Phenotype of the *Triplo-lethal* locus of *Drosophila melanogaster* and its suppression by hyperoxia

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Summary

The *Triplo-lethal* locus (*Tpl*) of *Drosophila* is both triplo-lethal and haploinsufficient, but the function of the locus is unknown. We have examined *Tpl*-aneuploid embryos and find that, in both trisomics and monosomics, the midgut shows extensive cell death and the tracheae are abnormal. Shortly thereafter, all tissues die. PCR-based genotyping of individual embryos and larvae show that this phenotype occurs in the trisomics after hatching and in the monosomics before hatching. Weak alleles of the interacting gene *Su(Tpl)* delay the death of *Tpl* trisomics, but they still show the same tracheal and midgut phenotypes before dying. Hyperoxia (45% oxygen) partially suppresses the phenotype of *Tpl* aneuploids, even though the use of a hypoxia reporter strain shows that dying *Tpl* aneuploids are not hypoxic. This is the first report of a phenotype associated with the *Tpl* locus and the first report of an environmental condition that suppresses the phenotype.

1. Introduction

Triplo-lethal (*Tpl*) is the only locus in the *Drosophila* genome that causes death when it is present in three copies (Lindsley *et al.*, 1972). *Tpl* is also one of a few loci that are haploinsufficient. Although the lethal gene-dosage effect shows that *Tpl* plays an essential role in the organism, the peculiar genetics of the region have frustrated efforts to understand the function of *Tpl*. Point mutations of *Tpl* have never been isolated despite many selection experiments with a range of mutagens (Dorer & Christensen, 1990; Keppy & Denell, 1979; Roehrdanz & Lucchesi, 1980). These experiments have only resulted in duplications and deficiencies for *Tpl*, and alleles of a dominant second-site suppressor, *Su(Tpl)* (Dorer *et al.*, 1995). Three explanations have been proposed to explain the inability to isolate point mutations or transposon insertions of *Tpl* (Dorer & Christensen, 1990; Keppy & Denell, 1979). These are that *Tpl* is very small (and thus presents a small target for mutagens), that *Tpl* does not encode a protein (and so its function is not

readily disrupted by single base changes) and that *Tpl* consists of locally repetitive or redundant genes. These possibilities are not mutually exclusive.

Little analysis of the morphology of dying *Tpl* aneuploids has been done, although Denell noted extensive embryonic lethality (Denell, 1974, 1976). Roehrdanz & Lucchesi (1981) noted that most of the lethality occurs during the embryonic stage but they did not look at the morphology of the dying embryos and their experiments were complicated by the partial rescuing effect of the X-linked *Isis* locus (Dorer *et al.*, 1993).

In this report, we show that tracheal development is abnormal in *Tpl* aneuploids and that the midgut is the first tissue to die. We have also found that hyperoxia (45% oxygen) partially suppresses the phenotype of *Tpl* aneuploids and acts additively with weak alleles of *Su(Tpl)* to suppress the phenotype further. However, *Tpl*-aneuploid embryos do not appear to be hypoxic, suggesting that hyperoxia might act indirectly by causing a compensatory change in some key step in the lethal pathway. This is the first report of a phenotype directly associated with *Tpl* and the first report of an environmental condition that suppresses the phenotype.

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2. Materials and Methods

(i) Drosophila *stocks and culture conditions*

Drosophila stocks were maintained on standard cornmeal–molasses medium supplemented with live yeast. Oregon-R wild-type flies were from the Bloomington Indiana *Drosophila* Stock Center. *Dp(3;3)Tpl/Df(3R)Tpl10* has been previously described (Keppy & Denell, 1979) and the strain *w; P{lacW}JK58/TM6C cu Sb e ca* was a gift of J. Kennison (NIH). The hypoxia reporter strain *UAS–GFP HEPO–GAL4/CyO* was a gift of E. Johnson (Stanford). Embryos were collected on orange-juice or grape-juice agar plates supplemented with a small drop of yeast paste.

(ii) *Microdissection and microscopy*

Embryos and larvae were dissected in PBS on a glass microscope slide. Fine metal forceps were used for dissecting larvae, and glass needles (pulled from Frederick Haer & Co., 3030-0-075 capillary tubes with a Kopf vertical pipette puller) were used for embryos. Trypan-Blue staining was done by a modification of the method of Krebs & Feder (1997). Embryos or larvae were dissected in 0.2 mg ml^{-1} Trypan Blue (Sigma) in PBS. After 30 minutes in Trypan Blue, the animals were rinsed briefly three times with PBS, allowed to stand for 15 minutes in PBS and then examined. Light microscopy was done with a Nikon SMZ800 stereoscope equipped with a Nikon CoolPix 950 digital camera. Fluorescence images were collected using a Bio-Rad MRC, 1024ES laser scanning confocal microscope.

(iii) *Gas chamber*

Gas mixtures were administered in glove bags (Instruments for Research and Industry, Cheltenham, PA) kept in a temperature controlled room (23–25 °C) under constant light. Gas was provided by mixing medical grade breathable air with either oxygen or nitrogen (Helget Gas, Omaha, Nebraska) using calibrated Supelco Rotameter flowmeters. The gas mixtures were humidified by bubbling with an air stone through water. Gas tanks were located outside the temperature-controlled room and outlet tubes from the bags were exhausted outside the room. To induce hypoxia, embryos were maintained in air until they were 14–18 h old, and then exposed to 1% oxygen for 6 h and allowed to recover in air for 2 h.

(iv) *Single embryo PCR*

New rearrangements of *Tpl* were isolated by crossing flies of the genotype $\Delta 2$-3;; *P{ry$^{+t7.2}$ = PZ}l(3)0108 6^{01086} ry506/ri Ki p^{p}* with *Dp(3;3)Tpl/Df(3R)Tpl10*.

Survivors were backcrossed to *Dp(3;3)Tpl/Df(3R)Tpl10* to generate a stock. Many of the resulting duplications and deficiencies flank the P element. Inverse PCR was used to sequence the novel junction flanking the P element (J. Engelman, J. A. Rudnick & A. Christensen, unpublished). One duplication (*Dp(3;3)Tpl^{JE19}/Df(3R)Tpl10*) extends from *Rm62* to *CG33202* (approximately 550 kb) and one deficiency (*Dp(3;3)Tpl/Df(3R)Tpl^{JE5}*) extends from *Rm62* to *Taf1* (approximately 640 kb). The phenotype of these rearrangements is indistinguishable from other *Tpl* duplications and deficiencies. These two rearrangements were crossed together to make a stock containing both new rearrangements, *Dp(3;3)Tpl^{JE19}/Df(3R)Tpl^{JE5}*. Primers were designed from the sequences of the junctions. One primer pair (5′-TTAATTCAAACCCCACGGAC-3′ and 5′-CCGACGACGAGATACATTTAAAG-3′) produces a 610 bp fragment only when the *Dp(3;3)Tpl^{JE19}* chromosome is the template, whereas the other (5′-CACACAACCTTTCCTCTCAAC-3′ and 5′-GGGTGATAAAATGGCGACAG-3′) produces a 599 bp fragment when *Dp(3R)Tpl^{JE5}* is the template. PCR was done with each primer pair separately on an aliquot of the same template preparation. Each run included one sample with Oregon-R DNA as a negative control and one sample of *Dp(3;3)Tpl^{JE19}/Df(3R)Tpl^{JE5}* as a positive control. Samples that did not amplify with either primer pair or that amplified with both primer pairs were discarded. Template preparation from single embryos or larvae was as previously described (Garozzo & Christensen, 1994).

3. Results

(i) *Visible phenotype of dying* Tpl *aneuploid embryos*

Flies heterozygous for a duplication of *Tpl* and a deficiency of *Tpl* (*Dp(3;3)Tpl/Df(3R)Tpl10*) were crossed to a wild-type strain (Oregon-R) and the progeny were examined by light microscopy. This cross produces equal numbers of embryos with three doses of *Tpl* and one dose of *Tpl*. Both classes of embryo will die, with some dying before hatching and others dying as first-instar larvae. Figure 1b, c shows two still-living first-instar larvae resulting from this cross. In contrast to the wild-type larva shown in Fig. 1a, these two display the typical features seen in most of the late embryos or early first instar larvae: the tracheae have turned brown and started to break apart, and the midgut has turned brown. Shortly after the midgut turns brown, movement ceases, the entire animal turns brown and the internal organs all deteriorate. Figure 1d shows an embryo that has died before hatching. We repeated these experiments but collected embryos over 2 hour periods and observed them at various intervals thereafter in order to see the
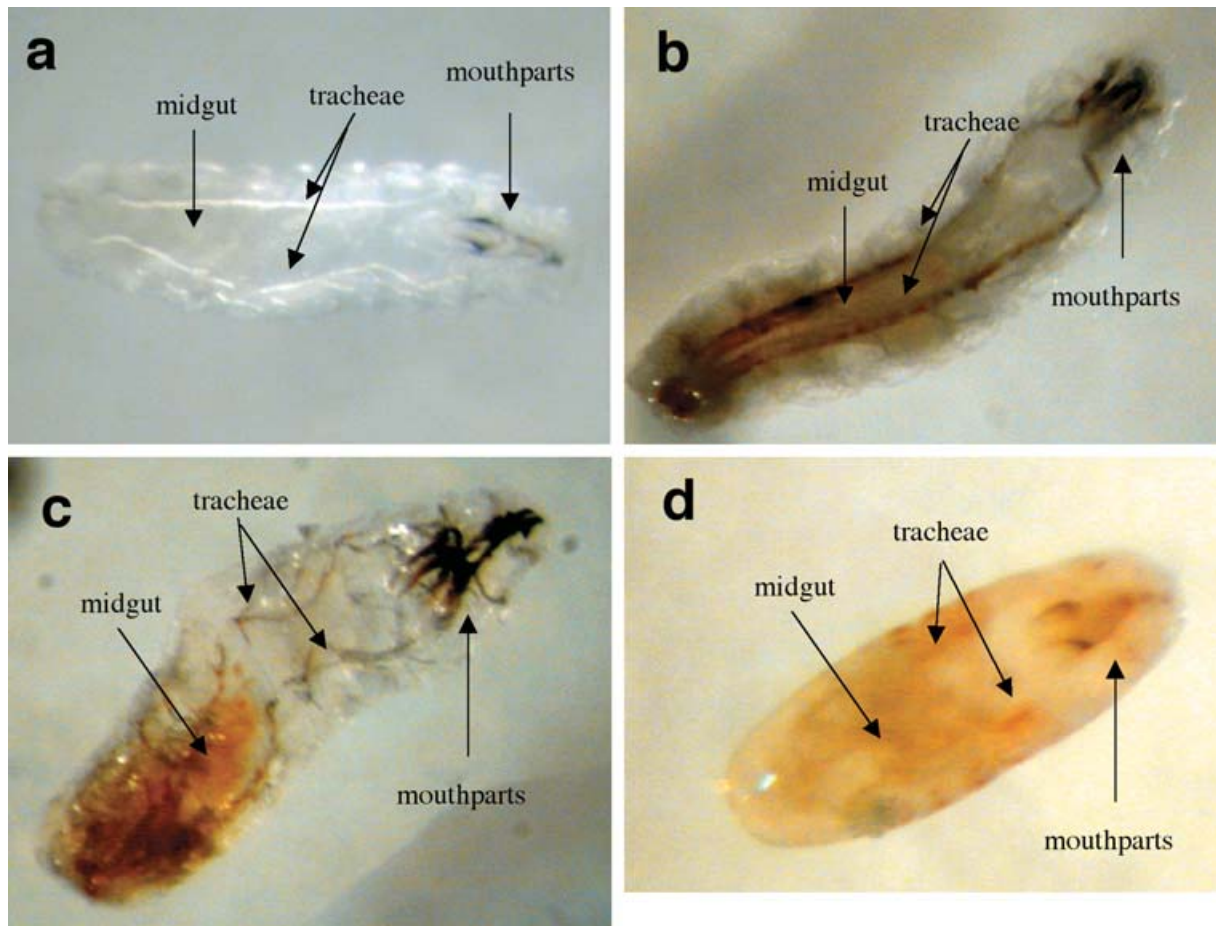
Fig. 1. Darkened tracheae and midgut in *Tpl*-aneuploids. (a) A wild-type first-instar larva. (b–d) Progeny from a cross of Oregon-R with *Dp(3;3)Tpl/Df(3R)Tpl10* showing two different newly hatched larvae (b, c) and an unhatched embryo that has stopped moving (d). All are oriented with the anterior to the right and show the mouthparts, the two dorsal branches of the tracheae and the midgut.

time course of the phenotype. At least 50 animals were scored at each time point. No hatching occurred until 28 hours after fertilization. From that point on, the embryos that did not hatch progressed through the stages of brown midgut, brown tracheae and death. By about 50 hours after fertilization, all the embryos had both brown tracheae and midgut, and were starting to turn completely brown. Larvae that did hatch looked normal until 56 hours after fertilization, when they started to move through the same stages of browning reactions (i.e. first the midgut, then the tracheae and then all tissues). By 60 hours, there were already dead larvae. None of the larvae moulted.

(ii) *Genotyping the dying animals*

In order to determine whether there was a difference between the phenotypes of animals with three doses of *Tpl* and animals with one dose of *Tpl*, we crossed Oregon-R females to *Dp(3;3)Tpl^{JE5}/Df(3R)Tpl^{JE19}*. Both chromosomes have unique junctions between P element and genomic sequence that allow us to use PCR to distinguish the duplication and deficiency. At

24 h, 36 h, 48 h and 60 h after fertilization, the DNA from 12–16 individual embryos and larvae was subjected to PCR. At 24 h, when none of the animals has hatched, both genotypes were present in the embryos. At all later times, however, the embryos all carried the *Df(3R)Tpl^{JE19}* chromosome and thus one dose of *Tpl*, and the larvae all carried the *Dp(3;3)Tpl^{JE5}* chromosome and thus three doses of *Tpl* (data not shown). Both genotypes therefore display the same progression of phenotypes, from brown midgut to brown tracheae to death. However, flies with one dose of *Tpl* have this phenotype before hatching (although at a time when the wild type would have already hatched) and flies with three doses of *Tpl* have the phenotype after hatching (although at a time when wild type would have already moulted to the second-instar stage).

(iii) *Identification of cell death by Trypan Blue staining*

In order to examine further the cause of death in *Tpl* aneuploids, we dissected and stained dying embryos
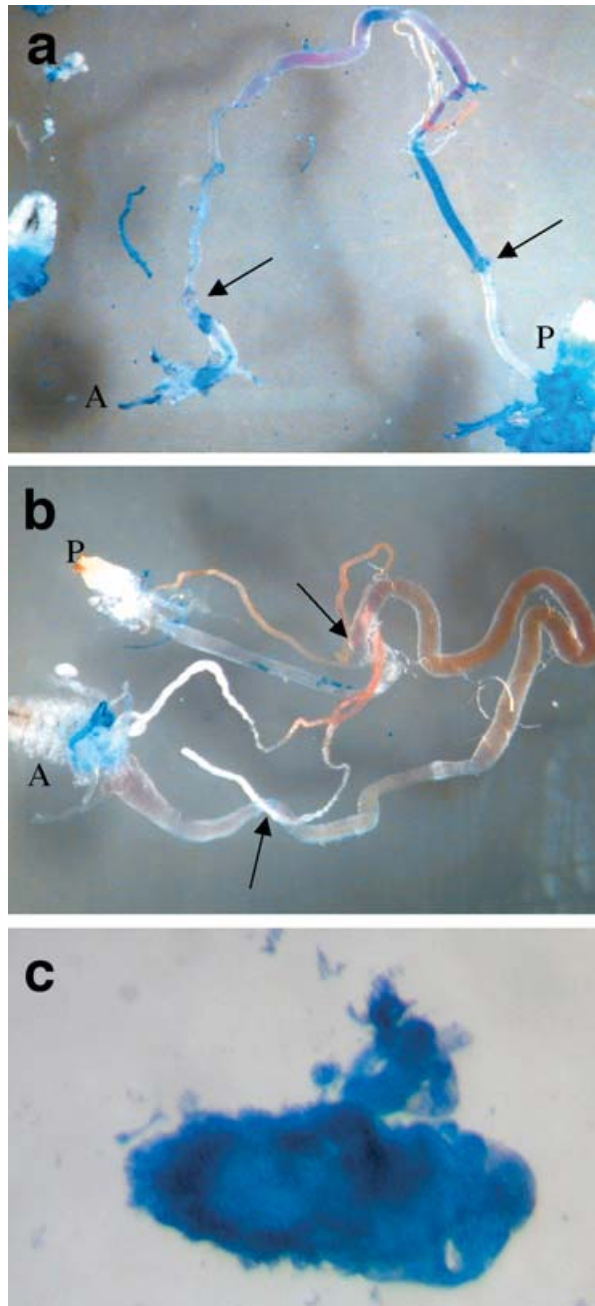
Fig. 2. Trypan Blue staining of *Tpl*-aneuploid and wild-type larvae. Newly hatched first-instar larvae were dissected and stained with Trypan Blue. (a) A larva resulting from a cross of Oregon-R with *Dp(3;3)Tpl/Df(3R)Tpl10*. (b) An Oregon-R larva. (c) An unhatched embryo dechorionated by hand and slit open in the presence of Trypan Blue. The ends of the midgut are denoted by arrows and the anterior and posterior ends of the dissected gut are indicated with A and P, respectively.
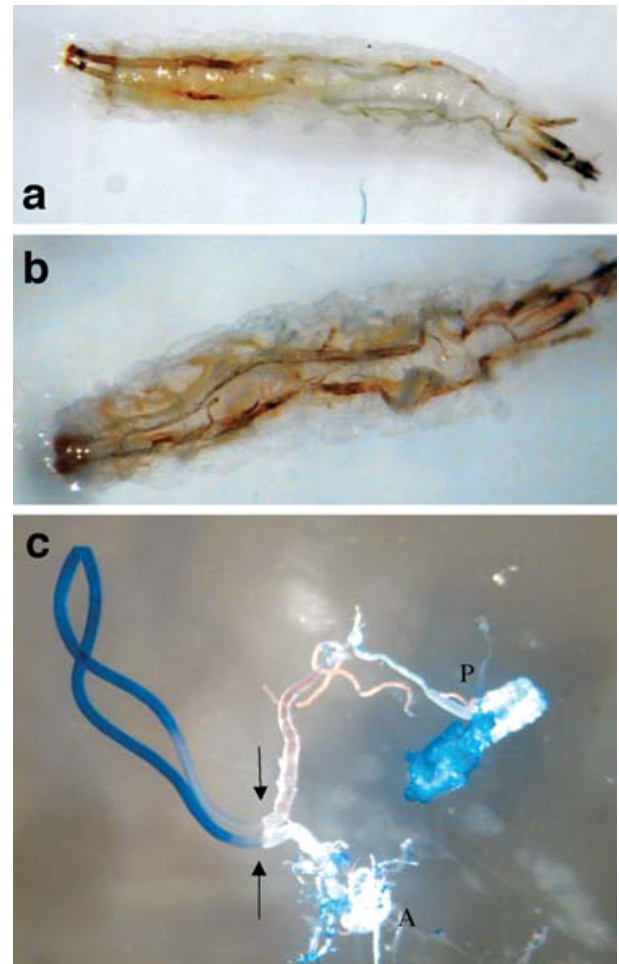

Fig. 3. *Tpl*-trisomic third-instar larvae partially suppressed by an allele of *Su(Tpl)*. (a–c) Third-instar larvae resulting from a cross of *Dp(3;3)Tpl/Df(3R)Tpl10* females with *w; Su(Tpl)^{JK58}/TM6C, cu Sb e ca* males. (a, b) Two different larvae oriented with anterior to the right. (c) A larva dissected and stained with Trypan Blue. The ends of the midgut are denoted by arrows and the anterior and posterior ends of the dissected gut are indicated with A and P, respectively.

and larvae with Trypan Blue, a stain that is excluded by living cells (Krebs & Feder, 1997). Figure 2a shows a first instar larva resulting from the *Dp(3;3)Tpl/Df(3R)Tpl10* × Oregon-R cross that has been dissected in the presence of Trypan Blue, compared with an Oregon-R larva in Fig. 2b. The only tissue that stains in the *Tpl* aneuploid is the midgut (apart from the

edges of the cuticle, which were damaged by dissection, a phenomenon that also occurs in the wild type). The tracheae were also often stained with Trypan Blue. However, we were unable to dissect the tracheae without damaging them, so we cannot exclude dissection damage as the cause of this staining. We dissected at least 50 *Tpl*-aneuploid first-instar larvae or embryos and never observed Trypan Blue staining of foregut, hindgut, Malpighian tubules, fat body, nervous system, salivary gland, muscles, gonads, dorsal vessel or imaginal discs, in any embryos or first instars that showed signs of life (such as movement). As the embryos die, they turn completely brown, the internal tissues become amorphous and all tissues stain with Trypan Blue (Fig. 2c). Embryos at this stage cannot be carefully dissected owing to their fragility, but they can be slit open to allow Trypan Blue staining. Before

it was stained, the embryo shown in Fig. 2c was comparable in appearance to the one in Fig. 1d.

(iv) *Phenotype of* Tpl *trisomic third-instar larvae with an allele of* Su(Tpl)

Mutations in the *Su(Tpl)* gene allow the survival to adulthood of flies with three doses of *Tpl* but not flies with one dose of *Tpl* (Dorer *et al.*, 1995; Roehrdanz & Lucchesi, 1980). In addition to being dominant suppressors of the triplo-lethality of *Tpl*, mutations of *Su(Tpl)* are recessive lethal. The *Su(Tpl)* locus encodes the transcriptional elongation factor dEll (Eissenberg *et al.*, 2002), although its mode of action in suppressing the lethality of *Tpl* might not be direct. One allele, *Su(Tpl)^{JK58}*, suppresses *Tpl* only weakly, resulting in many dying third-instar larvae and only very rare adults. By contrast, flies with the strong allele *Su(Tpl)^{10}* survive and are fertile, and the third-instar larvae display no morphological abnormalities (Dorer *et al.*, 1995). A cross of *Su(Tpl)^{JK58}/TM6C* with *Dp(3;3)Tpl/Df(3R)Tpl10* thus results in both dead embryos (those with only one dose of *Tpl* and those with three doses of *Tpl* and *TM6C*), and dying third-instar larvae with three doses of *Tpl* and *Su(Tpl)^{JK58}*. Figure 3a, b shows such dying third instars; they display the same tracheal and midgut phenotypes as the non-suppressed *Tpl* aneuploids shown in Fig. 1a, b. Figure 3c shows the dissection and Trypan Blue staining of a dying third-instar larva. It has the same midgut phenotype as the non-suppressed *Tpl*-aneuploid embryos and first instars. We obtained similar results with another weak suppressor allele, *Su(Tpl)^{JK53}* (data not shown).

(v) *Effects of changing oxygen concentration on the lethal phenotype*

Because the tracheae of dying *Tpl* aneuploids appeared to be abnormal, we thought that oxygen transport might be impaired. We tested this idea by exposing *Tpl*-aneuploid embryos to an increased oxygen concentration. We chose a concentration of 45% oxygen because we did not want to complicate the experiment with the toxicity of higher doses of oxygen (Smith & Gottlieb, 1975). When we crossed *Dp(3;3)Tpl/Df(3R)Tpl10* to the wild type under hyperoxic conditions, there were still no pupae or adults but those first-instar larvae that did hatch (Fig. 4a) were noticeably more vigorous and active. They often crawled to the walls of the vial, in contrast to those in the air, which usually died on the surface of the food, close to the chorion from which they had hatched. Not only were the larvae more active but they also did not show the tracheal breakdown and brown midgut until later than the larvae raised in air. Trypan blue staining of an approximately 48-h-old

first-instar larva raised in 45% oxygen is shown in Fig. 4b and there is little cell death in the midgut. Reducing the oxygen levels to 7–10% by mixing air with nitrogen had no noticeable rescuing effect on *Tpl* aneuploids.

Observations over time of hyperoxic embryos collected in a 2 h period revealed that the survival of both embryos and larvae was prolonged. In air, there were no healthy embryos at 36 h but, in 45% O_2, most of the embryos still appeared normal. After 48 h in air, all 210 embryos examined had the brown midgut and brown tracheal phenotypes, whereas, under hyperoxia, 14 of 40 embryos still had clear tracheae at 48 hours. Similarly, under normoxia, 10 of the 28 hatched larvae were dead by 60 h, whereas, in 45% oxygen, all of the 58 larvae examined had yellow midguts and none of them had brown tracheae or were dead. PCR analysis showed that, under hyperoxia, like in air, the animals that hatched had three doses of *Tpl*, whereas the animals that did not had one dose.

We also assessed the effect of hyperoxia on *Su(Tpl)^{JK58}/Dp(3;3)Tpl* third-instar larvae. The most obvious effect was that some of these larvae survived to adulthood in hyperoxia, but there was also a dramatic decrease in the visible tracheal and midgut browning phenotypes (Fig. 5a) and a reduction in cell death in the midgut (Fig. 5b).

(vi) *Are dying* Tpl-*aneuploid embryos hypoxic?*

If hyperoxia rescues *Tpl* aneuploids then the expected corollary is that *Tpl* aneuploids are hypoxic. We obtained a hypoxia reporter strain to test this idea. This strain carries two transgenes: the human erythropoietin (EPO) enhancer fused to the yeast transcriptional activator *GAL4* and the upstream activator sequence (UAS) from the yeast *GAL1* gene fused to green fluorescent protein (GFP). Under normoxia, hypoxia-inducible-factor 1α (HIF-1α) is hydroxylated using molecular oxygen as a substrate, which results in its ubiquitination and degradation (Dann *et al.*, 2002). Under hypoxia, this reaction does not occur and HIF-1α is transported to the nucleus, where it activates the transcription of target genes (Lavista-Llanos *et al.*, 2002; Semenza, 2001). This system is highly conserved between insects and mammals, and the human EPO promoter is induced by hypoxia in *Drosophila*, so hypoxic cells in the reporter strain express GFP (E. Johnson & M. Krasnow, personal communication). Figure 6a shows a control of the *HEPO–GAL4; UAS–GFP* strain viewed in a confocal microscope. The strong expression of GFP in the salivary glands and weak expression in the posterior spiracles is constitutive background expression. The fluorescence in the gut is auto-fluorescence from residual yolk and is yellow rather than the green of GFP. Figure 6b shows the response
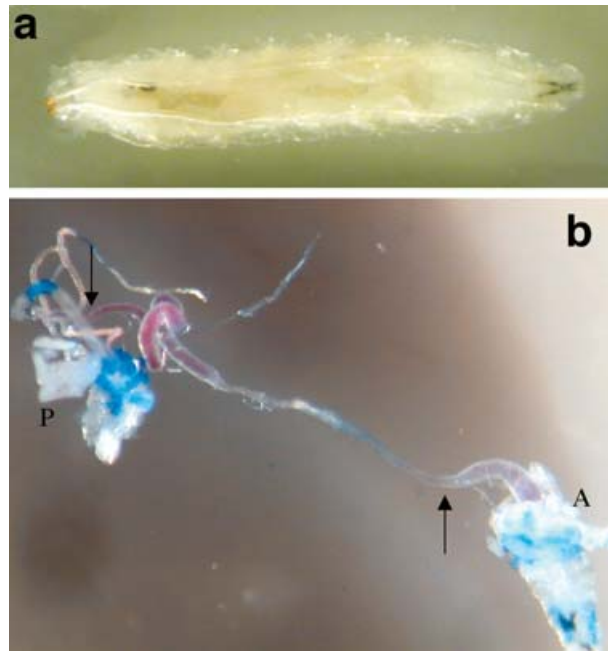
Fig. 4. *Tpl* aneuploids reared in 45% oxygen, showing progeny from a cross of Oregon-R with *Dp(3;3)Tpl/ Df(3R)Tpl10*. The cross was performed in 45% oxygen. (a) A newly hatched first-instar larva. (b) An embryo dissected and stained with Trypan Blue. The ends of the midgut are denoted by arrows, and the anterior and posterior ends of the dissected gut are indicated with A and P, respectively.

of the reporter strain to a period of hypoxia. To see whether *Tpl*-aneuploid embryos are physiologically hypoxic even when grown under normoxia, we collected embryos and first-instar larvae from a cross of *HEPO–GAL4; UAS–GFP* with *Dp(3;3)Tpl/ Df(3R)Tpl10*. The *Tpl*-aneuploid embryos resulting from this strain all had the appearance shown in Fig. 6c, and showed only the same background pattern of GFP expression as the normoxic wild-type control (Fig. 6a). Some first-instar larvae hatched from this cross and their patterns of GFP expression were also no different from the normoxic control (data not shown). We conclude from this that either *Tpl* aneuploids are not hypoxic or they cannot respond to hypoxia by activating HIF-1α.

To distinguish between these two possibilities, we subjected *Tpl* aneuploids carrying the hypoxia reporter to a period of actual hypoxia. Normal embryos subjected to a 6 h period of only 1% oxygen do not die (Fig. 6b) but *Tpl* aneuploids appeared to be very sensitive to hypoxia, as shown by an accelerated pattern of death. Most of the embryos in this experiment had already reached the stage where their internal organs had deteriorated, comparable to those in Figs 1c, 2c. There was some scattered GFP expression in many of them but usually not in any identifiable pattern, and a few embryos were as intact as those in Fig. 6d, e. In these embryos, it can be seen that even the salivary gland is starting to dissociate, but they also show GFP
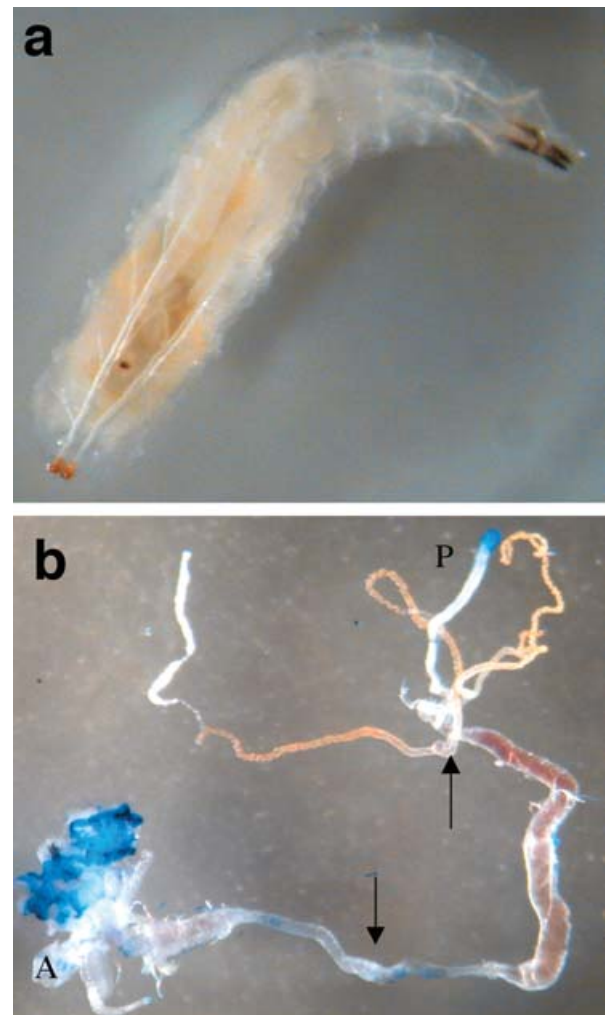

Fig. 5. *Tpl*-trisomic third-instar larvae partially suppressed by an allele of *Su(Tpl)* and reared in 45% oxygen, showing third-instar larvae resulting from a cross of *Dp(3;3)Tpl/Df(3R)Tpl10* females with *w; Su(Tpl)^{JK58}/ TM6C, cu Sb e ca* males. The cross was performed in 45% oxygen. (a) A live larva. (b) A larva dissected and stained with Trypan Blue. The ends of the midgut are denoted by arrows and the anterior and posterior ends of the dissected gut are indicated with A and P, respectively.

expression in the remnants of the tracheae (Fig. 6d) and in other unidentified cells (Fig. 6e). We conclude from this set of experiments that *Tpl* aneuploids can activate HIF-1α when they are hypoxic, that hypoxia enhances the lethality of *Tpl* aneuploidy and that *Tpl* aneuploids raised in air are not hypoxic.

4. Discussion

The *Triplo-lethal* locus is the most dosage-sensitive locus known in a metazoan, and its function remains a mystery. Although it has been known for many years that the lethal phase of *Tpl* aneuploids was in the late embryo or early first instar, it was not known whether there was any tissue specificity, nor whether *Tpl* trisomy and monosomy have different phenotypes, nor
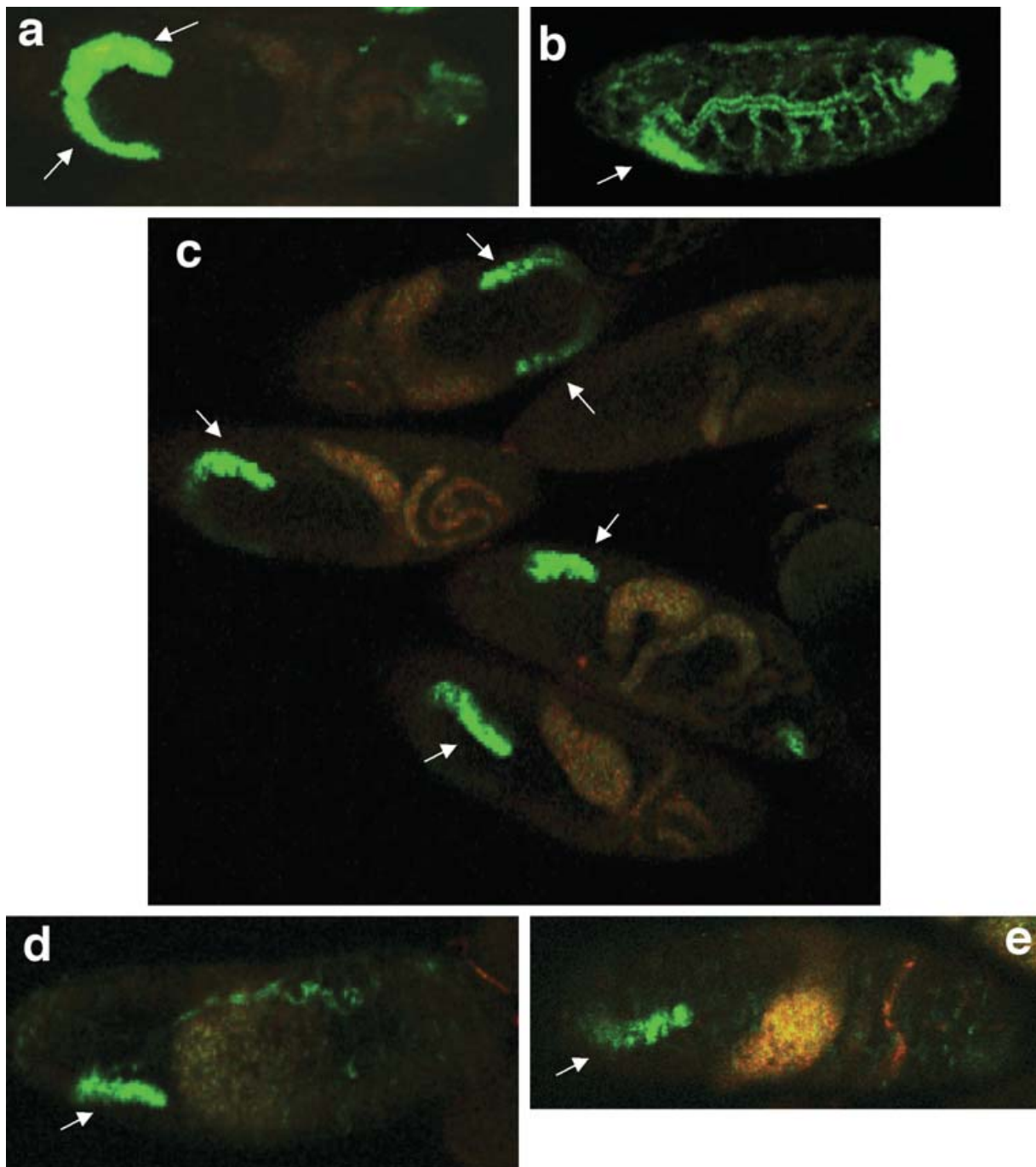
Fig. 6. Detection of hypoxia using a GFP reporter. (a) A dorsal view of an embryo from the *HEPO–GAL4; UAS–GFP* strain. (b) An embryo from the *HEPO–GAL4; UAS–GFP* strain that was raised in air for 14–18 h, then exposed to 1% oxygen for 6 h and allowed to recover for 2 h in air. (c) Several progeny from the cross *HEPO–GAL4; UAS–GFP × Dp(3;3)Tpl/Df(3R)Tpl10* raised in air. (d, e) Lateral views of progeny from the cross *HEPO–GAL4; UAS–GFP × Dp(3;3)Tpl/Df(3R)Tpl10* exposed to the same hypoxia treatment as in (b). Salivary glands are indicated by arrows. In lateral views, only one salivary gland is visible.

whether *Tpl* aneuploidy is cell-lethal. The results described here show that two morphological abnormalities appear in *Tpl* aneuploids: first, the midgut turns brown, accompanied by cell death, and then the tracheae appear to break up and turn brown. Soon thereafter, the entire animal will die. Strong mutant alleles of *Su(Tpl)* entirely eliminate the abnormalities

and death caused by *Tpl* trisomy, resulting in viable, fertile adults. Weak alleles of *Su(Tpl)* postpone the death of *Tpl* trisomics until the third-instar stage, but the dying third instars still display the same tracheal and midgut phenotypes as the unsuppressed *Tpl* aneuploids do in the embryonic or early first-instar stage. Both *Tpl* trisomy and monosomy produce the

same phenotype but at different times: monosomy shows in the late embryo, whereas trisomy does so in the first-instar larva.

We also found that hyperoxia partially rescues *Tpl* aneuploids. Hyperoxia appears to have similar effects on *Tpl* aneuploids and on *Tpl* trisomic larvae whose death has been delayed by a weakly suppressing allele of *Su(Tpl)*. In both cases, the tracheal and midgut phenotypes are delayed. Our initial hypothesis was that the tracheal defects of *Tpl* aneuploids prevented efficient oxygen transport, which in turn resulted in cell death. By this hypothesis, cell death would begin in the midgut because it is such a metabolically active tissue and hyperoxia would overcome the oxygen starvation resulting from inefficient transport. However, our use of a hypoxia reporter strain shows that *Tpl* aneuploids raised in air are not hypoxic enough to induce HIF-1α, even though hypoxia accelerates the death of *Tpl* aneuploids. If they are not hypoxic enough to induce HIF-1α then they are unlikely to be hypoxic enough to die of oxygen starvation, particularly because controls show that the dying embryos are capable of inducing HIF-1α. This result suggests that hyperoxia rescues *Tpl* aneuploids indirectly, perhaps by inducing a multifaceted oxidative stress response that would also alleviate the consequences of *Tpl* aneuploidy. Hypoxia or anoxia have been shown to arrest the development of *Drosophila* embryos for as much as 3 days, and normal development resumes on restoration of oxygen (Teodoro & O'Farrell, 2003; Wingrove & O'Farrell, 1999). Nitric oxide signalling appears to be involved in this response. By contrast, *Tpl* aneuploids are accelerated into the lethal phase by a brief period of hypoxia, suggesting that the nitric-oxide-mediated response to hypoxia is abnormal. We still do not know the function of the *Triplo-lethal* locus but, for the first time, we have observed a developmental phenotype associated with it, and have found an environmental condition (hyperoxia) that suppresses that phenotype.

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