr-1(tm395)III*	S. Mitani, NBP-Japan
BR3225	*pdr-1(tm598)III*	S. Mitani, NBP-Japan
RB545	*pek-1(ok275)X*	(Shen et al., 2001)
	ire-1(v33)II/mnC1; pek-1(ok275)X	(Shen et al., 2001)
RB772	*atf-6(ok551)X*	CGC
TK22	*mev-1(kn1)III*	(Honda et al., 1993)
SJ4005	*lin-15(n765ts); zcIs4[hsp-4::gfp;lin-15]V*	(Calfon et al., 2002)
SJ30	*ire-1(zc14)II; zcIs4[hsp-4::gfp]V*	(Calfon et al., 2002)
SJ6	*upr-1(zc6)X; zcIs4[hsp-4::gfp]V*	(Calfon et al., 2002)
CL2070	N2; *dvIs70[hsp-16.2::gfp]* (not mapped)	(Link et al., 1999)
BY200	N2; *byIs200[P$_{dat-1}$::gfp;rol-6(su1006)]V*	(Nass et al., 2002)
WG3	N2; *Is[P$_{aex-3}$::α-syn(WT);P$_{dat-1}$::gfp]* (not mapped)	(Lakso et al., 2003)
WG8	N2*; Is[P$_{aex-3}$::α-syn(A53T);P$_{dat-1}$::gfp]IV*	(Lakso et al., 2003)

Table 7. List of all Used *C. elegans* Strains.

Strains constructed by micro-injection

Strain	Genotype	DNA	Marker
BR897	N2; *byEx[$P_{unc-119}$::α-syn(WT);P_{dat-1}::gfp]*	pBY456	pBY266
BR898	N2; *byEx[$P_{unc-119}$::α-syn(WT);P_{dat-1}::gfp]*	pBY456	pBY266
BR899	N2; *byEx[$P_{unc-119}$::α-syn(WT);P_{dat-1}::gfp]*	pBY456	pBY266
BR900	N2; *byEx[$P_{unc-119}$::α-syn(A53T);P_{dat-1}::gfp]*	pBY457	pBY266
BR901	N2; *byEx[$P_{unc-119}$::α-syn(A53T);P_{dat-1}::gfp]*	pBY457	pBY266
BR968	N2; *byEx [$P_{unc-119}$::α-syn(A30P);P_{dat-1}::gfp]*	pBY458	pBY266
BR1846	N2; *byEx170 [P_{dat-1}::α-syn(A30P);P_{dat-1}::gfp]*	pBY1170	pBY266
BR1847	N2; *byEx171 [P_{dat-1}::α-syn(A30P);P_{dat-1}::gfp]*	pBY1170	pBY266
BR1905	N2; *byEx172 [P_{sel-12}::α-syn(WT);P_{sel-12}::gfp]*	pBY1158	pBY1153
BR1906	N2; *byEx173 [P_{sel-12}::α-syn(WT);P_{sel-12}::gfp]*	pBY1158	pBY1153
BR1907	N2; *byEx174 [P_{sel-12}::α-syn(WT);P_{sel-12}::gfp]*	pBY1158	pBY1153
BR1908	N2; *byEx176 [P_{sel-12}::α-syn(A30P);P_{sel-12}::gfp]*	pBY1160	pBY1153
BR1909	N2; *byEx177 [P_{sel-12}::α-syn(A30P);P_{sel-12}::gfp]*	pBY1160	pBY1153
BR1912	N2; *byEx178 [P_{sel-12}::α-syn(A30P);P_{sel-12}::gfp]*	pBY1160	pBY1153
BR1913	N2; *byEx175[P_{sel-12}::α-syn(WT);P_{sel-12}::gfp]*	pBY1158	pBY1153
BR1948	N2; *byEx179[P_{pdr-1}::gfp;rol-6(su1006)]*	pBY1013	pRF4
BR2317	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2318	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2319	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2320	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2321	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2322	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2323	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2324	*pdr-1(lg101); byEx[PCR-RBRB850/RB1070]*	PCR	pBY1153
BR2325	*pdr-1(lg101); byEx [K08E3]*	K08E3	pBY1153
BR2326	*pdr-1(lg101); byEx [K08E3]*	K08E3	pBY1153
BR2327	*pdr-1(lg101); byEx [K08E3]*	K08E3	pBY1153
BR2726	*pdr-1(lg103); byEx429[K08E3]*	K08E3	pBY1153
BR2727	*pdr-1(lg103); byEx430[K08E3]*	K08E3	pBY1153
BR2728	*pdr-1(lg103); byEx431[K08E3]*	K08E3	pBY1153
BR2729	*pdr-1(lg103); byEx432[K08E3]*	K08E3	pBY1153
BR2730	*pdr-1(lg103); byEx433[K08E3]*	K08E3	pBY1153
BR2768	*pdr-1(lg103);byEx434[pdr-1/K08E3.8;sel-12::gfp]*	pBY1500	pBY1153
BR2769	*pdr-1(lg103);byEx435[pdr-1/K08E3.8;sel-12::gfp]*	pBY1500	pBY1153
BR2770	*pdr-1(lg103);byEx436[pdr-1/K08E3.8;sel-12::gfp]*	pBY1500	pBY1153
BR2789	*pdr-1(lg101)III;byEx437[pdr-1(lg103);sel-12::gfp]*	pBY1569	pBY1153
BR2790	*pdr-1(lg101)III;byEx438[pdr-1(lg103);sel-12::gfp]*	pBY1569	pBY1153
BR2791	*pdr-1(lg101)III;byEx439[pdr-1(lg103);sel-12::gfp]*	pBY1569	pBY1153

Strain	Genotype	DNA	Marker
BR2792	*pdr-1(lg101)III;byEx440[pdr-1(lg103);sel-12::gfp]*	pBY1569	pBY1153
BR3136	*pdr-1(lg103); byEx417[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3137	*pdr-1(lg103); byEx418[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3138	*pdr-1(lg103); byEx419[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3139	*pdr-1(lg103); byEx420[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3140	*pdr-1(lg103); byEx421[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3141	*pdr-1(lg103); byEx422[pdr-1;sel-12::gfp]*	pBY1908	pBY1153
BR3045	N2; *byEx411 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3046	N2; *byEx412 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3047	N2; *byEx413 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3048	N2; *byEx414 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3049	N2; *byEx415 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3050	N2; *byEx416 [P_{pdr-1}::gfp::pdr-1;rol-6]*	pBY1794	pRF4
BR3187	N2; *byEx[P_{pdr-1}::gfp;rol-6]*	pBY1909	pRF4
BR3188	N2; *byEx[P_{pdr-1}::gfp;rol-6]*	pBY1909	pRF4
BR3189	N2; *byEx[P_{pdr-1}::gfp;rol-6]*	pBY1909	pRF4
BR3190	N2; *byEx[P_{pdr-1}::gfp;rol-6]*	pBY1909	pRF4
BR3191	N2; *byEx[P_{pdr-1}::gfp;rol-6]*	pBY1909	pRF4

Table 8. List of Transgenic *C. elegans* Strains Obtained by Micro-injection.

Strains constructed by crossing

Strain	Genotype
BR2429	*pdr-1(lg101)III* (9^{th} outcross)
BR2430	*pdr-1(lg103)III* (7^{th} outcross)
BR2775	*ire-1(v33)II* (1^{st} outcross)
BR2766	*pdr-1(lg103)III; pek-1(ok275)X*
BR2767	*pdr-1(lg101)III; pek-1(ok275)X*
BR2785	*ire-1(v33)II; pdr-1(lg101)III*
BR2786	*ire-1(v33)II; pdr-1(lg103)III*
BR3226	*ire-1(v33)II; pdr-1(tm395)III*
BR3227	*ire-1(v33)II; pdr-1(tm598)III*
BR3177	*pdr-1(lg103)III; atf-6(ok551)X*
BR2763	*pdr-1(lg103)III; ls[$P_{hsp-16.2}$::gfp]*
BR2764	*pdr-1(lg103)III; byIs200[P_{dat-1}::gfp]V*
BR2765	*pdr-1(lg101)III; byIs200[P_{dat-1}::gfp]V*
BR2783	*pdr-1(lg103)III; zcIs4[P_{hsp-4}::gfp]V*
BR3143	*pdr-1(lg103)III; ls[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]*
BR3144	*pdr-1(lg103)III; ls[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*

BR3183	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]*
BR3184	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3228	*pdr-1(tm395)III; Is[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]*
BR3229	*pdr-1(tm395)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3230	*pdr-1(tm598)III; Is[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]*
BR3231	*pdr-1(tm598)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3178	*ire-1(v33)II; Is[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]*
BR3179	*ire-1(v33)II; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3180	*Is[P_{aex-3}::α-syn(WT);P_{dat-1}::gfp]; zcIs4[P_{hsp-4}::gfp]V*
BR3181	*Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV; zcIs4[P_{hsp-4}::gfp]V*
BR3182	*atf-6(ok551)X; zcIs4[P_{hsp-4}::gfp]V*
BR3205	*rrf-3(pk1426)II; pdr-1(lg103)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3233	*pdr-1(lg103)III; atf-6(ok551)X; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3232	*pdr-1(lg101)III; atf-6(ok551)X; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*
BR3235	*pdr-1(lg103)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV; zcIs4[P_{hsp-4}::gfp]V*
BR3234	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV; zcIs4[P_{hsp-4}::gfp]V*
BR3236	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T)]IV; byEx437[pdr-1(lg103)]*
BR3237	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T)]IV; byEx438[pdr-1(lg103)]*
BR3238	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T)]IV; byEx439[pdr-1(lg103)]*
BR3239	*pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T)]IV; byEx440[pdr-1(lg103)]*
BR3242	*rrf-3(pk1426)II; pdr-1(lg101)III; Is[P_{aex-3}::α-syn(A53T);P_{dat-1}::gfp]IV*

Table 9. List of *C. elegans* Strains Obtained By Crossing.

6.2 Antibodies

Antibodies used in this study

Name	Epitope	Description	Reference
9E10	c-myc	mouse monoclonal	Boehringer
3F10	HA	rat monoclonal	Boehringer
Z-5	GST	rabbit polyclonal	Santa Cruz
1510	ubiquitin	mouse monoclonal	Chemicon
83722	GFP	rabbit polyclonal	Clontech
15G7	α-synuclein	rat monoclonal	(Kahle et al., 2000)

Table 10. List of Used Antibodies.

6.3 Vectors and Constructs

6.3.1 Vectors

C. elegans specific vectors were obtained from Dr. Andrew Fire (ftp://www.ciwemb.edu/pub/FireLabInfo/FireLabVectors/), genomic cosmids were obtained from the Sanger Center (http://www.sanger.ac.uk/Projects/C_elegans/).

Plasmids used in this study

Name	Description	Reference
pSKII	Standard cloning vector; Amp^R	Stratagene
pCRScript	Vector for blunt-end cloning; Amp^R	Stratagene
pGEM-T	Vector for T/A-overhang cloning; Amp^R	Promega
pPD95.75	Promoterless *gfp* vector; Amp^R	Fire Vector Kit 1995
pPD117.01	Promoterless *gfp* vector; Amp^R	Fire Vector Kit 1999
pPD129.36	T7 for production of dsRNA *in vitro* and *in vivo*; Amp^R	Fire Vector Kit 1999
pRF4	Dominant marker [*rol-6(su1006)*] ; Amp^R	(Mello et al., 1991)
pBY1153	*sel-12::gfp* co-injection marker; Amp^R	(Wittenburg et al., 2000)
pGBKT7	Yeast GAL4 DNA bdg. domain vector; Kan^R	Clontech
pGADT7	Yeast GAL4 activation domain vector; Amp^R	Clontech
pET-21a(+)	6xHIS *E. coli* expression vector; Amp^R	Novagen
pET-28b(+)	6xHIS *E. coli* expression vector; Kan^R	Novagen
pGST-parallel 3	GST *E. coli* expression vector; Amp^R	(Sheffield et al., 1999)
pCite-4a(+)	*In vitro* translation vector; Amp^R	Novagen
pET21-UbcH7	Purification of rec.UbcH7 from *E. coli*; Amp^R	M. Scheffner
pBY1875	pGEX4T1 GST::CHN-1; Amp^R	(Hoppe et al., 2004)
pBY1898	modified Baculovirus vector pAcUW51; Amp^R	(Lakowski et al., 2003)
pBY1884	GST::myc-CHN-1 in pET-21a(+);Amp^R	(Hoppe et al., 2004)
pBY1877	pGADT7 *chn-1*; Amp^R	(Hoppe et al., 2004)
pG77#AL2	P_{dat-1}*::gfp*	H. Huttner
pBY456	$P_{unc-119}$*::α-syn(WT)*	M. Okochi
pBY457	$P_{unc-119}$*::α-syn(A53T)*	M. Okochi
pBY458	$P_{unc-119}$*::α-syn(A30P)*	M. Okochi

Table 11. List of Used Plasmids.

6.3.2 Constructs

Phages containing *C. elegans* cDNA clones were obtained from Dr. Yuji Kohara, and were excised by plasmid rescue method described in the experimental procedures section.

Plasmids constructed in this study

Name	Size in bp	R/M	Vector	Cloning size in bp	Insert	Cloning size in bp	Description	tags	T	aa, kDa	Notes/Usage
pBY 1013	8570	Amp	pPD95.75	SphI/SalI 4486	PCR RB850/851 Ce-N2 genomic	SphI/SalI 4084	Ce-prk 4070bp Promoter incl AS 1-4	intron GFP	C		Ce-prk 4070 bp Promoter GFP-expression vector
pBY 1018	6981	Amp	pBluescript SK II	SacII/SalI 2885	PCR RB850/851 Ce-N2 genomic	SacII/SalI 4090	Ce-prk 4070bp Promoter				cloning vector
pBY 1019	3589	Amp	pBluescript SK II	SacII/SalI 2885	PCR RB853/852 Ce-N2 genomic	SacII/SalI 698	Ce-prk 3'part Promoter +genomicORF(AS 1-115)				cloning vector
pBY 1020	4872	Amp	pBluescript SK II	SacII/SmaI 2926	PCR RB853/839 Ce-N2 genomic	SacII/SmaI 1944	Ce-prk 3'part Promoter + fulllength-genomicORF				cloning vector
pBY 1021	4119	Amp	pBluescript SK II	SpeI/SmaI 2951	PCR RB838/839 Ce-N2 cDNA	NheI/SmaI 1172	Ce-prk cDNA				cloning vector
pBY 1022	11066	Kan LEU	pDBLeu	NheI/StuI 9894	PCR RB838/839 Ce-N2 cDNA	NheI/SmaI 1172	Ce-prk cDNA	Gal4DB	N		60mM 3AT Yeast-2-Hybrid-screening vector
pBY 1023	9038	Amp	pBY1013	StuI/SalI 8455	RB853/852 Ce-N2 genomic	StuI/SalI 583	Ce-prk 3'part Promoter +genomicORF(AS 1-115)	intron GFP	C		Ce-prk 4070bp Promoter + genomicORF (AS 1-115) GFP-expression vector
pBY 1037	4142	Amp	pCRScript Amp SK +	SrfI 2961	PCR RB838/839 Ce-N2 cDNA	blunt 1181	Ce-prk cDNA				cloning vector
pBY 1038	9830	Kan TRP	pDBTrp	NheI/NotI 8630	pBY1022-Fragment	NheI/NotI 1200	Ce-prk cDNA	Gal4DB	N		60 mM 3AT Yeast-2-Hybrid-screening vector
pBY 1039	11020	Kan LEU	pLeu VlHADB	SmaI/SacI 9839	PCR SE02/RB916 pBY1022	SmaI/SacI 1181	Ce-prk cDNA	Gal4DB	C		15 mM 3AT Yeast-2-Hybrid-screening vector
pBY 1040	3998	Amp	pLitmus28	EcoRV 2823	pBY1037-Fragment	SmaI 1175	Ce-prk cDNA	flanked by T7			T7-transcription vector for production of Ce-prk dsRNA
pBY 1062	9800	Kan TRP	pDBTrp	MluI/NotI 5304	pBY1039-Fragment	MluI/NotI 4496	Ce-prk cDNA	Gal4DB	C		15 mM 3AT Yeast-2-Hybrid-screening vector
pBY 1113	10278	Amp	pBY1023	NheI/SalI 8503	PCR RB853/916 Ce-N2 genomic	NheI/SalI 1775	Ce-prk 3'part Promoter + fulllength-genomicORF	intron GFP	C		Ce-prk 4070bp Promoter + fulllength-genomicORF GFP-expression vector
pBY 1114	10272	Amp	pBY1023	NheI/SmaI 8484	PCR RB853/916 Ce-N2 genomic	NheI/SmaI 1788	Ce-prk 3'part Promoter + fulllength-genomicORF	intron GFP	C		like pBY1113
pBY 1115	~ 8300	Kan TRP	pBY1062	KpnI/SmaI 6865	pADH1001-Fragment	KpnI/SmaI ~1400	like pBY1062 with stronger pADH	Gal4DB	C		Yeast-2-Hybrid-screening vector with stronger ADH-promoter
pBY 1116	10580	Amp	pBluescript SK II	SmaI 2961	Ce-K08E3-fragment-I	SnaBI/SmaI 7619	Ce-K08E3 subclone I (7619 bp)				cloning vector Ce-prk genomic locus subclone
pBY 1117	8849	Amp	pBluescript SK II	BamHI/SmaI 2957bp	Ce-K08E3-fragment-II	BamHI/SmaI 5896	Ce-K08E3 subclone II (5896 bp)				cloning vector Ce-prk genomic locus subclone
pBY 1118	6496	Kan	pET28b(+)	NheI/SacI 5328	pBY1039-Fragment	NheI/SacI 1176	Ce-prk cDNA	flanked by HIS	N C		bacterial expression vector His-CePARKIN-His
pBY 1119	6483	Kan	pET28b(+)	NheI/SalI 5317	pBY1039-Fragment	NheI/SalI 1166	Ce-prk cDNA	HIS	N		bacterial expression vector His-CePARKIN
pBY 1120	4872	Amp	pBluescript SK II	SacII/SmaI 2926	PCR RB853/839 Ce-N2 genomic	SacII/SmaI 1944	Ce-prk 3'part Promoter + fulllength-genomicORF				cloning vector
pBY 1121	8431	Amp	pPD95.75	BamHI/SmaI 4487	pBY1114-Fragment	BamHI/SmaI 3944	Ce-prk 2212bp Promoter + fulllength-genomicORF	intron GFP	C		Ce-prk 2212bp Promoter + fulllength-genomicORF GFP-expression vector
pBY 1122	4068	Amp	pPD49.26	PstI/SmaI 3396	PCR RB1021/1022 Ce-N2 genomic	PstI/blunt 672	Ce-prk 648bp Promoter				cloning vector short Ce-prk promoter (648 bp)
pBY 1123	9841	Amp	pPD49.26	SphI/SacI 3276	pBY1114-Fragment + pBY622-Fragment	SphI/SalI 5792 SalI/SacI 773	Ce-prk 4070bp Promoter + fulllength-genomicORF	EGFP	C		Ce-prk 4070bp Promoter + fulllength-genomicORF GFP-expression vector
pBY 1124	8000	Amp	pPD49.26	BamHI/SacI 3296	pBY1114-Fragment + pBY622-Fragment	BamHI/SalI 3931 SalI/SacI 773	Ce-prk 2212bp Promoter + fulllength-genomicORF	EGFP	C		Ce-prk 2212bp Promoter + fulllength-genomicORF GFP-expression vector
pBY 1125	4374	Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1033/1034 Hs-brain cDNA	blunt 1413	Hs-parkin-cDNA				cloning vector H.sapiens parkin cDNA
pBY 1126	5145	Amp	pBY1122	SmaI/SalI 3974	pBY1039-Fragment	SmaI/SalI 1171	Ce-prk cDNA				short Ce-prk promoter (648 bp) + Ce-prk cDNA
pBY 1127	5373	Amp	pBY1122	SmaI/SalI 3974	PCR RB1033/1034 Hs-brain cDNA	SmaI/SalI 1399	Hs-parkin-cDNA				short Ce-prk promoter (648 bp) + Hs-parkin cDNA
pBY 1128	7475	Amp	pBY1122	SphI/StuI 3506	pBY1114-Fragment	SphI/StuI 3969	Ce-prk 4070bp Promoter				Ce-prk 4070bp Promoter cloning vector
pBY 1129	5621	Amp	pBY1122	BamHI/StuI 3513	pBY1116-Fragment	BamHI/StuI 2108	Ce-prk 2212bp Promoter				Ce-prk 2212bp Promoter cloning vector
pBY 1130	4688	Amp	pBY1122	XhoI/StuI 3518	pBY1116-Fragment	XhoI/StuI 1170	Ce-prk 1275bp Promoter				Ce-prk 1275bp Promoter cloning vector
pBY 1155	8552	Amp	pBY1126	SphI/StuI 4583	pBY1113-Fragment	SphI/StuI 3969	Ce-prk 4070bp Promoter				Ce-prk 4070bp Promoter + Ce-prk cDNA
pBY 1156	6698	Amp	pBY1126	BamHI/StuI 4590	pBY1114-Fragment	BamHI/StuI 2108	Ce-prk 2212bp Promoter				Ce-prk 2212bp Promoter + Ce-prk cDNA
pBY 1157	8780	Amp	pBY1128	SmaI/SalI 7381	PCR RB1033/1034 Hs-brain cDNA	SmaI/SalI 1399	Hs-parkin cDNA				Ce-prk 4070bp Promoter + Hs-parkin cDNA

Name	Size in bp	R/M	Vector	Cloning size in bp	Insert	Cloning size in bp	Description	tags	T	aa, kDa	Notes/Usage
pBY 1158	6656	Amp	pBY871	MscI/NcoI 6142	pBY456-Fragment	MscI/NcoI 514	Hs-ASN (wt) cDNA				Ce-sel12 Promoter(I) + Hs-ASN (wt) cDNA
pBY 1159	6656	Amp	pBY871	MscI/NcoI 6142	pBY457-Fragment	MscI/NcoI 514	Hs-ASN (A53T) cDNA				Ce-sel12 Promoter(I) + Hs-ASN (A53T) cDNA
pBY 1160	6656	Amp	pBY871	MscI/NcoI 6142	pBY458-Fragment	MscI/NcoI 514	Hs-ASN (A30P) cDNA				Ce-sel12 Promoter(I) + Hs-ASN (A30P) cDNA
pBY 1167	4383	Amp	pCRScript Amp SK +	SrfI 2961	RB1048/1049 Dm-embryo cDNA	blunt 1422	Dm-parkin cDNA				cloning vector D.melanogaster parkin cDNA
pBY 1168	10231	Amp	pPD49.26	PstI/NcoI 3303	pBY266-Fragment + pBY456-Fragment	PstI/MscI ??? MscI/NcoI 514	Ce-DAT (T23G5.5) Prom. + Hs-ASN (wt) cDNA				Ce-DAT (T23G5.5) Promoter + Hs-ASN (wt) cDNA
pBY 1169	10231	Amp	pPD49.26	PstI/NcoI 3303	pBY266-Fragment + pBY457-Fragment	PstI/MscI ??? MscI/NcoI 514	Ce-DAT (T23G5.5) Prom. + Hs-ASN (A53T) cDNA				Ce-DAT (T23G5.5) Promoter + Hs-ASN (A53T) cDNA
pBY 1170	10231	Amp	pPD49.26	PstI/NcoI 3303	pBY266-Fragment + pBY458-Fragment	PstI/MscI ??? MscI/NcoI 514	Ce-DAT (T23G5.5) Prom. + Hs-ASN (A30P) cDNA				Ce-DAT (T23G5.5) Promoter + Hs-ASN (A30P) cDNA
pBY 1171	5382	Amp	pBY1122	SmaI/SalI 3974	PCR RB1048/1049 Dm-embryo cDNA	SmaI/SalI 1408	Dm-parkin cDNA				short Ce-prk promoter (648 bp) + Dm-parkin cDNA
pBY 1172	8789	Amp	pBY1128	SmaI/SalI 7381	PCR RB1048/1049 Dm-embryo cDNA	SmaI/SalI 1408	Dm-parkin cDNA				Ce-prk 4070bp Promoter + Dm-parkin cDNA
pBY 1176	8466	Kan TRP	pGBKT7	XhoI/StuI	PCR SE02/RB916 pBY1022	SmaI/SalI 1171	Ce-prk cDNA cloned without stop	Gal4DB myc	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1233
pBY 1177	8786	Amp LEU	pGADT7	SmaI/SacI 7957	PCR RB1054/1055 pGAD424-pal1	blunt/SacI 829	Ce-pal1 cDNA cloned without stop	Gal4AD HA	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1245
pBY 1178	7899	Kan TRP	pGBKT7	SmaI/SalI 7295	PCR RB1064/916 pBY1022	SmaI/SalI 604	Ce-prk cDNA fragment RING-IBR-RING	Gal4DB myc	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1237
pBY 1179	8178	Kan TRP	pGBKT7	SmaI/SalI 7295	PCR RB1065/916 pBY1022	SmaI/SalI 883	Ce-prk cDNA fragment deletion-UBI	Gal4DB myc	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1238
pBY 1180	7390	Amp	pBY1122	XhoI/StuI 3518	pBY1116-Fragment	SalI/StuI 3872	Ce-prk 4070bp Promoter genomic fragm. vs. PCR				Ce-prk 4070bp Promoter exchange genomic fragment vs. PCR-product
pBY 1190	9841	Amp	pBY1123	Bst1107I/NheI 6171	pBY1116-Fragment	Bst1107I/NheI 3670	Ce-prk 4070bp Promoter genomic fragm. vs. PCR				Ce-prk 4070bp Promoter + fulllength-genomicORF GFP-expression vector
pBY 1191	6926	Amp	pBY1129	SmaI/SalI 5527	pBY1157-Fragment	SmaI/SalI 1399	Hs-parkin cDNA				Ce-prk 2212bp Promoter + Hs-parkin cDNA
pBY 1192	6935	Amp	pBY1129	SmaI/SalI 5527	pBY1172-Fragment	SmaI/SalI 1408	Dm-parkin cDNA				Ce-prk 2212bp Promoter + Dm-parkin cDNA
pBY 1193	4373	Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1033/1034 Hs-skeletmusc.cDNA	blunt 1413	Hs-parkin cDNA (aus skeletal muscle)	T3 -> T7			cloning vector Hs-parkin cDNA no polymorphism
pBY 1194	4373	Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1033/1034 Hs-SMART cDNA	blunt 1413	Hs-park cDNA (aus SMART-library)	T3 <- T7			cloning vector Hs-parkin cDNA no polymorphism
pBY 1195		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk58h10	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1196		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk450a8	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1197		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk82e5	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1198		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk228e8	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1199		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk175e9	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1200		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk268h9	EcoRI/XhoI	Ce-K08E3.7 cDNA				cloning vector
pBY 1205		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk504f1	EcoRI/XhoI	Ce-F44G4.1 cDNA				cloning vector
pBY 1206		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk452e10	EcoRI/XhoI	Ce-F44G4.1 cDNA				cloning vector
pBY 1229	6433	Kan	pET 28b(+)	NcoI/SacI 5263	PCR RB1152/916 pBY1022	NcoI/SacI 1178	Ce-prk cDNA w/o stop	HIS	C	408 46,57	bacterial expression vector CePARKIN-His
pBY 1230	6475	Kan	pET 28b(+)	NdeI/SalI 5310	PCR RB1091/1153 pBY1022	NdeI/SalI 1163	Ce-prk cDNA with stop	HIS	N	406 46,28	bacterial expression vector His-CePARKIN
pBY 1231	7548	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1154 pBY1116	NcoI/SalI 271	Ce-prk Ex1+In1+Ex2	Gal4DB myc	N		Yeast-2-Hybrid vector to test with prk-interactors
pBY 1232	7503	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1154 pBY1022	NcoI/SalI 226	Ce-prk Ex1+Ex2 (UBI-domain)	Gal4DB myc	N	250 28,47	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1233	8446	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1153 Ce-N2 mixed cDNA	NcoI/SalI 1169	Ce-prk cDNA I (1158bp)	Gal4DB myc	N	564 64,49	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1234	8355	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1153 Ce-N2 mixed cDNA	NcoI/SalI 1078	Ce-prk cDNA III (1067bp)	Gal4DB myc	N	421 47,85	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1235	8404	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1153 Ce-N2 mixed cDNA	NcoI/SalI 1127	Ce-prk cDNA IV (1116bp)	Gal4DB myc	N	264 30,37	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1236	7981	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1153 Ce-N2 mixed cDNA	NcoI/SalI 704	Ce-prk cDNA II (693bp)	Gal4DB myc	N	409 46,91	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1237	7900	Kan TRP	pGBKT7	SmaI/SalI 7295	PCR RB1064/1153 pBY1022	SmaI/SalI 605	Ce-prk cDNA fragment RING-IBR-RING	Gal4DB myc	N	382 44,12	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1238	8179	Kan TRP	pGBKT7	SmaI/SalI 7295	PCR RB1065/1153 pBY1022	SmaI/SalI 884	Ce-prk cDNA fragment deletion-UBI	Gal4DB myc	N	475 54,54	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1239	8389	Amp LEU	pGADT7	NdeI/SacI 7927	PCR RB1143/1144 Ce-N2 cDNA	NdeI/EcoRI 462	Ce-R01H2.6 cDNA cloned without stop	Gal4AD HA	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1243
pBY 1240	8373	Amp LEU	pGADT7	NdeI/SacI 7927	PCR RB1145/1146 Ce-N2 cDNA	NdeI/SacI 446	Ce-M7.1 cDNA cloned without stop	Gal4AD HA	N		Yeast-2-Hybrid vector insert cloned without stop -> see pBY1244
pBY 1243	8431	Amp LEU	pGADT7	NdeI/EcoRI 7968	PCR RB1143/1166 Ce-N2 cDNA	NdeI/EcoRI 463	Ce-R01H2.6 cDNA cloned with stop	Gal4AD HA	N	313 35,31	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1244	8412	Amp LEU	pGADT7	NdeI/EcoRI 7968	PCR RB1145/1167 Ce-N2 cDNA	NdeI/EcoRI 444	Ce-M7.1 cDNA cloned with stop	Gal4AD HA	N	307 34,36	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1245	8790	Amp LEU	pGADT7	BamHI/XhoI 7976	PCR RB1054/1169 pal1-Klon E und D	BamHI/XhoI 814	Ce-pal1 cDNA cloned with stop	Gal4AD HA	N		cloned out of frame new:-> see pBY 1407
pBY 1246		Amp	pBY1180	SmaI/NotI 7385	pd2EGFP-1 Fragment	SmaI/NotI 872	d2EGFP	d2EGFP			Ce-prk 4070bp Promoter + destabilized EGFP
pBY 1247		Amp	pBY1180	SmaI/NotI 7385	pDsRed1-N1 Fragment	SmaI/NotI 702	DsRed1	DsRed1			Ce-prk 4070bp Promoter + DsRed1

Name	Size in bp	R/M	Vector	Cloning size in bp	Insert	Cloning size in bp	Description	tags	T	aa, kDa	Notes/Usage
pBY 1248	3891	Amp	pPD129.36	NcoI/SalI 2722	PCR RB1152/1153 Ce-N2 cDNA	NcoI/SalI 1169	Ce-prk cDNA	flanked by T7			T7-transcription vector for production of Ce-prk dsRNA
pBY 1249		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1160/1153 Cb-VT847 cDNA	blunt 1388	Cb-prk cDNA isoform I fulllength (1158bp)	T3 -> T7		385	cloning vector C.briggsae parkin cDNA I (385AS)
pBY 1250		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1160/1153 Cb-VT847 genomic DNA	blunt	Cb-prk genomicORF (1389bp)				cloning vector C.briggsae parkin genomic ORF (1389bp)
pBY 1281		Amp	pET 21a(+)	NdeI/SalI 5384	PCR RB1091/1153 pBY1022	NdeI/SalI 1163	Ce-prk cDNA			44,12	bacterial expression vector untagged CePARKIN
pBY 1282		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1160/1153 Cb-VT847 cDNA	blunt	Cb-prk cDNA isoform II D bp: 454-645 + 7bp-Ins.aus InII (972bp)	T3 -> T7		151	cloning vector (Insertion TCTTCCAG) C.briggsae parkin cDNA II (AS 1-151)
pBY 1283		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1160/1153 Cb-VT847 cDNA	blunt	Cb-prk cDNA isoform III D bp: 301-645 (814bp)	T3 <- T7		270	cloning vector C.briggsae parkin cDNA III (D AS 101-215)
pBY 1284		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1160/1153 Cb-VT847 cDNA	blunt	Cb-prk cDNA isoform IV D bp: 70-645 (582bp)	T3 -> T7		193	cloning vector C.briggsae parkin cDNA IV (D AS 24-215)
pBY 1348	9180	Amp LEU	pGADT7	KpnI/BamHI 7523	RB1233/916 pBY1022 RB1234/1235 annealed RB1236/1237 pGBKT7	KpnI/SacI 1178 SacI/AgeI 32 AgeI/BamHI 447		myc Gal4DB	C		intermediate cloning vector
pBY 1349	8464	Kan TRP	pGBKT7	BsaBI/BamHI 6263	pBY1348-Fragment	BsaBI/BamHI 2201	Ce-prk cDNA	myc Gal4DB	C	590 67,18	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1357	7774	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1243-Fragment	NdeI/XhoI 503	Ce-R01H2.6 cDNA	Gal4DB myc	N	327 37,62	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1358	7755	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1244-Fragment	NdeI/XhoI 484	Ce-M7.1 cDNA	Gal4DB myc	N	321 36,68	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1359	8133	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1245-Fragment	NdeI/XhoI 862	Ce-pal1 cDNA cloned with stop	Gal4DB myc	N		cloned out of frame new:-> see pBY 1408
pBY 1360	9103	Amp LEU	pGADT7	NdeI/XhoI 7928	pBY 1233-Fragment	NdeI/SalI 1175	Ce-prk cDNA	Gal4AD HA	N	550 62,18	Yeast-2-Hybrid vector to test with prk and prk-interactors
pBY 1361	8664	Kan TRP	pGBKT7	NdeI/SmaI 7280	PCR RB1251/1252 Ce-N2 cDNA	NdeI/blunt 1384	Ce-unc59 cDNA	Gal4DB myc		633 72,89	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1362	9342	Amp LEU	pGADT7	NdeI/SmaI 7958	PCR RB1251/1252 Ce-N2 cDNA	NdeI/SmaI 1384	Ce-unc59 cDNA	Gal4AD HA	N	619 70,57	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1363	8670	Kan TRP	pGBKT7	NdeI/SmaI 7280	PCR RB1253/1254 Ce-N2 cDNA	NdeI/blunt 1390	Ce-unc61 cDNA	Gal4DB myc		635 72,90	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1364	9348	Amp LEU	pGADT7	NdeI/SmaI 7958	PCR RB1253/1254 Ce-N2 cDNA	NdeI/SmaI 1390	Ce-unc61 cDNA	Gal4AD HA	N	621 70,58	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1394	3230	Amp	pPD129.36	NheI/NotI 2785	pBY456-Fragment	NheI/NotI 445	Hs-ASN (wt) cDNA	flanked by T7			T7-transcription vector for production of Hs-ASN (wt) dsRNA
pBY 1395	8939	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1300/1301 Ce-N2 cDNA	NdeI/SalI 1010	Ce-F44C4.3 cDNA	Gal4AD HA	N	495 54,15	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1396	8944	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1302/1303 Ce-N2 cDNA	NdeI/XhoI 1016	Ce-T03E6.7 cDNA	Gal4AD HA	N	497 55,77	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1397	9070	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1304/1305 Ce-N2 cDNA	NdeI/SalI 1142	Ce-C25B8.3 cDNA	Gal4AD HA	N	539 60,06	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1398	9133	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1306/1307 Ce-N2 cDNA	NdeI/XhoI 1205	Ce-F57F5.1 cDNA	Gal4AD HA	N	560 61,85	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1399	9127	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1308/1309 Ce-N2 cDNA	NdeI/SalI 1199	Ce-H22K11.1 cDNA	Gal4AD HA	N	558 61,07	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1400	7686	Amp	pBY1180/ 1122 neu	SmaI/NotI	pBY 1348-Fragment + pBY622-Fragment	SmaI/AgeI 1206 AgeI/Bsp120I 779	Ce-prk cDNA-myc + EGFP	myc EGFP	C		Ce-prk bp Promoter + Ce-prk cDNA + myc + EGFP
pBY 1401	7601	Amp	pBY1180/ 1122 neu	SmaI/NotI	pBY 1348-Fragment + pd2EGFP1-Fragment	SmaI/AgeI 1206 AgeI/NotI 864	Ce-prk cDNA-myc + d2EGFP	myc d2EGFP	C		Ce-prk bp Promoter + Ce-prk cDNA + myc + destabilized EGFP
pBY 1402	7516	Amp	pBY1180/ 1122 neu	SmaI/NotI	pBY 1348-Fragment + pDsRed1N1-Fragment	SmaI/AgeI 1206 AgeI/NotI 694	Ce-prk cDNA-myc + DsRed1	myc DsRed1	C		Ce-prk bp Promoter + Ce-prk cDNA + myc + DsRed1
pBY 1403		Amp Kan	pCR TOPO 2.1	T/A-cloning	PCR RB639/1153 Ce-N2 cDNA	T/A-cloning	Ce-prk cDNA with SL1 splice-leader				pWS118
pBY 1404		Amp Kan	pCR TOPO 2.1	T/A-cloning	PCR RB1287/916 Cr-EM464 cDNA	T/A-cloning	Cr-prk cDNA I (1161bp)				cloning vector C.remanei parkin cDNA I (387AS)
pBY 1405		Amp	pCRScript Amp SK +	SrfI 2961	PCR RB1287/916 Cr-EM464 genomic DNA	blunt	Cr-prk genomic ORF (1495bp)				cloning vector C.remanei parkin genomic ORF (1495bp)
pBY 1406	???	Amp	pPD129.36	EcoRV/HindIII	PCR RB1238/1194 Ce-N2 cDNA	blunt/HindIII	Ce-K08E3.8 cDNA	flanked by T7			T7-transcription vector for production of Ce-K08E3.8 dsRNA
pBY 1407	8794	Amp LEU	pBY1245	BamHI 8790	Klenow fill in + religation	shift to correct frame	Ce-pal1 cDNA	Gal4AD HA	N	447 49,80	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1408	8141	Kan TRP	pBY1359	BamHI 8133	Klenow fill in + religation	shift to correct frame	Ce-pal1 cDNA	Gal4DB myc	N	461 52,11	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1409	???	Amp	pPD95.75	SphI/SmaI 4467	pBY1400-Fragment	SphI/SmaI					
pBY 1414	7774	Kan TRP	pGBKT7	NcoI/SalI 7277	PCR RB1152/1153 prk-KO3 (lg103) cDNA	NcoI/SalI 497	Ce-prk KO3 (lg103) cDNA	Gal4DB myc	N	340 39,32	Yeast2Hybrid vector to test with prk-interactors
pBY 1415	7792	Kan TRP	pBY1349	KpnI/SacI 7286	PCR RB1233/1916 prk-KO3 (lg103) cDNA	KpnI/SacI 506	Ce-prk KO3 (lg103) cDNA	myc Gal4DB	C	366 42,01	Yeast2Hybrid vector to test with prk-interactors
pBY 1418	8281	Kan TRP	pGBKT7	NdeI/PstI 7261	pBY1395-Fragment	NdeI/PstI 1020	Ce-F44C4.3 cDNA	Gal4DB myc	N	509 56,46	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1419	8287	Kan TRP	pGBKT7	NdeI/PstI 7261	pBY1396-Fragment	NdeI/PstI 1026	Ce-T03E6.7 cDNA	Gal4DB myc	N	511 58,09	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1420	8413	Kan TRP	pGBKT7	NdeI/PstI 7261	pBY1397-Fragment	NdeI/PstI 1152	Ce-C25B8.3 cDNA	Gal4DB myc	N	553 62,37	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1421	8476	Kan TRP	pGBKT7	NdeI/PstI 7261	pBY1398-Fragment	NdeI/PstI 1215	Ce-F57F5.1 cDNA	Gal4DB myc	N	574 64,16	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1422	8621	Kan TRP	pGBKT7	NdeI/SmaI 7280	pBY1399-Fragment	NdeI/MscI 1341	Ce-H22K11.1 cDNA	Gal4DB myc	N	572 63,38	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1423	9445	Amp LEU	pGADT7	SmaI/BamHI 7970	PCR RB1335/1336 Ce-N2 cDNA	blunt/BamHI 1475	Ce-T12E12.1 cDNA	Gal4AD HA	N	660 75,39	Ce-Ariadne 2 in Yeast2Hybrid screening vector
pBY 1424	8734	Kan TRP	pGBKT7	NcoI/BamHI 7283	PCR RB1335/1336 Ce-N2 cDNA	NcoI/BamHI 1469	Ce-T12E12.1 cDNA	Gal4DB myc	N	658 75,98	Ce-Ariadne 2 in Yeast2Hybrid screening vector
pBY 1425	6861	Amp	pPD95.75	SalI/SmaI 4475	PCR RB1021/916 Ce-N2 genomic	XhoI/SmaI 2386	Ce-prk 650bp-Promoter + genomic ORF no stop	intron GFP	C		full-length prk-GFP construct for injection

Name	Size in bp	R/M	Vector	Cloning size in bp	Insert	Cloning size in bp	Description	tags	T	aa, kDa	Notes/Usage
pBY 1440		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk501h4 insert 1313bp	EcoRI/XhoI 1313	Ce-K08E3.7 cDNA				cloning vector
pBY 1441		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk677c9 Insert 1201 bp	EcoRI/XhoI 1301	Ce-K08E3.7 cDNA				cloning vector
pBY 1442		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk269h2 Insert 1392 bp	EcoRI/XhoI 1392	Ce-K08E3.7 cDNA				cloning vector
pBY 1443		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk97d10 Insert 1305 bp	EcoRI/XhoI 1305	Ce-K08E3.7 cDNA				cloning vector
pBY 1444		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk191a6 Insert 1439 bp	EcoRI/XhoI 1439	Ce-dur135 cDNA				cloning vector
pBY 1445		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk253f12 Insert 1313 bp	EcoRI/XhoI 1313	Ce-dur135 cDNA				cloning vector
pBY 1446		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk146g9 Insert 1213 bp	EcoRI/XhoI 1213	Ce-dur135 cDNA				cloning vector
pBY 1447		Amp	pBluescript SK II	EcoRI/XhoI	Ce-yk102c7 Insert 1224 bp	EcoRI/XhoI 1224	Ce-dur135 cDNA				cloning vector
pBY 1456	5500	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1243-Fragment HA-R01H2.6	NcoI/XhoI 539	HA-tagged Ce-R01H2.6 cDNA-ORF	HA	N	395 45,97	bacterial expression vector: GST-HA-R01H2.6
pBY 1457	5481	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1244-Fragment HA-M7.1	NcoI/XhoI 520	HA-tagged Ce-M7.1 cDNA-ORF	HA	N	389 45,02	bacterial expression vector: GST-HA-M7.1
pBY 1458	8848	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1420/1421 F49C12.9 cDNA	NdeI/XhoI 920	Ce-F49C12.9 cDNA-ORF	Gal4AD HA	N	465 52,39	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1459	8518	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1422/1423 F52C6.2 cDNA	NdeI/XhoI 590	Ce-F52C6.2 cDNA-ORF (+85 bp)	Gal4AD HA	N	355 39,25	Yeast-2-Hybrid vector (85bp länger) to test interaction with Ce-Parkin
pBY 1460	9124	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1424/1425 C44B12.5 cDNA	NdeI/XhoI 1196	Ce-C44B12.5 cDNA-ORF	Gal4AD HA	N	557 60,28	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1461	9294	Amp LEU	pGADT7	NdeI/SmaI 7958	PCR RB1426/1427 F29G9.5 cDNA	NdeI/blunt 1336	Ce-F29G9.5 cDNA-ORF	Gal4AD HA	N	603 67,38	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1462	8632	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1428/1429 F28D1.5 cDNA	NdeI/XhoI 704	Ce-F28D1.5 cDNA-ORF	Gal4AD HA	N	393 42,10	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1465	6052	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1395-Fragment HA-F44C4.3	NcoI/XhoI 1091	Ce-F44C4.3 cDNA-ORF	GST HA	N	577 64,81	bacterial expression vector: GST-HA-F44C4.3
pBY 1466	6055	Amp	pGST-parallel 3	BamHI/XhoI 4967	pBY1396-Fragment HA-T03E6.7	BglII/XhoI 1088	HA-tagged Ce-T03E6.7 cDNA-ORF	GST HA	N	593 67,95	bacterial expression vector: GST-HA-T03E6.7
pBY 1467		Amp	pGST-parallel 3	BamHI/	pBY1397-Fragment HA-C25B8.3	BglII/	Ce-C25B8.3 cDNA-ORF	GST HA	N	642 72,91	bacterial expression vector: GST-HA-C25B8.3
pBY 1468	6202	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1398-Fragment HA-F57F5.1	NcoI/XhoI 1241	Ce-F57F5.1 cDNA-ORF	GST HA	N	642 72,51	bacterial expression vector: GST-HA-F57F5.1
pBY 1469	6393	Amp	pGST-parallel 3	NcoI/StuI 5016	pBY1399-Fragment HA-H22K11.1	NcoI/MscI 1377	Ce-H22K11.1 cDNA-ORF	GST HA	N	640 71,73	bacterial expression vector: GST-HA-H22K11.1
pBY 1470	8431	Amp LEU	pGADT7	NdeI/XhoI 7928	pBY1414-Fragment prk-KO3-cDNA	NdeI/SalI 503	Ce-parkin-KO3 cDNA-ORF	Gal4AD HA	N	326 37,00	Yeast-2-Hybrid vector to test with prk-interactors
pBY 1484	8191	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1458-Fragment F49C12.9 cDNA	NdeI/XhoI 920	Ce-F49C12.9 cDNA	Gal4DB myc	N	479 54,70	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1485	7861	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1459-Fragment F52C6.2 cDNA	NdeI/XhoI 590	Ce-F52C6.2 cDNA	Gal4DB myc	N	369 41,57	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1486	8467	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1460-Fragment C44B12.5 cDNA	NdeI/XhoI 1196	Ce-C44B12.5 cDNA	Gal4DB myc	N	571 62,60	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1487	8616	Kan TRP	pGBKT7	NdeI/SmaI 7280	pBY1461-Fragment F29G9.5 cDNA	NdeI/SmaI 1336	Ce-F29G9.5 cDNA	Gal4DB myc	N	617 69,69	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1488	7975	Kan TRP	pGBKT7	NdeI/SalI 7271	pBY1462-Fragment F28D1.5 cDNA	NdeI/XhoI 740	Ce-F28D1.5 cDNA	Gal4DB myc	N	407 44,42	Yeast-2-Hybrid vector to test interaction with Ce-Parkin
pBY 1489	5917	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1458-Fragment F49C12.9 cDNA	NcoI/XhoI 956	HA-tagged Ce-F49C12.9 cDNA	GST HA	N	547 63,05	bacterial expression vector: GST-HA-F49C12.9
pBY 1490	5587	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1459-Fragment F52C6.2 cDNA	NcoI/XhoI 626	HA-tagged Ce-F52C6.2 cDNA	GST HA	N	437 49,92	bacterial expression vector: GST-HA-F52C6.2
pBY 1491	6193	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1460-Fragment C44B12.5 cDNA	NcoI/XhoI 1232	HA-tagged Ce-C44B12.5 cDNA	GST HA	N	639 70,94	bacterial expression vector: GST-HA-C44B12.5
pBY 1492	6388	Amp	pGST-parallel 3	NcoI/StuI 5016	pBY1461-Fragment F29G9.5 cDNA	NcoI/SmaI 1372	HA-tagged Ce-F29G9.5 cDNA	GST HA	N	685 78,04	bacterial expression vector: GST-HA-F29G9.5
pBY 1493	5701	Amp	pGST-parallel 3	NcoI/XhoI 4961	pBY1462-Fragment F28D1.5 cDNA	NcoI/XhoI 740	HA-tagged Ce-F28D1.5 cDNA	GST HA	N	475 52,76	bacterial expression vector: GST-HA-F28D1.5
pBY 1494	4869	Amp	pCite-4a(+)	NcoI/NotI 3657	Fragment myc-Ce-parkin	NcoI/NotI 1212	myc-tagged Ce-parkin cDNA-ORF	myc	N	434 49,50	in vitro-translation vector: myc-PARKIN
pBY 1495	5029	Amp	pBY 1456	NdeI/SmaI 5027		Klenow Fill-in + 2 bp		GST HA	N	259 30,27	bacterial expression vector: GST-HA (control)
pBY 1496	5836	Amp	pBY 1456	NdeI/XhoI 4997	PCR RB1671/1672 D1022.1-cDNA	NdeI/XhoI 839	S.cerevisiae Ubc6 homolog I (41%/60%)	GST HA	N	520 59,56	bacterial expression vector: GST-HA-D1022.1
pBY 1497	5656	Amp	pBY 1456	NdeI/XhoI 4997	PCR RB1673/1674 Y110A2AR.2-cDNA	NdeI/XhoI 659	S.cerevisiae Ubc6 homolog II (52%/68%)	GST HA	N	460 52,86	bacterial expression vector: GST-HA-Y110A2AR.2
pBY 1498	5512	Amp	pBY 1456	NdeI/XhoI 4997	PCR RB1675/1676 Y87G2A.9-cDNA	NdeI/XhoI 515	S.cerevisiae Ubc7 homolog I (58%/75%)	GST HA	N	412 47,37	bacterial expression vector: GST-HA-Y87G2A.9
pBY 1499	5494	Amp	pBY 1456	NdeI/XhoI 4997	PCR RB1677/1678 F58A4.10-cDNA	NdeI/XhoI 497	S.cerevisiae Ubc7 homolog II (51%/66%)	GST HA	N	406 47,25	bacterial expression vector: GST-HA-F58A4.10
pBY 1500	16668	Amp	pCRScript Amp SK +	SrfI 2961	Ce-K08E3 Fragment	EcoRV 13707					Cosmid subclone contains genes K08E3.7 and K08E3.8
pBY 1524	8767	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1671/1672 D1022.1-cDNA	NdeI/XhoI 839	S.cerevisiae Ubc6 homolog I (41%/60%)	Gal4AD HA	N	438 48,90	Yeast-2-Hybrid vector UBC-6 to test interaction with Ce-Parkin
pBY 1525	8587	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1673/1674 Y110A2AR.2-cDNA	NdeI/XhoI 659	S.cerevisiae Ubc6 homolog II (52%/68%)	Gal4AD HA	N	378 42,20	Yeast-2-Hybrid vector UBC-15 to test interaction with Ce-Parkin
pBY 1526	8443	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1675/1676 Y87G2A.9-cDNA	NdeI/XhoI 515	S.cerevisiae Ubc7 homolog I (58%/75%)	Gal4AD HA	N	330 36,71	Yeast-2-Hybrid vector UBC-14 to test interaction with Ce-Parkin
pBY 1527	8425	Amp LEU	pGADT7	NdeI/XhoI 7928	PCR RB1677/1678 F58A4.10-cDNA	NdeI/XhoI 497	S.cerevisiae Ubc7 homolog II (51%/66%)	Gal4AD HA	N	324 36,59	Yeast-2-Hybrid vector UBC-7 to test interaction with Ce-Parkin
pBY 1528	8110	Kan TRP	pGBKT7	NdeI/SalI 7271	PCR RB1671/1672 D1022.1-cDNA	NdeI/XhoI 839	S.cerevisiae Ubc6 homolog I (41%/60%)	Gal4DB myc	N	452 51,22	Yeast-2-Hybrid vector UBC-6 to test interaction with Ce-Parkin
pBY 1529	7930	Kan TRP	pGBKT7	NdeI/SalI 7271	PCR RB1673/1674 Y110A2AR.2-cDNA	NdeI/XhoI 659	S.cerevisiae Ubc6 homolog II (52%/68%)	Gal4DB myc	N	392 44,51	Yeast-2-Hybrid vector UBC-15 to test interaction with Ce-Parkin
pBY 1530	7786	Kan TRP	pGBKT7	NdeI/SalI 7271	PCR RB1675/1676 Y87G2A.9-cDNA	NdeI/XhoI 515	S.cerevisiae Ubc7 homolog I (58%/75%)	Gal4DB myc	N	344 39,03	Yeast-2-Hybrid vector UBC-14 to test interaction with Ce-Parkin
pBY 1531	7768	Kan TRP	pGBKT7	NdeI/SalI 7271	PCR RB1677/1678 F58A4.10-cDNA	NdeI/XhoI 497	S.cerevisiae Ubc7 homolog II (51%/66%)	Gal4DB myc	N	338 38,91	Yeast-2-Hybrid vector UBC-7 to test interaction with Ce-Parkin

Name	Size in bp	R/M	Vector	Cloning size in bp	Insert	Cloning size in bp	Description	tags	T	aa, kDa	Notes/Usage
pBY 1532	8933	Kan TRP	pGBKT7	NdeI/SmaI 7280	pBY1233-Fragment prk-KO1 short Fragment	NdeI/HindIII 319 HindIII/SspI 1334	prk-KO1 ORF A from short transcript	Gal4DB myc	N	723 80,48	
pBY 1533	9611	Amp LEU	pGADT7	NdeI/SmaI 7958	pBY1233-Fragment prk-KO1 short Fragment	NdeI/HindIII 319 HindIII/SspI 1334	prk-KO1 ORF A from short transcript	Gal4AD HA	N	709 78,17	
pBY 1534	7693	Kan TRP	pGBKT7	NdeI/NcoI 7298	pBY1233-Fragment prk-KO1 long Fragment	NdeI/HindIII 319 HindIII/BspHI 76	prk-KO1 ORF B from long transcript	Gal4DB myc	N	322 36,75	
pBY 1535	8350	Amp LEU	pGADT7	NdeI/XhoI 7928	pBY1534-Fragment	NdeI/SalI 422	prk-KO1 ORF B from long transcript	Gal4AD HA	N	309 34,66	
pBY 1569	15534	Amp	pBY1500	StuI/Bsp1407 I 14404	RB853/1856 parkin-KO3 genomic	StuI/Bsp1407I 498					parkin-KO3 (lg103) Cosmid subclone contains genes K08E3.7 and K08E3.8
pBY 1644		Amp	pACUW51-GSTmycP A	BglII/NotI	RB1414/1415 parkin-KO3 cDNA	BamHI/NotI 516					Baculovirus expression vector GST-myc-PARKIN KO3
pBY 1645	4197	Amp	pCite-4a(+)	NcoI/NotI 3657	pBY 1644-Fragment myc-parkinKO3 cDNA	NcoI/NotI 540					in vitro translation vector myc-PARKIN KO3
pBY 1772		Amp	yk484g5	EcoRI/XhoI	Ce-K08E3.8 cDNA	Insert:					
pBY 1773		Amp	yk620a8	EcoRI/XhoI	Ce-K08E3.8 cDNA		fulllength				~ 300 bp not sequenced
pBY 1774		Amp	yk600e8	EcoRI/XhoI	Ce-K08E3.8 cDNA		fulllength				~ 400 bp not sequenced
pBY 1775		Amp	yk420h7	EcoRI/XhoI	Ce-C17H11.6 cDNA						
pBY 1776		Amp	yk543d6	EcoRI/XhoI	Ce-C17H11.6 cDNA						
pBY 1777		Amp	yk81a2	EcoRI/XhoI	Ce-C17H11.6 cDNA		fulllength				~ 2000 bp not sequenced
pBY 1778		Amp	yk465b7	EcoRI/XhoI	Ce-F29G9.5 cDNA		first 24 bp missing				~ 300 bp not sequenced
pBY 1779		Amp	yk450d11	EcoRI/XhoI	Ce-F29G9.5 cDNA		first 9 bp missing				~ 300 bp not sequenced
pBY 1780		Amp	yk162f5	EcoRI/XhoI	Ce-F49C12.9 cDNA		fulllength				
pBY 1781		Amp	yk63a6	EcoRI/XhoI	Ce-F49C12.9 cDNA		fulllength				
pBY 1782		Amp	yk102b5	EcoRI/XhoI	Ce-C44B12.5 cDNA		first 192 bp missing				
pBY 1783		Amp	yk93d2	EcoRI/XhoI	Ce-C44B12.5 cDNA		first 191 bp missing				
pBY 1784		Amp	yk31b9	EcoRI/XhoI	Ce-C39D10.7 cDNA		fulllength contains Intron?!				~ 2400 bp not sequenced
pBY 1785		Amp	yk73c11	EcoRI/XhoI	Ce-T24D1.3 cDNA		fulllength				~ 120 bp not sequenced
pBY 1786		Amp	yk575c10	EcoRI/XhoI	Ce-F56C6.2 cDNA		first 329 bp missing				
pBY 1787		Amp	yk94g9	EcoRI/XhoI	Ce-F28D1.5 cDNA		first 195 bp missing				
pBY 1788		Amp	yk342h3	EcoRI/XhoI	Ce-T01G1.3 cDNA						
pBY 1789		Amp	yk150g2	EcoRI/XhoI	Ce-F57F5.1 cDNA		first 124bp missing				
pBY 1790		Amp	yk538d4	EcoRI/XhoI	xbp1 cDNA						clone into pPD129.36 for xbp-1 RNAi
pBY 1791		Amp	yk169h3	EcoRI/XhoI	xbp1 cDNA						clone into pPD129.36 for xbp-1 RNAi
pBY 1792	22382	Amp	pBY1500	SalI/Bsp120I 16653	pBY232-Frg	SalI/NotI 5729	unc-119 Rescue Fragment				
pBY 1793	21248	Amp	pBY1569	SalI/Bsp120I 15519	pBY232-Frg	SalI/NotI 5729	unc-119 Rescue Fragment				
pBY 1794	14529	Amp	pPD117.01	SalI/StuI 2324	pBY1500 Fragment PCR RB1021/1022 GFP aus pPD117.01 PCR RB838/1856	BsrGI/MscI 8435 XhoI/NotI 660 NotI/NheI 944 NheI/BsrGI 2162					N-terminal gfp::pdr-1 translational fusion
pBY 1795	4770	Amp	pBY1494	NdeI/XhoI 3568	HA-tag RB1741/1742 pBY1233-Fragment	NdeI/NcoI: 33 NcoI/SalI: 1169	HA-tagged Ce-parkin ORF	HA	N	404 46,18	in vitro-translation vector: HA-PARKIN
pBY 1802	9276	Amp LEU	pGADT7	EcoRI/XhoI 7948	RB2147/1419	EcoRI/SalI 1328	K08E3.8 cDNA-ORF				
pBY 1803	8616	Kan TRP	pGBKT7	EcoRI/SalI 8616	RB2147/1419	EcoRI/SalI 1328	K08E3.8 cDNA-ORF				
pBY 1908	16672		pBY1500	Acc65I	Mutagenesis	4bp insertion GTAC	after 191 bp of K08E3.8 shift after aa1-63				Rescue construct pdr-1 frameshift mutation in K08E3.8
pBY 1909			pPD117.01	4400	RB1021/1022	660	genomic pdr-1 promoter short	GFP			pdr-1 promoter contruct Ppdr-1
pBY 1910	4867	Amp	pBY1909	NotI/EcoRI 3462	RB2289/2290 H.s.parkin ORF	NotI/EcoRI 1405	Human parkin cDNA				Rescue contruct Ppdr-1::human parkin (short promoter)
pBY 1911	6341	Amp	pBY1456	BamHI/NotI	RB2196/2197	BclI/NotI	K08E3.8 ORF	GST-HA	N		bacterial expression vector: GST-HA-K08E3.8
pBY 1912	6741	Amp	pET21(a)+	BamHI/NotI		BclI/NotI	K08E3.8 ORF	6xHIS	N		bacterial expression vector: 6xHIS-K08E3.8

Table 12. List of Constructed Plasmids.

6.4 Oligonucleotides

Oligonucleotides for sequencing or PCR were purchased at a desalted grade from Metabion GmbH (Martinsried) or HPLC-purified from Thermo Electron Corporation (Ulm).

Oligonucleotides used in this study

Name	Sequence 5' → 3'	Restriction sites	Description
RB639	ACG TGG ATC CGG TTT AAT TAC CCA AGT TTG AG		SL1 specific primer
RB640	ACGTGGATCCGGTTTTAACCCAGTTACTCAAG		SL2 specific primer
RB839-parkin-rev	CCCCCCGGGTTAAATATTAAACCAATGGTCCCATTGACACTC	SspI, SmaI	pdr-1 cloning primer SspI before, SmaI after stop
RB 915-Park-rev	CGCCCGGGAGCTCGTCTACCTATTAAACCAATGGTCCCATTGACACTC	SacI, SmaI	pdr-1 cloning primer, contains stop
RB 916-Park-rev3	CGCCCGGGAGCTCGTCGACCTATTAAACCAATGGTCCCATTGACACTC	Sal I, SacI, SmaI	pdr-1 cloning primer, without stop
RB1021-Park-Prom-for	GGGCTGCAGGATCCTCGAGCGAACATTGCCAGCTTCTTGCGAGC	PstI, XhoI, BamHI	5´Parkin-Promoter Primer
RB1022-Park-Prom-rev	GCGGCCGCCCCGGGCTTCATGAGAGCTGAAAATTTGAC	SmaI, NotI	3´Primer Parkin-Promoter
RB1033-h/m-Park-for	GCGCCCGGGCATGATAGTGTTTGTCAGGTTCAACTCCAGC	SmaI	human/mouse parkin cloning primer
RB1034-hPark-rev	CCCGTCGACACGTCGAACCAGTGGTCCCC	SalI	human parkin cloning primer
RB1035-mPark-rev	CCCGTCGACACGTCAAACCAGTGATCTCCCATGC	SalI	mouse parkin cloning primer
RB1036-hPark-5´for	GGAGGATTACCCAGGAGACCG		5´upstream forward Primer for nested PCR
RB1037-hPark-3´rev	GGTAGACACTGGGTATGCTCCC		3´downstream reverse Primer for nested PCR
RB1038-mPark-5´for	CGTAGGTCCTTCTCGACCCGC		5´upstream forward Primer for nested PCR
RB1039-mPark-3´rev	CCTTGAGGTTGTGCGTCCAGGG		3´downstream reverse Primer for nested PCR
RB1048Dros-Park-for	GCGCCCGGGCATGCTGGAGCTGTTGCAATTTGGAGGG		5`-forward Primer for Fly Parkin-cDNA
RB1049Dros-Park-rev	GGCGTCGACCCGAACCAGTGGGCTCCCATGC		3`-reverse Primer for Drosophila Parkin-cDNA
RB1054-pal1-for	GGGACCCATGGATCCCATGTCGGTCGATGTCAAGTCGG	SmaI, NcoI, BamHI	pal1-cDNA-forward Primer
RB1055-pal1-rev	GGGAGCTCCCTCGAGCCGAATCTTCTGTTTGTCACG	SmaI, NcoI, BamHI	pal1-cDNA-reverse Primer
RB1056-D.m.Park-in-for	GGACTTGCAGCTGGAAAGCG		internal Primer for Drosophila Parkin-cDNA
RB1057-humPark-in-for	CCAGGGTCCATCTTGCTGGG		internal Primer for Homo sapiens Parkin-cDNA
RB1064-Park-RING-for	GGGGATGACCGAGTGCTGTGTTTGTGATGG		cloning of RING box domain forward primer
RB1065-Park-delUB-for	GGGGATGACAGATTCTTCAATTCTCGGAAGC		cloning of del UBL domain construct
RB1070-Park-3ÚTR-rev	GGGACTAGTGGGCCCGTACGGTATGAAATGAGCGAAATACTCAGC	BsiWI, ApaI, SpeI	Cloning of Parkin 3´UTR
RB1087-T7-Term	GCTAGTTATTGCTCAGCGG		T7-Terminator-Primer
RB1088-pGBKT7-3`Seq	GGAATTAGCTTGGCTGCAAGCG		3´-reverse Sequencing primer for pGBKT7
RB1091-Park-for	GGGGCATATGTCTGATGAAATCTCTATATTAATACAAG	SmaI, NdeI	Parkin 5´forward Primer contains ATG
RB1105-Park-C.brigg-rev1	GACTGGCAGCTGATATGATTACATCC		C.briggsae pdr-1 cloning primer
RB1106-Park-C.brigg-for1	GGATTCACGGTCTCCTGTGTTTATCC		C.briggsae pdr-1 cloning primer
RB1138-Cb-Park-rev2	GGGCTGCAGGAGTCAGCGACAAATCC	PstI, SmaI	C.briggsae pdr-1 cloning primer
RB1139-Cb-Park-for2	GGGCGATCCGCGAGTCAACTTCGGTGTTGG	BamHI	C.briggsae pdr-1 cloning primer
RB1143-R01H2.6-for	GGGCCATATGTCAGCGACACGGCGTCTTCAG	NdeI	Cloning of R01H2.6 ORF
RB1144-R01H2.6-rev	GGGAATTCGAGCTCAGGCCGCTTTTCGGCG	SacI, EcoRI	Cloning of R01H2.6 ORF
RB1145-M7.1-for	GGGCCATATGGCTCTCAAAAGAATCCAGAAG	NdeI	Cloning of M7.1 ORF
RB1146-M7.1-rev	GGGAATTCGAGCTCATAGCGTACTTTTGCGTCC	SacI, EcoRI	Cloning of M7.1 ORF
RB1147-Cb-Park-rev3	GAGAGAACCGAGGAAGCACTCGC		C.briggsae pdr-1 cloning primer
RB1148-Cb-Park-for3	CTTTCCATAGCTTCCGGAGACGC		C.briggsae pdr-1 cloning primer

Name	Sequence 5' → 3'	Restriction sites	Description
RB1149-Cb-Park-rev4	GGTACACCCAGGAGTTACCACG		C.briggsae pdr-1 cloning primer
RB1150-Cb-Park-for4	CGATGGTGAAACTGCTCCATCG		C.briggsae pdr-1 cloning primer
RB1151-Cb-Park-for5	CTAGCTACACGTGGCATATCCTAACC		C.briggsae pdr-1 cloning primer
RB1152-Park-for	GGGGCCCATGGCTATGTCTGATGAAATCTCTATATTAATACAAG		pdr-1 forward cloning primer, contains ATG
RB1153-Park(Stop)-rev	GGGACTAGTCGACTTAATTAAACCAATGGTCCCATTGACACTC	SalI, SpeI	pdr-1 forward cloning primer, contains stop
RB1154-Park-Ubi-rev	GGGTCGACTACGTTGCAGGTGTCAGTGAC		pdr-1 reverse primer cloning of UBL domain
RB1160-Cb-Parkin-ATG-for	GGGGCCCATGGCTATGTCAAATGAAGTCACAGTTGTTTTACAGG		C.briggsae Parkin ATG-Start-Primer
RB1166-R01H2.6-rev2	GGGAATTCTATTCAGGCCGCTTTTCGGCG		Cloning of R01H2.6 ORF
RB1167-M7.1-rev2	GGGAATTCACATAGCGTACTTTTGCGTCC		Cloning of M7.1 ORF
RB1168-H.s.-Park-rev2	GGGGTCGACTACACGTCGAACCAGTGGTCCC		human parkin cloning primer
RB1169-pal1-rev2	GGGCTCGAGTTATAGCCGAATCTTCTGTTTGTC		pal1-cDNA-reverse Primer
RB1170-Park-Ubi-rev2	GGGGTCGACTACGTTGCAGGTGTCAGTGAC		pdr-1 reverse primer cloning of UBL domain
RB1180-pGADT7-rev	AGATGGTGCACGATGCACAG		3' reverse sequencing primer pGADT7
RB1193-M13-rev	GGAAACAGCTATGACCATG		M13 reverse sequencing primer
RB1194-K08E3.8-rev	GGGAATTCTTACTCCATCATTTCGACGTCATC		K08E3.8 reverse cloning primer, stop
RB1195-gIII2382-rev	GGGAATTCAATTGAATGCTCGACGAACG		gIII2382 reverse cloning primer, stop
RB1204-T7long	GTAATACGACTCACTATAGGGC		T7 Promoter Primer
RB1205-T3	AATTAACCCTCACTAAAGGG		T3 Promoter Primer
RB1233-Park-for	CCGGTACCCCCGGGATGTCTGATGAAATCTCTATATTAATACAAG	SmaI, KpnI	cloning of Parkin cDNA, contains ATG
RB1234-myc-for	CTGAGCAGAAGCTGATCTCAGAGGAGGACCTA	5' SacI overhang	together with RB1235-myc-rev a c-Myc
RB1235-myc-rev	CCGGTAGGTCCTCCTCTGAGATCAGCTTCTGCTCAGAGCT	3' AgeI overhang	together with RB1234-myc-for a c-Myc
RB1236-GAL4DB-for	GGGACCGGTGATGAAGCTACTGTCTTCTATCG	AgeI	cloning of Gal4DB, after RB1234/1235 myc
RB1237-GAL4DB-rev	GGGGATCCGATACAGTCAACTGTCTTTGACC	BamHI	cloning of Gal4DB
RB1238-K08E3.8-for	GGGAAGCTTATGTCAGGACAAGGACCTCCACC		K08E3.8 primer
RB1239-C.b.-Park-rev	TGTGGAAGCAGGAATTAAGGCG		C.briggsae pdr-1 cloning primer
RB1251-unc59-cDNA for	GGGGCATATGAGCAGTCGGACTGCAAATAGC		unc-59 cloning primer
RB1252-unc59-cDNA rev	GGGTTAGTTTCGATTAAACAATCCGAGACC		unc-59 cloning primer
RB1253-unc61 cDNA for	GGGCATATGTCCGACATCGAGCATAAGTTAC		unc-61 cloning primer
RB1254-unc61 cDNA rev	GGGTCACTTTCTTAACTTCTTTGACACTTTG		unc-61 cloning primer
RB1818-K08E3.8in-for	CCACAGATGATGCAACAGCAAATGG		K08E3.8 primer
RB1819-K08E3-rev	TTTGCATGGATGTACCACTTGCCGG		K08E3.8 primer
RB1847-K08E3-for	CGCGACTTCTCATTTATATACCACAGAAGCG		K08E3.8 primer
RB1848-K08E3-rev	CGTGTATCTCAAAAACGGTTGGTCCAGC		K08E3.8 primer
RB1856-K08E3-rev	GCCCACTCCAAAACTGAGATATATCCC		K08E3.8 primer
RB1857-act-for	TCACGATCATGAGACCATTCAAA		act-1 cloning primer
RB1858-act-rev	GCAAATTGTAGTGGGGTCTTCTTATG		act-1 cloning primer
RB1859-act1/3-for	ATGTGTGACGACGAGGTTGCCGC		act-1 cloning primer
RB1860-act1/3-rev	TTAGAAGCACTTGCGGTGAACGATGG		act-1 cloning primer
RB1260-C.r.Prk-for1	CAACGAAAGGCAACTGAACGGC		C.remanei pdr-1 cloning primer
RB1261-C.r.Prk-rev1	CAGATACATGTGATTGCATCCG		C.remanei pdr-1 cloning primer
RB1287-C.r.Park-ATG-for	GGGCCATGGCCATGCCGAATGTCGTCACAATACTTCTGC	SmaI, NcoI	C.remanei cloning primer, contains ATG
RB1288-C.r.Park-rev	CAGTCAAGTTCGGTTGCTGTCCC		sequencing primer C.remanei Parkin
RB1300-F44C4.3-for	GGGGCATATGAAATACCTCATTCTTGCTGC	NdeI	F44C4.3 cloning primer
RB1301-F44C4.3-rev	GGGGGTCGACTTAGACTTTTGGGACTCCTCCG	SalI	F44C4.3 cloning primer
RB1302-T03E6.7-for	GGGGCATATGAACCGATTCATTCTTCTGGC	NdeI	T03E6.7 cloning primer
RB1303-T03E6.7-rev	GGGGCTCGAGTTAGACCAATGGATAACTGGCC		T03E6.7 cloning primer

Name	Sequence 5' → 3'	Restriction sites	Description
RB1304-C25B8.3-for	GGGGCATATGAAGACGTTGCTCTTCCTTTCC		C25B8.3 cloning primer
RB1305-C25B8.3-rev	GGGGGTCGACTCAGATGTTGTCATCGTAGACG		C25B8.3 cloning primer
RB1306-F57F5.1-for	GGGGCATATGCCTAATTCTTATCAGCAATATTC		F57F5.1 cloning primer
RB1307-F57F5.1-rev	GGGGCTCGAGTTACAATTTTGGAATTCCTCCG		F57F5.1 cloning primer
RB1308-H22K11.1-for	GGGGCATATGTCGGGCCGCGTTTTCCTTC		H22K11.1 cloning primer
RB1309-H22K11.1-rev	GGGGGTCGACTTATTTTCCGGTTCTAGAGGTG		H22K11.1 cloning primer
RB1311-3'RACE-rev	GGCCACGCGTCGACTAGTGATATCTTTTTTTTTTTTTTTT	NotI, MluI, SalI, SpeI, + EcoRV	3' RACE reverse Primer, hybridizes to polyA-tail
RB1312-5'RACE-for	GGCCACGCGTCGACTAGTGATATCGGGIIGGGIIGGGIIG	NotI, MluI, SalI, SpeI, + EcoRV	5'RACE forward Primer, homopolymeric dCTP tailed
RB1313-RACE-universal	GGCCACGCGTCGACTAGTGATATC	NotI, MluI, SalI, SpeI, + EcoRV	for nested PCR of 3' and 5' RACE products
RB1321-C.r.-Park-for3	AATCAGAACCGCAGAATTGGG		C.remanei pdr-1 cloning primer
RB1322-C.r.-Park-for4	ACGTTGTCCAAGATGTAATGC		C.remanei pdr-1 cloning primer
RB1323-C.r.-Park-rev3	GTAATATTTCGTCGCTGATCC		C.remanei pdr-1 cloning primer
RB1334-C.r.-Parkin-rev4	CCATCATCGTCATATGGTACCC		C.remanei pdr-1 cloning primer
RB1335-T12E11.1-ATG	GGGGCCCATGGACGATGAAGACATGAGCTGC	NcoI, SmaI	T12E12.1 cloning primer
RB1336-T12E11.1-Stop	CGGGATCCTTAAAAAGAAAAAGTCGTGAAGAAGTGTCTGACG	BamHI	T12E12.1 cloning primer
RB1337-KO31-inDel-rev	GAGTGATTCTCGAATCGTTCG		internal deletion primer T12E12.1 KO31
RB1403-hsp16.2-for	ATGTCACTTTACCACTATTTCCG		hsp-16.2 cloning primer
RB1403-hsp16.2-rev	TTATTCAGCAGATTTCTCTTCGACG		hsp-16.2 cloning primer
RB1405-hsp3-for	ATGAAGACCTTATTCTTGTTGGGC		hsp-3 cloning primer
RB1406-hsp3-rev	TTAGAGCTCGTCCTTGTCGTCAG		hsp-3 cloning primer
RB1407-hsp4-for	ATGAAAGTTTTCTCGTTGATTTTGATTGCC		hsp-4 cloning primer
RB1408-hsp4-rev	TTACAGTTCATCATGATCCTCCGATGG		hsp-4 cloning primer
RB1409-hsp70-for	ATGAGTAAGCATAACGCTGTTGG		hsp-70 cloning primer
RB1410-hsp70-rev	TTAGTCGACCTCCTCGATCGTTCC		hsp-70 cloning primer
RB1416-C.b.-prk-for7	AACGAATGCGGTCTCATGGCG		C.briggsae pdr-1 cloning primer
RB1417-C.r.-prk-rev5	GAGACAGAAAGAAGCTCTAGCG		C.remanei pdr-1 cloning primer
RB1418-C.r.-prk-for5	ATGTCAGGGCAGGGACAGCAACCG		ATG-Start Primer for C.remanei K08E3.8
RB1419-K08E3.8-rev	GGGGCCATGGTCGACTCCATCATTTCGACGTCATC	SalI, NcoI	Cloning of K08E3.8-ORF c-terminal
RB1420-F49C12.9-for	GGGGCATATGGTTAAATTGCATTTAAAAACCACC	NdeI	F49C12.9 cloning primer, contains ATG
RB1421-F49C12.9-rev	GGGCTCGAGTTAATTTTGGAGATCAATAAGAAACTCC	XhoI	F49C12.9 cloning primer, contains Stop
RB1422-F52C6.2-for	GGGGCATATGCTGCTCTCCATCAAAACGTCG	NdeI	F52C6.2 cloning primer, contains ATG
RB1423-F52C6.2-rev	GGGGCTCGAGCTAACAAGATCGAGACTTGTGC	XhoI	F52C6.2 cloning primer, contains Stop
RB1424-C44B12.5-for	GGGGCATATGGTGCAAATCTATCGATCATC	NdeI	C44B12.5 cloning primer, contains ATG
RB1425-C44B12.5-rev	GGGGCTCGAGTTAATAAGCATTGGAAGCAGCAACTGG	XhoI	C44B12.5 cloning primer, contains Stop
RB1426-F29G9.5-for	GGGGCATATGGGGCAACAACAGTCAGGTTTCG	NdeI	F29G9.5 cloning primer, contains ATG
RB1427-F29G9.5-rev	GGGTTACAAATAGAGTTCTTCTGGAGC	1/2 SmaI	F29G9.5 cloning primer, contains Stop
RB1428-F28D1.5-for	GGGGCATATGGCCCTTGTCAAGCTCACTC	NdeI	F28D1.5 cloning primer, contains ATG
RB1429-F28D1.5-rev	GGGGCTCGAGTTAACAGAATTGAACAGTGTATGAAGC	XhoI	F28D1.5 cloning primer, contains Stop
RB1430-K08E3.8-intern-for	TTGACGACAGTATTGGAGACAGCG		K08E3.8 internal primer
RB1431 Cr-K08E3.8-for	ATGTCAGGGCAGGGACAGCAACCG		C.remanei K08E3.8 primer
RB1470-K08E3.8-intern-for	CCGAATGAGGAGCAAATTCGAATGG	SalI	K08E3.8-3' part for cloning of fulllength
RB1471-T03E6.7-GFP-for	GGGGGCATGCTACCCAAACACAAGCCATGCTCC	SphI	cloning of T03E6.7-fulllength GFP fusion
RB1472-T03E6.7-GFP-rev	GGGGGCTCGAGACCAATGGATAACTGGCCTTGG	SalI	cloning of T03E6.7-fulllength GFP fusion
RB1473-F44C4.3-GFP-for	GGGGGGCATGCTGCCTTATAGAAGTTGTCTCCATGTC	SphI	cloning of F44C4.3-fulllength-GFP fusion
RB1474-F44C4.3-GFP-rev	GGGGGGTCGAGGGGACTTTTGGGACTCCTCCGACAACG	SalI	cloning of F44C4.3-fulllength-GFP fusion
RB1549-K08E3.8-intern-for	AGTATGACGCCGCAGCAACAAC		K08E3.8 internal primer

Name	Sequence 5' → 3'	Restriction sites	Description
RB1550-K08E3.8-intern-rev	TTCTGTCGTCTTCGCATACTTTCC		K08E3.8 internal primer
RB1616-Cb-K08E3.8-for	ATGTCGGGACAAGGACCGCCATCG		ATG-Primer for C.briggsae K08E3.8
RB1617-SP6-long	CGCCAAGCTATTTAGGTGACACTATAGAA		SP6 promoter primer long
RB1618-Cb-K08E3.8-rev	TCACTCCGCCATTTCAGCGTCTTCG		Stop-rev Primer for C.briggsae K08E3.8
RB1654-pGSTparallel-rev	TTCACCGTCATCACCGAAACGC		sequencing primer pGST-parallel 3 reverse
RB1655-ire1-OF	AAGAAGATGTGACTGGGGGTGAG		ire-1 (v33) KO Primer extern forward
RB1656-ire1-OR	CGAAGAAGATAAAGTGCAACTACAGG		ire-1 (v33) KO Primer extern reverse
RB1657-ire1-IF	GATAGGACGAAGCGAGGAAGAG		ire-1 (v33) KO Primer intern forward
RB1658-ire1-IR	ATATCCATGCGACGACGATGC		ire-1 (v33) KO Primer intern reverse
RB1659-ire1-Del IF	AGATGAGAGCAACATTTCATCTATTCACATTT		ire-1 (v33) KO Primer extern forward
RB1660-ire1-Del IR	GAGGCAGGGCAGATTCTATTCCGCTGACGCTG		ire-1 (v33) KO Primer extern reverse
RB1661-pek1-OF	CCTTGGTACCATTCAACGCT		pek-1 (ok275) KO Primer extern forward
RB1662-pek1-OR	CTGAGCCATCGACAAACTCA		pek-1 (ok275) KO Primer extern reverse
RB1663-pek1-IF	ATCACCGCTACTCTGGATGG		pek-1 (ok275) KO Primer intern forward
RB1664-pek1-IR	CTGAGAAGGCAACGCTCTCT		pek-1 (ok275) KO Primer intern reverse
RB1665-pek1-Del IF	GAGATGAGTGTGTATTATATAGTTTTAGCTGGGTTC		pek-1 (ok275) KO Primer in deletion forward
RB1666-pek1-Del IR	GAGCCGTATCTCCGGGTCCACAAGACTCC		pek-1 (ok275) KO Primer in deletion reverse
RB1671-D1022.1-for	GGGGGGCATATGAGTGAGCAGTACAACACTAAAAATGC	NdeI	D1022.1 cloning primer
RB1672-D1022.1-rev	GGGGCTCGAGTTAAAGGGTATAGTCAAAGTTTGTTGATGCC	XhoI	D1022.1 cloning primer
RB1673-Y110A2AR.2-for	GGGGGGCATATGCTGAATTTGGGTCCCGGC	NdeI	Y110A2AR.2 cloning primer
RB1674-Y110A2AR.2-rev	GGGGCTCGAGTTAATAAGAAAAAACGGGAAAAATCGATG	XhoI	Y110A2AR.2 cloning primer
RB1675-Y87G2A.9-for	GGGGGGCATATGGCTGGTTACGCTTTGAAGCGG	NdeI	Y87G2A.9 cloning primer
RB1676-Y87G2A.9-rev	GGGGCTCGAGTTAGACTTCCGAAGCGGGAAGACAC	XhoI	Y87G2A.9 cloning primer
RB1677-F58A4.10-for	GGGGGGCATATGGAGCAATCCTCCCTACTTCTG	NdeI	F58A4.10 cloning primer
RB1678-F58A4.10-rev	GGGGCTCGAGTCATTCTTCTTGACTTCTGCGAACAC	XhoI	F58A4.10 cloning primer
RB1713-F57F5.1-in-for	GATAAATTGGTCGACTGTCGAATTTCCCAATTCTCCAGCCAATCG		F57F5.1 internal primer
RB1739-Cr-K08E3.8-infor	AACTTGACTGCACAGCAGCAACAG		C.remanei K08E3.8 internal cloning primer
RB1740-Cb-K08E3.8-infor	TCGAACTTAACTCCTCAGCAGCAACAT		C.briggsae K08E3.8 internal cloning primer
RB1741-HA-for	TATGTACCCATACGATGTTCCAGATTACGCTAC		HA forward
RB1742-HA-rev	CATGGTAGCGTAATCTGGTTCATCGTATGGGTACA		HA reverse
RB1778-cln3.3-Ex-for	CTCTCCCACCTCAATTTTCC		KO-Screening-Primer cln3.3 (gk118)
RB1779-cln3.3-Ex-rev	ACCAATTGCTCCACAGGAAC		KO-Screening-Primer cln3.3 (gk118)
RB1780-cln3.3-In-for	AGACACGGCAGTTTGTTGGT		KO-Screening-Primer cln3.3 (gk118)
RB1781-cln3.3-In-rev	GGGATTGATACTCCTGCTGC		KO-Screening-Primer cln3.3 (gk118)
RB1925-T24D1.3-for	GGGGAATTCATGCCATCACCAGCACGCAGGTCT		T24D1.3 cloning primer
RB1926-T24D1.3-rev	GGGCTCGAGTTATTTGGAAGTTATTCGGCGCTT		T24D1.3 cloning primer
RB1947-Cr-K08E3.8-rev	AGCTGCGATCCAACTAATGGAG		C.remanei K08E3.8 cloning primer
RB1948-Cr-K08E3.8-for	TAATGCTCCTGGATCTGTCCCAG		C.remanei K08E3.8 cloning primer
RB1975-pek1-PF5-for	CCAATTGGAGCAATACATAGGAAAC		pek-1(ok275) KO primer
RB1976-pek1-PR2-rev	CTCTTGACAGCGTACTCGTTC		pek-1(ok275) KO primer
RB2022-prk-N-GFP-mut-for	CAAATTTTCAGCTCTCATGAAGCATGGCACCGGTTACGTACTCTGATGAAATCTCTATATTAATAC	mutagenesis primer	
RB2023-prk-N-GFP-mut-rev	GTATTAATATAGAGATTTCATCAGAGTACGTAACCGGTGCCATGCTTCATGAGAGCTGAAAATTTG	mutagenesis primer	
RB2024-prk-C-GFP-mut-for	GTGTCAATGGGACCATTGGTTTAATGCACCGGTTACGTACTAATAACTCTCTAAAATTCATCTTTTC	mutagenesis primer	
RB2025-prk-C-GFP-mut-rev	GAAAAGATGAATTTTAGAGAGTTATTAGTACGTAACCGGTGCATTAAACCAATGGTCCCATTGACAC	mutagenesis primer	
RB2147-K08E3.8-for	GGGGAATTCATGTCAGGACAAGGACCTCCACC	EcoRI	cloning of K08E3.8 Start primer

Name	Sequence 5' → 3'	Restriction sites	Description
RB2172-Cr-K08E3.8-rev	GCAATGGAAGTCTCAATTGAAGC		C.remanei K08E3.8 cloning primer
RB2173-CR-for	TATGGGCCTGGATCTGTGCAACC		C.remanei K08E3.8 cloning primer
RB2174-Cb-K08E3.8-rev	TCACTCCGCCATTTCAGCGTCTTCG		C.briggsae VT847 K08E3.8 cDNA Primer
RB2175-Cr-K08E3.8-rev	TTATTCAATCATTTCGACATCCTCG		C.remanei EM464 K08E3.8 cDNA Primer
RB2176-CR-K08E3.8-for	ACGTCAGAAAAAGTGGGAGGACTTGC		C.remanei EM464 K08E3.8 cDNA Primer
RB2186-Cb-K08E3.8-for	ACCACAACAACCACTTTCTCGTCCTGG		C.briggsae VT847 K08E3.8 cDNA Primer
RB2196-K08E3.8-for	GGGGGGTGATCAATGTCAGGACAAGGACCTCCACC	BclI	K08E3.8 cloning primer
RB2197-K08E3.8-rev	CCCGCGGCCGCCTCCATCATTTCGACGTCATC	NotI	K08E3.8 cloning primer
RB2204-Cr-K08e3.8-for	TAACGAGGATGTCGAAATGATTG		C.remanei K08E3.8 cloning primer
RB2210-CR-K08e3.8-rev	GGACAGATCCAGGAGCATTAAGC		C.remanei K08E3.8 cloning primer
RB2289-Hs-parkin-for	GGGGCGGCCGCATGATAGTGTTTGTCAGGTTCAACTCCACC		H.sapiens parkin cloning primer
RB2290-Hs-parkin-rev	GGGGAATTCCTACACGTCGAACCAGTGGTCCCC		H.sapiens parkin cloning primer
RB2291-KO1.09	GAAAAATGCGTGAAAACCGT		pdr-1 KOs primer extern forward
RB2292-KO1.10	CTGTGCTCCAACTAGAGGGC		pdr-1 KOs primer extern reverse
RB2293-KO1.11	GCACATGACTGCGAGGACTA		pdr-1 KOs primer intern forward
RB2294-KO1.12	GATGCATTTGGAGATGAGCA		pdr-1 KOs primer intern reverse
RB2295-KO31EL	TGGACGATGAAGACATGAGC		Ariadne-2 KO#31extern forward
RB2296-KO31ER	TCCACAGTACGCTACGATGC		Ariadne-2 KO#31extern reverse
RB2297-KO31IL	TGCCGGTTATGGAGATGG		Ariadne-2 KO#31 intern forward
RB2298-KO31IR	GATCCAAAACCGTTTCATGC		Ariadne-2 KO#31 intern reverse
RB2316-atf6-OL	GGCGGGAGTTTAGGAGATTC		atf-6(ok551) KO primer extern forward
RB2317-atf6-OR	AAAGGCACGGAAATTGAGAA		atf-6(ok551) KO primer extern reverse
RB2318-atf6-IL	AATGACCAGGAAATGTGGGA		atf-6(ok551) KO primer intern forward
RB2319-atf6-IR	AAGTGTCAATTGGCCAGTCC		atf-6(ok551) KO primer intern reverse
RB2323-atf6-indel	CTAAGAACTGAGAATGCCGC		atf-6(ok551) KO primer internal deletion
RB2331-rrf3-ex-for	T CGGAAACAGTTGCGAAGACG		rrf-3(pk1426) deletion primer extern forward
RB2332-rrf3-ex-rev	ATCGGAGCTTCATCTGCATC		rrf-3(pk1426) deletion primer extern reverse
RB2333-rrf3-in-for	ATGCTAAGCT CATTGGCAGC		rrf-3(pk1426) deletion primer intern forward
RB2334-rrf3-in-rev	ATCTCCGAGCCCTAGACGAATC		rrf-3(pk1426) deletion primer intern reverse
RB2335-rrf3-indel-for	TCAAGCCACA GAAGAGACTC		rrf-3(pk1426) deletion primer internal
RB2350-HA-SalI-for	GGGGGGTCGACTACCCATACGACGTACCAGATTACGCT	SalI	HA tag
RB2351-K08E3.8-NotI-rev	GGGGGCGGCCGCTTACTCCATCATTTCGACGTCATCAT	NotI	K08E3.8 reverse cloning primer
RB2355-pdr-1 for	GAGTGGTACAAGATGTGCACC		pdr-1 KO primer internal deletion

Table 13. List of Used Primers.

7 Appendix

7.1 DNA Sequences

7.1.1 *C. elegans*

7.1.1.1 *pdr-1*/K08E3.8 Genomic Locus

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LOCUS       pdr-1       12000 bp    DNA
DEFINITION  bp 26714-38713 from cosmid K08E3
SOURCE      N2 var. Bristol
ORGANISM    C. elegans

FEATURES             Location/Qualifiers
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                     /product="210 bp"
     CDS             complement (1627..2379)
                     /gene="K08E3.6 Exon V"
                     /product="753 bp"
     CDS             complement (2567..2938)
                     /gene="K08E3.6 Exon IV"
                     /product="372 bp"
     CDS             complement (2989..3481)
                     /gene="K08E3.6 Exon III"
                     /product="493 bp"
     CDS             complement (3532..3643)
                     /gene="K08E3.6 Exon II"
                     /product="112 bp"
     CDS             complement (3688..3793)
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     CDS             4101..4169
                     /gene="pdr-1 Exon I"
                     /product="69 bp"
     CDS             4173..5304
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     CDS             4215..4360
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     CDS             4889..5368
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     CDS             4930..5020
                     /gene="pdr-1 Exon IV"
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                /gene="pdr-1 Exon V"
                /product="205 bp"
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                /product="75 bp"
CDS             5679..5820
                /gene="pdr-1 Exon VII"
                /product="142 bp"
CDS             6040..6087
                /gene="K08E3.8 Exon I"
                /product="48 bp"
CDS             6526..6633
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                /product="108 bp"
CDS             7129..7338
                /gene="K08E3.8 Exon III"
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                /product="120 bp"

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7.1.1.2 *pdr-1* cDNA ORF

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7.1.1.3 K08E3.8 cDNA ORF

1326 bp

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 301 TCGTCGGTGC CCACTGCCGC AAATCCGACC ACTCCACAGA TGATGCAACA GCAAATGGGC
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1261 ATGCGAAGAC GACAGAAGAA ATGGGAGGAT CAACACAAAA ATGATGACGT CGAAATGATG
1321 GAGTAA
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7.1.2 *C. briggsae*

7.1.2.1 *pdr-1*/K08E3.8 Genomic Locus

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LOCUS       pdr-1       7962 bp    DNA
DEFINITION
SOURCE      VT847
ORGANISM    C. briggsae

FEATURES             Location/Qualifiers
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 121 AAGAGCCACA GATTCATTTC GAGCAGTTTC GATTTGAAGT GAAGCAGTGG ACGTTCCCAG
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7.1.2.2 *pdr-1* cDNA ORF

1158 bp

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7.1.2.3 K08E3.8 cDNA ORF

1461 bp

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1441 GACGCTGAAA TGGCGGAGTG A
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7.1.3 *C. remanei*

7.1.3.1 *pdr-1*/K08E3.8 Genomic Locus

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LOCUS       pdr-1          5589 bp    DNA
DEFINITION
SOURCE      EM464
ORGANISM    C. remanei

FEATURES             Location/Qualifiers
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                /gene="K08E3.8 Exon III"
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CDS             3229..3495
                /gene="K08E3.8 Exon IV"
                /product="267 bp"
CDS             3542..3919
                /gene="K08E3.8 Exon V"
                /product="378 bp"
CDS             3966..4226
                /gene="K08E3.8 Exon VI"
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7.1.3.2 *pdr-1* cDNA ORF

1164 bp

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7.1.3.3 K08E3.8 cDNA ORF

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7.2 Prediction of *pdr-1* Transcription Regulators

The identified sequences immediately 5' of the *pdr-1* translational start from the three nematode species were searched for binding sites of transcription factors using MatInspector (Quandt et al., 1995).

List of Regulatory sites in the *C. elegans pdr-1* Promoter

Family / matrix	Further Information	Opt.	Position from - to	Position anchor	Str.	Core sim.	Matrix sim.	Sequence
V$HOXF/HOX1-3.01	Hox-1.3, vertebrate homeobox protein	0.83	2 - 18	10	(-)	1.000	0.882	aaaacactATTAtttca
V$FKHD/FREAC4.01	Fork head related activator-4 (FOXD1)	0.78	8 - 24	16	(-)	1.000	0.780	gtttagaaAACActatt
V$MEF2/MEF2.05	MEF2	0.96	10 - 32	21	(+)	1.000	0.961	tagtgttttcTAAAcacgtaaac
V$FKHD/FREAC2.01	Fork head related activator-2 (FOXF2)	0.84	14 - 30	22	(+)	1.000	0.878	gttttcTAAAcacgtaa
V$EBOR/XBP1.01	X-box-binding protein 1	0.86	18 - 32	25	(-)	1.000	0.895	gtttACGTgtttaga
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein	0.86	21 - 31	26	(-)	1.000	0.870	tTTACgtgttt
V$FKHD/FREAC2.01	Fork head related activator-2 (FOXF2)	0.84	22 - 38	30	(+)	1.000	0.974	aacacgTAAAcatgaaa
N$CEDS/CES2.01	cell-death specification 2, bZIP factor involved in programmed cell death in C.elegans	0.77	45 - 55	50	(-)	1.000	0.873	cttgcGTAAgt
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein	0.86	46 - 56	51	(+)	1.000	0.870	cTTACgcaagt
V$TTFF/TTF1.01	Thyroid transcription factor-1 (TTF1) binding site	0.92	47 - 61	54	(+)	1.000	0.922	ttacgCAAGtgcttc
V$NKXH/HMX3.01	H6 homeodomain HMX3/Nkx5.1 transcription factor	0.89	49 - 61	55	(+)	1.000	0.891	acgcAAGTgcttc
V$GATA/GATA1.03	GATA-binding factor 1	0.95	71 - 83	77	(-)	1.000	0.952	tgcaGATAtgaga
V$OCT1/OCT1.02	Octamer-binding factor 1	0.82	73 - 87	80	(-)	1.000	0.853	tgaATGCagatatga
V$STAT/STAT6.01	STAT6: signal transducer and activator of transcription 6	0.84	96 - 114	105	(+)	1.000	0.937	ttggaTTCCtcggaatctt
V$BCL6/BCL6.01	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma	0.76	98 - 114	106	(+)	1.000	0.791	ggaTTCCtcggaatctt
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	128 - 138	133	(+)	1.000	0.965	gagACGCtcaa
V$PAX5/PAX5.02	B-cell-specific activating protein	0.75	121 - 149	135	(-)	1.000	0.750	gattgatgagattgAGCGtctccgcaagc
V$HOXF/HOXA9.01	Member of the vertebrate HOX - cluster of homeobox factors	0.87	136 - 152	144	(-)	1.000	0.922	tttGATTgatgagattg
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site	0.79	139 - 151	145	(-)	1.000	0.830	tTGATtgatgaga
V$PBXC/PBX1_MEIS1.02	Binding site for a Pbx1/Meis1 heterodimer	0.77	138 - 154	146	(-)	1.000	0.774	cattTGATtgatgagat
V$PBXF/PBX1.01	Homeo domain factor Pbx-1	0.78	141 - 153	147	(+)	1.000	0.997	tcatCAATcaaat
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	151 - 163	157	(-)	1.000	0.983	ggaatGTTTcatt
V$PAX2/PAX2.01	Zebrafish PAX2 paired domain protein	0.78	149 - 171	160	(+)	1.000	0.805	caaatgaaacattcctgAAACac
V$TEAF/TEF1.01	TEF-1 related muscle factor	0.84	156 - 168	162	(+)	1.000	0.922	aaCATTcctgaaa
V$STAT/STAT.01	Signal transducers and activators of transcription	0.87	155 - 173	164	(-)	1.000	0.911	acgtgtttcaGGAAtgttt
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	162 - 174	168	(-)	1.000	0.981	gacgtGTTTcagg
V$EBOR/XBP1.01	X-box-binding protein 1	0.86	163 - 177	170	(-)	1.000	0.943	aatgACGTgtttcag
V$CREB/ATF6.02	Activating transcription factor 6, member of b-zip family, induced by ER stress	0.85	161 - 181	171	(-)	1.000	0.977	ttttaatGACGtgtttcagga
V$PDX1/ISL1.01	Pancreatic and intestinal lim-homeodomain factor	0.82	167 - 187	177	(-)	1.000	0.876	ttcaaatttTAATgacgtgtt
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	179 - 191	185	(+)	1.000	0.926	aaatTTGAaaatg
N$CSKN/SKN1.02	maternal gene product, similar to bZIP proteins	0.99	185 - 197	191	(-)	1.000	0.993	aattATCAttttc
V$GATA/GATA2.01	GATA-binding factor 2	0.92	187 - 199	193	(+)	1.000	0.923	aaatGATAattga
V$OCT1/OCT1.06	Octamer-binding factor 1	0.80	186 - 200	193	(+)	1.000	0.900	aaaatgatAATTgaa
V$HOMS/S8.01	Binding site for S8 type homeodomains	0.97	192 - 200	196	(-)	1.000	0.997	ttcaATTAt
V$NKXH/DLX3.01	Distal-less 3 homeodomain transcription factor	0.91	190 - 202	196	(+)	1.000	0.958	tgaTAATtgaaac

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
V$IRFF/ISRE.01	Interferon-stimulated response element	0.81	190 - 204	197	(+)	1.000	0.879	tgataattGAAActa
V$CART/CART1.01	Cart-1 (cartilage homeoprotein 1)	0.84	191 - 207	199	(+)	1.000	0.856	gaTAATtgaaactaacc
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)	0.79	195 - 211	203	(-)	1.000	0.831	tataggTTAGtttcaat
I$DHOM/FTZ.01	fushi tarazu, involved in body segmentation of the drosophila embryo	0.81	213 - 225	219	(-)	1.000	0.837	ttcgatATTAagg
V$NKXH/NKX25.02	Homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites	0.88	213 - 225	219	(+)	1.000	0.884	cctTAATatcgaa
V$HOXF/PTX1.01	Pituitary Homeobox 1 (Ptx1)	0.79	237 - 253	245	(+)	1.000	0.820	gtgagtTTAGaatcatg
I$DHSF/HSF.03	heat shock factor (Drosophila)	0.75	245 - 265	255	(+)	1.000	0.772	AGAAtcatgttgaaaatgtga
V$CREB/CREB.04	cAMP-response element binding protein	0.87	257 - 277	267	(+)	1.000	0.911	aaaatgTGACgcgagttttcg
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	262 - 272	267	(+)	1.000	0.962	gtgACGCgagt
I$DDVL/DL.02	Dorsal, protein for dorso-ventral axis formation, homologous to vertebrate c-rel	0.91	269 - 279	274	(+)	1.000	0.925	gagtTTTCgcc
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	269 - 283	276	(-)	1.000	0.763	gtgcggcGAAAactc
V$HOXF/CRX.01	Cone-rod homeobox-containing transcription factor / otx-like homeobox gene	0.94	325 - 341	333	(+)	1.000	0.955	aaatgATTAgaaaacga
V$IRFF/ISRE.01	Interferon-stimulated response element	0.81	334 - 348	341	(+)	1.000	0.867	gaaaacgaGAAActc
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	346 - 360	353	(+)	1.000	0.849	ctcgcccGAAAataa
V$EVI1/EVI1.02	Ecotropic viral integration site 1 encoded factor	0.83	350 - 366	358	(+)	1.000	0.837	cccgaaaatAAGAgaaa
I$DDVL/DL.02	Dorsal, protein for dorso-ventral axis formation, homologous to vertebrate c-rel	0.91	360 - 370	365	(-)	1.000	0.943	cattTTTCtct
V$PAX6/PAX6.01	Pax-6 paired domain binding site	0.75	360 - 378	369	(-)	1.000	0.834	ttttcACGCatttttctct
V$AHRR/AHRARNT.02	Aryl hydrocarbon / Arnt heterodimers, fixed core	0.77	360 - 382	371	(+)	1.000	0.779	agagaaaaatGCGTgaaaaccgt
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	367 - 381	374	(+)	1.000	0.909	aatgcgtGAAAaccg
I$DDVL/DL.02	Dorsal, protein for dorso-ventral axis formation, homologous to vertebrate c-rel	0.91	371 - 381	376	(-)	1.000	0.959	cggtTTTCacg
V$VMYB/VMYB.02	v-Myb	0.90	376 - 386	381	(-)	1.000	0.927	tgaAACGgttt
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	376 - 388	382	(+)	1.000	0.985	aaaccGTTTcaaa
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	379 - 391	385	(-)	1.000	0.956	aaatTTGAacgg
V$IRFF/IRF2.01	Interferon regulatory factor 2	0.80	379 - 393	386	(-)	1.000	0.818	cgaaatttGAAAcgg
I$DSTA/STAT.01	signal transducers and activators of transcription	0.82	387 - 401	394	(-)	1.000	0.963	gttttccacGAAAtt
V$STAT/STAT.01	Signal transducers and activators of transcription	0.87	385 - 403	394	(+)	1.000	0.922	caaatttcgtGGAAaacag
V$NFAT/NFAT.01	Nuclear factor of activated T-cells	0.97	392 - 402	397	(+)	1.000	0.971	cgtgGAAAaca
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	407 - 419	413	(+)	1.000	0.937	gaatTTGAagctc
I$DHAR/HAIRY.01	Hairy, transcriptional repressor	0.88	432 - 442	437	(+)	1.000	0.948	tcaCACGcgac
V$AHRR/AHR.01	Aryl hydrocarbon / dioxin receptor	0.80	427 - 449	438	(-)	1.000	0.816	gggtcgcgtcGCGTgtgagacaa
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	438 - 448	443	(+)	1.000	0.961	gcgACGCgacc
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor	0.95	464 - 474	469	(-)	1.000	0.962	atgtGCGCcct
V$HESF/HES1.01	Drosophila hairy and enhancer of split homologue 1 (HES-1)	0.92	465 - 479	472	(-)	1.000	0.950	cagtcatGTGCgccc
V$MITF/MIT.01	MIT (microphthalmia transcription factor) and TFE3	0.81	464 - 482	473	(-)	1.000	0.847	tcgcagtCATGtgcgccct
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)	0.74	509 - 529	519	(-)	1.000	0.746	aaactagtcgaggCCTTgaag
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	527 - 539	533	(+)	1.000	0.942	ttttTTGAattta
V$PDX1/ISL1.01	Pancreatic and intestinal lim-homeodomain factor	0.82	529 - 549	539	(+)	1.000	0.886	ttttgaattTAATgtttaaaa
V$FKHD/FREAC2.01	Fork head related activator-2 (FOXF2)	0.84	536 - 552	544	(-)	1.000	0.890	cagtttTAAAcattaaa
V$MEF2/MEF2.05	MEF2	0.96	534 - 556	545	(-)	1.000	0.986	cttgcagtttTAAAcattaaatt
V$TBPF/MTATA.01	Muscle TATA box	0.84	540 - 556	548	(+)	1.000	0.876	atgttTAAAactgcaag

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
I$DE74/E74A.01	E74A early ecdysone-inducible gene in onset of Drosophila metamorphosis	0.88	564 - 578	571	(+)	1.000	0.887	gctagcaGGAAattt
V$ETSF/ETS1.01	c-Ets-1 binding site	0.92	565 - 581	573	(+)	1.000	0.927	ctagcAGGAattttt
I$DBRC/BRCZ1.01	Broad-Complex Z1 Zinc Finger isoform	0.88	576 - 592	584	(-)	1.000	0.905	agaaattaACAAaaaaa
V$OCT1/OCT1.06	Octamer-binding factor 1	0.80	578 - 592	585	(+)	1.000	0.874	tttttgttAATTtct
V$NKXH/DLX3.01	Distal-less 3 homeodomain transcription factor	0.91	582 - 594	588	(+)	1.000	0.924	tgtTAATttctaa
V$HNF1/HNF1.01	Hepatic nuclear factor 1	0.78	582 - 598	590	(+)	1.000	0.847	tGTTAatttctaagtca
V$AP1F/TCF11MAFG.01	TCF11/MafG heterodimers, binding to subclass of AP1 sites	0.81	583 - 603	593	(-)	1.000	0.839	aaattTGACttagaaattaac
I$DTLL/TLL.01	Drosophila gap gene tailless, involved in embryonic segmentation	0.93	591 - 599	595	(+)	1.000	0.939	ctaagTCAA

Table 14. List of Putative Transcription Factor Binding Sites in the *C. elegans pdr-1* Promoter.

619 bp inspected, 83 matches found.

List of Regulatory sites in the *C. briggsae pdr-1* Promoter

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
I$DSTA/STAT.01	signal transducers and activators of transcription	0.82	1 - 15	8	(-)	1.000	0.823	tcttttcaGAAAag
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)	0.85	6 - 20	13	(+)	1.000	0.940	ctgaaaaaGAAAcat
V$FKHD/HNF3B.01	Hepatocyte Nuclear Factor 3beta (FOXA2)	0.95	8 - 24	16	(+)	1.000	0.968	gaaaaagaAACAttatg
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	11 - 23	17	(-)	1.000	0.983	ataatGTTTcttt
V$TALE/TGIF.01	TG-interacting factor belonging to TALE class of homeodomain factors	1.00	43 - 49	46	(-)	1.000	1.000	tGTCAaa
V$HEAT/HSF1.01	Heat shock factor 1	0.93	99 - 109	104	(-)	1.000	0.936	AGAAraatcga
V$OCT1/OCT1.01	Octamer-binding factor 1	0.77	110 - 124	117	(-)	1.000	0.780	gcTATGgaaagagtc
V$CLOX/CDPCR3.01	cut-like homeodomain protein	0.75	117 - 133	125	(-)	1.000	0.797	tctccggaagctATGGa
V$ETSF/CETS1P54.01	c-Ets-1(p54)	0.94	118 - 134	126	(-)	1.000	0.970	gtctcCGGAagctatgg
I$DE74/E74A.01	E74A early ecdysone-inducible gene in onset of Drosophila metamorphosis	0.88	121 - 135	128	(-)	1.000	0.893	cgtctccGGAAgcta
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	130 - 140	135	(+)	1.000	0.965	gagACGCtcaa
V$CREB/ATF.01	activating transcription factor	0.90	134 - 154	144	(-)	1.000	0.909	tctcatTGACgaaattgagcg
V$HMTB/MTBF.01	muscle-specific Mt binding site	0.90	158 - 166	162	(-)	1.000	0.901	aggaATTTt
V$STAT/STAT.01	Signal transducers and activators of transcription	0.87	157 - 175	166	(-)	1.000	0.892	ggaatttttaGGAAttttt
V$BCL6/BCL6.01	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma	0.76	159 - 175	167	(+)	1.000	0.840	aaaTTCCtaaaaattcc
V$MEF2/AMEF2.01	Myocyte enhancer factor	0.80	158 - 180	169	(+)	1.000	0.829	aaaattccTAAAaattccactag
V$HMTB/MTBF.01	muscle-specific Mt binding site	0.90	168 - 176	172	(-)	1.000	0.922	tggaATTTt
I$DHOM/FTZ.01	fushi tarazu, involved in body segmentation of the drosophila embryo	0.81	217 - 229	223	(-)	1.000	0.837	ttcgatATTAagg
V$NKXH/NKX25.02	Homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites	0.88	217 - 229	223	(+)	1.000	0.884	cctTAATatcgaa
V$CDEF/CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.87	227 - 239	233	(+)	1.000	0.929	gaatCGCGatttt
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	261 - 275	268	(-)	1.000	0.909	ctcgcgaCAAAtgta
V$CDEF/CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.87	266 - 278	272	(+)	1.000	0.875	ttgtCGCGagttg
V$EVI1/EVI1.01	Ecotropic viral integration site 1 encoded factor	0.72	285 - 301	293	(-)	1.000	0.724	tcaaAAGAaaagctatg
V$CEBP/CEBPB.01	CCAAT/enhancer binding protein beta	0.94	312 - 330	321	(+)	1.000	0.946	ttcattctGCAAtttagaa
V$PAX2/PAX2.01	Zebrafish PAX2 paired domain protein	0.78	312 - 334	323	(+)	1.000	0.791	ttcattctgcaatttagAAACca
V$PCAT/CAAT.01	cellular and viral CCAAT box	0.90	328 -	333	(+)	1.000	0.929	gaaaCCAAtta

Family / matrix	Further Information	Opt.	Position from - to	Position anchor	Str.	Core sim.	Matrix sim.	Sequence
			338					
V$ECAT/NFY.02	Nuclear factor Y (Y-box binding factor)	0.91	327 - 341	334	(+)	1.000	0.952	agaaaCCAAttagaa
V$HOMS/S8.01	Binding site for S8 type homeodomains	0.97	331 - 339	335	(+)	1.000	0.999	accaATTAg
V$NKXH/MSX.01	Homeodomain proteins MSX-1 and MSX-2	0.97	329 - 341	335	(-)	1.000	1.000	ttcTAATtggttt
V$PLZF/PLZF.01	Promyelocytic leukemia zink finger (TF with nine Krueppel-like zink fingers)	0.86	340 - 354	347	(+)	1.000	0.882	aaaTACAggtttaac
V$PBXF/PBX1.01	Homeo domain factor Pbx-1	0.78	350 - 362	356	(+)	1.000	0.790	ttaaCAATcagcg
V$SORY/SOX5.01	Sox-5	0.87	349 - 365	357	(+)	1.000	0.986	tttaaCAATcagcgatc
V$CLOX/CDP.02	transcriptional repressor CDP	0.81	354 - 370	362	(-)	1.000	0.810	atattgATCGctgattg
V$CLOX/CDPCR3HD.01	cut-like homeodomain protein	0.94	358 - 374	366	(-)	1.000	0.978	ttttatattGATCgctg
V$CDXF/CDX2.01	Cdx-2 mammalian caudal related intestinal transcr. factor	0.84	362 - 380	371	(-)	1.000	0.858	ttctactTTTAtattgatc
V$TBPF/TATA.01	cellular and viral TATA box elements	0.90	365 - 381	373	(+)	1.000	0.967	caataTAAAagtagaaa
I$DSUH/SUH.01	Suppressor of Hairless, linked to notch pathway	0.83	381 - 393	387	(+)	1.000	0.865	aatGTGGaaaacg
V$NFAT/NFAT.01	Nuclear factor of activated T-cells	0.97	383 - 393	388	(+)	1.000	0.972	tgtgGAAAacg
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	393 - 407	400	(+)	1.000	0.909	gtagcgaGAAAcagt
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	397 - 409	403	(-)	1.000	0.994	aaactGTTTctcg
V$ETSF/ELK1.02	Elk-1	0.92	402 - 418	410	(-)	1.000	0.940	aaatccGGAAaactgtt
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	404 - 418	411	(-)	1.000	0.749	aaatccgGAAAactg
I$DE74/E74A.01	E74A early ecdysone-inducible gene in onset of Drosophila metamorphosis	0.88	405 - 419	412	(-)	1.000	0.941	caaatccGGAAaact
V$PAX2/PAX2.01	Zebrafish PAX2 paired domain protein	0.78	403 - 425	414	(+)	1.000	0.822	acagttttccggatttgAAACtc
V$IRFF/ISRE.01	Interferon-stimulated response element	0.81	411 - 425	418	(+)	1.000	0.829	ccggatttGAAActc
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	413 - 425	419	(+)	1.000	0.951	ggatTTGAaactc
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	439 - 449	444	(-)	1.000	0.951	gcgACGCggct
V$XBBF/RFX1.02	X-box binding protein RFX1	0.90	443 - 461	452	(+)	1.000	0.943	gcgtcgcgaccGCAAcgct
V$ZBPF/ZF9.01	Core promoter-binding protein (CPBP) with 3 Krueppel-type zinc fingers	0.87	467 - 481	474	(-)	1.000	0.897	gctgCCGCgcctcat
V$XBBF/RFX1.02	X-box binding protein RFX1	0.90	469 - 487	478	(+)	1.000	0.932	gaggcgcggcaGCAAcaat
V$SORY/SOX5.01	Sox-5	0.87	479 - 495	487	(+)	1.000	0.983	agcaaCAATggcctagg
B$SIGF/SIGMAP54.01	Subgroup of bacterial promoters specifically recognized by sigma p54 Polymerase subunit	0.85	484 - 504	494	(+)	1.000	0.856	caaTGGCctaggtcttgcggc
I$DHOM/DFD.01	Deformed, homeotic gene in drosophila development	0.99	502 - 514	508	(-)	1.000	0.997	ctagTAATctgcc
V$HNF6/HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)	0.82	526 - 540	533	(-)	1.000	0.835	tgaaaTCAAaagac
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.88	535 - 547	541	(-)	1.000	0.891	aaaAAGTtgaaat
B$CRBS/CRP.01	CRP binding site, cAMP - dependent catabolite repression in bacteria	0.71	533 - 559	546	(-)	1.000	0.770	aaaatatTGTGaaaaaagttgaaatca
V$IRFF/IRF1.01	Interferon regulatory factor 1	0.86	563 - 577	570	(-)	1.000	0.895	aaaaaactGAAAatt
I$DCAD/CAD.01	Drosophila homeodomain protein caudal, vertebrate homolog cdx	0.98	572 - 582	577	(+)	1.000	0.982	ttttTTTAaat
V$HOXF/EN1.01	Homeobox protein engrailed (en-1)	0.77	572 - 588	580	(+)	1.000	0.771	ttttTTTAaatgttttt
V$FKHD/HFH2.01	HNF-3/Fkh Homolog 2 (FOXD3)	0.93	577 - 593	585	(-)	1.000	0.975	ctgaaaaaAACAtttaa

Table 15. List of Putative Transcription Factor Binding Sites in the *C. briggsae pdr-1* Promoter.

595 bp inspected, 60 matches found.

List of Regulatory sites in the *C. remanei pdr-1* Promoter

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
V$VMYB/VMYB.02	v-Myb	0.90	17 - 27	22	(-)	1.000	0.925	cgaAACGgtag
V$PAX1/PAX1.01	Pax1 paired domain protein, expressed in the developing vertebral column of mouse embryos	0.61	20 - 38	29	(+)	1.000	0.643	CCGTttcgtagagtatata
V$NKXH/NKX31.01	Prostate-specific homeodomain protein NKX3.1	0.84	33 - 45	39	(-)	1.000	0.868	gaagAAGTatata
V$E4FF/E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter	0.82	56 - 68	62	(-)	1.000	0.977	atgACGTaactgg
N$CEDS/CES2.01	cell-death specification 2, bZIP factor involved in programmed cell death in C.elegans	0.77	58 - 68	63	(-)	1.000	0.902	atgacGTAAct
V$CREB/CREB.01	cAMP-responsive element binding protein	0.86	53 - 73	63	(-)	1.000	0.988	tgattaTGACgtaactggaga
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein	0.86	59 - 69	64	(+)	1.000	0.987	gTTACgtcata
O$RPOA/DTYPEPA.01	PolyA signal of D-type LTRs	0.78	107 - 127	117	(+)	1.000	0.797	tCCATcaaatgtggatgtctt
V$LEFF/LEF1.01	TCF/LEF-1, involved in the Wnt signal transduction pathway	0.86	120 - 136	128	(-)	1.000	0.928	ggtgcaaCAAAgacatc
V$VMYB/VMYB.02	v-Myb	0.90	133 - 143	138	(-)	1.000	0.905	cgaAACGggtg
I$DKNI/KNI.01	Drosophila gap gene knirps, involved in embryonic segmentation	0.91	143 - 155	149	(+)	1.000	0.919	gagtatGTTCgaa
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)	0.84	155 - 169	162	(+)	1.000	0.863	actaGTGGttcatga
V$PAX2/PAX2.01	Zebrafish PAX2 paired domain protein	0.78	155 - 177	166	(+)	1.000	0.800	actagtggttcatgaagAAACtg
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)	0.85	169 - 183	176	(+)	1.000	0.982	aagaaactGAAActg
V$HMTB/MTBF.01	muscle-specific Mt binding site	0.90	212 - 220	216	(-)	1.000	0.922	tggaATTTt
V$OCT1/OCT1.01	Octamer-binding factor 1	0.77	217 - 231	224	(-)	1.000	0.785	gcTATGaaaaatgga
V$TBPF/ATATA.01	Avian C-type LTR TATA box	0.81	230 - 246	238	(+)	1.000	0.835	gcatcttTAAGctgttt
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	238 - 250	244	(+)	1.000	0.994	aagctGTTTctcg
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	240 - 254	247	(-)	1.000	0.909	ctcgcgaGAAAcagc
V$SRFF/SRF.01	Serum response factor	0.66	252 - 270	261	(-)	1.000	0.702	ctgaacgTATAtgagactc
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	274 - 286	280	(-)	1.000	0.925	ggctTTGAaactg
V$LEFF/LEF1.02	TCF/LEF-1, involved in the Wnt signal transduction pathway	0.94	273 - 289	281	(+)	1.000	0.977	ccagtttCAAAgccttg
V$MOKF/MOK2.01	Ribonucleoprotein associated zinc finger protein MOK-2 (mouse)	0.74	272 - 292	282	(+)	1.000	0.754	tccagtttcaaagCCTTgttt
V$FKHD/FKHRL1.01	Fkh-domain factor FKHRL1 (FOXO)	0.83	283 - 299	291	(-)	1.000	0.886	tggaggaaAACAaggct
V$NFAT/NFAT.01	Nuclear factor of activated T-cells	0.97	288 - 298	293	(-)	1.000	0.976	ggagGAAAaca
V$ZF5F/ZF5.01	Zinc finger / POZ domain transcription factor	0.95	303 - 313	308	(+)	1.000	0.950	atgcGCGCatg
V$MITF/MIT.01	MIT (microphthalmia transcription factor) and TFE3	0.81	303 - 321	312	(+)	1.000	0.863	atgcgcgCATGtgcttctg
V$CLOX/CDP.02	transcriptional repressor CDP	0.81	328 - 344	336	(+)	1.000	0.842	tttcacATCGatgtcaa
V$TALE/TGIF.01	TG-interacting factor belonging to TALE class of homeodomain factors	1.00	339 - 345	342	(+)	1.000	1.000	tGTCAaa
V$PBXC/PBX1_MEIS1.03	Binding site for a Pbx1/Meis1 heterodimer	0.76	335 - 351	343	(-)	1.000	0.814	gcgatgttTGACatcga
V$BARB/BARBIE.01	barbiturate-inducible element	0.88	351 - 365	358	(-)	1.000	0.895	ctcgAAAGaagttgg
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	355 - 369	362	(-)	1.000	0.849	aaagctcGAAAgaag
V$BCL6/BCL6.02	POZ/zinc finger protein, transcriptional repressor, translocations observed in diffuse large cell lymphoma	0.77	364 - 380	372	(-)	1.000	0.775	caagtttTAGAaaagct
V$TBPF/MTATA.01	Muscle TATA box	0.84	367 - 383	375	(+)	1.000	0.851	ttttctAAAacttgtgg
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.88	370 - 382	376	(-)	1.000	0.895	cacAAGTttaga
V$HAML/AML3.01	Runt-related transcription factor 2 / CBFA1 (core-binding factor, runt domain, alpha subunit 1)	0.84	376 - 390	383	(+)	1.000	0.887	acttGTGGttgtaaa
V$LTUP/TAACC.01	Lentiviral TATA upstream element	0.71	376 - 398	387	(-)	1.000	0.734	attgaatatttacAACCacaagt
V$FKHD/FREAC3.01	Fork head related activator-3 (FOXC1)	0.84	381 - 397	389	(+)	1.000	0.877	tggttGTAAatattcaa
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor	0.86	388 - 398	393	(+)	1.000	0.891	aaatATTCaat
V$CART/XVENT2.01	Xenopus homeodomain factor Xvent-2; early BMP signaling response	0.82	386 - 402	394	(-)	1.000	0.838	ttTAATtgaatatttac

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
V$HNF6/HNF6.01	Liver enriched Cut - Homeodomain transcription factor HNF6 (ONECUT)	0.82	389 - 403	396	(+)	1.000	0.820	aatatTCAAttaaag
V$HOMS/S8.01	Binding site for S8 type homeodomains	0.97	393 - 401	397	(+)	1.000	0.999	ttcaATTAa
V$NKXH/MSX.01	Homeodomain proteins MSX-1 and MSX-2	0.97	391 - 403	397	(-)	1.000	0.995	cttTAATtgaata
V$HOXF/EN1.01	Homeobox protein engrailed (en-1)	0.77	390 - 406	398	(-)	1.000	0.871	tttcTTTAattgaatat
V$RBIT/BRIGHT.01	Bright, B cell regulator of IgH transcription	0.92	392 - 404	398	(+)	1.000	0.958	attcaATTAaaga
N$CEDS/CES2.01	cell-death specification 2, bZIP factor involved in programmed cell death in C.elegans	0.77	408 - 418	413	(-)	1.000	0.851	cgtacGTAAca
V$CREB/CREBP1.01	cAMP-responsive element binding protein 1	0.80	404 - 424	414	(+)	1.000	0.879	aaactgttACGTacgctagag
V$VBPF/VBP.01	PAR-type chicken vitellogenin promoter-binding protein	0.86	409 - 419	414	(+)	1.000	0.933	gTTACgtacgc
V$HIFF/HIF1.01	Hypoxia induced factor-1 (HIF-1)	0.87	409 - 421	415	(-)	1.000	0.875	tagcgtACGTaac
V$VMYB/VMYB.01	v-Myb	0.90	454 - 464	459	(-)	1.000	0.936	aaaAACGgttg
V$ETSF/ELK1.02	Elk-1	0.92	465 - 481	473	(-)	1.000	0.921	agagtcGGAAgaatcaa
V$OCT1/OCT1.01	Octamer-binding factor 1	0.77	476 - 490	483	(-)	1.000	0.780	gcTATGgaaagagtc
V$CLOX/CDPCR3.01	cut-like homeodomain protein	0.75	483 - 499	491	(-)	1.000	0.797	gctccggaagctATGGa
V$ETSF/CETS1P54.01	c-Ets-1(p54)	0.94	484 - 500	492	(-)	1.000	0.970	ggctcCGGAagctatgg
I$DE74/E74A.01	E74A early ecdysone-inducible gene in onset of Drosophila metamorphosis	0.88	487 - 501	494	(-)	1.000	0.896	cggctccGGAAgcta
V$VMYB/VMYB.02	v-Myb	0.90	496 - 506	501	(-)	1.000	0.922	ttgAACGgctc
V$IRFF/IRF7.01	Interferon regulatory factor 7 (IRF-7)	0.86	498 - 512	505	(-)	1.000	0.879	atGAAAttgaacggc
I$PRDH/PRD_HD.01	Drosophila paired homeodomain	0.70	502 - 522	512	(+)	1.000	0.851	ttcaatttcatcGATTagatg
V$CLOX/CDP.02	transcriptional repressor CDP	0.81	506 - 522	514	(-)	1.000	0.962	catctaATCGatgaaat
V$TEAF/TEF1.01	TEF-1 related muscle factor	0.84	524 - 536	530	(+)	1.000	0.922	aaCATTcctgaaa
V$STAT/STAT.01	Signal transducers and activators of transcription	0.87	523 - 541	532	(-)	1.000	0.911	tcgtatttcaGGAAtgttc
I$DKNI/KNI.01	Drosophila gap gene knirps, involved in embryonic segmentation	0.91	541 - 553	547	(+)	1.000	0.914	acatttGTTCaaa
V$SORY/SOX5.01	Sox-5	0.87	552 - 568	560	(-)	1.000	0.996	ataaaCAATatgtattt
V$FKHD/HFH8.01	HNF-3/Fkh Homolog-8 (FOXF1)	0.92	557 - 573	565	(-)	1.000	0.975	ggtgaatAAACaatatg
V$FAST/FAST1.01	FAST-1 SMAD interacting protein	0.81	559 - 573	566	(+)	1.000	0.916	tattgttTATTcacc
V$PIT1/PIT1.01	Pit1, GHF-1 pituitary specific pou domain transcription factor	0.86	563 - 573	568	(+)	1.000	0.862	gtttATTCacc
V$ECAT/NFY.03	Nuclear factor Y (Y-box binding factor)	0.80	567 - 581	574	(+)	1.000	0.808	attcaCCAAccaata
V$PCAT/ACAAT.01	Avian C-type LTR CCAAT box	0.86	572 - 582	577	(+)	1.000	0.895	ccaaCCAAtat
V$ECAT/NFY.02	Nuclear factor Y (Y-box binding factor)	0.91	571 - 585	578	(+)	1.000	0.934	accaaCCAAtatctt
V$GATA/GATA1.01	GATA-binding factor 1	0.96	575 - 587	581	(-)	1.000	0.960	taaaGATAttggt
V$SATB/SATB1.01	Special AT-rich sequence-binding protein 1, predominantly expressed in thymocytes, binds to matrix attachment regions (MARs)	0.93	577 - 593	585	(+)	1.000	0.954	caatatcttTAATatcg
V$GFI1/GfI1B.01	Growth factor independence 1 zinc finger protein Gfi-1B	0.86	592 - 606	599	(-)	1.000	0.929	aaaAATCacggttcg
V$HOXT/MEIS1_HOXA9.01	Homeobox protein MEIS1 binding site	0.79	598 - 610	604	(+)	1.000	0.839	gTGATttttgtga
I$DHUB/HB.02	Hunchback, early maternal and zygotic zinc finger gene, activated by bicoid	0.98	599 - 611	605	(-)	1.000	0.981	ctcacAAAAatca
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	627 - 641	634	(-)	1.000	0.927	ttcgcggCAAAtgta
V$E2FF/E2F.03	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.78	632 - 646	639	(+)	1.000	0.818	ttgcCGCGaatttttc
V$SORY/HMGIY.01	HMGI(Y) high-mobility-group protein I (Y), architectural transcription factor organizing the framework of a nuclear protein-DNA transcriptional complex	0.92	634 - 650	642	(+)	1.000	0.977	gccgcgAATTttcgccg
I$DDVL/DL.02	Dorsal, protein for dorso-ventral axis formation, homologous to vertebrate c-rel	0.91	639 - 649	644	(+)	1.000	0.925	gaatTTTCgcc
V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	639 - 653	646	(-)	1.000	0.777	atgcggcGAAAattc

Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
			from - to	anchor				
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.88	649 - 661	655	(-)	1.000	0.894	gaaAAGTtatgcg
V$EVI1/EVI1.01	Ecotropic viral integration site 1 encoded factor	0.72	651 - 667	659	(-)	1.000	0.724	tcaaAAGAaaagttatg
V$HOMS/S8.01	Binding site for S8 type homeodomains	0.97	696 - 704	700	(-)	1.000	0.997	ttcaATTAt
V$NKXH/DLX3.01	Distal-less 3 homeodomain transcription factor	0.91	694 - 706	700	(+)	1.000	0.949	aaaTAATtgaaaa
V$BRNF/BRN3.01	POU transcription factor Brn-3	0.78	693 - 709	701	(+)	1.000	0.800	gaaATAAttgaaaaatt
V$CART/XVENT2.01	Xenopus homeodomain factor Xvent-2; early BMP signaling response	0.82	696 - 712	704	(-)	1.000	0.917	cgTAATttttcaattat
V$NKXH/NKX25.02	Homeo domain factor Nkx-2.5/Csx, tinman homolog low affinity sites	0.88	701 - 713	707	(-)	1.000	0.895	tcgTAATttttca
V$PAX4/PAX4.01	Pax-4 homeodomain binding site, together with PAX-6 involved in pancreatic development	0.97	702 - 712	707	(+)	1.000	0.980	gaaaAATTacg
V$OCT1/OCT1.06	Octamer-binding factor 1	0.80	703 - 717	710	(-)	1.000	0.897	tctatcgtAATTttt
N$CEDS/CES2.01	cell-death specification 2, bZIP factor involved in programmed cell death in C.elegans	0.77	706 - 716	711	(-)	1.000	0.773	ctatcGTAAtt
I$PRDH/PAX6_HD.01	Drosophila PAX6 P3 homeodomain binding site	0.78	717 - 737	727	(+)	1.000	0.825	aatagttaaaataATTAtgaa
I$DHOM/FTZ.01	fushi tarazu, involved in body segmentation of the drosophila embryo	0.81	724 - 736	730	(+)	1.000	0.823	aaaataATTAtga
V$HOMS/S8.01	Binding site for S8 type homeodomains	0.97	726 - 734	730	(+)	1.000	0.990	aataATTAt
V$HOXF/HOX1-3.01	Hox-1.3, vertebrate homeobox protein	0.83	723 - 739	731	(-)	1.000	0.904	ttttcataATTAtttta
V$NKXH/DLX1.01	DLX-1, -2, and -5 binding sites	0.91	725 - 737	731	(+)	1.000	0.982	aaatAATTatgaa
V$OCT1/OCT1.04	Octamer-binding factor 1	0.80	730 - 744	737	(+)	1.000	0.814	atTATGaaaagattt
I$DSTA/STAT.01	signal transducers and activators of transcription	0.82	740 - 754	747	(-)	1.000	0.960	gctttccacGAAAtc
V$STAT/STAT.01	Signal transducers and activators of transcription	0.87	738 - 756	747	(+)	1.000	0.922	aagatttcgtGGAAagcga
V$GATA/LMO2COM.02	complex of Lmo2 bound to Tal-1, E2A proteins, and GATA-1, half-site 2	0.96	751 - 763	757	(+)	1.000	0.974	aagcGATAacggt
V$E2TF/E2.02	Papilloma virus regulator E2	0.87	750 - 766	758	(+)	1.000	0.901	aagcgataaCGGTttc
V$VMYB/VMYB.02	v-Myb	0.90	755 - 765	760	(+)	1.000	0.977	gatAACGgttt
I$DELF/ELF1.01	Drosophila Elf-1 (NTF-1), vertebrate homolog CP2 (human, mouse)	0.91	755 - 771	763	(+)	1.000	0.924	gataacgGTTTcggaat
V$AREB/AREB6.04	AREB6 (Atp1a1 regulatory element binding factor 6)	0.98	757 - 769	763	(+)	1.000	0.988	taacgGTTTcgga
V$HMTB/MTBF.01	muscle-specific Mt binding site	0.90	766 - 774	770	(+)	1.000	0.931	cggaATTTg
V$PAX2/PAX2.01	Zebrafish PAX2 paired domain protein	0.78	760 - 782	771	(-)	1.000	0.786	gggaatttcaaattccgAAACcg
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)	0.85	766 - 780	773	(+)	1.000	0.865	cggaatttGAAAttc
V$CHRF/CHR.01	Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.92	768 - 780	774	(+)	1.000	0.959	gaatTTGAaattc
V$NFKB/NFKAPPAB.02	NF-kappaB	0.81	770 - 784	777	(-)	1.000	0.871	caGGGAatttcaaat
V$IKRS/IK3.01	Ikaros 3, potential regulator of lymphocyte differentiation	0.84	774 - 786	780	(-)	1.000	0.877	agcagGGAAttc
V$BARB/BARBIE.01	barbiturate-inducible element	0.88	778 - 792	785	(-)	1.000	0.885	aacaAAAGcagggaa
V$GKLF/GKLF.01	Gut-enriched Krueppel-like factor	0.91	779 - 793	786	(-)	1.000	0.934	aaacaaaagcAGGGa
V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	788 - 802	795	(-)	1.000	0.927	ggtgcggCAAAacaa
V$CDEF/CDE.01	Cell cycle-dependent element, CDF-1 binding site (CDE/CHR tandem elements regulate cell cycle dependent repression)	0.87	797 - 809	803	(-)	1.000	0.874	gtgtCGCGgtgcg
I$DHAR/HAIRY.01	Hairy, transcriptional repressor	0.88	804 - 814	809	(+)	1.000	0.882	cgaCACGcatc
V$AHRR/AHRARNT.01	Aryl hydrocarbon receptor / Arnt heterodimers	0.92	799 - 821	810	(-)	1.000	0.924	ggactgcgatgCGTGtcgcggtg
V$PAX5/PAX9.01	Zebrafish PAX9 binding sites	0.78	803 - 831	817	(+)	1.000	0.804	gcgacaCGCAtcgcagtccgtagagcgca
V$CMYB/CMYB.01	c-Myb, important in hematopoesis, cellular equivalent to avian myoblastosis virus oncogene v-myb	0.99	827 - 835	831	(-)	1.000	0.990	caGTTGcgc
V$IRFF/IRF3.01	Interferon regulatory factor 3 (IRF-3)	0.85	827 - 841	834	(+)	1.000	0.852	gcgcaactGAAAgtg
V$NKXH/HMX3.01	H6 homeodomain HMX3/Nkx5.1 transcription factor	0.89	833 - 845	839	(+)	1.000	0.911	ctgaAAGTgcggg
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.88	845 - 857	851	(-)	1.000	0.990	gaaAAGTttagtc

	Family / matrix	Further Information	Opt.	Position		Str.	Core sim.	Matrix sim.	Sequence
				from - to	anchor				
	V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	850 - 864	857	(-)	1.000	0.805	ttcgctgGAAAAgtt
	V$STAT/STAT6.01	STAT6: signal transducer and activator of transcription 6	0.84	850 - 868	859	(+)	1.000	0.876	aacttTTCCagcgaatgtt
	V$MZF1/MZF1.01	MZF1	0.98	871 - 877	874	(-)	1.000	0.985	gcGGGGa
	V$PLZF/PLZF.01	Promyelocytic leukemia zink finger (TF with nine Krueppel-like zink fingers)	0.86	878 - 892	885	(-)	1.000	0.918	aaaTACAgtttgctt
	I$DDVL/DL.02	Dorsal, protein for dorso-ventral axis formation, homologous to vertebrate c-rel	0.91	888 - 898	893	(+)	1.000	0.947	tattTTTCtct
	V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	888 - 902	895	(-)	1.000	0.808	tgcgagaGAAAaata
	V$MZF1/MZF1.01	MZF1	0.98	904 - 910	907	(-)	1.000	1.000	gtGGGGa
	V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	920 - 934	927	(+)	1.000	0.849	gccgagcCAAAttcc
	V$MYOF/MYOGNF1.01	Myogenin / nuclear factor 1 or related factors	0.71	913 - 941	927	(-)	1.000	0.750	gaaaagtggaatTTGGctcggcggcgaga
	V$HMTB/MTBF.01	muscle-specific Mt binding site	0.90	927 - 935	931	(-)	1.000	0.953	tggaATTTg
	V$MYT1/MYT1.01	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.75	929 - 941	935	(-)	1.000	0.756	gaaAAGTggaatt
	V$E2FF/E2F.01	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.74	934 - 948	941	(-)	1.000	0.770	ttcaccaGAAAagtg
	V$FKHD/FKHRL1.01	Fkh-domain factor FKHRL1 (FOXO)	0.83	939 - 955	947	(+)	1.000	0.872	ttctggtgAACAactta

Table 16. List of Putative Transcription Factor Binding Sites in the *C. remanei pdr-1* Promoter.

959 bp inspected, 132 matches found.

7.3 Identified PDR-1 Interaction Partners

For the three independent Yeast-Two-Hybrid screens performed in this study, GAL4-activation domain *C. elegans* cDNA libraries (RB1 and RB3) were used.

PDR-1 interaction partners identified in this study

clone	gene name	ORF (bp/aa)	insert size (bp)	domains	predicted biochemical function	involved in	predicted localization	orthologs, homologs		
								C. elegans	*S. cerevisiae*	*H. sapiens*
Clones from 1st Yeast-Two-Hybrid Screen (pYS-I)										
a	**F29G6.3 (A)**	5787 1929	198- 5787	ATP/GTP binding motif A (P-loop), YLP motif	gametogenesis, osmoregulation			H11E01.3		
b	**F44C4.3** ***cpr-4***	1005 335	48-1005	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-5		Cathepsin B
c	**F28D1.5**	699 233	74- 699	Zinc finger (C2H2-type) thaumatin-like (sweet-tasting)	arabidopsis pathogenesis	cell stress		F28D1.3		
d	**C55B7.2** ***gly-2***	2007 669	449- 2007	alpha-1,3(6)-mannosyl-glycoprotein	beta-1,6-N-acetyl-glucosaminyltransferase	protein modification				MGAT5
e	**F29G9.5** ***rpt-2***	1329 443	3- 1329	AAA ATPase domain, ATP/GTP binding motif A	proteasome subunit, hydrolase, ATPase	protein degradation	ER, nucleus		Rpt2p	PSMC1, regul. subunit #4
h	**T27E9.1**	900 300	full- length	Mitochondrial Carrier Family ADP/ATP carrier protein	active transporter, secondary	small molecule transport, apoptosis	mitochondrial in membrane	T01B11.4, K01H12.2	Pet9p	ANT2
i	**K04H4.1** ***clb-2, emb-9***	5274 1744	3882- 5274	type IV collagen (alpha1-collagen)	structural protein, extracellular matrix	cell structure	basement membrane	LET-2, MEC-5, BLI-2, SQT-3		COL4A2,5
Clones from 2nd Yeast-Two-Hybrid Screen (pYS-II)										
1	**F57F4.3** ***gfi-1***	6459 2153	1325- 2686	ET module family		vesicle transport	Secr. vesicles peripheral membrane	F57F4.4		*Mucin*
2	**T03E6.7** ***cpl-1***	1011 337	375- 1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
3	**T03E6.7** ***cpl-1***	1011 337	207- 1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
4	**C44B12.5**	1191 397	306- 1191	transmembrane domain	unknown			C44B12.1		TFIID subunit4
5	**C44B12.5**	1191 397	285- 1191	transmembrane domain	unknown			C44B12.1		
6	**F26H11.2**	5133 1713	4332- 5133	PHD zincfinger, DDT domain DNA-binding (A+T-hook)	unknown HMG-I and HMG-Y	transcriptional regulation	nucleus			FALZ
7	**T03E6.7** ***cpl-1***	1011 337	360- 1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
8	**C02B10.1**	1257 419	520- 1257	isovaleryl-CoA dehydrogease	acyl-CoA oxidoreductase	lipid, fatty-acid & sterol metabolism	mitochondria		Pox1p	IVD
10	**F52C6.2**	333 110	full- length	domain related to ubiquitin	protein modification			F52C6.1,.3,.4	NEDD-8	NEDD-8
11	**W04A8.7** ***taf-1***	5379 1792	3381- 5379	Bromodomain, CCHC cytochrome C heme binding	transcription factor, DNA-binding protein	Pol-II transcription	nuclear		TFIID subunit	TAF2A
12	**C04F6.1** ***vit-5***	4809 1603	4127- 4530	vitellogenin family	170 kDa yolk protein	progeny nutrition	cytoplasmic in particles	VIT-4,-3,-2		TECTA
15	**Y94H6A.7**	1395 465		put. paralog of Y94H6A.D					Ltp1p	ACP1
16	**T03E6.7** ***cpl-1***	1011 337	452- 1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
17	**F29C12.1** ***pqn-32***	1776 592	570- 1776	DB module family Q/N-rich (Prion) domain	unknown			C49F8.3		M.m. RPTN
18	**F42C5.7** ***grl-4***	633 210	297- 750	Ground-like (Grl) domain protein secretion motif	hedgehog-related protein		extracellular soluble			Trithorax homolog2
19	**K04D7.1**	975 325	full- length	G-protein beta WD-40 repeat Beta-transducin family	translation factor guanine nucleotide bdg	aa-metabolism, protein synthesis, Pol-II transcription	cytoplasmic		Asc1p	RACK1
21	**C39D10.7**	3558 1185	971- 3558	chitin-binding Peritrophin-A domain	unknown		extracellular			Mucin-2 precursor

clone	gene name	ORF (bp/aa)	insert size (bp)	domains	predicted biochemical function	involved in	predicted localization	orthologs, homologs		
								C. elegans	*S. cerevisiae*	*H. sapiens*
22	**C25B8.3** ***cpr-6***	1137 379	371-1137	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-5		Cathepsin B
23	**T01G1.3**	3114 1083		G-protein beta WD-40 Yesat transport prot. WEB1	component of COPII coat of secretory vesicles	ER to Golgi transport	Secr. vesicles peripheral membrane		Sec31p	SEC31B
24	**ZK632.11**	1359 453	full-length	ZincFinger CCHC Proline rich (PSP)	unknown	nucleic acid binding		Y34D9A.7		DKFZP434
25	**C39D10.7**	3393 1131	2355-3393	chitin-binding Peritrophin-A domain	unknown	chitin metabolism	extracellular			

Clones from 3rd Yeast-Two-Hybrid Screen (pYS-III)

1	**B0336.7a**	1506 501	60-1506	Prenyl group binding site (CAAX box), C2H2 finger	unknown			Y54G11A.14		
3	**C25F6.3**	3252 1084	1854-3252	4Fe-4S binding domain Transmembrane domain	Dihydropyrimidine dehydrogenase	de novo pyrimidine biosynthesis			Ura3p	DPYD
5	**T03E6.7** ***cpl-1***	1011 337	114-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
6	**T03E6.7** ***cpl-1***	1011 337	375-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
7	**F37C4.5**	1668 556	282-1668		unknown			ZK1055.7, ZK1055.6		
8	**T24D1.3**	1047 349	full-length	ZF RING finger (C3HC4)	unknown	regulation of apoptosis		T24D1.5, C34F11.1		LOC51283
10	**F57F5.1**	1200 400	193-1200	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-6,-5,-4		Cathepsin B
11	**F57F5.1**	1200 400	302-1200	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-6,-5,-4		Cathepsin B
13	**M01E11.7 isoform A**	3333 1112	1115-3333	SH2-Src homology domain PTB (Phosphotyrosine-bdg.)		intracellular signaling		Y48G1C.F		Tensin
14	**F57F5.1**	1200 400	193-1200	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble			Cathepsin B
15	**F10C1.7** ***ifb-2***	1647 549	full-length	intermediate filament protein IF-tail	structural protein	cell structure	cytoskeletal	IFA-1,IFB-1 F10C1.2B		LMNA
16	**Y39B6A.1**	2205 735	1092-2205	HMW kininogen						
17	**F25B5.7 isoform A**	1683 561	179-1683	RNA-binding, RNP-1 motif PTB-associated	Polypyrimidine tract-binding-associated splicing factor	mRNA splicing	nucleus		Pab1p	SFPQ
19	**K12G11.3**	1047 349	full-length	Zinc-binding	alcohol deyhdrogenase, oxidoreductase	metabolism	cytoplasmic		Adh3p	ADH4
21	**T03E6.7** ***cpl-1***	1011 337	360-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
23	**F44C4.3** ***cpr-4***	1005 335	302-1005	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-5		Cathepsin B
24	**T03E6.7** ***cpl-1***	1011 337	87-1001	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
25	**T03E6.7** ***cpl-1***	1011 337	369-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
26	**F15G9.4a/b** ***him-4***	15594 5198	14143-15594	Immunglobulin domain, Ca2+ -binding EGF-like	DNA-associated (direct or indirect)	recombination	nuclear membrane?			Hemicentin
27	**F23H11.1** ***bra-2***	642 214	12-642	MYND finger protein ZincFinger	BMP receptor-associated molecule			BRA-1		BRAM1
28	**H22K11.1** ***asp-3***	1194 398	433-1194	aspartyl (acidic) protease	protease, hydrolase (other than proteasomal)	protein degradation	soluble cytoplasmic	ASP-4	Pep4p	Cathepsin D
29	**T03E6.7** ***cpl-1***	1011 337	96-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
30	**F49C12.9**	915 305	full-length	UBA / TS-N domain (ubiquitin associated)	unknown			F15C11.2b		
31	**F44B9.7** ***pqn-38***	1401 466	182-1401	ATP/GTP binding site motif A (P-loop)	unknown Q/N-rich (Prion) domain			ZC487.4		salivery prolin rich protein
33	**F57F5.1**	1200 400	193-1200	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	cytoplasmic soluble	CPR-6,-5,-4		Cathepsin B
34	**C07A12.3** ***nhr-35***	1629 542	542-1629	DNA-binding domain, Zinc finger C4-type	nuclear hormone receptor, transcription factor	Pol II transcription signal transduction	nuclear	NHR-64/-49, T23H4.2		HNF4A
35	**F29G9.5** ***rpt-2***	1329 443	63-1329	AAA ATPase domain, ATP/GTP binding motif A	proteasome subunit, hydrolase, ATPase	protein degradation	ER, nucleus	regulatory subunit P45	Rpt2p	PSMC1
36	**T03E6.7** ***cpl-1***	1011 337	87-1011	cysteine protease papain family, thiol protease	protease, hydrolase (other than proteasomal)	protein degradation	extracellular matrix	F41E6.6, R09F10.1		Cathepsin L
37	**F15G9.4a/b** ***him-4***	15594 5198	14143-15594	Immunglobulin domain, Ca2+ -binding EGF-like	DNA-associated (direct or indirect)	recombination	nuclear membrane?			Hemicentin

Table 17. Clones of the PDR-1 Yeast-Two-Hybrid Protein Interaction Screen.

7.4 List of *C. elegans* Genes and Human Homologs

gene	ORF	name/description	homolog
act-1	T04C12.6	Actin, (loading control)	Actin
abu-	11 genes	activated in blocked UPR family	Keratin-associat.
aex-3	C02H7.3	Aboc, Expulsion defective	DENN
ama-1	F36A4.7	amanitin resistant (loading control)	RNA pol II
asp-3	H22K11.1	aspartyl protease	cathepsin D
atf-6	F45E6.2	activating transcription factor	ATF6α
ces-2	ZK909.4	cell-death specification	D-site-bdg protein
chn-1	T09B4.10	C-term of Hsp70-interacting protein	CHIP
cpl-1	T03E6.7	cathepsin L family	cathepsin L
cpr-4	F44C4.3	cysteine protease related	cathepsin B
cpr-6	C25B8.3	cysteine protease related	cathepsin B
cyk-4	K08E3.6	cytokinesis defective	GAP
dat-1	T23G5.5	dopamine (DA) transporter	DAT-1
hsp-1	F26D10.3	heat-shock protein (cytosol)	Hsp-70
hsp-3	C15H9.6	heat-shock protein (ER)	Grp78/BiP
hsp-4	F43E2.8	heat-shock protein (ER)	Grp78/BiP
hsp-16.2	Y46H3A.3	heat-shock protein (cytosol)	Hsp20
hsp-70	C12C8.1	heat-shock protein (cytosol)	Hsp-70
ire-1	C41C4.4	IRE1 kinase related	IRE1
mdt-29	K08E3.8	MeDiaTor gene class protein	PQCAP
mev-1	T07C4.7	methylviologen sensitive	cytochrome *b*
nhr-111	F44G3.9	nuclear hormone receptor	
pdr-1	K08E3.7	Parkinson's disease related gene 1	Parkin
pek-1	F46C3.1	human PERK kinase homolog	PERK
pqn-	> 100 genes	Q/N-rich 'prion' domain genes	diverse
rol-6	T01B7.7	roller, helically twisted	collagen
rpt-2	F29G9.5	proteasome regulatory particle ATPase-like	19S subunit 4
rrf-3	F10B5.7	RNA-dependent RNA polymerase family	RdRP
sel-7	K04G11.2	suppressor/enhancer of lin-12	novel
sel-12	F35H12.3	suppressor/enhancer of lin-12	Presenilin 1
skn-1	T19E7.2	skinhead	NRF1

gene	ORF	name/description	homolog
ubc-2	M7.1	ubiquitin-conjugating enzyme	UbcH4/5
ubc-6	D1022.1	ubiquitin-conjugating enzyme	Ubc6
ubc-7	F58A4.10	ubiquitin-conjugating enzyme	Ubc7
ubc-9	F29B9.6	ubiquitin-conjugating enzyme (SUMO)	Ubc9
ubc-12	R09B3.4	ubiquitin-conjugating enzyme (Nedd8)	Ubc12
ubc-14	Y87G2A.9	ubiquitin-conjugating enzyme	Ubc7
ubc-15	Y110A2AR.2	ubiquitin-conjugating enzyme	Ubc6
ubc-18	R01H2.6	ubiquitin-conjugating enzyme	UbcH7/8
unc-59	W09C5.2	uncoordinated	CDCrel-1
unc-61	Y50E8A.4	uncoordinated	CDCrel-1
unc-119	M142.1	uncoordinated	HRG4
upr-1	uncloned	unfolded protein response abnormal	?
xbp-1	R74.3	X-box binding protein	XBP1

Table 18. List of *C. elegans* Genes and Human Homologs.

Listed and explained are all *C. elegans* gene names mentioned in this study. The gene names are given, according to the genetic nomenclature for *Caenorhanditis elegans* either on the basis of a mutant phenotype or on the basis of the predicted gene product. The corresponding *C. elegans* ORFs as well as the human homologs are listed.

7.5 List of Figures

7.6 List of Tables

7.7 Abbreviations

°C	degree celcius
3-AT	3-aminotriazol
5-HT	serotonin
6-OHDA	6-hydroxydopamine
6xHIS	hexa-histidine epitope tag
19S	regulatory subunit complex of the 26S proteasome
20S	core particle of the 26S proteasome
26S	26S proteasome, multicatalytic proteinase complex
β-ME	β-mercaptoethanol
Δ	deletion
µg	microgram
µl	microlitre
µM	micromolar
A30P	α-synuclein pathogenic mutation, alanine30->proline
A53T	α-synuclein pathogenic mutation, alanine53->threonine
aa	amino acid
AD	autosomal dominant
ADEs	*C. elegans* anterior deirids (DA containing neurons)
AR	autosomal recessive
AR-JP	autosomal-recessive juvenile parkinsonism
ATP	adenosine triphosphate
B. taurus	*Bos taurus*
bp	base pair
BSA	bovine serum albumine
C. briggsae	*Caenorhabditis briggsae*
C. elegans	*Caenorhabditis elegans*
C. remanei	*Caenorhabditis remanei*
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CEPs	*C. elegans* cephalic cells (DA containing neurons)
D. melanogaster	*Drosophila melanogaster*
DA	dopamine

DAPI	4',6'-diamidino-2-phenylindole hydrochloride
DNA	deoxyribonucleic acid
Dpy	dumpy (*C. elegans* mutant phenotype)
ds	double stranded
DTT	dithiothreitol
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
E4	multiubiquitin chain assembly factor
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Egl-D	egg-laying defective (*C. elegans* mutant phenotype)
ER	endoplasmatic reticulum
ERAD	ER-associated degradation
GFP	green fluorescent protein
GST	glutathione S-transferase
H. sapiens	*Homo sapiens*
HA	hemagglutinin epitope tag
HSP	heat-shock protein (chaperone)
IBR	C_6HC in-between RING-finger domain
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
L1-4	*C. elegans* larval stages 1-4
LBs	Lewy bodies
L-DOPA	levodopa
M. musculus	*Mus musculus*
Mb	megabase
ml	millilitre
mM	millimolar
MPTP/MPP^+	1-methyl-4-phenylpyridinium
mRNA	messenger ribonucleic acid
myc	*c*-myc epitope tag
n	number
n.d.	not determined
NGM	nematode growth media

nt	nucleotide
NPG	n-propyl gallate
OP50	*Escherichia coli* strain, food source for *C. elegans*
ORF	open reading frame
PCR	polymerase chain reaction
PD	Parkinsons's disease
PDEs	*C. elegans* posterior deirids (DA containing neurons)
PDR-1	Parkinson's disease related protein 1
R. norvegicus	*Rattus norvegicus*
RING	C_3HC_4 zinc finger domain (really interesting new gene)
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute
RT-PCR	reverse transcriptase polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SL	splice leader
SNpc	Substantia Nigra pars compacta
SW-PCR	single worm-PCR
TCA	trichloracetic acid
TM	transmission
TMP	trimethylpsoralen
Ub	ubiquitin
UBA	ubiquitin-associated domain
UBC	ubiquitin-conjugation enzyme, E2
UBL	ubiquitin-like
UCH	ubiquitin C-terminal hydrolase
Unc	uncoordinated (*C. elegans* mutant phenotype)
UPD	unique Parkin domain
UPR	unfolded protein response
UTR	untranslated region
WT	wild type
x-α-Gal	5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside

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(William Shakespeare, *Macbeth*, 5.8.73)

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