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Parkin and relatives: the RBR family of ubiquitin ligases

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Parkin and relatives: the RBR family of ubiquitin ligases. *Physiol Genomics* 17: 253–263, 2004; 10.1152/physiolgenomics.00226.2003.—Mutations in the *parkin* gene cause autosomal-recessive juvenile parkinsonism. *Parkin* encodes a ubiquitin-protein ligase characterized by having the RBR domain, composed of two RING fingers plus an IBR/DRIL domain. The RBR family is defined as the group of genes whose products contain an RBR domain. RBR family members exist in all eukaryotic species for which significant sequence data is available, including animals, plants, fungi, and several protists. The integration of comparative genomics with structural and functional data allows us to conclude that RBR proteins have multiple roles, not only in protein quality control mechanisms, but also as indirect regulators of transcription. A recently formulated hypothesis, based on a case of gene fusion, suggested that RBR proteins may be often part of cullin-containing ubiquitin ligase complexes. Recent data on Parkin protein agrees with that hypothesis. We discuss the involvement of RBR proteins in several neurodegenerative diseases and cancer.

Parkinson disease; protein quality control; transcriptional regulation

THE INTEGRATION OF GENOMIC and proteomic data is providing new perspectives on the origin of genes and the functions of their products. This review is focused on the information generated by the combination of functional and comparative genomic analysis of the RBR gene family. RBR genes are being extensively studied since the discovery that mutations in one of them, named *parkin*, are a frequent cause of familial autosomal-recessive juvenile parkinsonism (AR-JP). Since then, evidence has been obtained suggesting that RBR proteins are ubiquitin-protein ligases that function as key components of the cellular machinery that controls protein quality. They are also involved in other processes, including regulation of transcription factors such as p53 or the androgen receptor.

Although the last years have brought significant advances, our knowledge of RBR protein functions is still extremely fragmentary. One of the main conclusions obtained from the comparative genomic data is that this is an ancient and complex family. Multiple members are present in all eukaryotic species for which we have significant sequence information. The structures of several RBR proteins have diversified by incorporating new protein domains and rare cases of fusions of RBR genes with totally unrelated genes have been found as well. In conclusion, we can expect RBR proteins to play a wide repertoire of functions in all eukaryotic organisms. Much of that diversity has been only superficially explored. The information hitherto available will be summarized here.

A final aspect that we discuss in this work is the fact that comparative genomics may be used, in favorable cases, to obtain precise hints of the functions of proteins or even protein families. As we will show below, some of the characteristic features of the RBR proteins, such as their involvement in protein ubiquitination or even their interaction with particular types of proteins to generate ubiquitin-ligase complexes, can be deduced, in absence of any biochemical data, from the precise examination of the structure and the evolutionary origin of a single member of the family. This powerful example of the strength of eukaryotic comparative genomics to provide interesting functional information suggests that deep evolutionary and structural analyses of complex gene families may be a most profitable starting point for the experimental exploration of the biochemical roles of novel or barely studied proteins.

Parkin: a Ubiquitin Ligase Involved in Parkinson Disease

In 1997, a major locus involved in familial AR-JP was mapped to the long arm of chromosome 6 (58). Subsequently, the locus was determined to be *parkin*, also called *PARK2* (45). Since then, it has been established that mutations in *parkin* are also a frequent cause of sporadic early-onset Parkinson disease (PD; reviewed in Ref. 16) and, when heterozygous, may confer susceptibility to both early- and late-onset PD (21, 29, 71, 74). Kitada and coworkers (45) showed that *parkin* is expressed in multiple tissues and particularly in different regions of the brain, including the substantia nigra. In AR-JP as in other parkinsonian syndromes, including the most common late-onset, sporadic PD, there is loss of dopaminergic neurons, most significantly those of the substantia nigra. These results strongly suggest that *parkin* function is normally required, at least in humans, for long-term survival of dopaminergic neu-

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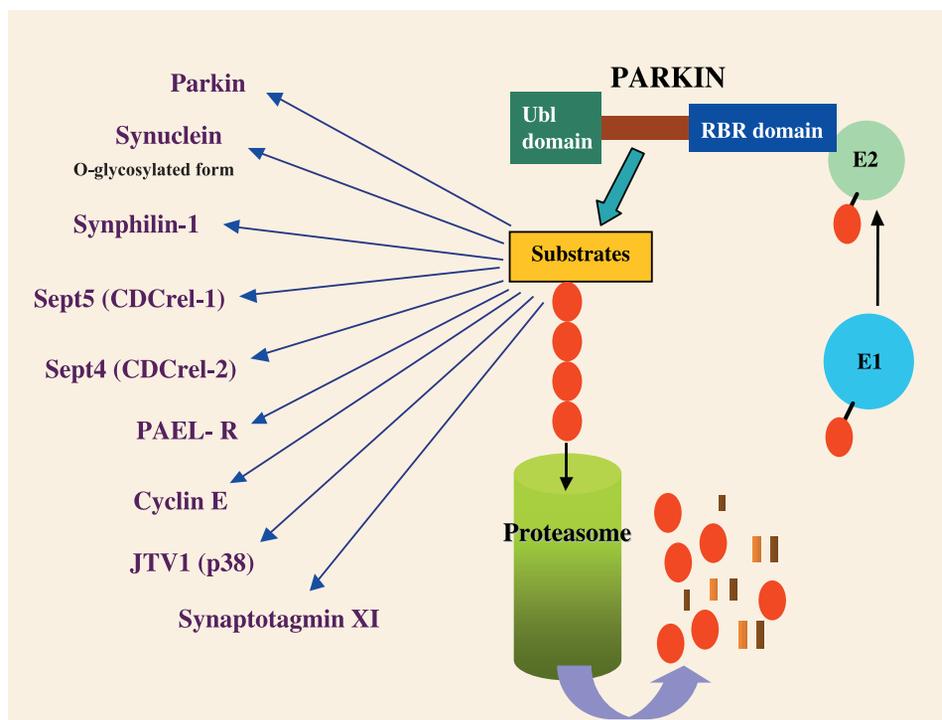
rons, although it is likely to have functions in other tissues as well. Parkin protein was described (45) as containing an ubiquitin-like domain in its NH₂-terminus and a COOH-terminal cysteine-rich region that included a motif similar to a RING finger (53; reviewed in Refs. 3 and 4). Later, it was established that the COOH-terminal region actually contains two RING fingers, characterized by the signature C₃HC₄ (that is, three conserved cysteine residues followed by a conserved histidine and then four additional conserved cysteines) plus an intermediate, cysteine-rich region (characterized by a C₆HC pattern), that was called IBR (63) or DRIL domain (89). Multiple proteins with the RING1-IBR/DRIL-RING2 signature were found in many eukaryotic species, including animals, plants, fungi, and protists such as *Plasmodium* or *Dictyostelium* (63, 89). For reasons that we will explain below, we recently renamed this characteristic protein structure as RBR domain (55).

A major breakthrough in the understanding of Parkin protein function was the discovery that it interacts with the ubiquitin-conjugating (E2) enzymes UbcH7 and UbcH8 and it has ubiquitin-protein ligase (E3) activity (Fig. 1; Refs. 35, 80, 94). Several reports had already demonstrated that RING finger-containing proteins act as ubiquitin ligases in organisms as different as yeasts and mammals (reviewed in Refs. 22, 41, and 87). Moreover, several proteins containing the RING1-IBR/DRIL-RING2 signature typical of Parkin had been previously shown to interact with E2 enzymes (57, 64). The finding that Parkin was a ubiquitin ligase was therefore not totally unexpected. However, the importance of this discovery must not be underestimated. Dopaminergic neurons in PD-affected individuals have characteristic cytoplasmic proteinaceous inclusions called Lewy bodies, in which one of the two main components is ubiquitin, suggesting that anomalies in protein metabolism might underlie parkinsonian neurodegeneration. This hypothesis was strengthened with the discovery of Parkin function as

a ubiquitin ligase and the characterization of its functional relationships to the products of two other genes, *UCH-L1* and *α-synuclein*, in which particular missense mutations causing familial PD have been found. *UCH-L1* (*PARK5*) encodes a protein able to act as ubiquitin carboxy-terminal hydrolase and ubiquitin ligase (50, 52), while *α-synuclein* (*PARK1*) encodes the other main component, with ubiquitin, of Lewy bodies. It is well established today that the products of these three genes belong to the same metabolic network: a particular *O*-glycosylated form of *α-synuclein* is a substrate of Parkin (81), and *UCH-L1* has been shown to coprecipitate with *α-synuclein* in brain extracts and is able to add ubiquitin to mono- or diubiquitylated *α-synuclein* in vitro (52). More recently, *UCH-L1* protein has been shown to contribute in vivo to the maintenance of cellular ubiquitin levels, probably by inhibiting ubiquitin degradation (72). Thus the evidence strongly suggests that Parkin, *UCH-L1*, and *α-synuclein* are functionally closely related and linked to the ubiquitin-proteasome pathway. However, whether failure of that pathway is the primary cause of neurodegeneration in both familial and sporadic PD or just a collateral effect of some other type of primary damage (i.e., oxidative stress) that would be the direct cause of cell death is still under discussion (reviewed in Refs. 7, 15, 23, 28, and 46; see also *Parkin function in an evolutionary context*, below).

That Parkin has multiple cellular roles is strongly suggested by its substrates (Fig. 1). They include not only *O*-glycosylated *α-synuclein*, as already indicated, but also the *α-synuclein*-interacting protein synphilin-1 (10), the septins Sept5 (formerly called CDCrel-1 or PNUTL1; Ref. 94) and Sept4 (formerly called CDCrel-2 or PNUTL2; Ref. 9), a seven transmembrane G protein-coupled receptor called Pael-R (34), cyclin E (82), *α/β*-tubulin (79), JTV1 (also known as p38), a structural subunit of the aminoacyl tRNA synthetase complex that also contributes to *c-myc* regulation (13, 43, 44, 78), and synaptotagmin XI (32). Parkin is also able to catalyze its own

Fig. 1. Parkin substrates and the ubiquitin-proteasome system. Ubiquitin (red) is first activated by the E1 (ubiquitin-activating) enzyme. Then it is transferred to the ubiquitin-conjugating enzyme (E2). From E2, ubiquitin is finally added to the substrate of a ubiquitin-protein ligase (E3), in this case Parkin. Polyubiquitinated substrates bind to the proteasome and are degraded, liberating short peptides and free ubiquitin, ready to be used again. The nine known substrates of Parkin are discussed in the text.



ubiquitination (94). Although our understanding of most of these interactions is still superficial, several results suggest a fundamental role of Parkin in protection against the accumulation of unfolded or misfolded proteins. Thus the damage caused by accumulation of abnormal forms of α -synuclein or unfolded Pael-R is relieved by Parkin function in cellular and animal models (34, 76, 92). Parkin has been also shown to interact with a human, polyglutamine-expanded, huntingtin protein expressed in transgenic mice, and it contributes, in cell culture assays, to the degradation of polyglutamine protein fragments (86). Apart from this characteristic function as protective agent, it is likely that Parkin is acting in neurons in other, more subtle, ways. For example, the interactions with septins and synaptotagmin XI suggest that Parkin may be indirectly involved in regulation of vesicle transport and exocytosis (32, 94). Finally, the interaction with cyclin E suggests that Parkin may be a direct inhibitor of apoptosis, because accumulation of cyclin E is known to trigger apoptosis in neurons (82; see also Ref. 14).

In conclusion, Parkin is a ubiquitin ligase probably involved in several cellular processes, and most especially protection against unfolded protein stress, and mutations in the *parkin* gene are one of the best-established causes of familial PD, with a likely role also as susceptibility alleles in sporadic PD. An obvious question is then whether the genes evolutionarily related to *parkin* have similarly important roles in this or other human diseases. Also in a more general way, we may ask whether an integrated knowledge of the data available for the gene family that includes *parkin* may provide additional clues about the cellular functions of *parkin* and its relatives.

The RBR Domain

The definition of the gene family that we called RBR, and that includes *parkin* and other genes with the RBR domain, required some precise analyses (55). A substantial difficulty was that the two reports that independently described the RBR domain (63, 89) were partially conflicting. For example, Parkin was considered to have a RING1-IBR-RING2 structure (63) but lacking a RING1-DRIL-RING2 structure (89), despite the fact that both reports were clearly defining very similar signatures. That inconsistency could be due either to slightly different definitions of the domains or to the fact that several, evolutionary unrelated, cysteine-rich protein domains existed that could be confused as a single entity. In fact, RING fingers are quite similar to other cysteine-rich domains known as PHD or LIM domains (4), and thus it was reasonable to envisage that several independent Cys-rich domains could be juxtaposed to generate quite similar signatures. To avoid these problems, we decided to generate a database of potential RBR proteins by permissively including all sequences that contained a Cys-rich region resembling the C₃HC₄-C₆HC-C₃HC₄ pattern of conserved residues that characterizes the RBR domain, but without doing any effort to eliminate proteins that did not perfectly fit that pattern. In that way, we reasoned that we could find all similar domains potentially present in the databases. After some detailed analyses, we found that most of the selected proteins actually had very similar domains, except for the fact that some of them were lacking one or a few of the key C or H residues and that distances among residues were substantially variable. Thus the complex pattern of more than 20

cysteine and histidine residues that characterizes the RBR domain seems to have arisen only once in evolution (55). We also concluded that Parkin does indeed contain one of these patterns, as first suggested by Morett and Bork (63).

One of the first significant results obtained when a large database of RBR proteins was generated is that there are obvious structural differences between RING1 and RING2. RING1 turned to be much larger and variable than RING2. Moreover, in many proteins, RING2 was lacking one (most often the characteristic histidine) or even several of the highly conserved residues that characterize the RING finger. Finally, some other characteristic, semiconservative, residues often found in canonical RING fingers (4) are absent in RING2. These results strongly suggested that both RING fingers in the RBR domain are functionally distinct, with RING2 being less constrained to have a canonical RING finger structure. As we have already indicated, several ubiquitin ligases contain a single RING finger, and thus the simplest explanation for this difference could be that one of the RINGs, generally RING2, is dispensable for protein function. This is, however, highly unlikely. It is true that in several RBR proteins only RING1 interacts with the ubiquitin-conjugating (E2) enzyme (1, 2, 38, 56, 64). However, Parkin and some other RBRs require both RINGs for E2 interaction (35, 68, 80) and point mutations in both Parkin RING fingers may cause AR-JP (reviewed in Ref. 93). Moreover, data from *Drosophila* RBRs in which only RING1 is required for that interaction also demonstrated that loss of function or severely hypomorphic mutations can be obtained by mutating either of the RING fingers (1). In summary, it is likely that in most cases RING2 is essential for the correct function of RBR proteins. For these reasons, we recently proposed an alternative hypothesis, namely that the differences in conservation in both RING fingers could be due to the fact that RBRs may work either as independent E3s or as part of E3 complexes (see below), and functioning in a complex may relax the need for a second canonical RING finger (55).

The sophisticated structure of the RBR domain favors the existence of gain-of-function missense mutations. Perhaps those types of mutations may contribute to explain the effect of some heterozygous mutations in humans. In fact, missense mutations in both RING fingers of *parkin* that dramatically alter its cellular localization and generate both cytoplasmic and nuclear inclusions in neuronal cell culture assays have been recently described (12, 27).

Comparative Genomics of the RBR Family

In a previous study, we used multiple alignments of the RBR signature plus structural comparisons to establish a classification of the proteins of the RBR family in seven model organisms (four animals: human, mouse, *Drosophila melanogaster*, and *Caenorhabditis elegans*; the plant *Arabidopsis thaliana*; and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*; Ref. 55). Using three independent methods of phylogenetic reconstruction (that generated largely identical results), we described 7 main subfamilies containing 69 of the 74 proteins found in those organisms. The subfamilies were named Parkin, Ariadne, Dorfin, ARA54, XAP3, Plant I, and Plant II (55). Only the subfamilies ARA54 and Ariadne were found to be present in fungi, animals, and plants, whereas three

(Parkin, Dorfin, XAP3) were animal specific and the other two, as their names indicate, were plant specific. Multiple genes were found in all species, ranging from 2 in model yeasts to no less than 37 in *Arabidopsis*. In this initial analysis, we detected 11 human RBR genes. Mladek et al. (61) obtained similar results in an analysis focused on *Arabidopsis*, showing that both genome duplication and retroposition may account for the surprisingly large number of RBR genes in that plant species.

We recently generated an updated (December 2003) version of the phylogenetic tree shown in our previous work, including all ($n = 237$) currently available sequences with complete or almost complete RBR domains. Results are summarized in Fig. 2. The seven subfamilies described in our previous work were detected again in this new, more comprehensive, analysis. In addition, five new subfamilies, statistically highly supported, became evident when more sequences were added (Fig. 2; other subfamilies are likely to exist for which available infor-

mation is still too insufficient to allow a formal description). Two of the additional subfamilies only include animal genes. We have called them RNF144 (which contains the human gene *RNF144*, also known as *KIAA0161*) and PAUL (which includes the recently characterized mammalian *PAUL* gene, formerly known as *FLJ10111*; see Ref. 5). These two families contain orthologous genes in both deuterostome and protostome species. Interestingly, most fungi seem to have many more RBR genes than the model yeast species considered in our previous work. For example, *Neurospora crassa* has at least seven RBR genes in contrast to the two genes found in *S. cerevisiae* or *S. pombe*. As a consequence of this larger number of genes and the fact that several fungal genomes have been recently fully or almost fully sequenced, we have been able to define two novel fungal-specific subfamilies (Fungal I and Fungal II; see Fig. 2). The fifth novel subfamily, TRIAD3, includes the mammalian *TRIAD3* gene plus similar genes in

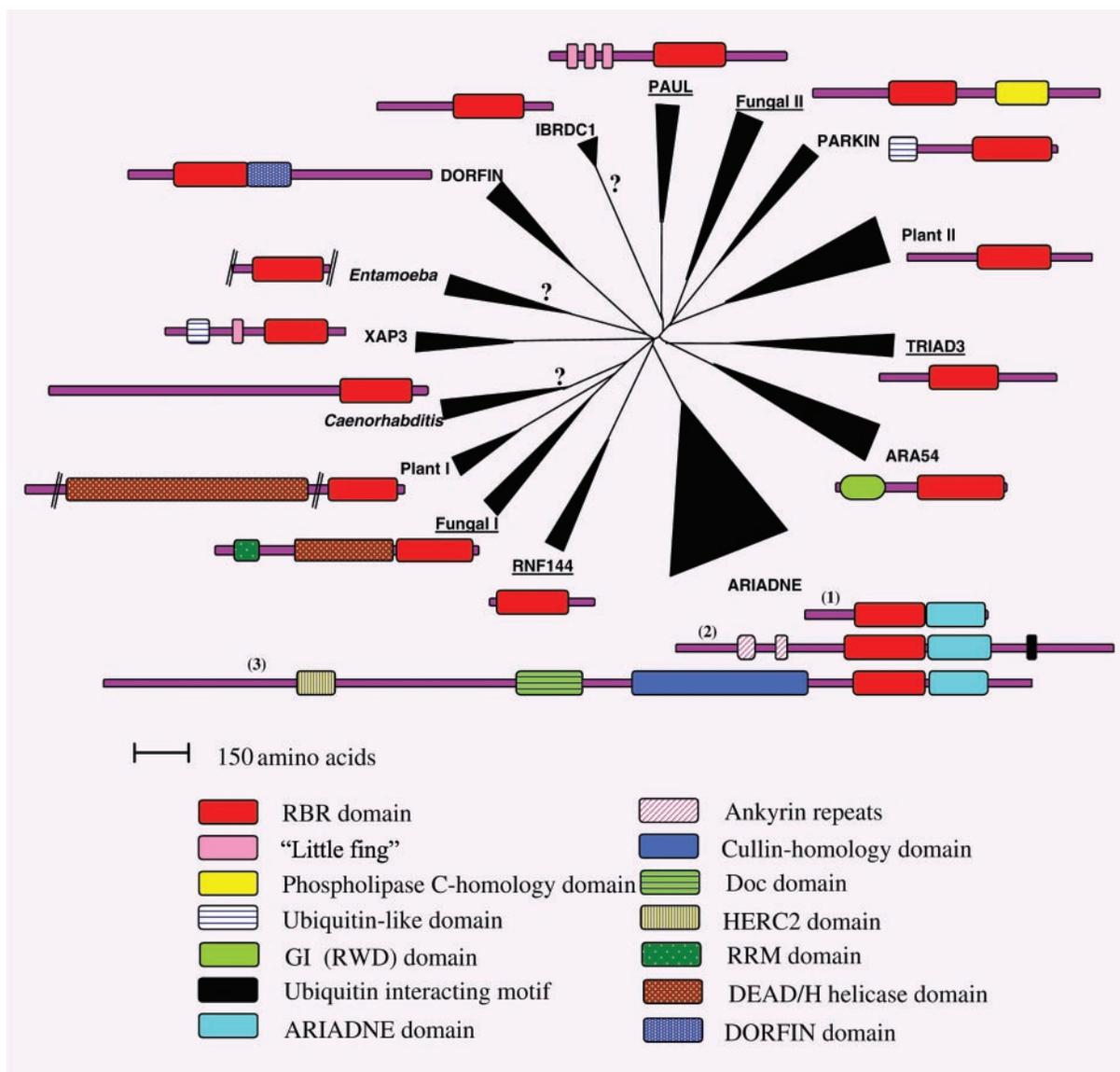


Fig. 2. Phylogenetic tree and structures of the main RBR subfamilies. The tree was generated using the “neighbor-joining and maximum parsimony” methods implemented in MEGA 2.1 (Ref. 48). Question marks refer to potential additional subfamilies. Domains found in RBR proteins are detailed. Three different structures are found in the Ariadne subfamily: 1) the most common, standard RBR + Ariadne domain structure; 2) structure of KIAA1386; 3) structure of Parc.

fungi. Interestingly, we found 4 additional human genes, making for a total of 15 in our species (Table 1). Another significant addition with respect to our previous analyses is the inclusion of sequences from several protist species that are located in three separate regions of the tree.

In this more comprehensive analysis we found as in our previous study (55) that the topology for many of the most internal branches of the tree is unclear. However, the statistical support for most of the defined subfamilies being monophyletic is strong. There are only two cases where such monophyly is weakly supported, namely the Ariadne and ARA54 subfamilies. Additional structural data, however, confirm that they both have been correctly defined (see Ref. 55). Figure 2 also shows a summary of the characteristic structures of the members of the different subfamilies. Because the acquisition or loss of a protein domain is a rare event, those proteins that have similar RBR domains (appearing together in the tree) and, in addition, share one or several additional domains must have necessarily a common origin and thus can be defined as belonging to the same subfamily. We found that ARA54 proteins have a characteristic GI (also called RWD) domain (18, 47), whereas Ariadne subfamily proteins share a highly conserved domain, located COOH-terminally with respect to the RBR domain, that we described as Ariadne domain (55). In our most recent analyses, we found that not only animal, fungal, and plant sequences, but also some protist RBR proteins, have Ariadne domains. These results confirm that Ariadne is the RBR subfamily whose origin can be traced back earlier in time.

Functions of RBR Proteins

Evolutionary, structural, and functional data can be combined to obtain a clearer picture of the cellular roles of RBR proteins. As we will see, RBRs emerge as multitask proteins able to act in different cellular contexts, from protein quality control to indirect regulation of transcription, and are most likely involved in multiple human pathologies.

Ariadnes. Ariadnes form the largest, most diverse and oldest RBR subfamily among those that we have described (Fig. 2). Functional data are so far restricted to animal Ariadnes. Sev-

eral members of the family derived from humans, mice, or *Drosophila* interact through their RING1 with the highly similar E2 enzymes UbcD10 (*Drosophila*), UbcM4 (mouse), or human UbcH7 and UbcH8 (1, 2, 56, 57, 64). Recently, an alternative interaction through the same RING1 has been reported between human Ari1 and 4EHP, the translation initiation factor 4E homolog (84). Since the same Ari motif mediates the interaction with 4EHP and the E2 enzyme, both interactions are mutually exclusive. However, despite the competition for the binding site, 4EHP is ubiquitinated in the presence of human Ari1 (84). Information of the phenotypes of mutants of Ariadne subfamily genes is only available for the *ari-1* and *ari-2* *Drosophila* genes. The lack, as well as the excess, of function of *ari-1* result in lethality at metamorphosis. Mutations in *ari-2* are also lethal (1).

Some members of the Ariadne subfamily have interesting structural features. Most RBR proteins, including most Ariadnes, are about 500 amino acids in length. Exceptional, however, are two animal Ariadne genes that in humans are called *KIAA1386* and *Parc* (formerly *KIAA0708*), which generate much larger products (Fig. 2). *KIAA1386* protein contains ankyrin repeats plus a short domain found in multiple proteins known to interact with ubiquitin (31). *Parc* contains characteristic HERC2, Doc, and Cullin domains. The structure of *KIAA1386* does not provide any special information about its function. *Parc* structure, however, contains very significant information. We demonstrated that *Parc* originated from an ancient gene fusion among an *ariadne* gene and a duplicate of another gene called *Cul7* (*Cullin7*, formerly *KIAA0076*) that also contains the HERC2, Doc, and Cullin domains, but lacks an RBR domain (55). Not only are the sequences of *Parc* and *Cul7* are very similar, but they also have an almost identical disposition of exons and introns (see Fig. 3A). These two genes are about 260 kb apart in both the human and mouse genomes (Fig. 3B). In fact, we have found evidence that a *Parc* gene exists in the urochordate *Ciona intestinalis*, suggesting that this gene fusion occurred more than 500 million years ago (J. I. Lucas and I. Marín, unpublished data).

Table 1. *Human RBR Genes*

Name	Alternative Names	Subfamily	Location	Evidence for E3 Function	Additional Data
<i>Parkin</i>	<i>PARK2</i>	Parkin	6q25.2–6q27	Direct	
<i>ARI1</i>	<i>ARIH1, HHARI</i>	Ariadne	15q24	Direct	
<i>ARI2</i>	<i>ARIH2, TRIAD1</i>	Ariadne	3p21.2–3p21.3	Murine ortholog interacts with E2	
<i>KIAA1386</i>		Ariadne	7q21.3		
<i>PARC</i>	<i>KIAA0708, H7-API</i>	Ariadne	6p21.1	Direct	
<i>RNF144</i>	<i>KIAA0161</i>	RNF144	2p25.2	Murine ortholog interacts with E2	
<i>IBRDC2</i>	<i>P53RFP</i>	RNF144	6p22.3	Direct	<i>RNF144</i> paralog. Mammalian-specific
<i>XAP3</i>	<i>C20orf18, RNF54, HOIL-1</i>	XAP3	20p13	Direct	Alzheimer disease locus located at 20p.
<i>Dorfin</i>	<i>RNF19</i>	Dorfin	8q22	Direct	
<i>DJ1174N9.1</i>		Dorfin	1p34–1p35.3		<i>Dorfin</i> paralog. Mammalian-specific. <i>PARK6</i> located at 1p35–1p36.
<i>IBRDC1</i>	<i>FLJ16403</i>	Not assigned	6q22.33		
<i>PAUL</i>	<i>FLJ10111, RNF31, ZIBRA</i>	PAUL	14q11.2–14q21.3		
<i>TRIAD3</i>	<i>ZIN</i>	TRIAD3	7p22.2		
<i>ARA54</i>	<i>RNF14</i>	ARA54	5q23.3–q31.1	Direct	
<i>ARA54_2</i>		ARA54	7q33		<i>ARA54</i> paralog. Found so far only in the human genome. A likely pseudogene.

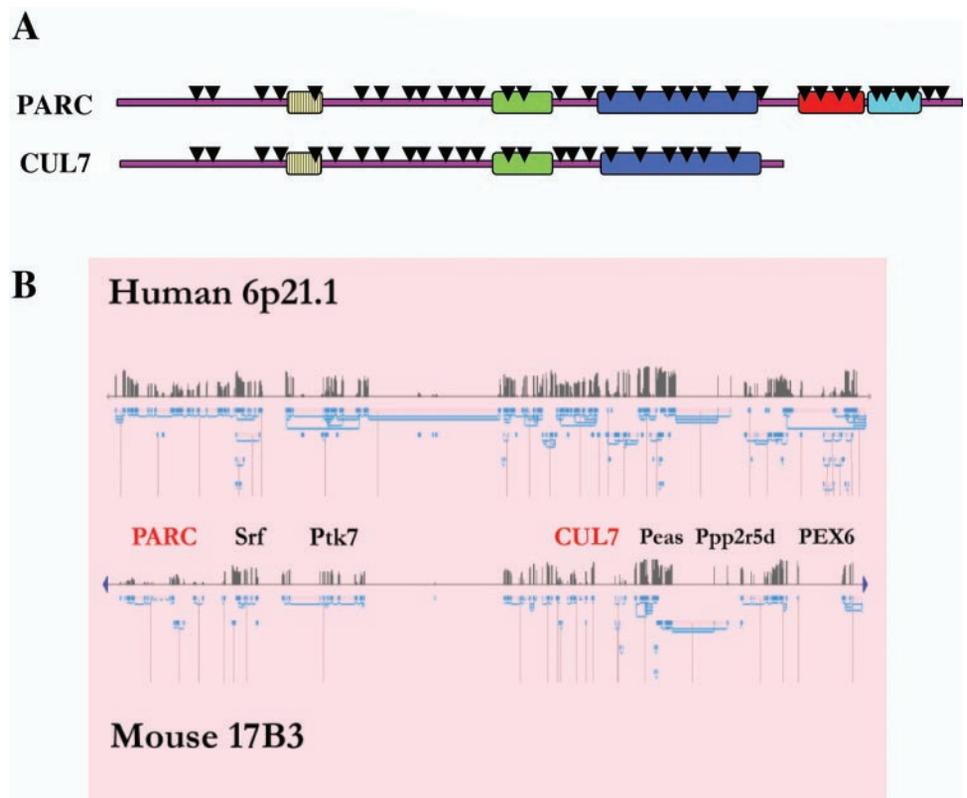


Fig. 3. *A*: comparison of the structures of the Ariadne protein *Parc* and the cullin-like protein *Cul7*. Triangles indicate intron positions (from Ref. 55). *B*: comparison of the genes and gene structures found in the regions where *Parc* and *Cul7* are localized in the human (6p21.1) and mouse (17B3) genomes. Names refer to conserved genes found in these regions. It is evident that the fusion that generated *Parc* is more ancient than the human-mouse divergence.

Ubiquitin ligases may function independently or as part of protein complexes able to act as E3s (24, 77, 90). It is still an open question whether RBRs generally work as independent E3s. In vitro, both Parkin and Dofin has been shown to be able to function independently (35, 69, 80, 94). However, the presence of a cullin domain in *Parc* led us to hypothesize that Ariadnes could function in the context of E3 complexes (55). It is well established that gene fusions tend to occur between genes that encode functionally related, often interacting proteins, and thus important insights about the functions of the gene generated by the fusion can be provided by considering those of both ancestral genes (the “Rosetta Stone strategy”; Ref. 54). Cullin proteins are well-known members of E3 complexes that also include RING finger-containing proteins in both yeasts and mammals. For example, mammalian Cullin1 and Cullin2 are part of complexes that include the RING finger protein Roc1 (reviewed in Ref. 87). In fact, Roc1 interacts with at least six cullins (70). All these data led to our hypothesis that the presence of a cullin domain in *Parc* must be an indication of it participating in similar complexes. An additional hint in favor of our suggestion was the demonstration that *Cul7* is also a member of an E3 complex that includes Roc1 (17). Indeed, immediately after our proposal, *Parc* was described as an E3 ligase that forms part of a large, still uncharacterized, cytoplasmic complex (67).

Parc ubiquitinates the tumor suppressor p53, preventing its translocation from the cytoplasm to the nucleus (67). As a sensor of DNA damage and other stresses, p53 must enter the nucleus to deter cell cycle progression and to induce apoptosis. In some tumor cells, by contrast, p53 remains and accumulates in the cytoplasm (62). Thus the downregulation of *Parc*, which would favor p53 translocation to the nucleus, is an interesting

target for therapy. Its role as a p53 negative regulator suggests that *Parc* might behave as an oncogene.

Parkin function in an evolutionary context. As already indicated, several experiments demonstrated that Parkin may act as an ubiquitin ligase on its own, and, until recently, it was assumed that Parkin was an independent E3 in vivo. Although it was already known that Parkin-mediated ubiquitination of the Pael-R is enhanced by association of Parkin with CHIP (33), a U-box protein that is also able to work independently as a ubiquitin ligase in conjunction with chaperones (reviewed in Ref. 65), it seemed unlikely that the hypothesis of Ariadnes acting in cullin-containing complexes could be generalized to Parkin or any other RBR proteins. However, Staropoli et al. (82) recently demonstrated that Parkin may be also found in a typical E3 complex containing Cullin1, and that cyclin E is a substrate for that complex. Thus it remains to be determined to what degree Parkin acts alone or as part of E3 complexes. Actually, Staropoli et al. (82) cited unpublished data suggesting that Parkin is generally part of a large complex when isolated from cerebral cortex and favored the idea that all actions of Parkin may be mediated by complexes related to the one they isolated. Thus at this point the possibility exists that not only Ariadnes and Parkin, but perhaps many or even all RBRs, may generally function in cullin-containing E3 complexes. If that turns to be correct, it would mean that the analysis of a single gene fusion, the one that generated *Parc*, could have provided enough information to predict the function (ubiquitin ligases) and partners (cullin proteins and other members of cullin-containing E3 complexes) for a whole gene family.

Another intriguing relationship among *ariadne* and *parkin* genes is the fact that *parkin* may be also, as *Parc*, an indirect

regulator of malignant progression. Cesari et al. (6) have found substantial evidence that *parkin* may act as a tumor suppressor gene. It is reasonable to hypothesize that one or several substrates of Parkin may be involved in regulation of cell cycle or apoptosis and that downregulation of *parkin* leading to an excess of those substrates could promote tumor development. Interestingly, this is the exact opposite of what we would expect to occur if one of the known substrates of Parkin, JTV1 (p38), is downregulated. JTV1 is a negative regulator of the oncogene *c-myc* (44). Overexpression of *c-myc* is often associated with cancer, but lack of Parkin leading to an excess of JTV1 protein should lead not to overexpression but to downregulation of *c-myc* expression. These results suggest that Parkin may be indirectly involved, perhaps in different tissues, in alternative and potentially contradictory aspects of cell cycle control. Finally, a very recent report suggests that *parkin* may be involved in susceptibility to leprosy (59). It is still to be determined how this result fits with the known functions of the gene.

The Parkin subfamily is animal specific and its relationships with other subfamilies are unclear, because it appears similarly distant from several of them (Fig. 2). Phylogenetic and structural analyses have so far detected putative *parkin* orthologs in several mammals, a fish, and some invertebrate species (*Drosophila*, *Anopheles*, *Caenorhabditis*). The involvement of the human gene in PD pathology and the presence of orthologs in other organisms strongly suggested that useful information could be obtained by mutagenizing these orthologs in different model organisms and testing whether the phenotypes resembled a parkinsonian syndrome. However, neither *Drosophila* nor, notably, mouse null *parkin* mutants show signs of neuronal degeneration. *Drosophila parkin* mutants have extensive anomalies in mitochondrial function that lead to varied phenotypes, from muscle defects to male sterility (26). *Parkin* mutant mice were described as almost normal, just exhibiting some anomalies in dopamine release and in behavioral tests (25, 37). Levels of several of the known substrates of Parkin (Sept5, synphilin-1, α -synuclein) were unchanged in these mutants. However, very recently it has been shown that the brain of *parkin* null mice have decreased levels of some mitochondrial proteins and proteins involved in control of oxidative stress, leading to reduced respiration and increased oxidative damage (73). These results are very interesting, because they suggest that dysfunction of Parkin, with anomalies in the ubiquitin-proteasome system, may lead to increased oxidative damage, thus linking together the two pathways that so far had been hypothesized to be primary causes of PD.

In any case, at face value, these results suggest that Parkin function may be distinct in humans vs. these two model organisms. We could even hypothesize that the dopaminergic-specific effects are due to a recent acquisition by human Parkin of a novel function. However, there is interesting evidence, most especially in the fly, suggesting otherwise. As we said above, Pael-R is a Parkin substrate. It has been shown that overexpression of Pael-R causes endoplasmic reticulum-associated stress (34). Most interestingly, pan-neural expression of human Pael-R in *Drosophila* leads to selective death of dopaminergic neurons, a phenotype that can be ameliorated by overexpression of human Parkin, or, significantly, it can be exacerbated by downregulating the expression of the endogenous *Drosophila parkin* gene in dopaminergic neurons (92).

Similarly, neural expression of human α -synuclein is also able to generate neuronal death in *Drosophila* (20), and this phenotype is suppressed by coexpression with human Parkin (92). It is reasonable then to conclude that several aspects of the parkinsonian syndrome leading to dopaminergic cell death can be mimicked in *Drosophila*. Moreover, the related effects on human Pael-R of the human and *Drosophila* Parkin proteins suggest that they retain similar dopaminergic-specific functions. Thus, although the expectation of dopaminergic-specific cell death phenotypes in animal *parkin* mutants has turned out to be unrealistic, we can still hypothesize that significant similarities in Parkin cellular functions in model animals and humans will be found. Indeed, the recent results in mutant mice described above (73) may be a first hint of the primary effects of Parkin mutations in our species.

Members of other subfamilies. Functional information on other RBR family members is accumulating and, consistent with data on *parkin* and *ariadne* genes, the involvement in ubiquitination often emerges as a common theme. Particularly interesting data have been obtained for *Dorfin*. The parallelism in the actions of Dorfin and Parkin is striking. Dorfin interacts with the E2 enzymes UbcH7 or UbcH8 through its RBR domain, whereas the COOH-terminal end of the protein binds the target substrate (68). It is likely that Dorfin acts, like Parkin, as a controller of protein quality and, similarly, it may be involved in the genesis of neurodegenerative diseases. Dorfin protein is present in the Lewy bodies found both in PD and in dementia with Lewy bodies patients, as well as in other proteinaceous aggregates found in individuals suffering multiple system atrophy or amyotrophic lateral sclerosis (ALS) (30). In addition, similarly to Parkin, Dorfin interacts with the α -synuclein-binding protein synphilin-1 through its COOH-terminal region, leading to its ubiquitination and proteasomal degradation (39). However, a direct interaction with α -synuclein has not been detected (30). In addition, Dorfin interacts with a mutated version, but not the normal one, of superoxide dismutase 1 (SOD1). This interaction leads to the ubiquitination of inactive SOD1 and its proteasomal degradation (69). Mutations on the *SOD1* gene have been implicated in the motor neuron disease ALS, that, as mentioned above, is also characterized by the formation of protein inclusion bodies. Although the mechanisms causing either sporadic or familial ALS disease are still only poorly understood, some familial ALS cases are due to *SOD1* mutations, and it has been suggested that, in those cases, misfolded SOD1 proteins may form aggregates, finally causing cytotoxicity and cell death (reviewed in Refs. 7, 88). It was reasonable then to hypothesize that Dorfin may have a protective effect against the cytotoxicity induced by the accumulation of mutant SOD1 (69), a suggestion that has recently obtained experimental support: Dorfin decreases the accumulation of mutant SOD1 in the mitochondria, thus diminishing the levels of cell-death signals released by the organelle (83). In this context, the finding that Dorfin expression is upregulated in spinal cords of patients with sporadic ALS is significant (36). It is very likely that Dorfin has additional functions, as suggested by the available information on its orthologs in mouse and invertebrates (75, 95).

Another RBR protein of clinical interest is the androgen receptor (AR) coactivator protein ARA54 (42). AR is a nuclear receptor that mediates the action of androgen on multiple

tissues of male mammals. Null AR mutant males suffer anomalies in sexual development. On the other hand, expanded polyglutamine tracts in the AR cause a neurodegenerative disease, spinobulbar muscular atrophy (Kennedy disease), characterized by the loss of motor neurons (49). It has been shown that ARA54 functions as an E3 ligase in conjunction with the E2 protein UbcH6, among other related E2s, and ubiquitinates itself for proteasomal degradation (38). Interestingly, a single copy of a mutated version of ARA54 can block the activity of the endogenous normal complement avoiding the AR-mediated transcriptional activation. Tumors in androgen-responsive tissues, such as prostate, frequently express AR and advanced prostate cancer is often treated with androgen ablation treatment. Interestingly, the dominant-negative form of ARA54 prevents cell proliferation of prostate cancer cells, suggesting that regulation of ARA54 activity is essential for AR-dependent cell growth and opening venues for cancer treatment (60). This is thus the third case, together with those previously mentioned of Parc and Parkin, of an RBR protein being potentially involved in cancer. The facts that blocking proteasome activity inhibits nuclear translocation of the AR (51) and that AR levels are precisely regulated by ubiquitination and proteasome degradation (51) suggest that ARA54 may be a key controller of AR action through the ubiquitination of AR or any of its coregulators. Significant is also the finding (66) that a paralog of ARA54 (called *IBRDC2* or *p53RFP*), regulated by *p53*, also encodes a protein with ubiquitin ligase activity and seems to negatively regulate the level of the cyclin/Cdk-interacting protein p21, suggesting an additional involvement of ARA54 subfamily genes in cell cycle control.

Another interesting recent study that emphasizes the connections of RBR proteins with transcriptional regulation refers to TRIAD3 (also called ZIN). Chen et al. (8) demonstrated that TRIAD3 protein interacts with a protein kinase, RIP, that is involved in activation of the transcription factor NF- κ B after stimulation of the tumor necrosis factor receptor. Overexpression of *TRIAD3* inhibits NF- κ B activation and sensitizes the cells to apoptotic signals (8). Finally, a recent report (91) implicates human XAP3 (also called HOIL-1) in ubiquitination leading to degradation of IRP2, a protein that acts as posttranscriptional regulator of genes involved in iron metabolism, by inhibiting translation of their transcripts at low iron levels (reviewed in Refs. 19, 40). Action of XAP3 depends on IRP2 being oxidized, and it has been suggested that XAP3 could act as a general controller of oxidative damage (40). It is known also that XAP3 proteins interact with protein kinase C, again suggesting multiple roles for members of this subfamily (11, 85).

Future Prospects

We can conclude that RBR proteins constitute an ancient and highly diversified family of proteins and that members of this family are able to perform very diverse tasks. Although a main role of the RBRs in control of protein quality through their action as ubiquitin ligases is quite well established, alternative roles, most especially as indirect transcriptional regulators, are starting to emerge. Significant clues point to RBRs as being involved in multiple human pathologies, in particular neurodegenerative diseases, with PD being just the most obvious example, and cancer. Two of the main venues of

research for the future are the characterization of the substrates of additional RBR proteins and the different protein complexes in which RBRs participate as ubiquitin ligases. We can expect also discoveries of alternative roles, different from tagging proteins for destruction, for several RBR members.

Comparative genomics can be used to select model organisms in which to study the functions *in vivo* of RBR proteins. If we put the emphasis on understanding their roles in humans, then the phylogenetic range of most of the RBR subfamilies suggests that interesting biomedical experiments could be performed not only in the mouse, but also in invertebrates, as has been shown already for *parkin* in *Drosophila*. On the contrary, fungal or plant RBRs are so distantly related and structurally different from animal RBRs that in general we cannot expect to obtain significant information from these organisms. An exception may be the Ariadne subfamily. Quite similar members of this extremely ancient ensemble seem to be present in all eukaryotes, and thus interesting information could be provided from the comparative functional study of Ariadnes in very diverse species.

Final questions that remain are when the RBR domain emerged and which were the characteristics of the early RBR proteins. Although different answers are compatible with the available data, an attractive possibility is that the ancestral RBR gene was similar to a modern Ariadne and that the RBR domain has been since then occasionally recruited to be used as part of other proteins. The alternative is that a few different RBRs (at most three or four) existed before the diversification of eukaryotes. Additional data provided by "exotic" genomes (e.g., other protists) may contribute to sort out between these two options.

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