Mol Cell Biochem
DOI 10.1007/s11010-010-0511-9

Zebrafish Ubc13 is required for Lys63-linked polyubiquitination and DNA damage tolerance

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Received: 23 March 2010 / Accepted: 2 June 2010

Abstract Ubiquitination is an important post-translational protein modification that functions in diverse cellular processes of all eukaryotic organisms. Conventional Lys48-linked poly-ubiquitination leads to the degradation of specific proteins through 26S proteasomes, while Lys63-linked polyubiquitination appears to regulate protein activities in a non-proteolytic manner. To date, Ubc13 is the only known ubiquitin-conjugating enzyme capable of poly-ubiquitinating target proteins via Lys63-linked chains, and this activity absolutely requires a Ubc variant (Uev or Mms2) as a co-factor. However, Lys63-linked poly-ubiquitination and error-free DNA damage tolerance in zebrafish are yet to be defined. Here, we report molecular cloning and functional characterization of two zebrafish *ubc13* genes, *ubc13a* and *ubc13b*. Analysis of their genomic structure, nucleotide and protein sequence indicates that the two genes are highly conserved during evolution and derived from whole genome duplication. Zebrafish Ubc13 proteins are able to physically interact with yeast or human Mms2 and both zebrafish *ubc13* genes are able to functionally complement the yeast *ubc13* null mutant for spontaneous mutagenesis and sensitivity to DNA damaging agents. In addition, upon DNA damage, the expression of zebrafish *ubc13a* and *ubc13b* is induced during embryogenesis and zebrafish Ubc13 is associated with nuclear chromatin. These results suggest the involvement of Lys63-linked poly-ubiquitylation in DNA damage response in zebrafish.

Keywords Zebrafish · Ubiquitin-conjugating enzyme · Lys63-linked poly-ubiquitination · Protein–protein interaction · DNA damage tolerance

Introduction

Ubiquitination is an essential biochemical reaction for post-translational modification of target proteins with ubiquitin [1]. This process appears to occur in all eukaryotic organisms, from yeast to human, and plays crucial roles in many cellular events including signal transduction [2–4], transcriptional regulation [5, 6], ribosomal protein synthesis [7], cell cycle progression [8, 9], receptor endocytosis [10], and DNA repair [11–13]. Ubiquitination leads to the attachment of a single Ub or poly-Ub chain to a protein and requires formation of an isopeptide bond between the C-terminal Gly76 on Ub and a lysine residue on target proteins [14, 15]. Ubiquitination of a protein contains several steps that are catalyzed by distinct enzymes. First, Ub is activated by an Ub-activating enzyme (Uba or E1) in the presence of ATP, forming a high-energy E1-Ub thiolester bond. Second, the activated Ub is transferred from E1 to a specific thiol of an Ub-conjugating

Electronic supplementary material The online version of this article (doi:10.1007/s11010-010-0511-9) contains supplementary material, which is available to authorized users.

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Published online: 16 June 2010

enzyme (Ubc or E2) to form an E2-Ub thiolester. Third, the Ub of E2-Ub is donated to the target protein either alone or with the help of an Ub ligase (E3). E3 acts as a primary substrate recognition factor, whereas E2 is thought to be involved in the reaction largely through its association with a given E3. Most eukaryotic organisms have one functional E1 enzyme and many of E2 and E3 enzymes. All known E2s belong to the same family that contains a conserved catalytic domain with an active Cys residue to form a thiolester bond with Ub [16], while, E3s known so far belong to several protein families, including HECT, RING and U-box [17].

Multiple rounds of ubiquitination may occur using the surface Lys of additional Ub as a substrate. Conventional poly-Ub via Gly76-Lys48 leads to the degradation of target proteins through 26S proteasomes, but Gly76-Lys63 poly-Ub appears to regulate protein activities in a non-proteolytic manner [16]. So far, Ubc13 is the only known E2 enzyme capable of catalyzing the Lys63-linked polyubiquitylation through its interaction with an Ubc/E2 variant (Uev) [13]. In the budding yeast *Saccharomyces cerevisiae*, Ubc13 forms a stable complex with an Uev called Mms2 [13], and promotes error-free DNA damage tolerance (DDT, also known as postreplication repair) [18] by poly-ubiquitinating the proliferating cell nuclear antigen (PCNA) via Lys63-linked chains [19]. Intracellular signaling for DDT consists of two parallel pathways that are mediated by Polζ (Rev3 and Rev7) or Ubc13–Mms2, deemed to be error-prone or error-free, respectively [20, 21]. The error-prone pathway mainly functions in translesion DNA synthesis (TLS) through low fidelity and non-essential DNA polymerases, whereas the error-free pathway works through template switch [22]. The current model suggests that mono-ubiquitination of PCNA by Rad6–Rad18, a E2–E3 complex, leads to TLS, whereas Mms2–Ubc13–Rad5, another E2–E3 complex, further poly-ubiquitinates PCNA at the same Lys164 residue and promotes error-free DDT [19]. Ubc13 homologs have been found in many eukaryotic organisms; however, their functions remain largely unknown due to the lack of an efficient means of targeted gene disruption in most multicellular eukaryotes and the intolerance of mammals to genomic instability.

Zebrafish (*Danio rerio*) has become one of the most useful and well-known model organisms because of the following advantages. First, the transparent body makes the zebrafish easy to observe the morphology alteration of cells and organs. Second, the early zebrafish embryos go through DNA replication and cell division rapidly [23]. Third, DNA, mRNA or proteins can be easily transferred into zebrafish embryos using microinjection [24]. Importantly, zebrafish is suitable for large scale analysis of forward and reverse genetics. These features not only attract geneticists and developmental biologists but also become a favoured model for research in cancer and genome integrity. For example, Ku80, an essential component of the non-homologous end joining pathway, is responsible for DNA damage repair in zebrafish embryos [25] and expression of the Ku70 subunit (XRCC6) is able to protect zebrafish embryogenesis from low dose ionizing radiation [26]. Furthermore, the zebrafish apurinic/apyrimidine endonuclease 1 functions in both DNA repair and regulation of embryonic development [27]. Here, we report the cloning and functional characterization of two zebrafish *Ubc13* genes and demonstrate that both of them play critical roles in Lys63-linked poly-ubiquitination and DNA damage response.

Materials and methods

Zebrafish care and maintenance

AB inbred strain of zebrafish (*Danio rerio*) was reared in a constant flow-through water system and maintained under standard conditions. Naturally fertilized zebrafish embryos were staged by hours post-fertilization (hpf) or by morphological features.

Cloning of zebrafish ubc13 cDNAs

Total RNA was isolated from 500 mg of zebrafish intestine with TRIZOL Reagent from Invitrogen following the manufacturer's instruction. First-strand cDNA was synthesized using the RevertAid^{TM} First-Strand cDNA Synthesis Kit from Fermentas. PCR primers were designed according to predicated full-length cDNA sequences for zebrafish *ubc13* genes in GenBank database (NP_998651 and NP_956636). Primers for zebrafish *ubc13a* are 5′-CCGCTCGAGGCCG GAAGGCGTGTGTCT-3′ and 5′-CGCGGATCCCAGAA AGAACAGAAATATGTGTGTGGAC-3′. Primers for zebrafish *ubc13b* are 5′-CCGCTCGAGACGCGCATGCGCTG TC-3′ and 5′-CGCGGATCCCAGATTCAATTTTCCCAG ACGAT-3′. All primers contain specific restriction sites (underlined) for subcloning zebrafish *ubc13* cDNAs into the pBluescript vector.

Yeast strains, culture and transformation

The haploids of yeast *S. cerevisiae* strain used in this study are listed in Fig. 4b. Yeast cells were grown at 30°C in either rich YPD or in a synthetic dextrose (SD) medium (0.67% bacto-yeast nitrogen base without amino acids, 2% glucose). To make the solid plates, 2% agar was added to either YPD or SD medium prior to autoclaving. Yeast cells were transformed using a LiAc method as described [28].

The source and preparation of *ubc13Δ::HIS3* cassettes were as previously described [21]. All the gene deletion strains were confirmed by Southern hybridization.

Complementation assay

Yeast strain HK578-10D and its *ubc13* single mutants were transformed with pGBT9, pGBT-DrUbc13a or pGBT-DrUbc13b. Transformants were selected on SD-Leu plates. After 3 days, five colonies were re-streaked onto a SD-Leu plate and subsequently used to inoculate liquid SD minimal medium. Following an overnight incubation, cell density was determined and equal numbers of cells from the transformants together with untransformed controls were imprinted onto YPD alone or YPD gradient plates containing 0.025% methyl methanesulfonate (MMS). A MMS gradient was formed by pouring 30 ml of YPD + MMS medium in a tilted square Petri dish. The Petri dish was placed flat after solidification and a top layer of 30 ml YPD was poured. 0.1 ml of overnight culture, mixed with 0.4 ml sterile water and 0.5 ml of molten YPD agar, and printed onto the plates using a sterile microscope slide. Plates were incubated at 30°C for 3 days before taking photograph.

Spontaneous mutagenesis assay

Yeast strain DBY747 and its isogenic *ubc13* derivative WXY849 bear a *trp1-289* amber mutation that can be reverted to Trp^+ by several different mutation events [29]. DBY747 was transformed with pGAD424E and WXY849 was transformed with pGAD-DrUBC13b or pGAD424E. Transformants were selected on SD-Leu plates. Each set of experiments contained five independent cultures of each strain. Overnight yeast cultures were counted using a hemocytometer and 5 ml of YPD liquid medium was inoculated to a final concentration of 20 cell/ml and incubated at 30°C for 3 days. Cells were spun down at 4,000 rpm, resuspended in sterile ddH_2O and were plated onto SD-Leu to score for total survival and onto SD-Leu-Trp to count the number of Trp^+ reversions. Spontaneous mutation rates were calculated as described [30].

Yeast two-hybrid analysis

The yeast two-hybrid strain PJ69-4A [31], received from Dr. P. James (University of Wisconsin, Madison, USA), was co-transformed with different combinations of yeast two-hybrid Gal4 binding domain and Gal4 active domain constructs. The co-transformed colonies were initially selected on SD-Leu-Trp plates. For each transformation, at least five independent colonies were plated onto SD-Leu-His with various concentrations of 1,2,4-amino triazole (3-AT) to test the activation of the *GAL1-HIS3* gene.

Protein expression and purification

The zebrafish *ubc13b* ORF was cloned into vector pET30 (Invitrogen, Carlsbad, CA, USA) and hUev1A and hMms2 were cloned into vector pGEX6p (Amersham Biosciences, Piscataway, NJ, USA). The resulting pET-DrUbc13b, pGEX-hUev1A and pGEX-hMms2 were transformed into *E. coli* strain BL21-CodonPlus (DE3)-RIL carrying extra copies of the *argU*, *ileY* and *leuW* tRNA genes (Stratagene, La Jolla, CA, USA). All above fusion protein was produced and purified following the instruction of manufacturers.

GST pull-down assay

The protein concentrations of purified, dialyzed pET-DrUbc13b, pGEX-hUev1A and pGEX-hMms2 were measured using a Bradford assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The GST-pulldown assay was carried out using Glutathione Sepharose 4B Microspin^{TM} GST Purification Columns as previously described [32]. Purified GST-hUev1A, GST-hMms2 or GST was added to individual columns and incubated for 1 h at 4°C. They were spun down and washed three times with 500 µl of PBS. His-DrUbc13B was then added and the columns were incubated for 1 h at 4°C. After centrifugation and washing, proteins were eluted by addition of 100 µl of glutathione elution buffer followed by centrifugation. 10 µl of each samples was loaded to the SDS-PAGE.

In vitro poly-ubiquitination assay

In vitro ubiquitin conjugation reactions were carried out using the purified DrUbc13a and GST-hMms2 proteins. Other components for this reaction such as Ub, Ub-K48R, Ub-K63R, ATP, and reaction buffer were purchased from Boston Biochem. For the reaction, a 20-ml reaction mixture contained 225 nM E1 enzyme, 450 mM Ub, 1 mM MgATP, 1 mM Ubc13, and 1 mM Uev1 in the supplied reaction buffer. The conjugation reactions were incubated at 37°C for 2 h. Samples were subjected to SDS-PAGE (12%), and Ub and poly-Ub were detected through Western blot using polyclonal rabbit anti-Ub antibodies from Sigma-Aldrich.

Immunofluorescence staining

ZF4 represents a fibroblast-like cell line that derived from 1-day-old zebrafish embryos [33]. ZF4 cells were grown in DMEM/F12 from GIBCO supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, and incubated at 28°C in 5% CO_2 and 95% air. Cells grown to 50–60% confluence on 35 mm Petri dishes

were kept on ice and treated with 5 μM camptothecin (CPT) or 0.01% MMS for 4 h, permeabilized in Triton buffer for 3 min, blocked for 15 min in a buffer containing 1% BSA and 0.09% sodium azide, pre-extracted with 0.2% NP40 for 3 min, and then incubated with the primary mouse anti-human Ubc13 antibody (1:400 dilution of 4E11 ascites fluid) at room temperature for 1 h. Cells were then incubated with the secondary antibody (Goat anti-mouse-FITC) for 1 h at room temperature. After three times of washing with 1× PBS buffer, cells were stained with DAPI and imaged under a fluorescent microscope.

Quantitative real-time RT-PCR

Right after fertilization, embryos were treated with different concentrations of MMS for 6 h. Total RNA was exacted from 50 MMS-treated embryos at 6 hpf with TRIZOL reagent from Invitrogen. 5 μg of total RNAs was treated with RNase-free DNase and reversely transcribed in the presence of random primers. First-strand cDNA was analyzed in triplicates with gene-specific primers including *β*-actin-FOR 5′-GATGATGAAATTGCCGCACTG-3′ and *β*-actin-REV 5′-ACCAACCATGACACCCTGATGT-3′ for *β*-actin, DrUbc13a-FOR 5′-AAAGCGGAGCCAGATGAG GG-3′ and DrUbc13a-REV 5′-GTCCCACATTGGGATG GTAGATTT-3′ for zebrafish *ubc13a*, and DrUbc13B-FOR 5′-TCTCGTGTCCAGCACAGTCC-3′ and DrUbc13b-REV 5′-TTTCCGCAAACATTCCCAC-3′ for zebrafish *ubc13b*. Real-time PCR was performed using an ABI prism 7000 sequence-detection system and the SYBR Green PCR Master Mix from TOYOBO. Fold-induction of gene expression was calculated by taking the level of *β*-actin gene expression as one. The relative gene expression was determined by the $2^{-\Delta\Delta C_T}$ method [34].

Results

Zebrafish ubc13 genes

To identify zebrafish *ubc13* genes, the amino acid sequence of human Ubc13 was used to blast the NCBI protein database (http://www.ncbi.nlm.nih.gov). Two proteins that are currently named ubiquitin-conjugating enzyme E2N (symbol: Ube2n) and ubiquitin-conjugating enzyme E2N-like (symbol: Ube2nl) were found to share a high degree of similarity with human Ubc13 (*E*-values <2e−82 and <2e−81, respectively). According to the guideline for naming duplicated zebrafish genes (http://zfin.org/zf_info/nomen.html), we named these two genes as *ubc13a* and *ubc13b*. DrUbc13a and DrUbc13b were used in the study to distinguish zebrafish Ubc13s with Ubc13 from other species by addition of a prefix "Dr".

Amino acid sequences of DrUbc13s were aligned with Ubc13s from six other species (Fig. 1a). Both DrUbc13 proteins consist of 154 amino acids with a difference of only one amino acid (Thr131 in DrUbc13a and Ser131 in DrUbc13b). This residue is not located in any known functional domains of Ubc13. DrUbc13a shares 98% amino acid sequence identity with hUbc13 and mUbc13, 70% identity with ScUbc13, 78% identity with SpUbc13, 87% identity with CeUbc13, 81% identity with DmUbc13a, and 78% identity with DmUbc13b. Importantly, DrUbc13s contain all critical residues that are necessary for three functional domains as defined in hUbc13 (Fig. 1a). These residues include the active site Cys87 for Ub thioester formation, Met64 required for the interaction

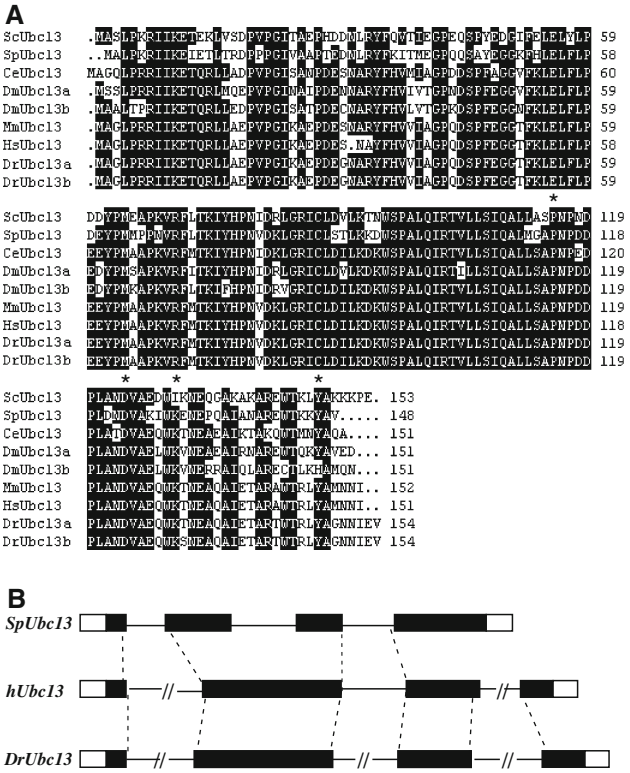


Fig. 1 Analysis of zebrafish *ubc13* and its orthologs. **a** Amino acid sequence alignment of Ubc13 from different organisms. Identical residues shared by the majority (6 out of 9) of Ubc13s are highlighted. The source of sequences (GenBank accession No) are: *Sc*, *S. cerevisiae* = NP_010377; *Sp*, *S. pombe* = NP_594929; *Ce*, *Caenorhabditis elegans* = NP_500272; *Dm*, *D. melanogaster* (DmUbc13a - *Bendless* = NP_511150; DmUbc13b = NP_609715); *Mm*, *M. musculus* = NP_542127; *Hs*, *H. sapiens* = NP_003339; *Dr*, *D. rerio* (DrUbc13a = NP_998651; DrUbc13b = NP_956636). Critical residues for Ubc13 functions are indicated with *asterisks* underneath the residue. Sequences were aligned and edited using a BioEdit program version 5.0.9 [59]. **b** Genomic organization of *S. pombe*, human and Zebrafish *ubc13*. *Filled boxes* exons, *open boxes* untranslated region, *solid lines* introns. Identical intron–exon locations between different *Ubc13* orthologs relative to their coding sequences are indicated by a connection line

with an E3 (TRAF6) [35], and three "pocket" residues (Glu55, Phe57 and Arg70) that determine binding specificity for Mms2 [32].

Zebrafish *ubc13a* and *ubc13b* are located on chromosomes 18 and 4, respectively. Full-length cDNAs of these two genes were cloned by RT-PCR. Our zebrafish *ubc13* cDNA sequences agree with the predicted genomic structures and sequences for both genes on the Ensemble database (http://www.ensembl.org/Danio_rerio/Info/Index). The genomic locus ID numbers are ENSDARG00000008748 for *Drubc13a* and ENSDARG00000045877 for *Drubc13b*. Both of zebrafish *ubc13* genes contain four exons and three introns (Fig. 1b), whereas their ORFs share only 71.8% nucleotide sequence identity with differences mainly in the third nucleotide of codons, suggesting that zebrafish *ubc13a* and *ubc13b* were derived from the whole genome duplication. This notion was supported by the evidence that promoter and downstream regions of zebrafish *ubc13a* and *ubc13b* share high sequence similarity and their downstream genes are also duplicated.

Next, we compared the genomic structure of zebrafish *ubc13s* with *ubc13* genes from the fission yeast and human. As shown in Fig. 1b, zebrafish *ubc13s* have same numbers of exons and introns, and identical intron/exon borders as *hUbc13*. *SpUbc13* shares two intron–exon borders with both *hUbc13* and zebrafish *ubc13s*. These results further indicate that *ubc13* was evolved early in the eukaryotic kingdom and that at least some of its introns predate the speciation event among yeast, zebrafish and mammal. In addition, a clustal analysis of Ubc13 proteins from these species indicated that sequence of Ubc13 proteins could be used as one of reliable parameters for phylogenetic analysis (Fig. S1).

Zebrafish Ubc13s physically interact with yeast Mms2 or human Uevs

Ubc13s from diverse species are able to form a complex with Uevs in yeast and human to promote the Lys63-linked poly-ubiquitylation. Since two DrUbc13 proteins differ in only one amino acid that is not located in known functional domains, DrUbc13b was used to investigate whether DrUbc13 has the same function as yeast and human Ubc13. The protein–protein interactions between DrUbc13 and yeast Mms2 or human Uevs, including hMms2 and Uev1A, were analyzed in a yeast two-hybrid assay [36]. The zebrafish *ubc13b* ORF was cloned in-frame into a Gal4 DNA-activation domain vector pGAD424E and the yeast Mms2 and human Uevs were cloned in-frame into a Gal4 DNA-binding domain vector pGBT9E. As shown in Fig. 2a, co-expression of $Gal4_{AD}$-DrUbc13b with $Gal4_{BD}$-yMms2, $Gal4_{BD}$-hMms2 or $Gal4_{BD}$-hUev1A in yeast cells resulted in the strong expression of endogenous P_{GAL1}-*HIS3* reporter gene, indicating that DrUbc13s are capable of interacting with yeast and human Uevs.

To further confirm the physical interaction between DrUbc13 and Uevs from human, a GST-affinity pull-down assay was carried out. As shown in Fig. 2b, DrUbc13b was eluted with GST-hMms2 or GST-hUev1A, but not with GST alone. This result indicates that DrUbc13b is able to form a stable complex with hMms2 or hUev1A in vitro.

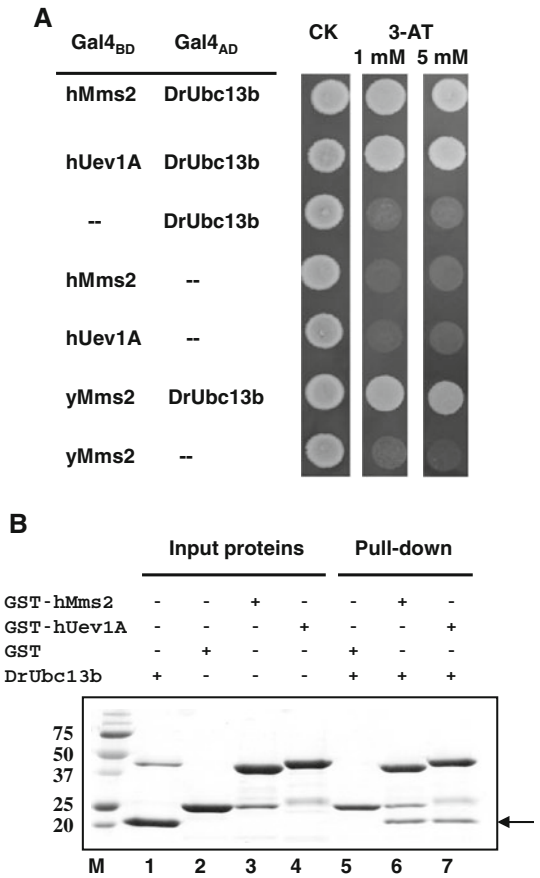


Fig. 2 Interaction between DrUbc13 and Uevs from yeast and human. **a** Yeast two-hybrid assays. *AD* Constructs in the Gal4 activation domain vector pDAD424. *BD* Constructs in the Gal4 DNA-binding domain vector pGBT9. The transformants carrying one AD construct and one BD construct were plated on non-selective SD-Leu-Trp plates (CK) and incubated for 3 days (*left panel*), or SD-His-Leu-Trp + 1 mM 3-AT (*middle panel*), SD-His-Leu-Trp + 3 mM 3-AT plates (*right panel*). **b** DrUbc13b forms a complex with hMms2 and hUev1A in a GST-pulldown assay. Purified GST, GST-hMms2 and GST-hUev1A were added to microspin columns. Following incubation, columns were spun and washed, and the purified DrUbc13b was added to the column. After further incubation and washing, the columns were eluted with reduced glutathione and subjected to SDS-PAGE gel analysis. *Lanes 1–4* show the purified His6-DrUbc13B, GST, GST-hMms2 and GST-hUev1A. *Lanes 5–7* show the eluent from the column preloaded with GST (*lane 5*), GST-hMms2 (*lane 6*) and GSThUev1A (*lane 7*). *Arrow* points to the His6-DrUbc13b band

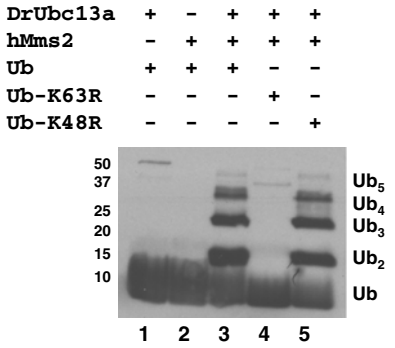
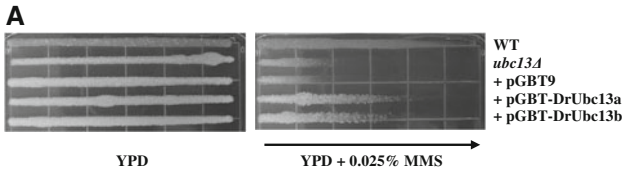


Fig. 3 DrUbc13 and hMms2-mediated Ub conjugation in vitro. The Ub conjugation assay was performed as indicated in "Materials and methods". Samples were subjected to SDS-PAGE, and a western blot using an anti-Ub antibody was carried out to monitor poly-Ub formation. Single Ub and different chains are indicated in the *right panel*



B

S. cerevisiae strains	Mutation rate ($\times 10^{-8}$)	Standard deviation	Fold increase
Wild type	3.19	0.18	1
ubc13Δ	60.8	4.4	19.1
ubc13Δ/DrUbc13b	4.56	0.75	1.43

Fig. 4 Functional complementation of the yeast *ubc13* mutant by zebrafish *ubc13*. **a** A gradient plate assay. The YPD control and YPD + 0.025% MMS gradient plates were incubated at 30°C for 3 days. All strains grew well on the YPD plate without MMS; on YPD + 0.025% MMS plate, zebrafish *ubc13* genes rescued yeast *ubc13* mutants to close to the wild type level while the vector alone had no effect. The *arrow* points towards the higher MMS concentration. **b** A spontaneous mutation assay. *WT*, DBY747 was transformed with vector pGAD424E. *ubc13Δ*, the yeast *ubc13* null mutant WXY849 transformed with vector pGAD424E only. *ubc13/zebrafish ubc13b*, WXY849 transformed with pGAD-DrUbc13b. Spontaneous mutation rates represent the average of three independent experiments with standard deviations. Fold-increase is relative to the wild type mutation rate

Zebrafish Ubc13 promotes Lys63-linked poly-ubiquitylation

Ubc13s from yeast and human are able to promote Lys63-linked poly-ubiquitylation in vitro [37] or in vivo [19] and a cofactor Uev is absolutely required for this biochemical reaction. We have previously reported that Arabidopsis Ubc13 and Uev1s are required for the assembly of Lys63-linked poly-Ub chains [38, 39]. To determine whether DrUbc13s have the same function as Ubc13s from other species, we performed an in vitro ubiquitination assay. As shown in Fig. 3, DrUbc13 or hMms2 alone was unable to create free poly-Ub chains (Lanes 1 and 2, respectively). However, DrUbc13 plus hMms2 (Lane 3) can generate double-, triple-, tetra- and penta-Ub chains. Moreover, poly-Ub conjugates were not detected when using an Ub-K63R mutant that lacks Lys63 (Lane 4), but were detected when using an Ub-K48R mutant that lacks Lys48 for conjugation but retains the Lys63 residue (Lane 5). These results indicate that formation of these poly-Ub chains by DrUbc13-hMms2 is mediated through Lys63, but not Lys48.

Zebrafish ubc13s functionally complement yeast ubc13 null mutants

The budding yeast *Ubc13* is involved in an error-free damage tolerance pathway [40]. To characterize roles of DrUbc13 in this pathway, a functional complementation experiment and a spontaneous mutagenesis assay were carried out. The yeast *ubc13* mutant displays an increased sensitivity to a variety of DNA damaging agents including MMS, which can be assessed by a gradient plate assay. Expression of either zebrafish *ubc13a* or *Ubc13b* from the yeast two-hybrid plasmid partially rescued the *ubc13* mutant from killing by MMS; in contrast, the pGBT9 vector alone did not confer any MMS resistance to the *ubc13* mutant (Fig. 4a).

The most important phenotype of *mms2* [18] or *ubc13* [40] mutant is its massive increase in spontaneous mutagenesis, indicating that both Ubc13 and Uev play essential roles in maintaining the genome integrity. The spontaneous mutation rate in the *ubc13* null mutant increased nearly 20-fold compared to the wild type cells, while expression of zebrafish *ubc13b* in the *ubc13* mutant reduced it to the near wild type level (Fig. 4b). From the above data, we infer that DrUbc13s have the same function as yeast Ubc13.

Zebrafish Ubc13 is involved in DNA damage response

Many DNA repair and response enzymes, including hUbc13 [41], are able to form stable focal localizations at sites of damaged DNA in the nuclei. To determine whether DrUbc13s are involved in DNA repair, we examined nuclear focus formation after DNA damage. ZF4 cells were exposed to DNA damaging agents including CPT and MMS. CPT is a topoisomerase I inhibitor, which converts a

single-stranded DNA break into a double-stranded break (DSB) at the replication fork [42, 43]; hence, CPT treatment is always used to induce highly specific and predictable DNA lesions. MMS is a DNA alkylating agent, which has been widely used to cause replication blocks and induce S-phase checkpoints [44]. Since Ubc13 appears to be freely translocated between the cytoplasm and the nucleus, we attempted to refine its localization using an in situ cell fractionation procedure before fixation, which is a method frequently applied to identify nuclear localization of DNA repair proteins [45]. We first test the specificity of the Ubc13 antibody against DrUbc13. As shown in Fig. S2, the antibody we previously raised for hUbc13 detected two specific bands from the ZF4 cell lysate, while only one specific band was found in lysates of human tumor cells HepG2 and Saos-2. The molecular weight of the lower band is as expected, but the upper band is thought to be due to the autoubiquitination of Ubc13, which has been observed in human Ubc13 from a previous study [46]. Hence, we were confident that this antibody is also specific for DrUbc13s. We then performed immunofluorescence staining assays with ZF4 cells. Comparing with the control in which Ubc13 were mainly distributed in cytoplasm, signals for Ubc13 were markedly accumulated in the nuclei after treatment with CPT or MMS (Fig. 5a). When detergent extraction under a stringent condition was performed to remove soluble components from cytoplasm as well as the nucleus, nuclear foci for Ubc13 were observed in nuclei of cells treated with CPT or MMS, but not with untreated cells (Fig. 5b). These data indicate that DrUbc13s are involved in DNA damage response and are associated with damaged chromatin. It is also noted that the Ubc13 protein level in zebrafish ZF4 cells is much higher than in cultured human cells examined in this study (Fig. S2), suggesting a potentially high level Lys63-linked poly-ubiquitination activity.

Previous study has shown that the yeast *Ubc13* expression is induced by DNA damage [40]. To ask whether the expression of zebrafish *ubc13* is able to respond to DNA damage agents during zebrafish embryogenesis, we first looked at the expression profiles of zebrafish *ubc13* genes during development by extraction of a relative expression of zebrafish *ubc13* genes from a web-based resource [55]. Both zebrafish *ubc13* genes appear to be universally expressed with the highest level shown before 18 somite stage and DrUbc13a as the dominant form during zebrafish embryogenesis (Fig. 6a). Next, we performed real-time PCR assays to detect zebrafish *ubc13* transcriptional expression in developing embryos treated with different concentrations of MMS. As shown in Fig. 6b, both zebrafish *ubc13a* and *ubc13b* transcript levels were significantly induced by MMS treatment ($P < 0.05$ or $P < 0.01$), although zebrafish *ubc13a* appears to be induced more than zebrafish *ubc13b*. These results support a notion that DrUbc13s are involved in DNA damage response.

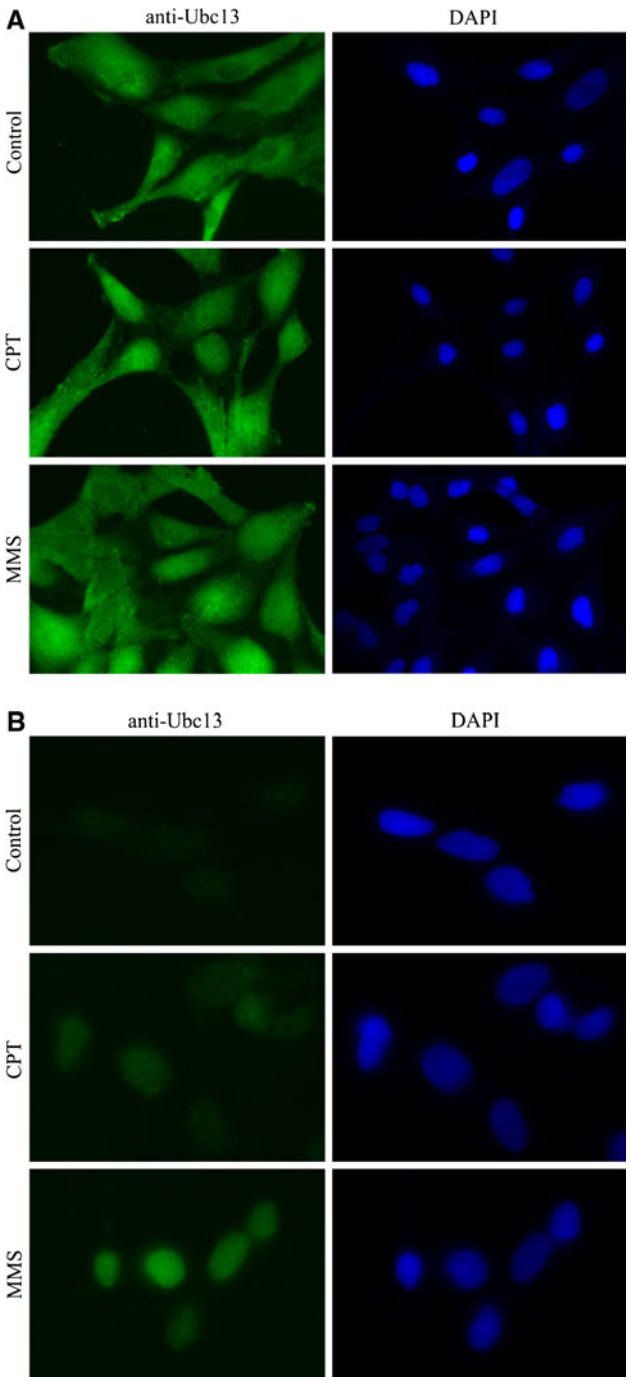


Fig. 5 MMS-induced DrUbc13 nuclear focus formation. ZF4 cells on ice were treated with 5 μM CPT or 0.01% MMS for 4 h and immunofluorescence staining were performed according to the protocol in "Materials and methods". FITC signals indicate the distribution of endogenous DrUbc13 proteins in cells and nuclei were shown by DAPI staining. **a** Cells were not treated with detergent. **b** Cells were treated with detergent after fixation to remove soluble proteins

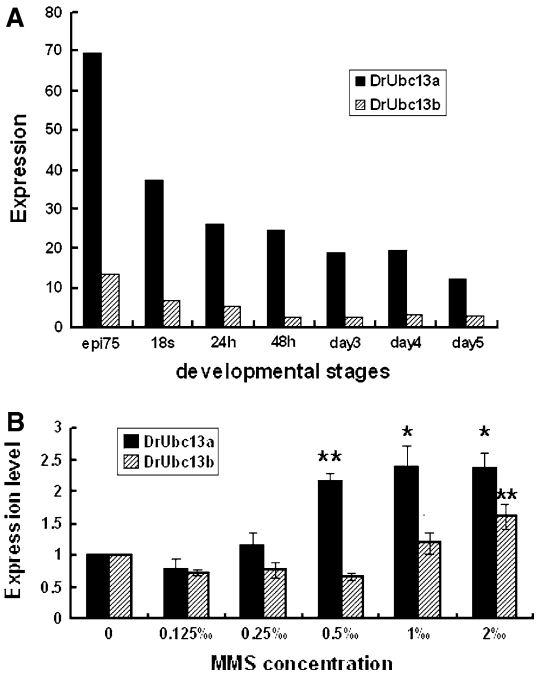


Fig. 6 MMS induces transcriptional expression of zebrafish *ubc13* genes during zebrafish embryonic development. **a** Expression profiles of zebrafish *ubc13* genes during development. Relative expression of zebrafish *ubc13* genes in different developmental stages was extracted from a web-based resource containing zebrafish gene microarray files of development (http://serine.umdnj.edu/~ouyangmi/cgibin/zebrafish/profile.htm) [55]. Accession numbers are BI877866 for zebrafish *ubc13a* and BE016083 for zebrafish *ubc13b*. *epi75*, 75% epiboly stage; *18s*, 18 somite stage. **b** Relative transcript levels of zebrafish *ubc13a* and *ubc13b* induced by MMS. Embryos were treated with MMS at indicated concentrations and collected at 6 hpf. Data represent mean ± SD from three independent experiments. Student's *t* test was performed using GraphPad Prism version 3.0 for Windows from GraphPad Software. Statistical significance was defined at $P < 0.05$. $*P < 0.05$ versus untreated embryos and $**P < 0.01$ versus untreated embryos

Discussion

Lys63-linked poly-ubiquitination is a post-translational modulation of target protein activities, which plays crucial roles in many cellular events, including stress response [47], DNA repair [13], homologous recombination [48], mitochondrial inheritance [49], plasma membrane protein endocytosis [50], and innate immunity [51]. As the only known E2 enzyme that promotes Lys63-linked Ub chain assembly, Ubc13 has been shown to regulate activation of NF-κB by poly-ubiquitinating NEMO/IKKγ in mammals [52], DDT in yeast [19] and possibly mammals [41] and plants [39] through the covalent modification of PCNA, and neuronal connectivity in *Drosophila* [53, 54]. However, the non-canonical poly-ubiquitination and its biological functions remain to be characterized in zebrafish. In this study, we identified two duplicated *ubc13* genes in zebrafish, isolated their full-length cDNAs, and investigated their functions in Lys63-linked poly-ubiquitination and DNA damage responses.

Zebrafish contains two highly conserved and duplicated *ubc13* genes encoding nearly identical proteins with only one residue difference. Although DrUbc13s have a minimum of 70% sequence identity with Ubc13s of other eukaryotic species from yeast to human, they have all known functional motifs. Moreover, *ubc13* is likely an ancient gene with housekeeping functions during the evolution, since certain intron–exon borders for Ubc13s from distant organisms are also highly conserved. In addition, transcriptional expression of zebrafish *ubc13*s is ubiquitous and DrUbc13a appears to be the major functional protein during the development [55]. It would be of great interest to examine the spatiotemporal expression patterns of zebrafish *ubc13*s and their developmental functions.

The first goal of our investigation is to determine whether Ubc13-mediated Lys63 poly-ubiquitination exists in zebrafish. It is known that Ubc13 alone is able to form a thiolester bond with Ub, while the formation of Lys63-linked poly-Ub chain requires a Uev as a binding partner [13]. In this study, we first examined the physical interaction between DrUbc13s and Uevs from other species by yeast two-hybrid and pull-down assays. Our data indicate that DrUbc13s are able to form a stable complex with yeast Mms2, human Uev1A or Mms2. Although neither assay employed is quantitative, the cross-species DrUbc13–Uev interaction is as strong as yeast and human intra-species Ubc13–Uev interactions. We have further shown that in vitro formation of various poly-Ub chains by DrUbc13-hMms2 is mediated through Lys63, but not Lys48. Thus, we infer that zebrafish also utilizes Ubc13-mediated Lys63 poly-ubiquitination as a means of gene regulation in one or more cellular pathways.

Ubc13 from yeast is a key member in the error-free branch of the PRR pathway, which requires Mms2 as a co-factor [13]. In human, Ubc13 interacting with hMms2 is also involved in DNA repair and protects cells from genomic instability [41]. Zebrafish is able to repair chromosomal lesions to a much greater extent than the human population, but the underling mechanisms remain unclear [56]. Given our findings that DrUbc13s are able to promote poly-Ub chain assembly with hMms2 through Lys63 linkage in vitro, zebrafish may utilize Ubc13-mediated Lys63 poly-ubiquitination as a means of regulation in DNA damage tolerance. This hypothesis was supported by several lines of evidence from this study. First, zebrafish *ubc13s* can functionally complement the cellular activity of corresponding yeast gene and expression of zebrafish *ubc13* in the yeast *ubc13* mutant restored the wild type Ubc13 activities, including resistance to DNA damaging agents and suppression of spontaneous mutagenesis. Second, upon DNA damage by

MMS or CPT, DrUbc13s are involved in formation of nuclear foci in treated cells. Third, zebrafish *ubc13* transcript levels were induced by MMS treatment during embryonic development. These observations suggest that Ubc13 is necessary for DNA damage response and may play crucial roles in maintaining genome stability in zebrafish like their counterparts in other eukaryotes.

Ubc13-mediated poly-ubiquitination appears to be primarily involved in environmental stress responses. In yeast, Ubc13 expression is DNA damage-inducible and involved in DDT [40]. Mms2 is required in Ubc13-dependent DNA damage response in mammalian cells, while the Ubc13–Uev1A complex functions in TRAF6-mediated stress response pathway following activation by proinflammatory cytokines [57, 58], as well as bacterial and viral infections [41, 52]. In the study, we demonstrate that zebrafish contains two *ubc13* genes encoding two proteins with only one conserved amino acid variation. In addition, zebrafish appears to be the only non-mammalian experimental animal model that contains two Uev homologs (Wen et al., personal communications). Therefore, zebrafish would be an ideal candidate to study functions of Lys63-linked ubiquitination in multiple cellular signaling pathways.

Acknowledgments We thank various laboratories for kindly providing reagents and plasmids, Drs. Z. Zhu and H. Dai for their valuable comments and suggestions, and all other members in Cui and Xiao Laboratories for helpful suggestions and technical assistance. This work was supported by grants from the National Basic Research Program of China (#2009CB941200) and the Canadian Institutes of Health Research MOP-53240 to WX.

References

1. Hershko A, Ciechanover A (1992) The ubiquitin system for protein degradation. Ann Rev Biochem 61:761–807
2. Tokunaga F, Sakata S, Saeki Y et al (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. Nat Cell Biol 11:123–132
3. Moynagh PN (2009) The Pellino family: IRAK E3 ligases with emerging roles in innate immune signalling. Trends Immunol 30:33–42
4. Haglund K, Dikic I (2005) Ubiquitylation and cell signaling. EMBO J 24:3353–3359
5. Kao CF, Hillyer C, Tsukuda T et al (2004) Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. Genes Dev 18:184–195
6. Geng F, Tansey WP (2008) Polyubiquitylation of histone H2B. Mol Biol Cell 19:3616–3624
7. Finley D, Bartel B, Varshavsky A (1989) The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature 338:394–401
8. Kotoshiba S, Kamura T, Hara T et al (2005) Molecular dissection of the interaction between p27 and Kip1 ubiquitylation-promoting complex, the ubiquitin ligase that regulates proteolysis of p27 in G1 phase. J Biol Chem 280:17694–17700
9. Wei W, Ayad NG, Wan Y et al (2004) Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. Nature 428:194–198
10. Mukhopadhyay D, Riezman H (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 315:201–205
11. Jentsch S, McGrath JP, Varshavsky A (1987) The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. Nature 329:131–134
12. Pastushok L, Xiao W (2004) DNA postreplication repair modulated by ubiquitination and sumoylation. Adv Protein Chem 69:279–306
13. Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell 96:645–653
14. Hochstrasser M (1996) Ubiquitin-dependent protein degradation. Annu Rev Genet 30:405–439
15. Jentsch S (1992) Ubiquitin-dependent protein degradation: a cellular perspective. Trends Cell Biol 2:98–103
16. Pickart CM (2001) Mechanisms underlying ubiquitination. Annu Rev Biochem 70:503–533
17. Hatakeyama S, Nakayama KI (2003) Ubiquitylation as a quality control system for intracellular proteins. J Biochem 134:1–8
18. Broomfield S, Chow BL, Xiao W (1998) MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway. Proc Natl Acad Sci 95:5678–5683
19. Hoege C, Pfander B, Moldovan GL et al (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 419:135–141
20. Ulrich HD, Jentsch S (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. EMBO J 19:3388–3397
21. Xiao W, Chow BL, Broomfield S et al (2000) The *Saccharomyces cerevisiae* RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. Genetics 155:1633–1641
22. Broomfield S, Hryciw T, Xiao W (2001) DNA postreplication repair and mutagenesis in *Saccharomyces cerevisiae*. Mutat Res 486:167–184
23. Kimmel CB, Ballard WW, Kimmel SR et al (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310
24. Takahashi N, Dawid IB (2005) Characterization of zebrafish Rad52 and replication protein A for oligonucleotide-mediated mutagenesis. Nucleic Acids Res 33:e120
25. Bladen CL, Lam WK, Dynan WS et al (2005) DNA damage response and Ku80 function in the vertebrate embryo. Nucleic Acids Res 33:3002–3010
26. Bladen CL, Navarre S, Dynan WS et al (2007) Expression of the Ku70 subunit (XRCC6) and protection from low dose ionizing radiation during zebrafish embryogenesis. Neurosci Lett 422:97–102
27. Wang Y, Shupenko CC, Melo LF et al (2006) DNA repair protein involved in heart and blood development. Mol Cell Biol 26:9083–9093
28. Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168
29. Xiao W, Samson L (1993) In vivo evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells. Proc Natl Acad Sci 90:2117–2121
30. Williamson MS, Game JC, Fogel S (1985) Meiotic gene conversion mutants in *Saccharomyces cerevisiae*. I. Isolation and characterization of pms1-1 and pms1-2. Genetics 110:609–646
31. James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144:1425–1436

32. Pastushok L, Moraes TF, Ellison MJ et al (2005) A single Mms2 "key" residue insertion into a Ubc13 pocket determines the interface specificity of a human Lys63 ubiquitin conjugation complex. J Biol Chem 280:7891–17900
33. Driever W, Rangini Z (1993) Characterization of a cell line derived from zebrafish (*Brachydanio rerio*) embryos. In vitro Cell Dev Biol 29(A):749–754
34. Winer J, Jung CK, Shackel I et al (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270:41–49
35. Wooff J, Pastushok L, Hanna M, Fu Y et al (2004) The TRAF6 RING finger domain mediates physical interaction with Ubc13. FEBS Lett 566:229–233
36. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340:245–246
37. Hofmann RM, Pickart CM (2001) In vitro assembly and recognition of Lys-63 polyubiquitin chains. J Biol Chem 276:27936–27943
38. Wen R, Newton L, Li G, Wang H et al (2006) *Arabidopsis thaliana* UBC13: implication of error-free DNA damage tolerance and Lys63-linked polyubiquitylation in plants. Plant Mol Biol 61:241–253
39. Wen R, Torres-Acosta JA, Pastushok L et al (2008) Arabidopsis UEV1D promotes Lysine-63-linked polyubiquitination and is involved in DNA damage response. Plant Cell 20:213–227
40. Brusky J, Zhu Y, Xiao W (2000) UBC13, a DNA-damage-inducible gene, is a member of the error-free postreplication repair pathway in *Saccharomyces cerevisiae*. Curr Genet 37:168–174
41. Andersen PL, Zhou H, Pastushok L et al (2005) Distinct regulation of Ubc13 functions by the two ubiquitin-conjugating enzyme variants Mms2 and Uev1A. J Cell Biol 170:745–755
42. Ryan AJ, Squires S, Strutt HL, Johnson RT (1991) Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. Nucleic Acids Res 19:3295–3300
43. Tsao YP, Russo A, Nyamuswa G et al (1993) Interaction between replication forks and topoisomerase I-DNA cleavable complexes: studies in a cell-free SV40 DNA replication system. Cancer Res 53:5908–5914
44. Myung K, Kolodner RD (2003) Induction of genome instability by DNA damage in *Saccharomyces cerevisiae*. DNA Repair 2:243–258
45. Andegeko Y, Moyal L, Mittelman L et al (2001) Nuclear retention of ATM at sites of DNA double strand breaks. J Biol Chem 276:38224–38230
46. McKenna S, Spyracopoulos L, Moraes T et al (2001) Noncovalent interaction between ubiquitin and the human DNA repair protein Mms2 is required for Ubc13-mediated polyubiquitination. J Biol Chem 276:40120–40126
47. Arnason T, Ellison MJ (1994) Stress resistance in *Saccharomyces cerevisiae* is strongly correlated with assembly of a novel type of multiubiquitin chain. Mol Cell Biol 14:7876–7883
48. Zhao GY, Sonoda E, Barber LJ et al (2007) A critical role for the ubiquitin-conjugating enzyme Ubc13 in initiating homologous recombination. Mol cell 25:663–675
49. Fisk HA, Yaffe MP (1999) A role for ubiquitination in mitochondrial inheritance in *Saccharomyces cerevisiae*. J Cell Biol 145:1199–1208
50. Galan JM, Haguenauer-Tsapis R (1997) Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. EMBO J 16:5847–5854
51. Keating SE, Bowie AG (2009) Role of non-degradative ubiquitination in interleukin-1 and toll-like receptor signaling. J Biol Chem 284:8211–8215
52. Zhou H, Wertz I, O'Rourke K et al (2004) Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. Nature 427:167–171
53. Muralidhar MG, Thomas JB (1993) The Drosophila bendless gene encodes a neural protein related to ubiquitin-conjugating enzymes. Neuron 11:253–266
54. Oh CE, McMahon R, Benzer S et al (1994) bendless, a Drosophila gene affecting neuronal connectivity, encodes a ubiquitin-conjugating enzyme homolog. J Neurosci 14:3166–3179
55. Ouyang M, Garnett AT, Han TM et al (2008) A web based resource characterizing the zebrafish developmental profile of over 16,000 transcripts. Gene Expr Patterns 8:171–180
56. Sussman R (2007) DNA repair capacity of zebrafish. Proc Natl Acad Sci 104:13379–13383
57. Deng L, Wang C, Spencer E et al (2000) Activation of the I-kappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103:351–361
58. Wang C, Deng L, Hong M et al (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346–351
59. Hall A (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98