# Molecular characterization and 3D Modeling of p62 in Grass Carp (Ctenopharyngodon Idella)

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**Abstract.** P62 is a ubiquitin-binding protein that plays a key role in biological pathways including autophagy and protein degradation. In this study, we analyzed the protein sequence of grass carp p62 (gcp62) and its 3D structure was predicated. The full-length cDNA of gcp62 was 1425 bp, and encoded a 474-aa protein. Multiple sequence alignment analysis showed that gcp62 shared 40.82~41.80% amino acid identities with its orthologues of human, mouse and tropical clawed frog, as well as relatively high identities (63.28 and 78.71% respectively) with yellow catfish and zebrafish p62. The phylogenetic analysis showed that gcp62 has close relationship with its orthologues in common carp and zebrafish, but differs from its homologs in mammalian species. Like in mammals, gcp62 was predicted to contain three conserved domains, the N-terminal Phox and Bem1p (PB1) domain, the ZZ domain and the C-terminal ubiquitin-associated (UBA)domain. This is the first time to construct the 3D structure of fish p62 and the high similarity of p62 structure between human and fish indicates the functional conservation in vertebrates.

Keywords: grass carp; p62; 3D modeling; Structure characterization.

#### 1. Introduction

All proteins in eukaryotic cells are degraded by the following two systems: ubiquitin-proteasome system (UPS) and the autophagy-lysosome system [1]. The UPS is highly effective in selectively degrading short-lived proteins as well as misfolded proteins labeling with ubiquitin tag, while the lysosome prefers to degrade long-lived proteins and remove damaged organelles through autophagy to recycle nutrients and generate energy [2]. Autophagy, as an evolutionarily conserved process is mediated by over 30 autophagy-related proteins and receptors in eukaryotes. During autophagy, a double membrane structure called autophagosomes selectively recognize and engulf the cargoes and subsequently fuse with acidic lysosomes for degradation [3]. These two distinct and interacting pathways maintain the homeostasis of intracellular substances and ensure the fundamental biological activity.

p62, also known as sequestosome 1(SQSTM1), is a multifunctional ubiquitin-binding protein that participates in two protein degradation systems, especially autophagy. The human p62 contains six prominent functional domains including a N-terminal PB1 domain, a central ZZ and TB modules, a LC3-interacting region (LIR), a Keap1-binding region (KIR), and the C-terminal UBA domain [4]. Multiple domains with special functions show the multifunctional features of p62 protein and the complexity of its regulatory mechanism. As a classic scaffold and aptamer protein, p62 mediates autophagy based on its multiple functional domains by interacting with other proteins. Firstly, p62 binds non-covalently with the ubiquitinated cargoes through its C-terminal ubiquitin-associated (UBA) domain and then target the ubiquitinated cargoes to the inner membrane surface of phagophore by the LC3-interacting region (LIR) motif. Finally, the polymerization ability of PB1 domain promotes the tight interaction between the cargoes and phagophore [5]. In addition, p62 has also been reported to involve in delivering ubiquitinated proteins to proteasome. Although the structure and function of p62 are well delineated in many mammalian species, most studies concerning teleost p62 only stay at gene cloning and more information about fish p62 are needed to be elucidated.

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In this study, the protein sequence of gcp62 has been analyzed by bioinformatics analysis and the predicted 3D structures of gcp62 presented high conservation compared with human p62, which has been deduced to play the similar functions such as autophagy in grass carp.

## 2. Method and Materials

#### 2.1 Sequence Analysis of gcp62

The signal peptide of gcp62 was predicted by SignalP6.0 (https://services.healthtech.dtu.dk/service.php?SignalP). Theoretical pI and molecular weight were calculated by ExPASy (https://web.expasy.org/compute pi/). The multiple sequence alignments of gcp62 with other orthologues were performed by DNAMAN software (Lynnon Biosoft, Pointe-Claire, Canada). The neighbor-joining phylogenetic tree was generated by MEGA7.0 software with the bootstrapping of 1000 repetitions. The accession numbers of p62 used in this research were listed as follows: human (NP 003891.1), mouse (NP 035148.1), tropical clawed frog (NP 001007894.1), zebrafish (AHA93916.1), yellow catfish (AQY15982.1), rainbow trout (XP 021439759.2), common carp (XP 042593729.1), grass carp (QHZ59459.1), yeast (033769075.1)

#### 2.2 Conserved Domain Analyzation and Homology Modelling

The protein domains of gcp62 was analyzed by SMART (<u>http://smart.embl-heidelberg.de/</u>). Using human p62 PB1 (PDB ID: 2kkc.1.A), ZZ (PDB ID: 6mj7.1A) and UBA(PDB ID: 2k0b.1A) as templates, three structural models of gcp62 domains were built by SWISS-MODEL (<u>https://swissmodel.expasy.org/</u>) respectively and visualized by VMD 1.9 software.

## 3. Results

#### 3.1 Sequence Analysis of gcp62

Although the sequence of gcp62 was cloned and upload to the NCBI by our laboratory before, its molecular and structural information remained to be unclear. As shown in Figure 1, the full-length cDNA sequence of grass carp was 1425 bp, which encodes a 474-aa protein without signal peptide. Theoretical pI and molecular weight of gcp62 was 5.77 and 51.2 kDa, respectively. The multiple sequence alignment was made by using DNAMAN software. And we found that gcp62 shares low identities with its mammalian homologs and relatively high identities (78.7% and 63.2% respectively) with zebrafish and yellow catfish. There is also a conserved serine phosphorylation site 403 with mammals (Figure 1). Phylogenetic analysis showed that gcp62 protein was clustered with common carp and zebrafish and distant from its homologs in mammalian species (Figure 2).

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human	MA <mark>SITVKAYIIGKELAAREIRRE</mark> SFCCSPEPEAEAEAAGPGPCPRULSRVAALFEATRPGCFQAH <mark>YRDEDG</mark> DL MASFTVKAYILGKEEATREIRRESFCFSPEPEAEAÇAAGPGPCPRULSRVAVLFPTIRPGCFQAHYRDBDCDL MPVTVKAYILGKDESHREIRREQLELPAAGKEKASVSSCPLLANKVTGVFQCLKGCAFQMFYKDBBCDL MSMTVKAYILGKEDCNKEIRREAVDQDVSTSFEY <mark>IL</mark> QRKVLDVFVCLRTAFFQMYYKDBDCD	VAFS <mark>S</mark> D 80
mouse	MASTTVKAY ILLGKEATRE IRRESECTSPE PEAEAQAAAGPGPCPRILLSRVAVILPTIRPGGFQAHTRDBIGDL	VAFS <mark>S</mark> D 80
tropical clawed frog	MPVTVKAY IIIGKDESHRETRRFQLELPAAGKEKASVSSOPIIIANKVTGVFQGTRGGAFOMFYRDPEODL	VAFSTD 75
zebrafish	MSMTVKAYIIGKEDCNKEIRRHAVDQDVSTSFPY QRKVLDVFVGIRTAPFOMYYKDPDGDM	IAFS <mark>S</mark> D 68
yellow fish	MSVTVKAYLIGKEDCHKEIRREAIDHDVSSSFEYLSRKLVDVESNIQNV&FQTYYKDEDDDM	
grass carp	M <mark>S</mark> MTVKAYI <mark>VGKE</mark> DCNKEIRREAVDQDVSTSFEYIKRKVIDVEVGIGTAFFÇMFYKDE <mark>DG</mark> DM	
Consensus	tvkaylgk eirrf el flfqyded	afs d
human	EELTYAMSYVKDDIFRIYIKEKKECR <mark>EDHRP</mark> PCAQEA <mark>P</mark>	118
mouse	EELTMAMSYVKDDIFRIYIKEKKECRREHRPPCAQEAP	118
tropical clawed frog	E <mark>PLTYAMSYVKDDIFRIYIKEKKE</mark> CRREHRPCAQEAP. E <mark>PLHYGLSLINEDVFRIYIKERKE</mark> CKRDHRGHCGQETP.	113
zebrafish	DPLMVGIALVKDDTFRLFIKQRKPHKRDSSPHGIPGFPFTTPHFSPPGPCHMGPSPMGPPGPPHMG.	134
yellow fish	DEIMEGLTMVKLATERIFIKEKKEHKRDFPHYMDQGFAVHPPGPHHMGPPGHPTMCP	125
grass carp	D <mark>P PMMGIAL VKDD</mark> T FRLFIK <mark>ORKP</mark> HK <mark>RD</mark> SS PHGF PGF <mark>P</mark> GF SFAGPHGASHF SPGPHHMGP PPMGP PGP PHMGP	QGPPHM 148
Consensus	e m fr ik ke r	
human	RNMVH PNVICDGCNG PVVGTRYKC SVCE DYDLG SVGECKGIHRGHTKIAFPSPFGHLSEGFSHSRWLR RNMVH PNVICDGCNG PVVGTRYKC SVCE DYDLG SVGEGKGIHREHSKLIFPN PFGHLSDSFSHSRWLR QNVVH PNVTCDGCDG PVVGNFKCLICFDYDLC STGEKKGIHKEHNMIMFPTPLVYPRSRWFR GPPHHPHPMVFGVTCDGCDG PVAGTRYKCTVCFDYDLOPTCQSKGIHKEHALLPIFHFMANVFEWLPRGKLW .PPHHPSSIVHLGVTCDGCEGCQAAGTRFKCTVCFNYDLGATCGAKGIHKEHALLPIFHPMANMYEWLPRGKLW	K <mark>VKH</mark> GH 192
mouse	RNMVHPNVICDGCNGPVVGTRYKCSVCPDYDLCSVCEGKGLHRPHSKLIFPNFFGHLSDSFSHSRWLR	K <mark>LK</mark> HGH 192
tropical clawed frog	QNVVHPNVTCDGCDGPVVGNRFKCLICFDYDLCSTCEKKGIHKEHNMIMFPTFLVYPRSRWFR	K <mark>MH</mark> HG <mark>V</mark> 182
zebrafish	GPPHHPHPMVHPGVTCDGODGFVAGTRYKCTVCPDYDLOPTCQSKGLHKEHALLPIFHEMANVFEWLPRGKFWR	KMR <mark>H</mark> CM 214
yellow fish	. PPHHPSSLVHLGVTCDGOEGQVAGTRFKCTVCPNYDLCATCCAKGLHKEHALLPIFHEMANMYEWLPRGKLLR	K <mark>MRH</mark> CM 204
grass carp	GPPPHHPPMVHPGVTCDGOBGPVAGTREKCTVCHDYDLOSSCQGKGLHKEHPLLPIEHPMANLFEWPPRGKEWR	K <mark>MR</mark> HCM 228
Consensus		k h
human	FGWPGWEMGPPG <mark>N</mark> WSPRPPRAGEARPGPTAESASGPSEDPSVNF <mark>IKN</mark> V <mark>GE</mark> SVAAALSPIGIEV	DIDVEH 261
mouse	FGWPGWEMGPPGNWSPRPPRAGDGRPCPTAESASAPPEDPNVNFLKNVGPSVAAALSPLGIEV	
tropical clawed frog	PPFPWMQGWAYPPRGYPCQNFQCAQESTPPQNETFAEEAAATSDSQSSQNP <mark>N</mark> VAF <mark>LKNVGE</mark> SVAA <mark>MLSPLGID</mark> V	
zebrafish	WANACACAONOPOPGPSGAOONOCAPENPNENGATASSCANVEYLKNIGEEVAAMLSPLGIDV	DIDVEH 283
yellow fish	WAG. AÇAQAHAÇAQ <mark>N</mark> ÇAQPGSSGGQPSS QÇANSQESÇAQTEASCAQT <mark>NMEYLKNIGFGVAAILSPLGID</mark> V	DIDVEH 279
grass carp	WANNÇAÇAHAÇAÇAÇ <mark>N</mark> ÇAÇPGPSGDÇQNQLASENMREDGATASSÇA <mark>N</mark> MEYLKNI <mark>GEGVAAMLSPLGID</mark> V	
Consensus	lkn ge vaa lsplgi v	didveh
human	CGKRSRLTPVSEESSTEEKSSSQPSSCCSDPSKPGGNV.EGATQSIAEQMRKIALES.EGRPEEQME.SDN	C <mark>SGG</mark> D 336
mouse	G <mark>GKRSRLTPTTFESS</mark> STGTEDK <mark>S</mark> NTQP <mark>S</mark> SCS <mark>SEVSKPDGAG</mark> .EGFAQSLTEQMKKIALES.VGQPEEQME.SGN	C <mark>SGG</mark> DD 338
tropical clawed frog	GKRTKVSTSHPOSVDGDSOPNSSTSFTETODIASRSDSAPNLMETEHTAKRMKDIALNSPLPODNEOGENSSS	ASGGDD 342
zebrafish	EGKRTKVTPTPEASSGPSSARSDSGSVGLLSRGSGPGSÇATEESVSEGTKKDQCS	<mark>D</mark> E 340
yellow fish	EGKKAKVTPTPFVSSGPFSGRSDS.SSGGISKG.AAGSPSSVGSTGDGAKMVLLDQKEKSN	<mark>D</mark> E 340
grass carp	EGKRSKVTPTPFASSGPFSARSDSGSVGLLSRGSGPGSÇATEGSITEGAKGQKDQGS	<mark>D</mark> E 362
Consensus	gk ps s s	d
human	DWTH <mark>IS</mark> SKEVDPSTGELQSIQMPESEGPSSLDPSQEGPTGIKEAALYPHLPPEADPRIIESLSQMLS	
mouse	DWTH <mark>LSS</mark> KEVDPSTGELQSLQMPESEGPSSLDPSQE <mark>CPT</mark> GL <mark>KEAALYPHLP</mark> PEADPRL <mark>I</mark> ESLSQMLS	MGE <mark>S</mark> DE 411
tropical clawed frog	DWTHVSSKEVDPSTGELQSIQLMETGQPCSLDPTRSSALPT.HAPTGIREAALYPHLFPEADPRLIESLSQMLS	MGF <mark>T</mark> DE 421
zebrafish	EWTH <mark>I</mark> SAKEVDPSTGELQSL <mark>RLEQDGADLPAPLNTASGTSTRDG</mark> PTGL <mark>R</mark> EAALYPHLPQDADPRL <mark>V</mark> ESLSQMLS	
yellow fish	EWTH <mark>IS</mark> SKEVDPSTGELQSL <mark>RMEVDGANDNPSPRSHSSTLN.QC</mark> ESGL <mark>REAALYPHLEQL</mark> ADPRL <mark>V</mark> ESLSQMLS	
grass carp	EWTH <mark>ISS</mark> KEVDPSTGELQSI <mark>RLEQDGADLPTPLSTAS</mark> STSNTQCPTGIREAALYPHLFQTADPRIVESLSQMLS	
Consensus	wth s kevdpstgelgsl p gl eaalyphlp adprl eslsomls: Identity	mgf de
human	GGWLTRLIQTKNYDICZALDTIQYSKHPPPL 40.82%	440
mouse	GGWLTRLIQTKNIDICFALDTUCISHEFFL 40.02% GGWLTRLIQT <mark>KNYDICF</mark> ALD <mark>TIC</mark> YSKHPPPL 41.21%	440
tropical clawed frog	GGWLTRLIEAKQFDIGSALLAMQSIRHIPPS 41.80%	452
zebrafish	GOVERNMENT COLUMN STREET ST	452
yellow fish	GGWLTRLI <mark>HTKNYDIGGALDTICYSKTF</mark> GQQK 78.71% GGWLTRLI <mark>HTKNYDIGA</mark> ALD <mark>TICYAKAF</mark> GQRK 63.28%	451
grass carp	GGWLTRLLHAKNFDIC <mark>SALDTICYSKTPGPKK</mark>	474
Consensus	ggwltrll k dig ald q	

Figure 1: Multiple sequence Alignment of gcp62 with other orthologues. The classical phosphorylation site of mammals was indicated in red arrow. The conserved amino acid residues of grass carp were indicated in blue letters.

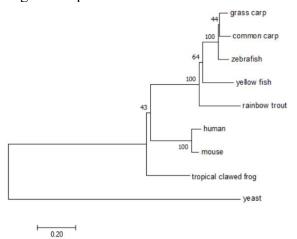


Figure 2: Phylogenetic tree analysis of p62 homologues. The numbers indicated the bootstrap confidence values obtained for each node after 1000 replications.

#### **3.2 3D Modeling of PB1, ZZ, and UBA domain of gcp62**

By using SMART, gcp62 had been predicted to have three structurally conserved domains: a PB1 domain (2–90), a ZZ-domain (158–201), and a UBA domain (427–466) (Figure 3A). The N-terminal PB1 domain of gcp62 contained secondary structures of 2  $\alpha$  helices and a mixed five-stranded  $\beta$  sheet in the order 2-1-5-3-4. Followed by PB1 scaffold was a 43-residue ZZ-domain, which consist an  $\alpha/\beta$ -fold that binds two atoms of zinc. The UBA domain in the C-terminal was made up of 3  $\alpha$  helices and contained a conserved phosphorylation of S406 corresponding to human S403 which is reported to enhance the affinity to ubiquitinated cargoes. 3D modeling results show highly similarity with corresponding domains of human p62 protein.

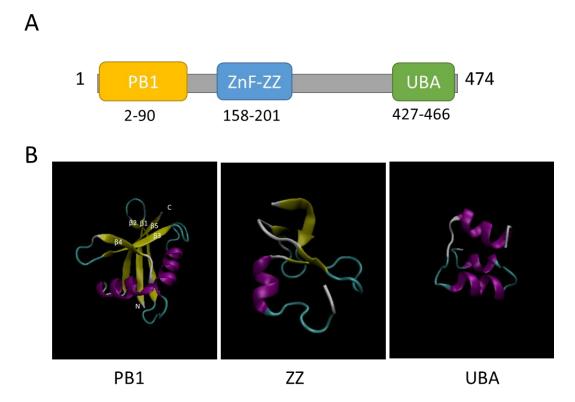


Figure 3: Domain architecture of gcp62 PB1, ZZ, and UBA domain. (A) The predicted conserved domains including PB1 (2-90 aa), ZZ (158-201 aa) and UBA domain (427-466 aa) were presented in the primary structure. (B) The structural models of gcp62 domains were constructed based on the human p62 PB1 (PDB ID: 2kkc.1. A), ZZ (PDB ID: 6mj7.1A) and UBA (PDB ID: 2k0b.1A) domains respectively. Different secondary structures were presented in distinct color.

#### 4. Discussion

In this study, the protein sequence of gcp62 has been identified and the 3D structures of three gcp62 domains were predicted for the first time. Although our laboratory cloned gcp62 in 2019, the molecular and structural information about gcp62 are not completely clear. Regardless of the multifunction of mammalian p62, few function related studies have been reported in teleost except zebrafish. Some studies demonstrate that zebrafish p62 as an autophagy receptor, can colocalize with pathogens such as Staphylococcus aureus and mycobacteria and target them to autophagy for degradation [6, 7]. However, in grass carp and common carp, the p62 only serve as an autophagy marker [8, 9] and the evidences of the specific function of p62 in autophagy are unknown. In this regard, we use bioinformatics methods to predict the protein structure of gcp62 and compare it with human p62 for learning its molecular characterization.

By binding to numerous interaction partners with its multifunctional domains, p62 is widely involved in several important cellular processes, such as protein ubiquitination degradation, autophagy and a series of signal pathways. By using SMART, we found that gcp62 has only three

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conserved functional domains, a PB1 domain (2–90), a ZZ-domain (158–201), and a UBA domain compared with human p62. In mammalian p62, the PB1 domain can bind to the corresponding PB1 domain in other proteins, or it can self-assemble into homologous complexes to form its own oligomerization and polymerization, leading to autophagic degradation of proteins [10]. The ZZ-domain is involved in binding RIP protein and is associated with tumor necrosis factor signal transduction and NF- κB activation pathway [11]. P62 has a strong affinity for polyubiquitin chains and can act as a receptor for binding to polyubiquitin chains of ubiquitinated proteins through the UBA domain. On one hand, polyubiquitinated cargoes serve as a target for degradation through UPS. On the other hand, they can also be transported to autophagic vesicles for degradation [12]. Finally, we found a conserved phosphorylation of the 403 serine residue in the UBA domain of gcp62 which can significantly accelerate the autophagy process in human [13]. The conserved domain, similar protein structure and phosphorylation site of gcp62 imply its possible conserved function during evolution.

Our studies analyzed the sequence of gcp62 protein and built the 3D structure with three conserved domains for the first time. These results suggested that gcp62 may exhibit similar abilities in protein degradation, autophagy and some signaling pathways as seen in mammals.

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