Metabolic rewiring in mutant Kras lung cancer

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Abbreviations: GTP, Guanosine triphosphate; KO, Knock out; KRAS, Kirsten rat sarcoma viral oncogene homolog; LKB1, Liver kinase B1; NSCLC, Non-small cell lung cancer; PDAC, Pancreatic ductal adenocarcinoma; ROS, Reactive oxygen species; SCC, Squamous cell carcinoma; TCA, Tricarboxylic acid; p53, transformation related protein 53 (Trp53).

Keywords: Lung cancer, mutant Kras, metabolism, mouse models, therapy

Abstract

Lung cancer is the leading cause of cancer related death worldwide, reflecting an unfortunate combination of very high prevalence and low survival rates, as most cases are diagnosed at advanced stages when treatment efficacy is limited. Lung cancer comprises several disease groups with non-small cell lung cancer (NSCLC) accounting for ~85% of cases and lung adenocarcinoma being its most frequent histological subtype. Mutations in *KRAS* affect ~30% of lung adenocarcinomas but unlike other commonly altered proteins (EGFR and ALK, affected in ~14 and 7% of cases, respectively), mutant KRAS remains untargetable. Therapeutic strategies that rely instead on the inhibition of mutant KRAS functional output or the targeting of mutant KRAS cellular dependencies (i.e. synthetic lethality) are an appealing alternative approach. Recent studies focused on the metabolic properties of mutant KRAS lung tumours have uncovered unique metabolic features that can potentially be exploited therapeutically. We review these findings here with a particular focus on *in vivo,* physiologic, mutant KRAS activity.

Cancer metabolism: the general picture

The reprogramming of cellular metabolism is now a widely recognised hallmark of cancer [1], involving a complex rearrangement of metabolic and energy producing networks to support the high proliferation rate of tumour cells and their unique metabolic demands. Arguably, the most striking and well-characterised metabolic feature of tumours is their altered glucose metabolism. In the 1920s, Otto Warburg showed that tumour cells exhibit an enhanced avidity for glucose, a critical cell nutrient, compared to normal tissue [2] and this metabolic phenotype has since been confirmed in a large proportion of tumours. In fact, it is routinely exploited in the clinic to both diagnose and stage tumours through positron emission tomography (PET)-based tumour imaging, which relies on the enhanced uptake of a radioactive fluorine-labelled glucose analogue (^{18}F-fluorodeoxyglucose) by tumour cells, relative to normal tissue [3].

Warburg also noted that under aerobic conditions, conversion of glucose to lactate (aerobic glycolysis) is significantly more prevalent in tumour cells than in normal ones. Furthermore, he argued that tumour cells rely more on aerobic glycolysis to metabolise glucose than on the more energy efficient process of mitochondrial oxidative phosphorylation, preferentially utilised by normal cells [2]. This glycolytic switch, known as the “Warburg effect”, was initially described as a compensation mechanism for mitochondria dysfunction in tumours [4], but its causes are now thought to be more complex. While mitochondrial damage can contribute to this metabolic rewiring, mitochondria remain functional in the majority of tumours, but aerobic glycolysis is nevertheless observed in such cases. Instead, glucose metabolism rewiring is more likely driven by the high demand of cancer cells for reducing equivalents and molecular precursors of proteins, lipids and nucleotides, the “building blocks” required to maintain their enhanced growth and proliferation [5].

Multiple additional metabolic changes have since been reported in cancer cells including the reprogramming of amino acid metabolism (to supplement the tricarboxylic acid (TCA) cycle as well as amino acid and nucleotide biosynthesis); and altered lipid metabolism (to support increased membrane synthesis and provide energy storage) [6-8]. The rewiring of glucose, amino acid and lipid metabolism can also alter cell signalling and oxidative stress management [9]. Reactive oxygen species (ROS) are a natural by-product of oxygen metabolism [10] and can have an important impact on cell fate. ROS are generated through both enzymatic and non-enzymatic reactions in the mitochondria, endoplasmic reticulum and peroxisomes and can also be produced by exogenous sources. High ROS levels are toxic to cells and therefore, in normal cells, ROS production (e.g. by the respiratory chain) and scavenging (e.g. through glutathione and superoxide dismutases) are tightly regulated to ensure survival [11].

Nevertheless, at well-tolerated levels, ROS can act as intracellular signalling molecules and play important regulatory roles, potentially providing selective advantages to cells [9, 12-17]. Cancer cells frequently display increased ROS levels but their role in tumourigenesis remains

controversial. Persistent ROS exposure was reportedly associated with DNA damage and predisposition to cancer [18]. However, attempts to treat tumours with antioxidants have been largely unsuccessful and there is evidence that they can in fact promote cancer in certain contexts [19]. In agreement, recent studies suggest that tumours frequently exhibit enhanced antioxidant capacity and that this phenotype may be positively selected during tumour development [20-22].

The general picture of cancer metabolism reprogramming is thus one of complex rearrangements where contradictions abound. Part of the difficulty in interpreting these changes derives from attempts to generalise observations acquired under defined, and often artificial conditions. While most cancers undergo metabolic rewiring, mounting evidence suggests that tumour metabolism signatures are context dependent, being influenced by a wide range of factors including oncogenic signalling, tissue of origin, microenvironment and tumour grade [21, 23-25]. Tumour type specific *in vivo* analyses of metabolism are thus fundamental to the improved understanding of their metabolic rewiring.

NSCLC metabolism: an *in patient* perspective

Advances in technology are now providing unique opportunities to study metabolic fluxes *in situ.* Through the combined use of ^{13}C-labelled substrate infusions in tissues and comprehensive profiling of carbon flux by metabolomics analysis [26] Fan and colleagues were able to demonstrate metabolic rewiring in fresh NSCLC surgical resections of mixed histology [27]. These lung tumours showed increased levels of lactate, demonstrating the expected increase in glycolysis relative to normal tissue. Tumour samples also exhibited an increase in glucose-derived TCA cycle intermediates, such as citrate and succinate, indicating that TCA cycle activity is enhanced in NSCLC relative to normal lung.

Typically, glycolysis is linked to the TCA cycle by the activity of pyruvate dehydrogenases, which mediate the conversion of pyruvate (the output of glycolysis) to Acetyl-CoA, that then enters the TCA cycle. Fan and colleagues reported that an alternative TCA cycle entry route,

mediated by pyruvate carboxylase activity, was upregulated in NSCLC samples. Pyruvate carboxylase catalyses the irreversible carboxylation of pyruvate to oxaloacetate, a TCA metabolite that is also utilized in biosynthetic reactions. Pyruvate carboxylase activity allows TCA cycle replenishment of oxaloacetate whenever its levels are reduced due to biosynthetic reactions (anaplerosis) [27], and its enhanced activity may play an important role in fulfilling the high anabolic demands of these tumours. The anaplerotic metabolism of pyruvate was also observed in early-stage NSCLC tissue and lung cancer slices *ex vivo* [28] (i.e. "Warburg slice" method [29]). Furthermore, knockdown of pyruvate carboxylase in NSCLC cells resulted in decreased tumour growth in a xenograft model, suggesting a dependence of lung tumours on pyruvate carboxylase-dependent anaplerosis [28]. Together, these studies demonstrate that the reprogramming of glucose metabolism in lung cancers involves both an aerobic glycolytic switch and the channelling of glucose-derived metabolites through the TCA cycle (Figure 1).

Through combined multimodal imaging analysis and ^{13}C-glucose flux profiling of NSCLC *in situ*, Hensley and colleagues independently confirmed the enhancement of glycolysis, TCA cycle activity and pyruvate carboxylation in tumour tissue relative to normal lung [23]. Increased activity of pyruvate dehydrogenase was also reported and shown to contribute to enhanced glucose oxidation across different histological tumour subtypes (Figure 1). Strikingly, this study uncovered a high degree of glucose metabolism heterogeneity within NSCLC, both between and within tumours. The authors showed that tissue perfusion (low versus high, as determined by tumour vasculature status), dictated preferential nutrient utilization in a given region, as well as its metabolic profile. Accordingly, areas of low perfusion preferentially utilized glucose, while highly perfused regions relied more on other nutrients. These data suggest a role for the tumour microenvironment on the metabolic heterogeneity of lung tumours, as recently reported for mouse models of pancreatic cancer [30-32].

Overall, the abovementioned studies provide strong evidence of metabolic rewiring in NSCLC. However, they show that in lung tumours, metabolic rewiring is defined by common tumour

features, but also by a high degree of intra and inter-tumour heterogeneity. From patient data it is unclear whether other factors, besides tissue perfusion, can contribute to NSCLC metabolic heterogeneity. Given that lung cancers exhibit a high mutation burden [33, 34], it is possible that their genetic heterogeneity is also reflected at the metabolic level, particularly since oncogene activation and loss of tumour suppressors can alter cellular metabolism [12]. Interestingly, Hensley and colleagues reported similar metabolic profiles in lung tumours with distinct driver mutations (KRAS, EGFR or no known driver) [23]. However, the number of tumours analysed for each group was relatively small, potentially preventing the detection of mutation-specific signatures. Mouse models can overcome these sample limitations and are therefore a powerful tool to address the relative impact of specific lung cancer mutations on tumour metabolism.

Mutant Kras lung adenocarcinoma: from man to mouse

KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is the most frequently mutated oncogene in lung adenocarcinoma and a member of the RAS family of GTPases, which also includes HRAS and NRAS [33, 35, 36]. KRAS is activated through GTP binding and once GTP-bound, can trigger multiple signalling transduction pathways and impact a wide range of cellular processes, including proliferation, cell survival and metabolism. *KRAS* is typically mutated in human cancer through missense mutations on codons 12, 13 and 61 that alter its protein conformation, resulting in the accumulation of constitutively active, GTP-bound KRAS protein [36, 37].

KRAS mutations are highly prevalent in NSCLC, being present in approximately one third of lung adenocarcinomas [33, 38, 39]. However, while in other tumour types (e.g. colorectal cancer) *KRAS* mutations were directly linked to patient survival [40, 41], the prognostic relevance of *KRAS* mutations in NSCLC is unclear [42], highlighting our lack of understanding of the oncogenic impact of these mutations in lung cancer. The development of mutant Kras lung tumour mouse models [43-46] helped define *Kras* mutations as oncogenic drivers in lung

adenocarcinoma. Indeed, independent groups showed that the activation of a single, endogenously regulated mutant *Kras* allele (*Kras*^{G12V} [44]; *Kras*^{G12D} [43]) is sufficient to promote lung tumour initiation (adenoma) in mice. Additional mutations are nevertheless required to promote the development of lung adenocarcinoma. This progression can be accelerated in these models by combining two most frequent genetic alterations found in human lung adenocarcinoma [33]: mutant Kras activation and Trp53 (p53) inactivation/loss [47].

Mutant Kras-driven lung tumour mouse models are widely used in mechanistic studies of tumour development and pre-clinical trials, for their ability to closely recapitulate the human disease. Since lung lesions from *Kras*^{mutant}*;p53*$^{-/-}$ mice progress rapidly, this genetic model is frequently used to study lung adenocarcinoma phenotypes. In contrast, lung tumours from mutant Kras mice (that retain wild-type p53) typically only mimic early disease stages (hyperplasias, adenomas). If allowed to age, these lesions can nevertheless evolve to adenocarcinomas, at which stage p53 is frequently inactive [43, 46, 47]. Given that p53 can have a major impact on cellular metabolism (e.g. it can inhibit glucose uptake and glycolysis, promote mitochondrial respiration, regulate oxidative stress) [48, 49], the metabolic distinctions between these models are potentially significant and should be considered in the interpretation of mutant Kras lung tumour metabolic findings derived from distinct models.

The progression of mutant Kras-driven murine lung tumours to adenocarcinoma can be accelerated by alternative cooperating mutations, namely loss of the Liver kinase B1 (Lkb1, also known as Stk11) [50, 51]. Lkb1 mediates mTOR signalling via activation of AMPK (AMP-activated protein kinase), a key metabolic sensor [52-55]. Hence, similarly to p53, Lkb1 loss can have a direct effect on the metabolic phenotypes of mutant Kras tumours. The use of different variations of these and other murine models is enabling the identification of the metabolic changes that mutant *Kras* lung tumours undergo during their development, and their potential therapeutic implications.

Metabolic rewiring in murine mutant Kras lung tumours

Mutant Kras activity leads to increased proliferation in multiple modelling systems both *in vitro* and *in vivo*. Thus, unsurprisingly, mutant Kras cancer cells exhibit metabolic signatures typically associated with high energetic and anabolic needs, including enhanced nutrient uptake and rewiring of their metabolism, as well as increased autophagy and macropinocytosis, relative to non-mutant cells [36, 56]. Glucose metabolic flux analysis carried out independently by different groups revealed clear similarities between mutant *Kras;p53*$^{-/-}$ murine lung adenocarcinomas [21, 57] and human NSCLC metabolism [23, 27, 28], confirming the relevance of these autochthonous murine models for the study of human lung cancer metabolism. Among these shared phenotypes are enhanced glucose uptake and its processing through glycolysis and the TCA cycle (Figure 2). Crucially, murine models are also uncovering novel metabolic properties of mutant Kras lung tumours, as well as new layers of metabolic heterogeneity. These findings are summarised below.

- **Amino acid utilization**

Multiple *in vitro* studies suggest a major role for glutamine utilization in cancer metabolism. In agreement, human pancreatic ductal adenocarcinoma (PDAC) cell lines and subcutaneous xenograft models were shown to rely on glutamine metabolism rewiring to maintain redox balance. In these models glutamine derived aspartate was converted into oxaloacetate (in an aspartate transaminase-dependent manner), which was then further processed into malate and pyruvate, increasing the NADPH/NADPH+ ratio in cells [58]. To what extent spontaneous PDAC may rely on this metabolic rewiring is however unclear.

However, unlike the majority of tumour cells grown in culture and PDAC xenografts [56, 58, 59], glutamine utilization was shown to be minimal in murine *p53*$^{-/-}$*;Kras*^{G12D} lung tumours and not increased relative to normal lung tissue [57]. These data are consistent with human NSCLC findings, where no preferential glutamine utilization was observed in tumours relative to the surrounding lung [28]. These data indicate that glutamine is not a major contributor to

lung cancer metabolism. Moreover, these findings emphasize the relevance of *in vivo,* autochthonous models for the study of tumour phenotypes and suggest that metabolic signatures identified *in vitro* may fail to recapitulate *in vivo* phenotypes. These discrepancies are likely due to the effects of tissue culture stress, non-physiological levels of nutrients and absence of tumour cell extrinsic factors (e.g. tumour microenvironment) in these cultures.

Amino acid metabolism is nevertheless altered in $Kras^{G12D/+};p53^{-/-}$ lung tumours. In particular, increased uptake of branched-chain amino acids was observed in these adenocarcinomas and conversely, lung tumour bearing animals exhibited decreased levels of branched-chain amino acids in their plasma [25]. Branched-chain amino acids, such as leucine and valine, are involved in multiple metabolic and cellular processes, and flux analysis in $Kras^{G12D/+};p53^{-/-}$ lung tumours demonstrated their utilization in tissue protein biosynthesis and as a nitrogen source (Figure 2). Depletion of the branched-chain amino acid transaminase *Bcat1* and *Bcat2* genes impaired the ability of lung tumour cell lines to form subcutaneous and orthotopic tumours in recipient mice, suggesting that branched-chain amino acid transamination is required for lung tumour development. Interestingly, these phenotypes were not recapitulated in pancreatic tumours driven by the same oncogenic drivers, providing direct evidence of tumour specificity regarding metabolic dependences.

- **Lipid biosynthesis and β-oxidation**

In vivo models indicate that lipid metabolism plays an important role in the development and maintenance of mutant Kras lung tumours. Acyl-coenzyme A (CoA) synthetase long-chain family member 3 (Acsl3) plays a key role in lipid metabolism by converting fatty acids into fatty Acyl-CoA esters, the substrates for lipid synthesis and β-oxidation. Acsl3 was found upregulated in mutant Kras tumours (*Tet-op-*$Kras^{G12D}$ model [60]) compared to normal lung tissue, and is also highly expressed in human lung cancers [61]. While *Acsl3*-loss had no deleterious effects on normal lung, *LSL-*$Kras^{G12D}$*;Acsl3*$^{-/-}$ mice showed a decrease in the number of lung lesions, and the ones that formed were more benign than those from *LSL-*

Kras^{G12D} mice. These data indicate that Acsl3 activity is important for mutant-Kras driven lung tumour initiation.

Lipid metabolism was also enhanced in advanced mutant Kras lung tumours. Deuterium-labelling experiments suggest that lung adenocarcinomas from two distinct spontaneous mutant Kras models (*p53*$^{-/-}$ and *Lkb1*$^{-/-}$) exhibit high levels of *de novo* fatty acid synthesis [62] (Figure 2). Moreover, these tumours showed a high dependence on Acetyl-CoA carboxylase, (the rate limiting enzyme for fatty acid synthesis [63]) for their maintenance. Accordingly, genetic depletion and pharmacological inhibition of Acetyl-CoA carboxylase inhibited tumour growth in xenografts and autochthonous murine lung tumour models (*p53*$^{-/-}$*;Kras*$^{G12D/+}$; and *Lkb1*$^{-/-}$*;Kras*$^{G12D/+}$) [62].

Autophagy inhibition, through loss of the autophagy related gene 7 (*Atg7)*, also exposed a potential lipid dependence of *Kras*$^{G12D/+}$*;p53*$^{-/-}$ lung tumours. Autophagy inhibition had a major therapeutic effect in this model by blocking tumour growth, inducing cell death, and converting existing lesions into benign oncocytomas [64]. These oncocytomas exhibited damaged mitochondria and lipid accumulation, suggestive of defective fatty acid oxidation. Interestingly, lipid accumulation was only seen in tumours that lacked p53, while the oncocytoma phenotype was also observed in the *Kras*$^{G12D/+}$ model. It is unclear how p53-loss may contribute to this lipid accumulation or if disruption of oxidation of other substrates (i.e. glucose) contributed to these phenotypes. Nevertheless, these data show a differential regulation of lipid metabolism in tumours that lack p53, which are typically more advanced, suggesting that metabolic rewiring may co-evolve with tumours.

- **Glucose metabolism and lung tumour progression**

As discussed above, both murine and human lung tumours show evidence of glucose metabolism rewiring. However, our lab recently showed that in *Kras*^{G12D}*;p53*$^{-/-}$ lung tumours, glucose metabolism is significantly distinct in low and high grade lesions, and modulated by mutant *Kras* allelic content [21]. These data imply that tumour grade and/or mutant *Kras*

content can significantly contribute to the metabolic heterogeneity of mutant Kras lung tumours.

As lung tumours from *Kras*$^{G12D/+}$;*p53*$^{-/-}$ mice progress from low to high grade they frequently acquire additional copies of the mutant allele (*Kras*^{G12D}/*Kras*$^{wild\text{-}type}$ alleles > 1). Mutant *KRAS* allelic gains are also frequent in human lung cancer and NSCLC cell lines. Indeed, ~49% of mutant KRAS NSCLC cell lines are homozygous for the mutant copy [21, 65, 66]. We and others previously showed that these mutant gene copy gains can lead to p53 activation and may contribute to p53 counter-selection in lung cancer (Figure 3) [67, 68]. Our recent data show that these copy gains also affect glucose metabolism and redox management. Indeed, *Kras*$^{G12D/G12D}$;*p53*$^{-/-}$ cells showed increased expression of glycolytic genes and altered glucose metabolism relative to *Kras*$^{G12D/+}$;*p53*$^{-/-}$ cells. Glucose flux analysis revealed that homozygous mutant Kras cells underwent a glycolytic switch, which was coupled to increased channelling of glucose-derived metabolites into the TCA cycle and towards the biosynthesis of glutathione [21], a key cellular antioxidant [69] (Figure 2 and 3). A similar metabolic rewiring was observed in high grade lung tumours, which acquire extra copies of mutant *Kras*. In contrast, low grade tumours, which remain heterozygous for mutant Kras, showed a similar glucose-derived carbon flux to that seen in normal tissues, providing evidence of metabolic rewiring during the malignant progression of this lung tumour model.

Mutant *KRAS* content-dependent differences in glucose metabolism or in the expression of glycolytic and glutathione biosynthesis genes were also observed in NSCLC cell lines and human lung adenocarcinoma, respectively [21]. It is however unclear what factors drive the selective pressure for these mutant gains or the resulting metabolic rewiring. Murine models showed no difference in the proliferative capacity of mutant Kras heterozygous and homozygous cells, suggesting that other factors may initially promote the outgrowth of mutant Kras homozygous cells. The increased antioxidant capacity of homozygous mutant Kras cells [21] may directly contribute to their selective growth during lung tumour progression. However, the effects of ROS on lung tumour development remain somewhat controversial, as discussed

below. Extrinsic factors, such as presence/absence of particular stromal cells, variations in nutrient availability [23] or varying oxygen levels within the growing tumour mass may also contribute to this selection. In support of the latter, enhanced expression of the hypoxia-inducible factor Hif2a (*Hif2a* knock-in) promoted tumour growth and malignant progression of $Kras^{G12D}$ lung tumours [70].

ROS management in mutant Kras lung tumours

Multiple studies have shown an effect of Kras mutations on ROS management *in vivo* but the implications of these effects varied depending on experimental context. On one hand, it has been proposed that mutant Kras can promote ROS generation [71, 72]. A pro-tumourigenic effect of mutant Kras-mediated ROS induction in pancreatic cells was also proposed. Accordingly, upregulation of mitochondrial ROS generation by endogenously regulated $Kras^{G12D}$ in pancreatic acinar cells was shown to promote the formation of precancerous lesions [73]. In contrast, DeNicola and colleagues showed that endogenously expressed mutant Kras can reduce oxidative stress through the induction of the key antioxidant programme regulator Nfe2l2 (Nrf2) [22]. Furthermore, *Nrf2* loss inhibited the development of benign lung tumour lesions in $Kras^{G12D/+};Nrf2^{-/-}$ mice, demonstrating that the antioxidant activity of this gene can promote mutant Kras-driven lung tumour initiation. Together, these data indicate that the effects of mutant Kras on ROS production are tissue specific; or that oncogenic Kras regulates redox management through modulation of both ROS generation and scavenging.

In support of a lung tumour promoting role for reducing agents, ROS inhibition through administration of the antioxidants N-acetylcysteine (NAC) and Vitamin E significantly enhanced lung tumour burden and decreased the survival of (p53 wild-type) $Kras^{G12D/+}$ mice [19]. NAC and Vitamin E treatment contributed to lung tumour development by reducing ROS, DNA damage and promoting tumour proliferation but interestingly, these tumour promoting effects were lost in $p53^{-/-}$ mice. The authors concluded that antioxidant treatment promoted

tumour growth by preventing p53-mediated anti-proliferative responses [19]. It is however unclear how tumour ROS levels were affected by p53 loss or antioxidants in this context.

ROS regulation by p53 is complex and poorly understood in the context of mutant Kras. On one hand p53 can promote antioxidant responses through different mechanisms. However, when levels of damage are high, p53 can induce a pro-oxidant state that leads to cell death [48]. Under normal conditions inactivation of p53 can, in principle, result in enhanced ROS but it would be important to establish to what extent mutant Kras activity may modulate this effect in lung tumours. The enhanced levels of the glutathione and its precursors (glutamate, serine and glycine) seen in advanced *Kras*^{G12D};*p53*$^{-/-}$ lung tumours [21] suggests that efficient antioxidant strategies may be actively selected for within this genotype, which in turn could diminish the potential effect of antioxidant treatment in these tumours.

Data from human NSCLC reinforces the notion that enhanced ROS management is positively selected during lung tumour progression (Figure 3). Mutations in KEAP1, a NRF2 inhibitor are present in ~20% of human lung adenocarcinomas [33]. Moreover, NRF2 signalling was shown to regulate the expression of serine biosynthesis genes in human NSCLC cells and enhanced expression of these genes correlated with poor overall survival [74], suggesting that NRF2 activity may directly contribute to lung cancer malignant progression.

It is unclear whether improved ROS management contributes to tumour progression by simply increasing cellular fitness/survival under high ROS conditions [75-77] or through other mechanisms. Studies in melanoma showed that cells in the blood and visceral organs experience increased levels of oxidative stress relative to established subcutaneous tumours [78]. Metastatic cells may therefore need to be able to cope with high ROS levels and thus, improved ROS management may increase the metastatic potential of tumour cells. In agreement, *p53*-null;*Kras*$^{G12D/G12D}$ lung tumour cells, which showed lower ROS and increased antioxidant capacity relative to heterozygous cells, were more metastatic in transplantation assays than *p53*-null;*Kras*$^{G12D/+}$ cells [21]. Notably, in other contexts, increased ROS levels

were shown to promote metastasis [79-81], suggesting that altered redox management may provide distinct benefits to different cells.

Interestingly, the ROS levels of mutant Kras lung tumours can vary significantly depending on their additional driver mutations or histological subtype. Accordingly, in *Kras*$^{G12D/+}$*;Lkb1*$^{-/-}$ mice, ROS levels were shown to increase during lung adenocarcinoma progression, and the corresponding adenocarcinomas exhibited significantly higher ROS levels, than those from *Kras*$^{G12D/+}$*;p53*$^{-/-}$ animals [53]. Expression of *Nrf2* and its target *Nqo1* were conversely significantly lower in Lkb1-deficient adenocarcinomas relative to p53$^{-/-}$. Besides adenocarcinomas, *Kras*$^{G12D/+}$*;Lkb1*$^{-/-}$ animals also develop lung squamous cell carcinomas (SCC) and adeno-squamous cell carcinomas and remarkably, ROS levels were significantly lower in SCC relative to adenocarcinomas. These data suggest that changes in ROS levels can modulate adenocarcinoma to SCC transdifferentiation, and this plasticity can potentially affect therapeutic responses.

Collectively, the abovementioned studies show that redox management plays an important role in mutant KRAS lung cancer, potentially modulating progression mechanisms and therapeutic responses.

Targeting the metabolism of mutant Kras lung tumours

As mutant Kras remains clinically undruggable and its prognostic value in lung cancer unclear, the status of this locus in lung tumours is not even routinely assessed outside the clinical trial setting [42]. Recent progress regarding the targeting of the G12C mutant KRAS isoform with small molecule inhibitors has renewed hope that the clinical relevance of mutant KRAS status may soon change [82, 83]. If indeed effective, these inhibitors will be of particular interest for the treatment of lung adenocarcinoma, where G12C is the most frequent KRAS mutation [84]. The recently described metabolic phenotypes of mutant Kras tumours provide a novel and appealing alternative targeting opportunity. Metabolic targeting strategies that have been pre-

clinically validated in lung models are discussed below and summarised in Figure 4.

Reassuringly, mouse models suggest that targeting liabilities inherent to mutant KRAS metabolic rewiring may constitute a feasible “synthetic lethal” approach. The high glucose dependence of advanced mutant Kras lung tumours is a major area of interest. Due to their reliance on glucose metabolism for enhanced antioxidant potential, high grade lung tumours from $Kras^{G12D/+};p53^{Fx/Fx}$ mice showed a high sensitivity to combined glucose and glutathione depletion [21]. The same treatment had no significant effect on low grade tumours of the same model, where glucose metabolism was more comparable to that of normal tissues. While this heterogeneity is not ideal from a tumour-response perspective, it indicates that there is likely a therapeutic window for targeting glucose metabolism rewiring in these tumours when normal tissue can be spared. Importantly, since this rewiring was associated with high grade tumours, these therapeutic strategies have the potential to target a significant proportion of human lung tumours, as these are typically diagnosed late.

Glucose depletion may nevertheless pose significant toxicity risks in the clinic. Instead, a better efficiency to tumour specificity balance may be achieved through the targeting of tumour-specific phenotypes associated with this metabolic reprogramming. Multiple glycolytic genes are upregulated in lung adenocarcinoma, representing potential targets, including Glucose transporter type 1 (*SLC2A1* or *GLUT1*), Phosphoglycerate kinase 1 (*PGK1)*, Enolase (*ENO1*), Phosphofructokinases (*PFKL/PFKM),* Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH),* Pyruvate kinase *(PKM2)* and lactate dehydrogenase *(LDH)*. Given that increased expression of SLC2A1, PGK1, ENO1, GAPDH and PKM2 correlate with poor prognosis [85-89], inhibition of these proteins may be particularly beneficial. In agreement, inhibition of SLC2A1 and PKM2 showed promising results in NSCLC cells *in vitro* and xenograft models [90-92].

Hexokinase 2 (Hk2) knockout models suggest that the inhibition of this key glycolytic enzyme may also provide therapeutic benefit to mutant Kras lung tumours. Accordingly, tumour latency

of $Kras^{G12D/+}$ mice was increased upon Hk2 loss [93], while systemic ablation of Hk2 decreased the proliferation of established $Kras^{G12D}$-driven lung tumours. The conversion of pyruvate to lactate by Ldh enzymes has also been targeted *in vivo* in spontaneous $Kras^{G12D}$-driven lung tumours [94]. Ldha depletion or inhibition resulted in decreased tumourigenesis and regression of established lesions, suggesting a role for Ldha in tumour initiation and proliferation. Interestingly, the main metabolic changes observed *in vivo* were decreased lactate fermentation and glutathione synthesis, suggesting that either or both metabolites are relevant for tumour initiation.

Oxidative pathways represent additional opportunities for lung cancer targeting. In light of the effects of autophagy inhibition on mitochondrial functionality and tumour maintenance [64, 95] (discussed above), the direct targeting of mitochondrial activity may be therapeutically relevant. In agreement, phenformin, a mitochondrial complex I inhibitor showed promising (albeit genotype-specific) therapeutic efficacy, decreasing tumour burden in $Kras^{G12D/+}$;$Lkb1^{-/-}$ mice (but not in $Kras^{G12D/+}$;$p53^{-/-}$) [96]. Moreover, tissue-specific deletion of the mitochondrial transcription factor A (*Tfam*), which is required for mitochondrial DNA replication and transcription, inhibited lung tumour formation and growth in $Kras^{G12D/+}$ mice [72]. The multiple mutant Kras pre-clinical models discussed above demonstrate that mutant Kras tumours also exhibit unique dependences regarding fatty acid synthesis and oxidation, TCA-dependent glucose metabolism, and branched-chain amino acid utilization, which can potentially be exploited therapeutically [21, 25, 61, 62, 64].

While the unique metabolic vulnerabilities of mutant KRAS tumours represent important opportunities for therapeutic targeting, their metabolic heterogeneity can potentially influence therapeutic responses beyond direct metabolic targeting. Hence, it would be important to determine to what extent lung tumour metabolic heterogeneity may affect the efficacy of standard therapy and subsequently, whether it can be involved in resistance to conventional cancer treatments.

The tumour microenvironment perspective

Data from different groups point to significant metabolic heterogeneity within mutant Kras tumours. This heterogeneity is seen between mutant Kras tumours from different tissues (pancreatic and lung tumours of a comparable genotype) [25]; lung tumours *in vivo* and their corresponding cell lines *in vitro* [57]; and low and high grade lung tumours within the same model [21]. While some of this metabolic variation may be tumour cell autonomous, microenvironmental factors are likely important players in this heterogeneity.

The tumour microenvironment is now increasingly recognised as a crucial element in tumour development, maintenance and response to therapy. The lung tumour stroma consists of multiple non-cancerous cells, such as immune cells and fibroblasts, which can provide structural support, but also immune protection and even promote invasion and metastasis [50, 97]. Stromal cells can affect tumour cell metabolism through a plethora of mechanisms, including competition for nutrients [98]; provision of alternative metabolic substrates [99] or modulation of tumour cell signalling via cell to cell contacts [98-100]. This interplay between stroma and tumour cells can thus potentially contribute another layer of complexity and heterogeneity to tumour metabolism, but it remains poorly studied in the context of mutant Kras lung tumours.

The tumour microenvironment is also of relevance from a lung tumour therapy perspective. Indeed, there is strong evidence that targeting the tumour-stroma crosstalk can be beneficial for lung cancer patients, with immune checkpoint inhibitors (anti-PD-L1, anti-PD-1) already showing therapeutic promise in advanced NSCLC [101-103]. However, resistance to these checkpoint antagonists has also been reported and the potential effects of tumour metabolism heterogeneity should be considered in this context. For instance, since altered metabolic programs can affect T-cell fate and differentiation [104, 105], tumours with distinct metabolic properties can potentially modulate T-cell responses differently ([106-108]), increasing the likelihood of resistance to treatment. Likewise, agents that target tumour cell metabolism may

yield unexpected results due to their potential effects on stroma cells and stroma-tumour cell communication. *In vivo* models will likely prove invaluable in the evaluation of stromal effects on metabolic targeting and resistance to therapy in lung tumours.

Closing remarks

The development of tools that enable the analysis of metabolic flux *in vivo* revolutionised the cancer metabolism field. Rather than a simplistic, unified model, we are now confronted with tumours as evolving metabolic entities, where both general and context-specific metabolic requirements need to be considered. This metabolic heterogeneity may in fact have contributed to differential therapeutic responses in seemingly comparable tumours, confounding our assessments of their efficacy. Despite this intrinsic heterogeneity, the therapeutic potential of metabolic targeting remains high, as demonstrated in the multiple *in vivo* mutant Kras lung tumour studies discussed here. Improved understanding of the unique metabolic dependencies of lung tumours *in vivo* is essential for the design of therapies that efficiently target this heterogeneous and evolving disease. By exposing such novel and much needed vulnerabilities in the large proportion of NSCLC affected by *KRAS* mutations we may finally be able to change the course of this often deadly disease.

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Figure legends

Figure 1: Human NSCLC metabolism based on *in situ* glucose flux analysis.

Representation of glucose-derived carbon flux in human NSCLC following ^{13}C-glucose infusion [23, 27, 28]. Metabolites referred to in the main text (*), as well as others discussed in the references are indicated. Enhanced glucose metabolism (orange) and alternative pathway fuels (purple) are shown. PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase.

Figure 2: Metabolic rewiring in lung tumours from *Kras$^{G12D/+}$; p53$^{-/-}$* mice.

Schematic representation of metabolic networks in lung tumours from Kras$^{G12D/+}$;p53$^{-/-}$ mice based on *in vivo* flux/tracing analyses. Enhanced flux from different labelled substrates is shown as indicated, with orange depicting glucose metabolism (based on ^{13}C-Glucose [21, 57]); green: amino acid metabolism (^{13}C-Leucine/Valine; solid), ^{15}N-Leucine; dashed [25]); and purple: fatty acid metabolism ($^{2}H_2O$ [62]). Grey arrow depicts similar flux relative to normal tissue [57]. Slc2a1, Glucose transporter 1; Acc, Acetyl-Co A carboxylase; Gcs, glutamylcysteine synthetase; Pdh, pyruvate dehydrogenase; Pc, pyruvate carboxylase.

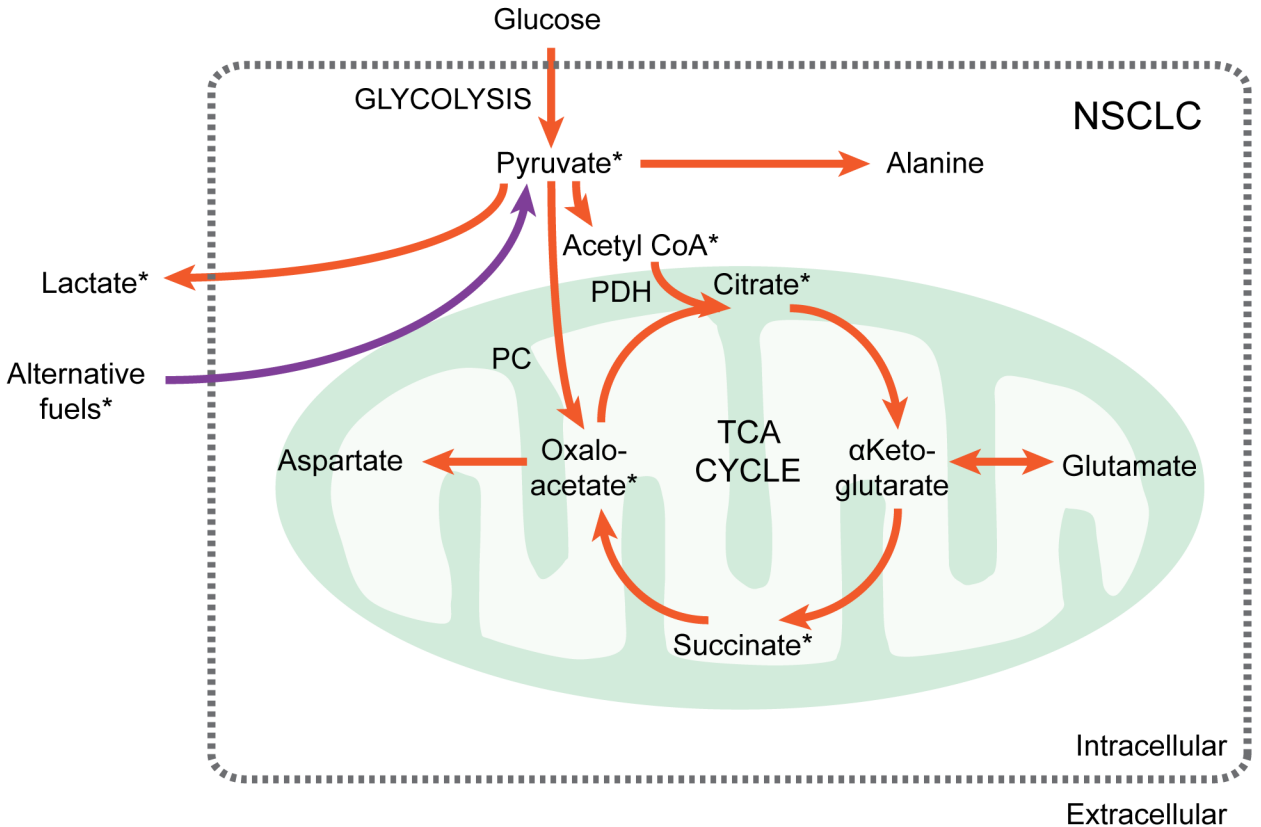
Figure 3: Metabolic reprogramming during mutant *Kras*-driven lung tumour progression.

Representation of cellular phenotypes altered during the progression of low (adenoma, Grade I and II adenocarcinoma) to high grade (Grade III and IV adenocarcinoma) lung tumours in *Kras$^{G12D/+}$;p53$^{-/-}$* mice.

Figure 4: Metabolic targets of mutant Kras lung tumours validated *in vivo.*

Schematic representation of metabolic targeting strategies that showed therapeutic efficacy in spontaneous mutant Kras lung tumours (GEMM KO and/or pharmacologic inhibition (*)) or NSCLC cell line xenografts. Metabolites and enzymes are shown, with genes overexpressed in human NSCLC samples indicated in red. Targeting approach and method of inhibition are shown in blue. ETC: Electron Transport Chain; 2DG: 2-deoxyglucose; BSO: Buthionine Sulfoximine; GEMM KO: Genetically engineered mouse model knock-out; CRISPR KO: Clustered regularly interspaced short palindromic repeats knock-out; shRNA: short-hairpin RNA; SMI: Small molecule inhibitor.

Figure 1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Lactate*
PDH
Citrate*
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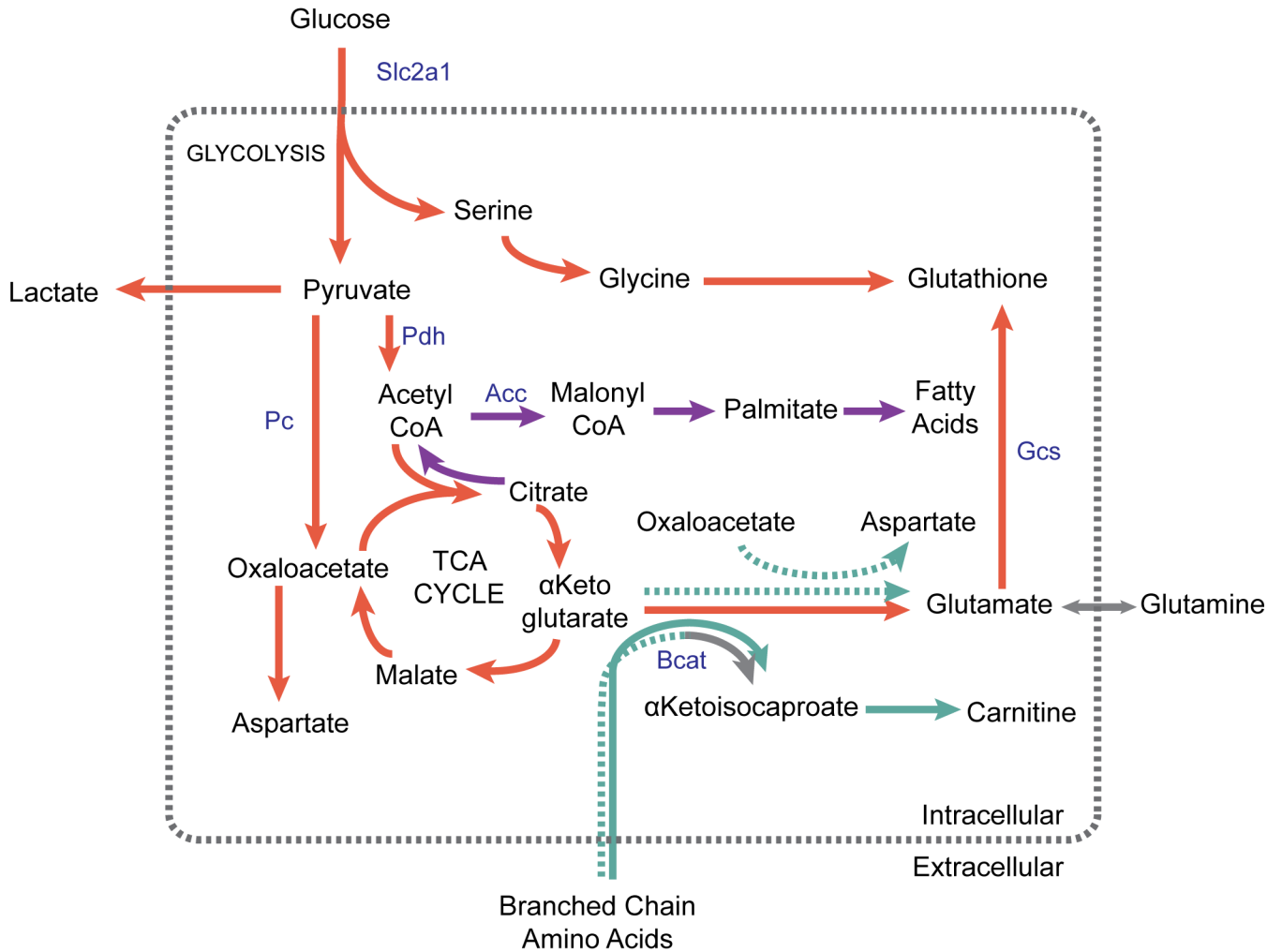
TCA CYCLE
αKeto-glutarate
Glutamate
Succinate*
Intracellular
Extracellular

Figure 2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Serine
Lactate
Pyruvate
Glycine
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Pdh
Pc
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Acc
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Palmitate
Fatty Acids
Gcs
Citrate
Oxaloacetate
Aspartate
Oxaloacetate
TCA CYCLE
αKeto glutarate
Glutamate
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Carnitine
Aspartate
Intracellular
Extracellular
Branched Chain Amino Acids

Figure 3

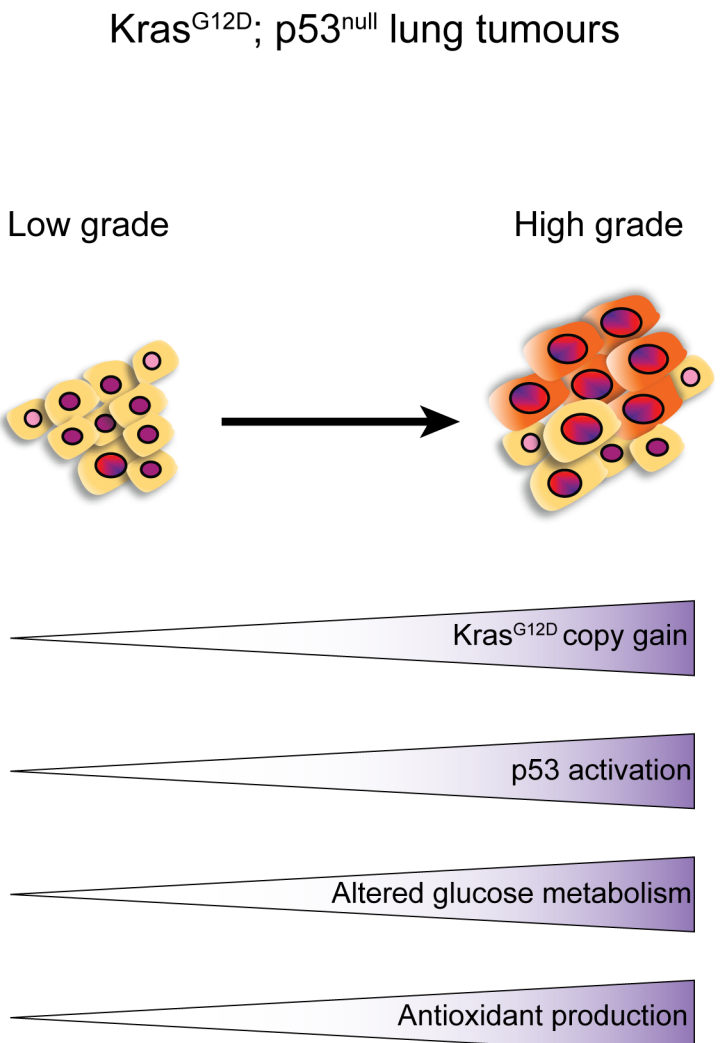
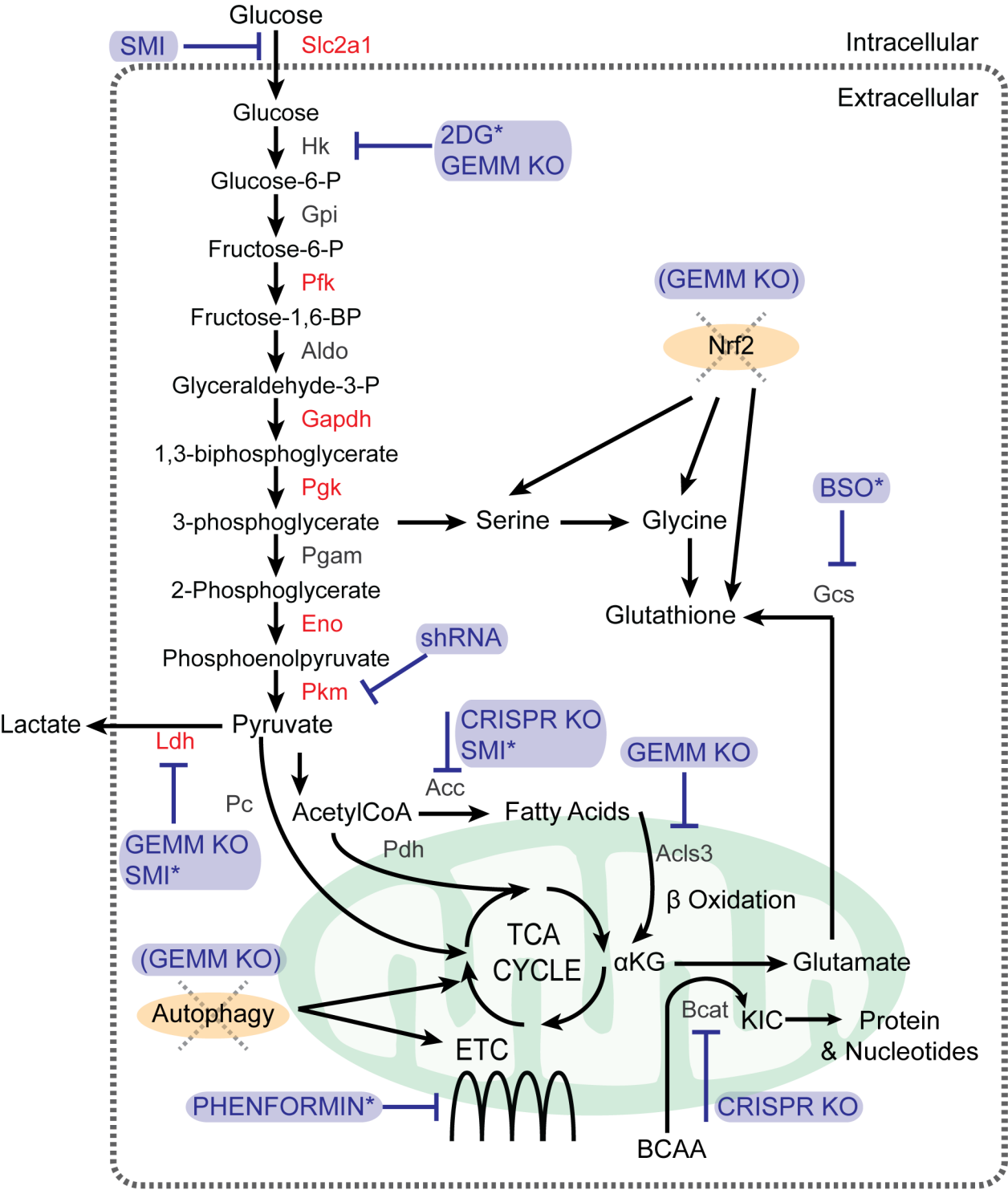

Kras^{G12D}; p53^{null} lung tumours
Low grade
High grade
Kras^{G12D} copy gain
p53 activation
Altered glucose metabolism
Antioxidant production

Figure 4


Glucose
SMI
Slc2a1
Intracellular
Extracellular
Glucose
Hk
2DG*
GEMM KO
Glucose-6-P
Gpi
Fructose-6-P
Pfk
Fructose-1,6-BP
Aldo
Glyceraldehyde-3-P
Gapdh
1,3-biphosphoglycerate
Pgk
3-phosphoglycerate
Serine
Glycine
Pgam
2-Phosphoglycerate
Eno
Phosphoenolpyruvate
shRNA
Pkm
Pyruvate
Lactate
Ldh
GEMM KO
SMI*
Pc
AcetylCoA
Acc
CRISPR KO
SMI*
Fatty Acids
GEMM KO
Acls3
Pdh
β Oxidation
TCA
CYCLE
αKG
Glutamate
(GEMM KO)
Nrf2
BSO*
Gcs
Glutathione
(GEMM KO)
Autophagy
ETC
Bcat
KIC
Protein
& Nucleotides
PHENFORMIN*
CRISPR KO
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