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Review

The HERC proteins: functional and evolutionary insights

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Abstract. HERC proteins are defined as containing both HECT and RCC1-like domains in their amino acid sequences. Six HERC genes have turned up in the human genome which encode two different sorts of polypeptides: while the small HERC proteins possess little more than the two abrementioned domains, the large ones are giant proteins with a plethora of potentially important regions. It is now almost 10 years since the discovery of the first family member and information is starting to accumulate pointing to a general role for these proteins as ubiquitin ligases involved in membrane-trafficking events. In this review, the available data on these six members are discussed, together with an account of their evolution.

Key words. HERC proteins; RCC1-like domain; HECT domain; ubiquitin ligase.

Definition

According to the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC), all proteins containing both HECT and RCC1-like domains in their amino acid sequences shall be referred to as HERC proteins and numbered according to the order in which they are approved at HGNC [1].

The HECT domain

HECT (homologous to E6AP COOH terminus) domains are protein domains showing a high degree of similarity (usually around 50%) with the carboxyl-terminal region of E6-associated protein (E6AP). The latter was originally identified as the cellular protein mediating the association between the tumor suppressor p53 and the E6 oncoprotein of tumorigenic human papillomaviruses HPV-16 and -18 [2, 3]. Shortly afterwards, it was discovered that the E6-E6AP complex functions as a ubiquitin ligase for p53, thereby inducing its proteasomal degradation [4]. This and later studies also showed that E6AP's ubiquitin ligase activity is not restricted to pathological conditions, but that instead it constitutes the normal function of this protein, with the sole difference that under physiological conditions it is not p53 but other proteins which undergo E6AP-mediated ubiquitination [5–7]. In addition, a few years after the initial identification of E6AP, a number of proteins were found that possess C-terminal regions very similar to that in E6AP. What is more, several of these proteins were shown to resemble E6AP not only in the sequence of its approximately last 350 amino acid residues, but also in its ability to form thioester bonds with ubiquitin, indicating that these proteins may also be active ubiquitin ligases [8, 9]. Thus, the HECT domain, as this C-terminal stretch of conserved amino acids came to be known, was established as a structural feature that endowed the proteins containing it with the ability to act as ubiquitin ligases. Protein ubiquitination is accomplished through a hierarchical enzymatic cascade consisting of a single ubiquitin-activating enzyme (E1), a limited number of ubiquitin-conjugating enzymes (E2) and many ubiquitin

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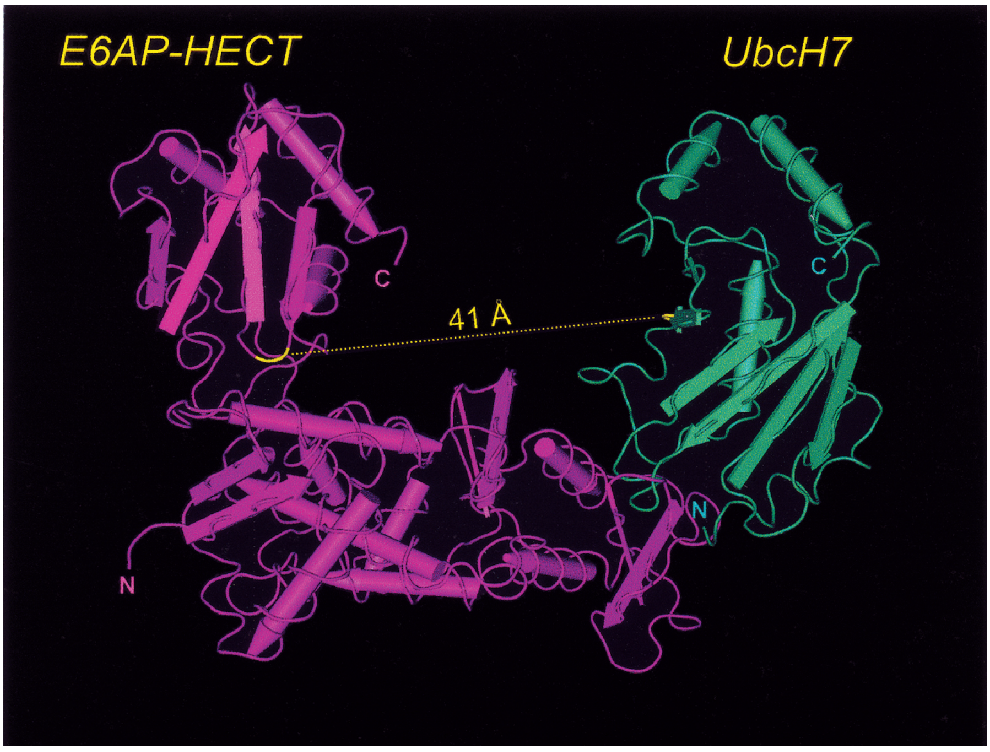

Figure 1. HECT domain structure. The 2.6 Å-resolution structure of the HECT domain of E6-associated protein (E6AP) bound to the E2 ubiquitin-conjugating enzyme UbcH7 is shown [11]. The HECT domain (residues 495–852 of human E6AP, shown in pink) consists of a large, mostly α-helical, N-terminal lobe (N-lobe, residues 495–737), which is connected by a three-residue hinge (residues 738–740) to the smaller C-lobe (residues 741–852, top left), which displays an α/β structure and contains the active site cysteine that forms the thioester bond with ubiquitin (Cys-820). Regarding UbcH7, it also has a mixed α/β structure and binds to the N-lobe of the HECT, in a position that allows its catalytic cysteine residue (Cys-86) to directly face the HECT Cys-820, which is 41 Å away in an open line of sight, as shown in the figure (both catalytic cysteines have been depicted in yellow). All data were obtained from [11]. The figure was generated using the program Cn3D (www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). The protein data bank (PDB) entry for the E6AP-UbcH7 structure is 1D5F.

ligases (E3). The great abundance of E3s enables them to specifically recognize substrates, this being the most important function of ubiquitin ligases. Currently, HECT domain proteins are regarded as one of two major classes of E3s (for a review on the ubiquitin system see [10]). Unlike all other E3s, HECT ubiquitin ligases utilize a covalent mechanism that involves the formation of a thioester bond between a highly conserved cysteine residue in the HECT active site and the C-terminus of ubiquitin [9]. Some insight into how this actually happens can be obtained from the crystal structure of the HECT domain of E6AP bound to the E2 UbcH7 (fig. 1 and [11]). In this structure, the HECT consists of two lobes with the active site cysteine located at the interface between the two, directly facing the active site cysteine in UbcH7, from which ubiquitin must be transferred to the HECT. For this to take place, though, the long distance separating the two cysteines in the crystal (41 Å) must first be surmounted, which presumably entails a conformational change induced by ubiquitin upon its binding to UbcH7 [11]. Once the HECT-ubiquitin conjugate has been formed, ubiquitin must be transferred to a substrate's lysine residue to form a stable isopeptide bond. However, since it has been shown that substrates usually bind outside the HECT [12], ubiquitin conjugation to substrates probably requires an intramolecular rearrangement so that the target lysine residue in the substrate can get close enough to the HECT active site. So, it seems clear that both the structure and function of HECT domains have been conserved during evolution. As will be seen now, the same does not appear to have occurred with RCC1-like domains.

The RCC1-like domain

The RCC1-like domain, or RLD, is a structural feature found in many proteins which displays high similarity to the sequence of the RCC1 protein (regulator of chromosome condensation-1). The RLD is characterized by the presence of several (usually seven) repeats of 51–68 aa each, thus making for a domain of up to 400 aa residues. The three-dimensional structure of the RCC1 RLD reveals a seven-bladed β-propeller fold wherein each blade corresponds to the previously identified sequence repeats

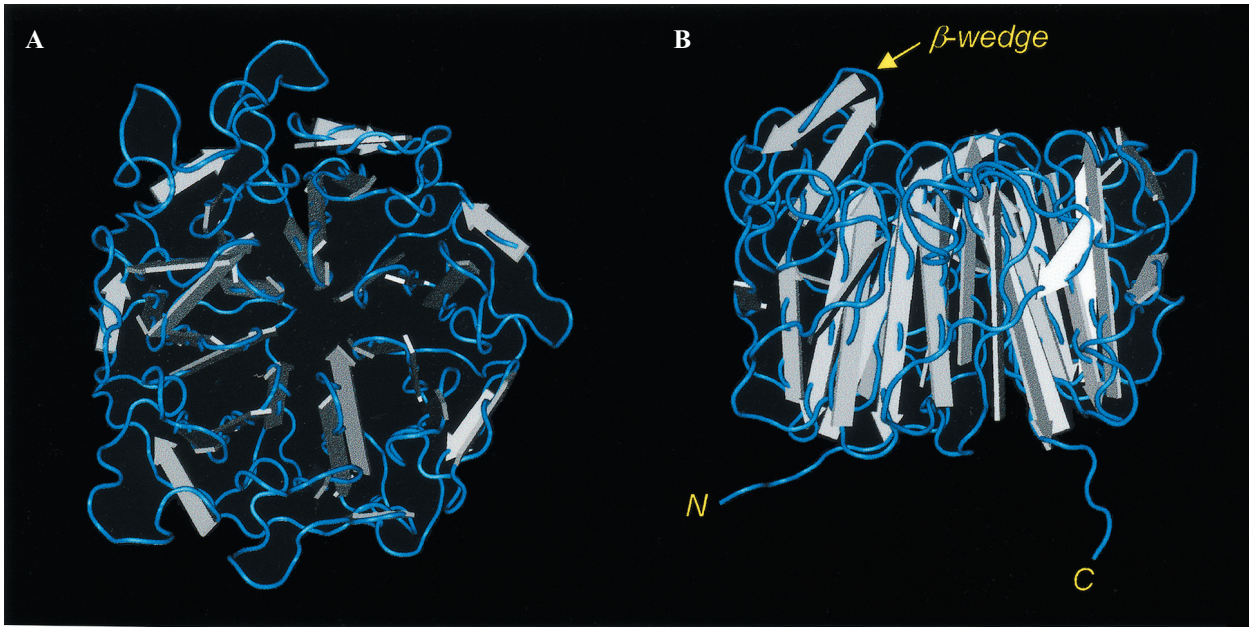

Figure 2. Human RCC1 structure. The 1.7 Å-resolution structure of the regulator of chromosome condensation-1 (RCC1) is shown [13]. (*A*) Frontal view of the β-propeller that allows visualization of its seven blades. (*B*) Lateral view of the β-propeller showing the β-wedge and the N- and C-termini of the protein. The side of RCC1 containing the N- and C-termini is the one interacting with chromatin, while the opposite side interacts and catalyzes nucleotide exchange on Ran. The β-wedge is a crucial player in the nucleotide exchange mechanism due to its ability to wedge itself between residues of the switch II (a loop in Ran that undergoes major conformational changes upon activation of this GTPase) and the P-loop of Ran (i.e. the loop in Ran which binds to the β-phosphate group of GDP·Mg^{2+}). This is thought to lead to Mg^{2+} release from Ran, which in turn decreases Ran's affinity for GDP, thus prompting nucleotide release [14]. Following nucleotide release, exchange is believed to be completed by the spontaneous binding of a GTP molecule (much more abundant in cells than GDP) to Ran's empty active site, which in turn triggers dissociation of RCC1 from Ran·GTP [82]. The figure was generated using the Cn3D program (see fig. 1). The PDB entry for the human RCC1 structure is 1A12.

(fig. 2 and [13, 14]). Up to now, close to 20 proteins harboring RLDs have been described in the literature, even though many more are present in databases [15]. The first to be discovered was RCC1 itself, which is also the best known. RCC1 was identified as the labile component in thermosensitive baby-hamster kidney tsBN2 cells which, when placed at the restrictive temperature, were unable to enter S-phase or, if they had already entered it, went into precocious chromosome condensation and mitosis without completing DNA replication [16, 17]. Further studies showed RCC1 to be a nuclear, chromatin-associated protein which interacts with the Ras-related GTP-binding protein Ran and catalyzes guanine nucleotide exchange upon it [18–20]. More recent studies have established that RCC1, through its ability to activate Ran, regulates a panoply of cellular processes that include nucleocytoplasmic transport, mitotic spindle assembly and nuclear envelope formation (for a recent review on the functions of Ran refer to [21]). Therefore, the RLD of RCC1 has a double role: while one face of the β-propeller binds to Ran and acts as a guanine nucleotide exchange factor (GEF) for this small GTPase [14], the opposite face associates with histones H2A and H2B and thus tethers RCC1 to chromatin, which is essential for its proper function (fig. 2 and [21, 22]). The enzyme activity of RCC1 raises the question of whether other RLD-containing proteins may also function as GEFs for small GTPases. In fact, a few years after it was demonstrated for RCC1, the HERC1 protein (see below) was also shown to stimulate guanine nucleotide dissociation on ADP-ribosylation factor (ARF) and Rab family small GTPases [23]. Although at the time this was taken to mean that HERC1, through one of its two RLDs, was a GEF for these GTPases, a recent report has challenged this view, since the stimulation of GDP dissociation induced by HERC1 is not accompanied by a concomitant increase in GTP uptake by the small G protein [24]. Moreover, nucleotide dissociation activity in HERC1 has been shown to rely on its RLD1 binding to phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$], setting HERC1 still further apart from RCC1 [24]. Aside from RCC1 and HERC1, two other RLDs have been purported to behave as GEFs: these are the RLDs of PRAF, a phosphoinositide-binding *Arabidopsis thaliana* protein [25], and of Claret, a protein involved in eye pigment granule biogenesis in *Drosophila* [26]. Both cases share the fact that the RLD substrates belong to the Rab family (plant Rab8 and the fly equivalent of human Rabs 32 and 38, respectively) and that their GEF

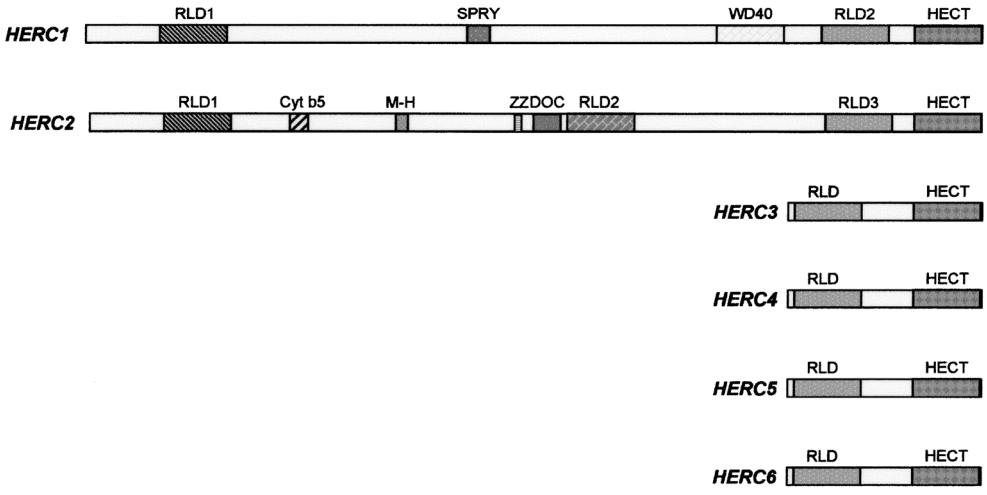

Figure 3. The human HERC family of proteins. The HERC proteins can be divided into two subfamilies, the small and large HERCs. Whereas the former possess little more than the RLD and HECT domains, the latter are giant proteins with more than one RLD, a HECT and several other conserved regions. Protein domains were identified using the InterProScan program at www.ebi.ac.uk/InterProScan/. CRLD, RCC1-like domain [13]; HECT, homologous to E6AP C-terminus [8, 11]; SPRY, spl A and RyR [44]; WD40, G protein β subunit-like repeats [40]; Cyt b5, cytochrome b5-like domain [63]; M-H, mind bomb-Herc2 domain [62]; ZZ, ZZ-type zinc finger [83]; DOC, DOC1/APC10 domain [57]).

activity has not been proven conclusively. In the first case it has been taken for granted on the basis of nucleotide dissociation experiments only, whereas in the second only a preferential binding for the GDP-bound form of Rab32/38 has been shown. Even though it is likely that these two proteins are bona fide GEFs, definitive proof of it should include GTP incorporation experiments. Although it is possible that other RLD-based GEFs await discovery, the actual fact is that no more of them have been reported so far (if we allow for the exception of Alsin, a GEF for Rac1 and Rab5 that uses other conserved domains for these activities and whose RLD has no known function [27]). Instead, RLDs have been shown to fulfil other roles, all of which involve interactions with other proteins. Examples of this include the RLD2 of HERC1, which binds to clathrin [28], and the RLDs of Nercc1, PAM, RPGR and DelGEF. The case of the Nercc1 kinase is a curious one: even though its RLD binds to the inactive, GDP-bound form of Ran, this most probably does not lead to guanine nucleotide exchange, since Nercc1 RLD lacks key catalytic residues which are needed for the GEF activity of RCC1 [29, 30]. Also interesting is the case of PAM, a giant protein involved in synaptogenesis and nociception, whose RLD2 specifically binds to and strongly inhibits adenylate cyclase type V [31]. On the other hand, RPGR, the protein affected in X-linked retinitis pigmentosa (RP3), uses its RLD to interact with two other proteins, namely RPGRIP and the δ subunit of cyclic GMP (cGMP) phosphodiesterase. These interactions, especially the first one, have been shown to be important for retinal function and are disrupted by all known RP3 mutations [32–35]. Finally, DelGEF has been shown to associate through its RLD with the human orthologue of yeast Sec5 as well as with a very small 9-kDa protein called DelGIP1. Both interactions appear to have a role in proteoglycan secretion [36, 37]. Despite other RLD proteins having been reported, nothing is known about the function of their RLDs, so they will not be dealt with in this review. Altogether, then, the available information indicates that the RLD is a structurally conserved, yet functionally very versatile domain, whose roles may include interactions with other proteins or phospholipids and in some cases GEF activity on small G proteins.

The HERC proteins

The human genome encodes more than 20 proteins containing a HECT domain in their C-termini [15]. The N-terminal regions of these proteins are diverse, but even so, many of these polypeptides can be placed in one of two subfamilies. In particular, the Nedd4 family of HECT ubiquitin ligases includes nine human members, all of which have similar N-termini containing a single calcium-binding C2 domain and two to four WW domains [38]. On the other hand, the second subfamily of HECT ligases is made up of the 6 human HERC proteins, which, as noted above, all possess one or more RLDs upstream of their HECTs. The HERC family members can in turn be subdivided into two subgroups according to their sizes and domain architecture (fig. 3 and table 1). All these members have already been published and characterized to varying extents.

Table 1. The human HERC family. Gene information was obtained from [56, 71, 80, 81] and from GenBank (www.ncbi.nlm.nih.gov/Genbank/) and the Ensembl Genome Browser (www.ensembl.org). Pairwise protein alignments were done using the Align program at www.ebi.ac.uk/emboss/align, whereas protein molecular weights were calculated using the Protein Parameters tool at www.expasy.ch. It should be noted that there is a discrepancy between the HERC3 chromosomal localization reported by different sources. According to the Ensembl Genome Browser the human HERC3 gene is located downstream of HERC6 and HERC5 in 4q22.1, whereas [81] and GenBank place it at 4q21 (n.a., not applicable; HERC5 does not exist in mouse).

Family member	Chromosomal localization	Gene size (kb)	Exon number	Human-Mouse identity (%)	Protein size (kDa)
HERC1	15q22	225	78	96	532
HERC2	15q13	211	93	95	528
HERC3	4q21–22	116	26	92	117
HERC4	10q21	153	29	91	118
HERC5	4q22	49	23	n.a.	117
HERC6	4q22	64	25	66	115

HERC1

The HERC1 protein can be regarded as the founding member of the family, insofar as it was the first to be discovered. HERC1, originally named p619 and later also p532, was identified during a search for human oncogenic sequences from breast cancer cells using so-called nude mouse tumorigenicity assays [39]. As a result of this search, a 150-kb DNA sequence containing fragments of at least two different human loci was isolated which preserved the ability to induce tumors in nude mice [23]. Subsequently, this DNA fragment, referred to as *oncH*, was used for the identification of expressed sequences with exon-trapping techniques. These yielded a single exon whose sequence encoded part of a protein with similarity to RCC1 (HERC1 RLDs display ~40% similarity to RCC1), and therefore a good candidate for a cell-cycle regulatory protein. Thus, the sequence of this exon was used to retrieve the full-length complementary DNA (cDNA) sequence of this novel gene from a human fetal brain library. So, after seven rounds of screening, eight overlapping clones were obtained which encompassed a 15-kb cDNA sequence containing a single open reading frame (ORF) encoding a predicted protein of 4861 aa and 532 kDa [23]. Analysis of the HERC1 sequence reveals the presence of a number of regions which are conserved in other proteins. These include two RLDs (RLD1 and RLD2), the C-terminal HECT, a WD40 domain homologous to the β subunits of heterotrimeric G proteins, a SPRY (spl A and RyR) domain and other minor motifs such as putative SH3-binding proline-rich sequences, a potential leucine zipper and several regions enriched in polar and acidic side chains. While the WD40 domain is well known both structurally, it adopts a seven-bladed β-propeller fold similar to the one found in RLDs [40], and functionally, it usually mediates protein-protein interactions, sometimes even with other WD40 proteins [41], not much is known of the SPRY domain, even though it has been suggested that it might fold in a similar way to immunoglobulin-like domains [42] (the latter are found in many proteins of diverse function, including many immune system and cell adhesion molecules, and adopt a β-sandwich fold [43]) and that it may likewise participate in interactions with other proteins or even RNAs [44]. Although the functions of all these domains in HERC1 have not yet been fully elucidated, several studies have contributed interesting insights which will now be summarized.

It has been shown that the messenger RNA (mRNA) of HERC1 displays a ubiquitous expression pattern with slightly higher levels in brain and testis [23]. Similarly, HERC1 mRNA [23] and protein [F. R. Garcia-Gonzalo and J. L. Rosa, unpublished data] have been detected in all tested cell lines, with the potentially relevant observation that mRNA levels are significantly higher in a number of tumor cell lines with respect to two normal cell lines of fibroblast origin [23]. This observation, together with the way in which HERC1 was originally discovered, raise the possibility of HERC1 having oncogenic properties. However, unlike the original *oncH* fragment (see above), HERC1 cDNA does not induce tumor formation in nude mouse assays. There are at least three potential explanations for this, namely that (i) out of the two human loci in oncH, only one contained an oncogene and this was not *HERC1*; (ii) *oncH* expressed a mutated oncogenic version of *HERC1* different from the one eventually cloned and (iii) HERC1 overexpression in the nude mouse assays was not sufficient for it to induce efficient tumor formation [23]. Therefore, the issue of the potential oncogenic capacity of HERC1 remains open to this day. At the subcellular level, the HERC1 protein has an exclusively cytoplasmic localization, where it can be found soluble or associated with vesicular membranes and with the Golgi apparatus, from which HERC1 is dislodged upon treatment with the fungal metabolite brefeldin A [23].

As to its function, the first studies were oriented to determine whether HERC1, like RCC1, could act as a GEF for small GTPases. In accordance with its observed subcellular distribution, HERC1 was found to stimulate guanine nucleotide dissociation from ARF and Rab family GTPases (ARF1, Rab3a and Rab5), both involved in

membrane trafficking, while no stimulation was observed for other GTPases such as Ran or R-Ras2/TC21. This activity was found to reside in HERC1's RLD1 [23] and, although it originally looked like the RLD1 acted as a GEF for these GTPases, a more recent paper has shown that HERC1 RLD1, rather than acting as a GEF, acts as a guanine nucleotide release factor (GRF), since it prevents rather than stimulates GDP/GTP exchange on the active site of these GTPases [24]. Moreover, the GRF activity of HERC1 has been shown to require the presence of $PI(4,5)P_2$ bound to the RLD1 of HERC1 [24]. On the other hand, HERC1 RLD2 does not possess either GEF or GRF activity over any of the above small G proteins. Instead, RLD2 has been shown to bind to ARF1 as well as to the heavy chain of clathrin [23, 28].

These two interactions support the idea of HERC1 playing an important role in intracellular membrane trafficking both in the Golgi, where ARF1 is a crucial player, and elsewhere in the cytoplasm (clathrin is a major coat component of many sorts of intracellular vesicles). Some clue as to how this role for HERC1 in vesicle traffic is accomplished may lie in several observations related to the clathrin-HERC1 interaction. Thus, only the cytosolic fraction of HERC1, but not the membrane-associated pool, was shown to be bound to clathrin heavy chain (CHC). Moreover, the chaperone Hsp70, which has been involved in the uncoating of clathrin-coated vesicles (CCVs) [45, 46], was also found in this complex, from which it dissociates in the presence of ATP [28]. This multiprotein complex also contains clathrin light chain (CLC), thus ruling out the initially considered possibility that the CHC-HERC1 interaction might compete with CHC-CLC binding (the RLD2 binding site in CHC was mapped very close to the CLC binding site [28]) [F. R. Garcia-Gonzalo and J. L. Rosa, unpublished data].

Aside from the observed functions for HERC1 RLDs, several data are also available concerning the HECT domain. In particular, the HECT of HERC1 has been shown to conjugate ubiquitin through its active site cysteine in a reaction that requires the presence of the E2 UbcH5, but that does not take place with other E2s such as UbcH1, UbcH6 or UbcH7, thus indicating that HERC1 is probably a functional E3 ubiquitin ligase that specifically transfers ubiquitin from UbcH5 on to the substrates [9]. Unfortunately, none of the proteins found to interact with HERC1 have been shown to undergo HERC1-mediated ubiquitination. Apart from the ones already mentioned, HERC1 has been shown to bind to the glycolytic isoenzyme pyruvate kinase M2 [47]. This protein was originally identified by its ability to associate with the HECT of HERC1 in the yeast two-hybrid system, and even though the interaction was confirmed in vitro and in vivo, no effect of HERC1 on either its enzyme activity or its ubiquitination could be found [47]. Finally, HERC1 has also been found to be recruited to sites of active actin polymerization at the plasma membrane induced upon activation of the GTPase ARF6 [48]. Even though HERC1 can act as a GRF for ARF6 in vitro [24], HERC1 does not appear to be regulating ARF6 activity in HeLa cells, since HERC1 overexpression neither activates nor prevents activation of this GTPase. Instead, HERC1 appears to be recruited to these membrane protrusions as a consequence of ARF6 activation [48]. Given the ability of ARF6 to activate $PI(4,5)P_2$ synthesis at the plasma membrane [49], it has been postulated that HERC1 might be recruited to ARF6-induced protrusions as a result of the ability of its RLD1 to interact with this phosphoinositide. Accordingly, HERC1, $PI(4,5)P_2$ and the $PI(4,5)P_2$-synthesizing enzyme, PI(4)P-5-kinase, all colocalize in these structures. Although the role of HERC1 in ARF6-induced, actin-rich protrusions has not yet been addressed, it might have to do with the active macropinocytosis occurring at these locations [48].

HERC2

Although HERC1 was the first HERC protein to be identified, the history of HERC2 can be traced back much earlier. Indeed, the discovery of HERC2 was the result of complementation studies carried out with mutant alleles of the pink-eyed dilution (*p*) locus in mouse chromosome 7C. The first mutants to be reported from this locus displayed varying degrees of coat and eye hypopigmentation but were otherwise fully viable and fertile. However, already in 1960 [50] some mutants were described which, besides the typical hypopigmentation phenotype, presented a much graver set of abnormalities, including reduced viability, smaller size, a jerky gait and sterility (a phenotype that would later become known as the *rjs* syndrome: runty, jerky, sterile). Even though initial reports found it difficult to believe that such pleiotropic effects could be due to the dysfunction of a single gene [50], later studies eventually proved this to be the case. In particular, the first clear indication of the monogenic origin of the *rjs* phenotype came from complementation analyses of a number of recessive *p*-locus alleles in which it was established that all alleles leading to the *rjs* syndrome when in homozygosis fail to complement each other when in heterozygosis, whereas they are fully complemented by alleles involved in the milder, exclusively pigmentation-related phenotype [51]. However, definitive proof of the existence of a single gene underlying the *rjs* phenotype still had to wait until a chemical mutagenesis protocol involving the use of N-ethyl-N-nitrosourea allowed Rinchik et al. to generate single base-pair mutants of this locus boasting a full-fledged *rjs* syndrome [52]. Once it had been demonstrated that a single gene accounted for all *rjs*-related symptoms, it was only a matter of time before the *rjs* gene was finally cloned. This was achieved

in 1998 by Lehman et al. [53], who showed that three of the previously studied *rjs* mutants contained deletions in a gene encoding a giant 528-kDa protein, later to be renamed *HERC2*. Of the three deletions described, two were deletions of large chunks of DNA which gave rise to severely truncated HERC2 mRNAs and proteins, whereas the third one was an intragenic deletion yielding a protein lacking 321 internal amino acid residues. While the first two deletions were too large to rule out the possibility of other genes downstream of *HERC2* also being deleted, the third deletion was confined within *HERC2* and thus strongly suggested that alterations in *HERC2* underlie the *rjs* disorder. Nevertheless, the possibility still remained that *rjs* was due to deletion of another gene located inside an intron of the *HERC2* gene. Therefore, ultimate proof of *HERC2* mutations being the causal agent of the *rjs* syndrome still had to wait one more year until it was shown that some of the previously reported single base-pair mutants leading to *rjs* were in fact splice junction mutations exclusively affecting *HERC2* [54].

In this same paper, Ji et al. also cloned the human orthologue of mouse *HERC2* and showed that recent (i.e. during the last 20 million years) duplication and translocation events involving fragments of the *HERC2* gene gave rise to the transcribed low-copy repeats, also called duplicons, which are found at the deletion breakpoint hotspots in human chromosome bands 15q11 and 15q13, involved in most cases of Prader-Willi syndrome (PWS). The latter is a so-called genomic imprinting disorder caused by lack of expression of a group of genes located within a 2-Mb stretch of DNA in the region between 15q11 and 15q13. In normal individuals, these genes are expressed only from paternal chromosome 15, since the maternal counterparts become silenced during oogenesis or early embryonic development in a process known as genomic imprinting. In patients with PWS, though, paternal expression of these genes also fails to take place owing, in 70% of cases, to a 4-Mb deletion occurring during spermatogenesis. More precisely, this deletion appears as a result of a mistake in homologous recombination during meiotic prophase I, and *HERC2* duplicons play a crucial role in increasing the likelihood of this mistake happening. However, despite *HERC2* having been found altered in some PWS patients and the symptoms of PWS resembling those of murine *rjs* to some extent, it appears that *HERC2* mutations do not underlie PWS, since the *HERC2* gene does not undergo imprinting and therefore PWS patients express their maternal *HERC2* allele, which, given the recessive nature of *HERC2* mutations in mice, should be sufficient to avoid complications (for a review of PWS and the role of *HERC2* duplicons in its etiopathogenesis see [55]). In any case, *HERC2* duplicons, despite being transcribed, should be regarded as pseudogenes given their high mutation rates (equivalent to those of introns), the presence of premature stop codons in all reading frames and the fact that they constitute but parts of the only functionally relevant, ancestral *HERC2* gene found in 15q13 (the region equivalent to mouse 7C by conservation of synteny) [56].

Concerning the HERC2 protein, it is, as mentioned above, a giant, highly conserved, 528-kDa protein containing a number of conserved regions, including the HECT, three RLDs, a DOC domain, an M-H domain, a cytochrome b5-like region and a ZZ-type zinc finger. The DOC domain was initially described as a roughly 200 aa region homologous to the APC10/DOC1 subunit of the APC ubiquitin ligase complex involved in progression through mitosis [57]. Recently, APC10/DOC1 was shown to adopt a β-sandwich fold and to significantly enhance the affinity of the APC complex for its substrates, thereby stimulating their polyubiquitination [58–61]. Since all proteins with DOC domains have also been found to possess either HECT or cullin domains, both involved in protein ubiquitination, it seems reasonable that the function described for APC10/DOC1 could be conserved in the other DOC-domain proteins. If so, it might then be expected that the DOC domain in HERC2 stabilized the association between HERC2 and its substrates. Another domain which might be involved in HERC2 activity as an E3 is the M-H domain (Mib-Herc2), which is also found in a zebrafish RING-finger-containing ubiquitin ligase called Mind Bomb (Mib) [62]. Regarding the cytochrome b5-like structural motif, the absence of the two histidine residues involved in heme coordination in cytochrome b5 makes it extremely unlikely that HERC2 also binds a heme group. Nonetheless, as has been shown for membrane-associated progesterone receptors (MAPRs), it might well be that the original heme-binding cytochrome b5 domain has in HERC2 turned into a hydrophobic binding pocket for some non-heme ligand [63]. On the other hand, HERC2 also contains a ZZ-type putative zinc finger motif with six conserved cysteines and two outlying histidines that may allow it to bind Zn^{2+} ions. Even though no studies have yet been performed with the HERC2 protein, interesting data are available from the analysis of its mRNA as well as the effects of its mutations in mice. Murine HERC2 mRNA is expressed ubiquitously but at significantly higher levels in brain and testis [53]. While most studied mutations have been shown to give rise to drastically truncated polypeptides, two of them are of special interest since they yield proteins that lack only a limited number of internal residues. The fact that one of these *rjs* mutants lacks only part of the HECT (aa 4428–4748) clearly attests to this domain's importance in HERC2 function and suggests that the *rjs* phenotype arises as a result of defective ubiquitination of one or more HERC2 substrates. By contrast, the second *rjs* mutant has an intact HECT but lacks a stretch of 53 aa (3716–3768) located right after the RLD2. Therefore, it seems likely that these 53 aa are required for the binding of HERC2 substrates (curiously, this mutation, in contrast to all other

studied, also leads to increased HERC2 mRNA levels, although the significance of this is currently unknown [64]). Finally, the physiological events for which HERC2-mediated ubiquitination might be important can be vaguely outlined on the basis of *rjs* symptoms. Thus, reduced growth and genital hypoplasia in *rjs* mice might be due to problems in hormonal secretion in the pituitary, which in turn might be due to dysfunction of hypothalamic neurosecretory neurons whose axons have been found degenerated in a *HERC2* mutant [65]. Likewise, other neurons might account for the jerky gait and maternal behavior defects also seen in *rjs* mice. On the other hand, it has been shown that defects in spermatogenesis are intrinsic to the germ line [66] and arise as a result of formation of an abnormal acrosome, a secretory organelle derived from the Golgi complex. Therefore, all data are consistent with a role for HERC2 in the secretory trafficking pathways of mainly neurons and sperm cell precursors.

HERC3

The cDNA of human HERC3, by then still referred to as D25215, was originally identified in 1994 in a random search for cDNAs larger than 2 kb [67]. However, it was not until 7 years later that the protein encoded by this cDNA was described for the first time [68]. HERC3 is a 117-kDa protein that is located in the cytosol and in cytoplasmic vesicular-like structures in all tested cell lines, where it colocalizes with markers of intracellular membrane trafficking pathways such as β-COP, Rab5 and ARF, but not with lysosomal (LIMP-II) or Golgi (GMPt1) markers [68]. Even though it cannot be ruled out that the RLD domain of HERC3 has dissociation activity for some as yet unidentified small GTPase(s), such activity was not detected for any of nine of these proteins, namely Ran, Rab3a, Rab5, Rab8, ARF1, ARF6, Ras, Rac and RhoA [68]. On the other hand, the HECT domain of HERC3 has been shown to be fully functional, insofar as it can form a thioester bond with ubiquitin, as long as the latter is supplied by an appropriate E2 (in vitro, both UbcH7 and UbcH5 have been shown to act as ubiquitin donors for HERC3, although the former does it more efficiently) [9]. As a matter of fact, HERC3 has been shown to bind ubiquitin, albeit with a reduced affinity, even when its thioester-forming active site cysteine (C1018) has been mutated to alanine [68]. This C1018-independent binding may be due to the non-covalent association of ubiquitin to either the HECT active site or, alternatively, to a different ubiquitin-binding site elsewhere in the HERC3 molecule. In any event, there is still another way in which HERC3 binds to ubiquitin. Indeed, not only does HERC3 appear to be a ubiquitination enzyme for other so far undiscovered proteins, but it also undergoes ubiquitination itself in one or more of its lysine residues, as shown by in vitro ubiquitination assays performed in the rabbit reticulocyte lysate system [68]. In other words, HERC3 appears to be both a ubiquitin ligase and a ubiquitination substrate. This raises the possibility of HERC3 ubiquitinating itself. However, this seems unlikely, since HERC3 ubiquitination is not at all affected by the C1018A mutation. In any case, it has been established that HERC3 undergoes polyubiquitination and that it is degraded in the proteasome [68]. Regarding the expression of HERC3 mRNA, a recent report shows that, in mouse, it is expressed throughout the brain, with especially high levels in the piriform cortex, the hippocampus and the amygdala [69]. HERC3 expression in other tissues has also been found [9, 69]. In summary, then, HERC3 is probably a ubiquitin ligase involved in membrane traffic whose own levels seem to be regulated by ubiquitin-dependent proteasomal degradation.

HERC4

Similar to what has just been said for HERC3, HERC4 cDNA was originally identified during a search for new human brain cDNAs encoding large proteins [70]. However, the initially reported sequence [70] was later found to be incomplete, and publication of the full-length HERC4 cDNA sequence still had to wait until a very recent paper [71]. This last article also reports all currently available knowledge on the HERC4 protein [71]. The mRNA of HERC4 has been found in all examined tissues, with its levels being significantly higher in brain and testis than in placenta and heart. Analogous to what has been seen for other HERCs, the subcellular localization of the overexpressed HERC4 protein appears in immunocytochemical studies as a cytoplasmic, punctuate staining indicating its association with membranous structures. A shocking aspect of the work on HERC4 is undoubtedly the complexity in the processing of its pre-mRNA. Thus, the HERC4 gene contains 29 potential exons, out of which only 25 or 26 usually end up in the mature mRNA (4.45 kb), which gives rise to two proteins of 1049 and 1057 aa which constitute the major forms of HERC4 in the cell. However, albeit with a lower frequency, the pre-mRNA can be spliced in different ways from those already mentioned. These alternative splicings may include events such as the exclusion of the first five exons and substitution of them by another containing an alternative translation initiation codon (which renders a protein lacking part of its RLD), the exclusion of exons 24 and 25 (creating a protein with a deletion in its HECT), the inclusion after exons 3 or 11 of an additional exon containing a stop codon (which generates severely truncated proteins) or the exclusion of exons 5 and 9, which alters the reading frame and gives rise to a small protein of only 118 aa. The physiological relevance of all these splicings is currently unknown, as is HERC4 protein function.

HERC5

HERC5, initially described as Ceb1 (cyclin E-binding protein-1) [72] and later also designated HERC4 [47, 48, 68], is a 117-kDa protein whose mRNA is highly expressed in testis and fetal brain and is found at much lower levels in other organs such as the ovaries, pancreas, heart, placenta and skeletal muscle. The HERC5 protein was originally identified in a yeast two-hybrid screen using cyclin E as bait [72]. More recently, HERC5 was also found during a search for genes upregulated after pro-inflammatory cytokine treatment of human skin microvascular endothelial cells (HSMECs) [73]. Both papers provide very valuable insights into the function of HERC5, which will now be reviewed in some detail.

Aside from the above-stated tissular distribution, HERC5 subcellular localization is analogous to what has already been shown for the other small HERCs, i.e. cytoplasmic with both a soluble and an inner membrane-bound component [72]. Interestingly, HERC5 gene expression has been shown to be the object of a fine regulation. Indeed, even though HERC5 levels are normally very low in most tissues, its expression increases considerably when cells are subject to certain treatments, such as expression of viral oncoproteins that inactivate the tumor suppressors p53 and Rb [72]. Similarly, HERC5 mRNA levels also rise in response to pro-inflammatory stimuli [73]. In particular, a clear increase in the amount of HERC5 mRNA is seen 8 h after treatment of HSMECs with either lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α) or interleukin 1 beta (IL-1β). This upregulation fails to occur if either NFκB activation or protein synthesis are blocked, thus probably indicating that HERC5 gene expression depends on some transcription factor whose own expression is in turn regulated by nuclear factor kappa B (NFκB). Moreover, this interpretation is in agreement with the observation that the proximal region of HERC5 gene promoter does not contain any consensus NFκB-binding site but instead harbors sites for other inflammation-related transcription factors [73]. Nevertheless, the changes observed in HERC5 mRNA after pro-inflammatory cytokine treatment of HSMECs are not accompanied by a concomitant increase in HERC5 protein levels. This apparent paradox can be explained on the basis of an independent effect of LPS on HERC5 protein half-life. This effect is much faster than the one affecting the mRNA and entails a very swift degradation of the protein, whose half-life descends from 9 h in the absence of LPS to a mere 2 h in its presence. As a consequence of this dual effect, HERC5 protein levels fall rapidly in response to LPS and are not restored until about 12 h later, thanks to the rise in the amount of mRNA [73].

From all the above data, a broad picture of HERC5 protein function starts to emerge. First, its high expression in testis suggests that HERC5 may play a special role at this location. In this regard, HERC5, by virtue of its E3 ubiquitin ligase activity, may participate in the massive protein ubiquitination and destruction taking place during spermatogenesis [74, 75]. On the other hand, the fine regulation of HERC5 during inflammation also points to this protein having an important say in this process. As has been seen, HERC5 disappears during the early phase of inflammation, which might lead to temporal substrate stabilization, only to reappear several hours later, presumably in order to contribute to the end phases of inflammation: this would be in accordance with the proved importance of ubiquitin-mediated protein degradation during the resolution of inflammation [76]. Finally, the regulation of HERC5 by p53 and Rb, together with its interaction with cyclin E and other cyclins [72], makes it appealing to think of a possible role for HERC5 in cell cycle progression. However, no one has yet shown any change in the level or activity of HERC5 during the cell cycle. Therefore, there is plenty of work ahead before the functions of this interesting protein are fully unveiled.

HERC6

The HERC6 gene, which was identified due to its high similarity to HERC5 (~50% nucleotide sequence identity in the HECT domain region) [71], has been preliminarily characterized, together with HERC4, in a very recent paper [71]. Analogous to what has already been said for HERC4, the most bewildering aspect of HERC6 studies concerns the multiple splicing products of its pre-mRNA. The initial HERC6 transcript contains 25 potential exons, of which normally 23, all but numbers 9 and 17, end up in the mature messenger molecule (3.89 kb). This mRNA encodes the most common form of HERC6 protein, containing 1022 aa. Less frequently, the primary HERC6 transcript undergoes alternative splicings, leading to at least three other mRNAs, where either exons 9, 16 and 17, exons 9, 10 and 14 or exons 7, 9, 10, 14 and 17 are missing. As a result, the proteins encoded by these mRNAs have internal deletions (36 non-conserved amino acid residues are deleted due to absence of exon 16) or are truncated (connection between exons 8 and 11 causes frameshift, leading to proteins of only 322 and 364 aa). As with HERC4, it is not known whether these minor forms of HERC6 have any functional relevance, although it is tempting to speculate that the truncated proteins containing only the RLD domain may act as dominant negative regulators of HERC6-mediated ubiquitination, since they would be expected to bind to substrates without ubiquitinating them. Finally, expression analyses show that HERC6 mRNA levels are higher in brain and testis than in placenta and heart [71], while subcellular distribution studies with the overexpressed HERC6 protein show that, like other family members, it is located in cytoplasmic, vesicular-like structures [71].

Evolution of *HERC* genes

The impressive development experienced by the genomics field in the course of the last decade is providing researchers with an increasingly profound comprehension of the ways in which the genomes of living creatures have evolved during the history of life on Earth. A recent article using the available genomic data on *HERC* genes in order to create a broad picture of their evolution [71] has some important implications. First, the elaboration of a phylogenetic tree with the sequences of all published *HERC* genes from different species firmly establishes the existence of the two aforementioned *HERC* subfamilies, i.e. the large and small *HERCs* [71]. Interestingly, the *Caenorhabditis elegans HERC4* orthologue emerges directly out of the tree's basal line [71]. This observation, together with the fact that *HERC4* is the only *HERC* gene to be found in the nematode genome, has led to the suggestion that *HERC4* might represent the most ancient family member, from which all others are derived [71]. Nevertheless, a more detailed analysis of the available data on animal as well as *HERC* gene evolution suggests another possibility. In particular, the fact that both chordates and arthropods possess at least one member of each *HERC* subfamily (e.g. *HERC4* and *HERC2* in *Drosophila* and all six members in humans) indicates that the last common ancestor of both phyla (i.e. the so-called Urbilateria [77]) must already have had one representative of each of these two subfamilies. In view of this, the absence of large *HERC* genes in nematodes (the last common ancestor of nematodes and arthropods was a protostome that lived much later than Urbilateria) should be interpreted as the result of secondary gene loss in the direct ancestors of nematodes, rather than as the small *HERC* genes having arisen first in evolution (fig. 4 and [78]). If this turns out to be true, then both *HERC* subfamilies would have already appeared by the time the first bilateral animals existed. Whether the small *HERC* genes gave rise to the large ones (or vice versa) or both arose independent of one another cannot be at present. On the other hand, the fact that *HERC* genes are missing from the genomes of other eukaryotes, such as fungi and plants, which, however, do possess RLD and HECT domains in separate proteins, suggests that the first *HERC* gene may have appeared as a result of a gene fusion event very early in animal evolution. Concerning the more modern family members, the first to appear were *HERC1* and *HERC3*, which may have arisen from *HERC2* and *HERC4* as a consequence of whole genome duplication events known to have occurred early in vertebrate evolution [71, 79]. The next member to appear was *HERC6*, owing to the duplication of the *HERC3* gene at some time during tetrapod evolution [71]. Finally, *HERC5* was the last family acquisition, existing in primates but not in rodents. Like *HERC6*, *HERC5* must have appeared after a gene duplication event, probably from *HERC6*, to which it is most closely related (*HERC3*, *HERC5* and *HERC6* are all located within a 330-kb cluster in human chromosome 4) [71]. As the genomes from other taxonomic groups within the animal kingdom are made public, it will be possible to learn more details about the key steps in the evolutionary history of this gene family.

Final remarks

Almost 10 years have elapsed since the initial description of HERC1 in 1996 and more than 40 since obtaining the first HERC2 mutants in 1960. During these years the cloning and initial characterization of all existing human HERCs has been achieved. In addition to fascinating insights into the evolutionary history of this gene family, the last years have witnessed an accumulation of evidence suggesting that these proteins may be active ubiquitin ligases (HERC1, HERC3 and HERC5 have been shown to form thioester bonds with ubiquitin [9, 73], while a mouse HERC2 mutant lacking only the HECT domain has been shown to display full-fledged *rjs* syndrome [53]). Nevertheless, since no ubiquitination substrates have yet been reported for any of these proteins, the possibility that they may have other functions, which may or may not be related to ubiquitin-dependent processes, should not be ruled out. On the other hand, evidence suggests that at least some family members may have important roles in intracellular membrane trafficking (the

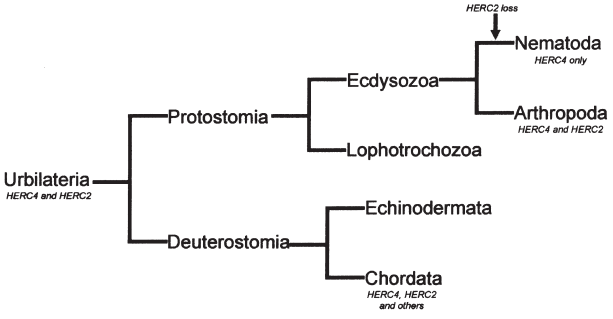

Figure 4. *HERC* family evolution. The fact that both arthropods and chordates possess small and large *HERC* genes suggests that the last common ancestor of all bilateral animals must already have possessed such genes. In turn, this indicates that the absence of large *HERCs* in nematodes must be due to a more recent gene loss event (arrow) having taken place during the evolution of nematodes or their immediate ancestors [*HERC2* orthologues have been found in at least four arthropod species, namely *Drosophila melanogaster* (GenBank accession number NP_608388), *Drosophila pseudoobscura* (EAL32685), *Anopheles gambiae* (EAA00368) and *Apis mellifera* (XP_395007). Only *HERC4* orthologues have been found in nematodes: *Caenorhabditis elegans* (NP_490834) and *Caenorhabditis briggsae* (CAE63916)]. The evolutionary tree, which shows only major taxa, has been adapted from [77]. Distances in the tree bear no relation to actual evolutionary time

evidence for this is especially compelling for HERC1 [23, 24, 28], although it is also available for HERC2 [53] and HERC3 [68], let alone the fact that all members appear to localize in cytoplasmic vesicle-like structures). Also of potential interest is the observation that most HERC proteins, or at least their mRNAs, appear to be highly expressed in brain and/or testis, where they could have specialized functions (this has been proven for HERC2, which is essential for mouse spermatogenesis [53, 54, 66], but it may also be the case for other members [23, 69, 71, 72]). In any case, there is still a very long way ahead until the workings of these proteins can be known in detail. Many new discoveries on the HERC proteins should be expected in the forthcoming years.

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