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Published in:
Biochimica et Biophysica Acta - Gene Regulatory Mechanisms

DOI:
10.1016/j.bbagrm.2023.194925

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
The moonlighting of RAD23 in DNA repair and protein degradation

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ARTICLE INFO
Keywords:
Protein degradation
Proteasome
Ubiquitin
ERAD
UBL
UBA

ABSTRACT
A moonlighting protein is one, which carries out multiple, often wholly unrelated, functions. The RAD23 protein is a fascinating example of this, where the same polypeptide and the embedded domains function independently in both nucleotide excision repair (NER) and protein degradation via the ubiquitin-proteasome system (UPS). Hence, through direct binding to the central NER component XPC, RAD23 stabilizes XPC and contributes to DNA damage recognition. Conversely, RAD23 also interacts directly with the 26S proteasome and ubiquitylated substrates to mediate proteasomal substrate recognition. In this function, RAD23 activates the proteolytic activity of the proteasome and engages specifically in well-characterized degradation pathways through direct interactions with E3 ubiquitin-protein ligases and other UPS components. Here, we summarize the past 40 years of research into the roles of RAD23 in NER and the UPS.

1. Introduction

RAD23 was first discovered in budding yeast cells based on the UV-sensitivity phenotype of a deletion mutant [1,2]. Later, the two human homologs, RAD23A and RAD23B, were purified and cloned along with their interaction partner XPC [3] and mapped to the genome [4]. Hence, RAD23 was initially linked to nucleotide excision repair (NER). Subsequently, however, various other RAD23 interaction partners, which are not associated with NER, have been identified [5–17]. Today, after 40 years of research into RAD23, we have learned that this protein not only has a well-established function in NER, but strikingly, also takes part in targeted protein degradation via the ubiquitin-proteasome system (UPS) [12,18–20]. These dual roles in NER and the UPS [19,20] makes RAD23 a textbook example of a moonlighting protein [21,22], i.e. a protein which exhibits more than one function within the same polypeptide chain. As a testimony to its role in these fundamental processes, RAD23 is phylogenetically highly conserved in eukaryotes [6,23]. Conservation is also evident from multiple reports of interspecies complementation [5,6,23].

Similar to their budding yeast homolog, the human RAD23A and RAD23B proteins consist of four distinct domains (Fig. 1A), which are connected by stretches of less conserved intrinsically disordered regions (Fig. 1B) [6,25]. From the N- to the C-terminal the first domain is a ubiquitin-like domain (UBL), which is followed by a ubiquitin-associated domain (UBAI), an XPC-binding domain (XPCB) and finally another UBA domain (UBA2) [26,27] (Fig. 1A).

The N-terminal UBL domain shares sequence similarity to ubiquitin [28], allowing RAD23 to bind the proteasome [12,13,29,30], a large and highly abundant protease complex found in the cytosol and nucleus of all eukaryotic cells [31–33]. The XPCB domain, on the other hand, is capable of binding XPC [27], called Rad4 in yeast [34], and the deglycosylating enzyme, PNGase [7,35]. The XPCB domain shows distant relationship to the STI1-like domain in the Dsk2/UBQLN family and the HSP70 co-chaperoneSacsin [36–38]. The UBA domains are three-helix bundle structures that associate with ubiquitin chains, allowing RAD23 to bind the ubiquitin chain of proteins targeted for proteasomal degradation [12,39,40].

According to the human protein atlas database (https://www.proteinatlas.org), human RAD23A and RAD23B are broadly expressed across most tissue types, with RAD23B being 10-fold more abundant than RAD23A [41]. The homologs in human [7,42,43], S. pombe [23], S. cerevisiae [44,45] and plant cells [46] are predominantly localized in the nucleus and to a lesser extent the cytosol.

In the following, we provide a detailed overview of RAD23s functions in nucleotide excision repair and ubiquitin-dependent protein degradation. As for the nomenclature, we will refer to the protein as

* Special issue on “The ubiquitin system”; Edited by Hai Rao, Michael Glickman, Feng Rao.
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https://doi.org/10.1016/j.bbagrm.2023.194925
Received 17 December 2022; Received in revised form 20 February 2023; Accepted 23 February 2023
Available online 28 February 2023
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RAD23 when reporting general and species independent aspects of the protein, while the *S. cerevisiae* protein will be referred to as Rad23, the *S. pombe* protein as Rhp23, and the human orthologues as RAD23A and RAD23B. When referring to proteasome subunits, we will mention the human protein names, but otherwise use the more logical yeast nomenclature throughout.

2. Nucleotide excision repair

The main role of RAD23 in NER lies in the stabilization and stimulation of XPC. However, to truly appreciate its role in NER, one must first understand the context in which it functions. The NER pathway is responsible for dealing with various structurally unrelated DNA lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and (6–4) photoproducts (6,4-PPs) or bulky chemical adducts [47] and results in the excision of ~30 nucleotides around the damaged site [48,49]. Subsequent recruitment of the replication machinery will then fill in and repair the damaged region.

The initial steps of NER occur by two different mechanisms, and are divided into either global genome (GG)-NER, which detects DNA damage throughout the genome, and transcription-coupled (TC)-NER, which is responsible for dealing with various structurally unrelated DNA lesions, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and (6–4) photoproducts (6,4-PPs) or bulky chemical adducts [47] and results in the excision of ~30 nucleotides around the damaged site [48,49]. While XPC is capable of binding 6,4-PPs damage sites directly [65,66], it often relies on DDB1-DDB2 for proper recruitment to the DNA damaged site [66]. To perform its functions, the DDB1-DDB2 heterodimer forms a complex with CUL4A and RBX1 [67]. This E3 complex ubiquitylates histones in response to UV-damage which loosens the interaction between histones and DNA thus facilitating recruitment of XPC and subsequent NER steps [68–70]. Additionally, CUL4-dependent ubiquitylation and subsequent proteasomal degradation of DDB2 seem to facilitate recruitment of XPC [71–74]. As DDB2 is solely responsible for the DDB1-DDB2 heterodimer binding to the DNA lesion [54], its degradation likely causes the DDB1-CUL4A complex to dissociate from the damaged area, allowing downstream NER-proteins to perform their functions. However, it is also possible that XPC competes for binding to the damaged site [75–77]. Lastly, XPC is also subject to CUL4A-dependent ubiquitylation which increases its affinity for damaged and undamaged DNA [73]. This, along with other posttranslational modifications such as sumoylation [78] and casein kinase II-dependent phosphorylation [79], adds more layers of regulation to NER that remain somewhat unclear and will not be further elaborated on here. Finally, the mentioned E3 activities of the DDB-CUL4A complex are negatively regulated by the COP9 signalosome (CSN) [67], which dissociates from the E3 complex when DDB2 binds to the damaged DNA [80,81].

3. RAD23 in nucleotide excision repair

Amidst these multiple interactions, RAD23A or RAD23B bind to XPC. This interaction stimulates XPC activity [27,82,83] and stabilizes the

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**Fig. 1.** The RAD23 domain organization and structure.
(A) Domain organization of RAD23 orthologues in human, budding yeast and fission yeast. UBL and UBA domain locations as described in UniProt. XPCB domains co-located as described previously [36,57]. The sequence identity between the regions is shown. (B) The structure of human RAD23B as predicted by AlphaFold [298,299] (AF-P54727-F1) with its domains highlighted.
XPC protein [41,84,85]. The RAD23 homologs enhance the XPC binding affinity specifically to damaged DNA [86]. Importantly, only a fraction of the total cellular RAD23B is found in complex with XPC [42,82]. The RAD23-XPC complex is well characterized biochemically and structurally. More recently, with a cryo-EM structure of a yeast NER initiation complex containing Rad23 [87]. A fragment of RAD23B spanning amino acids 277–332, which covers the XPCB domain, was shown to be responsible for binding to XPC, and necessary and sufficient for NER functionality, although this truncated protein performed less well than full-length protein [27]. This domain forms five aliphatic α-helices, which bind to XPC largely via hydrophobic residues [88], which may explain the resistance of the XPC-RAD23B complex to high salt concentrations [3,27]. Similar results were found for structures of the RAD23A XPCB domain (residues 231–287) [25,36]. The NMR structure of RAD23B-XPCB is quite similar to that of RAD23A, as might be expected given the sequence similarity between RAD23A and RAD23B [88]. Considering that the UBQLN family is related to RAD23, it is possible that these proteins can stabilize XPC as well. However, this remains to be tested.

In addition to RAD23, the protein Centrin2 also binds to the C-terminal part of XPC, resulting in a trimeric RAD23–XPC–Centrin2 complex [84,89] and improving NER [84,89–91]. However, while Centrin2 seems to function downstream of the damage recognition step in the recruitment of TFIIH [92,93], RAD23 quickly dissociates from XPC upon formation of the final damage-bound XPC nucleoprotein complex (Fig. 2) [86]. As the DNA- and RAD23-binding regions of XPC partially overlap, it has been suggested that RAD23 dissociation is necessary for XPC-DNA damage complex formation [86] and possibly lowers the energetic cost of nucleoprotein rearrangements necessary to reach the final conformation [56,86].

While the RAD23B homolog is most often found bound to XPC [3,42], the RAD23A homolog can substitute for RAD23B binding to XPC in vitro [83,84,94]. Indeed, RAD23A has been shown to be functionally redundant with RAD23B in regard to NER both in a cell-free system [83] and in mouse embryonic fibroblasts [24,41,85]. Hence, in mice, the genes encoding both RAD23A and RAD23B must be deleted to observe fibroblast NER deficiency, indicating that either RAD23A or RAD23B is sufficient to fulfill the NER role [85]. However, RAD23A is less abundant compared to RAD23B and accordingly it has been observed that in mouse fibroblasts, RAD23A needs to be overexpressed to substitute RAD23B in XPC stabilization [41].

The notion that RAD23 function in NER is exclusively mediated through its interaction with XPC, is supported by the fact that RAD23AB deletions give the same phenotype as an XPC knock-out, with impaired GG-NER and apparently normal TC-NER [85].

While the above section has focused on human RAD23A and RAD23B in NER, budding yeast Rad23 appears to function similarly in that it stabilizes and potentiates its XPC homolog Rad4 [95], thereby aiding the UV-damage repair mechanism. The budding yeast proteins necessary and sufficient for NER are: Rad14 (human XPA), Rad4-Rad23 complex.

Fig. 2. Overview of the GG-NER pathway.
(1) In the absence of damage, the complex consisting of DDB1, DDB2, CUL4A and RBX1 [49,260] is inactive, due to binding of the COP9 signalosome (CSN). (2) When UV-induced DNA damage occurs, the DNA lesion is recognized by the DDB-CUL4A complex. The COP9 signalosome (CSN) then dissociates from the complex, activating the E3 ligase complex, and an E2 (ubiquitin-conjugating enzyme) [67]. (3) The CUL4A E3 ligase complex ubiquitylates histones, XPC and DDB2, which leads to easier access to the damaged site for repair proteins [68,69], improved affinity of XPC to damaged DNA [73] and dissociation followed by degradation of DDB2 [71,73,74]. Aided by the above processes, the Centrin2:XPC:RAD23B complex is recruited to the damage site. RAD23 likely dissociates prior to or during the formation of the final XPC-DNA damage complex [56,86]. (4) Upon XPC binding, the TFIIH-complex is recruited to the damage site. This unwinds the DNA strands, positioning it for excision. (5) XPC likely leaves the site prior to recruitment of the excision-complex endonucleases XPF-ERCC1 and XPG. (6) After excision around the damaged site [261], a DNA polymerase can resynthesize the strand of nucleotides, before DNA ligase finally seals the resulting nick. We refer to the following reviews for further information [49,77,260,262]. Figure prepared using BioRender.com.
(XPC-RAD23AB), Rad2 nuclease (Human XPG/ERCC5), Rad1-Rad10 nuclease (Human XPF/ERCC1), replication protein A and TFIIH. These are structurally and functionally conserved between budding yeast and human [96].

The Rad4-Rad23 complex seems to bind 6,4-PPs, but not cyclobutane pyrimidine dimers, similar to human XPC-RAD23B [97]. In fission yeast, two XPC homologs, Rhp41 and Rhp42 [98], operate in NER along with Rhp23. The regulatory E3 DDB1-CUL4 is conserved in most eukaryotes, including fission yeast [99]. In budding yeast, which lacks a CUL4 orthologue, its functions appear instead to be carried out by the distantly related Rtt101 [100]. For further information regarding the role of RAD23 in NER as well as the more general role of the UPS in NER we refer to the following reviews [101–104].

4. Intracellular protein degradation via the ubiquitin-proteasome system

Most protein degradation in the cytosol and nucleus of eukaryotic cells occurs via the ubiquitin-proteasome system (UPS) [105–107]. Here, proteins are first conjugated to ubiquitin through an enzymatic cascade consisting of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-protein ligase [108,109]. Repeated cycles of this so-called ubiquitylation reaction result in the formation of a target protein conjugated to a chain of ubiquitin moieties (Fig. 3A). As conjugation occurs on the ε-amino group in a lysine side-chain, and since ubiquitin itself contains seven lysine residues, different types of ubiquitin chains can be formed, depending on which of the ubiquitin lysine residues engage in chain formation [110]. The preferred signal for proteasomal degradation is ubiquitin chains linked through K48. The ubiquitin chain on the target protein allows for specific interaction with subunits of the 26S proteasome [31–33]. The 26S proteasome is composed of two large sub-complexes, the 20S core proteasome and the 19S regulatory complex. The proteolytic activity resides within the cylindrical 20S proteasome, while the 19S regulatory complex binds the ubiquitylated target proteins. Once bound, ATPase subunits in the 19S regulatory complex catalyze the unfolding of the protein substrate and the opening of the “gate” into the 20S core [111,112]. Then the substrate is deubiquitylated by specific 19S subunits and associated deubiquitylating enzymes (DUBs) [113], and threaded into the lumen of the 20S proteasome particle where the target protein is ultimately degraded (Fig. 3A).

5. RAD23 in the ubiquitin-proteasome system

As mentioned above, RAD23 plays a central role within the UPS and binds to the proteasome and its ubiquitylated substrates via the UBL and the UBA domains, respectively (Fig. 3 ABC).

Together with other so-called UBL/UBA proteins, such as the human
ubiquilins (UBQLN1–4) and their yeast orthologue Dsk2 [114], RAD23 acts as a proteasomal substrate shuttle. These proteins bind substrates targeted for degradation and tether them to the proteasome, thus promoting their degradation [12,18,30,115–119].

In addition to the UBL/UBA shuttle factors, the intrinsic proteasome subunits Rpn1 (PSMD2 in humans), Rpn10 (PSMD4 in humans), and Rpn13 (ADRM1 in humans) of the regulatory particle may also bind to ubiquitin chains directly [111]. The relative contribution of the intrinsic (proteasome subunits) and extrinsic ubiquitin receptors (UBL/UBA proteins) is still largely unknown and likely to depend on the substrate protein [120] and the cellular conditions. The importance of RAD23 in the degradation of specific substrates has been demonstrated biochemically in vitro [121,122]. Also, non-denaturing gel electrophoresis has shown the formation of a proteasome-Rad23-poly-ubiquitylated substrate complex, which was dependent on functional UBL and UBA domains, indicating that Rad23 facilitates substrate-proteasome interactions [123]. Likewise, a decrease in proteasome-associated ubiquitin conjugates has been observed in a rad23Δdsk2Δ double knockout strain [124], hinting that many proteasome substrates are degraded via Rad23 and Dsk2. The importance of extrinsic shuttle factors is further emphasized by accumulation of ubiquitin-protein conjugates and other severe phenotypes observed in mutants lacking these proteins [122,44,85,125–127] (Box 1). Also, RAD23A has been found to outcompete ubiquitin for binding to the proteasome at certain sites [128] and human RAD23A and RAD23B stimulate proteasome peptidase activity [129,130]. Interestingly, free mono-ubiquitin did not facilitate increased degradation, while ubiquitin chains did [129,130]. This stimulatory effect was caused by the UBL domain and is also observed for many other UBL domain proteins [129–131]. Notably, the UBL domains could replace the need for a ubiquitin chain for 26S proteasome activation through allosteric mechanisms leading to opening of the gate into the 20S particle and stimulation of the 19S ATPase activity [129–131]. Additionally, full length RAD23B and its UBL domain were shown to activate latent proteasomes [130,131].

While UBL domains may mimic ubiquitin, their binding affinities to the intrinsic ubiquitin binding subunits are distinct from those of ubiquitin. Similarly, one must assume the positioning of a ubiquitylated substrate relative to the 26S proteasome is different, depending on whether a UBL/UBA shuttle is involved [132]. Thus, shuttle factors enable a new level of substrate regulation.

In humans, the proteasomal ubiquitin receptor Rpn10 has two ubiquitin interaction motifs, UIM1 and UIM2, which were shown to bind ubiquitin [133]. One study found that the UIM2 motif preferably binds to the RAD23A UBL domain over ubiquitin, and demonstrated the formation of a ternary RPN10-RAD23A-ubiquitin complex [128]. Based on these observations, and previous findings that the RAD23A UBL binds UIM2, but not UIM1 [29], the authors suggested a model in which the substrate-ubiquitin chain initially binds to the UBA domain of RAD23A, and consequently becomes associated with the proteasome as the UBL domain binds the Rpn10 UIM2. This, in turn, would facilitate human Rpn10 UIM1 binding of the poly-ubiquitin chain, which would likely help position the substrate aptly for further proteasomal processing (Fig. 3C) [128]. Importantly, since the proteasome contains multiple ubiquitin-binding subunits, a similar situation can be reached if RAD23 rather interacts with Rpn1 or even Rpn13. In yeast, Rpn10 [134,135] and Rpn13 [136] only contain one UIM and a ubiquitin-binding Prudom domain, respectively [137,138]. Rpn1, however, contains nearly a dozen proteasome/cyclosome (PC) repeats (Fig. 3B). These were shown to not only interact with poly-ubiquitylated species, but also with the UBL domains of Rad23, Dsk2 as well as the UBL domain of the proteasome-associated deubiquitylating enzyme Ubp6 (USP14 in humans) [137,138]. Accordingly, there is a suggested model for S. cerevisiae in which, through binding of the UBL domain to Rpn1, and ubiquitin chains to Rpn10, a substrate is anchored to the proteasome at multiple locations and in an orientation suitable for the subsequent degradation of the substrate [137]. Interestingly, it has been shown that Rpn1 can also simultaneously accommodate both Rad23, Ubp6 and K48-linked ubiquitin [112], with the proteins likely cooperating to facilitate ubiquitin binding and deubiquitylation in a coordinated manner [112,137] (Fig. 3B). However, the system seems to exhibit a large degree of redundancy, with UBL proteins and ubiquitin-chains being able to bind the same sites of the proteasome subunits both in yeast [137] and humans [128]. This promiscuity is possibly important for the proteasome shuttle system to function efficiently.

6. UBL-proteasome interaction

In the 26S proteasome, multiple UBL domain binding sites have been

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**Box 1**

*Phenotypes of RAD23 knock-outs.*

Despite the mentioned UV-sensitivity, yeast strains lacking Rad23 appear largely as the wild-type. They display normal growth at various temperatures [127,264] and show no notable accumulation of ubiquitylated substrates [264]. Since the 26S proteasome is essential, this indicates that although Rad23 is broadly involved in the UPS, under normal conditions RAD23 function is not strictly required. However, *rad23Δ* strains do exhibit sensitivity to the amino acid analog canavanine [127], and some model substrates are stabilized in this background [119,127]. More notable phenotypes are observed when other ubiquitin binding factors are deleted as well. Thus, in budding yeast the *rad23Δ* strain displays severely slowed growth and is inviable at increased temperatures [127,264] and show no notable accumulation of ubiquitylated substrates [264]. Since the 26S proteasome is essential, this in...
reported, including the intrinsic ubiquitin receptors Rpn1, Rpn10, and Rpn13. Hence, human RAD23A and RAD23B have been reported to bind the C-terminal ubiquitin-interaction motif UIM2 of the human Rpn10 subunit [25,29,139,140] and the ubiquitin-binding Pru-like domain of Rpn13 [136]. The S. cerevisiae and S. pombe orthologues of RAD23, however, have been reported to bind the proteasome-cyclosome (PC) repeats or toroid ubiquitin binding site on the Rpn1 subunit [13,30,137,141,142].

The toroid binding site binds two ubiquitins in tandem. Rad23-UBL binds exclusively the high affinity site corresponding to helix 28/30. In the interaction, the UBL domain mimics the classical basic and hydrophobic residues of ubiquitin, but binding is further enhanced compared to ubiquitin by conserved positive (K10 and K11) and hydrophobic (V50 and V69) residues not found in ubiquitin [141]. These data, however, do not exclude the possibility of RAD23 binding to other ubiquitin-binding proteasome subunits as well, since in vivo yeast Rad23 is ubiquitylated at multiple positions in the UBL domain [149], an effect which is countered by the DUB Ubp12 that also interacts with the Rad23 UBL domain [144]. The ubiquitylation of the Rad23 UBL domain appears to stimulate its function in protein degradation [144], but will likely also affect interactions with other ubiquitin-binding proteins. In addition, although the ubiquitin-binding proteasome subunits in general appear to display greater affinity for ubiquitin chains than for free (mono) ubiquitin, most still bind mono-ubiquitin, albeit with reduced affinity [133,145-147]. Hence, binding of the structurally related UBL domain to these sites seems reasonable. Accordingly, yeast Rad23 has been shown to bind Rpn10, although weakly compared to Rpn1 [148].

Indeed, structural studies found that S. cerevisiae Rad23 is evolutionarily fine-tuned to bind Rpn1, thus showing binding preference towards this site [141]. Other UBL-domain proteins such as the human UBQLNs and their yeast orthologue Dsk2 also interact with Rpn1. While Dsk2 prefers to bind Rpn13 [141], it also interacts with Rpn1 [137,149], and accordingly, Rad23 and Dsk2 compete for binding to the proteasome regulatory particle [30,150]. Similarly, S. pombe Rhp23 competes with another UBL domain protein, Bag101, for proteasome binding [151].

Finally, binding of the UBL domain to the proteasome may also be subject to regulation. In yeast, the ubiquitin-binding proteasome subunit Rpn10 is produced in excess of the other proteasome subunits. The resulting extra-proteasomal Rpn10 prevents the UBL/UBA protein Dsk2 from docking at the proteasome [152]. A similar mechanism may potentially restrict the RAD23-proteasome interaction. Budding yeast Rad23 also interacts with peptidyl-tRNA hydrolase Pth2, which inhibits Rad23-proteasome interaction, and accordingly ubiquitin-dependent degradation is accelerated in a pth2Δ strain and retarded by overexpression of Pth2 [153]. In addition, in S. cerevisiae the Rad23-proteasome interaction is also more directly regulated through phosphorylation [154]. Hence, phosphorylation of serine residues in the Rad23 UBL domain reduces proteasome binding [154], and as human RAD23A and RAD23B are also phosphorylated [155,156], this mechanism may be conserved [154]. In conclusion, there appears to be some redundancy as to which proteasomal subunits RAD23 interacts with. However, in general yeast Rad23 seems to prefer binding to Rpn1, while human RAD23 interacts with Rpn10 (Fig. 3 BC).

7. UBA-ubiquitin interaction

The two UBA domains in RAD23 [26,27,157] allow it to bind ubiquitin with either UBA domain [12,40]. These domains bind K48-linked poly-ubiquitin chains [39,158] and poly-ubiquitylated substrates [115,159]. Optimal binding affinity is achieved at ubiquitin chain lengths of 4-6 moieties [39]. Work on budding yeast Rad23 has shown that UBA1 binds ubiquitin better than UBA2 [28,40].

While the UBA domains show high binding preference for K48-linked ubiquitin chains, they also bind K63- and K29-linked chains [39,158], and distinct binding conformations have been reported between K48 and K63 di-ubiquitin chains [39,160,161]. When K48-linked ubiquitin chains bind to RAD23A, high binding affinity is achieved by the formation of a UBA:di-ubiquitin complex where UBA2 is sandwiched between two ubiquitin moieties [162].

Interestingly, ubiquitin binding differs between Rad23 and other ubiquitin binding domains such as Rpn10, as point mutations in conserved, buried residues of ubiquitin (L67S and L69S), fail to bind the UIM of Rpn10, while retaining strong binding to Rad23 and Rad23A [163].

Notably, the RAD23 ubiquitin-binding preferences differ from that of the UBL/UBA protein UBQLN1, which shows no preference between K63 and K48-linked ubiquitin chains [164]. These different binding preferences most likely provide substrate specificity between the UBL/UBA proteins. One study has attempted to map the observed distribution of ubiquitin chain types in S. cerevisiae for shuttle proteins and other ubiquitin binding domains more systematically [124]. However, relatively little work has been done investigating shuttle factor-specific substrates. Currently known Rad23 substrates comprise the cyclin-dependent kinase inhibitors S. cerevisiae Sic1 and Far1 [121], S. pombe Rum1 [12], and various others, including artificial substrates [119,127,165]. Finally, by engaging in several well-described degradation pathways (see below), the degradation of a wide variety of proteasome substrates appears to depend on RAD23.

8. RAD23 interaction with PNGase and function in ER-associated degradation

Budding yeast Rad23 also functions in the ubiquitin-proteasome dependent degradation of misfolded secretory proteins through the so-called endoplasmic reticulum (ER)-associated degradation (ERAD) pathway [166]. In this pathway, secretory proteins that fail to attain a native folded conformation in the ER are retrotranslocated into the cytosol and degraded by the UPS. This translocation is mediated by an ER membrane residing protein complex containing E3 ligases that associates with the hexameric ring-shaped ATPase complex known as p97 or Cdc48, which ultimately powers the extraction of the misfolded ER or transmembrane proteins [167–170]. Within this system, RAD23 interacts with the highly conserved [171] peptide:N-glycosylase (PNGase) through interactions of its XPCB domain [172]. This interaction has been demonstrated in human [7,172], mouse [173,174], budding yeast [17,35] and plant cells [175]. PNGase is involved in ERAD of N-glycosylated proteins, by ensuring that any substrate N-glycosyl groups are removed from the protein, before undergoing proteasome-dependent degradation [176–179]. By virtue of its ability to bind the proteasome and PNGase, RAD23 thus effectively couples deglycosylation and degradation.

X-ray diffraction studies on yeast Rad23 and PNGase showed the interaction to occur between the XPCB-domain of Rad23 and the PNGase N-terminal Rad23 binding domain, although the C-terminal tail of PNGase also connects with Rad23 [17]. Overall, this agrees with biochemical assays [7,8,180]. Interestingly, X-ray crystallography on the mouse RAD23:PNGase complex [174] showed variations from the yeast structure [17], with different sides of the yeast and mouse XPCB interaction to occur between the XPCB-domain of Rad23 and the PNGase N-terminal Rad23 binding domain, although the C-terminal tail of PNGase also connects with Rad23 [17]. Overall, this agrees with biochemical assays [7,8,180].

By virtue of its ability to bind the proteasome and PNGase, RAD23 thus effectively couples deglycosylation and degradation.
cleft, which obstructs accessibility of native glycoproteins, but not of more flexible denatured glycoproteins [17], which explains reported preference of PNGase for denatured substrates [17,181–184].

The PNGase N-terminal extension is only found in metazoan homologs [185], and contains a PUB (PNGase/UBA or UBX) domain. This domain is found in proteins also containing UBA and UBX domains, and the domain is associated with ERAD and the UPS [186,187]. The PUB domain allows PNGase to interact with other ERAD components [9,173], the UBL domain of RAD23A [172], and most notably the C-terminal tail of p97 [188–190].

The metazoan PNGase C-terminal extension contains a mannose-binding carbohydrate recognition domain (CRD), which likely binds glycosylations of translocated glycoprotein substrates in an orientation that favors deglycosylation [191,192]. Together, all these interactions allow for efficient ERAD-substrate processing (Fig. 4). While de-glycosylation is not necessary for proteasomal degradation [181], and yeast mutants lacking PNGase are viable and capable of degrading various glycosylated substrates [171], deglycosylation likely makes the degradation process more efficient [181,193]. This is supported by the increased half-life of the CPY* model ERAD substrate in a PNGase deletion mutant [171]. However, the ricin A chain (RTA) ERAD substrate seems to rely on both PNGase and Rad23, for efficient degradation. Functional Rad23 UBA domains were also needed for proper RTA degradation [180]. Hence, in this case substrate binding through PNGase cannot substitute for Rad23 ubiquitin interaction.

9. RAD23 in the UFD degradation pathway

In addition to ERAD, RAD23 has also been implicated in the so-called ubiquitin fusion degradation (UFD) pathway, which was first characterized using proteins artificially fused to ubiquitin that are rapidly degraded via the UPS [115,119,165,166,194]. In this pathway, the p97/Cdc48 ATPase complex again plays a central role. Along with its cofactors Npl4 and Ufd1, it catalyzes the unfolding of poly-ubiquitylated substrates [195]. Unlike the 26S proteasome ATPase ring which engages substrates via disordered tails or internal flexible loops in the substrate protein [196–198], the p97/Cdc48 ATPase, bound to the Npl4 and Ufd1 cofactors, initiates substrate processing through the conjugated ubiquitin chain [199,200]. This leads to translocation of the substrate through p97/Cdc48 ATPase ring, resulting in mechanical unfolding of the substrate. The process is strictly dependent on the substrate being conjugated to K48-linked ubiquitin chains [124,199,200] and the unfolding thus leads to dissociation/segregation of ubiquitylated target proteins from their binding partners and thus stimulates their proteasomal degradation [201].

Within the UFD pathway, the ubiquitin ligase Ufd2 catalyzes the ubiquitylation of UFD substrates [202,203]. In turn, this allows them to be bound by RAD23 and shuttled to the proteasome for degradation [194]. Yeast Rad23 interacts with the N-terminal region of Ufd2 [194,204], which in turn binds p97/Cdc48 [203], resulting in a Rad23/Ufd2/Cdc48 complex [165,205] (Fig. 5). Since the Rad23 UBL domain separately binds to both Ufd2 and the proteasome, it needs to dissociate from Ufd2 before it can interact with the proteasome [194]. Accordingly, it has been shown that p97/Cdc48 promotes disassembly of the Rad23-Ufd2 complex [205]. Intriguingly, the ability to bind Ufd2 seems essential for the degradation of the UFD substrate UbV76-V-gal, since adding the Rad23 UBL to another shuttle protein, Ddi1, or otherwise allowing Ddi1 to bind Ufd2, seems to restore UbV76-V-gal degradation in a rad23Δ background [194].

While the UFD pathway is often investigated using artificial fusion proteins, it also includes endogenous targets that are not ubiquitin fusion proteins but simply proteins where their degradation pathway involves the UFD components. Indeed the fusion of ubiquitin may mimic ubiquitylation, and many cellular proteins are thus expected to follow the UFD pathway. Well studied UFD substrates include the yeast proteins Hmg2 [205] and Spt23 [165], and via an adapter protein Ufd2 has also been linked to poly-ubiquitylation of unfolded myosin [206]. However, the involvement of RAD23 for the degradation of these proteins has not been tested. Although the UFD-pathway and Cdc48-Ufd2-Rad23 degradation-axis have been predominantly studied in yeast, the human RAD23A and RAD23B homologs likely perform similar functions. At the very least, one study, mapping the interactome of the RAD23B UBL domain, found that it interacts with human p97, Ufd1 and Npl4 [207]. Co-precipitation studies also found an interaction between RAD23B and human Ufd2 (UBE4B) [208,209].

10. Intra- and intermolecular associations between the UBL/UBA domains

Given the nature of their domains, one might expect RAD23 and its fellow UBL/UBA proteins to interact with one another [210]. Indeed, S. cerevisiae Rad23 and the UBL/UBA protein Ddi1 form heterodimers with their respective UBL and UBA domains [211]. Human RAD23A forms heterodimers with UBQLN1 through similar domain interactions [212]. Additionally, budding yeast Rad23 forms homodimers through its C-terminal part (XPCB and UBA2 domains) in vitro [211,213]. However, no such homodimers have been observed for the human RAD23A [25].

Finally, RAD23A forms intramolecular contacts leading to a closed and likely inactive [122,172,214] conformation with the UBL domain interacting with one of either UBA domains [25,214]. Importantly, this conformation is abrogated in the presence of either Rpn10 or ubiquitin, allowing RAD23A to unwind and efficiently perform its shuttle functions [25,215].

It has been suggested that the inactive, “closed” dimer conformation
The foci formation appears to be relevant only under stress conditions. Nuclear foci such as nucleoli, PML bodies, or DNA damage foci [221] contain K48-conjugated ubiquitin [221]. Importantly, these foci are distinct from other known proteasomes with their surroundings, and contain K48-conjugated ubiquitin [165].

UBQLN2, which forms condensates in vivo, the follicular region related to liquid-liquid phase separation (LLPS). Indeed, for the human heteromeric complexes of RAD23 and other UBL-UBA shuttles may be becoming available for binding the proteasome after the UBA domains - much like the above starvation in human cells. These inclusions, called starvation-induced proteasome, it was shown that RAD23B-dependent nuclear foci form in response to stress [218]. These foci, which among other roles appear to regulate the degradation of hyperosmotic stress-induced orphan ribosomal proteins, also contained K48-linked, but not K63-linked, proteins as well as other UPS components such as p97 and the E3 ligase E6AP. Notably, knocking out RAD23B greatly attenuated the foci formation, whereas a knockdown of the UBQLNs, RAD23A or XPC had no effect. Foci formation could be partially restored in knock-out cells upon introduction of a UBL-defective (L8A) RAD23B, but not by a UBA-defective (L225A, L401A) mutant, indicating that the UBA-domains are essential for the foci formation. Dependent on the UBA domains, RAD23B formed condensates in vitro; the presence of ubiquitin chains and poly-ethylen glycol as crowding reagent [218]. This is similar to the situation with UBQLN2, where the UBA-domain also promotes phase-separation [217].

UBQLN2 is likely explained by multiple factors. For instance, UBQLN2’s LLPS-promoting interactions have been reported to rely on their STI1 domains [222-225] of which there are four, compared to RAD23’s single STI1-like XPCB domain. In addition, differences in the ubiquitin chain binding preferences of RAD23 and UBQLN2 may play a role, since for UBQLN2 it has been shown that K11- or K48-linked tetra-ubiquitin inhibits LLPS, while K63- or M1-linked chains enhance LLPS [226].

Finally, RAD23B has also been observed to become sequestered in intracellular inclusions upon overexpression of aggregation-prone poly-Q proteins, including poly-Q expanded huntingtin. The sequestration effect is dependent on the UBA domains and sufficiently strong to confer a reduced abundance of XPC (presumably by destabilization as a result of stripping XPC of RAD23) [227]. However, unlike the above LLPS-like foci which appear to depend on RAD23-RAD23 interactions, these inclusions likely represent insoluble protein aggregates, which may form upon accumulation of any severely misfolded protein.

11. RAD23 as a stabilizer and negative regulator of poly-ubiquitin chain assembly

Through their binding to ubiquitin chains, the RAD23 UBA1 and UBA2 domains can prevent K48 chain elongation [40,158,159], due to K48 being a part of the binding surface recognized by the UBA domain [214,215]. In contrast, K29- and K63-linked chains are not occluded by UBA binding and consequently their elongation is not inhibited by RAD23A binding [158]. Additionally, Rad23 overexpression has been shown to inhibit protein degradation in budding yeast [159,228]. The apparent explanation for these observations is that a surplus of Rad23 decreases the probability of a given Rad23 protein interacting simultaneously with both a substrate and the proteasome [229]. Some studies suggested the UBA domains (especially UBA2) of Rad23 to be responsible for the observed protein stabilization [122,158,228], but the UBL domain is equally capable of preventing protein degradation [129,131]. However, the mechanisms are likely different, as the UBL domain is expected to outcompete binding to the proteasome, whereas the UBA domains shield the ubiquitin chains from recognition by other proteins and subsequent degradation.

While the stabilizing effects of RAD23 did cause some confusion around its role and caution the interpretation of data relying on overexpression, RAD23 function as a facilitator of protein degradation appears undisputed. It has been suggested that the binding of the UBA domain of RAD23 and of other shuttle proteins to ubiquitin chains, help prevent excessive ubiquitin chain elongation or premature de-
ubiquitylation. Thus, directionality to the process may be provided by shielding the substrate from DUBs until appropriate interactions with the proteasome can be initiated. It is important to notice that such degradation regulation, through the presence of ubiquitylated and degraded. Accordingly, across eukaryotes, RAD23 and other proteasomal substrate shuttles (including UBQLN1-4) display a remarkable lack of lysine residues. In human RAD23A, a central 198-residue stretch, devoid of lysine, spans 54% of the protein from the UBL domain to the UBA domain. Introducing lysine residues by mutagenesis of this so-called lysine desert results in RAD23A ubiquitylation and reduced UV tolerance. Thus, the lysine desert appears important for function. However, despite being ubiquitylated at two native lysine residues in the UBL domain and at lysines residues introduced in the lysine desert, RAD23A is not rapidly degraded.

The stability of RAD23 has been noted before. Thus, despite its association with the proteasome, RAD23 generally appears to be a stable protein in both yeast and humans, somehow evading the destiny of degradation that befalls its substrates. Indeed, artificial binding of model proteins to the 26S proteasome is sufficient to trigger their ubiquitin-independent degradation. Accordingly, it has been demonstrated that the presence of a UBL domain can increase the degradation of proteins, supposedly on account of the domain’s ability to bind the proteasome. The stability of RAD23 seems to be tied to its stable, well-folded domains, which leave no unstructured region available for initiation of the ATPase-dependent substrate-unfolding and subsequent threading into the proteolytic particle. Indeed, fusing a disordered region to the C-terminus of S. cerevisiae Rad23 did cause its rapid degradation, whereas substituting the C-terminal UBA2 domain with another stably folded domain did not affect stability. Interestingly, internally unstructured regions also seemed to facilitate degradation, although only when present at lengths much longer than any of those found in RAD23.

It is important to notice that such degradation regulation, through the presence or absence of unstructured initiation sites, is likely a common property of protein degradation rather than a specific trick utilized by RAD23 and its fellow protein shuttles. Accordingly, studies on three substrates undergoing non-canonical ubiquitin-independent proteasomal degradation also concluded that only proteasomal association and the presence of an unstructured region in either end of the protein were necessary to achieve proteasome substrate degradation. Moreover, the sequence composition may play a role in degradation evasion. It has been shown, that disordered tails of proteasome substrates have sequences unfavorable for protein degradation initiation. While such degradation-evading sequence traits are found in many of the proteasomes substrates and the principle also has been demonstrated for proteasome substrates, the sequences and mechanisms are still poorly understood.

Given that the linker regions between the folded domains in RAD23 possess disordered stretches, it is tempting to imagine these sequences also contribute to stability. Indeed, when fused onto an artificial model substrate, these linkers rendered the substrates stable compared to a control sequence. However, since replacing the loops with peptides of different and unbiased sequences does not substantially destabilize Rad23, their contribution to stability appears minor.

In spite of its resilience to proteasomal degradation while working as a shuttle factor, RAD23 will eventually become a substrate itself. Human RAD23A and RAD23B bind to the E3 ligase E6AP, and undergo E6AP-dependent ubiquitylation and degradation. Hence, perhaps the presence of a ubiquitin chain may stimulate unfolding and degradation of RAD23. Likewise, RAD23 might be positioned differently at the proteasome when it interacts as substrate rather than as a co-factor.

RAD23A is degraded in a cell cycle dependent manner, with levels being highest during M-phase before gradually dropping fivefold over late G1 and S-phase after which the levels return in M-phase. Overexpression of E6AP did not lead to increased degradation of RAD23A during mitosis, suggesting the underlying regulation is not merely a result of E6AP upregulation. A study on endogenous RAD23B in HeLa cells also showed a drop in nuclear RAD23B levels during S-phase, and this cell-cycle dependent regulation pattern has also been reported in yeast. Indeed, RAD23 could be linked to functions in either the UPS or in DNA repair.

13. Concluding remarks

Here, we have reviewed the roles RAD23 in NER and the UPS. Although it appears that these functions are independent, and that RAD23 thus truly performs separate functions, it is important to note that the general nature of the UPS links degradation indirectly to most cellular processes, including NER. Thus, RAD23’s role in protein degradation may affect DNA repair processes indirectly, through regulatory degradation of various DNA repair proteins. For instance, in yeast the XPC homolog Rad4 is ubiquitylated and degraded by the proteasome. Potentially this could provide a regulatory mechanism to attenuate the DNA repair system. This may also explain why the RAD23 UBL domain has been reported to have some role in NER function. Conversely, Rad4 has also been linked to protein degradation via the UFD pathway, and XPC has been shown to regulate turnover of p53.

In addition, other studies have linked XPC to additional functions, including chromatin remodeling and cell signaling. It is possible that RAD23 is also involved in these processes, through its interaction with XPC. As for the function of RAD23 and other UBL/UBA domain proteins, one may wonder why these are necessary at all. In particular since the 26S proteasome is highly abundant and already equipped with multiple ubiquitin-binding substrates. However, having multiple mutually redundant substrate receptors may make the system more robust. In particular, since the shuttle proteins have different ubiquitin chain preferences and may interact with the proteasome at different sites, this may effectively broaden the substrate specificity of the proteasome, which essentially must be able to target the entire proteome.

We discuss, RAD23 and other shuttle-proteins counteract unnecessary ubiquitylation after an appropriate ubiquitin chain length has been reached, and may shield ubiquitylated substrates from DUBs, thus adding directionality to the pathway. Perhaps most importantly, the shuttle proteins allow for regulation of the 26S proteasome. Thus, upon binding to proteasomes, RAD23 (and other UBL/UBA shuttles) allosterically stimulate the ATPase activity of the 26S proteasome and increase its proteolytic activity. Regulation of degradation therefore appears to not only occur at the level of target ubiquitylation, but also downstream of ubiquitylation at the last possible step before degradation. It is tempting to speculate that perhaps tightly folded substrate proteins may rely more heavily on RAD23’s ability to activate the proteasomal ATPase activity. In this respect, it is important to note the rather subtle phenotypes of cells with deletions in the RAD23 encoding genes (Box 1). Hence, under normal conditions the UPS appears perfectly functional without RAD23. Future studies on RAD23 and other
UBL/UBA domain proteins will likely reveal under which conditions this regulation becomes important and identify specific substrates. Such studies, along with a deeper characterization of the RAD23-containing foci may unravel important new insights. Furthering our mechanistic understanding of RAD23-mediated substrate delivery at the 26S proteasome will require detailed structural and single molecule studies [257]. An increased mechanistic and structural understanding of how RAD23 collects ubiquitylated target proteins at E3 is also warranted, as are more detailed studies on RAD23 in NER. Finally, as more and more proteins are being characterized in ever increasing detail, it will be interesting to follow how wide-spread the phenomena of moonlighting proteins is.

Abbreviations

CPD cyclobutane pyrimidine dimer
CRD carbohydrate recognition domain
CSN COP9 signalosome
DUB deubiquitylating enzyme
ERAD ER-associated degradation
GG-NER global genome NER
LLPS liquid-liquid phase separation
NER nucleotide excision repair
PC proteasome/cyclosome
UBL ubiquitin-like
UFD ubiquitin fusion degradation
UPS ubiquitin-proteasome system
XPCB XPC-binding

6,4-PP (6–4) photoproduct

Funding
We are supported by grants from Novo Nordisk Foundation (https://novonordiskfonden.dk) challenge programme PRISM (NNF18OC0033950), REPIN (NNF18OC0033926), and NNF21OC0071057, the Independent Research Fund Denmark (Natur og Univers, Det Frie Forskningsråd) (https://dff.dk/) 10.46540/2032-00007B, and the Villum Foundation (https://veluxfoundations.dk/) 40526. The funders had no role in the preparation of the manuscript or decision to publish.

CRediT authorship contribution statement

Martin Gronbaek-Thygesen: Writing – original draft, Writing – review & editing, Visualization. Caroline Kampaemeyer: Writing – review & editing, Visualization. Kay Hofmann: Writing – review & editing, Conceptualization. Rasmus Hartmann-Petersen: Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors thank Asta B. Andersen for helping with the illustrations, and apologize to those authors whose work we were unable to cite due to space constraints. Some figures were prepared using BioRender.com.

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11

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