Metabolic flux prediction in cancer cells with altered substrate uptake

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Abstract

Proliferating cells, such as cancer cells, are known to have an unusual metabolism, characterized by an increased rate of glycolysis and amino acid metabolism. Our understanding of this phenomenon is limited but could potentially be used in order to develop new therapies. Computational modelling techniques, such as flux balance analysis (FBA), have been used to predict fluxes in various cell types, but remain of limited use to explain the unusual metabolic shifts and altered substrate uptake in human cancer cells. We implemented a new flux prediction method based on elementary modes (EMs) and structural flux (StruF) analysis and tested them against experimentally measured flux data obtained from ^{13}C-labelling in a cancer cell line. We assessed the quality of predictions using different objective functions along with different techniques in normalizing a metabolic network with more than one substrate input. Results show a good correlation between predicted and experimental values and indicate that the choice of cellular objective critically affects the quality of predictions. In particular, lactate gives an excellent correlation and correctly predicts the high flux through glycolysis, matching the observed characteristics of cancer cells. In contrast with FBA, which requires *a priori* definition of all uptake rates, often hard to measure, atomic StruFs (aStruFs) are able to predict uptake rates of multiple substrates.

Metabolic flux prediction in cancer cells

Cancer cells are known to exhibit unusual metabolic activity, characterized by high rates of glucose consumption and lactate production even under aerobic conditions (the Warburg effect [1]), as well as increased glutamine catabolism and amino acid metabolism [2,3]. The conversion of glucose to lactate is less efficient for ATP production than mitochondrial oxidative phosphorylation, which is mainly used by normal cells, but this process is able to achieve high rates and supports the rapid proliferation of tumours [4]. For some time, it was believed that defects in mitochondrial respiration were the main cause of the down-regulation of oxidative phosphorylation and the converse up-regulation of glycolysis. Yet normal mitochondrial activity has been observed in some tumours, leading to renewed interest into the causes and mechanisms of metabolic reprogramming in cancer cells [5,6].

Computational modelling can play an important role in understanding the mechanisms of metabolic flux redistribution in cancer cells. Techniques, such as flux balance analysis (FBA), have been widely used to predict fluxes in various cell types ranging from bacteria to human cells [7]. FBA relies on a stoichiometric model of the metabolic network, a set of constraints on exchange fluxes and the definition of a metabolic objective (e.g. maximum biomass production) to predict intracellular fluxes using linear programming. Although FBA has been very successfully applied to unicellular organisms for applications in biotechnology and metabolic engineering, its use in human cells is more challenging due to the difficulty to precisely characterize exchange fluxes between cells and their environment.

The concept of control effective flux (CEF) was introduced to understand changes in transcriptional regulation [8] and later modified for metabolic flux estimation [9]. CEFs consist in estimating the efficiency of direct metabolic routes or elementary modes (EMs) [10], towards a given cellular objective. The contributions of all EMs are then weighed accordingly and summed up to predict the flux distribution, under the assumption that more efficient routes are likely to be favoured. Structural fluxes (StruFs) were inspired from CEFs and used to identify gene deletion strategies in conjunction with a cellular objective [11]. An important advantage of this technique is to take into consideration not only the optimal route but also the full set of possible routes, thereby accounting for redundancy and flexibility in the metabolic network. Moreover, constraints on exchange fluxes do not need to be pre-defined. So far, CEF and StruF have been applied to situations where a single substrate input was used [11–13]. In this work, we investigate whether this technique can be successfully applied

Key words: atomic structural flux, biological objectives, cancer metabolism, elementary modes, metabolic flux, multiple substrates.

Abbreviations: aStruF, atomic StruF; CEF, control effective flux; EM, elementary mode' FBA, flux balance analysis; StruF, structural flux; TCA cycle, tricarboxylic acid cycle.

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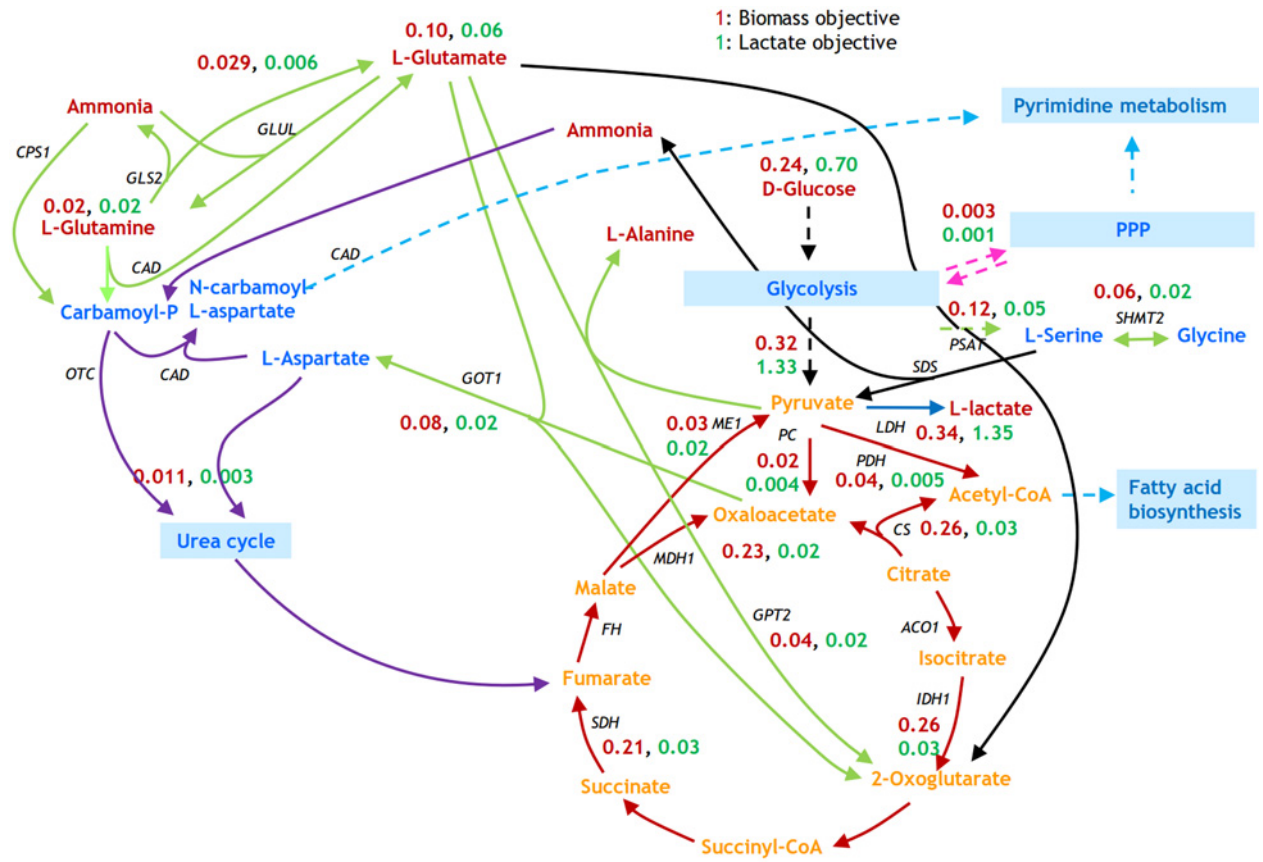
Figure 1 | **Metabolic network used for this study**

Extracellular metabolites (red), reactions in glycolysis (black), amino acid metabolism (green), lactate production (blue), TCA cycle (red) and biomass (blue). The numbers indicate predicted fluxes using biomass as an objective (red) and lactate (green).



to more complex cell types, such as in cancer, and to contexts where multiple substrate inputs occur. We present an extension of StruFs termed atomic StruFs (aStruFs), which allows the consideration of multiple substrate uptakes. We compare predictions derived from aStruF and classical FBA to experimental flux measurements obtained through carbon isotope labelling and we compare the predictive value of aStruFs for different cellular objectives in order to identify the best-suited characterization of cancer cells.

Metabolic model

The metabolic model was reconstructed starting from a central model for carbon metabolism for Chinese hamster ovary cells [14] and manually adapted for reactions present or absent from human cancer cells. All reactions were checked against the Recon2 model [15]. The reconstructed model contains 114 reactions of central metabolism representing glycolysis, tricarboxylic acid cycle (TCA cycle), pentose phosphate pathway, urea cycle and amino acid metabolism (Figure 1); 95 internal metabolites and 25 eternal metabolites were included in the model. Lumped reactions were added to represent incorporation of central metabolites into proteins, nts and lipids and to represent breakdown of haeme. The biomass reaction was also taken from Zamorano [14]. EMs were calculated using efmtool [16]; 167226 EMs were obtained in our model. The model is provided in Supplementary File 1 in Metatool format [17].

Parsimonious FBA simulations were carried out using OptFlux [18]. In order to create comparable conditions between pFBA and aStruFs simulations, only glucose uptake was set to 1 and all other inputs were left unconstrained.

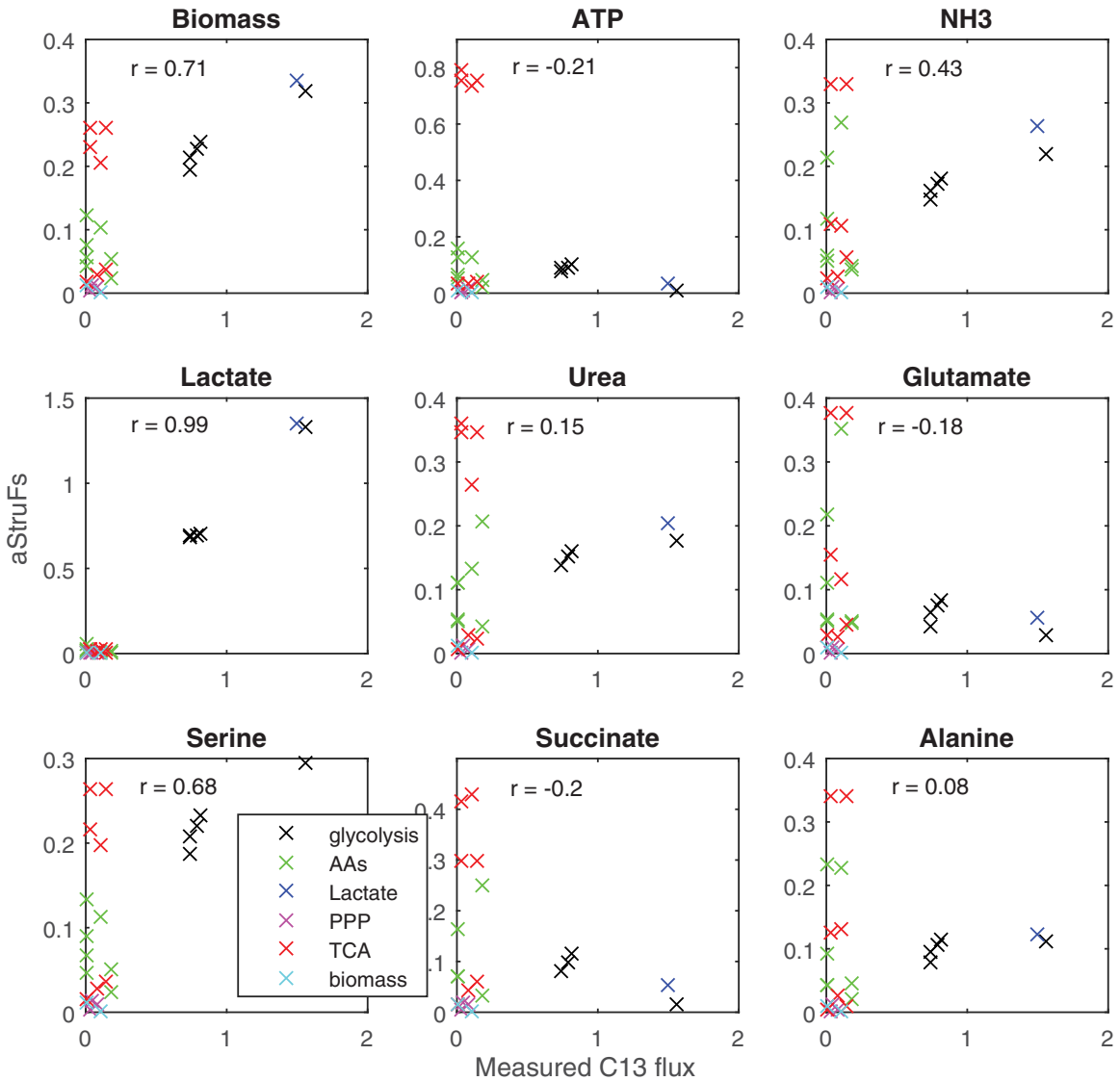
Atomic structural fluxes

The efficiency ε_i of each EM i is defined as the ratio of the EM's objective to the investment required to establish the EM:

$$\varepsilon_i = \frac{e_i^{objective}}{\sum_k |e_i^k|} \quad (1)$$

Where $e_i^{objective}$ is the yield of production of the cellular objective by EM i normalized for substrate uptake. Since the system possesses more than one substrate uptake (glucose, 13 amino acids, choline and ethanolamine), a choice is needed to

Figure 2 | Comparison between predicted (vertical axis) and measured fluxes (horizontal axis) for different cellular objectives



quantify yields with respect to different substrates: we chose to use the number of carbon atoms of each substrate (e.g. glucose = 6, glutamine = 5):

$$e_i^k = \frac{c_i^k}{\sum_{s \in \{substrates\}} Cmoles_{s,i}} \quad (2)$$

Where c_i^k is the coefficient of reaction k in EM i and $Cmoles_{s,i}$ is the number of carbon atoms in substrate s for EM i.

The StruF of reaction k is obtained by weighting each EM containing the reaction by its efficiency and summing up all these contributions:

$$aSF_k = \frac{\sum_i \varepsilon_i \cdot |e_i^k|}{\sum_i \varepsilon_i} \quad (3)$$

CEF and *aStruF* values are not comparable across networks [11,12]. Appropriate normalization is necessary if one intends to compare StruFs across metabolic networks of different sizes, for example in the search of deletion targets specifically harming the cancer cell:

$$aStrufF_k = \frac{aSF_k}{aSF_{\text{Total Sub}}} \quad (4)$$

Biological objectives

The choice of cellular objective affects the values of control-effective fluxes, since the efficiency of each EM is estimated by determining its contribution towards the objective relative to the costs required to establish the mode [8]. Although the assumption of optimality towards maximum growth of a wild-type microorganism is justifiable, the choice of the

most relevant biological objective for human cancer cells is still an open question. We tested nine objectives to account for a range of biological processes in cancer cells:

- Biomass is the most frequently used objective for metabolic flux analysis. This objective is relevant to simulate the fast growth of cancer cells, but may be questionable because cancer cells seemingly waste many substrates as measured in cell cultures by Jain et al. [19].
- Lactate is a clear objective for cancer cells, since it is well known that they produce high amounts of lactate (Warburg effect).
- Glutamate, alanine, ammonia or urea: Many cancers show an elevated uptake of glutamine [20], which exceeds the cellular demands of biomass formation. This excess nitrogen may be removed from the cell by excretion of amino-acids like alanine and glutamate, frequently coupled with ammonia excretion or coupled with urea excretion.
- The serine synthesis pathway has been shown essential in several cancer sub-types to funnel glutamate into TCA [21].
- Succinate accumulation has been shown to induce hypoxia response, associated with tumour formation [22,23].

Comparison between predicted and experimental fluxes

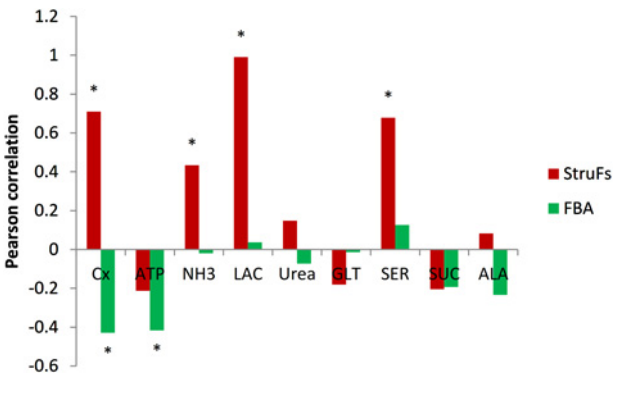
Predictions were tested against experimentally measured flux data obtained from ^{13}C-labelling in an A549 carcinoma cell line [24]. Cells were cultured in high-glucose medium supplemented with glutamine. For flux measurements ^{13}C-labelled glucose and glutamine were used for 24 h to achieve isotopic steady state.

Results show that the best correlations between predicted and experimental fluxes are obtained using lactate (0.99) or biomass production (0.71) as the cellular objective (Figure 2). The predicted fluxes for these pathways are illustrated in more detail in Figure 1. Serine also shows a reasonably good prediction (0.68) but other objectives produce a weak or no correlation. In particular, lactate gives an excellent correlation and correctly predicts the high flux through glycolysis, matching the observed characteristics of cancer cells. When biomass is used as the objective, the predicted glycolytic flux is lower, but a higher flux is directed through the TCA cycle and towards fatty acid production; predicted fluxes for glycolysis and lactate production still reasonably match observed values, however TCA cycle and amino acid production tend to be overestimated (Figure 2). Using serine as the objective gives similar characteristics but tends to overestimate the TCA flux even more, whereas glutamate and alanine objectives predict most of the flux going towards amino acid production through further elevated uptake of other amino acids and are no longer able to predict correct glycolysis and lactate fluxes.

We additionally compared the values predicted by aStruFs with those predicted by FBA. The results show that FBA is not able to account for the observed flux distribution (Figure 3), irrespectively of the cellular objective used. This illustrates the fact that FBA requires precise constraints on exchange fluxes to be defined *a priori* in order to make good predictions, but when such constraints are unknown it cannot predict respective import levels in the case of multiple substrate uptakes. On the contrary, StruFs are able to predict relative import levels of multiple substrates when the correct cellular objective is used.

Figure 3 | Correlation coefficients between measured fluxes and values predicted values using structural fluxes (red) and parsimonious FBA (green)

The asterisk (*) indicates a significant correlation between predictions and measurements.



Concluding remarks

We have shown that aStruFs are a promising technique for metabolic flux prediction in complex cellular contexts such as cancer. FBA has been successfully applied in unicellular organisms such as bacteria or yeast, where exchange fluxes can be more easily determined. However in multicellular and heterogeneous systems such as human tissues, exchange rates between cells and extracellular environment are more difficult to measure experimentally. The lack of tissue-specific metabolite uptake and secretion rates urges the development of techniques to understand substrate usage of cells. The advantage of aStruFs is to rely only on the definition of a cellular objective and on the assumption that more efficient routes towards that objective are favoured, but not on the definition of constraints for exchange fluxes; unlike FBA, exchange fluxes do not belong to the inputs but belong to the results.

aStruFs offer the potential to develop more precise simulations to predict the effects of mutations, identify drug targets and understand regulatory mechanisms of metabolic flux in diseases such as cancer. This technique could be further improved by taking into consideration more information about the intracellular state of tumours, such as gene and protein expression. Currently, possibilities to develop this method remain hampered by the limited availability of multi-omics datasets for human cell types,

but these are likely to improve with the ever-growing development of experimental techniques. Single cell and MS-imaging techniques in particular offer promising perspectives to collect the data needed to fully characterize a cell's state [25,26]. Modelling will be essential to interpret these data in an integrated way.

References

1 Warburg, O. (1956) On the origin of cancer cells. Science **123**, 309–314 CrossRef PubMed
2 Jang, M., Kim, S.S. and Lee, J. (2013) Cancer cell metabolism, implications for therapeutic targets. Exp. Mol. Med. **45**, e45 CrossRef PubMed
3 Kim, M.H. and Kim, H. (2013) Oncogenes and tumor suppressors regulate glutamine metabolism in cancer cells. J. Cancer Prev. **18**, 221–226 CrossRef PubMed
4 Vazquez, A., Liu, J., Zhou, Y. and Oltvai, Z.N. (2010) Catabolic efficiency of aerobic glycolysis: The Warburg effect revisited. BMC Syst. Biol. **4**, 58 CrossRef PubMed
5 Seyfried, T.N. and Shelton, L.M. (2010) Cancer as a metabolic disease. Nutr. Metab. **7**, 7 CrossRef
6 Ferreira, L.M.R., Hebrant, A. and Dumont, J.E. (2012) Metabolic reprogramming of the tumor. Oncogene **31**, 3999–4011 CrossRef PubMed
7 Mardinoglu, A., Gatto, F. and Nielsen, J. (2013) Genome-scale modeling of human metabolism – a systems biology approach. Biotechnol. J. **8**, 985–996 CrossRef PubMed
8 Stelling, J., Klamt, S., Bettenbrock, K., Schuster, S. and Gilles, E.D. (2002) Metabolic network structure determines key aspects of functionality and regulation. Nature **420**, 190–193 CrossRef PubMed
9 Beurton-Aimar, M., Beauvoit, B., Monier, A., Vallée, F., Dieuaide-Noubhani, M. and Colombié, S. (2011) Comparison between elementary flux modes analysis and 13C-metabolic fluxes measured in bacterial and plant cells. BMC Syst. Biol. **5**, 95 CrossRef PubMed
10 Schuster, S., Fell, D.A. and Dandekar, T. (1999) A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. Nat. Biotechnol. **18**, 326–332 CrossRef
11 Soons, Z., Ferreira, E.C., Patil, K.R. and Rocha, I. (2013) Identification of metabolic engineering targets through analysis of optimal and sub-optimal routes. PLoS One **8**, e61648 CrossRef PubMed
12 Çakir, T., Kirdar, B., Onsan, Z.I., Ulgen, K.O. and Nielsen, J. (2007) Effect of carbon source perturbations on transcriptional regulation of metabolic fluxes in *Saccharomyces cerevisiae*. BMC Syst. Biol. **1**, 18 CrossRef PubMed
13 Taylor, P.N. and Schwartz, J.M. (2014) *In silico* prediction of elementary mode fluxes. In International Work-Conference on Bioinformatics and Biomedical Engineering (IWBBIO 2014), Granada, Spain, vol. 1, pp. 183–191, University of Manchester, Manchester
14 Zamorano, F. (2012) Metabolic Flux Analysis of CHO Cell Cultures. Ph.D. Thesis, Université de Mons, Mons, Hainaut, Belgium
15 Thiele, I., Swainston, N., Fleming, R.M., Hoppe, A., Sahoo, S., Aurich, M.K, Haraldsdottir, H., Mo, M.L., Rolfsson, O., Stobbe, M.D. et al. (2013) A community-driven global reconstruction of human metabolism. Nat. Biotechnol. **31**, 419–425 CrossRef PubMed
16 Terzer, M. and Stelling, J. (2008) Large-scale computation of elementary flux modes with bit pattern trees. Bioinformatics **24**, 2229–2235 CrossRef PubMed
17 von Kamp, A. and Schuster, S. (2006) Metatool 5.0: fast and flexible elementary modes analysis. Bioinformatics **22**, 1930–1931 CrossRef PubMed
18 Rocha, I., Maia, P., Evangelista, P., Vilaca, P., Soares, S., Pinto, J., Nielsen, J., Patil, K., Ferreira, E. and Rocha, M. (2010) OptFlux: an open-source software platform for *in silico* metabolic engineering. BMC Syst. Biol. **4**, 45 CrossRef PubMed
19 Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A.L., Kafri, R., Kirschner, M.W., Clish, C.B. and Mootha, V.K. (2012) Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science **336**, 1040–1044 CrossRef PubMed
20 DeBerardinis, R.J. and Cheng, T. (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene **29**, 313–324 CrossRef PubMed
21 Possemato, R., Marks, K.M., Shaul, Y.D., Pacold, M.E., Kim, D., Birsoy, K., Sethumadhavan, S., Woo, H., Jang, H.G., Jha, A.K. et al. (2011) Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature **476**, 346–353 CrossRef PubMed
22 Selak, M.A., Armour, S.M., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B. and Gottlieb, E. (2005) Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- prolyl hydroxylase. Cancer Cell **7**, 77–86 CrossRef PubMed
23 Frezza, C., Zheng, L., Folger, O., Rajagopalan, K.N., MacKenzie, E.D., Jerby, L., Micaroni, M., Chaneton, B., Adam, J., Hedley, A. et al. (2011) Haem oxygenase is synthetically lethal with the tumour suppressor fumarate hydratase. Nature **477**, 225–230 CrossRef PubMed
24 Walther, J.L., Metallo, C.M., Zhang, J. and Stephanopoulos, G. (2012) Optimization of 13C isotopic tracers for metabolic flux analysis in mammalian cells. Metab. Eng. **14**, 162–171 CrossRef PubMed
25 Mannello, F., Ligi, D. and Magnani, M. (2012) Deciphering the single-cell omic: innovative application for translational medicine. Expert Rev. Proteomics **9**, 635–648 CrossRef PubMed
26 Jiang, H., Favaro, E., Goulbourne, C.N., Rakowska, P.D., Hughes, G.M., Ryadnov, M.G., Fong, L.G., Young, S.G., Ferguson, D.J.P., Harris, A.L. and Grovenor, C.M. (2014) Stable isotope imaging of biological samples with high resolution secondary ion mass spectrometry and complementary techniques. Methods **68**, 317–324 CrossRef PubMed

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