

Protein family review

The ring between ring fingers (RBR) protein family

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Summary

Proteins of the ring between ring fingers (RBR)-domain family are characterized by three groups of specifically clustered (typically eight) cysteine and histidine residues. Whereas the amino-terminal ring domain (N-RING) binds two zinc ions and folds into a classical cross-brace ring finger, the carboxy-terminal ring domain (C-RING) involves only one zinc ion. The three-dimensional structure of the central ring domain, the IBR domain, is still unsolved. About 400 genes coding for RBR proteins have been identified in the genomes of uni- and multicellular eukaryotes and some of their viruses, but the family has not been found in archaea or bacteria. The RBR proteins are classified into 15 major subfamilies (besides some orphan cases) by the phylogenetic relationships of the RBR segments and the conservation of their sequence architecture. The RBR domain mediates protein-protein interactions and a subset of RBR proteins has been shown to function as E3 ubiquitin ligases. RBR proteins have attracted interest because of their involvement in diseases such as parkinsonism, dementia with Lewy bodies, and Alzheimer's disease, and in susceptibility to some intracellular bacterial pathogens. Here, we present an overview of the RBR-domain containing proteins and their subcellular localization, additional domains, function, specificity, and regulation.

Gene organization and evolutionary history

The ring between ring fingers (RBR) proteins are a large and diverse group of proteins characterized by a compact sequence module that is predicted to form three ring finger-type, or 'ring', domains separated by loops [1,2]. The RBR domain usually occurs as part of a multidomain protein with diverse functional modules, and appears to mediate protein-protein interaction. The function of most of the family members has not yet been explored experimentally, but a subset of RBR proteins is known to have E3 ubiquitin ligase activity.

The sequence of each ring domain in the RBR region contains a cluster of, typically, eight cysteine and histidine residues that potentially bind metal ions. The amino-terminal ring domain

(RING1 or N-RING) is thought to bind two zinc ions and to fold into a classical cross-braced ring finger, whereas the carboxy-terminal ring (RING2 or C-RING) appears to bind only one metal ion and forms a hydrophobic core different from that of classical ring fingers [3]. The central cysteine/histidine cluster is likely to also form a ring finger-type structure. Morett and Bork [4] derived a general sequence profile-based characterization of this domain and called it IBR (in between rings). Independently, van der Reijden *et al.* [5] identified this domain - with a more restricted PROSITE-like pattern [6] C₆HC - as DRIL (double ring finger linked) domain, and called the family of RING-DRIL-RING-containing proteins TRIAD. The two definitions are largely overlapping but not identical. The approach of

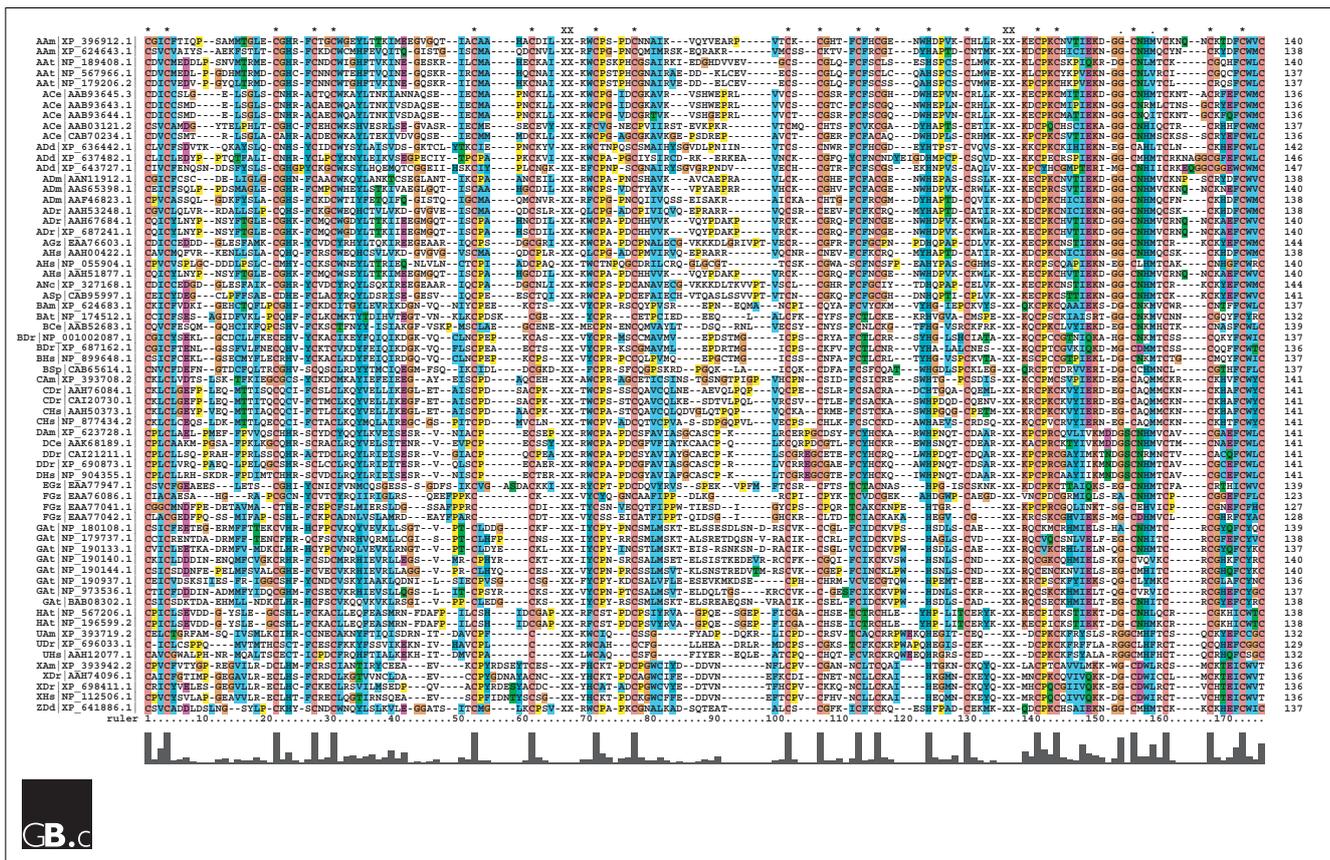


Figure 1

Multiple alignment of RBR domain segments. The figure includes the sequences of 64 representative RBR segments. This alignment is part of the grand alignment used to create the phylogenetic tree in Figure 2. The sequence identifier gives the subfamily code (first letter) followed by the taxon and the accession number in GenBank. The loop segments between the three ring-like domains are indicated by 'XX'. The gray bars at the bottom represent the degree of amino acid type conservation of alignment positions. The alignment was primarily generated with CLUSTALX [70] and edited manually. Taxon abbreviations: Am, *Apis mellifera*; At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Gz, *Gibberella zeae*; Hs, *Homo sapiens*; Nc, *Neurospora crassa*; Sp, *Schizosaccharomyces pombe*.

Morett and Bork [4] is consistent with the concept of sequence homology and the criterion of statistically significant sequence similarity and is therefore the preferred one.

RBR proteins make up a large, diverse family with, at present, around 400 representatives in sequence databases. On the basis of sequence conservation within the RBR segment, RBR proteins are assigned to 15 subfamilies (A-I, P, S, T, U, X, Z; Figure 1). There are also some 10 orphan sequences that could be considered as their own groups. Subfamily A (Ariadne-like proteins) can be further subdivided into around 10 clusters (Ao-A9). Similarly, several subgroups can be distinguished for subfamilies S (found only in viruses), T (the TRIAD3 proteins), and Z (found only in protozoa). The alignment of RBR segments from sequence representatives of the various groups is shown in Figure 1 and details with respect to the conservation of the cysteine/histidine pattern and loop sizes are given in Additional data

file 1, available online with this article. Additional information (sequence lists, subgroup alignments, annotations, and supplementary text and tables) is also available at our website [7].

Genome sequencing projects have added considerably to the information on RBR genes; they comprise a complex and ancient gene family that has been found in all eukaryotes examined and in some of their viruses. There are two RBR proteins in the yeast *Saccharomyces cerevisiae*, six in *Drosophila melanogaster*, 10 in *Caenorhabditis elegans*, about 40 in *Arabidopsis thaliana*, around 23 in the zebrafish (*Danio rerio*), and about 15 in humans (Additional data file 1). Genes of RBR proteins are dispersed throughout the genome. They vary in chromosomal location and exon number. For example, Mladek *et al.* [8] have studied the Ariadne (A) subfamily in *Arabidopsis*: the 16 *AtARI* genes are distributed between all five chromosomes at 10 loci. Despite the conserved sequence, they have distinct gene

structures. The number of exons varies between one (*AtARI3*) and 15 (*AtARI5*, *AtARI7* and *AtARI8*). The *AtARI* genes are differentially expressed during plant development and in an organ-specific manner.

Splicing isoforms of RBR proteins are widespread [8-10]. At least for some RBR proteins, alternative splicing leads to shortened isoforms that control the cellular localization and function of a respective parental larger isoform [9]. For the rat transcription factor RBCK1, the RBR domain has a crucial role in its transcriptional activity [11]. RBCK2 has been identified as an alternative splice variant of RBCK1 that lacks the carboxy-terminal part of RBCK1, including the RBR region [11]. Yoshimoto *et al.* [9] showed that RBCK2 represses the transcriptional activity of RBCK1 by tethering it within the cytoplasm. A similar alternative splice variant lacking the RBR region has been reported recently for parkin [12].

The genetic module coding for the RBR region has apparently been reused in several gene contexts during evolution and has been sequentially modified by point mutations and insertions/deletions and shuffled into new genomic locations. A retrotransposition-based mechanism has been proposed to underlie these changes and evidence hinting at this comes from *Arabidopsis* [8]. A putative phylogenetic relationship between the RBR segments in various taxonomic ranges is presented in Figure 2, and it is notable that some RBR protein subfamilies are found only in specific taxonomic groups (Additional data file 1); for example, subfamilies C (such as RING finger protein 144), I (IBR domain-containing protein 1, IBRDC1), P (parkin), U (Ariadne-like E3 ubiquitin ligase, PAUL) and X (human hepatitis B virus X-associated protein, XAP) have been found only in animals. Several other subfamilies are specific for fungi (subfamilies E and F) and plants (subfamilies G and H). Besides the large number of RBR protein genes in higher eukaryotes, they also notably occur in unicellular eukaryotes and in entomopoxviruses and iridoviruses [7] (Additional data file 1). Interestingly, there are no genes in archaeal or eubacterial genomes that could truly be called RBR family members.

Characteristic structural features

The whole RBR segment typically contains some 200 consecutive amino acids. The ring-like sequence domains tend to get smaller going from the amino to the carboxyl terminus - N-RING around 60 residues, IBR around 50 residues, and C-RING around 40 residues [1,2]. Loops on the amino-terminal side of the RBR region have a higher sequence and size variability. In contrast, the cysteine/histidine positions are more strongly conserved in N-RING and the IBR. Substitutions with non-cysteine or non-histidine residues at cysteine/histidine pattern positions are observed only in the C-RING domain, which is known to

bind only one zinc ion. Some RBR proteins seem to remain functional without C-RING, but parkin and others apparently require this part of the RBR region for their correct function as E3 ubiquitin ligases [2].

Unfortunately, little direct structural information is available for the RBR region. Capili *et al.* [3] reported the three-dimensional structure (PDB accession number 1WD2) of the C-RING in the human Ariadne protein HHARI (residues 326-395 of UNIPROT accession number Q9Y4X5, subfamily A1/Ari1), which folds into a novel structure binding a single zinc ion (Figure 3a). Also, a three-dimensional structure (PDB 1WIM, structure report unpublished) that essentially represents the N-RING (residues 20-100) in the human UbcM4-interacting protein 4 (subfamily C/RNF144; UNIPROT accession number P50876) is very similar to a classical ring-finger domain (Figure 3b). No structural information is known for any IBR domain.

As well as conservation within the RBR segment, the sequence architecture conserved among distant taxa is an important criterion for the classification of RBR proteins, as shown in Figure 4. The functional significance of the additional segments and their cooperation with the RBR part of the sequence is most often not clear, and is certainly an urgent research task for the near future. For example, it would be of interest to determine whether the two hydrophobic regions in the dorfin (subfamily D), each the length of a transmembrane helix, do function as membrane-attachment modules or whether they are critical for protein complex formation. The available data are conflicting. The two long transmembrane helices (each 30-35 residues long with conserved prolines) are predicted by the programs TMHMM [13] and DAS-TMfilter [14,15] at the carboxy-terminal side of the dorfin RBR region. A carboxy-terminal deletion that includes the hydrophobic segment but leaves the RBR region untouched results in an enzyme unable to bind ubiquitinated substrates [16], suggesting that this region is responsible for ubiquitin binding. On the other hand, localization of the protein near the nuclear membrane and the centrosome, co-localization with vimentin, and interaction with the calcium-sensing receptor CaR (an integral membrane protein) support membrane-embedding [16,17]. RNF144 (subfamily C) and IBRDC1 (subfamily I) also have a predicted transmembrane helix at the carboxy-terminal side of the RBR region, and localization at the Golgi membrane has been shown for RNF144 [18].

The combination of the RBR region with domains and motifs known from the ubiquitination pathway (such as ubiquitin-associated UBA, ubiquitin UBQ, and ubiquitin-interacting motif, UIM) is not a real surprise, whereas the association with helicase domains (DEXDc and HELICc), nucleic-acid-binding domains (KH and RRM) and protein-binding domains (RWD, Armadillo/HEAT repeats and ankyrin segments) would make sense in the context of the involvement of RBR

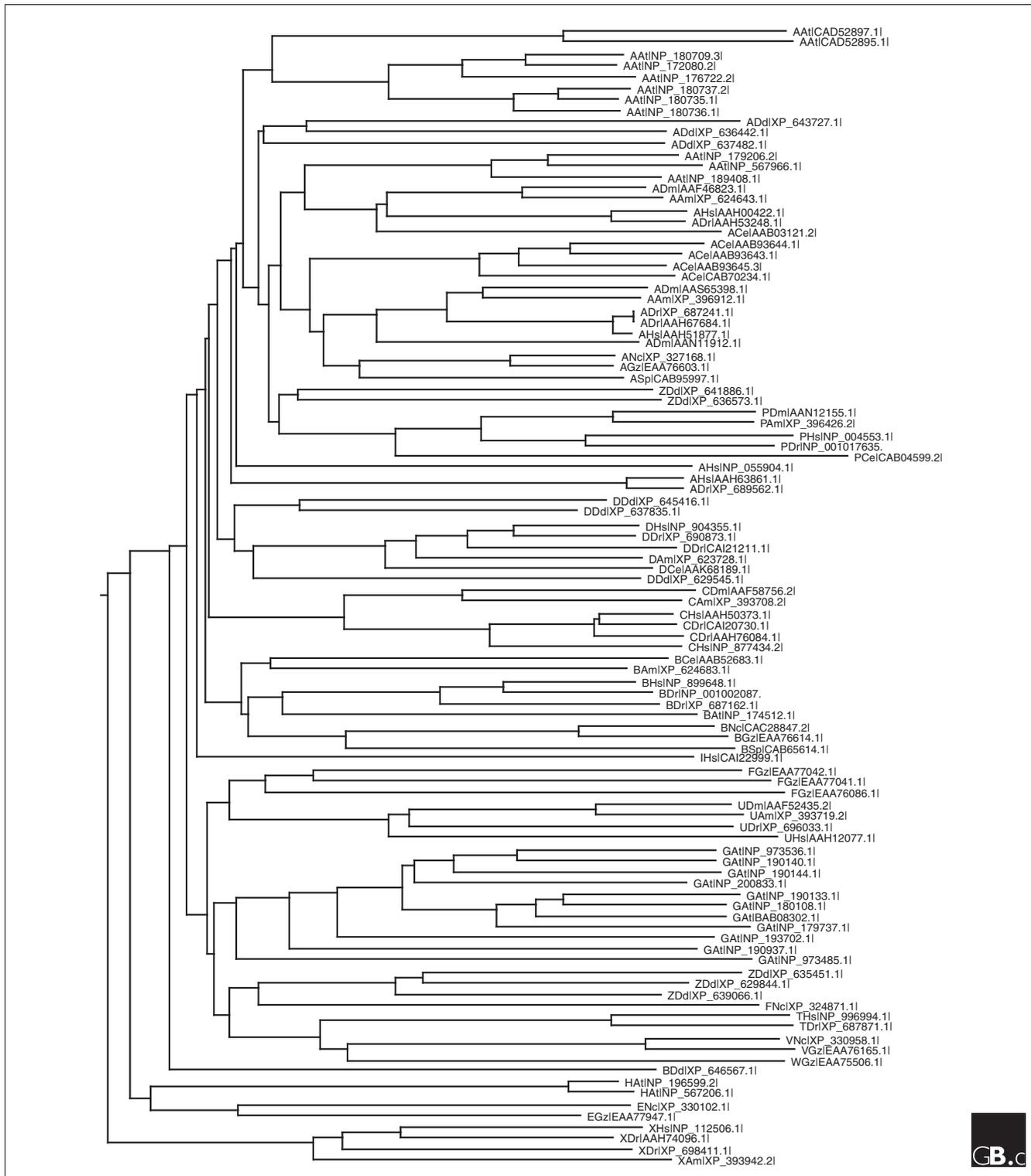


Figure 2
 Phylogenetic tree of RBR domain segments. A grand alignment of RBR segments from 102 proteins representative of the most populated subgroups was used to create the tree. We used the program SEAVIEW and the tool ATV from the Forester package [71-73]. Each entry is labeled as in Figure 1. Typically, subfamily members cluster nicely together and the phylogenetic relationships within subfamilies are determined with significance. Closer to the root of the tree, the branching becomes increasingly uncertain. Some groups of fungal and protozoan sequences are more heterogeneous and appear at several tree positions. The *D. discoideum* sequence XP_646567.1 does not appear together with other ARA54 sequences but was assigned to the group 'B (ARA54)' because of the RVD domain in the sequence architecture.

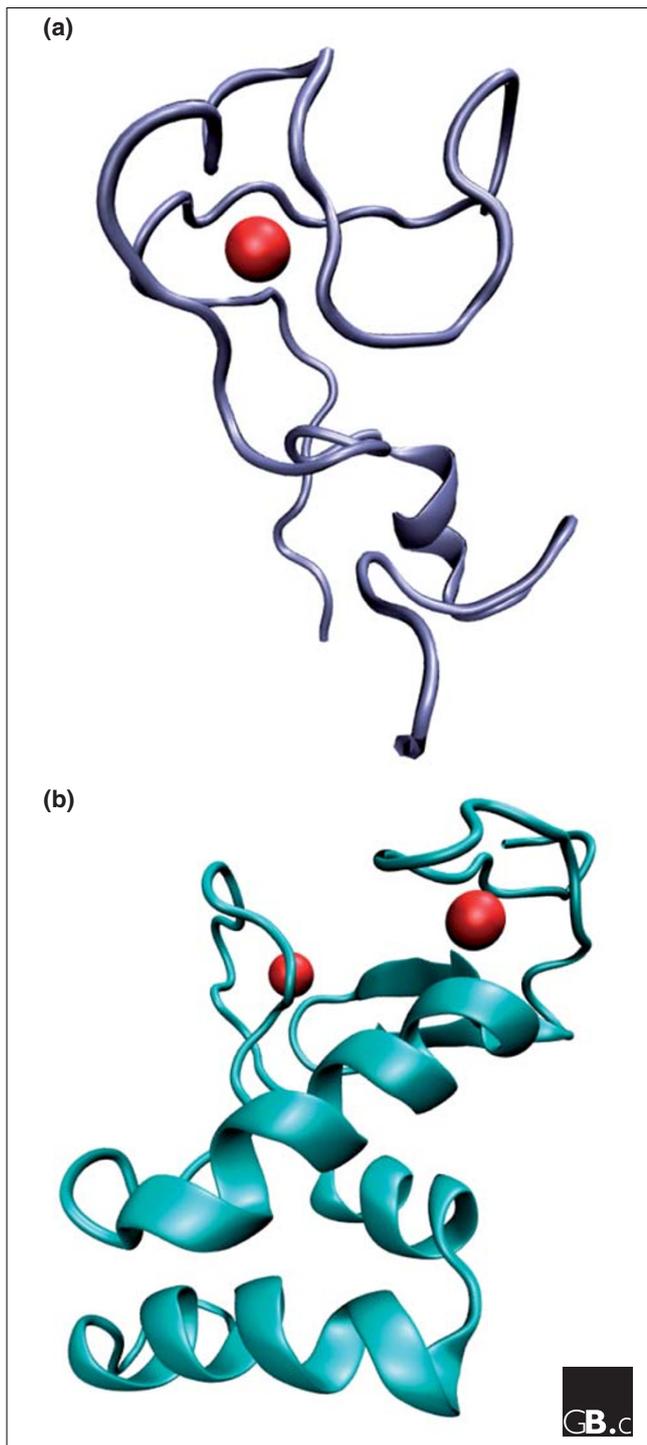


Figure 3
Structures of ring domains in the RBR segments. The structure graphics were generated with the program VMD [74]. The backbone trace and the secondary structure are shown as ribbons and the zinc ions as red spheres. **(a)** Carboxy-terminal part of the RBR region (C-RING) in the human Ariadne-I homolog protein HHARI (PDB accession number 1WVD2; residues 326-395 of UNIPROT accession number Q9Y4X5; subfamily A1/Ari1). **(b)** The N-RING of the RBR segment of the human UbcM4-interacting protein 4 (PDB accession number 1WIM1; residues 20-100 of UNIPROT accession number P50876; subfamily C/RNF144).

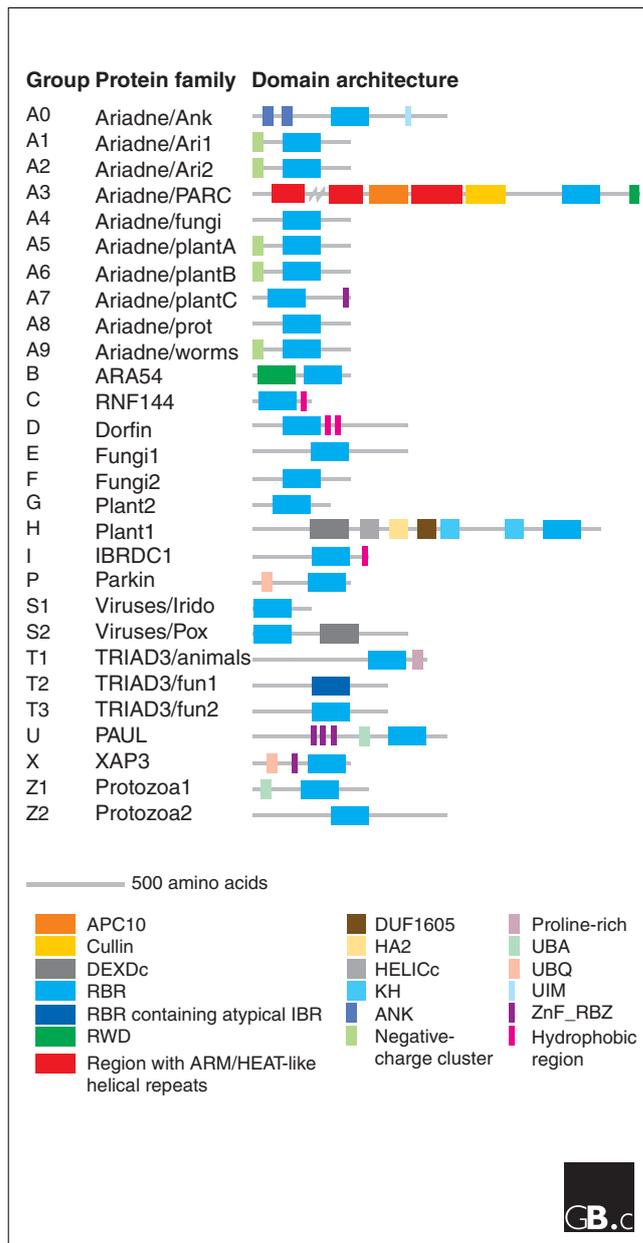


Figure 4
Sequence architecture of RBR proteins. The detailed sequence architecture of the subfamilies of RBR proteins is shown. The globular domains (such as cullin) and non-globular regions (negative-charge clusters or proline-rich regions) are color-coded as shown in the key. It should be noted that the typical sequence architecture is shown. There are several exceptions: for example, PAUL proteins mostly contain three additional zinc fingers, but a few representatives have only one. Similarly, dorfins have two predicted hydrophobic helices with the exception of the protozoan members, which have only one or none. The two sequences of the Fungi1 group are very diverse and have only the RBR segment in common. Among the Plant1 representatives, one protein contains two RRM domains instead of the usual two KH domains. Domain accession numbers: APC10, PF03256; cullin, SM00182; DEXDc, SM00487; IBR, SM00647; RWD, SM00591; DUF1605, PF07717; HA2, PF04408; HELICc, SM00490; KH, SM00322; RRM, SM00360; ANK, SM002481; UBA, SM00165; UBQ, SM00213; UIM, SM00726; and ZnF_RBZ, SM00547. The first two letters 'PF' and 'SM' indicate the PFAM and SMART databases, respectively.

proteins in the regulation of gene expression. A functional link between ubiquitination and RNA metabolism appears to be a general phenomenon [19].

Localization and function

RBR proteins fulfill diverse functions, ranging from the control of protein quality to the regulation of translation and signaling [10,20-22]. This diversity is highlighted by the manner in which some RBR proteins were originally discovered. Protein-protein interaction studies first attracted attention to the RBR proteins. For example, XAP3 [23] and rat RBCK1 [11] were discovered with the regulatory domain of protein kinase C- β -interacting protein as a bait, and the Ariadne proteins were found as interaction partners of ubiquitin-conjugating enzymes (E2s) in fruit flies, mice and humans [24-27]. The putative Ariadne-like E3 ubiquitin ligase PAUL appeared in a complex extracted with the cytoplasmic domain of the muscle-specific kinase (MuSK) as bait [28]. Parkin is the best characterized RBR protein functionally [29,30]. In a clinical context, mutations in the gene encoding parkin are associated with sporadic early-onset parkinsonism and autosomal recessive juvenile parkinsonism [29-30] and parkin has recently also been associated with susceptibility to intracellular pathogens such as *Salmonella typhi*, *S. paratyphi* and *Mycobacterium leprae* and cancer [31-35]. Several other RBR proteins are involved in human neurodegenerative diseases, susceptibility to infections, and cancer [2, 21,36,37].

E3 ubiquitin ligase activity has been reported for 15 RBR proteins, and 25 of their substrates and more than 70 interactors have been identified so far (Figure 5; Additional data file 1). As typical E3 enzymes, RBR proteins interact with ubiquitin-conjugating enzymes (E2s) and catalyze the covalent attachment of ubiquitin to target proteins [38]. For most of the characterized RBR proteins, the N-RING is essential for recruiting specific E2s and binding substrates. There are exceptions to this rule, however, and substrate interactions have been defined for non-RBR regions as well (Figure 5; Additional data file 1). RBR proteins are considered single-molecule E3 ubiquitin ligases (E3s). However, parkin and the parkin-like cytoplasmic protein (PARC) interact with components of the SCF-like E3 ubiquitin ligase complex, such as cullin and F-box proteins [39,40].

Parkin and dorfin protect dopaminergic neurons from the consequences of mitochondrial damage [41-43] and from harmful levels of aggregation-prone proteins by ubiquitin-mediated proteasomal degradation and/or subcellular tethering of such proteins [29,30,44]. They are also involved in the endoplasmic reticulum-associated degradation pathway (ERAD). This is supported by their interaction with E2s associated with the endoplasmic reticulum and by their role in promoting the degradation of unfolded or misfolded forms of transmembrane proteins,

such as the parkin-associated endothelin receptor-like receptor (Pael-R) [45], synaptotagmin XI [46], the AAA-ATPase valosin-containing protein [47], and the dopamine transporter [48], before they accumulate in the endoplasmic reticulum. Moreover, plasma membrane receptors such as the Toll-like receptors TLR4 and TLR9 are substrates for the RBR protein TRIAD3A [10]. This led to the hypothesis that TRIAD3A controls the intensity and duration of pro-inflammatory responses mediated by Toll signaling. A recent study proposes a novel role for parkin in regulating signaling from the epidermal growth factor receptor (EGFR) through its ability to bind EGFR and the EGFR pathway substrate 15 (EPS15) and to ubiquitinate EPS15, consequently regulating the internalization and degradation of EGFR [22].

At least three RBR proteins are involved in the regulation of the cell cycle and apoptosis. PARC acts as negative regulator of the tumor suppressor protein p53. Overexpression of PARC was shown to sequester p53 in the cytoplasm without ubiquitinating it for degradation [49]. In contrast, the RBR protein p53RFP, a member of the RNF144 subfamily (C), targets an inhibitor of cell cycle progression (p21^{WAF1}) for degradation. The zinc finger protein inhibiting NF- κ B protein (ZIN), a splicing variant of TRIAD3, similarly supports the degradation of an inhibitor of NF- κ B activation (RIP). Overexpression of p53RFP or RIP induces apoptosis [50,51].

The protein Vif, encoded by the human immunodeficiency virus (HIV), induces the translocation of ZIN to the nucleus, suggesting that RBR proteins might be attractive candidates for interfering with virus replication. Vif is important for the assembly of HIV-1 particles and the stability of the reverse transcription complex [37]. Another possible regulator of virus infection is the RBR protein heme-oxidized IRP2 ubiquitin ligase (HOIL-1), which interacts with the X protein of hepatitis B virus and enhances its ability to activate X-responsive promoters [23].

RBR proteins of the Ariadne subfamily are probably involved in translational regulation, as the Ariadne-subfamily protein HHARI ubiquitinates the eukaryotic mRNA cap-binding protein 4EHP [20], which apparently alters the binding efficiency of 4EHP to mRNA caps. 4EHP ubiquitination may also be a signal for the intracellular compartmentalization of specific mRNA populations.

RBR proteins can be recruited by their interaction partners to particular subcellular compartments. For example, RBCK1 is translocated to the nucleus via an interaction with its RBR-domain-deficient splicing variant RBCK2 [9]. Such nucleo-cytoplasmic shuttling is frequent with RBR proteins and is supported by the protein-interaction map of *Drosophila* [52], which revealed an interaction of the RBR protein ARI-2 with a classical nuclear transport receptor, karyopherin 3. On the other hand, some RBR members may be able to regulate nucleo-cytoplasmic transport themselves.

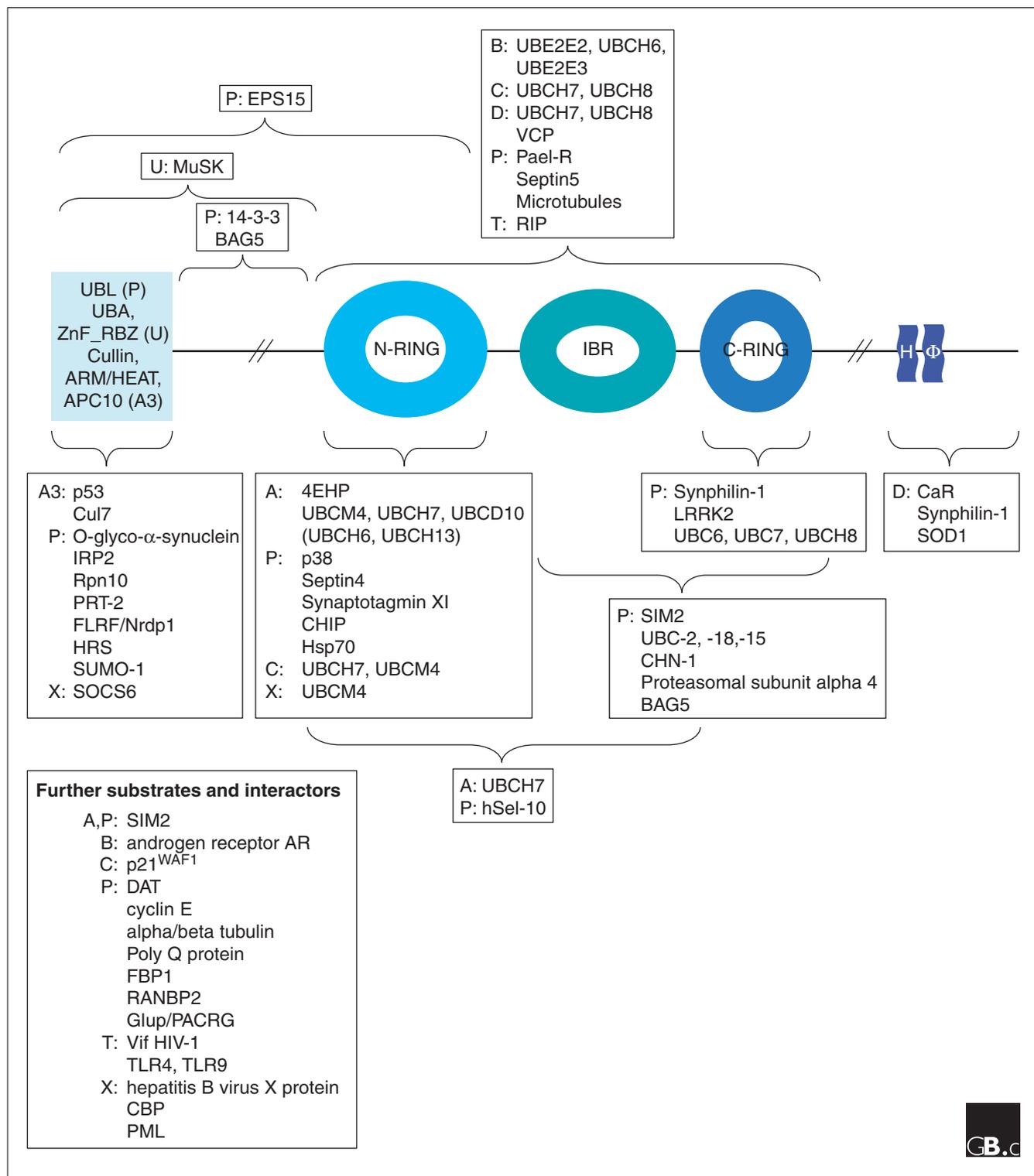


Figure 5
 Known interactors with the RBR protein. The interacting proteins for each region are boxed and are preceded by the name of the subfamily of RBR proteins with which they interact: A, Ariadne; A3, Parc; B, ARA54; D, dorfin; P, parkin; C, RNF144; X, XAP; U, Paul; T, TRIAD3. The additional amino-terminal domains present in some subfamilies of RBR proteins are highlighted in light blue and indicated by the domain abbreviation with the subfamily in which they are found in parentheses. UBL, ubiquitin-like domain; UBA, ubiquitin-associated domain; ZnF_RBZ, zinc finger; cullin, cullin-like domain; ARM/HEAT, Armadillo and HEAT repeats; APC10, anaphase-promoting complex subunit 10. A carboxy-terminal hydrophobic segment is present in the dorfins. Additional substrates and interactors are listed in Additional data file 1.

For example, parkin interacts with and ubiquitinates Ran-binding protein 2 (RanBP2), which is related to the small ubiquitin-related modifier (SUMO) E3 ligase family and is a component of the nuclear pore complex [53]. Binding of parkin to SUMO-1 enhances parkin's nuclear translocation and auto-ubiquitination, indicating that both its E3 activity and subcellular localization are modulated through association with SUMO-1 [54].

Mechanisms of regulation

RBR proteins are also subject to posttranslational regulation that predominantly tends to inhibit their E3 activity. Parkin's stability is controlled by the RING finger domain E3 ligase FLRF/Nrdp1, which interacts with the amino terminus and reduces parkin's half-life and enzymatic activity [55]. Auto-ubiquitination has been shown to inhibit E3 activity in members of the Ariadne, androgen receptor-associated protein 54 (ARA54), RNF144, dorfin, parkin and TRIAD3 subfamilies [10,16,49,56-62] (Additional data file 1). Parkin's E3 activity is also suppressed through the binding of the chaperone-like protein 14-3-3 η to its linker region (Figure 5). 14-3-3 η is released from this inhibitory complex upon its tight binding of α -synuclein. Thus, parkin's activity is mutually regulated by 14-3-3 η and α -synuclein [63]. A comparable antagonistic regulation of parkin by the carboxyl terminus of the Hsc-70-interacting protein (CHIP) and the chaperone Hsp70 is involved in the degradation of unfolded Pael-R [45]. Whereas Hsp70 inhibits parkin's E3 activity by forming a complex with unfolded Pael-R and parkin, CHIP induces the dissociation of Hsp70 and enhances parkin's activity and the degradation of Pael-R. Parkin is also inhibited through its enhanced sequestration to protein aggregates on interaction with Bcl-2-associated athanogene 5 (BAG5) and Hsp70 [64].

Phosphorylation is involved in the negative regulation of parkin, as stress-induced reduction of phosphorylation results in an increase in its activity [65]. Parkin is controlled by nitric-oxid (NO) modifications in a biphasic manner [66,67]. Within the first two hours of *S*-nitrosylation, parkin's catalytic activity increases. Then it declines gradually and is inhibited 24 hours after NO exposure [68]. Whereas phosphorylation and *S*-nitrosylation are reversible, a novel irreversible covalent adduct, a dopamine-derived catechol modification of parkin, has been detected that decreases E3 activity and solubility. Parkin is particularly sensitive to this modification, as the adduct could not be transferred to the Ariadne-subfamily member HHARI [69].

Frontiers

RBR proteins and their interaction partners appear to be involved in nearly all major cellular events: transcription and RNA metabolism, translation, subcellular tethering, regulation of posttranslational modification and protein stability, cellular and stress signaling, cell-cycle control, and

the course of microbial infection. These glimpses into the diverse functions of RBR proteins have mainly been gained by detailed analyses of the multipurpose neuroprotective agent parkin, some ten other animal RBR proteins and a single member in *Arabidopsis*. Essentially nothing is known about the remaining 350 or so RBR proteins, in particular the animal-specific IBRDC1, and the plant-, fungus-, protozoa- and virus-specific subfamilies (H, G, F, E, Z and S). It is likely that some of these RBR proteins also have E3 activities. It is not yet clear, however, whether RBR proteins act as single molecules and/or in SCF-like E3 ligase complexes, and whether they catalyze mono- or polyubiquitination and lysine 48- or 63-types of linkages. Apart from the E3 ligase function, RBR segments might serve as activation domains, interact with cytoskeletal components, and act as tethering modules. Therefore, one future challenge is to analyze their changing subcellular distributions.

Several RBR proteins are associated with neurodegenerative and infectious diseases, and the three-dimensional structures of the IBR and the complete RBR domain, and knowledge of the residues responsible for interactions and structural stabilizations, will be a prerequisite for identifying specific target sites for the possible design of therapeutic drugs. Future work should reveal the functional significance of the additional domains and their cooperation with the RBR domain. This is particularly important for members of the neglected organism-specific subfamilies, as these are found in organisms that pose pathogenic, agricultural and aquacultural threats (for example, the human pathogenic protozoan *Entamoeba histolytica* and fungus *Aspergillus fumigatus*, the plant pathogenic fungi *Gibberella zeae* and *Magnaporthe grisea*, and the fish and amphibian viruses lymphocystis disease virus and grouper iridovirus). More surprises and astonishing and valuable discoveries are expected from the future analysis of RBR proteins.

Additional data file

Additional data file 1, containing supplementary tables and additional references, is available online.

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