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## Research Paper

Sources of superoxide/H<sub>2</sub>O<sub>2</sub> during mitochondrial proline oxidation

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## ABSTRACT

p53 Inducible gene 6 (PIG6) encodes mitochondrial proline dehydrogenase (PRODH) and is up-regulated several fold upon p53 activation. Proline dehydrogenase is proposed to generate radicals that contribute to cancer cell apoptosis. However, there are at least 10 mitochondrial sites that can produce superoxide and/or H<sub>2</sub>O<sub>2</sub>, and it is unclear whether proline dehydrogenase generates these species directly, or instead drives production by other sites. Amongst six cancer cell lines, ZR75-30 human breast cancer cells had the highest basal proline dehydrogenase levels, and mitochondria isolated from ZR75-30 cells consumed oxygen and produced H<sub>2</sub>O<sub>2</sub> with proline as sole substrate. Insects use proline oxidation to fuel flight, and mitochondria isolated from *Drosophila melanogaster* were even more active with proline as sole substrate than ZR75-30 mitochondria. Using mitochondria from these two models we identified the sites involved in formation of superoxide/H<sub>2</sub>O<sub>2</sub> during proline oxidation. In mitochondria from *Drosophila* the main sites were respiratory complexes I and II. In mitochondria from ZR75-30 breast cancer cells the main sites were complex I and the oxoglutarate dehydrogenase complex. Even with combinations of substrates and respiratory chain inhibitors designed to minimize the contributions of other sites and maximize any superoxide/H<sub>2</sub>O<sub>2</sub> production from proline dehydrogenase itself, there was no significant direct contribution of proline dehydrogenase to the observed H<sub>2</sub>O<sub>2</sub> production. Thus proline oxidation by proline dehydrogenase drives superoxide/H<sub>2</sub>O<sub>2</sub> production, but it does so mainly or exclusively by providing anaplerotic carbon for other mitochondrial dehydrogenases and not by producing superoxide/H<sub>2</sub>O<sub>2</sub> directly.

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## Introduction

A hallmark of cancer cells is their ability to overcome signaling pathways that trigger cell death [1]. Programmed cell death and cell cycle arrest are partly controlled by p53 expression [2,3], therefore p53 in tumor cells is either mutated or sequestered in the cytoplasm by its negative modulator MDM2 [4]. Apoptosis

induced by p53 activation is preceded by the expression of PIGs (p53 induced genes) [5]. One of the most prominent is PIG6, which encodes the mitochondrial enzyme proline dehydrogenase (PRODH) [5]. In most tumor cells proline dehydrogenase levels are low [6,7]. Proline dehydrogenase overexpression in certain cancer cell lines is associated with apoptosis through mechanisms involving proline-dependent mitochondrial production of reactive oxygen species (ROS) [6,8–10]. Since proline dehydrogenase overexpression is also associated with reduced tumor formation *in vivo*, it has been suggested as a direct mediator of p53-induced tumor suppression [7]. Paradoxically, some cancer cells may use proline oxidation to drive proliferation and protection against stress, and inhibition of proline dehydrogenase in such proline-addicted cells may be therapeutically useful [7,11–13].

Cancer cells utilize a variety of metabolic pathways to support the high energy demands of proliferation and biosynthesis [14]. Recently, proline catabolism has gained attention as one such pathway, particularly under nutrient stress [11,12,15]. Proline and hydroxyproline comprise ~20% of the amino acids in collagen [16], the main constituent of the extracellular matrix. Collagen

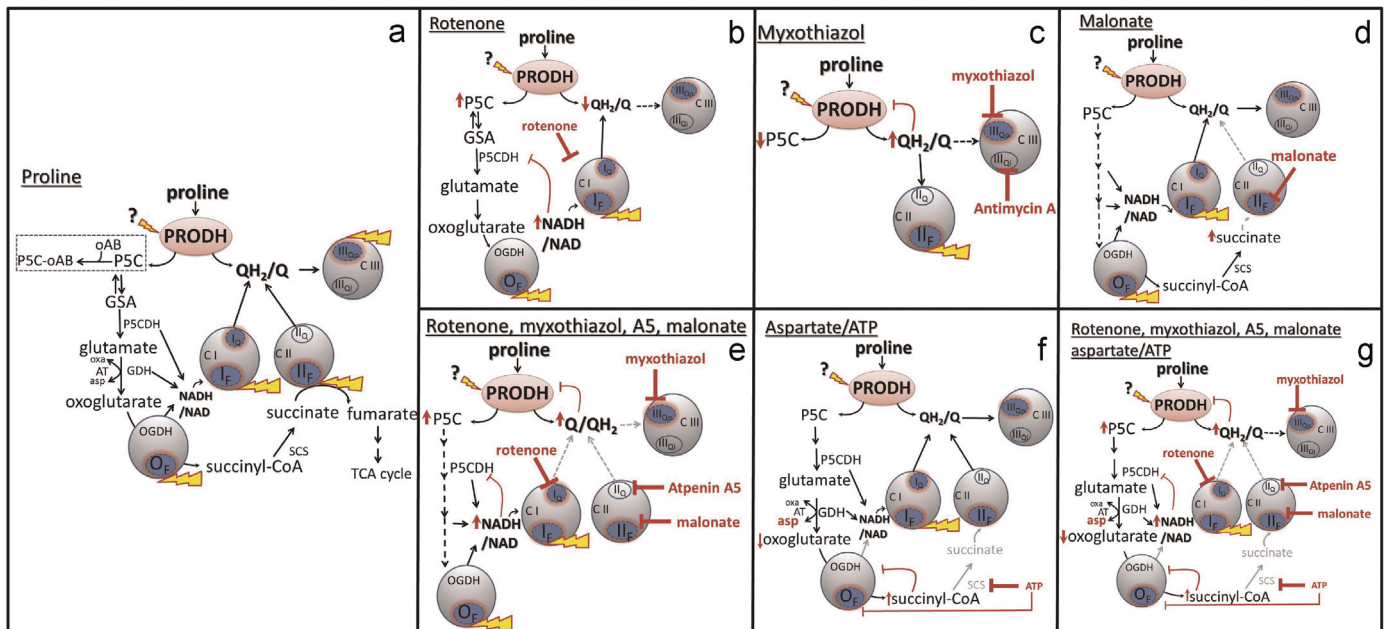
**Abbreviations:** PIG6, proline dehydrogenase inducible gene 6; PRODH, proline dehydrogenase; GDH, glutamate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase complex; SCS, succinyl-CoA synthase; AT, aminotransferase; ROS, reactive oxygen species; P5C, Δ<sup>1</sup>-pyrroline-5-carboxylate; Oxa, oxaloacetate; Asp, aspartate; TCA, tricarboxylic acid; A5, atpenin A5; GSA, glutamic semi-aldehyde; oAB, o-aminobenzaldehyde; I<sub>F</sub>, flavin of complex I; I<sub>IF</sub>, flavin of complex II; Ill<sub>Qo</sub>, quinone binding site on the outer/cytosolic face of complex III; O<sub>F</sub>, Flavin of the oxoglutarate dehydrogenase complex

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**Fig. 1.** Metabolic pathways and sites of superoxide/H<sub>2</sub>O<sub>2</sub> production during mitochondrial proline oxidation. Electron transport chain complexes I, II, and III and the 2-oxoglutarate dehydrogenase complex (OGDH) are shown as gray circles; sites that can generate superoxide and/or H<sub>2</sub>O<sub>2</sub> as blue ovals; sites that may produce superoxide/H<sub>2</sub>O<sub>2</sub> in particular conditions by yellow tags; sites of inhibition by red blunted arrows. (a) Control conditions. Proline dehydrogenase (PRODH) oxidizes proline to  $\Delta^1$ -pyrroline-5-carboxylate (P5C), directly reducing ubiquinone (Q) to ubiquinol (QH<sub>2</sub>) and indirectly reducing NAD to NADH through the dehydrogenases of P5C (P5CDH), glutamate (GDH) and 2-oxoglutarate (OGDH). P5C spontaneously forms glutamate semi-aldehyde (GSA) and its formation can be monitored colorimetrically with o-aminobenzaldehyde (oAB) (dotted rectangle). Sites active in the presence of: (b) rotenone to inhibit I<sub>0</sub>; (c) myxothiazol or antimycin A to inhibit complex III; (d) malonate to inhibit II<sub>F</sub>; (e) rotenone, myxothiazol, malonate and A5, leaving only site I<sub>F</sub> (and O<sub>F</sub>) active to produce ROS; (f) aspartate and ATP to inhibit OGDH [22]; (g) rotenone, myxothiazol, malonate, A5, aspartate and ATP to inhibit superoxide/H<sub>2</sub>O<sub>2</sub> production from I<sub>0</sub>, III<sub>Q0</sub>, II<sub>F</sub> and (partially) O<sub>F</sub>, leaving only I<sub>F</sub> and proline dehydrogenase active to produce ROS. Here we examine whether proline dehydrogenase can generate superoxide/H<sub>2</sub>O<sub>2</sub>, as indicated by "?". Oxa, oxaloacetate; asp, aspartate; AT, aminotransferase; SCS, succinyl-CoA synthase; TCA, tricarboxylic acid; A5, atpenin A5.

may be degraded to provide amino acids and energy for growing and proliferating cells. In eukaryotes, proline is catabolized to glutamate in the mitochondrial matrix through proline dehydrogenase and  $\Delta^1$ -pyrroline-5-carboxylate (P5C) dehydrogenase (Fig. 1a). Proline dehydrogenase oxidizes proline to P5C and reduces flavin adenine dinucleotide (FAD) to FADH<sub>2</sub>. *In vitro*, FAD is regenerated using artificial electron acceptors [17]. The natural electron acceptor is unknown but may be ubiquinone in the mitochondrial respiratory chain [18]. P5C dehydrogenase then oxidizes P5C to glutamate and reduces NAD<sup>+</sup> to NADH + H<sup>+</sup>. The glutamate is metabolized to 2-oxoglutarate, succinate and other tricarboxylic acid intermediates; oxoglutarate and succinate levels have been shown to change upon proline dehydrogenase expression [7]. Thus proline oxidation feeds electrons into the electron transport chain at multiple sites: (1) at ubiquinone from succinate dehydrogenase and probably proline dehydrogenase, and (2) at NAD from the dehydrogenases for P5C, 2-oxoglutarate and glutamate (Fig. 1a). The two enzymes involved in the oxidation of proline to glutamate are shared amongst different organisms. Therefore, proline catabolic pathway was evolutionarily conserved between microorganisms, insects and mammals [11,17,18].

Overexpression of proline dehydrogenase in different cancers increases ROS production and apoptosis [8,9,19]. *In vitro*, proline dehydrogenases from different microorganisms can reduce oxygen directly, but 16–2500 times more slowly than artificial electron acceptors [17,20]. There are at least ten sites linked to mitochondrial electron transport that can also produce superoxide and/or H<sub>2</sub>O<sub>2</sub> [21–23], and it is unclear whether proline dehydrogenase generates these species directly, or indirectly by providing substrate to other sites. The mitochondrial sites with the greatest maximum capacities for superoxide/H<sub>2</sub>O<sub>2</sub> production are the outer quinone-binding site of respiratory complex III (site III<sub>Q0</sub>), the flavin of complex II (site II<sub>F</sub>), the quinone-binding site of complex I

(site I<sub>0</sub>) and the E3 flavin of the 2-oxoglutarate dehydrogenase (site O<sub>F</sub>), pyruvate dehydrogenase (site P<sub>F</sub>) and branched-chain 2-oxoacid dehydrogenase complexes (site B<sub>F</sub>) [22–24].

Here, we use mitochondria isolated from *Drosophila* and the breast cancer cell line ZR75-30 to identify the sites involved in superoxide/H<sub>2</sub>O<sub>2</sub> production during proline oxidation. We show that sites I<sub>F</sub>, II<sub>F</sub> and O<sub>F</sub> dominate the observed H<sub>2</sub>O<sub>2</sub> production. Proline dehydrogenase itself may not generate superoxide/H<sub>2</sub>O<sub>2</sub>, but proline oxidation does so indirectly from other sites.

## Materials and methods

### Flies

*Drosophila melanogaster* strain w<sup>1118</sup> were raised on standard lab food [25]. 10–15 Day old adult males were used.

### Cells

All cells were from ATCC. ZR75-30, SJS-1 and DU4475 cells were cultured in RPMI, MCF7 and RKO cells in DMEM, and PC3 cells in DMEM/F-12 + GlutaMAX. All media were supplemented with 10% (v/v) fetal bovine serum (Gibco).

### Western blotting

Cells were harvested at ~70% confluence, washed with ice-cold PBS, lysed, and sonicated. Protein (15  $\mu$ g, determined using BCA (Thermo Scientific)) was separated on 4–12% NUPAGE gels using 1  $\times$  MES buffer (Invitrogen). Anti-PRODH 68 kDa (Santa Cruz: 376,401) was used at 1:1000 dilution. Anti  $\beta$ -actin (Santa Cruz:

47,778) was used at 1:1000 dilution. Chemiluminescence was normalized to  $\beta$ -actin.

### Mitochondria

~200 Flies were homogenized and mitochondria were isolated [26]. The final mitochondrial pellet was resuspended in KHE (120 mM KCl, 3 mM HEPES, 5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2) and kept on ice. Protein was determined by Bradford assay (Biorad).

ZR75-30 cells were harvested from 2 to 4 500  $\text{cm}^2$  dishes at ~90% confluence, washed three times in ice-cold STE (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4), scraped off, suspended in 50 ml STE and centrifuged for 10 min at 500g. The pellet was resuspended in STE supplemented with 1% (w/v) fatty acid-free bovine serum albumin and homogenized (up to 20 strokes). The suspension was brought to 50 ml and centrifuged for 5 min at 1000g. The supernatant was centrifuged for 10 min at 11,000g. The pellet was resuspended in STE and centrifuged for 10 min at 10,000g. The mitochondrial pellet was resuspended in STE and kept on ice. Protein was determined by Bradford assay (Biorad).

### Respiration

Oxygen consumption was measured at 37 °C for ZR75-30 mitochondria and 25 °C for *Drosophila* mitochondria using an XF24 extracellular flux analyzer (Seahorse Bioscience) [27]. 10  $\mu\text{g}$  Mitochondrial protein in 20  $\mu\text{l}$  was added to each well and centrifuged for 15 min at 2000g. Volume was brought to 500  $\mu\text{l}$  with KHE containing 5 mM  $\text{K}_2\text{HPO}_4$ , 2 mM  $\text{MgCl}_2$ , 0.3% (w/v) fatty acid-free bovine serum albumin (KHEPMB) and 1 mM ADP. Proline was added from port A and the “phosphorylating” rate of oxygen consumption was determined.

### Proline oxidation to P5C

Mitochondria (0.1 mg protein/ml) were incubated for 10 min in KHE containing 1 mM ADP, 5  $\mu\text{M}$  carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), 2.5  $\mu\text{g}/\text{ml}$  oligomycin and 5 mM *o*-aminobenzaldehyde (oAB). 5 mM Proline was added  $\pm$  4  $\mu\text{M}$  rotenone or 2  $\mu\text{M}$  myxothiazol. At 30 min reaction was stopped by adding 0.5% (v/v) trichloroacetic acid. pH was brought to 7.1 using KOH. Tubes were centrifuged for 10 min at 10,000g and the supernatants were transferred to new tubes containing an extra 5 mM oAB to fully develop the assay [28].  $A_{440}$  of the P5C-oAB complex was measured and the concentration was calculated using  $\epsilon_M=2580$ .  $A_{440}$  without mitochondria or proline was ~14% of the standard value.

### Superoxide/ $\text{H}_2\text{O}_2$

$\text{H}_2\text{O}_2$  was detected using 5 U/ml horseradish peroxidase to catalyze the oxidation of 50  $\mu\text{M}$  Amplex UltraRed (Molecular Probes) by  $\text{H}_2\text{O}_2$  to its fluorescent product. Exogenous superoxide dismutase (25 U/ml) converted any superoxide to  $\text{H}_2\text{O}_2$ . Fly mitochondria (0.075 mg mitochondrial protein/ml) were suspended in KHE at 25 °C with 1 mM ADP, 2.5  $\mu\text{g}/\text{ml}$  oligomycin, 5  $\mu\text{M}$  FCCP, and the detection system. Fluorescence was monitored using a microplate reader (Pherastar, BMG Labtech) at  $\lambda_{\text{excitation}}=540$  nm,  $\lambda_{\text{emission}}=590$  nm. ZR75-30 mitochondria (0.15 mg protein/ml) were resuspended in KHEPMB with 1 mM ADP plus 2.5  $\mu\text{g}/\text{ml}$  oligomycin. Fluorescence was recorded using a Varian, Cary Eclipse spectrofluorimeter ( $\lambda_{\text{excitation}}=560$  nm,  $\lambda_{\text{emission}}=590$  nm) with constant stirring at 37 °C. Fluorescence was calibrated using  $\text{H}_2\text{O}_2$  standards under the exact conditions of the assay.

### NAD(P)H redox state

NAD(P)H was measured using 0.3 mg fly mitochondrial protein/ml at 25 °C in KHE plus 1 mM ADP, 2.5  $\mu\text{g}/\text{ml}$  oligomycin and 5  $\mu\text{M}$  FCCP, or 0.3 mg ZR75-30 mitochondrial protein/ml at 37 °C in KHEPMB plus 1 mM ADP and 2.5  $\mu\text{g}/\text{ml}$  oligomycin. The reduction state of endogenous NAD(P)H was determined by autofluorescence using the Pherastar microplate reader at  $\lambda_{\text{excitation}}=340$  nm,  $\lambda_{\text{emission}}=460$  nm. NAD(P)H was assumed to be 0% reduced after 5 min without added substrate, then rotenone was added. With *Drosophila* mitochondria, rotenone increased the reduction of NAD(P)H to ~80% due to endogenous substrates. Addition of proline further increased NAD(P)H reduction to 98%. The 100% reduced level was established with saturating substrate (e.g. 5 mM malate, glutamate and proline) and 4  $\mu\text{M}$  rotenone. Intermediate values were determined as % reduced NAD(P)H relative to the 0% and 100% values.

### Cytochrome $b_{566}$ reduction state

The reduction state of endogenous cytochrome  $b_{566}$  was measured using 0.75 mg mitochondrial protein/ml in KHE plus 2.5  $\mu\text{g}/\text{ml}$  oligomycin and 1 mM ADP with constant stirring at 25 °C in an Olis DW 2-dual wavelength spectrophotometer at  $A_{566}-A_{575}$  nanometers [29,30]. Cytochrome  $b_{566}$  was assumed to be 0% reduced after 5 min without added substrate, then 4  $\mu\text{M}$  rotenone, 5 mM malonate and 2  $\mu\text{M}$  antimycin A were added to report reduction caused by endogenous substrates. 5 mM Proline was subsequently added and %cytochrome  $b_{566}$  reduction was determined relative to the 0% and 100% values. Cytochrome  $b_{566}$  was assumed to be 100% reduced after addition of 5 mM succinate plus 5 mM glycerolphosphate.

### Curve fitting

Data in Figs. 3e and 4d were fitted by nonlinear regression to an exponential, yielding the parameter values in Eq. (1) for *Drosophila* mitochondria

$$\text{Rate of } \text{H}_2\text{O}_2 \text{ production}_{(\% \text{NAD(P)H})} = 9.85 + e^{(0.019 \times \% \text{NAD(P)H})} \quad (1)$$

and Eq. 2 for ZR75-30 mitochondria:

$$\text{Rate of } \text{H}_2\text{O}_2 \text{ production}_{(\% \text{NAD(P)H})} = 19.46 + e^{(0.028 \times \% \text{NAD(P)H})} \quad (2)$$

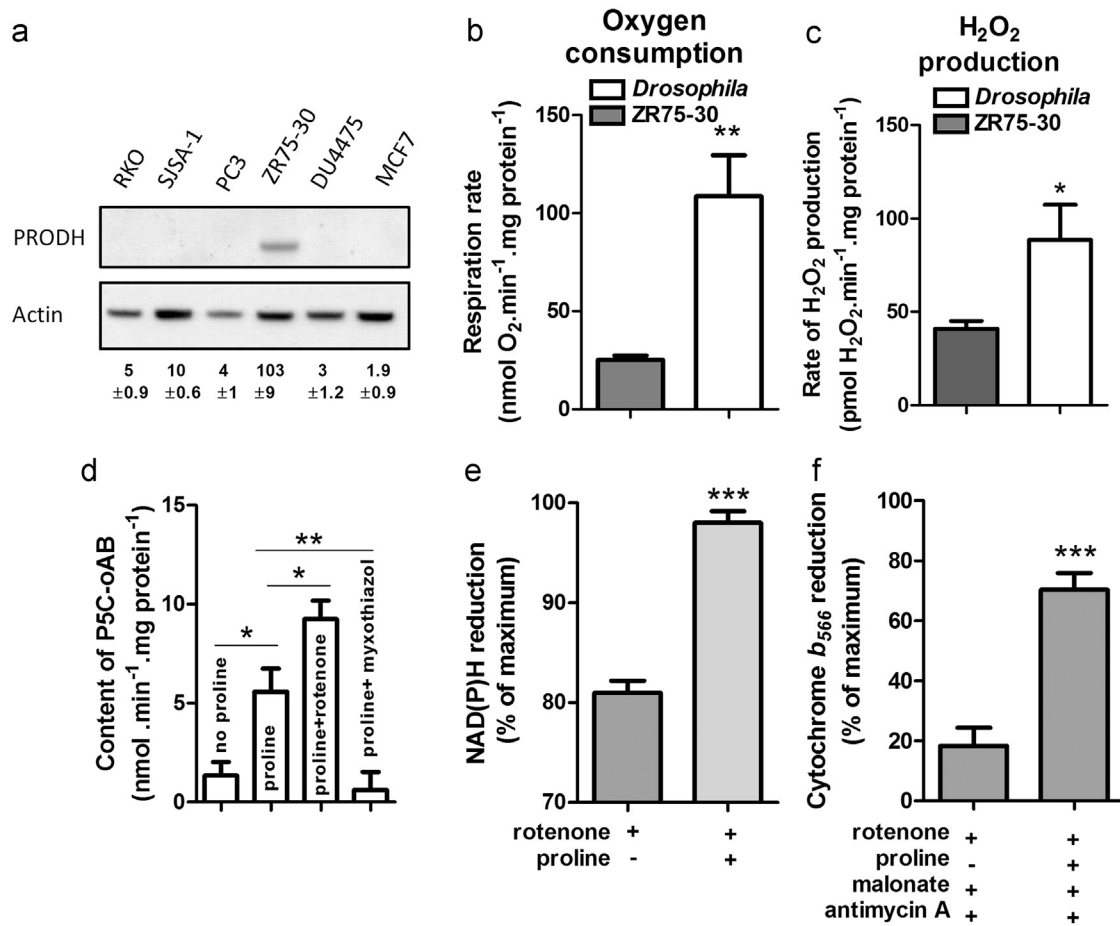
where rate is in  $\text{pmol } \text{H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$ . S.E.M. values for predicted rates of  $\text{H}_2\text{O}_2$  production in Figs 3f-i and 4e,f were calculated using error propagation [31].

### Statistics

All data are means  $\pm$  S.E.M ( $n \geq 3$ ). Differences between two conditions were analyzed by Student's *t*-test or (where the variance was assumed to be unequal, when comparing measured and predicted values) Welch's *t*-test [31]. One-way ANOVA with Dunnett's post-hoc test was used to compare multiple conditions.  $p < 0.05$  was considered significant (Prism).

### Results

Tumor cells generally have very low endogenous levels of proline dehydrogenase [6,32], complicating evaluation of its direct production of superoxide/ $\text{H}_2\text{O}_2$ . Basal levels of proline dehydrogenase in different tumor cell lines were analyzed by Western blot. Levels in five lines were low relative to the breast cancer cell line ZR75-30 (Fig. 2a). Mitochondria isolated from ZR75-30 cells had



**Fig. 2.** Proline dehydrogenase levels in cancer cells and effects of proline oxidation in mitochondria isolated from ZR75-30 cells and *Drosophila*. (a) Western blot analysis of proline dehydrogenase (PRODH) levels in different cancer cell lines. Numbers are PRODH intensities normalized to actin. (b) ADP-stimulated oxygen consumption by ZR75-30 and *Drosophila* mitochondria oxidizing proline. (c) H<sub>2</sub>O<sub>2</sub> production by ZR75-30 and *Drosophila* mitochondria oxidizing proline in the presence of rotenone, myxothiazol, atpenin A5 and malonate. (d)  $\Delta^1$ -pyrroline-5-carboxylate (P5C) in *Drosophila* mitochondria after oxidation of proline for 20 min. (e) NAD(P)H and (f) cytochrome b<sub>566</sub> reduction state in *Drosophila* mitochondria. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 by unpaired *t*-test (one-way ANOVA with Dunnett's post-test in d). Additions where indicated were 5 mM proline, 4  $\mu$ M rotenone, 5 mM malonate, 2  $\mu$ M antimycin A, 2  $\mu$ M myxothiazol, and 1  $\mu$ M atpenin A5.

measurable proline-dependent respiration in the presence of ADP (Fig. 2b, gray bar) and measurable proline-dependent H<sub>2</sub>O<sub>2</sub> production when several other sites of superoxide/H<sub>2</sub>O<sub>2</sub> production were inhibited (Fig. 2c, gray bar). However, to investigate superoxide/H<sub>2</sub>O<sub>2</sub> production from proline dehydrogenase itself we first examined insect flight muscle, which is rich in proline dehydrogenase because proline is a major fuel used for flight [33]. Mitochondria prepared from the fruit fly *Drosophila melanogaster* come mostly from flight muscle, and their H<sub>2</sub>O<sub>2</sub> production has been previously characterized [26]. Their proline-dependent respiration rates at 25 °C were 4-fold higher than those of mitochondria from ZR75-30 cells at 37 °C (Fig. 2b, white bar) and their proline-dependent H<sub>2</sub>O<sub>2</sub> production rates were more than double (Fig. 2c, white bar). The rate differences would be even more marked if temperature corrected. Therefore, we first characterized proline-dependent H<sub>2</sub>O<sub>2</sub> production in *Drosophila* mitochondria before doing so in mitochondria isolated from ZR75-30 cells.

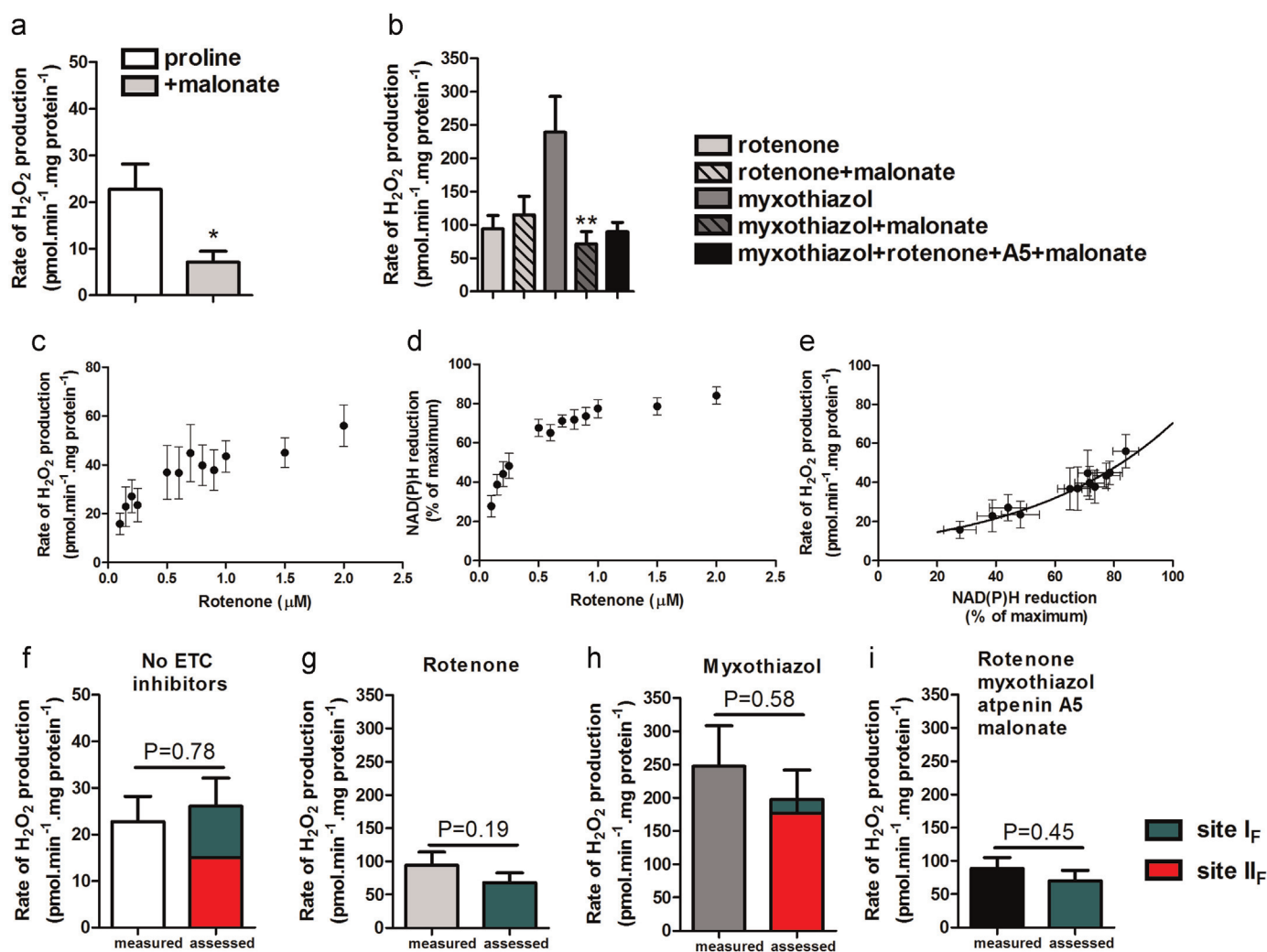
#### Proline dehydrogenase is a ubiquinone reductase

The crystal structures and kinetics of proline dehydrogenase from different microorganisms are well characterized [17,34–36]. Less is known about proline dehydrogenases from eukaryotes, particularly humans [37]. Proline oxidation by proline dehydrogenase can be monitored when the product P5C forms a colored

adduct with o-aminobenzaldehyde (oAB) (Fig. 1a) [38]. P5C-oAB formation, in the presence of different inhibitors of the electron transport chain, was used to define the endogenous electron acceptor used by proline dehydrogenase (Figs. 1 and 2d). *Drosophila* mitochondria were incubated with 1 mM ADP (allosterically stimulates proline dehydrogenase [39]), 2.5  $\mu$ g/ml Oligomycin (inhibits ADP consumption) and 5  $\mu$ M FCCP (promotes rapid substrate utilization). In this condition (Fig. 1a), proline induced formation of P5C (Fig. 2d, proline). In the absence of exogenous proline P5C accumulation was indistinguishable from zero (Fig. 2d, no proline); therefore endogenous proline levels are low.

NADH is formed during proline oxidation (Fig. 1a). In the presence of rotenone to block NADH reoxidation (Fig. 1b) proline addition increased the reduction level of NADH (Fig. 2e). High NADH/NAD inhibits NAD-linked dehydrogenases. If proline dehydrogenase uses NAD as its electron acceptor, rotenone should slow formation of P5C-oAB, whereas if P5C dehydrogenase uses NAD as electron acceptor and proline dehydrogenase does not, P5C-oAB levels will increase as P5C consumption through P5C dehydrogenase is prevented. Fig. 2d shows that rotenone increased P5C-oAB formation, indicating that proline dehydrogenase *in situ* does not use NAD directly as its electron acceptor.

Myxothiazol and antimycin A inhibit complex III and block reoxidation of ubiquinol (QH<sub>2</sub>) formed during proline oxidation (Fig. 1c). Addition of proline in the presence of rotenone, malonate (complex II inhibitor) and antimycin A increased Q<sub>2</sub>-pool reduction,



**Fig. 3.** Superoxide/H<sub>2</sub>O<sub>2</sub> production at 25 °C during proline oxidation by *Drosophila* mitochondria. (a,b) H<sub>2</sub>O<sub>2</sub> production with proline plus different inhibitors. (c) Dependence of superoxide/H<sub>2</sub>O<sub>2</sub> production driven by endogenous substrates on rotenone concentration. (d) Dependence of %NAD(P)H reduction on rotenone concentration. (e) Relationship between the rate of superoxide production from site I<sub>F</sub> and %NAD(P)H reduction, replotted from (c) and (d). (f–i) Measured and predicted rates of superoxide/H<sub>2</sub>O<sub>2</sub> production during oxidation of proline with inhibitors as shown. Left-hand bars represent the measured rates from panels (a) and (b). Colored bars represent predicted rates for site I<sub>F</sub> (green) and site II<sub>F</sub> (red). \**p* < 0.05; \*\**p* < 0.01 by unpaired one-tailed *t*-test (by Welch's *t*-test assuming unequal variances in f–i). Additions where shown were 5 mM proline, 5 mM malonate, 4 μM rotenone, 2 μM myxothiazol, 1 μM atpenin A5.

**Table 1**

NAD(P)H reduction and predicted rates of superoxide production by site I<sub>F</sub> in *Drosophila* mitochondria.

Substrates and inhibitors	NAD(P)H reduction (% of maximum)	Predicted rate of superoxide Production from site I <sub>F</sub> (pmol min <sup>-1</sup> mg protein <sup>-1</sup> )
Proline (Fig. 3a)	5.5 ± 1.9	11.0 ± 1.0
Proline, malonate (Fig. 3a)	2.6 ± 1.8	10.3 ± 0.6
Proline, rotenone (Fig. 3b)	98.0 ± 1.0	67.8 ± 14.7
Proline, myxothiazol (Fig. 3b)	37.9 ± 5.6	20.8 ± 8.7
Proline, myxothiazol, malonate (Fig. 3b)	20.8 ± 4.9**	17.7 ± 5.8
Proline, myxothiazol, rotenone, A5, malonate (Fig. 3b)	100	70.4 ± 15.4

\*\* *p* = 0.007 comparing proline, myxothiazol and proline, myxothiazol and malonate (paired *t*-test, one-tailed).

reported by the reduction state of cytochrome *b*<sub>566</sub>[29] (Fig. 2f), indicating that proline oxidation reduces ubiquinone directly (and not only through complex I). High Q<sub>H2</sub>/Q inhibits

ubiquinone-linked dehydrogenases. If proline dehydrogenase uses ubiquinone as its electron acceptor, complex III inhibitors should slow adduct formation. Addition of myxothiazol decreased P5C accumulation (Fig. 2d), showing that proline dehydrogenase uses ubiquinone as its electron acceptor in intact mitochondria.

#### H<sub>2</sub>O<sub>2</sub> production during proline oxidation by uncoupled *Drosophila* mitochondria

Fig. 3a shows that in the absence of inhibitors of electron transport, 5 mM proline induced H<sub>2</sub>O<sub>2</sub> production by uncoupled *Drosophila* mitochondria. Proline oxidation can increase ubiquinol and NADH levels (Fig. 2e,f) and generate oxoglutarate and succinate [7,40]. Therefore, several different sites could be active during proline oxidation, including proline dehydrogenase but also sites I<sub>F</sub>, I<sub>Q</sub>, II<sub>F</sub>, III<sub>Q0</sub> and O<sub>F</sub> (Fig. 1a). To test the contribution of site II<sub>F</sub>, complex II was inhibited by malonate [41] (Fig. 1d). H<sub>2</sub>O<sub>2</sub> production decreased by ~70% (Fig. 3a, +malonate), showing that site II<sub>F</sub> was probably responsible for much of the H<sub>2</sub>O<sub>2</sub> produced during proline oxidation under these conditions.

To maximize any production of superoxide/H<sub>2</sub>O<sub>2</sub> from proline dehydrogenase itself, we added proline and manipulated electron

transport flow with inhibitors. First, complex I was inhibited with rotenone, thereby increasing NADH/NAD (Fig. 2e, Table 1), slowing P5C dehydrogenase, raising P5C concentration (Fig. 2d, proline+rotenone) and stalling proline dehydrogenase (Fig. 1b). The rate of H<sub>2</sub>O<sub>2</sub> generation (Fig. 3b, first bar) increased >4-fold compared to Fig. 3a. However, under this condition sites I<sub>F</sub> and O<sub>F</sub> will be active [22] and may contribute to the signal. Site II<sub>F</sub> was inactive, since malonate did not decrease the rate (Fig. 3b, second bar).

Next, myxothiazol was added to inhibit complex III, thereby reducing ubiquinone (Fig. 2f) and stalling proline dehydrogenase (Fig. 1c). This gave high rates of H<sub>2</sub>O<sub>2</sub> production (Fig. 3b, third bar). However, NAD was 38% reduced (Table 1), so site I<sub>F</sub> might be active. Furthermore, addition of malonate strongly decreased the signal (Fig. 3b, fourth bar). Therefore, during proline oxidation plus myxothiazol most superoxide/H<sub>2</sub>O<sub>2</sub> production arises from complex II at site II<sub>F</sub>.

When sites I<sub>Q</sub>, II<sub>F</sub> and III<sub>Q<sub>0</sub></sub> were all inhibited by addition of rotenone, myxothiazol, malonate and atpenin A5 (inhibits site II<sub>Q</sub>) (Fig. 1e), a measurable rate remained (Fig. 3b, fifth bar). Thus, after inhibiting most of the sites predicted to be active during proline oxidation, some site was still able to produce superoxide/H<sub>2</sub>O<sub>2</sub> at a measurable rate.

#### Rates of superoxide/H<sub>2</sub>O<sub>2</sub> production from specific sites during proline oxidation by *Drosophila* mitochondria

Complex I produces superoxide through sites I<sub>F</sub> and I<sub>Q</sub>[42]. Site I<sub>Q</sub> can be blocked by rotenone, but there are no suitable inhibitors of site I<sub>F</sub>. However, the rate of superoxide production from site I<sub>F</sub> is uniquely related to NADH redox state [31,43]. Fig. 3c shows the rate of H<sub>2</sub>O<sub>2</sub> generation driven by endogenous substrates during titration with rotenone in *Drosophila* mitochondria. This signal was assumed to be from site I<sub>F</sub> since all other putative sites were presumably oxidized and inactive; it was not from proline dehydrogenase itself, since any endogenous proline was insufficient to drive significant proline dehydrogenase activity (Fig. 2d, no proline). Fig. 3d shows the corresponding NAD(P)H reduction state. Fig. 3e replots the rate of H<sub>2</sub>O<sub>2</sub> production as a function of NAD(P)H reduction. This curve was used to assess the contribution of site I<sub>F</sub> at a given NAD(P)H reduction state.

We then measured NAD(P)H during proline oxidation. Table 1 shows the NAD(P)H levels and assessed rates from site I<sub>F</sub> under the conditions of Fig. 3a and b. Fig. 3(f-i) shows the contribution of site I<sub>F</sub> to the rates observed in Fig. 3a and b.

The contributions of other sites were assessed using inhibitors of electron transport after correcting for any alterations in NAD(P)H and the rate of site I<sub>F</sub>[31]. Fig. 3(f-i) shows that under the conditions of Fig. 3a without malonate, sites I<sub>F</sub> (green) and II<sub>F</sub> (red) fully accounted for the measured H<sub>2</sub>O<sub>2</sub> production; there was no evidence for any direct contribution of site O<sub>F</sub> or proline dehydrogenase.

Under the conditions of Fig. 3b plus rotenone (Fig. 1b), NAD was ~98% reduced (Table 1). Fig. 3g shows that under this condition site I<sub>F</sub> fully accounted for the measured rate of H<sub>2</sub>O<sub>2</sub> production; there was no evidence for any direct contribution of proline dehydrogenase. Similarly, Fig. 3h shows that when proline was oxidized in the presence of myxothiazol (Fig. 1c) H<sub>2</sub>O<sub>2</sub> production was dominated by site II<sub>F</sub>, a small fraction was attributed to site I<sub>F</sub>, and there was no evidence for any direct contribution of proline dehydrogenase.

To maximize any superoxide/H<sub>2</sub>O<sub>2</sub> production by proline dehydrogenase itself, the enzyme was pharmacologically isolated by blocking sites I<sub>Q</sub>, II<sub>F</sub> and III<sub>Q<sub>0</sub></sub> with rotenone, malonate, atpenin A5 and myxothiazol (Figs. 1e and 3b, fifth bar). Fig. 3i shows that even under these conditions H<sub>2</sub>O<sub>2</sub> production was dominated by site I<sub>F</sub>,

which was not inactivated under these conditions; there was no evidence for any direct contribution of proline dehydrogenase.

Thus, *Drosophila* mitochondria oxidizing proline through proline dehydrogenase generated superoxide/H<sub>2</sub>O<sub>2</sub> at substantial rates from other sites, but there was no evidence of superoxide/H<sub>2</sub>O<sub>2</sub> production from proline dehydrogenase itself even under conditions designed to maximize such production.

#### Mitochondria isolated from the breast cancer cell line ZR75-30 produce H<sub>2</sub>O<sub>2</sub> during proline oxidation

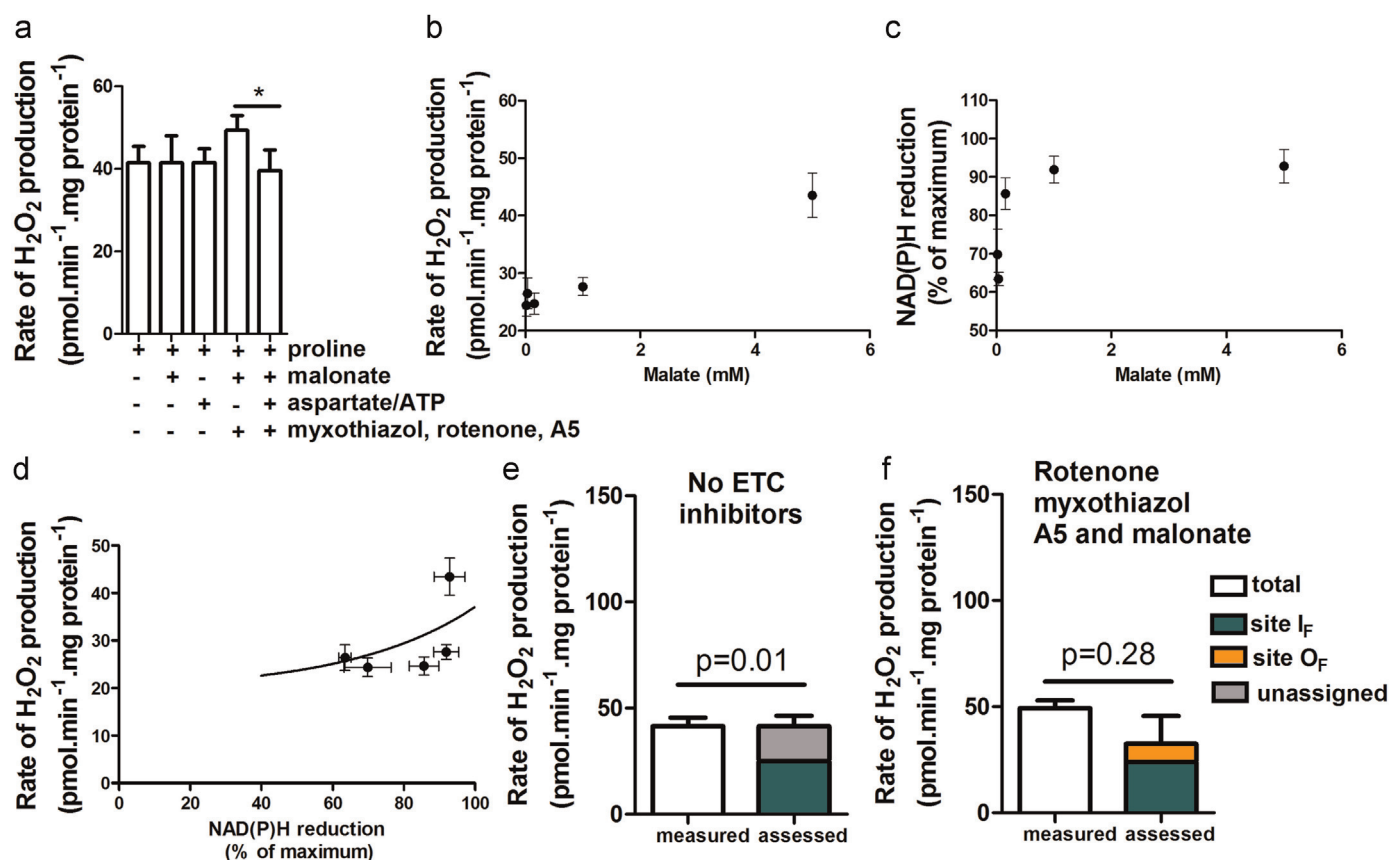
To investigate whether proline dehydrogenase generates superoxide/H<sub>2</sub>O<sub>2</sub> directly or indirectly in cancer cell mitochondria, we repeated the analysis using mitochondria from ZR75-30 human breast cancer cells.

Fig. 4a (proline) shows that ZR75-30 mitochondria produced 41.5 ± 4.0 pmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> when oxidizing proline in the absence of inhibitors of electron transport. Under these conditions sites I<sub>F</sub>, I<sub>Q</sub>, II<sub>F</sub>, III<sub>Q<sub>0</sub></sub> and O<sub>F</sub> might be active (Fig. 1a). However, inhibiting sites I<sub>Q</sub>, II<sub>F</sub>, III<sub>Q<sub>0</sub></sub> and O<sub>F</sub> did not decrease the rate of H<sub>2</sub>O<sub>2</sub> production (Fig. 4a). Specifically, blocking site II<sub>F</sub> using malonate did not decrease the rate of H<sub>2</sub>O<sub>2</sub> production (Fig. 4a, proline + malonate). The 2-oxoglutarate dehydrogenase complex is an important source of superoxide/H<sub>2</sub>O<sub>2</sub> in rat skeletal muscle mitochondria [22], and in cancer cells there is a positive relationship between proline dehydrogenase levels and 2-oxoglutarate concentration [7], so site O<sub>F</sub> might produce superoxide/H<sub>2</sub>O<sub>2</sub> during proline oxidation (Fig. 1a). To decrease its activity, aspartate and ATP were added (Figs 1f and 4a) (aspartate removes 2-oxoglutarate by transamination; ATP inhibits the 2-oxoglutarate complex directly and by increasing succinyl-CoA levels [22]). Fig. 4a (proline+aspartate/ATP) shows that H<sub>2</sub>O<sub>2</sub> production was not decreased, although this might be because aspartate/ATP failed to suppress site O<sub>F</sub> when 2-oxoglutarate was produced by proline metabolism (Fig. 1a). However, H<sub>2</sub>O<sub>2</sub> production during proline oxidation was sensitive to aspartate/ATP when sites I<sub>Q</sub>, III<sub>Q<sub>0</sub></sub> and II<sub>F</sub> were inhibited by rotenone, myxothiazol, atpenin A5 and malonate, respectively (Figs. 1e and 4a, fourth and fifth bars), so site O<sub>F</sub> was active under this specific condition, and aspartate/ATP was a valid test of the contribution of site O<sub>F</sub> in the initial condition (Fig. 4a, proline).

To determine the contribution of site I<sub>F</sub> in ZR75-30 mitochondria oxidizing proline (Fig. 4a, proline), we measured NAD(P)H. Fig. 4b and c shows the dependence of H<sub>2</sub>O<sub>2</sub> production and NAD(P)H reduction levels on the concentration of added malate in the presence of rotenone, aspartate and ATP to suppress other sites [22]. Fig. 4d shows the relationship between the rate of superoxide/H<sub>2</sub>O<sub>2</sub> production from site I<sub>F</sub> and NAD(P)H reduction. This relationship was used to assess the rates of superoxide/H<sub>2</sub>O<sub>2</sub> production from site I<sub>F</sub> during proline oxidation by ZR75-30 mitochondria in Fig. 4e and f. Table 2 shows the levels of NAD(P)H reduction and the rates of superoxide/H<sub>2</sub>O<sub>2</sub> production from site I<sub>F</sub> during proline oxidation.

With proline alone (Fig. 1a), site I<sub>F</sub> was responsible for 60% of measured H<sub>2</sub>O<sub>2</sub> production (Fig. 4e). Under this condition the measured rate was significantly different from the rate assessed for site I<sub>F</sub> ( $p=0.01$ , white bar vs site I<sub>F</sub> green) suggesting that another site was active. The remaining 40% may be from site O<sub>F</sub> (because suppression of site O<sub>F</sub> by aspartate/ATP may be incomplete with proline as substrate) or from other sites, including proline dehydrogenase itself. If proline dehydrogenase did produce superoxide/H<sub>2</sub>O<sub>2</sub> directly under this condition, the rates were low, < 16 ± 7 pmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup> (Fig. 4e, unassigned gray).

When proline dehydrogenase was pharmacologically isolated by inhibiting sites I<sub>Q</sub>, II<sub>F</sub>, II<sub>Q</sub> and III<sub>Q<sub>0</sub></sub> with rotenone, malonate,



**Fig. 4.** Sites involved in superoxide/ $H_2O_2$  production at 37 °C during proline oxidation by ZR75-30 mitochondria. (a)  $H_2O_2$  production. (b–d) Relationship between the rate of superoxide production by site  $I_F$  and the reduction state of NAD(P)H in the presence of different concentrations of malate plus rotenone, ATP and aspartate. (b) Dependence of  $H_2O_2$  production rate on malate concentration. (c) Dependence of %NAD(P)H reduction on malate concentration. (d) Relationship between the rate of superoxide production from site  $I_F$  and %NAD(P)H reduction, replotted from (b) and (c). (e,f) Measured and predicted rates of superoxide/ $H_2O_2$  production in the presence of proline (e) in the absence of electron transport chain (ETC) inhibitors and (f) in the presence of inhibitors as shown. White bars represent rates from (a). Colored bars represent predicted rates for sites  $I_F$  (green) and  $O_F$  (orange) and unassigned rates (gray). \* $p < 0.05$  by paired one-tailed  $t$ -test (by Welch's  $t$ -test assuming unequal variances in (e), and (f). In (e)  $p = 0.01$  when comparing the measured rate to the rate predicted from site  $I_F$  (green). Additions where indicated were 5 mM proline, 5 mM malonate, 2.5 mM ATP, 1.5 mM aspartate, 2  $\mu$ M myxothiazol, 4  $\mu$ M rotenone, and 1  $\mu$ M atpenin A5.

**Table 2**

NAD(P)H reduction and predicted rates of superoxide production by site  $I_F$  in ZR75-30 mitochondria.

Substrates and inhibitors	NAD(P)H reduction (% of maximum)	Predicted rate of superoxide production from site $I_F$ ( $\text{pmol min}^{-1} \text{mg protein}^{-1}$ )
Proline	$59.5 \pm 8.0$	$24.9 \pm 2.8$
Proline, aspartate and ATP	$60.0 \pm 5.0$	$25.0 \pm 2.65$
Proline, rotenone, myxothiazol, malonate, A5	$51.9 \pm 1.4$	$23.8 \pm 2.1$
Proline, rotenone, myxothiazol, malonate, A5, Aspartate and ATP	$41.3 \pm 3.9^*$	$22.8 \pm 1.8$

Conditions are described in Fig. 4a.

\*  $p = 0.04$  vs proline, rotenone, myxothiazol, malonate and A5 (paired  $t$ -test, one-tailed).

atpenin A5 and myxothiazol (Fig. 1g), site  $I_F$  produced 50% of the  $H_2O_2$  (Fig. 4f). Another 20% was sensitive to aspartate and ATP, suggesting that it came from site  $O_F$ . The sum of the rates from sites  $I_F$  and  $O_F$  was not significantly different from the measured rate, so there was no evidence for any direct contribution of proline dehydrogenase to  $H_2O_2$  production. If proline

dehydrogenase did contribute under this condition, its contribution was small,  $< 17 \pm 13 \text{ pmol min}^{-1} \text{mg protein}^{-1}$ .

Thus, ZR75-30 mitochondria oxidizing proline through proline dehydrogenase generated superoxide/ $H_2O_2$  at measurable rates from other sites, but there was little evidence of superoxide/ $H_2O_2$  production from proline dehydrogenase itself even under conditions designed to maximize such production.

## Discussion

The role of proline dehydrogenase as a direct source of superoxide or  $H_2O_2$  has not been explored *in situ*, although proline oxidation generates ROS in cells [6,8]. Because proline oxidation feeds electrons into multiple sites of the citric acid cycle and electron transport chain, it has been unclear whether this ROS arises directly from proline dehydrogenase or from downstream mitochondrial sites. Purified proline dehydrogenases from humans and different microorganisms react directly with molecular oxygen in the absence of endogenous electron acceptors to produce superoxide/ $H_2O_2$  [17,20,44], supporting the view that proline dehydrogenase produces mitochondrial ROS and promotes apoptosis in cancer cells [6,10]. However, under certain circumstances including nutrient and oxidative stress and hypoxia, proline and proline dehydrogenase may exert protective effects by acting as an alternative energy source [11–13]. In this context, depletion of proline dehydrogenase is detrimental [44].

It has been challenging to understand the roles of proline and proline dehydrogenase in cellular metabolism and cancer, and whether ROS production directly by the enzyme is responsible for triggering apoptosis in cancer cells. To address this issue, we measured the rates of production of superoxide/H<sub>2</sub>O<sub>2</sub> from different candidate sites during proline oxidation in isolated mitochondria *in situ*.

Initially, *Drosophila* mitochondria were chosen as a model because they have abundant proline dehydrogenase and oxidize proline faster than cancer cell mitochondria (Fig. 2b). In insects proline is used as the source of carbon to power flight [33]. We confirmed that the initial acceptor for electrons from proline dehydrogenase *in situ* is ubiquinone, not NAD (Fig. 2d–f). The sites involved in superoxide/H<sub>2</sub>O<sub>2</sub> production during proline oxidation under a variety of conditions were quantified. Although proline oxidation caused substantial rates of superoxide/H<sub>2</sub>O<sub>2</sub> production from other sites, specifically sites II<sub>F</sub> in complex II and I<sub>F</sub> in complex I (Fig. 3f), there was no significant unassigned rate and no evidence of superoxide/H<sub>2</sub>O<sub>2</sub> production from proline dehydrogenase itself even under conditions designed to maximize such production (Fig. 3i).

Next, the breast cancer line ZR75-30 was chosen as a model to investigate the role of proline dehydrogenase in cancer cells because of its high basal amounts of proline dehydrogenase relative to other cancer cell lines tested (Fig. 2a). Mitochondria from ZR75-30 cells consumed oxygen and produced H<sub>2</sub>O<sub>2</sub> with proline as substrate (Fig. 2b and c). The sites involved in superoxide/H<sub>2</sub>O<sub>2</sub> production during proline oxidation under a variety of conditions were quantified. In the absence of inhibitors of electron transport, most of the measured H<sub>2</sub>O<sub>2</sub> production was from site I<sub>F</sub>. However, a significant proportion was unassigned, and may have arisen from site O<sub>F</sub> in the 2-oxoglutarate dehydrogenase complex or from proline dehydrogenase (Fig. 4e). Under conditions designed to maximize any contribution from proline dehydrogenase itself (Fig. 4f), proline oxidation caused measurable superoxide/H<sub>2</sub>O<sub>2</sub> production from sites I<sub>F</sub> and O<sub>F</sub>. There was no significant unassigned rate and no evidence of superoxide/H<sub>2</sub>O<sub>2</sub> production from proline dehydrogenase itself. Together the results in the two systems show that proline oxidation by isolated mitochondria generates significant superoxide/H<sub>2</sub>O<sub>2</sub> from downstream sites, specifically I<sub>F</sub>, II<sub>F</sub> and O<sub>F</sub>, but there was little evidence for superoxide/H<sub>2</sub>O<sub>2</sub> production from any other site, and no evidence for any superoxide/H<sub>2</sub>O<sub>2</sub> production by proline dehydrogenase itself.

Our conclusion that proline dehydrogenase is not a significant source of superoxide/H<sub>2</sub>O<sub>2</sub> in mitochondria contrasts with the observations cited above that the isolated enzyme can generate ROS. This might be because production by the enzyme itself is insufficient to register in the context of other much more active mitochondrial sites. Alternatively, proline dehydrogenase *in situ* may have different radical production properties than the purified enzyme. Evidence comes from kinetic analyses showing that the K<sub>m</sub> of proline dehydrogenase for proline depends greatly on the electron acceptor [17,18,20,45]. Ubiquinone may suppress the ability of the enzyme to donate electrons directly to oxygen *in situ* and *in vivo*.

We conclude that proline dehydrogenase does not directly produce superoxide or H<sub>2</sub>O<sub>2</sub> at significant rates in flight muscle or cancer cell mitochondria. Instead, proline oxidation feeds electrons into the respiratory chain directly through ubiquinone reduction and indirectly through further metabolism, and triggers superoxide/H<sub>2</sub>O<sub>2</sub> production from other downstream sites. This does not preclude proline dehydrogenase from playing important roles in cancer cell metabolism, but it is not itself a significant source of superoxide/H<sub>2</sub>O<sub>2</sub>.

## Authors' contributions

Conception and design: MDB, RLSG, CLQ, CCB, GKS; Development of methodology: RLSG, CLQ, MDB; Acquisition of data: RLSG, DER; Analysis and interpretation of data: RLSG, CLQ, MDB; Writing, review, and/or revision of the manuscript: RLSG, MDB; Administrative, technical, or material support: MDB, CCB; Study supervision: MDB.

## Conflict of interest

The funding sources had no role in study design; in collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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