Glycolytic flux occurs in *Drosophila melanogaster* recovering from camptothecin treatment

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Camptothecin (CPT) and CPT-derived drugs are widely used against gynaecological and colorectal cancers. On account of their mechanism of action these drugs target rapidly dividing cells and may have an adverse effect on normal tissues. We sought to investigate their impact on normal cells by using *Drosophila* as a model. We investigated the possible involvement of *Drosophila* homologue of p53 (Dmp53) and a member of the retinoblastoma binding protein 6 family, known as Snama. On account of its molecular features and experimental evidence gleaned from mammalian studies we propose Snama as a candidate in Dmp53 regulation. We have used proteomics and core molecular biology techniques on embryos and on adult flies. We found that flies that recover from CPT treatment display a metabolic programme characterized by glycolytic flux, depletion of Dmp53 and increase of Snama transcripts. When we introduced methyl pyruvate in the diet to bypass the glycolytic pathway, we noticed differential expression of Dmp53 and Snama and improvement in reproduction and embryonic development. The development of embryos into the pupal stage was significantly improved to 40% ($P=0.02$) when CPT was given to mothers in combination with methyl pyruvate. This investigation highlights the importance of energy production mechanisms in cells that recover from chemotherapy and differences between the metabolic programmes used by recovering cells and those adopted by cancer cells. *Anti-Cancer Drugs* 21:945–957 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Camptothecin (CPT) and its structural analogues are effective anticancer agents used against ovarian and colorectal cancers (CRC), but CPT has some adverse toxic effects. CPT structural analogues, such as irinotecan, are highly effective agents against CRC and ovarian cancer [1–3]. On account of poor solubility and high toxicity of CPT, water-soluble analogues were developed, and consequently, irinotecan and topotecan were approved for use as anticancer drugs in the mid-1990s [4–6]. These drugs of the CPT family are topoisomerase poisons that inhibit DNA topoisomerase I (Topo1), a nuclear enzyme that introduces transient single-strand breaks on DNA, thereby relaxing supercoils during replication. Topo1 religates the nicked strand forming an intact DNA helix again. A key step in the process is the formation of a cleavable complex between the enzyme and DNA through the covalent linkage between a tyrosine in the enzyme active site and the 3′ end of the DNA strand. Drugs of the CPT family turn Topo1 into a cytotoxic poison by stabilizing the cleavable complex preventing religation. This results in double-strand breaks during replication. Topo1 is the sole cellular target of CPT and acts at the DNA synthesis stage of the cell cycle [6–8]. Although these drugs are intended for the rapidly dividing cancer cells, normal cells are also affected. Topo1 is an essential protein during *Drosophila* and mammalian embryonic development [9]. In *Drosophila* it is concentrated in the ovaries and is maternally inherited by the early embryo (0–2 h) with maximal zygotic expression in the 6–12 h period [10].

The *Drosophila* homologue of p53 (Dmp53) acts downstream of the cell cycle checkpoint kinases of the phosphoinositide 3-kinase related family, ataxia telangiectasia mutated and the ataxia telangiectasia mutated and Rad3-related in response to DNA damage and is known to induce apoptosis by upregulating the transcription of downstream effectors, such as *reaper*, *head involution defective* and *sickle* [11]. The CPT derivative, irinotecan, is known to induce apoptosis in a p53-dependent manner [12]. CPT also causes apoptosis by generating free radicals [13].

The pathways that lead to CPT-induced apoptosis are largely dependent on the biological context. In a *myeloblastosis viral oncogene*-transformed chicken monoblast cell line CPT induced lysosome-dependent cell death that is often associated with oxidative stress rather than apoptosis [14]. Furthermore, the prolonged administration of these drugs may result in the development of resistance and concomitant reduction in p53 expression. For example, treatment of a human hepatocellular carcinoma cell line with an active metabolite of irinotecan, induced increased p53 transcription and translation and

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promoted mitochondria-dependent apoptosis in a short-term culture (24 h), but after prolonged treatment (for 6 days) p53 expression in these cells was reduced and apoptosis was inhibited. This was attributed to cellular efflux of the drug through the drug transporter protein, P-glycoprotein, which was concurrently increased [15]. In the human breast cancer cell line, MCF-7, CPT induces apoptosis through p53-dependent and p53-independent pathways, but this induction involves the elevation of the downstream effector p21 waf1/cip1 in both the cases [16].

The p53-dependent pathway mediating a *Drosophila* response to CPT is further complicated by the apparent differences between invertebrate and vertebrate p53 regulatory mechanisms. The oncoprotein murine double-minute 2 (Mdm2) is known to promote proteasome-dependent degradation of p53 in vertebrate cells in a tightly regulated manner, but no homologue has been identified in *Drosophila* despite concerted efforts to identify it by many groups worldwide [17]. There is, however, another mechanism of p53 regulation that may be shared by both vertebrates and invertebrates. This involves the p53-associated cell testis (PACT) protein, also known as retinoblastoma binding protein 6 (RBBP6), which has been shown to enhance the activity of Mdm2 during mouse embryonic development [18] and to associate with p53 and Rb1 in many vertebrate systems [19,20]. RBBP6 was found to be strongly expressed in oesophageal cancer cells, but not in the surrounding normal oesophageal epithelium [21]. In MCF-7 cells, the overexpression of truncated RBBP6 promotes CPT-induced apoptosis when the p53-binding domain is included [22]. Mammalian Mdm2 also promotes apoptosis in *Drosophil*a when overexpressed although molecular partners in this interaction are not known [23]. Excess Mdm2 has been shown in many studies using cultured mammalian cells and the mouse as a model in some cases, to cause disruption of cell differentiation and increased tumourigenicity. These observations are interpreted to indicate that Mdm2 may have other molecular targets, especially at higher concentrations [24]. RBBP6 (PACT) was shown in the mouse model to enhance the activity of Mdm2 [18]. It was suggested that the absence of a *Drosophila* homologue of Mdm2 could mean either that the *Drosophila* gene has no significant sequence similarity with vertebrate Mdm2 or that Dmp53 does not require tight regulation [11]. An alternative view is that Dmp53 turnover is regulated by a different molecule in *Drosophila*. A good candidate for this is the *Drosophila RBBP6* homologue, *Snama*, also known as *mini-me*, which has been characterized and found to behave like a suppressor of apoptosis [25,26].

The purpose of this study is to explore the impact of CPT on normal cells using *Drosophila* as a model system and to assess the role of RBBP6 proteins. The fruitfly, being an amenable model organism, could provide a useful tool for studying the effects of CPT on normal and abnormal cells and perhaps for use as a model to study the side effects of cancer drug treatments. We have investigated p53 and RBBP6 expression in wild-type flies upon treatment and the effect of the drug on the reproductive system of flies.

Methods

Fly maintenance

Drosophila melanogaster (Canton S) were reared at 25°C on standard cornmeal agar food and yeast.

Camptothecin treatment and methyl pyruvate supplementation

Flies were fed CPT at a 1 mmol/l concentration in yeast blobs by placing dimethylsulfoxide (DMSO)-dissolved CPT in a yeast paste on a cornmeal agar vial using a syringe. A negative control with nothing added to the yeast was set up in a separate vial. This procedure was followed when exposing the flies to 10% methyl pyruvate supplement.

Mating flies and treatment with camptothecin

To investigate the effect of CPT on the reproductive biology of flies mating experiments were set up. Virgin female flies were mated with males of the same age and duplicate vials were used for each experimental group. Typically, flies were given food treated with CPT as above or with DMSO for 2 days and then removed to new vials containing untreated food. Five female and one male fly of each of the following experimental groups were placed in nine vials: untreated female flies with untreated male flies; treated female with untreated male flies; untreated female flies with treated male flies; treated female with treated male flies; DMSO-fed female flies with DMSO-fed male flies; DMSO-fed female flies with untreated male flies; untreated female with DMSO-fed male flies; DMSO-fed female with CPT-fed male flies; CPT-fed female with DMSO-fed male flies.

The number of embryos present on the feeding plates was counted every 24 h after mating and the flies were transferred to untreated food. Embryos from day 1 were transferred to cornmeal media and allowed to develop further. The pupae developing from these embryos and the adults that finally emerged were counted. The development was recorded as a percentage of embryos that reached the pupal stage and the percentage of embryos that reached adulthood.

Mating experiments were repeated with methyl pyruvate supplementation in combination with CPT and methyl pyruvate alone using the same experimental groups as above.

Mortality assays

The mortality rates of flies exposed to CPT, methyl pyruvate and a combination of these were also observed. One hundred newly enclosed male and female flies were exposed to CPT and the number of dead flies was recorded. To estimate the effect of CPT treatment on survival probability, a Kaplan–Meier product limit estimate of the survival functions of each fly grouped according to sex and treatment was made at a 95% confidence interval.

Acridine orange staining

To show the occurrence of apoptosis the embryos were stained with acridine orange as described by Abrams *et al.* [27]. The slides were viewed and photographed using the axioskopmot 2 fluorescence microscope (Carl Ziess Pty. Ltd, Oberkochen, Baden-Württemberg, Germany).

Western blot analysis

Typically, 10 flies in 50 µl of a 5 × SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer were homogenized in various 1.5 ml microtubes. The samples were centrifuged at 13 000 *g* for 30 min and the supernatant was moved to a fresh tube. After separation by SDS-PAGE, the proteins were transferred to a Hybond-P, a PVDF membrane (Amersham Bioscience, Amersham, Buckinghamshire, UK; catalogue#1487), in Towbin buffer using a Hoefer mini VE blotting cassette (Amersham Bioscience catalogue# PH 80-6418-96). Nonspecific binding was prevented by blocking in SuperBlock Dry Blend Blocking buffer (Pierce, Rockford, Illinois, USA; catalogue #37545). The blot was probed with a 1 : 3000 dilution of anti-Dmp53 [Santa Cruz Biotechnology (Santa Cruz, California, USA) p53 dD-21, 200 µg/ml] for 1 h. The primary antibody was removed and the membrane was washed with PBS–Tween twice for 10 min. The membrane was then incubated for 1 h in a 1 : 40 000 dilution of peroxidase-conjugated rabbit antigoat immunoglobulin secondary antibody (Pierce catalogue #31407) for 1 h. To remove the secondary antibody the membrane was washed six times for 10 min each with PBS–Tween. The bound probe was detected by using the SuperSignal West Pico chemiluminescent substrate (Pierce catalogue#37077) according to the manufacturer's instructions and exposed to X-ray film.

Two-dimensional gel electrophoresis

Typically, samples of protein extracts from five flies ground in an radioimmunoprecipitation assay buffer were run on a two-dimensional (2D) PAGE gel. The first dimension was performed on immobilised pH gradient strips for use in a Protean Isoelectric Focusing (IEF) system (BioRad, Laboratories Inc., Hercules, California, USA). Protein concentration was determined by using the Bradford assay (BioRad catalogue#500–0001). Equal amounts of each sample were purified and concentrated using a 2D PAGE sample preparation kit (BioRad catalogue#163–2130) or through trichloroacetic acid precipitation. The resulting pellet was resuspended in 125 µl of a 2D rehydration buffer [8 mol/l urea, 2% 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, 50 mmol/l dithiothreitol, 0.2% Bio-lyte, 0.5% bromophenol blue]. The resuspended samples were placed along the side of the groove in a 7 cm rehydration tray. The IEF strips (BioRad catalogue#1632099) were placed face down in the sample and covered with mineral oil and passive rehydration was allowed to occur for 12 h. IEF was performed according to the following programme: an initial low voltage (250 V) 20 min linear ramping step, followed by a high voltage (4000 V) 2 h linear ramping step. The final step was a rapid ramping step for 10 000 V–h. On completion of isolelectric focusing the strip equilibrated for 10 min in solution 1 (6 mol/l urea, 2% SDS, 0.375 mol/l Tris–HCl pH 8.8, 20% glycerol, 2% dithiothreitol), which was replaced with solution 2 (6 mol/l urea, 2% SDS, 0.375 mol/l Tris–HCl pH 8.8, 20% glycerol, 2.5% iodoacetamide) for a further 10 min. The strips were then placed briefly in an SDS running buffer and placed on top of a 12% polyacrylamide separating gel and overlaid with agarose. The proteins were separated by electrophoresis for 2 h at 150 V and stained with Coomassie blue.

Image analysis

The two gels representing CPT-treated and CPT-untreated samples, were digitized using the PDQuest 2D Analysis Software version 6.2 (BioRad catalogue #170–9630). Spot detection was carried out with a sensitivity setting of 112.21 using the CPT-treated sample gel as the master gel. This resulted in 327 spots being detected in the CPT gel image and 314 spots in the untreated gel image. The spots were initially matched using the automated matching function. The classical matching function was used for extended matching. Both methods match spots based on the position of the landmark spots. This was followed by manual spot matching and analysis across images. Eventually, there was a final count of 150 spots in the master gel and 122 and 74 spots in the CPT and untreated images, respectively.

Normalization was carried out automatically by the programme, based on total quantity in all the valid spots option. This method assumes that changes in density average out across the gels being analysed.

Briefly, the normalization formula used is as follows:

$$\text{Normalized spot quantity} = \frac{\text{Raw spot quantity} \times \text{scaling factor}}{\text{Normalization factor (total quantity in all valid spots)}}$$

The scaling factor used was 10^6 parts per million.

Protein identification using matrix-laser desorption-time-of-flight mass spectrometry

Mass spectroscopy analysis was carried out at the Plateforme Protéomique of the Institut de Biologie Moléculaire et Cellulaire in Strasbourg. The spots that were validated by the PDQuest software were excised from the gel and subjected to tryptic in-gel digestion as described earlier [28]. The proteins were then analysed by the matrix-laser desorption-time-of-flight and were identified in the *Drosophila* database through peptide fingerprinting using the Mascot search engine (London, UK).

Glutathione S transferase assay

A glutathione S transferase (GST) assay was carried out to confirm that the changes in the optical density values observed in the 2D PAGE gels did reflect real changes in the protein amount and therefore in activity. The assay was carried out as described earlier [29]. One unit of the enzyme will conjugate 10.0 nmol of 1-chloro-2,4-dinitrobenzene with reduced glutathione per minute and the glutathione dinitrobenzene conjugate extinction coefficient at 340 nm is 0.0096/μmol/l/cm.

Semiquantitative reverse transcription-polymerase chain reaction

Total RNA samples were collected from the specified experimental groups and time periods. The following primers were used:

Dmp53 forward 5′-TAGCCGGAATTCATGTATATATCACAGCCAATG-3′

Dmp53 reverse 5′-CCAGATGAGCTCTCATGGCAGCTCGTA-3′

Reaper forward 5′-AAACCAGAATTATGGCAGTGGCATTCTAC-3′

Reaper reverse 5′-TTACTCGAGCTCTCATTGGATGGCTTGCG-3′

Snama forward 5′-TTCATATGGATCCATGTCGGTACACTAT-3′

Snama reverse 5′-CGAACAAAGCTTCTCCTTGCAATCG-3′

Rp49 forward 5′-TGTTGTGTCCTTCCAGCTTAA-3′

Rp49 reverse 5′-ACTGATATCATCCAGATAATG-3′.

All reactions were obtained over 32 cycles. This cycle number at which exponential amplification occurs was established by amplifying Rp49 for 25, 30, 35 and 40 cycles. These PCR products were visualized on a 1% agarose gel and digitized on a GS800 calibrated densitometer (BioRad catalogue#170–7980). The resulting images were analyzed using the Quantity one software (BioRad). The average densities of the individual Rp49 bands obtained at the different cycle numbers, as determined by the densitometer, were used to plot a curve of the product formed versus cycle number. The resulting graph was then used to determine the optimum cycle number for the test reactions.

The same master mix was used for all reactions, with a final reaction concentration of $1 \times Mg^{2+}$ free standard Taq buffer (New England Biolabs, Ipswich, Massachusetts, USA; catalogue#B9015), 1.5 mmol/l $MgCl_2$, 400 μmol/l dNTPs, 1 mmol/l of each primer and 1.25 units of *Taq* DNA polymerase (New England Biolabs catalogue#M0320). For the *Dmp53*, *Snama* and *reaper* transcripts the cycling conditions were as follows: 50°C for 30 min; 94°C for 5 min; followed by 32 cycles 94°C for 30 s, 60°C for 40 s, 68°C for 70 s and a final extension at 68°C for 5 min. For Rp49 the annealing temperatures were lowered to 56°C.

Statistical analysis

Statistical analyses were carried out using Statistix 7.0 (Analytical Software, Tallahasee, Florida, USA). The differences were evaluated by a one-way analysis of variance followed by a Tukey's HSD (Honestly Significant Difference) test. *P* values less than 0.05 were considered significant and led to the rejection of the null hypothesis of no significant difference between means.

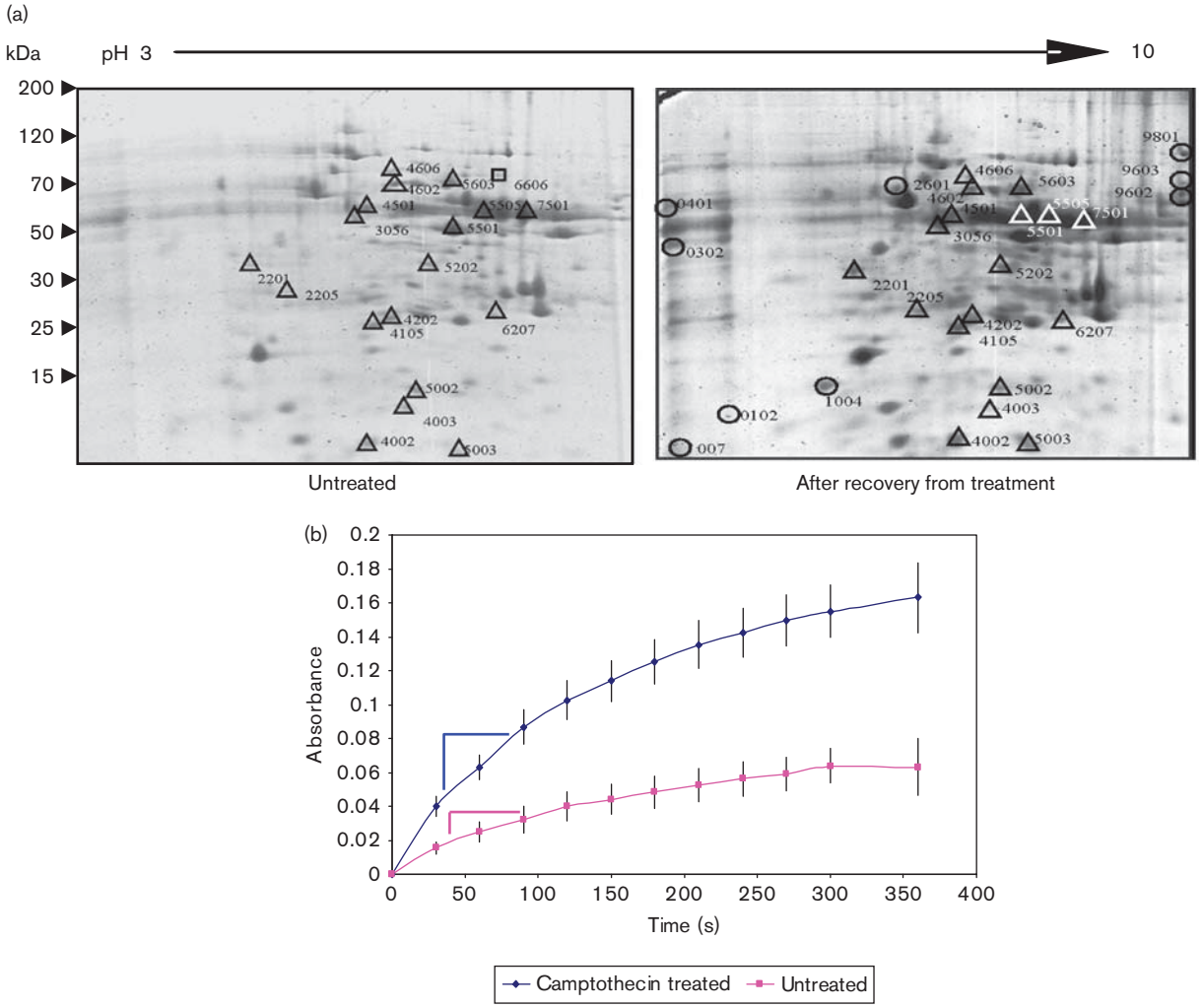
Results

Proteomic analysis of *Drosophila melanogaster* after exposure to camptothecin

To identify changes in protein expression after treatment of normal *Drosophila* cells with CPT, the extracts from flies that were recovering from CPT treatment and from untreated flies were resolved by 2D PAGE and analysed by PDQuest software to determine upregulated and downregulated spots (Fig. 1a). Many proteins, whose expression was altered, are involved in biological processes that include the glycolytic pathway, the tricarboxylic acid (TCA) cycle, the cytoskeleton, protein folding and a variety of others (Table 1).

Glycolytic enzymes whose expression was elevated included fructose-1,6-bisphosphate aldolase, enolase and pyruvate kinase. Notably, triosephosphate isomerase (TPI) was downregulated. TPI is an important enzyme that catalyses the isomerization of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate during glycolysis. Mutations that cause *TPI* deficiency result in haemolytic anaemia, neuromuscular degeneration, susceptibility to infections and premature death [30–32]. Enzymes involved in oxidative phosphorylation (Krebs cycle) such as aconitase, and dihydrolipoamide dehydrogenase mitochondrial precursor were also upregulated indicating active mitochondrial respiration. Aconitase is at the entry level of Kreb's cycle and dihydrolipoamide dehydrogenase is part of the pyruvate dehydrogenase complex, which plays a pivotal role in the oxidation of pyruvic acid to acetyl-CoA [33]. Among other important

Fig. 1



Altered protein expression in response to camptothecin (CPT) treatment. (a) Two-dimensional gel electrophoresis of *Drosophila melanogaster* crude protein extract from untreated flies and from flies recovering from CPT treatment. Proteins that occur in both samples are marked with a triangle. Those that only appear after CPT exposure are marked with a circle. Finally those that disappear after CPT exposure are marked with a rectangle. (b) Glutathione S transferase enzyme activity assay using equal protein amounts from untreated flies and from flies recovering form CPT treatment. Enzyme activity was calculated from the indicated gradient equations. Enzyme activities were 0.15 and 0.051 μmol/min/ml in samples from treated and untreated flies, respectively.

and upregulated enzymes is uridine diphosphate glucose phosphorylase, which is important for glycogen metabolism and bellwether the *Drosophila* mitochondrial ATP synthase, and is active across the inner membrane catalysing ATP synthesis during oxidative phosphorylation; it also provides further evidence of active mitochondria.

One of the key differences between cancer and normal cells is their different metabolic programmes, especially glucose uptake and energy production; cancer cells consume much higher levels of glucose and prefer the faster but less efficient process called glycolysis to oxidative phosphorylation, which is more efficient but slower. These results indicate that there are significant changes in metabolic pathways during recovery from CPT treatment. Altered expression of aconitase can be used as an indicator of mitochondrial function [34]. Reduced aconitase levels would indicate compromised mitochondrial function. On the basis of the elevated levels of aconitase the TCA is deemed to be elevated during recovery. Increased glycolysis is reported to positively promote tumourigenesis and to be directly influenced by the hypoxia-inducible factor in CRC. Hypoxia-inducible factor is a transcription factor that can activate virtually all the genes that encode the glycolytic pathway proteins [35].

Other important enzymes with altered expression include arginine kinase (AK) and ATP synthetases whose increased expression suggests a mechanism for providing

Table 1 Upregulated and downregulated proteins upon recovery from camptothecin treatment

Sample number	Identity	Accession number	Molecular weight	Isoelectric point	OD before	OD after
Oxidative metabolism						
0302	Chain D, fructose-1,6-bisphosphate aldolase	CG31692-RA	39 231	6.67	5	24
4105	Triose phosphate isomerase	CG2171-RA	26 942	6.00	47	14
5501	Enolase, isoform A	CG17654-PA	46 861	6.10	152	401
9602	UGP, isoform A	CG4347-PA	58 944	6.92	0	32
9602	Lethal (1) G0030, isoform A	CG3861-PA	51 713	8.89	0	32
9602	Pyruvate kinase	RH07636p	57 951	7.13	12	42
9801	Aconitase	CG9244-PB	86 200	8.49	5	41
0401	ATP synthase β subunit		53 544	5.19	12	42
0302	Arginine kinase	CG32031-PD	40 126	6.04	5	24
5603	Dihydrolipoamide dehydrogenase mitochondrial precursor	CG7430-PA	53 565	6.41	19	72
9602	Bellwether	CG3612-PA	59 612	9.09	0	32
9801	Bellwether	CG3612-PA	59 612	9.09	5	41
Protein folding						
2601	Protein disulfide isomerase, isoform A	CG6988-PA	56 031	4.72	0	13
4606	Chaperonin, isoform C	CG7033-PC	58 223	5.68	8	41
4602	ERp60, isoform B	CG8983-PB	55 678	5.62	50	31
Antioxidant activity and detoxification						
2201	Glutathione S transferase S1, isoform C	CG8938-PC	27 653	4.57	9	57
5002	Superoxide dismutase	CG11793-PA	15 974	5.67	12	63
4202	Ferritin 2 light chain homologue	CG1469-PB	25 483	5.90	8	30
6606	Transferrin precursor		72 964	6.69	4	0
3506	Glutamine synthetase 2, isoform C	CG1743-PC	41 962	5.42	15	96
Cytoskeleton						
3506	Actin 88F	CG5178-PA	42 072	5.29	15	96
2205	Flightin	CG7445-PA	20 643	5.30	3	44
Other						
4501	Yolk protein 1	CG2985-PA	48 739	7.19	28	105
5505	Yolk protein 2	CG2979-PA	49 744	7.74	119	341
7501	Yolk protein 2	CG2979-PA	49 744	7.74	119	342
5003	Odorant-binding protein 99a	CG18111-PA	16 384	6.19	45	48
3506	Mitochondrial processing peptidase	CG3731-PA	52 525	5.67	15	96
2205	20S proteasome α subunit		26 995	4.83	3	44

OD, optical density; UGP, uridine diphosphate-glucose pyrophosphorylase.

additional energy-producing pathways. Arginine is a phosphagen found in insects and is akin to the creatinine kinase in animals. These enzymes are required when ATP demand exceeds what can be supplied by the mitochondria. The increased levels of AK during recovery from CPT may suggest another mechanism that normal cells use to increase the efficiency of oxidative phosphorylation. AK provides a temporary energy buffer in insects [36,37]. Overall, these results suggest that normal cells use multiple pathways to produce energy when recovering. It may be difficult to assess the impact of these mechanisms in further inducing tumourigenesis using *Drosophila* as a model but it seems that this model organism could be an important tool for assessing the impact of anticancer drugs on both normal and cancer cells.

CPT treatment also generates free radicals and the cells may respond to this by the evidently increased expression of proteins that are involved in detoxification and antioxidant activity, such as superoxide dismutase and GST. GST expression was confirmed by using an assay to support the proteomic results (Fig. 1b). Superoxide dismutase converts superoxide to hydrogen peroxide and water, thus clearing the organism of free radicals that may cause DNA damage.

Camptothecin treatment increases transcription of Dmp53 level and reduces Snama

The tumour suppressor p53 known to be induced by CPT in both normal and cancer cells inhibits glycolysis while promoting oxidative phosphorylation [38,39]. Mdm2 is the key regulator of p53 in vertebrates but no homologue has been identified in invertebrates so far. The mechanism by which p53 is regulated in invertebrates is therefore still not well understood. We propose that Snama could play the role of Mdm2 in flies based on two pertinent observations. First, vertebrate orthologues of Snama, variously known as proliferation potential protein-related 2, PACT, RBBP6, Domain With No Name are known to bind p53 [19,20,40,41]. Second, the mouse, RBBP6, negatively regulates p53 by enhancing the activity of Mdm2. Null *rbbp6* mouse mutants have a phenotype that is reminiscent of *mdm2*$^{-/-}$. They die at E7.5 as they are small and developmentally retarded. This phenotype is partially rescued in a p53 null background [18]. In the developing *Drosophila* eye *Snama* is transcriptionally controlled by the hedgehog and is required for cell proliferation and survival [25]. Snama is essential for *Drosophila* embryonic development, probably acting as a suppressor of apoptosis. Disruption of *Snama* causes ectopic and early apoptosis in the *Drosophila* embryos

[26]. The apparent absence of a *Drosophila* Mdm2 homologue raises speculation about an alternative pathway for Dmp53 regulation. Although direct interaction between Dmp53 and Snama has not been shown evidence obtained in other systems, which strengthens speculation that Snama is a candidate that mediates an alternative pathway to Mdm2.

We measured the transcriptional activation of *Snama*, *Dmp53* and *reaper* in adult flies during treatment and after recovery from CPT treatment. Reaper is a downstream effector of and is transcriptionally controlled by Dmp53 [42]. Our results (Fig. 2a), show that CPT treatment causes an increase in the transcription of Dmp53 but there is no concomitant increase in *reaper* mRNA. Transcription of *Snama* is reduced during treatment but is upregulated upon recovery.

Given the glycolytic flux observed from proteomic studies we tested the effect of bypassing the glycolytic pathway by adding methyl pyruvate to the food, thereby providing the endproduct of glycolysis. The methyl pyruvate supplement accelerated the decline of *Dmp53* and the activation of *Snama* transcription. When added to the food alone, methyl pyruvate seems to reduce the expression of all three genes. Inexplicably, this supplementation virtually abolishes *reaper* expression when not combined with CPT such that no mRNA is detectable by PCR. Similar results were obtained in sample larvae whose mothers were treated with CPT (Fig. 2b). As p53 is known to inhibit glycolysis the decline in *Dmp53* transcription may be an important factor in promoting the observed glycolytic flux in flies that are recovering from CPT treatment.

Camptothecin effect on oogenesis and development

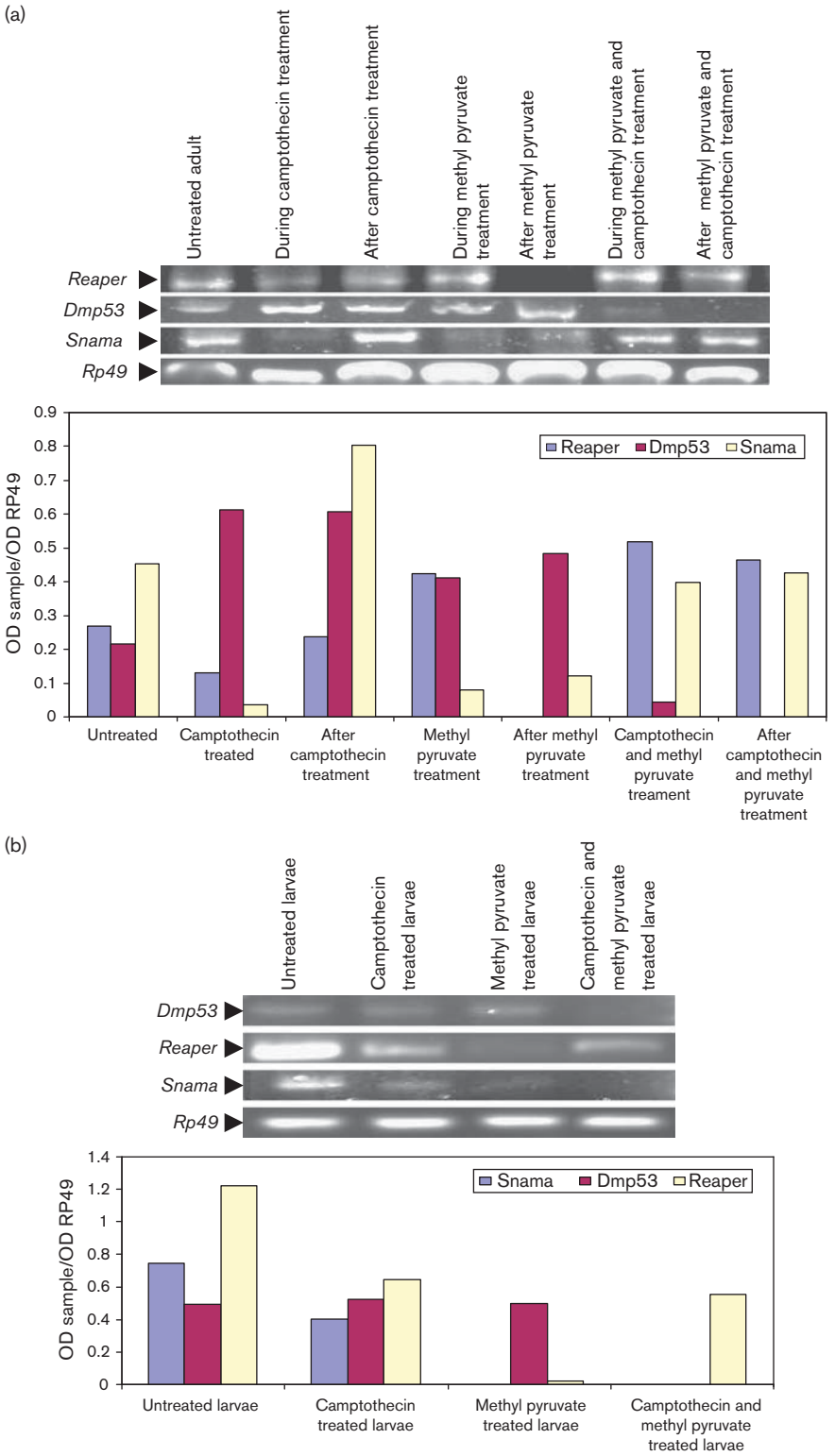
As a Topo 1 inhibitor CPT targets nucleic acid functions related to transcription and replication. CPT is, therefore, likely to adversely affect multiple organs, especially those with high mitotic rates. *Drosophila* Topo1 is an essential enzyme required for oogenesis and early embryonic development but it is not required for gamete viability [10,43,44]. Patients, including young women, who are treated with CPT, suffer severe ovarian toxicity in addition to the normal adverse effects, such as myeloid suppressions, neutropenia and anaemia [45]. Symptoms of toxicity caused by clinically approved CPT derivatives and those in clinical trials, include resorptions and death of foetuses, reduced litter size, stillborns, postnatal mortality and delays in physical development when applied in high dose, but milder effects in medium doses indicating severe adverse effects on the female reproductive system [45–47]. Although research continues on the development of delivery systems that specifically target cancer cells [48], other methods aiming at protecting healthy organs during chemotherapy are desirable.

We exposed male and female *Drosophila* to CPT, measured their fecundity and monitored the development of their offspring. The newly enclosed flies were separated first according to sex, by which every one was of the same age and then were further divided into three experimental groups. In the first group female flies were treated and then mated with healthy male flies. In the second group the treated male flies were mated with healthy female flies; and third, both female and male flies were treated and mated. For each experimental group the number of embryos produced was counted after the removal of the adults to new vials and the pupae that finally developed from these embryos were counted. These numbers were used as an indication of development time and success. The numbers of adult flies of both sexes that died were recorded as an indication of the effect of CPT on mortality rates.

CPT treatment has pronounced adverse effects on the reproductive system of female flies. This was measured by the number of embryos laid and by their development (Fig. 3a–c). CPT dramatically reduced the number of embryos laid by treated females that were mated with either treated or untreated male flies. Treating male flies with CPT did not affect the number of embryos laid by untreated female flies. Generally, treated flies died earlier than control flies (Fig. 3b). Approximately 50% of the flies died by day 10 after treatment compared with approximately 30 and 10% of untreated male and female flies, respectively. The life span of treated flies is therefore shorter than that of untreated flies. Methyl pyruvate was again used as a supplement alone and in combination with CPT to test whether a mechanism that bypasses the glycolytic pathway could alleviate the adverse effects that were observed. When given alone, methyl pyruvate did not elicit serious adverse effects on fecundity and development but there was an improvement when combined with CPT. For example, the number of embryos laid by female flies given the CPT treatment and the methyl pyruvate supplement was significantly ($P = 0.002$) improved compared with CPT treatment alone (Fig. 3a). Moreover, approximately 20% of the embryos from CPT-treated mothers developed into pupae whereas approximately 40% of embryos from mothers treated with the combination developed into pupae (Fig. 3c).

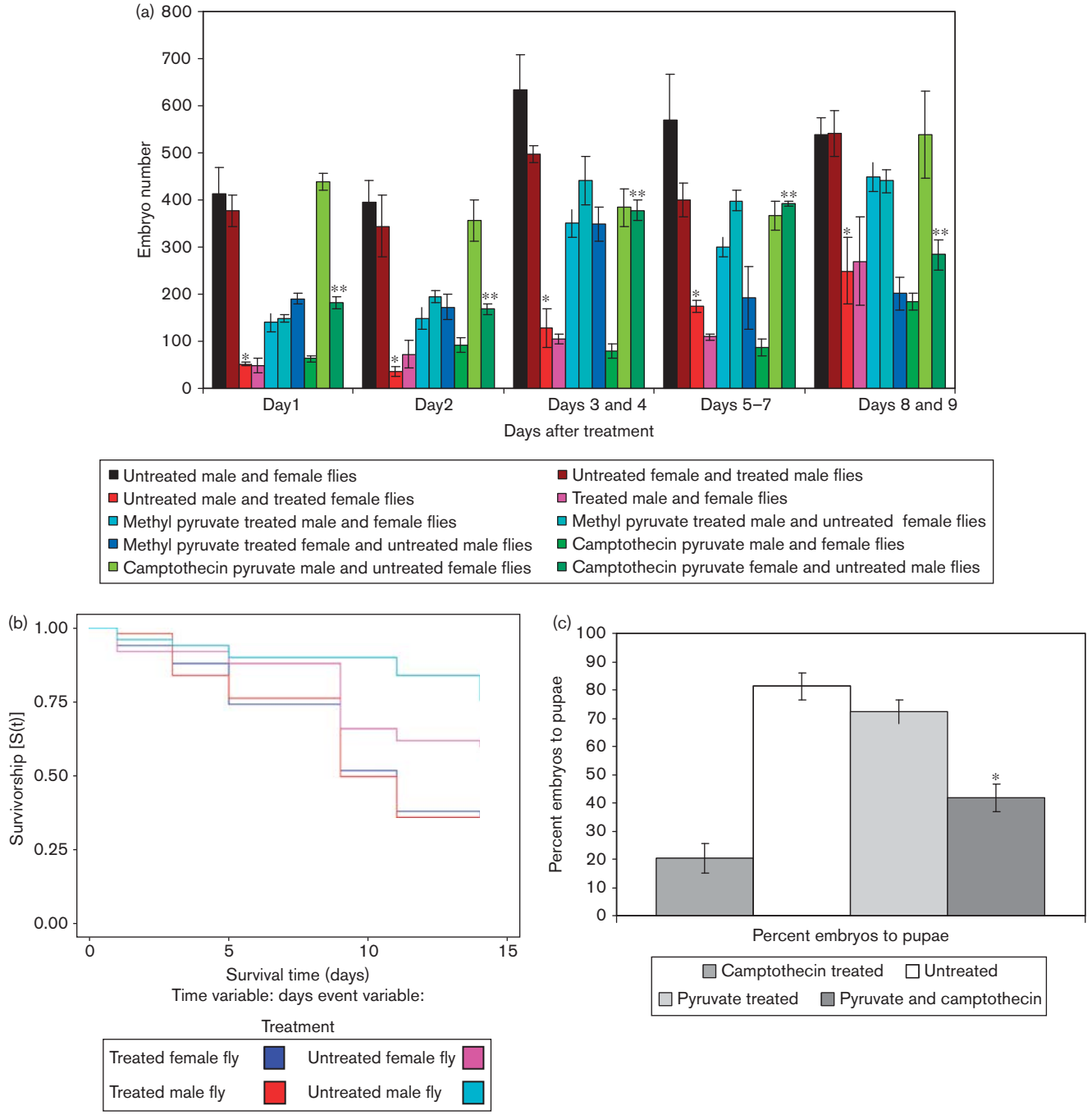
Western blot analysis using the Santa Cruz anti-Dmp53 antibody (dD-21) shows several bands with a differential profile based on treatment status. There are three theoretical isoforms of Dmp53: a short Dmp53n (123 amino acids), a long Dmp53L (495 amino acids) and Dmp53 (385 amino acids) [49,50]. Their sizes should be approximately 13.5, 54.5 and 42 kDa, respectively. It is, however, often difficult to determine the correct p53 size because of the extensive posttranslational modifications that often occur in p53 proteins [51]. The 42-kDa band is present after CPT treatment but was absent when the

Fig. 2



The semi-quantitative reverse transcriptase-PCR analysis using gene specific primers for *reaper*, *Dmp53* and *Snama* on total RNA obtained from (a) adult flies and (b) from larvae whose mothers were treated with camptothecin. OD, optical density.

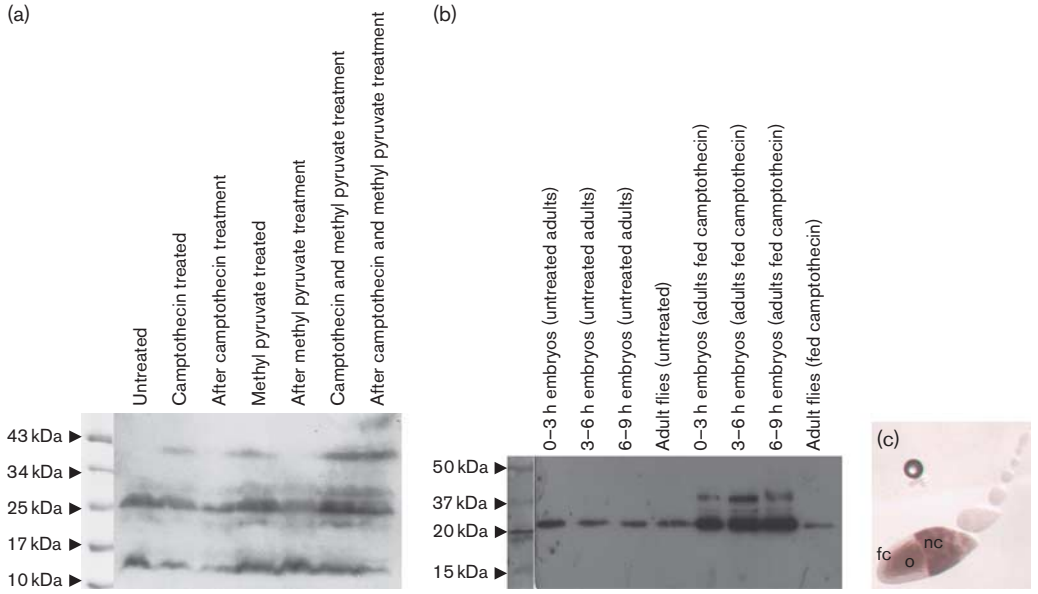
Fig. 3



The effect of camptothecin and methyl pyruvate on mortality, fecundity and development. (a) Number of embryos laid by each experimental group as indicated. A significant comparison is made between embryos laid by camptothecin treated female flies without supplement (*) and with supplement (**) $P=0.002$. (b) Mortality rates. (c) Percentage of embryos that develop to pupal stage in each experimental group as indicated. Comparisons between larvae produced by treated against those from untreated female flies has $*P=0.02$.

flies were allowed to recover. This isoform is controlled by the internal promoter and lacks the transactivating domain [50]. It also appears during and after treatment with a combination of methyl pyruvate and CPT (Fig. 4a) and in embryos laid by CPT-treated mothers only (Fig. 4b). Spatial expression of p53 isoforms in normal cells has been reported [52]. This suggests differential activation of these isoforms. Furthermore, selective expression of p53 isoforms in normal cells responding to DNA-damaging agents, such as actinomycin D, has been shown experimentally [53]. This provides evidence that these isoforms have specific regulated functions.

Fig. 4



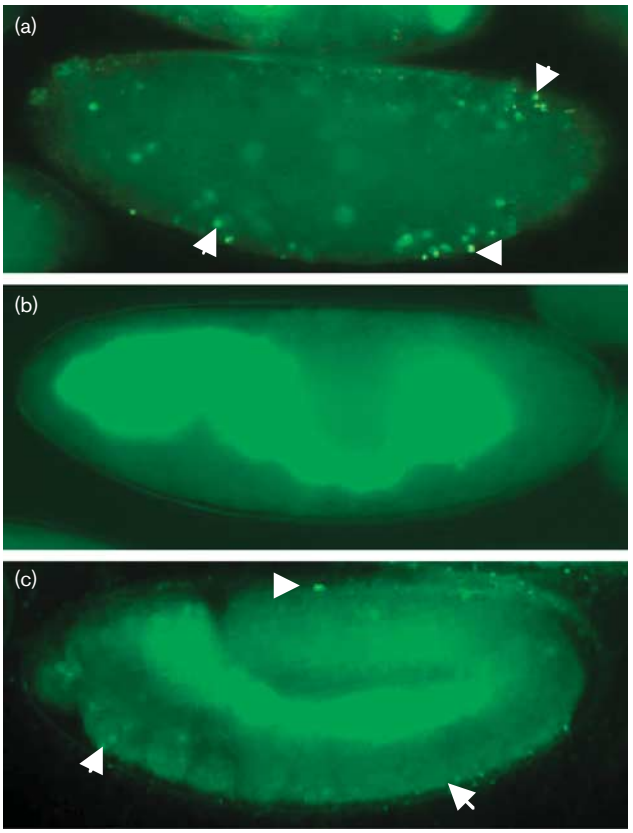
Differential expression of dp53 and effect of camptothecin. Western blot analysis of protein samples from (a) treated and untreated adult flies using the anti-Dmp53 antibody, (b) from embryos laid by treated and untreated mothers as indicated and (c) immunocytochemical staining of an ovariole with the anti-Dmp53 antibody showing spatial expression of dp53 during oogenesis in nc (nurse cells), o (oocyte) and none in fc (follicle cells).

Using the same antibody to stain ovaries, Dmp53 protein was detected in nurse cells and in the oocytes of wild-type flies but not in follicle cells (Fig. 4c). An earlier report showed localization of *Dmp53* transcripts in nurse cells, but not in the oocyte and in follicle cells at stage 10. However, maternal contribution was assumed to be present in embryos as abundant *Dmp53* transcripts were detectable at the blastoderm stage [54]. This immunocytochemical experiment, showing the presence of the *Dmp53* protein in the oocyte, indicates that it is required during oogenesis. Staining embryos with the vital dye acridine orange, targeting apoptotic cells shows that embryos whose mothers were treated with CPT unexpectedly undergo pronounced apoptosis early in embryogenesis (Fig. 5a) and none in the late stages of development when normal apoptosis should occur (Fig. 5b). Normal embryonic apoptosis appears initially at the dorsal region of the head, approximately 7 h after egg laying when the germ band is fully extended and spreads throughout the embryo in later stages ([55]; and Fig. 5c). In addition to the abnormal apoptosis pattern, these embryos exhibit clear abnormalities in the central nervous system and in the gut. This abnormal pattern of embryonic cell death could be related to changes in Dmp53 expression in embryos laid by treated mothers.

Discussion

We have shown by proteomics that glycolytic flux occurs when wild-type fruit flies recover from CPT treatment. Although glycolysis is the preferred energy production mechanism for cancer cells to oxidative phosphorylation, the metabolic programme in recovering wild-type flies has important differences, such as functional TCA cycle and upregulated glycogenesis, as both of these have been shown to be downregulated in CRC cells [56]. This suggests that the glycolytic flux that is observed is different from the Warburg effect. It has been shown that disruption of *p53* gene function causes a shift from mitochondrial respiration to glycolysis, which is observed in cancer cells and is referred to as the Warburg Effect [57,58]. It is worth noting that the metabolic programme that is observed here is accompanied by a reduction in *Dmp53* transcription and by the currently inexplicable appearance of new protein bands raising speculation about the rather complex mechanisms of *Dmp53* regulation. In mammalian systems p53 has been shown to regulate glycolysis by intervening at key points in the pathway. First, p53 can inhibit glucose uptake by repressing the expression of glucose transporters such as Glut 1. Second, p53 can directly inhibit the glycolytic pathway by activating the expression of TP53-induced glycolysis and apoptosis regulator, which functions to decrease the activity of the glycolytic enzyme, 6-phosphofructose-1-kinase. Third, p53 can directly repress the expression of another glycolytic enzyme, phosphoglycerate mutase. Furthermore, p53 plays an important role in influencing mitochondrial respiration or oxidative phosphorylation through transcriptional control of the *Synthesis of cytochrome c oxidase 2* gene [38]. The observed downregulation and upregulation of *Dmp53* transcripts and their putative negative regulator, *Snama*, respectively, could be a mechanism by which glycolytic flux is achieved

Fig. 5



Apoptosis in embryos laid by camptothecin treated mothers. Embryos were stained for 3–6 h with acridine orange as described in the 'Methods' section. (a) A poorly developed embryo laid by camptothecin treated mother looking younger than expected and showing apoptosis (arrows). (b) An embryo at germ band extension showing no apoptosis and (c) an embryo from an untreated mother at germ band extension showing apoptosis (arrows).

by recovering the flies. Moreover, these conditions are accelerated by the addition of methyl pyruvate, the end-product of glycolysis, and a substrate for oxidative phosphorylation. Another important enzyme whose expression is downregulated during recovery from CPT treatment is TPI whose deficiency in humans results in haemolytic anaemia and neuromuscular impairment [30]. Interestingly, this enzyme is upregulated in CRC cells [56] providing another indicator that the metabolic programme involved here is different from that used by cancer cells.

CPT-induced apoptosis has been studied widely in cancer cells and some studies have been conducted on *Drosophila*, albeit to a limited extent. Regulation of Dmp53, which evidently is required for apoptosis, is compounded by the apparent absence of Mdm2 in invertebrates. In vertebrate cells, p53 is regulated by various mechanisms, primarily ubiquitin-dependent degradation through the proteasome system. This ubiquitination is catalysed mainly by the really interesting new gene finger protein, Mmd2. We propose RBBP6, a protein that is known to enhance the activity of Mmd2 and to interact with p53 in vertebrates as a good candidate for p53 regulation in the invertebrate system, perhaps without the cooperation of Mdm2. The RBBP6 family is characterized by a highly conserved ubiquitin-like amino-terminal domain, a really interesting new gene finger-like motif and a p53-binding domain [19,22,26,40,41,59,60]. Furthermore, we have experimental evidence showing that Snama has ubiquitin ligase activity (unpublished). All these characteristics are present in Mdm2 and are required for the regulation of p53. We have shown here that on recovery from CPT treatment flies express high levels of *Snama* and low levels of *Dmp53* transcripts. The nature of the interaction between Snama and Dmp53 must await good antiSnama antibodies, which we are currently developing, and the use of appropriate *dmp53* and *snama* mutants. Nevertheless, taken together, our findings and the published features of the RBBP6 family support the proposed association of Snama and Dmp53 and indicate that Snama may be involved in the cellular response to CPT treatment.

Recurrent ovarian and cervical cancers are often treated with CPT-derived anticancer agents, such as topotecan. These drugs target, mainly, the haematopoetic system, the lymphatic tissue, the gastrointestinal tract, hair bulbs and reproductive organs such as testicles and ovaries [3]. They are designed to provide better safety than CPT but they still cause severe toxicity in reproduction and in embryonic development as animals that are treated during pregnancy experience foetal deaths, postimplantation losses and reduced litter sizes [46,47,61]. The reproductive system of the flies is evidently affected adversely by CPT, as the numbers of embryos laid and offspring that develop to adulthood are severely reduced. It is inexplicable at this stage why apoptosis appears in early embryos and is absent in older embryos. The obvious candidate for investigation in matters surrounding apoptosis is the known inducer, Dmp53, which is also known to be involved in biological processes such as reproduction, genomic repair, fidelity and recombination, the regulation of metabolic processes, longevity, and surveillance of the stability of development [62]. The role that is played by Dmp53 and Snama in the observed phenotypes can be identified by careful use of appropriate mutants and key reagents such as antibodies. Snama, which is shown to be upregulated upon recovery while Dmp53 is downregulated, could act as a regulator of Dmp53. These results also suggest that methyl pyruvate improves the development of embryos. This can also be confirmed by focused study on the role of pyruvate supplementation.

Our results indicate that the glycolytic flux that occurs in normal cells during recovery from CPT treatment is different from the Warburg Effect seen in cancer cells. Perhaps the differences could be exploited to reduce

stress on normal cells during chemotherapy. Furthermore, the RBBP6 family protein, Snama, may play an important role in DNA damage response caused by CPT. Overall, these results point to the possible use of *Drosophila* as a model system to study CPT pharmacodynamics.

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References

1 Schulz C, Boeck S, Heinemann V, Stemmler H-J. UGT1A1 genotyping: a predictor of irinotecan-associated side effects and drug efficacy? *Anticancer Drugs* 2009; **20**:867–879.
2 Schulz C, Heinemann V, Schalhorn A, Moosmann N, Zwingers T, Boeck S, *et al.* UGT1A1 gene polymorphism: impact on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer. *World J Gastroenterol* 2009; **15**:5058–5066.
3 Lorusso D, Pietragalla A, Mainenti S, Masciullo V, Di Vagno G, Scambia G. Review role of topotecan in gynaecological cancers: current indications and perspectives. *Crit Rev Oncol Hematol* 2009; **74**:163–174.
4 Gilmour DS, Elgin SC. Localization of specific topoisomerase I interactions within the transcribed region of active heat shock genes by using the inhibitor camptothecin. *Mol Cell Biol* 1987; **7**:141–148.
5 Chung M-K, Han S-S, Kim J-C. Evaluation of the toxic potentials of a new camptothecin anticancer agent CKD-602 on fertility and early embryonic development in rats. *Regul Toxicol Pharmacol* 2006; **45**:273–281.
6 Hsiang Y-H, Hertzberg R, Hechtj S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase. *J Biol Chem* 1985; **260**:14873–14878.
7 Nitiss J, Wang JC. DNA topoisomerase-targeting antitumour drugs can be studied in yeast. *Proc Natl Acad Sci U S A* 1988; **85**:7501–7505.
8 Covey JM, Jaxel C, Kohn KW, Pommier Y. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res* 1989; **49**:5016–5022.
9 Larsen AK, Gobert C. DNA topoisomerase I in oncology: Dr Jerkyll or Mr Hyde? *Pathol Oncol Res* 1999; **5**:171–179.
10 Lee MP, Brown SD, Chen A, Hsieh T-S. DNA topoisomerase I is essential in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 1993; **90**:6656–6660.
11 Brodsky MH, Weinert BT, Tsang G, Rong YS, McGinnis NM, Golic KG, *et al.* Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. *Mol Cell Biol* 2004; **24**:1219–1231.
12 Takeba Y, Kumai T, Matsumoto N, Nakaya S, Tsuzuki Y, Yanagida Y, *et al.* Irinotecan activates p53 with its active metabolite, resulting in human hepatocellular carcinoma apoptosis. *J Pharmacol Sci* 2007; **104**:232–242.
13 Troyano A, Fernandez CF, Sancho P, Blas ED, Aller P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. *J Biol Chem* 2001; **276**:47107–47115.
14 Ondrousková E, Soucek K, Horváth V, Smarda J. Alternative pathways of programmed cell death are activated in cells with defective caspase-dependent apoptosis. *Leuk Res* 2008; **32**:599–609.
15 Takeba Y, Sekine S, Kumai T, Matsumoto N, Nakaya S, Tsuzuki Y, *et al.* Irinotecan-induced apoptosis is inhibited by increased P-glycoprotein expression and decreased p53 in human hepatocellular carcinoma cells. *Biol Pharm Bull* 2007; **30**:1400–1406.
16 Liu W, Zhang R. Upregulation of p21WAF1/CIP1 in human breast cancer cell lines MCF-7 and MDA-MB-468 undergoing apoptosis induced by natural product anticancer drugs 10-hydroxycamptothecin and camptothecin through p53-dependent and independent pathways. *Int J Oncol* 1998; **12**:793–804.
17 Moll UM, Petrenko O. The MDM2-p53 interaction. *Mol Cancer Res* 2003; **1**:1001–1008.
18 Li L, Deng B, Xing G, Teng Y, Tian C, Cheng X, *et al.* PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proc Natl Acad Sci U S A* 2007; **104**:7951–7956.
19 Witte MM, Scott RE. The proliferation potential protein-related (P2P-R) gene with domains encoding heterogeneous nuclear ribonucleoprotein association and Rb1 binding shows repressed expression during terminal differentiation. *Proc Natl Acad Sci U S A* 1997; **94**:1212–1217.
20 Simons A, Melamed-Bessudo C, Wolkowicz R, Sperling J, Sperling R, Eisenbach L, *et al.* PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene* 1997; **14**:145–155.
21 Yoshitake Y, Nakatsura T, Monji M, Senju S, Matsuyoshi H, Tsukamoto H, *et al.* Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complimentary DNA microarray analysis. *Clin Cancer Res* 2004; **10**:6437–6448.
22 Gao S, Scott RE. Stable overexpression of specific segments of the P2P-R protein in human MCF-7 cells promotes camptothecin-induced apoptosis. *J Cell Physiol* 2003; **197**:445–452.
23 Sutcliffe JE, Brehm A. Of flies and men; p53, a tumour suppressor. *FEBS Lett* 2004; **567**:86–91.
24 Folberg-Blum A, Sapir A, Shilo B-Z, Oren M. Overexpression of mouse Mdm2 induces developmental phenotypes in Drosophila. *Oncogene* 2002; **21**:2413–2417.
25 Jones C, Reifegerste R, Moses K. Characterization of Drosophila mini-me, a gene required for cell proliferation and survival. *Genetics* 2006; **173**:793–808.
26 Mather A, Rakghotho M, Ntwasa M. SNAMA, a novel protein with a DWNN domain and a RING finger-like motif: a possible role in apoptosis. *Biochim Biophys Acta* 2005; **1727**:169–176.
27 Abrams JM, White K, Fessler LI, Steller H. Programmed cell death during *Drosophila* embryogenesis. *Development* 1993; **117**:29–43.
28 Rabilloud T, Strub J, Luche S, Van Dorsselaer A, Lunardi J. A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics* 2001; **1**:699–704.
29 Habig W, Pabst M, Jakoby W. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**:7130–7139.
30 Fermo E, Bianchi P, Vercellati C, Rees DC, Marcello AP, Barcellini W, *et al.* Triose phosphate isomerase deficiency associated with two novel mutations in TPI gene. *Eur J Haematol* 2010; **85**:170–173.
31 Ferenc O, Judit O, Judit O. Triosephosphate isomerase deficiency: facts and doubts. *IUBMB Life* 2006; **58**:703–715.
32 Seigle JL, Celotto AM, Palladino MJ. Degradation of functional triose phosphate isomerase protein underlies sugarkill pathology. *Genetics* 2008; **179**:855–862.
33 Park Y-H, Patel MS. Characterization of interactions of dihydrolipoamide dehydrogenase with its binding protein in the human pyruvate dehydrogenase complex. *Biochem Biophys Res Commun* 2010; **395**:416–419.
34 Paul A, Belton A, Nag S, Martin I, Grotewiel MS, Duttaroy A. Reduced mitochondrial SOD displays mortality characteristics reminiscent of natural aging. *Mech Ageing Dev* 2007; **128**:706–716.
35 Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 2008; **30**:393–402.
36 Schneider A, Wiesner RJ, Grieshaber MK. On the role of arginine kinase in insect flight muscle. *Insect Biochem* 1989; **19**:471–480.
37 Chamberlain ME. Mitochondrial arginine kinase in the midgut of the tobacco hornworm (*Manduca sexta*). *J Exp Biol* 1997; **200**:2789–2796.
38 Cheung EC, Vousden KH. The role of p53 in glucose metabolism. *Curr Opin Cell Biol* 2010; **22**:186–191.
39 Vousden KH, Ryan KM. p53 and metabolism. *Nat Rev Cancer* 2009; **9**:691–700.
40 Scott RE, Giannakouros T, Gao S, Peidis P. Functional potential of P2P-R: a role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix associated regions of DNA? *J Cell Biochem* 2003; **90**:6–12.
41 Pugh DJR, Eiso AB, Faro A, Lutya PT, Hoffmann E, Rees DJG. DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct Biol* 2006; **6**:1–12.
42 Lee JH, Lee E, Park J, Kim E, Kim J, Chung J. *In vivo* p53 function is indispensable for DNA damage-induced apoptotic signaling in *Drosophila*. *FEBS Lett* 2003; **550**:5–10.
43 Zhang CX, Chen AD, Gettel NJ, Hsieh T-S. Essential functions of DNA topoisomerase I in *Drosophila melanogaster*. *Dev Biol* 2000; **222**:27–40.
44 Morham SG, Kluckman KD, Voulomanos N, Smithies O. Targeted distribution of the mouse topoisomerase I gene by camptothecin selection. *Mol Cell Biol* 1996; **16**:6804–6809.
45 Tanaka T, Utsunomiya T, Utsunomiya H, Umesaki N. Irinotecan HCl, an anticancer topoisomerase I inhibitor, frequently induces ovarian failure in premenopausal and perimenopausal women. *Oncol Rep* 2008; **19**:1123–1133.

46 Chung M-K, Kim C-Y, Kim J-C. Reproductive toxicity evaluation of a new camptothecin anticancer agent, CKD-602, in pregnant/lactating female rats and their offspring. *Cancer Chemother Pharmacol* 2007; **59**:383–395.
47 Chung M-K, Kim J-C, Han S-S. Embryotoxic effects of CKD-602, a new camptothecin anticancer agent, in rats. *Reprod Toxicol* 2005; **20**:165–173.
48 Dharap SS, Wang Y, Chandna P, Khandare JJ, Qiu B, Gunaseelan S, *et al.* Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide. *Proc Natl Acad Sci U S A* 2005; **102**:12962–12967.
49 Bourdon J-C. p53 family isoforms. *Curr Pharm Biotechnol* 2007; **8**:332–336.
50 Bourdon J-C, Fernandes K, Murray-Zmijewski F, Liu G, Diot A, Xirodimas DP, *et al.* p53 isoforms can regulate p53 transcriptional activity. *Genes Dev* 2005; **19**:2122–2137.
51 Meek DW. Mechanisms of switching on p53: a role for covalent modification. *Oncogene* 1999; **18**:7666–7675.
52 Han K-A, Kulesz-Martin MF. Alternatively spliced p53 RNA in transformed and normal cells of different tissue types. *Nucl Acids Res* 1992; **20**:1979–1981.
53 Kulesz-Martin MF, Lisafeld B, Huang H, Kisiel ND, Lee L. Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal cells. *Mol Cell Biol* 1994; **14**:1698–1708.
54 Jin S, Martinek S, Joo WS, Wortman JR, Mirkovic N, Sali A, *et al.* Identification and characterisation of a p53 homologue in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 2000; **97**:7301–7306.
55 Bangs P, White K. Regulation and execution of apoptosis during *Drosophila* development. *Dev Dyn* 2000; **218**:68–79.
56 Bi X, Lin Q, Foo TW, Joshi S, You T, Shen H-M, *et al.* Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways. *Mol Cell Proteomics* 2006; **5**:1119–1130.
57 Ma W, Sung H, Park J, Matoba S, Hwang P. A pivotal role for p53: balancing aerobic respiration and glycolysis. *J Bioenerg Biomembr* 2007; **39**:243–246.
58 Bensaad K, Vousden KH. p53: new roles in metabolism. *Trends Cell Biol* 2007; **17**:286–291.
59 Gao S, Scott RE. P2P-R protein overexpression restricts mitotic progression at prometaphase and promotes mitotic apoptosis. *J Cell Physiol* 2002; **193**:199–207.
60 Gao S, White MM, Scott RE. P2P-R protein localizes to the nucleus of interphase cells and the periphery of chromosomes in mitotic cells which show maximum P2P-R immunoreactivity. *J Cell Physiol* 2002; **191**:145–154.
61 Chung M-K, Kim J-C, Han S-S. Effects of CKD-602, a new camptothecin anticancer agent, on pregnant does and embryo–fetal development in rabbits. *Drug Chem Toxicol* 2008; **28**:35–49.
62 Lane D, Levine A. P53 Research: the past thirty years and the next thirty years. *Cold Spring Harb Perspect Biol* 2010. doi: 10.1101/cshperspect.a000893:1-10.