FAST AND FURIOUS: METABOLIC PATHWAYS FUELLING DEVIL FACIAL TUMOUR DISEASE

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Summary:

Devil Facial Tumour Diseases (DFTD), threatening Tasmanian devils, consist of two distinct transmissible cancers, DFT1 and DFT2, with differing origins and geographic spread. We investigated the metabolic differences between DFT1 and DFT2, examining cell viability, metabolic outputs, and bulk gene expression. Using both DFT1 and DFT2 cell lines and biopsies, we found that glycolysis, oxidative phosphorylation, glutamate metabolism and fatty acid synthesis are all essential for the survival of both tumour types. However, DFT2 exhibited higher rates of glycolysis and lactate generation compared to DFT1. This coincided with elevated ATP production, cholesterol biosynthesis and ROS generation, as well as an increased reliance on fatty acid metabolism. Furthermore, DFT2 is less metabolically adaptable than DFT1, being unable to switch to oxidative phosphorylation as DFT1 can when required. These metabolic changes in DFT2 were associated with an increased expression of metastasis genes, and in conjunction with its higher growth rate, suggests a more aggressive cancer phenotype than DFT1. These findings provide a metabolic basis for developing new treatments that are applicable to both DFT1 and DFT2, as well as aid our understanding of the epidemiology and aetiology of DFTD to guide the development of conservation strategies.

Keywords Tasmanian devil, Transmissible Cancer, Metabolism, Transcriptomics, Pathway Enrichment, Glycolysis, Reactive Oxygen Species, Cholesterol Biosynthesis

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INTRODUCTION

In multicellular organisms, cell division is tightly regulated to maintain tissue integrity and function. Such regulatory mechanisms comprise, but are not limited to, cell cycle checkpoints[1], telomere length[2] and immunosurveillance[3]. When one or several of these safeguards are impaired, cells proliferate uncontrollably, which can lead to cancer. Traits tumour cells acquire on the path to becoming cancerous are known as the hallmarks of cancer[4,5]. One important hallmark is the deregulation of cellular metabolism, as tumour cells need to generate the necessary resources (nucleotides, building blocks for membranes, electron acceptors, etc.) to sustain high cell division rates. Cancer cells also evolve in a challenging environment where oxygen and nutrient availability varies over space and time with tumour size and vascularisation. Hence these cells must rewire their metabolism to produce enough precursors and energy, while also maintaining redox homeostasis by detoxifying excess reactive oxygen species (ROS) produced by the mitochondria[6].

The most common metabolic alteration found in cancer cells is increased glucose uptake and fermentation of glucose to lactate, also known as the "Warburg effect"[7]. Whilst normal cells completely oxidise glucose through the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC; i.e., oxidative phosphorylation or OXPHOS), cancer cells convert most glucose to lactate through aerobic glycolysis[7]. The Warburg effect allows cancer cells to produce energy in the form of adenosine triphosphate (ATP) along with reducing equivalents at a higher rate compared to OXPHOS. Moreover, aerobic glycolysis has been shown to: (i) promote tissue invasion and immune evasion, (ii) drive the biosynthesis of nucleotides, lipids, and proteins, and finally (iii) play a role in cell signalling through ROS and chromatin modulation[8]. Alternatively, glucose can enter the pentose phosphate pathway (PPP) and participate in nucleotide synthesis. The PPP is also a major source of nicotinamide adenine dinucleotide phosphate (NADPH), contributing to ROS detoxification[7].

Aside from glucose, other molecules fuel cancer metabolism with glutamine being the second most consumed nutrient by cancer cells[7]. Most glutamine is converted to glutamate which can then be further processed to amino acids, nucleotides, or α-ketoglutarate, respectively contributing to protein synthesis, nucleic acid production and fuelling the TCA cycle[7,9,10]. Furthermore, cancer cells harness lipid metabolism to obtain sufficient energy (ATP), building blocks for their membranes, and signalling molecules for proliferation, survival, invasion, and metastasis[11]. Precursors and products of the TCA cycle such as glucose, acetyl coenzyme A (acetyl-CoA) and citrate can be used to produce lipids including fatty acids and cholesterol[7,9]. Both enhanced lipid synthesis and lipid breakdown have been linked to cancer development[7].

These metabolic alterations occur not only during cancer development but also throughout the metastatic cascade (i.e., when cells detach from the extracellular matrix, travel through the bloodstream, and establish in a new tissue)[6]. An even more radical environmental change occurs in transmissible cancers where cancer cells are passed on from one individual to another as allo- or xeno-grafts[12–14]. Only two studies have investigated metabolic changes in

transmissible cancers, one in bivalve transmissible neoplasia (BTN)[15] that impacts a variety of shellfish species and the other in Devil Facial Tumour 1 (DFT1)[16] that is endangering Tasmanian devils[17,18]. The study on BTN used transcriptomics to identify key cancer drivers in bivalve neoplastic cells and found many metabolic pathways (including TCA and glycolysis) to be altered in BTN cells compared to healthy haemolymph cells[15]. The study on DFT1 used a more focused, assay-based approach and found that DFT1 depends on carbohydrate metabolism to proliferate[16]i. DFT1 has resulted in 60% of population loss[19] and resulted in the species being listed as endangered on the IUCN Red List.

Devil Facial Tumour Diseases (DFTD) are two transmissible facial cancers affecting the Tasmanian devil (*Sarcophilus harrisii*)[14]. The diseases are fatal and passed on through biting, which is frequent during social interactions[20]. DFTD consist of two distinct cancers that arose independently in separate founder animals: DFT1, estimated to have emerged in 1986 in Northeast Tasmania, and DFT2, estimated to have emerged in 2011 in the Southeast of the island[14,21,22]. Both cancers cause facial tumours that eventually lead to animals dying from starvation or organ failure due to metastatic disease within six to twelve months after the appearance of visible tumours[21,23,24]. Although both cancers cause similar clinical symptoms, a few notable differences between them exist that include (i) devil- and cell type-of-origin, (ii) geographical spread and (iii) *in vitro* growth dynamics. First, DFT1 originated from a myelinating Schwann cell of a female devil, while DFT2 originated from an immature or repair Schwann cell of a male devil[25,26]. Interestingly, repair Schwann cells show increased motility and proliferation compared to differentiated Schwann cells[27], potentially translating to more aggressive cancers prone to metastasise. Second, DFT1 has propagated to most of Tasmania while DFT2 has a restricted geographic range, only recently affecting devils outside of the peninsula where it originated to co-occur with DFT1[28]. This has led to three reported cases of co-infection with both DFT1 and DFT2 in devils, and competition between the two diseases for hosts has been hypothesised[29]. In a recent *in vitro* study, DFT1 cells were observed to grow slower in comparison to DFT2 cells, with DFT2 outcompeting DFT1 in co-cultures[30].

Recent work by Gérard et al.[30] hypothesised that differences in DFT1 and DFT2 growth dynamics could be explained by the distinct differentiation state of their Schwann cell-of-origin[26]. For instance, less differentiated repair Schwann cells dramatically increase glycolysis to assist in peripheral nerve repair[27]. This should translate into metabolic differences ultimately responsible for DFT1 and DFT2's diverging *in vitro* growth dynamics and competitiveness.

Here, we investigate similarities and differences between DFT1 and DFT2 metabolism by growing cell lines in the presence of metabolic inhibitors and measuring cell viability. We also measured outputs of major metabolic pathways such as cholesterol content, lactate, ATP, and ROS production. To link our functional assays to gene expression data, we generated full transcriptomes for DFT1 and DFT2 cell lines and reanalysed previously published transcriptomes from tumour biopsies taken from wild infected animals[26]. Lastly, we compared differential expression of metastasis-related genes in DFT1 and DFT2, given their

different cell type-of-origin. As previous work focused on finding a treatment against DFT1[16,31], this study could provide a basis for developing treatments applicable to both DFT1 and DFT2 and will enhance the understanding of transmissible cancer metabolism.

RESULTS

Many enriched pathways are shared between DFT cell lines and biopsies

In order to understand the metabolic differences between DFT1 and DFT2, we used bulk RNA-sequencing of DFT1 and DFT2 cell cultures and tumour biopsies. According to multidimensional scaling (MDS), tumour biopsies clustered into distinct groups, suggesting clear differences between DFT1 and DFT2 (Fig 1A). DFT1 and DFT2 cell lines also separated into two distinct clusters on the first dimension of the MDS plots (Fig. 1B). When combining both datasets, cell lines clustered separately from tumour biopsies on the first dimension, and DFT1 cell lines grouped away from DFT2 cell lines on the second dimension (Fig. 1C). Interestingly, the biopsies did not cluster according to DFT on the second dimension, indicating less variability between DFT1 and DFT2 biopsies than between DFT1 and DFT2 cell line gene expression profiles. Thus, DFT biopsies were more similar in gene expression than DFT cell lines. Biopsy tumour purity was comparable to cell line tumour purity (78.77% and 79.34% respectively; Table S1), indicating minimal contamination with healthy host tissue.

Differential gene expression analysis revealed nearly twice as many DEGs between DFT1 and DFT2 cell lines compared to biopsies (8,612 DEGs and 4,569 DEGs, respectively). Cell lines show 4,200 up-regulated and 4,412 down-regulated genes in DFT2 compared to DFT1, while biopsies show 2,536 up-regulated and 2,033 down-regulated genes. Overall, more genes were detected in the cell lines than in the biopsies (17,468 and 15,931). Heatmaps of the top 20 up- and down-regulated genes, ranked by log fold-change (logFC) show cell lines and biopsies clustering by DFT (Fig. 1D). This grouping is less striking for the DFT2 biopsies, where 13 of the top 20 upregulated genes are solely overexpressed in one of the two samples. The most upregulated gene in DFT2 cell lines was *PDGFRA*, a receptor for platelet derived growth factors, and the most downregulated one was *NTS*, which may be involved in the regulation of fat metabolism[32]. In DFT2 biopsies, these genes are *SMTNL1* and *UBE2M*, respectively (see Table S2 for a full list of gene symbols, names, and brief descriptions). However, these genes are solely differentially expressed in one of the two biopsy samples. Of the genes differentially expressed in both cell lines and biopsies, it was found that RFX4 and HOXD13, both of which encode transcription factors, and TNR, whose product is a neural extracellular matrix protein, were upregulated in both DFT2 cell lines and biopsies. Three genes, *CHRNA3*, *CLDN19* and *SSX2IP* were present within the top 20 downregulated genes in DFT2, both in cell lines and biopsies. CHRNA3 plays a role in neurotransmission, CLDN19 in tight junctions between cells and SSX2IP in eliminating ROS.

To understand pathway-level gene expression patterns, a multi-contrast enrichment analysis revealed 178 significantly enriched gene sets, i.e., sets with coordinated up or coordinated down regulation of genes (FDR < 0.05; Fig. 1E; Fig. S1). Most sets (153) showed concordant enrichment between cell line and biopsy data (top-right and bottom-left quadrants in Fig. 1E). From these, 145 were upregulated in both DFT2 cell lines and biopsies and 8 were downregulated. Conversely, 25 significantly enriched gene sets showed discordant

enrichment between cell lines and biopsies. Overall, although DFT cell lines diverge more in terms of gene expression than DFT biopsies, a vast majority of significantly enriched pathways are shared between both datasets.

Gene sets expected (or known) to be differentially expressed between DFT2 and DFT1 were concordantly upregulated in our analysis (Fig. S1): interferon gamma signalling, antigen presentation: folding, assembly and peptide loading of Major Histocompatibility Complex (MHC) class I, signalling by PDGF, nervous system development, axon guidance and somitogenesis[25,26,33,34].

Cholesterol biosynthesis and nucleotide metabolism show concordant enrichment in DFT2

Among the 178 enriched gene sets, a total of 14 pathways falls under the "metabolism" Reactome hierarchy (Fig. 2A). Two pathways showed concordant enrichment in DFT2 cell lines and biopsies: cholesterol biosynthesis ($s_{biopsies}$ = 0.16 and $s_{cell\ lines}$ = 0.47) and metabolism of nucleotides ($s_{biopsies}$ = 0.07 and $s_{cell\ lines}$ = 0.23). The cholesterol metabolism genes *SQLE*, *LSS* and *MVD* were upregulated in DFT2, in both datasets (Fig. 2B). Similarly, the *AK5*, *GMPR*, *TYMP*, *ENTPD4*, *NUDT18*, *IMPDH1*, *TXNRD1* and *GSR* genes, linked to the metabolism of nucleotides, were upregulated in DFT2 biopsies and cell lines. Within the same gene set, DFT2 cell lines and biopsies saw downregulated *AK9*.

Two metabolism related gene sets were concordantly enriched in DFT1 cell lines and biopsies: sulfur amino acid metabolism ($s_{biopsies}$ = -0.31 and $s_{cell\ lines}$ = -0.38) and degradation of cysteine and homocysteine (a subset of the sulfur amino acid metabolism gene set; $s_{biopsies}$ = -0.46 and $s_{cell\ lines}$ = -0.60; Fig. 2A). Sulfur amino acid genes, including *MTR*, *MPST*, *CTH*, and *ADO*, were downregulated in DFT2 cell lines and biopsies (Fig. 2D).

DFT2 cells have higher glycolysis rates than DFT1 cells

Cholesterol biosynthesis was observed to be enriched in DFT2 cells and biopsies. It is known from previous research that cholesterol homeostasis is important for DFT1 cell proliferation[16] and can stimulate a metabolic shift towards glycolysis. This prompted us to initially quantify the relative contribution of glycolysis in DFT1 and DFT2 cell proliferation. Lactate, a by-product of glycolysis, was firstly measured in the cell culture medium (Fig. 3A). DFT2 cells tended to produce more lactate than DFT1 cells, with two DFT2 cell lines (SN, TD549) showing significantly increased extracellular lactate production over time. SN produces 30.51 nmol of lactate, 95% CI [26.76, 34.59], whilst the highest lactate production by a DFT1 cell line was 2.7 nmol ($p < 0.01$; 95% CI [0.13, 11.85]). Remarkably, the DFT2 TD549 cell lines produced over a hundred times more lactate (174.83 nmol, 95% CI [139.51, 215.68]) compared to other cell lines. This higher lactate production by DFT2 cells indicates higher rates of glycolysis compared to the DFT1 cell lines.

We then tested how this difference in glycolysis rates would impact energy production in the form of ATP (Fig. 3B). To compare the amount of energy produced by glycolysis versus OXPHOS, we reported total, oxidative and glycolytic ATP production. Two DFT2 cell lines overall produced 1.5 times more ATP than DFT1 cells. RV cells produced 1.59 nmol of ATP (95% CI [1.27, 2.01]), SN cells 1.6 nmol (95% CI [1.28, 2]), with the highest ATP production in DFT1 cells of 0.98 nmol by 1426 ($p < 0.001$, 95% CI [0.73, 1.31]). Exposure of cells to 2-deoxyglucose [2DG, which is converted to 2-deoxyglucose-6-phosphate (2DG6P) and accumulates in the cell, ultimately inhibiting glycolysis[35]], significantly decreased oxidative ATP production between 0.33 to 0.65 nmol in all cell lines ($p < 0.001$) (except one DFT1 cell line, 1426). Inhibiting OXPHOS with oligomycin (glycolytic ATP production only) significantly decreased ATP production in two DFT1 cell lines (1426 by 0.43 nmol and C5065 by 0.36 nmol; $p < 0.001$), and one DFT2 cell line (TD549 by 0.32), suggesting that DFT1 relies more on OXPHOS for ATP generation than DFT2. Although glycolytic and oxidative ATP production did not differ significantly between the DFT2 SN and RV cell lines, both cell lines produced significantly higher amounts of ATP when cultured with 2DG and oligomycin than all other cell lines ($p < 0.05$; all cell lines except glycolytic ATP for 1426). Hence, higher rates of glycolysis in DFT2 cells resulted in higher ATP production, relative to DFT1.

To further investigate the importance of metabolic selection in DFT1 and DFT2, we measured cell viability in the presence of various substrates (Fig 3C). Both DFT1 and DFT2 showed a significant decrease in cell viability by at least 71.6 % ($p < 0.001$) when glycolysis was inhibited with 2DG, compared to growth in glucose alone. To test whether cells could switch to OXPHOS to obtain the ATP necessary for their survival, glucose was replaced by galactose. Galactose can enter glycolysis after being converted to glucose-6-phosphate through the Leloir pathway. However, this is less efficient and results in no net ATP gain[36]. Galactose significantly decreased viability in one DFT1 cell line (C5065 by 34.09 %; $p < 0.001$) and in two DFT2 cell lines (RV by 47.28 % and SN by 51.09 %; $p < 0.001$). Lastly, pyruvate was added to the 2DG and galactose treatments to assess whether this could restore cell viability by driving OXPHOS[37]. Pyruvate did not rescue viability in any cell line when added to 2DG. In contrast, when added to galactose, pyruvate significantly increased the survival of two DFT1 cell lines (4906 by 26.33 % and C5065 by 36.22 %; $p < 0.001$). Interestingly, the TD549 DFT2 cell line showed significantly higher survival compared to the other two DFT2 cell lines in most treatments (by at least 30.47 % in galactose, $p < 0.001$; 19.58 % in galactose and pyruvate, $p < 0.01$; and 22.8 % in 2DG, $p < 0.001$). Overall, it appears that DFT2 relies more on glycolysis than DFT1 for survival. Furthermore, DFT1 can switch to oxidative metabolism when required, but DFT2 cannot.

Linking the functional and transcriptomic data, we identified genes that could be responsible for DFT2's higher glycolytic rates. Although the glycolysis gene set was not enriched in DFT2 ($p > 0.05$), three glycolysis genes were upregulated in DFT2: *ENO3*, *PFKM* and *ADPGK* (Fig. 3D). One gene, *HKDC1*, was downregulated in DFT2 in both datasets, whereas *HK3* was upregulation in DFT2 biopsies but was downregulated in DFT2 cell lines. This

suggests that *ENO3*, *PFKM* and *ADPGK* are primarily driving the increase in glycolytic rates observed in DFT2 compared to DFT1.

DFT2 cells produce more ROS than DFT1 cells

We next investigated the effect of increased glycolysis and ATP production on ROS generation (Fig. 4A), as higher levels of glycolysis have been associated with elevated ROS production and oxidative stress[38]. Overall, ROS production tended to be higher in DFT2 cell lines, with RV producing the highest amount of ROS (70624 RFU, 95% CI [23282, 31874], $p < 0.001$), followed by SN (39949 RFU, 95% CI [32283, 47615]. These values were significantly higher than DFT1 lines 1426 (22679, 95% CI [18272, 27087], $p < 0.01$) and C5065 (12439 RFU, 95% CI [9479, 15400], $p < 0.01$). To identify the type of ROS being produced, we quantified mitochondrial superoxide ROS production (Fig. 4B), however, no significant difference in the production of mitochondrial ROS was observed between any cell line ($p > 0.05$). DFT2 thus produces more ROS than DFT1, with this increase being cytoplasmic (and not mitochondrial) in origin.

In line with the observed elevated ROS production, both DFT2 cell lines and biopsies show enrichment of ROS detoxification genes (Fig. 4C; Fig. S1). *SOD3*, *CYBA*, *GPX7*, *TXNRD1* and *GSR* are overexpressed in both DFT2 datasets. Interestingly, *NCF4* is upregulated in DFT2 biopsies but downregulated in DFT2 cell lines. This suggests that increased cytoplasmic ROS production by DFT2 is somewhat mitigated by the upregulation of ROS detoxification genes to avoid ROS-induced cell death.

DFT2 relies more on lipid metabolism than DFT1

Lipid metabolism is essential in rapidly dividing cells such as cancer cells[39], which led us to test the role of fatty acid metabolism in DFT1 and DFT2 proliferation. We used etomoxir, an irreversible carnitine palmitoyl-transferase 1A inhibitor (CPT1A), to inhibit fatty acid oxidation[40] (Fig. 5A). Etomoxir acts by causing lipid accumulation and decreased ATP and NADPH production, ultimately stopping cell proliferation[41]. Etomoxir significantly decreased the viability of two DFT2 cell lines (RV by 35.34 % and SN by 38.17 %; $p < 0.001$), but not in TD549 (by 1.58 %; $p > 0.05$). DFT1 cell lines had no change in survival when cultured in etomoxir in comparison to glucose only controls.

To investigate which aspect of fatty acid metabolism (anabolism or catabolism) DFTs rely on, cells were further treated with pioglitazone, an ACSL4 inhibitor blocking fatty acid synthesis[40] (Fig. 5B). When treated with this compound, all cell lines showed decreased viability compared to untreated cells ($p < 0.001$). Pioglitazone had a greater antiproliferative effect on DFT2 cell lines (by 89.29 %), compared to DFT1 (by 35.33 %; $p < 0.001$). We hence conclude that DFT2 relies more on both fatty acid oxidation and fatty acid synthesis than DFT1.

Linking these results to the transcriptomics data, we found that the following fatty acid metabolism genes were concordantly upregulated in DFT2 cell lines and biopsies: *THRSP*, *PTGIS*, *PTGS2*, *TBXAS1*, *FADS2*, *SCD5*, *MAPKAPK2* and *ACSL3* (Fig. 5C). The *ACSL1*, *PRKAG2*, *PRXL2B*, *ELOVL4*, *FAAH*, *ELOVL2* and *ELOVL3* genes were downregulated in DFT2. It is important to note however that the total fatty acid metabolism gene set is not significantly enriched in DFT2 ($p > 0.05$).

Based on our previous observation that cholesterol biosynthesis genes are enriched in DFT2, we measured cholesterol content in DFT1 and DFT2 cells (Fig. 5D). DFT2 cells contained 3 to 4 times more cholesterol than DFT1 cells ($p < 0.05$), consistent with DFT2 upregulating genes linked to cholesterol biosynthesis (Fig. 2A & B).

Both DFT1 and DFT2 rely on glutamate production and oxidative metabolism for survival

DFT1 and DFT2's reliance on glutamine, an essential substrate for cancer cell metabolism[42], was tested by incubating cells in glutamine depleted medium (Fig. 5E). This significantly decreased the viability of only one DFT1 cell line (4906 by 20.5 %; $p < 0.01$) and significantly increased the viability of another DFT1 cell line (1426 by 19.03 %; $p < 0.001$). DFT2 cell lines were not impacted by the absence of glutamine in their culture medium. The DFT1 and DFT2 cells were then grown with BPTES, an inhibitor preventing glutaminase-1 from converting glutamine to glutamate, and this treatment significantly decreased the viability of all cell lines, DFT1 and DFT2 alike (by 72.99 % on average; $p < 0.001$). This result suggests that DFT1 and DFT2 can generate endogenous glutamine, but both rely on the conversion of this glutamine to glutamate for survival.

We also investigated the importance of the pentose phosphate pathway (PPP) on DFT cell viability using oxythiamine[43] (Fig. 5A). This molecule prevents thiamine from transitioning to its active form, ultimately inhibiting transketolase, an essential enzyme in the PPP[44]. Oxythiamine only significantly decreased viability in one DFT2 cell line (RV by 32.66 %; $p < 0.001$), indicating that the PPP is unlikely to be vital for DFT cells.

Lastly, DFT1 and DFT2 cells were grown with oligomycin, an ATP synthase inhibitor. Inhibiting oxidative ATP generation significantly decreased cell viability for all six cell lines, compared to untreated controls (Fig 5B; by 42.5% on average, $p < 0.001$). This demonstrates that oxidative phosphorylation, and the ATP generated by this pathway, remains important for DFT cell survival.

Metastasis genes are upregulated in DFT2

We hypothesise that faster *in vitro* DFT2 growth, driven by the metabolic differences we observed, could lead to a more aggressive cancer phenotype. We thus included two custom gene sets to our multi-contrast enrichment analysis: one focusing on genes involved in metastasis and a second with genes involved in cell competition. Whilst the cell competition

gene set was not enriched in DFT2 compared to DFT1, the metastasis gene set did show significant concordant enrichment in DFT2 ($s_{biopsies}$ = 0.23 and $s_{cell\ lines}$ = 0.24; Fig S1). *PTGS2*, *DLC1*, *MTSS1*, *CASP8*, *CCN2*, *GAS1* and *DRG1* are upregulated in DFT2 cell lines and biopsies, although *PTGS2* and *MMP1* are solely overexpressed in one of the two DFT2 biopsies (Fig. 6). Lastly, *NECTIN3* is concordantly downregulated in DFT2 cell lines and biopsies.

We also note that pathways linked to collagen and the extra-cellular matrix (ECM) organisation, which could be important for tissue invasion and metastasis, show concordant upregulation in DFT2 cell lines and biopsies compared to DFT1: crosslinking of collagen fibrils, collagen degradation, collagen chain trimerization, collagen biosynthesis and modifying enzymes, assembly of collagen fibrils and other multimeric structures, collagen formation, and extracellular matrix organization (Fig. S1).

DISCUSSION

This work demonstrates that, *in vitro*, similar metabolic pathways drive DFT1 and DFT2 proliferation, with glycolysis, OXPHOS, glutamine metabolism and fatty acid synthesis being essential for their survival. However, the two transmissible tumours differ in the extent to which they rely on glycolysis. DFT2 has a higher rate of glycolysis relative to DFT1, which is accompanied by higher lactate generation and ATP production. This is consistent with DFT2's hypothesised cell-type of origin (i.e., a repair Schwann cell, whilst DFT1 originated from a myelinating Schwann cell[26,45]). During nerve injury, repair Schwann cells increase glycolysis in comparison to myelinating Schwann cells to support axons[27]. Our transcriptomic analysis revealed that DFT2 could achieve this by upregulating *ADPGK*, *PFKM*, and *ENO3*. ADPGK catalyses phosphorylation of glucose to glucose-6-phosphate using ADP as a phosphate donor. This initial step of glycolysis is typically catalysed by ATP-dependent hexokinases. Therefore, it is believed that ADPGK could assist in conserving ATP during hypoxia, thereby reducing the ATP consumption necessary for glucose phosphorylation[46]. Next, PFKM catalyses the phosphorylation of D-fructose 6-phosphate to fructose-1,6-bisphosphate using ATP, which is the first committed step of glycolysis. Finally, ENO3 is responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Because fructose-1,6-bisphosphate production has previously been identified as one of the key flux-controlling steps of glycolysis[47], we expect *ADPGK* and *PFKM* upregulation to play a major role in DFT2's higher rate of glycolysis in comparison to DFT1. Interestingly, we also observed upregulation of hexokinase 3 (*HK3*) in DFT2 biopsies, which further suggests this could be a mechanism underlying higher rates of glycolysis. Future work should investigate the effects of knock-down of these genes on DFT cell survival and glycolysis output to confirm their role in DFT2's higher glycolytic rates. Enzyme activity assays would also confirm whether increased RNA expression can be linked to increased protein steady-state levels.

This is the first study directly comparing DFT1 and DFT2 metabolism using tumour cell lines and biopsies. At the time of this work, DFT2 transcriptomes were scarce with only a single study conducting full RNA sequencing of one DFT1 and DFT2 cell line and two DFT1 and DFT2 biopsies simultaneously[26]. By conducting full transcriptome analyses of two additional DFT1 and DFT2 cell lines, this study provides valuable data for future *in vitro* work on DFTD. Overall, more genes were differentially expressed in the cell lines than in biopsies, likely due to differences in sequencing depth between the transcriptomes. In the future, re-sequencing both cell lines and biopsies on the same platform would offer a more robust approach, as this difference could have led to an underestimation of the number of differentially expressed genes in the biopsy data. Nevertheless, by integrating *in vitro* metabolic assays with the analysis of cell line and biopsy transcriptomes, we minimize the risk of identifying metabolic differences associated with adaptation to cell culture conditions. Consequently, we can confidently identify metabolic pathways crucial for these transmissible tumours.

Previous research[16] had demonstrated the importance of glycolysis for DFT1 proliferation. We confirm their findings by showing the reliance of both DFT1 and DFT2 on glycolysis for survival. Ikonomopoulou and colleagues[16] further linked cholesterol homeostasis to this metabolic shift towards glycolysis in DFT1. We observe a similar pattern in DFT2. Cholesterol biosynthesis genes are enriched in DFT2 cell lines and biopsies, which is consistent with DFT2 containing threefold more cholesterol than DFT1 and its higher rate of glycolysis. Indeed, the acidic environment caused by elevated glycolysis and ATP hydrolysis has been shown to increase cholesterol biosynthesis in human malignancies, allowing cancer cells to sustain proliferation, migrate and invade tissues[48].

Another finding of our study is that DFT2 produces more cytoplasmic ROS than DFT1. Current research[38] shows that increased glucose conversion to pyruvate can elevate cellular ROS levels. We thus hypothesise that higher glycolysis rates driven by *ADPGK* and *PFKM* upregulation could be responsible for both increased ROS production and upregulation of ROS detoxification genes in DFT2. Furthermore, we observed concordant enrichment of genes belonging to the degradation of cysteine and homocysteine pathway in DFT1 cell lines and biopsies. Degradation of these amino acids through the transulphuration pathway leads to the generation of glutathione (GSH) and taurine, both sustaining redox chemistry[49]. This could explain lower ROS production by DFT1 compared to DFT2 cell lines, although this needs to be verified by quantifying GSH production.

Within the DFT2 cell lines tested in this study we mostly observed comparable effects to the inhibitors used, although TD549's response tended to differ from the other two cell lines (RV and SN). As such, TD549 produced less ATP and ROS, more lactate, and had an overall better survival in the presence of inhibitors than the other DFT2 cell lines. We hypothesise that rearrangements on chromosome 2 distinguishing TD549 from RV and SN could explain such differences[50]. TD549 notably lacks one copy of the gene *FSHR*[50], the product of which is responsible for downstream PI3K-AKT signalling[51], a pathway implicated in altered carbohydrate metabolism in DFT1[16]. Our findings are somewhat limited due to the use of *in vitro* conditions that do not accurately reflect the genetic and vascular heterogeneity of an *in vivo* tumour[52]. For instance, replicating this work on the already existing DFT1 xenograft mouse model could address those caveats[53]. However, to the best of our knowledge, a DFT2 xenograft model remains to be developed. In addition, one might not capture the full extent of the diversity of tumours circulating currently in Tasmania, a problem which could be addressed by deriving more recent cell lines from primary tumours.

Increased glycolysis rates in cancer cells play an important role in immune evasion by limiting the availability of glucose to tumour-infiltrating lymphocytes[8]. DFT1 is thought to evade the immune system by downregulating MHC molecules, which are still expressed by DFT2[34,54]. Furthermore, certain DFT2 lineages carry a Y chromosome (as DFT2 originated from a male devil) and, likely because of its immunogenicity, appear to be less successful at infecting female devils[22,55]. Therefore, we hypothesise that increased lactate production in DFT2, because of increased glycolysis rates, might play a role in evading the devil's immune

system. Indeed, previous research reported that few tumour-infiltrating lymphocytes were observed within DFT2 biopsies[34].

ROS are a source of oxidative DNA damage and result in higher DNA mutation rates. We observed higher ROS generation in DFT2 cell lines compared to DFT1, and this concurs with the higher mutation rates observed in DFT2 tumours compared to DFT1 tumours[22]. High mutation rates in clonally reproducing organisms should lead to the accumulation of deleterious mutations, and eventually genomic decay[56,57]. However, the three types of transmissible cancers known so far all share mutation rates exceeding those of human cancers, and it is proposed that such a high mutation load could carry indirect positive fitness outcomes[58].

DFT2's higher rate of glycolysis explains its previously reported faster *in vitro* growth and could explain how it is able to outcompete DFT1 in co-culture assays[30]. Higher rates of cell division require increased deoxyribonucleotide triphosphate (dNTP) synthesis, which are not only rate-limiting for DNA replication and repair, but also for transcription, ribosome biogenesis and post-translational protein glycosylation[59]. In DFT2 cells, nucleotide metabolism and catabolism are also enriched, which would support these metabolic requirements for faster cell division[30].

Considering these observations, we hypothesise that DFT2 could be a more aggressive cancer than DFT1. Meta-analyses of human cancer patients have associated elevated aerobic glycolysis with poorer prognosis[60,61]. For instance, elevated expression of glycolysis-related genes was associated with aggressive phenotypes such as poor tumour differentiation, deeper invasion, lymph node metastasis and vascular invasion in high-grade brain tumours[61]. A second meta-analysis[60] demonstrated ultimately worse overall survival for hepatocellular carcinoma patients with elevated expression of glycolysis markers. Based on these human studies, it can be predicted that DFT2 tumours could have a higher potential to invade tissues and metastasise. This is in agreement with the overexpression of metastasis-related genes by DFT2 relative to DFT1, and it will be interesting to compare DFT1 and DFT2 tumour growth and metastasis rates in wild devils when data becomes available.

The fact that DFT1 and DFT2 appear to share some common pathways for their growth and survival is promising for future devil treatment. Atorvastatin, which has proven to be effective in reducing DFT1 tumour growth[16], is likely to also inhibit DFT2 tumour growth, underscoring its potential to assist in ongoing management plans for the conservation of Tasmanian devils. However, DFT2's increased glycolytic rate and potential metabolic plasticity could represent an important threat to Tasmanian devils if these metabolic features translate *in vivo*. If so, DFT2 is likely to have a faster growth rate, enhanced immune evasion and higher adaptive potential than DFT1. It is important to note that DFT2 has been progressing on a different epidemiological landscape than DFT1. DFT2 arose in a smaller, more disperse population that had already been exposed to DFT1 for almost 30 years[-]. However, DFT2's evolutionary trajectory remains unknown. Our results, taken together with DFT2's expansion outside of the peninsula where it originated, highlight the necessity to

further research the differences and similarities of these unique transmissible cancers and their endangered host.

RESOURCE AVAILABILITY

Lead Contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Anne-Lise Gérard (anne-lise.gerard@evobio.eu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

All data and code are publicly available as of the date of publication. Metabolic assay data have been deposited at [data-type-specific repository] as [Database: accession number]. All original code has been deposited on GitHub and is available at https://github.com/Anne-LiseGerard as of the date of publication. RNA-seq data have been deposited at NCBI's Sequence Read Archive (SRA) and are available under PRJNA1122388. This paper also analyzes existing RNA sequences, available under PRJEB28680 on the SRA.

ACKNOWLEDGEMENTS

This work was supported by an ARC Linkage (LP170101105), ARC Decra (DE170101116), ARC DP (DP230100162), an ANR TRANSCAN (ANR--18--CE35--0009), the Save The Tasmanian Devil Eric Guiler Research Funds, Deakin University's LES Blue Sky and SEBE Industry partnership funds, a CNRS International Research Project, a Morris Animal Foundation grant (D19ZO--413) and the Hoffmann Family. This research was supported by use of the Nectar Research Cloud, a collaborative Australian research platform supported by the NCRIS-funded Australian Research Data Commons (ARDC). The authors gratefully acknowledge the contribution to this work of the Victorian Operational Infrastructure Support Program received by the Burnet Institute.

AUTHOR CONTRIBUTIONS

Conceptualization: A.-L.G., F.P., A.G.S., M.M., M.Z., B.U. Data Curation: A.-L.G., F.P., C.V. Formal Analysis: A.-L.G., F.P., C.V., A.M.D. Funding acquisition: A.-L.G., R.H., F.T., B.U. Investigation: A.-L.G., F.P., C.V. Methodology: A.-L.G., F.P., M.M., M.Z. Project administration: A.-L.G., U.B. Resources: A.-L.G., F.P., C.V., A.G.S., H.S., M.M., M.Z. Software: A.-L.G., F.P., C.V., M.Z. Supervision: A.-L.G., F.T., B.U. Validation: A.-L.G., F.P., C.V. Writing – Original draft: A.-L.G. Writing – Reviewing & Editing. A.-L.G., F.P., C.V., A.M.D., A.G.S., R.H., H.S., F.T., M.M., M.Z., B.U.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used Apple Intelligence to improve the readability and language of some sentences in the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

FIGURE LEGENDS

Figure 1 *In vitro* DFT1 and DFT2 transcriptomes differ more than *in vivo* ones. **A** Multi-dimensional scaling (MDS) plot of biopsy RNA sequences (DFT1 in blue triangles and DFT2 in pink triangles). **B** MDS plot of cell line RNA sequences (DFT1 in blue circles and DFT2 in pink circles). **C** MDS plot of both cell line and biopsy RNA sequences. **D** Top 20 genes up- and top 20 genes down-regulated in DFT2, for biopsies and cell lines.. **E** Multi-contrast enrichment analysis results. Each dot represents one gene set and is plotted according to its enrichment score (s) within the cell line dataset and the biopsy dataset. Significantly enriched (FDR < 0.05) gene sets are shown in red.

Figure 2 Cholesterol biosynthesis and nucleotide metabolism show concordant enrichment in DFT2. **A** Multi-contrast pathway analysis, metabolism-related gene sets. Heatmaps of DEGs for **B** cholesterol biosynthesis genes, **C** nucleotide metabolism genes, **D** sulfur amino acid genes, and **E** degradation of cysteine and homocysteine genes. Only significant DEGs are represented (FDR < 0.05).

Figure 3 DFT2 cells show higher glycolysis rates than DFT1 cells. **A** Lactate quantity measured in cell culture supernatant, best fit model: lactate ~ cell line + day. **B** Total (glucose), oxidative (2DG) and glycolytic (oligomycin) ATP production. Best fit model: ATP ~ treatment * cell line + (1 | experiment). **C** Percent viability of DFT1 (blue) and DFT2 (red) cell lines grown in treatments targeting glucose metabolism relative to glucose control medium. Best fit model: % viability ~ treatment * cell line + (1 | experiment). Mean and 95 % confidence intervals shown in colour and data points in grey. Compact letter display represents post hoc contrasts for which p < 0.05. **D** Heatmaps of DEGs for glycolysis genes. Only significant DEGs are represented (FDR < 0.05).

Figure 4 DFT2 cells produce more ROS than DFT1 cells. **A** Overall ROS and **B** mitochondrial ROS production in relative fluorescence units (RFU) of DFT1 and DFT2 cell lines after 1 hour. Best fit model: ROS ~ cell line. **C** Heatmaps of DEGs for ROS detoxification genes. Only significant DEGs are represented (FDR < 0.05).

Figure 5 DFT2 relies more on lipid metabolism than DFT1, and other pathways of interest. Percent viability of DFT1 (blue) and DFT2 (red) cell lines grown in treatments targeting **A** fatty acid oxidation and the PPP, **B** fatty acid oxidation and OXPHOS, and **D** glutamine metabolism. Best fit models: % viability ~ treatment * cell line. **C** Cholesterol content of lipids extracted from 10^6 cells, best fit model: cholesterol ~ DFT. Mean and 95 % confidence intervals shown in colour and data points in grey. Compact letter display represents post hoc contrasts for which p < 0.05. **E** Heatmaps of DEGs for fatty acid metabolism. Only significant DEGs are represented (FDR < 0.05).

Figure 6 Metastasis genes are upregulated in DFT2. Heatmaps of DEGs for metastasis genes. Only significant DEGs are represented (FDR < 0.05).

STAR METHODS

EXPERIMENTAL MODEL

Cell Lines and Cell Culture

Devil Facial Tumour 1 (DFT1) cell lines 4906, 1426 and C5065 and DFT2 cell lines RV, SN and TD549 were grown *in vitro* as previously described[21,30,62]. DFT cell lines were derived from different animals and act as biological replicates. Cells were cultured in RPMI-1640 media (Gibco, 21870092; 2 g/L of glucose), supplemented with 1 % GlutaMAX (Gibco, 35050061), 10 % FBS (Gibco, 10099141) and 1 % 10,000 U/mL penicillin/streptomycin (Gibco, 15070063) at 35 °C and 5 % CO2. Upon reaching 80-90 % confluency, cells were washed with DPBS (Gibco, 14190250) detached using TrypLE Express (Gibco, 12605010), centrifuged at 500 x g for 5 minutes and passaged 1:3. Cells used in the present work were maintained below passage 30 and were tested for mycoplasma as described in Gérard et al.[30]. Available information on the cell lines used in the present study can be found in the table below.

Cell line	DFT	Sex of host	Date collected	Location (Tasmania)
4906	DFT1	Male	17/01/2007	Coles Bay
1426	DFT1	Female	10/10/2005	Fentonbury
C5065	DFT1	Unknown	23/07/2007	Bangor
RV	DFT2	Male	26/03/2014	Cygnet
SN	DFT2	Male	12/10/2014	Lower Snug
TD549	DFT2	Male	16/12/2015	Woodbridge

METHOD DETAILS

Sample Processing and RNA Sequencing

Sample Collection

All DFT cell lines were grown in 6-well plates (Corning, CLS3516), until 80-90 % confluency. Cells were then washed with DPBS (Gibco,14190250) and one well was harvested per cell line using 1 mL of TRI Reagent® (Sigma, T9624) per well. Samples were then stored at -80 °C until further processing.

RNA extraction

RNA was extracted from all samples using the Zymo Direct-zol™ RNA kit (Zymo Research, R2051) as per manufacturer instructions. Quantification and qualification of extracted RNA was performed on a Qubit RNA High Sensitivity (Invitrogen) and on a Tapestation 4200-RNA Screentape (Agilent). All samples had RNA integrity scores (RIN) scores greater than 9.5.

Library Preparation and Sequencing

Library preparation was performed using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs, E7760) with 400 ng of each sample, 5X Adapter dilution and a 10 cycle PCR. Finally, technical triplicates from all samples were sequenced at the Charles Rivers Laboratories on a NovaSeq6000 (Illumina) using 300 cycles and 150 paired end reads.

Previously published data

RNA sequences from two DFT1 and two DFT2 primary tumours published in Patchett et al.[26] were re-analysed to compare *in vitro* samples to *in vivo* ones, in order to determine if laboratory cell culture conditions substantially altered DFT cell metabolism. Briefly, these data consist of two biological replicates per DFT, sequenced in duplicate (i.e., technical replicates) using 100 base-pair single end read mRNA sequencing on the Hiseq-2500 platform (Illumina). FASTQ files were downloaded from the European Nucleotide Archive (project PRJEB28680).

Metabolic assays

Cell viability after metabolic inhibition

Three independent experiments were performed, each comprised of 6 technical replicates per cell line and per culture condition. Cell viability was approximated by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Cell Proliferation Kit I, Roche, 11465007001) following instructions from the manufacturer. For all experiments, 10,000 cells/well were seeded in 96-well cell culture plates (Corning, CLS3997) and were incubated for 24 h to allow cell adhesion. Next, culture medium was changed on all wells with either fresh culture medium (glucose controls) or culture medium spiked with a metabolic inhibitor (treatments; see table below). Here, the term “cell viability” is used loosely as MTT reduction can be affected by several factors, including metabolic and energy perturbations, and hence cannot be used reliably to discriminate between cell cycle arrest and cell death[63,64]. Concentration of inhibitors was determined based on common concentrations used in the literature[65–70]. Cells were incubated for an additional 72 h before MTT reduction was measured at 550 nm in a FLUOstar Omega plate-reader (BMG LABTECH) and the Omega software (version 3.00 R2). Controls were used to define 100 % viability and wells containing only media were used to blank the readings. Results are reported in % viability. Three independent experiments were performed, each comprised of 6 technical replicates per cell line and per culture condition.

Treatment	Composition
2DG	2-deoxy-D-glucose 1 mM (Sigma, D8375)
2DG + pyruvate	2-deoxy-D-glucose 1 mM + pyruvate 5 mM (Sigma, P2256)
BPTES	BPTES 5 µM (Sigma, SML0601)
DON	DON 25 µM (Sigma, D2141)

Etomoxir	etomoxir 0.01 mM (Sigma, E1905)
Galactose	RPMI, no glucose (Gibco, 11879020) + galactose 11 mM (Sigma, G5388)
Galactose + pyruvate	RPMI, no glucose + galactose 11 mM + pyruvate 5 mM
Glucose + pyruvate	pyruvate 5 mM
Inhibitor 968	inhibitor 968 25 µM (Sigma, SML1327)
No glucose	RPMI, no glucose
No glutamine	no GlutaMAX
Oxythiamine	oxythiamine 1 mM (Sigma, 0400)
Pioglitazone	pioglitazone hydrochloride 30 µM (Merck, PHR1632)
Oligomycin	oligomycin A 5 µM (Merck, 75351)

Measurement of ATP production

Cells were left to adhere to the culture plates for 24 h. Culture medium was then replaced with fresh culture medium for the controls and culture medium with 1 mM 2DG (Sigma, D8375; to measure oxidative ATP production) or 5 µM oligomycin (Sigma, 75351; to measure glycolytic ATP production), as previously described[71]. The plate was then incubated for 3 h. ATP was quantified using the ATPlite 1step kit (PerkinElmer, 6016731) according to instructions from the manufacturer. Luminescence of samples along with ATP standards was measured in a FLUOstar Omega plate-reader (BMG Labtech). Wells containing media only were used to blank the readings. Three independent experiments were performed, each comprised of 6 replicates per cell line and per culture condition.

Measurement of ROS production

Overall ROS production was measured using H2DCFDA (Sigma, D6883). Plates were incubated for 24 h to let cells adhere. Culture medium was removed, and cells were washed with DPBS. Cells were subsequently incubated with 10 µM H2DCFDA for 30 min in the dark at 35°C. Wells were washed with DPBS and incubated for 20 min with 100 µL of DPBS. Fluorescence at 480 nm excitation and 520 nm emission, was read on a FLUOstar Omega plate-reader for 1 h. Wells containing only media were used to blank the readings. Three independent experiments were performed, each comprised of 6 replicates per cell line.

Mitochondrial ROS production was quantified using MitoSOX™ Green (Thermo Fisher Scientific, M36006). Cell lines were seeded on black clear bottom 96-well plates (Interpath, 655090). Cells were left to adhere for 48 h after which culture medium was removed, and cells were washed with 100 µL DPBS. To each well, 100 µL MitoSOX™ Green 1 µM working solution was added, and the plate was incubated at 35°C and 5% CO_2 for 30 minutes. MitoSOX™ Green was removed, cells were washed with 100 µL DPBS, and plates were centrifuged at 400 x g for five minutes. A final 100 µL DPBS was added to each well, and plates were incubated one hour in the dark, prior to reading fluorescence intensity at 485 nm excitation/520 nm emission with a FLUOstar Omega plate-reader. Three independent experiments were performed with six replicates per cell line.

Measurement of extracellular lactate

Cells were plated and incubated as described above. Cell culture supernatants were harvested after 2, 6 and 8 days, deproteinised using 10 kDa MWCO spin filters (Merck, UFC5010), and stored at -80 °C until further analysis. After each collection, cell number per well was measured by MTT. Cell culture supernatants were thawed, and lactate was measured using a Lactate Assay Kit (Sigma, MAK064) according to the manufacturer's protocol. Absorbance was measured at 570 nm on a FLUOstar Omega plate-reader. Wells containing only media were used to blank the readings. Two independent experiments were performed, each comprised of duplicates for each cell line. Lactate content was standardised by cell number and calculated for 10^6 cells.

Measurement of cholesterol content

One million cells were pelleted and stored at -80 °C until further processing. Samples were thawed and were resuspended in 200 µL of a 7:11:0.1 chloroform:isopropanol:IGEPAL mix (Merck, 1.02445; Chem Supply, AH323-4; Merck, CA-630) to extract lipids. Samples were centrifuged for 10 min at 13,000 x g to remove insoluble material. The organic phase was transferred to a new tube and left to air dry at 50 °C for 1 h. Then samples were dehydrated under a vacuum for 30 min at 50 °C using a SpeedVac (Savant). Dried lipids were resuspended in 200 µL of Cholesterol Assay Buffer from the Cholesterol Quantitation Kit (Sigma, MAK043). Manufacturer's directions were followed to measure cholesterol content. Absorbance was recorded at 570 nm on a FLUOstar Omega plate-reader. Three samples per cell line, each from independent cell subcultures, were used for this experiment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequence analysis

Sequence reads were filtered (3' end quality trimming with a quality threshold of 10) and trimmed using skewer[72] (v0.2.2) and pseudo-aligned to the Tasmanian devil reference genome (mSarHar 1.11) using kallisto[73] (v0.50.0), both using Bash[74] scripts. Technical replicates were combined and genes with a mean count of less than 10 across all samples were removed to exclude genes with extremely low expression. Human homologs of Tasmanian devil genes were obtained from Ensembl (release 109) using BioMart[75]. Differential gene expression analysis between DFT1 and DFT2 cell lines, and between DFT1 and DFT2 tumour biopsies was performed using DESeq2[76] (v1.42.0). Differential expression p-values underwent false discovery rate (FDR) adjustment to account for multiple testing[77]. An adjusted p-value (FDR) < 0.05 was considered significant. To identify groups of genes (pathways) that are statistically over-expressed, a multi-contrast pathway enrichment analysis was performed using mitch[78] (v1.14.0) and Reactome[79] gene sets (V85). This allowed to directly compare differential gene expression between DFT1 and DFT2 cell lines to DFT1 and DFT2 biopsies. To the Reactome gene sets, we added two custom gene sets curated from the literature to investigate genes involved in metastasis and in cell competition (Table S3). We also interrogated significant differentially expressed genes (DEGs) belonging to Reactome pathways investigated in our

metabolic assays. Differential gene expression and pathway enrichment analyses were performed in R[80] (v4.3.3). For pathway enrichment analysis, an adjusted p-value (FDR) < 0.05 was considered significant.

Tumour purity calculation

To ensure tumour biopsies were not contaminated by host tissue, tumour purity was calculated and compared to that of the cell lines (which are clonal, hence not contaminated by host tissue). Tumour biopsy and cell line sequences were trimmed using skewer[72] then aligned to the mSarHar1.11 genome using the STAR aligner[81]. Files were converted from .sam to .bam, sorted by genomic location and indexed using SAMtools[82]. Mean coverage was then calculated over arbitrary 1kb non overlapping windows over the entire genome and over two large deletions proper to DFT1 and DFT2, using the BEDtools[83] coverage function. The DFT1 specific deletion is found at 3:192,161,000-216,866,000 (as per Stammnitz et al.[84]) and the DFT2 specific deletion at 5:141461000-175266000 (chosen from Stammnitz et al.[33]). Tumour purity was then calculated as (coverage deletion / overall coverage) x 100. Tumour purity calculations were performed using Bash[74] scripts and are available in Table S1.

Mixed effects models

All statistical analyses were performed in R[80] (version 4.3.0) using Gaussian mixed effects linear models. Response variables were either cell viability or metabolite production. To choose appropriate explanatory variables (cell lines, DFT), models were built following the Zuur protocol[85] (Table S4). Briefly, models with or without experimental replicate as a random intercept were built to account for possible batch effects between experiments. The most parsimonious fixed effects model was then selected. Models with the lowest Akaike's Information Criterion (AIC) and highest AIC weights were selected. Model assumptions (i.e., homogeneity of variances and normality of residuals) were verified using diagnostic plots. Model selection showed all experiments required cell lines to be considered separately and could not be pooled by DFT due to variability in the results among cell lines of the same DFT. Model means and 95 % confidence intervals are reported in Table S5. Contrasts were performed using Tukey's post hoc test, with Bonferroni correction for multiple testing (Table S5).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TRI Reagent®	Sigma	Cat#T9624
2-deoxy-D-glucose	Sigma	Cat#D8375
pyruvate	Sigma	Cat#P2256
BPTES	Sigma	Cat#SML0601
DON	Sigma	Cat#D2141

etomoxir	Sigma	Cat#E1905
galactose	Sigma	Cat#G5388
inhibitor 968	Sigma	Cat#SML1327
oxythiamine	Sigma	Cat#0400
pioglitazone hydrochloride	Merck	Cat#PHR1632
oligomycin A	Merck	Cat#75351
H2DCFDA	Sigma	Cat#D6883
MitoSOX™ Green	Thermo Fisher Scientific	Cat#M36006
chloroform isopropanol	Merck Chem Supply	Cat#1.02445 Cat#AH323-4
IGEPAL	Merck	Cat#CA-630
Critical commercial assays		
Zymo Direct-zol™ RNA kit	Zymo Research	Cat#R2051
NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina	New England Biolabs	Cat#E7760
Cell Proliferation Kit I	Roche	Cat#11465007001
ATPlite 1step kit	PerkinElmer	Cat#6016731
Lactate Assay Kit	Sigma	Cat#MAK064
Cholesterol Quantitation Kit	Sigma	Cat#MAK043
Deposited data		
DFT1 and DFT2 primary tumours RNA sequences	Patchett et al.[26]	http://www.ebi.ac.uk/ena/data/view/PRJEB28680
DFT1 and DFT2 cell line RNA sequences	This paper	https://dataview.ncbi.nlm.nih.gov/object/PRJNA1122388?reviewer=r0gk5jji5gp2u8bffsu0aevt9r
Tasmanian devil reference genome mSarHar1.11	Stammnitz et al.[22]	https://ftp.ensembl.org/pub/release-109/fasta/sarcophilus_harrisii/
Reactome	Gillespie et al.[79]	https://reactome.org
Experimental models: Cell lines		
4906 (DFT1)	Pyecroft et al.[62]	N/A
C5065 (DFT1)	Pyecroft et al.[62]	N/A
1426 (DFT1)	Pyecroft et al.[62]	N/A
RV (DFT2)	Pye et al.[21]	N/A
SN (DFT2)	Pye et al.[21]	N/A
TD549 (DFT2)	Pye et al.[21]	N/A
Software and algorithms		
skewer	Jiang et al.[72]	https://github.com/relipmoc/skewer
kallisto Bash	Bray et al.[73] GNU[74]	https://github.com/pachterlab/kallisto https://www.gnu.org/software/bash/
BioMart	Smedley et al.[75]	https://asia.ensembl.org/info/data/biomart/index.html

DESeq2	Love et al.[76]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
mitch	Kaspi and Ziemann[78]	https://www.bioconductor.org/packages/release/bioc/html/mitch.html
R	R Core Team[80]	https://www.r-project.org
STAR	Dobin et al.[81]	https://github.com/alexdobin/STAR
SAMtools	Li et al.[82]	http://www.htslib.org
BEDtools	Quinlan and Hall[83]	https://bedtools.readthedocs.io/en/latest/
Other		
R scripts	This paper	https://github.com/Anne-LiseGerard/dftd_rnaseq/
Bash scripts	This paper	https://github.com/Anne-LiseGerard/dftd_rnaseq/

SUPPLEMENTAL INFORMATION

Figure S1. Multi-contrast pathway enrichment analysis, all significantly enriched pathways (FDR < 0.05).
Table S1. Excel file containing the results of the tumour purity calculations for cell line and biopsy sequences.
Table S2. Excel file containing for all genes mentioned in text: gene symbols, gene names and brief description.
Table S3. Excel file containing custom gene sets used in the pathway enrichment analysis and references for genes included in those sets.
Table S4. Excel file containing model selection for the mixed effects models: degrees of freedom, Akaike's Information Criterion (AIC) and AIC weights, and which model was selected at each step.
Table S5. Excel file containing mixed effects models outputs: estimates, lower confidence levels, upper confidence levels, and compact letter display of significant differences ($p < 0.05$). Significant differences calculated using Tukey's post hoc test, with Bonferroni correction for multiple testing.

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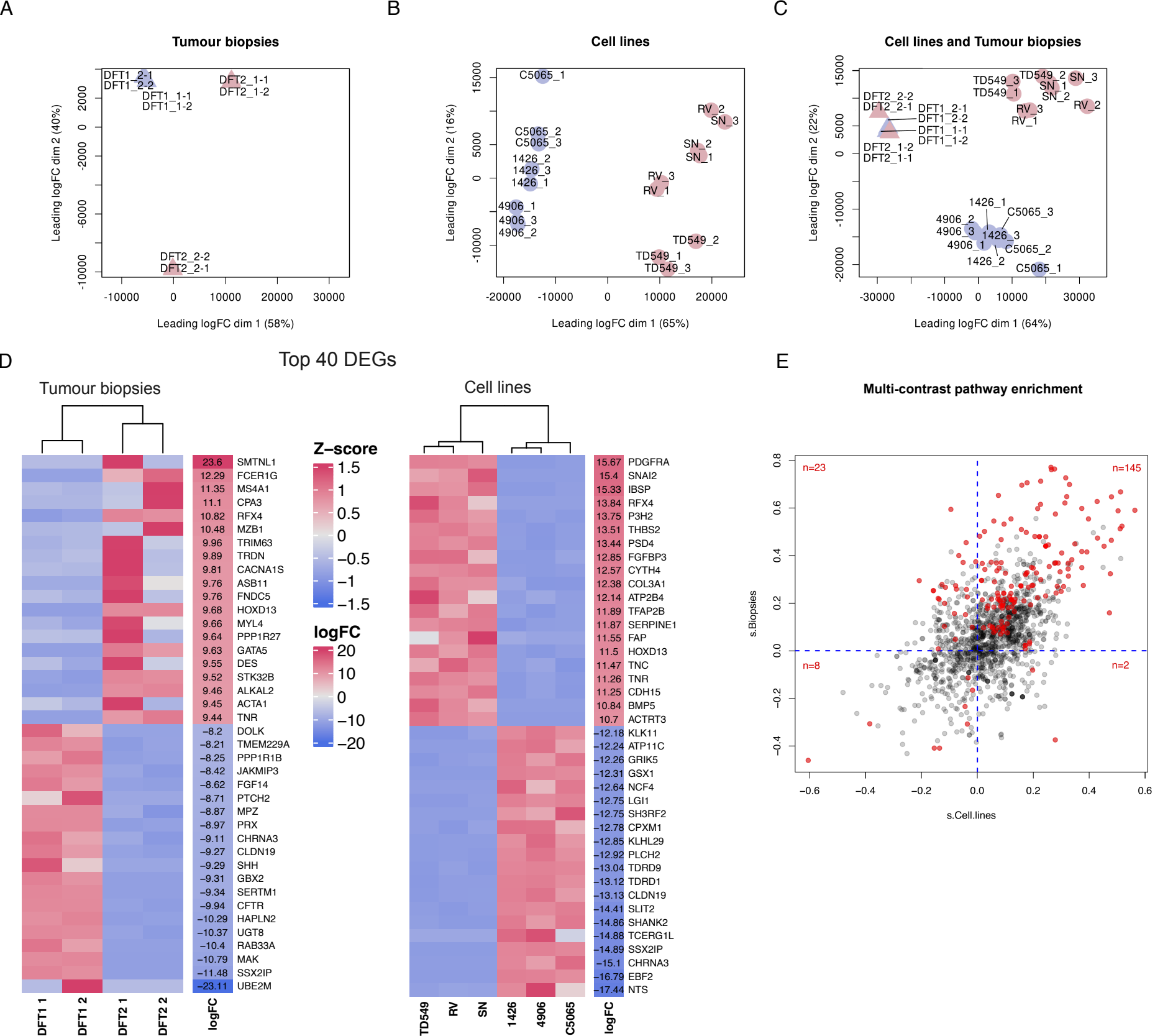
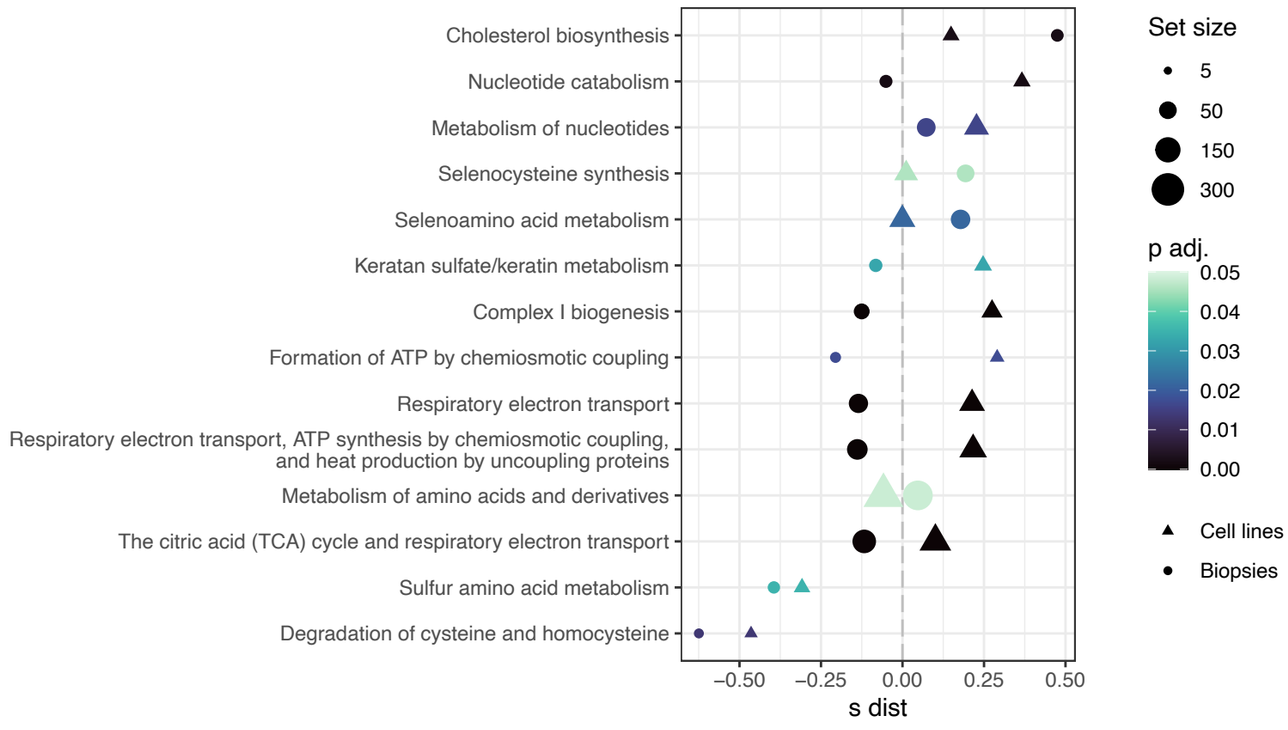
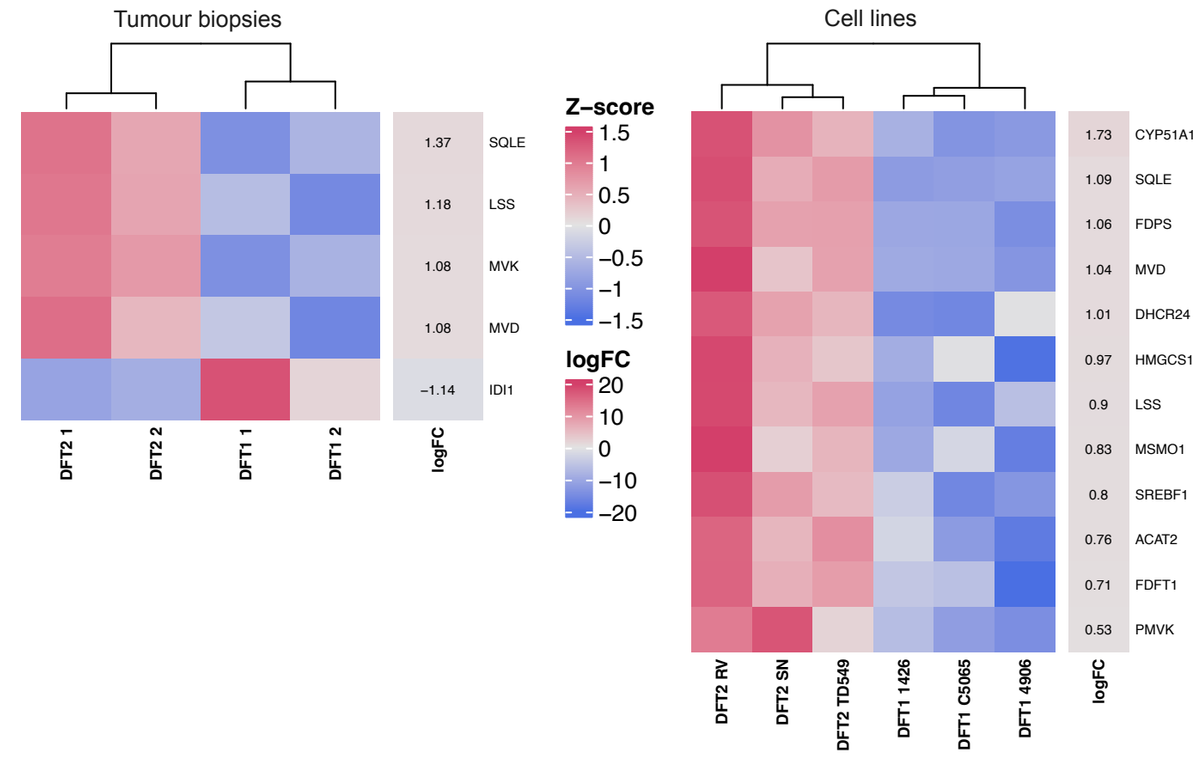

A
Tumour biopsies
Leading logFC dim 1 (58%)
Leading logFC dim 2 (40%)
DFT1_2-1
DFT1_2-2
DFT1_1-1
DFT1_1-2
DFT2_1-1
DFT2_1-2
DFT2_2-2
DFT2_2-1
B
Cell lines
Leading logFC dim 1 (65%)
Leading logFC dim 2 (16%)
C
Cell lines and Tumour biopsies
Leading logFC dim 1 (64%)
Leading logFC dim 2 (22%)
D
Top 40 DEGs
Tumour biopsies
Cell lines
Z–score
logFC
DFT1 1
DFT1 2
DFT2 1
DFT2 2
logFC
TD549
RV
SN
1426
4906
C5065
logFC
E
Multi-contrast pathway enrichment
n=23
n=145
n=8
n=2
s.Biopsies
s.Cell.lines

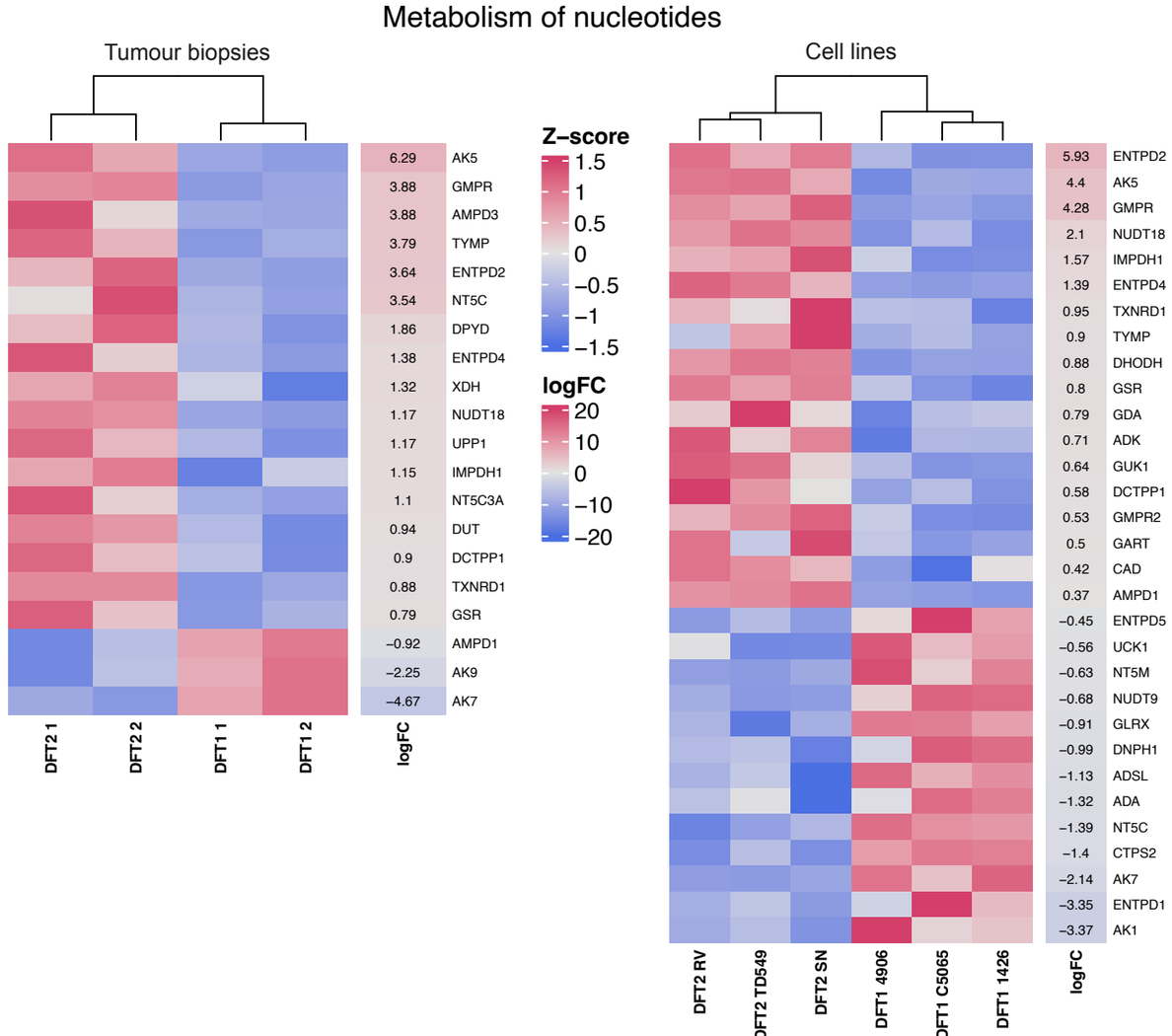
A


Multi−contrast pathway analysis
Cholesterol biosynthesis
Nucleotide catabolism
Metabolism of nucleotides
Selenocysteine synthesis
Selenoamino acid metabolism
Keratan sulfate/keratin metabolism
Complex I biogenesis
Formation of ATP by chemiosmotic coupling
Respiratory electron transport
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins
Metabolism of amino acids and derivatives
The citric acid (TCA) cycle and respiratory electron transport
Sulfur amino acid metabolism
Degradation of cysteine and homocysteine
−0.50
−0.25
0.00
0.25
0.50
s dist
Set size
5
50
150
300
p adj.
0.05
0.04
0.03
0.02
0.01
0.00
Cell lines
Biopsies

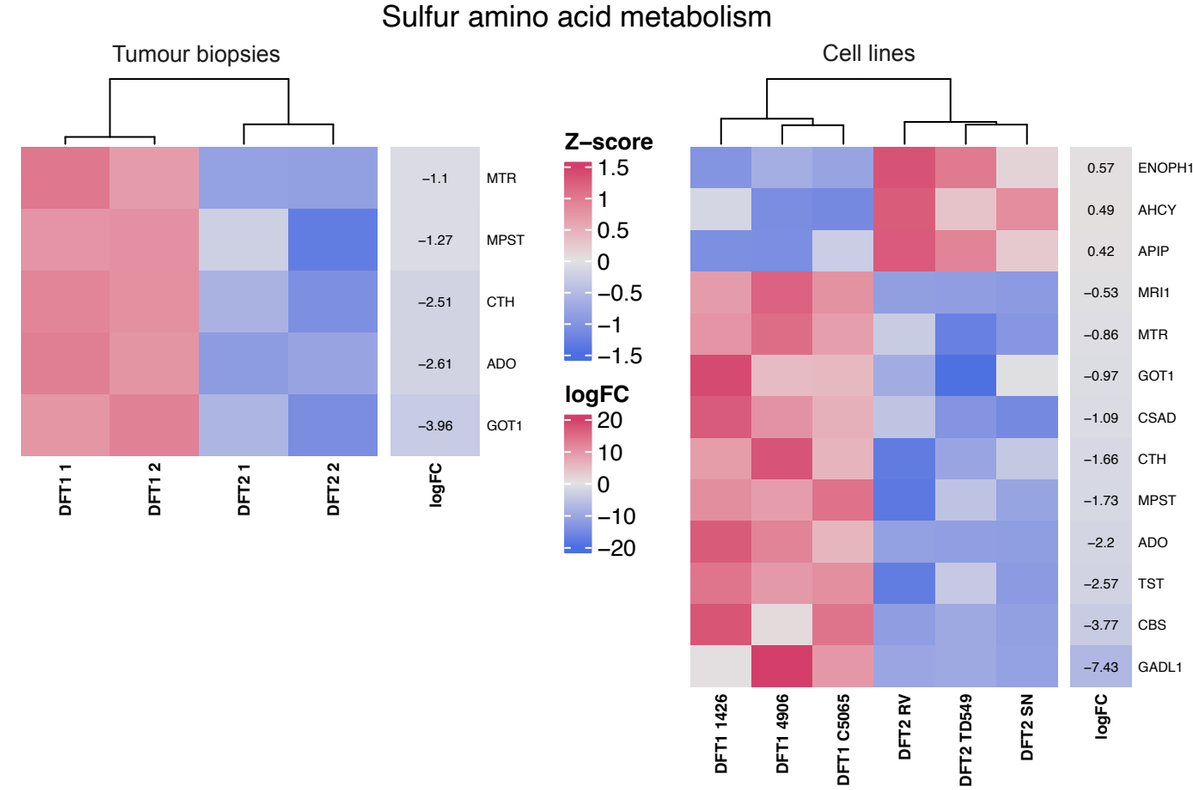
B

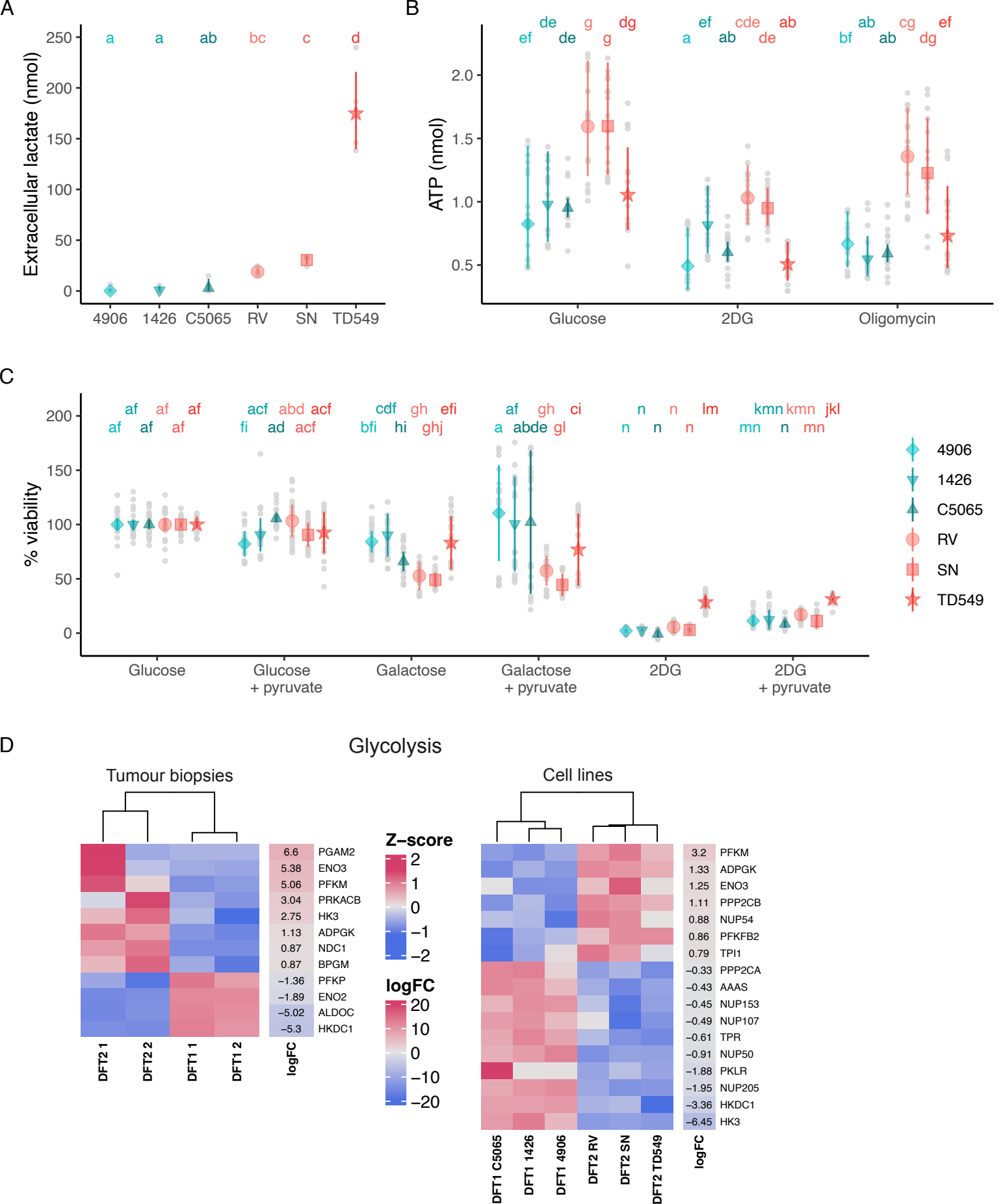

Cholesterol biosynthesis
Tumour biopsies
Cell lines
Z−score
1.5
1
0.5
0
−0.5
−1
−1.5
logFC
20
10
0
−10
−20
1.37 SQLE
1.18 LSS
1.08 MVK
1.08 MVD
−1.14 IDI1
DFT2 1
DFT2 2
DFT1 1
DFT1 2
logFC
1.73 CYP51A1
1.09 SQLE
1.06 FDPS
1.04 MVD
1.01 DHCR24
0.97 HMGCS1
0.9 LSS
0.83 MSMO1
0.8 SREBF1
0.76 ACAT2
0.71 FDFT1
0.53 PMVK
DFT2 RV
DFT2 SN
DFT2 TD549
DFT1 1426
DFT1 C5065
DFT1 4906
logFC

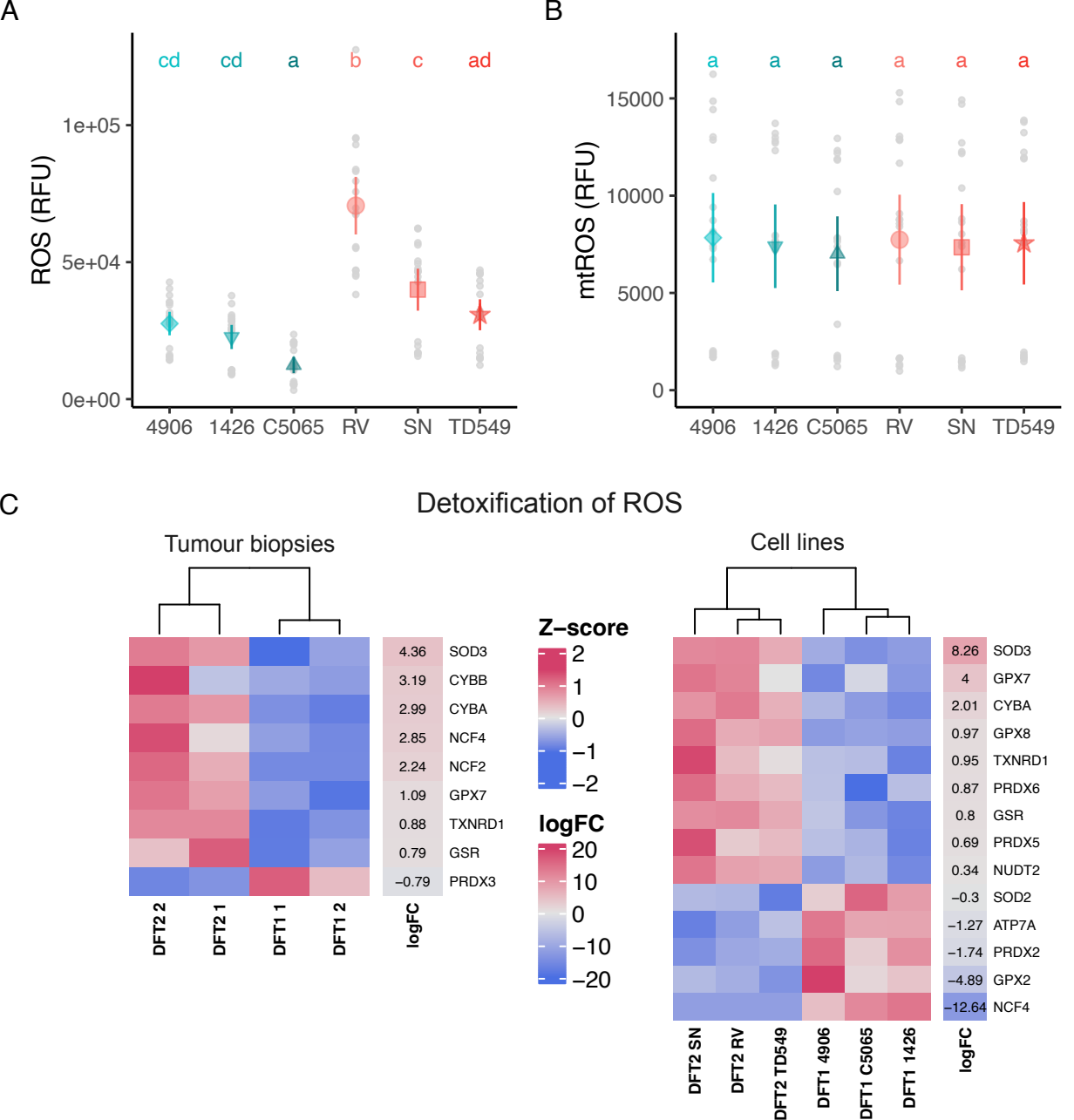
C

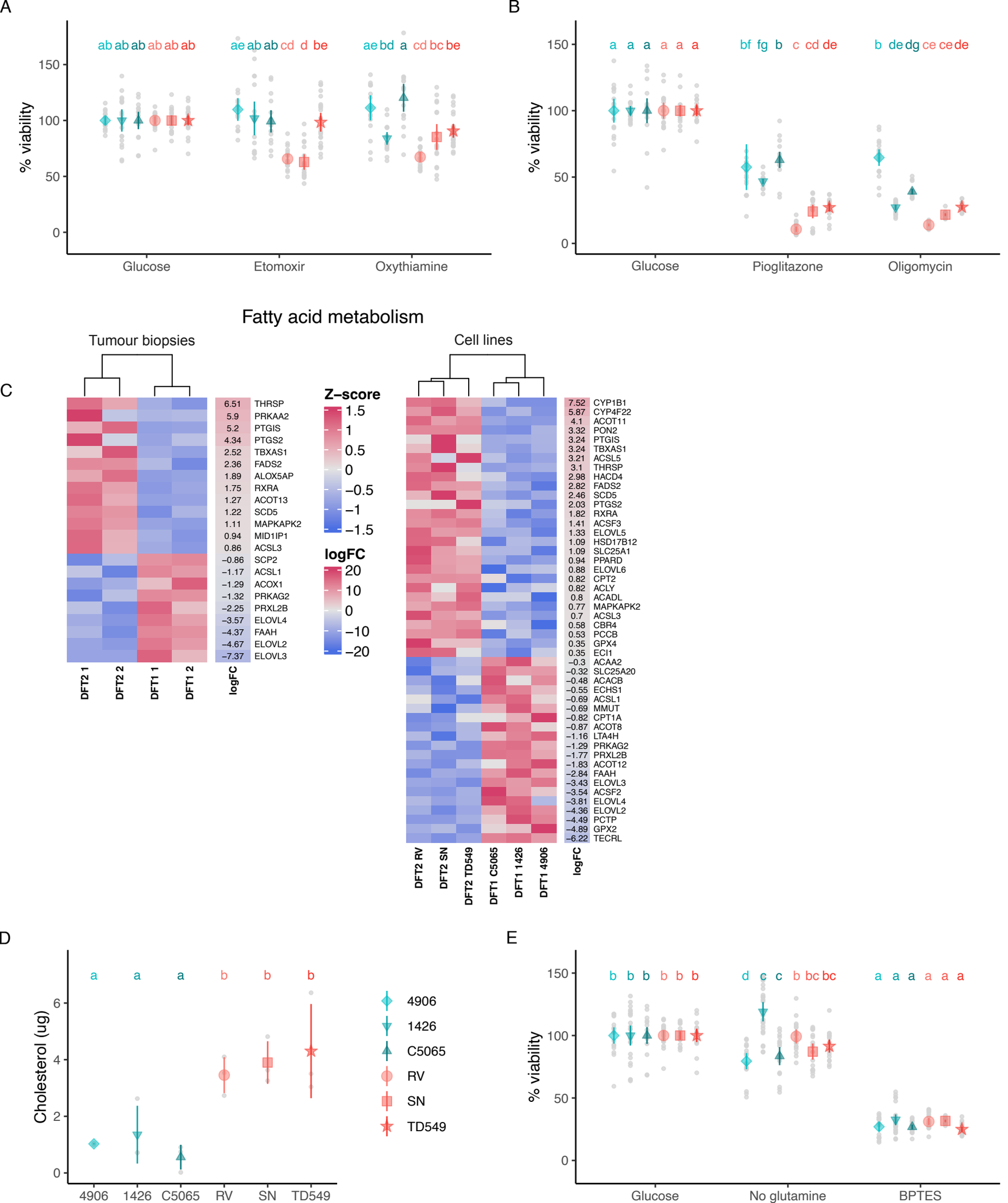

Metabolism of nucleotides
Tumour biopsies
Cell lines
Z−score
1.5
1
0.5
0
−0.5
−1
−1.5
logFC
20
10
0
−10
−20
6.29 AK5
3.88 GMPR
3.88 AMPD3
3.79 TYMP
3.64 ENTPD2
3.54 NT5C
1.86 DPYD
1.38 ENTPD4
1.32 XDH
1.17 NUDT18
1.17 UPP1
1.15 IMPDH1
1.1 NT5C3A
0.94 DUT
0.9 DCTPP1
0.88 TXNRD1
0.79 GSR
−0.92 AMPD1
−2.25 AK9
−4.67 AK7
DFT2 1
DFT2 2
DFT1 1
DFT1 2
logFC
5.93 ENTPD2
4.4 AK5
4.28 GMPR
2.1 NUDT18
1.57 IMPDH1
1.39 ENTPD4
0.95 TXNRD1
0.9 TYMP
0.88 DHODH
0.8 GSR
0.79 GDA
0.71 ADK
0.64 GUK1
0.58 DCTPP1
0.53 GMPR2
0.5 GART
0.42 CAD
0.37 AMPD1
−0.45 ENTPD5
−0.56 UCK1
−0.63 NT5M
−0.68 NUDT9
−0.91 GLRX
−0.99 DNPH1
−1.13 ADSL
−1.32 ADA
−1.39 NT5C
−1.4 CTPS2
−2.14 AK7
−3.35 ENTPD1
−3.37 AK1
DFT2 RV
DFT2 TD549
DFT2 SN
DFT1 4906
DFT1 C5065
DFT1 1426
logFC

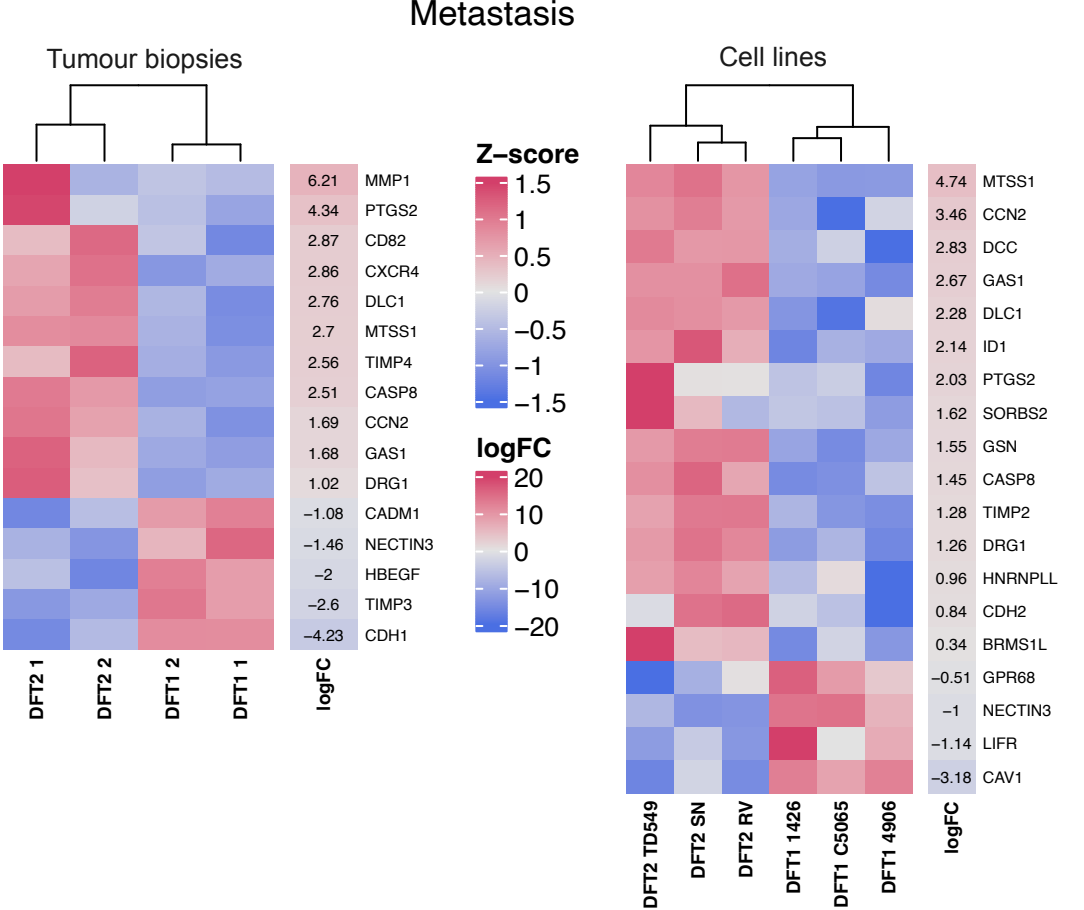
D


Sulfur amino acid metabolism
Tumour biopsies
Cell lines
Z−score
1.5
1
0.5
0
−0.5
−1
−1.5
logFC
20
10
0
−10
−20
−1.1 MTR
−1.27 MPST
−2.51 CTH
−2.61 ADO
−3.96 GOT1
DFT1 1
DFT1 2
DFT2 1
DFT2 2
logFC
0.57 ENOPH1
0.49 AHCY
0.42 APIP
−0.53 MRI1
−0.86 MTR
−0.97 GOT1
−1.09 CSAD
−1.66 CTH
−1.73 MPST
−2.2 ADO
−2.57 TST
−3.77 CBS
−7.43 GADL1
DFT1 1426
DFT1 4906
DFT1 C5065
DFT2 RV
DFT2 TD549
DFT2 SN
logFC


A
Extracellular lactate (nmol)
250
200
150
100
50
0
a a ab bc c d
4906 1426 C5065 RV SN TD549
B
ATP (nmol)
2.0
1.5
1.0
0.5
de g dg
ef de g
ef cde ab
a ab de
ab cg ef
bf ab dg
Glucose
2DG
Oligomycin
C
% viability
200
150
100
50
0
af af af
af af af
acf abd acf
fi ad acf
cdf gh efi
bfi hi ghj
af gh ci
a abde gl
n n lm
n n n
kmn kmn jkl
mn n mn
Glucose
Glucose + pyruvate
Galactose
Galactose + pyruvate
2DG
2DG + pyruvate
4906
1426
C5065
RV
SN
TD549
D
Glycolysis
Tumour biopsies
Cell lines
Z-score
2
1
0
−1
−2
logFC
20
10
0
−10
−20
6.6 PGAM2
5.38 ENO3
5.06 PFKM
3.04 PRKACB
2.75 HK3
1.13 ADPGK
0.87 NDC1
0.87 BPGM
−1.36 PFKP
−1.89 ENO2
−5.02 ALDOC
−5.3 HKDC1
DFT2 1
DFT2 2
DFT1 1
DFT1 2
logFC
3.2 PFKM
1.33 ADPGK
1.25 ENO3
1.11 PPP2CB
0.88 NUP54
0.86 PFKFB2
0.79 TPI1
−0.33 PPP2CA
−0.43 AAAS
−0.45 NUP153
−0.49 NUP107
−0.61 TPR
−0.91 NUP50
−1.88 PKLR
−1.95 NUP205
−3.36 HKDC1
−6.45 HK3
DFT1 C5065
DFT1 1426
DFT1 4906
DFT2 RV
DFT2 SN
DFT2 TD549
logFC


A
cd
cd
a
b
c
ad
ROS (RFU)
1e+05
5e+04
0e+00
4906
1426
C5065
RV
SN
TD549
B
a
a
a
a
a
a
mtROS (RFU)
15000
10000
5000
0
4906
1426
C5065
RV
SN
TD549
C
Detoxification of ROS
Tumour biopsies
Cell lines
Z–score
2
1
0
−1
−2
logFC
20
10
0
−10
−20
4.36 SOD3
3.19 CYBB
2.99 CYBA
2.85 NCF4
2.24 NCF2
1.09 GPX7
0.88 TXNRD1
0.79 GSR
−0.79 PRDX3
DFT2 2
DFT2 1
DFT1 1
DFT1 2
logFC
8.26 SOD3
4 GPX7
2.01 CYBA
0.97 GPX8
0.95 TXNRD1
0.87 PRDX6
0.8 GSR
0.69 PRDX5
0.34 NUDT2
−0.3 SOD2
−1.27 ATP7A
−1.74 PRDX2
−4.89 GPX2
−12.64 NCF4
DFT2 SN
DFT2 RV
DFT2 TD549
DFT1 4906
DFT1 C5065
DFT1 1426
logFC


A
% viability
Glucose
Etomoxir
Oxythiamine
B
% viability
Glucose
Pioglitazone
Oligomycin
Fatty acid metabolism
C
Tumour biopsies
Cell lines
Z–score
logFC
DFT2 1
DFT2 2
DFT1 1
DFT1 2
DFT2 RV
DFT2 SN
DFT2 TD549
DFT1 C5065
DFT1 1426
DFT1 4906
D
Cholesterol (ug)
4906
1426
C5065
RV
SN
TD549
E
% viability
Glucose
No glutamine
BPTES


Metastasis
Tumour biopsies
Cell lines
Z–score
1.5
1
0.5
0
−0.5
−1
−1.5
logFC
20
10
0
−10
−20
6.21 MMP1
4.34 PTGS2
2.87 CD82
2.86 CXCR4
2.76 DLC1
2.7 MTSS1
2.56 TIMP4
2.51 CASP8
1.69 CCN2
1.68 GAS1
1.02 DRG1
−1.08 CADM1
−1.46 NECTIN3
−2 HBEGF
−2.6 TIMP3
−4.23 CDH1
DFT2 1
DFT2 2
DFT1 2
DFT1 1
logFC
4.74 MTSS1
3.46 CCN2
2.83 DCC
2.67 GAS1
2.28 DLC1
2.14 ID1
2.03 PTGS2
1.62 SORBS2
1.55 GSN
1.45 CASP8
1.28 TIMP2
1.26 DRG1
0.96 HNRNPLL
0.84 CDH2
0.34 BRMS1L
−0.51 GPR68
−1 NECTIN3
−1.14 LIFR
−3.18 CAV1
DFT2 TD549
DFT2 SN
DFT2 RV
DFT1 1426
DFT1 C5065
DFT1 4906
logFC