Identification of four SUMO paralogs in the medaka fish, *Oryzias latipes*, and their classification into two subfamilies.

Daisuke Seki\(^1,\,2\), Shinpei Obata\(^1\), Tsuyoshi Shirozu\(^1\), Takeshi Kitano\(^1\) and Hisato Saitoh\(^1,\,2,\,3\)

\(^1\)Department of Biological Sciences, Graduate School of Science and Technology, and
\(^2\)Global COE (Centers of Excellence) Program, Global Initiative Center for Pulsed Power Engineering, Kumamoto University, Kumamoto, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

\(^3\)To Whom correspondence should be addressed:

Email: hisa@kumamoto-u.ac.jp,

Full Postal Address: Department of Biological Sciences, Graduate School of Science and Technology,

Kumamoto University, Kumamoto, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

Phone: +81-96-342-3450, Fax: +81-96-342-3450
Abstract  At least four paralogs of the small ubiquitin-related modifier (SUMO) exist in humans, but there is limited information about SUMO paralogs from other vertebrate species. We isolated the four cDNA encoding proteins, similar to human SUMOs, from the medaka fish, *Oryzias latipes*: *OlSUMO*-1, *OlSUMO*-2, *OlSUMO*-3, and *OlSUMO*-4. The amino acid sequences of *OlSUMO*-2, *OlSUMO*-3 and *OlSUMO*-4 are 89‒94% identical, but they share only 45% identity with *OlSUMO*-1. Phylogenetic analysis, transient expression of *OlSUMOs* in cultured cells and *in vitro* binding of *OlSUMOs* with two different SUMO-interacting proteins demonstrated that the medaka SUMO paralogs can be grouped into two subfamilies, *OlSUMO*-1 and *OlSUMO*-2/3/4, respectively. Furthermore, this is the first report of all four *OlSUMO* transcripts being expressed in medaka embryos, implying that they have a role in fish development. The study will improve understanding of the relationship between structural and functional diversity of SUMO paralogs during vertebrate evolution.

Keywords  Posttranslational modification; Small ubiquitin-related modifier (SUMO); medaka fish
Introduction

Post-translational modifications are an important mechanism by which structures and functions of cellular proteins are controlled. Among post-translational protein modifications, sumoylation is a unique type in which the small ubiquitin-related modifiers (SUMOs) are covalently conjugated to lysine residues in a wide variety of target proteins in eukaryotic cells. Sumoylation is important in regulating numerous cellular processes, including transcription, epigenetic gene control, genomic instability, and protein degradation (Geiss-Friedlander and Melchior 2007; Wilson and Heaton 2008; Wang and Dasso 2009). The SUMO modification pathway is regulated markedly not only by multiple enzymes involving SUMO proteases (SEPNs), SUMO-activation E1 enzyme (Aos1/Uba2), SUMO-conjugation E2 enzyme (Ubc9) and SUMO-E3 ligases, such as the protein inhibitor of the activated STAT (PIAS) family of proteins and Ran-binding protein 2/nucleoporin 358kDa (RanBP2/Nup358), but also by diverse SUMO-interacting proteins that recognize conjugated SUMO moieties via SUMO-interacting motifs (SIMs), also known as SUMO-binding domains (Geiss-Friedlander and Melchior 2007; Wilson and Heaton 2008; Wang and Dasso 2009).

SUMOs are highly conserved from yeast to humans. At least three paralogs have been reported in human and mice: SUMO-1/SMT3C, SUMO-2/SMT3A and SUMO-3/SMT3B. SUMO-2 and SUMO-3 are more closely related to each other (95% amino acid identity) than they are to SUMO-1 (≈50% identity). Although SUMO-1 and SUMO-2/3 can be equally conjugated to a subset of proteins, several lines of evidence indicate that SUMO-1 and SUMO-2/3 are conjugated to different proteins, and represent unique signals regulating different cellular functions (Saitoh and Hinchey 2000; Tatham et al. 2001; Rosas-Acosta et al. 2005; Vertegaal et al. 2006). Intriguingly, in humans but not in mice, there is another SUMO paralog, designated as SUMO-4, which differs from SUMO-1/2/3 in that it not only seems to be expressed mainly in the kidney, lymph node and spleen, but is also unable to form covalent modification with substrates because of a unique proline residue at position 90 (Pro-90) (Guo et al. 2004; Owerbach et al. 2005). To date, there is limited information on a SUMO paralog that is similar to human SUMO-4 in other vertebrate species, and when the structural and functional diversification of SUMO paralogs occurred during vertebrate evolution remains uncertain. Thus, it is important to identify and investigate other examples of vertebrate SUMOs.
Here we report the isolation of four cDNAs of medaka SUMO paralogs, termed: *OlsUMO-1, OlsUMO-2, OlsUMO-3 and OlsUMO-4*. Medaka, *Oryzias latipes*, is a small egg-laying freshwater teleost fish with several advantages for biological experiments (Ozato et al. 1986; Wada et al. 1995; Ishikawa et al. 2000; Loosli et al. 2000; Kasahara et al. 2007; Shiraishi et al. 2008). Our data, including sequence comparison and quantitative analysis of transcripts during medaka embryogenesis, protein expression and subcellular localization in cultured cells, and comparison of binding affinities to paralog-specific SUMO-binding proteins, suggest a possible classification of medaka SUMOs into two subfamilies, *OlsUMO-1* and *OlsUMO-2/3/4*, and imply the absence of a functionally important proline residue in medaka SUMO paralogs which corresponds to the Pro-90 in human SUMO-4, arguing the divergence and/or specialization of structure and function of human SUMO-4 during vertebrate evolution.

**Materials and Methods**

**Fish samples**
The orange-red variety of medaka, the FLFII strain, was selected for the experiments. Fish embryos were maintained in ERM (17 mM NaCl, 0.4 mM KCl, 0.27 mM CaCl$_2$$\cdot$2H$_2$O, 0.66 mM MgSO$_4$, pH 7.0) at 26°C under a 14 hours light and 10 hours dark cycle. Developmental stages of the embryos were determined according to the description by Iwamatsu (Iwamatsu et al. 1994).

**Database analysis and construction of the phylogenic tree**
BLAST searches for DNA and protein identities were conducted using the medaka transcription database (www.blast.ddbj.nig.ac.jp/top-j.html and www.ensembl.org/Oryzias_latipes/index.html) and the Medaka Genome Initiative (www.park.itc.u-tokyo.ac.jp/K-medaka/MGI2/MGI.html). Analyses of the predicated protein sequences were conducted using BLAST. Phylogenic tree are generated using ClustalW server at the DDBJ (clustalw.ddbj.nig.ac.jp/top-j.html) with standard setting. The GeneBank protein sequences and accession numbers used in these analyses were as follows: human SUMO-1, P63165; human SUMO-2, P61956; human SUMO-3, P55854; human SUMO-4, BAH05006; human ubiquitin, P62988; human Aos1, AAD23902; human Uba2, CAG33037; human Ubc9, CAA05359; human PIAS1,
O75925; human PIAS2, O75928; human PIAS4, AAH04389; human RanBP2/Nup358, P49792; human SENP1, Q9P0U3; human SENP3, Q9H4L4; human SENP5, Q96H10; human SENP6, Q9GZR1; human SE\nRNBP2/Nucleoporin 358kDa (RanBP2/Nup358), P49792; human MBD1-containing chromatin-associated factor 1 (MCAF1), Q6VMQ6; human Ring finger protein 4 (RNF4), P78317; human thymine DNA glycosylase (TDG), Q13569; human histone H3-K9 methyltransferase SETDB1, Q15047.

RNA extraction, cDNA cloning, and sequence analysis

Total RNA was extracted from 0-, 3-, 6- and 9-dpf embryos of FLFII medaka using ISOGEN (Nippongene), and the first strand cDNA was synthesized from the total RNA by oligo(dT) priming with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen). cDNAs encoding the open reading frames of OISUMO-1, OISUMO-2, OISUMO-3 and OISUMO-4 were amplified by PCR using the following primers:

OISUMO-1 forward (fw) 5'-CGCACACAGTCAGGATAAAC-3'/reverse (rv) 5'-AAAAACATCAGAAATTGTGGCT-3',

OISUMO-3 fw 5'-TCCCCGTCAATTCACCAGAC-3'/rv 5'-AGGGATGTGGAAAGAAAACAGT-3',

OISUMO-4 fw 5'-GCAGCATGTGTGGCTGA-3',

respectively.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To quantitatively compare the amount of transcripts of medaka SUMO paralogs, real-time RT-PCR was performed using LightCycler 350S (Roche) and LightCycler FastStart DNA Master™ SYBR Green I (Roche) according to the manufacturer’s protocols. The sequences of the primer used in this assay were as follows: OISUMO-1 fw 5'-GAGGCGACAAGAAGATGGA-3'/rv 5'-TTTGGGGTTGTGTTATCTGC-3',

OISUMO-2 fw; 5'-AAAACGACACATCAACCTG-3'/ rv 5'-GGCTGTCAATTCACCAGAC-3'/rv

5'-GGCTGTCAATTCACCAGAC-3'/rv

5'-GAAGATTGTTGCTGC-3'/rv

5'-GGCGAAAAAGACTGAGC-3'/rv

5'-GGCGAAAAAGACTGAGC-3'/rv

5'-GGCGAAAAAGACTGAGC-3'/rv

5'-GGCGAAAAAGACTGAGC-3'/rv

respectively.

5'-GCAGCATGTGTGGCTGA-3', and medaka β-actin (the GeneBank accession
number; S74868) fw 5'-TCCACCTCCAGCAGATGTG-3'/ rv
5'-AGCATTTGCGGTGGACGAT-3'. We also used the transcripts derived from the GAPDH, RPL7 and 18srRNA genes as internal controls; the relative ratios of the SUMO paralogs were almost the same (data not shown).

Construction of expression plasmids
To generate pEGFP-OISUMO-1/2/3/4gg and pEGFP-OISUMO-1/2/3/4g mammalian expression plasmids, the DNA fragments of medaka SUMO paralogs were amplified by PCR using the following oligonucleotide primers:

**OISUMO-1gg fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-1g fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-2gg fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-2g fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-3gg fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-3g fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-4gg fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

**OISUMO-4g fw**
5' - GA GAATTC ATGTCAGACGGAGAC - 3'/rv
5' - TA GTCGAC TTATCCGCCGGTCTGTTC - 3',

*Construction of expression plasmids*

To generate pEGFP-OISUMO-1/2/3/4gg and pEGFP-OISUMO-1/2/3/4g mammalian expression plasmids, the DNA fragments of medaka SUMO paralogs were amplified by PCR using the following oligonucleotide primers: OISUMO-1gg fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-1g fw 5'-GAGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-2gg fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-2g fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-3gg fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-3g fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-4gg fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3', OISUMO-4g fw 5'-GGAATTCATGTCAGACGGAGAC-3'/rv
5'-ATGTCAGACGGAGAC-3' (the EcoRI and SalI sites are underlined for the fw and rv primers, respectively). PCR fragments were digested with EcoRI-SalI and inserted into EcoRI-HindIII-digested pEGFP-C2 (Clontech) or pGEX-4T-1 (Amersham Pharmacia Biotech). pEGFP-human SUMO-1 and SUMO-3 plasmid constructs as described previously (Saitoh et al. 1998; Saitoh and Hinchey 2000; Uchimura et al. 2006; Uwada et al. 2010).

Cell culture, transfection, and immunoblotting
Hela cells (maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% CO2 incubator) were transfected with the appropriate pEGFP-expression plasmids using GeneJuice (TaKaRa). After 24 hours the
cells were washed with PBS and lysed in two pellet volumes of 3xSDS sample buffer. Proteins were separated on SDS-PAGE followed by Western blotting using anti-GFP antibody (Santa Cruz). For detection of GFP signals under the microscope, the cells transfected with GFP-constructs were grown on coverslips and fixed with 4% paraformaldehyde. The cells were permeabilized with 0.2% Triton X-100, mounted under coverslips, and analyzed using Biorevo BZ-9000 (Keyence).

Glutathione S-transferase(GST)-pulldown assay

pGEX, pGEX-human SUMO-1/3, pGEX-Ol/SUMO-1/2/3/4, pET28-RanBP2-IR and pET28-MCAF1 plasmids were introduced into E. coli BL21(DE3) and Rosetta(DE3) strains. The recombinant GST, GST-human SUMO-1/3, GST-Ol/SUMO-1/2/3/4, (His)$_6$-RanBP2-IR and (His)$_6$-MCAF1 fusion proteins were expressed as described previously (Uchimura et al. 2006; Uwada et al. 2010). A GST-pulldown assay was carried out as described previously (Uchimura et al. 2006; Uwada et al., 2010). The proteins were separated on SDS-PAGE followed by immunoblot analysis using anti-(His)$_6$-tag antibody (Roche) and anti-GST antibody (Santa Cruz).

Results

The SUMO pathway components in the medaka data base

For a comprehensive analysis of the SUMO modification pathway in lower vertebrate species, we used human genes encoding various components in the SUMO pathway to search for orthologs in medaka protein and DNA databases. Our initial search identified at least four distinct medaka SUMO genes: _Olsumo-1_, _Olsumo-2_, _Olsumo-3_ and _Olsumo-4_, which contain multiple small introns (Fig.1A). In addition, there were at least: one gene similar to the human Aos1 (SUMO-E1) gene, one gene similar to human Uba2 (SUMO-E1) gene, and a gene similar to the human Ubc9 (SUMO-E2) gene. At least five distinct genes encoding genes similar to human de-sumoylation protease SENPs, genes similar to several human SUMO-E3s, including RanBP2/Nup358, PIAS1, PIAS2 and PIAS4, and genes similar to currently identified human SUMO-interacting/binding proteins, such as MCAF1, RNF4, TDG and SETDB1 were also detected (Table 1). These results strongly suggest the existence of a protein-conjugation pathway in medaka analogous to the human SUMO pathways.
Isolation of four different SUMO transcripts: *OlSUMO*-1, *OlSUMO*-2, *OlSUMO*-3, and *OlSUMO*-4

To confirm that all four *OlSUMO* genes can be transcribed in medaka, we designed primer pairs and used them to amplify the cDNA fragments using total RNA prepared from fertilized medaka embryos (see Materials and methods section for details). The cDNA fragments for all of the primer pairs, corresponding to full-length *OlSUMO*-1/2/3/4, amplified efficiently; we then cloned them and determined their DNA sequences. The GeneBank accession numbers of *OlSUMO*-1, *OlSUMO*-2, *OlSUMO*-3 and *OlSUMO*-4 are as follows: GQ463435, GQ463436, GQ463438 and GQ463437, respectively. We found three sequences identical to GQ463435, GQ463437, GQ463438 in the Ensemble’s medaka gene database (www.ensembl.org/Oryzias_latipes/index.html) and have deposited them as SUMO-1, SUMO-2 and SUMO-4, respectively. We thus gave the nomenclatures for GQ463435, GQ463436, GQ463438 and GQ463437 as *OlSUMO*-1, *OlSUMO*-2, *OlSUMO*-3 and *OlSUMO*-4, respectively. Although it appears that designation in the database does not consider any biological criteria such as homology search and phylogenetic alignment (see below), we followed the *OlSUMO* nomenclature (as above) to avoid confusion.

The amino acid sequences deduced from each cDNA are shown in Fig. 1B. Comparisons of the amino acid sequences showed that *OlSUMO* are only 16–17% similar to ubiquitin. The similarities of *OlSUMO*-1 versus *OlSUMO*-2, *OlSUMO*-1 versus *OlSUMO*-3, *OlSUMO*-1 versus *OlSUMO*-4, *OlSUMO*-2 versus *OlSUMO*-3, *OlSUMO*-2 versus *OlSUMO*-4 and *OlSUMO*-3 versus *OlSUMO*-4 were 48, 46, 48, 92, 89 and 94%, respectively. Thus, *OlSUMO*-2, *OlSUMO*-3 and *OlSUMO*-4 proteins are highly homologous, and they are approximately 50% identical to *OlSUMO*-1.

Phylogenetic analysis showed that *OlSUMO*-1 had the highest similarity to human SUMO-1 (*HsSUMO*-1), and *OlSUMO*-3 was closely related to human SUMO-3 (*HsSUMO*-3); *OlSUMO*-2 and *OlSUMO*-4 were equally related to human SUMO-2 (*HsSUMO*-2) (Fig. 1C). The relationship of human SUMO-4 to *OlSUMO*-2/4 is no closer than that of human SUMO-2 to *OlSUMO*-2/4. It should be noted that a proline at 90 amino acid residue in human SUMO-4, which appears critical for this paralog’s function (Guo et al. 2004; Owerbach et al. 2005), is not conserved in either *OlSUMO* paralog.
OlSUMO proteins have potential to serve in the SUMOylation pathway

To confirm that the proteins encoded by the four medaka SUMO genes attach to other proteins similarly to SUMO proteins from other organisms, cDNAs of the OlSUMOs were expressed in Hela cells to see whether OlSUMO proteins could conjugate to cellular proteins. We first generated the constructs that lack the C-terminal amino acids from the highly conserved di-glycine (gly-gly) residues of each OlSUMO (Fig. 1B). The generated fragments, OlSUMO-1gg, OlSUMO-2gg, OlSUMO-3gg and OlSUMO-4gg, were fused to the C-terminus of an enhanced green fluorescent protein, generating EGFP-OlSUMO1gg, EGFP-OlSUMO2gg, EGFP-OlSUMO3gg and EGFP-OlSUMO4gg fusion proteins, respectively. EGFP-tagged OlSUMOgg constructs were then expressed in Hela cells, and total cell lysates prepared from the transfected cells were analyzed by Western blot with anti-GFP antibody. Non-conjugated forms of EGFP-OlSUMOgg proteins that migrate at around 45 kDa (EGFP~27kDa+SUMO~18kDa) were clearly identified (arrowheads in Fig. 2). In addition, all EGFP-OlSUMOgg proteins form a number of conjugates that migrate at higher molecular mass than the 45 kDa-monomer band. It should be noted that the signal intensities at higher molecular mass varied among the cells expressing different constructs, suggesting functional heterogeneity among OlSUMO paralogs. Removal of the C-terminal invariant gly residues from the EGFP-OlSUMOgg constructs, termed EGFP-OlSUMO1g, EGFP-OlSUMO2g, EGFP-OlSUMO3g and EGFP-OlSUMO4g, completely removed the high molecular mass bands. Thus these data indicate that all OlSUMO proteins have the potential to conjugate to other proteins, and suggest that activation of the gly residue at the C-terminal end is critical for transfer of SUMO to target proteins, arguing that all OlSUMO paralogs are logically active in mammalian cultured cells.

Biochemical distinctions among OlSUMOs

To improve understanding of biochemical and physiological properties of OlSUMO paralogs, we tested subcellular localization of four OlSUMO paralogs. This was carried out by transient transfection assay using EGFP-OlSUMOs. A marked concentration of green fluorescence at the nuclear rim was observed in many of the EGFP-OlSUMO1-transfected Hela cells shown (Fig. 3A). In contrast, a mild concentration of the signal at the nuclear rim in the EGFP-OlSUMO2/3/4-transfected
Hela cells implies that OISUMO-1 and OISUMO-2/3/4 have different requirements in their subcellular localization at the nuclear rim. It should be noted that we observed some punctuate concentrations in the nucleus of both the EGFP-OISUMO-1 and EGFP-OISUMO-2/3/4 transfected cells, indicating the possibility of colocalization of OISUMO-1 and OISUMO-2/3/4 in the nuclear punctuate structures in interphase cells.

Next, we conducted a GST-pulldown assay to compare the affinity of two types of SIM-containing SUMO-interacting proteins, RanBP2 and MCAF1, to the OISUMO paralogs. RanBP2, a SIM-containing protein localized at the nuclear pore, binds more readily to human SUMO-1 than it does to SUMO-2/3 (Saitoh et al. 1998; Song et al. 2004; Hecker et al. 2006), and MCAF1, a SIM-containing protein involved in nuclear/chromatin function, binds more readily to human SUMO-2/3 than it does to SUMO-1 (Hecker et al. 2006; Uchimura et al. 2006; Sekiyama et al. 2008; Uwada et al. 2010). The (His)$_6$-RanBP2-IR protein was detected in the GST-OISUMO-1-pulldown fraction, but not in GST-OISUMO2/3/4-pulldown fractions (Fig. 3B). In contrast, the (His)$_6$-MCAF1 protein was detected in the GST-OISUMO-2/3/4-pulldown fractions, but not in the GST-OISUMO-1-pulldown fraction. These results indicate that OISUMO1 and OISUMO2/3/4 paralogs can be distinguished by their subcellular localization, and differentiated biochemically by SUMO-1-specific and SUMO-2/3-specific SIM-containing proteins, supporting the notion that OISUMO paralogs can be grouped into two subfamilies.

Quantitative analysis of transcripts of OISUMOs during medaka embryogenesis

To elucidate functional heterogeneity or differential regulation among OISUMO paralogs, or both, we assessed the relative amounts of OISUMO transcripts in medaka embryos at different developmental stages using real-time RT-PCR. All OISUMO transcripts were reasonably abundant in 0-dpf embryos (stage 8-9), 3-dpf embryos (stage 29), 6-dpf embryos (stage 36), and 9-dpf embryos (stage 39) (Fig. 4). While the expression levels of OISUMO-2/4 in 0-dpf embryos and OISUMO-2/3/4 in 3-dpf embryos were significantly higher than they were in the others, all of the OISUMO transcripts seemed to undergo small changes in abundance through embryogenesis. To compare the relative amounts of the subfamilies at each embryonic stage, we integrated the values of the OISUMO-2/3/4 transcripts and compared them with those of OISUMO-1. The levels of the transcripts from the OISUMO-2/3/4 subfamily were
constantly higher than those of OlSUMO-1 at any of embryonic stages (Fig. 4 and data not shown). The OlSUMO-2/3/4 transcripts were approximately 8-fold more abundant than the OlSUMO-1 transcript, especially at 3-dpf. These data indicate that transcripts of all four SUMO paralogs exist during medaka embryogenesis, and that the amount of OlSUMO-2/3/4 transcript is greater than that of OlSUMO-1 at any stage of development.

Discussion

By searching the medaka genome and EST databases, we discovered gene loci and cDNA sequences for multiple components of the SUMO modification system, four SUMO paralogs: OlSUMO-1, OlSUMO-2, OlSUMO-3 and OlSUMO-4; one E1 (one OlAos1 and one OlUba2); one E2 (OlUbc9); three OlPIAS families of E3s; Ol/RanBP2/Nup358; another class of E3; and a five-gene family encoding putative SUMO proteases (OlSENPs). We also found several mammalian orthologs of downstream effector proteins from the SUMO modification pathway, including MCAF1 (Uchimira et al. 2006), TDG (Baba et al. 2005), SETDB1 (Ivanov et al. 2007), RNF4 (Häkli et al. 2005), and others (data not shown). These SUMO pathway components in the medaka database suggest that there is a SUMO modification pathway in medaka. Given that we and others also found multiple SUMO paralogs and SUMO modification enzymes in the zebrafish (Danio reio) database (data not shown; Nowak and Hammerschmidt 2006; Yuan et al. 2009), our search implies sumoylation as an important signaling mechanism in fishes.

To date, in humans, four SUMO paralogs, HsSUMO-1 to HsSUMO-4, have been identified; HsSUMO-1, HsSUMO-2 and HsSUMO-3 can act as protein modifiers, whereas HsSUMO-4 seems to be expressed only in restricted tissues and may not have the ability to be conjugated to other proteins (Guo et al. 2004; Owerbach et al. 2005; Geiss-Friedlander and Melchior 2007; Wilson and Heaton 2008), indicating that HsSUMO-4 constitutes a subgroup that is distinct from HsSUMO-1/2/3. Our biochemical studies using SIM-containing proteins, RanBP2 and MCAF1, support the notion of two SUMO subfamilies in medaka (Fig. 3B). With regard to their subcellular localization, we also found subfamily-specific properties (Fig. 3A). These findings suggest that OlSUMOs can be grouped into two subfamilies; OlSUMO-1 and OlSUMO-2/3/4 subfamilies. In addition, all OlSUMOs are expressed throughout
medaka embryogenesis (Fig. 4). When transiently expressed in Hela cells, all
OlSUMOs were incorporated into higher molecular mass regions, suggesting that they
all have ability to be conjugate to cellular proteins in vivo (Fig. 2). An amino acid
sequence alignment experiment suggested that all medaka SUMO paralogs do not
contain a unique proline residue located at position 90 in HsSUMO-4 (Fig. 1). Thus we
suppose that all OlSUMOs appear distinct from HsSUMO-4 and suggest the emergence
of human SUMO-4 paralog after mammalian evolution.

The SUMO modification system is essential in most organisms including S.
cerevisiae, C. elegans, Arabidopsis thaliana and mice (Geiss-Friedlander and Melchior
2007), and may play a critical role in some parasitic diseases (Cabral et al. 2008).
Whether individual SUMO proteins are essential in organisms that have multiple
SUMO paralogs remains unclear. Genetic studies have linked SUMO-1
haploinsufficiency to the cleft lip or palate condition in humans, indicating that
SUMO-1 and SUMO-2/3 play a role in development (Alkuraya et al. 2006; Pauws and
Stanier 2007). On the other hand, mice lacking SUMO-1 develop without any apparent
abnormalities, implying that humans and mice may be different in their specific
requirements for SUMO paralogs (Evdokimov et al. 2008; Zhang et al. 2008). In future
experiments using the medaka system, it is important to investigate which
developmental processes can be compensatory among SUMO paralogs, to elucidate
whether more subtle phenotypic differences are involved in the development of tissues
and organs lacking either SUMO paralogs, and to identify targets for SUMOylation by
different OlSUMO paralogs during embryogenesis and organogenesis. We believe our
study provides a basis for such experiments.

Acknowledgments
We thank Ms. Maki Kuroki for help with extraction of RNA from medaka embryos. We
appreciate the helpful discussions with the members of the Saitoh Laboratory. This
work was supported by grants to Hisato Saitoh from the Naito Foundation, the Novartis
Foundation, the Ministry of Education, Culture, Sport, Science and Technology
(MEXT), and the Japan Society for the Promotion of Science (JSPS). Daisuke Seki is a
Junior Research Associate of the global COE Program at the Global Initiative Center for
Pulsed Power Engineering.
Table 1. Components of the medaka SUMO pathway.

<table>
<thead>
<tr>
<th>Protein activity</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Accession number</th>
<th>Expression (ESTs)</th>
<th>Expression (RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO</td>
<td>OISUMO-1</td>
<td>2</td>
<td>ENSORLG00000000622</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISUMO-2</td>
<td>1</td>
<td>ENSORLG00000003279</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISUMO-3</td>
<td>22</td>
<td>ENSORLG00000013523</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISUMO-4</td>
<td>8</td>
<td>ENSORLG00000009773</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>E1</td>
<td>OISAE1</td>
<td>13</td>
<td>ENSORLG0000001538</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OISAE2</td>
<td>6</td>
<td>ENSORLG00000012575</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td>E2</td>
<td>OIJBC9</td>
<td>8</td>
<td>ENSORLG00000013514</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td>E3</td>
<td>OIPhas1a</td>
<td>6</td>
<td>ENSORLG0000006728</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OIPhas1b</td>
<td>3</td>
<td>ENSORLG0000002077</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OIPhas2</td>
<td>9</td>
<td>ENSORLG00000017353</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OIPhas4</td>
<td>4</td>
<td>ENSORLG00000004970</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td>SENP</td>
<td>OISENP1</td>
<td>7</td>
<td>ENSORLG00000007817</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISENP2</td>
<td>22</td>
<td>ENSORLG00000013689</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISENP5</td>
<td>17</td>
<td>ENSORLG00000009124</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISENP6</td>
<td>24</td>
<td>ENSORLG00000017309</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISENP7a</td>
<td>21</td>
<td>ENSORLG00000011710</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OISENP7b</td>
<td>21</td>
<td>ENSORLG00000011701</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>SUMO-interacting proteins</td>
<td>OIRanBP2/ OINup358</td>
<td>21</td>
<td>ENSORLG00000015523</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OIMCAF1</td>
<td>1</td>
<td>ENSORLG00000004183</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OIRNF4</td>
<td>Unknown</td>
<td>ENSORLG00000020573</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>OITDG</td>
<td>5</td>
<td>ENSORLG00000001329</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>OSETDB1</td>
<td>16</td>
<td>ENSORLG00000010602</td>
<td>Yes</td>
<td>NT</td>
</tr>
</tbody>
</table>

a. Accession number, ensemble medaka database accession number.

b. Expression confirmed by the presence of EST in the database (www.ensembl.org/Oryzias_latipes).

c. Expression confirmed by RT-PCR using total RNA from 9- days after fertilization (daf) of the medaka embryo.

d. NT, not-tested by RT-PCR.
e. ND, not-determined.

References


Owerbach D, McKay EM, Yeh ET, Gabbay KH, Bohren KM (2005) A proline-90 residue unique to SUMO-4 prevents maturation and sumoylation. Biochem Biophys Res Commun 337: 517-520


Figure Legends

Figure 1. Identification and characterization of the medaka OISUMO gene family.
A, schematic representation of organization of the medaka OISUMO genes. Black boxes and lines indicate exons and introns, respectively. The numbers of chromosome on which OISUMO-1, OISUMO-2, OISUMO-3 and OISUMO-4 gene loci are located are shown on the left side.
B, amino acid sequence alignment of medaka OISUMO paralogs by the ClustalW method. The GeneBank accession numbers of OISUMO-1, OISUMO-2, OISUMO-3 and OISUMO-4 are as follows: GQ463435, GQ463436, GQ463438 and GQ463437, respectively. Potential sumoylation sites (ϕKXE) are boxed. The Ub domain present in all OISUMO paralogs is outlined. The arrowhead represents the potential processing site by SENPs that expose the highly-conserved gly-gly residues (reverse type) involved in SUMO conjugation. The asterisk denotes a proline at 90 amino acid residue in human SUMO-4, which appears critical for paralog function (grey box).
C, phylogenetic relationship among medaka OISUMO-1/2/3/4, human SUMOs (HsSUMO-1/2/3/4), C. elegans SUMO (CeSUMO) and D. melanogaster SUMO (DrSUMO). Phylogenetic tree are generated using ClustalW server at the DDBJ (clustalw.ddbj.nig.ac.jp/top-j.html) with standard setting. Bootstrap values (1,000 replicates) are shown at the branches.

Figure 2. Ability of OISUMO paralogs to conjugate to cellular proteins.
Hela cells were transfected with EGFP vector (lane 1), pEGFP-HsSUMO-1gg (lane 2), pEGFP-HsSUMO-1g (lane 3), pEGFP-HsSUMO-3gg (lane 4), pEGFP-HsSUMO-3g (lane 5), pEGFP-OISUMO-1gg (lane 6), pEGFP-OISUMO-1g (lane 7), pEGFP-OISUMO-2gg (lane 8), pEGFP-OISUMO-2g (lane 9), pEGFP-OISUMO-3gg (lane 10), pEGFP-OISUMO-3g (lane 11), pEGFP-OISUMO-4gg (lane 12) or pEGFP-OISUMO-4g (lane 13). After 24 hours, total cell lysates were analyzed by immunoblotting using anti-EGFP antibody. The position of the EGFP-OISUMO monomer (~45 kDa) is indicated by an arrowhead. The high molecular mass bands are indicated by a bracket with an asterisk. Molecular mass standards are expressed in kilodaltons (kDa).
Figure 3. Distinct biochemical properties among OISUMO-1/2/3/4 proteins.

A, subcellular localization of OISUMOs. Hela cells were transfected with pEGFP-HsSUMO-1gg, pEGFP-HsSUMO-3gg, pEGFP-OlSUMO-1gg, pEGFP-OlSUMO-2gg, pEGFP-OlSUMO-3gg and pEGFP-OlSUMO-4gg. After 24 hours, the cells were fixed and EGFP signals were detected under the fluorescent microscope. DAPI-stained images are also indicated (inset).

B, interaction among OISUMOs and SIM-containing proteins. A bacterial lysate expressing recombinant (His)$_6$-RanBP2-IR (upper panel) or (His)$_6$-MCAF1 (bottom panel) was incubated with beads of GST-OlSUMO-1gg (lane 3), GST-OlSUMO-2gg (lane 4), GST-OlSUMO-3gg (lane 5) and GST-OlSUMO-4gg (lane 6). Following incubation, a GST-pulldown assay was carried out and the proteins associated with the beads were subjected to immunoblot analysis using anti-(His)$_6$ antibody. For positive controls, GST-HsSUMO-1 (lane 1) and GST-HsSUMO-3 (lane 2) were used. Amounts of GST-SUMOs used in the pull-down assay were visualized by immunoblot analysis with anti-GST antibody (bottom).

Figure 4. Expression of OISUMO paralogs embryos at different developmental stages.

Quantitative detection of the transcripts of OISUMO paralogs in 0-, 3-, 6- and 9-dpf embryos. Quantitative RT-PCR was used to compare the amounts of OISUMO-1 (black), OISUMO-2 (gray), OISUMO-3 (white) and OISUMO-4 (dark gray) transcripts using total RNA prepared from at least three individual embryos at the different developmental stages. In this experiment we used β-actin for the internal control; it showed a 2-fold reduction during medaka embryogenesis.
Fig. 1
Fig. 2
Fig. 3
Fig. 3