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Reversion of lethality and growth defects in Fatiga oxygen-sensor mutant flies by loss of Hypoxia-Inducible Factor-α/Sima

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Hypoxia-Inducible Factor (HIF) prolyl hydroxylase domains (PHDs) have been proposed to act as sensors that have an important role in oxygen homeostasis. In the presence of oxygen, they hydroxylate two specific prolyl residues in HIF-α polypeptides, thereby promoting their proteasomal degradation. So far, however, the developmental consequences of the inactivation of PHDs in higher metazoans have not been reported. Here, we describe novel loss-of-function mutants of *fatiga*, the gene encoding the *Drosophila* PHD oxygen sensor, which manifest growth defects and lethality. We also report a null mutation in dHIF-α/*sima*, which is unable to adapt to hypoxia but is fully viable in normoxic conditions. Strikingly, loss-of-function mutations of *sima* rescued the developmental defects observed in *fatiga* mutants and enabled survival to adulthood. These results indicate that the main functions of Fatiga in development, including control of cell size, involve the regulation of dHIF/Sima.

Keywords: *Drosophila*; hypoxia-inducible factor; oxygen sensor; Sima

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INTRODUCTION

Recent work has led to the definition of widely operative signalling systems that control the transcriptional response to hypoxia through hypoxia-inducible factor (HIF; Maxwell *et al*, 1993; Wang & Semenza, 1993). HIF proteins are a family of α/β-heterodimers in which the common β-subunit is constitutive and the α-subunits are oxygen-regulated by mechanisms that include transcriptional co-activator recruitment, subcellular localization and protein stabilization (Semenza, 2001; Bruick, 2003; Schofield & Ratcliffe, 2004). The regulation of proteasomal degradation of α-subunits has been well characterized in cell culture and in *in vitro* systems. In the presence of oxygen, a series of 2-oxoglutarate and iron-dependent dioxygenases termed PHDs (prolyl hydroxylase domains) hydroxylate specific prolyl residues in the HIF-α oxygen-dependent degradation domain (ODDD; Bruick & McKnight, 2001; Epstein *et al*, 2001), enabling its ubiquitination and proteasomal degradation (Maxwell *et al*, 1999; Ivan *et al*, 2001; Jaakkola *et al*, 2001). As molecular oxygen is absolutely required in the prolyl hydroxylation reaction and enzyme activity is sensitive to mild hypoxia, the PHDs have suitable characteristics that enable them to function as bona fide oxygen sensors that determine the half-life of HIF-α proteins (Semenza, 2001), thereby controlling hypoxia-dependent transcription. Analyses of 'knockout' mouse strains have shown developmental roles of mammalian HIF proteins (Iyer *et al*, 1998; Adelman *et al*, 2000; Tomita *et al*, 2003; Covello & Simon, 2004; Pfander *et al*, 2004). They are required for the normal formation of the heart, brain, vasculature, cartilage and placenta, suggesting that fetal oxygen availability might have a role in these processes. However, this question remains open, and the developmental effects of genetic inactivation of the oxygen-sensitive PHD pathways have not yet been defined (Pugh & Ratcliffe, 2003).

We have previously reported that the *Drosophila* bHLH-PAS proteins Similar (Sima) and Tango (Tgo) are, respectively, the functional homologues of HIF-α and HIF-β in the fly (Lavista-Llanos *et al*, 2002; Gorr *et al*, 2004). We also described a lethal P-element insertional mutation (l(3)02255) in the *Drosophila* PHD gene (CG1114 in FlyBase) that fails to down-regulate Sima protein in normoxia, thus driving constitutive activation of the transcriptional response to hypoxia. The aim of the present work was to investigate the developmental role of *Drosophila* PHD, which we have named *fatiga* (*fga*; Spanish for 'fatigue') after its lack-of-oxygen phenotype.

RESULTS AND DISCUSSION

As a first step in the study of the functions of *fga* in development, we generated new loss-of-function mutations by mobilizing the l(3)02255 P-element, which is located between the second and

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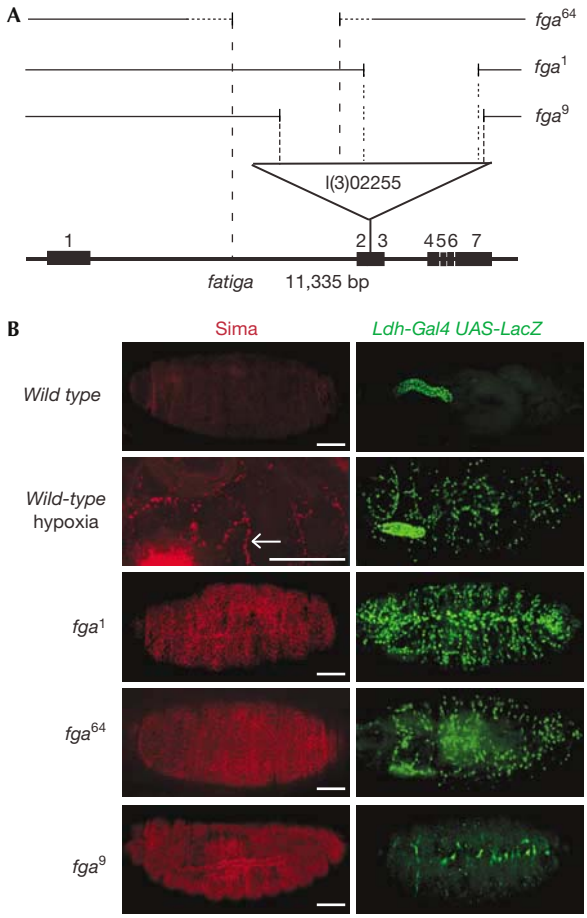

Fig 1 | Phenotypes of *fatiga* mutations. (**A**) Schematic representation of the *fga* locus; the P-element insertional allele fga^{02255} and the three novel alleles fga^{1}, fga^{9} and fga^{64} generated by imprecise excisions are indicated. (**B**) Sima protein (red) and *Ldh-Gal4 UAS-LacZ* reporter expression (green) were not observed in wild-type embryos in normoxia, but were turned on in hypoxia (5% O_2) in a pattern that followed tracheal branches (arrow). In *fga* mutants, Sima protein accumulation and induction of the reporter occurred in normoxia. On the basis of the expression of the reporter, the relative strength of the alleles was $fga^{1} > fga^{64} > fga^{9}$, although Sima levels looked similar in fga^{1} and fga^{64} embryos. Scale bars, 50 μm.

third exons of the *fga* gene. Precise excisions of the transposon led to a reconstitution of the wild-type hypoxic response, as shown by hypoxia-inducible expression of transcriptional reporters that are based on the murine *LDH-A* enhancer (*Ldh-LacZ* and *Ldh-Gal4*; Lavista-Llanos *et al*, 2002). Imprecise excisions of the P-element resulted in three novel *fga* alleles (fga^{1}, fga^{9} and fga^{64}; Fig 1A) that were characterized at the molecular level by Southern blot analysis and PCR experiments. fga^{9} conserved a 1.4 kb fragment of the original transposon; in fga^{64}, a large genomic portion upstream of the insertion site was removed, and fga^{1} conserved a fragment of about 9 kb of the original P-element (Fig 1A). In normoxic wild-type embryos, Sima protein and induction of the *Ldh-Gal4* reporter were not detected; upon exposure to hypoxia (5% O_2), both Sima protein and reporter expression were observed, mainly in the tracheal system (Fig 1B; Lavista-Llanos *et al*, 2002). The molecular basis of this pattern of induction is now under investigation. Interestingly, *fga* loss-of-function alleles showed different levels of accumulation of Sima protein in normoxia, which was widely expressed in fga^{1} and fga^{64}, and showed some prevalence in the tracheal system in fga^{9}, correlating with the constitutive induction of the hypoxic reporter (Fig 1B). fga^{02255}, fga^{1} and fga^{64} were lethal at the first larval instar and fga^{9} died in the pupal stage. As overexpression of Sima through a ubiquitous *Gal4* driver provokes lethality in the larval stages, we reasoned that lethality in *fga* mutants could be due to overaccumulation of Sima protein in normoxia. To test this hypothesis and to determine if Fga is a dedicated regulator of Sima or whether, alternatively, it might modulate other molecular targets (Frei & Edgar, 2004; Frei *et al*, 2005), we sought to analyse *fga* phenotypes in a *sima*-free genetic background. Loss-of-function mutations of *sima* have not been reported so far, but two different P-element insertions mapping within the *sima* locus were available from the Public Stock Centers. One of these insertion lines was able to respond to hypoxia and, thus, was indistinguishable from the wild type (data not shown). In contrast, embryos homozygous for the other insertion, $sima^{07607}$ (Fig 2A), did not express *sima* mRNA (Fig 2B) and failed to induce the *Ldh-LacZ* reporter in hypoxia (Fig 2C). Introduction of a *UAS-Sima* transgenic element under the control of an *hs-Gal4* driver was able to rescue induction of reporter expression, which was expressed in a wild-type pattern (Fig 2C), indicating that the absence of Sima was indeed responsible for the lack of hypoxic response. Altogether, these results indicate that $sima^{07607}$ is a *sima* loss-of-function allele.

To explore whether the absence of Sima protein, and thus the inability to respond to hypoxia, affects developmental progression, we analysed phenotypes in $sima^{07607}$ mutants. Homozygous mutant embryos developed without any obvious difference from the controls, and the first-instar larvae looked healthy and motile. Next, we placed homozygous mutant or control larvae in vials containing fresh food, which were then exposed to 21% or 5% O_2 until individuals attained the pupal stage. We observed that $sima^{07607}$ mutants were viable and fertile in normoxia, but virtually unable to develop in hypoxia (Fig 2D). Precise excision of the P-element totally reverted hypoxia-dependent lethality, which indicated that the insertion was indeed responsible for this phenotype (Fig 2D). Thus, we conclude that, unlike Tango that participates as a common bHLH-PAS partner in several developmental processes in normoxia (Sonnenfeld *et al*, 1997), Sima is necessary for developmental progression in hypoxia but not in normoxia.

Recently, Frei & Edgar (2004) reported that *fga* mutations caused a reduction in cell size, but it is unclear whether this effect depends on overaccumulation of Sima. The availability of $sima^{07607}$ as a *sima* loss-of-function allele enabled us to answer this particular question and, more generally, to address the extent to which the developmental defects of *fga* loss-of-function mutations are due to the de-regulated accumulation of Sima protein. As expected, in $fga^{1}sima^{07607}$ double homozygous mutants, Sima protein was undetectable and embryos did not show any expression of hypoxia-inducible reporters in normoxia (Fig 3A). Consistent with previously described growth defects of *fga* mutants (Frei & Edgar, 2004), fga^{9} pupae were smaller than their heterozygous siblings (Fig 3B,C) and, interestingly, they exhibited a delay in larval development, taking 2 additional days

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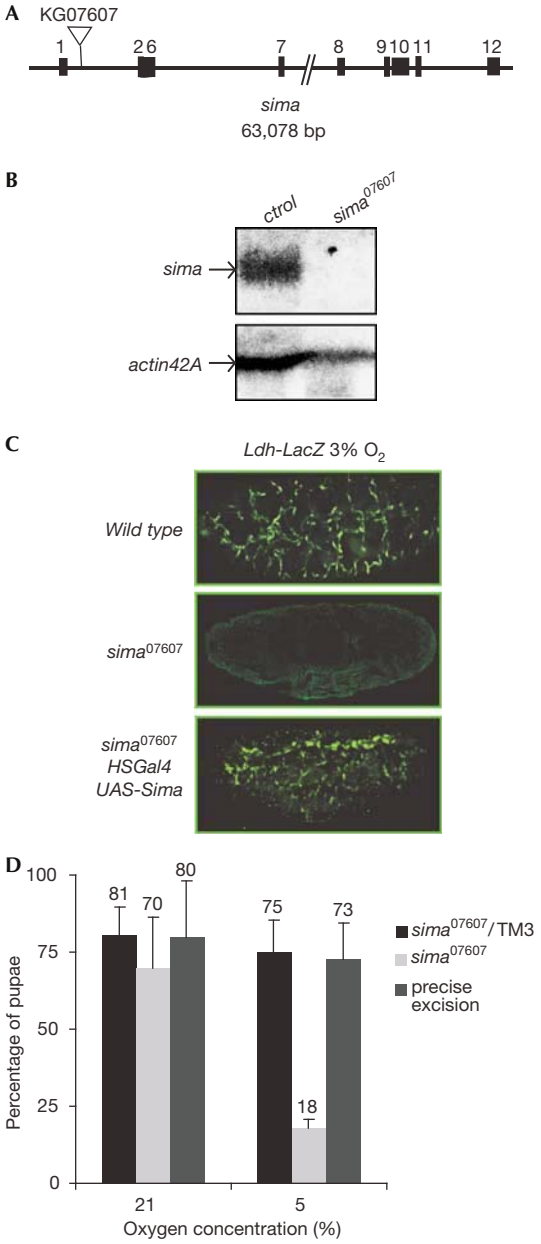

Fig 2 | Characterization and phenotypes of *sima* mutants. (**A**) Schematic representation of the *sima* locus showing *sima*^{07607} P-element insertion. (**B**) Northern blot analysis showing that *sima* mRNA is expressed in wild-type but not in *sima*^{07607} homozygous flies in normoxia. (**C**) The *Ldh-LacZ* reporter was induced following 5 h exposure to 3% O_2 in control embryos but not in *sima*^{07607} homozygotes; a *UAS-Sima* element rescued reporter induction in *sima*^{07607} mutants. (**D**) Only a small proportion of *sima*^{07607} homozygotes attained the pupal stage at 5% O_2 as compared with the control larvae. Precise excision of the P-element led to complete rescue of survival in hypoxia (5% O_2).

to reach the pupariation stage (Fig 3D). Strikingly, *fga*^{1}*sima*^{07607} double homozygous mutants were indistinguishable from the controls, both in their pupal weight and in the duration of larval development (Fig 3B–D). Thus, the loss of Sima provoked the complete reversion of growth defects occurring in *fga* mutants. To answer whether overaccumulation of Sima is sufficient to account for the autonomous reduction in cell size that was reported for *fga* mutant cells (Frei & Edgar, 2004), we overexpressed Sima protein in random clones using the flipase-induced recombination (FLP-OUT) technique, and the effect on cell size was analysed. As shown in Fig 3E (see also supplementary Fig S1 online), overexpression of Sima in isolated cells caused a marked autonomous reduction of cell size, which correlated with smaller nuclei, as shown by 4,6-diamidino-2-phenylindole (DAPI) staining. Taken together, these results indicate that Sima is a downstream effector of Fga as a regulator of cell growth. Further analyses were carried out on the tracheal system; once again defects (particularly, air-filling impairment) that were observed in *fga* mutants, were corrected in *fga sima* double mutants (see the supplementary information online and supplementary Fig S2 online).

Given the reversion of the analysed *fga* phenotypes in *fga sima* double mutants, we wanted to test whether lethality that occurred following *fga* loss-of-function is also due to overaccumulation of Sima. Data in Fig 4 show that this is indeed the case, as, in normoxia, *fga*^{1} *sima*^{07607} double homozygous mutants were viable to adulthood, even when many of these adults failed to complete emergence from the pupal case, and those that emerged (24%; $N > 160$) looked weak and frequently died shortly afterwards. As expected, in hypoxia, this reversion of lethality did not occur, and *fga* or *sima* single mutants, as well as *fga sima* double mutant flies, died in the first larval stage. Overall, these results show that *Drosophila* development can proceed in the absence of PHD oxygen sensors, provided that the HIF-α subunit is absent and oxygen availability is not compromised. Thus, we conclude that the most fundamental functions of Fatiga/PHD in development probably involve the downregulation of Sima protein levels. However, *fga sima* exarate adults show defects in wing and ovary development (supplementary Fig S3 online), which may imply that Fga is involved in patterning these organs in a Sima-independent manner. Detailed genetic and molecular analysis of *fga sima* double mutants should help to define Sima-independent developmental functions of the oxygen sensor in *Drosophila*.

The relationship between developmental processes and inducible systems dedicated to maintaining an organism's homeostasis following environmental challenges is only beginning to be explored (Britton *et al*, 2002). The current work on oxygen signalling shows that although aberrant activation of the hypoxia-responsive machinery has the potential to disturb development and cause lethality, total inactivation of the system is compatible with developmental progression that is sufficient to maintain life—at least in the basal state. The work suggests the operation of a hierarchical organization, in which inducible oxygen signalling is superimposed on 'hard-wired' developmental programmes that already make a significant contribution to the physiological challenge of oxygen homeostasis (Jarecki *et al*, 1999; Frazier *et al*, 2001). We speculate that a similar hierarchy and common functionality between ontogenic processes and inducible-response pathways may apply to other systems and may be an important principle facilitating adaptation. Interestingly, several other examples have been reported in which removal of one regulatory component of a pathway that is crucial for maintenance of homeostasis leads to the constitutive activation of a downstream

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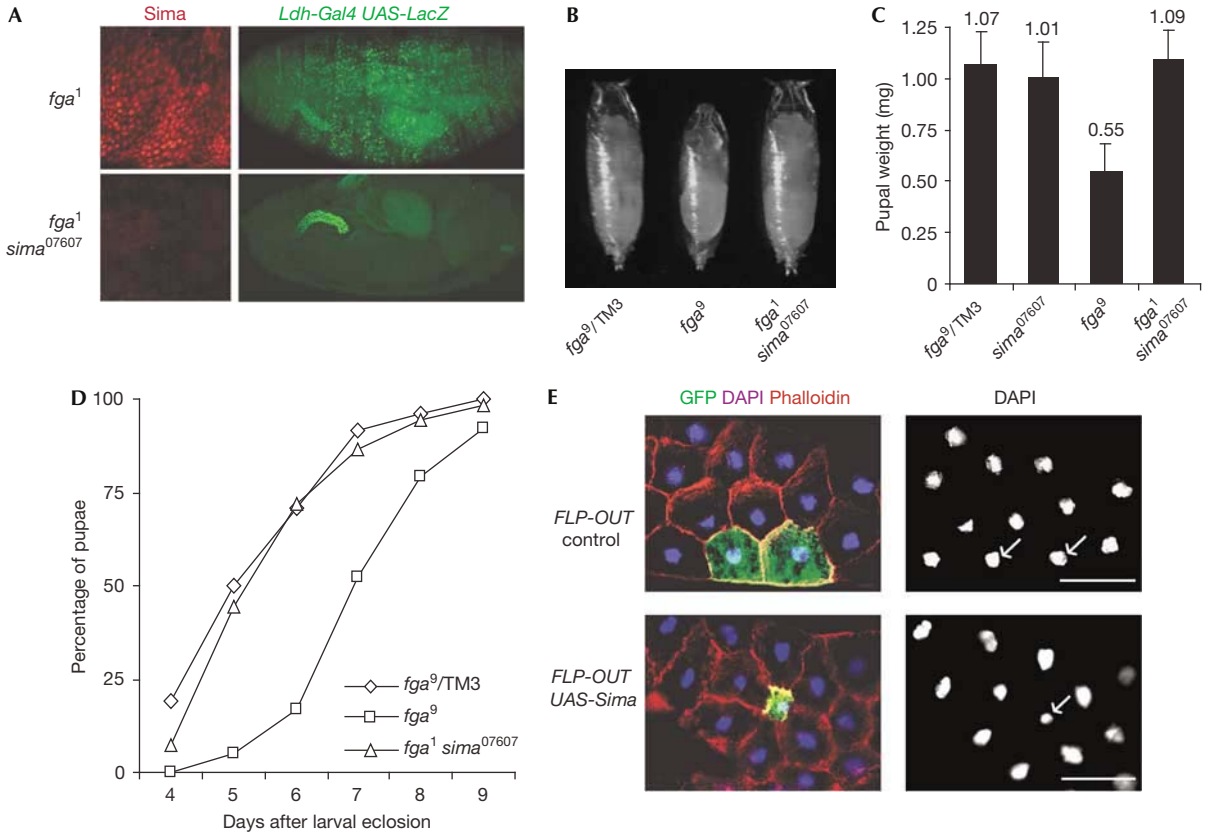

Fig 3 | *sima* loss-of-function reverts phenotypic defects of *fatiga* mutants. (**A**) Normoxic accumulation of Sima and induction of the *Ldh-Gal4* hypoxia-inducible reporter occurred in *fga*^{1} homozygous embryos but not in *fga*^{1} *sima*^{07607} double mutants. (**B,C**) *fga*^{9} homozygous pupae were smaller than their heterozygous siblings and (**D**) larval development until pupariation lasted 2 days more than that in the controls (100% refers to the individuals from each genotype that attained the pupal stage). Both pupal weight ($N > 13$) and duration of larval stages of *fga*^{1}*sima*^{07607} ($N > 120$) were indistinguishable from the those of the controls (**B–D**). (**E**) Sima was randomly overexpressed in isolated cells using the 'flip-out' method. Sima-expressing cells in the fat body (marked with green fluorescent protein (GFP)) were smaller than neighbouring cells that did not express Sima (cells unmarked with GFP). Similar flip-out control experiments in which GFP alone was expressed in random cells did not cause any growth alteration. Note that nuclei from cells overexpressing Sima were smaller than those from neighbouring cells. DAPI, 4,6-diamidino-2-phenylindole. Scale bars, 50 μm.

effector, causing lethality, whereas the simultaneous ablation of the effector together with the regulator restores the ability of the organism to develop: (i) Mdm2 knockout mice fail to develop beyond embryonic day 6.5, but concomitant genetic ablation of its dedicated target molecule, p53, allows the development of normal and fertile adult mice (Jones *et al*, 1995; Montes de Oca Luna *et al*, 1995). (ii) *Drosophila* PTEN mutations are lethal but if such mutations are combined with mutations in the protein kinase B PH domain, developmental progression is largely restored (Stocker *et al*, 2002). Thus, the de-regulation of pathways that maintain cell homeostasis is apparently more detrimental than inactivating the whole pathway, suggesting that despite the ability of these pathways to perturb development, their physiological operation is essentially superimposed on a relatively sufficient 'hard-wired' programme.

METHODS

Fly strains and genetics. Fly stocks used in this study were *Ldh-Gal4 UAS-GFPLacZ* and *Ldh-LacZ* (Lavista-Llanos *et al*, 2002), *Hs-Gal4*, *btl-Gal4* (Shiga *et al*, 1996), *Term-Gal4* (Jarecki *et al*, 1999), *escargot-Gal4* (Steneberg *et al*, 1998), *UAS-Sima* (Bacon *et al*, 1998), *sima*^{07607} (kindly provided by Hugo Bellen), *fga*^{02255} (Spradling *et al*, 1999), *Hs-FLP*^{122}, *act>CD2>GAL4* and the TM3 *act-GFP* balancer chromosome (Bloomington Stock Center, Bloomington, IN, USA).

Generation of fatiga alleles: *fga*^{02255} P [*lacZ*, *ry*$^{+}$] element was mobilized through crosses with a Δ2-3 line, overexpressing transposase. *rosy*$^{-}$ males from the offspring were individually crossed with females carrying a TM3*ry* balancer to establish balanced stocks, and were tested for lethality against the chromosomal deficiency Df(3)R3-4.

Precise excision of P{SUP or P}sima^{07607}: the *sima*^{07607} homozygous viable P[$y^{+}w^{+}$] element was mobilized as above and the resulting y^{-} w^{-} males were used to establish independent lines. Homozygous flies from these stocks were tested for survival at 5% O_2.

Generation of fga^{1}*sima*^{07607}*double mutants*: heterozygous *y w*; *fga*^{1}/TM3*Sb* flies were crossed with *y w*; *sima*^{07607} individuals and non-*Sb* females from the offspring were crossed with *y w*;

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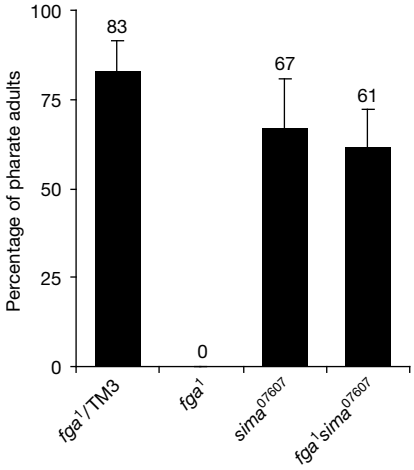

Fig 4 | *sima* loss of function reverts lethality of *fatiga* mutants in normoxia. A large proportion of control or $sima^{07607}$ homozygous first-instar larvae, but none of the fga^{1} homozygotes, reached the adult stage in normoxia. Viability to adulthood was restored in fga^{1} $sima^{07607}$ double homozygous mutants ($N > 160$).

Xa/TM3 males. Males carrying the $sima^{07607}$ mutation, identified by dark body colour and red eyes, were tested for lethality by crossing them individually with Df(3)R3-4/TM3*Sb* females. The presence of the fga^{1} mutation in candidate double mutant strains was confirmed by Southern blot.

Molecular analysis of *fatiga* mutations. For Southern blots, genomic DNA extracted from fga^{1}, fga^{9}, fga^{64} and fga^{02255} heterozygous larvae or from wild-type controls was digested with different restriction enzymes, run on agarose gels, transferred to a Zeta-Probe GT Genomic membrane (Bio-Rad Laboratories, Hercules, CA, USA) and hybridized with ^{32}P-labelled genomic fragments flanking or including the P-element insertion site.

Immunostaining and bright-field microscopy. Rabbit anti-β-gal antibody (Cappel MP Biomedicals, Irvine, CA, USA) was used at a dilution of 1:1200 and rat anti-Sima antibody (Lavista-Llanos *et al*, 2002) was used at a 1:150 dilution. Cy^{TM}2-conjugated anti-rabbit and Cy^{TM}3-conjugated anti-rat secondary antibodies were used at a 1:150 dilution (Jackson Laboratories, West Grove, PA, USA). Observations were made by fluorescence microscopy in a BX-60 Olympus microscope or by confocal microscopy in a Zeiss LSM5 Pascal microscope.

Northern blot. Total RNA was isolated from $sima^{07607}$ or *y w* adult flies using the Trizol reagent (Life Technologies, Carlsbad, CA, USA); 25 μg of total RNA was run on a formaldehyde gel, transferred to Zeta-Probe GT Genomic membrane (Bio-Rad) and hybridized with ^{32}P-CTP-labelled *sima* or *actin42A* probes. A storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) was used to detect ^{32}P emission.

Hypoxia treatment. Hypoxia was applied in a Forma Scientific 3131 incubator, by regulating the proportions of oxygen and nitrogen at 25 °C. For studies of survival, adults carrying a mutation balanced with TM3 act-GFP (green fluorescent protein) were placed in egg-laying bottles and the first-instar larvae from the offspring were sorted under a fluorescence microscope. The same number of homozygous mutants or GFP-expressing heterozygous larvae were placed in vials containing fresh food, which were then exposed to 21% or 5% O_2 until individuals attained pupal or adult stages.

Analysis of growth defects. The first-instar larvae of different genotypes were sorted and placed in fresh vials, as described above. To measure the duration of larval development, pupae were counted daily until day 10 after the first-instar larval sorting. For the experiments described in Fig 3C, pupae were weighed 24 h after pupariation using a Mettler AE 240 electronic balance. For 'flip-out' experiments, *hsFLP; act>CD2>Gal4/UASGFP* or *hsFLP; act>CD2>Gal4/UAS-GFP UAS-Sima* flies were heat shocked during embryogenesis for 15 min at 35 °C and then allowed to develop at 25 °C; third-instar larvae were dissected, fixed in 4% paraformaldehyde, stained with 4,6-diamidino-2-phenylindole and/or phalloidin and mounted in 40% glycerol.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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