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# Cflarb Complemented the Function of Cflara to Allow Cflara Knock out Zebrafish to Normal Development

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## Abstract

Cellular FLICE-inhibitory protein (cFLIP, cflara) is a regulator of death receptor (DR)-induced apoptosis and NF-κB activation. cFLIP is known to prevent activation of the caspase cascade by binding to FADD/caspase-8. Up-regulated cFLIP has been identified in many tumor types, and therefore restoring apoptosis by silencing cFLIP may be one of the more potent strategies in cancer therapeutics. The zebrafish cFLIP gene, cflara, has 2 death effector domains (DEDs) and a single caspase-like domain. Expression of cflara was detected in the zebrafish embryo by RT-PCR and whole-mount *in situ* hybridization. To study the *in vivo* function of cflara, we generated a cflara knockout mutant zebrafish using transcription activator-like effector nucleases (TALENs). Frame shift mutation is caused by a 10 bp deletion in the first DED domain. By inbreeding the F1 generation, a homozygous mutant fish was produced and confirmed by PCR. Knockout of cflara leads to abnormal heart development and embryonic lethality in mice. However, mutant zebrafish did not show any differences from wild type in heartbeat rate, survival rate or development. Zebrafish have analogues of both cflara and cflarb. Quantitative PCR showed that cflarb mRNA levels of mutant zebrafish were higher than those in the wild type. In a chemical exposure experiment, mutant zebrafish larvae showed a longer survival rate compared with wild type after $CoCl_2$ treatment. However, no significant difference was observed from cisplatin treatment. This data suggests that cflarb may contribute to normal development and causes a difference in chemical resistance.

**Keywords:** Zebrafish; cflar; TALEN; Cisplatin; Cobalt chloride

## Introduction

Apoptosis is known as programmed cell death and various stresses induce apoptosis through death receptor (DR) signaling [1,2]. DRs, TNF-α, TNFR1, Fas and TRAIL bind with their ligand and subsequently initiate apoptotic signaling by oligomerization with the adaptor protein FADD, via its death domain (DD) [2]. FADD has a death effector domain (DED) that binds to the DED of the intracellular protein, such as procaspases. During apoptotic signaling, receptors, adaptor proteins, and procaspases form a complex called the death-inducing signaling complex (DISC). After binding to DISC, procaspases homodimerize and generate active caspase by autocatalytic cleavage [3].

Cellular FLICE-inhibitory protein (cFLIP) is a well-known inhibitor of death receptor mediated apoptosis [4] and aberrantly expressed in various cancers. The cFLIP gene has 13 splice variants and 3 protein isoforms, cFLIPR (24 kDa), cFLIPS (short form; 26 kDa) and cFLIPL (long form; 55 kDa) [5–7]. cFLIP has 2 DEDs and cFLIPL has 1 more domain, called the caspase-like domain [8]. cFLIP has an anti-apoptotic role by binding to DISC with its DED and inhibiting the recruitment or caspase cleavage of procaspase 8 [3]. Upon heterodimerization with procaspase 8, cFLIP cleaves and produces p43-FLIP and p22-FLIP [5,6,9]. In addition to its anti-apoptotic role, these cleavage products have roles in the regulation of NF-κB signaling [9,10].

By protecting apoptosis from DR signaling, cFLIP plays various role in embryonic development and the maintenance of homeostasis [11,12]. cFLIP is required for normal development in mice [11]. Previous studies have shown that mice deficient in FADD, caspase-8 and cFLIP undergo embryonic lethality at embryonic day 10.5 (E10.5) because of a failure in yolk sac vascularization [11,13,14]. cFLIP has a role in the homeostasis of various tissues, such as the skin, intestine, and liver, by preventing apoptosis [15]. Additionally, in various cancers, increased expression levels of cFLIP have been observed [16]. cFLIP expression decreases apoptosis caused by anticancer drugs, such as cisplatin and death ligands [17].

Zebrafish have been used as a model animal to better understand the function of human disease-related genes [18–20]. Up to 71.4% of human genes have a zebrafish orthologue, including cflara an orthologue of the human cFLIP gene [21]. The alignment of the amino acid sequences of human and zebrafish cFLIP showed high similarity in 2 DED motifs and an inactive caspase-like domain. Prediction of the protein structure indicates that the tertiary structure of the cFLIP is well conserved from zebrafish to human and zebrafish cFLIP is able to induce NF-κB activation and inhibit the extrinsic apoptotic pathway [22].

Transcription activator-like effector nucleases (TALENs) are sequence-specific nucleases that are composed of FokI endonuclease and DNA-binding domains [23]. TALENs induce site-specific DNA double-strand breaks (DSBs) and occur in homologous recombination (HR) or non-homologous end-joining (NHEJ). NHEJ reconnects DNA and causes small insertions or deletions. In zebrafish, TALEN-mediated mutant fish can be generated by microinjection of fertilized egg [24,25].

To study the *in vivo* function of the cflara protein in tumor formation, we generated cflara knockout mutant zebrafish using TALEN. The survival rate, development and heart rate of mutant fish were evaluated. Furthermore, we identified the gene expression and response to chemical stimuli. Mutant zebrafish had normal development and showed high mRNA and protein expression level of the cflara analogue gene, cflarb. In summary, we demonstrated that cflara mutant zebrafish have analogue gene expression and have a different stress response to wild type.

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## Materials and Methods

### Zebrafish maintenance

Wild type AB strains of zebrafish were maintained in aged tap water at 28°C and under a controlled light cycle (14 h light /10 h dark cycle). Fish were fed brine shrimp twice daily. Zebrafish embryos were produced by natural spawning and raised in the egg water (0.6 g/L Instant Sea Salt, 0.01 mg/L methylene blue) at 28°C. All zebrafish experiments were conducted according to the ethical guidelines of Konkuk University Institutional Animal Care and Use Committee, South Korea. Embryonic stages were determined by the number of hour post-fertilization (hpf), days post-fertilization (dpf), and microscopic observation of morphology.

### Isolation of the zebrafish cflara gene

Zebrafish cflara (ZFIN:ZDB-GENE-030826-3) was amplified using PCR (forward primer 5'-GAAGCTCGCTGCTGGCAAAACC-3', reverse primer 5'-GACGCGCTCCTTTCTGAGAATTCC-3'). The amplified PCR product was cloned into a T-easy vector (Promega, USA).

### Establishment of the zebrafish cflara mutant

The TALEN vectors were designed for targeting the third exon of cflara and constructed by ToolGen (Korea, www.toolgen.com). TALEN vectors were linearized and subsequently purified by ethanol precipitation. TALEN mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion, USA) and purified using phenol/chloroform precipitation. One-cell embryos were microinjected with 750 pg of TALEN mRNAs and developed to 2~3 dpf. Genomic DNA of each larva and adult fins were extracted with a genomic DNA isolation buffer (10 mM Tris, 50 mM EDTA, 200 mM NaCl, 0.5% SDS, 0.5 mg/ml Proteinase K) for 12 h at 55℃ and purified using phenol-chloroform precipitation. PCR was performed using purified genomic DNA and cflara exon 3 region primers (forward primer 5'-GTGACCGCATCTCTTAGCAGCG-3', reverse primer 5'-GACGCGCTCCTTTCTGAGAATTCC-3'). PCR products were purified using a Gel extraction kit (Elpisbiotech, Korea) and a T7 endonuclease I (New England Biolabs, USA) assay was performed.

### Whole-mount *in situ* hybridization

A DIG-RNA labeling kit (Roche, Germany) was used to synthesize the digoxigenin-labeled antisense RNA probe for cflara according to the manufacturer's instruction. Embryos were fixed with 4% paraformaldehyde (PFA) in PBS with 0.1% Tween 20 (PBST) overnight at 4℃ and were subsequently washed in PBST. Embryos were then prehybridized for 1 h at 65°C in hybridization buffer [formamide (50%), saline-sodium citrate (SSC; 5X), heparin (50 µg/mL), total RNA (500 µg/mL), Tween 20 (0.1%)]. DIG-RNA probes with embryos were hybridized in hybridization buffer for 16 h at 65°C. Solutions were removed and washed in 2X, 0.2X SSC and PBST. For antibody staining, embryos were incubated with AP-conjugated anti-DIG antibody (Roche, Basel, Switzerland) overnight at 4℃. Embryos were then washed for 30 min, 10 times in PBST and the solution was finally replaced with staining buffer [Tris–HCl (100 mM; pH 9.5), NaCl (100 mM), and $MgCl_2$ (50 mM)] for 30 min, 3 times. Antibody was detected by incubation of AP staining solution [Tris–HCl (100 mM; pH 9.5), NaCl (100 mM), $MgCl_2$ (50 mM), NBT (4.5 µL/mL), BCIP (3.5 µL/mL)] with the embryos. Embryos were mounted in glycerol and visualized under dissecting microscope.

### Quantitative PCR and western blotting

Zebrafish samples were collected at various ages to extract total RNA using the RNeasy Mini Kit (Qiagen). 1 µg of total RNA was used to generate cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using primers for cflara (forward primer 5'-AGGAGATTTGCTGGCCTTTGC-3', reverse primer 5'-CACATCTGCCATTAGAACCCTG-3') and cflarb (forward primer 5'-CTGCACACACTCGCTGTGC -3', reverse primer 5'-CAGGTCCTCCGTGTCCATCTCC-3'). The quantitative real-time reaction was performed using the Fast SYBR Green Master Mix Real-Time PCR Master Mix (Applied Biosystems).

For the western blot analysis, Zebrafish embryo and adult were collected and washed in ice cold PBS, and then homogenized in RIPA buffer and stand on ice for 10 min. Homogenates were centrifuged in 13,000 rpm at 4°C. Supernatants were collected and protein concentration was measured by BCA assay. Equal amount of proteins were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 10% β-mercaptoethanol) and boiled at 95°C in 5 min. Protein was separated on SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were blocked with 5% skimmed milk in TBST buffer (20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 30 min at room temperature. And membranes were incubated with the polyclonal peptide antibodies and anti-GAPDH (LF-PA0018, Ab frontier) for overnight at 4℃ and then probed with secondary antibodies. The cflara and cflarb polyclonal antibodies were raised against synthetic peptides derived from the intermediate region of zebrafish cFLIP proteins (TLVNKVTASLSSDESKILLYLCTDLF for cflara, CGALDTHSELTHSSELRVCTHSLLCR for cflarb; ZDB-GENE-091204-464). The probed membranes were visualized using an ECL detection kit by Luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

### Survival rate

Zebrafish embryos at 1 dpf were manually decorionated using a stereomicroscope and transferred to 6-well plates. Cisplatin and cobalt chloride ($CoCl_2$) stock solutions were made by dilution with deionized water. Each group was exposed to several doses of Cisplatin/$CoCl_2$. The treatment was refreshed daily and continued until 7 dpf. Each group has at least 20 embryos.

### Heart rate

Zebrafish larvae at 2, 3 dpf were used for heart rate measurements. To minimize the effect of outside temperature on heart rate, embryos were kept in a 28°C incubator, and moved individually for measurement. Heart rates were measured by direct observation under a stereomicroscope for each embryo for 1 min.

### Statistical analysis

Data are summarized as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using a Student's *t*-test, and *p* values of less than 0.05 were considered to be statistically significant.

## Results

### Expression pattern of cflara during early development of zebrafish

To define the temporal expression pattern of cflara gene in zebrafish, we examined zebrafish egg, embryo, and larvae by RT-PCR. The cflara gene was expressed in fertilized eggs (1-cell stage) and continued to the protruding-mouth stage (72 h) (Figure 1A). This result suggests that zebrafish cflara transcripts originated from maternal transcription, and zygotic transcription continued during later development. Whole-

mount *in situ* hybridization analyses revealed that cflara transcripts were ubiquitously and uniformly expressed in the embryo during the early embryonic stages (Figure 1B). At 36 h, cflara transcripts showed expression restricted to the brain region. From 48 h to 72 h, cflara was expressed in the heart and gut regions (Figure 1B). This expression pattern was similar to those observed in a previous cFLIP mouse [11].

## TALEN induced mutagenesis in cflara gene

To establish the zebrafish cflara mutant, we used the TALEN method, wherein the cflara gene consists of 11 exons and a TALEN target site located in exon 3. Consequently, we obtained the cflara$^{cnu024}$ line with a 10 base pair deletion form in the TALEN target sequence and cflara$^{cnu024}$ mRNA translated into 61 amino acids, whereas cflara mRNA translated into 418 amino acids (Figure 2A). By inbreeding the heterozygous F1 generation, 7 knockouts homozygous F2 mutant embryo was produced (Figure 2B). Only three representative F2 generation adult fish for each genotype (male and female KO, and heterozygous) were confirmed by PCR (Figure 2C, right). The schematic indicates the PCR band size of the cflara gene (Figure 2C, left). PCR results showed a 10-bp smaller band size in mutant fish.

## Characterization of cflara mutant zebrafish

All mutant larvae exhibited normal morphology, compared to wild type fish. Furthermore, the mutant fish did not show marked differences compared with wild type in terms of their survival rate (Figure 3A). No morphological abnormalities were observed (Supplementary Figure 1). Previous experiments showed cflara expression in the heart region (Figure 1B); however in the current study the heart rate of mutants showed no marked difference compared with wild type fish (Figure 3B). Even though

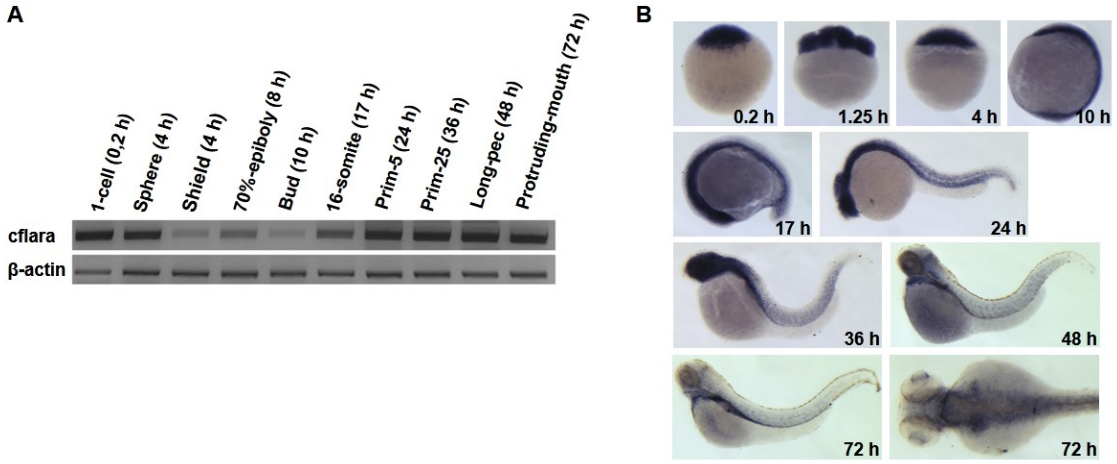


**Figure 1:** Expression profiles of cflara during early development of zebrafish. (A) Cflara transcript was detected from the 1-cell stage to the protruding-mouth stage (72 h). β-actin was used as a loading control. (B) *In situ* hybridization of cflara transcript. Cflara was ubiquitously expressed from the 1-cell stage to 10 h. From 17 h to 36 h, cflara expression was most intense in the head region. At 72 h, gene expression was observed in the heart and gut regions.

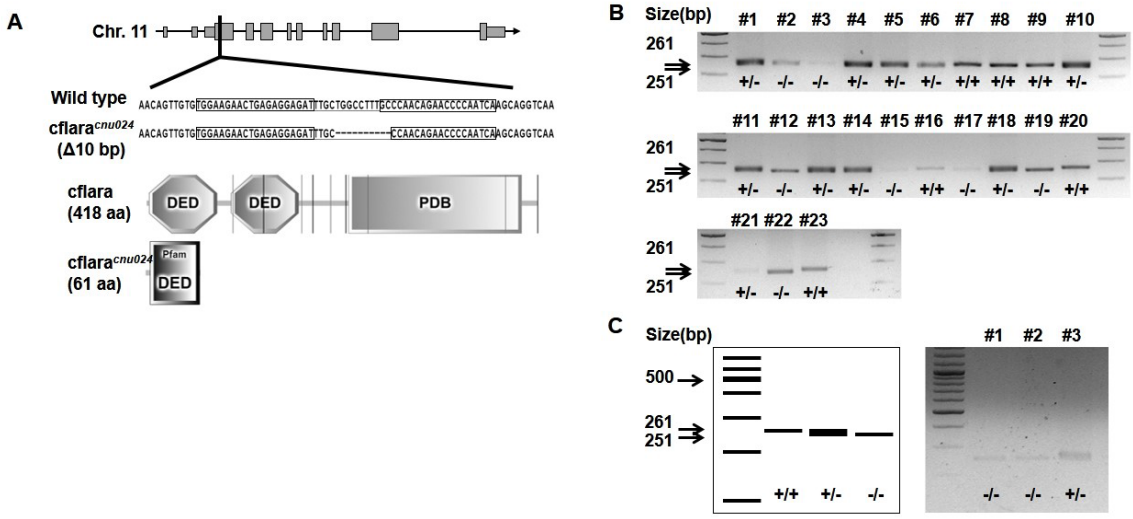


**Figure 2:** Schematic diagram of zebrafish *cflara*$^{cnu024}$ mutant. (A) Schematic representation of the genomic structure of cflara, with coding and untranslated exons described as solid and open boxes, respectively. The location of the TALEN target site in exon 3. Left and right TALEN binding sites marked as box and target sequences underlined with a dotted line. Domain architecture of wild type and mutant forms. The wild type cflara protein consists of 418 amino acids and 2 DED domains (upper). The cflara$^{cnu024}$ protein consists of 61 amino acids and 1 truncated DED domain (bottom). (B) Genotyping result of each F2 embryo. (C) Genotyping schema of the cflara gene (left). PCR result of the genotype of 3 adult F2s (right).

we did not perform histological analysis, these results suggest that heart function and development in mutant fish were normal. Next, we performed Cisplatin and $CoCl_2$ treatment and determined the survival number of fish daily. In the $CoCl_2$ treated group, mutant fish showed a significantly longer survival time, compared with wild type fish ($p<0.05$). However, the Cisplatin treated group showed no difference in survival time between mutant and wild type fish. The mechanism by which $CoCl_2$ treated mutant fish underwent an increase in survival rate, compared with wild type, remains to be elucidated and will require further study.

## Gene expression change in mutant zebrafish

Zebrafish has an analogue of the cflara gene, cflarb. Since mutant fish showed normal development, we believe that there is a possibility of cflarb expression in mutant fish. To investigate the expression levels of cflara and cflarb in zebrafish relative to β-actin and GAPDH, quantitative PCR and western blotting were performed in embryos and adult zebrafish. As shown in Figures 4A and 4D, the cflara expression levels of mRNA and protein were higher in wild type adults, compared to embryonic expression level. Furthermore, expression of

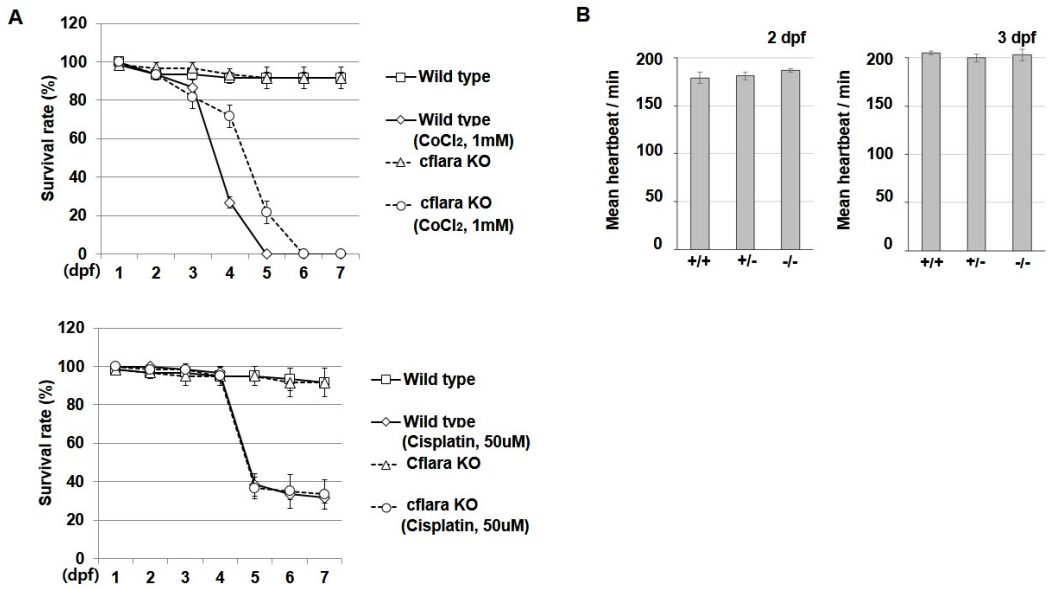


**Figure 3:** Survival rate and heart rate measurement. (A) Survival rate curve of $CoCl_2$ and Cisplatin exposure treatment. In the $CoCl_2$ group, mutants had a longer survival term than wild type fish. (B) Heart rate measurement of mutant embryo to 3 dpf.

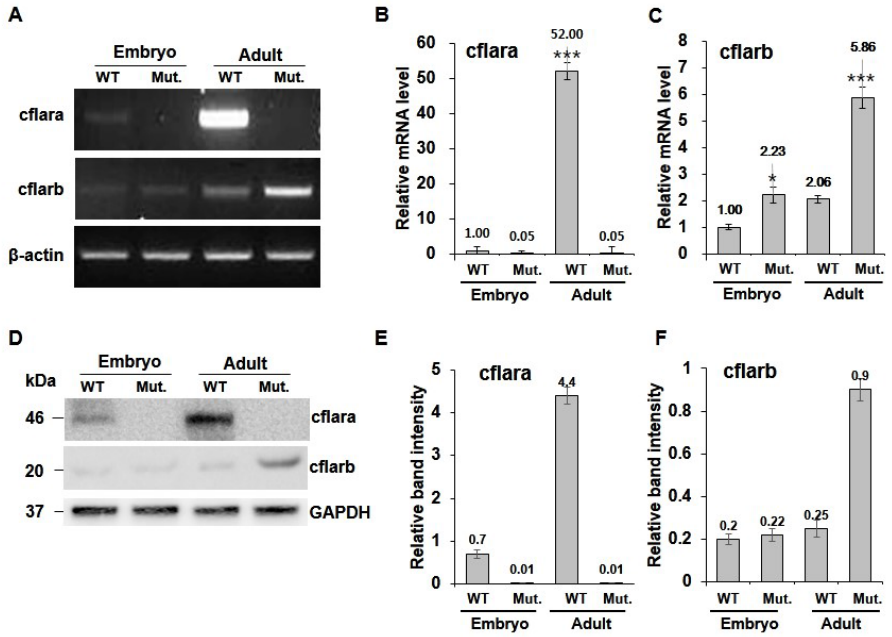


**Figure 4:** Cflara and cflarb expression in wild type and mutant zebrafish. (A) Quantitative PCR results. (B) Transcription level of Cflara gene relative to β-actin. (C) Transcription level of Cflarb gene relative to β-actin. (D) Western blots results. (E) Protein level of Cflara gene relative to GAPDH. (F) Protein level of Cflarb gene relative to GAPDH. Mutant had no expression of the Cflara gene, but shows higher expression level of Cflarb than the mutant. Data are expressed as Mean ± S.E.M (n=6). *** $p<0.001$ compared with adult mutant zebrafish group, * $p<0.05$ compared with wild type embryo group and *** $p<0.001$ compared with wild type of adult zebrafish group.

cflara were absent in mutant fish. Both wild type and mutant fish had cflarb expression, but mutant fish exhibited a higher cflarb level than wild type in embryo and adult zebrafish. Band intensities revealed a statistically significant increase in the expression of cflara in adult wild type fish ($p<0.001$) (Figures 4B and 4E). With respect to cflarb, there was significantly higher expression in embryo and adult mutant fish compared with wild type expression, respectively ($p<0.05$ and $p<0.001$) (Figures 4C and 4F). This data suggests that cflarb may compensate for the function of cflara in normal development.

## Discussion

In this study, we have described the generation and characterization of cflara mutant zebrafish because cFLIP was aberrantly overexpressed in various cancers [16]. In mice embryos, cflara has intense expression in the heart and is required for proper heart development [11,26]. Deletion of cflara leads to embryonic lethality in mice past 10.5 days associated with a failure to remodel the yolk sac vasculature [11].

Similar to the mouse, cflara was expressed in the heart, gut and brain regions. Zebrafish embryos reach stages of heart development up to 48 hpf and depletion of cflar transcripts in zebrafish resulted in developmental abnormalities accompanied by edema and irregular red blood cell flow, caused by fluid leakage from the vascular system [27]. However, cflara mutant zebrafish displayed no abnormalities of development, heart development, or heart rate to 72 hpf.

Zebrafish are teleost fish and whole-genome duplication (WGD) took place in teleost ancestors, called the teleost-specific genome duplication (TSD) [28]. An average of 2.28 zebrafish genes have a human analogue gene, and this is probably the reason for the TSD [29]. cflara is located on chromosome 11 and produces 418 amino acids (a.a.). A predicted cflara analogue gene in zebrafish, cflarb (ZDB-GENE-091204-464), is located on chromosome 9, produces a small amino acid product (197 a.a.) and its amino acid sequence identity shows a 35% similarity with cflara by NCBI BLASTP (protein-protein BLAST). Q-PCR and western blotting results showed that cflarb is relatively highly expressed in cflara mutant fish. Previous research explained that cFLIP has a role in inhibiting the death ligand induced apoptosis signal during development [30]. As such, it is possible that the cflarb gene product has an anti-apoptotic function in developmental stages, like cflara. However, this may be due to the difference between oviparous and viviparous animals, since caspase 8 deficient embryos are rescued by *ex vivo* whole-embryo culture [26].

Mutant fish had a longer survival term than wild type fish after $CoCl_2$ treatment and no difference in the Cisplatin treated group was observed. Previous reports showed that Cisplatin stimulated apoptosis via intrinsic and extrinsic pathways [31] and cFLIP knockdown increased sensitivity to Cisplatin [17]. $CoCl_2$ is a chemical inducer of hypoxia-inducible factor-1 (HIF1) [32]. Under hypoxia, impaired expression of anti-apoptotic genes, including cFLIP [33] and NF-κB [34], have been reported. Human cFLIP produces cFLIP long/short form proteins. The long form has an anti-apoptotic function and could regulate NF-κB activation by a catalytic product of its caspase-like domain. However, the short form has no caspase-like domain but has an anti-apoptotic role by preventing catalysis of caspase-8. In the prediction of cflarb protein, cflarb has one DED and no caspase-like domain. Due to a lack of a caspase-like domain, cflarb may not be able to induce NF-κB activation, but could prevent apoptosis via its one DED domain. If cflarb has an anti-apoptotic function, the difference with wild type and mutant in $CoCl_2$ treatment test may be due to impaired NF-κB activation function of cflarb. Until now, the function of cflarb as putative gene has not been known yet, but as shown in the results of this experiment, cflarb complemented cflara function and cflara mutant zebrafish normally developed.

In conclusion, our results show that a zebrafish cflara mutant has increased expression of cflarb gene, and has no developmental abnormalities, but did show marked differences following $CoCl_2$ treatment, when compared to wild type. Why $CoCl_2$ treated mutant fish showed longer survival term, compared with wild type, requires further study. Furthermore, in-depth studies of the role and function of cflarb's compensation of the function of cflara in normal development in primary cell cultures, including DR ligand treatment, tumor formation, and fish inflammation assay, are essential.

## Bioethics on the Experimental Animals

All procedures in the present study were approved by the Konkuk University Institutional Animal Care and Use Committee (Permit Number: KU14022) and ethical guidelines of Konkuk University Institutional Animal Care and Use Committee were followed.

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### Conflict of Interest

All authors have declared no conflict of interest.

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