Published online 25 August 2011

Nucleic Acids Research, 2011, Vol. 39, No. 20 ***e139***
doi:10.1093/nar/gkr645

Transgenic zebrafish model to study translational control mediated by upstream open reading frame of human *chop* gene

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Received January 13, 2011; Revised July 21, 2011; Accepted July 22, 2011

ABSTRACT

Upstream open reading frame (uORF)-mediated translational inhibition is important in controlling key regulatory genes expression. However, understanding the underlying molecular mechanism of such uORF-mediated control system *in vivo* is challenging in the absence of an animal model. Therefore, we generated a zebrafish transgenic line, termed huORFZ, harboring a construct in which the uORF sequence from human CCAAT/enhancer-binding protein homologous protein gene (huORF^{chop}) is added to the leader of GFP and is driven by a cytomegalovirus promoter. The translation of transgenic *huORF^{chop}-gfp* mRNA was absolutely inhibited by the huORF^{chop} cassette in huORFZ embryos during normal conditions, but the downstream GFP was only apparent when the huORFZ embryos were treated with endoplasmic reticulum (ER) stresses. Interestingly, the number and location of GFP-responsive embryonic cells were dependent on the developmental stage and type of ER stresses encountered. These results indicate that the translation of the huORF^{chop}-tag downstream reporter gene is controlled in the huORFZ line. Moreover, using cell sorting and microarray analysis of huORFZ embryos, we identified such putative factors as Nrg/ErbB, PI3K and hsp90, which are involved in huORF^{chop}-mediated translational control under heat-shock stress. Therefore, using the huORFZ embryos allows us to study the regulatory network involved in human uORF^{chop}-mediated translational inhibition.

INTRODUCTION

The upstream open reading frame (uORF) is an mRNA element in the 5′-untranslated region (UTR), which affects the translational efficiency of many eukaryotic genes. A uORF is present in approximately half of human and mouse transcripts, and it is conserved among mammals (1,2). The transcripts of genes containing uORF are involved in growth, differentiation and proliferation (1–3). Knockout of the uORF start codon of the C/EBPβ gene in mice results in hyperactivation of acute-phase response genes, persists repression of E2F-regulated genes and impairs osteoclast differentiation (4). Although these results demonstrate that uORF-mediated translational control plays important roles in mediating gene expression, no knock-in animal model has been developed to study its underlying molecular mechanisms.

Transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP) is a member of the C/EBP family and plays important roles during proliferation, differentiation and endoplasmic reticulum (ER) stress (5–7). Stimuli that disrupt the homeostasis of the ER affect proper protein folding and lead to the accumulation of unfolded and misfolded proteins in the ER lumen, finally inducing ER stress (8). The *chop* is slightly expressed and present in the cytosol under non-stressed conditions. However, excess stress leads to induction of *chop* and its accumulation in the nucleus to suppress the transcription of the *Bcl-2* gene, finally inducing cellular apoptosis (7,9,10). Nevertheless, *chop* expression is highly regulated and can be rapidly induced in response to different stress stimuli, including oxidative and reductive stress (11,12). Interestingly, induction of *chop* involves both transcriptional and post-transcriptional mechanisms (13), and a uORF located at the 5′-UTR of *chop* mRNA

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inhibits the rate of translation (14,15). Again, however, since no *in vivo* animal model is available, we cannot fully understand the molecular regulation of uORF^{chop}-mediated translational control during ER-associated stresses.

We have addressed this problem by generating a zebrafish transgenic line harboring a plasmid construct in which the uORF sequence derived from the human *chop* (huORF^{chop}) is added to the leader of the reporter GFP mRNA and is driven by a cytomegalovirus (CMV) promoter. This zebrafish line, termed huORFZ, provides a novel *in vivo* system to study the mechanism of uORF-mediated translational control. In addition, we demonstrated that huORFZ embryos are specific and sensitive in their translation of the GFP reporter under various stresses. As such, this zebrafish transgenic line can also serve as a model for monitoring ER stresses.

MATERIALS AND METHODS

Zebrafish husbandry and microscopy

Zebrafish were raised as described (16). Fluorescence was visualized with a fluorescent stereomicroscope (MZ FLIII, Leica) and a confocal spectral microscope (TCS SP5, Leica).

Plasmid construction

The scheme of constructing all the plasmids used in this study was illustrated in Figure 1A. Plasmids phuORF^{chop} and phuORF^{chop}-Lu containing huORF^{chop} fused with luciferase (Lu) were described previously (15). BikDD is a Bik mutant having mimic phosphorylation at T33D and S35D residues to enhance their binding affinity with the anti-apoptotic proteins Bcl-X_L and Bcl-2. Consequently, BiKDD is more potent than wild-type Bik and other Bcl-2 family proapoptotic genes in inducing apoptosis (17). The zebrafish uORF^{chop} fragment (zfuORF^{chop}) was obtained from PCR using a forward primer (zfuORF^{chop} -F: ACAA AGCTTATGGTTAACATGAGCGATC) and a reverse primer (zfuORF^{chop}-R: TGTGCATTTCACGCTCTCCA CAAGAAGA). The zfuORF^{chop} fragment was inserted at the upstream of red fluorescent protein reporter *DsRed* (Clontech). Then, plasmid pzfuORF^{chop}-DsRed was constructed after the huORF^{chop} fragment in phuORF^{chop} was replaced by the zfuORF^{chop}-DsRed fragment. Total RNAs of zebrafish embryos at 72 hours post-fertilization (hpf) were extracted to generate the cDNA pools by Superscript III Reverse Transcriptase Kit (Invitrogen). After the coding sequence of zebrafish *Nrg* was cloned by RT-PCR, it was inserted into plasmid pGEMTeasy (Promega), as confirmed by sequencing, and the subcloned *Nrg* coding sequence was inserted into pCS2+ vector to generate pCS2 + zNrg.

Microinjection and generation of transgenic zebrafish

Microinjection was performed as described (18). All the mRNAs were generated by the SP6 Message Machine Kit (Ambion), and they were diluted to working concentrations of 44 ng/μl prior to each 2.3 nl injection. Plasmids phuORF^{chop}-GFP and pzfuORF^{chop}-DsRed were linearized by PvuI and injected into one-celled stage embryos with a volume of 2.3 nl of a DNA solution (15 ng/μl). Founders (G0) with germline transmission of the transgene were identified by pair-crossing with wild-type zebrafish after they reached adulthood, and their progeny were treated with heat stress at 24 hpf and screened for GFP expression at 48 hpf. Progeny that were GFP-positive (F1 generation) were raised to adulthood to establish the transgenic lines.

Zebrafish brain injection was performed as described (19), except that we used embryos at 72 hpf; anisomycin (4 M) and rapamycin (500 μM) were mixed with 0.1% (v/v) phenol red prior to microinjection, and ~2.3 nl was injected into the embryo's brain.

Cell culture, transfection and drug treatment

Cell lines of HEK293T and HeLa were cultured and transfected as described (15). After 24 h, the cultures were changed with fresh medium for 2 h, and the following were added: dimethyl sulphoxide (DMSO), 2.5 μg/ml tunicamycin (Sigma), 1 μM thapsigargin (Sigma), 0.5 μM anisomycin, 600 μM indomethacin or 10 mM sodium salicylate (Sigma). Cells were harvested 0–4 h post-treatment.

WISH, western blot analysis and dual Lu assay

The procedures of Whole-mount *in situ* hybridization (WISH) were previously described (20), except that the coding sequences of *chop* and double-stranded RNA-activated protein kinase-like ER kinase (*PERK*) were isolated by RT–PCR and labeled with DIG to serve as riboprobes. Western blot analysis and dual Lu assay were previously described (15), except that deyolked samples were dissolved in 2 μl of 2× sodium dodecyl sulfate (SDS) sample buffer per embryo and incubated for 5 min at 95°C. After full-speed centrifugation for 1 min in a microcentrifuge to remove insoluble particles, the samples were loaded on a 12% SDS gel (seven embryos per lane). Antibodies used were 1:750 anti-GFP (Santa Cruz), 1:2000 anti-DsRed (abcam) and 1:750 anti-β-actin (abcam).

Cell death assay

Stable transfectants of phuORF^{chop}-GFP or phuORF^{chop}-BikDD (cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 100 μg/ml of penicillin/streptomycin) were seeded on a 10-cm dish the day before drug treatment. Fresh medium containing 600 μM indomethacin was added to the culture. After 0–4 h, cells were harvested for cell death assay. Trypsinized cells were resuspended in phosphate buffered saline (PBS), and 10 μl of trypan blue was added to 90 μl supernatants. Cell death was determined on a 10-μl aliquot of mixture using a hemocytometer. Survival rate (%) is presented as the number of live cells divided by the number of total cells. The mean values of three independent experiments were represented.

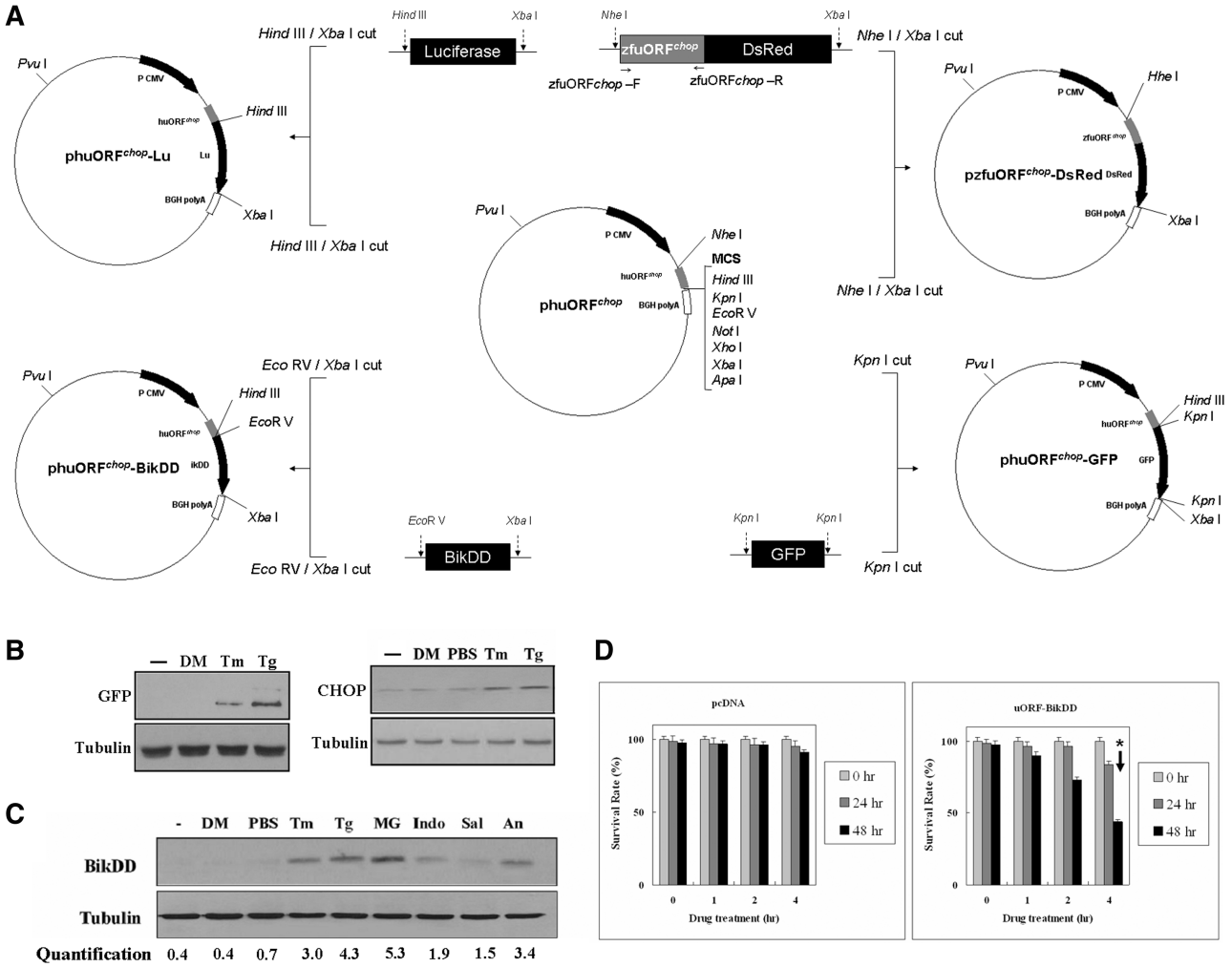

Figure 1. Characterization of uORF^{chop} *in vitro*. (**A**) The scheme of plasmids' construction. (**B**) Western blot analysis of the GFP (left) or CHOP (right) in HEK293T cells transfected with plasmid phuORF^{chop}-GFP and treated with tunicamycin (Tm; 2.5 μg/ml), thapsigargin (Tg; 1 μM) or DMSO (DM; as solvent control). (**C**) Western blot analysis of the BikDD protein in HEK293T cells transfected with plasmid phuORF^{chop}-BikDD and treated with various drugs [DMSO(DM), PBS, 2.5 μg/ml tunicamycin (Tm), 1 μM thapsigargin (Tg), 0.5 μM MG132 (MG), 600 μM indomethacin (Indo), 10 mM sodium salicylate (Sal) and 0.5 μM anisomycin (An)]. These signals were quantified as indicated. (**D**) The stable transfectants [pcDNA (left) or phuORF^{chop}-BikDD (right)] were treated with indomethacin (Indo) and analyzed by cell death assays. Asterisk indicates the significant difference at $P < 0.05$ level.

Stress treatment

For heat-shock and cold-shock experiments, a 2 ml centrifuge tube filled with 30 dechorionated embryos at 72 hpf was subjected to 40°C for 1 h and 5°C for 12 h, respectively. The treated embryos were collected into a 3-cm Petri dish and incubated at 28.5°C until they developed at 96 hpf. To examine the responsiveness of stress strength, 30 dechorionated embryos were subjected to heat-shock at 37–40°C for 1 h or at 40°C for 20–60 min. Adult fish were heat-shocked by incubation at 40°C for 2 h in a 500-ml tank filled with water and supplied with air, and subsequently transferred to a 28.5°C incubator for 24 h. For drug treatment, the concentrations of inhibitors we used were dependent on the drug toxicity of embryos at 72 hpf treated for 24 h. Thapsigargin (Sigma), 17AAG (Sigma), AG1478 (Calbiochem) and LY294002 (Calbiochem) were dissolved in DMSO. $CoCl_2$ (Sigma) was dissolved in embryo medium. Deoxygenated water was prepared by boiling the embryo medium. All drugs were diluted to the desired final concentrations by embryo medium. We treated embryos with thapsigargin at 1 μM for 24 h, 17AAG at 10 μM for 24 h, LY294002 at 30 μM for 5 h, $CoCl_2$ at 4 mM for 24 h, alcohol at 1.5% for 24 h, AG1478 at 5 μM for 16 h and deoxygenated water for 4 h. Control embryos were treated with the equivalent amount of DMSO solution. The embryos at 72 hpf were incubated at 28.5°C when they were treated with these drugs. Treatment was performed in a 3-cm Petri dish filled with 30 embryos in a total volume of 3 ml embryo medium. After drug treatment, we removed drug by washing twice with embryo medium. Then, embryos were collected into a new 3-cm Petri dish and incubated until they developed at the 96-hpf stage.

Dissociation of embryonic cells and FACS

Four hundred embryos at 120 hpf from the huORFZ line were collected into a 2-ml Eppendorf, resuspended with 1.2 ml pre-heated (28°C) protease solution (PBS, pH 8; 0.25% trypsin; 1 mM EDTA) and incubated for 24 min at 28°C. During the incubation period, the mixture was pipetted 50 times every 12 min with a 1-ml pipette tip. After all cells were completely separated, 0.2 ml stop solution (30% Fetal bovine serum (FBS); 6 mM $CaCl_2$; PBS) was added, and the mixture was pipetted 50 times and incubated at 28°C for 5 min to stop trypsin activity. Cells were then centrifuged at 3000 rpm for 5 min and re-suspended in 1 ml sorting solution (5% FBS, 50 U/ml penicillin, 0.05 mg/ml streptomycin and PBS). Fluorescence activated cell sorting (FACS) was performed at room temperature under sterile conditions using a FACSAria cell sorting system (BD Bioscience, CA, USA), according to standard protocols described previously (21). Following sorting, when cell viability was >95%, 5×10^5 GFP-positive cells were isolated and 1300 pg of total RNA were extracted to prepare microarray analysis.

Microarray analysis

The Zebrafish Oligo Microarray Kit (Agilent, Taiwan), which contained 95 000 probes covering 45 000 genes, was used, and the microarray data were analyzed by Welgene Biotech Co. (Agilent, Taiwan) using an Agilent Certified Service Provider Program.

Quantitative RT–PCR

Equal numbers of GFP-positive and GFP-negative cells were centrifuged, resuspended in 200 µl of Trizol Reagent (Invitrogen) and stored at −80°C. Total RNA was isolated according to manufacturer's instructions. For quantitative PCR, first-strand cDNA was generated using 1 µg total RNA. Both cDNA concentrations were adjusted to 200 ng/µl and qPCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. PCR primers were as follows: *Arr,* GACCA CCAGGCAGTTCTC and TGGACGTTGACGCTGAT G; *bip*, AAGAGGCCGAAGAGAAGGAC and AGCAG CAGAGCCTCGAAATA; *ef1a*, CTCCTCTTGGTCGC TTTGCT and CCGATTTTCTTCTCAACGCTCT; MMP13: ACTCGTCGATGCATGCN and TGACTGT ACTGATTTAGCAA; *Hsp90*, ATCGTGGTGCATGC GTCC and TTCATGCTACGATCC; *Nrg*, AGGGA GATGCTGGAT and CGGCCAAGACATCCAG. Expression levels were determined by comparison with a standard curve from total RNA isolated from whole embryo. Since the amount of *ef1a* mRNA was equally displayed in both GFP-positive and GFP-negative cells, values from GFP-negative and GFP-positive cells were normalized to *ef1a* to obtain relative expression levels. Standard deviations were calculated from triplicate measurements.

Genomic DNA extraction and Southern blot analysis

Genomic DNA and Southern blot analysis followed the methods described by Chou *et al*. (22) with some modifications. Fifty zebrafish embryos at 72 hpf were digested with proteinase K (200 µg/ml) solution containing 0.5% SDS and 25 mM EDTA for 16 h at 55°C. Genomic DNAs extracted from either wild-type embryos or F3 huORFZ embryos were digested with HindIII or XbaI and transferred onto a nylon membrane (Amersham, USA). Hybridization was carried out using an EGFP-specific DIG-labeled probe (500 bp), which was prepared via PCR using a forward primer (ATGGTGAGCAAGG GCGAGGA) and a reverse primer (AGAAGATGGTGC GCTCCTGG). Following the hybridization of the DIG-labeled probe, positive signals were visualized 2 h following the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

RESULTS

Under ER-stress-inducing drug treatment, huORF^{chop}-mediated translational inhibition is repressed *in vitro*

We investigated the response of huORF^{chop}-mediated translational inhibition under ER stress conditions *in vitro*. To accomplish this, HEK293T cells were transfected with the plasmid phuORF^{chop}-GFP and subsequently treated with tunicamycin and thapsigargin, which are ER-stress-inducing drugs by the release of calcium from the ER of cells. The GFP was hardly detected under normal condition, whereas the appearance of GFP was greatly induced if cells were treated with either tunicamycin or thapsigargin (Figure 1B). Similarly, compared to untreated cells, the endogenous CHOP protein in cells was increased significantly after cells were treated with either drugs (Figure 1B). These data suggested that huORF^{chop}-mediated translational inhibition is blocked by the ER stress-inducing drugs *in vitro*.

We next constructed an expression plasmid, phuORF^{chop}-BikDD, in which a constitutive active form of BikDD (17), driven by huORF^{chop}, was translated. The functionality of the BikDD protein is demonstrated by its apoptotic effect. Accordingly, results showed that the BikDD protein was detected only when cells were treated with various ER-stress-inducing drugs (Figure 1C), suggesting that the huORF^{chop} cassette lost its inhibitory capacity, resulting in the translation of a functional BikDD encoded by the downstream mRNA. Citing another example, HeLa cells were transfected with phuORF^{chop}-BikDD, and apoptosis was observed after a 4-h treatment of indomethacin (Figure 1D). Moreover, the degree of cell death was positively correlated with the expression level of BikDD. In both cases, the apoptotic effect, which was mediated by expression of BikDD, indicated that the translated BikDD is functional. This line of evidence indicates that downstream genes behind the huORF^{chop} sequence are

inducible to translate into functional proteins under ER stress-inducing drug treatment.

The huORF^{chop} and zfuORF^{chop} sequences inhibit the translation of the downstream reporter gene *in vivo*

We microinjected the linearized plasmids pGFP and phuORF^{chop}-GFP into zebrafish embryos. While the GFP signal could be detected in the control embryos injected with plasmid pGFP (Figure 2A), it was only weakly observed in the embryos injected with plasmid phuORF^{chop}-GFP (Figure 2B). We constructed plasmid pzfuORF^{chop}-DsRed, in which the huORF^{chop} and GFP reporter were replaced by zfuORF^{chop} and DsRed, respectively. Results showed that the zfuORF^{chop} sequence also repressed the expression of the DsRed reporter (Figure 2C versus D). RT–PCR and western blot analysis were further employed to demonstrate that the reporter gene in the uORF^{chop}-containing plasmids was transcribed (Figure 2I), but was not translated (Figure 2J). To rule out the possibility that reduced fluorescent protein expression may have been controlled at the transcriptional level, we microinjected mRNA transcribed from the plasmids with or without the uORF^{chop} sequence. In both cases, although the mRNAs of *gfp* and *DsRed* were distributed evenly throughout the control embryos (Figures 2E and G), the GFP proteins were not detectable in the embryos injected with mRNA containing the uORF^{chop} sequence (Figures 2F and H). RT–PCR showed that both *huORF^{chop}-GFP*-mRNA and *DsRed*-mRNA were present in embryos, but western blot analysis showed only the presence of DsRed protein (Figure 2K), indicating that the translation of the downstream reporter gene was inhibited by the uORF^{chop} sequence. Taken together, the uORF^{chop} cassette can repress the translation of downstream genes, irrespective of whether uORF^{chop} is human or zebrafish in origin.

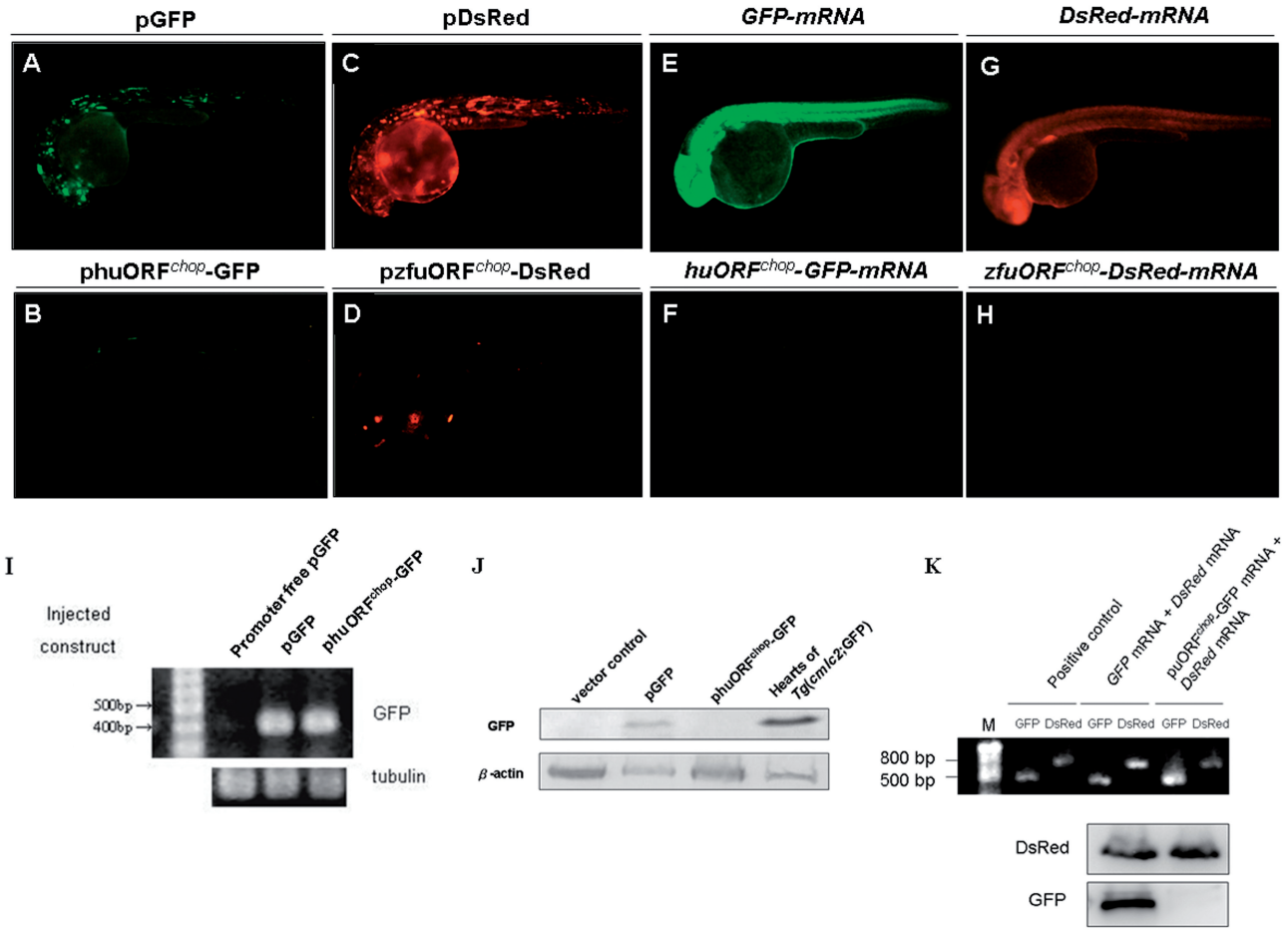

Figure 2. Translational inhibition directed by the uORF^{chop} segment *in vivo*. The expression of reporter gene at 28 hpf was observed in embryos injected with plasmid pGFP (**A**), phfuORF^{chop}-GFP (**B**), pDsRed (**C**) or pzfuORF^{chop}-DsRed (D) under fluorescence microscopy. The reporter gene was greatly repressed in the embryos injected with plasmids containing the uORF^{chop} segment. Embryos at the one-celled stage were injected with mRNA encoding GFP (**E**), huORF^{chop}-GFP (**F**), DsRed (**G**) or zfuORF^{chop}-DsRed (**H**), and observed fluorescent signals at 28 hpf. (**I**) RT–PCR detection of GFP cDNA in embryos injected with indicated plasmids. The promoter-free pGFP, in which the promoter region was deleted by cutting with PvuI and HindIIII, served as negative control. (**J**) Western blot detection of GFP in embryos injected with indicated plasmids. Embryos whose hearts were tagged with GFP were derived from transgenic line *Tg(cmlc2::GFP)* and used as positive control. (**K**) RT–PCR and western blot analysis were used to detect reporter cDNA and protein level, respectively, in embryos injected with indicated mRNA. The templates of plasmid pGFP or pDsRed served as positive control.

Development of transgenic line huORFZ harboring the huORF^{chop} sequence

Transgenic lines harboring huORF^{chop}-GFP were generated by microinjection of phuORF^{chop}-GFP linearized by PvuI digestion into zebrafish embryos. We screened the putative founders to determine if the GFP signal appeared at 48 hpf after the embryos at 24 hpf were treated with heat-shock. Seven F1 transgenic lines were obtained from 375 G0 founders screened. Thereafter, seven hereditarily stable F2 heterozygotic strains were generated individually. All embryos derived from these seven transgenic lines shared a common characteristic in that they did not exhibit GFP signals unless they were treated with heat-shock stress. Based on the stronger intensity of GFP signals, the higher number of GFP-positive cells and the Mendelian inheritance percentage of GFP expression in embryos that responded to heat-shock stress, we chose one transgenic line for further study, designating it as line huORFZ.

The transmitted percentages of exogenous DNA fragment in the next generations of transgenic line huORFZ were 48.7% (38 GFP-positive embryos out of 78 embryos examined) at F2 and 75.9% (192 GFP-positive embryos out of 253 embryos examined) at F3, which were in accordance with Mendelian inheritance, indicating that the transgene was inserted at a single locus in the huORFZ genome. Genomic DNAs from huORFZ embryos were then extracted and digested with HindIII or XbaI. When Southern blotting was performed with an EGFP-specific DIG-labeled probe, two major positive bands with molecular weights of 6.1 kb and 9.2 kb appeared on the gel when genomic DNA was digested with HindIII, whereas two major positive bands with 6.1 kb and 4.8 kb appeared when genomic DNA was digested with XbaI (Supplementary Figure S1), indicating that head-to-tail, tail-to-tail and head-to-head concatemer of transgenic DNA fragments had been formed. Based on the intensity of positive signal against that of known concentration (10 ng) of PvuI-cut phuORF^{chop}-GFP, we calculated there were ~200 copies of the plasmid per haploid genome (Supplementary Figure S1).

The translational inhibition of transgenic line huORFZ harboring the huORF^{chop} sequence can be controlled by ER and ER-associated stresses

In the huORFZ embryos, we found that the *huORF^{chop}-gfp* mRNA was ubiquitously transcribed from 10 hpf to 96 hpf (Figure 3A). However, the GFP signal was not apparent in these embryos from one-cell stage to 96 hpf (data not shown) or at 96 hpf (Figure 3B) under normal condition. It was only when the huORFZ embryos were treated with ER-associated stresses that we found an apparent GFP signal (Figure 3B). When the huORFZ embryos were treated with heat-shock stress at 72 hpf, the GFP signal was observed in brain, spinal cord and head mesenchyme during 96 hpf (Figure 3B), but the distribution patterns and expression level of *huORF^{chop}-gfp* mRNA were unchanged in both huORFZ control embryos and heat-shock-treated embryos (Figure 3C). In fact, the GFP signal was first detected in the head region after heat-shock stress for 8 h, and it then gradually increased in the brain and spinal cord (Figure 3D). Moreover, the responsiveness of the GFP signal in huORFZ embryos seemed to depend on the strength of stresses they encountered. We monitored the stress strength and set a time course to analyze the intensity of GFP controlled by the uORF^{chop}-mediated translational inhibition. We found that the GFP signal increased gradually after heat-shock treatment from 37°C to 40°C for 1 h (Figure 3E). In addition, when treatment was accompanied by a longer induction time at 40°C, the stronger intensity of the induced GFP also appeared in the huORFZ embryos (Figure 3F), suggesting that the degree of translation of downstream genes positively depends on the strength of stress we applied. Moreover, we observed that huORF^{chop}-mediated translational control was responsive to heat-shock stress in adult huORFZ fish (Supplementary Figure S2). Thus, we concluded that the translation of the huORF^{chop}-tag downstream reporter gene could be controlled in the huORFZ line.

We also examined the GFP expression in the huORFZ embryos that were exposed to various ER and ER-associated stresses, including cold-shock, thapsigargin, alcohol, deoxygenated water and $CoCl_2$ (hypoxia-mimetic agents). When the huORFZ embryos were treated with different stresses, we found that the GFP expression patterns were not necessarily identical (Figure 4). For example, after embryos were treated with either cold-shock or alcohol, GFP was strongly detected in brain and spinal cord. When embryos were treated with thapsigargin, GFP was strongly detected in the brain, but weakly in the cranial mesenchyme. When embryos were incubated with deoxygenated water, GFP was strongly detected in the brain and spinal cord. Finally, when embryos were incubated with $CoCl_2$, GFP was strongly detected in the brain and spinal cord, but only slightly detected in the tail muscle tissues. In some cases, we could inject drugs directly into huORFZ embryos to induce reporter gene expression. For instance, after huORFZ embryos were injected with anisomycin, GFP was greatly detected in the brain and spinal cord, but only slightly detected in the kidney primordia (Figure 4B). After huORFZ embryos were injected with rapamycin, GFP was only detected in the hindbrain region and spinal cord (Figure 4B). This evidence suggested that different tissues responded differently to the different stresses they encountered. In addition, we found that neuronal tissues were responsive to heat-shock if the embryos at 72 hpf were treated, whereas the cardiac cells, eye cells and muscle cells were responsive to same stress if the embryos at 96 hpf were treated (Supplementary Figure S3) Thus, different tissues are also responsive to stress at different embryonic stages. Taken together, we concluded that the translational inhibition of the huORF^{chop} sequence responds to stresses in a stage- and tissue-dependent manner.

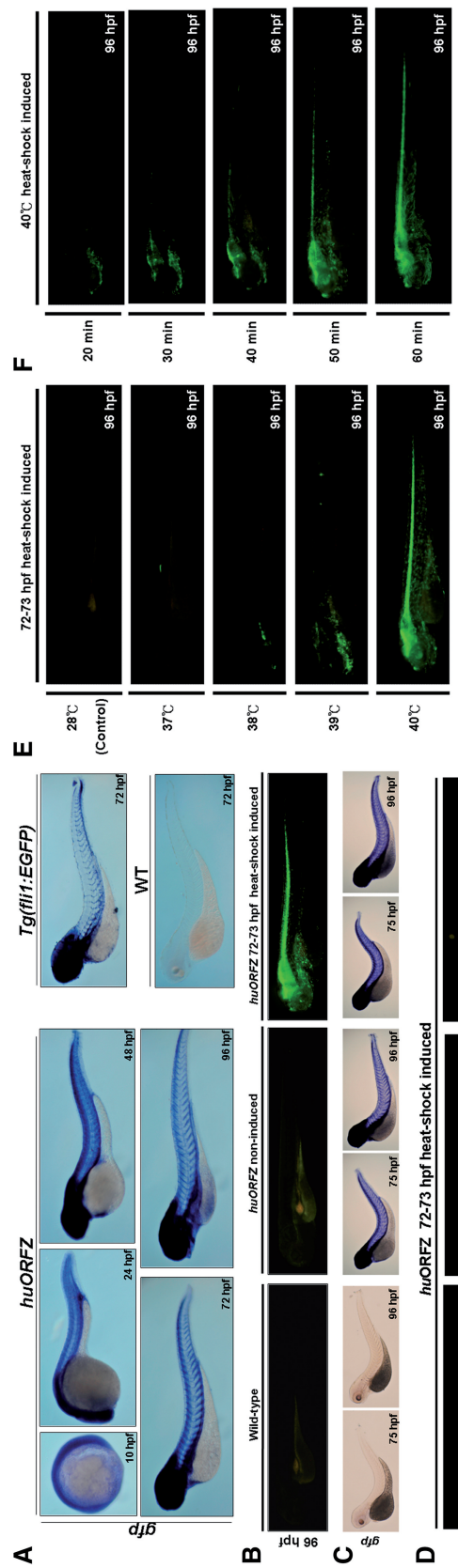

Figure 3. Characterization of the transgenic line huORFZ. (**A**) WISH analysis of *gfp* mRNA driven by $uORF^{chop}$ in huORFZ embryos. The $uORF^{chop}$-*gfp* mRNA was ubiquitously expressed throughout the embryos at 10, 24, 48, 72 and 96 hpf. Embryos derived from line *Tg(fli::GFP)* served as positive control, whereas embryos from the wild-type strain served as negative control. (**B**) Wild-type and huORFZ embryos were exposed to normal temperature (28°C) or heat-shock (40°C for 1 h) at 72 hpf, and fluorescence activity was analyzed during 96 hpf. (**C**) WISH of *gfp* mRNA in wild-type and huORFZ embryos exposed to normal temperature or heat-shock. Compared to embryos at normal temperature, no change in the distribution pattern of $uORF^{chop}$-*gfp* mRNA was observed, even when these same embryos were treated with heat-shock stress at 72 hpf. However, in the huORFZ embryos exposed to heat-shock stress, GFP signals were apparent. (**D**) The fluorescence images of GFP intensity were taken at 2-h intervals after the embryos at 72 hpf from huORFZ line were exposed to heat-shock stress at 40°C for 1 h. The fluorescence signals were undetected between 72 hpf and 78 hpf. (**E**) The huORFZ embryos were exposed to various temperatures from 37°C to 40°C for 1 h at 72 hpf, and fluorescence signals were observed at 96 hpf. As temperature increased, the GFP signals in heat-shock-treated embryos became stronger. (**F**) At 72 hpf, the huORFZ embryos were exposed from 20 to 60 min at a temperature stress of 40°C, and the fluorescence signals were observed at 96 hpf. Similar to the above result, GFP signals in huORFZ embryos became stronger as exposure time to heat-shock stress increased.

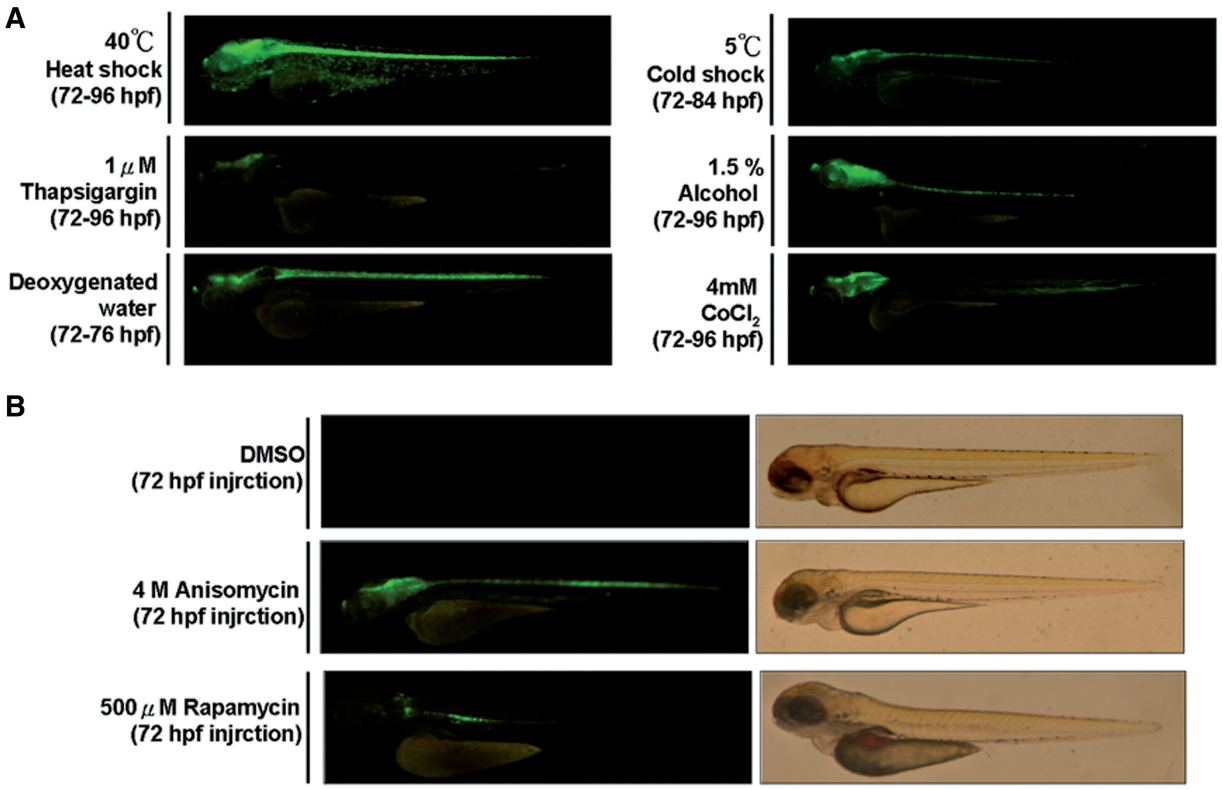

Figure 4. huORF^{chop} is responsive to various stresses in zebrafish embryos. (**A**) The fluorescence activity of GFP was observed in the huORFZ embryos at 96 hpf when embryos were treated with heat-shock (40°C) or cold-shock (4°C), or incubated with thapsigargin, alcohol or deoxygenated water. The incubation condition and drug concentration were indicated on each panel. (**B**) The fluorescence activity of GFP was observed at 96 hpf in the huORFZ embryos when we directly injected anisomycin and rapamycin into the brain of embryos at 72 hpf. Embryos injected with DMSO served as a negative control.

Transcription and translation of the endogenous *chop* gene are increased under ER and ER-associated stresses

We determined if the detection of GFP signals in huORFZ embryos under ER and ER-associated stresses could be correlated with endogenous *chop* expression. To accomplish this, embryos were immersed in medium containing either the ER-stress-inducing drug thapsigargin or alcohol, or embryos were administered with heat-shock and fixed immediately. Later, WISH was processed for the *chop* probe. Compared to the control embryos, the *chop* transcripts were greatly increased throughout the head region in the embryos treated with thapsigargin (Figure 5B), heat-shock (Figure 5C) or alcohol (Figure 5D). Western blot analysis also demonstrated that the CHOP protein was greatly increased when embryos were treated with either thapsigargin or heat-shock (Figure 5I). Meanwhile, we also analyzed the transcripts of *PERK*, which is another proximal effector responding to ER stress, and we found that the *PERK* transcripts were greatly increased throughout the head region in embryos treated with various stresses (Figure 5E vs. F–H). Thus, the genes involved in ER stresses in zebrafish embryos are sensitive to increase under ER and ER-associated stresses, both at transcription and translation levels.

Nrgs/ErbB signaling, PI3K signaling and Hsp90 are involved in uORF^{chop}–mediated translational inhibition *in vivo*

Using the huORFZ embryos allowed us to study the regulatory network involved in uORF^{chop}-mediated translational inhibition. Particularly, FACS of cells from huORFZ transgenic zebrafish coupled with microarray analysis could be employed to compare the expression profiles between the GFP-positive and GFP-negative cells. As a consequence, any genes that specifically express in the GFP-positive cells would be identified. To prove this hypothesis, we used FACS to collect GFP-positive cells from the heat-shock-treated huORFZ embryos. Although FACS revealed that ~1.9% (n = 530 000) of cells displayed strong GFP fluorescence (Figure 6A), no GFP-positive cells were detected from the huORFZ embryos in the absence of heat-shock treatment (Figure 6A). Diagnostic FACS of the population of sorted cells showed that the GFP-positive and GFP-negative cells displayed 93.1% and 100% purity, respectively (Figure 6B). After collecting and comparing the expression profiles of GFP-positive and GFP-negative cells (Figure 6C), we performed a microarray analysis. A total of 2137 under-represented transcripts (1841 annotated genes) and 724 over-represented transcripts (276 annotated genes) were identified in GFP-positive

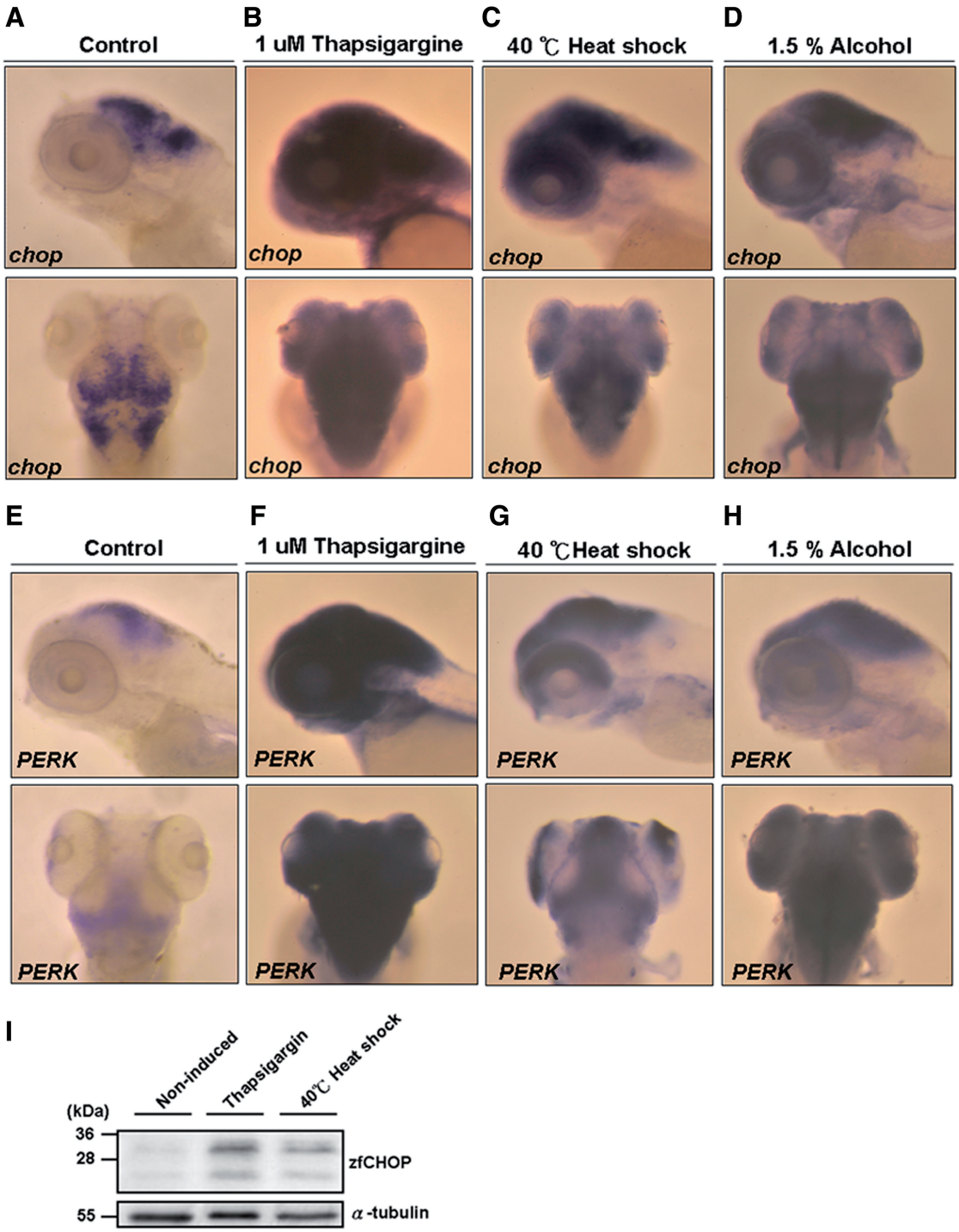

Figure 5. Endogenous *chop* and *PERK* were induced at the head region by different stresses. WISH of *chop* (**A–D**) and *PERK* (**E–H**) in control embryos (A and E), thapsigargin-treated embryos (**B** and **F**), heat-shock-treated embryos (**C** and **G**), and alcohol-treated embryos (**D** and H) at 96 hpf. (A) Endogenous *chop* transcripts were detected at the hindbrain and tectum. (B–D) The *chop* transcripts were greatly expressed throughout the eyes and head region in the embryos treated with 1 μM thapsigargin (B), heat-shock at 40°C (C) and 1.5% alcohol (D). (E) *PERK* transcripts were slightly expressed in the tectum and hindbrain lip at 96 hpf. (F–H) The expression of PERK was greatly induced at eyes and head region when embryos were treated with 1 μM thapsigargin (F), heat-shock at 40°C (G) and 1.5% alcohol (H). (**I**) Western blot detection of the CHOP protein in embryos treated with 1 μM thapsigargin or heat-shock at 40°C. α-Tubulin was used as the internal standard.

cells as compared with GFP-negative cells. Particularly, microarray analysis exhibited several genes that are known to express in the nervous system, such as *arrestin (Arr), developing brain homeobox 1 b (dbx1b), neuregulin (Nrg), Erythroblastic Leukemia Viral Oncogene Homolog B* (*erbB*), as well as astrocyte markers *apolipoprotein Eb (apoeb)* and *glial fibrillary acidic protein (gfap)*, which presented 2.5-fold greater read values after GFP-positive

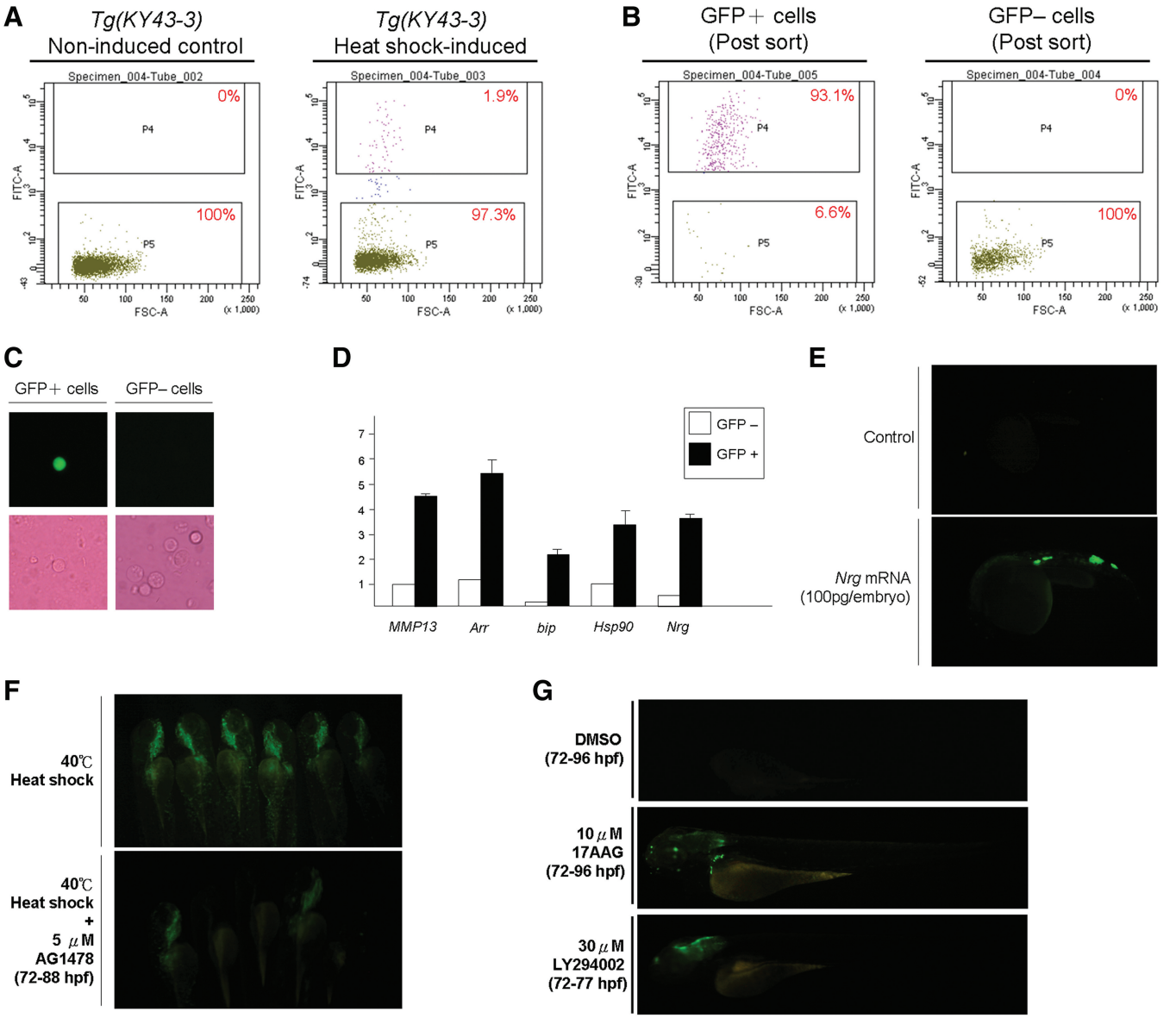

Figure 6. Enrichment of GFP-positive cells from heat-shock-treated huORFZ embryos. Diagnostic fluorescence-activated cell sorting (FACS) of dissociated huORFZ embryos. (**A**) Cells from non-induced huORFZ control embryos. (**B**) Cells from heat-shock-induced huORFZ embryos. (**C**) GFP-positive and GFP-negative cells were sorted by FACS. (**D**) Using quantitative RT–PCR to determine the relative expressions of genes as indicated in GFP-positive (black bars) and GFP-negative (white bars) cells. (**E**) The fluorescence activity of GFP was observed in the huORFZ embryos at 20 hpf when embryos were injected with *Nrg* mRNA. (**F**) The fluorescence activity of GFP was observed in the huORFZ embryos at 96 hpf when embryos were treated with heat-shock at 40°C for 30 min and then incubated with AG1478 for 16 h. (**G**) The fluorescence activity of GFP was observed in the huORFZ embryos at 96 hpf when embryos were incubated with DMSO, 17AAG or LY294002.

cells were enriched by sorting out. These data indicated that ER-stress-responsive cells are GFP-positive cells and that most GPF-positive cells are neuronal cell types. In addition, some ER-stress-associated genes such as *heat-shock protein 90* (*hsp90*), *PERK*, and *immunoglobulin binding protein* (*Bip*), were also up-regulated in GFP-positive cells (Supplementary Table S1). Real-time PCR was performed from the same total RNA used for array hybridization. The pattern of expression was similar in both analyses (Figure 6D), indicating that these GFP-positive cells are nerve cells that respond to ER stress.

To confirm whether these putative genes shown on microarray are truly involved in uORF-mediated translational inhibition, we selected several genes for further study. First, we found that the GFP signal was apparent in the huORFZ embryos injected with *Nrg* mRNA (Figure 6E). The epidermal growth factor (EGF) family consists of four related ErbB receptors involved in cell–cell communication during development of the nervous system (23). Therefore, after treating huORFZ embryos with heat-shock, we next added AG1478 to inhibit ErbB activity. Since the GFP signal was greatly reduced (Figure 6F), we concluded that Nrg/ErbB signaling is

involved in uORF^{chop}-mediated translational inhibition as a negative modulator. Second, PI3K/Akt signaling, which impairs ER function, plays an important role in stress condition (24,25). Genes such as *matrix metalloproteinase 13* (*MMP*) (26), *arr* (27) and *Nrg* (28) that have been reported to interact with PI3K signaling were also strongly upregulated in GFP-positive cells. When we treated huORFZ embryos with LY294002 to inhibit PI3K activity, the GFP signal was strongly induced in brain (Figure 6G), indicating that PI3K signaling is also involved in uORF^{chop}-mediated translational inhibition as a positive modulator. Third, when we treated huORFZ embryos with an Hsp90 inhibitor, 17AAG, the GFP signal was strongly induced in brain (Figure 6G), suggesting that heat-shock proteins are also responsible for uORF^{chop}–mediated translational inhibition. Since we demonstrated that Hsp90, Nrg/ErbB signaling and PI3K signaling are all involved in uORF^{chop}-mediated translational inhibition *in vivo,* we concluded that huORFZ embryos would be useful in studying the signaling network involved in uORF^{chop}-mediated translational control *in vivo*.

DISCUSSION

Under ER stresses, GFP expression in the huORFZ embryos is detected only in specific tissues, such as brain and spinal cord, but not throughout the whole embryos, not even in the skin upon initial and direct contact with ER-stress-related drugs, alcohol or heat-shock. We speculate that neural tissue responds with particular sensitivity to various ER stresses. Although all cells of huORFZ embryos contain *huORF^{chop}-gfp* mRNA, it is noteworthy that the GFP is not translated by every cell because not all cells in the tissues respond equally to stress. For example, the *huORF^{chop}-gfp* mRNA is detected in all cells of brain, muscles, heart and eyes, but GFP is only displayed by some cells, suggesting a possible mechanism by which each tissue may activate some cells as 'first responders', even when all cells encounter the same environmental cue. As such, the huORFZ line may provide valuable information regarding dynamic cell-specific response to ER stress during development or under pathological conditions.

Furthermore, the huORFZ line provides an excellent model to search for specific genes that are involved in uORF^{chop}-mediated translational control using FACS coupled with microarray. In our study, we found that HSP90 is involved in regulating uORF^{chop}-mediated translational inhibition during heat-shock stress, suggesting that HSPs not only function as chaperones, but also play a role in uORF^{chop}-mediated regulation when cells encounter heat-shock stress. PI3K/Akt signaling mediates multiple cellular functions, such as cell survival (25,29). However, again, our studies also found that inhibition of PI3K activity represses uORF^{chop}-mediated translational inhibition and initiates the translation of downstream genes. Thus, we propose that PI3K/Akt signaling controls cell survival by *chop* expression through positively mediating uORF^{chop}-mediated translational inhibition during ER stress. Nrg/ErbB signaling plays important roles in growth and differentiation of glial, neuronal and cardiac cells (23,28). The relationship between Nrg/ErbB signaling and stress is still unknown. In heart, Nrg1 protects ventricular myocytes from anthracycline-induced apoptosis via ErbB4-dependent activation of PI3K/Akt (28). However, when we inhibited Nrg/ErbB signaling in heat-shocked huORZF embryos, the GFP signal was greatly reduced, indicating that Nrg/ErbB signaling is negatively involved in uORF^{chop}-mediated translational inhibition in a manner independent of PI3K signaling. These accumulated lines of evidence demonstrate that this approach shows promising potential as a predictive *in vivo* model for the identification of genes involved in uORF-mediated translational control during ER-associated stresses. Additionally, in the event that uORF-mediated translational inhibition occurs at the tissue or organic level, crossing the huORFZ line with other transgenic fish could be used to analyze the tissue-specific genes involved in uORF^{chop}-mediated translational control and ER stress in specific cell types. Therefore, double fluorescence-positive cells could be captured and sorted for further study by taking advantage of high-throughput analyses of microRNAs, RNA, peptides, proteins and a zebrafish-based *in vivo* assay.

ER dysfunction is involved in a variety of human diseases (30). Although the relationship between ER stress and some disease-related proteins has been studied using animal models of these diseases, few *in vivo* data concerning ER stress are available. Only one mouse model has been established for monitoring ER stress (31). However, problems with leaky fluorescence have developed, and, generally, real-time analysis of mammalian embryos is challenging. Therefore, developing an alternative animal model, such as our huORFZ line, to monitor ER stress *in vivo* is critical. Aside from its use in the dynamic monitoring of ER stresses, the huORFZ embryos have additional biomedical applications. First, the huORFZ model shows no evidence of leaky fluorescence signal under normal condition, and the response of GFP signals depends on the strength of stress. Thus, the huORFZ line could be applied to examine whether a given drug has toxic sideeffects. Second, different tissues of huORFZ embryos respond differently to the different stresses they encounter. Therefore, this line could be used to test whether a given tissue is sensitive to sideeffects resulting from the application of novel ER-stress-related drugs. Third, the zebrafish is already increasingly popular as a model animal to study many human diseases. Thus, huORFZ could be crossed with most any transgenic line that expresses disease-related human proteins to analyze the status of ER stress during the course of disease, or even serve as a drug screening model for a specific human disease.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank the service providing by the Cell Sorting Core Facility of the First Core Laboratory, National Taiwan University College of Medicine.

FUNDING

The National Science Council of Taiwan (NSC100-2321-B-002-036) and the Innovative Research Program from National Taiwan University (NHRI-EX98-9201EI). Funding for open access charge: National Science Council, Taiwan, ROC.

Conflict of interest statement. None declared.

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