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1 # Transcriptome analysis of FOXO-dependent hypoxia gene expression identifies
2 # Hipk as a regulator of low oxygen tolerance in *Drosophila*

3

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13 Running head: Transcriptomic analysis of hypoxia in *Drosophila*

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17 ribosome, mitochondria

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## Summary

**When exposed to low oxygen or hypoxia, animals must alter their metabolism and physiology to ensure proper cell-, tissue- and whole-body level adaptations to their hypoxic environment. These alterations often involve changes in gene expression. While extensive work has emphasized the importance of the HIF-1 alpha transcription factor on controlling hypoxia gene expression, less is known about other transcriptional mechanisms. We previously identified the transcription factor FOXO as a regulator of hypoxia tolerance in *Drosophila* larvae and adults. Here we use an RNA-sequencing approach to identify FOXO-dependent changes in gene expression that are associated with these tolerance effects. We found that hypoxia altered the expression of over 2000 genes and that approximately 40% of these gene expression changes required FOXO. We discovered that hypoxia exposure led to a FOXO-dependent increase in genes involved in cell signaling, such as kinases, GTPase regulators, and regulators of the Hippo/Yorkie pathway. Among these, we identified homeodomain-interacting protein kinase (Hipk) as being required for hypoxia survival. We also found that hypoxia suppresses the expression of genes involved in ribosome synthesis and egg production, and we showed that hypoxia suppresses tRNA synthesis and mRNA translation and reduces female fecundity. Among the downregulated genes, we discovered that FOXO was required for suppression of many ribosomal protein genes and genes involved in oxidative phosphorylation, pointing to a role for FOXO in limiting energetically costly processes such as protein synthesis and mitochondrial activity upon hypoxic stress. This work uncovers a widespread role for FOXO in mediating hypoxia changes in gene expression.**

## Introduction

Animals often live in conditions where environmental oxygen levels fluctuate (CLEGG 1997; DANOVARO *et al.* 2010; PARK *et al.* 2017). As a result, they must coordinate their physiology and metabolism with changes in oxygen availability to maintain proper homeostasis. This coordination can occur through alterations in gene expression and is essential for ensuring organismal survival in low oxygen (BICKLER AND BUCK 2007; RAMIREZ *et al.* 2007; HARRISON AND HADDAD 2011; PADILLA AND LADAGE 2012; NAKAZAWA *et al.* 2016; SAMANTA *et al.* 2017; HARRISON *et al.* 2018; SCHITO AND REY 2018; HOLDSWORTH AND GIBBS 2020).

Across all metazoans, perhaps the best-described and most intensively studied mechanism of gene regulation in hypoxia involves the HIF-1 alpha transcription factor (SEMENZA 2011; SEMENZA 2014b). When cells encounter low oxygen conditions, HIF-1 alpha protein is stabilized and translocates to the nucleus to induce gene expression. HIF-1 alpha-regulated genes encompass a diverse array of genes

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53 that are involved in biological processes such as metabolism, cell signaling and transcription, and that
54 together coordinate cell-, tissue- and whole-body level adaptations to low oxygen (SEMENZA 2011;
55 SAMANTA *et al.* 2017). Studies in model organisms have identified how HIF-1 alpha is a key regulator of
56 hypoxia in both normal physiology and in pathological disease states (SEMENZA 2014a; SEMENZA
57 2014b). However, compared to our understanding of HIF-1 alpha biology, less is known about other
58 transcriptional mechanisms that contribute to both cellular and systemic oxygen homeostasis.
59
60 *Drosophila* have provided a versatile and informative model system for investigating organismal
61 responses to hypoxia. In their natural ecology, *Drosophila* live and grow on rotting, fermenting food rich
62 in microorganisms – an environment characterized by low ambient oxygen (CALLIER *et al.* 2015;
63 MARKOW 2015; HARRISON *et al.* 2018). They have therefore evolved mechanisms to tolerate hypoxia.
64 For example, larvae and adults can tolerate severe hypoxia (~1% oxygen) for up to 24 hours with little
65 impact on viability (BARRETTO *et al.* 2020), while embryos can survive complete anoxia (0% oxygen) for
66 several days (FOE AND ALBERTS 1985; TEODORO AND O'FARRELL 2003). Genetic studies have shown
67 that flies can survive oxygen deprivation by increasing tracheal branching to expand oxygen supply to
68 tissues (CENTANIN *et al.* 2008; WONG *et al.* 2014), and by remodelling their physiology and metabolism
69 through both HIF-1 alpha-dependent and independent mechanisms (WINGROVE AND O'FARRELL 1999;
70 LAVISTA-LLANOS *et al.* 2002; TEODORO AND O'FARRELL 2003; CENTANIN *et al.* 2005; ROMERO *et al.* 2007;
71 HARRISON AND HADDAD 2011; MORTON 2011; LI *et al.* 2013; BANDARRA *et al.* 2014; HARRISON *et al.*
72 2018; LEE *et al.* 2019; TEXADA *et al.* 2019; BARRETTO *et al.* 2020). Relatively few studies, however, have
73 used genome-wide approaches to identify gene expression changes associated with adaptation to
74 hypoxia in *Drosophila* (LIU *et al.* 2006a; LI *et al.* 2013). One study examined transcriptome changes
75 associated with larval hypoxia and identified widespread changes in metabolic and gene expression (LI
76 *et al.* 2013). This study also showed that of the hundreds of gene expression changes, over half were
77 independent of HIF-1 alpha, emphasizing the importance of additional transcriptional mechanisms in
78 the control of hypoxia gene expression (LI *et al.* 2013).
79
80 Using *Drosophila* larvae and adults, we previously identified the transcription factor, Forkhead Box O
81 (FOXO), as a regulator of hypoxia tolerance (BARRETTO *et al.* 2020). FOXO is a conserved regulator of
82 stress responses and animal aging (WEBB AND BRUNET 2014). Studies in *Drosophila* have shown it is
83 induced by stressors such as starvation, oxidative stress, pathogens, and ionizing radiation (JUNGER *et*
84 *al.* 2003; DIONNE *et al.* 2006; KARPAC *et al.* 2009; KARPAC *et al.* 2011; BORCH JENSEN *et al.* 2017).
85 Genetic studies have also shown that, in general, loss of *foxo* induces stress sensitivity and shortens
86 lifespan whereas increased FOXO activity, particularly in tissues such as gut, muscle and fat body can
87 promote stress resistance and extend lifespan (GIANNAKOU *et al.* 2004; HWANGBO *et al.* 2004;
88 TETTWEILER *et al.* 2005; KRAMER *et al.* 2008; DEMONTIS AND PERRIMON 2010; ALIC *et al.* 2014a; ALIC *et*

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89 *al.* 2014b; DOBSON *et al.* 2017). We showed that FOXO activity is rapidly induced in hypoxia and that it
90 is needed for hypoxia survival in both larvae and adults. We also identified the immune Relish/NF
91 Kappa B transcription factor as one target of FOXO important for its hypoxia tolerance effects.
92 However, it is unclear what other genes FOXO may regulate in hypoxia. Previous studies have shown
93 that under normal conditions FOXO can bind to thousands of genomic loci (ALIC *et al.* 2011; BIRNBAUM
94 *et al.* 2019) and can regulate the expression of hundreds genes in a tissue- and context-specific
95 manner (ALIC *et al.* 2011; ALIC *et al.* 2014a; ALIC *et al.* 2014b), raising the possibility that it may mediate
96 broad effects on gene expression in hypoxia.
97
98 In this report we describe our transcriptome analysis of hypoxia-mediated gene expression changes
99 upon hypoxia in adult flies. We show that FOXO is required for upregulation of genes involved in cell
100 signaling and we identify the kinase Hipk as a regulator of hypoxia tolerance. We also see that FOXO
101 suppresses expression of genes involved in protein synthesis and mitochondrial activity, suggesting it
102 plays an important role in limiting energetically costly processes in low oxygen stress.
103

104 ## Material and Methods

105

106 ### *Drosophila* Stocks and Culturing

107 Flies were grown on medium containing 150 g agar, 1600 g cornmeal, 770 g Torula yeast, 675 g
108 sucrose, 2340 g D-glucose, 240 ml acid mixture (propionic acid/phosphoric acid) per 34 L water. All
109 stocks were maintained at either 18°C or RT. For adult hypoxia exposures, flies were raised from
110 embryos to adults at 25°C and then, following eclosion, females were allowed to mate for 2 days before
111 being separated from males and aged for another 5-6 days, at which time point hypoxia experiments
112 were performed. For larval hypoxia exposures, hatched larvae were grown on food at 25°C until 96hrs
113 after egg-laying, at which time point hypoxia experiments were performed. The following *Drosophila*
114 strains were used: $w^{1118}$, $foxo^{D94}$*/TM6B, UAS-hipk* RNAi, *da-GS-Gal4 (daughterless-GeneSwitch)*. For
115 UAS gene induction using the GeneSwitch system, adult flies were fed food supplemented with RU486
116 (200μM) for seven days. Control (non-induced flies) were maintained on normal food supplemented
117 with ethanol (vehicle control for RU486).
118

119 ### Hypoxia exposure and measurement of hypoxia survival

120 Vials of adult flies or larvae were placed into an airtight glass chamber into which a mix of 1%
121 oxygen/99% nitrogen gas continually flowed. Flow rate was controlled using an Aalborg model P gas
122 flow meter. Normoxic animals were maintained in vials in ambient air. For hypoxia survival experiments,
123 mated female adults were placed in placed into hypoxia (1% oxygen) for 20 hours in groups of 15 flies

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124 per vial. Then, vials were removed from hypoxia and the flies were allowed to recover before the
125 numbers of dead flies were counted.
126
127 **Total RNA isolation**
128 Adult flies (5 per group), adult tissues (from 10 animals per group), or larvae (10 per group) were snap
129 frozen on dry ice. Total RNA was then isolated using Trizol according to the manufacturer's instructions
130 (Invitrogen; 15596-018). Extracted RNA was then DNase treated (Ambion; 2238G) to be used for
131 subsequent qPCR or mRNA-sequencing.
132
133 **mRNA-sequencing and RNA-seq analyses**
134 Three to four independent biological replicates (5 flies per group) of normoxia- and hypoxia-exposed
135 groups of $w^{1118}$ and *foxo* mutants were prepared and analyzed. RNA-sequencing was conducted by the
136 University of Calgary Centre for Health Genomics and Informatics. The RNA Integrity Number (RIN)
137 was determined for each RNA sample. Samples with a RIN score higher than 8 were considered good
138 quality, and Poly-A mRNA-seq libraries from such samples were prepared using the Ultra II Directional
139 RNA Library kit (New England BioLabs) according to the manufacturer's instructions. Libraries were
140 then quantified using the Kapa qPCR Library Quantitation kit (Roche) according to the manufacturer's
141 directions. Finally, RNA libraries were sequenced for 100 cycles using the NextSeq 500 Sequencing
142 System (Illumina). Transcripts were quantified using kallisto (BRAY *et al.* 2016) referencing refSeq
143 mRNA (release: 2019-Oct-15) corresponding to dm6 annotation. Differential expression testing was
144 performed using sleuth (PIMENTEL *et al.* 2017).
145
146 **Gene Ontology, KEGG pathway, and tissue expression analyses**
147 Analyses of Gene Ontology and KEGG pathway enrichment of up- and down-regulated genes (>1.5-
148 fold, q-val (FDR corrected p-val) <0.05) were performed using G-profiler (RAUDVERE *et al.* 2019) and
149 Revigo (SUPEK *et al.* 2011).
150
151 **Quantitative RT-PCR measurements**
152 Total RNA was extracted from either whole flies, whole larvae, or isolated adult tissues. The RNA was
153 then DNase treated as described above and reverse transcribed using Superscript II (Invitrogen;
154 100004925). The generated cDNA was used as a template to perform qRT-PCRs (ABI 7500 real time
155 PCR system using SyBr Green PCR mix) using gene-specific primers. PCR data were normalized to
156 *beta tubulin* or *eIF2 alpha* mRNA levels. The following primers were used:
157
158 *tRNA ala* Forward: GCGGCCGCACTTTCACTGACCGGAAACG
159 *tRNA ala* Reverse: GCGGCCGCGCCCGTTCTAACTTTTTGGA

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160 *tRNA arg* Forward: GCGGCCGCGTCCGTCCACCAATGAA AAT
161 *tRNA arg* Reverse: GCGGCCGCCGGCTAGCTCAGTCGGT AGA
162 *tRNA eMet* Forward: GCGGCCGCCGTGGCAATCTTCTGAA ACC
163 *tRNA eMet* Reverse: GCGGCCGCTCAGTGGAAAACCATA TGTTCG
164 *tRNA iMet* Forward: AGAGTGGCGCAGTGGAAG
165 *tRNA iMet* Reverse: AGAGCAAGGTTTCGATCCTC
166 *beta tubulin* Forward: ATCATCACACACGGACAGG
167 *beta tubulin* Reverse: GAGCTGGATGATGGGGAGTA
168 *Hipk* Forward: CAACAATGTCAAGGCATC
169 *Hipk* Reverse: CAGGCTGCACAGTGTGGAAA
170 *eIF2 alpha* Forward: TCTTCGATGAGTGCAACCTG
171 *eIF2 alpha* Reverse: CCTCGTAACCGTAGCAGGAG
172

173 **Statistical analysis of qRT-PCR data**

174 All qRT-PCR data were analyzed by Student's t-test or two-way ANOVA followed by post-hoc Students'
175 t-test where appropriate. All statistical analyses and data plots were performed using Prism statistical
176 software. Differences were considered significant when p values were less than 0.05.
177

178 **Polysome Profiling**

179 Larvae were lysed in lysis buffer (25 mM Tris pH 7.4, 10 mM $MgCl_2$, 250 mM NaCl, 1% Triton X-100,
180 0.5% sodium deoxycholate, 0.5 mM DTT, 100 mg/ml cycloheximide, 1 mg/ml heparin, 16 Complete mini
181 roche protease inhibitor, 2.5 mM PMSF, 5 mM sodium fluoride, 1 mM sodium orthovanadate and 200
182 U/ml ribolock RNAse inhibitor (Fermentas)) using a Dounce homogenizer. Lysates were then
183 centrifuged at 15,000 rpm for 20 minutes and the supernatant was removed carefully using a fine
184 syringe to avoid the floating fat content. For each condition, lysates containing 300 mg of total RNA
185 were then layered on top of a 15-45% w/w sucrose gradient (made using 25 mM Tris pH 7.4, 10 mM
186 $MgCl_2$, 250 mM NaCl, 1 mg/ml heparin, 100 mg/ml cycloheximide in 12 ml polyallomer tube) and
187 centrifuged at 37,000 rpm for 150 minutes in a Beckmann Coulter Optima L-90K ultracentrifuge using a
188 SW-41 rotor. Polysome profiles were obtained by pushing the gradient using 70% w/v sucrose pumped
189 at 1.5 ml/min into a continuous OD254 nm reader (ISCO UA6 UV detector).
190

191 **Fecundity Assay**

192 1-2 day old virgin $w^{1118}$ females were allowed to mate with males for 2 days and then the females were
193 separated and either maintained in normoxia (controls) or exposed to hypoxia for either 8 or 12 hours
194 before being returned to normoxia. Twenty four hours later, the females were transferred in groups of 3
195 to new vials and allowed to lay eggs for a 24 hour period (day 1), and then transferred to a second set

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196 of vials to lay eggs for a further 24 hour period (day 2). Fecundity was then assessed by measuring the
197 number of viable pupae per female that emerged from eggs laid on day 1 and day 2.
198
199 ## Results
200
201 **Hypoxia leads to upregulation of transcription factor and kinase gene expression.**
202
203 Adult mated $w^{1118}$ (control) or $foxo^{\Delta 94}$ (foxo null mutant)(SLACK *et al.* 2011) females were either
204 maintained in normoxia or exposed to hypoxia (1% oxygen) for 16 hours and we then isolated whole-
205 body RNA for RNA-seq analysis (Figure 1A). We first examined the gene expression changes induced
206 by hypoxia in the control animals. Using a cut-off of +/-1.5 fold and a false-discovery rate corrected p-
207 value <0.05, we identified 1081 genes with reduced mRNA expression and 1257 genes with increased
208 mRNA expression in $w^{1118}$ animals (Figure 1B, Supplemental Table 1). Among the upregulated genes,
209 we saw increased expression of several genes previously shown to be induced upon hypoxia exposure
210 in larvae and/or adults. For example, we saw increased expression of the fly fibroblast growth factor
211 homolog, *branchless (bnl),* the glycolytic enzyme, *Lactate dehydrogenease (Ldh/ImpL3)*, and the
212 transcriptional repressor, *hairy (h)*, each of which has been shown to be upregulated upon hypoxia
213 exposure (CENTANIN *et al.* 2008; ZHOU *et al.* 2008; LI *et al.* 2013) (Figure 1C). We previously showed
214 hypoxia induces rapid nuclear localization and increased transcriptional activity of the transcription
215 factor FOXO, which we found promoted hypoxia tolerance by increasing expression of the innate
216 immune transcription factor Relish (*Rel*) (BARRETTO *et al.* 2020). Consistent with this, our transcriptome
217 data showed that hypoxia led to increased expression of two FOXO target genes, *Thor* and *InR*, and
218 increased expression of *Rel* and antimicrobial peptide genes (e.g., *CecA1, CecA2, AttB, AttA, Dro)*,
219 which are known targets of Relish (Figure 1C). Together these changes in gene expression confirm that
220 our low oxygen exposure protocol induced a robust hypoxic response. Two previous studies used
221 genome-wide transcriptome analyses to examine hypoxia regulated genes in *Drosophila* (LIU *et al.*
222 2006a; LI *et al.* 2013). Like us, Liu *et al*. examined hypoxia in adult female flies, and they used DNA
223 microarray hybridization to identify genes that showed significantly increased expression after 6 hours
224 of severe (0.5% oxygen) hypoxia exposure. They identified 79 genes, of which 47 (59%) were also
225 identified in our RNAseq analysis (significant overlap, $p = 7.3 \times 10^{-29}$) (Supplemental Table 1). Li *et al.*
226 also used DNA microarray hybridization to detect hypoxia-regulated genes, in this case, in late L3
227 larvae using milder hypoxia (4% oxygen). They identified 627 significantly (p<0.01) upregulated (>1.5
228 fold) genes, of which 130 (21%) were also identified as upregulated in our RNA-seq analysis (significant
229 overlap, $p = 2.2 \times 10^{-19}$), and they identified 417 significantly (p<0.01) downregulated (>1.5 fold) genes,
230 of which 80 (19%) were also identified in our RNA-seq analysis (significant overlap, $p = 4.8 \times 10^{-13}$)
231 (Supplemental Table 1). Thus, even though this study analyzed hypoxia at a different stage of the life

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232 cycle and at a different concentration of oxygen, one-fifth of the genes that were identified as being
233 hypoxia-regulated in larvae were also identified in our study in adults. The differences in the number of
234 genes identified in our study versus the previous studies likely reflect differences in biology between
235 larvae and adults, and the greater sensitivity of RNA-seq approaches to detect differentially expressed
236 genes compared to DNA microarrays.
237
238 We used Gene Ontology analysis to examine the genes that showed upregulated expression upon
239 hypoxia. This analysis showed that the upregulated genes were particularly enriched for gene
240 categories related to chromatin modification and transcription, small G-protein regulators, and kinases
241 (Figure 2A). In addition, KEGG pathway analysis of the upregulated genes showed enrichment for
242 genes involved in Hippo, Notch, FOXO, and MAPK signaling (Figure 2B). We saw hypoxia-induced
243 increases in gene expression for 132 regulators of transcription and chromatin, and 61 kinases (Figure
244 2C). Together, these analyses suggest that hypoxia leads to widespread upregulation of different
245 signaling pathways and transcriptional responses.
246
247 **FOXO is required for hypoxia-induced upregulation of signaling molecules and regulators of the Hippo**
248 **pathway.**
249
250 To identify FOXO-dependent hypoxia-induced genes we identified genes that showed a significant (q-
251 val <0.05), >1.5-fold upregulation upon hypoxia in *w*$^{1118}$ but not *foxo*$^{\Delta 94}$. Using this criteria, we found that
252 of the 1257 genes that were upregulated upon hypoxia exposure, 551 (44%) were not significantly
253 upregulated in *foxo* mutants, suggesting that a large proportion of hypoxia-induced gene expression
254 requires FOXO activity (Supplemental Table 1). Two previous studies used ChIP-chip and ChIP-seq
255 approaches to identify FOXO genomic binding sites in young female adult flies, and, between them,
256 identified 3925 loci that bound FOXO and were within 1kb of a protein coding gene (ALIC *et al.* 2011;
257 BIRNBAUM *et al.* 2019). Interestingly, we saw that of the 553 FOXO-dependent hypoxia upregulated
258 genes that we identified, 265 (48%) overlapped with these FOXO-bound genes (Figure 3A), suggesting
259 that almost half the FOXO-dependent hypoxia genes may be induced by direct FOXO transcriptional
260 activation (Supplemental Table 1). We used Gene Ontology analysis to examine the 553 FOXO-
261 dependent hypoxia-upregulated genes. The main classes of genes identified were largely related to
262 signaling regulators, such as kinases, GTPase regulators, and guanine-nucleotide exchange factors
263 (Figure 3C). For example, we saw that many GTPase regulators required FOXO for their hypoxia
264 upregulation and many of these contained FOXO binding sites within 1Kb of their gene coding region.
265 In addition, almost half the kinases that we saw were induced in hypoxia were dependent on FOXO for
266 their induction. Interestingly, among these signaling molecules, we saw enrichment in regulators of the

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267 Hippo signaling pathway, many of which were previously shown to be enriched among FOXO-bound
268 genes, suggesting that they may be directly regulated by FOXO (Figure 3D).
269

270 **Hipk is upregulated in hypoxia and modulates hypoxia tolerance.**
271

272 One regulator of the Hippo pathway we saw upregulated and associated with a FOXO DNA binding site
273 was Homeodomain interacting protein kinase (Hipk). In *Drosophila*, Hipk has been shown to control
274 metabolism and growth in epithelial tissues, and has been shown to function as a regulator of several
275 signaling pathways including Hippo, Wingless, Notch, JAK/STAT and JNK (LEE *et al.* 2009a; LEE *et al.*
276 2009b; CHEN AND VERHEYEN 2012; POON *et al.* 2012; VERHEYEN *et al.* 2012; BLAQUIERE *et al.* 2014;
277 TETTWEILER *et al.* 2019; WONG *et al.* 2019; KINSEY *et al.* 2021; STEINMETZ *et al.* 2021). In addition, a
278 recent study in *C. elegans* showed that the worm homolog of Hipk, hpk1, was a regulator of worm
279 survival in low oxygen (DOERING *et al.* 2022). We therefore examined the role of Hipk in *Drosophila*
280 hypoxia in more detail. Using qRT-PCR we confirmed that hypoxia exposure led to an increase in *hipk*
281 mRNA levels in whole animals. We also saw hypoxia-induced increases in *hipk* mRNA levels in specific
282 tissues such as the head, thorax (which is enriched in muscle), ovaries, and abdomen (which is
283 enriched in adipose tissues), suggesting that the hypoxia-mediated increase in Hipk expression
284 occurred across many tissues (Figure 3E). To explore the functional role for Hipk in hypoxia, we used
285 the RNAi to knockdown *hipk* in flies and examined the effects on hypoxia tolerance. We used the
286 *daughterless-GeneSwitch-Gal4 (da-GSG)* driver to induce ubiquitous expression of the dsRNA and to
287 restrict RNAi-mediated knockdown of *hipk* to adult stages. We fed *da-GSG>hipk RNAi* females either
288 normal food (control) or RU486-containing food to induce RNAi (*hipk RNAi*) and then examined the
289 effects on hypoxia survival. We found that following 20 hours of hypoxia exposure the *hipk RNAi*
290 animals had significantly reduced hypoxia survival compared to the control flies, suggesting that Hipk is
291 required for hypoxia tolerance (Figure 3F).
292

293 **Hypoxia downregulates expression of protein synthesis and egg production genes and leads to reduced**
294 **translation and fecundity.**
295

296 We used Gene Ontology analysis to examine the genes that showed significantly reduced (>1.5 fold
297 decrease) expression upon hypoxia in control $w^{1118}$ animals. We saw enrichment in genes involved in
298 ribosome function, egg formation, and proteolysis (Figure 4A, B). Almost all the proteolysis genes were
299 proteases that showed enriched expression in either the intestine or fat body (Supplemental Table 1).
300 The decreased expression of genes involved in ribosome function is consistent with suppressed protein
301 synthesis, a widely seen response to hypoxia in different organisms. We previously showed that
302 regulation of tRNA synthesis was a key mechanism for regulating protein synthesis in *Drosophila*,

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303 particularly in response to nutrient starvation (MARSHALL *et al.* 2012; RIDEOUT *et al.* 2012;
304 SRISKANTHADEVAN-PIRAHAS *et al.* 2018). When we examined tRNA levels by qPCR, we saw a strong
305 reduction following hypoxia exposure (1% oxygen) in adults (Figure 4C). Furthermore, we saw that
306 exposure of larvae to 1% oxygen also led to a strong reduction in tRNA levels that was observed at both
307 2 and 24 hours of hypoxia exposure (Figure 4D, E). We also saw that hypoxia larvae showed a similarly
308 rapid decrease in overall translation compared to normoxic animals as shown by a decrease in
309 polysome:monosome ratios in polysome profiles from whole animal lysates (Figure 4F). These results
310 indicate that global suppression of protein synthesis is a common response to extreme hypoxia in both
311 larvae and adults.
312
313 Given the decreased expression of egg formation genes, we also examined whether hypoxia might
314 impact female fecundity. To do this, we exposed mated $w^{1118}$ females to hypoxia for either 8 or 12
315 hours, allowed them to recover for a day, and then measured how many viable progeny they produced
316 in the subsequent two days. We saw that when exposed to either 8 or 12 hours of hypoxia, females
317 produced significantly fewer viable progeny compared to normoxic control females (Figure 4G). These
318 results indicate that a brief exposure to hypoxia can suppress fecundity in female flies.
319
320 **FOXO is needed for hypoxia-mediated decreases in ribosome and mitochondrial gene expression.**
321
322 We then examined which downregulated genes were dependent on FOXO, by identifying genes that
323 were significantly downregulated (<1.3 fold) in $w^{1118}$ flies but not *foxo* mutants. We chose a lower fold
324 change value because the significantly downregulated genes tended to be less affected than the
325 upregulated genes. From this analysis, we identified 529 genes (39% of 1343 total downregulated
326 genes) (Supplemental Table 1). Of these, 87 were previously shown to be bound to FOXO (ALIC *et al.*
327 2011; BIRNBAUM *et al.* 2019), suggesting that FOXO-mediated decreases in gene expression in hypoxia
328 are largely indirect (Figure 5A) (Supplemental Table 1). We used GO analysis to examine the functional
329 categories of FOXO-dependent downregulated genes and identified strong enrichment in two main
330 classes - ribosomal proteins and mitochondrial regulators (Figure 5B). We saw that genes coding for
331 ribosomal proteins for both the small and large subunits showed reduced expression in $w^{1118}$ but not
332 *foxo* mutant animals (Figure 5C). We also found that many mitochondrial genes required FOXO for their
333 downregulation in hypoxia, including known or predicted mitochondrial ribosome proteins, cytochrome-
334 C oxidase subunits, complex I subunits, ATP synthases subunits, and mitochondrial transporters
335 (Figure 5C). These results suggest that an important role for FOXO in hypoxia is to suppress both
336 mitochondrial and protein synthetic activity.
337

## 338 Discussion

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339
340 We previously showed that the FOXO transcription factor was required for hypoxia tolerance
341 (BARRETTO *et al.* 2020). One focus of this current study was to identify which genes might be FOXO-
342 regulated in hypoxia. Our results indicate that hypoxia exposure alters (+/- 1.5 fold or greater) the
343 transcript levels of ~2300 genes in our control ($w^{1118}$) line, indicating a widespread modification of gene
344 expression. To identify which gene expression changes are FOXO dependent, we chose to identify
345 which genes had significantly altered expression in $w^{1118}$ but not *foxo* mutants. This analysis showed
346 that approximately 40% of hypoxia regulated genes required FOXO. Furthermore, using data from
347 previous genome wide FOXO ChIP studies (ALIC *et al.* 2011; BIRNBAUM *et al.* 2019) we saw that
348 approximately half the FOXO dependent upregulated genes were directly bound by FOXO. These
349 results suggest that FOXO is needed for widespread transcriptional changes upon hypoxia. A previous
350 report examining genome-wide changes in gene expression upon hypoxia exposure in larvae showed
351 that HIF-1 alpha was required for just under half of the changes in gene expression, and that the
352 transcription factor estrogen-related receptor (ERR) was also important for mediating many of the
353 effects of hypoxia on gene expression (LI *et al.* 2013). This study and our findings suggest that HIF-1
354 alpha, ERR, and FOXO may mediate many of the widespread changes in gene expression when flies
355 are in low oxygen conditions. For example, we previously showed that FOXO was required for hypoxia
356 tolerance in larvae (BARRETTO *et al.* 2020), suggesting it may cooperate or work in parallel with HIF-1
357 alpha and/or ERR to regulate hypoxia-mediated changes in gene expression at this developmental
358 stage. Interestingly, we also saw that ERR mRNA levels were significantly increased upon hypoxia in
359 adults (1.48-fold), although this was below our cut-off of 1.5-fold. Nevertheless, this suggests that ERR
360 may also be important for hypoxia-mediated gene expression changes in the adult.
361
362 Hypoxia-upregulated genes were enriched for kinases, regulators of small GTPases, and regulators of
363 gene expression such as transcription factors and chromatin modifiers. This suggests that a major
364 response to hypoxia is widespread alterations in cell-cell signaling pathways and their downstream
365 transcriptional effectors. We found that upregulation of many of these signaling genes was dependent
366 on FOXO and likely direct, since many of these bound FOXO. Interestingly, regulators of the Hippo
367 pathway were among the FOXO dependent upregulated genes. The Hippo pathway has been best
368 studied in the context of cell growth and proliferation especially in epithelial, neural, and stem cells (MA
369 *et al.* 2019; WU AND GUAN 2021). In these cells, the pathway often functions to couple cell-to-cell
370 adhesion and cell polarity cues to the regulation of the downstream transcription factor Yorkie. Among
371 the hypoxia-upregulated genes were several cell polarity/cell adhesion factors (*Ed, dlg1, sdt, baz)* and
372 signaling molecules (*hpo, mats, pez, mts)* that function to negatively regulate Yorkie, suggesting that
373 this may be an important regulator of hypoxia-mediated transcriptional responses. This regulation of
374 Yorkie-mediated transcription may be important for regulation of stem or germ cell division upon

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375 hypoxia in adult flies. Yorkie can also regulate the processes of tracheal formation and immune
376 signaling, which are both important in hypoxia. Recent studies have also shown that the mammalian
377 homolog of Yorkie, Yap1, controls hypoxia-mediated angiogenesis in bone, suggesting that regulation
378 of Hippo/Yorkie signaling may be a conserved hypoxia response (SIVARAJ *et al.* 2020).
379
380 One kinase that showed FOXO-dependent increase in hypoxia was Hipk. We saw that this increase
381 occurred across multiple tissues and was required for flies to survive hypoxia. These results point to
382 Hipk as a regulator of hypoxia tolerance. As well as regulating Hippo/Yorkie signaling, Hipk can
383 modulate other signaling pathways such as JNK, JAK/STAT, Wingless signaling(LEE *et al.* 2009b;
384 HUANG *et al.* 2011; CHEN AND VERHEYEN 2012; POON *et al.* 2012; VERHEYEN *et al.* 2012; BLAQUIERE *et*
385 *al.* 2014; TETTWEILER *et al.* 2019; KINSEY *et al.* 2021; STEINMETZ *et al.* 2021), as well as Notch signaling
386 (LEE *et al.* 2009a), a pathway that we saw enriched in the KEGG analysis of hypoxia-upregulated
387 genes. Thus, Hipk's role in hypoxia tolerance may rely on regulation of any one of these pathways. Hipk
388 has also been shown to induce glycolysis in larval epithelial tissues where it promotes tumor-like
389 overgrowth (WONG *et al.* 2019). Hence, the hypoxia-mediated induction of Hipk may also be needed to
390 induce glycolysis, a widely described metabolic response to low oxygen. Interestingly, a recent report
391 showed that the *C. elegans* homolog of Hipk, hpk1, was needed for survival in low oxygen (DOERING *et*
392 *al.* 2022), suggesting a common role for Hipk in organismal hypoxia tolerance in both worms and flies.
393
394 Among the genes showing reduced expression in hypoxia, we saw strong enrichments for genes
395 involved in egg production and translation. Furthermore, we saw that hypoxia suppressed female
396 fecundity, and reduced translation and tRNA synthesis in both larvae and adults. Egg production is an
397 energetically costly process, and therefore may be suppressed to ensure appropriate allocation of
398 energetic resources to promote survival during stress. This type of trade-off between fecundity and
399 stress responses has been seen in *Drosophila* in response to other environmental challenges. For
400 example, upon infection with bacteria, fungi or viruses, flies have been shown to reduce their
401 reproductive output and capacity (SCHWENKE *et al.* 2016). Moreover, germline deficient females that
402 cannot produce eggs have enhanced immunity compared to fertile flies (SHORT *et al.* 2012). Similarly,
403 nutrient starvation leads to reduced germline stem cell division and reduced egg production in females
404 (DRUMMOND-BARBOSA AND SPRADLING 2001; LAFEVER *et al.* 2010; ABLES *et al.* 2012).
405
406 Protein synthesis is also an energetically costly process that has been estimated to account for at least
407 one-third of a cell's ATP use (BUTTGEREIT AND BRAND 1995). Hence, it is not surprising that suppression
408 of protein synthesis is a conserved response to hypoxia that is seen in many animals and that can
409 promote hypoxia tolerance (HOFMANN AND HAND 1994; HOCHACHKA *et al.* 1996; LIU *et al.* 2006b; VAN
410 DEN BEUCKEN *et al.* 2006; ANDERSON *et al.* 2009; SCOTT *et al.* 2013). Our transcriptomic analyses

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suggest that one way that hypoxia suppresses protein synthesis is by reducing expression of ribosome protein genes via FOXO. We also saw that FOXO was required for hypoxia-mediated suppression of many mitochondrial genes, including mitochondrial ribosomal proteins, mitochondrial transporters, and regulators of oxidative phosphorylation, such as subunits of ATP synthase, Cytochrome oxidase C, and Complex I. These results suggest that FOXO may also contribute to hypoxia tolerance by limiting energetically costly metabolic processes. FOXO suppression of ribosome protein and mitochondrial genes has also been seen in muscle following nutrient starvation in *Drosophila* larvae (TELEMAN *et al.* 2008). Furthermore, a recent study showed that the FOXO homolog in *C. elegans*, daf-16, promotes a hypoxia tolerant phenotype by suppressing ribosomal protein gene expression and partially suppressing genes involved in oxidative phosphorylation (HEMPHILL *et al.* 2022). Hence, reducing both ribosome gene expression and mitochondrial oxidative phosphorylation may be common FOXO-mediated stress responses.

In conclusion, our transcriptome analysis supports a model in which FOXO promotes hypoxia tolerance through controlling the upregulation of cell signaling pathways while suppressing the energetically costly processes of protein synthesis and mitochondrial activity. Given the conserved roles for FOXO in mediating hypoxia tolerance in different animals (SCOTT *et al.* 2002; MENDENHALL *et al.* 2006; MENUZ *et al.* 2009; LIU *et al.* 2016; BARRETTO *et al.* 2020; HEMPHILL *et al.* 2022) and the alterations of FOXO transcription factor activity in diseases associated with hypoxia, such as cancer, stroke, and ischemia (MAIESE *et al.* 2008; FUKUNAGA AND SHIODA 2009; MAIESE *et al.* 2009; LIU *et al.* 2022), our findings highlight processes that may contribute to low oxygen adaptations in both normal and disease states.

## Data availability

The RNA-sequence data has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number: GSE206206 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE206206)


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447

## 448 Conflict of interests

449 The authors declare no competing interests.
450

## 451 Author contributions.

452 K.D., E.C.B and B.L, carried out genetic and molecular experiments in *Drosophila*. M.J. performed
453 bioinformatic analyses. K.D., E.C.B, B.L, and S.S.G. analyzed the data. M.G. and S.S.G. obtained
454 funding. S.S.G. directed the study and wrote the manuscript. All authors helped edit the final
455 manuscript.
456

## 457 Figure Legends

458

459 **Figure 1. Hypoxia-induced alterations in whole-body gene expression.** A) Schematic outline of our
460 experimental approach. B) Volcano plot showing the up- (orange) and down-regulated (blue) genes
461 following hypoxia exposure. Genes were considered differentially expressed if they showed a significant
462 (q-val (FDR corrected p-val) <0.05) change in expression that was > +/- 1.5-fold different in hypoxia vs
463 normoxia. Dashed line indicates q-val = 0.05. C) Heatmap depicting the change in expression (Log2
464 fold change, hypoxia vs normoxia conditions) of previously described hypoxia-induced genes.
465

466 **Figure 2. Hypoxia upregulates mRNA expression of transcription factors and kinase genes.** A) GO
467 analysis (molecular function category), and B) KEGG pathway analysis of genes showing >1.5-fold
468 increase following hypoxia exposure. C) Heatmap depicting the increases in mRNA expression (Log2
469 fold change hypoxia vs normoxia) of transcription factor genes. D) Heatmap depicting the increases in
470 mRNA expression (Log2 fold change hypoxia vs normoxia) of kinase genes.
471

472 **Figure 3. Hipk is a hypoxia-induced gene required for organismal hypoxia tolerance.** A) Venn diagram
473 showing overlap between genes previously shown to have FOXO binding within 1kb, as detected by
474 ChIP, and FOXO-dependent upregulated genes identified in the present study. B) GO analysis
475 (molecular function category) of FOXO-dependent hypoxia induced genes (genes showing a significant
476 >1.5-fold increase in mRNA expression following hypoxia exposure in $w^{1118}$ but not *foxo* mutants). C)
477 Heatmap depicting the increases in mRNA expression (Log2 fold change, hypoxia vs normoxia) of
478 GTPase regulators and kinases in $w^{1118}$ and *foxo* mutants. Blue squares indicate genes previously
479 shown to have FOXO binding within 1kb of the gene as measured by ChIP. D) Heatmap depicting the

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480 increases in mRNA expression (Log2 fold change, hypoxia vs normoxia) of Hippo pathway genes in
481 *$w^{1118}$* and *foxo* mutants. Blue squares indicate genes previously shown to have FOXO binding within
482 1kb of the gene as measured by ChIP. E) qPCR analysis of *hipk* mRNA levels from normoxia vs
483 hypoxia exposed animals. RNA was isolated from either whole animals or specific tissues. Bars
484 represent mean +/- SEM. Symbols represent individual data points, n=4 per condition. * p<0.05,
485 Students t-test. F) Hypoxia survival of control (*daGSG > hipk RNAi*, no RU486) vs *hipk* RNAi (*daGSG >*
486 *Hipk RNAi*, RU486-treated) adult flies. Data are presented as box plots (25%, median and 75% values)
487 with error bars indicating the min and max values, n = 14 groups of flies per condition.
488
489 **Figure 4. Hypoxia downregulates mRNA expression of protein synthesis and egg formation genes and**
490 **leads to reduced translation and decreased fecundity.** A, B) GO analysis (A, biological process category
491 and B, cellular component category) of genes showing >1.5-fold decrease in expression following
492 hypoxia exposure. C, D) qRT-PCR measurement of tRNA levels following C, 2hrs or D, 24hrs of
493 hypoxia exposure in developing larvae. Bars represent mean +/- SEM. Symbols represent individual
494 data points, n=4 per condition. * p<0.05, Students t-test. F) Polysome profiles of normoxia (left) and
495 hypoxia (right) exposed larvae. Plots indicate continuous OD 254nm measurements from fractionated
496 whole-body lysates. Peaks corresponding to 40S, 60S, 80S and polysomes are indicated. The top and
497 bottom lysate fractions from the centrifuged sucrose gradients are indicated. G) Fecundity
498 measurements from mated females exposed to normoxia or 8 or 12 hrs of hypoxia. Data show the
499 mean number of viable pupae per female that developed from eggs laid on day 1 or day 2 following the
500 hypoxia exposure. Bars represent mean +/- SEM. Symbols represent individual data points, n=4 per
501 condition. * p<0.05, Students t-test following two-way ANOVA.
502
503 **Figure 5. Hypoxia downregulation of ribosomal protein and mitochondrial regulator gene expression**
504 **requires FOXO.** A) GO analysis (cell component category) of FOXO-dependent hypoxia suppressed
505 genes (genes showing a significant decrease in mRNA expression following hypoxia exposure in *$w^{1118}$*
506 but not *foxo* mutants). B) Heatmap depicting the decreases in mRNA expression (Log2 fold change,
507 hypoxia vs normoxia) of ribosomal protein genes and mitochondrial regulator genes in *$w^{1118}$* and *foxo*
508 mutants. Colored circles indicate different classes of mitochondrial genes (blue: ATP synthase subunits;
509 orange: mitochondrial ribosomal proteins; green: Cytochrome C oxidase subunits; grey: Complex I
510 subunits; black: mitochondrial transporters).
511
512 **Supplemental Table 1**
513 Processed RNA-seq data, including lists of up- and down-regulated genes.
514
515 # References

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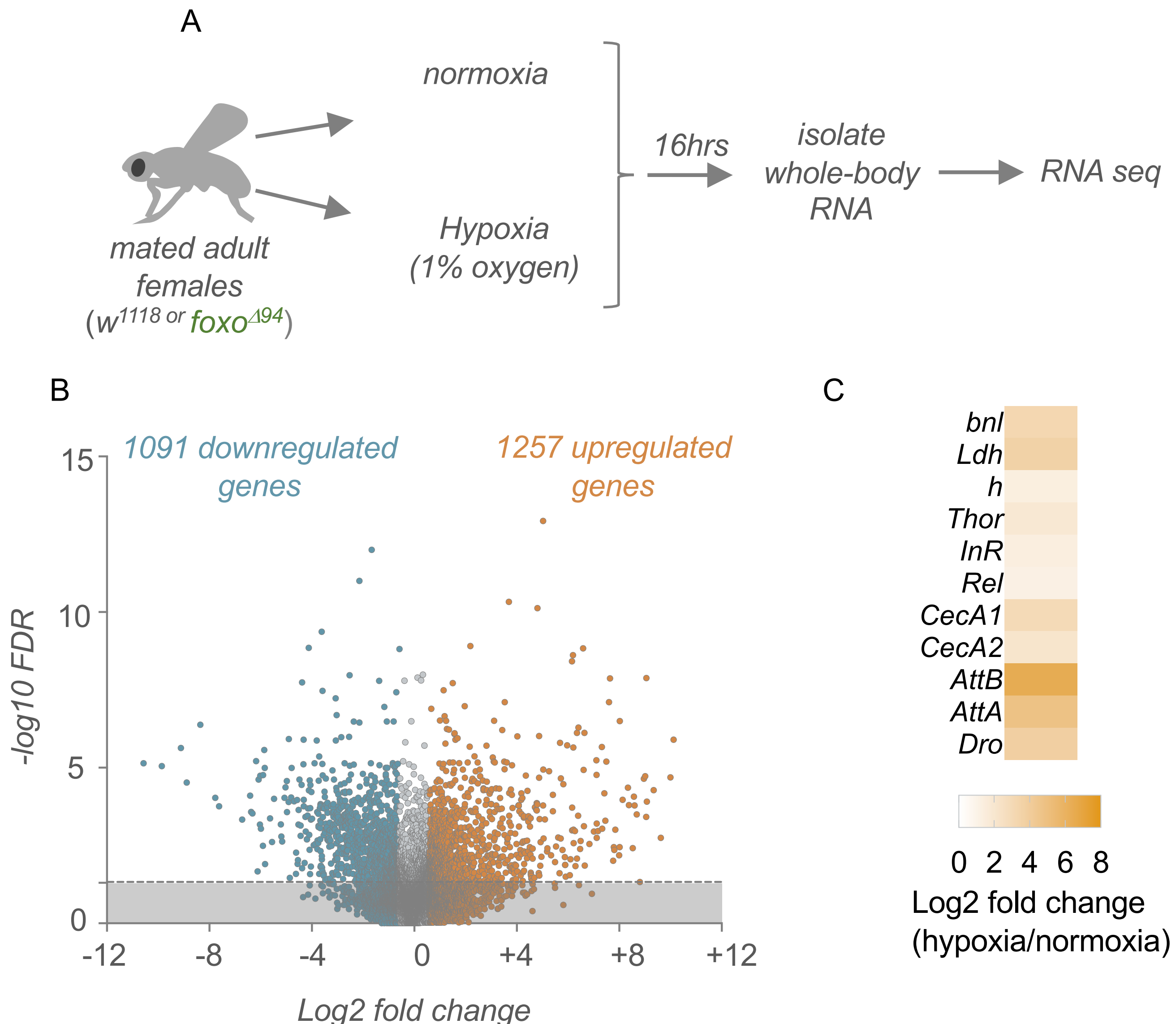


**Figure 1. Hypoxia-induced alterations in whole-body gene expression.** A) Schematic outline of our experimental approach. B) Volcano plot showing the up- (orange) and down-regulated (blue) genes following hypoxia exposure. Genes were considered differentially expressed if they showed a significant (q-val (FDR corrected p-val) <0.05) change in expression that was > +/- 1.5-fold different in hypoxia vs normoxia. Dashed line indicates q-val = 0.05. C) Heatmap depicting the change in expression (Log2 fold change, hypoxia vs normoxia conditions) of previously described hypoxia-induced genes.

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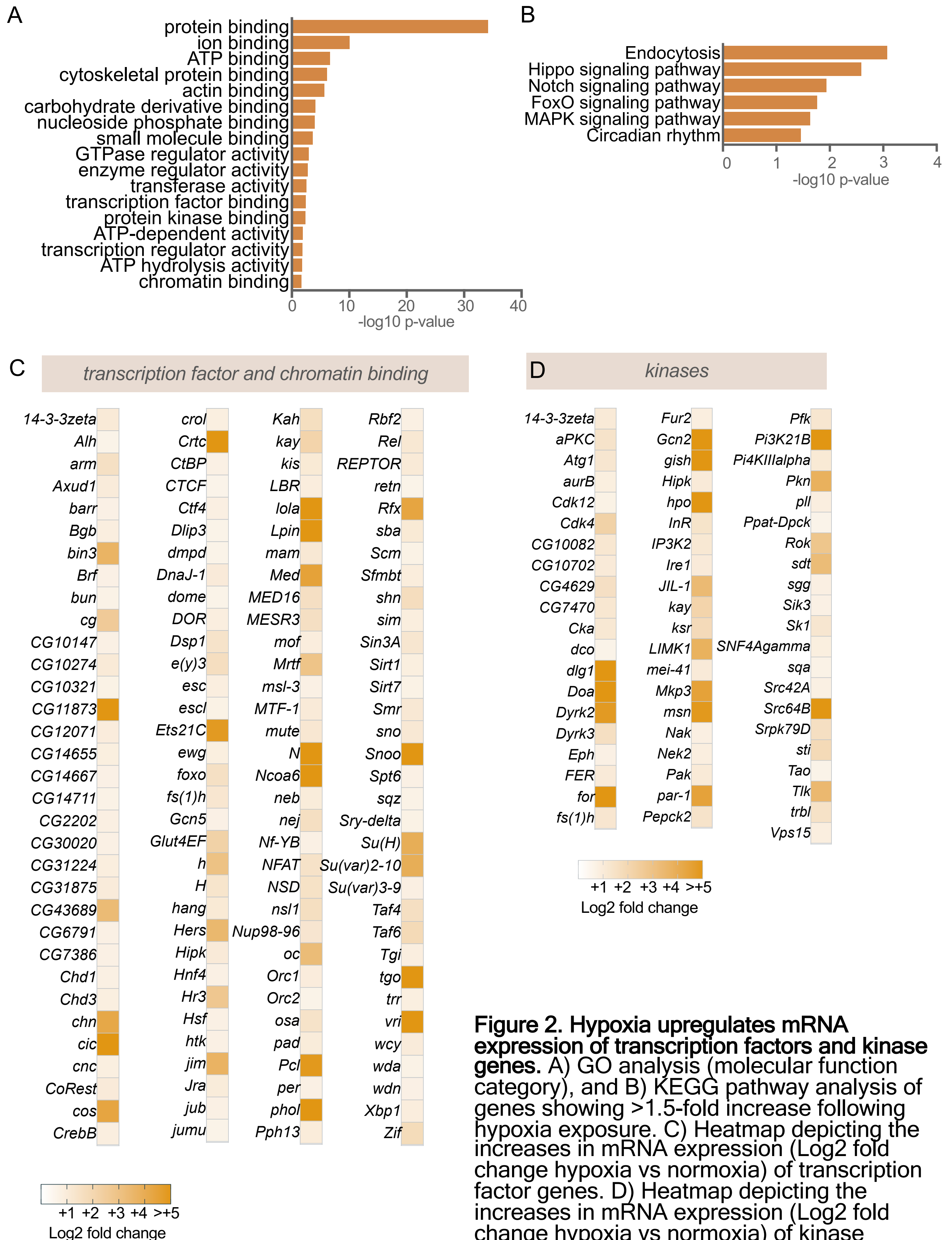


**Figure 2. Hypoxia upregulates mRNA expression of transcription factors and kinase genes.** A) GO analysis (molecular function category), and B) KEGG pathway analysis of genes showing >1.5-fold increase following hypoxia exposure. C) Heatmap depicting the increases in mRNA expression (Log2 fold change hypoxia vs normoxia) of transcription factor genes. D) Heatmap depicting the increases in mRNA expression (Log2 fold change hypoxia vs normoxia) of kinase genes.

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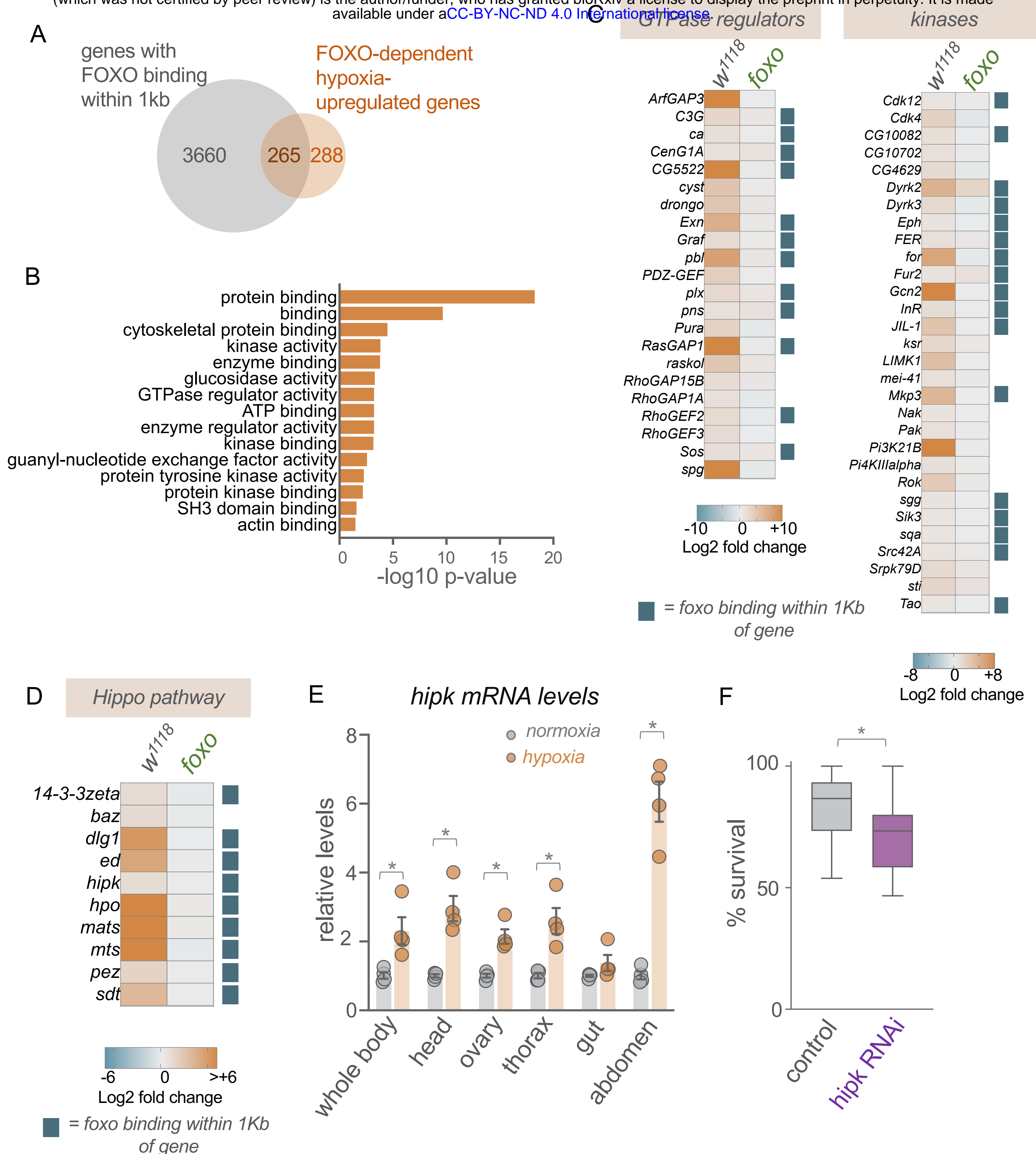


**Figure 3. Hipk is a hypoxia-induced gene required for organismal hypoxia tolerance.** A) Venn diagram showing overlap between genes previously shown to have FOXO binding withing 1kb, as detected by ChIP, and FOXO-dependent upregulated genes identified in the present study. B) GO analysis (molecular function category) of FOXO-dependent hypoxia induced genes (genes showing a significant >1.5-fold increase in mRNA expression following hypoxia exposure in *w*$^{1118}$ but not *foxo* mutants). C) Heatmap depicting the increases in mRNA expression (Log2 fold change, hypoxia vs normoxia) of GTPase regulators and kinases in *w*$^{1118}$ and *foxo* mutants. Blue squares indicate genes previously shown to have FOXO binding within 1kb of the gene as measured by ChIP. D) Heatmap depicting the increases in mRNA expression (Log2 fold change, hypoxia vs normoxia) of Hippo pathway genes in *w*$^{1118}$ and *foxo* mutants. Blue squares indicate genes previously shown to have FOXO binding within 1kb of the gene as measured by ChIP. E) qPCR analysis of *hipk* mRNA levels from normoxia vs hypoxia exposed animals. RNA was isolated from either whole animals or specific tissues. Bars represent mean +/- SEM. Symbols represent individual data points, n=4 per condition. * $p<0.05$, Students t-test. F) Hypoxia survival of control (*daGSG > hipk RNAi*, no RU486) vs *hipk* RNAi (*daGSG > hipk RNAi*, RU486-treated) adult flies. Data are presented as box plots (25%, median and 75% values) with error bars indicating the min and max values, n = 14 groups of flies per condition.

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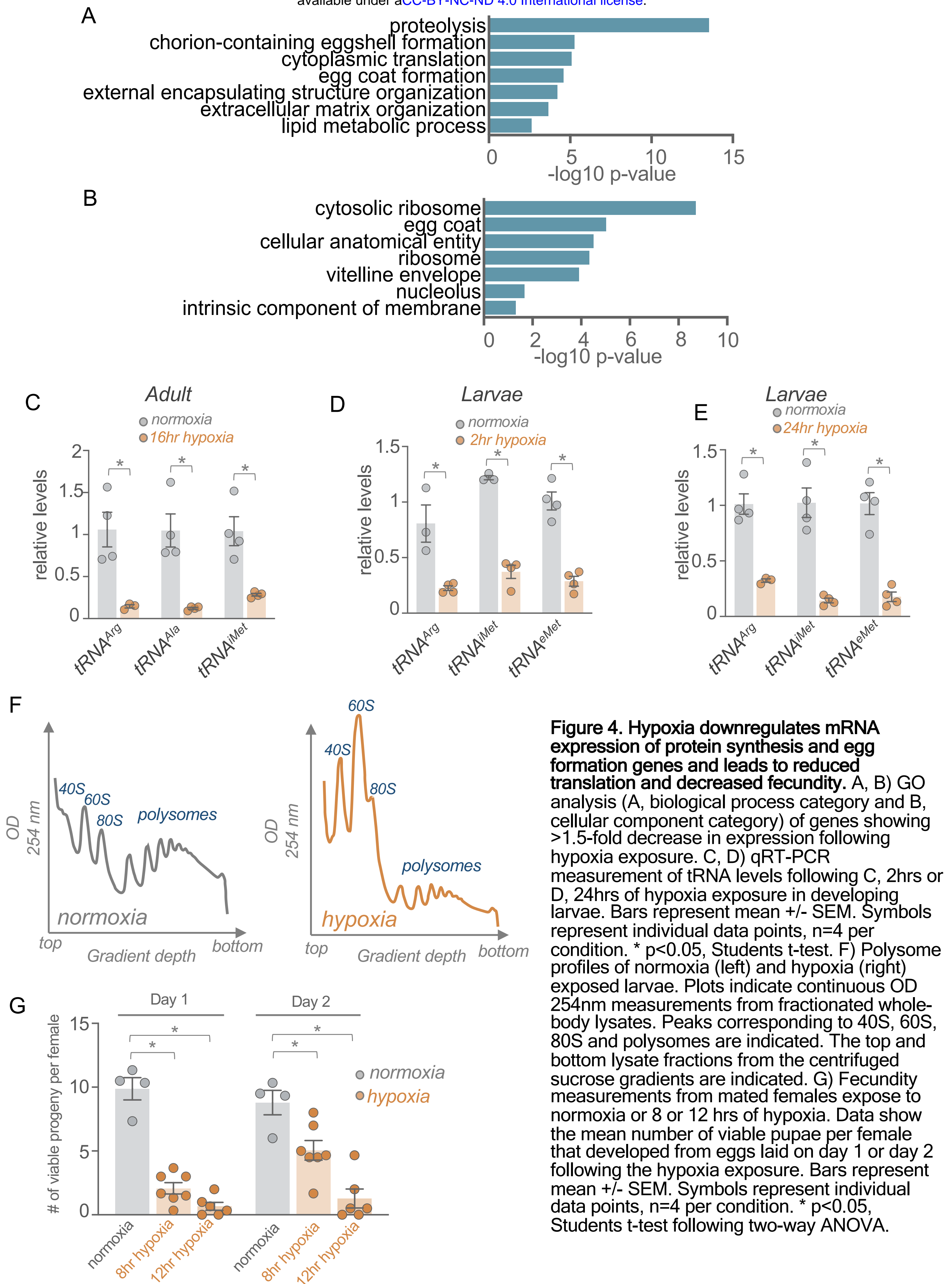


**Figure 4. Hypoxia downregulates mRNA expression of protein synthesis and egg formation genes and leads to reduced translation and decreased fecundity.** A, B) GO analysis (A, biological process category and B, cellular component category) of genes showing >1.5-fold decrease in expression following hypoxia exposure. C, D) qRT-PCR measurement of tRNA levels following C, 2hrs or D, 24hrs of hypoxia exposure in developing larvae. Bars represent mean +/- SEM. Symbols represent individual data points, n=4 per condition. * p<0.05, Students t-test. F) Polysome profiles of normoxia (left) and hypoxia (right) exposed larvae. Plots indicate continuous OD 254nm measurements from fractionated whole-body lysates. Peaks corresponding to 40S, 60S, 80S and polysomes are indicated. The top and bottom lysate fractions from the centrifuged sucrose gradients are indicated. G) Fecundity measurements from mated females expose to normoxia or 8 or 12 hrs of hypoxia. Data show the mean number of viable pupae per female that developed from eggs laid on day 1 or day 2 following the hypoxia exposure. Bars represent mean +/- SEM. Symbols represent individual data points, n=4 per condition. * p<0.05, Students t-test following two-way ANOVA.

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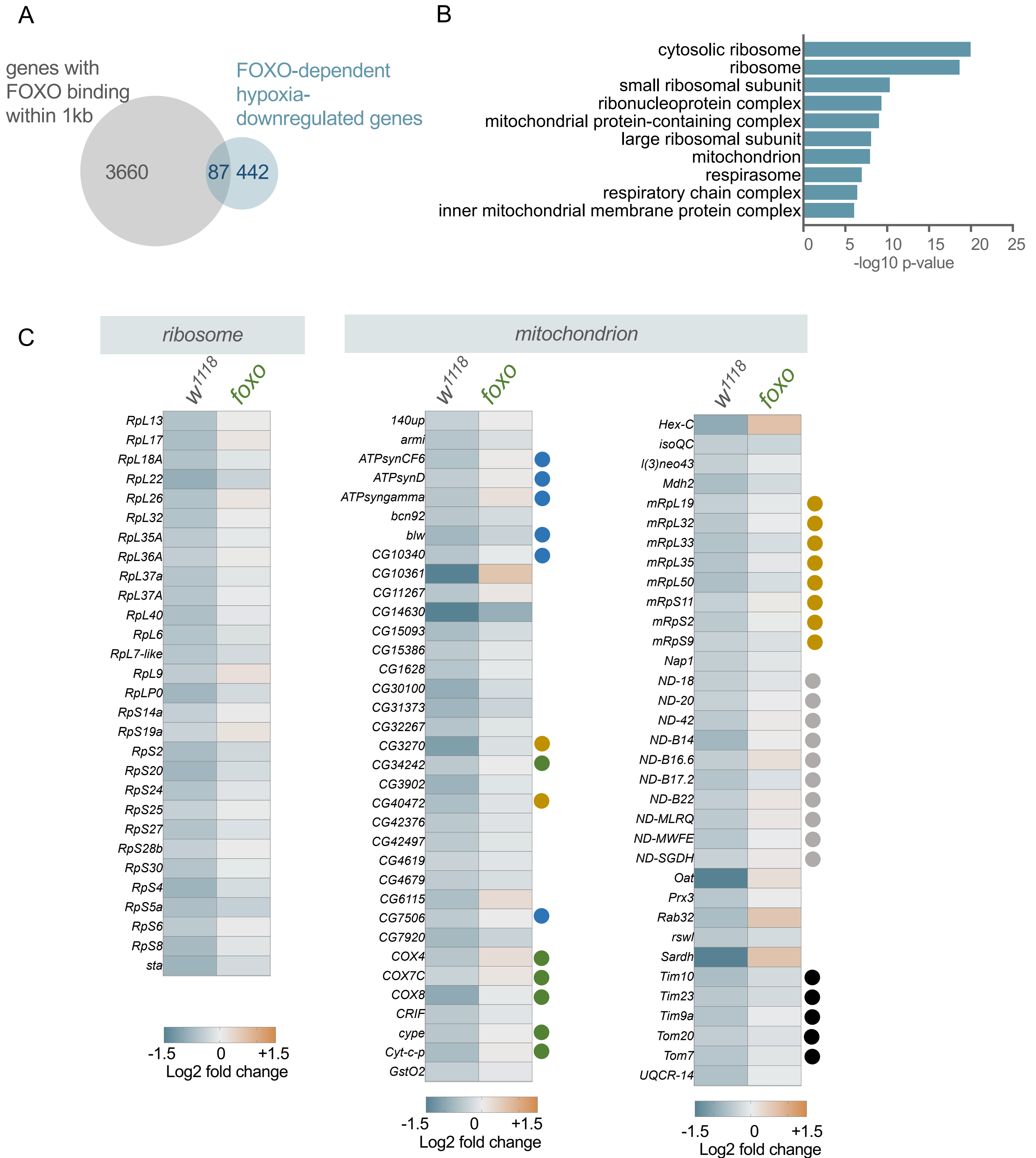


**Figure 5. Hypoxia downregulation of ribosomal protein and mitochondrial regulator gene expression requires FOXO.** A) GO analysis (cell component category) of FOXO-dependent hypoxia suppressed genes (genes showing a significant decrease in mRNA expression following hypoxia exposure in $w^{1118}$ but not *foxo* mutants). B) Heatmap depicting the decreases in mRNA expression (Log2 fold change, hypoxia vs normoxia) of ribosomal protein genes and mitochondrial regulator genes in $w^{1118}$ and *foxo* mutants. Colored circles indicate different classes of mitochondrial genes (blue: ATP synthase subunits; orange: mitochondrial ribosomal proteins; green: Cytochrome C oxidase subunits; grey: Complex I subunits; black: mitochondrial transporters).