A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

An Attempt To Reestablishing The Lost Function Of Ascorbic Acid Biosynthesis In Zebrafish (*Danio rerio*), By Constructing A Transgenic Zebrafish With A Functional L-Guluno-γ-Lactone Oxidase (*gulo*) Isolated From Cloudy Catshark (*Schliorhinus torazame*).

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GRADUATE SCHOOL

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Summary

The vitamin C is one of the essential micronutrients in living organisms. Vitamin C, also known as ascorbic acid, is a water-soluble low molecular weight carbohydrate which acts as a strong antioxidant. It involves a vast array of biological processes in the body such as collagen synthesis, gene regulation, epigenetic regulation, hormonal regulation, in metabolic energy synthesis, and immunity. Both plants and animal kingdoms synthesize vitamin C in their body except for few exceptions. In animal kingdom including human, anthropoid primates, guinea pigs, some bats, some passerine birds, and teleost fish have lost the ascorbic acid biosynthesis ability throughout the evolution. This functional loss is caused by a genetic lost in those animal species. The animals mentioned above have lost the *gulo* (L-gulono-γ-lactone oxidase) gene that codes for the L-gulono lactone oxidase enzyme which catalyzes the final enzymatic reaction of the animal ascorbic acid synthesis pathway. Up to date scientists were unable to elucidate a proper solid reason for the fluctuated *gulo* loss throughout the evolution, there are several hypotheses around the science community, but those hypotheses are clashing each other. So, up to now the *gulo* loss remain as a paradox in science. With the modern development of molecular biology and transgenic studies, we tend to address this problem from a new approach. We attempt to revoke the ascorbic acid biosynthesis in teleost fish using one of the most popular vertebrate model *Danio rerio* (zebrafish). In search of *gulo* gene, we found an ascorbic acid synthesizing fish species which was available in fish markets in Korea. The cloudy catshark (*Scyliorhinus torazame*) *gulo,* which was designated as *Sgulo,* was isolated from the catshark kidney and used to revoke the ascorbic acid biosynthesis in zebrafish. In the process, we constructed a Tol2 based expression clone which was included a b-actin promoter, *Sgulo* and mcherry reporter

Tg(bactin2:Sgulo-mcherry). We used the multi-site gateway cloning technique in constructing the expression clone. The constructed expression clone was microinjected to zebrafish embryos and selected the F0 positive embryos by the red fluorescent, which was given by the mcherry. Though we used b-actin promoter F0 generation gave a mosaic expression of the fluorescent construct. This mosaic expression was due to the different construct integration time at the embryo development. Afterward, we checked for the germline transmission of F0 generation and obtained the F1 generation by mating the F0 positive male with wild-type female. The positive F1 was used to make the F2 generation. After making the transgenic F1 zebrafish line, we confirmed the Sgulo genomic insertion and the Sgulo mRNA expression in the transgenic fish. Furthermore, we checked the physiological and developmental differences, growth differences, endogenous ascorbic acid levels, and Sgulo enzyme activity in transgenic and wild-type zebrafish. The physiological and development properties between the transgenic and wild-type zebrafish did not show any difference between the two groups, but the growth and the weight of the fish, endogenous ascorbic acid levels and Sgulo enzyme activity showed a significant difference in Tg with compared to Wt group. With these results, we could confirm that we were able to create a transgenic zebrafish which consist of a *Sgulo* gene in its genome, which expresses ubiquitously and able to re-establish the ascorbic acid biosynthesis. As future aspects, further, analysis can be done using the developed transgenic model. High precision and high-tech analysis like HPLC could confirm our results at a high precision level and could clear the roads to use the *Sgulo* transgenic model as a regenerative, toxicological, immune, behavioral, therapeutic and cancer research model.

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1. Introduction

1.1. Ascorbic acid

Ascorbic acid, also is known as vitamin C, is a water-soluble low molecular weight carbohydrate, which could donate H^+ to generate ascorbate monoanion or dianion (dehydroascorbic acid) (Figure 1). These two consecutive electron oxidations allow it to become one of the most effective donor antioxidants in organisms [1]. At physiological pH, the existing dominant derivative of vitamin C is ascorbate (99%) with low concentrations of ascorbic acid (0.1%) and dehydroascorbate (0.005%) [2]. Since the discovery of ascorbic acid by the Albert Szent-Györgyi in 1928, ascorbic acid has been the most industrially synthesized vitamin of all time [3]. Albert Szent-Györgyi received the Nobel prize in Physiology or Medicine in 1937 for his tremendous work on vitamin C isolation and identification in the process of studying the metabolic processes and effect of catalysts-substances in cellular nutrient break down [4].

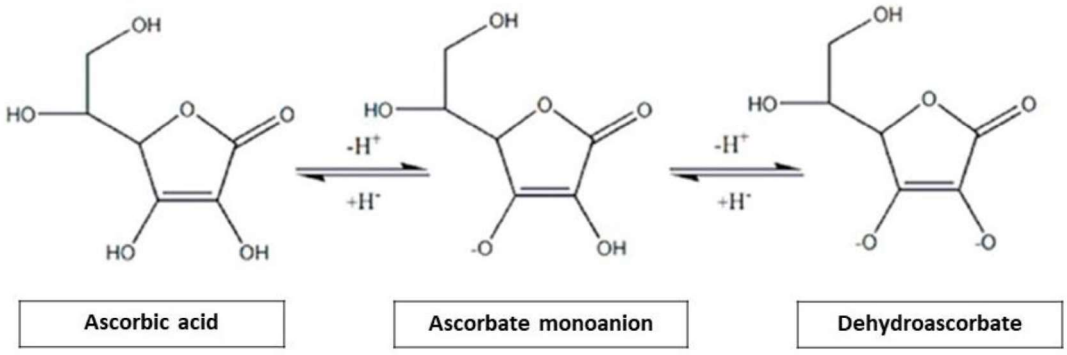

Figure 1: Chemical structure of ascorbic acid and the equilibrium between its oxidation states.
In physiological pH ascorbate monoanion concentration increases, and an acidic pH equilibrium shifts towards the ascorbic acid.

1.2. Biological importance of vitamin C

Vitamin C is an essential vitamin for human health as well as for other animals. Ascorbate (vitamin C) involved in a vast range of biological processes. The primary functions of ascorbate can be separated as enzymatic and non-enzymatic [5]. Ascorbic acid is a major cofactor for the family of gene regulatory and biosynthetic monooxygenase and dioxygenases. The main enzymatic function in ascorbate is shown in collagen synthesis. Collagen is the main component of the extracellular matrix. The function of the ascorbate is the major factor which regulates the proper folding of collagen. Ascorbate acts as an enzymatic cofactor for Fe^{2+}-2-oxoglutarate-dependent family of dioxygenases and maintains the optimum activity of the enzyme [6]. These dioxygenases catalyze the hydroxylation of proline and lysine residues in protocollagen to hydroxyproline and hydroxylysine [7]. This hydroxylation enables the proper assembly of collagen precursor to form a triple helical structure of functional mature collagen. Moreover, hypoxia-inducible transcription factor (HIF) prolyl and asparaginyl hydroxylases also a member of Fe^{2+}-2-oxoglutarate-dependent dioxygenase family, which require ascorbate as a reducing agent in maintaining the ferrous ion in the enzyme [8]. Through HIF system ascorbate influence in regulating several genes involved in cellular processes including cell survival, glucose uptake, angiogenesis, iron homeostasis, and glycolysis [9]. The demethylases which contain Jumonji catalytic domains which are involving in demethylation of trimethylated lysine also a novel member of Fe^{2+}-2-oxoglutarate-dependent dioxygenase family [10]. Which indicate that ascorbate is the main cofactor which catalyzes the histone demethylation acting as an essential epigenetic regulator [2,11].

Vitamin C is involved in carnitine biosynthesis, in carnitine biosynthesis pathway vitamin C is used as a cofactor by two hydroxylases, these synthesized carnitine involved in transporting

fatty acid into mitochondria to generate metabolic energy [6]. Moreover, vitamin C serves as a cofactor for hydroxylases involved in synthesizing catecholamine and amidated peptide hormones (e.g.-norepinephrine and vasopressin), which regulates cardiovascular responses upon severe infections [12]. All this evidence exhibit the importance of ascorbic acid in cellular biochemical machinery by maintaining the optimum function of the enzyme.

Numerous previous studies have shown the protective role of vitamin C against the development of cancer [13,14]. Also, it has been shown that the plasma ascorbate concentration inversely associated with cancer risk [15]. One of the main nonenzymatic function of vitamin C is the antioxidant function towards the oxidative damage induced by peroxyl radicals in lipid peroxidation [16]. Vitamin C maintains the vitamin E levels in the body by regenerating vitamin E from α-tocopheroxyl radicals [17]. Vitamin E is the primary antioxidant in lipid membrane oxidation and LDL [18]. Vitamin E undergoes a one-electron oxidation to form α-tocopheroxyl radicals which are reduced by the ascorbate to regenerate vitamin E [2]. Vitamin C has modulated the vasorelaxation by elevating the bioavailability of nitric oxide (NO) levels using several mechanisms, mainly by elevating the endothelial NO synthase (eNOS) and eliminating the intracellular superoxide which has the potential to deactivate NO [19]. All these evidence indicate the ability of ascorbate to readily donate electrons, which allows it to protect important biomolecules by oxidative damages.

Vitamin C has shown a significant role in immunity [20]. In Table 1**,** it has been shown various mechanisms of vitamin C function, which have the potential to leads to immune-modulation.

Table 1. Vitamin C role in immunity

Immune system component	Vitamin C function
Epithelial barriers	Enhances collagen synthesis and stabilization [21–23] Speeding up the wound recovery [24] Protect by ROS- induced damage [25–27] Enhances fibroblast proliferation and migration [28,29]
B and T-lymphocytes	Enhances the antibody levels [30,31] Enhances cell differentiation and proliferation [32,33]
Phagocytes (macrophages, neutrophils)	Antioxidant activity, act as an electron donor [34,35] Enhance phagocytosis and ROS generation [36,37] Facilitate the apoptosis and clearance [37,38] Increase the motility/chemotaxis [32,39]
Inflammatory mediators	Decrease the histamine levels [40,41] Modulates cytokine production [41,42]

All the studies mentioned above exhibit the vitamin C involvement in biological processes in organisms. This manifest biological importance has made vitamin C an essential micronutrient for animals.

1.3. Ascorbic acid (vitamin C) biosynthesis

Animals (most of vertebrates and invertebrates) and plants both have acquired the ability to synthesize vitamin C through different biosynthetic pathways (Figure 2), but including humans, some animals have lost the ability to synthesize it (the reason for the functional loss will

be discussed under the next subtopic) [43,44]. Animals synthesize vitamin C using glucose in the glucuronic acid pathway, but in plants, it synthesized using mannose [44]. In the animal ascorbic acid synthesis pathway, L-gulonolactone is oxidized to 2, Keto-L-gulonolactone by the L-gulonolactone oxidase enzyme and 2, Keto-L-gulonolactone spontaneously enolized to L-ascorbate [45].

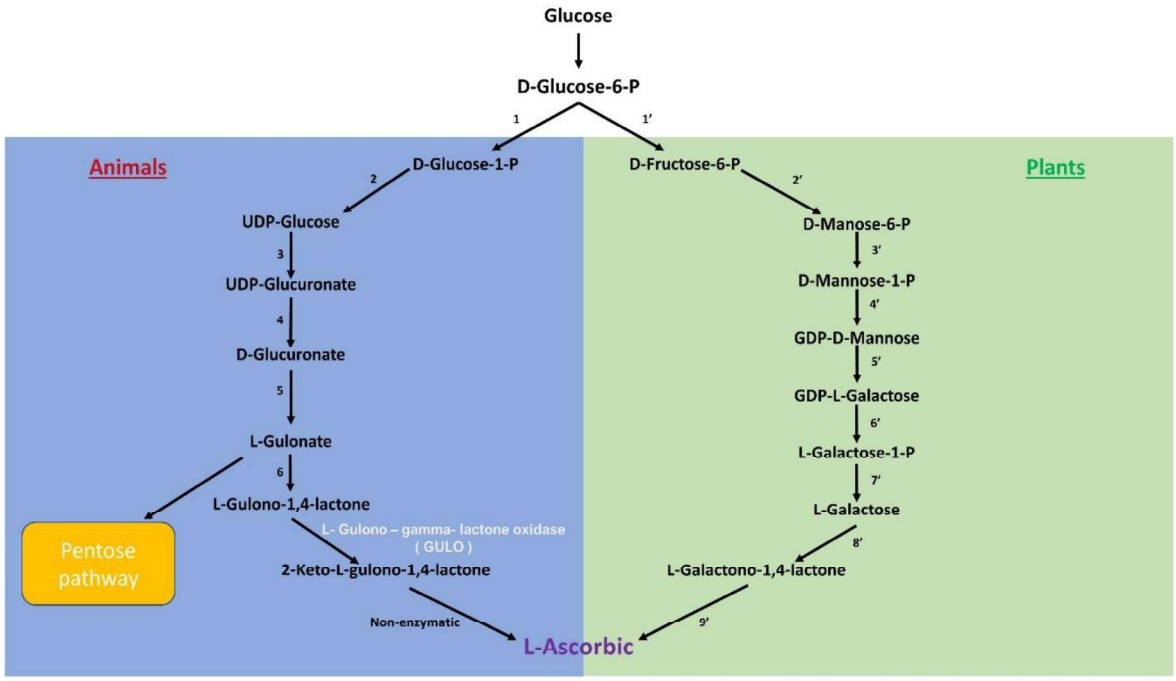

Figure 2. Vitamin C biosynthesis in animals and plants.
Numbers represent the following enzymes 1. Phosphoglucomutase, 2. UDP-glucose pyrophosphorylase, 3. UDP-glucose dehydrogenase, 4. UDP-glucuronidase, 5. Glucoronate reductase, 6. Gluconolactonase, 1'. Glucose-6-phosphate isomerase, 2'. Mannose-6-phosphate isomerase, 3'. Phosphomannomutase, 4'. GDPD-mannose pyrophosphorylase, 5'. GDP-D-mannose-3,5-epimerase, 6'. Phosphodiesterase, 7'. Sugar phosphatase, 8'. L-galactose dehydrogenase, 9'. L-galactonolactone oxidase.

In animals, L-Gulonate enters the pentose phosphate pathway through 3-keto-L-gulonate and synthesize the precursors for the nucleotides [46]. The crucial step in the vitamin C biosynthesis is the final enzymatic step of oxidization of L-gulono-1,4-lactone to 2- Keto-L-

gulonolactone, which is governed by the key enzyme L-gulonolactone oxidase. The formation of L-ascorbic acid from the 2- Keto-L-gulonolactone is the only spontaneous non-enzymatic reaction in ascorbic acid biosynthesis.

1.4. The loss of vitamin C biosynthesis

Vitamin C (ascorbic acid) is biosynthesized by the majority of the animal kingdom (vertebrate and invertebrates), but some vertebrates including anthropoid primates [47], guinea pigs [48], some bat species [49], certain passerine birds [50], and teleost fishes [51] have lost the ability of vitamin C biosynthesis. The final enzymatic step of the vitamin C biosynthesis is catalyzed by L-gulono-gamma-lactone oxidase (GULO, EC1.1.3.8) (Figure 2). The loss of the vitamin C biosynthesis in organisms mentioned above is due to the lack of functional *GULO* gene in their genomes [43].

1.4.1. The genetics and the loss of *GULO* throughout vertebrate evolution

The functional GULO activity has fluctuated throughout the vertebrate evolution. The *GULO* orthologs have got 11 highly conserved exons [52]. Phylogenetic and synteny analysis has shown that some species (guinea pigs, humans, some bats) contain the traces of *GULO* pseudogenes and some vertebrates such as zebrafish have entirely lost the gene from its genome [52]. The teleost fish and higher primates cannot synthesize ascorbic acid due to the loss of *GULO* genes in their remote ancestors. In fish, it has been shown that all the cartilaginous and non-teleost bony fish can synthesize ascorbic acid [53,54]. However, the common ancestor of teleost fish have lost the *gulo,* and as a result, teleost fish were unable to synthesize ascorbic acid [43,51,55]. The occurrence of this mutation estimated to occur between 200-210 MYA [43]. Although *GULO* has loss in the teleost genome, the gulonolactonase gene which encodes for the

penultimate enzyme in the ascorbic acid synthesis pathway of animals could be found in teleost genomes such as zebrafish, the salmon and the European flounder [43]. This evidence shows that the loss of vitamin C biosynthesis is exclusively due to the loss of *gulo* from the teleost genome. During vertebrate evolution, the organ used to synthesize ascorbic acid have changed from kidney to liver [52]. Vitamin C production of fishes, amphibians, reptiles, and ancient bird orders have occurred in the kidney, whereas recent bird orders and mammals produce their vitamin C in the liver [56]. This organ shift has occurred due to the selective pressures of higher stressful conditions in high active species. Because ascorbic acid mainly involved in maintaining the biochemical homeostasis in animals [57,58].

1.4.2. Vitamin C deficiency

Vitamin C has been an essential nutrient in vertebrate due to the vast array of function in the body. As mentioned in the above sub-topic, some vertebrates have lost the ability of ascorbic acid synthesis due to the lack of *GULO.* Those animals fulfilled their vitamin C requirement by the diet. Without the proper dietary supply of vitamin C vertebrates could develop scurvy; this is the major defect from vitamin C deficiency and could be a fatal condition if not treated [9]. In teleost vitamin C deficiency, it could show an impaired wound healing, reduced growth and survival rate, reduced immune competence, and suboptimal embryonic and larval development [59].

1.5. Previous attempts on restoring vitamin C biosynthesis in scurvy prone animals

The importance of ascorbic acid to the survival of the organism has triggered an enormous spotlight in the scientific community. Several studies have conducted to restore the ascorbic acid synthesis in the vertebrates, which lacks the vitamin C synthesis capability.

Primates are entirely depended on the external dietary intake of vitamin C, so there has been a study to correct the metabolic loss of vitamin C synthesis in human cells, by infecting the HEK293 cells using adenoviral construct containing murine L-gulono-gamma-lactone oxidase, this study have able to express a functional *Gulo* gene in human cells [60]. Furthermore, a previous study was conducted to restore the ascorbic acid synthesis in *Gulo*$^{-/-}$ mice by expressing gulonolactone oxidase using a helper-dependent adenovirus, in this study scientist have able to produce ascorbic acid endogenously in human and *Gulo*$^{-/-}$ transgenic mice [61]. In another experiment which conducted in McMaster University, guinea pigs have been able to express the Gulo and produce endogenous vitamin C; this study has been conducted by a gene therapeutic approach using lentiviral vectors which comprise mouse *Gulo* under murine cytomegalovirus (mCMV) promoter [62]. However, a study done in Finland has shown gene transfer technique which transfers rat gulonolactone oxidase cDNA (*Rgulo*) into rainbow trout have neither indicated a *Rgulo* transcription, Gulo protein or enzyme activity [63]. In the experiment, they have concluded that the conditions which needed for the translation and stability of Rgulo are absent in rainbow trout cells. However, the research team has assumed that a *gulo* gene transfer from a lower fish group would be more successful in restoring the vitamin C biosynthesis.

1.6. Zebrafish (*Danio rerio*) and Cloudy catshark (*Scyliorhinus torazame*)

Zebrafish (*Danio rerio*) are fish water species which originate from South Asia. In the past 40 years, these small fish has become a powerful and popular model in a vast array of researchers, including research areas such as vertebrate development, genetics, regeneration, and toxicology [64]. The popularity of zebrafish as a model organism was boosted due to several specific reasons, including the optical clarity of embryogenesis, high fecundity, rapid growth and

the short generation time, small size, ease of care and manipulation [64,65]. In the current scientific world, zebrafish used as a development model, genetic model, regenerative model, behavioral model, and toxicology model [64,66]. The synteny analysis has confirmed the complete loss of *gulo* in zebrafish genome [52]. This genetic loss has made zebrafish a potentially unique and a novel model for studying ascorbic acid function. Studies have shown the vitamin C deficiency in zebrafish activates the purine nucleotide cycle and also results in elevated AMPD activity [67]. Furthermore, another study has shown the higher specific growth rate, survival rate, and fecundity in zebrafish groups which fed vitamin C supplemented diets compared to the controls which did not supplement vitamin C [68].

Cloudy catshark (*Scyliorhinus torazame*) is a cartilaginous shark species distributed in the Northwest Pacific sea (Japan, Korea, China, and Taiwan) [69]. Cloudy catshark has a functional L-gulono-γ-lactone oxidase gene and can synthesize vitamin C; a study has confirmed the presence of *GULO* (GLO) gene and the enzyme activity. Furthermore, it has shown the kidney-specific expression in cloudy catshark [53].

1.7. Objectives and the summary of the project

The main objective of this project was to express a functional *gulo* gene in zebrafish, which was isolated from a vitamin C synthesizing fish species. Our attempt was to create a vitamin C synthesizing zebrafish model by reestablishing the lost gene and the function in the ascorbic acid biosynthesis pathway. We isolated the *gulo* gene (*Sgulo*) from cloudy catshark kidney and made a construct containing three elements (beta-actin promoter, isolated *Sgulo,* and mcherry sequence) using Tol2 based Gateway cloning technique. The construct was co-microinjected to zebrafish embryos with transposase mRNA. The transgenic embryos were

selected using a fluorescent microscope. Thus, mcherry act as a reporter in the construct the positive embryos gave a red fluorescent. The selected embryos were raised until the sexual maturity of the fish. The mature transgenic fish were crossed with AB control fish and obtained the F1 generation. This F1 generation was again selected using fluorescent microscopy and genotype confirmed using PCR afterward used for the developmental and physiological parameter assessments, ascorbic acid assay, and GULO enzyme activity assay. Afterward, the differential gene expression was analyzed in several genes using qPCR.

2. Materials and Methods

2.1. Zebrafish husbandry

The adult zebrafish were maintained according to the standard protocols[70]. In summary, the fish was subjected to 14:10 h light/dark cycles (LD), and the temperature was maintained at 28^0C. Fish were fed with live artemia, up to three months feeding was done trice a day, and after three months, fish were fed twice a day. The embryos were obtained by mating the male and female zebrafish in a breeding tank and collected the embryos within the first hour of lights on; collected embryos were placed in E3 medium and raised in 28^0C incubator providing the 14:10 h LD cycle.

2.2. Isolation of *Sgulo* from cloudy catshark

2.2.1. In-silico analysis of *Sgulo*

First, the in-silico analysis of the *Sgulo* was performed. The nucleotide protein sequences of the *Sgulo* and the orthologs were obtained from the National Center for biotechnology information (https://www.ncbi.nlm.nih.gov/). The open reading frame, putative amino acid sequence, the multiple sequence alignment, and hydrophobicity of *Sgulo* and orthologs were analyzed using Bioedit software [71]. The conserved domain search programs CDD, NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), ExPaSy PROSITE (https://prosite.expasy.org/), Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) were used to delineate the domain architecture of *Sgulo*. The I-TASSER protein model prediction server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the tertiary structure of Sgulo, mcherry fusion protein, and the PyMOL v1.5 software was used to visualize the 3-D structure. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method in

MEGA (ver 7.0). The identity and the similarity percentages between the gulo orthologs were calculated by the MatGat software. Furthermore, transmembrane probabilities of the Sgulo and the Sgulo, mcherry fusion were analyzed using the TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.2.2. Dissection of cloudy catshark to obtain kidney

The cloudy catshark (*Scyliorhinus torazame*) was obtained by the local market in Hamdeok (Jeju, South Korea). The fish was dissected and obtained the kidney tissues, immediately snap frozen the tissues in liquid nitrogen.

2.2.3. Total RNA extraction

The total RNA was extracted from the collected catshark kidney tissues using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA). The purity and the concentration were determined by using Multiskan GO microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.2.4. The cDNA synthesis

The extracted RNA was used to synthesize first strand cDNA using PrimeScript™ First-strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. Briefly, RNA was incubated with 1 µl of 50 µM oligo $(dT)_{20}$ and 1 µl of 10mM dNTP for 5 min at 65^0C. Subsequently, the mixture was cooled on ice and 4 µl of the 5xPrimeScript™ buffer, 0.5 µl of RNase inhibitor (20U), and 1 µl of PrimeScript™ reverse transcriptase (200 U) were added, and the solution was incubated for 1 h at 42^0C. The reaction was terminated by incubating the solution at 70^0C for 15 min. The synthesized cDNA samples were diluted 20-fold and stored at -20^0C for the later experiments.

2.3. Constructing the Expression construct

We used the Tol2kit to construct the desired construct.[72] The primer designing and the selection of the vectors were done compatibly with Multisite Gateway® Three-Fragment Vector Construction Kit.[73]

2.3.1. Primer designing

The primer designing was done following the manufacturer's instruction; in brief, the attB PCR primers were designed by adding attB flanking regions to the forward and reverse primers. Also, the Kozak consensus sequence was added to the forward primer. Also, for analyzing the differential gene expression of CAT, SOD1, SOD2, HIFI, cyb5a, and procollagen, we designed the qPCR primers, as shown in Table 2.

Table 2. Primer details

Purpose	Name	Primer sequence (5'-3')
Sgulo Amplifying	Sgulo F1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGATCAAGGCACCATGGGA
	Sgulo F2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGAAAAAGGTCTTTTCCAGGTAATTGTTGAGG
qPCR Analysis	CAT	TGCCAAACCACCAGAATCCCTTGTA
		TCAAGGTGACGATTACAGCAGCCA
	SOD1	AGCCAGTGAAGGTGACTGGTGAAA
		TATCGGTTGGCCCACCATGAGTTT
	SOD2	TCACAGCAAGCACCATGCAACA
		CACCGCCATTGGGTGACAGATTT
	HIF1	GTGTGCAGCCTGTAGTGCAACATAAC
		AGGACATCTGAAGAGGGCAACATGAC
	Cyb5a	TGCCAAACCACCAGAATCCCTTGTA
		TCAAGGTGACGATTACAGCAGCCA
	Procollagen	ACCGATGGCTTCCAGTTCGAGTATG
		TTGCCAGAAGCCTGGTCCATGTAT

2.3.2. Gateway cloning vectors

As mentioned above, we used the Tol2kit gateway-based technique for the cloning. In constructing the vector construct, we used several Tol2kit vectors such as pDONR221, p5E-bactin2, p3E-mcherrypA and pDestTol2pA

2.3.3. Amplifying attB-PCR products

The *Sgulo* was amplified using attB PCR Sgulo primers (Table 2). The PCR conditions were as follows one cycle of 94^0C for 5 min, 30 cycles of 95^0C for 30 s, 58^0C for 30 s, 72^0C for 90 s, and a final extension of 72^0C for 7 minutes. The PCR products were confirmed by gel electrophoresis, and PCR products were purified using Accuprep® Gel Purification Kit (BIONEER, Korea).

2.3.4. Constructing entry clones using the BP recombination reaction

The entry clone was constructed by using multisite gateway cloning BP recombination reaction according to the manufacturer's instruction with slight modifications. In brief, 1µl of pDONR™ 221 plasmid (150ng/ µl) and 1 µl of purified attB PCR products (150 ng/µl) was mixed in 6 µl of TE buffer (pH=8.00), 2 µl of BP clonase was added to the mixture. The mixture was vortexed and spun down and incubated at 25^0C overnight. After the incubation 1 µl of proteinase K (Sigma-Aldrich, USA) was added and incubated at 37^0C for 10 min. The mixture was stored at -20^0C and used for the LR reaction in the following day. The constructed entry clone was transformed into the One Shot® Top10 chemically competent *E.Coli* following the heat shock transformation protocol provided by the manufacturer and entry clone plasmids were extracted using AccuPrep® Plasmid Mini Extraction Kit (BIONEER, Korea). A sequence confirmation was done using sequencing primers before constructing the expression clone.

2.3.5. Constructing expression clones using the LR recombination reaction

In the construction of the expression clone, we used the Gateway LR recombination reaction and followed the manufacturer's instructions with slight modification. In brief, p5E-bactin2 (50 ng), pME-Sgulo (50 ng) (The constructed entry clone with the confirmed *Sgulo*), p3E-mcherrypA (50 ng) and pDestTol2pA (150 ng) were mixed in 4 µl of TE buffer (pH=8.0).To the mixture, 2 µl of LR clonase II was added and vortexed the mixture followed by a spin down. The mixture was incubated for 48 hours at 25^0C; proteinase K 1 µl was added and incubated for 10 min at 37^0C. The clone was then transformed into the One Shot® Top10 chemically competent *E.Coli* following the heat shock transformation protocol provided by the manufacturer. The plasmid extraction was done using the QIAGEN® Plasmid purification Kit (Hilden, Germany)

2.4. Synthesizing the capped transposase mRNA

The pCS2FA-transposase plasmid was linearized by Not I (NEB®) and purified using Accuprep® PCR Purification Kit (BIONEER, Korea). From the linearized DNA 2 µg of DNA was used for the In-vitro transcription carried out by mMESSAGE mMACHINE™ SP6 Transcription kit (Ambion™, Life Sciences, Inc.). The In-vitro transcribed RNA was purified using the Qiagen RNeasy Mini Kit, and subsequently, ethanol precipitation was conducted. The RNA concentration was quantified using a Multiskan GO microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). Stored at -80^0C until further use.

2.5. Construction of transgenic zebrafish

2.5.1. Microinjection

For the microinjection PV820, Pneumatic pico pump (WPI, Inc, Sarasota, USA) was used, and we determined the micro-injecting volume by micro scale using standard oil drop method (measuring the oil drop diameter). The microinjecting mixture was made by mixing expression clone (2 µl), purified transposase mRNA (1 µl), KCl (1 µl) and Phenol red (1 µl) in 5 µl of nuclease-free water (final mixture volume = 10 µl). The concentrations of the expression clone and the transposase mRNA were made to appropriate values that would reach the requirement of the experiment. These concentrations were optimized by conducting pre-trails, and the optimum amount of the genetic materials that were suitable for the injection was determined as 75 pg of constructed expression clone and 25 pg of transposase mRNA per embryo. Then the 3 µl of the mixture was inserted into the micro-injecting needle. For the microinjection zebrafish embryos were obtained as mentioned in section 2.1, the embryos were mounted in a mold which was prepared by 2% agarose. With the use of the microinjector needle and the microscope, the genetic materials were injected into the first cell stage of the embryos; the injected embryos were collected into E3 medium and incubated at 28 ^{0}C.

2.5.2. Selection of transgenic F0 embryos

In the 18 hours of postfertilization, the microinjected embryos were selected under the fluorescent microscope (Leica, DM600B). The red fluorescent embryos were selected and raised at 28^{0}C.

2.5.3. Raising the F0 transgenic zebrafish

The selected F0 transgenic embryos were kept at 28^0C for five days in E3 medium. After 5 d.p.f (days post fertilization) the hatched zebrafish larvae were transferred into the zebrafish nurturing unit. In the nurturing unit, larvae were fed by manually ground powder form fish feed (Tera Bits complete fish feed) until one month after the one-month artemia feeding was started. The zebrafish larvae were kept at the nurturing unit for three months. Ones the larvae become the adults after 3 months post fertilization, the fish were transferred into the circulation unit. When transferring the adult zebrafish to circulation unit, the male and female fish were separated and raised in separate tanks. To select the germline transmission in transgenic F0 zebrafish, we crossed the F0 males with the Wt female and obtained the embryos as shown in Figure 3. Subsequently, after 24 hpf embryos were checked for the fluorescent and the male zebrafish which gave the positive embryos were selected as the germline transmitting F0.

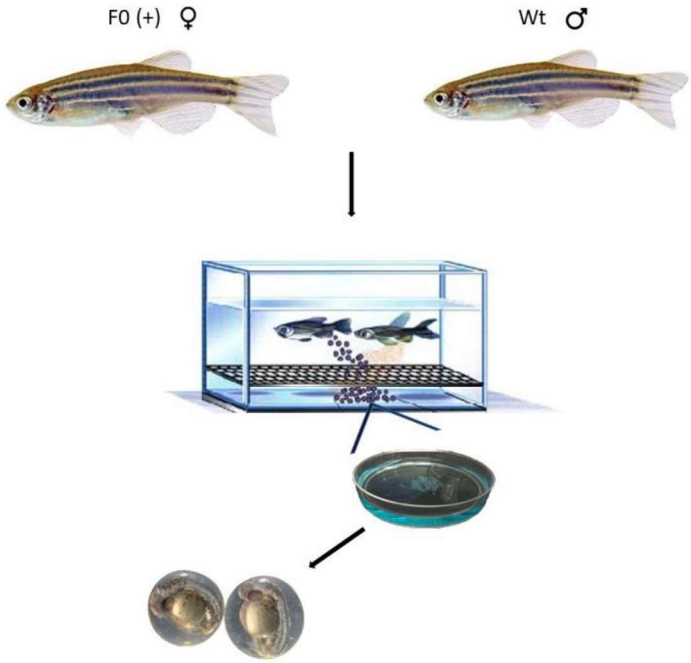

Figure 3. Obtaining embryos to check the germline transmission

The positive male F0 zebrafish was crossed with Wt female fish and obtained the F1 embryos.

2.5.4. Making the F1 transgenic zebrafish line

In the making of the F1 generation, we crossed the AB wild-type female and a founder male fish and obtained the F1 embryos. The selection of transgenic F1 was conducted under the fluorescent microscope, as mentioned in the above section. The selected embryos were nurtured and raised to the adulthood, the adult zebrafish were separated according to sex and transferred to the circulation tanks separately.

2.5.4. Making the F2 transgenic zebrafish line

In the making of the F2 generation, we crossed the AB wild-type female and F1 males to obtain the F2 generation, the selection and the raising of the F2 generation were done as mentioned previously in section 2.5.4. The F2 transgenic line was kept progressing by breeding.

2.5.5. Confirmation of transgenic fish by genotyping

The confirmation of the transgenic fish was conducted by genotyping. The zebrafish were anesthetized using Tricaine (4ml for 100ml water). Fins were clipped, and 50 µl of lysis buffer and 1 µl proteinase K was mixed with the clipped fin and incubated at 60^0C for 1 hour and 95^0C for 10 minutes while vortexing in every 15 min time interval. After the incubation period, the samples were stored at -20^0C. The extracted genomic DNA was used as the template DNA and performed the PCR using *Sgulo* specific primers using the thermal cycle mentioned in section 2.3.3. The PCR products were subjected to gel electrophoresis and visualized under UV illuminator.

2.6. Development and physiological assessment between transgenic and control fish groups

For comparing the physiological and developmental differences between the Tg and Wt group, two experiments were designed. The first experiment was to observe the physiological differences. The both transgenic (Tg) and wild type (Wt) larvae of 24 hpf, 48 hpf, 72 hpf, and 96 hpf were observed and photographed the differences under the light microscope; we used five embryo samples for each group and time point. The second experiment was to assess the heart function, for that we counted the beats per minutes (bpm) in the 96 hpf zebrafish larvae. The larvae heart was observed under the light microscope, and video photographed the heart, from

video analysis bpm was counted, and the average bpm was taken for both Tg and Wt group, 5 larvae zebrafish was used for each sample, and three replicates of the experiment was conducted.

2.7. Analysis of growth rate between transgenic and control zebrafish

The average growth rate of Tg and Wt adult zebrafish were assessed. The Tg and Wt zebrafish larvae were raised separately for 3 months. The experiment was set to perform 3 replicates using 10 fish per sample group. Therefore 30 zebrafish were used for one group (Tg and Wt). The same conditions were provided to the Tg and Wt groups, same tank size and fish number, an equal amount of feed per diet, same light cycle, temperature, and aeration. After 3 months of raising under above conditions, the fish (sample size of 10) were separately weighed using a chemical balance (A&D weighing, Inc, USA) and averaged the weight; three replicates were performed for the experiment by each group.

2.8. The *Sgulo* expression in transgenic zebrafish

The expression of *Sgulo* was confirmed using extracted RNA of Tg zebrafish. The RNA extraction was performed using the QIAGEN® RNEASY Mini kit (Germany) in briefly, 30 mg of Tg and Wt zebrafish muscle tissues were homogenized using homogenizer (taco™prep bead beater) and Total RNA extraction was performed following the manufacturer's instruction. The purity of the extracted RNA was assessed by running the samples on a 1.5 % agarose gel and the concentrations were measured at 260 nm in a μDrop plate (Thermo Scientific, Waltham, MA, USA). From the extracted RNA, cDNA was synthesized using the PrimeScript first strand cDNA synthesis Kit (TaKaRa, Japan). The synthesized cDNA was used as the template and performed the PCR using *Sgulo* specific primers. The PCR products were run on a 1.5% agarose gel.

2.9. Gene expression analysis

The gene expression analysis of superoxide dismutase 1 and 2 (SOD1,2), catalase (CAT), hypoxia-inducible factor-1 (HIF1), cytochrome 5b (cyb5), and procollagen were assessed in liver, kidney, brain, and muscle tissues using qPCR. The qPCR analysis was carried out in a 10 µl reaction volume containing 3 µl cDNA, 0.4 µl of each forward and reverse primers (Table 2), 5 µl of 2x TaKaRa Ex Taq SYBR premix and1.2 µl H_2O. The thermal cycler, model Dice TP950 (TaKaRa, Japan) was used under the following thermal cycle parameters: one cycle of 95 ^{0}C for 5 s, then 45 cycles of 95 ^{0}C for 5 s, 58 ^{0}C for 10 s, 72 ^{0}C for 20 s, and a final cycle of 95 ^{0}C for 15 s, 60 ^{0}C for 30 s, and 95 ^{0}C for 15 s. The transcription of the genes mentioned above was determined using the 2(-ΔΔC(T)) method [74] and as an internal reference gene elongation factor 1a (*ef1a*) in zebrafish was used (Gene bank ID:AM422110). The gene transcriptional data was presented as expression fold relative to *ef1a* expression. Furthermore, the gene transcription in the Tg group was compared to the Wt transcription making Wt transcription as the basal transcriptional level.

2.10. GULO assay

The Sgulo enzyme activity was assessed according to the earlier used standard GULO activity assessment [75] with some modifications. Briefly, first, we made the standard curve for the ascorbate concentration using a series of ascorbic acid dilutions. Afterward, the two zebrafish groups Tg and Wt were weighed separately (5 fish per each group). The whole fish was homogenized using 5 ml of 0.05 M sodium phosphate buffer (pH=7.4, 0.2% deoxycholate), the homogenates were centrifuged for 30 min at 4 ^{0}C at 20000g in (Avanti® J-E high-speed centrifuge, Beckman colter, Inc), 4 ml of the supernatant was added 1 ml of 5.6 mM L-

gulonolactone (SIGMA-ALDRICH®, USA) and incubated at 25 ^{0}C for 30 minutes. The reaction was stopped by adding 2ml of the stopping solution which contained 18% metaphosphoric acid and 16% of trichloroacetic acid (SIGMA-ALDRICH®, USA), afterward 0.1 g acid washed charcoal (SIGMA-ALDRICH®, USA) was added to the mixture and subsequently filtered using filter papers (570 mm, Whatman). A 4 ml of the filtrate was taken and added 1 ml of DNPH reagent, covered the test tubes with aluminum foil and incubated at 47 ^{0}C for 90 min. the samples were then cooled with swirling in an ice bath while 5 ml of 85% H_2SO_4 (SIGMA-ALDRICH®, USA) was added dropwise. The mixture was kept for 20 min at room temperature, and the absorbance was measured at 524 nm wavelength taking Wt sample as the blank. The activity was calculated using the standard curve, which was constructed previously using standard ascorbic acid.

2.11. Statistical analysis

All the functional and transcriptional analysis were conducted in triplicate reactions, and the significant differences were presented as means ± SD. Statistically significant differences ($P<0.05$) were determined by the two-tailed unpaired t-test using GraphPad program (GraphPad Software, Inc., La Jolla, CA, USA)

3. Results and discussion

3.1. In-silico analysis

The sequence analysis (Figure 4), multiple sequence alignment (Figure 5), 3-D structure prediction (Figure 7), phylogenetic analysis identity/similarity analysis Table 3 and the transmembrane probabilities (Figure 8) of the *Sgulo* and Sgulo +mcherry fusion was performed.

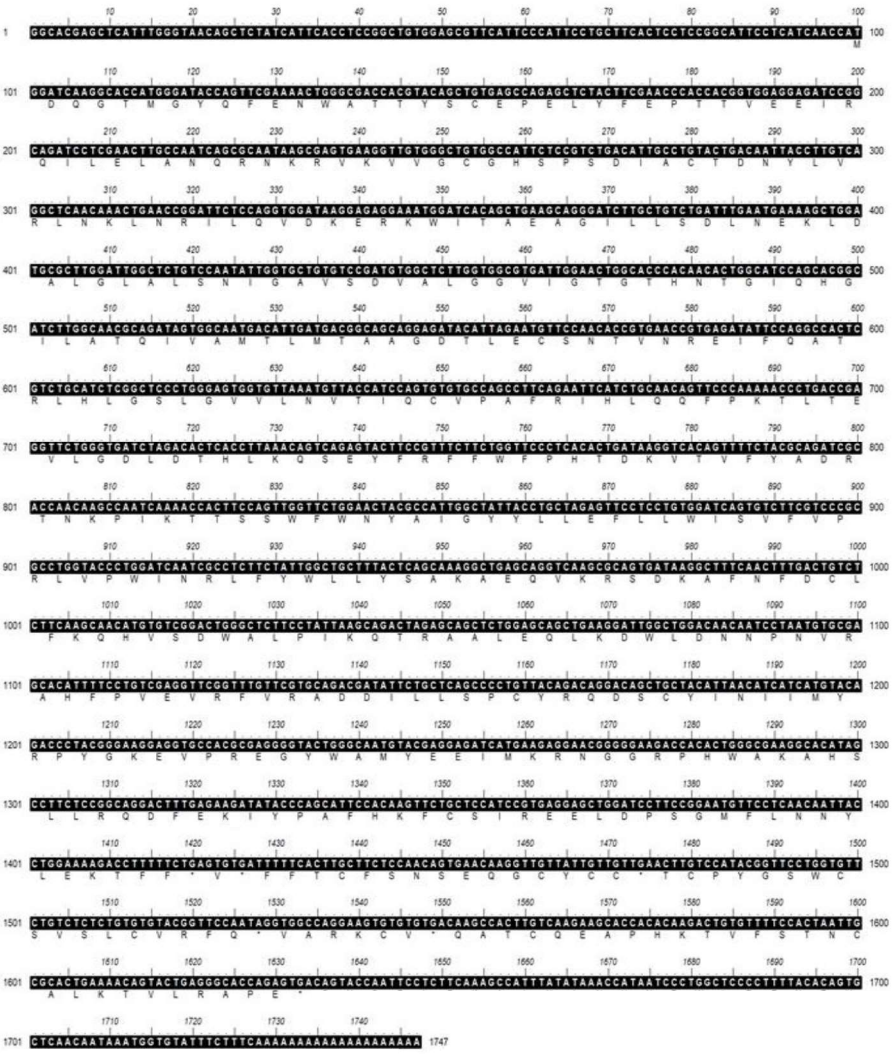

Figure 4. **Sequence analysis of *Sgulo*.**
The sequence analysis and the graphic view was obtained by Bio-edit software, the complete *Sgulo* (Accession no- AY039838) sequence is highlighted, and one letter code for the protein translation is shown in the figure.

The *Sgulo* gene sequence consists of 1323 bp coding sequence, which codes for 440 amino acids, as shown in Figure 4. The predicted molecular weight of the Sgulo protein was 50.9 kDa while giving a 7.17 theoretical iso-electric electric point.

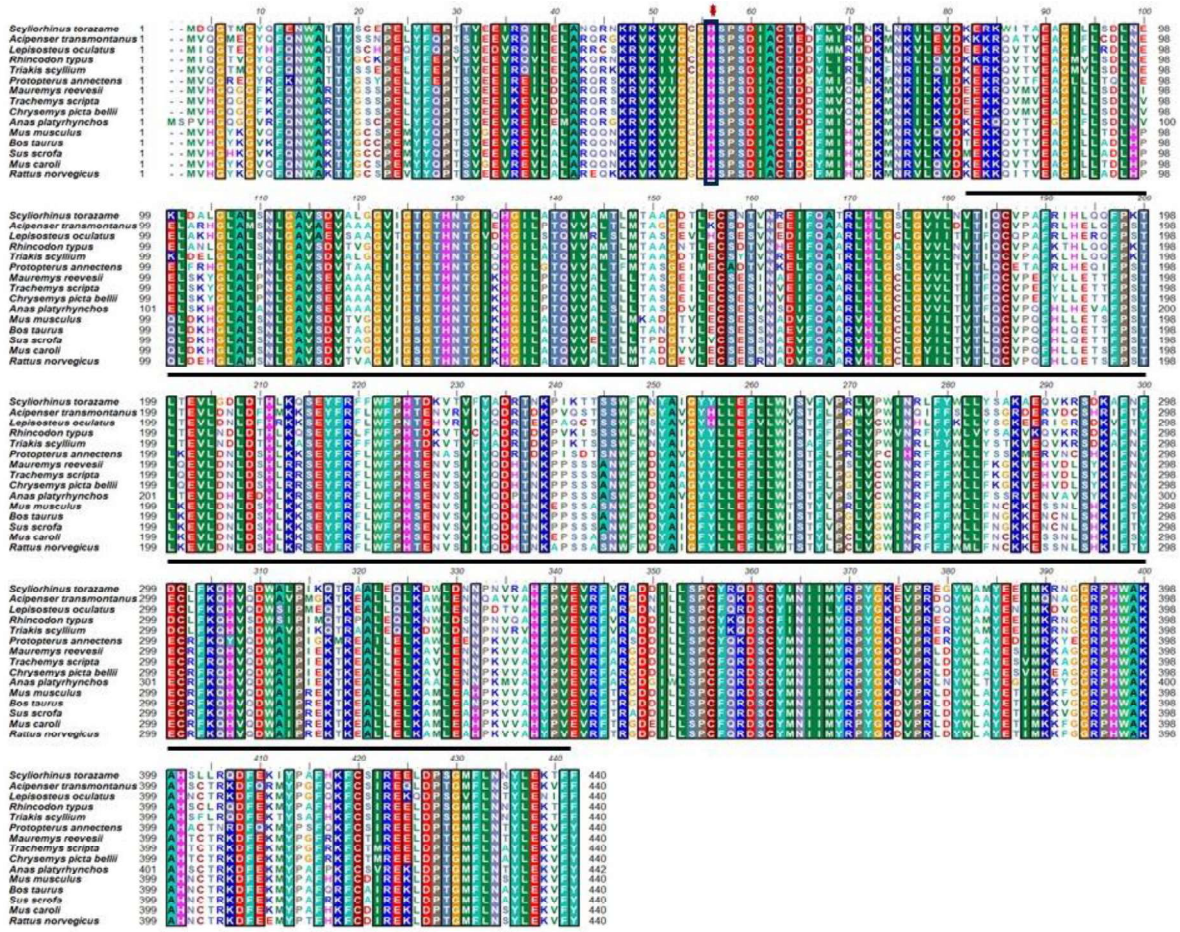

Figure 5. Multiple sequence alignment of the gulo orthologs.
The multiple sequence alignment was constructed using Bio-edit software, and the gulo sequences of *Scyliorhinus torazame* (AAK73281.1), *Acipenser transmontanus* (ABO15549.1), *Lepisosteus oculatus* (XP_015207781.1), *Rhincodon typus* (XP_020372305.1), *Triakis scyllium* (ABO15547.1), *Protopterus annectens* (AGQ16461.1), *Mauremys reevesii* (AET14635.1), *Trachemys scripta* (AET14636.1), *Chrysemys picta bellii* (XP_008172130.1), *Anas platyrhynchos* (XP_021135031.1), *Mus musculus* (NP_848862.1), *Bos Taurus* (Q3ZC33), *Sus scrofa* (Q8HXW0), *Mus caroli* (XP_021036878.1), *Rattus norvegicus* (BAA02232.1) were used for the alignment. The star and the black box showed the conserved histidine residue, which is the active site in the gulo, and the black color bar indicate the ALO domain.

The multiple sequence alignment of the gulo orthologs showed high conservation of the sequence among other vertebrates, as shown in Figure 5. The histidine residue at the 54th position, which acts as the active site shows high conservation. Also, throughout the ALO domain, it shows high conservation among the gulo orthologs.

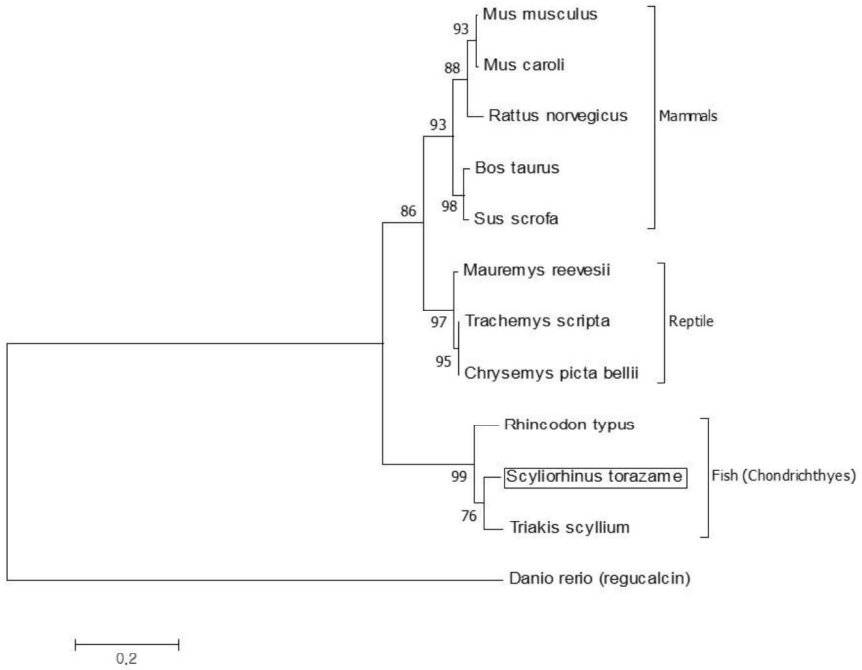

Figure 6. Phylogenetic tree of selected gulo orthologs.
The Neighbor-joining tree was constructed using MEGA 7.0 following a 5000 Bootstrap method and using the Poisson model. The sequences of the organisms which was used for the analysis were obtained from the NCBI database and the accession no are as follows *Mus musculus* (NP_848862.1), *Mus caroli* (XP_021036878.1), *Rattus norvegicus* (BAA02232.1), *Bos Taurus* (Q3ZC33), *Sus scrofa* (Q8HXW0), *Mauremys reevesii* (AET14635.1), *Trachemys scripta* (AET14636.1), *Chrysemys picta bellii* (XP_008172130.1), *Rhincodon typus* (XP_020372305.1), *Scyliorhinus torazame* (AAK73281.1), *Triakis scyllium* (ABO15547.1) and as the out-group the regucalcin *of Danio rerio* (AAH75882) was used in the analysis.

Figure 6 showed the phylogenetic relationship of Sgulo with other vertebrate counterparts. The Sgulo group with main fish clade (Chondrichthyes) and deviated from other vertebrates. Also, it indicates that the gulo is evolved from a common ancestor. The identity and the

similarity results are shown in Table 3, and it reveals the Sgulo conservation throughout the animal kingdom, the highest identity and the similarity to Sgulo is respectively 92% and 95.9% shown by the gulo ortholog of *Triakis scyllium.* Also, the gulo ortholog of *Mus musculus* gave an identity and similarity percentage of 64.3% and 81.8% respectively. These obtained results of multiple sequence analysis, phylogenetic analysis and identity, and similarity percentages implicate the evolutionary conservation of the Sgulo throughout the evolution.

Table 3. Identity and similarity percentages between gulo orthologs

Gulo orthologs			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Organism	Accession no		Identity%														
1. Scyliorhinus torazame	AAK73281.1			68	65.2	85.9	92	68.2	65.5	65.2	65.2	66.1	64.8	64.5	64.3	64.5	63.2
2. Acipenser transmontanus	ABO15549.1		86.1		78.4	68	68.4	74.5	74.5	73.9	73.9	74.2	72.3	70.5	71.1	71.1	71.6
3. Lepisosteus oculatus	XP_015207781.1		83.9	91.4		65.9	64.8	68	72.5	71.4	71.4	72.6	70.7	69.8	69.1	68.9	68.9
4. Rhincodon typus	XP_020372305.1		95	86.6	85.5		87.7	67.3	64.5	64.8	64.8	65.8	65.8	64.9	65	65.2	63.8
5. Triakis scyllium	ABO15547.1		95.9	86.1	84.1	95		68.9	65.7	65.5	65.5	66.7	64.5	64.1	63.9	64.1	63.0
6. Protopterus annectens	AGQ16461.1		85.7	89.8	87.3	87.3	85.7		75.9	75	74.8	74	74.5	73.2	73.2	73.4	72.3
7. Mauremys reevesii	AET14635.1		82.7	88.9	86.8	84.1	83.2	89.3		98	97.7	85.5	83.6	82.3	80.2	79.8	79.3
8. Trachemys scripta	AET14636.1		82.7	88.9	86.8	84.1	83.2	89.1	99.3		99.8	84.6	84.1	82.7	79.8	79.8	78.9
9. Chrysemys picta bellii	XP_008172130.1	Similarity%	82.5	88.9	87	83.9	83	89.1	99.3	100		84.4	83.9	82.5	79.5	79.5	78.6
10. Anas platyrhynchos	XP_021135031.1		82.1	87.6	85.5	83.3	82.4	88.5	94.1	93.4	93.4		80.8	80.1	78.7	78.5	77.6
11. Bos taurus	sp|Q3ZC33		82.3	86.4	85.9	84.3	82	88	92.5	92.7	92.7	91		96.6	92.3	92	90.0
12. Sus scrofa	sp|Q8HXW0		81.6	85.5	85	83.4	81.1	87.3	91.6	91.8	91.8	90	98.9		92	91.8	89.8
13. Mus musculus	NP_848862.1		81.8	85	83.9	83.4	81.8	87.3	91.6	91.4	91.4	90	96.8	95.7		98.6	95.0
14. Mus caroli	XP_021036878.1		81.8	84.8	83.9	83.4	81.8	87.3	91.8	91.6	91.6	90.3	96.8	95.7	99.3		
15. Rattus norvegicus	BAA02232.1		81.6	85.5	84.5	83.6	82	87.3	91.8	91.6	91.6	90.7	96.6	95.5	97.5	98	94.8

The conservation of the active sites and the main domains implies the conservation of the function of the protein while the phylogenetic tree, multiple sequence alignment and the identity and the similarity percentages confirm the protein relationship with other gulo orthologs.

The activity of the protein directly relies on its folding and the localization [76]. If the enzyme activity is directly affected if the active site is not appropriately folded in the correct orientation to makes a perfect binding site for the substrate and co-factors. This hindrance of the active site and the main domains of a protein would cause an inactive protein.

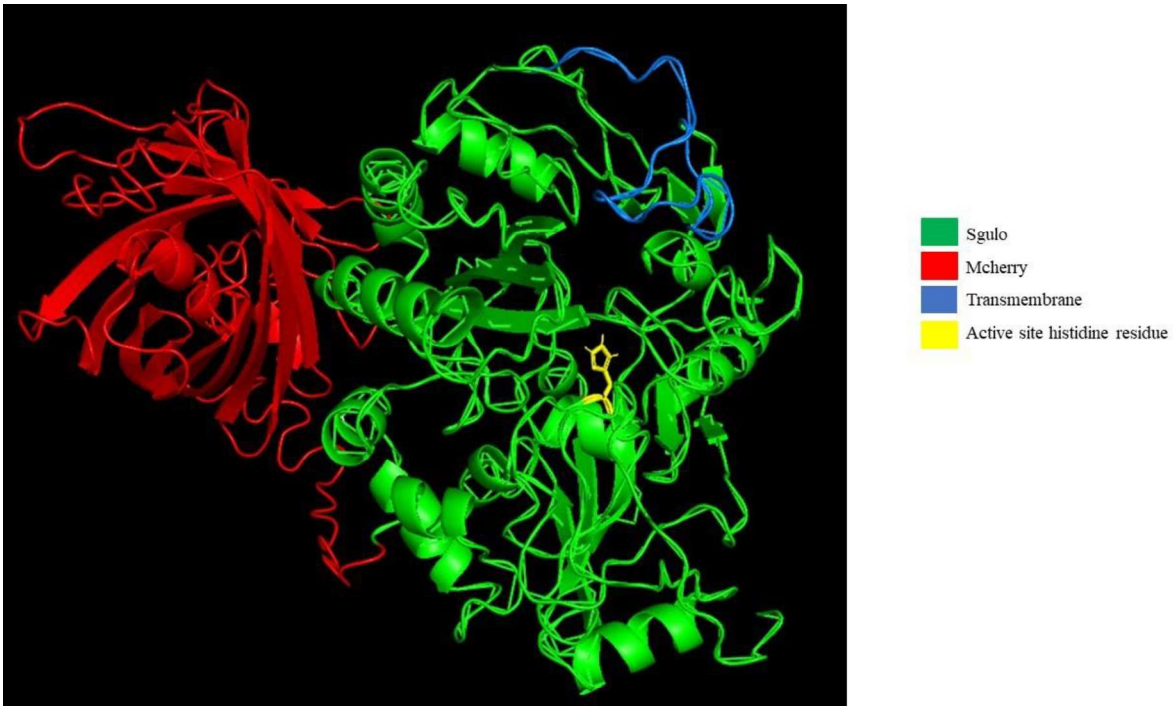

Figure 7. The predicted 3-D structure of Sgulo + mcherry fusion protein.
The 3-D structure of the Sgulo and mcherry fusion was predicted by I-TASSER online server. The green color showed the Sgulo, and the red color indicates the mcherry, the yellow residue demonstrates the active site histidine residue, and the blue chain is the transmembrane region of the Sgulo.

The Sgulo protein contains its active site histidine in the 54th position, and according to the 3-D structure prediction of Sgulo, mcherry fusion protein the active site histidine and the transmembrane region residues are not disturbed by the mcherry. This non-hindrance structure guaranteed the non-disturbed function of the Sgulo.

The gulo is a transmembrane protein which localized into the endoplasmic reticulum. For the function of the protein, this translocation and the transmembrane properties are inevitable. As shown in Figure 8, the transmembrane probability of the Sgulo is higher in the 250-275 aa region, and the N-terminal is predicted inside the membrane, and the C-terminal is predicted to be outside the membrane.

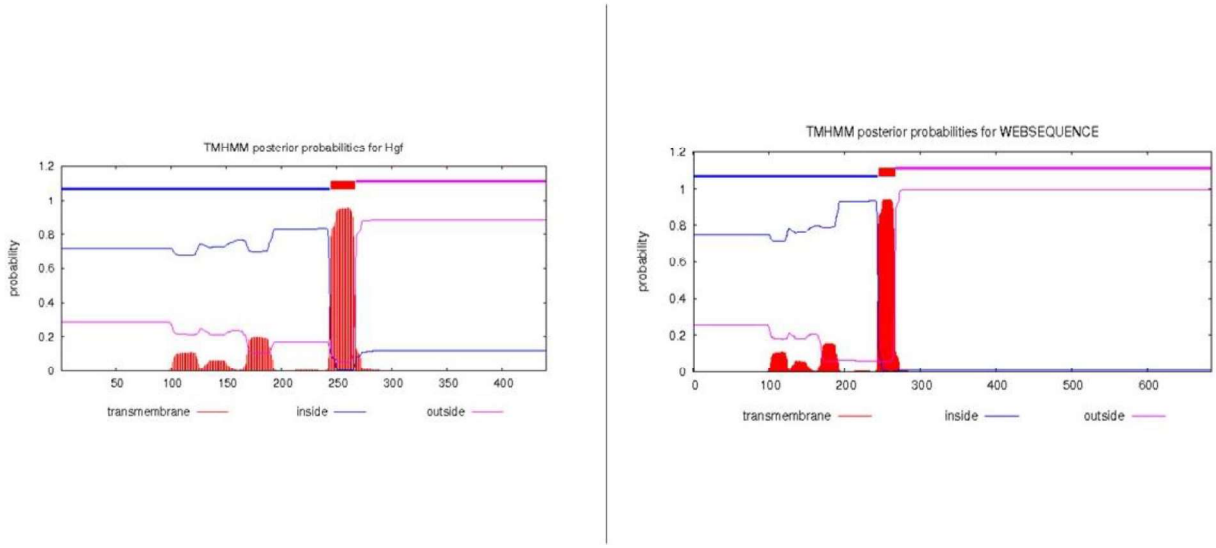

Figure 8.The transmembrane probability of both Sgulo and Sgulo + mcherry fusion.
The prediction of transmembrane helices in the Sgulo and the Sgulo fusion was assessed by the TMHMM server. The red spikes in the predicted graph show the transmembrane regions of the protein. The blue color bar indicates the protein part, which is in the inside of the membrane, and the pink color bar showed the region where the residues are located outside the membrane.

This result shows that the Sgulo is a transmembrane protein while around 251-273 residues are transmembrane. The transmembrane probability of the Sgulo, mcheery fusion showed a similar result which indicates that the mcherry also at the outside of the membrane like the C-terminal of the Sgulo and it did not affect for the localization or to the transmembrane region of the Sgulo protein.

3.2. Gulonolactonase expression

The penultimate step of the animal vitamin C biosynthesis is catalyzed by the gulonolactonase or regucalcin (SMP30) enzyme; it catalyzes the step which turns L-gulonate to L-gulono-1,4, lactone (Figure 2). The gulonolactonase expression of the healthy wild type zebrafish could be observed in all tested tissues except for the gill, as shown in Figure 9.

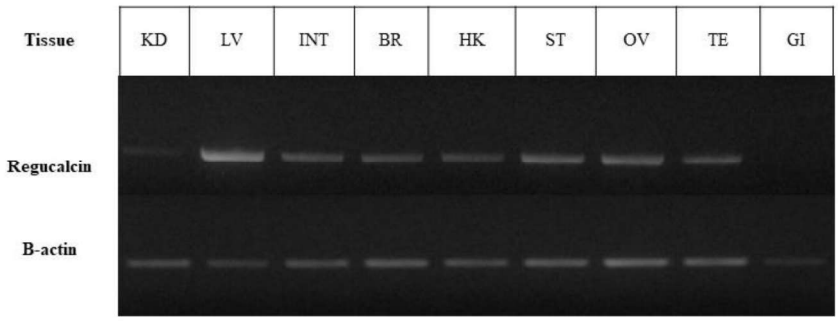

Figure 9. The relative expression of *gulonolactonase* (*Danio rerio)* in different tissues.
The zebrafish gulonolactonase expression in KD (kidney), LV (liver), INT (intestine), BR (brain), HK (head-kidney), ST (stomach), OV (ovary), TE (testis), GI (gill). The B-actin expression was used as the reference gene expression.

The selection of the b-acting promoter as our construct promoter was based on this result, because this result guides us to use a ubiquitous promoter as our construct promoter to express the Sgulo, mcherry fusion protein ubiquitously in the transgenic zebrafish.

3.3. Construct expression clone

As shown in Figure 10, the expression construct consists of 5'element, middle element, and 3'element. The poly A tail section is included after the 3' element, and the whole construct is flanked by two Tol2 elements.

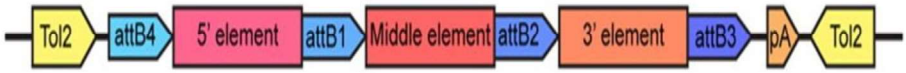

Figure 10.The diagram of construct expression clone.

The bactin2 promoter sequence was used as the 5' element and the *Sgulo* sequence as the middle element also the mcherry was used as the 3' element which acts as the reporter in the construct *Tg(bactin2:Sgulo-mcherry)*. The poly A segment includes to produce G cap mRNA

when the construct transcript its mRNA and the Tol2 sequences act as the transposable segment sequence, which needed to integrate the construct into the zebrafish genome.

3.4. Transgenic zebrafish

3.4.1. F0 generation

The transgenic zebrafish were obtained as described in the materials and method section 2.5. The F0 generation of the transgenic zebrafish was selected, and Figure shows the difference between the positive and negative F0 zebrafish larvae. The positive samples gave a mosaic mcherry expression which indicated by the mosaic pattern of the red fluorescent. The negative larvae did not show any red fluorescent. The red fluorescent implies the expression of the mcherry reporter, and it indicated the integration of the construct into the zebrafish genome. The mosaic nature of the positive larvae is due to the integration time of the construct into the zebrafish genome. Though we micro-inject the construct into the first cell stage of the zebrafish embryos, the integration could occur after the cell division. As a result, the expression could be mosaic because every cell will not receive the construct and the transposase mRNA.

The integration of the construct using the Tol2 sequence occur at random because large scale ET screen analysis has shown that there are around 338 Tol2 insertion sites in the zebrafish genome [77]. Therefore, after obtaining the F0 generation, we used only one F0 male to maintain the transgenic line. For this purpose, we identified the germline transmission of F0 generation, as shown in Figure , which was described in the materials and method section 2.5.3.

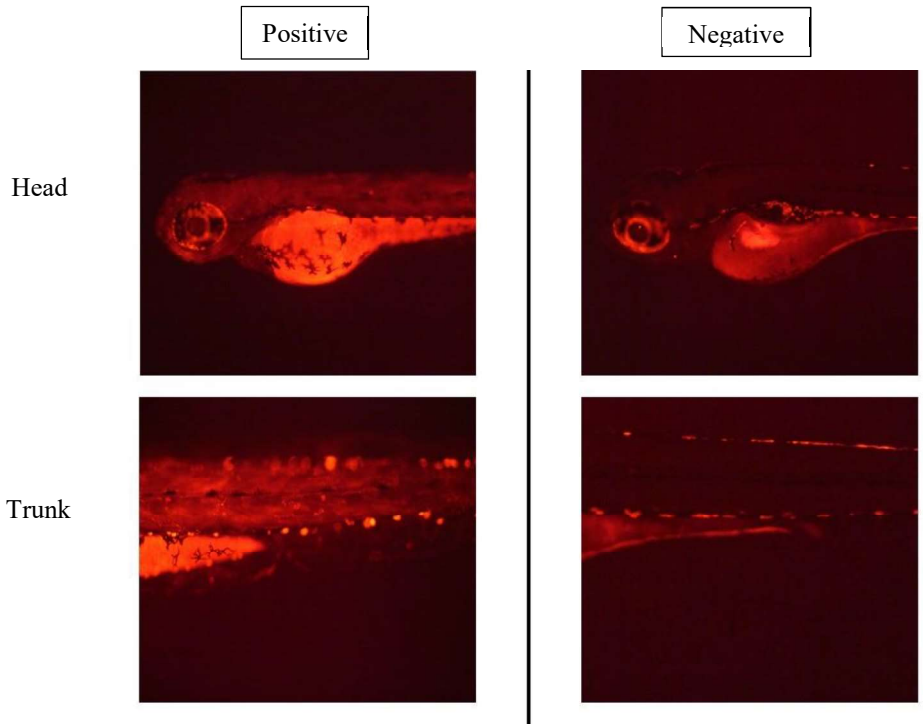

Figure 11. The F0 positive and negative selection.
The positive and negative F0 generation at 48 hpf, embryos were anesthetized using the tricaine and planted on a microscope slide using 2% cellulose afterward observed under the fluorescent microscope (Leica, DM600B) using (100x and 200x) magnifications.

Though the construct integrated into the zebrafish genome, the mosaic nature of the expression facilitates the non-integration of the construct into the germline cells. The integration of the construct into the germline cells plays a vital role in maintaining the transgenic zebrafish line.

F1 embryos

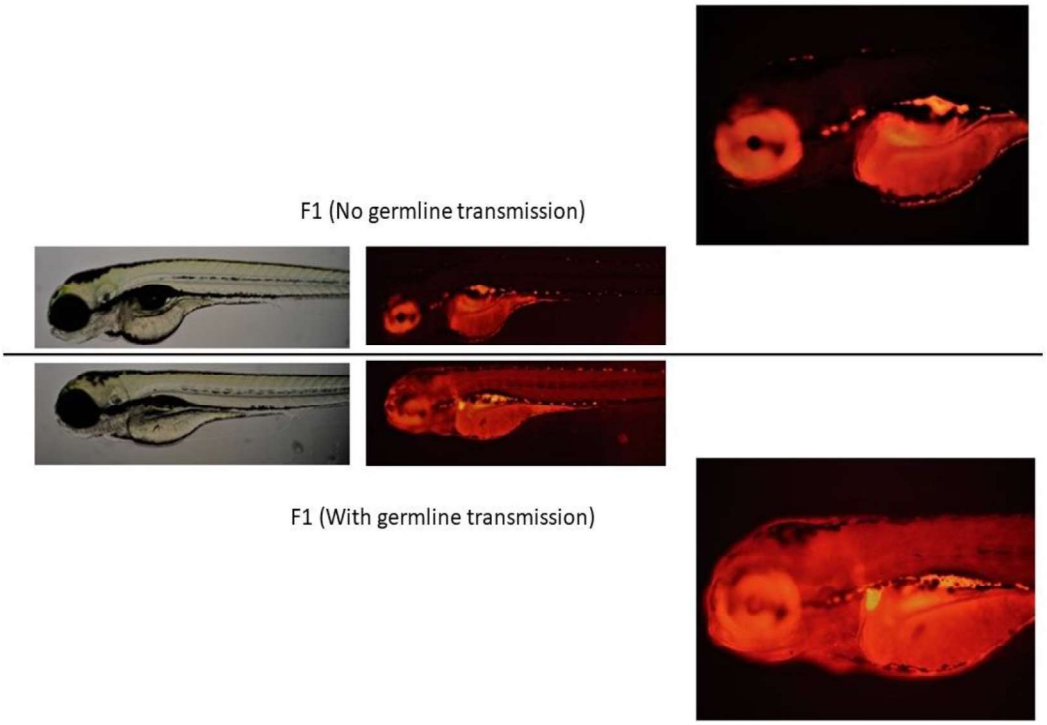

Figure 12. The identification of the F0 germline transmission.
The F1 generation observed under the fluorescent microscope, embryos were anesthetized using the tricaine and planted on a microscope slide using 2% cellulose and search for the red fluorescent. The F0 which gave the F1 with red fluorescent was identified as the germline transmitting F0

The F1 positive larvae indicate the germline transmission because we use a wild type of female fish for the breeding with F0 transgenic male. 36% of the F0 male gave a germline transmission, and from that, we used only one healthy F0 to obtain the F1 generation.

3.4.2. F1 generation

The results show the ubiquitous expression of mcherry in the F1 transgenic zebrafish larvae, while, wild type did not show any mcherry expression.

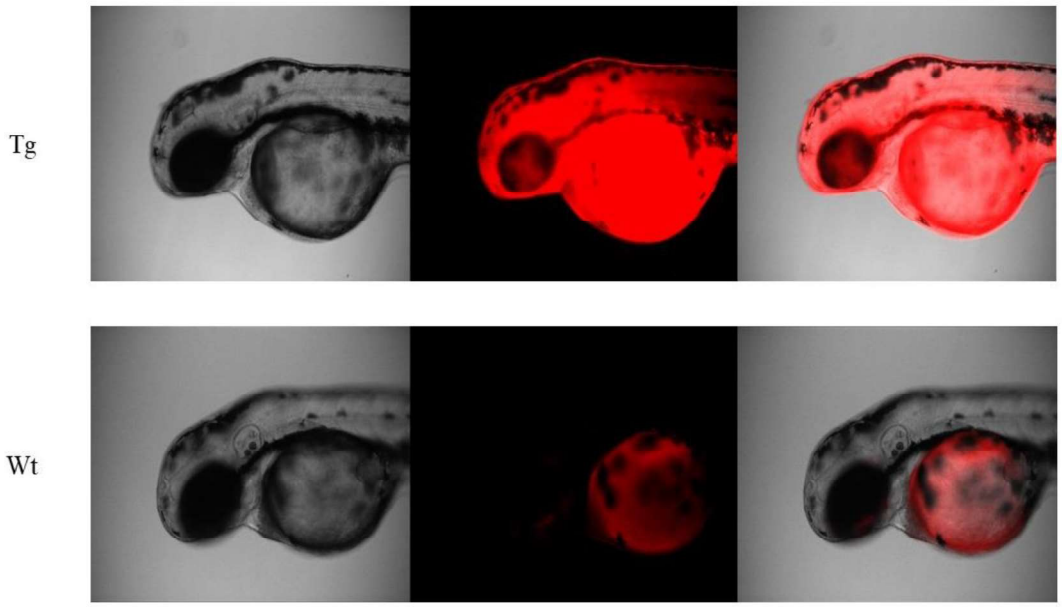

Figure 11. The F1 generation under a fluorescent microscope.
The 24 hpf embryos were anesthetized using the tricaine and planted on a microscope slide using 2% cellulose and observed under the fluorescent microscope (Leica, DM600B) in (100x) magnification.

These results indicate the Ubiquitous expression of Sgulo, mcherry fusion in every cell type of the F1 transgenic zebrafish larvae.

3.5. Genotyping and expression confirmation

The red fluorescent indicated the expression of mcherry. Also, the *Sgulo* gene integration in genomic level was confirmed by the genotyping. As shown in Figure 12**,** transgenic F1 generation gave the PCR band of *Sgulo* amplification while wild type did not give any PCR product which was conducted using *Sgulo* amplification primers with isolated genomic DNA.

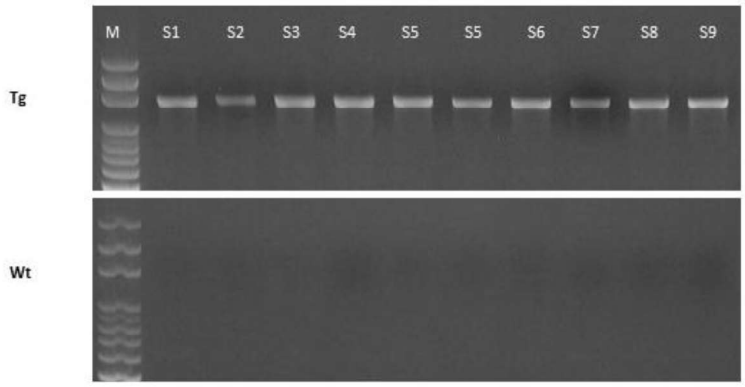

Figure 12. The gel image of the Tg and Wt F1 zebrafish genotyping
The PCR results obtained by the genotyping of transgenic (Tg) and wild type (Wt) zebrafish with M-100 bp marker, S1-S9 indicates the sample numbers.

These results revealed the exclusive genomic integration of the *Sgulo* into the zebrafish genome. The confirmation of the integration of *Sgulo* in genomic level led us to confirm the *Sgulo* expression in mRNA level. As shown in Figure 13, the transgenic zebrafish F1 showed the expression in RNA level while wild type showed negative results.

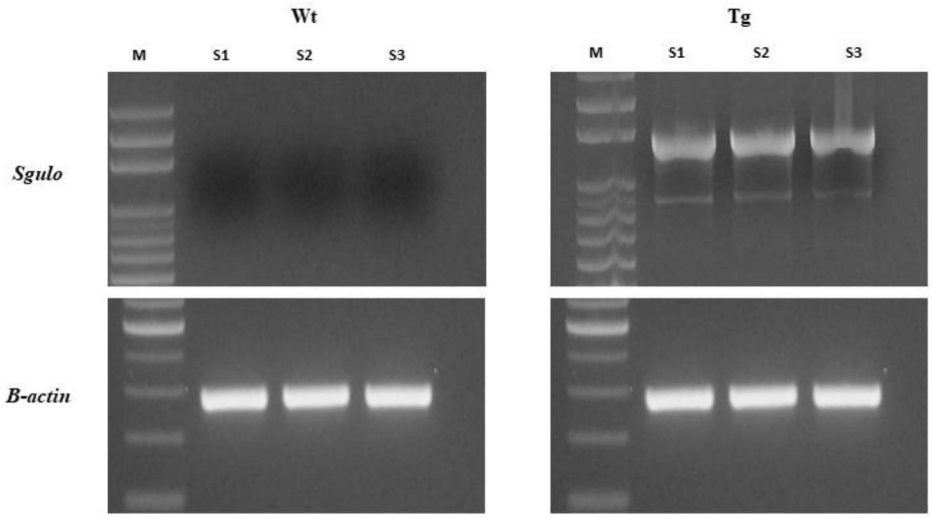

Figure 13. The Sgulo expression confirmation.
The gel images of the Sgulo expression confirmation of Tg and Wt zebrafish, b-actin was used as the reference gene for the experiment. M- 100 bp marker, S1- S3 indicates the sample numbers.

The results showed that the while b-actin was expressed in both Tg and Wt zebrafish, the *Sgulo* expression was only observed in Tg samples indicating both genomic integration and the transcriptional expression of *Sgulo* in transgenic F1 zebrafish. These genotyped confirmed transgenic and wild type F1 zebrafish was used to examine the developmental and physiological differences, Ascorbic acid content, and the differential gene expression between the transgenic and wild type zebrafish.

3.6. Development and physiological assessment

The developmental and the physiological differences between the transgenic and wild type zebrafish were assessed. The first experiment was to compare the physiological differences using zebrafish larvae under the microscope, and in the second experiment, the heart rate of the two groups was assessed.

3.6.1. The physiological difference

The zebrafish larvae of both transgenic and wild type zebrafish were observed under the light microscope, as shown in Figure 14. In all tested stages of the development (48,72, 96 hpf), there was no significant physiological difference between the transgenic and wild type group. These results indicate that the *Sgulo* expression in the Tg zebrafish larvae did not affect its early stage development positively or negatively. This may be because the *Sgulo* shows a neutral effect for the early stage development of the zebrafish. Also, even the *Sgulo* expression occurred in the zebrafish early stage development; we could not observe any physiological change due to the presence of enough ascorbic acid in zebrafish embryo yolk, which got by its mother. The embryonic yolk sac is the nutrient pool of zebrafish embryo [78], and it may be provided the sufficient amount of ascorbic acid in the early developmental stages so that, even though Sgulo expression occurred and produce ascorbic acid in the larvae, the excess ascorbic acid would remove from the body by the excretion mechanism. So as a result, there will not be any physiological changes between the transgenic and wild type zebrafish.

We compared the heart rate difference with Tg and Wt, because previous studies have shown that ascorbic acid increases the catecholamine levels, including norepinephrine and vasopressin [12]. The heart rate of the normal wild-type embryonic zebrafish closes to the human heart rate 120-180 beats per minutes (bpm) [79].

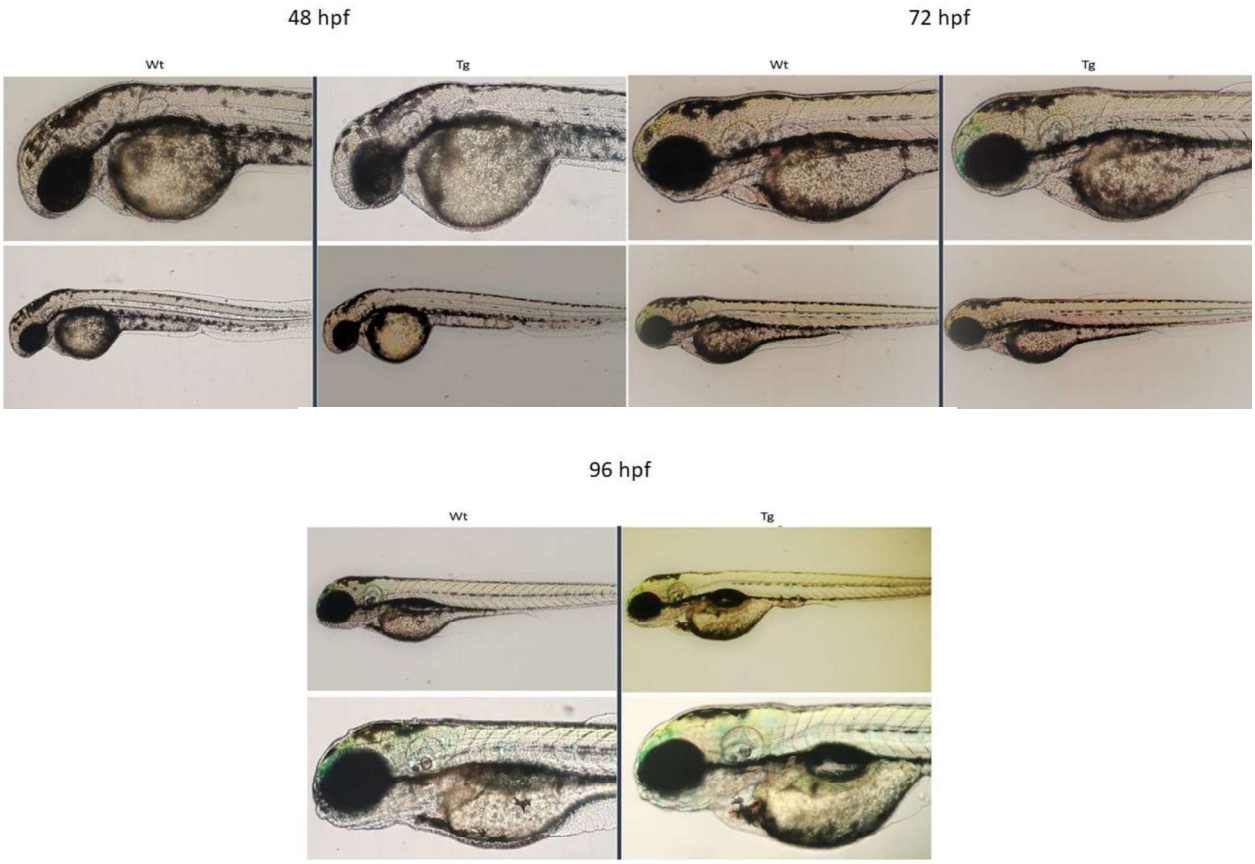

Figure 14. The developmental and physiological difference between Tg and Wt.
The Physiological differences in the developmental stages of zebrafish at 48, 72, and 96 hpf was observed under the light microscope (Leica, DM600B) at (100x and 200x) magnifications.

As shown in Figure 15, we analyzed the heart rate of both transgenic and wild-type zebrafish. The results were 142 ± 6 bpm for the transgenic group and 136± 4 for wild-type group. The results showed no difference in the heartbeat of the Tg and Wt group with compared to the normal recorded zebrafish embryonic heartbeat. These results showed that there is no significant physiological difference suggesting that *Sgulo* expression could not affect the early embryonic development of the zebrafish.

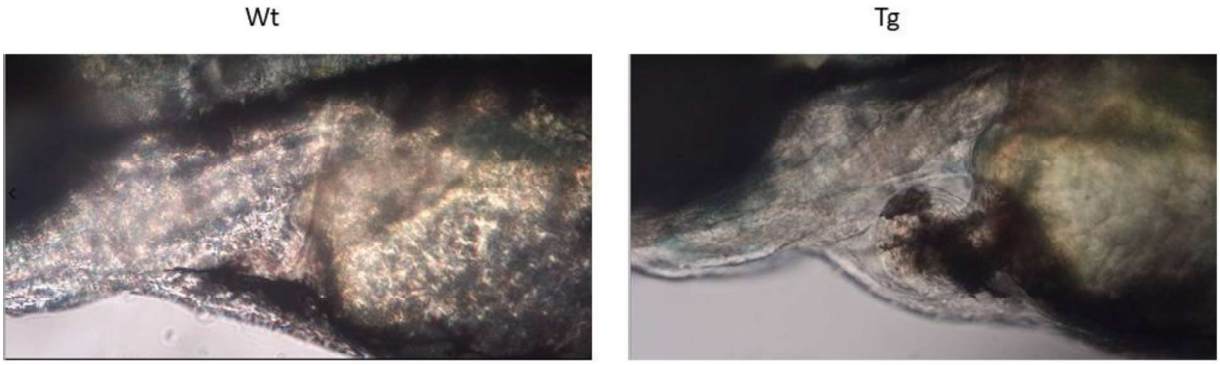

Figure 15. The images of the heart rate analysis video.
The images of the video photographed Wt and Tg heart function using Leica, DM600B microscope, and 400x magnification. The heart rate was counted for a minute using the video. The 96 hpf zebrafish were used for the experiment with 5 replicate samples.

3.7. Growth analysis

The weight difference of the transgenic and wild-type 3 months old adult zebrafish is shown in Figure 16. The growth analysis shows a 122 mg of average weight in wild-type while 151.3 mg weight in the transgenic zebrafish F1 generation. This average weight difference is a significant difference in three months old adult zebrafish.

The main function of ascorbic acid is to act as an antioxidant and cofactor for enzymes. It acts as the main cofactor for the ferrous and 2-oxoglutarate dependent dioxygenases[9]. The collagen synthesis is carried out by the family of two 2-oxoglutarate dependent dioxygenases where the lysin and proline residues of the procollagen hydroxylated [2]. Due to the *Sgulo* expression, vitamin C biosynthesis may be increased in Tg zebrafish, and as mentioned previously, it directly affects the collagen synthesis.

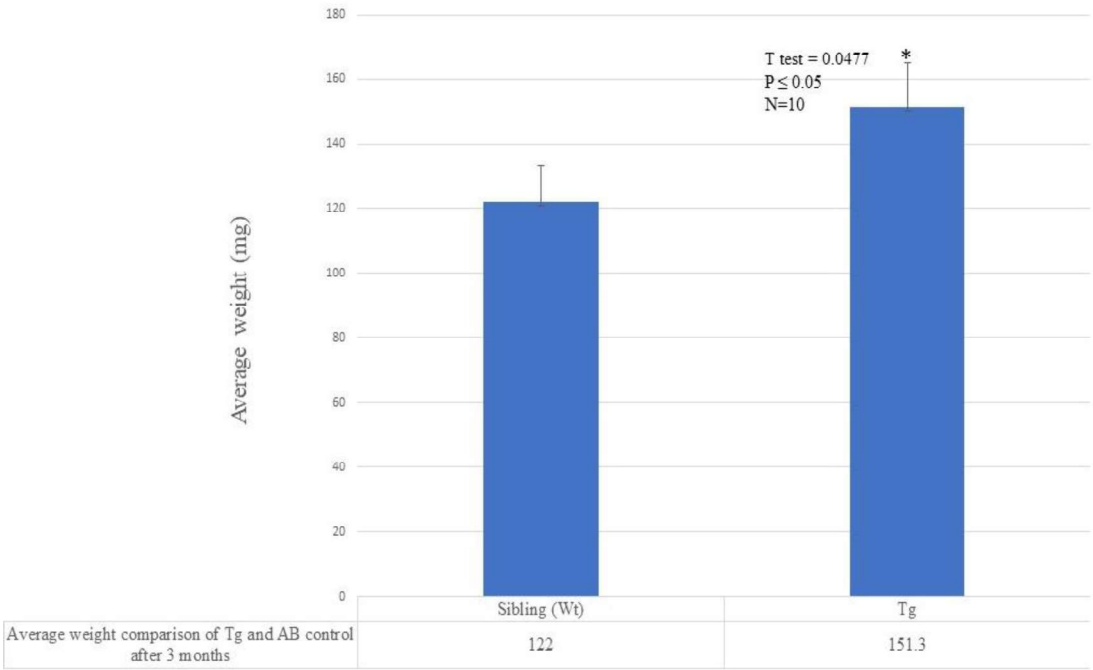

Figure 16. Average growth analysis.
The average growth was measured by the total body weight of 3 months old zebrafish (Wt and Tg). The error bars represent the standard deviation with experimental replicates (n=10). The significant difference between the Wt and Tg group is indicated by an asterisk (*) ($P<0.05$)

Also, collagen acts as the main component of the extracellular matrix, which gives a significant contribution to the weight of the organism. As a result, the weight of the transgenic zebrafish increased significantly.

3.8. Endogenous ascorbic acid

The endogenous ascorbic acid in the transgenic and wild-type zebrafish was quantified as mentioned in the materials and method section. It results in a significant ascorbic acid level difference between the wild type and transgenic groups.

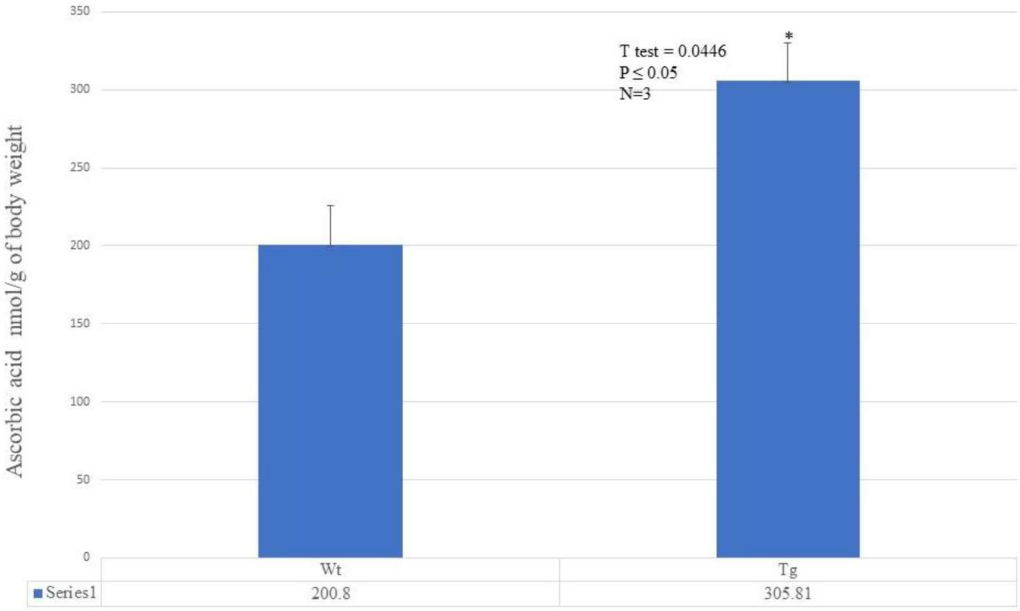

Figure 17. The endogenous ascorbic acid level in zebrafish.
The whole homogenized zebrafish was used to detect the endogenous ascorbic acid level. The experiment was carried out by the Oxi Select ascorbic acid detection kit, and the difference between the ascorbic acid levels of Wt and Tg fish shown by the graph. The error bars represent the standard deviation with experimental replicates (n=3). The significant difference between the Wt and Tg group is indicated by an asterisk (*) ($P<0.05$).

As shown in Figure 17, wild type fish showed 200.8 nmol/g ascorbic acid content while transgenic fish contains a 305.8 nmol/g ascorbic acid in the body. This significant difference in the ascorbic acid level in transgenic zebrafish could be occurred due to the production of ascorbic acid in its body. Previous studies on the ascorbic acid level in zebrafish also have shown a similar ascorbic acid level between 100-200 nmol/g in wild type zebrafish [80]. In our results, the increment of the ascorbic acid in transgenic zebrafish could be elaborated by the expression of *Sgulo,* which leads to the synthesis of ascorbic acid in the zebrafish body cells. Because the *gulo* is the only enzyme lack in the animal vitamin C synthesis pathway. The expression of *Sgulo*

fulfill that scarcity in the pathway, and we can conclude the synthesis of endogenous ascorbic acid in the transgenic zebrafish.

3.9. Differential gene expression

The gene expression profiles of catalase (CAT), hypoxia-inducible factor 1 (HIF1), superoxide dismutase 1 and 2 (SOD1, SOD 2), cytochrome b5a (cyb5a), and procollagen were analyzed using qPCR. Both wild type and transgenic zebrafish groups were used to analyze the gene expression in brain, kidney, liver, and muscle tissues. Figure 18 shows the differential gene expression results that we obtained from the analysis. The graph A shows the CAT expression between Wt and Tg.

Catalase is the main antioxidant in the organism's body, and it has been recorded that the liver shows higher expression in teleost fish [81]. It may be due to the higher metabolism rate in liver. The transgenic group shows higher expression in the liver than the Wt group. The catalase mainly involved in the decomposition of hydrogen peroxide [82]. As mentioned in the introduction ascorbic acid is involved in collagen synthesis, and it produces hydrogen peroxide as a by-product, also in the synthesis of ascorbic acid hydrogen peroxide is formed therefore to neutralize the hydrogen peroxide the CAT level could be increased in the transgenic zebrafish. The HIF1 differential expression is shown in graph B, which indicated significant downregulation in the liver and upregulation in the muscles in transgenic fish.

The HIF1 expression is essential in the hypoxic (low O_2) conditions where the molecular oxygen level is down in the cells [83].

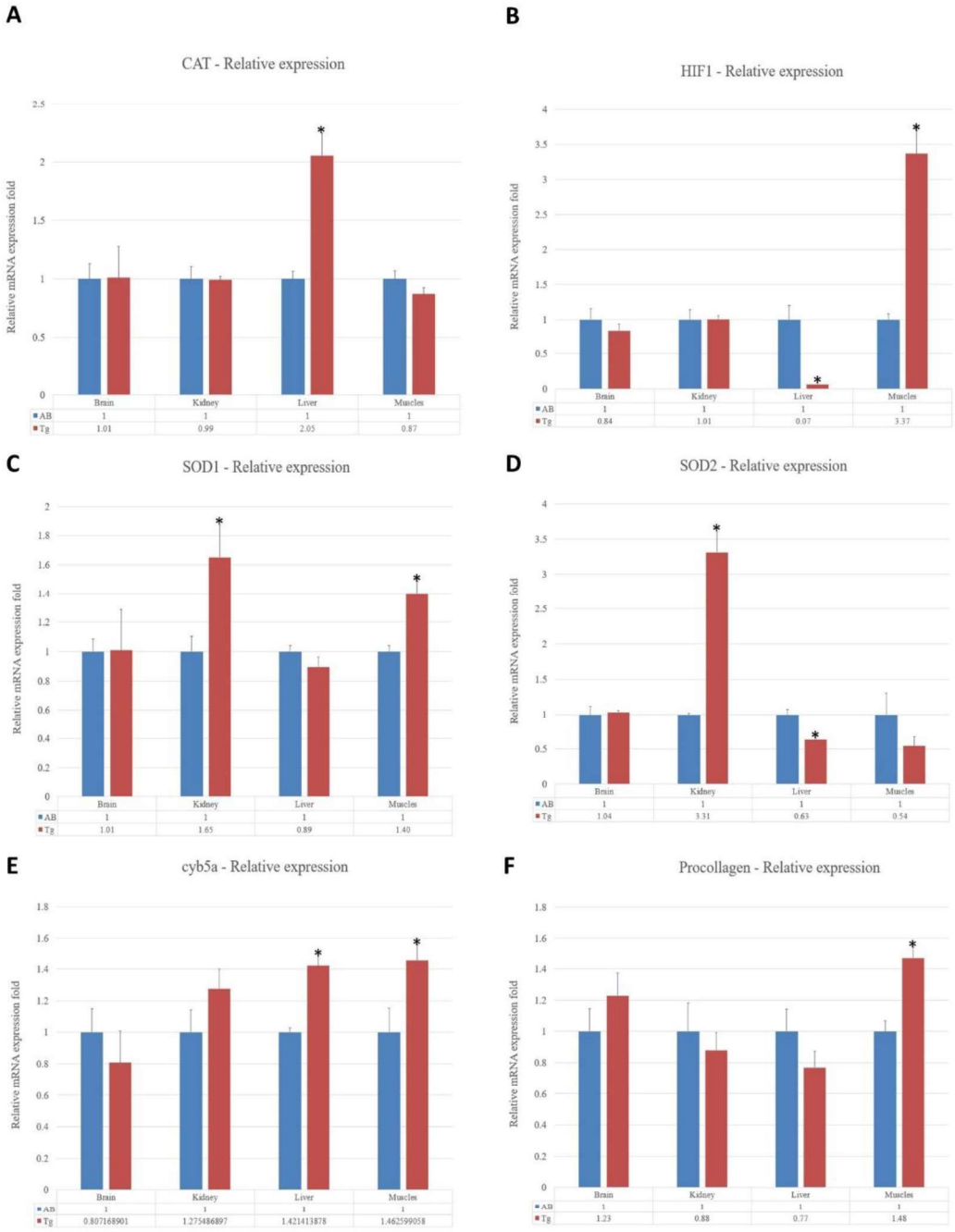

Figure 18. Deferential gene expression profile of AB wild-type and Tg fish

The differential gene expression profiles of Wt and Tg zebrafish in brain, kidney, liver, and muscle tissues. The mRNA expression was measured by qRT-PCR and evaluated by the Livak method [74]. *Danio rerio* EF1-α was used as an internal control. Error bars represent the standard deviation with experimental replicates (n=3). Significant differences between Tg groups and AB wild type control groups are indicated by an asterisk (*) ($P<0.05$)

The reason for the down-regulation of HIF1 in the Tg liver could be the high oxygen concentration in the liver, which resulted in the high decomposition of hydrogen peroxide from catalase. When the hydrogen peroxide decomposes it produces water and O_2 as products, this O_2 concentration could be the result of the down-regulation of HIF1 in the liver of Tg zebrafish. Also, the up-regulation of HIF1 in muscles of the Tg fish could be due to the O_2 deprivation from the muscles. Because the collagen synthesis needs O_2 to synthesize collagen and collagen is essential for the muscle tissues, and also, the energy metabolism in the muscles are higher in the fish due to the constant swimming. Due to these reasons, the O_2 level in the muscle tissues of the Tg zebrafish could be lower than the Wt fish, and as a result, HIF1 could be upregulated to maintain the homeostasis. The previous studies also have shown the HIF1 upregulated expression in more oxidative muscles [84]. The graph C and D clearly shows the differential SOD expression in the Wt and Tg groups. The SOD1 is upregulated in kidney and liver also SOD 2 is upregulated in the kidney and down-regulation in muscles. SOD's are potent anti-oxidants which oxidized the superoxide radicals in the cellular environment. The increment of SOD's in Tg zebrafish rivaled an increased antioxidant property in Tg fish, which have been shown in the previous study by providing dietary vitamin C to yellow catfish [85]. In this study, researchers have shown a significant increment of the antioxidant capability of catfish in terms of SOD's by providing dietary ascorbic acid. The high levels of SOD in the kidney may result because of the high oxidative stress in the Tg zebrafish. Because, as previously mentioned, catalase activity is higher in the liver, which indicates a high O_2 production in the liver. The produce O_2 will be converted to superoxide by NADPH oxidase this produced superoxide could be converted to hydrogen peroxide by the SOD's [86]. Due to these reasons, the kidney SOD levels could increase to balance the oxidative stress in the kidney of Tg zebrafish. The cyb5a

(cytochrome b5a) is a major enzyme which reconverts the partially oxidized form of ascorbate (semi-dehydroascorbate) into ascorbate [87]. This may be the reason for cyb5a upregulation in the transgenic zebrafish liver and muscles in Figure 18 graph E because the liver and the muscles are the main organs which provide us the evidence of ascorbic acid biosynthesis in transgenic zebrafish.

The procollagen is the primary precursor for the synthesis of collagen; Figure 18 graph F shows an exclusive increment of procollagen in transgenic zebrafish muscles than the Wt. Several previous studies have shown the enhancement of procollagen mRNA due to the involvement of ascorbic acid [22,23]. With all these results of differential gene expression of CAT, SOD1 and SOD2 revealed increase antioxidant capacity in transgenic zebrafish, and HIF1 expression gave substantial evidence on oxygen concentration and the production inside the cells of liver and muscle. The procollagen mRNA expression demonstrated a significant upregulation in transgenic zebrafish muscle tissues indicating the increment of collagen synthesis. With all these results, the gene expression analysis provides substantial evidence for the synthesis of ascorbic acid in the transgenic zebrafish model.

3.10. Sgulo enzyme activity

In section 3.8, we showed the endogenous ascorbic acid level in the transgenic zebrafish. Also, we examined the Sgulo enzyme activity using an in-vitro experiment. The isolated crude protein solution showed an activity of 535 $\mu M\ g^{-1}\ h^{-1}$ ascorbate concentration, while the wild-type protein extract did not show any activity.

The wild type group did not show any significant activity because Wt group do not consist the gulo activity in its proteome profile, but in the transgenic zebrafish in its proteome profile Sgulo

is present, and it showed the gulo function by converting the substrate L-guluno-1,4-lactone to L-ascorbic acid.

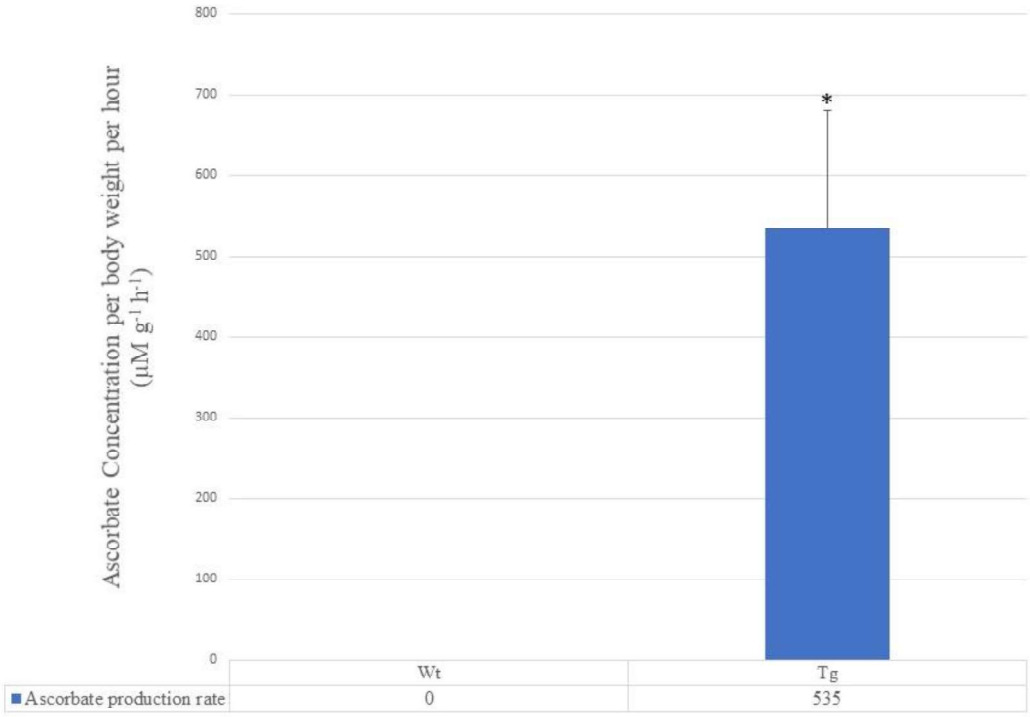

Figure 19. The Sgulo enzyme activity.
The enzyme activity was measured by a colorimetric method established in a previous study using DNPH. The concentration values were obtained by a standard curve constructed before the experiment, and the absorbance values of the Tg group was measured taking Wt group as the blank and reducing the endogenous ascorbic amount from each Wt and Tg group. Error bar represents the standard deviation with experimental replicates (n=3).

The produced ascorbic acid (ascorbate) react with the DNPH solution and form a colorful osazone which gives a maximum absorbance at 524 nm [75,88,89]. With these results, we can confirm the activity of Sgulo in the transgenic zebrafish model.

4. Conclusion

We were able to create a transgenic zebrafish by integrating L-gulonolactone oxidase gene to its genome, which was isolated by cloudy catshark (*Scyliorhinus torazame*). The integrated Sgulo was expressed in the transgenic zebrafish in ubiquitously using b-actin promoter and was observed the expression using the red fluorescent of the mcherry reporter. The developmental and physiological stages of the early developmental stage did not show any difference between the Wt and the Tg fish. The introduced Sgulo was showing its activity in-vitro, and we observed the increment of endogenous ascorbic acid levels in transgenic zebrafish also, in our differential gene expression analysis Tg zebrafish showed a potential degree of influence towards the gene expression of CAT, HIF1, SOD1, SOD2, cyb5a, and procollagen. With all these experiments and results, we could conclude the biosynthesis of ascorbic acid in transgenic zebrafish, which was introduced a foreign *gulo* gene to its genome and filled the blank in the ascorbic biosynthesis pathway. We have succeeded in our attempt to re-establishing the lost function of ascorbic acid biosynthesis by introducing the lost gene in the pathway.

The future aspect of this research would be to analyze the samples with high precision quantification methods such as HPLC and establish the transgenic model as regenerative, toxicological, immune and behavioral, therapeutic and cancer research model. Also, these findings have potential to be used the economic model in fish aquaculture because in fish aquaculture, vitamin C is heavily used in feed production and this brings up an alternative aspect to reduce the dietary vitamin C in fish feed.

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