Identification of Genes Underlying Hypoxia Tolerance in Drosophila by a P-element Screen

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ABSTRACT Hypoxia occurs in physiologic conditions (e.g. high altitude) or during pathologic states (e.g. ischemia). Our research is focused on understanding the molecular mechanisms that lead to adaptation and survival or injury to hypoxic stress using Drosophila as a model system. To identify genes involved in hypoxia tolerance, we screened the P-SUP P-element insertion lines available for all the chromosomes of Drosophila. We screened for the eclosion rates of embryos developing under 5% O2 condition and the number of adult flies surviving one week after eclosion in the same hypoxic environment. Out of 2187 lines (covering ~1870 genes) screened, 44 P-element lines representing 44 individual genes had significantly higher eclosion rates (i.e. >70%) than those of the controls (i.e. ~7–8%) under hypoxia. The molecular function of these candidate genes ranged from cell cycle regulation, DNA or protein binding, GTP binding activity, and transcriptional regulators. In addition, based on pathway analysis, we found these genes are involved in multiple pathways, such as Notch, Wnt, Jnk, and Hedgehog. Particularly, we found that 20 out of the 44 candidate genes are linked to Notch signaling pathway, strongly suggesting that this pathway is essential for hypoxia tolerance in flies. By employing the UAS/RNAi-Gal4 system, we discovered that genes such as osa (linked to Wnt and Notch pathways) and lqf (Notch regulator) play an important role in survival and development under hypoxia in Drosophila. Based on these results and our previous studies, we conclude that hypoxia tolerance is a polygenic trait including the Notch pathway.

Whether in pathological conditions or at high altitude, hypoxia can severely affect survival, early development, and fitness of an organism (Mishra and Delivoria-Papadoopoulos 1999; Shimoda and Semenza 2011; Webster and Abela 2007). Depending on the duration and severity of hypoxia, cell type, tissue, or organism, the injury caused by hypoxia could be significant and irreversible. Hence, it can result in long-term morbidity and mortality in humans, especially in infants (Ramachandrapa et al. 2011). To maintain function and homeostasis, cells sense and respond to inadequate oxygen levels (De Bels et al. 2011; Kappler et al. 2011; Semenza 2011). Some aspects of the response involve changes in gene expression, and a number of studies have identified various sensitivities of cells and organisms to hypoxic stress (Anderson et al. 2009; Clerici and Planes 2009; De Bels et al. 2011; Koyama et al. 2011; Larson and Park 2009), including a variety of genetic pathways and mechanisms that can potentially affect the response to hypoxia.

Hypoxia-tolerant organisms, such as the African naked mole-rats, Crucian carp, aquatic turtles, and fruit flies, provide a unique opportunity to study the effect of genes influencing hypoxia tolerance or injury in vivo (Hochachka et al. 1997; Larson and Park 2009; Nilsson and Renshaw 2004). The added advantages of using Drosophila as a model system is that their genome has been sequenced, many human disease genes are conserved in Drosophila, and a number of genetic tools and stocks are available for manipulation of genes in vivo. In particular, there is a vast array of single transposon insertions covering almost the entire Drosophila genome (Bellen et al. 2004; Spradling et al. 1999). We have chosen to perform an unbiased screen of P-Sup P-element lines covering a large portion of the Drosophila genome to determine the potentially interesting genes in hypoxia tolerance.
Table 1 Percentage eclosion and number of adult flies surviving in controls (CS, yw) and P-element lines at 5% O2

| Gene Symbol | Chr | % Eclosion | Adult Flies | % Pupration | Molecular Function | Human Orthologs |
|-------------|-----|------------|-------------|--------------|-------------------|-----------------|
| CS(control) |       | 6.8 ± 0.67 | 1 ± 0.03    | 85.7 ± 5.68 | Guanyl-nucleotide exchange factor activity | Pleckstrin homology domain containing, family F (with FYVE domain) member 2/ PLEKHF2 |
| yw(control) |       | 7.5 ± 2.15 | 0 ± 0.67    | 81.5 ± 10.25 |                   |                 |
| CG14782     | X   | 75 ± 10.5  | 10 ± 5.4    | 97 ± 6.7     |                   |                 |
| CG15742     | X   | 75 ± 13.3  | 4 ± 0.9     | 89 ± 10.12   | Unknown           |                 |
| CG9413      | X   | 80 ± 8.9   | 10 ± 5.8    | 78 ± 5.15    | Amino acid trasmembrane transporter activity | Solute carrier family 7 (glycoprotein-associated amino acid transporter light chain, bo,+ system), member 9/ SLC7A9 |
| Dip1        | X   | 72 ± 9.9   | 8 ± 2.3     | 75 ± 3.22    | Double-stranded RNA binding |                 |
| CG10700     | 2   | 84.5 ± 0.95| 20 ± 2.5    | 78 ± 10.2    | Electron carrier activity; FAD binding |                 |
| CG2915      | 2   | 74 ± 12    | 21 ± 1.8    | 69 ± 5.67    | Metalloproteinase activity, zinc ion binding |                 |
| CG30169     | 2   | 76 ± 23    | 5 ± 1.2     | 72 ± 12.35   | Unknown           |                 |
| CG4612      | 2   | 71 ± 0.45  | 22 ± 6.7    | 89 ± 10.42   | mRNA binding; poly(A) binding; nucleotide binding |                 |
| CG6230      | 2   | 88 ± 3.5   | 47 ± 10.6   | 82 ± 12.5    | ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism; ATP binding | ATPase type 13A1/ ATP13A1 |
| CG6860      | 2   | 90.47 ± 5.7| 23 ± 2.5    | 80 ± 13.45   | Protein binding | Leucine-rich and calponin homology (CH) domain containing 1/ LRCH1 |
| CG8677      | 2   | 82.1 ± 7.4 | 3 ± 0.5     | 73.1 ± 9.4   | Transcription repressor activity; protein binding; zinc ion binding | Cat eye syndrome chromosome region, candidate 2/ CECR2 |
| cpa         | 2   | 90 ± 3.6   | 42 ± 9      | 85 ± 11.5    | Actin binding     | Capping protein (actin filament) muscle Z-line, alpha 1/ CAPZA1 |
| CycE        | 2   | 70.4 ± 4.8 | 14 ± 6.8    | 72 ± 4.2     | Cyclin-dependent protein kinase regulator activity |                 |
| Drp1        | 2   | 72.5 ± 7.5 | 12 ± 4.8    | 73 ± 6.77    | GTP binding; GTPase activity |                 |
| Fak56D      | 2   | 75.19 ± 0.57| 5 ± 0.99   | 72.0 ± 10.22 | Protein tyrosine kinase activity |                 |
| mRp518B     | 2   | 88 ± 3.5   | 3 ± 1.3     | 76 ± 11.34   | Mitochondrial ribosomal protein, structural constituent of ribosome |                 |
| Mys45A      | 2   | 89 ± 6     | 20 ± 7.9    | 81 ± 12.6    | Binding           | SDA1 domain containing 1/ SDA1 |
| Rep2        | 2   | 87.2 ± 2.25| 39 ± 2.6    | 75.3 ± 10.27 | Protein binding | Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)/ MLLT10 |
| Alln        | 3   | 76 ± 4.5   | 5 ± 0.77    | 75 ± 8.77    | Transcription factor activity | Unc-51-like kinase 2 (C. elegans)/ ULK2 |
| Atg1        | 3   | 88 ± 2.99  | 20 ± 3.6    | 93 ± 6.90    | Protein kinase activity; protein serine/threonine kinase activity; kinase binding; kinase activity; ATP binding | Core-binding factor, beta subunit/ CBFB |
| Bgb         | 3   | 87 ± 3.78  | 7 ± 1.3     | 77 ± 5.12    | Positive regulation of transcription from RNA polymerase II promoter | GULP, engulfment adaptor PTB domain containing 1/ GULP1 |
| ced-6       | 3   | 73 ± 10.6  | 3 ± 0       | 83 ± 9.9     | Protein binding |                 |
| CG14185     | 3   | 83 ± 5.66  | 8 ± 3.44    | 69 ± 14.65   | Protein binding | Adenylosuccinate synthase activity; ADSS |
| CG17273     | 3   | 86.7 ± 20.1| 10 ± 2.3    | 82.7 ± 6.8   | GTP binding | Adenylosuccinate synthase/ ADSS |
| CG32064     | 3   | 84.4 ± 4.5 | 30 ± 2.6    | 80 ± 9.23    | Proteolysis | Monoxygenase, DBH-like 1/ MOX1 |
| CG33169     | 3   | 76.5 ± 7.99| 11 ± 2.7    | 96.5 ± 10.55 | Unknown |                 |
| CG5233      | 3   | 89 ± 9.7   | 16 ± 5.6    | 77 ± 12.6    | Dopamine beta-monooxygenase activity |                 |
| CG6028      | 3   | 75 ± 10.89 | 20 ± 2.45   | 72 ± 9.8     | GTP binding | Fumarylacetocacetate hydrolase domain containing 2A/ FAHD2A |
| CG8116      | 3   | 89.2 ± 6.25| 26 ± 12.7   | 92.2 ± 17.5  | Unknown | Transmembrane protein 216/ TMEM216 |
| CG8177      | 3   | 79 ± 8.97  | 10 ± 3.33   | 73 ± 3.2     | Anion exchanger activity; inorganic anion exchanger activity | Solute carrier family 4, anion exchanger, member 3/ SLC4A3 |
| CG8180      | 3   | 86 ± 1.33  | 7 ± 2.3     | 78 ± 7.8     | Unknown |                 |
| CG9737      | 3   | 77.6 ± 8.9 | 9 ± 2.2     | 80.6 ± 4.5   | Proteolysis; phagocytosis, engulfment |                 |
| chb         | 3   | 70.8 ± 1.22| 15 ± 1.2    | 90.2 ± 13.75 | GTP binding; microtubule binding | Cytoplasmic linker associated protein 1/ CLASP1 |

(continued)
Table 1, continued

| Gene Symbol | Chr | % Eclosion | Adult Flies | Pupariation | Molecular Function | Human Orthologs |
|-------------|-----|------------|-------------|-------------|--------------------|----------------|
| Chro        | 3   | 80 ± 7.9   | 7 ± 2       | 93 ± 6.49   | Chromatin binding  |                |
| l(3)mbn     | 3   | 85 ± 6.79  | 32 ± 3.9    | 79 ± 8.5    | Plasmacyte differen-|                |
| liq         | 3   | 90.3 ± 3.5 | 3 ± 0.22    | 93 ± 15.2   | Regulation of Notch |                |
| Manf        | 3   | 86 ± 3.57  | 5 ± 2.22    | 92 ± 9.2    | Neuron maintenance; |                |
|             |     |            |             |             | neuron projection  |                |
|             |     |            |             |             | development        |                |
| osa         | 3   | 86.3 ± 9.9 | 58 ± 10.2   | 98.5 ± 10.3 | DNA binding; trans- |                |
|             |     |            |             |             |cription coactivator |                |
| polo        | 3   | 80 ± 2.35  | 11 ± 1      | 99 ± 10.34  | Cell cycle; protein |                |
|             |     |            |             |             | kinase activity     |                |
|             |     |            |             |             | Cell cycle; establish- |                |
|             |     |            |             |             | ment or mainten-     |                |
|             |     |            |             |             |ance of chromatin    |                |
|             |     |            |             |             | architecture; chro- |                |
|             |     |            |             |             |mosome organiza-     |                |
|             |     |            |             |             |tion                |                |
| Scrib       | 3   | 90 ± 2.1   | 18 ± 2      | 86 ± 10.7   | Protein binding     |                |
| sec8        | 3   | 85 ± 2     | 36 ± 6.9    | 77 ± 7.89   | Neurotransmitter     |                |
|             |     |            |             |             | secretion           |                |
| tna         | 3   | 89 ± 9.86  | 20 ± 4.22   | 85 ± 12.5   | Chromatin-mediated   |                |
|             |     |            |             |             | maintenance         |                |
|             |     |            |             |             | of transcription    |                |
| ci          | 4   | 89 ± 6.77  | 5 ± 1.77    | 95 ± 8.95   | Protein binding, cell|                |
|             |     |            |             |             | cycle regulation    |                |

Also shown are human orthologs of the candidate genes.

MATERIALS AND METHODS

Fly stocks

P{SUPor-P} (Roseman et al. 1995) P-element set for chromosomes X, 2, 3, and Y were obtained from the Bloomington Drosophila Stock Center (Bloomington, Indiana, USA). A list of all the genes included in our P-element screen is attached as supporting information, Table S2. The UAS, TRIP, and RNAi lines were obtained from the Bloomington Drosophila Stock Center and Vienna Drosophila RNAi Center (Vienna, Austria), respectively. Osa gene stocks were kindly provided by Dr. Jessica Treisman (NYU School of Medicine). The Gal4 drivers da, Eaat1, Elav, P[GawB]c739, P[GawB]D667, He, and Hml were obtained from the Bloomington Drosophila Stock Center.

P-element screening for hypoxia tolerance

The P-element lines were tested for hypoxia tolerance based on two phenotypes: (1) eclosion rates at 5% O2 and (2) adult flies that survived post eclosion at 5% O2.

Eclosion rates at 5% O2: For each P-element line, 50 females and males were put in a vial with standard corn-meal food. After allowing females to lay eggs for about 6 hr (to obtain about ≥100 eggs), the vials were cleared and the eggs were put under 5% O2 for 4 weeks in specially designed computerized chambers (Model A44x0, Biospherix, Redfield, NY) and ANA-Win2 Software (Version 2.4.17, Watlow Ana- faze, CA). After 4 weeks, the number of eclosed and un-eclosed pupae was counted, and the percentage eclosion was calculated for each P-element line tested. Percentage eclosion was determined by calculating the ratio of the number of empty pupae to the total number of pupae in each culture vial. In our screen, we maintained a minimum pupariation of 50% to ensure that the percentage eclosion rate was not biased based on pupae number. We and others have shown that in the Drosophila life cycle, the pupal stage is a critical oxygen-sensitive stage, and hence, we chose this phenotype for our screen (Heinrich et al. 2011; Peck and Maddrell 2005; Zhou et al. 2007). Particularly, we have observed that eclosion under hypoxia for controls is severely affected by hypoxia (eclosion rate less than 10%). The lines that showed percentage eclosion >70% were re-tested at least three times, starting with 100–150 eggs at 5% O2, to confirm the results. We chose a 70% cut off since it was significantly higher than all the control fly types (7–8%) and driver fly stocks (45–50%).

Adult flies that survived post eclosion at 5% O2: For each line (each P-element line retested as well as controls), we started with 100–150 eggs in the vial and kept them at 5% O2 for 4 weeks, and then counted the average number of adults that survived one week after eclosion.

Real-time PCR analysis of P-element lines

Total RNA was extracted from flies (yw-control and P-elements) under normoxia, using Trizol (Invitrogen, Carlsbad, CA). cDNA was produced from total RNA through RT-PCR using Superscript III First-Strand Synthesis system (Invitrogen).

Real-time PCR was performed using a GeneAmp 7500 sequence detection system using POWER SYBR Green chemistry (Applied Biosystems, Foster City, CA). The expression level of Actin was used to normalize the results (fwd: CTAACCTCGCCCTCTCCTCT; rev: GCAGCCAAGTGTGAGTGTGT). The fold change was calculated using expression level of yw in normoxia as well as hypoxia, which was used as control for all the P-element lines. P-elements with eclosion rate of greater than 85% were tested with real-time PCR. The primer information for the P-elements genes is provided in Table S1.

Tissue-specific upregulation or downregulation of genes from P-element screen

Depending on the expression of genes in the P-element lines, UAS or RNAi stock of genes were used to overexpress or knockdown the expression of the genes ubiquitously or in specific tissues in the F1 progeny using various Gal4 drivers. The Gal4 drivers used were da (expresses in all tissues), Eaat1 (glial cells), elav-Gal4 [neurons-nervous system (CNS and PNS)], P[GawB]c739 [strong expression in alpha and beta lobe Kenyon cells (intrinsic neurons) of the Mushroom bodies], P[GawB]D667 (adult muscles), He-Gal4 (hemocytes), and Hml-Gal4 (larval hemocytes). In the F1 progeny, eclosion rates were calculated after 4 weeks under 5% O2 for one developmental cycle (egg-adult). Unpaired Student t-tests were used to calculate significant differences in the percentage eclosion of each P-element line, or F1-progeny of UAS, TRIP, or RNAi lines and Gal4 drivers compared with the controls.
Data analysis and statistical tests
For selection of strongly hypoxia tolerant line we chose a cutoff of >70% eclosion which was 10-fold higher than CS control eclosion rate. The gene ontology (GO)-based analyses were performed using GenMAPP software (Dahlquist et al. 2002). The pathway analysis of the candidate genes was done using DAVID program utilizing KEGG and PANTHER, as well as FLIGHT, databases (Huang da et al. 2009; Mourikis et al. 2010; Saj et al. 2010; Sims et al. 2006).

RESULTS
Genome-wide P-element screen for hypoxia tolerance genes
To identify genes involved in hypoxia tolerance, we screened P-element insertion lines generated by BDGP Gene Disruption Project (Bellen et al. 2004; Roseman et al. 1995). We specifically chose SuP or P insertion lines because these P-elements were designed to maximally disrupt genes (Bellen et al. 2004; Lukacsovich et al. 2001; Roseman et al. 1995). Out of 2187 lines (covering ~1870 genes) screened, 44 P-element lines (44 genes) had rather high eclosion rates (>70% eclosion). Table 1 and Figure 1 show the eclosion rates (each line was retested starting with 100–150 eggs in each vial) and the average number of adult eclosed flies surviving under 5% O₂ for the P-elements lines that were hypoxia-tolerant. Table 1 also shows the human orthologs of the genes found in our screen. In this screen, we found certain interesting candidate genes, such as sec8, cpa, cyclin E, osa, l(3)mbn, Alb, and tna, which show remarkable (70–80%) eclosion rates and hypoxia tolerance during all stages of the developmental cycle (egg to adult) (Table 1 and Figure 1). The eclosion rate of the controls and P-element lines was 98–100%, in normoxia.

Functional categorization of candidate genes
GO and pathway analyses were performed to determine the predominant biological processes and pathways that are potentially regulated by the candidate genes and play a role in hypoxia tolerance. The biological process categories in which these candidate genes were overrepresented include spindle organization, synaptic vesicle endocytosis and transport, regulation of transcription, and cell cycle (Figure 2A). The molecular function of the mutated genes in the hypoxia-resistant P-element insertion lines ranged from...
transcriptional co-regulators, to DNA or protein binding, to ATP and GTP binding, to carrier activity, to metalloexopeptidase and exopeptidase activity (Figure 2B and Table 1). For example, we found that P-element lines of a number of transcriptional regulators, such as osa, Alh, and tna, had a strong hypoxia resistance phenotype. Starting with 100–150 eggs, we observed that the downregulated osa line had a high eclosion rate (86%) and that the average number of flies that survived after eclosion are ~58 flies (>50%), which is significantly higher compared with controls (eclosion rate 7%, and average number of adults surviving after eclosion <2). Table 2 shows the pathways related to the 44 candidate genes found in our screen. It is intriguing that we find a strong link to Notch pathway (20 genes/44 genes), but at the same time, we also discovered other pathways, such Wnt, Erk, Hedgehog or JAK/STAT, and VEGF pathways, that seem to play important roles in hypoxia tolerance.

Overexpression or knockdown of single genes and hypoxia tolerance

Before we studied the role of each differentially expressed gene, we performed real-time PCR, as shown in Figure 3. PCR showed that in these P-elements, the expression of some of the genes was indeed significantly altered under normoxia and hypoxia (Figure 3). For example, sec8, osa, and tna were significantly downregulated, and l(3)mbn, Alh, lqf, CG5235, atg1, and ci were more than 1.5-fold upregulated. To understand the mechanisms underlying hypoxia tolerance in vivo, we overexpressed (using the UAS-Gal4 system) or knocked down (RNAi) these genes ubiquitously (e.g. da-gal4 drivers) or in specific tissues, depending on whether these particular genes were upregulated or downregulated in the P-elements (Figure 4). We chose to study in detail 4 genes (out of the 44 from our initial screen) based on the following criteria: a) they showed a strong hypoxia phenotype [e.g. the osa gene had a percentage eclosion of 86.3 ± 9.9 and had the highest average number of flies (58 ± 10.2) that survived post eclosion]; b) they showed a clearly significant upregulation or downregulation in the P-element line by real-time PCR; and c) availability of fly lines (either UAS or RNAi) and mutants to further study their effect in vivo. Hence, we decided to further study the following genes: osa, lqf, tna, and sec8 (Figure 4). Indeed we found that the upregulation or downregulation of these genes in these P-element lines had a functional significance under hypoxia. When we upregulated or knocked down the genes using UAS, TRIP, or RNAi lines and ubiquitous da-GAL4 driver, the resulting F1 progeny and mutant stocks matched the phenotype we observed in the P-element lines under hypoxia. For example, we found that knockdown of osa (either by a TRIP RNAi or with a hypomorph mutant) leads to a tremendous increase of eclosion of flies in hypoxia (P < 0.05; Figure 4). We also tested the artificial constructs of osa gene in which the gene was attached to a constitutive activating or repressor domain (Collins et al. 1999). Our results showed that constitutive repression of osa (as seen in F1-UAS-osarDXdaga4) leads to better eclosion under hypoxia, whereas upregulation of osa (F1-UAS-osaxdaga4 or F1-UAS-osadxada4) leads to significantly lower eclosion rate under hypoxia. This is consistent with the hypothesis that knockdown or loss of osa expression leads to significantly better eclosion of flies at 5% O2, indicating that osa is a repressor of genes that are important in hypoxia tolerance. Similarly, we found that an in vivo loss of sec8 and tna function gives a survival advantage for eclosion in 5% O2. In contrast, an upregulation of the lqf gene (F1-UASlqfxdaga4) significantly increases (98% eclosion compared with controls with eclosion rate of 7%) the eclosion rate of flies under hypoxia. Knockdown of lqf in mutant stocks IqflAR1, FDDR9, and F1-TRIP RNAIXda-gal4) tremendously reduced eclosion rates (Figure 4). This is very intriguing as lqf is a Notch regulator, and we have previously shown the importance of Notch in hypoxia adaption in flies (Zhou et al. 2011).

Tissue-specific overexpression or knockdown of osa and lqf genes

To determine whether there is any tissue-specific effect of knockdown or overexpression in various tissues such as the central nervous system, we utilized progenies of crosses made with specific GAL4 drivers. We then subjected the F1 progeny of such crosses to 5% O2 and quantified eclosion rates. As depicted in Figure 5, our data show that the specific knockdown of osa in the nervous system (elav-gal4) and mushroom body (MB) of the brain has an opposite effect on eclosion, as compared with increasing its expression ubiquitously (i.e. its knockdown in these tissues decreased eclosion rates) (Figures 4 and 5). This suggests that osa has a specific role in
| Symbol     | Gene     | Signaling Pathway |
|------------|----------|-------------------|
| CG15742    | CG15742  | JNK modifier      |
| Dip1       | CG15367  | 1) Innate immunity 2) Notch signaling |
| CG14782    | CG14782  | 1) JNK modifier 2) Notch signaling |
| CG9413     | CG9413   | Not detected      |
| CG2915     | CG2915   | Not detected      |
| mRps18B    | CG10757  | Notch signaling   |
| Mys45A     | CG8070   | 1) Lipid storage 2) Notch signaling 3) Cardiogenic genes |
| CG6230     | CG6230   | Notch signaling   |
| spa        | CG10540  | 1) M. fortuitum infection 2) Morphogenesis 3) Phagocytosis |
| CG4612     | CG4612   | 1) JNK modifier 2) Mito Ca²⁺ and H⁺ regulation |
| CycE       | CG3938   | 1) M. fortuitum infection 2) Morphogenesis 3) Lipid storage 4) miRNA pathway 5) cell cycle 6) p53 pathway 7) Ubiquitination pathway |
| Drp1       | CG3210   | 1) Mito morphology 2) Notch signaling 3) Ca²⁺ signaling (Ca²⁺ entry) 4) Endocytosis |
| Rep2       | CG1975   | Notch signaling   |
| CG8680     | CG6860   | Not detected      |
| Fak56D     | CG10023  | 1) Angiogenesis 2) Integrin signaling pathway 3) VEGF signaling pathway |
| CG10700    | CG10700  | Not detected      |
| CG30169    | CG30169  | Not detected      |
| CG8677     | CG8677   | Not detected      |
| osa        | CG7467   | 1) Wnt signaling 2) Mito Ca²⁺ and H⁺ regulation 3) Notch signaling |
| CG32064    | CG32064  | 1) Glutathione metabolism 2) Sesquiterpenoid and triterpenoid biosynthesis in Urea cycle metabolism  |
| CG8116     | CG8116   | Notch signaling   |
| seeB       | CG2095   | 1) E. coli/S. aureus infection 2) Phagocytosis |
| Atg1       | CG10967  | 1) Cell cycle kinase 2) Notch pathway 3) Regulation of autophagy 4) mTOR signaling pathway |
| l(3)mbn    | CG12755  | ERK signaling     |
| CG5235     | CG5235   | Not detected      |
| CG8177     | CG8177   | 1) Multipolar division 2) Ca²⁺ signaling (Ca²⁺ entry inhibition) |
| CG33169    | CG33169  | Notch signaling   |
| CG17273    | CG17273  | 1) Innate immunity 2) Purine metabolism 3) Alanine-aspartate and glutamate metabolism 4) Wnt signaling pathway 5) De novo purine biosynthesis 6) Metabolic pathways |
| CG9737     | CG9737   | Phagocytosis      |
| Chro       | CG10712  | 1) M. fortuitum infection 2) Hedgehog signaling 3) Notch signaling |
| pzm        | CG7752   | 1) JAK/STAT signaling 2) ERK signaling 3) E2F signaling 4) Notch signaling 5) Hedgehog signaling 6) M. fortuitum infection 7) Ca²⁺ signaling |
| ced6       | CG11804  | 1) C. trachomatis infection 2) Ca²⁺ signaling |
| polo       | CG12306  | 1) Cell cycle kinase 2) Kinase cell progression 3) Centrosome number 4) Mitosis 5) Morphogenesis 6) Cytoskeletal morphogenesis 7) DFoxO signaling 8) Notch signaling 9) Phagocytosis 10) Apoptosis pathway 11) Progesterone-mediated oocyte maturation 12) Endocytosis |
| Bgb        | CG7959   | Not detected      |
| Iqf        | CG8532   | 1) Insect dengue virus infection 2) Endocytosis 3) Notch signaling |
| chb        | CG32435  | 1) ERK signaling 2) Tubulin flux 3) Mitosis |
| Scrib      | CG42614  | 1) Innate immunity 2) Cardiogenic genes 3) Notch signaling 4) Ca²⁺ signaling |
| CG8180     | CG8180   | 1) JAK/STAT signaling 2) ERK signaling |
| Alh        | CG10700  | 1) Cell growth and viability 2) Mito Ca²⁺ and H⁺ regulation 3) Notch signaling |
| CG6028     | CG6028   | Not detected      |
| tna        | CG7958   | 1) Cell growth and viability 2) Wnt signaling 3) Notch signaling 4) Hedgehog signaling 5) Ca²⁺ signaling 6) Dpp signaling 7) Interferon-gamma signaling pathway 8) JAK/STAT signaling pathway |
| CG14185    | CG14185  | Notch signaling   |
| Manf       | CG7013   | Not detected      |
| ci         | CG2125   | 1) Hedgehog signaling 2) Notch signaling |

*Signaling pathways are based on DAVID (KECK and PANTHER database) and FLIGHT database.*
the central nervous system and that under hypoxia its loss of function decreases eclosion rates. Knockdown of osa using the muscle-specific driver shows a similar phenotype of strong eclosion rate (90%) as ubiquitous expression (Figures 4 and 5).

Figure 6 shows data related to the lqf gene. We have observed that upregulation of lqf in glial cells leads to a significantly higher eclosion (93%, P < 0.001). Furthermore, specific upregulation of lqf in larval hemocytes increased eclosion (99%, P < 0.001 vs. controls), and its knockdown had an opposite effect. Under 5% O2, knockdown of lqf specifically in the muscles tremendously reduces eclosion rates. This may be linked to the abnormalities in wings and legs caused by loss of lqf expression (Cadavid et al. 2000), but we do not observe any significant lowering of eclosion rates under normoxia. This suggests that under hypoxia, the knockdown of lqf in muscles has a significant impact on development.

DISCUSSION

In the present study, we used a genome-wide P-SUP transposable element screen for hypoxia tolerance during all developmental stages in flies, starting from embryos at 5% O2. Out of 1870 genes screened, 44 genes showed strong hypoxia tolerance phenotype. This is intriguing because this is a relatively small number of genes that show a relation to hypoxia, indicating that there is some specificity between phenotype and genotype. This phenotype of hypoxia tolerance of these P-element lines was strong as they did not only show increased eclosion rate but also the number of flies that survived after eclosion was impressive compared with the wild-type flies. This result indicates that these candidate genes not only help in hypoxia tolerance across development but also in the adult after eclosion. We have examined the role of sec8, osa, tna, and lqf genes in hypoxia tolerance in vivo. These genes have varied molecular and biological functions but have not been previously studied in the context of survival in hypoxia. For instance, sec8 is a part of an evolutionarily conserved eight-subunit protein complex that is required for tethering exocytic carriers to target membranes in eukaryotic cells (Oztan et al. 2007). The liquid facets locus (lf) encodes epsin, a vertebrate protein associated with the clathrin endocytosis complex (Cadavid et al. 2000). Recent studies support the view that many proteins governing membrane sorting during endocytosis participate also in nuclear signaling and transcriptional regulation, mostly by modulating the activity of various nuclear factors (Pyrzynska et al. 2009). A number of these proteins are implicated in the regulation of cell proliferation and tumorigenesis (Pyrzynska et al. 2009). In addition, besides endocytosis, sec8 is also involved in the regulation of synaptic microtubule formation and glutamate receptor trafficking (Liebl et al. 2006). Hence, these genes through their endocytic, neuronal, or transcriptional regulatory function significantly help in hypoxia tolerance.

Osa gene may also be acting as a transcriptional regulator. Indeed it is genetically linked to three other genes found in our present screen (i.e. CycE, All, and tna) (Baig et al. 2010; Gutierrez et al. 2003). Recent studies have suggested an intriguing role for osa, which is to establish a chromatin environment in the regulatory regions of EGFR as well as WNT target genes, making them available for both activators and repressors and facilitating transcription in response to signaling (Collins and Treisman 2000; Terriente-Felix and de Celis 2009). Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes (Collins et al. 1999; Collins and Treisman 2000; Treisman et al. 1997). This osa-mediated repression acts on Groucho/Pangolin complex and specific downstream genes, such as Dpp, nubbin, and ubx of the Wg pathway (Collins et al. 1999; Collins and Treisman 2000; Lopez et al. 2001; Vazquez et al. 1999). It is also noteworthy that osa showed tissue specificity, as its effect in the nervous system is opposite to that when it is expressed ubiquitously. A previous study has shown that osa can negatively regulate proneuronal development through pannier and chip genes through chromatin remodeling (Heitzler et al. 2003). Hence, we can infer that it can act both as a positive...
and negative regulator of transcription, depending on its location and physiological function.

In previous studies in our laboratory, we have shown an effect of Notch on survival and adaptation of flies selected over generations under hypoxia (Fan et al. 2005; Gustafsson et al. 2005; Zhou et al. 2011). Interestingly, in this study, we also find genes linked to Notch pathway as shown in Table 2. This is remarkable as there is no a priori reason for the screen to be biased to one pathway or another. Besides, in our current study no selection or adaptation to long-term hypoxia has been utilized. Nevertheless, a link to the Notch pathway for hypoxia tolerance during one generation is very interesting and would indicate that the Notch pathway is not only important for hypoxia survival in long-term (transgenerational) conditions but also in shorter-term hypoxia, including during development.

It is known that osa and lqf are strongly linked to the Notch pathway (Armstrong et al. 2005; Kankel et al. 2007; Vaccari et al. 2008; Windler and Bilder 2010). In fact, lqf (ortholog of Mammalian Epsin) is a Notch regulator through Delta (Overstreet et al. 2004). Epsin modulates Notch pathway activity in Drosophila and C. elegans (Tian et al. 2004). It interacts with the Notch pathway during multiple Notch-dependant events in Drosophila (Tian et al. 2004). Ligands of the Delta and Serrate must normally be endocytosed in signal-sending cells to activate Notch (Overstreet et al. 2004; Wang and Struhl 2005). It has been shown that only those molecules of Ser and DI that are targeted by ubiquitination to enter the Epsin (vertebrate lqf)-dependent pathway have the capacity to activate Notch (Todi and Paulson 2011; Wang and Struhl 2005). Genetic studies have shown that the BRM complex (composed of brm, osa, and moira) shows a close functional connection with Notch signaling (Armstrong et al. 2005). Hence, these genes could be functioning through the Notch signaling pathway to provide strong hypoxia tolerance. For example, osa is known to affect wing tissue, independent of its effect on the Wnt pathway. This might be related to Notch signaling in these cells as osa is also required to promote DI (Notch ligand) expression in vein territories (Terriere-Felix and de Celis 2009). In addition, through its chromatin-remodeling activity, osa is known to regulate the cell cycle by coordinating cell-cycle progression through downstream genes, such as CycE interaction or string/cdc25 expression, in normal vs. cancer cells (Baig et al. 2010; Brumby et al. 2002; Moskkin et al. 2007). This cell-cycle arrest of cells requires the function of several signaling pathways: Wg, Egfr, and Notch as well as chromatin-remodeling controlling cell proliferation through the Notch pathway. Indeed, in our screen we found that P-element lines affecting CycE as well as Alh (polycistrom gene controlling chromatin-structure) also had strong eclosion under hypoxia. This might be linked to Osa-CycE interaction or Osa-Alh chromatin modeling mediated by Notch regulation (Saj et al. 2010). To study the effect of CycE overexpression in proliferation of bristle lineage in Drosophila, Simon et al. (2009) have shown that Notch acts as a repressor, whereas in Scrib mutants, Notch aids cooperatively in cell proliferation and survival with the Scrib gene (Brumby and Richardson 2003). The Notch signaling pathway and its interaction with ATG1 may be related to the function of Notch in macroautophagy during fly metamorphosis (Kiffin et al. 2006). In a recent study, it has been shown that in...
Drosophila crystal cells, HIF1α/sima activates Notch receptor signaling, which promotes hematocyte survival during both normal hematopoietic development and hypoxic stress (Mukherjee et al. 2011). Hypoxia-inducible factor is considered to be one of the primary regulatory pathways involved in hypoxia tolerance (Wang and Semenza 1993). Our screen included HIF1α/sima P-element line, and we found that the loss of sima in the P-element line showed similar phenotype of eclosion under hypoxia as controls. This is consistent with the previous study that showed that sima loss of function affects development under hypoxia (Centanin et al. 2005). As our screen is based on the phenotype of hypoxia tolerance, it is reassuring to see that the sima mutant did affect hypoxia tolerance and had low eclosion rates (less than 70%). This explains why we could not detect the role of HIF1α/sima, which is a major hypoxia-sensitive pathway, in our study. We also discovered that that hypoxia tolerance is polygenic as other pathways, such as Wnt, JNK, or Hedgehog, were linked to the candidate genes and played a role in hypoxia tolerance (Table 2). Our future goal is to study the mechanism(s) of hypoxia tolerance as mediated by these genes through genetic epistasis or interaction studies.

Other possibilities may also regulate the interplay of Osa and Wg signaling, such as mutual transcriptional regulation of common target genes (Baig et al. 2010). In vertebrates, direct transcriptional regulation of cyclins by SWI/SNF complex (Osa mammalian ortholog) components has been implicated, and mammalian BRG1 (Osa-Brm complex) and β-catenin (the vertebrate ortholog of Armadillo) interact with each other to activate Wnt target genes (Baig et al. 2010). Similarly, other mechanism(s) may be responsible for our observed hypoxia-tolerant phenotype. Our observation of the specific role of lqf in larval hemocytes in eclosion under hypoxia may be related to its autophagic function (Csikos et al. 2009). During the larval stage, hemocytes play an important role in adult and pupal structural remodeling involving both their phagocytic (apoptotic cells) as well as their immune function (Holz et al. 2003). Furthermore, in our screen, we found the tumor suppressor gene, lethal(3) malignant, which is required for the differentiation of hemocytes (Konrad et al. 1994). The P-element line in which this gene was upregulated showed strong eclosion under hypoxia, which reinforces the role of specifc genes affecting hematocyte functions and thereby altering hypoxia tolerance (Azad et al. 2009).

In summary, the P-element screen is a distinct method for identifying genes that lead to hypoxia tolerance in Drosophila. Indeed, by screening 2187 lines, we identified 44 strong hypoxia-tolerant lines (44 genes). The genes found in our screen not only play a role in hypoxia tolerance during development but also help in adult survival one week post eclosion. Of interest, we found that among the 44 lines that seemed hypoxia tolerant, a few genes (Drp1, CG10700, CG30169, l(3)mbn, CG5235, polo, lqf, CG6028, sec8, Cyclin E, Atp1, mRpS18B, and ci) were similar to those in our previous work on the hypoxia-adapted adult flies as well on the adapted Drosophila larvae (Zhou et al. 2007, 2008). This clearly reinforces the potential role of such genes in hypoxia tolerance. Furthermore, in this screen, for the first time we have discovered the distinct role of osa and lqf genes in hypoxia tolerance by over expressing or knocking down these genes in vivo ubiquitously or in specific tissues in Drosophila.

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