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Title: Ubiquitin-dependent regulation of translesion polymerases

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Abbreviations used: APC, anaphase promoting complex; BRCT, BRCA1 C-terminal; CPDs, cyclobutane pyrimidine dimers; PAD, polymerase-associated domain; PCNA, proliferating cell nuclear antigen; PIP, PCNA interacting peptide; pol, polymerase; RIR, REV1-interacting region; TLS, translesion synthesis; UBM, ubiquitin binding motif; UBZ, ubiquitin-binding zinc finger; USP1, ubiquitin specific protease 1; XPV, xeroderma pigmentosum variant.

Abstract

In response to DNA damage, TLS (translesion synthesis) allows replicative bypass of various DNA lesions, which stall normal replication. TLS is achieved by low-fidelity polymerases harboring less stringent active sites. In human, Y-family polymerases together with Pol ζ (polymerase ζ) are responsible for TLS across different types of damage. Protein-protein interaction contributes significantly to the regulation of TLS. REV1 plays a central role in TLS because it interacts with all other Y-family members and Pol ζ. Ubiquitin-dependent regulatory mechanisms also play important roles in TLS. Ubiquitin binding domains have been found in TLS polymerases and they might be required for TLS activity. Monoubiquitination of PCNA (proliferating cell nuclear antigen), the central scaffold of TLS polymerases, is thought to promote TLS. In addition, both non-proteolytic and proteolytic polyubiquitination of PCNA and TLS polymerases has been demonstrated. Owing to their low fidelity, the recruitment of TLS polymerases is strictly restricted to stalled replication forks.

Introduction

Transmission of genetic materials from one generation to the next is the essence of life. Organisms have evolved accurate machineries to ensure faithful replication of DNA genome. Replicative DNA polymerases are characteristic for their high processivity and high fidelity which are achieved by their stringent active sites and the 3′-5′ proofreading exonuclease activity. DNA, however, is constantly subject to damage caused by both extrinsic and intrinsic agents, such as UV light and reactive oxygen species. In order to maintain the integrity of genome, cells are endowed with elaborate DNA damage repair mechanisms, including nucleotide excision repair, base excision repair, mismatch repair, homologous recombination and non-homologous end joining, to remove DNA lesions before replication starts. However, some damages may escape from the repair machinery and persist in the genome. Such lesions on DNA template hinder the incorporation of nucleotides by replicative DNA polymerases because the stringent active sites of these polymerases accommodate normal template bases and correct complementary nucleotides only. Consequently, unrepaired DNA lesions cause replication block. Since stalled replication forks can lead to major chromosome abnormalities and cell death, replicative arrests have to be circumvented to maintain cell survival. Cells have evolved DNA damage tolerance or post-replicative repair mechanisms to bypass damages, restoring DNA replication. One proposed lesion bypass pathway involves template switching in which the undamaged sister chromatid, instead of the damaged strand is used as a template for DNA replication in order to restore the correct sequence opposite the lesion. This pathway is generally considered to be error-free and the proposed models are discussed in a recent review [1].

Another DNA damage tolerance pathway which is extensively studied is TLS (translesion synthesis), in which nucleotides are inserted opposite the lesion, without

repairing it. TLS employs specialized DNA polymerases which lack proofreading activity and have low processivity as well as low fidelity. In contrast to replicative polymerases, their active sites are more relaxed and thus can accommodate bulky distorted damaged bases [2]. TLS is evolutionarily conserved and the proteins involved can be found in bacteria and in both lower and higher eukaryotes. Most of these proteins belong to Y-family of DNA polymerases, a new family following five preceding ones (A, B, C, D and X). In bacteria there are DNA Pol (polymerase) IV and Pol V. In budding yeast, there are two members, REV1 and Pol η. In mammals, Y-family polymerases expand to four members, REV1, Pol η, Pol ι and Pol κ, implicating the complexity of TLS in higher eukaryotes [3]. In addition to Y-family polymerases in eukaryotes, Pol ζ is a member of the B-family DNA polymerases, which include high-fidelity replicative polymerases such as Pols α, δ and ε, yet displays TLS activity. Pol ζ is capable of extending terminally mismatched or distorted primers opposite to DNA lesions more efficiently than Pol α. Like Y-family polymerases, Pol ζ has low processivity [4] and does not possess 3′-5′ proofreading exonuclease activity [5].

Mammalian translesion polymerases

Like high-fidelity replicative polymerases, Y-family DNA polymerases contain the typical catalytic core, including thumb, fingers and palm domains, in the central region of the proteins. Structural studies of TLS DNA polymerases, however, reveal that the thumb and finger domains are remarkably shorter than those in replicative DNA polymerases. This structure gives a more open and relaxed active site to accommodate bulky DNA lesions and to allow mismatched base pairing to occur. Such loose conformation generates a considerably smaller interface between the DNA buries and the catalytic core. Consequently, the “induced-fit” conformational change

around the active site is apparently destroyed, conferring low fidelity on Y-family polymerases [6]. In addition to the catalytic core, Y-family DNA polymerases also harbor a little finger domain, also referred as PAD (polymerase-associated domain), at the C terminus of the catalytic core. PAD extends from the fingers domain to mediate DNA binding and the movement of primer-template duplex [2]. The interaction between PAD and the primer-template duplex after nucleotide insertion opposite the damaged base is proposed to trigger polymerase switch, likely through conformational change [7]. Although the amino acid sequence of PAD is relatively diverse among the members of Y-family polymerases, its structural conformation is conserved. Noticeably, the diversity of PAD sequence may contribute to the differences in lesion specificity among the members in the family [2].

In general, TLS is considered to be a mutagenic process owing to the low fidelity of polymerases involved. However, some TLS polymerases are capable of replicating over certain lesions efficiently and with considerable accuracy; one prominent error-free example of TLS is carried out by Pol η. Pol η is exclusively found in eukaryotes and it is particularly important to human because of its protective role against skin cancer. Loss of Pol η activity is identified in a genetic disorder known as XPV (xeroderma pigmentosum variant), which is more vulnerable to skin cancer and has higher sensitivity to sunlight [8]. Pol η is able to accurately and efficiently replicate through CPDs (cyclobutane pyrimidine dimers), a major UV-induced DNA lesion [9]. Elevated UV-induced mutation frequency is observed in Pol η knockout mouse embryonic fibroblasts [10]. On the other hand, overexpression of Pol η does not increase spontaneous mutation in human cells [11]. It is apparent that Pol η is specialized for the bypass of CPD in an error-free manner, and this accurate process is less likely to be substituted by other polymerases in the family. In support of this, human Pol η has low processivity with undamaged DNA whereas the processivity

increases remarkably when it bypasses *cis-syn* thymine dimer, and it preferentially inserts dATP opposite both thymines [7]. In primary fibroblasts of Pol η knockout mice, UV-induced mutagenesis is largely suppressed by depletion of Pol ι [12]. It implies that individual mammalian TLS polymerase may target specific lesions induced by various DNA damaging agents.

In spite of the conservation of polymerase catalytic core, REV1 is a dCMP transferase with restricted activity in inserting dCMP not only opposite template G but also opposite other template bases [13]. At the N terminus of REV1, there is a BRCT (BRCA1 C-terminal) domain, which is unique among Y-family members. BRCT domain is required for REV1 to bind PCNA (proliferating cell nuclear antigen). Deletion or inactivation of BRCT domain abrogates the localization of REV1 to replication foci in nucleus. BRCT domain is also required for cell survival and DNA damage-induced mutagenesis in yeast and chicken DT40 cells [3]. Notably, BRCT domain and dCMP transferase activity of human REV1 are not required for effective DNA damage tolerance [14]. It is therefore obvious that BRCT domain provides another layer of regulation for REV1, which is absent from other members of Y-family polymerases.

REV1 plays a pivotal role in maintaining genomic integrity by TLS and it works closely with Pol ζ for most spontaneous and induced mutagenesis in yeast [3]. REV1 physically binds to many proteins in the TLS pathway. The C-terminal 100-150 amino acids of mammalian REV1 can interact with other Y-family polymerases including Pols η, κ and ι (Figure 1), whilst PAD of REV1 binds Pol η in yeast [15]. The sequence homology in the C-terminal region of REV1 is more conserved among higher vertebrates (such as human and mouse) than among lower eukaryotes (for example, fungi and yeast) [16]. This may explain the variation of binding targets between human and yeast REV1 orthologues. Very recently a novel RIR

(REV1-interacting region) has been identified in the C-terminal region of human Pols κ, ι, and η. Pol κ mutant deficient in RIR is unable to restore UV resistance of Pol κ null cells [17]. BRCT domain, PAD as well as the C-terminal region of yeast REV1 are capable of physically interacting with REV7, the accessory subunit of Pol ζ [18]. Besides binding to REV7 [19], the C-terminus of yeast REV1 can also bind to REV3, the catalytic subunit of Pol ζ, and this interaction promotes the proficiency of Pol ζ for mismatch extension and extension opposite DNA lesions [20]. Interaction between REV1 and REV7 is conserved in all organisms including yeast, mouse and human, implicating the important role of REV1 in TLS by Pol ζ. It is noteworthy that dCMP transferase activity of yeast REV1 is not required for UV mutagenesis because dCMP is rarely inserted opposite UV-induced lesions [21]. Further, the transferase activity of REV1 is dispensable for the bypass of abasic sites [22] and lesion modified by *N*-2-acetylaminofluorene [23]. Both yeast wild-type REV1 and its polymerase-deficient mutant are capable of interacting with REV3 and REV7 independently and these complexes are localized to DNA lesions [24]. Taken together, REV1 likely serves a structural role in TLS by Pol ζ.

Pol ζ is a heterodimeric complex comprising REV3 and REV7. Upon binding to REV7, the catalytic activity of REV3 is enhanced [4]. Like REV1, Pol ζ is a TLS enzyme conserved in eukaryotes, from yeast to human. Nevertheless, polymerase activity has not been demonstrated in mammalian Pol ζ, in contrast to its yeast and *Drosophila* counterparts [4, 25]. Both REV3 and REV7 belong to B-family of DNA polymerases, and Pol ζ possesses a relatively higher fidelity than Y-family polymerases though REV3 is devoid of 3′-5′ proofreading exonuclease activity [4]. Whilst Pol ζ is not efficient in replicating through DNA lesions, it is competent to extend from mispaired primer termini. Therefore, the primary role of Pol ζ in TLS is proposed to extend from the nucleotides inserted opposite the lesion by other TLS

enzyme(s) [3]. One example is that human Pol ι and Pol ζ cooperate sequentially in a way that the former inserts nucleotides opposite damage sites and the latter acts as an primer extension enzyme [26]. We have demonstrated that suppression of human REV7 in cultured cancer cells using RNAi (RNA interference) technique results in hypersensitivity to DNA-damaging agents, impaired sister chromatid exchange, reduction in frequencies of spontaneous and drug-induced mutations, and increase in chromosome aberration in response to DNA damage [27]. These findings agree with the observations in avian cells, implicating the evolutionarily conserved role of REV7 in DNA damage pathway in eukaryotes. Reduction of REV3 expression in yeast substantially decreases UV-induced mutation frequency [5] and disruption of *REV3* gene in mice is embryonic lethal. Mouse cells with *REV3* gene interrupted have lower survival rate and more cells arrested in S or G2/M phase of cell cycle [28]. Chicken DT40 cells with homologous deletion of *REV3* are sensitive to DNA-damaging agents and cells are accumulated in G2 phase [29]. These data suggest that the functional role of REV3 in TLS is conserved among species. In yeast, *REV3*-deleted cells arrest in G2 upon UV irradiation [30]. Intriguingly, yeast REV1 is expressed in a cell cycle-dependent manner, in which REV1 mRNA and protein levels peak at G2/M phase and this expression profile does not alter drastically after DNA damage by UV [2]. Given the indispensability of REV1 in TLS by Pol ζ, yeast and mammalian models suggest that REV3 functions after S phase, conceivably in G2/M, and this coincides with the expression pattern of REV1, highlighting the tight functional relationship between these two TLS proteins.

Apart from its role in TLS, REV7 has another identity in cell cycle regulation. Human REV7 is also called MAD2B, MAD2L2 or MAD2β for its high sequence homology with a mitotic checkpoint protein MAD2 [31, 32]. Like MAD2, MAD2B is able to bind CDH1 and/or CDC20, activators of APC (anaphase promoting complex),

thereby inhibiting APC activation. Although MAD2B is homologous to MAD2, it does not bind to MAD1, an anchoring protein for MAD2 at the kinetochore at the onset of mitotic checkpoint, suggesting that MAD2B may deliver signal other than mitotic checkpoint activation to APC-mediated proteolytic pathway [31, 32]. The role of MAD2B in mitotic checkpoint is also demonstrated in the infection process of intestinal epithelial cells by *Shigella*. IpaB, an effector protein in *Shigella* for bacterial invasion of host cells, interacts with MAD2B in G2/M phase, leading to unscheduled activation of APC^{Cdh1} and subsequent degradation of APC substrates such as cyclin B and CDC20, which in turn elicits G2/M arrest [33]. The novel role of REV7 in mitotic checkpoint, which in particular is associated with APC-mediated proteolysis, hints that REV7 may act as a mediator to link between ubiquitin-mediated proteolysis and DNA damage pathways [34]. Identification of REV7 targets for protein degradation therefore can provide evidence to support the crosstalk between these two important pathways in cells (Figure 1).

Role of ubiquitin in translesion synthesis

In order to implement TLS, the access and recruitment of TLS proteins to a damaged site are prerequisites. Eukaryotic PCNA is a homotrimeric protein which constitutes a donut-shaped structure surrounding double-stranded DNA. It slides along DNA during replication, providing a platform to tether the replicative polymerases to the DNA template. Since replication fork is stalled at DNA lesions, it is perceived that PCNA also serves as a docking site for TLS proteins. Indeed all eukaryotic Y-family polymerases, except REV1, contain PIP (PCNA interacting peptide) which allows them to interact with PCNA while the binding with PCNA requires BRCT domain of REV1 [3]. REV1 protein localizes to the nucleus exclusively and colocalizes with PCNA in replication foci, the replication machineries in S-phase cells, only when

BRCT domain is intact. Considering that Y-family polymerases can associate with PCNA and mammalian REV1 is able to interact with other members in the Y-family and Pol ζ (Figure 1), it is prone to speculate that TLS polymerases display high avidity toward PCNA, like replicative polymerases. In fact the recruitment of TLS polymerases is regulated rigidly through posttranslational modification of PCNA by ubiquitin. “Polymerase switch” is a generally accepted model in which PCNA acts as a platform where replicative polymerases are switched to TLS polymerases to carry out lesion bypass [35]. In response to DNA damage, a replication fork is stalled at a lesion where single-stranded DNA is generated and this triggers the activation of an E3 ubiquitin ligase RAD18. RAD18 together with an E2 ubiquitin-conjugating enzyme RAD6 forms an E2-E3 complex which monoubiquitinates PCNA at K164. Meanwhile USP1 (ubiquitin specific protease 1), a deubiquitinase, is degraded by an autocleavage mechanism to prevent the removal of ubiquitin from PCNA [36]. The activation of ubiquitin ligase and inactivation of deubiquitinase deliberately promote the formation of monoubiquitinated PCNA persistently, which consequently promotes TLS [37]. Although PCNA is thought to be monoubiquitinated only when it is loaded at stalled replication fork, PCNA monoubiquitination is dispensable for fork progression on damaged DNA, but required for post-replicative gap filling [38]. Noticeably, monoubiquitinated PCNA particularly activates Pol η and REV1, concurring with a role in promoting TLS [35]. In addition, PCNA is monoubiquitinated in a RAD18-dependent manner upon UV irradiation of human cells, and Pol η only interacts with monoubiquitinated PCNA, but not unmodified one [39]. PCNA monoubiquitination is also required for Pol η-dependent error-free incorporation of dCMP opposite 8-oxoguanine [40].

UBM (ubiquitin binding motif) and UBZ (ubiquitin-binding zinc finger) domains have been found in TLS polymerases [41]. These domains are evolutionarily

conserved in all Y-family polymerases and are located at the C-terminal region of the proteins [3]. With possible exceptions [42], UBM and UBZ are generally required for polymerases to bind ubiquitin and to localize to replication foci [43, 44]. In particular, they promote robust interaction between TLS polymerases and monoubiquitinated PCNA [45, 46]. In the absence of DNA damage, monoubiquitination of PCNA is inhibited by deubiquitinase USP1, thereby prohibiting the switch from replicative to TLS polymerases. The timely monoubiquitination of PCNA during DNA damage therefore confers a spatial and temporal regulation on recruitment of TLS proteins to bypass lesions. Structural analysis reveals that PIP of Pol η interacts with PCNA stronger than UBM does [47]. The relative affinity between different PCNA binding motifs hence could limit the binding of Pol η to monoubiquitinated PCNA, rather than other ubiquitinated products induced by DNA damage. Since all three monomers of PCNA can be monoubiquitinated simultaneously and monoubiquitinated PCNA does not alter the activity of replicative DNA polymerase, it is possible that replicative DNA polymerases may not be displaced from PCNA during TLS at lesion sites [42]. On the other hand, each monoubiquitinated monomer may bind to different TLS polymerase which attempts to access the stalled primer terminus on a trial and error basis [3]. However, the exact mechanism of polymerase switching remains to be elucidated.

The massive interactions among the members of TLS polymerases, and between these members and PCNA via ubiquitin, together with distinct efficacies of TLS polymerases toward various lesions, impose the idea of a two-step model in which two polymerases are involved in TLS. For example, Pol η binds to and targets Pol ι to replication foci [48]. Pol ζ is not efficient in inserting nucleotides opposite DNA lesions while it efficiently extends from nucleotides. It thus cooperates with other polymerases for incorporating nucleotide to achieve TLS [22, 26, 49] and various

combinations of the two polymerases also determine the activation of either error-free or error-prone pathways [49].

It is not surprising that PCNA and TLS polymerases can also be regulated by polyubiquitination. Modification of PCNA at lysine 164 with lysine 63-linked polyubiquitin chain by yeast RAD5 and mammalian HLTF (helicase-like transcription factor)/SHPRH (SNF2 histone linker PHD ring helicase) does not cause proteolysis, but prevents TLS by promoting error-free damage avoidance [50-52]. On the other hand, polyubiquitination-dependent proteasomal degradation of yeast Pol η has been demonstrated [53]. This raises the interesting question as to whether the stability of other TLS polymerases might also be regulated by polyubiquitination and degradation. Particularly, because REV7 interacts with REV1 and REV3 [19] on one hand, and regulates APC^{CDH1} or APC^{CDC20} on the other hand, it will be of great interest to investigate whether REV1 and REV3 might undergo APC-dependent proteolysis through their interaction with REV7 (Figure 1). Plausibly, polyubiquitination of PCNA and TLS polymerases both associated and not associated with proteasome-dependent protein degradation might serve important regulatory roles in TLS.

Conclusion

TLS is an important DNA damage tolerance pathway and it employs low fidelity polymerases to replicate across lesions, which arrest replication by replicative polymerases. Identification of different binding domains, such as RIR for REV1 binding, and PIP plus UBM for PCNA binding, in TLS polymerases enables us to comprehend the functional relationship among these proteins. TLS polymerases have low fidelity, introducing mutations in the genome. Therefore, strict control of TLS polymerases is essential. Ubiquitin-dependent regulation of TLS polymerases is known to execute at different levels. Monoubiquitination of scaffold protein PCNA and its subsequent interaction with TLS polymerases are a key regulatory step that recruits TLS polymerases to the lesions. However, further investigations are required to elucidate the detailed mechanism of polymerase switching. Yeast REV1 protein is expressed in a cell cycle-dependent manner in which the level peaks at G2/M phase. Yeast Pol η has fast protein turnover, which is regulated by proteosome-mediated degradation. It remains elusive whether expression levels of other TLS polymerases in yeast and human are regulated in response to DNA damage. Posttranslational modification of proteins, such as ubiquitination and phosphorylation, can modulate protein stability, localization, activity, protein interaction, and direct the target proteins to different signaling pathways. Hence, a comprehensive repertoire of TLS protein modifications would lend us insights into the regulation of and the interrelation of the components in TLS. Pol ζ is a key protein in TLS and its subunit REV7 is also participating in mitotic checkpoint in which it inhibits APC-proteasome pathway. If TLS polymerases are able to be covalently linked to ubiquitin, they can be our targets of study to investigate whether there is a crosstalk between DNA damage tolerance and ubiquitination machinery.

Figure legend

Figure 1 Multilayered interactions of mammalian REV1 between components in TLS and APC-mediated proteolysis.

Mammalian Y-family polymerases consist of REV1, Pol κ, Pol ι and Pol η. REV1 interacts with Pol κ, Pol ι and Pol η using its C-terminal 100-150 residues. Two UBMs (UBM1 and UBM2) of REV1 are required for the association with PCNA which is monoubiquitinated at lysine 164 (K164) during DNA damage. Similarly, ubiquitin binding domains at the C-termini of other Y-family polymerases (UBZ of Pol κ and Pol η, and UBM of Pol ι) are able to interact with monoubiquitinated PCNA. PCNA is a homotrimer and it is plausible that all subunits may be monoubiquitinated, thus interacting with three proteins at the same time. All Y-family polymerases, except REV1, possess PIP which can bind PCNA regardless of the ubiquitination state of PCNA. Although REV1 lacks PIP, BRCT domain at the N-terminus of REV1 is responsible for interacting with PCNA. REV7, the accessory subunit of Pol ζ, interacts with the catalytic subunit REV3 and promotes its activity. REV7 binds to the C-terminal region of REV1 without affecting its activity. REV7 can interact with the activator of APC, CDH1 and/or CDC20, thereby inhibiting the activation of APC. APC is an E3 ubiquitin ligase which covalently conjugates ubiquitin moiety to target substrates subject to proteasomal degradation.

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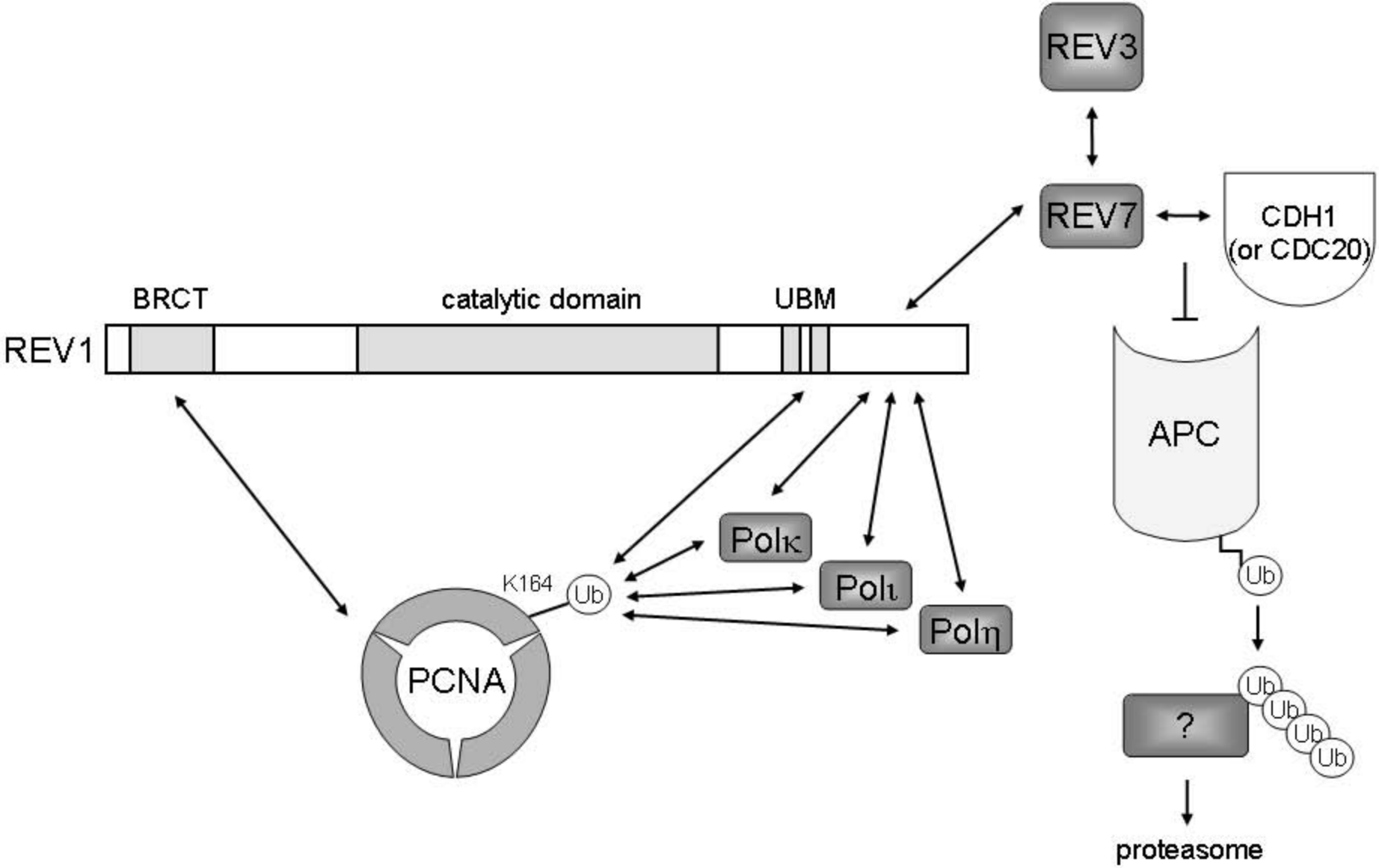
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