Mitochondrial dysfunction generates a growth-restraining signal linked to pyruvate in Drosophila larvae

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ABSTRACT

The Drosophila bang-sensitive mutant tk0 25t, manifesting a global deficiency in oxidative phosphorylation due to a mitochondrial protein synthesis defect, exhibits a pronounced delay in larval development. We previously identified a number of metabolic abnormalities in tk0 25t larvae, including elevated pyruvate and lactate, and found the larval gut to be a crucial tissue for the regulation of larval growth in the mutant. Here we established that expression of wild-type tko in any of several other tissues of tk0 25t also partially alleviates developmental delay. The effects appeared to be additive, whilst knockdown of tko in a variety of specific tissues phenocopied tk0 25t, producing developmental delay and bang-sensitivity. These findings imply the existence of a systemic signal regulating growth in response to mitochondrial dysfunction. Drugs and RNAi-targeted on pyruvate metabolism interacted with tk0 25t in ways that implicated pyruvate or one of its metabolic derivatives in playing a central role in generating such a signal. RNA-seq revealed that dietary pyruvate-induced changes in transcript representation were mostly non-coherent with those produced by tk0 25t or high-sugar, consistent with the idea that growth regulation operates primarily at the translational and/or metabolic level.

Introduction

Mitochondrial dysfunction is a common underlying cause or manifestation of human disease [1–3]. Whilst mammalian models such as the mouse have provided insights into the underlying processes, the use of Drosophila to understand mitochondrial pathophysiology has been relatively neglected, despite its versatility and the availability of a wide variety of easily applied genetic tools.

Deficient mitochondrial protein synthesis is frequently associated with mitochondrial diseases [4], and Drosophila provides a valuable model to study the physiological effects of limitations on mitochondrial translation in the context of animal development. The Drosophila tk0 gene, encoding mitoribosomal protein S12, a core component of the mitoribosomal decoding centre, has been a particular object of study in this regard. The canonical mutant tk0 25t displays a range of phenotypic features that resemble mitochondrial disease in humans, including developmental delay, impaired sound-responsiveness, bang-sensitivity (paralytic seizures induced by mechanical shock) and antibiotic sensitivity [5]. Other tk0 25t phenotypes are unique to Drosophila, such as male courtship defect [5]. These phenotypic features reflect an underlying deficiency of mitoribosomes [5] and consequent global deficiency of the enzymatic functions of oxidative phosphorylation (OXPHOS) that depend upon mitochondrial translation products, manifesting in both adults [5] and larvae [6]. All of these phenotypes are reversed by ubiquitous expression of a transgenic copy of the wild-type tk0 gene, using the UAS/GAL4 system [6]. Since developmental delay occurs during the larval stages [5], this prompts the question as to which of the larval tissues mediates the crucial signalling that regulates growth in response to limitations on mitochondrial protein synthesis, and by what mechanism.

In a follow-up study [7], we obtained some relevant clues as to the underlying mechanism(s) whereby the growth rate of tk0 25t larvae is adjusted, so as to take account of the decreased capacity for processing...
nutritional resources caused by mitochondrial dysfunction. In particular, we observed that components of the apparatus of cytosolic protein synthesis and secretion were down-regulated in tko<sup>25t</sup> larvae, both at the transcript level and via a key regulatory step of cytosolic protein synthesis, the ribosomal protein S6 kinase (S6K). tko<sup>25t</sup> larvae were also found to be unaffected by low levels of the cytosolic protein synthesis inhibitor cycloheximide, which retarded the development of wild-type larvae, also implicating the cyto- 

In the same study [7] we determined that the strength of the tko<sup>25t</sup> phenotype depends upon the culture conditions, specifically the sugar content of the growth medium. When tko<sup>25t</sup> flies were cultured on high-sugar medium, their growth was further impaired compared with those grown on low-sugar medium, and many of the observed changes in gene expression were more pronounced. The effect of high sugar was accompanied by increases in the level of pyruvate and lactate in the larvae, whilst supplementation of the medium with pyruvate or lactate exacerbated developmental delay. tko<sup>25t</sup> larvae also manifested low levels of ATP and a greatly decreased NADPH/NADP ratio, both of which were enhanced by high-sugar medium [7].

A key result from the previous study was the observation that expression of wild-type tko specifically in portions of the larval gut, a major secretory tissue, partially alleviated the developmental delay of tko<sup>25t</sup> [7]. Since the driver used in this experiment did not express at a high level in all regions of the gut, we reasoned that other gut-specific drivers used in combination might provide a more complete rescue of the phenotype. To embark on such a study, we initially implemented what we assumed would be negative controls, directing wild-type tko expression in other regions of the larva with high specificity. However, this produced the unexpected result that partial rescue of developmental delay was conferred by expression in each of the tissues tested (muscle, fat-body, neurons, as well as gut), with some evidence of additive effects. Conversely, tko knockdown in each specific tissue produced a partial developmental delay, accompanied by bang sensitivity, the canonical adult phenotype of tko<sup>25t</sup>.

These new findings indicate the existence of a systemic and possibly metabolic signal integrating growth across the entire larva, in response to limitations on mitochondrial translational or OXPHOS capacity. Given the previous findings implicating pyruvate and/or lactate as key regulatory metabolites, we studied the effects of drugs and RNAi targeted on pyruvate and its metabolic transactions. The findings are consistent with pyruvate metabolism playing a central role in generating the signal that links growth and mitochondrial function in Drosophila larvae.

Materials and methods

Drosophila strains and culture

Drosophila strains were procured from stock centres, supplied by colleagues or maintained long term in our laboratory. A full list of GAL4 drivers, RNAi lines and other strains used in the study are provided in Tables 1, 2 and 3, respectively. Markers carried on standard balancers were used to distinguish experimental from control progeny. Except where stated, flies were cultured on standard high-sugar medium (HS), as detailed in [7]. A variant, ‘zero-sugar’ medium (ZS), containing standard dietary supplements but no added sugars [7], was used where indicated. Note that HS and ZS media are not isocaloric: in an earlier study [7] the extent of developmental delay in tko<sup>25t</sup> flies was shown to depend only on the sugar content of the medium, not its calorific value. Sodium pyruvate, dichloroacetate or UK5099 (Sigma-Aldrich), were added to these media from aqueous stock solutions after the medium had been cooled to below 65°C, giving the final concentrations indicated in the figures.

Developmental and bang-sensitivity assays

Mean developmental time to eclosion and bang-sensitivity were measured as previously [5,7], in temperature-controlled incubators, with temperature verified daily throughout the experiment. In all crosses where developmental time to eclosion was measured, at least 3 (usually 4) replicate vials were studied, and the entire experiment was repeated to validate the findings.
RNA analysis

Quantitative reverse-transcription PCR (qRTPCR) to confirm the effectiveness of RNAi was conducted as previously \[7,37\], using RpL32 as an internal standard and verified primer pairs (all shown 5' to 3') as follows: for \(Pdk\) – GGATTCGGAACAGATGCAAT and CGCGATAGAACTTTGAGCTTG, for \(Mpc1\) – GCCGACACACAAAAGAGTCC and GCTGGACCTTGTAGGCAAAT, for \(Men\) – ACTCGATCCTACGACGCTGT and TGAGGAAGGACTCTGCGAAT. RNA sequencing and data analysis were carried out as previously \([7]\), using RNA from Oregon R or \(tko^{2St}\) L3-stage larvae cultured on different media. Note that initial statistical filtering by Cuffdiff (chipster. csc.fi) excludes genes in any pairwise comparison where the differences fail significance testing, regardless of their magnitude. For further analysis (see Results), arbitrary thresholds were then applied to restrict the analysis to genes showing substantial differences in expression, as measured by either of two parameters: >8 fold change.

### Table 1. GAL4 drivers used in the study.

| Name* | Stock centre ID\(\text{a}\) or source | Chromosome | Expression pattern | References\(\text{d}\) |
|-------|---------------------------------|-------------|-------------------|-------------------|
| da-GAL4 | BL 8641 | 3 | ubiquitous | [8] |
| gut-GAL4 | KY 113094 | 2 | midgut, proventriculus, gastric ceca, salivary glands | [7,9], Fig. S1 |
| elav-GAL4 | BL 458 | X | neurons, embryonic neuroblasts and glioblasts | [10,11] |
| nrz2-GAL4 | BL 6800 | 2 | glial cells, weak expression in some neurons | [12–16], Fig. S1 |
| G14 | kind gift of John Sparrow | G14-Gal4 | muscle, salivary glands | [17–19] |
| Me2F2-GAL4 | BL 27390 | 3 | muscle | [20] |
| Kr-GAL4 | kind gift of John Sparrow | | early embryo, larval midgut | [21,22], Fig. S1 |
| Lsp2-GAL4 | BL 6357 | 3 | Fat body (third larval instar and adult) | [23,24], Fig. S1 |

*a as used here.

\(\text{b} BL =\) Bloomington, KY = Kyoto.

*c consensus or most recent revision from published literature, or based on figures of this paper.

*d literature citation(s) or figure of this paper.

### Table 2. RNAi lines used in the study.

| Symbol of targeted gene | Stock centre ID\(\text{a}\) | Chromosome (insertion) | Reference |
|-------------------------|--------------------------|------------------------|-----------|
| tko | BL 38251 | TRiP 2 | [25,26] |
| Mpc1 | BL 67817 | TRiP 2 | [25,27] |
| Pdk | BL 28613 | TRiP 3 | [25,26] |
| Men | BL 38256 | TRiP 2 | [25,26] |
| Men | VDRC 330428 | shRNA | [28] |
| Men | VDRC 104016 | KK | [28,29] |
| Men-b | BL 57489 | TRiP 2 | [25,27] |
| Men-b | VDRC 100812 | KK | [28,29] |
| Ldh | VDRC 110190 | KK | [29] |

*a BL = Bloomington, VDRC = Vienna Drosophila Research Centre.

*b literature citation(s).

### Table 3. Other Drosophila strains used in the study.

| Name\(\text{a}\) | Stock centre ID\(\text{b}\) | Chromosome (insertion, mutation or balancer) | Other features\(\text{c}\) | References\(\text{d}\) |
|-----------------|------------------|---------------------------------|------------------|-------------------|
| tko\(2St\) | n/a | X | Bang-sensitive; coding-region mis-sense mutant | [30–32] |
| UAS-tko\(\text{a}\) (1) | n/a | 3 | transgenic for wild-type copy of tko cDNA; without driver does not alleviate bang sensitivity of tko\(2St\) | [6] |
| UAS-tko\(\text{a}\) (8) | n/a | 2 | transgenic for wild-type copy of tko cDNA; without driver does not alleviate developmental delay of tko\(2St\) | [6] |
| FM7 | BL 995 | X | balancer chromosome | [33] |
| CyO | BL 4959 | 2 | balancer chromosome | [34] |
| TM35b | n/a | TM3-5b | 3 | balancer chromosome, currently available from stock centre combined with CyO | [34] |
| UAS-Stinger | BL 65402 | UAS-GFP, UAS-Stinger | transgenic expressor of nuclear-targeted GFP | [35] |
| UAS-mCD8-GFP | KY 108068 | mCD8-GFP, + many variants | transgenic expressor of membrane-targeted GFP | [36] |

*a as used here.

*b BL = Bloomington, KY = Kyoto, n/a not currently available from stock centres.

*c consensus or most recent revision from published literature, or based on figures of this paper.

*d literature citation(s) or figure of this paper (Figure 1).
or >100 units of FPKM (mass fraction). Raw sequence data have been deposited at ArrayExpress (www.ebi.ac.uk/ArrayExpress/).

**Metabolite analysis**

Batches of 20 larvae were homogenized in 100 μl of 6M guanidine hydrochloride on ice. The homogenate was incubated at 95°C for 5 min and centrifuged at 12000 g_{max} for 5 min at 4°C. The supernatants were stored at −80°C, and later diluted 1:10 and with PBS (pH 7.4) for analysis. Pyruvate and lactate were measured with commercially available fluorescence-based determination kits (Abcam) according to manufacturer’s instructions. 10 μl of sample was combined with 50 μl of either lactate or pyruvate reaction mix, incubated at room temperature for 30 min after which fluorescence was measured (excitation at 535 nm, emission at 590 nm) using a plate reader. Lactate and pyruvate standards were used to generate standard curves and concentrations were normalized to soluble protein as measured using the Bradford method.

**Statistics**

For pairwise comparisons between groups, the two-tailed (unpaired) Student’s t test (Microsoft Excel) was applied. For multiple comparisons, we used one-way ANOVA with Tukey post hoc HSD test online (astatsa.com). For comparisons where multiple factors were being assessed, two-way ANOVA (GraphPad Prism or online tool at vassartstats.net/anova2u.html, as indicated) was used, together with Dunnett’s or Tukey’s post hoc multiple comparisons tests as indicated, where interactions were detected, or where more than two levels were compared.

**Results**

**Wild-type tko expression in diverse tissues alleviates developmental delay in tko^{25t}**

Using the line UAS-tko’^{(8)} [6], in which a wild-type tko transgene is expressed under the control of GAL4, we tested the tissue-specificity of developmental delay in the tko^{25t} background, by combining it with different GAL4 drivers directing distinct tissue patterns of expression. In addition to almost complete rescue using da-GAL4 and the partial rescue with gut-GAL4 documented previously, we found that drivers specific for the fat body (Lsp2-GAL4), muscle (G14 and Mef2-GAL4) and CNS (neurons, elav-GAL4) all gave a partial rescue (Figure 1), although this was most pronounced or significant in each case at different characteristic temperatures (Table S1).

We confirmed the specificity of patterns of expression of these drivers using GAL4-dependent constructs for GFP (Fig. S1). The alleviation of the phenotype was due to a positive effect of the transgene/driver combination, and not due to a negative effect of the CyO balancer chromosome in the tko^{25t} background (Fig. S2). Note, however, that this was not true of the TM3Sb balancer (Fig. S2), the use of which was therefore avoided in all experiments described.

The partial rescue produced by several drivers appeared to be additive, based on two lines of evidence. First, we attempted to combine pairs of drivers and the UAS-tko’^{(8)} transgene. This experiment was technically challenging for several reasons: the problematic nature of the TM3Sb balancer precluded its use; some transgenic combinations had poor viability, especially as homozygotes, and the drivers did not all perform optimally at the same temperature. However, the combination of the fat-body and muscle drivers Lsp2-GAL4 and G14 appeared to give an additive enhancement at 22°C (Fig. S3), although this should be interpreted cautiously, due to the imperfect experimental design. The second piece of evidence for a combinatorial effect was that a second transgenic line, UAS-tko’^{(1)}, which already showed a one-day alleviation of developmental delay compared with tko^{25t} flies bearing no transgene [6], showed a further alleviation of developmental delay when combined with different GAL4 drivers (Figure 2). These findings suggest the operation of a systemic signal that integrates the degree of mitochondrial dysfunction across tissues, calibrating growth to the ability of the organism to process nutritional resources.

**RNAi-mediated tko knockdown in diverse tissues phenocopies tko^{25t}**

To further test this hypothesis, we used RNA-mediated knockdown of tko to profile the tissues in which the resulting mitochondrial translational deficit leads to a tko^{25t}-like phenotype. Ubiquitous tko knockdown using the da-GAL4 driver and the
Bloomington TRiP line 38251 targeted on tko resulted in a phenotype resembling an exaggerated version of tko\textsuperscript{25t}. At 25°C tko knockdown was lethal, whilst at 22°C, it was lethal to males and semilethal to females, which eclosed with a long delay (5–9 d) and were too weak to permit a meaningful test of their bang-sensitivity. At 18°C females eclosed with a 7 to 8-d delay (Figure 3(a)) and were highly bang-sensitive (Figure 4(a)), whilst the few males that eclosed were even more delayed (Figure 3(a)) and extremely weak. Knockdown using tissue-specific drivers produced a milder version of the same phenotype, whether knockdown was targeted specifically to neurons (using elav-GAL4, Figures 3(b), 4(b)) or muscle (Mef2-GAL4 at 18°C, Figures 3(c), 4(c)). Even more limited knockdown targeted on the early embryo, and portions of the midgut (Kr-GAL4, Figure 3(d), Fig. S1) also produced a significant, though very modest developmental delay, but without bang-sensitivity (Figure 4(e)). Mef2-GAL4-driven tko knockdown at higher temperatures (22, 25°C) again gave...
semilethality and severe weakness, which was more severe in males. *elav*-GAL4-driven knockdown also gave sex- and temperature-dependent bang-sensitivity: at 25°C flies were extremely weak, whilst developmental delay was seen at all temperatures but was generally significant only in males (Figure 4(b)). Driving *tko* knockdown with the glial driver *nrv2*-GAL4 gave no bang sensitivity (Figure 4(d)).

**Drugs that affect pyruvate metabolism impact larval growth**

In principle, a systemic signal regulating growth according to mitochondrial function could be endocrine or metabolic in nature. Candidate metabolites for such a role that were previously shown to be markedly abnormal in *tko* larvae, include pyruvate and lactate, which were approximately threefold elevated, and NADPH and ATP, which were highly depleted [7]. Since ATP and NADPH may be considered too labile to perform an intercellular role, we focused our attention on pyruvate and lactate, which are interconvertible through lactate dehydrogenase, and which were previously found to exacerbate and phenocopy the developmental delay of *tko*, when added to the medium ([7], Figure 5(a)). We investigated the developmental effect of two drugs known to affect pyruvate metabolism, dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (Pdk) and UK5099, an inhibitor of the mitochondrial pyruvate carrier, which could be predicted to have

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**Figure 2.** Alleviation of *tko* developmental delay in a second UAS-*tko* line. Time to eclosion at 25°C (means ± SD, n ≥ 3 replicate vials for each cross), of flies of the indicated genotypes, using various drivers plus the UAS-*tko* transgene, shown previously to confer a modest rescue of developmental delay without any driver [6]. Controls were FM7 balancer flies with transgene but without driver, as shown. Horizontal lines denoted by asterisks (*, **, *** ) indicated significant differences in pairwise comparisons of flies of a given sex and *tko* genotype, with and without actively driven *tko* (Student’s t test, p < 0.05, 0.01, 0.001, respectively. The specificity of each driver was confirmed by parallel crosses in which it was used to direct the synthesis of nuclear- or membrane-localized GFP (see Figure S1). Note that we avoided the use of the TM3 balancer because we established that it conferred a developmental delay in conjunction with *tko*, whereas the chromosome 2 balancer CyO did not (Fig. S2).
We tested different concentrations of pyruvate and DCA for their effects on the development of wild-type and tko females in ZS medium (Figure 5(b)), and performed a more extensive study at single, effective concentrations on different media and both sexes, including heterozygous tko females (Figure 5(a)). Like pyruvate, DCA produced an additional, dose-dependent developmental delay in both tko as well as in wild-type flies (Figure 5(a, b, S5)).

UK5099 (25 μg/ml) also exacerbated the developmental delay of tko, but had no significant effect on the eclosion timing of wild-type flies (Figure 5(a, S5)).

**Genetic manipulations that affect pyruvate metabolism impact larval growth and survival**

Next, we analyzed the effects of knocking down the genes coding for the key proteins of pyruvate metabolism targeted by these drugs. The mitochondrial pyruvate carrier is a heterodimer of the ubiquitous subunit Mpc1 (CG14290) and a differentially expressed second subunit, Mpc2, encoded in Drosophila by a small gene family (CG9396, CG9399 and CG32832, the latter being testis specific). Pdk is encoded by a single-copy gene (Pdk, CG8808). Using the available RNAi lines for Mpc1 and Pdk from the Harvard Medical School TRiP library, we first confirmed that knockdown for each of the two genes using the ubiquitously acting da-GAL4 driver gave viable flies, and used qRTPCR to verify that knockdown was effective at the RNA level (Figure 6(a)). We then evaluated the effects of knockdown on wild-type and tko flies grown in standard high-sugar medium, or on medium supplemented with 25 mg/ml pyruvate (Figure 6(b)). Mpc1 or Pdk knockdown produced no effect on eclosion timing in wild-type flies cultured on standard medium. However, when pyruvate was

![Figure 3. RNAi knockdown of tko by different drivers results in developmental delay. Times to eclosion (means ± SD, n ≥ 3 replicate vials for each cross) for flies of the indicated sex and genotype, using the various drivers at the temperatures shown. Horizontal lines denoted by asterisks (*, **, *** ) indicate significant differences in pairwise comparisons between knockdown and control flies of a given sex, using a given driver (Student’s t test, p < 0.05, 0.01, 0.001, respectively). Note that males were in general more severely affected and in some cases (e.g. da-GAL4) too few males eclosed to permit a statistically meaningful analysis. Note that most GAL4 drivers exhibit the classic pattern of temperature dependence [38], i.e. increased activity at higher temperature. However, for the strongest drivers this may also lead to highly deleterious effects at high temperature, such that, at a lower temperature, results are more informative.](image)
added to the medium, \( \text{Mpc1} \) knockdown exacerbated the developmental delay produced in wild-type flies, but not that of \( \text{tko}^{25\text{t}} \) (Figure 6(b), panel i), whilst \( \text{Pdk} \) knockdown had no effect on eclosion timing of wild-type flies on either medium, but mildly alleviated the developmental delay of \( \text{tko}^{25\text{t}} \) (Figure 6(b), panel ii) on pyruvate-supplemented medium (see Fig. S6 for summary of the relevant statistical analyses by two-way ANOVA).

Knockdown of two other genes of pyruvate metabolism, coding, respectively, for the cytosolic and mitochondrial isoforms of malic enzyme, produced more dramatic results in combination with \( \text{tko}^{25\text{t}} \). In our previous study, we observed that knockdown of \( \text{Men} \), the gene encoding the cytosolic isoenzyme, produced no significant effects on \( \text{tko}^{25\text{t}} \) (Figure 4(c) of [7]). In the present study we were able to make use of more potent and specific TRiP lines for \( \text{Men} \), as well as one further dsRNA line from the VDRC collection, all of which gave a strong knockdown of the gene at the RNA level under the control of \( \text{da-GAL4} \) (Figure 7(a)), but had no significant effect on the development of otherwise wild-type flies (Figure 7(b)). However, in combination with \( \text{tko}^{25\text{t}} \) they were all developmentally lethal or semilethal at both 29°C and 25°C (Figure 7(c)). Both of the \( \text{Men-b} \) knockdown lines produced a developmental delay in wild-type flies, and were again synthetically lethal with \( \text{tko}^{25\text{t}} \) (Fig. S4), but this result should be interpreted cautiously, since we were not able to demonstrate convincing and consistent knockdown of \( \text{Men-b} \) at the RNA level by qRT-PCR, using several different primer sets. Knockdown of lactate dehydrogenase (\( \text{Ldh} \)) was lethal to both wild-type and \( \text{tko}^{25\text{t}} \) larvae, making comparable experiments uninformative.

Despite their similar effects on eclosion timing (Figure 5), the addition of pyruvate and DCA to the low-sugar culture medium had opposite effects on the tissue levels of pyruvate and lactate in L3 larvae (Figure 8), although the changes were only significant for pyruvate levels (Tables S3). Pyruvate addition increased both lactate and pyruvate to levels comparable with those seen in high-sugar medium, whilst DCA lowered them, in accord with the expectation

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**Figure 4.** RNAi knockdown of \( \text{tko} \) by different drivers results in bang-sensitivity. Box-plots of recovery times from mechanical shock (bold black or red lines – medians, respectively, for controls and \( \text{tko} \) knockdown flies, with filled boxes representing first to third quartiles) of flies of the indicated genotype, sex and culture temperature. Note the different scales required to plot these data. White bars for control flies were in most cases not plottable, since median and both quartiles were at or very close to zero.
that it would augment pyruvate metabolism by removing inhibition from (i.e. activating) PDH. The different effects of pyruvate and DCA imply that growth rate cannot be determined directly or solely by pyruvate concentration averaged across the tissues. Instead, a metabolite of pyruvate, or pyruvate in a specific intracellular or tissue-compartment, is likely to be instrumental.

**Gene expression changes induced by pyruvate are non-coherent with those induced by tko^{25t}**

Growth of tko^{25t} in high sugar was previously observed to downregulate some components of the protein synthetic and secretory machinery, as well as a number of key genes involved in developmental progression. These effects were seen also in zero-sugar medium but were less pronounced. We used RNA-seq to test whether pyruvate addition produced the same changes in gene expression, applying similar criteria as in the previous study [7]. Using each of two parameters (i) absolute magnitude of changes measured by mass fraction (FPKM), and (ii) fold-change, we created lists of the genes showing the most pronounced alterations in expression due to pyruvate supplementation (Table S4). We considered both protein-coding and non-coding RNAs, as well as increases and decreases. From these ‘base-comparison’ lists of pyruvate-regulated genes (198 in the mass-fraction list and 248 in the fold-change list, applying arbitrary thresholds of >100 FPKM units or >eightfold change, Tables S4) we asked how many are regulated similarly in each of
six additional comparisons (Figure 9). In each output we considered the genes from the base-comparison list in five categories: those regulated in the same direction, either (i) above or (ii) below the arbitrary thresholds, those regulated in the opposite direction, again (iii) above or (iv) below threshold, and (v) those missing from the compared list, due to the initial statistical filtering. This analysis enabled us to draw the following conclusions, illustrated in Figure 9. First, most of the genes up- or down-regulated by pyruvate in wild-type larvae were regulated similarly by pyruvate in tko<sup>25T</sup> (comparison 1 in Figure 9). Second, only a minor subset of genes (≤15%) were regulated in the same direction by high-sugar (comparisons 2 and 3). Many genes were oppositely regulated by the presence of tko<sup>25T</sup> (comparisons 4 and 5), and many pyruvate-regulated genes in wild-type showed either no effect or a change in the opposite direction in tko<sup>25T</sup> larvae (comparison 6). The two approaches (mass-fraction and fold-change) gave similar outcomes. The functionally identified genes regulated similarly by pyruvate and by sugar were mostly linked to development.
(cuticular, muscle and a few gut proteins: Tables S5, sheet 1). Many of them, or others in the same functional categories, were oppositely altered in tko\textsuperscript{25t} (Tables S5, sheet 2). Many of the remaining genes regulated by dietary pyruvate, that were not affected by sugar or by tko\textsuperscript{25t}, code for components of the gene expression machinery or for OXPHOS and other mitochondrial functions (Table S4).

**Discussion**

**Developmental delay in tko\textsuperscript{25t} depends on a systemic signal**

Three lines of evidence presented in this paper support the idea that a systemic signal links mitochondrial function to growth rate in *Drosophila* development. First, the developmental delay of tko\textsuperscript{25t} mutant larvae could be partially corrected (Figure 1) by expression of the wild-type allele via any of several different tissue-specific drivers, showing little or no overlap in the specificity of their expression (Fig. S4A). Second, as far as could be demonstrated, these effects were additive (Figure 2, S4). Third, RNAi-mediated knockdown of tko using similarly specific drivers regenerated the mutant phenotype of tko\textsuperscript{25t}, including both developmental delay as well as (at least in some cases) bang-sensitivity. These observations imply that diminished mitochondrial function in a specific tissue is somehow integrated across the entire larva, presumably by

![Figure 7. RNAi-mediated knockdown of Men (malic enzyme is synthetically (semi)lethal with tko\textsuperscript{25t}. (a) Men RNA levels measured by qRTPCR and (b) time to eclosion of flies of the indicated genotypes (i.e. with relevant Men RNAi construct or balancer as shown), means ± SD, n ≥ 3 vials from each cross. Eclosion timing is shown for the temperature at which flies were also cultured for qRTPCR (25°C or 29°C as shown). Eclosion timing at the other temperature was qualitatively similar, as shown in Fig. S4A. Horizontal bars denoted by asterisks (***) indicate significant differences, in pairwise comparisons of RNAi and balancer control flies of each given genotype and sex analyzed (Student’s t test, \(p < 0.001\)). (c) Proportion (%) of eclosing RNAi male progeny that also carried tko\textsuperscript{25t}, as opposed to the FM7 balancer, from crosses of the general type: tko\textsuperscript{25t}/FM7; daGAL4 x FM7/Y; RNAi. Means ± SD, n ≥ 3 vials from each cross. Asterisks (***) above the bars denotes significant deviation from expected frequency of 50% (chi-squared test with Yates’ continuity correction, \(p < 0.001\)). See also Fig. S4. For a summary diagram of the reactions targeted by RNAi and by various drugs see Figure 10.](image-url)
the sharing of metabolites and their processing. Furthermore, this is not an all-or-none phenomenon, where growth is switched between two alternate programs whenever mitochondrial function crosses some threshold: the amount of developmental delay is variable, implying that the overall metabolic capacity of the larva is somehow being measured physiologically, and growth rate adjusted accordingly. These findings could be further strengthened by the use of additional drivers, especially in combination, although the problematic nature of the TM3 balancer means that we would need first to identify those carried on chromosome 3 that were viable as homozygotes (also with no maternal effects) and which had no unreported off-target expression patterns.

As indicated above, we hypothesized that such a signal could be either endocrine or metabolic in nature, or a combination of both. Endocrine signals of metabolic – specifically mitochondrial stress are well established in other organisms, such as FGF21 or GDF15 in mammals [39]. Although both of these growth-factor superfamilies have representatives in insects, neither has a clear orthologue in Drosophila. The closest match to human GDF15, Glass bottom boat, is much more similar to other members of the BMP/TGFβ superfamily, whilst none of the three Drosophila FGFs (Pyramus, Thisbe and Branchless) is a convincing match to human FGF21, and each of them plays a well-studied role in specific developmental programs (see [40] for review).

Two other well-known endocrine systems in Drosophila do provide a more direct connection between growth regulation and mitochondrial metabolism, namely the insulin-like peptides and the steroid hormone ecdysone. The insulin/IGF signalling system in the fly [41,42] is responsive to metabolic and nutritional signals to co-ordinate growth and behaviour, whilst ecdysteroids, partly synthesized in mitochondria [43,44], regulate progression between the different developmental stages, especially the onset of metamorphosis [45]. It will be worthwhile to study whether either interacts with tko25t.

Tissue-specificity of the bang-sensitive phenotype

Bang-sensitivity is reported as a feature of mutants in two other genes for core functions of mitochondria, namely sesB (adenine nucleotide translocase) [46] and kdn (citrate synthase) [47]. These mutants, as well as tko25t, show ATP depletion [47–49]. Global tko knockdown was here shown to phenocopy both the developmental delay (Figure 3) and bang-sensitivity (Figure 4) of the original tko25t mutant, supporting the view that tko25t is a simple hypomorph. However, the two phenotypes are clearly separable, based on the fact that the transgenic line UAS-tko*(8) shows no rescue of developmental delay in the absence of a GAL4 driver, but does exhibit effective rescue of bang-sensitivity [6], due, presumably, to an insertional effect [50]. The use of tissue-specific drivers to knock down tko sheds further light on this, since exclusively neuronal or muscle drivers both gave a bang-sensitive phenotype (Figure 4(a–c)), whereas a glial driver or one active in embryogenesis and in parts of the larval midgut (Fig. S1A) did not (Figure 4(d,e)). These findings imply
that adequate mitochondrial OXPHOS capacity in muscle and in neurons, but not in other tissues, is required to avoid the prolonged paralytic seizures characteristic of *tko*<sup>25t</sup>. The severity of the bang-sensitive phenotype produced by knockdown of *tko* using different GAL4 drivers is comparable with, or in some cases stronger, than that produced by *tko*<sup>25t</sup>. However, since the relative strength of the various tissue-specific drivers is not easily measured, the significance of this cannot be assessed.

**Lactate and pyruvate metabolism is linked to growth regulation in *tko*<sup>25t</sup>**

Candidates for a metabolic signal of mitochondrial dysfunction were already suggested by the previous
observation of elevated pyruvate and lactate levels in \textit{tko}^{25t} larvae [7], and the fact that their addition to the culture medium phenocopied the effects of high-sugar on \textit{tko}^{25t}. Serum lactate is already considered a key biomarker for diagnosing OXPHOS disorders in humans [39], whilst pyruvate has been identified as a crucial regulator of stemness and growth in mammalian cells [51]. In flies, the enzyme that interconverts lactate and pyruvate, lactate dehydrogenase (Ldh, or ImpL3) has previously been implicated as a target of growth signalling by the estrogen-related receptor [52]. This offered an attractive starting point for the present study.

Drugs and genetic manipulations that affect pyruvate metabolism (summarized in Figure 10) impacted the development of \textit{tko}^{25t} (and, in some cases, wild-type flies), broadly supporting the view that pyruvate accumulation is a marker for a metabolic process that limits growth. The growth-inhibiting effects of pyruvate and of DCA were also dose-dependent. However, measurements of lactate and pyruvate levels in flies grown on different media (Figure 8) implied that neither was a direct predictor of developmental phenotype, which may instead depend on a downstream product of pyruvate metabolism. Thus, pyruvate or lactate supplementation resulted in only a modest increase in the steady-state level of pyruvate, whereas DCA, which substantially decreased pyruvate and lactate levels (Figure 8), produced paradoxical effects on developmental timing, accelerating the growth of \textit{tko}^{25t} on zero-sugar medium, but retarding that of wild-type flies, whilst in high-sugar medium it compounded rather than alleviated \textit{tko}^{25t} developmental delay (Figure 5(b)). Pdk knockdown exacerbated the developmental delay of \textit{tko}^{25t} (Figure 6(b), ii), but only under conditions of pyruvate overload. Although DCA is a potent inhibitor of Pdk [53], it may also have other targets [54–56] and is accumulated in cells via the plasma-membrane monocarboxylate transporters, MCTs [57]. Moreover, in cancer cells, it has been reported to inhibit the pentose phosphate pathway [58] and thereby decrease the level of NADPH, which is already depleted in \textit{tko}^{25t}, especially when grown on high-sugar medium [7]. Such off-target effects may account for the different outcomes of DCA treatment and Pdk knockdown.

UK5099, predicted to limit mitochondrial pyruvate utilization by inhibiting the mitochondrial pyruvate carrier, produced a clear exacerbation of the \textit{tko}^{25t} developmental phenotype (Figure 5(b)), whilst \textit{Mpc1} knockdown produced different effects, slowing the growth of wild-type flies under pyruvate overload, but exhibiting only minor effects on \textit{tko}^{25t} development that were essentially epistatic to the effect of added pyruvate (Figure 6(b), i). Although originally identified as an inhibitor of the mitochondrial pyruvate carrier [59], UK5099 was subsequently shown to block MCTs as well [60–63]. If it blocks lactate efflux \textit{in vivo}, its net effect may be to increase cytosolic lactate and/or pyruvate independently of its effects on mitochondria, potentially accounting for the different outcome of \textit{Mpc1} knockdown. Other, more specific inhibitors, such as PS10 for Pdk [64], or GW604714X and GW450863X for the mitochondrial pyruvate carrier [65], may prove useful in disentangling these effects, although their efficacy and specificity would need to be verified for \textit{Drosophila}.

The findings with \textit{Men} are also consistent with pyruvate being a critical metabolite, although the effect on \textit{tko}^{25t} was lethality rather than extended development. One possibility is that a high level of pyruvate facilitates NADPH depletion via the NADPH-dependent conversion of pyruvate to malate catalyzed by Men. If, as hypothesized earlier, NADPH depletion is a critical element of growth signalling in \textit{tko}^{25t} [7], a decreased capacity of pyruvate to malate conversion could undermine this signalling, with catastrophic consequences for a larva with severe limitations on TCA-cycle and biosynthetic flux, and ATP depletion.

Overall, whilst we can infer that manipulations affecting pyruvate and lactate metabolism affect growth-regulation in \textit{tko}^{25t}, possible off-target or secondary effects of the drugs and knockdowns preclude a definitive mechanistic conclusion at this time. Unravelling the many possibilities will require the development of methods for assaying fluxes of the key metabolites at the subcellular level \textit{in vivo}, considering separately the mitochondrial and cytosolic pools of pyruvate, malate, NADPH, ATP and their derivatives.

\textbf{Pyruvate modulates growth rate translationally and/or post-translationally}

Growth of \textit{tko}^{25t} in high-sugar medium was previously observed to affect gene expression in two
ways: first, via a post-translational mechanism affecting S6K, second, via changes in transcript representation [7]. In the present study, we found that only a minor fraction of the genes regulated at the RNA level by pyruvate were regulated in the same way by high sugar, whilst those responding to tko25t were mostly altered in the opposite direction. This was unexpected, given the fact that tko25t responded similarly to pyruvate and high sugar, and suggests that global growth regulation in response to mitochondrial dysfunction and metabolic disturbance occurs mainly at the (post-)translational level. Regulation at the protein level is also suggested by the fact that the list of similarly or oppositely regulated genes at the RNA level includes very few that are connected either to metabolism or to transcription. It remains possible that relevant changes remain buried in the list of transcriptional targets because they change only by rather modest amounts that are below the thresholds set here but are nevertheless critical to the regulation of growth. More plausibly, a global regulator like the cyclin-dependent kinases or AMPK, which would not have to compete with cell-specific transcriptional programs, can respond to a systemic signal in broadly similar ways in all cells.

In conclusion, this study provides evidence that mitochondrial dysfunction triggers a systemic response that curtails the rate of growth of Drosophila larvae, and confirms a key role for pyruvate or its metabolic products in eliciting this signal.

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No potential conflict of interest was reported by the authors. During the review process of the manuscript, corresponding author HTJ was appointed as Editor-in-Chief of the journal. However, he remained uninvolved in, and blinded to, the review and editorial process.

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