# Tolerance to Hypoxia Is Promoted by FOXO Regulation of the Innate Immunity Transcription Factor NF-κB/Relish in *Drosophila*

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**ABSTRACT** Exposure of tissues and organs to low oxygen (hypoxia) occurs in both physiological and pathological conditions in animals. Under these conditions, organisms have to adapt their physiology to ensure proper functioning and survival. Here, we define a role for the transcription factor Forkhead Box-O (FOXO) as a mediator of hypoxia tolerance in *Drosophila*. We find that upon hypoxia exposure, FOXO transcriptional activity is rapidly induced in both larvae and adults. Moreover, we see that *foxo* mutant animals show misregulated glucose metabolism in low oxygen and subsequently exhibit reduced hypoxia survival. We identify the innate immune transcription factor, NF-κB/Relish, as a key FOXO target in the control of hypoxia tolerance. We find that expression of Relish and its target genes is increased in a FOXO-dependent manner in hypoxia, and that *relish* mutant animals show reduced survival in hypoxia. Together, these data indicate that FOXO is a hypoxia-inducible factor that mediates tolerance to low oxygen by inducing immune-like responses.

**KEYWORDS** hypoxia; *Drosophila*; FOXO; NF-κB; glucose metabolism; immunity; HIF-1α

OXYGEN is essential for the normal growth, development, and functioning of tissues and organs. However, while the air we breathe contains ~20% oxygen, even under healthy physiological conditions, our cells and tissues receive considerably lower levels. These can be anywhere from 1 to 10% oxygen depending on the tissue (McKeown 2014). Hence, our tissues and organs need to function and maintain homeostasis at low levels of oxygen. This aspect of normal physiology is often neglected in tissue culture experiments where cells are routinely maintained in 20% oxygen. In addition, many pathologies such as heart disease, stroke, and chronic lung disease are characterized by severe oxygen deprivation (hypoxia) (Semenza 2011). This hypoxia has deleterious effects on tissue metabolism and function, and can lead to death. Understanding how cells, tissues, and organisms adapt to low oxygen is therefore an important question in biology.


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One central hypoxic mechanism involves induction of the hypoxia-inducible factor (HIF)-1α transcription factor, which can control the expression of a diverse array of target genes that maintain cellular homeostasis in low oxygen (Semenza 2014). The importance of HIF-1α has been shown by loss-of-function genetic analyses in model organisms such as *Caenorhabditis elegans*, *Drosophila*, and mice. For example, in *C. elegans* and *Drosophila*, which are normally quite hypoxia-tolerant, HIF-1α mutants die when exposed to low oxygen (Jiang *et al.* 2001; Centanin *et al.* 2005; Li *et al.* 2013). Tissue-specific mouse knockouts have also shown how HIF-1α can control organ-level and whole-body adaptation to low oxygen in both physiological and pathological conditions (Schipani *et al.* 2001; Cramer *et al.* 2003; Tomita *et al.* 2003; Huang *et al.* 2004; Mason *et al.* 2004; Boutin *et al.* 2008). However, compared to our understanding of HIF-1α, less is known about other transcription factors that are important in mediating hypoxia adaptation in animals.

The conserved transcription factor Forkhead Box-O (FOXO) is an important mediator of adaptation to stress in animals (Webb and Brunet 2014). Studies in *Drosophila* have provided important insights into the role of FOXO as a regulator of organismal physiology. Here, different environmental stressors—such as starvation, oxidative stress, pathogens,

and ionizing radiation—have been shown to induce FOXO transcriptional activity (Junger *et al.* 2003; Dionne *et al.* 2006; Karpac *et al.* 2009, 2011; Borch Jensen *et al.* 2017). Once induced, FOXO then directly controls the expression of an array of metabolic and regulatory genes that together function to maintain organismal homeostasis and survival (Gershman *et al.* 2007; Teleman *et al.* 2008; Alic *et al.* 2011; Birnbaum *et al.* 2019). Indeed, genetic upregulation of FOXO is sufficient to promote stress resistance in *Drosophila*, and it is one of the most effective ways to extend life span (Giannakou *et al.* 2004; Hwangbo *et al.* 2004; Kramer *et al.* 2008; Demontis and Perrimon 2010; Alic *et al.* 2014).

In this paper, we report our work using *Drosophila melanogaster* to explore hypoxia tolerance. In their natural ecology, *Drosophila* grow in rotting, fermenting food rich in microorganisms, an environment likely characterized by low ambient oxygen (Callier *et al.* 2015; Markow 2015; Harrison *et al.* 2018). Probably as a consequence of this environment, they have evolved mechanisms to tolerate hypoxia (Centanin *et al.* 2008; Li *et al.* 2013; Lee *et al.* 2019). Here, we explore whether induction of FOXO is one such mechanism. Previous work has shown that reduced insulin signaling and FOXO induction confers hypoxia tolerance in *C. elegans* and zebrafish (Scott *et al.* 2002; Mendenhall *et al.* 2006; Menuz *et al.* 2009; Liu *et al.* 2016). Moreover, the mammalian FOXO homolog FOXO3a can be induced in cell culture upon hypoxia exposure, where it regulates metabolic responses and cell death (Bakker *et al.* 2007; Jensen *et al.* 2011). In this paper, we show that FOXO is required for hypoxia tolerance in *Drosophila*, and that it functions by regulating the immune transcription factor NF-κB. Thus, the induction of FOXO is a conserved mechanism of hypoxia tolerance in animals.

## Materials and Methods

### *Drosophila stocks*

Flies were raised on medium containing 150 g agar, 1600 g cornmeal, 770 g Torula yeast, 675g sucrose, 2340 g D-glucose, and 240 ml acid mixture (propionic acid/phosphoric acid) per 34 liter water and maintained at 25°, unless otherwise indicated. The following fly stocks were used: $w^{1118}$, $sima^{KG07607}$*/TM3,Ser,GFP* [Bloomington *Drosophila* Stock Center (BDSC) #14640] (Centanin *et al.* 2008), *Df (3R)X3F* (BDSC #2352), $foxo^{\Delta 94}$*/TM3,Ser*, *GFP* (Slack *et al.* 2011), *thor-LacZ* (BDSC #9558) (Bernal and Kimbrell 2000), $Relish^{E38}$ (BDSC #9458) (Hedengren *et al.* 1999) $Relish^{E20}$ (BDSC #9457) (Hedengren *et al.* 1999) *hsflp*; *UAS-dp110* (Britton *et al.* 2002), *act* > *CD2* > *Gal4,UAS*-GFP (Britton *et al.* 2002), UAS-*Fatiga RNAi* (VDRC #103382), *UAS-PTEN* (Britton *et al.* 2002), and *daughterless-GSG* (Sun *et al.* 2014), *r4-GAL4* (BDSC #33832).

### *Hypoxia exposure*

For all hypoxia experiments, vials containing *Drosophila* were placed into an airtight glass chamber into which a premix of 5% oxygen/95% nitrogen, 1% oxygen/99% nitrogen, or 100% nitrogen continually flowed (Lee *et al.* 2019). Flow rate was controlled using an Aalborg model P gas flow meter. Alternatively, for some experiments, *Drosophila* vials were placed into a Coy Laboratory Products *in vitro* $O_2$ chamber that was maintained at fixed oxygen levels of 1% or 5% by injection of nitrogen gas.

### *Immunofluorescence staining*

Larvae were inverted using fine forceps in 1× PBS. Inverted larvae were fixed in 8% paraformaldehyde for 30 min, washed in 1× PBS/0.1% Triton X-100 (PBST), and blocked for 2 hr at room temperature in 1× PBS/0.1% Tween 20/1% bovine serum albumin (PAT). Larvae were then incubated overnight with primary antibody diluted in PAT at 4°, washed three times with 1× PBS with 3% Triton X-100 (PBT) and 2% fetal bovine serum (FBS), and incubated with secondary antibody diluted 1:4000 in PBT with FBS for 2 hr at room temperature. Larvae were washed with PBT and stained with 1:10,000 Hoechst 33342 dye for 5 min, then washed three times more with PBT. Larval tissues were isolated using fine forceps and then mounted on glass slides with cover slips using Vectashield mounting media (Vector Laboratories, Burlingame, CA). The rabbit anti-FOXO antibody was used at 1:500 dilution (a gift from Marc Tatar). Goat anti-rabbit Alexa Fluor 568 (Invitrogen, Carlsbad, CA) was used as the secondary antibody. Hoechst 33,342 (Invitrogen) was used to stain nuclei. Quantification of FOXO nuclear staining was done by scoring cells with prominent nuclear FOXO staining, as observed in the representative images of hypoxia-treated (5 and 1% oxygen) fat-body cells in Figure 1A.

### *Quantitative PCR*

Total RNA was extracted from either 96-hr after egg laying (AEL) larvae or from 1-week-old mated female adults, using TRIzol according to the manufacturer's instructions (catalog number 15596–018; Invitrogen). RNA samples were then subjected to DNase treatment according to the manufacturer's instructions (2238 G; Ambion) and reverse transcribed using Superscript II (catalog number 100004925; Invitrogen). The generated complementary DNA was used as a template to perform quantitative (q)RT-PCRs (ABI 7500 real time PCR system using SyBr Green PCR mix) using specific primer pairs. PCR data were normalized to β-tubulin levels. Each experiment was independently repeated a minimum of three times. The following primers were used:

β-tubulin: forward 5′-ATCATCACACACGGACAGG-3′; reverse 5′ GAGCTGGATGATGGGGAGTA-3′.
4e-bp: forward 5′-GCTAAGATGTCCGCTTCACC-3′; reverse: 5′ CCTCCAGGAGTGGTGGAGTA-3′.
relish: forward 5′-TCCTTAATGGAGTGCCAACC-3′; reverse 5′-TGCCATGTGGAGTGCATTAT-3′.
dorsal: forward 5′-TGTTCAAATCGCGGGCGTCGA-3′; reverse 5′-TCGGACACCTTCGAGCTCCAGAA-3′.

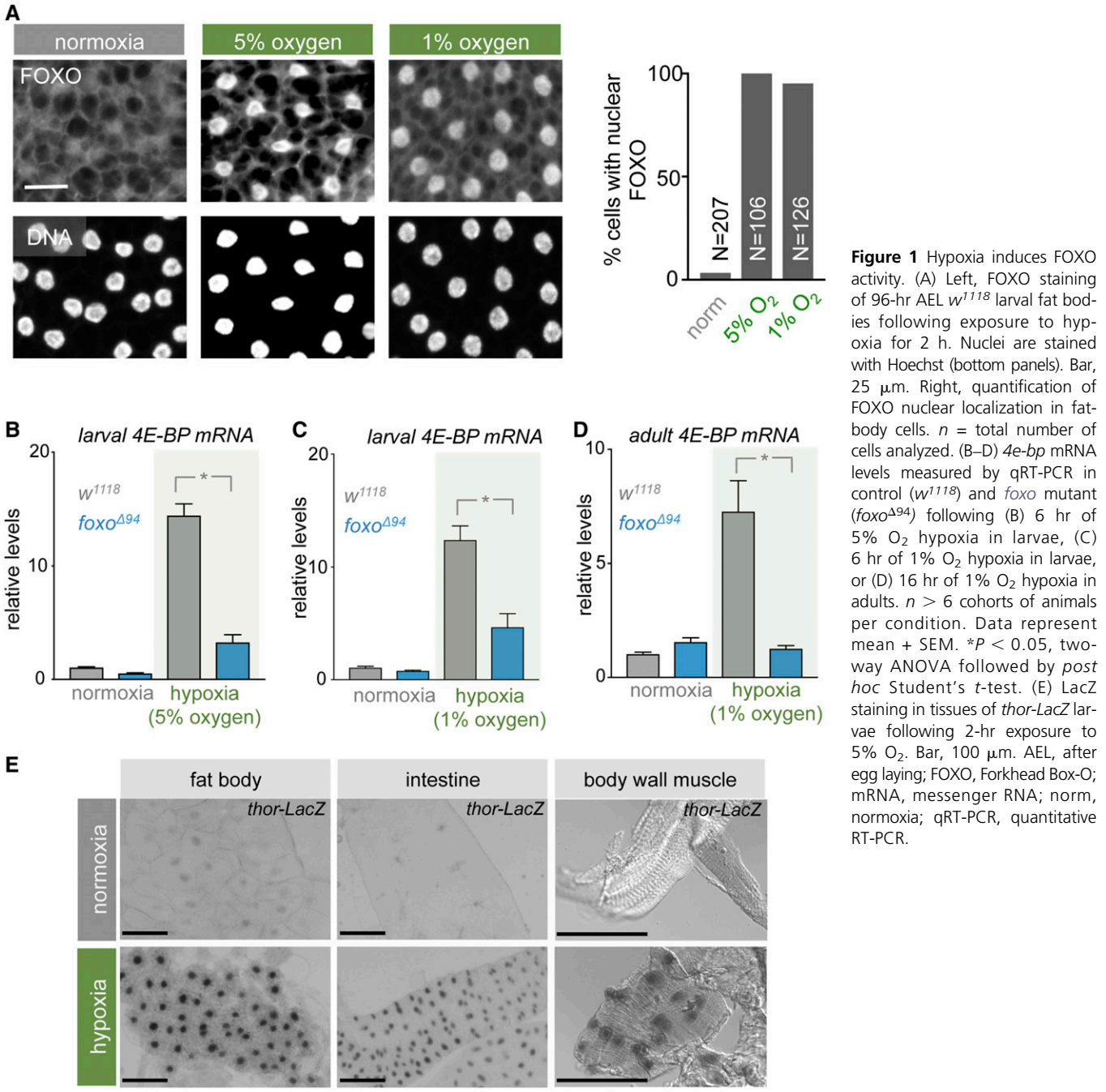



**Figure 1** Hypoxia induces FOXO activity. (A) Left, FOXO staining of 96-hr AEL $w^{1118}$ larval fat bodies following exposure to hypoxia for 2 h. Nuclei are stained with Hoechst (bottom panels). Bar, 25 μm. Right, quantification of FOXO nuclear localization in fat-body cells. *n* = total number of cells analyzed. (B–D) *4e-bp* mRNA levels measured by qRT-PCR in control ($w^{1118}$) and *foxo* mutant ($foxo^{\Delta 94}$) following (B) 6 hr of 5% $O_2$ hypoxia in larvae, (C) 6 hr of 1% $O_2$ hypoxia in larvae, or (D) 16 hr of 1% $O_2$ hypoxia in adults. $n > 6$ cohorts of animals per condition. Data represent mean + SEM. $^*P < 0.05$, two-way ANOVA followed by *post hoc* Student's *t*-test. (E) LacZ staining in tissues of *thor-LacZ* larvae following 2-hr exposure to 5% $O_2$. Bar, 100 μm. AEL, after egg laying; FOXO, Forkhead Box-O; mRNA, messenger RNA; norm, normoxia; qRT-PCR, quantitative RT-PCR.

dif: forward 5′-CGGACGTGAAGCGCCGACTTG-3′; reverse 5′ CAGCCGCCTGTTTAGAGCGG-3′.
attacin A: forward 5′-AGGAGGCCCATGCCAATTTA-3′; reverse 5′ CATTCCGCTGGAACTCGAAA-3′.
cecropin A: forward 5′-TCTTCGTTTTCGTCGCTCTCA-3′; reverse 5′ ATTCCCAGTCCCTGGATTGTG-3′.
ldh: forward: 5′-AGATCCTGACTCCCACCGAA-3′; reverse: 5′-GCCTGGACATCGGACATGAT-3′.
fatiga: forward: 5′-ATTGAGCCCAAGTTTGATCG-3′; reverse: 5′-AGCTGCCAGATTGTTCGTCT-3′.
Def: forward: 5′-TGAAGTTCTTCGTTCTCGTGG-3′; reverse: 5′-CACCAGGACATGATCCTCTG-3′.
Mtk: forward: 5′-CGATTTTTCTGGCCCTGCT-3′; reverse: 5′-CCGGTCTTGGTTGGTTAGGAT-3′.
Cec C: forward: 5′-TCATCCTGGCCATCAGCATT-3′; reverse: 5′-CGCAATTCCCAGTCCTTGAAT-3′.
Dros: forward: 5′-TTTGTCCACCACTCCAAGCAC-3′; reverse: 5′-ATGGCAGCTTGAGTCAGGTGA-3′.

### *Lac Z staining*

Larvae were inverted using fine forceps in 1× PBS. Inverted larvae were fixed in 8% paraformaldehyde for 30 min, washed in PBST, and then incubated in 500 μl of an X-Gal solution containing 10 mM sodium phosphate buffer, pH 7.2,

150 mM NaCl, 1mM $MgCl_2$, 10 mM $K_4[Fe^{II}(CN)_6]$, 10 mM $K_3[Fe^{III}(CN)_6]$, and 0.1% Triton X-100 with 12.5 μl of 8% X-Gal solution (in DMSO) added immediately prior to incubation. Samples were then incubated at 37° until the X-Gal staining was visible.

#### Measurement of hypoxia survival

***Larvae:*** Newly hatched larvae were placed in food vials (50 larvae per vial) and then maintained in either normoxia or hypoxia (5% oxygen). Larvae exposed to hypoxia were maintained in this environment until ~80% of larvae had pupated. Then, vials were removed from hypoxia and the numbers of eclosing adults were counted.

***Adults:*** First, 4–5 days posteclosion, mated female adults were placed into hypoxia (1% oxygen) for 24 hr in cohorts of 20 flies per vial. Then, vials were removed from hypoxia and the flies were allowed to recover for 48 hr before the numbers of dead flies were counted.

***Starvation:*** At 4–5 days posteclosion, mated female adults were subjected to starvation by transferring them from food vials to vials containing 0.4% agar/PBS for 24 hr. The numbers of dead flies were then counted.

#### Glucose, glycogen, trehalose, and TAG assays

Adult female *Drosophila* were either exposed to hypoxia (1% oxygen) for 16 hr or maintained in normoxia, and then frozen on dry ice. Colorimetric assays for each of the metabolites were then conducted using the methods described in detail in Tennessen *et al.* (2014).

#### Preparation of protein extracts and western blotting

First, 96-h AEL *Drosophila* whole larvae were lysed with a buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM EDTA, 25% glycerol, and 1% NP-40 with the following inhibitors: 50 mM NaF, 1 mM PMSF, 1 mM DTT, 5 mM sodium orthovanadate ($Na_3VO_4$), and Protease Inhibitor cocktail (Roche, catalog number 04693124001) and Phosphatase inhibitor (Roche, catalog number 04906845001), according to the manufacturer's instructions. Protein concentrations were measured using the Bio-Rad (Hercules, CA) Dc Protein Assay kit II (5000112). Protein lysates (15–30 μg) were resolved by SDS-PAGE and electrotransferred to a nitrocellulose membrane, subjected to western blot analysis with specific antibodies, and visualized by chemiluminescence [enhanced ECL solution (Perkin-Elmer)]. Primary antibodies used in this study were: anti-Akt (1:500 dilution; Cell Signaling, catalog number #9272), anti-pAkt-T342 (1:1000 dilution; gift from Michelle Bland), and anti-pAkt-S505 (1:1000 dilution; Cell Signaling, catalog number 4504). Goat secondary antibodies were purchased from Santa Cruz Biotechnology (sc-2030, 2005, and 2020). For experiments looking at Akt phosphorylation, total Akt levels were used as a loading control because the level of this protein was unaffected by hypoxia.

#### Statistical analyses

Data were analyzed by Student's *t*-test or two-way ANOVA. All statistical analysis and data plots were performed using Prism software. In all figures, statistically significant differences are presented as * and indicate $P < 0.05$.

#### Data availability

All reagents are available on request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, main figures, and supplemental figures. Supplemental material available at figshare: https://doi.org/10.25386/genetics.12429398

## Results

#### Hypoxia induces FOXO activity

The main way that FOXO is regulated is through nuclear–cytoplasmic shuttling. To determine if hypoxia exposure could induce FOXO, we transferred third-instar larvae growing on food to either moderate (5% oxygen) or severe hypoxic environments (1% oxygen), and then stained for FOXO localization using an anti-FOXO antibody (Figure 1A). We saw that exposure to hypoxia caused FOXO relocalization from the cytoplasm to the nuclei of fat-body cells (Figure 1A and Supplemental Material, Figure S1A). This effect was rapid; nuclear relocalization occurred within 15 min of exposing larvae to hypoxia (Figure S1, B and C). We next examined the effects of hypoxia on the expression of *4e-bp*, a well-characterized FOXO target gene. We measured messenger RNA (mRNA) levels of *4e-bp* using qRT-PCR in whole third-instar larvae exposed to either 5 or 1% oxygen. We saw that *4e-bp* levels were strongly increased in control ($w^{1118}$) larvae exposed to both hypoxic conditions (Figure 1, B and C). As with the FOXO nuclear localization, this increase in *4e-bp* was rapid and was seen within 15–30 min following hypoxia exposure (Figure S1D). However, the hypoxia-induced increase in *4e-bp* mRNA levels was largely abolished in $foxo^{\Delta 94}$, a deletion line that is a null mutant for the *foxo* gene (Slack *et al.* 2011) (Figure 1, B and C). We also examined the effects of hypoxia in adults. We exposed adult females to 1% $O_2$ and found that, as in larvae, *4e-bp* levels were increased in control ($w^{1118}$) animals and that this effect was blunted in *foxo* mutants (Figure 1D). Finally, we examined the tissue pattern of *4e-bp* induction by examining LacZ staining in *thor-LacZ* flies, which is a LacZ-enhancer trap in the *4e-bp* gene locus (Bernal and Kimbrell 2000). We found that larvae exposed to 2 hr of 5% $O_2$ showed increased LacZ staining in the majority of larval tissues including the fat body, the intestine, and the body wall muscle (Figure 1E), suggesting that the hypoxia induction of FOXO activity is not tissue-restricted. Together, these data indicate that exposure to hypoxia in both *Drosophila* larvae and adults results in rapid induction of FOXO transcriptional activity.

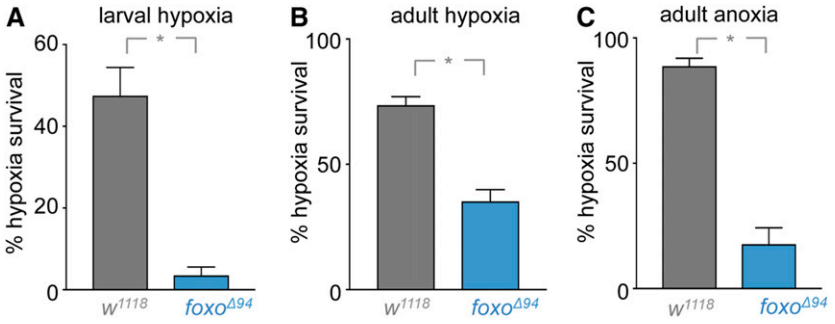



**Figure 2** FOXO is required for hypoxia tolerance. (A) Control ($w^{1118}$) and *foxo* mutant ($foxo^{\Delta 94}$) animals were exposed to hypoxia (5% $O_2$) throughout their larval period, before being returned to normoxia as pupae. The percentage of flies that eclosed as viable adults was then counted. (B and C) Adult control ($w^{1118}$) or *foxo* mutant ($foxo^{\Delta 94}$) flies were exposed to either (B) 24 hr of 1% $O_2$ or (C) 6 hr of 0% $O_2$, before being returned to normoxia. The percentage of viable flies was then counted. Data represent mean + SEM. $^*P < 0.05$, Student's *t*-test. $n > 4$ cohorts of animals per condition. FOXO, Forkhead Box-O.

### *FOXO is required for hypoxia tolerance*

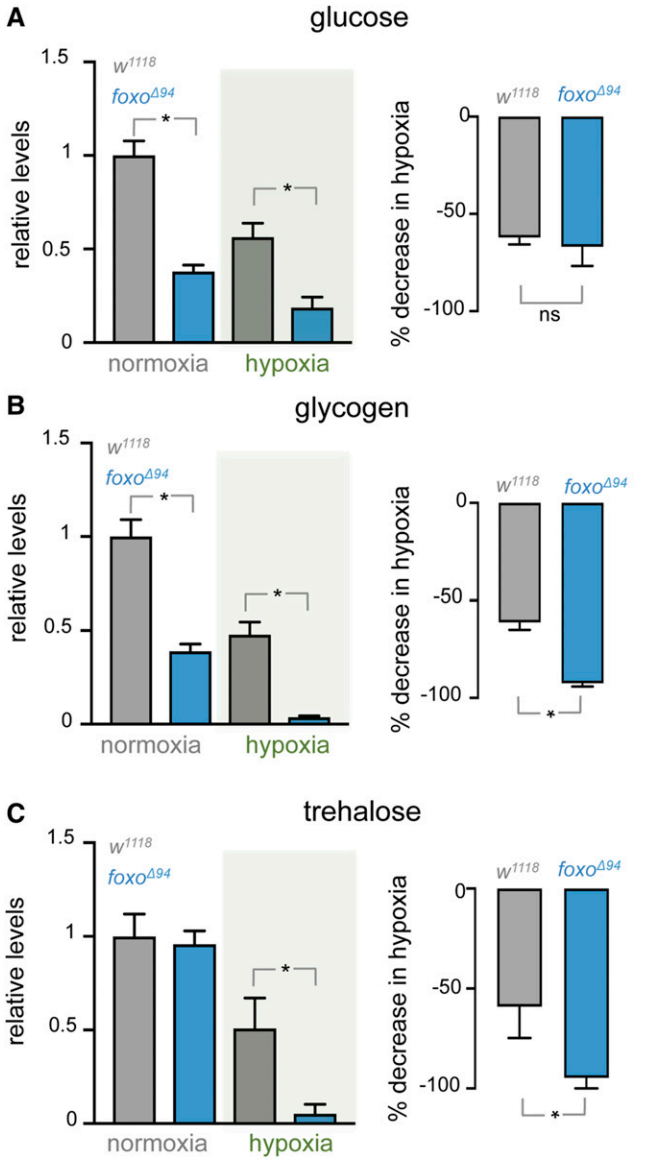
Is FOXO activation required for *Drosophila* survival in low oxygen? To find out, we measured hypoxia survival in $foxo^{\Delta 94}$ animals. Under standard laboratory conditions (rich food and normoxia) *foxo* mutant animals are viable (Slack *et al.* 2011). Therefore, we examined how well these mutants tolerate low oxygen. We first examined hypoxia survival in larvae. Control ($w^{1118}$) and *foxo* mutant embryos were allowed to develop in normoxia, and then newly hatched larvae were transferred to hypoxia (5% oxygen) for the duration of their larval period, before being returned to normoxia. We then counted the number of animals that developed to viable adults. We found that the *foxo* mutant animals reared in hypoxia had a significant decrease in viability compared to control animals (Figure 2A). In contrast, survival of *foxo* animals to adulthood in normoxia was no different to control animals (Figure S2A). We next examined hypoxia survival in female adults. Control ($w^{1118}$) and *foxo* mutant animals were exposed to either severe hypoxia (1% oxygen) for 24 hr or anoxia (0% oxygen) for 6 hr. After these low oxygen exposures, flies were returned to normoxia and the number of surviving animals counted. As observed in larvae, we found that the adult *foxo* mutant animals showed significantly decreased survival in both the hypoxic and anoxic conditions (Figure 2, B and C), while survival in normoxia over the same time periods was unaffected (Figure S2B). During severe hypoxia and anoxia, adult flies become immobile. However, when *foxo* adults were exposed to starvation instead of hypoxia for 24 hr there was no effect on viability, indicating that the decrease in hypoxia survival in *foxo* mutants is not simply a consequence of reduced nutrient intake as a result of immobility (Figure S2C). Together, our data indicate that FOXO activation is required for organismal survival in low oxygen in both developing larvae and adults.

Cells, tissues, and organisms adapt to low oxygen by altering their metabolism (Semenza 2011). In particular, a key adaptation is the upregulation of glycolysis. Therefore, we checked whether FOXO might be important for controlling glucose metabolism in hypoxic animals. We first measured total glucose levels in adult animals exposed to hypoxia. Control animals exhibited a decrease in glucose levels after 16 hr of hypoxia (Figure 3A). *foxo* mutant flies had lower levels of total glucose in normoxia and these levels were even further depleted upon exposure to hypoxia (Figure 3A, left panel); however, the relative decrease in total glucose levels in hypoxia was similar between control and *foxo* animals (Figure 3A, right panel). In contrast, we saw a different pattern when we measured levels of glycogen (the stored form of glucose) and trehalose (the circulating form of glucose in *Drosophila*). As with total glucose, both glycogen and trehalose levels in normoxia were lower in *foxo* mutant animals compared to control animals, and the levels of both forms of glucose were decreased in hypoxia (Figure 3, B and C, left panels). However, *foxo* mutants showed a significantly greater decrease in both glycogen and trehalose in hypoxia compared to control animals (Figure 3, B and C, right panel).

Finally, we investigated expression of lactate dehydrogenase (*ldh*)—a key glycolytic enzyme—in $w^{1118}$ and $foxo^{\Delta 94}$ adult females. We saw that control animals had increased *ldh* mRNA levels when exposed to hypoxia, as has been reported before (Lavista-Llanos *et al.* 2002; Li *et al.* 2013) and is consistent with an upregulation of glycolysis. In contrast, *foxo* mutant animals had increased *ldh* levels in normoxia, and this expression increased significantly further in hypoxia (Figure 3D). Taken together, these data indicate that *foxo* mutants show deregulated control over normal glucose metabolism in hypoxia; they show overproduction of *ldh* and they exhibit a larger depletion of both stored and circulating glucose in hypoxia compared to control animals.

### *Hypoxia induces FOXO by inhibiting phosphoinositide 3-kinase/Akt signaling*

We next examined how hypoxia induces FOXO. The best-studied cellular response to hypoxia involves induction of the HIF-1α transcription factor (called *sima* in *Drosophila*). HIF-1α induces expression of metabolic and regulatory genes required for hypoxia adaptation, and HIF-1α is required for organismal tolerance to low oxygen in both *Drosophila* and *C. elegans* (Jiang *et al.* 2001; Centanin *et al.* 2005). However, we found that FOXO was still relocalized to the nucleus upon hypoxia in fat-body cells from both $sima^{KG7607}$ homozygote mutant larvae (Figure 4A and Figure S3A) and $sima^{KG7607}/Df$ larvae (Figure S3, B and C). We also found that fat-body knockdown of fatiga, a prolyl hydroxylase that functions in normoxia to hydroxylate sima and target it for degradation, did not lead to increased FOXO nuclear localization (Figure



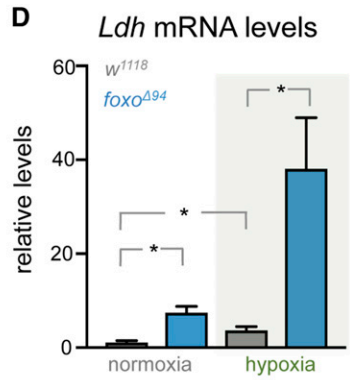




**Figure 3** *foxo* mutants have altered glucose homeostasis in hypoxia. (A–C) Levels of free glucose (A), glycogen (B), or trehalose (C), in adult control ($w^{1118}$) and *foxo* mutant ($foxo^{\Delta 94}$) flies exposed to normoxia or 1% $O_2$ hypoxia for 16 hr. $n = 15$. Left panels indicate relative metabolite levels in normoxia and hypoxia. Right panels indicate the % change in levels in hypoxia. Data represent mean + SEM. Left panels, $^*P < 0.05$, Student's *t*-test following significant two-way ANOVA; right panels, $^*P < 0.05$, Student's *t*-test. (D) *Ldh* mRNA levels measured by qRT-PCR in control ($w^{1118}$) and *foxo* mutants ($foxo^{\Delta 94}$) following 16 hr of 1% $O_2$ hypoxia in adults. Data represent mean + SEM. $^*P < 0.05$, two-way ANOVA followed by *post hoc* Student's *t*-test. $n > 10$ per condition. FOXO, Forkhead Box-O; mRNA, messenger RNA; ns, not significant; qRT-PCR, quantitative RT-PCR.

S3D). Finally, we found that hypoxia induction of the sima target gene, *fatiga*, but not the FOXO target gene, *4e-bp*, was suppressed in *sima* mutant larvae (Figure 4C). Together, these data suggest that induction of FOXO is independent of the classic HIF-1α response.

One main way that FOXO can be regulated is via the conserved insulin/phosphoinositide 3-kinase (PI3K)/Akt pathway (Webb and Brunet 2014). This is best seen in response to nutrient availability in *Drosophila*. In rich nutrients, insulin signaling via PI3K to Akt kinase is high and Akt can phosphorylate FOXO, leading to its cytoplasmic retention. However, during starvation, insulin/PI3K/Akt signaling is low, thus reducing phosphorylation of FOXO and allowing it to relocalize to the nucleus to induce transcription. We investigated whether decreased Akt activation was involved in FOXO induction during hypoxia exposure. Akt is activated by phosphorylation at two sites: threonine 342 and serine 505. We measured the relative amounts of Akt phosphorylated at each site after exposure to hypoxia using phospho-specific antibodies. We saw that when third-instar larvae were exposed to hypoxia, there was a reduction in phosphorylation of Akt at both sites (Figure 5, A and B). To determine if suppression of Akt signaling was mediating the induction of FOXO, we used the flp-out technique to induce mosaic expression of the catalytic subunit of PI3K, *dp110*, to maintain Akt activity in fat-body cells. We found that during hypoxia, expression of *dp110* was sufficient to prevent FOXO nuclear relocalization (Figure 5C). To further explore whether hypoxia induces FOXO by inhibiting Akt signaling, we compared the effects of hypoxia to other manipulations that suppress Akt. We first examined nutrient deprivation, which suppresses systemic insulin signaling and inhibits the Akt pathway. We found that nutrient starvation led to a similar increase in FOXO nuclear localization compared to hypoxia

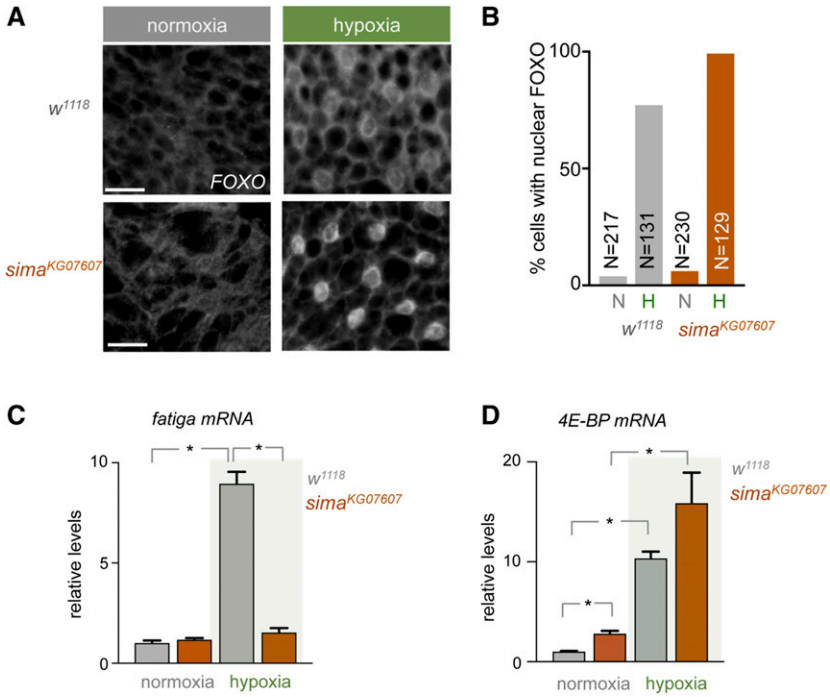



**Figure 4** Hypoxia induces FOXO independently of *sima*/HIF-1α. (A) FOXO staining in fat bodies of control ($w^{1118}$) and *sima* mutant ($sima^{KG07607}$) larvae exposed to either normoxia or 5% $O_2$ hypoxia for 2 hr. Bar, 25 μm. (B) Quantification of FOXO nuclear localization in fat-body cells of control ($w^{1118}$) and *sima* mutant ($sima^{KG07607}$) larvae exposed to either normoxia or 5% $O_2$ hypoxia for 2 hr at 96-hr AEL. n = total number of cells analyzed. (C and D) *fatiga* mRNA and *4E-BP* mRNA levels measured by qRT-PCR in control ($w^{1118}$) and *sima* mutant ($sima^{KG07607}$) third instar larvae maintained in normoxia or exposed to hypoxia (5% $O_2$ hypoxia) for 6 hr. Data represent mean + SEM. $^*P < 0.05$, two-way ANOVA followed by *post hoc* Student's *t*-test. $N > 4$ per condition. AEL, after egg laying; FOXO, Forkhead Box-O; H, hypoxia; HIF, hypoxia-inducible factor; mRNA, messenger RNA; N, normoxia; qRT-PCR, quantitative RT-PCR.

(Figure 5D). Moreover, we found that this localization was not stronger when we exposed larvae to simultaneous starvation and hypoxia exposure (Figure 5D and Figure S4A), suggesting that both starvation and hypoxia share a common mechanism to induce FOXO. We also examined the effects of genetic suppression of Akt signaling. To do this, we overexpressed PTEN, a phosphatase that reverses the effects of PI3K to suppress Akt. We found that PTEN expression in the fat body led to an increase in FOXO nuclear localization that was similar to that following hypoxia (Figure S4, B and C). Taken together, these data suggest that hypoxia induces FOXO by suppressing Akt signaling.

### *FOXO induces Relish-dependent hypoxia survival*

In *Drosophila*, FOXO maintains tissue and organismal homeostasis in response to various stresses, including starvation, oxidative stress, irradiation, and infection. In each case, FOXO functions by regulating diverse and often distinct target genes. We surveyed potential FOXO targets that might be important for hypoxia tolerance and we identified a role for the NF-κB transcription factor *relish*.

In *Drosophila* there are three NF-κB transcription factors, Relish, Dorsal, and Dif. They have been best characterized as effectors of immune signaling downstream of the Immune Deficiency, IMD (Relish) and Toll (Dorsal and Dif) pathways, where they induce expression of antimicrobial peptides and promote innate immune responses (Buchon *et al.* 2014). We found that when exposed to hypoxia, adult *Drosophila* showed an increase in *relish* [(as reported previously by Liu *et al.* (2006) and Bandarra *et al.* (2014)], but not *dorsal* or *dif*, mRNA levels (Figure 6, A–C). Furthermore, we found that this hypoxia-induced increase in *relish* mRNA was blocked in both *foxo* mutant adults (Figure 6D) and larvae (Figure S5). Finally, we found that hypoxia could induce strong expression of Relish-regulated antimicrobial peptides in both adults (Figure 6, E and F) and larvae (Figure S6), and that this was also blocked in *foxo* mutants. These data suggest that in hypoxia, FOXO can induce an immune-like response via upregulation of Relish.

To test whether this immune-like response was important for hypoxia survival, we examined hypoxia survival in two independent *relish* null mutants, $rel^{E38}$ and $rel^{E20}$ (Hedengren *et al.* 1999). We found that both $rel^{E38}$ and $rel^{E20}$ adult flies showed a significant decrease in viability after hypoxia exposure (Figure 7, A and B), while survival in normoxia was unaffected (Figure S6). We also found that the reduction in hypoxia survival seen in *relish*, *foxo* double mutants was similar to that seen in either mutant alone (Figure 7C) suggesting that they function in the same genetic pathway to control hypoxia tolerance. Together, these data point to FOXO activation as a meditator of hypoxia tolerance via induction of an immune-like response through the NF-κB -like transcription factor Relish. We then tested whether induction of Relish-mediated transcription was sufficient to mediate the effects of FOXO on hypoxia tolerance. To do this we examined the effects of expression of a constitutively active version of Imd (ImdCA) (Petkau *et al.* 2017), the upstream activator of Relish. We used a ubiquitous gene-switch driver to express ImdCA in all tissues of adult flies. We found that this was sufficient to induce strong expression of relish target AMP genes (Figure S7). However, expression of ImdCA was not sufficient to reverse the decrease in hypoxia survival seen in *foxo* mutants (Figure 7D).

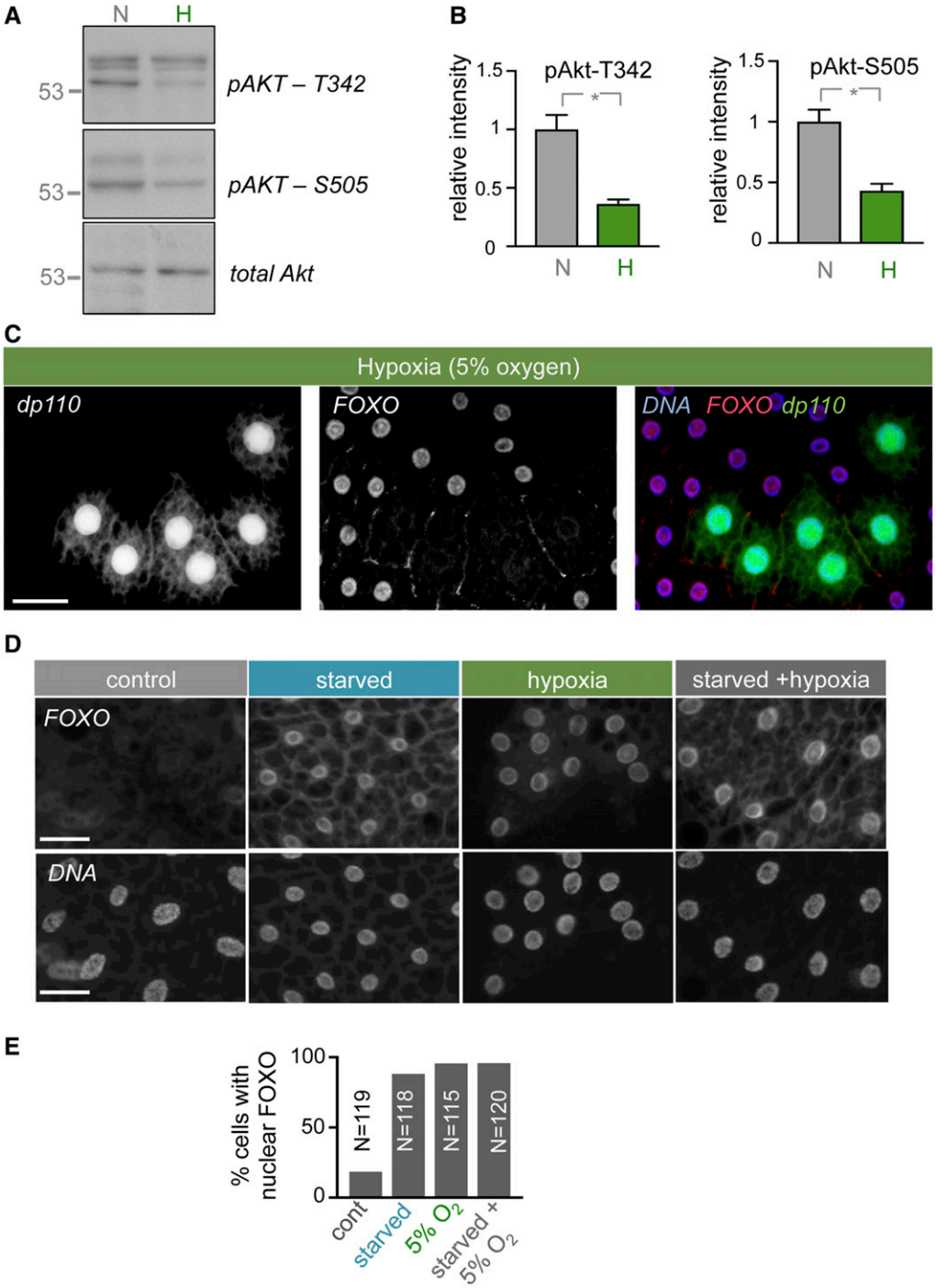



**Figure 5** Hypoxia induces FOXO by inhibiting PI3K/Akt. (A and B) Western blot analysis of phosphorylated T342 and S505 Akt, and total Akt in control ($w^{1118}$) larvae following 2 hr of normoxia or 5% $O_2$ hypoxia. Quantification of blots (relative phospho-Akt intensity/total Akt intensity) is shown in (B). $n = 4$ per condition. $^*P < 0.05$, Student's *t*-test. (C) FOXO staining in UAS-*dp110*-overexpressing fat-body clones (GFP-positive). Nuclei are stained with Hoechst dye (blue). Bar, 50 μm. (D) FOXO staining in 96-hr AEL larvae that were maintained in normoxia, starved (PBS only), exposed to hypoxia, or simultaneously starved and exposed to hypoxia. Top images show FOXO staining, while bottom images show corresponding nuclear staining (Hoechst dye). Bar, 50 μm. (E) Quantification of FOXO nuclear localization in fat body cells from 96-hr larvae that were maintained in normoxia, starved (PBS only), exposed to hypoxia, or simultaneously starved and exposed to hypoxia. *n* = total number of cells analyzed. AEL, after egg laying; cont, control; FOXO, Forkhead Box-O; H, hypoxia; N, normoxia; PI3K, phosphoinositide 3-kinase.

## Discussion

In this paper, we report that FOXO is a HIF required for organismal survival in low oxygen, and we show that one way that FOXO functions is through upregulation of Relish/NF-κB (Figure 8). We saw that the hypoxia induction of FOXO occurs via suppression of PI3K/Akt signaling. This response is most likely induced by hypoxia-mediated reduction of insulin release and signaling, the main activator of PI3K/Akt, as previously reported in *Drosophila* larvae (Wong *et al.* 2014; Texada *et al.* 2019). Together with previous studies in *C. elegans* and Zebrafish showing that reduced insulin signaling and FOXO induction confer hypoxia tolerance (Scott *et al.* 2002; Mendenhall *et al.* 2006; Menuz *et al.* 2009; Liu *et al.* 2016), our work suggests that FOXO is a conserved mediator of hypoxia responses.

Interestingly we found that the induction of FOXO upon hypoxia occurs in *sima* mutants, suggesting that the FOXO hypoxic response occurs independently of the classically described HIF-1α response. Work in mammalian cell cultures has reported that upon hypoxia, HIF-1α can induce FOXO3a function (Jensen *et al.* 2011). However, *in vivo* genetic

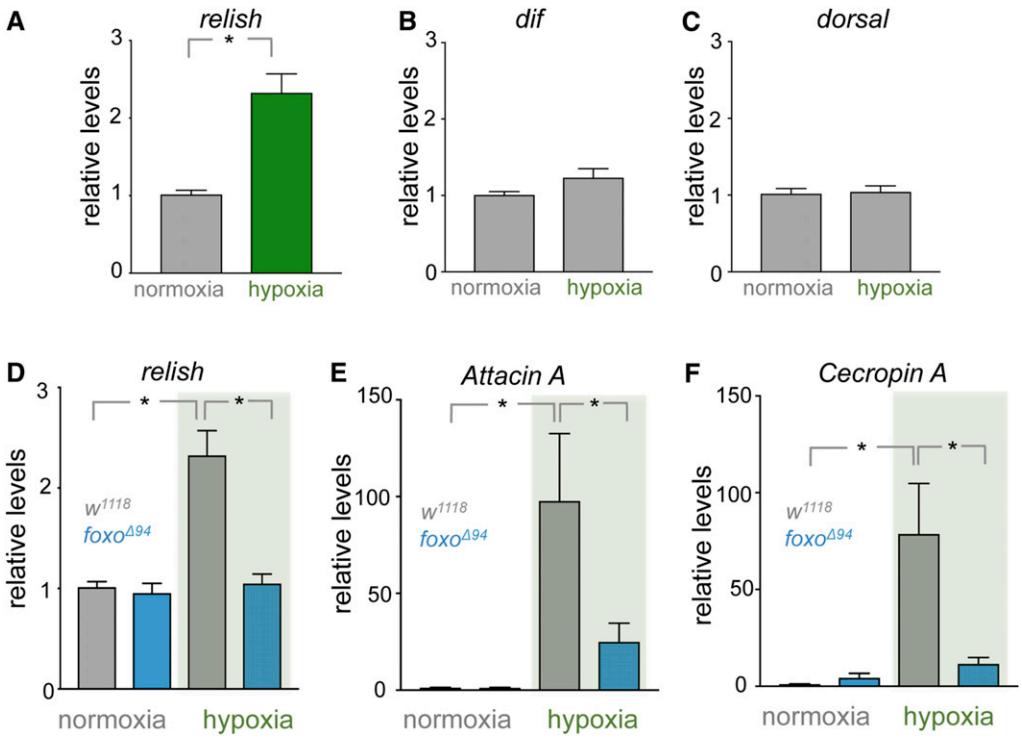



**Figure 6** FOXO induces Relish-dependent transcription in hypoxia. (A–C) Expression levels of *relish* (A), *dif* (B), and *dorsal* (C) mRNA in $w^{1118}$ adult females exposed to either normoxia or 16 hr of 1% $O_2$. Data represent mean + SEM, $n = 10$, $^*P < 0.05$, Student's *t*-test. (D–F) Expression levels of *relish* (D), *attacin A* (E), and *cecropin A* (F) mRNA in $w^{1118}$ and $foxo^{\Delta 94}$ adult females exposed to either normoxia or 16 hr of 1% $O_2$. Data represent mean + SEM, $n = 10$, $^*P < 0.05$, two-way ANOVA followed by Student's *t*-test. FOXO, Forkhead Box-O; mRNA, messenger RNA.

studies in model organisms suggest that the HIF-1α and FOXO transcription factors may act in parallel to mediate responses to hypoxia. For example, in *Drosophila*, a hypoxia-induced HIF-1α pathway that leads to target of rapamycin inhibition functions independently of FOXO (Reiling and Hafen 2004). In *C. elegans*, the extension of life span caused by hypoxia and increased HIF-1α protein levels occurs in the absence of FOXO nuclear localization and function (Mehta *et al.* 2009; Müller *et al.* 2009; Zhang *et al.* 2009; Leiser *et al.* 2011). Finally, both HIF-1α and FOXO have been shown to act in parallel in *C. elegans* to control iron homeostasis (Ackerman and Gems 2012). These genetic studies, and our work presented here, suggest that inductions of both FOXO and HIF-1α are two parallel responses to hypoxia in animals.

One key way that cells, tissues, and organisms adapt to low oxygen is by altering their glucose metabolism to maintain homeostasis (Nakazawa *et al.* 2016; Xie and Simon 2017). Our data suggest that one reason that *foxo* mutants may show reduced hypoxia tolerance is that they have deregulated control over glucose metabolism. Thus, we saw that *foxo* mutant animals had low levels of glucose in normoxia and that both stored and circulating forms of glucose were significantly decreased under hypoxia compared to controls. These results suggest that FOXO is needed for either gluconeogenesis during stress, as has been reported in *C. elegans* (Hibshman *et al.* 2017), or for proper control of glycolysis. Indeed, we saw that expression of *ldh* is markedly increased in *foxo* mutants. Ldh is a rate-limiting enzyme involved in the conversion of pyruvate to lactate, which is a key metabolic event that can drive increased glycolysis, and *ldh* levels have been shown to increase in larvae upon hypoxia exposure (Li *et al.* 2013). Thus, one possibility is that *foxo* mutant animals may engage in abnormally high levels of glycolysis in low oxygen, leading to depletion of glucose and reduced hypoxia tolerance. This is consistent with previous studies in *Drosophila* showing a major role for FOXO as a regulator of metabolic homeostasis in the context of other stress responses such as starvation and pathogenic infection (Dionne *et al.* 2006; Teleman *et al.* 2008). For example, FOXO often functions in a tissue-specific manner to control systemic sugar and lipid metabolism (Wang *et al.* 2011; Karpac *et al.* 2013; Borch Jensen *et al.* 2017; Zhao and Karpac 2017; Molaei *et al.* 2019). These effects have been shown to be important for FOXO to extend life span and to promote increased tolerance to stress.

A central finding of our work is that one way that FOXO provides protection in low oxygen is through induction of the immune transcription factor Relish. In *Drosophila*, there are two main immune effector pathways that respond to pathogen infection and that work through induction of NF-κB transcription factors: the IMD pathway, which targets the NF-κB homolog Relish, and the Toll pathway, which works via the Dorsal and Dif NF-κB transcription factors (Buchon *et al.* 2014). We found that hypoxia specifically induced Relish via FOXO, and that this response was required for hypoxia tolerance. These data, together with previous work showing hypoxia induction of Relish (Liu *et al.* 2006; Bandarra *et al.* 2014), suggest that induction of an immune-like transcriptional response may be a protective mechanism in low oxygen in *Drosophila*. In the context of animal immunity, there is

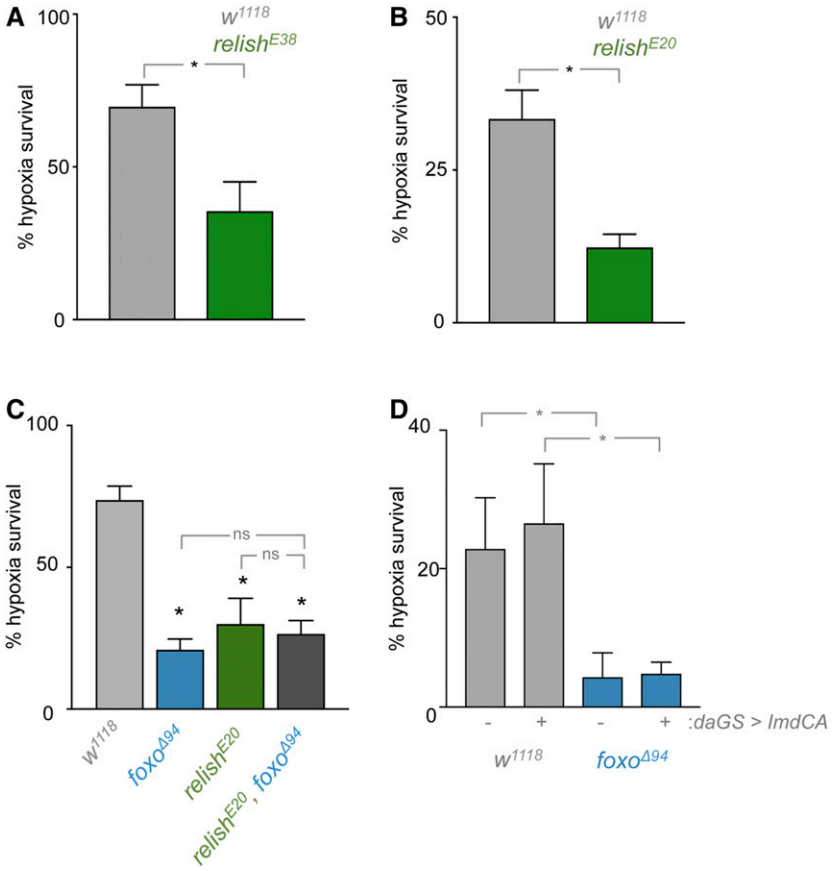



**Figure 7** Relish is required for hypoxia survival. (A and B) Survival of adult female $w^{1118}$, (A) $relish^{E38}$, or (B) $relish^{E20}$ flies after exposure to 24 hr of 1% $O_2$. Data represent mean + SEM, $n$ = *$P < 0.05$, Student's $t$-test. (C) Survival of adult female $w^{1118}$, $foxo^{\Delta 94}$, $relish^{E38}$, or $foxo^{\Delta 94}$, $relish^{E38}$ mutant flies after exposure to 24 hr of 1% $O_2$. Data represent mean + SEM, $n$ = *$P < 0.05$, Student' $t$-test, compared to $w^{1118}$ control group. (D) Hypoxia survival (24 hr at 1% $O_2$) of adult female $w^{1118}$ or $foxo^{\Delta 94}$ $relish^{E20}$ flies with (+) or without (−) expression of *UAS-ImdCA* with a ubiquitous *da-GeneSwitch* driver. Data represent mean + SEM, $n$ = *$P < 0.05$, two-way ANOVA followed by Student's $t$-test. ns, not significant.

increasing appreciation of the role for infection tolerance as a defense strategy against pathogens (Ayres and Schneider 2012; Medzhitov *et al.* 2012; Lissner and Schneider 2018). This tolerance is often mediated via alterations in systemic metabolism and physiology to limit infection-induced tissue damage (Wang *et al.* 2016; Weis *et al.* 2017; Ganeshan *et al.* 2019). Our findings suggest that tolerance to hypoxia may share some of these metabolic and physiological functions. In *Drosophila*, this interplay between hypoxia and innate immune responses may reflect the natural ecology of flies. In the wild, *Drosophila* grow on rotting, fermenting food, an environment rich in microorganisms, including pathogenic bacteria. In these anaerobic conditions, low ambient oxygen may "prime" animals to deal with subsequent pathogenic bacterial encounters. Hence, one speculative idea is that experimental exposure of *Drosophila* to hypoxia may induce Relish and provide protection against the detrimental effects of subsequent pathogenic infection. This concept of hypoxia preconditioning has been observed in *C. elegans*, where it is important in protecting against cell death and damage induced by pore-forming toxins (Dasgupta *et al.* 2007; Bellier *et al.* 2009).

It is possible that the effects of FOXO on metabolism in hypoxia could be mediated via Relish. For example, a recent report showed that Relish was required to control metabolic responses to nutrient deprivation in *Drosophila* (Molaei *et al.* 2019). Furthermore, constitutive activation of IMD signaling, which signals via Relish, was shown to lead to decreased circulating sugars in adult *Drosophila* (Davoodi *et al.* 2019). In mammals, NF-κB is activated in response to cytokines, and it

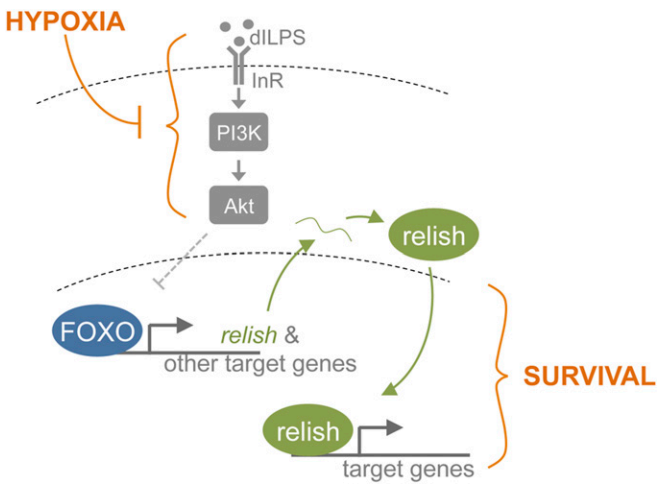



**Figure 8** A model for FOXO- and Relish-dependent hypoxia survival. Upon hypoxia exposure, the PI3K/Akt pathway is inhibited and FOXO is able to relocalize to the nucleus. FOXO can then upregulate target genes, including the NF-κB factor Relish, to promote hypoxia survival. FOXO, Forkhead Box-O; PI3K, phosphoinositide 3-kinase; dILPs, Drosophila insulin-like peptides; InR, insulin receptor.

functions as a central regulator of immune and inflammatory responses (Zhang *et al.* 2017). Several studies have shown that an important way that NF-κB works to mediate these effects is through the control of glycolysis and mitochondrial metabolic activity (Mauro *et al.* 2011; Tornatore *et al.* 2012). Indeed, links between immunity and metabolism are emerging as important components of infection tolerance in animals (Ayres and Schneider 2012). Our data suggest the possibility that organisms may also coopt some of these immune–metabolism interactions to tolerate low oxygen.

Relish has also been shown to influence systemic metabolism in response to different stresses by controlling endocrine signaling. For example, in response to radiation damage, Relish activity in the larval fat body can control systemic insulin signaling (Karpac *et al.* 2011). In addition, Relish can function in the adult muscle in response to mitochondrial stress to control expression of the TGFβ ligand activin, which in turn regulates fat-body lipid metabolism (Song *et al.* 2017). Hence, it is possible that these types of endocrine signaling effects may explain how Relish functions to control metabolism and survival in hypoxia.

Functional interactions between FOXO and Relish have been described in response to other stressors in *Drosophila*. For example, nutrient starvation induces Relish in larvae via FOXO and this is important for controlling systemic insulin signaling (Karpac *et al.* 2011). In addition, as adults age, FOXO is induced in the intestine and it, in turn, upregulates Relish to control intestinal homeostasis and life span (Karpac *et al.* 2013; Guo *et al.* 2014). Interestingly, Relish and FOXO have an antagonistic relationship in adult fat, and these interactions are important for metabolic adaptation and survival upon starvation (Molaei *et al.* 2019). Hence, the links between FOXO and Relish are likely to be tissue-specific, but they may have evolved to function as a general mediator of stress responses. Functional links between NF-κB and FOXO have also been reported in mammalian cells (Lin *et al.* 2004; Thompson *et al.* 2015), and together with the reported induction of NF-κB in hypoxia in mammalian cell culture (Rius *et al.* 2008; Fitzpatrick *et al.* 2011), they suggest that the hypoxia-FOXO-NF-κB regulation that we see in *Drosophila* may operate in mammalian cells too.

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## Literature Cited

Ackerman, D., and D. Gems, 2012 Insulin/IGF-1 and hypoxia signaling act in concert to regulate iron homeostasis in Caenorhabditis elegans. PLoS Genet. 8: e1002498. https://doi.org/10.1371/journal.pgen.1002498

Alic, N., T. D. Andrews, M. E. Giannakou, I. Papatheodorou, C. Slack *et al.*, 2011 Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. Mol. Syst. Biol. 7: 502. https://doi.org/10.1038/msb.2011.36

Alic, N., J. M. Tullet, T. Niccoli, S. Broughton, M. P. Hoddinott *et al.*, 2014 Cell-nonautonomous effects of dFOXO/DAF-16 in aging. Cell Rep. 6: 608–616. https://doi.org/10.1016/j.celrep.2014.01.015

Ayres, J. S., and D. S. Schneider, 2012 Tolerance of infections. Annu. Rev. Immunol. 30: 271–294. https://doi.org/10.1146/annurev-immunol-020711-075030

Bakker, W. J., I. S. Harris, and T. W. Mak, 2007 FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. Mol. Cell 28: 941–953. https://doi.org/10.1016/j.molcel.2007.10.035

Bandarra, D., J. Biddlestone, S. Mudie, H. A. Muller, and S. Rocha, 2014 Hypoxia activates IKK-NF-κB and the immune response in Drosophila melanogaster. Biosci. Rep. 34: e00127. https://doi.org/10.1042/BSR20140095

Bellier, A., C. S. Chen, C. Y. Kao, H. N. Cinar, and R. V. Aroian, 2009 Hypoxia and the hypoxic response pathway protect against pore-forming toxins in C. elegans. PLoS Pathog. 5: e1000689. https://doi.org/10.1371/journal.ppat.1000689

Bernal, A., and D. A. Kimbrell, 2000 Drosophila Thor participates in host immune defense and connects a translational regulator with innate immunity. Proc. Natl. Acad. Sci. USA 97: 6019–6024. https://doi.org/10.1073/pnas.100391597

Birnbaum, A., X. Wu, M. Tatar, N. Liu, and H. Bai, 2019 Age-dependent changes in transcription factor FOXO targeting in female Drosophila. Front. Genet. 10: 312. https://doi.org/10.3389/fgene.2019.00312

Borch Jensen, M., Y. Qi, R. Riley, L. Rabkina, and H. Jasper, 2017 PGAM5 promotes lasting FoxO activation after developmental mitochondrial stress and extends lifespan in Drosophila. Elife 6: e26952 [corrigenda: Elife 7: e37316 (2007)]. https://doi.org/10.7554/eLife.26952

Boutin, A. T., A. Weidemann, Z. Fu, L. Mesropian, K. Gradin *et al.*, 2008 Epidermal sensing of oxygen is essential for systemic hypoxic response. Cell 133: 223–234. https://doi.org/10.1016/j.cell.2008.02.038

Britton, J. S., W. K. Lockwood, L. Li, S. M. Cohen, and B. A. Edgar, 2002 Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev. Cell 2: 239–249. https://doi.org/10.1016/S1534-5807(02)00117-X

Buchon, N., N. Silverman, and S. Cherry, 2014 Immunity in Drosophila melanogaster–from microbial recognition to whole-organism physiology. Nat. Rev. Immunol. 14: 796–810. https://doi.org/10.1038/nri3763

Callier, V., S. C. Hand, J. B. Campbell, T. Biddulph, and J. F. Harrison, 2015 Developmental changes in hypoxic exposure and responses to anoxia in Drosophila melanogaster. J. Exp. Biol. 218: 2927–2934. https://doi.org/10.1242/jeb.125849

Centanin, L., P. J. Ratcliffe, and P. Wappner, 2005 Reversion of lethality and growth defects in Fatiga oxygen-sensor mutant flies by loss of hypoxia-inducible factor-alpha/Sima. EMBO Rep. 6: 1070–1075. https://doi.org/10.1038/sj.embor.7400528

Centanin, L., A. Dekanty, N. Romero, M. Irisarri, T. A. Gorr *et al.*, 2008 Cell autonomy of HIF effects in Drosophila: tracheal cells sense hypoxia and induce terminal branch sprouting. Dev. Cell 14: 547–558. https://doi.org/10.1016/j.devcel.2008.01.020

Cramer, T., Y. Yamanishi, B. E. Clausen, I. Forster, R. Pawlinski *et al.*, 2003 HIF-1alpha is essential for myeloid cell-mediated

inflammation. Cell 112: 645–657. https://doi.org/10.1016/S0092-8674(03)00154-5

Dasgupta, N., A. M. Patel, B. A. Scott, and C. M. Crowder, 2007 Hypoxic preconditioning requires the apoptosis protein CED-4 in C. elegans. Curr. Biol. 17: 1954–1959. https://doi.org/10.1016/j.cub.2007.10.017

Davoodi, S., A. Galenza, A. Panteluk, R. Deshpande, M. Ferguson *et al.*, 2019 The immune deficiency pathway regulates metabolic homeostasis in Drosophila. J. Immunol. 202: 2747–2759. https://doi.org/10.4049/jimmunol.1801632

Demontis, F., and N. Perrimon, 2010 FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell 143: 813–825. https://doi.org/10.1016/j.cell.2010.10.007

Dionne, M. S., L. N. Pham, M. Shirasu-Hiza, and D. S. Schneider, 2006 Akt and FOXO dysregulation contribute to infection-induced wasting in Drosophila. Curr. Biol. 16: 1977–1985. https://doi.org/10.1016/j.cub.2006.08.052

Fitzpatrick, S. F., M. M. Tambuwala, U. Bruning, B. Schaible, C. C. Scholz *et al.*, 2011 An intact canonical NF-κB pathway is required for inflammatory gene expression in response to hypoxia. J. Immunol. 186: 1091–1096. https://doi.org/10.4049/jimmunol.1002256

Ganeshan, K., J. Nikkanen, K. Man, Y. A. Leong, Y. Sogawa *et al.*, 2019 Energetic trade-offs and hypometabolic states promote disease tolerance. Cell 177: 399–413.e12. https://doi.org/10.1016/j.cell.2019.01.050

Gershman, B., O. Puig, L. Hang, R. M. Peitzsch, M. Tatar *et al.*, 2007 High-resolution dynamics of the transcriptional response to nutrition in Drosophila: a key role for dFOXO. Physiol. Genomics 29: 24–34. https://doi.org/10.1152/physiolgenomics.00061.2006

Giannakou, M. E., M. Goss, M. A. Junger, E. Hafen, S. J. Leevers *et al.*, 2004 Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science 305: 361. https://doi.org/10.1126/science.1098219

Guo, L., J. Karpac, S. L. Tran, and H. Jasper, 2014 PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell 156: 109–122. https://doi.org/10.1016/j.cell.2013.12.018

Harrison, J. F., K. J. Greenlee, and W. Verberk, 2018 Functional hypoxia in insects: definition, assessment, and consequences for physiology, ecology, and evolution. Annu. Rev. Entomol. 63: 303–325. https://doi.org/10.1146/annurev-ento-020117-043145

Hedengren, M., B. Asling, M. S. Dushay, I. Ando, S. Ekengren *et al.*, 1999 Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell 4: 827–837. https://doi.org/10.1016/S1097-2765(00)80392-5

Hibshman, J. D., A. E. Doan, B. T. Moore, R. E. Kaplan, A. Hung *et al.*, 2017 *daf-16*/FoxO promotes gluconeogenesis and trehalose synthesis during starvation to support survival. Elife 6: e30057. https://doi.org/10.7554/eLife.30057

Huang, Y., R. P. Hickey, J. L. Yeh, D. Liu, A. Dadak *et al.*, 2004 Cardiac myocyte-specific HIF-1alpha deletion alters vascularization, energy availability, calcium flux, and contractility in the normoxic heart. FASEB J. 18: 1138–1140. https://doi.org/10.1096/fj.04-1510fje

Hwangbo, D. S., B. Gersham, M.-P. Tu, M. Palmer, and M. Tatar, 2004 Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429: 562–566 [corrigenda: Nature 434: 118 (2005)]. https://doi.org/10.1038/nature02549

Jensen, K. S., T. Binderup, K. T. Jensen, I. Therkelsen, R. Borup *et al.*, 2011 FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. EMBO J. 30: 4554–4570. https://doi.org/10.1038/emboj.2011.323

Jiang, H., R. Guo, and J. A. Powell-Coffman, 2001 The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc. Natl. Acad. Sci. USA 98: 7916–7921. https://doi.org/10.1073/pnas.141234698

Junger, M. A., F. Rintelen, H. Stocker, J. D. Wasserman, M. Vegh *et al.*, 2003 The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J. Biol. 2: 20. https://doi.org/10.1186/1475-4924-2-20

Karpac, J., J. Hull-Thompson, M. Falleur, and H. Jasper, 2009 JNK signaling in insulin-producing cells is required for adaptive responses to stress in Drosophila. Aging Cell 8: 288–295. https://doi.org/10.1111/j.1474-9726.2009.00476.x

Karpac, J., A. Younger, and H. Jasper, 2011 Dynamic coordination of innate immune signaling and insulin signaling regulates systemic responses to localized DNA damage. Dev. Cell 20: 841–854. https://doi.org/10.1016/j.devcel.2011.05.011

Karpac, J., B. Biteau, and H. Jasper, 2013 Misregulation of an adaptive metabolic response contributes to the age-related disruption of lipid homeostasis in Drosophila. Cell Rep. 4: 1250–1261. https://doi.org/10.1016/j.celrep.2013.08.004

Kramer, J. M., J. D. Slade, and B. E. Staveley, 2008 Foxo is required for resistance to amino acid starvation in Drosophila. Genome 51: 668–672. https://doi.org/10.1139/G08-047

Lavista-Llanos, S., L. Centanin, M. Irisarri, D. M. Russo, J. M. Gleadle *et al.*, 2002 Control of the hypoxic response in Drosophila melanogaster by the basic helix-loop-helix PAS protein similar. Mol. Cell. Biol. 22: 6842–6853. https://doi.org/10.1128/MCB.22.19.6842-6853.2002

Lee, B., E. C. Barretto, and S. S. Grewal, 2019 TORC1 modulation in adipose tissue is required for organismal adaptation to hypoxia in Drosophila. Nat. Commun. 10: 1878. https://doi.org/10.1038/s41467-019-09643-7

Leiser, S. F., A. Begun, and M. Kaeberlein, 2011 HIF-1 modulates longevity and healthspan in a temperature-dependent manner. Aging Cell 10: 318–326. https://doi.org/10.1111/j.1474-9726.2011.00672.x

Li, Y., D. Padmanabha, L. B. Gentile, C. I. Dumur, R. B. Beckstead *et al.*, 2013 HIF- and non-HIF-regulated hypoxic responses require the estrogen-related receptor in Drosophila melanogaster. PLoS Genet. 9: e1003230. https://doi.org/10.1371/journal.pgen.1003230

Lin, L., J. D. Hron, and S. L. Peng, 2004 Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity 21: 203–213. https://doi.org/10.1016/j.immuni.2004.06.016

Lissner, M. M., and D. S. Schneider, 2018 The physiological basis of disease tolerance in insects. Curr. Opin. Insect Sci. 29: 133–136. https://doi.org/10.1016/j.cois.2018.09.004

Liu, G., J. Roy, and E. A. Johnson, 2006 Identification and function of hypoxia-response genes in Drosophila melanogaster. Physiol. Genomics 25: 134–141. https://doi.org/10.1152/physiolgenomics.00262.2005

Liu, X., X. Cai, B. Hu, Z. Mei, D. Zhang *et al.*, 2016 Forkhead transcription factor 3a (FOXO3a) modulates hypoxia signaling via up-regulation of the von Hippel-Lindau gene (VHL). J. Biol. Chem. 291: 25692–25705. https://doi.org/10.1074/jbc.M116.745471

Markow, T. A., 2015 The secret lives of Drosophila flies. Elife 4: e06793. https://doi.org/10.7554/eLife.06793

Mason, S. D., R. A. Howlett, M. J. Kim, I. M. Olfert, M. C. Hogan *et al.*, 2004 Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. PLoS Biol. 2: e288. https://doi.org/10.1371/journal.pbio.0020288

Mauro, C., S. C. Leow, E. Anso, S. Rocha, A. K. Thotakura *et al.*, 2011 NF-κB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. Nat. Cell Biol. 13: 1272–1279. https://doi.org/10.1038/ncb2324

McKeown, S. R., 2014 Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br. J. Radiol. 87: 20130676. https://doi.org/10.1259/bjr.20130676

Medzhitov, R., D. S. Schneider, and M. P. Soares, 2012 Disease tolerance as a defense strategy. Science 335: 936–941. https://doi.org/10.1126/science.1214935

Mehta, R., K. A. Steinkraus, G. L. Sutphin, F. J. Ramos, L. S. Shamieh *et al.*, 2009 Proteasomal regulation of the hypoxic response modulates aging in C. elegans. Science 324: 1196–1198. https://doi.org/10.1126/science.1173507

Mendenhall, A. R., B. LaRue, and P. A. Padilla, 2006 Glyceraldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans. Genetics 174: 1173–1187. https://doi.org/10.1534/genetics.106.061390

Menuz, V., K. S. Howell, S. Gentina, S. Epstein, I. Riezman *et al.*, 2009 Protection of C. elegans from anoxia by HYL-2 ceramide synthase. Science 324: 381–384. https://doi.org/10.1126/science.1168532

Molaei, M., C. Vandehoef and J. Karpac, 2019 NF-κB shapes metabolic adaptation by attenuating Foxo-mediated lipolysis in Drosophila. Dev Cell 49: 802–810.e6. https://doi.org/10.1016/j.devcel.2019.04.009

Müller, R.-U., F. Fabretti, S. Zank, V. Burst, T. Benzing *et al.*, 2009 The von Hippel Lindau tumor suppressor limits longevity. J. Am. Soc. Nephrol. 20: 2513–2517. https://doi.org/10.1681/ASN.2009050497

Nakazawa, M. S., B. Keith, and M. C. Simon, 2016 Oxygen availability and metabolic adaptations. Nat. Rev. Cancer 16: 663–673. https://doi.org/10.1038/nrc.2016.84

Petkau, K., M. Ferguson, S. Guntermann, and E. Foley, 2017 Constitutive immune activity promotes tumorigenesis in Drosophila intestinal progenitor cells. Cell Rep. 20: 1784–1793. https://doi.org/10.1016/j.celrep.2017.07.078

Reiling, J. H., and E. Hafen, 2004 The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. Genes Dev. 18: 2879–2892. https://doi.org/10.1101/gad.322704

Rius, J., M. Guma, C. Schachtrup, K. Akassoglou, A. S. Zinkernagel *et al.*, 2008 NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature 453: 807–811. https://doi.org/10.1038/nature06905

Schipani, E., H. E. Ryan, S. Didrickson, T. Kobayashi, M. Knight *et al.*, 2001 Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev. 15: 2865–2876.

Scott, B. A., M. S. Avidan, and C. M. Crowder, 2002 Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science 296: 2388–2391. https://doi.org/10.1126/science.1072302

Semenza, G. L., 2011 Oxygen sensing, homeostasis, and disease. N. Engl. J. Med. 365: 537–547. https://doi.org/10.1056/NEJMra1011165

Semenza, G. L., 2014 Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu. Rev. Pathol. 9: 47–71. https://doi.org/10.1146/annurev-pathol-012513-104720

Slack, C., M. E. Giannakou, A. Foley, M. Goss, and L. Partridge, 2011 dFOXO-independent effects of reduced insulin-like signaling in Drosophila. Aging Cell 10: 735–748. https://doi.org/10.1111/j.1474-9726.2011.00707.x

Song, W., E. Owusu-Ansah, Y. Hu, D. Cheng, X. Ni *et al.*, 2017 Activin signaling mediates muscle-to-adipose communication in a mitochondria dysfunction-associated obesity model. Proc. Natl. Acad. Sci. USA 114: 8596–8601. https://doi.org/10.1073/pnas.1708037114

Sun, X., C. T. Wheeler, J. Yolitz, M. Laslo, T. Alberico *et al.*, 2014 A mitochondrial ATP synthase subunit interacts with TOR signaling to modulate protein homeostasis and lifespan in Drosophila. Cell Rep. 8: 1781–1792. https://doi.org/10.1016/j.celrep.2014.08.022

Teleman, A. A., V. Hietakangas, A. C. Sayadian, and S. M. Cohen, 2008 Nutritional control of protein biosynthetic capacity by insulin via Myc in Drosophila. Cell Metab. 7: 21–32. https://doi.org/10.1016/j.cmet.2007.11.010

Tennessen, J. M., W. E. Barry, J. Cox, and C. S. Thummel, 2014 Methods for studying metabolism in Drosophila. Methods 68: 105–115. https://doi.org/10.1016/j.ymeth.2014.02.034

Texada, M. J., A. F. Jorgensen, C. F. Christensen, T. Koyama, A. Malita *et al.*, 2019 A fat-tissue sensor couples growth to oxygen availability by remotely controlling insulin secretion. Nat. Commun. 10: 1955. https://doi.org/10.1038/s41467-019-09943-y

Thompson, M. G., M. Larson, A. Vidrine, K. Barrios, F. Navarro *et al.*, 2015 FOXO3-NF-κB RelA protein complexes reduce proinflammatory cell signaling and function. J. Immunol. 195: 5637–5647. https://doi.org/10.4049/jimmunol.1501758

Tomita, S., M. Ueno, M. Sakamoto, Y. Kitahama, M. Ueki *et al.*, 2003 Defective brain development in mice lacking the Hif-1alpha gene in neural cells. Mol. Cell. Biol. 23: 6739–6749. https://doi.org/10.1128/MCB.23.19.6739-6749.2003

Tornatore, L., A. K. Thotakura, J. Bennett, M. Moretti, and G. Franzoso, 2012 The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. Trends Cell Biol. 22: 557–566. https://doi.org/10.1016/j.tcb.2012.08.001

Wang, A., S. C. Huen, H. H. Luan, S. Yu, C. Zhang *et al.*, 2016 Opposing effects of fasting metabolism on tissue tolerance in bacterial and viral inflammation. Cell 166: 1512–1525.e12. https://doi.org/10.1016/j.cell.2016.07.026

Wang, B., N. Moya, S. Niessen, H. Hoover, M. M. Mihaylova *et al.*, 2011 A hormone-dependent module regulating energy balance. Cell 145: 596–606. https://doi.org/10.1016/j.cell.2011.04.013

Webb, A. E., and A. Brunet, 2014 FOXO transcription factors: key regulators of cellular quality control. Trends Biochem. Sci. 39: 159–169. https://doi.org/10.1016/j.tibs.2014.02.003

Weis, S., A. R. Carlos, M. R. Moita, S. Singh, B. Blankenhaus *et al.*, 2017 Metabolic adaptation establishes disease tolerance to sepsis. Cell 169: 1263–1275.e14. https://doi.org/10.1016/j.cell.2017.05.031

Wong, D. M., Z. Shen, K. E. Owyang, and J. A. Martinez-Agosto, 2014 Insulin- and warts-dependent regulation of tracheal plasticity modulates systemic larval growth during hypoxia in Drosophila melanogaster. PLoS One 9: e115297. https://doi.org/10.1371/journal.pone.0115297

Xie, H., and M. C. Simon, 2017 Oxygen availability and metabolic reprogramming in cancer. J. Biol. Chem. 292: 16825–16832. https://doi.org/10.1074/jbc.R117.799973

Zhang, Q., M. J. Lenardo, and D. Baltimore, 2017 30 years of NF-κB: a blossoming of relevance to human pathobiology. Cell 168: 37–57. https://doi.org/10.1016/j.cell.2016.12.012

Zhang, Y., Z. Shao, Z. Zhai, C. Shen, and J. A. Powell-Coffman, 2009 The HIF-1 hypoxia-inducible factor modulates lifespan in C. elegans. PLoS One 4: e6348. https://doi.org/10.1371/journal.pone.0006348

Zhao, X., and J. Karpac, 2017 Muscle directs diurnal energy homeostasis through a myokine-dependent hormone module in Drosophila. Curr. Biol. 27: 1941–1955.e6. https://doi.org/10.1016/j.cub.2017.06.004

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