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Magdalena Hidalgo[1], Ronan Le Bouffant[2], Valérie Bello[2], Nicolas Buisson[2], Patrick Cormier[3], Michèle Beaudry[1,*], Thierry Darribère[2,*].

[1] Université Paris13, EA2363, UFR-SMBH, Laboratoire Réponses Cellulaires et Fonctionnelles à l'Hypoxie, 93017 Bobigny Cedex, France.

[2] UPMC Sorbonne Université (Université Pierre et Marie Curie), UMR CNRS 7622, Laboratoire de Biologie du Développement, 75252 Paris Cedex 05, France.

[3] UMPC Sorbonne Université (Université Pierre et Marie Curie), UMR CNRS 7150, Equipe Traduction cycle cellulaire et développement, Laboratoire Mer et Santé, Station Biologique de Roscoff, 29680 Roscoff, France.

* corresponding authors:
Thierry Darribère, UPMC Sorbonne Université (Université Pierre et Marie Curie), UMR CNRS 7622, Laboratoire de Biologie du Développement, 9 quai Saint-Bernard, 75252 Paris Cedex 05, France. E-mail : thierry.darribere@upmc.fr; Tel : 0033144273690.
Michèle Beaudry, Université Paris13, EA2363, UFR-SMBH, Laboratoire Réponses Cellulaires et Fonctionnelles à l'Hypoxie, 74 rue Marcel Cachin, 93017 Bobigny Cedex, France. E-mail : mbeaudry@yahoo.fr. Tel : 0033148388876.

Abstract

Cell growth, proliferation, differentiation, and survival are influenced by the availability of oxygen. The effect of hypoxia on embryonic cells and the underlying molecular mechanisms to maintain cellular viability are still poorly understood. In this study, we show that hypoxia during Xenopus embryogenesis rapidly leads to a significant developmental delay and to cell apoptosis after prolonged exposure. We provide strong evidence that hypoxia does not affect somitogenesis but affects the number of mitotic cells and muscle-specific protein accumulation in somites, without interfering with the expression of MyoD and MRF4 transcription factors. We also demonstrate that hypoxia reversibly decreases Akt phosphorylation and increases the total amount of the translational repressor 4E-BP, in combination with an increase of the 4E-BP associated with eIF4E. Interestingly, the inhibition of PI3-Kinase or mTOR, with LY29002 or rapamycin respectively, triggers the 4E-BP accumulation in Xenopus embryos. Finally, the overexpression of the non-phosphorylatable 4E-BP protein induces, similar to hypoxia, a decrease in mitotic cells and a decrease in muscle-specific protein accumulation in somites. Taken together, our studies suggest that 4E-BP plays a central role under hypoxia in promoting the cap-independent translation at the expense of cap-dependent translation and triggers specific defects in muscle development.

Keywords: hypoxia, 4E-BP, muscle, translation, development

Introduction

Oxygen (O_2) is an environmental and developmental signal regulator involved in several events such as energy homeostasis, development and progenitor cell differentiation. Hypoxia can arise from physiological events (*e.g.* exercise, travel at high altitude) as well as from pathophysiological conditions (*e.g.* tissue ischemia, inflammation, solid tumors) (Papandreou et al., 2005; Wouters et al., 2005). Understanding cellular responses to hypoxia is of great importance since hypoxia impacts normal development and the malignant progression of a solid tumor (Rivard, 1986; Wouters et al., 2005; Sharma et al., 2006; Simon and Keith, 2008; Wouters and Koritzinsky, 2008; Dunwoodie, 2009; Rodricks et al., 2010).

The cellular response to hypoxia depends on the severity of the hypoxic insult and may result in complex gene expression changes to maintain cellular viability through transcriptional and post-transcriptional events. This response is largely driven by a transcriptional program initiated by stabilization of hypoxia-inducible factors (HIF) (Guillemin and Krasnow, 1997; Semenza, 2003; Cummins and Taylor, 2005; Wouters et al., 2005). HIF-1 and HIF-2 promote transcription of more than 60 putative downstream genes that affect hypoxia tolerance, energy homeostasis, angiogenesis and tumor growth (Semenza, 2003).

Besides HIF stabilization, the PI3K/Akt signalling pathway is an important contributor to hypoxia tolerance (Mazure et al., 1997; Barry et al., 2007). Akt is a serine/threonine kinase that resides downstream of PI3K and phosphorylates a variety of substrates (Kandel and Hay, 1999; Brazil and Hemmings, 2001). Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC2), a negative regulator of the mammalian target of rapamycin (mTOR). Consequently mTOR is activated and stimulates the cap-dependent translation (Hay and Sonenberg, 2004).

Cap-dependent translation requires the assembly of an active eIF4F (eukaryotic initiation factor 4F) complex at the m^7GpppN cap structure present at the 5' terminus of the mRNA (Hay and Sonenberg, 2004; Kapp and Lorsch, 2004; Holcik and Sonenberg, 2005; Sonenberg and Hinnebusch, 2009). The eIF4F complex consists of the cap-binding protein, eIF4E, a scaffolding protein, eIF4G and an ATP-dependent helicase, eIF4A. Formation of eIF4F facilitates the recruitment of the pre-initiation complex, 43S, which is necessary to initiate translation. The assembly of the cap-binding complex eIF4F is a key step of the translational regulation and is regulated through a set of eIF4E-binding proteins (4E-BPs) controlled by mTOR-mediated phosphorylation (Hay and Sonenberg, 2004; Sonenberg and Hinnebusch, 2009). 4E-BPs reversibly bind to eIF4E in their hypophosphorylated form obstructing the interaction between eIF4E and eIF4G and consequently preventing the formation of eIF4F complex (Pause et al., 1994; Haghighat et al., 1995). Interestingly, several cellular mRNAs use an alternative mechanism of translation initiation, involving an internal ribosomal entry site (IRES) at the 5' untranslated region and are translated efficiently when cap-dependent translation is impaired under a variety of stress conditions including serum deprivation, irradiation, apoptosis and hypoxia (Holcik et al., 2000).

Hypoxia results in a rapid inhibition of protein synthesis through the repression of the initiation step of mRNA translation (Wouters et al., 2005). This can be regulated by a hypoxia dependent inhibition of eIF4F as evidence by a reduction of eIF4E/eIF4G association and a corresponding increase of eIF4E/4E-BP (Koritzinsky et al., 2006). In the same way, hypoxia rapidly and reversibly triggers hypophosphorylation of mTOR and its effector 4E-BP in human embryonic kidney cells (Arsham et al., 2003). In addition, hypoxia rapidly induces a reversible increase in 4E-BP protein levels in sea urchin embryos (Le Bouffant et al., 2006) and FoxO activates 4E-BP mRNA transcription in Drosophila embryos undergoing oxidative stress or after nutrient starvation (Tettweiler et al., 2005). While the 4E-BP translational inhibitory functions and mechanisms involved in its regulation have been extensively studied in vitro, the biological role of 4E-BP in developing organisms is unclear to date. Few studies have been performed regarding the effects of hypoxia on embryonic development, even though O_2 has been involved in the development of several pathologies of human pregnancy (Unger et al., 1988; Julian, 2011). Xenopus laevis embryos contain a 4E-BP protein (Fraser et al., 1999) but little is known about its role during embryonic development, particularly during hypoxic stress.

Our objective was to determine the effects of hypoxia on vertebrate embryonic development and muscle formation and to examine the involvement of 4E-BP in mediating these hypoxic effects. Here, we show that hypoxia causes oxygen-dependent growth retardation, apoptosis and lethality after prolonged exposure. Exposure to hypoxic conditions also decreases Akt phosphorylation and induces a reversible increase in 4E-BP downstream protein amount that associates with eIF4E in hypoxia. Moreover, analysis of muscle development provides strong evidence that hypoxia does not affect somitogenesis but affects the accumulation of muscle-specific proteins in somites, independently of mRNA variation. Finally, our data show for the first time that overexpression of the non-phosphorylatable 4E-BP protein causes effects similar to hypoxia on somite cells. Taken together, our studies suggest that 4E-BP plays a key role in mediating the hypoxia-induced defects in muscle formation, without affecting the expression of myogenic transcription factors.

Results

Hypoxia causes growth retardation, lethality and apoptosis

To investigate the effects of hypoxia on early development, we exposed Xenopus embryos to several levels of oxygen from the end of the gastrulation (stage 13) to late tailbud stages (stage 42). Control embryos were exposed to 21% O_2 corresponding to normoxia and hypoxic embryos were exposed to 10% or 5% O_2 that represent moderate and strong hypoxic stress, respectively. These conditions were validated by measurements of dissolved oxygen level and by quantitative real-time PCR (qRT-PCR) to determine *HIF-1α* transcription level which is tightly controlled by cellular oxygen tension (Gorlach, 2009) and its known VEGF target gene. The oxygen partial pressure in the medium was proportional to the oxygen level in the hypoxic chamber and *HIF-1α* and *VEGF* mRNA expression increased significantly in relation to oxygen availability (Fig. S1). Moreover, we performed Western blot analyses with an antibody against the phospho-p38-MAPK, a member of the class of mitogen-activated protein kinases that are responsive to stress stimuli. As shown in Figure 1A-B, phospho-p38-MAPK was undetectable at stage 21 and was detected at very low level at stage 25 in control embryos. When embryos were subjected to 10% O_2, levels of phospho-p38-MAPK were not significantly higher as compared to the control at any stage (Fig. 1A-B). However, levels were dramatically higher in embryos exposed to 5% O_2 at stages 21 and 25 when compared to the control embryos and to embryos subjected to 10% O_2 (Fig. 1A-B). These results show that exposure to 5% O_2 causes a strong cellular stress response during embryonic development whereas 10% O_2 seems to have less of an effect.

External embryo morphology was observed and compared to Nieuwkoop and Faber table stage illustrations (Nieuwkoop and Faber, 1994). As shown in Figure 1C-D, both moderate and strong hypoxia slowed down embryonic development rate. Forty-two hours of hypoxia treatment caused a significant retardation in Xenopus development without affecting external embryo morphology (Fig. 1D). When normoxic embryos have reached stage 30, embryos exposed to 10% O_2 have reached stage 28 and embryos exposed to 5% O_2 have reached stage 25. Moreover, hypoxic embryos maintain their delayed growth through development (Fig. 1C).

Depigmentation of embryos and dissociation of tissues were used as indicators to assess hypoxia related lethality. The number of dead embryos was counted (Fig. 1E). Xenopus embryos exposed for 24h to 10% O_2 had a survival rate of 77% while those exposed for 24h to 5% O_2 had a stark decrease in survival represented by a 48% survival rate. Since hypoxia causes significant embryonic death depending on oxygen availability, the apoptosis patterns in relation to the oxygen concentration was analysed. Embryos (n=15) were fixed at

stage 25 when the cellular stress marker phospho-p38-MAPK level was higher and sections were treated with TUNEL. TUNEL positive cells were counted on frontal sections of control and hypoxic embryos, and the average of positive cells per embryo is presented in Figure 1F. Apoptosis was 77% higher in embryos exposed to strong hypoxia (5% O_2) compared to the control. In embryos exposed to 10% O_2 the number of apoptotic cells was 43% higher compared to the control embryos suggesting that hypoxia causes apoptosis depending on the oxygen level.

Strikingly, embryos exposed to hypoxia and especially to 5% O_2 did not make any movement when pricked with fine pliers compared to control embryos, which ran off (data not shown). This observation suggests that hypoxia caused strong motility defects probably by affecting muscles. To further investigate the effect of hypoxia on muscle development, experiments were performed on embryos from stage 21 to stage 25, after the formation of the first somites (Nieuwkoop and Faber, 1994) and before death of the majority of embryos exposed to strong hypoxia.

Hypoxia decreases Akt phosphorylation in Xenopus embryos

The serine/threonine protein kinase Akt plays a critical role in metabolism, cell survival, mRNA translation, proliferation and cell cycle regulation. In addition, Akt signalling pathway has been shown to regulate myoblast proliferation and differentiation (Bodine et al., 2001; Wilson and Rotwein, 2007; Wu et al., 2010). Based on these data, the Akt signalling activation was analysed by Western blot using two anti-phospho-Akt antibodies (Fig. 2).

Akt was phosphorylated on Thr308 and Ser473 in control embryos suggesting an activation of Akt protein during the normal development. Interestingly, in embryos exposed to hypoxia, the level of phospho-Akt decreased in an oxygen concentration-dependent manner at stages 21 and 25. The phosphorylation status of Akt protein was significantly weaker under strong hypoxia (5% O_2) in both stages compared to control and to 10% O_2-exposed embryos, although each batch present variability and in some cases hypoxic embryos already presented phospho-Akt at stage 25 (Fig. 2). Therefore, the Akt signalling protein is inhibited in hypoxia during early embryonic development particularly in 5% O_2 context. Otherwise, the Akt phosphorylation level was more affected at stage 21 than stage 25.

Hypoxia increases the amount of 4E-BP protein, which binds to eIF4E

The translational repressor 4E-BP is known to be a downstream substrate of the Akt signalling pathway (Gingras et al., 1998). Akt mediated phosphorylation of the translational

repressor 4E-BP can subsequently lead to inactivation or degradation of 4E-BP (Elia et al., 2008). We then performed Western blot analyses to determine 4E-BP protein levels in embryos exposed to hypoxia. As shown in Figure 3A-B, 4E-BP protein levels were significantly higher in hypoxia-exposed embryos at stages 21 and 25, compared to the controls maintained under normoxic conditions. Moreover, 4E-BP protein levels were significantly higher in embryos subjected to 5% O_2 than to 10% O_2, suggesting that hypoxia-induced overexpression of 4E-BP depends on oxygen availability. To know if the increase of 4E-BP protein was due to transcriptional regulation, we performed qRT-PCR. As shown in Figure 3D, the relative *4E-BP* mRNA level did not change significantly under hypoxia.

In mammals, 4E-BP has been shown to bind to eIF4E in its hypophosphorylated form. Therefore, decrease of Akt activity induced by hypoxia should consequently increase the amount of 4E-BP associated with eIF4E. To validate this hypothesis, we used m^7GTP affinity beads to test if overexpressed 4E-BP is able to bind its target, eIF4E. Protein extracts were incubated with m^7GTP-conjugated Sepharose beads and bound proteins were analysed by Western blot with antibodies against 4E-BP and eIF4E. After m^7GTP purification, Western blot analysis revealed that the amount of 4E-BP associated with eIF4E increased significantly in embryos exposed to 5% and 10% O_2 (Fig. 3C). Taken together, these data demonstrate that hypoxia reduces Akt phosphorylation level and leads to 4E-BP accumulation, which is able to bind to eIF4E.

It was then important to show links between 4E-BP amount regulation and Akt signalling pathway activity in Xenopus embryos. Indeed, if an active Akt signalling pathway maintains a low level of 4E-BP, it is then conceivable that embryos treatment with LY294002, a well-established inhibitor of PI3-kinase acting upstream of Akt, will modify the amount of 4E-BP protein in normoxic condition. To test this hypothesis, 4E-BP protein amount was analysed by Western-blot in control and treated embryos. As expected, 2 hours treatment with 50 μM of LY294002 affected Akt phosphorylation state and triggered an increase of 4E-BP protein amount (Fig. 3E). In addition, since mTOR acts downstream Akt, we then tested the effect of rapamycin, a mTOR activity inhibitor. Interestingly, this drug induced a dose-dependent accumulation of 4E-BP in 3 hours treated embryos (Fig. 3F).

Taken together, these data suggest that the 4E-BP protein level is dependent of the PI3K/Akt/mTOR signalling pathway in Xenopus embryos.

Effects of hypoxia exposure are reversible upon reoxygenation

We investigated whether hypoxic effects are reversible by exposing embryos to 5% O_2 until stage 21 and then subjecting a subset of embryos to normoxia (*i.e.* reoxygenation; Fig. 4A). Protein levels of 4E-BP as well as the phosphorylation of p38-MAPK and Akt were analysed by Western blot. As shown in Figure 4B, phospho-p38-MAPK level decreased in reoxygenated embryos showing that the cellular stress was abolished. Moreover, embryonic death was prevented in the subset of embryos subsequently exposed to normoxia (9%) compared to the subset of embryos kept under hypoxic condition (54%). Interestingly, both phospho-Akt and 4E-BP protein levels returned to basal levels observed in controls (normoxia) suggesting that this signalling pathway was reactivated.

We have shown that the reoxygenation of the embryos triggers a decrease of the 4E-BP protein level and that 4E-BP amount is under a rapamycin-sensitive pathway. We then hypothesized that the decrease of 4E-BP amount triggered by reoxygenation of the embryos should be altered after rapamycin treatment. To test this idea, embryos were exposed to 5% O_2 until stage 21 and shifted to normoxia in absence or presence of rapamycin. After 15, 30 and 45 min of reoxygenation, the level of 4E-BP was analysed by Western blot. As shown in Figure 4C, 4E-BP amount was more important in reoxygenated embryos cultured for 30 and 45 min in presence of rapamycin compared to those cultured in absence of the drug. However, 4E-BP amount decreased in both cases suggesting that this time of exposure to rapamycin (less than 1 hour) seems to be not sufficient to induce the maximal effect of this drug and to maintain a high level of 4E-BP. In agreement with this, we show that 3 hours treatment with rapamycin was required to induce an increase of the 4E-BP amount in normoxia (Fig. S2).

Altogether, these results show that effects of hypoxia are reversible when oxygen is available, suggesting a correlation between oxygen concentration and embryonic survival as well as a correlation between oxygen concentration, the PI3K/Akt/mTOR signalling pathway and the stability of 4E-BP.

Hypoxia specifically decreases the number of mitotic cell in somites

Our data show that hypoxia decreased Akt activation and increased the levels of translational repressor 4E-BP. Since Akt have been shown to be involved in cell proliferation and that control of polypeptide synthesis plays an important role in this mechanism, we used phosphorylated-Histone H3 (phospho-H3) as a mitosis marker. Immunodetection was performed with the anti-phospho-H3 antibodies on cryostat sections of stage 25 embryos (n=8). The number of phospho-H3 positive cells was determined and the average of positive cells per tissues is presented in Figure 5. The number of mitotic cells in the neural tube, the

notochord and the epidermis was not significantly affected in strong hypoxia. However, the number of positive cells was strongly reduced (90%) in somites of embryos exposed to 5% O_2 as compared to control.

These results suggest that hypoxia specifically induces a decrease in the number of mitotic cell in somites, suggesting a decrease in somite cell proliferation.

Hypoxia does not affect somitogenesis but affects the accumulation of specific proteins in muscle cells

In vertebrate embryos, somites are regular transient structures repeated along the anterior/posterior axis of the embryo, which then differentiate into a part of the dermis, bone, cartilage and skeletal muscles. During somitogenesis, somitic cells are stacked in blocks and undergo a 90° rotation relative to the anteroposterior axis of the embryo to form myotome fibers that are aligned parallel to the notochord (Keller, 2000). Movements of nuclei were followed during progressive alignment and, at the end of rotation, nuclei are arranged in regularly ordered stripes so that one stripe corresponds to one somite.

In this study, we previously observed that embryos exposed to hypoxia did not make any movement compared to control embryos, suggesting muscle defects. In addition, we showed that hypoxia specifically decreases mitotic cell in somites and negatively regulates the serine/threonine protein kinase Akt that has been involved in muscle cell differentiation (Bodine et al., 2001; Wilson and Rotwein, 2007; Wu et al., 2010). To further investigate the effects of hypoxia on somitogenesis and muscle formation, we sectioned embryos and analysed the morphology of somites by immunostaining with the F59 antibodies against MHC (myosin heavy chain) protein and myotome/skeletal muscle-specific monoclonal 12/101 antibodies (Kintner and Brockes, 1984). On frontal sections, blocks of somitic cells were discernable, any changes in somite number was observed and segmentation of the presomitic mesoderm did not appear to be affected in hypoxia (Fig. 6A-D). Interestingly, we noticed that both the F59 (Fig. 6A-B) and the 12/101 antibodies (Fig. 6C-D) stainings were dramatically weaker in the embryos exposed to hypoxia as compared to the control. These data were confirmed by Western blot showing that the 12/101 protein level was weaker in embryos exposed to 10% and 5% O_2 as compared to the control (Fig. 6E).

To investigate whether hypoxic effects are reversible, embryos were exposed to 5% O_2 until stage 21 and then a subset of them was subjected to normoxia. As shown in Figure S3, the 12/101 staining was higher in reoxygenated embryos compared to hypoxic embryos, indicating that some hours of reoxygenation partially reversed the phenotype in muscles.

Since the expression of MHC protein and the 12/101 muscle marker were decreased in hypoxic embryos, it is possible that hypoxia affects the somite fate by inhibiting the expression of genes coding for transcription factors involved in muscle cell differentiation. Therefore we investigated whether hypoxia interfered with the myogenic signalling pathway by analysing the expression patterns of XMyoD and XMRF4, early and late markers of muscle cell differentiation respectively. The expression of these specific proteins was analysed by immunostaining of frontal sections. Comparison between hypoxic and control embryos did not reveal any significant differences in the expression of both proteins. Indeed, anti-XMyoD and anti-XMRF4 antibodies revealed identical nuclear stainings in both normoxic (21% O_2) and hypoxic (5% O_2) embryos at stage 25 (Fig. 6 F-Q).

Interestingly, the decrease of MHC protein amount induced by hypoxia was independent of *Myh 1, 4, 8* and *6* mRNAs variation (encoding for the MHC protein recognized by the F59 antibody) as shown by qRT-PCR analysis (Fig. 6R and S4). Moreover and as expected, qRT-PCR analysis also showed no significant variations of *MyoD* mRNA level for stage 25 under hypoxic conditions (Fig. 6S).

In the epidermis of hypoxic embryos, the expression of p63 transcription factor, one of the earliest markers of epidermis differentiation, as well as the acetylated tubulin protein, a late marker of ciliated cells differentiation, were not affected (Fig. 6T-W).

Together, these results show that hypoxia does not affect somitogenesis and the expression of at least two transcription factors involved in muscle cell differentiation. However, hypoxia affects the accumulation of muscle-specific proteins in somites without affecting stability or transcription of mRNAs.

Overexpression of a non-phosphorylatable 4E-BP protein affects the accumulation of muscle-specific proteins in somites

The results above show that hypoxia increases functional 4E-BP protein levels, as indicated by eIF4E protein binding. In addition, hypoxia affects the accumulation of muscle-specific proteins. We then hypothesized that maintained eIF4E sequestration by 4E-BP is involved in the muscle cell protein accumulation. To validate this possibility, both blastomeres of two-cell stage embryos were injected with mRNA encoding for full-length 4E-BP protein (WT-4E-BP) and Western blot analysis was used to establish 4E-BP protein levels at stage 25. Unexpectedly, in this condition, only a very small amount of WT-4E-BP was detectable (Fig. 7A), suggesting that the overexpressed protein was rapidly degraded. Since it has been recently suggested that phosphorylation of 4E-BP may promote its degradation (Elia

et al., 2008), we hypothesized that the injection of mRNA encoding for a non-phosphorylatable 4E-BP protein should maintain high overexpression of 4E-BP protein and efficiently sequester eIF4E. Most of the data available to date indicates that mTOR is the main kinase of 4E-BP (Brunn et al., 1997; Burnett et al., 1998). Therefore, both blastomeres of two-cell stage embryos were then injected with mRNA encoding for a 4E-BP mutated protein (4A-4E-BP), in which all the four mTOR regulated residues in 4E-BP were mutated to alanine (Martineau et al., 2012). Abolishing the possibility of phosphorylation on these four serine/threonine-to-alanine substitution has been shown to render 4E-BP constitutively active in sequestrating eIF4E (Oulhen et al., 2009). As expected, *4A-4E-BP* mRNA injection induced a significant increase in 4E-BP protein levels without exposure to hypoxia (Fig. 7A). Interestingly, overexpression of 4E-BP did not significantly affect developmental stage, apoptosis and embryo survival (data not shown).

We then checked that the 4A-4E-BP mutated protein associated efficiently with the endogenous eIF4E using m^7GTP affinity beads. Protein extracts were incubated with m^7GTP-conjugated Sepharose beads and bound proteins were analysed by Western blot with antibodies against 4E-BP and eIF4E. As shown in Figure 7B, WT-4E-BP and 4A-4E-BP proteins bind eIF4E, indicating that the mutation of the phosphorylation sites of 4E-BP did not alter its functionality other than stabilizing the protein.

To investigate the effects of overexpressing 4E-BP protein in somites, embryos were unilaterally injected at the four-cell stage into the presumptive somite region with mRNA encoding for 4A-4E-BP or WT-4E-BP as a control. Immunostaining of sectioned embryos revealed that the 4A-4E-BP protein was unilaterally detected in somites (Fig. 7C). Both the *4A-4E-BP* and *WT-4E-BP* mRNA injected embryos developed normally according to the external morphology (Fig. 7D-E). However, Figure 7F shows a significant decrease in the number of mitotic cells in somites of *4A-4E-BP* mRNA injected side compared to the control side of embryos, suggesting that overexpression of non-phosphorylatable 4E-BP protein induces a decrease in somite cell proliferation. Immunohistochemical analyses with 12/101 antibodies and Hoechst were performed. Interestingly, the 12/101 staining was dramatically weaker in the *4A-4E-BP* mRNA injected side compared to the control side as well as to the *WT-4E-BP* mRNA injected embryos (Fig. 7G-H) while qRT-PRC analyses show that the injection of *4A-4E-BP* mRNA did not alter the *Myh 1, 4, 8* and *6* mRNAs expression level (Fig. 7I and S4). Then, immunohistochemistry and qRT-PCR analysis of MyoD expression showed that this transcription factor is correctly expressed in *4A-4E-BP* mRNA injected embryos at both protein and mRNA levels (Fig. 7 J-L and M, respectively).

These results show that overexpression of the non-phosphorylatable 4E-BP protein leads to similar muscle effects compared to those observed in embryos exposed to hypoxia on the number of mitotic cells, the expression of muscle transcription factor and on the accumulation of muscle-specific proteins in the somite cells, independently of mRNAs variation.

Discussion

Our data show that exposure to hypoxia for 24 hours significantly reduced growth and delayed embryonic development in an oxygen-dependant manner. These effects are similar to those observed in mouse, chicken and sea urchin embryos (Le Bouffant et al., 2006; Sharma et al., 2006; Ream et al., 2008). Hypoxia also affected embryonic survival depending on the oxygen availability. During human pregnancy, acute hypoxia observed during retroplacental haemorrhage, intoxication with CO, and acute decompensation of maternal diabetes mellitus, has been linked with the sudden death of the fetus. Our model is much more linked with chronic hypoxia of the fetus. In such cases, chronic hypoxia leads to intrauterine growth retardation (IUGR; Vandenbosche and Kirchner, 1998) defined by a fetal weight corresponding that less than 10% of predicted fetal weight for gestational age. Its annual incidence is estimated between 4% to 7% of the total gestation number and the major conditions that can give rise to IUGR are maternal hypertension, maternal diabetes mellitus, maternal systemic lupus, smoking, certain infectious diseases, and lastly fetal chromosomal abnormalities like 21 trisomy or Turner syndrome. Interestingly, since Xenopus embryos develop externally, we show that growth retardation and embryonic death were independent on the maternal behaviour and placenta formation. Moreover, the growth retardation of embryos may reflect defects in timing of development suggesting alterations of developmental timer. However, this feature of hypoxia was not explored.

In the same way, we demonstrate that both strong and moderate hypoxia induce apoptosis. While the p38-MAPK has been shown to be involved in apoptosis (Bulavin et al., 1999; She et al., 2000; Zarubin and Han, 2005), in our hands there is no clear correlation between apoptotic patterns and phospho-p38-MAPK levels. Therefore, hypoxia may activate other mechanisms involved in apoptosis induction, such as the activation of pro-apoptotic factors. Since Akt was shown to be involved in cell survival (Dudek et al., 1997), the regulation of this kinase could be a good candidate to activate apoptosis in hypoxia. Indeed,

Bcl-2-Associated Death promoter (BAD) protein, caspase 9, and FoxO have been identified as targets of Akt. Activated Akt can phosphorylate and inactivate these factors, whereas the inhibition of Akt induces apoptosis (Datta et al., 1997; Kandel and Hay, 1999; Brazil and Hemmings, 2001). In agreement with this hypothesis, we show that Akt is reversibly dephosphorylated in hypoxia suggesting a link between this signalling pathway and the hypoxia-induced apoptosis in Xenopus embryos. However, we cannot exclude the involvement of other signalling pathways in the induction of apoptosis. Interestingly, the pro-apoptotic factor Bmf (Bcl-2-Modifying Factor), a member of the Bcl-2 family, has been shown to act as a sensor for stress that associates with the repression of the cap-dependent translation (Grespi et al., 2010).

Cap-dependent translation is correlated with the availability of the eukaryotic initiation factor eIF4E, which constitutes a major checkpoint for mRNA translation regulation (Richter and Sonenberg, 2005). The availability of eIF4E has been implicated in hypoxic responses in part by the phosphorylation of 4E-BP, a downstream target of the Akt signalling pathway (Wouters et al., 2005). Strikingly, we demonstrate that in Xenopus embryos hypoxia induced overexpression of a functional 4E-BP protein able to sequester eIF4E. The fact that 4E-BP association with eIF4E increases in hypoxia is in agreement with the Akt inhibition, since the 4E-BP/eIF4E dissociation complex is dependent of an active Akt signalling pathway and the phosphorylation state of 4E-BP. While the phosphorylation control of 4E-BP has been extensively studied (Armengol et al., 2007), little is known about the significant role of the variation in 4E-BP levels as a means of control of the availability of eIF4E (Cormier et al., 2003). The first demonstration that modification of 4E-BP levels may influence cell fate has been obtained in sea urchin where 4E-BP is fully degraded shortly after fertilization to allow embryonic development (Salaun et al., 2003). Moreover, hypoxia was shown to result in a reversible increase in 4E-BP protein levels in sea urchin embryos (Le Bouffant et al., 2006). In agreement with this, we show that the amount of the 4E-BP protein increases dramatically in Xenopus embryos following hypoxia therefore supporting the hypothesis that, besides its phosphorylation state, 4E-BP overexpression is also important to inhibit eIF4E availability and subsequent cap-dependent translation.

However, little is known about the mechanisms that control 4E-BP expression since the amount of protein in the cell reflects both protein synthesis and degradation. Therefore, a working model is proposed in Figure 8 to illustrate mechanisms that could be involved in 4E-

BP overexpression during embryonic development in hypoxia. On the one hand, deactivated Akt in hypoxia could prevent the phosphorylation of FoxO members resulting in nuclear translocation of these transcription factors and expression of FoxO-regulated genes such as 4E-BP, as shown in Drosophila embryos under dietary restriction and oxidative stress (Tettweiler et al., 2005). It is important to note that in pancreatic cells, *4E-BP* mRNA transcription is regulated by the transcription factor Smad4 after TGFβ treatment (Azar et al., 2009) and consequently, it cannot be excluded that *4E-BP* mRNA transcription is under the control of different transcription factors. However, qRT-PCR analyses did not show any significant variation in *4E-BP* mRNA levels in embryos exposed to 10% and 5% O_2, suggesting that the 4E-BP gene transcription was not responsible for the increase in 4E-BP protein amount at stages 21 and 25. On the other hand, deactivated Akt in hypoxia prevents the activation of its downstream mTOR protein and therefore prevents the phosphorylation of 4E-BP. Since it was shown that inhibition of proteasomal activity enhances the level of hypophosphorylated 4E-BP in Jurkat cells, 4E-BP protein can be activated and stabilized to accumulate in hypoxic cells via the inhibition of its degradation pathway. In agreement with this hypothesis, we show that inhibition of the PI3K/Akt pathway or mTOR activity, using LY294002 or rapamycin respectively, leads to an increase in 4E-BP amount in normoxic embryos, suggesting that the 4E-BP level is dependent of the Akt signalling pathway and under a rapamycin-sensitive pathway. Interestingly, since the inhibition of mTOR, known to phosphorylate 4E-BP, leads to its accumulation in normoxic embryos and slows its degradation in reoxygenated embryos, our data also suggests that the phosphorylation state of 4E-BP is associated with its stability, probably due to the inhibition of its degradation pathway. However, an additional increase in *4E-BP* mRNA translation could also participate in the accumulation of 4E-BP in hypoxic cells (Fig. 8).

Importantly, the inhibition of the initiation step of mRNA translation by 4E-BP has been proposed to act as a cellular survival mechanism involving decreased protein synthesis in order to conserve oxygen because protein synthesis is extremely energy costly. Teleman et al. (Teleman et al., 2005) and Tettweiler et al. (Tettweiler et al., 2005) provide support for the role of 4E-BP in mediating Drosophila embryonic survival under unfavourable conditions. In agreement with this idea, we demonstrate that effects of hypoxia exposure are reversible when oxygen is available and embryonic death was reduced with reoxygenation concomitant with the decrease in 4E-BP level. Moreover, inhibition of *4E-BP* mRNA translation with specific

Morpholino injection in two-cell stage embryos strongly induced lethality in normoxia, suggesting that a basal level of 4E-BP is required to regulate protein synthesis and ensure survival of embryos during the early embryonic development (data not shown).

In this study, we also observed that embryos exposed to hypoxia did not make any movement. In vertebrate embryos, somites are regular transient structures repeated along the anterior/posterior axis of the embryo, which then differentiate into a part of the dermis, bone, cartilage, tendon-cell lineages and skeletal muscles. Since the sclerotome is formed at stage 37 and the dermatome is a single layer of cells under the epidermis, somites of embryos at stages 21 and 25 are mainly composed of myotome cells which will give rise to skeletal muscle. Because premyoblast proliferation is known to be an early step of the myogenesis, one hypothesis was that hypoxia exposure affects cell proliferation in developing somites. In agreement with this, we found that hypoxia induces a 90% decrease in mitotic cell in somites. Interestingly, 4E-BP is known to be implicated in the inhibition of cell proliferation (Armengol et al., 2007; Azar et al., 2009) and, in the same way, we show that overexpression of a non-phosphorylatable 4E-BP protein in somites induced a decrease in cell proliferation. Consequently, hypoxia-induced 4E-BP overexpression is in agreement with the inhibition of myoblast proliferation. However, how is this only cell type affected by hypoxia remains an open question. One hypothesis is that the hypoxia-induced 4E-BP overexpression occurs selectively in myoblast precursors. Therefore, it would be interesting to perform in situ analysis of protein distribution in the embryos exposed to hypoxia.

In addition, we observed that the 12/101 monoclonal antibodies that recognize a not yet identified epitope of the differentiated muscle cells (Kintner and Brockes, 1984) produced a weaker signal in hypoxic embryos. We demonstrate that hypoxia does not affect mechanisms involved in the segmentation of the presomitic mesoderm but affects accumulation of muscle-specific proteins during the embryonic development. However, hypoxia does not disturb the protein expression pattern of both XMyoD and XMRF4, early and late markers of muscle cell fate respectively, suggesting that oxygen does not interfere with the events leading to the expression of the myogenic transcription factors in somite nuclei. Therefore, hypoxia could affect cap-dependent translation of mRNA produced downstream MyoD and MRF4 activation. The fact that MyoD and MRF4 were not affected following hypoxia treatment or 4E-BP overexpression lead us to propose that the translation of these two transcription factors mRNAs is under cap-independent control which is mostly mediated by mRNA structural element called IRES (Hellen and Sarnow, 2001). Interestingly,

IRES have been identified in several mammalian mRNAs, mainly in control genes such as transcription factors and growth factors (Vagner et al., 2001). For instance, functional IRESs have been recently identified in Fibroblast Growth Factor 1 (Martineau et al., 2004; Conte et al., 2009), which plays a crucial role in myoblast differentiation. It is noteworthy that a number of genes including the hypoxia-inducible genes encoding HIF-1α remain preferentially translated when eIF4E is sequestered by its repressor 4E-BP (Wouters and Koritzinsky, 2008) and we show that *HIF-1α* mRNA levels increased depending on the oxygen availability. Therefore, 4E-BP overexpression could represent an oxygen-sensitive pathway that occurs upstream changes in gene expression mediated by HIF-1 in hypoxia. This regulation of the translational machinery by hypoxia in Xenopus embryos might support mRNA-selective translation. In agreement with this hypothesis and with our data, it has been recently published that translation facilitates genes classes like transcription and signal transduction during acute hypoxia, whereas it represses the expression of genes involved in cell growth and protein metabolism (van den Beucken et al., 2011). Therefore, determination of the number and the nature of mRNAs translated in hypoxia condition is now an important goal.

Finally, an important step in muscle development is the abundant synthesis of specific proteins required for muscle cell contraction including actin and myosin that approximately occupy 80% of the cytoplasmic volume. To determine whether the translation machinery and particularly the availability of the cap binding protein eIF4E was involved in muscle defects, two constructs were used. The first one codes for full-length sea urchin 4E-BP protein (WT-4E-BP). The second one codes for 4E-BP mutated protein (4A-4E-BP) in which four phosphorylation sites of 4E-BP were mutated dramatically affecting the release of 4E-BP from eIF4E (Oulhen et al., 2009). Our data support that the phosphorylation state of the 4E-BP protein is crucial to ensure the stability of the protein in Xenopus embryo since the 4E-BP protein accumulate in *4A-4E-BP* injected embryos but not in *WT-4E-BP*. Moreover, since mTOR is the major kinase of these phosphorylatable sites, this is in good agreement that the regulation of 4E-BP amount is under the PI3K/Akt/mTOR axis. We also observed that the 12/101 monoclonal antibodies produced a weaker signal although MyoD was expressed in *4A-4E-BP* injected embryos. We therefore demonstrate that overexpression of the non-phosphorylatable 4E-BP protein leads to similar effect on muscle proteins than in embryos exposed to hypoxia (*i.e*. decrease in 12/101 signal but not in MyoD expression). The data

suggest for the first time that the hypoxia-induced 4E-BP could be responsible for defects in muscle formation during the early vertebrate development of the myotomes, without affecting specification by the myogenic transcription factors but preventing the abundant synthesis of muscle-specific proteins.

Materials and methods

Xenopus laevis embryos, hypoxia exposure and measurement of dissolved oxygen

Xenopus laevis embryos were obtained as previously described (Hidalgo et al., 2009). After the gastrulation events (stage 13 according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994)), embryos were cultivated in 0.1X MMR with several oxygen levels (10% and 5% O_2) in hypoxic chambers. The oxygen partial pressure in the medium was measured at several oxygen levels with an apparatus used to measure blood gases (Radiometer ABL520, Copenhagen, Denmark).

Plasmid constructs and mRNA injection experiments

The 342-bp fragment corresponding to sea urchin wild type 4E-BP or to mutant 4A-4E-BP was isolated from the pGex4T1 plasmid (Oulhen et al., 2009) and inserted into the pCS2+ vector digested by *Bamh1* and *Xho1*. Synthetic capped mRNAs were made by in vitro transcription as described in Djiane et al. (Djiane et al., 2000). All blastomeres or two blastomeres of two-cell stage or four-cell stage embryos were injected depending on the experiment.

Isolation of eIF4E and associated proteins

Xenopus embryos were collected and homogenized in lysis buffer (50 mM Tris HCl, 100 mM NaCl, pH 7,5, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 120 nM okadaic acid, 1 mM PMSF, 1% Triton X100) supplemented with protease inhibitors. Lysates were centrifuged for 15 minutes at 14,000 g at 4°C. Isolation of eIF4E and its partners from embryo extracts was performed using m^7GTP beads (Amersham Biosciences). Briefly, 2 mg proteins were incubated with 25 μg of m^7GTP sepharose beads for 1 hour at 4°C. The beads were washed with 2 ml of 1X binding buffer containing 100 mM NaCl. Laemmli sample buffer was added directly to the beads and denatured at 95°C for 5 minutes before Western blot analysis.

Western blot analysis

Xenopus embryos were collected and homogenized in lysis buffer (50 mM Tris HCl, 100 mM NaCl, pH 7,5, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 120 nM okadaic acid, 1 mM PMSF, 1% Triton X100) supplemented with protease inhibitors. Protein samples, separated by 12% SDS-PAGE, were transferred to nitrocellulose membranes (Hybond) as described by Towbin (Towbin et al., 1979). The membranes were blocked in 5% non-fat dry milk and incubated with the following primary antibodies: anti-α-Tubulin (T0926, Sigma), anti-4E-BP1 (9452, Cell Signaling), anti-eIF4E (9742, Cell Signaling), anti-p38-MAPK (9212, Cell Signaling), anti-phospho-p38-MAPK (9211, Cell Signaling), anti-phospho-Akt Ser473 (9271, Cell Signaling), anti-phospho-Akt Thr308 (9275, Cell Signaling), anti-Akt total (9272, Cell Signaling), anti-sea urchin 4E-BP (alpha-69, (Oulhen et al., 2010)), 12101 (DSHB). After washing they were incubated with the appropriate secondary antibodies: anti-rabbit HRP conjugated (111-036-045, Jackson ImmunoResearch), anti-mouse HRP conjugated (115-036-062, Jackson ImmunoResearch) and detected by chemiluminescence. All experiments were repeated at least 3 times. For quantification, blots were exposed to X-ray film for various time points and the films were scanned and analysed using the ImageJ software from the NIH.

Immunochemistry

Embryos were embedded with 15% cold-water fish gelatin (FLUKA, biochemika) and 15% sucrose. Tissues were sectioned at 14 μm thickness by a cryostat (Leica CM 3050S). Sections were blocked in 20% goat serum and the following primary antibodies were used: mouse monoclonal 12/101 (DSHB, 1:2000), F59 (DSHB, 1:100), alpha-69 ((Oulhen et al., 2010), 1:100), mouse anti-MyoD (D7F2, DSHB), guinea pig anti-MRF4 (kindly provided by Dr B. Della Gaspera, Université Paris 5, France, 1:50), mouse anti-P63 (Abcam, 1:50), mouse anti-acetylated tubulin (Sigma 6-11B-1, 1:500). After washing they were incubated with the appropriate secondary antibodies: anti-mouse CY3 conjugated (Sigma C2181, 1:100), anti-rabbit FITC conjugated (Jackson ImmunoResearch 111-095-144, 1:40), anti-guinea pig Alexa 488 conjugated (Invitrogen A11073, 1:500). Nuclei were stained with Hoechst H33258 (Sigma, 1:1000). Sections were washed and then mounted in Immunomount (Thermo electron corporation).

All imaging was done at room temperature. Immunofluorescent staining was imaged using a Nikon Eclipse E800 microscope equipped with a QEi Evolution camera (Media Cybernetics)

(ARC N° 7867). A 4x (Plan 0.1 NA), 10x (Plan-apochromat 0.45 NA) or 20x (Plan-apochromat 0.75 NA) were used and the image acquisition software was Image-Pro Plus.

Quantitative PCR

Total RNAs were isolated from 6 embryos (stages 21 and 25) using the RNeasy mini kit (Quiagen) according to the manufacturer instructions. All primers were designed using Primer Express Software. PCR reactions were carried out using SYBR green (Applied Biosystems) on a StepOnePlus (Applied Biosystems). All experiments were repeated at least 3 times on separate experiment and the real-time PCR was also performed in duplicate. The results were analysed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The relative expression of genes is shown normalized to the expression of the house-keeping gene RPL13 although several house-keeping genes were tested to validate the results. The real-time PCR primers are given Table S1.

TUNEL staining and proliferation assays

Embryos (n=15) were fixed and sectioned. TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridinetriphosphate nick end-labelling) staining was carried out following the protocol as previously described (Hidalgo et al., 2009). Sections were mounted in Immunomount (Thermo electron corporation) and positive cells were counted, on all sections of embryos in several tissues, by microscopy at a magnification of 100X, in non-overlapping fields.

Proliferation assays were performed on sections of embryos (n=8) stained with the rabbit anti-human phospho-Histone H3 antibody (Ser 10, mitosis marker, Euromedex H5110-14B, 1:500) and the anti-rabbit alkaline phosphatase-conjugated antibody (Jackson Immunoresearch 111-055-144, 1:5000). Positive cells were counted, on all sections of embryos in several tissues, in both the control and the hypoxic embryos by microscopy at a magnification of 100X, in non-overlapping fields.

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Figure legends

Fig. 1. Hypoxia causes growth retardation, lethality and apoptosis.

(A) Western blot analysis. Protein extracts at stages 21 and 25 from embryos exposed to 21%, 10% and 5% O_2 were studied by Western blot using anti-phopsho-p38-MAPK and anti-p38-MAPK. Anti-tubulin alpha antibodies were used as a loading control. A 5% O_2 exposure caused a strong cellular stress during the embryonic development. **(B)** Densitometric quantification of phospho-p38-MAPK from immunoblots in the different experimental conditions as described in material and method. **(C)** Growth of embryos at 21°C in functions of time and of oxygen rate. Less oxygen is available more the retardation is important and hypoxic embryos maintained their delay. **(D)** Lateral view of embryos 42 hours after fertilization. When control embryos were stage 30, embryos exposed to 10% O_2 were stage 28 and embryos exposed to 5% O_2 were stage 25. **(E)** Embryo survival in function of oxygen level. Moderate hypoxia permits survival but strong hypoxia causes significant embryonic death. **(F)** Stage 25 embryos were sectioned (n=15) and TUNEL stained. Positive cells were counted on frontal sections of control and hypoxic embryos. The average of positive cells per embryos is compared on the graph. Apoptosis was promoted with hypoxia. $*P<0.05$; $**P<0.005$; $***P<0.001$. Scale bar = 400 μm.

Fig. 2. Hypoxia decreases Akt phosphorylation.

(A) Western blot analysis. Protein extracts at stages 21 and 25 from embryos exposed to 21%, 10% and 5% O_2 were resolved by electrophoresis, blotted and reacted with anti-Thr308-Akt, anti-Ser473-Akt or anti-Akt antibodies. Anti-tubulin alpha antibodies were used as a loading control. **(B-C)** Densitometric quantification of Thr308-Akt (B) and Ser473-Akt (C) from immunoblots in the different experimental conditions as described in material and method. The level of phospho-Akt decreased in hypoxia. $*P<0.05$; $**P<0.005$.

Fig. 3. Hypoxia increases the amount of 4E-BP protein which binds to eIF4E.

(A) Western blot analysis. Protein extracts from embryos exposed to 21%, 10% and 5% O_2, at stages 21 and 25 were resolved by electrophoresis, blotted and reacted with anti-4E-BP antibodies (9452, Cell Signaling). Anti-α tubulin antibodies were used as a loading control. **(B)** Densitometric quantification of 4E-BP from immunoblots in the different experimental conditions as described in material and method. $*P<0.05$; $**P<0.005$; $***P<0.001$. **(C)**

Isolation of eIF4E and its partners from stage 25 embryo extracts was performed using m^7GTP Sepharose beads before Western blot analysis. Immunoblots with anti-4E-BP and anti-eIF4E antibodies were performed. An anti-tubulin alpha antibody was used as a purification control in m^7GTP samples. 4E-BP binds eIF4E. **(D)** qRT-PCR of *4E-BP* mRNA in normoxia (21% O_2) and hypoxia (10 and 5% O_2) at stages 21 and 25. No significant variation of the mRNA amount was detected. **(E)** Western blot analysis. Protein extracts from embryos exposed to several concentration of LY294002, a well-established inhibitor of PI3-kinase which acts upstream of Akt, were resolved by electrophoresis, blotted and reacted with anti-4E-BP and anti-Thr308-Akt antibodies. Anti-α tubulin antibodies were used as a loading control. Treatment with 50 μM LY294002 affected Akt phosphorylation state and triggered an increase of 4E-BP protein amount. **(F)** Western blot analysis. Protein extracts from embryos exposed to rapamycin, a compound known to block mTOR activity, were resolved by electrophoresis, blotted and reacted with anti-4E-BP and anti-Thr308-Akt antibodies. Anti-α tubulin antibodies were used as a loading control. Treatment with rapamycin induced a dose-dependent increase of the 4E-BP amount.

Fig. 4. Effects of hypoxia exposure are reversible upon reoxygenation.

(A) Experimental design. Embryos were exposed to 5% O_2 until stage 21 and then some of them were replaced under normal condition (reoxygenation) whereas the others still developed in hypoxia. Control embryos were exposed to 21% O_2 (normoxia). **(B)** Western blot analysis using anti-4E-BP, anti-phospho-p38-MAPK, anti-Thr308-Akt and anti-Ser473-Akt antibodies were performed. Anti-tubulin alpha antibodies were used as a loading control. When oxygen is available, the P-p38-MAPK, P-Akt as well as the 4E-BP protein levels return to the basal level observed in control. The embryonic death at stage 25 was reduced (9%) when replaced under normal conditions compared to hypoxia (54%). **(C)** Embryos were exposed to 5% O_2 until stage 21 and shifted to normoxia in absence (-) or presence (+) of rapamycin. The level of 4E-BP was analysed by Western blot after 15, 30, 45 min of reoxygenation. Anti-tubulin alpha antibodies were used as a loading control. 4E-BP amount was more important in reoxygenated embryos cultured in presence of rapamycin compared to those cultured in absence of the drug.

Fig. 5. Hypoxia specifically decreases the number of mitotic cell in somites.

Stage 25 embryos were sectioned (n=8) and immunodetections were performed with the anti-phospho-Histone H3 antibody. Positive cells were counted on frontal sections of control and hypoxic embryos. The average of positive cells per tissues is compared on the graph. The number of positive cells was specifically reduced in somites of embryos exposed to 5% O_2. *P<0.05.

Fig. 6. Hypoxia does not affect somitogenesis but affects the accumulation of specific proteins in muscle cells.

(A-D) Stage 25, control (A-C) and 5% O_2-exposed embryos (B-D). Sections were nuclei stained with Hoechst (blue) and immunostained with the F59 antibodies (red; A-B) or with the 12/101 antibodies (red; C-D). Both stainings disappeared in hypoxic embryos. **(E)** Western blot analysis using the 12/101 antibodies and anti-alpha tubulin antibodies as a loading control. The 12/101 protein level was weaker in embryos exposed to hypoxia. **(F-Q)** Stage 25 control embryo and 5% O_2-exposed embryos sectioned and stained with Hoechst (blue) and immunostained with the anti-MyoD (G, J) and anti-MRF4 (M,P) antibodies (red). No significant differences in the localisation and expression of MyoD and MRF4 were detected. **(R-S)** qRT-PCR of *Myh 1, 4 and 8* mRNAs (R) and *MyoD* mRNA (S) in normoxia (21% O_2) and hypoxia (10 and 5% O_2) at stage 25. The amount of mRNAs remained constant regardless of oxygen availability. **(T-W)** 21% and 5% O_2-exposed embryos, sectioned and stained with Hoechst (blue) and immunostained with the anti-P63 (red; T,U) and anti-acetylated tubulin antibodies (red; V, W). P63 was still expressed in nuclei of cells and acetylated tubulin in ciliated cells of the epidermis. Scale bar = 100 μm.

Fig. 7. Overexpression of a non-phosphorylatable 4E-BP protein affects the accumulation of muscle-specific proteins in somites.

(A-B) Embryos were injected at the two-cell stage on both blastomeres with mRNA coding for a full-length sea urchin 4E-BP protein (WT-4E-BP) and coding for a 4E-BP mutated protein (4A-4E-BP). (A) Protein extracts from stage 25 embryos were studied using an anti-4EBP antibody (alpha 69). Immunoblot with anti-tubulin alpha antibodies was used as a loading control. The *4A-4E-BP* injection induced an overexpression of the 4E-BP protein without hypoxia exposure while in the *WT-4E-BP* injected embryos the 4E-BP protein level was much lower. (B) Isolation of eIF4E and its partners from stage 25 embryo extracts was performed using m^7GTP Sepharose beads before Western blot analysis. Immunoblots with anti-4E-BP (alpha 69) and anti-eIF4E antibodies were performed. WT-4E-BP and 4A-4E-BP

proteins bind eIF4E. **(C)** Embryos were unilaterally injected at the four-cell stage into the presumptive somite region with the *WT-4E-BP* or *4A-4E-BP* constructs. At stage 25, embryos were fixed, sectioned and stained with an anti-4EBP antibody (alpha 69; red) and Hoechst (blue). 4E-BP protein was unilaterally detected in somites of *4A-4E-BP* injected embryos. **(D-E)** External views of embryos. Lateral views of both control (D) and *4A-4E-BP* injected (E) embryos. They developed normally. **(G-H)** At stage 25, control and injected embryos were fixed, sectioned and stained with the 12/101 antibodies (red) and Hoechst (blue). (G) Frontal section of *WT-4E-BP* injected embryo. (H) Frontal section of *4A-4E-BP* injected embryo. The 12/101 staining was weaker in the *4A-4E-BP* injected side (lower part) compared to the control side and compared to the *WT-4E-BP* injected embryos. **(I)** qRT-PCR of *Myh 1, 4, 8* mRNAs in normoxia (21% O_2) at stage 25 of non injected (CT), *WT-4E-BP* (WT) and *4A-4E-BP* (4A) injected embryos. The amount of mRNAs remained constant. **(J-L)** *4A-4E-BP* injected embryos at stage 25, sectioned and stained with the anti-MyoD antibody (red, K) and Hoechst (blue, L); merged image (J). **(M)** qRT-PCR of *MyoD* mRNA in normoxia (21% O_2) at stage 25 of non injected (CT), *WT-4E-BP* (WT) and *4A-4E-BP* (4A) injected embryos. The amount of mRNAs remained constant. Scale bar = 200 μm.

Fig. 8. Working model.

In normoxia (left part), phospho-Akt indirectly activates mTOR that stimulates protein translation notably through phosphorylation and inhibition of 4E-BP. It is possible that phospho-4E-BP is degraded via the proteasome pathway. Akt can also phosphorylate FoxO members resulting in cytoplasmic retention and inactivation of this transcription factor. Myoblasts can proliferate and differentiate in this context. In hypoxia (right part), 4E-BP overexpression can be explained by two different ways. One possibility is that deactivated Akt prevents the inhibition of FoxO members therefore resulting in the expression of FoxO-regulated genes such as 4E-BP. Another possibility is that deactivated Akt prevents the mTOR activation and therefore the phosphorylation and degradation of 4E-BP. Consequently, 4E-BP protein can accumulate in the cytoplasm of hypoxic cells to repress the initiation step of the mRNA translation. In this context, transcription factors are correctly expressed in nuclei to specify myoblast lineage but hypoxia-induced 4E-BP causes muscle defects preventing the synthesis of specific muscle proteins.

S1.

(A) The oxygen partial pressure in the medium was measured at several oxygen levels. The dissolved oxygen level (mmHg) was proportional to the oxygen level in the hypoxic chamber (%). **(B-C)** qRT-PCR analysis of *HIF-1α* (B) and *VEGF* (C) mRNA from embryos exposed to 21%, 10% and 5% O_2. A significant increase of *HIF-1α* and *VEGF* mRNA expression in relation to oxygen availability was observed. **P*<0.05.

S2.

Western blot analysis. Protein extracts from embryos exposed for several hours to 80 μM rapamycin were resolved by electrophoresis, blotted and reacted with anti-4E-BP and anti-Thr308-Akt antibodies. Anti-α tubulin antibodies were used as a loading control. 3 hours treatment with rapamycin triggered an increase of the 4E-BP amount without affecting Akt phosphorylation.

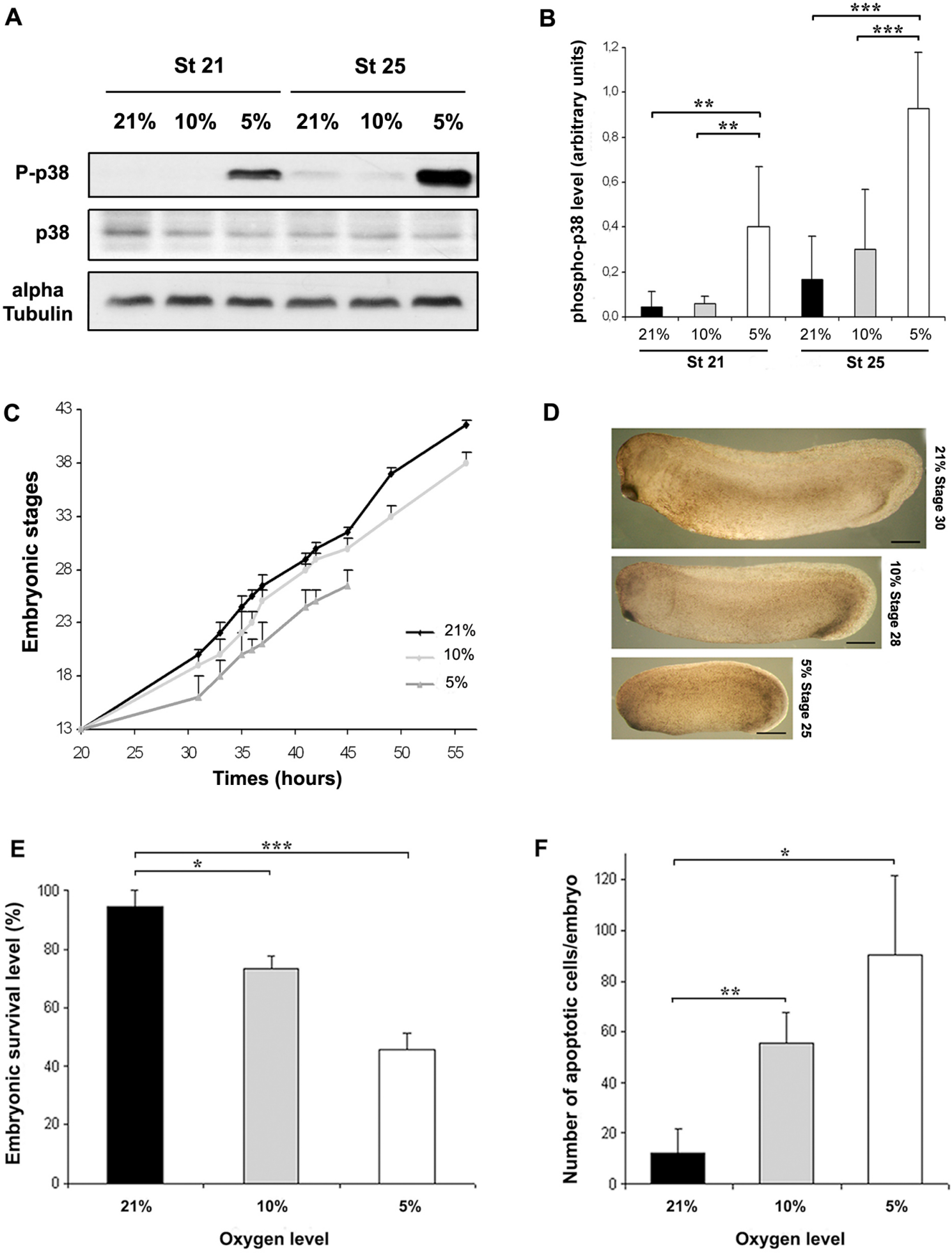
S3.

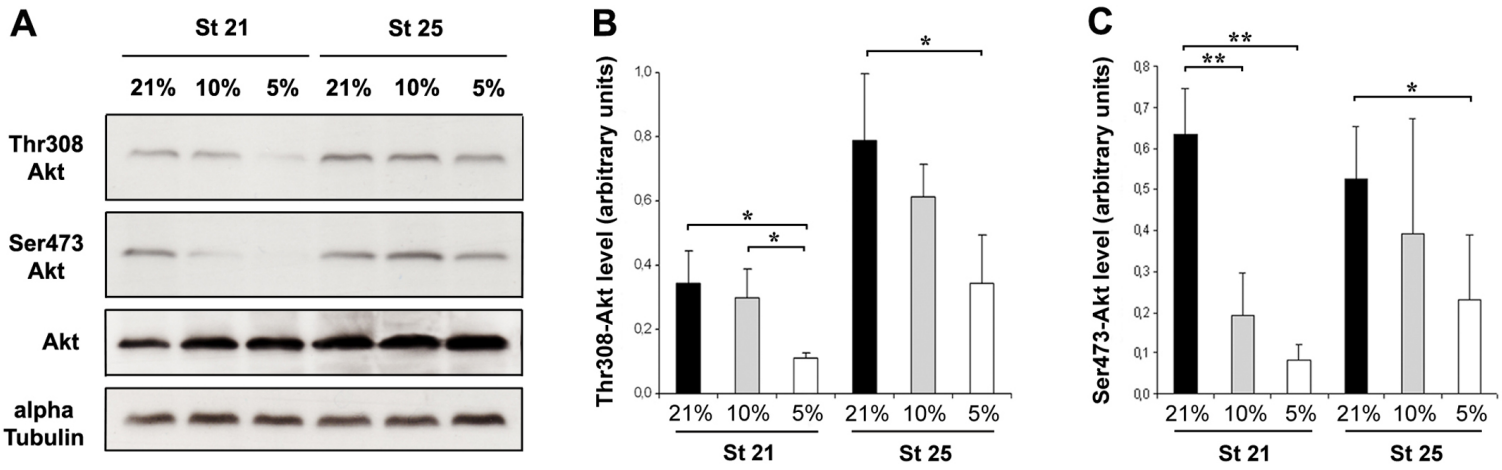
(A-C) Stage 25, control (A), reoxygenated (B) and 5% O_2-exposed embryos (C). Sections were nuclei stained with Hoechst (blue) and immunostained with the 12/101 antibodies (red). **(D)** Western blot analysis using the 12/101 antibodies and anti-alpha tubulin antibodies as a loading control. The 12/101 staining was higher in reoxygenated embryos compared to hypoxic embryos but did not return to basal levels observed in controls (normoxia). Scale bar = 100 μm.

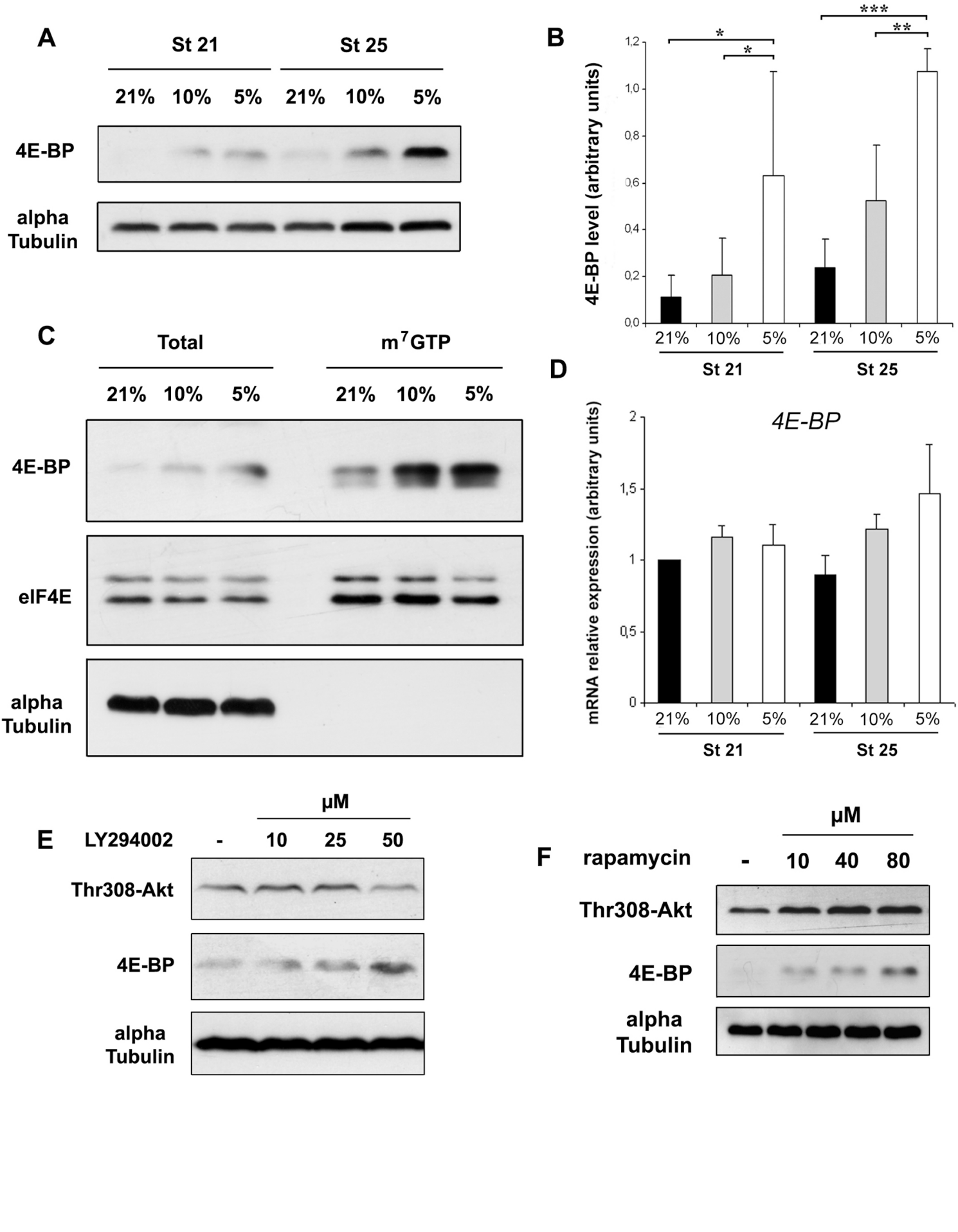
S4.

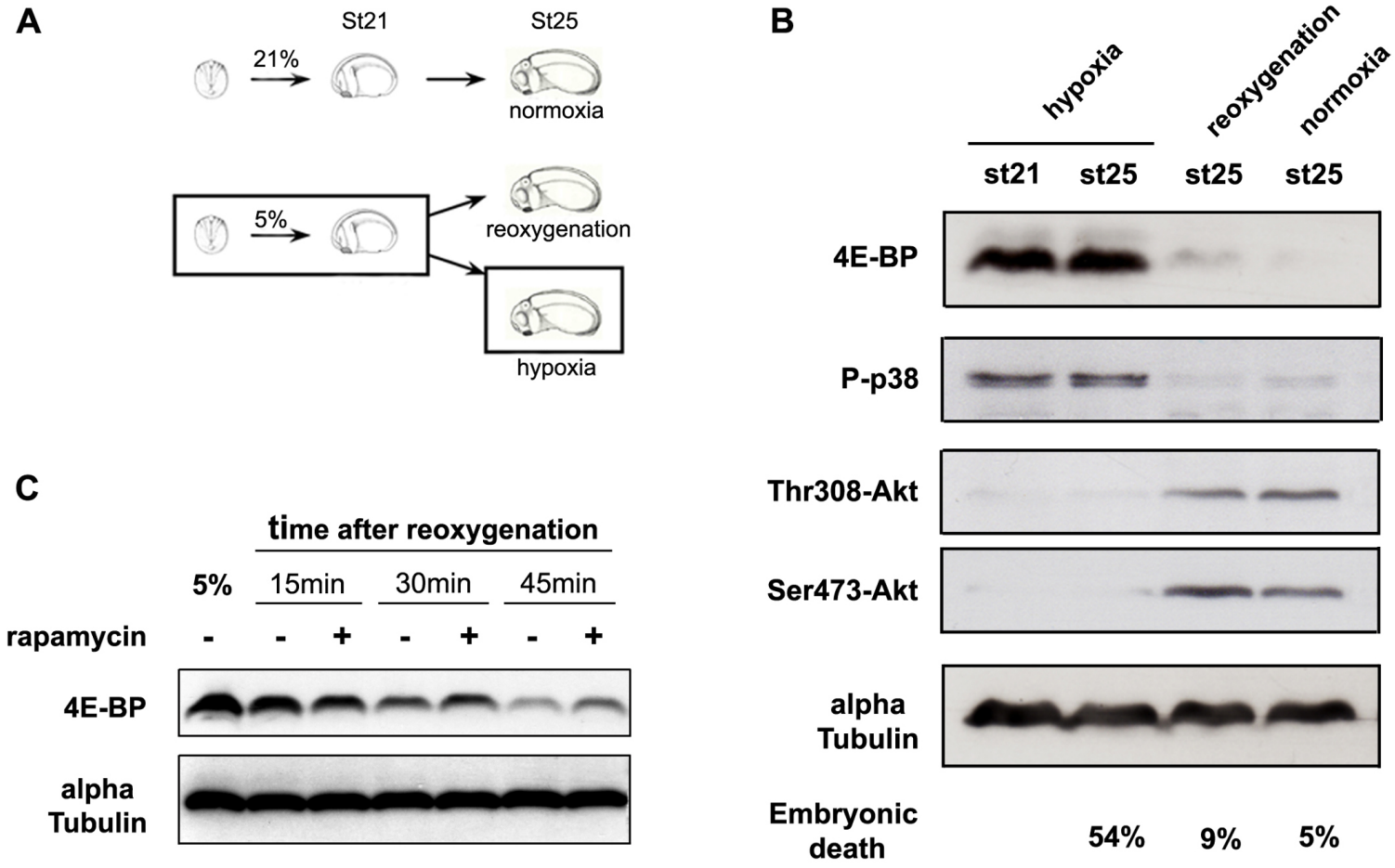
(A-B) qRT-PCR of *Myh 6* mRNAs from embryos at stage 25 (A) exposed to 21%, 10% and 5% O_2, and from embryos (B) non injected (CT), *WT-4E-BP* (WT) and *4A-4E-BP* (4A) injected embryos. The amount of mRNAs remained constant.

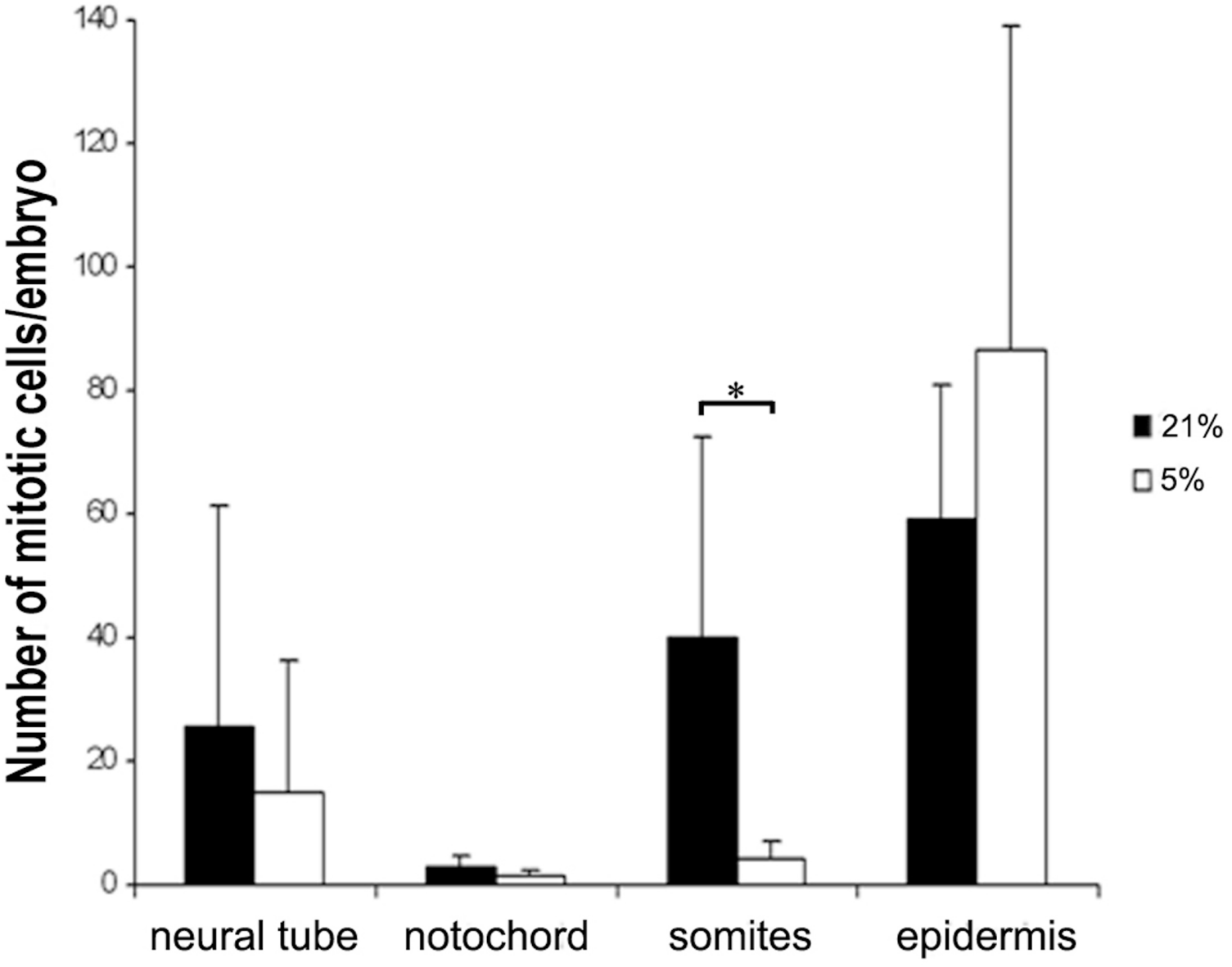
Table S1. qRT-PCR primer sequences.

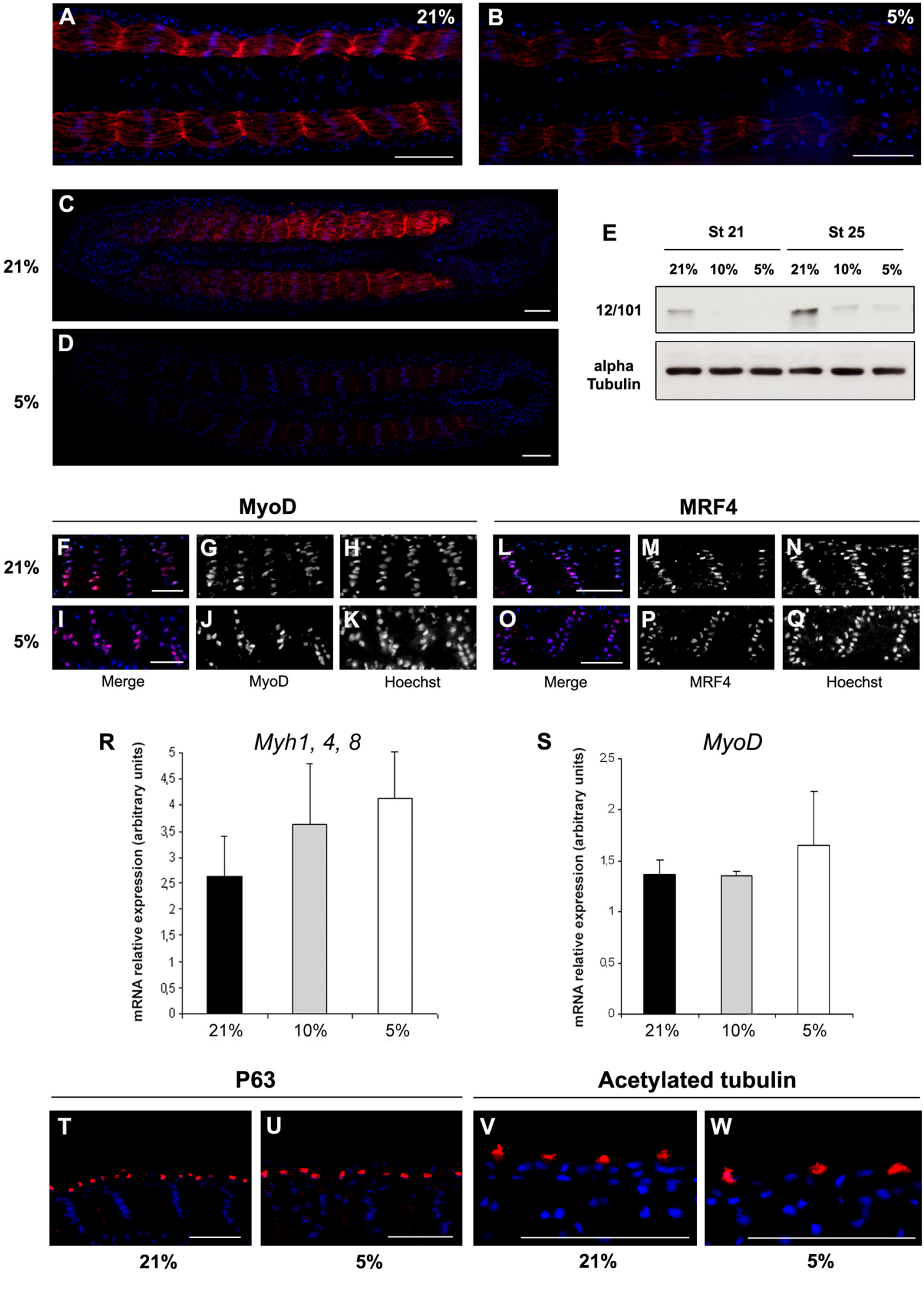

A
St 21
St 25
21%
10%
5%
21%
10%
5%
P-p38
p38
alpha Tubulin
B
phospho-p38 level (arbitrary units)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
**
**
***
***
21%
10%
5%
21%
10%
5%
St 21
St 25
C
Embryonic stages
43
38
33
28
23
18
13
20
25
30
35
40
45
50
55
Times (hours)
21%
10%
5%
D
21% Stage 30
10% Stage 28
5% Stage 25
E
Embryonic survival level (%)
100
80
60
40
20
0
*
***
21%
10%
5%
Oxygen level
F
Number of apoptotic cells/embryo
120
100
80
60
40
20
0
*
**
21%
10%
5%
Oxygen level

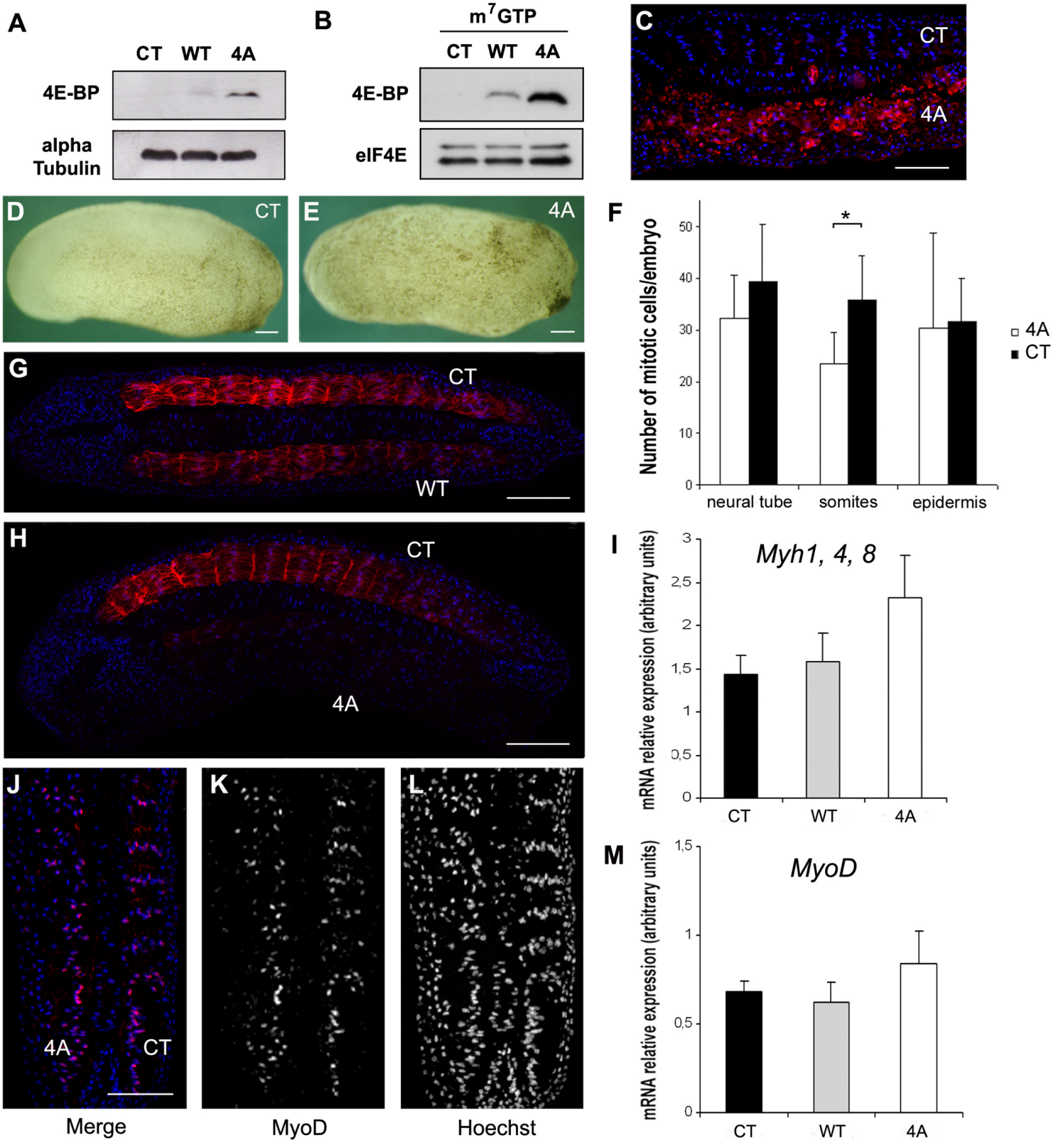

A
St 21
St 25
21%
10%
5%
21%
10%
5%
Thr308 Akt
Ser473 Akt
Akt
alpha Tubulin
B
Thr308-Akt level (arbitrary units)
1,0
0,8
0,6
0,4
0,2
0,0
*
*
*
21%
10%
5%
21%
10%
5%
St 21
St 25
C
Ser473-Akt level (arbitrary units)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
**
**
*
21%
10%
5%
21%
10%
5%
St 21
St 25

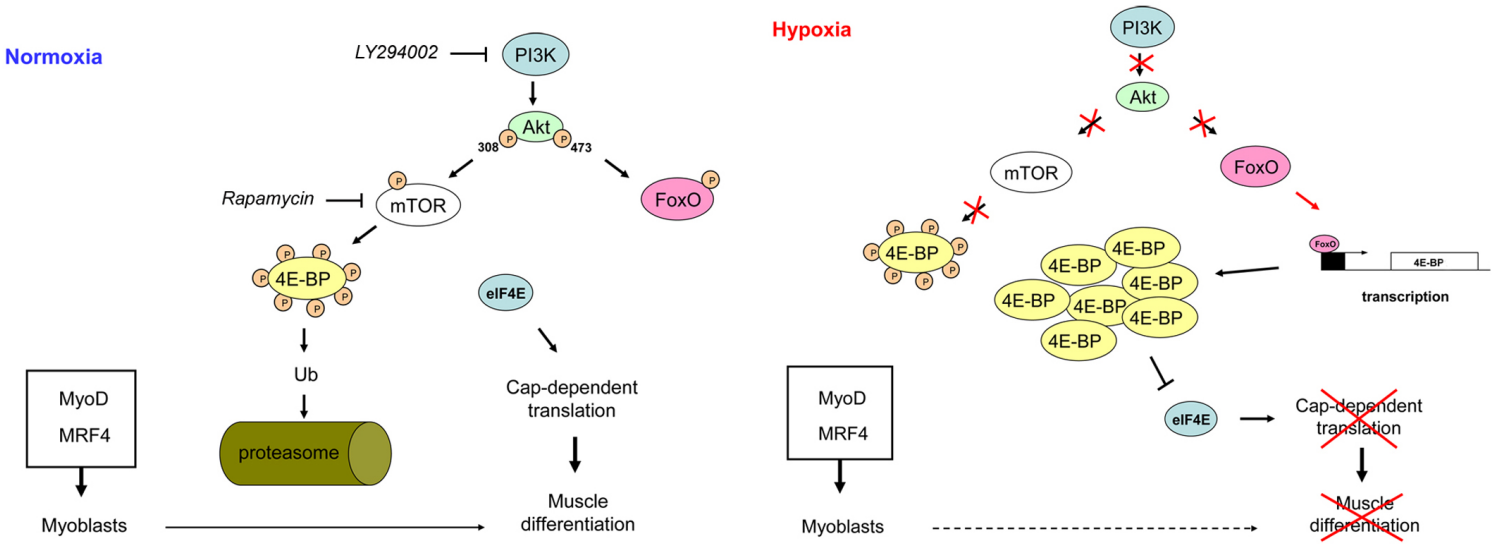

A
St 21
St 25
21%
10%
5%
21%
10%
5%
4E-BP
alpha Tubulin
B
4E-BP level (arbitrary units)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
*
***
**
21%
10%
5%
21%
10%
5%
St 21
St 25
C
Total
m7GTP
21%
10%
5%
21%
10%
5%
4E-BP
eIF4E
alpha Tubulin
D
4E-BP
mRNA relative expression (arbitrary units)
2
1,5
1
0,5
0
21%
10%
5%
21%
10%
5%
St 21
St 25
E
μM
LY294002
-
10
25
50
Thr308-Akt
4E-BP
alpha Tubulin
F
μM
rapamycin
-
10
40
80
Thr308-Akt
4E-BP
alpha Tubulin


A
St21
St25
21%
normoxia
5%
reoxygenation
hypoxia
B
hypoxia
reoxygenation
normoxia
st21
st25
st25
st25
4E-BP
P-p38
Thr308-Akt
Ser473-Akt
alpha Tubulin
Embryonic death
54%
9%
5%
C
time after reoxygenation
5%
15min
30min
45min
rapamycin
- - + - + - +
4E-BP
alpha Tubulin

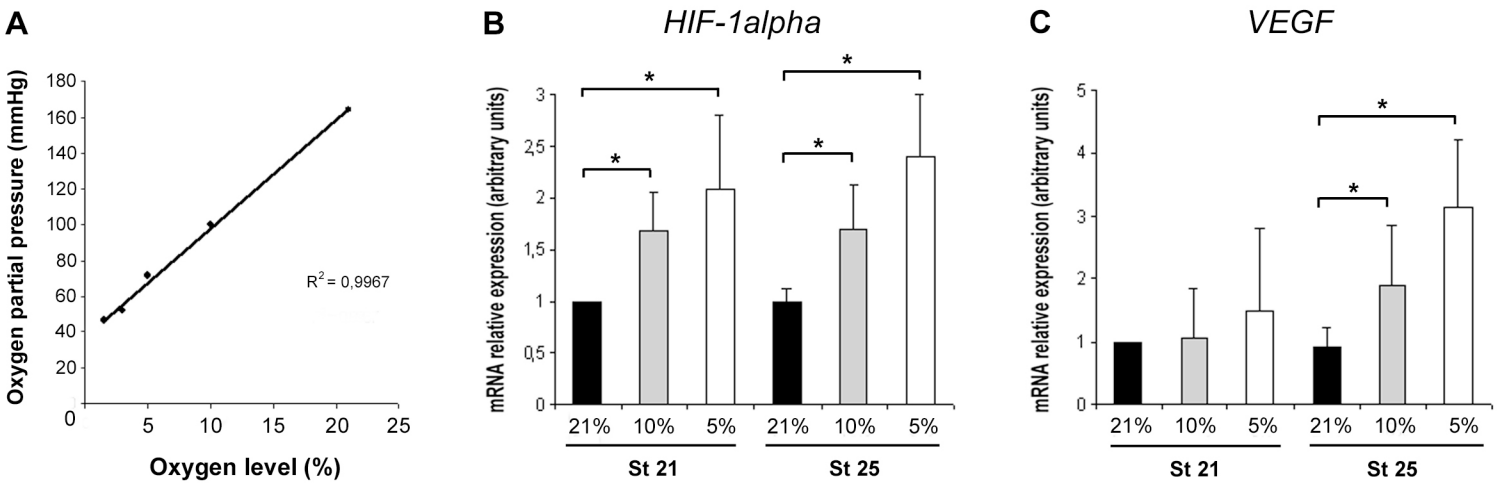

Number of mitotic cells/embryo
140
120
100
80
60
40
20
0
*
21%
5%
neural tube
notochord
somites
epidermis

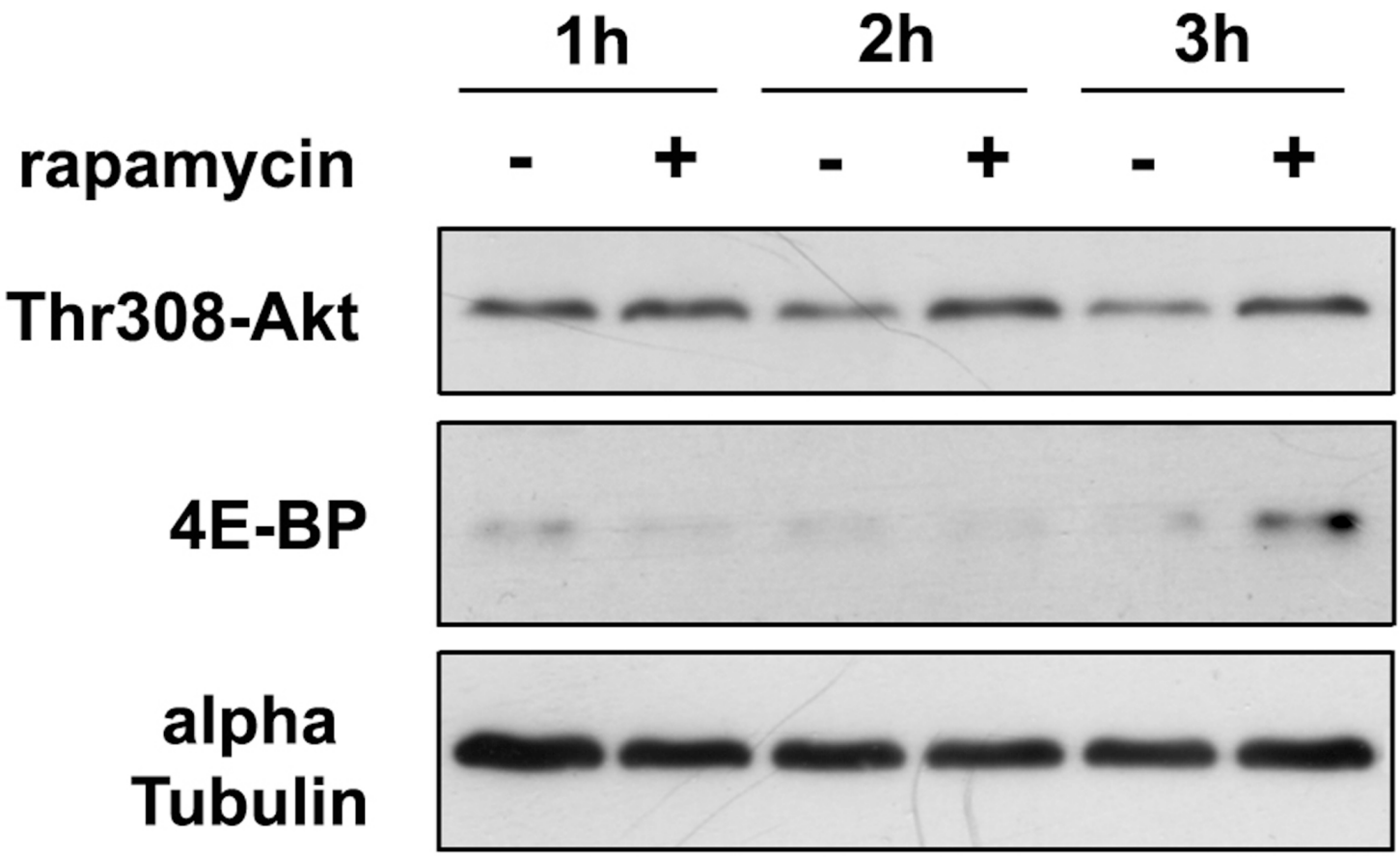

A
21%
B
5%
C
21%
D
5%
E
St 21
St 25
21% 10% 5% 21% 10% 5%
12/101
alpha Tubulin
MyoD
MRF4
F
G
H
L
M
N
21%
I
J
K
O
P
Q
5%
Merge
MyoD
Hoechst
Merge
MRF4
Hoechst
R
Myh1, 4, 8
mRNA relative expression (arbitrary units)
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
21%
10%
5%
S
MyoD
mRNA relative expression (arbitrary units)
0
0,5
1
1,5
2
2,5
21%
10%
5%
P63
Acetylated tubulin
T
U
V
W
21%
5%
21%
5%

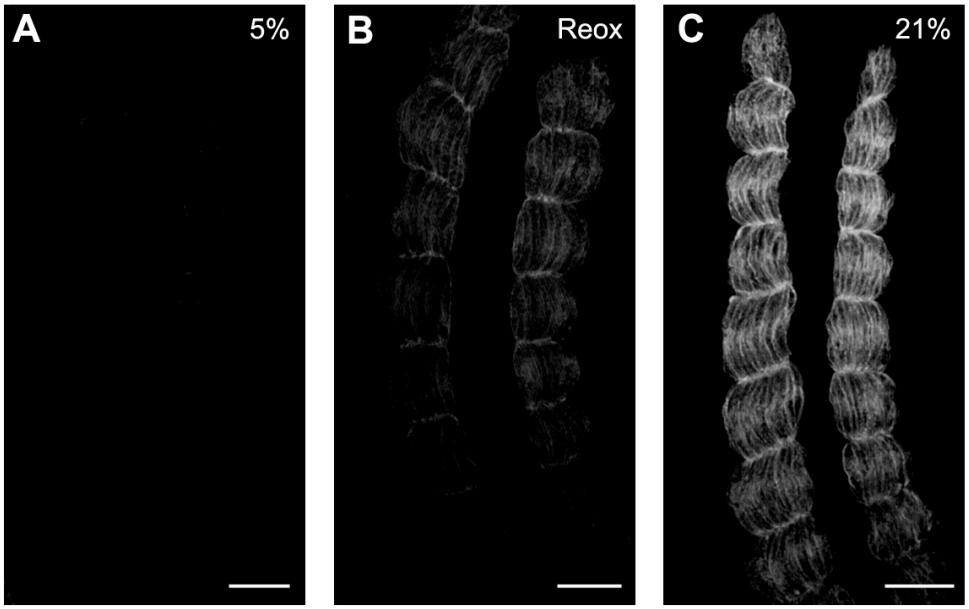

A
CT WT 4A
4E-BP
alpha Tubulin
B
m7GTP
CT WT 4A
4E-BP
eIF4E
C
CT
4A
D
CT
E
4A
F
Number of mitotic cells/embryo
50
40
30
20
10
0
*
neural tube
somites
epidermis
4A
CT
G
CT
WT
H
CT
4A
I
Myh1, 4, 8
mRNA relative expression (arbitrary units)
3
2,5
2
1,5
1
0,5
0
CT
WT
4A
J
4A
CT
K
L
M
MyoD
mRNA relative expression (arbitrary units)
1,5
1
0,5
0
CT
WT
4A
Merge
MyoD
Hoechst

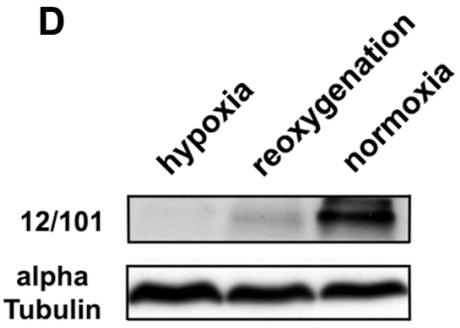

Normoxia
LY294002
PI3K
Akt
308
473
Rapamycin
mTOR
FoxO
4E-BP
eIF4E
Ub
proteasome
Cap-dependent translation
MyoD
MRF4
Myoblasts
Muscle differentiation
Hypoxia
PI3K
Akt
mTOR
FoxO
4E-BP
transcription
eIF4E
Cap-dependent translation
MyoD
MRF4
Myoblasts
Muscle differentiation

	Forward	Reverse
Myh 1/4/8	5'-TGCCACAGAAAACAAGGTCAAA-3'	5'-TCGAGAGCTGCCATTTCTTCA-3'
Myh 6	5'-ACCTTTCTGCATGAGCCAGC-3'	5'-TCACACAGAAGAGACCTGAATATGTG-3'
HIF1alpha	5'-CCAGGCTATTGGAATTGGTACC-3'	5'-GGCCCAGGATCAACATTTGT-3'
4E-BP	5'-GGCACCCTCTTCTCCACCA-3'	5'-CGACGGTCCAAAAGAAACTTG-3'
MyoD	5'-CTCCGATGGCATGATGGATT-3'	5'-CTTCTCCTGGAGCCGCAG-3'
ODC	5'-GGGCAAAGGAGCTTAATGTGG-3'	5'-TGCCAACATGGAAACTCACAC-3'
Actin b	5'-TCTATTGTGGGTCGCCCAAG-3'	5'-TTGTCCCATTCCAACCATGAC-3'
EEF1alpha	5'-CTTCTCAGGCCGACTGTGC-3'	5'-ATTCACCAACACCAGCAGCA-3'
RPL13	5'-GGAATCCCACCTCCCTATGAC-3'	5'-GCGCACAATCTTCAGAGCAG-3'
VEGF	5'-CTATGCCTGGAGAGGGAGACC-3'	5'-CATGCTGCGCTCGTACACTT-3'


A
Oxygen partial pressure (mmHg)
180
160
140
120
100
80
60
40
20
0
5
10
15
20
25
Oxygen level (%)
R2 = 0,9967
B
HIF-1alpha
mRNA relative expression (arbitrary units)
3
2,5
2
1,5
1
0,5
0
21%
10%
5%
21%
10%
5%
St 21
St 25
C
VEGF
mRNA relative expression (arbitrary units)
5
4
3
2
1
0
21%
10%
5%
21%
10%
5%
St 21
St 25


1h
2h
3h
rapamycin
- + - + - +
Thr308-Akt
4E-BP
alpha
Tubulin


A
5%
B
Reox
C
21%


D
hypoxia
reoxygenation
normoxia
12/101
alpha
Tubulin