Integration of CNS survival and differentiation by HIF2α

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Hypoxia-inducible factor (HIF) 1α and HIF2α and the inhibitor of apoptosis survivin represent prominent markers of many human cancers. They are also widely expressed in various embryonic tissues, including the central nervous system; however, little is known about their functions in embryos. Here, we show that zebrafish HIF2α protects neural progenitor cells and neural differentiation processes by upregulating the survivin orthologues birc5a and birc5b during embryogenesis. Morpholino-mediated knockdown of hif2α reduced the transcription of birc5a and birc5b, induced p53-independent apoptosis and abrogated neural cell differentiation. Depletion of birc5a and birc5b recapitulated the neural development defects that were observed in the hif2α morphants. The phenotypes induced by HIF2α depletion were largely rescued by ectopic birc5a and birc5b mRNAs, indicating that Birc5a and Birc5b act downstream of HIF2α. Chromatin immunoprecipitation assay revealed that HIF2α binds to birc5a and birc5b promoters directly to modulate their transcriptions. Knockdown of hif2α, birc5a or birc5b reduced the expression of the cdk inhibitors p27/cdkn1b and p57/cdkn1c and increased ccnd1/cyclin D1 transcription in the surviving neural progenitor cells. The reduction in elavl3/HuC expression and enhanced pcna, nestin, ascl1b and sox3 expression indicate that the surviving neural progenitor cells in hif2α morphants maintain a high proliferation rate without terminally differentiating. We propose that a subset of developmental defects attributed to HIF2α depletion is due in part to the loss of survivin activity.

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Hypoxia-inducible factors (HIFs), including HIF1, HIF2 and HIF3, are known to function in oxygen homeostasis by regulating the genes responsible for glucose uptake and metabolism, erythropoiesis, angiogenesis, apoptosis and cell proliferation.1 The HIFs are heterodimeric basic-helix-loop-helix-PAS transcription factors that consist of an oxygen-regulated α subunit and a constitutively expressed β subunit, which is also known as ARNT.1 In normoxic conditions, both HIF1α and HIF2α are targeted for proteosomal degradation by prolyl hydroxylase and the von Hippel–Lindau (VHL) E3 ligase complex.2 When cells are subjected to hypoxia, the HIF-α factors are stabilized and in turn associate with ARNT and activate target genes.2 HIF3α lacks a conventional C-terminal transactivation domain and it is postulated to act as a negative regulator of hypoxia-inducible gene expression.3

Despite their oxygen homeostatic functions in adult tissues, HIF-related pathways also have critical functions in embryos. Constitutive depletion of the mouse HIF1α gene (HIF1α−/−) results in developmental arrest and embryonic lethality, which is characterized by neural tube defects, cardiovascular malformations, dysregulated erythropoiesis signaling, and marked cell death within the cephalic mesenchyme.4–6 HIF2α null (HIF2α−/−) embryos died as a result of inadequate blood vessel fusion, remodeling and impaired fetal lung matura- tion.7,8 Inhibition of HIF2α expression also enhanced the generation of reactive oxygen species (ROS) and reduced transcription of primary anti-oxidant enzymes (AOEs), which in turn caused a syndrome of multiple-organ pathology.9 Neural cell-specific depletion of HIF1α resulted in hydrocephalus accompanied by an increase in neuron cell apoptosis and vascular regression in the telencephalon of mutant mouse embryos.10

Depending on the severity of hypoxia, hypoxic signals may induce different responses during cell death. For the pro-apoptotic pathway, HIF1α conspires with p53 and/or BNIP3 to promote apoptosis.11,12 However, hypoxia can also induce an anti-apoptotic response by increasing the expression of the anti-apoptotic protein IAP2 and suppressing the expression of the pro-apoptotic protein Bax through a HIF1α-independent mechanism.13 A recent study has shown that HIF2α may be
involved in the anti-apoptotic properties of tumor cells. Inhibition of HIF2α promoted p53 activity and induced tumor cell death by disturbing cellular redox homeostasis and promoting the accumulation of ROS.14

Survivin (Birc5) is the smallest member of the inhibitor of apoptosis proteins (IAPs) and contains a single baculovirus IAP repeat (BIR) domain and an extended C-terminal 2-helical coiled-coil domain.15 In addition to inhibiting apoptosis, survivin also has important functions in cell mitosis and cell-cycle progression.16 The mammalian survivin protein is widely expressed in embryonic cells, especially in neural progenitor cells, but it is barely detectable in quiescent adult cells.17

During brain development, survivin is highly expressed in neural precursor cells. Targeted deletion of survivin in neural precursor cells leads to massive apoptosis in the central nervous system (CNS) due to elevated caspase-3 and caspase-9 activities.18 Interestingly, survivin is widely expressed in all kinds of malignant tumors, making it a potent target for cancer therapy.15,18

There are multiple HIF-α factors, including HIF1α, HIF2α and HIF3α, in zebrafish that are widely expressed during development.20,21 Concurrent knockdown of these three factors leads to neural cell death and abrogates neuronal development, indicating that these HIF-α factors have critical roles in neural cell survival and differentiation.20 Nevertheless, the authentic HIF-α factor responsible for the fates of CNS neural progenitor cells (NPCs) remains to be elucidated. Here, we demonstrate that of the three HIF-α factors, HIF2α has a major role in maintaining cell survival and promotes neural progenitor cell differentiation. HIF2α depletion caused massive cell death and abrogated neural cell differentiation due to aberrant expression of the survivin homologs (birc5a and birc5b). The surviving NPCs remained in the cell cycle. The defects that appeared in hif2α morphant embryos were rescued by ectopic injection of the birc5a or birc5b mRNA, suggesting that survivins act downstream of HIF2α to protect neural progenitor cells and promote neural differentiation. Chromatin immunoprecipitation assay revealed that HIF2α binds to both birc5a and birc5b promoters directly to modulate their transcriptions.

Results

HIF2α knockdown induces p53-independent apoptosis. There are multiple HIF-α factors, including HIF1α, HIF2α and HIF3α, in zebrafish that are widely expressed during development.20,21 Concurrent knockdown of these three factors leads to neural cell death and abrogates neuronal development.20 To clarify the particular HIF-α factor that determines the fates of zebrafish CNS NPCs, we analyzed apoptotic events in individual hifα morphant embryos. We found that knockdown of hif2α by either of two distinct antisense morpholinos resulted in massive apoptosis at the 24- and 48-h post-fertilization (h.p.f.) stage (Figures 1a–c, g–h and t). Conversely, knockdown of hif1α and hif3α either individually or concurrently did not increase the number of apoptotic cells (Figures 1d–f, i and t), indicating that HIF2α has a unique function in protecting embryonic cells against apoptosis.

In addition to the massive apoptosis, a morphological abnormality with small and acellular head was observed in hif2α morphants at the 48-h.p.f. stage (Figure 1h). During early stage of development, massive apoptosis was elicited by hif2α morpholino at the 12-h.p.f. stage before any morphological defect observed (Figures 1j and k). Moreover, the pcna expression in the anterior proliferative region was diminished at the 12-h.p.f. stage, possibly due to the considerable cell death (Figures 1l and m). It suggests that the gross developmental abnormalities observed in hif2α morphants is possibly caused by extensive cell death occurred in the early stage of development.

A similar anti-apoptotic function has been observed for the human HIF2α protein. siRNA-mediated knockdown of HIF2α promoted p53 activity in human tumor cells and induced cell death by disturbing the cellular redox homeostasis and prompting the accumulation of ROS.14 To examine whether the hif2α morpholino-induced apoptosis in zebrafish embryos is mediated by p53 activity, we concurrently knocked down p53 and hif2α using translational morpholons. Our results show that the depletion of p53 expression did not block the massive apoptosis induced by treatment with hif2α morpholinos (Figures 1n and o). Similarly, severe apoptosis was observed in p53ΔM214K mutant embryos after injection with a hif2α translation-blocking MO (Figures 1p and q), indicating that the HIF2α depletion-induced apoptosis is not an off-target effect of the morpholino and that it is mediated through a p53-independent pathway.

To verify whether the apoptotic cells observed in hif2α morphants are undifferentiated NPCs, embryos were first in situ hybridized with fluorescence nestin (nes) RNA probe followed by TUNEL assay. We found that most of apoptotic cells were colocalized with nes-positive cells (as labeled yellow in Figures 1r and s), indicating that the apoptotic cells in hif2α morphants are indeed NPCs.

Knockdown of hif2α abrogates CNS differentiation. We next examined the fate of the surviving NPCs in the hifα morphant embryos using the terminal differentiation marker elavl3. Knockdown of hif2α caused a dramatic reduction in elavl3 transcription (Figures 2b, c and m) compared with wild-type embryos (Figure 2a) or the other two types of hifα morphants (Figures 2d–f and m), indicating that loss of hif2α expression not only stimulated NPC apoptosis but also abrogated the differentiation of these cells. The loss of neural differentiation in hif2α morphants was confirmed by the lack of HuC/D expression (Figure 2h) and acetylated γ-tubulin (Figure 2k). Consistent with this observation, the hif2α morphants had higher transcript levels of the neural progenitor markers, such as nes, sox3 and achaete-scute complex-like 1b (asc1b), compared with control embryos in the late stages of development (Figures 3a–f), suggesting that the NPCs are retained in the undifferentiation stage. The high level of PCNA expression in CNS suggests that the undifferentiated NPCs in the hif2α morphants maintain a high level of proliferation (Figures 3h and j). Moreover, the reduction in the expression of the cdk inhibitors cdkn1b (Figures 4a, b and g) and cdkn1c in the CNS region (Figures 4c, d and h) was accompanied by an increase in ccnd1 transcription (Figures 4e, f and i), indicating that the NPCs in the...
hif2α morphants did not exit normally from the cell cycle. It was further supported by double fluorescent in situ hybridization of nes and ccnd1 expression (Figures 4j–o). The colocalization of these two gene expression in the CNS of hif2α morphants (yellow in Figure 4o) suggesting that most of NPCs in these hif2α morphants were hyperproliferating cells.
Knockdown of *hif2a* impairs CNS development. (a–f) Lateral views of *elavl3* expression in WT (a), *hif2a* ATG-MO (b), *hif2a* SPL-MO (c), *hif1a* ATG-MO (d), *hif3a* ATG-MO (e) and *hif1a*, *hif3a* dual ATG-MO (f) 24 h.p.f. embryos. *elavl3* expression in the CNS was decreased greatly under the *hif2a* ATG-MO and *hif2a* SPL-MO treatments, indicating that these NPCs were not terminally differentiated. (g–i) Immunofluorescent staining of HuC/HuD (in red) in transverse brain sections of WT (g), *hif2a* ATG-MO (h) and *hif1a*, *hif3a* dual ATG-MO (i) 48 h.p.f. embryos. Nuclei are labeled by DAPI staining (in blue). Consistent with the mRNA transcriptional activity, knockdown of *hif2a* eliminated HuC expression, indicating a defect in neural differentiation. Knockdown of *hif2a* also caused morphological abnormality with small head. (j–l) Dorsal views of whole-mount immunostained images of acetylated α-tubulin in WT (j), *hif2a* ATG-MO (k) and *hif1a*, *hif3a* dual ATG-MO (l) 24 h.p.f. embryos. Axons were visualized by antibody staining for acetylated α-tubulin. The neural development defect following *hif2a* MO treatment was reflected by the loss of axon growth. (m) Percentages of normal, moderate and severe loss of *elavl3* expression in the WT control (*n* = 50), *hif1a* ATG-MO (*n* = 54), *hif2a* ATG-MO (*n* = 69), *hif2a* SPL-MO (*n* = 23), *hif3a* ATG-MO (*n* = 25) and (*n* = 64) embryos. Knockdown of *hif2a* with either translation-blocking or splicing-blocking MOs led to a severe loss of *elavl3* transcription, indicating impairment in neural differentiation.

Figure 2  Integration of CNS survival and differentiation by HIF2α

Cell Death and Differentiation

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Survivins function downstream of HIF2α to protect neural progenitor cells and promote neural differentiation. Survivin is the smallest member of the IAP family and has multiple functions in cell survival, mitosis and proliferation. Knockdown of the zebrafish survivin orthologues birc5a and birc5b causes profound neurodevelopmental, hematopoietic, cardiogenic, vasculogenic and angiogenic defects. Together, these results suggest that survivin functions in embryonic development. In various human cancer cells, survivin/BIRC5 transcription is upregulated as a result of increases in HIF1α. This raises the possibility that the survivin orthologues in zebrafish are also affected by one of the HIF factors. Here, we analyzed the transcriptional activities of the survivin orthologues birc5a and birc5b in various hif2α morphant embryos. In wild-type embryos, the survivin orthologues birc5a and birc5b and hif2α were all transcribed intensively in the CNS region (Figures 5a–c). Depletion of HIF2α resulted in a reduction in the transcription of both birc5a and birc5b (Figures 5d, e, h and i), indicating that hif2α correlates with survivin expression. Conversely, concurrent depletion of hif1α and hif3α did not affect the transcription of either birc5a or birc5b (Figures 5f–i). This suggests that zebrafish survivin orthologues are affected specifically by HIF2α but not by other HIF-α factors. To determine whether the survivin deficiency accounts for the HIF2α depletion-induced NPC apoptosis and aberrant neural differentiation, we examined NPC apoptosis and neural differentiation in birc5a and birc5b morphant embryos. Knockdown of either birc5a or birc5b caused considerable increases in apoptosis and morphological abnormality, which were similar to that observed in hif2α morphant embryos (Figures 6a, b, g, h, 7a–c and 8). Additionally, the birc5a and birc5b morphants exhibited much lower levels of elavl3 transcription compared with WT controls (Figures 6c, e and i–l). Likewise, birc5a and birc5b morphants exhibited higher levels of nes and pcna transcription compared with controls (Figures 9a–i). Moreover, the birc5a and birc5b morphant embryos had
lower levels of \(cdkn1b\) and \(cdkn1c\) transcription and higher levels of \(ccnd1\) transcription than control embryos (Figures 9j–r). Together, these observations indicate that knockdown of either \(birc5a\) or \(birc5b\) results in the induction of NPC apoptosis and the inhibition of cell-cycle exit in the NPCs, which is similar to the phenotype observed in the \(hif2a\) morphant embryos. Moreover, ectopic treatment with the \(birc5a\) or \(birc5b\) mRNA not only successfully prevented \(hif2a\) MO-induced apoptosis and morphological abnormality (Figures 6m, n, 7d and 8), but also restored \(elavl3\) expression to a nearly normal level (Figures 6o–r), indicating that the survivin orthologues indeed rescue the impairment in NPC development that is caused by \(HIF2a\) deficiency.

**HIF2a binds to \(birc5a\) and \(birc5b\) promoters.** Since depletion of HIF2a resulted in a reduction in the transcription of both \(birc5a\) and \(birc5b\), it raises a possibility that HIF2a indeed controls the survivin orthologues through direct interactions with their promoters. We retrieved the upstream sequence of both \(birc5a\) and \(birc5b\) genes from UCSC genome website (http://genome.ucsc.edu/) and identified multiple hypoxia-response element (HRE) core sequences (A/GCGTG) in the promoter regions (Figure 10c). Similar multiple HRE core sequences were also identified in human and mouse \(BIRC5\) promoters (Figure 10c). To determine if zebrafish HIF2a binds to the HRE specifically, oligonucleotides containing the HRE core sequence ACGTG...
were synthesized, biotin labeled and electrophoretic mobility shift assay (EMSA) was carried out (Figure 10a). When HIF2α was expressed alone or it was coexpressed with ARNT1a in COS-1 cells, a mobility complex was formed with wild-type HRE probe (lanes 3 and 4). This mobility complex was effectively competed by a 200-fold molar excess of the unlabeled HRE (lane 5). Conversely, the mobility complex was, however, unaffected by a 200-fold molar excess of the unlabeled mutated HRE (lane 6). These observations demonstrated the transfected HIF2α binds to HRE with a sequence specificity.

To examine whether HIF2α binds directly to birc5a and birc5b promoter in vivo, we immunoprecipitated crosslinked chromatin from adult brain tissue with a HIF2α-specific antibody. The precipitated chromatin was then analyzed by PCR using primer pairs that amplified modules spanning the birc5a and birc5b loci. The ChIP assay demonstrated that the module 5 of birc5a and modules 2 and 6 of birc5b were significantly enriched by the HIF2α antibody (Figures 10b and c), suggesting that HIF2α binds directly to both birc5a and birc5b promoters to modulate their expressions.

**Discussion**

HIF2α and survivin are prominent markers in many human cancers and have critical roles in cell apoptosis and proliferation. Previously, we found that depletion of all of the HIF α factors caused massive apoptosis in the brains of zebrafish embryos. Here, we showed that of the three HIF-α factors, HIF2α is the factor that modulates neural progenitor cell growth and differentiation through its downstream effects on the survivin orthologues, Birc5a and Birc5b. Depletion of HIF2α expression caused reductions in both of the birc5a and birc5b transcripts, which in turn elicited massive apoptosis and abrogated neural progenitor cell differentiation. Although human survivin (BIRC5)
Survivin orthologues function downstream of HIF2α to control CNS development. (a and b) Transverse brain sections with TUNEL staining in 48 h.p.f. wild-type (WT; a) and hif2α ATG-MO (b) embryos. (c and d) Lateral views of elavl3 expression in WT (c) and hif2α ATG-MO (d) 24 h.p.f. embryos. (e and f) Immunofluorescent staining of HuC/HuD (in red) in transverse brain sections of WT (e) and hif2α ATG-MO (f) 48 h.p.f. embryos. Nuclei are labeled by DAPI staining (in blue). (g and h) Transverse brain sections with TUNEL staining in birc5a ATG-MO (g) and birc5b ATG-MO (h) 48 h.p.f. embryos. Knockdown of birc5a and birc5b induced CNS apoptosis. (i and j) Lateral views of elavl3 expression in birc5a ATG-MO (i) and birc5b ATG-MO (j) 24 h.p.f. embryos. Compared with WT, birc5a and birc5b MO embryos exhibited lower elavl3 expression, and a similar pattern was found in hif2α MO embryos. (k and l) Immunofluorescent staining of HuC/HuD in transverse brain sections of birc5a ATG-MO (k) and birc5b ATG-MO (l) 48 h.p.f. embryos. The loss of neural differentiation in birc5a and birc5b MO embryos was confirmed by the deficiency in post-mitotic HuC/D expression. (m and n) Transverse brain sections with TUNEL staining in 48 h.p.f. hif2α ATG-MO embryos injected with 50 pg of birc5a mRNA (m) or birc5b mRNA (n). The intensive apoptosis observed in the hif2α morphants was effectively eliminated by either ectopic addition of birc5a or birc5b mRNA. (o and p) Lateral views of elavl3 expression in 24 h.p.f. hif2α ATG-MO embryos injected with 50 pg of birc5a mRNA (o) and birc5b mRNA (p). (q and r) Immunofluorescent staining of HuC/HuD in transverse brain sections of 48 h.p.f. hif2α ATG-MO embryos injected with 50 pg of birc5a mRNA (q) and birc5b mRNA (r). The loss of elavl3 expression in hif2α morphants was rescued by in vitro transcribed birc5a or birc5b mRNA.
zebrafish survivin orthologues (birc5a and birc5b) promoters are all enriched with multiple HREs, these survivin genes are modulated by different HIF factors. In human cancer cells, BIRC5 transcription is modulated by HIF1 through its direct binding to the HRE in the proximate promoter region. In zebrafish, we demonstrate that both birc5a and birc5b transcriptions are controlled by HIF2, but not HIF1. It is noteworthy that although zebrafish HIF1 does not involve in NPC growth and differentiation, it still have an essential role in other developmental events. Depletion of HIF1 expression abrogated erythropoiesis and reduced hemoglobin content (data not shown). It suggests that the sets of HIF1- and HIF2-modulated genes in human and zebrafish are slightly different due to the discriminative target selectivity of HIF1 and HIF2 factors cross species.
It has been shown that HIFs have important roles in embryonic cell differentiation and proliferation. Hypoxic preconditioning promotes embryonic stem cell neural differentiation and enhances cell survival through upregulating HIF1α and HIF2α. However, the functions of these HIFs in cell differentiation still have not been elucidated. Here, we...
undergo final differentiation. Ectopic treatment with the \textit{birc5a} or \textit{birc5b} mRNA not only successfully prevented \textit{hif2a}-MO-induced apoptosis and morphological abnormality, but also restored neural differentiation. It suggests that the abrogation of differentiation is a secondary effect elicited by HIF2α depletion due to the loss of its downstream effectors, Birc5a and Birc5b.

Previous studies have demonstrated a close relationship between the hypoxia-signaling pathway and cell-cycle arrest in mammalian cells. For instance, either overexpression of the stable forms of HIF1α and HIF2α in murine cells or treatment of murine cells with hypoxia directly resulted in an increase in p27 transcription and cell-cycle arrest at the G1 phase.\textsuperscript{26,27} Moreover, targeted depletion of HIF1α in chondrocytes resulted in decreased p57 expression, enhanced BrdU incorporation and cell death.\textsuperscript{28} We demonstrate that the loss of survivin could be responsible for the reductions in p27 and p57 expression and the aberrant neural development that is observed in the \textit{hif2a} morphant zebrafish embryos. Survivin also had a similar effect on cell differentiation in mouse erythroid development. Depletion of survivin from hematopoietic progenitor cells results in aberrant erythroid formation.\textsuperscript{29} Likewise, earlier studies have demonstrated that depletion of zebrafish \textit{birc5a} and \textit{birc5b} transcription evoked profound neuro-developmental, hematopoietic, cardiogenic, vasculogenic and angiogenic defects.\textsuperscript{22}

In \textit{Drosophila}, dying cells trigger compensatory proliferation through both initiator and effector caspase activities.\textsuperscript{30} In apoptotic proliferating tissues, the initiator caspase Dronc coordinates apoptosis and compensatory proliferation through the Jun N-terminal kinase and p53 pathways, which in turn induce secretion of the mitogens Decapentaplegic (Dpp) and Wingless (Wg).\textsuperscript{30,31} In apoptotic differentiating tissues, the effector caspases DrICE and Dcp-1 activate the hedgehog pathway to induce compensatory proliferation.\textsuperscript{32} Hedgehog signaling triggers the reentry of cells that had previously exited the cell cycle.\textsuperscript{32,33} In zebrafish embryos, knockdown of \textit{hif2a} resulted in the loss of expression of the \textit{survivin} orthologues, which in turn caused severe neural progenitor cell death, increased proliferation activity and abrogated neural differentiation. This phenomenon of cell death-associated proliferation and abrogation of differentiation in zebrafish embryos is analogous to what has been observed in \textit{Drosophila} apoptosis-induced compensatory proliferation. We hypothesize that reentry into the cell cycle causes descendant cells to remain in the progenitor stage without further differentiating. These findings suggest that a key function of HIF2α in developing embryos is related to inhibition of neural progenitor cell death and maintenance of neural differentiation through the functions of downstream survivin orthologues (Figure 11). Depletion of HIF2α and the survivin orthologues causes massive neural progenitor cell death, which induces compensatory proliferation and abrogates neural differentiation.

It has been demonstrated that inhibition of human HIF2α promotes tumor cell death through p53 activation. HIF2α depletion promotes p53-mediated responses in clear cell renal cell carcinoma (ccRCC) by disrupting cellular redox homeostasis, which thereby permits ROS accumulation and DNA damage.\textsuperscript{14} Although a similar level of p53 accumulation...
was also observed in hif2α morphant embryos (data not shown), we demonstrated that inhibition of p53 expression did not prevent massive apoptosis in hif2α morphant embryos. Similarly, HIF2α inhibition-induced massive apoptosis was also observed in the p53 mutant line. Instead, we demonstrated that massive apoptosis observed in hif2α morphant embryos is caused by depletion of the survivin orthologues, which in turn causes apoptosis and induces complementary proliferation in neighboring cells. The replenished proliferating cells remain in the cell cycle and do not terminate differentiation.

Figure 11 A proposed mechanism by which HIF2α deprivation induces NPC apoptosis and blocks neural differentiation. Knockdown of hif2α results in a reduction in the survivin orthologues, which in turn causes apoptosis and induces complementary proliferation in neighboring cells. The replenished proliferating cells remain in the cell cycle and do not terminate differentiation.

Flow cytometric DNA content analysis. After removal of the chorion and yolk, 20 embryos were washed in PBS and transferred to a collection tube. Embryos were digested with a trypsin (0.05%/EDTA (1 mM) solution for 10 min at room temperature and then completely dissociated into single-cell suspensions by pipetting. Trypsin was inactivated by incubation with CaCl2 (1 mM)/lamb serum (5%), and the whole suspension was passed through a 40-μm filter (BD Falcon, Franklin Lakes, NJ, USA). The cells were centrifuged at 2000 x g at 4°C, washed in PBS and fixed in 70% ethanol overnight at −20°C. After fixation, the cells were centrifuged and resuspended in PBS. The suspended cells were stained by treatment with RNase A (20 μg/ml) and propidium iodide (50 ng/ml) (Sigma, St. Louis, MO, USA) for at least 15 min at 4°C in the dark. In all, 30,000 events were acquired per sample and propidium iodide-positive cells were quantified by flow cytometry (BD FACSCanto, Franklin Lakes, NJ, USA). Cells with DNA content less than that of 2 N (G0/G1) cells were classified as apoptotic cells and cells distribute between 2 and 4 N (G2/M) cells were S-phase cells.

In situ hybridization. The hif2α (DQ375242, bases 2557–3137), birc5a (NM_194397, bases 38–485), birc5b (NM_145195, bases 2–421), elav3/Huc (BC065343, bases 55–554), res (XM_001918687, bases 54–754), pcna (BC064299, bases 255–1367), p27cdk1 (NM_212792, bases 611–1262), p57cdk1r1 (NM_001020240, bases 50–1133) and ccndf (NM_131025, bases 1094–2213) templates were used as cDNA and cloned into the pGEM T-easy vector (Roche Applied Science, Indianapolis, IN, USA) for in situ transcription with the DIG RNA labelling kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. Whole-mount in situ hybridization was performed essentially as described previously.20 Nitro blue tetrazolium/bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) served as the substrate for color development. Double fluorescent in situ hybridization was performed using DIG-UTP and FITC-UTP-labeled RNA probes followed by sequential detection using HRP-labeled anti-DIG and HRP-labeled anti-FITC antibodies (Roche, Indianapolis, IN, USA) and fluorescent tyramide amplification. Following RNA probe hybridization, embryos were blocked in 10% serum, 1% BSA and were then incubated with HRP anti-FITC at 1: 500 dilution for overnight in 1% serum, 1% BSA. After washing, anti-FITC antibody was visualized with TSA Plus Fluorescein solution (Perkin-Elmer, Waltham, MA, USA) for 45 min, dehydrated in methanol and quenched with 1% H2O2 in methanol for 30 min. After rehydrating, embryos were blocked again and incubated with HRP anti-DIG at 1: 500 dilution for 4 h, then washed and reacted with TSA Plus Cy3 solution (Perkin-Elmer) for 45 min. Embryos were cleared in glycerol, mounted and viewed on a Zeiss LSM 510 confocal fluorescence microscope (Zeiss, Jena, Germany).

RT-qPCR. Total RNA extracted from embryos using TRIzol (Invitrogen, Carlsbad, CA, USA) followed by DNases I treatment (Roche) was used for cDNA synthesis with Superscript-II (Invitrogen). Quantitative (q)PCR was performed using the Power SYBER Q-PCR Master Mix (BioNoVas, Toronto, Canada) in a Bio-Rad, iQ5 Gradient Real Time PCR System (Bio-Rad, Hercules, CA, USA). qPCR conditions were 95°C for 3 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The following primers were designed using the Primer Express Software version 3.0 (Applied Biosystems, Carlsbad, CA, USA):

birc5a forward, 5′-GGGCCACCGGGATTGAAA-3′; birc5a reverse, 5′-CAGTCTGGATCTGCTGTTCCTTCTT-3′; birc5b forward, 5′-GGAGGCACCTGCACCTACCAT-3′; birc5b reverse, 5′-ACCTTACACGAAAGATGCGAACAT-3′; cdkn1b forward, 5′-TGTTGTCGTGCACCACTAAT-3′; cdkn1b reverse, 5′-TGACATGCTGGTCACCA-3′; cdkn1c forward, 5′-AAGACGACGCGGCGAAAT-5′;
Relative quantification was determined using the 2−ΔΔCt method: relative expression = \( \frac{Ct_{\text{sample}}}{Ct_{\text{control}}} \).

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\begin{align*}
5\text{a-1:} & \quad \text{CTCATGCTGTGGTCGAAC} \quad \text{C} \quad \text{TTCCATGTGTGAACCAGC} \\
5\text{a-2:} & \quad \text{ACGTGCTGATTAAGG} \quad \text{C} \quad \text{CTTCAGAATTAAGAGACAG} \\
5\text{a-3:} & \quad \text{TGTTACAGGTTTCTAGC} \quad \text{C} \quad \text{TCCCAAGATAAAGGAGACAG} \\
5\text{a-4:} & \quad \text{GATGTGCTCCAGCAGTGGTT} \quad \text{C} \quad \text{TCTCTCGCTCCTCCTTCAAG} \\
5\text{a-5:} & \quad \text{AGCTAGTTAGCCAGGCAA} \quad \text{C} \quad \text{GCAGAGACCTCCTGCTGAA} \\
5\text{a-6:} & \quad \text{CTAACCTGACTGGCCACCTAA} \quad \text{C} \quad \text{GCTATAGATGTCGACGG} \\
5\text{a-7:} & \quad \text{GGCTTGGTGCGACCACACAC} \quad \text{C} \quad \text{AGGACTTACATACGGCGGAT} \\
5\text{a-8:} & \quad \text{ATCCAGTCGAACCGGCCAGA} \quad \text{C} \quad \text{AGAAGGCTTCAATACGGTCA}.
\end{align*}
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Duplicate mean values were calculated according to the Ct quantification method using β-actin transcript levels as the reference for normalization. Relative quantification was determined using the ΔΔCt method: relative expression = 2−ΔΔCt.

Apopotosis detection. Zebrafish embryos at 24 h.p.f. were dechorionated and incubated for 30 min in 5 μg/ml actinone orange in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄). The embryos were subsequently washed repeatedly in E3 medium. For microscopic examination, embryos were transfected with 0.01% ethyl 3-aminobenzoate methanesulfonate (Sigma, Cat. No. T3356). TUNEL assays were performed using an in situ cell death detection kit (POD, Roche) as described by the manufacturer. For combined fluorescent immunostaining, embryos were manually de-yolked and the images were captured on a Zeiss LSM 510 confocal microscope.

Chromatin immunoprecipitation. ChIP was performed by using Magna CHIP Protein A magnetic beads (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. Briefly, adult brain tissues were crosslinked with formaldehyde and chromatin was isolated. The isolated chromatin was sonicated to an average size of about 200–300 bp. Protein A magnetic beads were incubated with antibody against HIF2α (ChIP grade rabbit polyclonal antibody purchased from GeneTex (Irvine, CA, USA), GTX103707) or control IgG at 4°C for 1 h. Immunoprecipitation reactions were performed by incubating magnetic beads-linked antibody with the chromatin overnight at 4°C. The immunoprecipitated chromatin complexes were washed with TE buffer and the protein–DNA cross-links were reverse crosslinked by proteinase K digestion at 37°C for 2 h followed by 95°C for 10 min. The DNA was purified by spin columns and then eluted with elution buffer. Immunoprecipitated DNA and input DNA were used as templates for PCR amplification (30 cycles). The PCR primers used in ChIP assay are listed in Table 1.

Electrophoretic mobility shift assay. After COS-1 cells were transfected with the indicated plasmids for 48 h, nuclear extracts were prepared by minipreparation method as described previously. The nuclear extracts were preserved at −70°C until use. Oligonucleotide probe containing the HRE core sequence (5′-CATCGCTGTCAGTAAGGAACTAATGGTCA-3′) and mutant probe (5′-CATCGCTGTCAGTAAGGAACTAATGGTCA-3′) was synthesized and labeled with 5′-end biotin (Purigene, Taipei, Taiwan). For each EMSA reaction, 20 μg of nuclear extracts from normal or transfected COS-1 cells was mixed with biotin-labeled probes and premixed binding reagents (Thermo, Waltham, MA, USA) and incubated at room temperature for 30 min. For competitive experiments, 30× normal and mutant probes were added. Unbound DNA probes were resolved from protein–DNA complexes by electrophoresis on a 10% polyacrylamide gel. After electrophoresis, DNA oligonucleotides were transferred onto a nylon membrane (Pall, Washington, NY, USA) and UV crosslinked. Biotin-labeled DNAs were detected by using the Lightshift Chemoiluminescent EMSA Kit according to the manufacturer’s instructions (Thermo).

Immunostaining. Zebrafish acetylated tubulin IHC was performed as described in The Zebrafish Book. Briefly, embryos were fixed in 4% paraformaldehyde (PFA) at 4°C, dehydrated and stored in MeOH. After rehydration, embryos were treated with proteinase K, fixed in 4% PFA, washed in 0.3% Triton X-100 in PBS (PBT) and subsequently blocked for 1 h in PBT containing 5% horse serum. Embryos were incubated with mouse anti-acetylated tubulin antibody (1:1000, Sigma, T-6793). After several washes with PBT, embryos were blocked for 1 h and incubated with goat anti-rabbit Alexa Fluor 568 secondary antibody (Sigma). Images were acquired using a Zeiss Axioskop 2 fluorescence microscope (Zeiss). For cross-sectional analysis, embryos were embedded in OCT mount medium (Sakura Finetek, Torrance, CA, USA), Cryostat sectioned (CM-1900, Leica, Nussloch, Germany) and mounted on glass slides (Superfrost Plus, J1800AMNZ). The primary antibodies included mouse anti-HuC/HuD (Molecular Probes, Eugene, OR, USA, 1:1000) and monoclonal anti-ACNA (Sigma, P8825, 1:1000). After several washes with PBT, the slides were incubated with goat anti-rabbit Alexa Fluor 568 secondary antibody (Sigma). After repeated washes, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) at 10 ng/ml to visualize the nuclei. Fluorescence and UV light images were acquired using a fluorescence microscope (OLYMPUS BX 51, Olympus, Tokyo, Japan).

Conflict of interest
The authors declare no conflict of interest.

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