Zebrasish Mms2 promotes K63-linked polyubiquitination and is involved in p53-mediated DNA-damage response

Rui Wen a,b, Jie Li c, Xin Xu a, Zongbin Cui c,*, Wei Xiao a,b,**

a College of Life Sciences, Capital Normal University, Beijing, 100048, People’s Republic of China
b Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E5
c Institute of Hydrobiology, Chinese Academy of Sciences, Wuhu, 430072, Hubei, People’s Republic of China

A R T I C L E   I N F O

Article history:
Available online 3 November 2011

Keywords:
Zebrafish
Ubc13
Mms2
K63-linked polyubiquitination
DNA-damage tolerance
p53

A B S T R A C T

The ubiquitin-conjugating enzyme Ubc13 together with a Ubc/E2 variant (Uev) form a stable complex and mediate K63-linked polyubiquitination, which is implicated in DNA damage tolerance in yeast and mammalian cells. The zebrarsh Danio rerio is a lower vertebrate model organism widely used in the studies of vertebrate development and environmental stress responses. Here we report the identification and functional characterization of two zebrashf Uev genes, Drmms2 and Druev1. Their deduced amino acid sequences indicate that the two Uev genes evolved separately prior to the appearance of vertebrates. Both zebrashf Uevs form a stable complex with DrUbct13 as well as Ubc13s from yeast and human, and are able to promote Ubc13-mediated K63 polyubiquitination in vitro, suggesting that their biochemical activities are conserved. Despite the fact that both zebrashf Uev genes can functionally replace the yeast Mms2 DNA-damage tolerance function, they exhibited differences in DNA-damage response in zebrashf embryos: ablation of DrMms2, but not DrUev1, enhances both spontaneous and DNA-damage induced expression of p53 effectors p21 and mdm2. In addition, DrUbc13 specifically binds Drp53 in an in vitro assay. These observations collectively indicate that zebrashf Mms2 and Ubc13 form a stable complex, which is required for p53-mediated DNA-damage response.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Ubiquitination, the attachment of ubiquitin (Ub) to a target protein, is an essential process found in all eukaryotic cells from unicellular yeast to human. Ub conjugated to the target protein can alter protein stability, localization or activity [1]. Ubiquitination is involved in many cellular processes including ribosomal biogenesis [2], cell cycle progression [3], apoptosis [4], mitochondrial inheritance [5], transcriptional regulation [6] and DNA repair [7,8]. Three enzymes, Ub-activating enzyme (Uba or E1), Ub-conjugating enzyme (Ubc or E2), and Ub ligase (E3), are involved in this process. Ubiquitination can be divided into three types: monoubiquitination, multiple monoubiquitination and polyubiquitination and different Ub modifications play different roles in the regulation of cellular processes [9]. Poly-Ub chains formed via the C-terminal G76 and K48 of Ub play a role in targeting proteins for degradation by the 26S proteasome. K6-linked polyubiquitin chain on E3 enzyme BRCA1 is implicated in nuclear focus formation during DNA repair [10], and K63-linked polyUb chains are involved in DNA repair, endocytosis, and NF-κB activation [11]. So far, Ubc13 is the only known Ub-conjugating enzyme capable of catalyzing K63-linked polyubiquitination reaction and this function requires an interaction with an Ubc/E2 variant (Uev), either Mms2 or Uev1. Uev proteins constitute a distinct subfamily within the E2 protein family. They have sequence similarity to Ubcs but lack the conserved Cys residue that is critical for the catalytic activity of E2s. The first Uev gene, called Mms2, was isolated from the budding yeast Saccharomyces cerevisiae and is involved in error-free postreplication repair (PRR) [12–14]. The crystal structures of yeast and human Ubc13 and Mms2 complex exhibit that Mms2 is required for creating K63 poly-Ub chains. Different organisms contain different numbers of Uev genes: for example, yeast has only one [15]. Arabidopsis contain four Uev genes [16] and human contain two Uev genes, one of which with several isoforms [17]. Although Uev gene family members share great amino acid sequence similarity and act as a cofactor of Ubc13, they may have completely different biological functions. For example, human Ubc13–Mms2 is required for DNA-damage response but not for NF-κB activation, whereas Ubc13–Uev1A is involved in NF-κB activation but not...
DNA-damage response [18]. These observations suggest that Uev family members may have evolved to function in diverse cellular processes.

The zebrafish, Danio rerio, a tropical freshwater fish, is a widely used vertebrate model organism in scientific research, particularly in the studies of vertebrate development and gene functions [19]. D. rerio is a very good vertebrate model organism. The advantages of this model organism are: the genome sequence is fully obtained; characterized mutants are available for further research; DNA, mRNA or proteins can be easily transferred into zebrafish embryos using microinjection [20]; and target gene expression can be suppressed in embryos by interference technology. Our previous research showed that zebrafish contains two ubc13 genes, the products of which are able to physically interact with yeast or human Mms2, and are able to functionally complement the yeast ubc13 null mutant for spontaneous mutagenesis and sensitivity to DNA-damaging agents [21]. Here we report the functional studies of two zebrafish Uev genes, uev1 and mms2. Our results indicate that although both zebrafish genes are able to functionally complement the yeast mms2 null mutant and their proteins interact with yeast, human and zebrafish Ubc13s, they apparently evolved separately at an early stage. Interestingly, only Mms2 is involved in p53-dependent transcriptional activity.

2. Materials and methods

2.1. Zebrafish maintenance and yeast cell cultures

Wild-type AB inbred strain of zebrafish (D. rerio) was raised in a constant flow-through water system and maintained under standard conditions. The stages of naturally fertilized zebrafish embryos were defined by hours post-fertilization (hpf) or based on the morphological features.

The haploid yeast strains used in this study are listed in Table 1. 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) supplemented with necessary nutrients as recommended [22]. For 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 [23]. The sources and preparation of ubc13Δ::hisG-URA3-hisG [24] and mms2Δ::HIS3 [25] cassettes were as described previously.

2.2. Molecular cloning of zebrafish cDNAs and plasmid construction

To clone zebrafish Uev genes, 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 by using the ReverTra™ First-Strand cDNA Synthesis Kit from Fermentas. Each ORF was amplified by PCR from the above cDNA preparation by using gene-specific primers that were designed according to predicted full-length cDNA sequences. The flanking 5′ BamHI and 3′ SalI restriction sites (underlined in the primer sequences) were used to clone the PCR products into the yeast two-hybrid vector pGAD424 (for GAL4Δ fusion), which was derived from pGAD424 [26], with a 1-bp framseshift at the multiple cloning site. Primers for zebrafish Mms2 are 5′-CCGGGAATTCATGGCCGCCTCAGGGAG-3′ and 5′-CCGGTGCATCTATTTGCTGATGTGTCCTC-3′, and primers for zebrafish Uevs are 5′-CCGGGAATCCATGGCCGCCTCAGGGAG-3′ and 5′-CCGGTGCATCTATTTGCTGATGTGTCCTC-3′. The identity of each cloned ORF was verified by sequencing.

2.3. Yeast survival assay

Yeast strain HK580-10D and its isogenic mms2Δ single or ubc13Δ mms2Δ double mutants were either singly transformed with pGAD-DrUbc13 and pGAD-DrUbc13A. Transformants were selected on SD-Leu or SD-Leu-Trp plates, respectively. The gradient plate assay was performed as described previously [14].

2.4. Spontaneous mutagenesis assay

Yeast strain DBY747 and its mms2Δ derivative WXY642 bear a trp1-289 amber mutation that can be reverted to Trp+ by several different mutation events [27]. WXY642 was transformed with pGAD-DrMms2, pGAD-DrUev1 or pGAD424Bg, and 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 cells/ml and incubated at 30 °C until the cell titer reached 2 × 10⁷ cells/ml. Cells were spun down at 4000 rpm, resuspended in sterile ddH₂O and plated on YPD in duplicate to score total survivors and onto SD-Trp plates to score Trp+ revertants. Spontaneous mutation rates (number of revertants per cell per generation) were calculated as previously described [28].

2.5. Yeast two-hybrid analysis

To perform the yeast two-hybrid analysis, the yeast strain PJ69-4A [29] was co-transformed with different combinations of Gal4Δ and Gal4Δ constructs. The construction of pGBT-DrUbc13A, pGBT-DrUbc13B [21], pGBT-Ubc13 [30], and pGBT-Ubc13 [14] has been described previously. 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-Trp-His with various concentrations of the histidine biosynthesis inhibitor 1,2,4-aminotriazole (3-AT) to test the activation of the Pgal1·HIS3 gene and onto SD-Leu-Trp-Ado to detect the activation of the Pgal2·ADE2 reporter gene.

2.6. Protein expression and purification

Both zebrafish uev and p53 ORF were cloned into pGEX6p (Amersham Biosciences). The resulting pGEX-DrUevs and pGEX-Drp53 were transformed into Escherichia coli strain BL21 (DE3)·RII (Strategene). The GST–Uev fusion proteins were induced at 37 °C for 2 h and the GST–p53 fusion protein was induced at 16 °C for 24 h by 0.5 mM IPTG. The proteins were purified as described previously [13]. The DrUbc13 protein was produced and purified as described previously [21].
2.7. Affinity pull-down assays

MicroSpin GST Purification Modules (GE Healthcare) were used for DrUbc13 and DrUev glutathione S-transferase (GST) pull-down assays. 500 µl of bacterial crude cell extract containing GST–DrMms2 or GST–DrUev1A was loaded into the purification module and incubated for 1 h at 4°C with gentle rocking. The module was then washed three times with 500 µl PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.3); 40 µg of purified His6–DrUbc13 in PBS was added and the incubation was continued for another hour at 4°C. The module was washed again three times with 500 µl PBS, and then 80 µl of reduced glutathione buffer (10 mM glutathione in 50 mM Tris–HCl, pH 8.0) was added to elute proteins bound to the MicroSpin module. The elution samples were subjected to SDS-PAGE analysis.

The DrUbc13–Drp53 pull-down assay was carried out by using a His Spin Trap™ column (GE Healthcare). Purified DrUbc13 was added to individual columns and incubated for 1 h at 4°C. The columns were spun and washed three times using a binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). GST or GST–Drp53 was then added to the columns and incubated for 1 h at 4°C. After centrifugation and washing with the binding buffer, proteins were eluted by the addition of 80 µl of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) followed by centrifugation. A 15 µl sample was added to 15 µl of SDS-PAGE loading buffer and analyzed on a 12% SDS-PAGE gel.

2.8. Ub conjugation reaction

Ub conjugation reactions were performed by using purified DrUbc13A and GST–DrUev proteins. Ub thioester/conjugation initiation reagents were purchased from Boston Biochem. The reaction mixture contained 225 nM E1 enzyme, 450 mM Ub, 1 mM MgATP, 1 mM DrUbc13 and 1 mM DrUev in the supplied reaction buffer and the total reaction volume is 20 µl. The K63 mutant Ub was purchased from Boston Biochem (UM-K63R). The conjugation reactions were performed at 37°C for 2 h. Samples were subjected to 12% SDS-PAGE analysis; Ub and poly-Ub were detected through a horseradish peroxidase Western blot detection system by using polyclonal rabbit anti-Ub antibodies (Sigma–Aldrich).

2.9. Suppression of target gene expression by morpholino oligonucleotides

Morpholinos (MOs) were purchased from Gene Tools, LLC (Corvallis, OR, USA). Target sequences of two MOs for Drmms2 and DrUev1A are 5′-AGACAACCCTAGAGAGCGCCCATC-3′ and 5′-GGAGCACCAGCCACATTGACCATCGC-3′, respectively. Capped mRNAs for Mms2-EGFP and Uev1-EGFP were synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA). The standard control MO from Gene Tools was used to detect any non-specific interference in the transcriptional process. Each embryo at one/two-cell stage was microinjected with 10 ng MO or 10 ng MO plus 200 pg capped mRNAs. Injected embryos were treated with or without 0.1% MMS.

2.10. Real-time RT-PCR (qRT-PCR)

Total RNA of each group was extracted from 50 embryos at 12 hpf using TRIzol reagent from Invitrogen. The RNA quality was assessed by agarose gel electrophoresis and ultraviolet spectrophotometry. First-strand cDNA for each RNA sample was synthesized with the RevertAid™ First-Strand cDNA Synthesis Kit from Fermentas. 5 µg of total RNA was treated with RNase-free DNase and reverse transcribed in the presence of random primers. qRT-PCR was performed using an ABI prism 7000 sequence-detection system and the SYBR Green PCR Master Mix from TOYOBO. Gene-specific primers include 5′-CGGAGGTGAAGAC-GATCA-3′ and 5′-TGGTAGATGCTATTGATG-3′ for 18S ribosomal RNA, 5′-GCCCTGAAAGAAAACAC-3′ and 5′-GACCCGTCTCT-TGCCCTGAG-3′ for zebrafish p21, 5′-CGAGAGAGAGGAAG-AAGCA-3′ and 5′-GCCATCATGCCACCAAG-3′ for zebrafish mdm2, 5′-AGTGTTGCTTGCTTGCTG-3′ and 5′-GATGCCGAGGC-GCTGTC-3′ for zebrafish p53. The expression of p21 and mdm2 was normalized to 18S ribosomal RNA. The relative gene expression was determined by the 2−ΔΔCT method [31].

3. Results

3.1. Molecular cloning of zebrafish UEV genes

Zebrafish UEV ORFs were cloned from zebrafish intestine by RT-PCR using gene-specific primers to the yeast two-hybrid vector pGAD424. Sequencing results revealed that nucleotide sequences were identical to the annotated complete coding sequences in the NCBI database (http://www.ncbi.nlm.nih.gov). Based on the guideline for naming zebrafish genes (http://zfin.org/zf_info/nomen.html), we named these two genes Drmms2 and DrUev1 for reasons given below. The NCBI database indicates that Drmms2 is located in chromosome 24 at the position of 37030790 to 37015142 with 15568 bp of genomic DNA, whereas DrUev1 is located in chromosome 11 at the position of 2392518–2409583 with 17066 bp genomic DNA. The predicted ORFs of Drmms2 and DrUev1 are 438 and 444 bases, respectively, with 76% identity. The genomic structure analysis predicts that both Drmms2 and DrUev1 contain four exons and three introns; however, the 3′ UTR of Drmms2 is much longer than DrUev1 (Fig. 1A), resulting in the predicted mRNA length of Drmms2 and DrUev1 to be 2076 and 1104 bases, respectively. To know whether zebrafish Uevs are conserved among model organisms, the protein sequences of Drmms2 and DrUev1 were aligned with those of Uev proteins from six other eukaryotic organisms. As shown in Fig. 1B, amino acid sequence identity between Drmms2 and DrUev1 is approximately 94%, and between DrUev1 and those from other species ranges from 46% to 58%. Furthermore, critical residues involved in Uev activities, including F13 of hMms2 required for the physical interaction with Ubc13 [14], S32 and I62 of hMms2 [32] required for the noncovalent interaction with Ub, and poly-Ub chain assembly, are all conserved (Fig. 1B). To further investigate the evolution of zebrafish Uevs, the protein sequences of Drmms2 and DrUev1 were compared with human and mouse Uevs. Drmms2 shares 93–94.5% sequence identity with human and mouse Mms2, but only 84–87.5% identity with human and mouse Uev1. Similarly, the DrUev1 sequence is closer to Uev1s than to Mms2s from human and mouse (Fig. 1B and C). The phylogenetic analysis supports a notion that the two UEV genes evolved from a common ancestor prior to the formation of vertebrates (Fig. 1C), and hence that the zebrafish UEV genes may be an ideal model to study mammalian UEV gene functions.

3.2. DrUevs physically interact with Ubc13s from different organisms

Yeast and human Ubc13 and Uevs form a stable complex to mediate K63-linked polyubiquitination and play an essential role in preserving the genome from DNA damage [12–14]. To ask whether DrUevs physically interact with Ubc13 from different organisms, a yeast two-hybrid assay [33] between the cloned zebrafish UEV genes and UBC13 genes from different species was performed.
These studies revealed that DrUev proteins were able to interact with both human and yeast (Fig. 2A), as well as zebrafish (Fig. 2B) Ubc13s. The physical interactions between DrUevs and Ubc13 from different organisms are robust and deemed strong. In contrast, none of the negative controls displayed positive interactions under the same experimental conditions.

To further confirm the physical interaction between DrUevs and DrUbc13 in vitro, a GST-affinity pull-down assay was conducted. As shown in Fig. 3A, bacterial cell extracts from GST–DrMms2 (lane 3) and GST–DrUev1A (lane 4) transformants were able to pull down purified recombinant DrUbc13. In contrast, extract from cells expressing GST alone (lane 2) was unable to pull down detectable amount of DrUbc13. Hence, we are able to conclude that both zebrafish Uev proteins are able to form stable heterodimers with DrUbc13.

3.3. DrUev is required for Ubc13-mediated K63-linked polyubiquitination in vitro

Although all E2s are able to form active-site thioesters with Ub, up to now, Ubc13 is the only known E2 enzyme capable of mediating K63-linked poly-Ub chains, and a Uev is absolutely required for this poly-Ub chain assembly. The function of Uev is to orientate the acceptor Ub to the appropriate position and allow its K63 residue to...
be proximal to the C-terminus of the donor Ub linked to the Ubc13 active site [13]. To directly examine whether DrUevs are involved in Ubc13-mediated K63-linked polyubiquitination, an in vitro Ub conjugation assay was carried out and the results are shown in Fig. 3B. DrUbc13 (lane 1), DrMms2 (lane 2) and DrUev1 (lane 5) alone cannot generate free poly-Ub chains. DrUbc13 with DrMms2 (lane 3) or DrUev1 (lane 6) can generate di- and tri-Ub chains. In addition, the poly-Ub conjugates were not detected when using a Ub-K63R mutant to replace wild-type Ub (lanes 4 and 7). Hence, the above observations indicate that poly-Ub chains mediated by DrUbc13–DrMms2 or DrUbc13–DrUev1 are exclusively formed through G76–K63.

3.4. Zebrafish UEV genes functionally complement yeast mms2 null mutants

Yeast MMS2 is the first isolated and functionally characterized UEV gene [15]. It is a member of the error-free PRR pathway and plays a critical role in protecting the yeast genome from mutagenesis and cell death caused by DNA-damaging agents [15]. To ask whether DrUevs have the same function as yeast MMS2, two experimental approaches, namely yeast survival and spontaneous
Fig. 4. Functional complementation of the yeast mms2 mutation by zebrafish UEV genes. (A) Complementation of the mms2 single mutation by Drmms2 and Druev1. Yeast strain HK580-10D (wild type) and its mms2 single mutant WXY942 was transformed with pGAD-DrMms2 or pDAD-DrUev1. The transformants were grown overnight and printed onto YPD and YPD + 0.025% MMS gradient plates to test their abilities to complement the yeast mms2 defect. The plates were incubated at 30 °C for 2 days before being photographed. The arrow indicates higher MMS concentrations. Several transformants of each treatment were tested with the same result, and only one is shown here. (B) Complementation of the mms2 ubc13 double mutations by Drmms2, Druev1 and Drubc13. The mms2 ubc13 double mutant strain WXY955 was co-transformed with two plasmids as indicated and the experimental conditions were as described in (A).

mutagenesis assays, were employed, both of which showed that Drmms2 and Druev1 functionally complemented the error-free PRR defect in yeast. Druev genes cloned in the yeast two-hybrid plasmid rescued the mms2 mutant from killing by MMS to a level comparable to wild type cells, whereas mms2 mutant cells transformed with the vector alone did not acquire any MMS resistance (Fig. 4A). The role of yeast Mms2 in DNA-damage tolerance relies on its ability to form a heterodimer with Ubc13 that mediates K63-linked poly-Ub chain assembly [12]. In order to assess in vivo complex formation and functions between DrUevs and DrUbc13, the yeast mms2 ubc13 double mutant was created and co-transformed with Druev and Drubc13A. Expression of either zebrafish Druev with Drubc13A partially rescued the yeast mms2 ubc13 double mutant from MMS-induced killing. In contrast, the yeast mms2 ubc13 double mutant cells transformed with the combination of any single zebrafish and control vectors did not display enhanced MMS resistance (Fig. 4B), implying that both DrUb13 and DrUev are required for the DNA-damage tolerance in yeast.

One of the characteristic phenotypes of the yeast mms2 [15] or ubc13 [24] mutant is its massive increase in spontaneous mutagenesis, indicating that these genes play an important role in protecting cells from genomic instability, a hallmark of cancer. In this study, the mms2 mutant strain exhibited a 22-fold increase in the spontaneous mutation rate compared with wild type cells (Table 2). However, when the same mms2 mutant was transformed with a plasmid containing either Druev1 or Drmms2, the spontaneous mutation rate was reduced to a level similar to that of wild type cells (Table 2). The above functional complementation results collectively suggest that zebrafish UEV genes are able to replace yeast MMS2 to protect yeast cells from spontaneous and DNA-damage-induced genomic instability.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Key alleles</th>
<th>Rate (× 10⁻⁶)</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY747</td>
<td>Wild type</td>
<td>3.18 ± 0.18</td>
<td>1</td>
</tr>
<tr>
<td>WXY642/pGAD424</td>
<td>mms2Δ</td>
<td>70.2 ± 7.96</td>
<td>22.04</td>
</tr>
<tr>
<td>WXY642/Drubc13</td>
<td>mms2Δ/Drubc13</td>
<td>3.58 ± 0.56</td>
<td>1.13</td>
</tr>
<tr>
<td>WXY642/DrMms2</td>
<td>mms2Δ/DrMms2</td>
<td>4.32 ± 0.24</td>
<td>1.35</td>
</tr>
</tbody>
</table>

*All strains are isogenic derivatives of DBY747.

*The spontaneous mutation rate is the average of three independent experiments with standard deviations.

*Relative to the wild type mutation rate.

3.5. DrMms2 but not DrUev1 is involved in p53-dependent transcriptional activity

In order to determine roles of DrMms2 and DrUev1 in zebrafish embryonic development, two MOs were designed to target Drmms2 or Druev1. As shown in Fig. 5, MOs efficiently suppressed the EGFP fusion-gene expression. Under the above conditions, no obvious morphological defects were found in developing embryos injected with mms2-MO or uev1-MO (data not shown). It was previously reported that human Ubc13 elicits K63-linked polyubiquitination of p53, facilitates the formation of monomeric p53, increases its localization to the cytoplasm and decreases its transcriptional activity [34]. However, it remains unclear which cofactor of Ubc13 is involved in the regulation of p53 activity although either Mms2 or Uev1 can serve as a cofactor of Ubc13 to promote p53 polyubiquitination in vitro [34]. To answer the above questions, we measured the expression of two well-defined p53-responsive genes, p21 and mdm2 [35,36]. As shown in Fig. 6, MMS treatment resulted in a massive increase in p21 (Fig. 6A) and mdm2 (Fig. 6B) expression (p < 0.01 in both cases). This activation can be partially achieved through MO ablation of Mms2 (p < 0.01 in both cases). Interestingly, suppression of Mms2 in MMS-treated zebrafish embryos further enhanced p21 and mdm2 expression to approximately 37- and 55-fold, respectively, suggesting that Mms2 plays a critical role in negatively regulating the p53 activity. In contrast, MO suppression of Uev1 had no noticeable effects on p21 or mdm2 expression regardless of MMS treatment (Fig. 6), suggesting that Druev1 is not involved in p53-mediated DNA-damage response.

3.6. Direct interaction of Ubc13 with p53

There are at least two possibilities by which ablation of zebrafish Mms2 results in an enhanced expression of p53-dependent
genes. One is that lack of endogenous Mms2 causes spontaneous DNA damage that activates p53; another possibility is that the Mms2-Ubc13 complex binds to p53 and suppresses its transcriptional activity. To test the first hypothesis, we measured the p53 expression with or without DNA damage and meanwhile suppressed either Mms2 or Uev1 in zebrafish embryos by MO. As shown in Fig. 7A, in Mms2-ablated embryos the expression patterns of p53 parallel that of p21 and mmd2, suggesting that the p53 activation could be due to increased spontaneous DNA damage. In contrast, ablation of Uev1 does not enhance p53 expression in either spontaneous or MMS-treated conditions (Fig. 7A), indicating that the effect is gene specific. To test the second hypothesis, we attempted in vitro interaction between zebrafish Ubc13 and p53. As shown in Fig. 7B, His6-tagged DrUbc13 is able to pull down GST–Drp53, but not GST alone; hence, zebrafish Ubc13 directly interacts with p53 in vitro. Attempts to determine the above interaction in vivo were not successful to date because of our inability to ectopically express tagged genes in cultured ZF4 cells (data not shown).

4. Discussion

4.1. The evolution of UEV family genes

The Uev family protein is clearly derived from Ubc, highly conserved from yeast to human and surprisingly ubiquitously expressed. In budding [15] and fission [30] yeasts, there is only one UEV gene and its only known function to date is in DNA-damage tolerance (a.k.a. PRR in budding yeast). Other model eukaryotic organisms such as Drosophila and nematode also contain only one UEV gene, although their biological function(s) is currently unknown. In contrast, genome database analyses suggest that higher eukaryotes such as plants and mammals have multiple Uev genes [37]. Arabidopsis contains four UEV1 genes due to an apparently recent gene duplication of each of two UEV1 genes [16]. However, nucleotide and amino acid sequence analyses reveal no clear evolutionary lineage between the two pairs of Arabidopsis UEV1 genes and the two human UEV genes, MMS2 and UEV1A [16], indicating that the two UEV genes were independently evolved after the separation of animal and plant kingdoms. When did the single UEV gene duplicated and evolved independently? The discovery of two UEV genes from zebrafish and their respective similarities with mammalian MMS2 and UEV1 reveals an evolutionary
model in which UEV was duplicated prior to the appearance of vertebrates and then separately evolved to confer different biological functions. This model suggests that either these UEV genes are functionally redundant, or the organisms employ different Uev proteins in Ub conjugation to respond to diverse environmental and cellular signals.

4.2. Conserved biochemical and biological functions of Uev family proteins

Despite the Uev sequence diversity and early evolution in higher eukaryotes, it becomes clear that all UEV genes were from the same ancestor, as their genomic structures including intron–exon junctions are highly conserved. In this study, we demonstrated that both zebrafish Uev proteins are able to physically interact with Ubc13 proteins from zebrafish as well as from other organisms. More importantly, they are absolutely required for Ubc13-mediated polyubiquitination and this poly-Ub chain is deemed K63-linked. Based on these observations, we conclude that zebrafish Uev proteins function as cofactors of Ubc13 to modify target proteins with a K63-linked poly-Ub chain.

The similarly conserved biological function is the involvement in DNA-damage response by the Ubc13–Uev complex. In budding [38] and fission [39] yeasts, this is achieved through polyubiquitination of the replication clamp PCNA by the E2–E3 complex Mms2–Ubc13–Rad5. In Arabidopsis, recent genetic analyses suggest that a Uev1D–Ubc13–Rad5A complex is involved in DNA-damage tolerance [16,40,41], whereas in mammalian cells, Ubc13–Uev also appears to be involved in PCNA polyubiquitination [42], although its cognate E3 remains a subject of debate [43–47]. It is interesting to note that all UBC13 and UEV genes from different organisms are able to functionally complement the corresponding yeast mutants [16,21,41,48–52], suggesting that the PCNA polyubiquitination activity and the DNA-damage tolerance mechanism is highly conserved throughout eukaryotes.

4.3. Zebrafish Mms2 is involved in DNA-damage response

Although both Drrms2 and Druev1 are able to functionally complement the yeast defect in the DNA-damage response, it does not necessarily mean that they confer the same function in their own host. A good example is that while mammalian MMS2 and UEV1A both functionally complement the yeast mms2 defect, they have distinct functions in their own cells [18]. In mammalian cells, Ubc13 is also involved in other DNA-damage responses, such as double-strand break repair by using RFNB [51,52] and RFN168 [53,54] as cognate E3s. It is unclear whether a Uev and K63-linked poly-Ub chain is involved in this process [55]. Another reported involvement of Ubc13 in DNA-damage response is its negative regulation of p53 transcriptional activity in mammalian cells; this regulation is diminished upon DNA damage, allowing p53 activation [34]. This appears to be achieved through the direct association between Ubc13 and p53 on polysomes that results in p53 polyubiquitination and interference with its tetramerization [56]. In vitro studies suggest that both Mms2 and Uev1A can be potential cofactors and that Ubc13 requires K63 of Ub to stabilize p53 [34]. However, it is unclear whether a Uev is required for the process in vivo and if so which Uev is required. In this study, we found that suppression of DrMms2, but not DrUev1, alleviates both spontaneous and DNA-damage-induced p53 activation of downstream genes, suggesting that Mms2 is the cognate Uev for this DNA-damage response. In the attempts to further address roles of zebrafish Mms2–Ubc13 in the regulation of p53, we were able to demonstrate direct interaction between Ubc13 and p53, suggesting that it is the Mms2–Ubc13 complex, but not the Uev1–Ubc13 complex, that negatively regulates the p53 activity. The above observations raise two important questions. Firstly, if Mms2–Ubc13 directly binds and inactivates p53 as observed in mammalian cells, why is the expression of p53 gene itself regulated like its target genes? We do not have a definitive answer to this question; however, it has been reported that the human p53 promoter contains p53-binding sequences and its expression can be activated in a p53-dependent manner [57]. Hence, the enhanced p53 expression in Mms2-depleted embryos may be a secondary effect. Secondly, why does the Uev1–Ubc13 complex, which can also bind p53 through Ubc13, not inhibit p53 activity? This question is reminiscent of the phenomena as reported in mammalian cells [18]. Given the fact that DrMms2 and mammalian Mms2 share more sequence homology than to Uev1 proteins from the same organisms, it becomes conceivable that DrMms2 is a true ortholog of mammalian Mms2. By the same token, DrUev1 may be a true ortholog of mammalian Uev1, although additional experiments are needed to strengthen this possibility.

4.4. Involvement of Uev in pleiotropic cellular processes

Despite the putative functional diversity among UEV genes in higher eukaryotes, all Uev family proteins confer the same
biological chemistry, namely to promote Ubc13-mediated K63-
linked polyubiquitination. Among all Ubc/E2 proteins, Ubc13 is
unique since it is the only known Ubc/E2 specifically promoting
K63-linked polyubiquitination, which is believed to serve in cell-
ular signaling other than protein degradation, and this process
absolutely requires a Uev as cofactor [12,13]. In addition to the
well-defined roles in DNA-damage tolerance [12,13] and NF-κB
activation [58–60], K63-linked polyubiquitination has also been
implicated in other cellular processes such as plasma membrane
protein endocytosis [61], mitochondrial inheritance [5], ribosome
function [62], mitotic cell cycle checkpoint [63], neurodegeneration
[64,65] and apical dominance [66]. Although in several cases, Ubc13
was demonstrated to be required for the process, its cognate Uev
has not been explored. This is often due to the fact that in an in vitro
assay, either Uev appears to be sufficient to support the polyubiqui-
tination reaction, which often lead to misleading conclusions.

Since zebrafish is the only known non-mammalian organism that con-
tains two Uev genes with clear lineage to the two mammalian Uevs,
this study may provide an ideal vertebrate model to investigate how
the diverged UEV genes regulate K63-linked polyubiquitination for
various cellular processes.

Reference

[1] D. Finley, B. Bartel, A. Varshavsky, The tails of ubiquitin precursors are
ribosomal-proteins whose fusion to ubiquitin facilitates ribosome biogenesis,


markedly up-regulates β-defensin-2 expression in human airway epithelium


[7] L. Pastushok, W. Xiao, DNA postreplication repair modulated by ubiquitina-


ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA repli-

NFκB and the interleukin-6 promoter through NFκB-inducing kinase, Antioxid.
Redox Signal. 3 (2001) 293–304.

conjugating enzyme functions in assembly of novel polyubiquitin chains for

Ellison, Noncovalent interaction between ubiquitin and the human DNA repair
protein MMS2 is required for UBC13-mediated polyubiquitination, J. Biol.

[13] L. Pastushok, T.F. Moreau, M.J. Ellison, W. Xiao, A single Mms2 key residue inser-
tion into a Ubc13 pocket defines the interface specificity of a human Lys63

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors wish to thank both Xiao and Cui laboratory mem-
bers for technical assistance and helpful discussion, and Michelle
Hanna for proofreading the manuscript. This work is supported by
the Capital National University 211 Special Fund (No. 10531182313)
and the Canadian Institutes of Health Research operating grant
MOP-93612 to W.X., and the National Natural Science Foundation of
China (30871442 and 31171390) to Z.C.

References


