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Fasting enhances cold resistance in fish through stimulating lipid catabolism and autophagy

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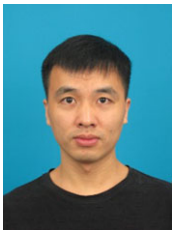
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Key points

- In a cold environment, mammals increase their food intake while fish decrease or stop feeding. However, the physiological value of fasting during cold resistance in fish is currently unknown.
- Fasting for more than 48 h enhanced acute cold resistance in zebrafish, which correlated with lipid catabolism and cell damage attenuation.
- Lipid catabolism and autophagy were necessary for cold resistance in fish and the inhibition of mitochondrial fatty acid β-oxidation or autophagy weakened the fasting-induced cold resistance.
- Repression of mechanistic target of rapamycin (mTOR) signalling pathway by rapamycin largely mimicked the beneficial effects of fasting in promoting cold resistance, suggesting mTOR signalling may be involved in the fasting-induced cold resistance in fish.
- Our study demonstrates that fasting may be a protective strategy for fish to survive under cold stress.

Abstract In cold environments, most homeothermic animals increase their food intake to supply more energy to maintain body temperature, whereas most poikilothermic animals such as fishes decrease or even stop feeding under cold stress. However, the physiological value of fasting during cold resistance in poikilotherms has not been explained. Here, we show that moderate fasting largely enhanced cold resistance in fish. By using pharmacological (fenofibrate, mildronate, chloroquine and rapamycin) and nutritional approaches (fatty acids diets and amino acids diets) in wild-type or specific gene knock-out zebrafish models (carnitine palmitoyltransferase-1b-deficient strain, CPT1b$^{-/-}$, or autophagy-related protein 12-deficient strain, ATG12$^{-/-}$), we verified that fasting-stimulated lipid catabolism and autophagy played essential roles in the improved cold resistance. Moreover, suppression of the mechanistic target of rapamycin (mTOR) pathway by using rapamycin mostly mimicked the beneficial effects of fasting in promoting cold resistance as either the physiological phenotype or transcriptomic pattern. However, these beneficial effects were largely reduced when the mTOR pathway was

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activated through high dietary leucine supplementation. We conclude that fasting helps fish to resist cold stress by modulating lipid catabolism and autophagy, which correlates with the mTOR signalling pathway. Therefore, fasting can act as a protective strategy of fish in resisting coldness.

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Introduction

Temperature is a prime environmental factor affecting living organisms. Colder temperatures reduce biochemical activities and significantly affect life processes. Temperature has been a powerful selection factor throughout animal evolution (Alfageeh & Smales, 2006; Pörtner & Somero, 2007). Vertebrates have developed complicated and effective adaptive mechanisms to tolerate cold temperatures, using different mechanisms in various species. Homeothermic animals, such as most mammals and birds, increase their food intake and physical activity to maintain their body temperature when exposed to cold stress (Cormarèche-Leydier, 1981; Köhler *et al.* 2010). Hibernation is another important strategy of some vertebrates, including some mammals, amphibians and reptiles, to resist coldness. Hibernating animals largely decrease their physical activity and food intake, resulting in decreased metabolic efficiency that favours nutrient transformation from carbohydrates to lipids, by which energy is saved to maintain a relatively stable body temperature and metabolic homeostasis (Boyer & Barnes, 1999; Carey *et al.* 2003).

Fish are the largest group of poikilothermic vertebrates that cannot maintain a constant body temperature. Water temperatures change depending on seasons and geographic location, which can cause cold stress to fish in some regions. For example, natural phenomena such as cold ocean currents and cold-air outbreaks can decrease rapidly local water temperatures (Donaldson *et al.* 2008). Acute decreases in temperature affect significantly the metabolism of fish and can cause massive annual mortality. This situation has led to large losses of production for many fish species around the world, both in aquaculture and wild environments (Overstreet, 1974; Donaldson *et al.* 2008; Wu *et al.* 2011). Viewed as either an economic or a research interest, studying the mechanisms of cold resistance in fish is fascinating. Several mechanisms related to cold adaptation in fish have been reported, including the alteration of cell-membrane fluidity (Johnston & Roots, 1964), recruitment of different muscle fibres (Gerlach *et al.* 1990), synthesis of molecular chaperones (Fader *et al.* 1994; Donaldson *et al.* 2008), and generation of antifreeze proteins in freezing environments (Devries, 1971; Cao *et al.* 2016). Moreover, several transcriptomic studies have indicated that numerous genes involved in energy metabolism are changed significantly in several fishes under cold stress (Gracey *et al.* 2004; Mininni *et al.* 2014), and a transgenic zebrafish (*Danio rerio*) strain expressing carp muscle-specific form III of creatine kinase showed enhanced cold resistance (Wang *et al.* 2014). The results of these studies suggest that energy metabolism correlates with cold resistance. However, the detailed metabolic strategy of fish to resist cold stress has not been well described.

It is well known that fish reduce their feeding rate as the ambient water temperature decreases (López-Olmeda & Sánchez-Vázquez, 2011) and they may even stop feeding if the water temperature is too low (Sala-Rabanal *et al.* 2003), contrary to isothermic animals, which commonly increase their food intake to resist environmental coldness. Fasting is an important means of metabolic adjustment. In many species, including yeast, flies, worms, fish, rodents, and rhesus monkey, dietary restrictions and short-term fasting have proven to be beneficial in resisting ageing and multiple stresses (Colman *et al.* 2009; Fontana *et al.* 2010; Mitchell *et al.* 2010). As reported, the benefits of fasting can include not only optimization of nutrient metabolism, but also reduction of cell damage by enhancing autophagy (Libert & Guarente, 2013). Therefore, by considering coldness as a stress factor, fasting can be hypothesized to improve tolerance to cold stress in some poikilothermic animals. However, no physiological mechanisms have been established to describe the correlation between fasting and cold resistance in fish. In other poikilothermic animals, only one work has described that the fly *Drosophila melanogaster* displayed enhanced resistance to cold stress after 24 h of fasting (Le Bourg, 2013), but no mechanisms were established.

In mammals, a number of studies have indicated that many diseases or metabolic dysfunctions are related to excess up-regulation of the mechanistic target of rapamycin (mTOR) pathway (Shaw *et al.* 2004; Bhat *et al.* 2013; Perl, 2015). Thus, inhibition of mTOR by drugs or genetic modification has multiple beneficial effects in extending longevity and resistance to diseases or environmental stresses (Thomson *et al.* 2009; Johnson *et al.* 2013; Lamming, 2016). Notably, fasting is a natural physiological status that inhibits mTOR signalling, and the positive effects of dietary restrictions or fasting in mammals are also correlated with mTOR inhibition. However, fasting and mTOR inhibition have different physiological effects, including insulin sensitivity, changes

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in mitochondrial function, fat deposition and fatty acid oxidation (Yu *et al.* 2015; Michael *et al.* 2016). Considering the beneficial effects of fasting and/or mTOR inhibition on multiple levels of stress resistance, we hypothesized that a reduction of food intake (fasting), is an important protective mechanism in fish to resist cold stress, specifically through metabolic adjustments in which mTOR signalling is likely to be involved.

Therefore, in the present study, we used normal (wild-type; WT) and several gene knock-out zebrafish to investigate the effects of fasting on cold stress resistance and its underlying mechanisms. A series of *in vivo* and *in vitro* experiments indicated that fasting for more than 48 h efficiently improves the survival rate of zebrafish under acute cold stress. Fasting-stimulated lipid catabolism and autophagy play essential roles in improving cold resistance in zebrafish. The fasting-enhanced cold tolerance in fish is mostly mimicked by mTOR inhibition, and its up-regulation reduces cold tolerance in fish. All these results enlighten our understanding on the underlying mechanisms of fasting in cold resistance in fish.

Methods

Ethical approval

All experiments were conducted strictly under the *Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the Ministry of Science and Technology of China*. This study was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (approval no.: F20140101). The study was done in compliance with ethical principles and standards of *The Journal of Physiology*. In all the *in vivo* experiments, the fish were individually anaesthetized with MS-222 (17 mg/L) (tricaine methanesulfonate, Sigma-Aldrich, St Louis, MO, USA) until the respiratory opercula stopped moving (4–5 min) before sampling was conducted.

Animal source, experimental design and treatments

Four-month-old adult zebrafish (0.3–0.4 g) used in the experiments were obtained from the Chinese National Zebrafish Resource Center (Wuhan, China). Before the formal experiments, the fish were maintained in a zebrafish breeding system under a 14 h light–10 h dark cycle at 28°C. The fish were fed twice daily (09.00 h and 17.00 h) using a commercial diet (Shengsuo, Yantai, China) containing 50% protein and 8% lipid. One week before the experiments, only female fish were selected for the treatments in order to avoid gender differences in lipid metabolism.

To delineate the effect of fasting on cold stress, fish were fasted for 24, 48, 72 and 96 h at 28°C. Fish in the fasting treatments were deprived of feed and the fasting time was counted from the last feeding, while control group were fed twice daily as described above. Liver samples were collected for further analysis after 72 h fasting period because all the three fasting durations (48, 72 and 96 h) enhanced zebrafish survival rate.

To evaluate the effect of lipid catabolism on cold stress, fenofibrate (6.67 g/kg diet, Sigma-Aldrich) and mildronate (25 g/kg diet, Micxy, Chengdu, China) were used to stimulate and inhibit fatty acid β-oxidation, respectively. Furthermore, zebrafish were fed with three fatty acid diets containing 7% crude lipid (4.9% palmitic acid (PA), 4.86% linoleic acid (LA) and 4.9% α-linolenic acid (ALA)). To study the effect of inhibited autophagy on cold stress, zebrafish were fed with a diet supplemented with 100 mg/kg dietary chloroquine (CQ). Finally, the contribution of the mTOR signalling pathway to cold stress resistance was studied by suppressing its activities using dietary rapamycin (RAPA; 25 and 50 mg/kg diet) and activating it using dietary leucine (control group: 22.5% protein, C; leucine group: 22.5% protein and 2% leucine, Leu^{+}). In all dietary treatments before the cold stress, zebrafish were maintained at 28°C and the feeding rate was set at 3% body weight per day. All the dietary treatments were conducted for 3 weeks. After 3 weeks, at least 20 liver samples from each treatment were collected and stored immediately at −81°C for further analysis.

The fish that remained from fasting and all dietary treatments were divided into two groups. In the first group, about 60–80 visually health fish from each experimental and control groups were divided into three to four replicates per group. They were then immediately subjected to cold stress by transferring them to an aseptic and transparent 8 L tanks containing cold water at 11°C. We selected 11°C for the cold stress experiments because it was a suitable lethal temperature (fish started to die at this temperature after 24 h) from our preliminary experimental results. The low water temperature in each experiment was maintained by a constant circulation of cold water by using a thermostat (Shunmatech, Nanjing, China). A fish was considered dead from cold shock when it stopped any movement and activities for at least 1 h. Dead fish were collected and counted every 12 h. In the second group, fish were reared in similar tanks whereby water was maintained at 28°C (normal temperature). About 20 liver samples from dietary treatments at cold and normal temperatures were collected after 6, 12 or 24 h and stored immediately at −80°C for further analysis.

Establishment of *atg12* and *cpt1b* mutant zebrafish lines and fish treatment

Zebrafish autophagy-related protein 12 (*atg12*) and carnitine palmitoyltransferase-1b (*cpt1b*) mutants were generated by the CRISPR/Cas9-induced gene knockout

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method. Briefly, the CRISPR/Cas9 target sites were designed by using the online tool ZiFiT Targeter version 4.2. The Cas9 mRNAs were generated by *in vitro* transcription from pCS2-nCas9n by using the mMESSAGE mMACHINE T3 kit (Invitrogen, Carlsbad, CA, USA). Double strand DNA for specific gRNA synthesis was amplified by PCR from pT7-gRNA. The target single-guide RNAs (sgRNAs) were synthesized using the mMESSAGE mMACHINE T7 kit. The Cas9 mRNA and sgRNA were co-microinjected into one-cell-stage embryos using a Picoliter Microinjector PLI-100A (Warner Instruments, Hamden, CT, USA). The F0 founders carrying mosaic mutations were identified by high-resolution melt analysis and heteroduplex motility assay and mated with WT fish to produce heterozygous F1 offspring. The *atg12* and *cpt1b* heterozygotes were allowed to self-cross to obtain the atg12$^{-/-}$, and cpt1b$^{-/-}$ homozygous mutants (F2), resulting in deletion of 8 base pairs (bp) and a 5 bp fragment on the target sites, respectively. The F2 adult fish were sampled to test the effect of autophagy and fatty acid β-oxidation after knock-out. To verify the relationship between loss of genes and fasting, the WT and F2 adult fish were either fed with commercial feed used in the acclimation or fasted for 3 days; subsequently they were subjected to acute cold stress and their survival rates were counted.

Transmission electron microscopy

Three liver samples from zebrafish in the fed and fasted treatments and those reared at normal and cold temperatures were collected and fixed immediately with 2.5% glutaraldehyde for 2–4 h. The samples were washed three times by using 0.1 M sodium phosphate buffer (pH 7.4) for 15 min each time. The resulting liver tissues were fixed with 1% osmium tetroxide for 2 h, then washed three times by using 0.1 M sodium phosphate buffer (pH 7.4). Blocks were then dehydrated in a graded series of ethanol (50–70–80–90–95–100–100%) followed by 100% acetone. The samples were then infiltrated in 1:1 acetone: SPI-Pon 812 resin for 3 h, followed by 1:2 acetone: SPI-Pon 812 resin overnight and SPI-Pon 812 resin for 6 h, and subsequently embedded in SPI-Pon 812 resin at 60°C for 48 h. Ultra-thin sections (60–80 nm) were cut using a Leica UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained using uranyl acetate and lead citrate, finally viewed on a Hitachi HT7700 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) and documented in digital images.

TUNEL analysis

Three pieces of liver samples were fixed in 4% paraformaldehyde, embedded in paraffin and subsequently cut into 5 μm sections. The sections were deparaffinized twice in dimethylbenzene and then graded in a series of ethanol, after repair with proteinase K (2 mg/mL) and permeabilized with 0.2% Triton X-100–phosphate-buffered saline (PBS). DNA fragmentation was determined by TdT and dUTP 2:29 for 2 h. The sections were washed with PBS, followed by staining using 4′,6-diamidino-2-phenylindole (DAPI; 0.3 mmol/L) for 10 min, and finally sealed and documented as digital images on a Nikon Eclipse Ti-SR inverted microscope (Nikon, Tokyo, Japan).

Cell culture and treatment

The zebrafish liver (ZFL) cell line was kindly provided by Prof. Zhi-Gang Zhou (Chinese Academy of Agricultural Sciences, Beijing, China). An LDF medium consisting of 50% Leibovitz's medium (L-15; Thermo Fisher Scientific, Waltham, MA, USA), 35% Dulbecco' modified Eagle's medium (DMEM; Thermo Fisher Scientific) and 15% Ham's nutrient mixture (F-12, Thermo Fisher Scientific) was used to prepare the medium for cell culture. Cells were cultured in LDF complete medium containing 6% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel), 15 mM HEPES buffer solution (Thermo Fisher Scientific) and 50 unit/mL penicillin and 50 μg/mL streptomycin (Thermo Fisher Scientific) at 28°C in 5% CO_2. Rapamycin (1 μM, LC Laboratories, Woburn, MA, USA) and hydroxychloroquine sulfate (5 μM, Selleckchem, Huston, TX, USA) were used to treat ZFL cells. After drug treatments, ZFL cells were transported to an incubator at 4°C to simulate acute cold shock stress. We used 4°C to challenge ZFL cells because it was a suitable lethal temperature for these cells (cells started to die at this temperature after 24 h) based on our preliminary results. After cold stress, the cell station was recorded by light microscopy. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Biotechnology, Haimen, China) was used to assay ZFL cell survival.

Fatty acid β-oxidation assay

β-Oxidation efficiency of five fatty acids, PA, oleic acid (OA), LA, ALA, arachidonic acid (ARA), in zebrafish liver samples was assayed as reported previously (Du *et al.* 2010). Briefly, five [1-^{14}C]fatty acids were complexed in a reaction system including liver homogenate (1:20 w/v), 0.2 mM L-carnitine, 10 μM cytochrome *c* and 0.2 mM malic acid separately. The reaction mixture was incubated in a thermo-shaker at 28°C for 2 h before the reaction was stopped by adding 1 mL of 10% (w/v) perchloric acid. The resulting contents were transferred into tubes in ice, and then was filtered using a Millipore (Billerica, MA, USA) filter (0.45 μm pore size). The water-soluble products

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containing ^{14}C from fatty acid oxidation were assayed for radioactivity in a liquid scintillation spectrometer, MicroBeta2 Plate Counter (Perkin Elmer, Waltham, MA, USA). β-Oxidation efficiency of livers from different treatments were measured using the same method, except only [1-^{14}C]palmitate was added in the reaction system to represent the β-oxidation efficiency of fatty acids.

Biochemical parameters and oxygen consumption rate measurements

Total protein of whole fish body was determined by a semi-automatic Kjeldahl System (FOSS, Hillerød, Denmark) after acid digestion. Hepatic biochemical parameters including triglyceride (TG) and glycogen were assessed by using specific commercial kits (Jiancheng Biotech Co., Nanjing, China). All analyses were performed according to the relevant kit protocols according to the manufacturer's guidance.

Oxygen consumption rate (OCR) of zebrafish at two temperatures and under three treatments (control, RAPA, fasting) at 12°C was measured using a Strathkelvin Instruments 782 Oxygen Meter system (North Lanarkshire, UK). We used 12°C to measure the oxygen consumption rate because lower temperature (e.g. 11°C) would cause zebrafish transient coma and affect the measurements. The oxygen consumption rate was calculated as: OCR = [ΔO_2 concentration (mg/L) × water volume (L)]/[fish mass (g) × time (h)].

Quantitative real time PCR and transcriptomic analysis

Total RNA of tissues and cells was isolated by using a Tri Pure Reagent (Aidlab, Beijing, China). The quality and quantity of total RNA were tested by NANODROP 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNAs were synthesized using a PrimerScript^{TM} RT reagent Kit with a gDNA Eraser (RR047A, Takara, Shiga, Japan). Quantitative real time PCR (qPCR) (20 μL) was performed using 2 × Ultra SYBR Mixture (Aidlab, China) in a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). The qPCR reaction consisted of 95°C for 10 min, 40 cycles of 95°C for 5 s and 60°C for 18 s. Melting curves of amplified products were generated to ensure the specificity of assays at the end of each PCR. Elongation factor 1 α (EF1α) and 18S RNA were used as the reference genes. The method of $2^{-\Delta\Delta Ct}$ was used for estimating the relative cDNA abundance.

Functional-enrichment analysis

In all treatments, the liver from zebrafish at both 28°C and 11°C were sampled for transcriptomic analysis. At least 12 liver samples in every treatment were pooled to prepare the RNA-seq transcriptome library. After quantification by using TBS380, a paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq 4000 (Illumina, Inc., San Diego, CA, USA; 2 × 150 bp read length). Differentially expressed genes (DEGs) between two different samples were calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. Functional-enrichment analysis including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed to identify the DEGs that were enriched significantly in GO terms and metabolic pathways at Bonferroni-corrected P-value ≤ 0.05 compared with the whole-transcriptome background.

Western blot

Homogenates of juvenile fish and liver tissues were cell lysed by using ice-cold RIPA lysis buffer (Beyotime Biotechnology) in the presence of 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotechnology) for 30 min. After 10,400 g centrifugation for 10 min, the resulting supernatant was mixed with 5 × SDS loading buffer and boiled at 95°C for 5 min. Proteins (20–50 μg) were loaded, separated on SDS-PAGE gels and transferred to nitrocellulose filter membranes. The membranes were blocked for 1 h by using 5% bovine serum albumin (BSA) in PBS supplemented with 0.1% Tween 20 and were incubated by using the following rabbit antibodies: LC3A/B (1:800, cat. no. 4108, Cell Signaling Technology, Danvers, MA, USA), p-AMPKα (1:1000, cat. no. 2535, Cell Signaling Technology), P62 (1:750, cat. no. 5114, Cell Signaling Technology), beclin-1 (1:600, CAT.NO.11306-1-AP, Proteintech, Rosemont, IL, USA), mTOR (1:1000, cat. no. 2972, Cell Signaling Technology), p-mTOR (1:1000, cat. no. 2971, Cell Signaling Technology), p-S6 (1:1000, cat. no. 4856, Cell Signaling Technology), S6 (1:1000, cat. no. 2217, Cell Signaling Technology). Bound antibodies were detected using goat anti-rabbit IgG (Li-Cor Biotechnology, Lincoln, NE, USA) by using the Odyssey CLx Imager (Li-Cor). The target proteins were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All antibodies were tested in a preliminary study (results not shown) and found to be specific for zebrafish samples before the formal experiments.

Statistical analyses

All values are reported as the mean ± standard error of the mean (SEM). Statistical difference for measured variables between two groups were tested by using Student's unpaired t test. Statistical comparisons for measured parameters containing more than two groups

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were compared by using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test for specific comparisons among groups whenever differences were obtained. A value of $P < 0.05$ was considered statistically significant. All analyses were conducted by using the SPSS Statistics v. 21 (IBM Corp., Armonk, NY, USA).

Results

Fasting-enhanced cold resistance correlated with nutritional metabolism and cellular repair

As mentioned above, many fishes decrease feed intake as the water temperature lowers. We likewise report that zebrafish decreased significantly their feed intake and then completely stopped feeding when the water temperature dropped from 28°C to 13°C (Fig. 1*A*). To determine whether fasting could promote resistance to acute cold stress, the zebrafish cultured at 28°C were fasted for 24, 48, 72 and 96 h, and then rapidly transferred into cold water (11°C); their survival rate was recorded and compared with fish that were fed normally (control) (Fig. 1*B*). We found that 48–96 h fasting enhanced significantly the survival rate of the zebrafish exposed to acute cold stress, but 24 h fasting did not change their cold stress-resistance ability (Fig. 1*C*). This result demonstrates that, a suitable duration of fasting can enhance cold resistance in zebrafish.

To explore the underlying mechanisms, we selected 72 h fasting as the fixed fasting treatment and 24 h after cold stress as the sampling time point for the succeeding experiments. The majority of assays were mainly performed on samples from fish liver or liver cells because the liver plays central roles in metabolism and homeostatic processes in fish (Dhanasiri *et al.* 2013). The fed fish (control), fed cold-stressed (control-cold), fasting and fasting cold-stress groups are designated C, CC, F and FC, respectively. We first tested the expression of genes involved in apoptosis and endoplasmic reticulum (ER) stress (Fig. 1*D*). Cold stress elevated significantly the mRNA expression of apoptosis-related genes, including *Caspase-9* and C/EBP homologous protein (*CHOP*), but not the ER-related genes, including activating transcription factor 6 (*ATF6*) and X-box binding protein 1 (*XBP1*) (C *vs.* CC). However, 72 h fasting significantly reduced the expression of these genes under cold stress (FC *vs.* CC), corresponding with the survival rate phenotype. Transmission electron microscopy (Fig. 1*E*) further indicated that cold stress impaired seriously ER structures and mitochondria in the fed state (C *vs.* CC); however, these cold-induced organelle impairments were largely relieved in the 72 h fasting fish (CC *vs.* FC). TUNEL staining of liver tissues also showed much apoptosis signalling in the CC group, but almost no apoptosis signal in the FC group (Fig. 1*F*).

To understand the underlying mechanisms on how fasting relieved the cold-induced damage, we performed transcriptomic assays of liver samples. Results of the RNA-seq assay indicated 21 main KEGG pathways that were significantly changed (Fig. 1*G*). Among them, 11 pathways are related to the regulation of metabolism, and the other 10 pathways are involved in either diseases or cell proliferation. The changed pathways between fed and fasting at 28°C were mainly related to metabolism and cell proliferation (C *vs.* F). In both fed groups (C *vs.* CC), cold stress caused changes mainly in diseases and cell proliferation, followed by metabolic pathways, including lipid and glucose metabolism. Of note, the lipid metabolism between the fed and fasting groups under cold stress (CC *vs.* FC) was changed significantly. We classified the changed genes by their different functions (Fig. 1*H*). Comparison of the changed genes mainly indicated that at transcriptomic levels the FC group, which had a higher survival rate, tended to have higher lipid catabolism, lower lipogenesis, lower carbohydrate and amino acid catabolism, comparable inflammation and apoptosis, and lower tissue and DNA repair than the CC group. A summary of the up-regulated and down-regulated transcriptomic changes among all four the treatments are shown in Fig. 1*I*. Notably, the conditions of fasting and cold stress each stimulated lipid catabolism and cellular repair, particularly lipid catabolism in the FC group as compared with in the CC group. These data suggest that metabolism, especially lipid catabolism, and cellular repair are two critical processes in the fasting-enhanced resistance to acute cold stress in zebrafish.

Enhancing lipid catabolism promoted cold resistance

To further identify the roles of different energy sources (carbohydrate, lipid and protein) in the fasting-enhanced cold resistance, we measured the mobilization of carbohydrate, lipid and protein after fasting for 1–4 days. Glycogen content and the expression of genes involved in glycolysis in the liver decreased significantly after 12 h fasting (Fig. 2*A*). Hepatic TG decreased significantly after 72 h. The expression of the key genes related to lipid catabolism [adipose triglyceride lipase (*ATGL*) and carnitine palmitoyl transferase 1b (*CPT-1b*)] increased significantly after 12 h and 48 h, respectively, while the key genes related to lipogenesis [fatty acid synthase (*FAS*) and acetyl-CoA carboxylase (*ACCα*)] decreased significantly after 12 h (Fig. 2*B*). Body protein content did not change during the 4 day fasting period, and the expression of the protein catabolism-related genes did not change after the 72 h fasting period, although some gene expression [oligopeptide transporter 1 (*PEPT1*)

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and asparagine synthetase (*Asns*)] decreased after fasting (Fig. 2*C*). These data illustrate that glycogen was first mobilized during the early fasting stage (within 24 h), and lipid was mostly utilized after glucose catabolism. Protein catabolism was not activated at least during the 72 h fasting period. Since 24 h fasting could not promote cold resistance, and the fasting-enhanced cold resistance appeared after 48 h fasting, the catabolism of lipid, but not glucose, is likely to be essential in the fasting-enhanced cold resistance of zebrafish.

We next examined whether modifying lipid catabolism would affect cold resistance in zebrafish. In clinical and fish studies, fenofibrate is widely used as an agonist of peroxisome proliferator-activated receptor α (PPARα) to stimulate fatty acid β-oxidation (Dreyer *et al.* 1993), while mildronate is commonly used as a competitive inhibitor of L-carnitine synthesis to suppress fatty acid β-oxidation (Simkhovich *et al.* 1988). We fed zebrafish with fenofibrate and mildronate for 3 weeks, using the optimal doses reported in our previous studies

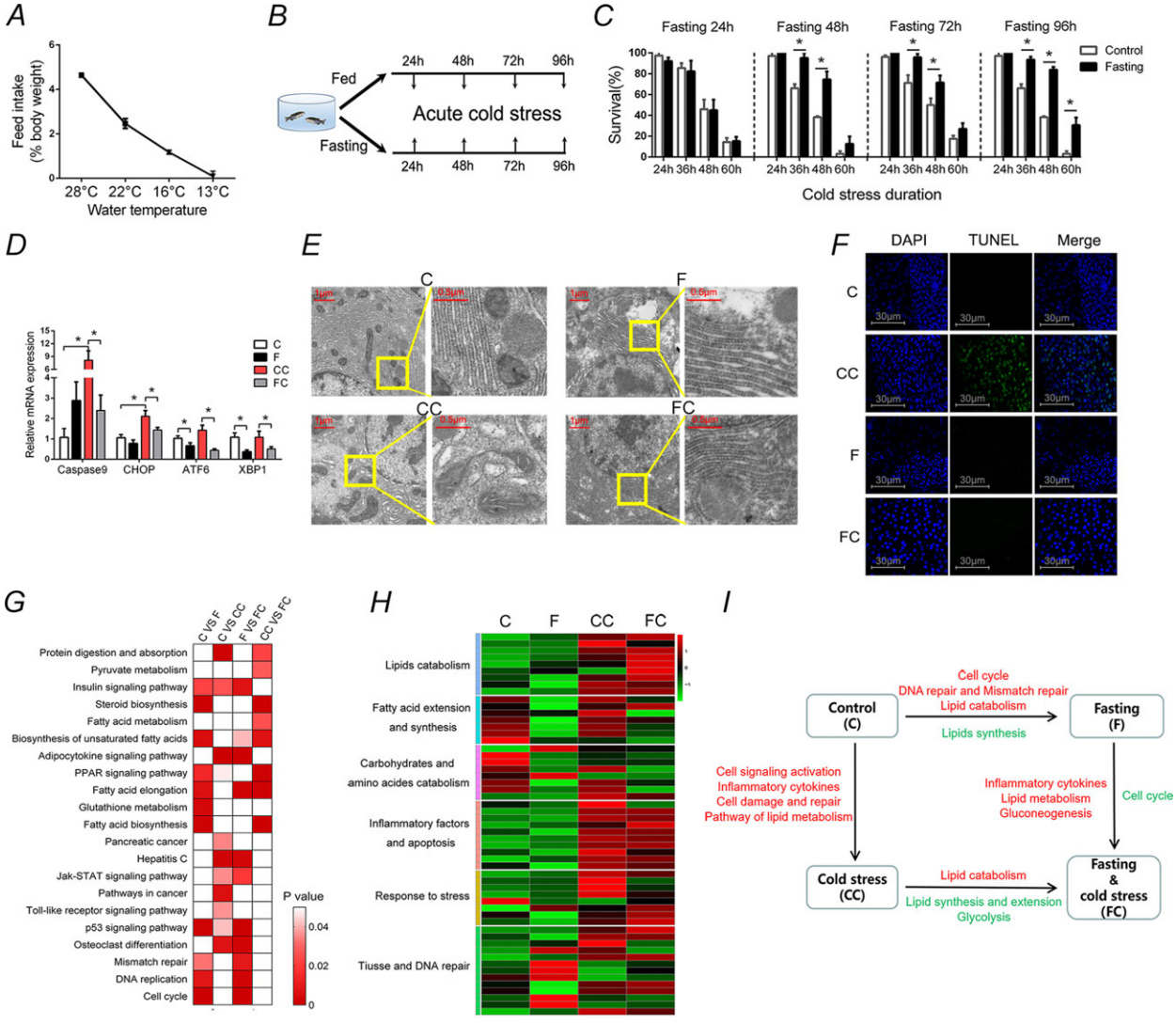


Figure 1. Fasting-enhanced cold resistance correlates with nutritional metabolism and cellular repair
A, feed intake (% body weight) of adult zebrafish at four temperatures. *B*, zebrafish were fasted for four durations and then exposed to acute cold stress synchronous with the corresponding fed groups. *C*, the survival rate of control (fed) and fasting under acute cold stress (three replicates per group, n = 13–51 animals). *D*, relative expression of genes associated with apoptosis and endoplasmic reticulum stress (n = 4–6). *E*, transmission electron microscopy of liver tissues (n = 3). *F*, DAPI and TUNEL double staining of zebrafish liver. *G–I*, transcriptome analysis of liver mRNA. *G*, significantly changed KEGG pathways; red ($P \leq 0.05$) and white ($P > 0.05$). *H*, heat map of gene expression involved in changed pathways classified by functions; red and green indicate higher and lower expression, respectively. *I*, summary of changed functions among the four treatments; red and green wording signify increased and decreased, respectively. Data are means ± SEM. Statistical significance was determined by Student's *t* test. $^*P \leq 0.05$.

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(Ning *et al.* 2016; Pan *et al.* 2017) (Fig. 3*A*). After 3 weeks, fenofibrate increased significantly fatty acid β-oxidation (Fig. 3*B*) and decreased significantly hepatic TG content (Fig. 3*C*). In contrast, mildronate reduced fatty acid β-oxidation (Fig. 3*B*) and increased liver TG content (Fig. 3*C*). The cold-stress test following indicated that fenofibrate increased significantly the survival rate, whereas mildronate exacerbated significantly the mortality rate (Fig. 3*D*). This indicates that lipid catabolism is necessary for cold resistance in zebrafish.

To further confirm the essential roles of mitochondrial β-oxidation in cold resistance in fish, we knocked out CPT1b (the key enzyme transporting long-chain fatty acid into mitochondria from cytosol) in zebrafish using CRISPR/Cas9 technology (Fig. 3*E*). The CPT1b$^{-/-}$ fish were visually healthy but had lower fatty acid β-oxidation activities in both their liver and muscle tissues (Fig. 3*F*). The cold-stress test showed that fasting increased the survival rate in both normal (WT) and CPT1b$^{-/-}$ fish, but the survival rate increased by fasting (net fasting effect) was lower in the CPT1b$^{-/-}$ fish than in the WT fish (Fig. 3*G*). More importantly, the CPT1b$^{-/-}$ fish survival rate in cold stress was significantly lower than WT fish in both the fed and fasting states (Fig. 3*G*). This shows that the impaired fatty acid β-oxidation largely reduced cold resistance, highlighting that lipid catabolism is required for the fasting-induced cold resistance.

To exclude the possible side effects of chemical drugs and gene knock-out, we hypothesized that the dietary supplementation of easily utilized lipid substrates would also promote the cold resistance of the

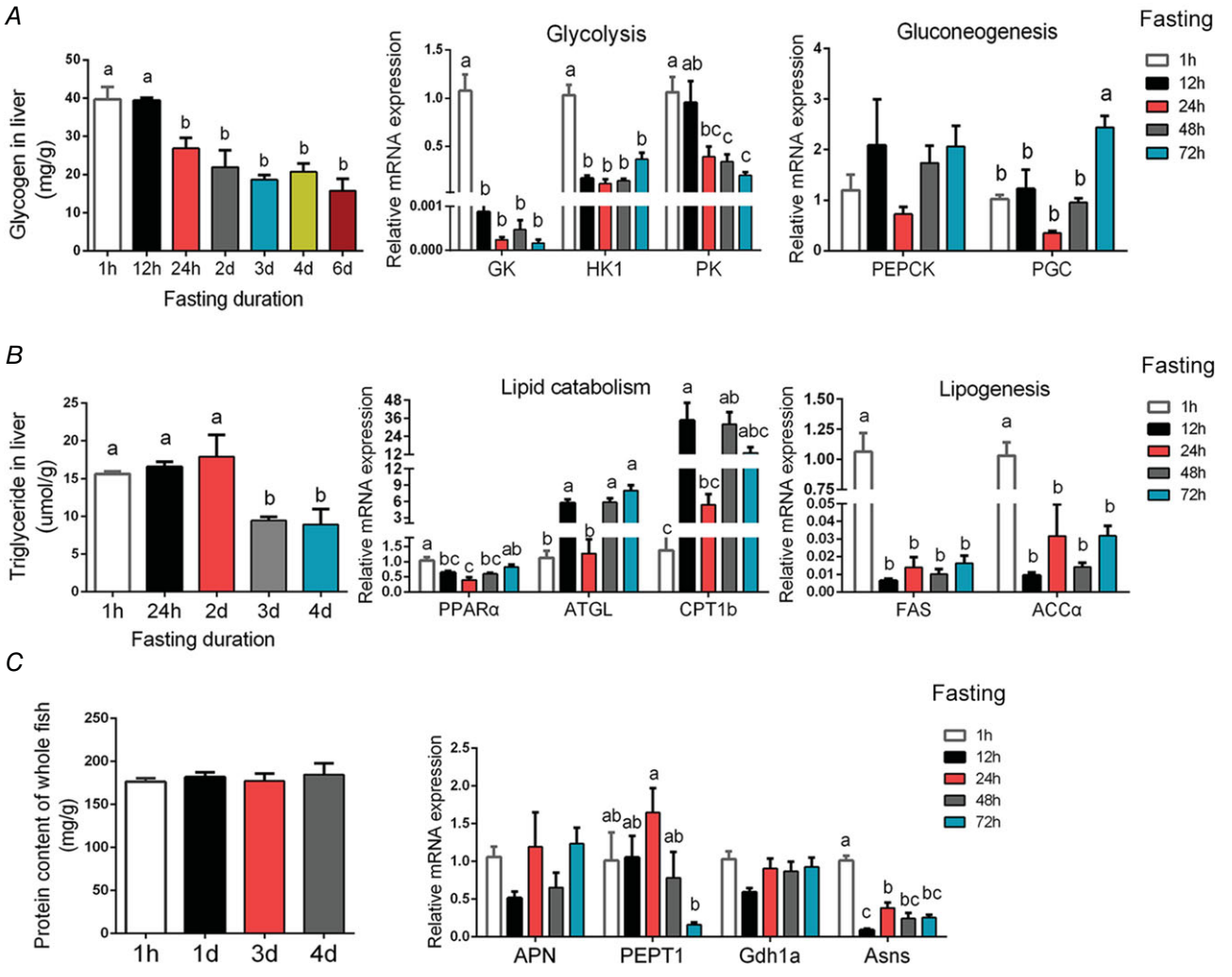


Figure 2. The mobilization of different energy sources during fasting
A, glycogen content (n = 3–5); gene expression of glycolysis and gluconeogenesis during fasting (n = 4–6). *B*, triglyceride level (n = 3–4); relative expression of lipid catabolism and lipogenesis during fasting (n = 4–6). *C*, protein content of whole fish (n = 3–4); and expression of genes associated with protein catabolism (n = 4–6). Data are means ± SEM. Statistical significance was determined by one-way ANOVA. Different letters signify significant differences among groups.

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zebrafish. Therefore, we first examined the *in vitro* β-oxidation efficiencies of five ^{14}C-labeled fatty acids (16:0, 18:1$n-9$, 18:2$n-6$, 18:3$n-3$ and 20:4$n-6$) by using liver homogenate and found that 18:2$n-6$ had the greatest β-oxidation efficiency (Fig. 3*H*). We then chose palm oil, safflower oil and perilla oil, which mainly contain around 70% of total fatty acids, as 16:0, 18:2$n-6$ and 18:3$n-3$, respectively, to create three diets, which were fed to fish for 3 weeks (Fig. 3*I*). During this feeding trial, the safflower oil diet caused a higher rate of oxygen consumption (Fig. 3*J*). Results of the cold-stress test showed that the group fed 18:2-enriched safflower oil attained the best survival rate (Fig. 3*K*). This result indicates that the type of dietary lipid can affect cold resistance in a fish. All these data verify that, in fish, lipid is an essential energy source for resisting coldness and lipid catabolism, especially fatty acid β-oxidation, plays an important role in the fasting-induced cold resistance.

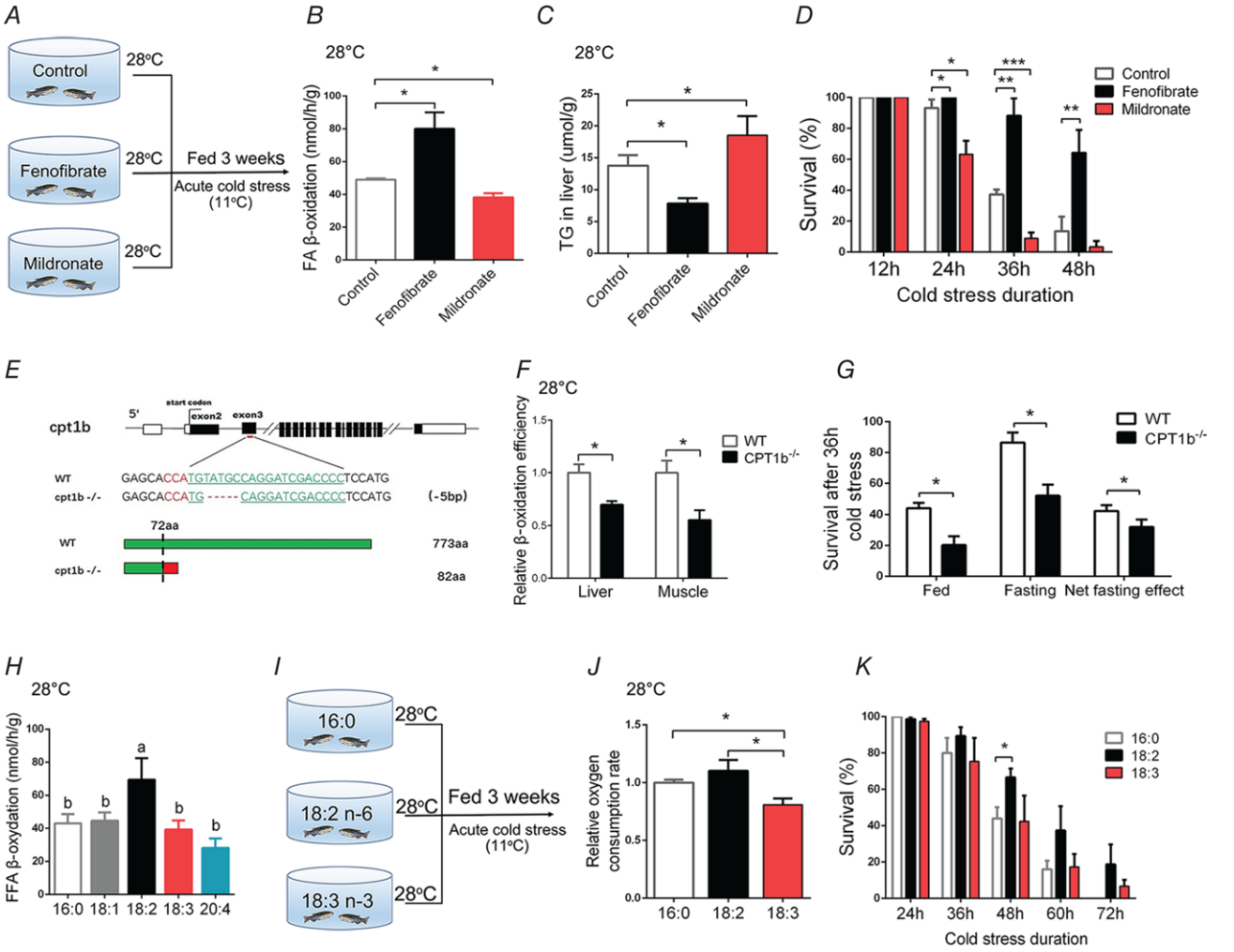


Figure 3. Enhancing lipid catabolism promotes cold resistance
A, experiment design: lipid metabolism dug treatments and cold stress treatment. *B*, oleic acid β-oxidation efficiency of zebrafish liver after treatments ($n = 4$). *C*, zebrafish triglyceride content in liver after treatments ($n = 4$). *D*, zebrafish survival rate under cold stress treatments (three replicates per group, $n = 25$–26 animals). *E*, comparisons of genes and amino acid sequences between WT and CPT1b$^{-/-}$ zebrafish. *F*, liver and muscle tissues relative β-oxidation efficiency of WT and CPT1b$^{-/-}$ zebrafish ($n = 3$–4). *G*, survival rate of WT and CPT1b$^{-/-}$ zebrafish after 36 h cold stress; net fasting effects are the differences between fasting and fed groups. WT and CPT1$^{-/-}$ fish were either fed or fasted for 3 days (three replicates per group, $n = 13$–18 animals). *H*, β-oxidation efficiency of five fatty acids in zebrafish liver ($n = 3$). Different letters signify significant differences among groups. *I*, experimental design: three fatty acids sources fed and cold stress treatment. *J*, relative oxygen consumption rate of zebrafish fed on three fatty acid feeds. *K*, survival rate of zebrafish treated by the three fatty acid feeds under cold stress ($n = 3$). Data are means ± SEM. Statistical significance was determined by Student's *t* test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

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Stimulation of autophagy improved cold resistance

In stress resistance, increasing energy supply reduces cell damage (Libert & Guarente, 2013). In the present study, cold stress caused cellular damage and fasting decreased the cell damage caused by cold stress in zebrafish liver (Fig. 1*E*). Autophagy is an important process to clean damaged organelles (Jisun *et al.* 2012), and fasting induces autophagy (Martinet *et al.* 2006; Mizushima & Komatsu, 2011). To verify whether, fasting-induced autophagy could promote cold resistance, we tested autophagy by using an inhibitor, chloroquine (CQ), and an activator, rapamycin (RAPA), in ZFL cells. Consistent with a previous report (Tekirdag *et al.* 2013), RAPA increased the levels of microtubule-associated protein 1 light chain 3 type II (LC3-II), a protein required for assembly of autophagosomes (Tanida *et al.* 2004), and decreased levels of sequestosome 1 (P62, a protein that usually accumulates when the autophagic process is blocked; Komatsu *et al.* 2007) (Fig. 4*A*). Exposing cells to CQ increased the LC3-II and P62 protein levels (Fig. 4*A*), thereby demonstrating the inhibition of autophagy as reported previously (Porter *et al.* 2013). In the cold-stress test, CQ reduced cell survival rate whereas RAPA increased it (Fig. 4*B*). Similarly, under cold stress, the expression of genes involved in apoptosis and ER stress were elevated by CQ treatment and reduced by RAPA treatment (Fig. 4*C*). These data show that autophagy contributed to the cold resistance in the ZFL cells.

To verify this conclusion using live fish, we fed adult zebrafish a feed containing CQ (100 mg/kg feed) for 3 weeks (Fig. 4*D*). Dietary CQ reduced significantly the expression of autophagy-related genes (i.e. *beclin-1* and *ATG12*) (Fig. 4*E*) and prevented reduction of the proteins P62 and LC3-II (Fig. 4*F*), as reported previously. However, CQ did not affect expression of the ER-stress-related genes (i.e. heat shock protein a5 (*HSPa5*) and *ATF6*) (Fig. 4*E*), suggesting the CQ feed helped to inhibit autophagy without causing cellular stress. Nevertheless, we found that the CQ treatment reduced significantly the survival rate of fish subjected to acute cold stress (Fig. 4*G*). Moreover, 24 h cold stress for CQ-fed fish reduced *beclin-1* and increased *P62* mRNA expression in the liver (Fig. 4*H*). Surprisingly, under cold stress, a number of ER stress-related genes were highly expressed in the fish fed on CQ (Fig. 4*H*), suggesting that the inhibition of autophagy caused a high degree of cellular damage in conditions of coldness and also decreased the capacity to resist cold stress.

To determine whether the autophagy pathway is required for fasting-induced cold resistance, we knocked out ATG12, a protein essential for the assembly of phagophore (Geng & Klionsky, 2008), in zebrafish using CRISPR/Cas9 technology, which resulted in gene-sequence differences when compared with WT fish, as shown in Fig. 4*I*. The higher level of P62 protein in the liver of ATG12$^{-/-}$ zebrafish than in WT, indicated that autophagy was inhibited in the ATG12$^{-/-}$ fish (Fig. 4*J*). Following cold stress, the survival rate of the ATG12$^{-/-}$ fish was significantly lower than that of WT fish. Although fasting improved survival rate in both WT and ATG12$^{-/-}$ fish, the net fasting effect in enhancing cold resistance was lower in the ATG12$^{-/-}$ fish than in the WT fish (Fig. 4*K*). Taken together, up-regulation or down-regulation of autophagy promotes or lowers cold resistance, respectively. Furthermore, autophagy plays an essential role in the fasting-induced cold resistance in fish.

Suppression of the mTOR signalling pathway correlated to the cold resistance

A number of studies in other organisms have demonstrated that fasting causes suppression of mTOR (Bhat *et al.* 2013), which has been connected with increased lipid catabolism and autophagy (Johnson *et al.* 2013). Hence, we hypothesized that the fasting-enhanced cold resistance may correlate with the mTOR signalling pathway. Thus, we first measured the activity of the mTOR signalling pathway in zebrafish in the fed and fasting states. We found that the phosphorylation of mTOR (S2448) and the downstream protein ribosomal protein S6 (p-S6; S235/236) decreased significantly after 3 days of fasting (Fig. 5*A*), indicating fasting suppressed mTOR activity in zebrafish. To verify whether the suppression of mTOR activity could help zebrafish to resist cold stress, we prepared two diets containing an mTOR signalling inhibitor (RAPA), at 25 or 50 mg/kg diet, and fed adult zebrafish for 3 weeks (Fig. 5*B*). At the end of the feeding trial, the phosphorylation of S6 (S235/236) decreased for both RAPA doses, but the phosphorylation of mTOR (S2448) decreased only for the high-dose RAPA treatment (Fig. 5*C*), implying that dietary supplementation with RAPA at 50 mg/kg efficiently reduced the activities of the mTOR signalling pathways. As expected, the post-cold-stress survival rate was RAPA-dose dependent, and it was significantly higher for fish fed on the higher dose compared to the control fish (Fig. 5*D*). Accordingly, the RAPA treatments also decreased significantly the expression of genes related to apoptosis and ER stress in the liver of fish reared at 28°C (Fig. 5*E*). These results demonstrate that suppression of mTOR by RAPA efficiently promote the cold resistance of zebrafish.

We then hypothesized that up-regulation of the mTOR signalling pathways would reduce the cold resistance in fish. Therefore, we prepared two diets containing a normal (2% diet) and high dose of leucine (4% diet), an amino acid that has been identified as an mTOR activator in many species (Proud, 2002), and fed zebrafish for 3 weeks (Fig. 5*F*). The phosphorylation of both mTOR (S2448) and S6 (S235/236) was elevated by the high-leucine feed (Fig. 5*G*), showing that the high leucine

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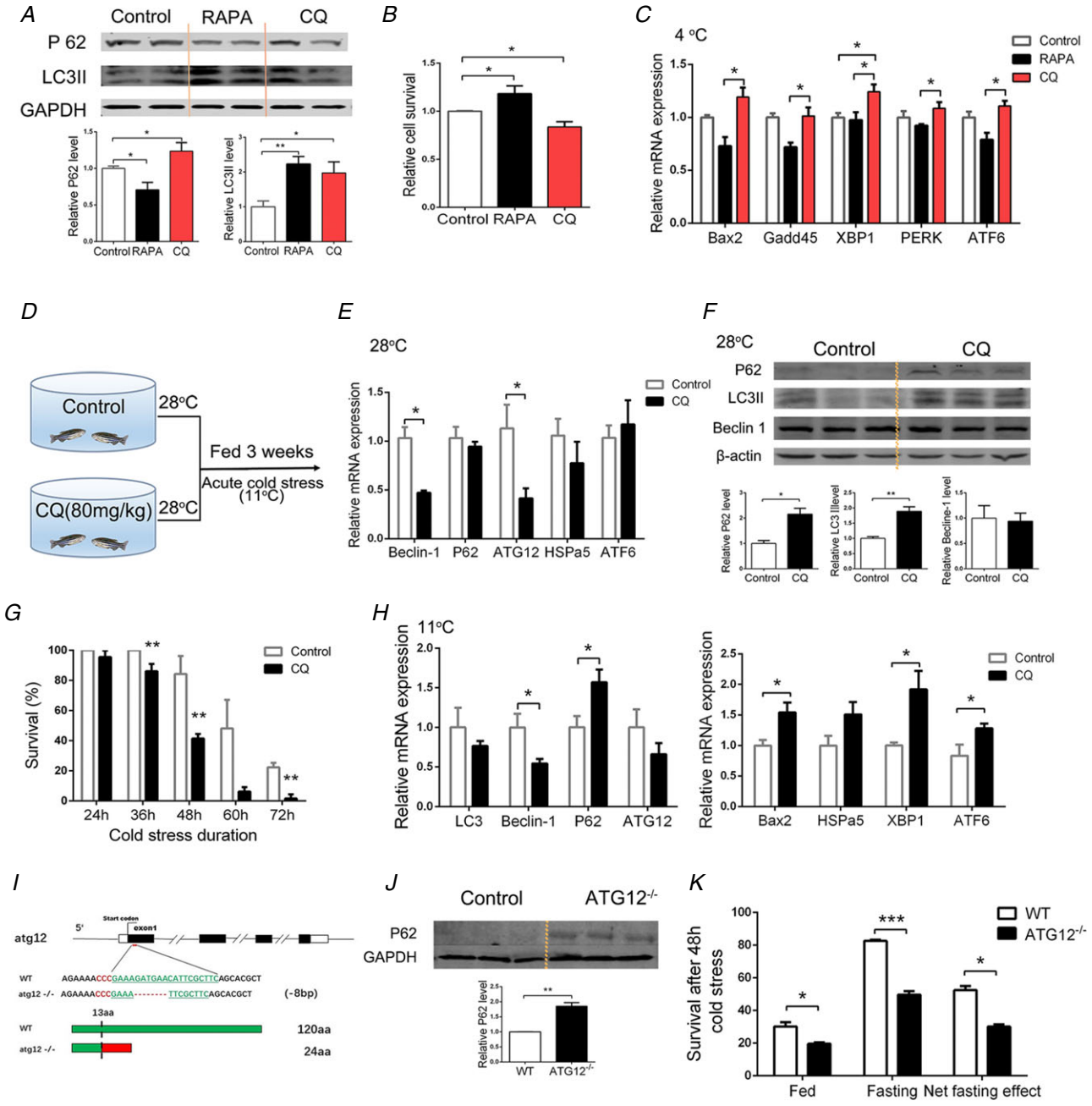


Figure 4. Stimulation of autophagy promotes cold resistance
A, western blotting and quantification of protein involved in autophagy ($n = 2$, experiment was done twice). *B*, relative survival of cells exposed to cold stress ($n = 3$). *C*, relative expression of genes associated with apoptosis and ER stress in ZFL cells under cold stress ($n = 4$–6). *D*, experimental design: zebrafish were fed with control and CQ feeds and then exposed to cold stress. *E*, relative expression of genes associated with autophagy and ER stress in livers of fish at 28°C ($n = 4$–6). *F*, western blotting and quantification of proteins associated with autophagy ($n = 3$). *G*, survival rate of zebrafish under cold stress (three replicates per group, $n = 20$–23 animals). *H*, relative expression of genes associated with autophagy (left) or apoptosis and ER stress (right) in livers of fish at 11°C ($n = 4$–6). *I*, comparisons of genes and amino acid sequences between WT and ATG12$^{-/-}$ zebrafish. *J*, P62 protein content in the liver of WT and ATG12$^{-/-}$ zebrafish ($n = 3$). *K*, survival rate of WT and ATG12$^{-/-}$ zebrafish after 48 h cold stress; net fasting effect are the differences between fasting and fed group (three replicates per group, $n = 13$–18 animals). Data are means ± SEM. Statistical significance was determined by Student's *t* test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

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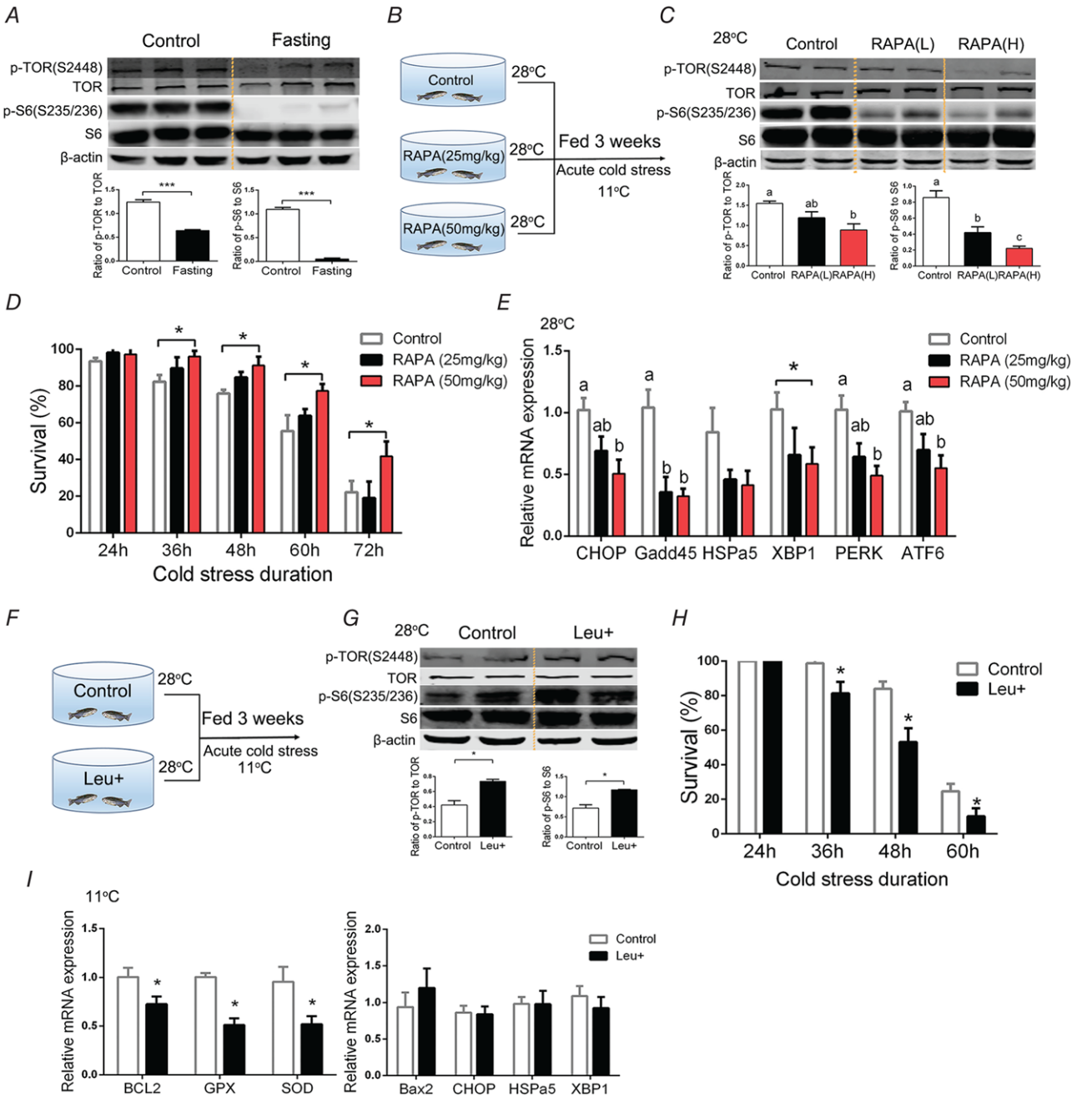


Figure 5. Suppression of mTOR signalling pathway correlates with the cold resistance
A, western blotting and quantification of mTOR signalling pathway proteins in liver of fed and fasted zebrafish ($n = 3$). *B*, experimental design: zebrafish were treated with feeds with three rapamycin concentrations (RAPA) and then exposed to cold stress. *C*, western blotting and quantification of mTOR signalling pathway proteins in livers of fish fed on RAPA feeds ($n = 2$, experiment was done twice). Different letters signify significant differences among groups. *D*, the survival rate of zebrafish under cold stress (four replicates per group, $n = 23$–28 animals). *E*, relative expression of genes associated with apoptosis and ER stress ($n = 6$–8). *F*, experimental design: zebrafish were fed with control (leucine 2%) and Leu$^+$ (leucine 4%) feeds and then exposed to cold stress. Different letters signify significant differences among groups. *G*, western blotting and quantification of mTOR signalling pathway proteins in liver of fish fed on leucine feeds ($n = 2$). *H*, survival rate of zebrafish exposed to cold stress (three to four replicates per group, $n = 26$–27 animals). *I*, relative expression of genes associated with anti-apoptosis and anti-oxidative stress (left) and apoptosis and ER stress (right); ($n = 5$–8). Data are means ± SEM. Statistical significance was determined by Student's *t* test or one-way ANOVA. $^*P \leq 0.05$, $^{***}P \leq 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

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feed efficiently activated mTOR signalling pathways. As expected, the high leucine feed decreased significantly the survival rate of fish under cold stress (Fig. 5*H*). Of note, high-leucine feed reduced significantly the mRNA levels of an anti-apoptosis gene [B-cell leukemia/lymphoma 2 (*BCL2*)] and the anti-oxidative enzymes glutathione peroxidase 1a (*GPX1a*) and superoxide dismutase 1 (*SOD1*), but did not affect expression of the apoptosis- and ER stress-related genes (Fig. 5*I*). Taken together, these results show that the suppression of the mTOR signalling pathways correlates with fasting in enhancing cold resistance in zebrafish. Furthermore, altered activities of mTOR signalling pathways by pharmacological or nutritional approaches affect significantly their resistance to cold stress.

mTOR suppression mimics the comprehensive effects of fasting in promoting cold resistance

Previous studies have indicated different effects between fasting and the direct repression of mTOR in relation to mammals' resistance to multiple stresses (Michael *et al.* 2016). However, whether the effects of fasting-enhanced cold resistance could be completely mimicked by mTOR suppression in fish remains unclear. Therefore, we fed adult zebrafish with control or RAPA (50 mg/kg) feed for 3 weeks. Some fish in the control group were fasted during the last 3 days, and the fish in the control (fed), RAPA and fasting groups were exposed to acute cold stress (Fig. 6*A*). During the first 36 h under cold stress, the fasting and RAPA groups displayed similar survival rates, which were higher than that of the control group; for the overall duration, however, zebrafish in the fasting group had a higher survival rate than the RAPA group (Fig. 6*B*), suggesting that fasting had a stronger effect than the RAPA treatment. We then measured the metabolic efficiency of fish at both 28°C and 11°C, and found that the fatty acid β-oxidation ability was elevated both by fasting and RAPA, but it was significant only for the fasting group as compared with the control fish (Fig. 6*C*). Similarly, at 11°C, the oxygen consumption rate was increased significantly in both treatments, with the highest value obtained in the fasting group (Fig. 6*D*). Moreover, we assayed key proteins involved in the mTOR signalling pathway, energy supply and autophagy in livers of fish under cold stress. The results indicated that both RAPA and fasting decreased the ratios of p-mTOR/mTOR, and increased the phosphorylation of AMP-activated protein kinase [p-AMPK (T172)], LC3-II and beclin-1. However, fasting generally had stronger effects than RAPA in these modulations, except in p-S6/S6, which was not reduced by fasting under cold stress (Fig. 6*E*). The above data indicate that RAPA and fasting promote cold resistance through similar modulation approaches; however, fasting has stronger effects than RAPA, and this correlates with the higher degree of lipid catabolism induced by fasting.

To further compare the global gene patterns between RAPA and fasting in cold stress, we performed the hepatic transcriptome assays before and after cold stress. The cluster analysis of the changed genes in significantly changed pathways indicated that, before cold stress, the gene patterns of the RAPA treatment (R) and the control group (C) were similar, while after cold stress, the RAPA (RC) and fasting (FC) treatments had more comparable gene patterns (Fig. 6*F*). We next collected the significantly changed genes associated with apoptosis, stress response and metabolism, and made comparisons among groups (Fig. 6*G*). The results indicated that under cold stress, RAPA treatment and fasting generally resulted in a similar pattern of decreased expression of the genes involved in apoptosis, stress response, lipid transport and synthesis, amino acid metabolism, and gluconeogenesis. These transcriptomic data indicate again that fasting and mTOR suppression has similar effects in promoting cold resistance.

Discussion

In fish, acute temperature decrease reduces the activities of various enzymes and the fluidity of biomembranes, consequently affecting many biological processes (Hazel, 1995; Viña, 2002). By inhibiting metabolic efficiency and destroying membrane structure of cell, cold stress may lead to an energy-supply shortage and cause cell damage, both of which can lead to fish mortality. The present study indicates that fasting helps fish to resist acute cold stress by stimulating lipid catabolism and autophagy, and these two processes were correlated with increased energy supply and reduced cellular damages, respectively. The inhibition of the mTOR signalling pathway mostly mimicked the beneficial effects of fasting in promoting cold resistance in fish. Our study highlights the important roles of lipid catabolism and autophagy in cold resistance in fish and demonstrates that fasting can act as a protective strategy in fish to resist cold. We illustrates the correlations among fasting, lipid catabolism, autophagy and mTOR repression in zebrafish under cold stress in Fig. 7.

Energy metabolism is a basic life characteristic that is involved in all physiological activities, including adaptations to cold stress. In cold environments, mammals increase their food intake to provide additional energy to balance the loss of heat (Lowell & Spiegelman, 2000). Shivering of skeletal muscle and non-shivering thermogenesis of brown adipose tissue are two important thermogenesis mechanisms in cold adaptation in mammals. Thermogenesis from skeletal muscle shivering mainly fuels muscle glycogen and plasma glucose (Haman *et al.* 2010), while non-shivering thermogenesis is mainly mediated by a brown-fat-specific mitochondrial

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protein (uncoupling protein 1) and also depends on the catabolism of lipids (Enerbäck *et al.* 1997). Recently, non-shivering thermogenesis has also been reported to rely on the supply of endogenous free fatty acid, which is regulated by ATGL in white adipose tissue and cardio muscle (Schreiber *et al.* 2017). Therefore, both glucose and free fatty acids are efficient fuels in resisting coldness in mammals. However, during long periods of cold, lipids are the main energy source in some animals. For example, hibernating animals shift the utilization of energy from carbohydrates to fat during torpor (Carey *et al.* 2003). In teleosts, although metabolic enzyme activities decrease with decreasing water temperature, fish strive to keep the body temperature between 0.1 and 1°C above the ambient water temperature (Beitinger *et al.* 2000). To maintain a higher body temperature, several compensation mechanisms are activated in fish, including increasing mitochondrial volume density and other

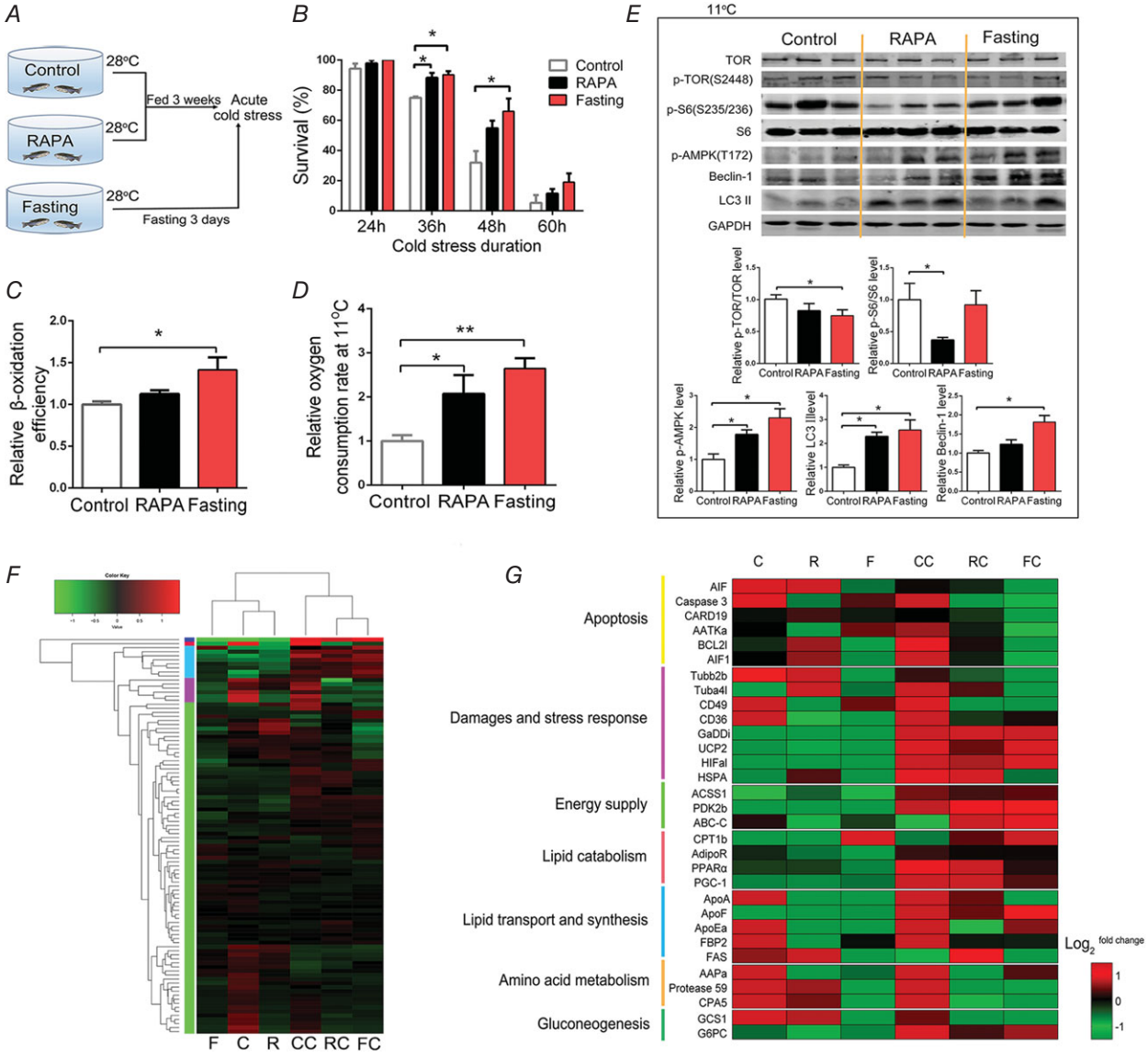


Figure 6. The mTOR suppression mimics the comprehensive effects of fasting in promoting cold resistance

A, experimental design: zebrafish treated with control, RAPA feeds and fasting, subsequently exposed to cold stress. *B*, survival rate of zebrafish under cold stress (three to four replicates per group, n = 14–23 animals). *C*, relative fatty acid β-oxidation efficiency of zebrafish. *D*, relative oxygen consumption rate of fish under cold stress (n = 3). *E*, western blotting and quantification of protein involved in the mTOR signalling pathway, energy supply and autophagy in livers of fish under cold stress (n = 3). *F* and *G*, liver transcriptome analysis of zebrafish livers. *F*, cluster analysis of the expression of mainly changed genes. *G*, heat map of the expression of genes associated with seven biggest changed functions. Data are means ± SEM. Statistical significance was determined by Student's *t* test. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

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mitochondrial properties (membrane phospholipids, enzymatic complement and cristae densities) to elevate the energy supply when exposed to lower temperatures (Guderley, 2004). Previous studies also found that lower temperatures decreased the whole fish triacylglycerol level (Lermen *et al.* 2004), increased the activity of fatty acid oxidation enzyme (Kyprianou *et al.* 2010), and increased the expression of lipid metabolism-related genes (Gracey *et al.* 2004). However, the dispensable roles of lipid metabolism in cold adaptation were never discussed in fish. It should be noticed that in the first 48 h of fasting, liver glycogen was reduced significantly and this indicates fish first used carbohydrate to supply energy, but after 48 h, lipid utilization was increased. Considering short fasting (less than 48 h) did not promote significantly cold resistance, the utilization of lipids was more important than that of carbohydrates for the cold stress resistance of zebrafish. Similarly, when mammals were fed on ketogenic diets (high fat, low carbohydrate diet), they switched metabolism from carbohydrate to lipid utilization by up-regulating the genes associated with oxidative phosphorylation and fatty acid oxidation pathways (Bough *et al.* 2006; Kennedy *et al.* 2007), the number of mitochondria and the electron transport chain proteins (Srivastava *et al.* 2012). This largely increase energy metabolites in the body of animals (Bough & Rho, 2007). Considering that fish are unable to utilize carbohydrates as efficiently as mammals (Hemre *et al.* 2002; Polakof *et al.* 2012), lipids are very likely to be a more efficient energy source to resist acute cold stress in fish.

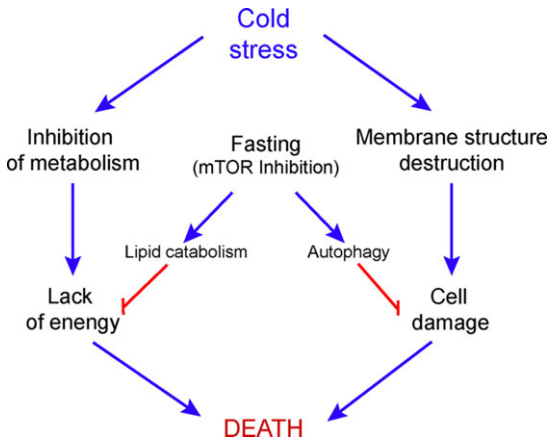


Figure 7. Summary of cold stress resistance mechanism of fasting
In zebrafish, cold stress inhibits metabolism and impairs membrane structure, which suppresses energy supply and induces cell damages, and finally leads to fish death. Fasting could stimulate lipid catabolism and autophagy, which were highly correlated to the energy supply and cellular damage attenuation, respectively, and result in enhancing the resistance to acute cold stress. The inhibition of the mTOR signalling pathway could mostly mimic the beneficial effects of fasting in promoting cold resistance in fish. [Colour figure can be viewed at wileyonlinelibrary.com]

Other than inhibition of the energy supply, our study also found fasting reduced the damage caused by cold stress through inducing autophagy. It is known that autophagy degrades the misfolded proteins and damaged organelles caused by stresses (Mizushima *et al.* 2008). However, whether these mechanisms are applied in conditions of thermal stress was unknown. In moderately cold environments, mammals maintain their body temperature by enhancing thermogenesis, without it causing perceptible damage. Therefore, the requirements for repairing damaged organelles are not likely to be high in mammals in cold conditions. In poikilotherms, autophagy has been found to help *Caenorhabditis elegans* survive heat stress (Kumsta *et al.* 2017), but a study on *D. melanogaster* showed a negative effect of autophagy caused by fasting on resisting cold stress (Le Bourg & Massou, 2015). The present study revealed that activation of autophagy due to fasting is an important mechanism of fish to resist cold stress.

Interestingly, fish can survive much longer periods of starvation than many homeotherms by adjusting their metabolic, physiological and morphological functions to adapt to the long starvation (Krumschnabel & Podrabsky, 2009). In addition, fish may initially decrease or even stop feeding when suffering from different stresses (Kulczykowska *et al.* 2010), especially changes in the environment, such as temperature, dissolved oxygen and salinity (Arnesen *et al.* 1998). When fish adapt to the new environment, their feed intake will recover (Liebert & Schreck, 2006). Considering the beneficial effects of fasting in promoting energy utilization and inducing autophagy, among other processes, it is reasonable to suggest that fasting is a protective strategy to resist a wide range of stresses in fish, including acute cold stress.

There is consensus that, in mammals, the positive effect of dietary restriction in causing life extension is probably correlated with a reduced mTOR signalling pathway (Miller *et al.* 2014), and its repression also plays positive roles in resisting multiple environmental stresses (Johnson *et al.* 2013). However, the correlation between mTOR repression and cold resistance has not been explained in fish. In the present study, we found that repression and stimulation of mTOR signalling by RAPA or leucine-enriched feed in zebrafish enhanced or weakened the resistance to acute cold stress, respectively. Moreover, treatment using RAPA also showed similar beneficial effects to fasting in stimulating the energy supply, reducing oxidative stress and inducing autophagy. However, the cold-resistant effect of RAPA treatment was relatively weaker than that of fasting; possibly, the experimental condition did not completely simulate the real physiological status. Despite this, the results obtained still indicate that mTOR repression may be involved

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in the mechanisms of fasting-assisted cold resistance. The different outcomes between RAPA treatment and fasting may result from the different degrees of mTOR inhibition. Some studies have indicated that fasting may regulate lipid catabolism and autophagy through other signalling pathways rather than mTOR. For examples, fasting stimulated the SIK2-p300-PPARα pathway to mediate glucagon signalling and regulated lipid metabolism (Zhang *et al.* 2016). Fasting also induced autophagy by activating AMPK and then directly activated phosphorylation of Forkhead box O3 (FoxO3) and the Unc-51-like kinase 1 (Ulk1) without repression of mTOR (Mammucari *et al.* 2007; Sanchez *et al.* 2012). We also noticed that p-S6 level was decreased by fasting at 28°C (Fig. 5*A*). However, at 11°C, the fasting-induced p-S6 inhibition was recovered (Fig. 6*E*). Of note, the fasting-induced p-mTOR inhibition was still maintained under cold stress. Considering p-S6 is regulated through several pathways, including mTOR–P70S6K, 3-phosphoinositide-dependent protein kinase-1 (PDK1)–P70S6K and RAS/extracellular signal-regulated kinase (ERK)–P90S6K (Alessi *et al.* 1998; Frödin & Gammeltoft, 1999; Magnuson *et al.* 2012), cold stress might up-regulate p-S6 level through mTOR-independent pathways in the fasting fish. As reported in overnight-fasted rats, acute cold exposure up-regulated expression of PDK1 and RAS (Wang & Wahl, 2014). This suggests that in the fasting fish, cold exposure might up-regulate p-S6 through PDK1 and RAS signalling. Although other signalling pathways than mTOR might be involved in fasting, the present study showed that the suppression or stimulation of mTOR signalling affected largely the cold tolerance in zebrafish. More importantly, the suppression of mTOR by RAPA also stimulated lipid catabolism and autophagy as fasting. It is reasonable to suggest that the fasting-stimulated lipid catabolism and autophagy may be regulated through the mTOR signalling pathway.

In summary, we found that fasting enhanced significantly the resistance to acute cold stress in zebrafish. This process may be derived from the mTOR inhibition-mediated stimulation of energy supply by increased lipid catabolism, as well as relief of cellular damage through increased autophagy. Considering that fish decrease their feed intake or stop feeding in cold environments, fasting could be a protective strategy for fish to survive under cold stress.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

D-L.L. and Z-Y.D. conceived the study and designed experiments; D-L.L., Q. M., J.W., L.-Y.L. and S.-L.H. performed experiments; D-L.L., Q. M. and S.M.L. analysed data; D.-L.L. generated the CPT1b and ATG12 knock-out zebrafish strains; M.-L.Z. and L.-Q.C. provided essential technical supports and helped to revise the manuscript; D-L.L., S.M.L. and Z-Y. D. wrote the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors

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