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Drosophila bendless catalyzes K63-linked polyubiquitination and is involved in the response to DNA damage

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ABSTRACT

In this study, we report the identification and functional characterization of the *Drosophila ben/ubc13* gene, encoding a unique ubiquitin-conjugating enzyme (Ubc or E2), in DNA-damage response. Ben forms a heterodimer with DmUev1a, the only Ubc/E2 variant (Uev) in *Drosophila*. Ben and DmUev1a act together to catalyze K63-linked polyubiquitination *in vitro*. *ben* can functionally rescue the yeast *ubc13* null mutant from killing by DNA-damaging agents. We also find that Ben^{P97S}, which was previously described to affect the connectivity between the giant fiber and the tergotrochanter motor neuron, fails to interact with the RING protein Chfr but retains interaction with DmUev1a as well as Uevs from other species. The corresponding yeast Ubc13^{P97S} interacts with Mms2 but fails to bind Rad5. Consequently, neither *ben*^{P97S} nor *ubc13*^{P97S} is able to complement the yeast *ubc13* mutant defective in error-free DNA-damage tolerance. More importantly, the *ben*^{P97S} mutant flies are more sensitive to a DNA-damaging agent, suggesting that Ben functions in a manner similar to its yeast and mammalian counterparts. Collectively, our observations imply that Ben-DmUev1a-promoted K63-linked polyubiquitination and involvement in DNA-damage response are highly conserved in eukaryotes including flies.

1. Introduction

Ubiquitin (Ub) is a 76-amino-acid polypeptide used to post-translationally modify substrate proteins through covalent attachment at one of its seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) or its N-terminal methionine (Met1) via an isopeptide bond. The transfer of ubiquitin to a substrate takes place through an enzymatic cascade involving a Ub activating enzyme E1, a Ub conjugating enzyme (Ubc or E2) and a Ub ligase E3 [1]. The ubiquitination system impacts upon nearly all cellular events. The most characterized function of Ub modification is the K48-linked poly-Ub chain that targets proteins for degradation by the 26S proteasome, which is called a ubiquitin proteasome system (UPS) [2,3]. In contrast, polyubiquitination through other Lys linkage may function as a reversible signal [4].

DNA damage is induced by exposure to environmental agents (e.g., ultraviolet irradiation, ionizing radiation and chemicals) and can also be generated spontaneously during cellular metabolism [5]. Because DNA damage would inhibit and/or alter fidelity of replication and transcription, there are several diverse and highly accurate repair processes including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and homologous recombination repair (HRR). In addition, DNA damage tolerance (DDT) does not actually remove the DNA lesions but allows bypass of unrepaired replication blocks [6]. In yeast and mammalian cells, proliferating cell nuclear antigen (PCNA) can be either monoubiquitinated by the E2-E3 complex Rad6-Rad18 at the Lys164 residue or further modified with Lys63-linked Ub chain by another E2-E3 complex, Mms2-Ubc13-Rad5 [7]. Ubc13 is the only identified E2 to mediate Lys63-linked poly-Ub chain formation in eukaryotes, and this enzymatic activity requires a Ubc variant (UEV) as a co-factor [8,9]. Mms2 is a member of the UEV family of proteins [10], which shares significant sequence similarity with other Ubcs but lack the active Cys residue [11]. PCNA monoubiquitination promotes translesion DNA synthesis (TLS), whereas PCNA polyubiquitination promotes error-free lesion bypass [7,12–14].

Ubc13 is an evolutionarily conserved protein. In budding yeast, the Ubc13-Mms2 complex is required for DNA damage response [8,11,15]. In mammalian cells, the Ubc13-Uev1a complex is involved in IκB/NF-κB signaling-mediated cell survival [16–18] and in breast cancer metastasis [19,20] and protein endocytosis [21,22]. In addition, Ubc13/Uev1a is required for TNFα-induced TRAF2 ubiquitination and GCKR/SAPK activation [23].

DmUev1a, the *Drosophila* ortholog of Uev, plays essential roles in regulating immune deficiency- and NF-κB-mediated innate immunity [24], NOPO (Drosophila homolog of human TRAF-interacting protein

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TRIP)-mediated genomic integrity [25], and TNF-JNK-mediated tumor progression and cell death [26,27], by forming an E2 complex with its heterodimer partner Bendless (Ben), the *Drosophila* ortholog of Ubc13 [28]. However, there is no report on the involvement of the Ben-Uev1a complex in DNA damage response in *Drosophila*.

In this study, we cloned and characterized the *ben* gene in *Drosophila* and demonstrated that Ben is highly conserved and involved in DNA damage response *in vitro* and *in vivo*. We found the *ben1* allele carries the point mutation ben^{C289T} (Ben-P97S) and is defective in the interaction with certain RING finger E3s. This point mutation is sufficient to compromise DNA damage response in flies.

2. Materials and methods

2.1. Escherichia coli and yeast cell culture

E. coli Trans10 (Transgen Biotech CD101) and BL21(DE3) (Transgen Biotech CD701) cells were used in this study. Yeast cells were cultured at 30 °C with 200 rpm/min shaking in YPAD or in a synthetic dextrose (SD) medium (0.67% Bacto-yeast nitrogen base without amino acid, 2% glucose) supplemented with necessary nutrients as recommended [29]. To make plates, 2% agar was added to either YPAD or SD medium prior to autoclaving. The lithium acetate method of yeast transformation was carried out as described [30]. The yeast two-hybrid strain used in this study is PJ69-4a [31] (*MATα trp1-901, leu2-3, ura3-52, his3-200, gal4Δ gal80Δ* P_{GAL2}*-ADE2 LYS2:*P_{GAL1}*-HIS3 met2:*P_{GAL7}*-lacZ*). Functional complementation was performed using the haploid yeast strain HK580-10D (*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) as the wild-type. The source and preparation of the *ubc13Δ:HIS3* cassette were as described previously [15].

2.2. Fly lines and husbandry

All stocks were grown on a standard medium at 25 °C unless otherwise indicated. Flies were transferred to new vials with fresh food every three days. The following fly stocks were used: ben^{1} [32] (BL9565) and w^{1118} (BL3605). Genetic crosses with two different strain backgrounds were conducted. C(1)A, or Compound (1) of Armentrout, contains an apparently stable, compound-ring-X chromosome and hence cannot produce single-X chromosome derivative by heterochromatic exchange. FM7, or In(1)FM7, contains X chromosomal inversion and results in a complete, or near complete, suppression of exchange along the length of the X chromosome.

2.3. Molecular cloning of ben and uev1a cDNAs, and site-specific mutagenesis

To clone *ben* and *DmUev1a* genes, total RNA was extracted from *D. melanogaster* adult flies using a TRIzol reagent (Invitrogen), and the ThermoScript RT-PCR kit (Invitrogen) was used to synthesize first-strand cDNA. The *ben* and *DmUev1a* ORFs were amplified by PCR from the above cDNA preparation using gene-specific primers. Yeast two-hybrid vectors *pGAD424Bg* and *pGBT9Bg* were derived from *pGAD424* and *pGBT9* [33]. Mutants $yUbc13^{P97S}$ and ben^{P97S} were made by using PCR-based site-specific mutagenesis and the recombinant plasmids as templates. The cloning and mutagenesis primers are listed in Supplementary Table S1.

2.4. Yeast two-hybrid analysis

PJ69-4a cells were co-transformed with a combination of Gal4 activation domain ($Gal4_{AD}$, from pGAD424Bg)- and Gal4 binding domain ($Gal4_{BD}$, from pGBT9Bg)-based vectors. The co-transformed colonies were selected on SD-Leu-Trp plates. Protein interaction was determined on synthetic complete medium lacking Trp, Leu and His, and supplemented with 3-amino-1,2,4-triazole (3-AT, Sigma-Aldrich).

2.5. Protein expression, purification, and GST pull-down assay

Full-length *ben* and *DmUev1a* were cloned in plasmid pET30a(+) and pGEX6p, respectively. The resulting pET-Uev1a and pGEX-Ben were transformed into *E. coli* BL21(DE3) cells. The His_6-Uev1a fusion protein was purified following a previously published protocol [34] and GST-Ben-K92R were produced and purified as previously described [9]. Cells in a 200-ml culture were grown at 37 °C to A_{590} = 0.3 in an LB medium containing 50 μg/ml ampicillin or 50 μg/ml kanamycin followed by induction with 0.2 mM isopropyl-β-D-1-thiogalacto pyranoside (IPTG) for 5 h at 37 °C. Cells were harvested by centrifugation and stored at −80 °C. All subsequent steps were performed at 4 °C. For the pull-down assay, crude cell extracts were loaded on Glutathione Sepharose™ 4 B beads and then 10 μg of purified His_6-DmUev1a was added. After incubation overnight at 4 °C and washing with a lysis buffer 5 times, the GST beads were boiled with SDS-PAGE 6 × loading buffer for 10 min before western blotting analysis.

2.6. Ub conjugation reaction

In vitro Ub conjugation reactions were performed by using purified GST-Ben and His_6-Uev1a proteins as describe above. Ub thioester conjugation initiation reagents were purchased from Boston Biochem. The 20-μl reaction mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP and 225 nM E1 enzyme in the supplied reaction buffer, plus the following components as indicated: 200 μM Ub, 10 μM Ben and 10 μM Uev1a. The His_6-HA-Ub (WT, K63R and K48R) fusion proteins were purified with Ni Sepharose. The conjugation reactions were performed at 37 °C for 1.5 h. Samples were subjected to 10% SDS-PAGE, and different forms of Ub molecules were detected by western blotting using a mouse anti-HA monoclonal antibody (Santa Cruz sc-7392).

2.7. Yeast survival assay

Yeast strain HK580-10D and its isogenic *ubc13Δ* were transformed with pGAD424, pGAD424-UBC13 or derivatives. Transformants were selected on SD-Leu plates. Gradient plate and serial dilution assays were performed as described previously [35]. A gradient plate was made by pouring molten YPAD agar with indicated MMS concentration into a tilted square Petri dish. After solidification, the Petri dish was returned flat and equal volume of YPAD agar without MMS was poured to form the top layer. Cells from an overnight culture were mixed with 1% molten YPAD agar and immediately imprinted onto freshly made gradient plates via a microscope slide.

2.8. Spontaneous mutagenesis assay

Yeast strain DBY747 and its derivative WXY849 (*ubc13Δ*) bear a *trp1-289* amber mutation that can be reverted to Trp^+ by different mutation events [36]. WXY849 cells were transformed with pGAD424, pGAD-UBC13 or derivatives. The transformants were selected on SD-Leu plates. Each set of experiments contained five independent cultures for each strain. Overnight yeast cultures were counted using a hemocytometer and five replicate cultures of 20 ml of SD-Leu medium for each strain with a start concentration of 20 cells/ml were incubated at 30 °C until the cell number reached 2×10^7 cells/ml (OD_{600} = 1.0). Cells were collected by centrifugation at 3000*g*, washed and plated onto YPAD or SD-Leu (for strains carrying pGAD424-based plasmids) in triplicate to score total survivors and onto SD-Trp or SD-Leu-Trp (for strains carrying pGAD424 plasmids) plates to score Trp^+ revertants. Spontaneous mutation rates (number of revertants per cell per generation) were calculated as described [37].