Role of IGF signaling in catch-up growth and accelerated temporal development in zebrafish embryos in response to oxygen availability

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SUMMARY

Animals respond to adverse environments by slowing down or arresting growth and development. Upon returning to normal conditions, they often show compensatory acceleration in growth and developmental rate. This phenomenon, known as 'catch-up' growth, is widely documented in the animal kingdom. The underlying molecular mechanisms, however, are poorly understood. Using the zebrafish embryo as an experimental model system, we tested the hypothesis that changes in IGF signaling activities play an important role in the accelerated growth and temporal development resulting from re-oxygenation following hypoxia. We show that chronic hypoxia reduced, and re-oxygenation accelerated, embryonic growth and developmental rate. Whereas hypoxia repressed the Igf1 receptor and its downstream Erk1/2 and Akt signaling activities, re-oxygenation restored their activities. Specific inhibition of Igf1 receptor signaling during re-oxygenation by genetic and pharmacological approaches attenuated catch-up growth. Further analysis showed that whereas PI3K-Akt is required in both normal and catch-up growth, Mek1/2-Erk1/2 activation induced by elevated IGF signaling during re-oxygenation is particularly crucial for catch-up growth. These results suggest that the evolutionarily conserved IGF signaling pathway coordinates growth and temporal development in zebrafish embryos in response to oxygen availability.

INTRODUCTION

Adverse environments cause developmental retardation and growth disorders in animal embryos and human fetuses (Gillooly et al., 2002; Kajimura et al., 2005; Saenger et al., 2007). Upon returning to normal conditions, they often show compensatory acceleration in growth and developmental rate. This phenomenon is referred to as 'catch-up' or 'compensatory' growth. Catch-up growth is found in premature human infants following recovery from fetal growth retardation caused by intrauterine growth restriction, Cushing's syndrome, growth hormone deficiency or hypothyroidism (Hales and Ozanne, 2003; Jensen et al., 2003; Saenger et al., 2007; Wit and Boersma, 2002). The catch-up phenomenon is observed not only in human infants but also in a wide variety of metazoans, ranging from *C. elegans* to mammals (Fielenbach and Antebi, 2008; Ozanne and Hales, 2004), suggesting that there are evolutionarily conserved mechanisms coordinating embryonic/fetal growth and developmental rate in response to a changing environment. To date, two leading models have been proposed to explain catch-up growth. The first is systemic regulation theory, according to which the central nervous system compares actual body size with an age-appropriate developmental stage and subsequently adjusts growth and the developmental rate to compensate via the endocrine system. The second model is based on autonomous regulation of cellular activity at each peripheral organ and tissue, such as occurs at the growth plate in the developing skeleton (Boersma et al., 2002; Gat-Yablonski et al.,

2008; Tanner, 1963; Wit and Boersma, 2002). Regardless of available models, the molecular basis underlying the catch-up growth process has been a long-standing mystery.

Insulin-like growth factors (IGFs) are evolutionarily conserved peptides that regulate a variety of cellular processes (LeRoith, 2008). The biological actions of IGFs are mediated through the Igf1 receptor (Igf1r). Ligand occupancy of Igf1r leads to tyrosine phosphorylation of its intracellular domain and subsequent activation of signaling cascades including the Ras-Mek1/2-Erk1/2 and phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB)/Akt pathways (LeRoith, 2008). In addition, a family of IGF-binding proteins (Igfbp1-6) can control IGF actions by promoting or inhibiting IGF binding to Igf1r depending on the physiological context and cell type, thus allowing temporal and spatial regulation of bioactive IGF availability in target tissues (Duan and Xu, 2005). The central importance of IGF signaling in embryonic growth and development is demonstrated by genetic studies showing that targeting disruption of the *Igf1*, *Igf2* or *Igf1r* genes in mice, natural mutations in these genes in human patients, and knocking down Igf1r in zebrafish embryos, result in severe dwarfism (Baker et al., 1993; Liu et al., 1993; Schlueter et al., 2007; Klammt et al., 2008). The signaling pathways downstream of Igf1r appear to be crucial for regulating embryonic growth and development in a variety of animal species, supporting the idea of an evolutionarily conserved regulatory system acting in an early phase of life (Brogiolo et al., 2001).

The goal of this study was to test the role of IGF signaling in catch-up growth in zebrafish embryos. The zebrafish embryo is an excellent model system for dissecting the molecular and cellular basis underlying altered embryonic growth and development in response to changes in environmental factors. Zebrafish are genetically tractable and their embryos develop externally, thus eliminating the confounding factor of maternal compensation. The

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rapid development and optical transparency of zebrafish embryos also make it possible to observe phenotypic changes in real time (Kimmel et al., 1995). Importantly, the IGF signaling system is evolutionarily conserved among vertebrates and the molecular and cellular basis of IGF/Igf1r actions during normal embryonic development has been well characterized in zebrafish (Duan and Xu, 2005; Schlueter et al., 2007). We provide several independent lines of evidence suggesting that the evolutionarily conserved Igf1r-Erk1/2 signaling plays an important role in catch-up growth resulting from re-oxygenation following hypoxia.

MATERIALS AND METHODS

Materials
Chemicals and reagents were purchased from Fisher Scientific unless noted otherwise. The Igf1r inhibitor NVP-AEW541 (Garcia-Echeverria et al., 2004) was kindly provided by Novartis Institutes for Biomedical Research (Basel, Switzerland). The Igf1r inhibitor BMS-754807 (Carboni et al., 2009) was purchased from JiHe Pharmaceutica (Beijing, China). SU5402 and AG-1478 were purchased from Tocris Bioscience (Ellisville, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA). The PI3K inhibitor LY294002 was purchased from Sigma-Aldrich (St Louis, MO, USA) and the MAPK inhibitor U0126 from Cell Signaling Technology (Danvers, MA, USA).

Experimental animals
Zebrafish (*Danio rerio*) were maintained at 28°C on a 14 hour:10 hour (light:dark) cycle, and fed twice daily. Embryos were generated from natural crosses and the fertilized eggs were raised at 28.5°C and staged according to Kimmel et al. (Kimmel et al., 1995). All experiments were conducted in accordance with guidelines approved by the University Committee on the Use and Care of Animals, University of Michigan.

Quantitative real-time RT-PCR (qRT-PCR)
RNA was isolated from zebrafish embryos by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After DNase treatment, total RNA (2.5 μg) was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). qRT-PCR was carried out in an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers used are shown in Table S1 in the supplementary material. Plasmid cDNAs for each target gene were used as controls. The absolute copy number of a particular gene transcript was calculated based on the standard curve, and the value was normalized to β-*actin* mRNA levels.

Generation of transgenic fish lines
To generate transgenic lines, cDNAs carrying the zebrafish *hsp70* promoter and encoding a dominant-negative form of zebrafish Igf1ra as an EGFP fusion (*dnIGF1R-GFP*) (Schlueter et al., 2007) or EGFP were subcloned into the pT2AL200R150G vector (Kawakami et al., 2004). After verifying the DNA sequence, 100 pg DNA was co-injected with 50 pg transposase mRNA into 1-cell stage embryos. The injected embryos were raised to adulthood. F0 fish were self-crossed to generate F1 progenies. F1 embryos were subjected to a 1-hour 37°C heat shock treatment. GFP-positive F1 embryos from different F0 founder fish were maintained separately as independent lines. The stable lines were maintained by inbred crossing. The F3 homozygous transgenic fish were crossed with wild-type fish to obtain embryos for experiments.

Hypoxia and re-oxygenation experiments
The dissolved oxygen level of the system water under ambient conditions (normoxia) was ~6.5±0.5 mg/liter. For the hypoxia treatment, the oxygen level was reduced to 0.6±0.1 mg/liter by bubbling nitrogen gas into the water. The oxygen concentration was measured using a dissolved oxygen meter (YSI Model 7800). The experimental design is illustrated in Fig. 1A.

Pharmacological inhibition of IGF signaling
NVP-AEW541 and BMS-754807, LY294002 and U0126 were used to inhibit the Igf1r, PI3K and Mek1/2 activities, respectively. SU5402 was used to inhibit fibroblast growth factor receptor 1 (Fgfr1) and AG-1478 was used to inhibit epithelial growth factor receptor (EGFR) signaling.

Flow cytometry analysis
Cell cycle analysis was carried out using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Sample preparation, flow cytometry and cell-cycle analysis were performed as previously described (Schlueter et al., 2007).

mRNA microinjection
Capped mRNAs were synthesized using the mMessage mMachine system (Ambion, Austin, TX, USA). Capped mRNA was injected into embryos as reported previously (Kajimura et al., 2005; Schlueter et al., 2007).

Biochemical analysis
Immunoprecipitation and immunoblotting were performed as described previously (Schlueter et al., 2007). The anti-phospho-tyrosine antibody (4G10) was purchased from Upstate (Billerica, MA, USA) and the Igf1r antibody (C-20) from Santa Cruz (Santa Cruz, CA, USA) The Fgfr1 antibody was purchased from AnaSpec (Fremont, CA, USA). Akt and Erk1/2 antibodies were from Cell Signaling Technology. Three to five independent experiments were performed and analyzed. In each experiment, each sample contained 20 or more embryos. An equal amount of total protein was used. The ratios of phosphorylated to total protein were calculated after the images had been analyzed by densitometry using a Fluor-S Imager (Bio-Rad) and ImageJ software (NIH, Bethesda, MD, USA). Caspase 3 activity was measured using the ApoAlert Caspase 3 Activation Assay Kit (BD Biosciences) according to the manufacturer's instructions. Final values are expressed as fold induction of caspase 3 activity over controls.

Growth and development measurements
Head-trunk angle (HTA) and body length, defined as the distance from the center of the retina to the end of the tail, were measured as reported (Kimmel et al., 1995). HTA is a well-established parameter for determining the developmental stage of a zebrafish embryo (Kimmel et al., 1995). Relative developmental rate was calculated using the following formula: $(_dy/_dt)=(y^n-y^0)/(t^n-t^0)$ where y^0 is the HTA at the starting time-point (t^0) and y^n is the HTA at a defined end time point (t^n). Values are presented as relative percentile of the control group. The integral change caused by Igf1r inhibition was analyzed by area under curve (AUC) analysis, for which the area under the developmental curve in the Igf1r inhibition group was subtracted from that of the control group $(AUC^{\Delta Igfr1}-AUC^{Control})$.

Statistics
Values are shown as mean ± s.e.m. Statistical significance among experimental groups was determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Student's *t*-test was used for comparisons between two groups. Statistical tests were performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA), and significance was at $P<0.05$.

RESULTS

Hypoxia followed by re-oxygenation results in catch-up growth
Experiments were carried out as depicted in Fig. 1A. Head-trunk angle (HTA) and body length were measured at various time points to quantify growth and developmental rates. There is a strong correlation between body size and HTA under all experimental conditions (see Fig. S1 in the supplementary material). Therefore, HTA was used as a quantitative indicator of catch-up growth thereafter. In the constant normoxia (Norm) group, the mean value of HTA increased gradually from 67.75±2.16° to 171.90±1.12° between 24 and 96 hpf (Fig. 1B). Hypoxia greatly diminished the HTA value. The difference between the Norm and hypoxia (Hypo) groups became significant at 30 hpf (after 6 hours of hypoxia) and remained so thereafter. The HTA value in the Hypo group only increased slowly to 84.62±4.21° at 48 hpf and no significant increase was found thereafter. Most embryos in the Hypo group
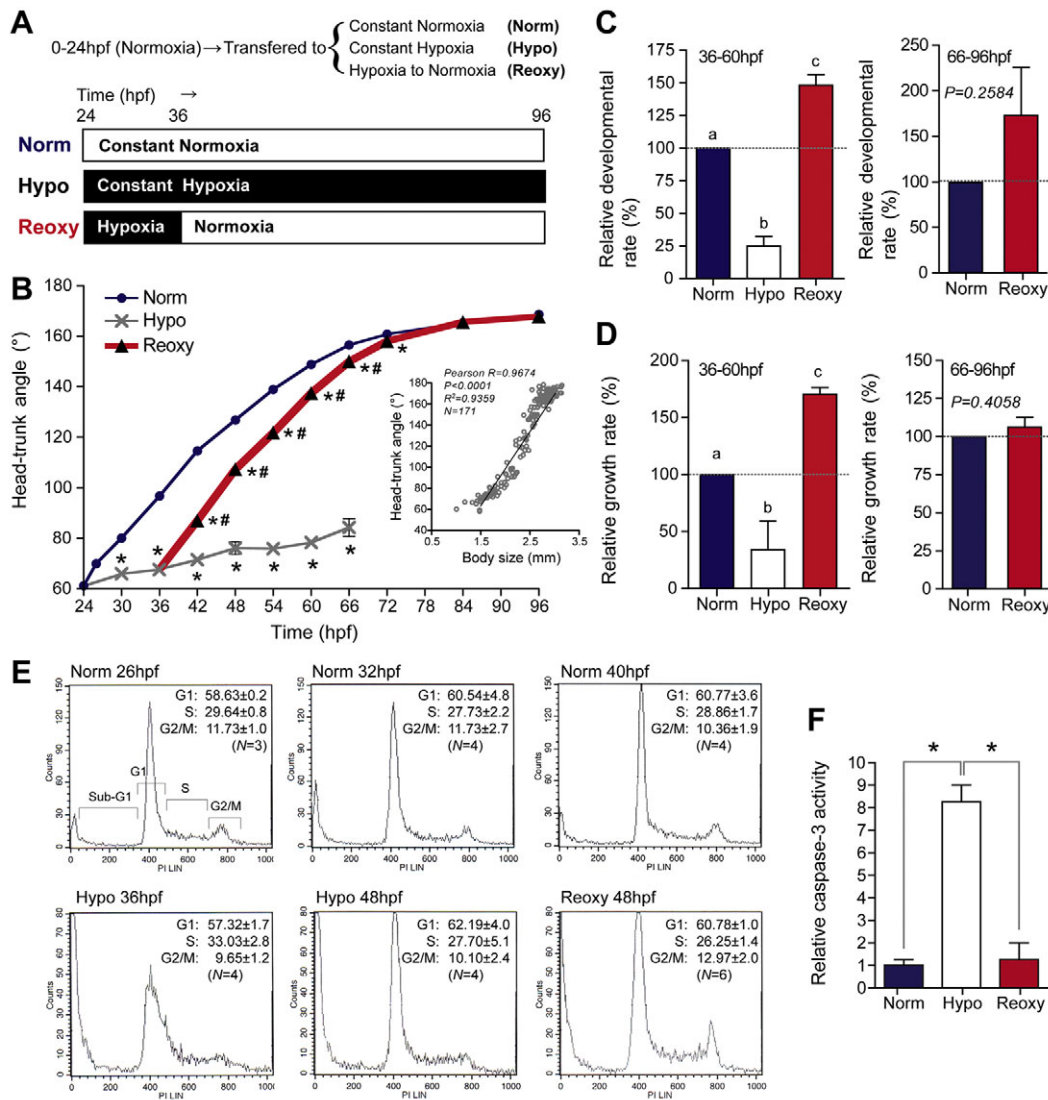
Fig. 1. Hypoxia followed by re-oxygenation accelerates growth and temporal development. (**A**) The experimental design. Zebrafish embryos were raised from 24 to 96 hpf under constant normoxia (Norm) or constant hypoxia (Hypo), or raised under hypoxia until 36 hpf and then transferred to normoxia until 96 hpf (Reoxy). (**B**) Analyses of changes in head-trunk angle (HTA) and body size under the conditions shown in A. Data are mean ± s.e.m., n=12-20; *, P<0.05 compared with the corresponding time in the Norm group; #, P<0.05 compared with the corresponding time in the Hypo group. Inset shows a correlation analysis of body size and HTA of the Norm embryo (P<0.0001, R^2=0.9359, n=171). (**C**) Analysis of relative developmental rate based on HTA between 36 and 60 hpf (left) and between 66 and 96 hpf (right). Norm group was set as 100%. Values shown are mean + s.e.m., n=3-5. Different letters represent statistically significant differences (P<0.05). (**D**) Analysis of relative growth rate. (**E**) Representative cell cycle histograms of embryos from the Norm group (26, 32 and 40 hpf), the Hypo group (36 and 48 hpf), and the Reoxy (48 hpf). The mean value in each phase of the cell cycle is given at the upper right of each histogram; the numbers (N) of independent flow cytometry analyses are also shown. (**F**) Effects of hypoxia and re-oxygenation on caspase 3 activity. 48 hpf wild-type embryos of the Norm, Hypo and Reoxy groups shown in Fig. 1A were assayed. Data are mean + s.e.m., n=2. *, P<0.05.

died at ~60-72 hpf (Fig. 1B). The re-oxygenation (Reoxy) group clearly showed an accelerated rate of increase. At ~72-84 hpf (36-48 hours following re-oxygenation), the HTA value of the Reoxy group 'caught up' with that of the Norm group (Fig. 1B). To further capture this accelerated rate of development and growth, the relative developmental and growth rates were calculated. Compared with the Norm group, the Hypo group had a significantly lower relative developmental rate, as well as a lower growth rate between 36 and 60 hpf. The values of the Reoxy group were significantly greater than those of either the Norm or Hypo groups during this period (P<0.05) (Fig. 1C,D). Since most

embryos in the Hypo group died after 66 hpf, only the relative developmental rates in the Norm and Reoxy groups were calculated between 66 and 96 hpf and compared (Fig. 1C,D). No significant difference was seen, suggesting that the Reoxy group had caught up with the Norm group at this time.

To understand the cellular basis underlying these global changes in growth and development, flow cytometry analysis was performed. As shown in Fig. 1E, there were no significant differences in the percentage of cells in the G_1, S or G_2/M phases among embryos in the Norm, Hypo or Reoxy groups. We further examined the effect of each treatment on apoptosis by measuring
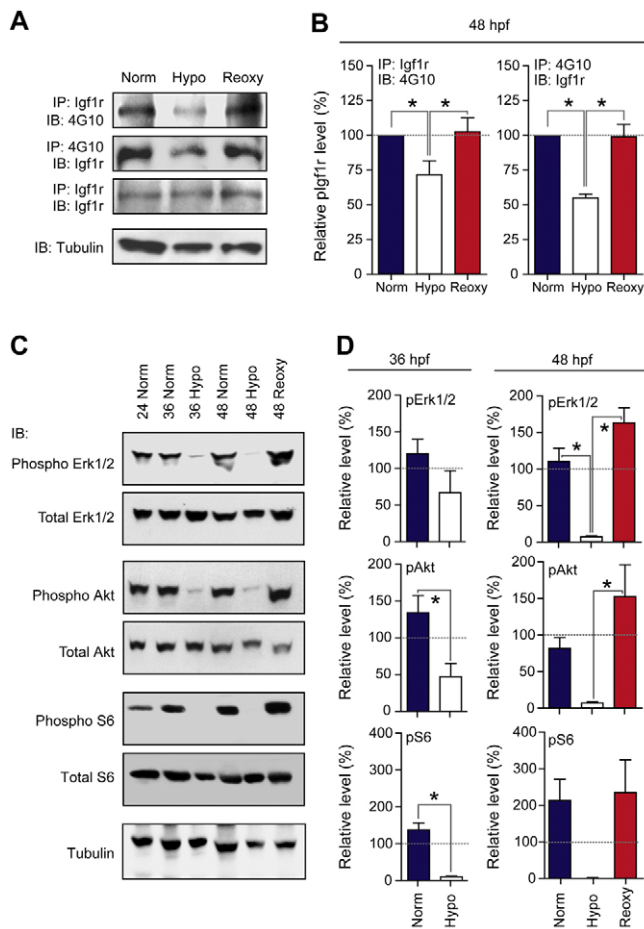
Fig. 2. Hypoxia represses and re-oxygenation restores IGF signaling activities. (**A**) Representative results of biochemical analysis. Zebrafish embryos of the Norm, Hypo and Reoxy group shown in Fig. 1A were sampled at 48 hpf. Equal amounts of total protein were subjected to immunoprecipitation (IP) followed by immunoblotting (IB) using the indicated antibodies. (**B**) The phosphorylated/total Igf1r ratio. Data are mean + s.e.m., n=3-5; *, $P<0.05$. (**C**) Embryos of the Norm, Hypo and Reoxy groups shown in Fig. 1A were sampled at 24, 36 and 48 hpf, respectively. The samples were lysed and blotted using the indicated antibodies. (**D**) The phosphorylated/total protein ratio was quantified and values are expressed relative to that of the 24 hpf Norm group. Data are mean + s.e.m., n=3. *, $P<0.05$.

the activity of caspase 3, an executioner caspase. As shown in Fig. 1F, hypoxia caused an 8-fold increase in caspase 3 activity and re-oxygenation reduced it to the basal level.

We have previously shown that hypoxia delays the timing of heart and head skeleton morphogenesis (Kajimura et al., 2005). To further investigate the impact of oxygen availability on organogenesis, we examined the effect of hypoxia on brain and skeletal muscle organogenesis by examining the mRNA expression patterns of several marker genes. The *pax2a* mRNA expression pattern at 48 hpf in hypoxia was similar to that in embryos at 32 hpf in normoxia, but no patterning abnormality was observed under hypoxia (see Fig. S2 in the supplementary material, upper panels). No patterning abnormality was observed in other tissue-specific marker genes, including the eye markers *rx1* and *rx2* and the muscle markers *myoD* (*myod1* – Zebrafish Information Network) and *myogenin* (see Fig. S2 in the supplementary material, lower

panels). These results suggest that hypoxia delays the timing of organogenesis and inhibits tissue differentiation and that re-oxygenation following hypoxia leads to catch-up growth and accelerated temporal development. These changes in embryonic growth and developmental rates in response to oxygen availability are associated with changes in apoptosis and timing of tissue differentiation but not with changes in cell cycle progression.

Hypoxia represses and re-oxygenation restores IGF signaling activities

Given the importance of IGF signaling in regulating embryonic growth and development, we investigated possible changes in the activities of the Igf1r signaling pathways during hypoxia and re-oxygenation. We have previously shown that the levels of *igfbp1* mRNA were significantly elevated under hypoxia (Kajimura et al., 2005; Kamei et al., 2008). Recent studies have suggested that the zebrafish genome contains two functional *igf1* genes, two *igf2* genes, and two *igf1r* genes (Schlueter et al., 2006; Zou et al., 2009). To determine whether hypoxia causes any changes in one or more IGF ligand and/or receptor genes, the mRNA levels of all known IGF ligands (*igf1a*, *1b*, *2a* and *2b*) and receptor genes (*igf1ra* and *b*) were determined. No significant changes were found in the mRNA levels of the IGF ligands or receptors, although hypoxia increased *igfbp1a* expression in these embryos (see Fig. S3 in the supplementary material).

We next analyzed the Igf1r protein using 48 hpf embryos from the Norm, Hypo and Reoxy groups. As shown in Fig. 2A, the Hypo group had lower levels of phosphorylated Igf1r than the Norm group and the ratio of phosphorylated to total Igf1r in the Hypo group was significantly lower than in the Norm group ($P<0.05$). The ratio of phosphorylated to total Igf1r in the Reoxy group was similar to that of the Norm group (Fig. 2B). Next, we analyzed the levels of phosphorylated and total Erk1/2 (Mapk3/1 – Zebrafish Information Network), Akt and ribosomal protein S6. Hypoxia strongly repressed Erk1/2 phosphorylation, and this effect was most obvious after 24 hours of hypoxia treatment (i.e. at 48 hpf) (Fig. 2C,D). There was a robust restoration of Erk1/2 phosphorylation following re-oxygenation (Fig. 2C,D), coinciding with the accelerated embryonic growth and developmental rates. Similar trends were found in Akt signaling activities (Fig. 2C,D) and S6 phosphorylation levels (Fig. 2C,D). These results suggest that chronic hypoxia strongly represses, and re-oxygenation restores, IGF signaling activities in zebrafish embryos.

Inhibition of IGF signaling abolishes re-oxygenation-induced catch-up growth

We postulated that the restoration of IGF signaling during re-oxygenation plays a role in catch-up growth. To inhibit Igf1r-mediated signaling in vivo in a temporally controlled manner, we generated transgenic zebrafish lines that express a dominant-negative form of zebrafish Igf1r (dnIGF1R-GFP) or GFP under the control of the heat shock-inducible *hsp70* promoter: *Tg(hsp70:dnIGF1R-GFP)* or *Tg(hsp70:GFP)*. Fluorescence microscopy indicated robust induction of dnIGF1R-GFP after heat shock treatment (37°C for 1 hour). Peak expression was observed 3-6 hours after induction, but the signal was easily detectable even 20 hours later (Fig. 3A). Successful expression of the dnIGF1R-GFP fusion protein and its effect in inhibiting Akt phosphorylation were further confirmed by immunoblotting (Fig. 3B). Subsequent immunoprecipitation analysis revealed that expression of the dnIGF1R-GFP fusion protein decreased the levels of phosphorylated Igf1r (Fig. 3C), whereas it had no such effect on
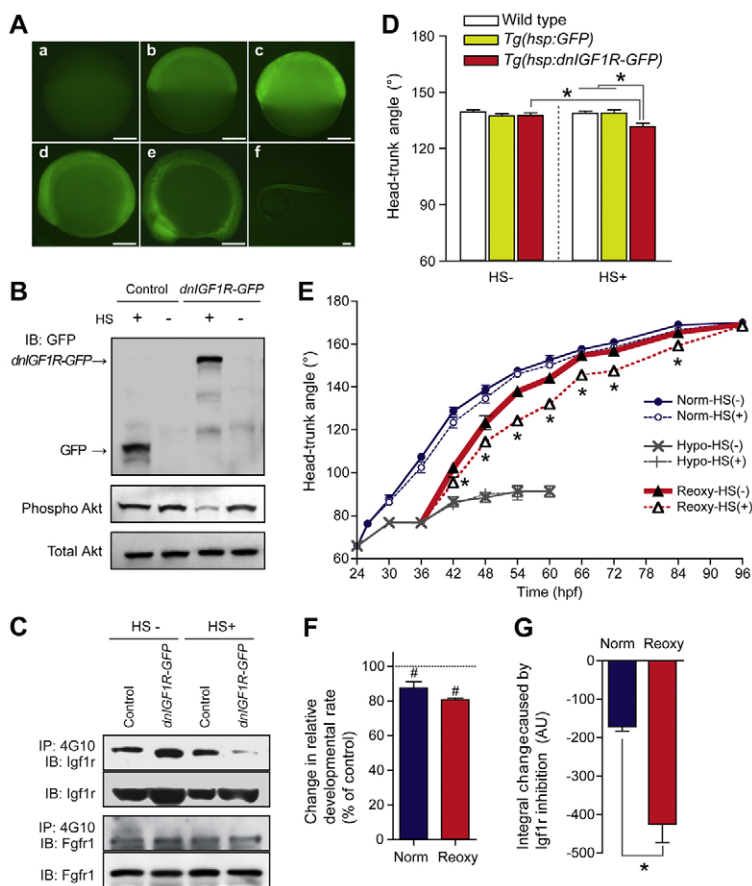
Fig. 3. Temporally controlled blockade of Igf1r signaling inhibits catch-up growth. (**A**) Inducible expression of a dominant-negative form of Igf1r (dnIGF1R-GFP). *Tg(hsp70:dnIGF1R-GFP)* zebrafish embryos were exposed to a 1-hour heat shock (37°C) at 4 hpf and GFP signal was observed at 1 (**b**), 3 (**c**), 6 (**d**), 10 (**e**) and 20 hours (**f**) post heat shock. (**a**) No treatment control. Scale bars: 200 μm. (**B**) *Tg(hsp70:dnIGF1R-GFP)* and *Tg(hsp70:GFP)* embryos were exposed (or otherwise) to a 1-hour heat shock (HS) treatment at 4 hpf and raised to 24 hpf and analyzed by western blot using an anti-GFP antibody. The effect on Akt signaling was also examined and is shown in the lower panel. (**C**) Expression of dnIGF1R-GFP inhibited Igf1r signaling but had no effect on Fgfr1 signaling. Wild-type embryos and *Tg(hsp70:dnIGF1R-GFP)* embryos were exposed (or otherwise) to a 1-hour heat shock and analyzed by IP followed by IB analysis using the indicated antibodies. (**D**) Inhibition of Igf1r signaling decreases the HTA under normoxia. *Tg(hsp70:dnIGF1R-GFP)*, *Tg(hsp70:GFP)* and wild-type embryos were exposed (or otherwise) to a 1-hour heat shock at 26 and 38 hpf and HTA was determined at 48 hpf. Data are mean + s.e.m., n=6-12; *, $P<0.05$. (**E**) Inhibition of IGF signaling blunts catch-up growth. *Tg(hsp70:dnIGF1R-GFP)* embryos were raised according to the experimental regime depicted in Fig. 1A. A subset of embryos from each treatment was subjected to a 1-hour heat shock every 12 hours starting at 26 hpf in the Norm group or 36 hpf in the Hypo and Reoxy groups. Owing to the developmental delay caused by hypoxia, the 36-hpf Hypo/Reoxy embryos were developmentally equivalent to those at 26 hpf in the Norm group. The HTA values were determined at the time points indicated. Data are mean ± s.e.m., n=9-17; *, $P<0.05$ between the Reoxy-HS(−) and Reoxy-HS(+) groups. (**F,G**) Inhibition of IGF signaling causes greater inhibition in the catch-up growth. Changes in relative developmental rate of *Tg(hsp70:dnIGF1R-GFP)* embryos were calculated in the Norm and Reoxy groups (F). The control (no heat shock) group was set as 100%. Data are mean + s.e.m., n=2; #, $P<0.05$ compared with the control. Cumulative change was calculated by area under curve (AUC) analysis (G). Values are shown as $AUC^{HS(+)}$ minus $AUC^{HS(−)}$. Data are mean + s.e.m., n=2; *, $P<0.05$.

fibroblast growth factor receptor 1 (Fgfr1) (Fig. 3C). As shown in Fig. 3D, heat shock treatments significantly decreased the HTA value in the *Tg(hsp70:dnIGF1R-GFP)* embryos, whereas no such effect was found in wild-type or *Tg(hsp70:GFP)* embryos. There was no significant difference in HTA among these groups without heat shock (Fig. 3D). These results suggest that the heat shock-induced expression of dnIGF1R-GFP inhibits IGF signaling and inhibits embryonic growth under normoxia.

Next, the possible role of IGF signaling in catch-up growth was examined using *Tg(hsp70:dnIGF1R-GFP)* embryos. Heat shock treatments significantly blunted catch-up growth as indicated by the significantly lower HTA values at multiple time points during the catch-up period (Fig. 3E). They also significantly inhibited the relative developmental rate under normoxia or during re-oxygenation (Fig. 3F). The data were further interrogated using area under curve (AUC) analysis, which provides an indication of the cumulative change caused by the inhibition of Igf1r-mediated signaling during the entire experimental period. The AUC value for the Reoxy group was significantly greater than that of the Norm group (Fig. 3G), suggesting that IGF signaling plays a more important role in catch-up growth. These effects were specific to dnIGF1R-GFP expression, as heat shock treatment had no such effects in *Tg(hsp70:GFP)* embryos (see Fig. S4 in the supplementary material).

As a complementary and independent approach, we investigated the role of IGF signaling in catch-up growth using NVP-AEW541, a well-established Igf1r inhibitor (Garcia-Echeverria et al., 2004).
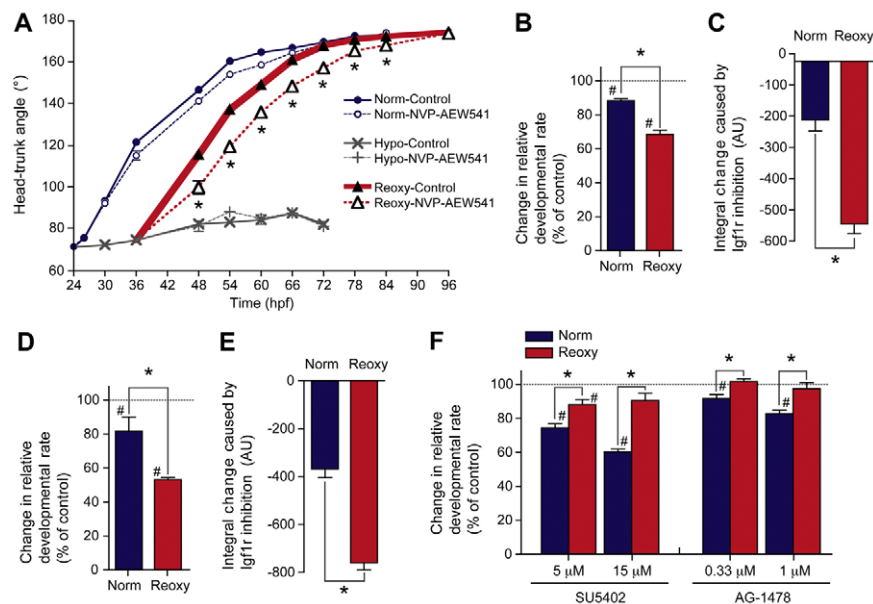
Fig. 4. Pharmacological blockade of IGF signaling inhibits catch-up growth. (**A-C**) Effects of the Igf1r inhibitor NVP-AEW541. Wild-type zebrafish embryos were raised following the experimental regime depicted in Fig. 1A. Embryos in the Norm group were treated with or without NVP-AEW541 (2 μM) from 26 to 96 hpf, and embryos in the Hypo and Reoxy groups were treated from 36 to 96 hpf. HTA was determined at the indicated time points (A). Data are mean ± s.e.m., *n*=11-21; *, *P*<0.05 between the Reoxy-Control and the Reoxy-NVP-AEW541 groups. (B) Changes in relative developmental rate of embryos in the Norm and Reoxy groups. The control (DMSO alone) group was set as 100%. Mean + s.e.m. of three independent experiments. #, *P*<0.05 compared with the DMSO control group. *, *P*<0.05 compared with the Norm group treated with the same inhibitor. Integral change was analyzed using AUC analysis (C). Values are AUC$^{NVP-AEW541}$ minus AUC^{DMSO} and the mean + s.e.m. of three independent experiments is shown. *, *P*<0.05. (**D,E**) Effects of the Igf1r inhibitor BMS-754807. The same experiments were performed in the presence or absence of BMS-754807 (0.5 μM). (**F**) Distinct effects of various RTK inhibitors. Wild-type embryos were raised as depicted in Fig. 1A. Embryos in the Norm and Reoxy groups were treated with the FGFR inhibitor SU5402 (5 and 15 μM) or the EGFR inhibitor AG-1478 (0.33 and 1 μM). The DMSO control group was set as 100% and relative effects of these inhibitors in inhibiting normal and catch-up growth were calculated as in B. Data are mean + s.e.m., *n*=14-15; #, *P*<0.05 compared with the respective DMSO control group; *, *P*<0.05 compared with the Norm group treated with the same inhibitor.

After its effectiveness in inhibiting IGF signaling and optimal doses were determined in pilot experiments, wild-type embryos, raised under normoxia, hypoxia, or the re-oxygenation regime, were treated with NVP-AEW541 (2 μM). As shown in Fig. 4A, inhibition of IGF signaling by NVP-AEW541 blunted the catch-up growth as indicated by the significantly lower HTA values at multiple time points during the catch-up period. NVP-AEW541 treatment resulted in a significant decrease in relative developmental rate in both the Norm and Reoxy groups (Fig. 4B), but it had a significantly greater effect in inhibiting catch-up growth compared with normal growth. Likewise, the AUC analysis revealed that NVP-AEW541 treatment resulted in a greater reduction in the cumulative changes in the Reoxy group (Fig. 4C). We performed additional pharmacological blockade experiments using the structurally distinct, newly developed Igf1r inhibitor BMS-754807 (0.5 μM) (Carboni et al., 2009). BMS-754807 treatment had a similar effect in blunting the HTA value during the catch-up period (Fig. 4D) and caused a greater reduction in the relative developmental rate and the cumulative changes in the Reoxy group as compared with the Norm group (Fig. 4E).

To determine whether the action is unique to IGF signaling or more widely spread among receptor tyrosine kinases (RTKs), we compared the effects of the Igf1r inhibitor with those of the EGFR inhibitor AG-1478 and the FGFR inhibitor SU5402 (Goishi et al., 2003; Tanaka et al., 2005). As shown in Fig. 4F, inhibition of EGFR signaling resulted in a considerable decrease in growth and developmental rates under normoxia, whereas it had little effect

during the catch-up growth (Fig. 4F). Inhibition of FGFR only had a small effect (9.7-12.3%) in inhibiting catch-up growth, but had a greater effect in reducing growth and developmental rates under normoxia (Fig. 4F), the opposite of what was seen with the Igf1r inhibitors. These results suggest that although all three RTKs are involved in normal and catch-up growth, Igf1r signaling plays a particularly important role in catch-up growth.

Activation of the Erk1/2 pathway is particularly crucial for catch-up growth

Igf1r belongs to the RTK family and utilizes two major intracellular signaling pathways: the PI3K-Akt cascade and the Ras-Mek1/2-Erk1/2 cascade (LeRoith, 2008). How these downstream signaling pathways are utilized in vivo under different conditions is not well understood. Interestingly, pharmacological blockade of Igf1r signaling caused a strong reduction in the levels of Erk1/2 phosphorylation in the Reoxy group (23.6±12.6% of the control), whereas it only had a modest inhibitory effect in the Norm group (88.5±10.2% of the control group). By contrast, pharmacological blockade of Igf1r signaling caused modest decreases in Akt phosphorylation levels in both the Norm and Reoxy groups (see Fig. S5 in the supplementary material). These data imply that Erk1/2 activation induced by elevated IGF signaling might play a greater role in catch-up growth and accelerated temporal development than Akt phosphorylation. To test this idea, we used LY294002, a PI3K inhibitor, and U0126, a Mek1/2 (Map2k1/2 – Zebrafish Information Network) inhibitor. As shown in Fig. 5A, U0126 treatment reduced
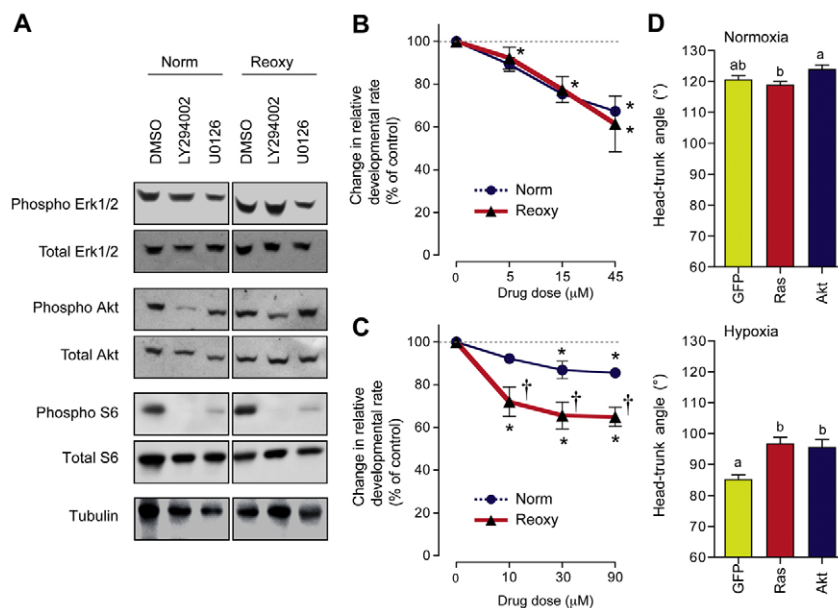
Fig. 5. Distinct roles for the PI3K-Akt and Mek1/2-Erk1/2 signaling pathways in normal and catch-up growth. (**A**) Wild-type zebrafish embryos were raised following the experimental regime depicted in Fig. 1A. Embryos in the Norm group were treated with or without LY294002 (5 μM) or U0126 (10 μM) from 26 to 38 hpf. Embryos in the Reoxy group were treated from 36 to 48 hpf. Embryos were sampled in the middle of the experiment (4 hours post drug treatment) and the lysates were assayed for Erk1/2, Akt, S6 and Tubulin by immunoblotting. (**B**) Effect of LY294002 on normal and catch-up growth. Embryos were treated with LY294002 for 12 hours at the indicated doses. Data are mean ± s.e.m., n=3; *, $P<0.05$ compared with the no treatment control group. (**C**) Effect of U0126 on normal and catch-up growth. Embryos were treated with U0126 for 12 hours at the indicated doses. Data are mean ± s.e.m., n=3; *, $P<0.05$ compared with the control group; †, $P<0.05$ compared with the corresponding Norm group. (**D**) Forced expression of constitutively active Ras or Akt increases catch-up growth but has no effect on normal growth. Embryos injected with GFP, HA-RasV12 or myr-Akt mRNA (50 pg/embryo) were raised under normoxia or hypoxia until 36 hpf and the HTA determined. Data are mean + s.e.m., n=16-30. Different letters represent significant differences at $P<0.05$.

Erk1/2 phosphorylation without affecting Akt phosphorylation levels in the Norm and Reoxy groups. LY294002 treatment specifically inhibited Akt signaling. Both U0126 and LY294002 treatments resulted in reduced levels of phosphorylated S6, but the effect of LY294002 appeared more profound (Fig. 5A). Inhibition of Igf1r or its downstream signaling resulted in 2- to 6-fold increases in caspase 3 activity (see Fig. S6 in the supplementary material). The effects of Erk1/2 or Akt inhibition on catch-up growth are shown in Fig. 5B,C. Inhibition of PI3K by LY294002 resulted in similar dose-dependent decreases in developmental rate in both the Norm and Reoxy groups (Fig. 5B). Inhibition of Erk1/2 signaling by U0126 also decreased the developmental rate in both the Norm and Reoxy groups, but the inhibitory effect was significantly greater following re-oxygenation (Fig. 5C). These data suggest that activation of the Mek1/2-Erk1/2 pathway is particularly important for the acceleration of growth and temporal development during the catch-up period, whereas the requirement for the PI3K-Akt pathway is similar for both normal growth and development and re-oxygenation-induced catch-up growth.

Forced activation of MAPK and Akt signaling increases growth and developmental rate under hypoxia

We next determined whether forced activation of IGF signaling would be sufficient to restore embryonic growth and normal temporal development during hypoxia. Capped mRNAs of a constitutively active form of Ras (HA-RasV12) and constitutively active Akt (myr-Akt) were introduced into zebrafish embryos by microinjection. The effectiveness of HA-RasV12 in activating

Erk1/2 was confirmed in developing zebrafish embryos and cultured mammalian cells (see Fig. S7 in the supplementary material). As shown in Fig. 5D, forced expression of HA-RasV12 did not cause any significant changes in the developmental rate under normoxia (Fig. 5D), presumably owing to the higher basal levels of Ras signaling under normoxia. In addition, forced expression of HA-RasV12 at this level did not alter embryo patterning under normoxia (see Fig. S7 in the supplementary material). By contrast, HA-RasV12 significantly alleviated the developmental retardation caused by hypoxia (Fig. 5D). Likewise, forced expression of myr-Akt at this level did not change the HTA value (Fig. 5D), nor did it alter patterning (see Fig. S7 in the supplementary material) under normoxia. However, myr-Akt significantly alleviated hypoxia-induced growth and developmental retardation (Fig. 5D). These results suggest that the activation of Ras-Erk1/2 and PI3K-Akt is sufficient to accelerate embryonic growth and temporal development under hypoxia.

DISCUSSION

In this study we show that chronic hypoxia slows down embryonic growth and development and delays the onset of organogenesis in zebrafish embryos. Re-oxygenation after hypoxia accelerates embryonic growth and developmental rate. Changes in oxygen availability do not alter embryonic patterning and have no significant effect on cell cycle progression but they do affect apoptosis. We further show that although hypoxia represses Igf1r, Erk1/2 and Akt signaling activities, these are restored during the re-oxygenation-induced catch-up growth phase. Specific inhibition of IGF signaling by genetic and pharmacological approaches
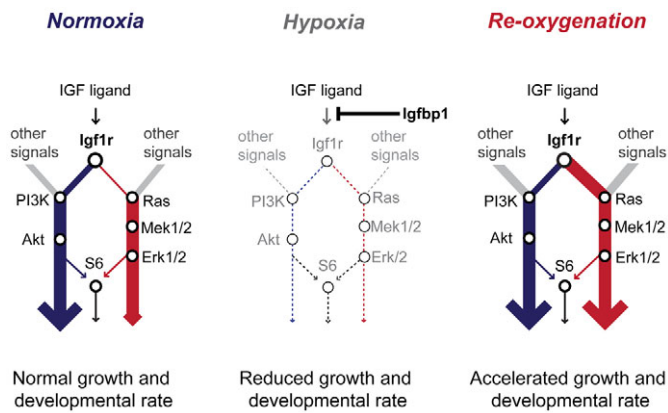
Fig. 6. Model of how IGF signaling coordinates embryonic growth and developmental timing in response to changing oxygen levels. Ligand binding by Igf1r activates both the PI3K-Akt and Mek1/2-Erk1/2 pathways. (Left) The PI3K-Akt pathway plays a more important role in embryonic growth and development under normoxia. (Middle) Under hypoxia, IGF signaling is reduced at the level of Igf1r via upregulation of inhibitory Igfbp1 and by suppression of the PI3K-Akt and Mek1/2-Erk1/2 pathways. This reduced signaling results in embryonic growth retardation and developmental delay. (Right) Upon re-oxygenation, IGF signaling is rapidly restored at all levels (indicated by bold letters). Stronger activation of the Igf1r-Ras-Mek1/2-Erk1/2 pathway plays a greater role in catch-up growth and accelerated developmental timing than normal growth. The line width represents the relative magnitude of activation within each pathway. The size of the arrow represents the relative importance of each signaling pathway.

impeded re-oxygenation-induced catch-up growth. These findings suggest that the evolutionarily conserved IGF signaling pathway plays a key role in catch-up growth in response to changing environmental oxygen availability (Fig. 6).

Hypoxia elicits both acute and chronic adaptive responses. Chronic hypoxic responses involve changes in gene expression (Semenza, 2007). Our previous work illustrated that one of the mechanisms by which chronic hypoxia inhibits zebrafish embryonic growth and temporal development is the inhibition of IGF signaling through inducing the expression of an inhibitory IGF-binding protein, such as Igfbp1 (Kajimura et al., 2005). In the current study, we have shown that hypoxia causes a reduction in the levels of Igf1r phosphorylation, providing direct evidence of cross-talk between hypoxia and IGF signaling in vivo. Since hypoxia and re-oxygenation did not result in any significant change in the mRNA levels of any IGF ligand or receptor genes, the reduction in the total Igf1r protein level is more likely to occur through changes at the translational and/or post-translational levels of regulation. Our further analysis revealed that hypoxia repressed Akt and Erk1/2 signaling activities in zebrafish embryos. Inhibition of either PI3K-Akt or Mek1/2-Erk1/2 signaling activities dampened embryonic growth and development under normoxia. Forced expression of a constitutively active Ras or Akt promoted embryonic growth under hypoxia but not under normoxia. These results suggest that hypoxia influences IGF signaling in the following ways: (1) by inducing the expression of the inhibitory Igfbp1; (2) by reducing Igf1r protein and phosphorylation levels; and (3) by altering intracellular signal transduction pathways downstream of Igf1r (Fig. 6).

A key finding of this study is that changes in Igf1r-Erk1/2 signaling activities play a pivotal role in the catch-up growth process in zebrafish. Although the role of IGF signaling in normal growth and development has been well documented (Duan and Xu, 2005; Schlueter et al., 2007; Schlueter et al., 2006; LeRoith, 2008), IGF involvement in catch-up growth in response to oxygen availability has not been studied in zebrafish. In this study, we have provided several lines of evidence to support the notion that IGF signaling is important for catch-up growth and accelerated temporal development in zebrafish. First, although hypoxia repressed Igf1r and its downstream Erk1/2 and Akt signaling activities, these activities were restored upon re-oxygenation. Specific inhibition of Igf1r following re-oxygenation by dnIGF1R-GFP blunted the acceleration in growth and developmental rate. Another independent line of evidence came from the pharmacological inhibition experiments. Pharmacological blockade of IGF signaling by NVP-AEW541 and BMS-754807, two structurally distinct Igf1r inhibitors, attenuated re-oxygenation-induced catch-up growth. It should be pointed out that genetic and pharmacological inhibition of IGF signaling also inhibited embryonic growth and temporal development under normal oxygen conditions, but caused a greater decrease in catch-up growth, as indicated by the AUC analysis results. By comparison, the EGFR and FGFR inhibitors had limited effects on catch-up growth, whereas both were able to cause significant decreases in embryonic growth and developmental rates under normoxia. These results suggest that IGF signaling plays a more important role in catch-up growth than during normal growth.

Although hypoxia repressed PI3K-Akt and Mek1/2-Erk1/2 signaling activities, these activities were restored upon re-oxygenation. Using specific inhibitors, we found that the PI3K-Akt pathway is required both in normal growth and in re-oxygenation-induced catch-up growth. Likewise, inhibition of the Mek1/2-Erk1/2 pathway also decreased growth and temporal development during normoxia and re-oxygenation, but a greater decrease was seen during re-oxygenation. Recent studies in invertebrate models suggest that both the Mek1/2-Erk1/2 and PI3K-Akt signaling pathways are indispensable for the temporal control of heterochronic gene transcription and translation, which is crucial for the regulation of developmental timing (Bateman and McNeill, 2004). In *C. elegans*, many heterochronic genes, including nuclear factor genes and microRNAs, have been characterized (Hristova et al., 2005; Morita and Han, 2006; Solomon et al., 2008). It would be of interest to determine how expression and translation of these so-called heterochronic genes are linked to IGF signaling in a vertebrate model system and whether they play any roles in catch-up growth.

Another intriguing observation made in this study is that inhibition of Igf1r in vivo resulted in a stronger reduction in the levels of Erk1/2 phosphorylation following re-oxygenation than under normoxic conditions. This implies a greater level of Erk1/2 activation following re-oxygenation, probably induced by restored Igf1r signaling activity. Moreover, specific inhibition of the Mek1/2-Erk1/2 pathway using U0126 had a more profound effect in stalling HTA increases during catch-up growth than during normal growth. The molecular mechanisms underlying this pronounced role of Igf1r-Erk1/2 signaling during re-oxygenation are still unclear. In cultured murine skeletal muscle cells, hypoxia has been shown to alter intracellular IGF signaling pathways by repressing Akt-mTOR and p38 MAPK (MAPK14 – Mouse Genome Informatics) (Ren et al., 2010). Hypoxia induces catabolic metabolism and the activation of sirtuins, which may affect the IGF1R-Ras-Erk1/2 signaling pathway by modifying insulin receptor substrate 2 (IRS2), an adaptor protein for IGF1R (Li et al., 2008). Alternatively, an increase in reactive oxygen species (ROS) during re-oxygenation may also influence the Igf1r-Ras-Erk1/2

action during the re-oxygenation period (Benassi et al., 2006; Yin et al., 2009). ROS activate many signaling molecules including p38 MAPK (Powell et al., 2004). Erk1/2 and p38 have been shown to cooperatively activate common downstream target molecules such as mitogen- and stress-activated protein kinases 1/2 (MSK1/2) (Anjum and Blenis, 2008). The activation of MSK1/2 plays an important role in the cellular stress response by modifying the functions or activities of nuclear factors including CREB, ATF1 and c-Fos and also by remodeling chromatin structure through phosphorylation of histone H3 and HMG-14 (Schuck et al., 2003; Soloaga et al., 2003; Wiggin et al., 2002). Future experiments will need to determine whether differences in metabolism and/or ROS preferentially affect the Igf1r-Erk1/2 signaling pathway.

Although our findings suggest that IGF signaling is important for catch-up growth, they do not necessarily imply that IGF signaling is the only signaling pathway involved. Blockade of IGF signaling has a robust inhibitory effect during the burst of initial catch-up growth and these embryos took longer to catch-up, but they did catch up eventually. There are several possible explanations for this. First, heat shock treatment induced robust but transient expression of dnIGF1R-GFP. This might explain the stronger effect of dnIGF1R-GFP on the initial phase of catch-up growth. Likewise, Igf1r inhibitors added to the water might be gradually degraded. However, these explanations are clearly insufficient because embryos eventually caught up even if we added inhibitors repetitively. Likewise, forced expression of either constitutively active Ras or Akt was able to increase the HTA value from 85° to close to 100°. These were significant increases but they were nonetheless lower than the HTA value of the Norm group at this stage. It is therefore likely that other signaling pathways are also involved in catch-up growth. Future studies are needed to elucidate these additional signaling mechanisms.

To conclude, we have provided genetic, biochemical and pharmacological evidence to support the notion that the restoration of IGF signaling following re-oxygenation is crucial for catch-up growth and accelerated temporal development. Our results suggest that PI3K-Akt and Mek1/2-Erk1/2 signaling, two major pathways downstream of Igf1r, play overlapping yet distinct roles in vivo. Whereas the PI3K-Akt pathway is required for both catch-up growth and normal growth, Mek1/2-Erk1/2 signaling activation is enhanced and plays a more important role in catch-up growth and accelerated temporal development (Fig. 6). These data suggest that activation of MAPK signaling might serve to fine-tune the IGF signaling pathway to coordinate developmental rate in response to environmental oxygen tension. Our results indicating a role for IGF signaling in coordinating zebrafish embryo growth and development in response to changes in oxygen availability are consistent with genetic studies in the mouse model. Studies using various knockout mice suggest that the imprinted expression of Igf2 regulates fetal growth by coordinating the balance between placental nutrient/calcium supply and fetal demand (Constancia et al., 2002; Constancia et al., 2005; Dilworth et al., 2010). Therefore, it appears that evolutionarily conserved IGF signaling plays an important role in coordinating embryonic/fetal growth and developmental rate in response to a changing environment.

Catch-up growth is often observed in the human fetus/infant following a period of growth and developmental retardation imposed by adverse conditions (Wit and Boersma, 2002). Growing evidence suggests that infants who experience catch-up growth have significantly higher risks for adult-onset diseases such as mental retardation, diabetes, cardiovascular disease and obesity (Hales and Ozanne, 2003; Saenger et al., 2007). Alterations in IGF

signaling cascades accompanied by developmental arrest and subsequent catch-up growth might well be related to the manifestation of these disorders. A better understanding of the signaling mechanisms that regulate catch-up growth and accelerated development might open a new avenue for exploring the causal relationship between catch-up growth and adult-onset metabolic diseases.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.056853/-/DC1

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