Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth

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Introduction

Several decades ago Otto Warburg first described that tumors exhibit glycolytic metabolism with a reduced rate of oxidative phosphorylation, despite the availability of adequate oxygen. This phenomenon, known as the ‘Warburg effect,’ has been proposed to be key driver of tumor progression (Warburg, 1956). Since then, several researchers have observed altered glucose metabolism associated with a high rate of lactate secretion in cancer cells and tumor tissues (Sauer et al, 1982; Steinberg et al, 1992). Furthermore, aerobic glycolysis represents a robust hallmark of cancer that is employed for tumor detection by positron emission tomography analysis with the 2-[18F]fluoro-2-deoxy-D-glucose tracer (Laing et al, 2009). However, we and others have recently provided evidence on the important anabolic role of glutamine in tumor cell proliferation (DeBerardinis et al, 2007; Yuneva et al, 2007; Gaglio et al, 2009). Indeed, by using different experimental approaches, we have also shown that glycolytic cancer cells consume more glutamine as compared with their normal counterparts to synthesize proteins, nucleotides and fatty acids and produce energy (Kovacevic and McGivan, 1983; Baggetto, 1992; DeBerardinis et al, 2007).

Several reports suggest that both nutrient uptake changes and metabolic alterations are under direct control of ras or myc oncogenes (Chiaradonna et al, 2006b; Wise et al, 2008; Vander Heiden et al, 2009). In particular, oncogenic Ras proteins, identified in 25% of human cancers (Bos, 1989), correlate with metabolic alterations, including increased glucose and glutamine consumption, lactic acid accumulation, altered expression of mitochondrial genes, increased reactive oxygen species (ROS) production and reduced mitochondrial activity (Bos, 1989; Vizan et al, 2005; Chiaradonna et al, 2006a; Yun et al, 2009; Weinberg et al, 2010). A result of this metabolic reprogramming is the dependence of K-Ras transformed cells on glucose and glutamine availability, since their withdrawal induces apoptosis and cell-cycle arrest, respectively (Ramanathan et al, 2005; Telang et al, 2006; Yun et al, 2009). However, the precise metabolic effects downstream of oncogenic Ras signaling in cancer cells have not been completely elucidated.

Due to the interconnected nature of metabolic pathways and the pleiotropic effects mediated by oncogenic signals, a
systems approach is required to elucidate the mechanisms of such transforming events. To date, most large-scale analyses of tumor cells have employed microarray technology that provides a robust means of analyzing transcriptional modifications associated with physio/pathological changes (Ross et al, 2000; Scherf et al, 2000). However, various levels of post-transcriptional controls may not be captured by these analyses (Mata et al, 2005; Metallo and Vander Heiden, 2010), and new approaches are now emerging to increase our knowledge about cancer cell physiology (Liotta and Petricoin, 2000; Laubenbacher et al, 2009; Kreeger and Lauffenburger, 2010).

Metabolomic techniques offer a more direct means of studying metabolism at the systems level. Measuring metabolite concentrations, indeed, represents a sensitive approach that generates detailed `snapshots' of biological processes (Hiller et al, 2009). Nevertheless, metabolic routes are best characterized by the measurement of fluxes, which describe the actual functionality of a given enzyme or pathway (Stephanopoulos and Vallino, 1991; Sauer, 2006). To this end, isotopic tracers and computational algorithms enable the quantitative estimation of intracellular fluxes and associated confidence intervals for a given system (Metallo et al, 2009; Hiller et al, 2010), and such methods are now effectively applied to mammalian cells (Vizan et al, 2005; Munger et al, 2008).

Therefore, to better understand the regulation of cancer cell metabolism and to identify key metabolic routes altered in K-Ras transformed cells, we have applied a systems-level approach based on the combined application of metabolic and transcriptional analyses. We have monitored the flux of 13C-labeled glucose and glutamine as well as [15N]glutamine into downstream metabolites in normal and transformed cells and performed a detailed comparison with the transcriptional profiles obtained from the same cell lines. Herein, we show that K-ras oncogene expression enhances glucose uptake but decreases its utilization in the tricarboxylic acid (TCA) cycle and associated anabolic pathways. Furthermore, we show that while K-Ras transformation decreases overall flux through the TCA cycle, it increases utilization of the carbon backbone and amino-nitrogen moieties of glutamine either through TCA cycle or transamination activities in order to sustain biosynthetic reactions, including amino-acid, nucleotide and glutathione synthesis. Finally, we present evidence describing the dependence of K-Ras on glutamine metabolism, as inhibition of key enzymes along this pathway specifically compromises the proliferation of transformed cells.

Results

K-Ras transformation enhances glycolytic flux and decreases oxidative TCA cycle flux in mouse fibroblasts

We have previously shown that transformed NIH3T3 mouse fibroblasts expressing an oncogenic K-Ras protein (G12V) (Shih et al, 1981; Pulciani et al, 1982; Bossu et al, 2000) exhibit elevated sensitivity to glucose availability, reduced mitochondrial function linked to a decrease of Complex I activity and protein level, and reduced proliferation in response to glucose, glutamine or galactose shortage as compared with immortalized NIH3T3 mouse fibroblasts (Chiaradonna et al, 2005, 2006a; Gaglioti et al, 2009; Baracca et al, 2010). NIH3T3 cells are a genetically well-characterized immortalized cell line that has long been established as a model of ‘normal’ cells for the study of cell transformation, as these cells undergo contact inhibition, exhibit no growth in soft agar, and do not form tumors in immunocompromised mice, in contrast to isogenic transformed lines (Bossu et al, 2000).

Therefore, we performed basic metabolic analyses to compare NIH3T3 cells (normal, N) and NIH3T3 K-Ras (transformed, T) 54 h (T 34) after plating. This time point was chosen since it corresponds to the time in which the two cell lines begin to show a different proliferative ability (Chiaradonna et al, 2006b). As shown in Figure 1A, cell-specific glucose (Glc) and glutamine (Gln) consumption as well as lactate (Lac) and glutamate (Glu) secretion were measured. Significant increases in Glc uptake and Lac production in T cells were observed (Figure 1A), consistent with other reports linking ras mutations to aerobic glycolysis (Eistrom et al, 2004; Vizan et al, 2005). Although previous studies have shown an increase of Gln uptake in other kinds of cancer cell lines (Yuneva et al, 2007; Wise et al, 2008; Gao et al, 2009), we did not detect any significant changes in Gln uptake and Glu secretion between the two cell lines. To further define N- and T-cell metabolism, we have determined the relative abundance of various intracellular amino and organic acids by performing a targeted metabolomic analysis (Figure 1B). Notably, metabolites associated with glycolysis and biosynthetic processes were present in larger quantities in T cells, including pyruvate (Pyr), Lac, citrate (Cit), and aspartate (Asp). On the other hand, TCA cycle intermediates such as α-ketoglutarate (AKG), succinate (Suc), and fumarate (Fum), which arise primarily from oxidative metabolism, were less abundant in T cells as compared with N cells.

Isotopic tracers provide an effective means of determining the relative contribution of a substrate to a particular metabolic pathway, and mass spectrometry enables quantification of the relative abundance of molecules with different numbers of isotopic label in each metabolite pool (i.e., M0, M1, M2, ... where M2 abundance includes all molecules with two 13C-labeled atoms). For instance, uniformly labeled Glc ([13C6]glucose) is converted to M3 Pyr (with three 13C atoms) and subsequently M2 Cit through the combined action of pyruvate dehydrogenase and citrate synthase. In the same way, M2 Cit, progressing along the TCA cycle, generates M4 AKG and Glu by TCA cycle-related reactions (i.e., transamination and reductive amination; Supplementary Figure S1). We cultured N and T cells in the presence of 13C-labeled glucose and quantified the mass isotopomer distribution (MID) of Cit and Glu via gas chromatography/mass spectrometry (GC/MS). These data indicated that T cells oxidize less Glc carbon to Cit and Glu, presumably diverting more Pyr to Lac, as evidenced by the significant decreases in M2 labeling observed in these metabolites (Figure 1C).

Consistent with the observation of decreased Glc oxidation, parallel [13C6]glutamine labeling experiments indicated that Glu carbon utilization for metabolic intermediate synthesis (e.g. Asp) was increased in T cells. We specifically observed a relative increase in M3 and M4 Asp abundance (Figure 1D), which are generated from M5 AKG downstream of reductive
carboxylation by isocitrate dehydrogenase and oxidation by oxoglutarate dehydrogenase, respectively (Supplementary Figure S2). These results support the notion that Gln entering the TCA cycle of T cells may follow both the canonical forward oxidative reaction and a reductive carboxylation (Yoo et al., 2008). Moreover, given the significant role of Asp in amino-acid and nucleotide synthesis, these results suggest that T cells are strongly dependent on Gln carbon for biosynthesis (Weinberg et al., 2010).

Mass isotopomer data alone provides a somewhat limited view of cellular metabolism. To better characterize N- and T-cell metabolism at the systems level, we integrated our extracellular uptake and secretion results with MID data obtained by culturing the cells with [U-13C5]glutamine. Using these data, we estimated intracellular fluxes in each cell type using an elementary metabolite unit (EMU)-based 13C metabolic flux analysis (MFA) algorithm (Young et al., 2008). Upon obtaining acceptable fits, we performed a sensitivity analysis to generate 95% confidence intervals for each flux in the network (Antoniewicz et al., 2006; Antoniewicz et al., 2007). Flux results for N and T cells are listed in Table I, and the full dataset and model description are provided in Supplementary information.

Figure 1 K-Ras transformed fibroblasts decouple glycolysis and the TCA cycle. (A) Extracellular uptake and secretion of Glc, Lac, Gln, and Glu in N and T cells for 54 h of growth. (B) Relative metabolite abundances in N and T cells measured by GC/MS. (C) M2 labeling of Cit and Glu in N and T cells cultured with a 1:1 mixture of [1-13C]glucose and [U-13C6]glucose. (D) Mass isotopomer distribution (MID) of Asp in cells cultured in the presence of [U-13C5]glutamine. Error bars indicate s.e.m. (n=3). (E) Schematic representation of the metabolic routes involved in M3-M4 Asp labeling by using [U13C5]glutamine tracer. In the scheme are represented the key cytoplasmic and mitochondrial enzymes involved in these metabolic routes. (F) Absolute expression values of some genes, involved in the pathways described in the scheme (E), in N and T cell lines. The two cell lines, grown in 25 mM Glc + 4 mM Gln (normal medium), were collected for transcriptional analysis at two different time points (48 and 72 h). The list of gene abbreviations is available in Supplementary information.
mentary information and Supplementary Figures S3 and S4. These data indicate that transformation induces a significant reorganization of central carbon metabolism. T cells reduce flux through the PDH complex by ~50% (i.e., flux values of Pyr→AcCoAmit+CO2). Furthermore, net fluxes throughout the TCA cycle were decreased by the same amount. Each of these changes was highly significant, as evidenced by the lack of overlap in confidence intervals between the two cell types. Although most exchange fluxes in reversible reactions of the TCA cycle could not be accurately determined, we observed no significant change in reductive flux through IDH or malic enzyme. Consistent changes in metabolic fluxes were observed when performing MFA using labeling data from cells cultured with13C-glucose (see Supplementary Information for detailed results), though the observability of some fluxes (e.g. IDH exchange flux) was significantly decreased when using these tracers. These findings provide evidence that transformation causes cells to decouple glycolysis and the TCA cycle, with an overall decrease in activity of oxidative reactions within the TCA cycle.

Transcriptional profiling of N and T fibroblasts is largely coherent with metabolic flux analysis Various reports have indicated that oncogenes alter the expression of genes encoding metabolic enzymes (Mathupala et al., 1997; Zhong et al., 1999). To identify oncogenic K-Ras-dependent transcriptional effects, we performed microarray transcriptional profiling of N and T cells grown as above for 48 and 72 h.

Table 1 Exponentially determined fluxes and 95% confidence intervals estimated from MFA model using [U-13C5]glutamine as a tracer

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Flux</th>
<th>Confidence interval</th>
<th>Flux</th>
<th>Confidence interval</th>
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<tr>
<td><strong>Glycolysis</strong></td>
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<td>Glucext→G6P</td>
<td></td>
<td></td>
<td>65.5</td>
<td>61.2, 69.9</td>
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<td>58.2, 66.9</td>
<td>83.1</td>
<td>77.6, 88.6</td>
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<td>18.6</td>
<td>0.0, Inf</td>
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<td>F6P→DHAP+GAP</td>
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<td>59.9, 68.5</td>
<td>82.4</td>
<td>76.9, 88.0</td>
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<tr>
<td>DHAP→GAP</td>
<td>64.2</td>
<td>59.9, 68.5</td>
<td>82.4</td>
<td>76.9, 88.0</td>
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<td>DHAP→GAP</td>
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<td>0.0, Inf</td>
<td>13.1</td>
<td>0.0, Inf</td>
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<tr>
<td>GAP→3PG</td>
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<td>120.6, 137.9</td>
<td>165</td>
<td>154.0, 176.1</td>
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<td>0</td>
<td>0.0, Inf</td>
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<td>3PG→Pyr</td>
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<td>165</td>
<td>154.0, 176.1</td>
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<td>Pyr→Lac</td>
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<td>105.9, 123.4</td>
<td>158.1</td>
<td>146.9, 169.2</td>
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<tr>
<td>Lac→Lacet</td>
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<td>105.9, 123.4</td>
<td>158.1</td>
<td>146.9, 169.2</td>
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<td><strong>Pentose phosphate pathway</strong></td>
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<td>6PG→P5P+CO2</td>
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<td>2.71, 3.29</td>
<td>1.00</td>
<td>0.90, 1.10</td>
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<td>P5P+P5P→S7P+GAP</td>
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<td>0.73, 0.93</td>
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<td>0.13, 0.20</td>
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<tr>
<td>S7P+GAP→F6P+E4P</td>
<td>0.83</td>
<td>0.73, 0.93</td>
<td>0.17</td>
<td>0.13, 0.20</td>
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<tr>
<td>P5P+E4P→F6P+GAP</td>
<td>0.83</td>
<td>0.73, 0.93</td>
<td>0.17</td>
<td>0.13, 0.20</td>
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<tr>
<td><strong>TCA cycle</strong></td>
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<tr>
<td>Pymit→CO2→OAA</td>
<td>0</td>
<td>0.0, 3.2</td>
<td>0</td>
<td>0.0, 1.9</td>
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<tr>
<td>Mal→Pymit+CO2</td>
<td>1.8</td>
<td>0.9, 5.9</td>
<td>1.8</td>
<td>0.7, 4.7</td>
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<tr>
<td>Mal→Pymit+CO2</td>
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<td>0.0, 3.2</td>
<td>1.6</td>
<td>0.0, 1.9</td>
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<tr>
<td>Pymit→AcCoAmit+CO2</td>
<td>16.1</td>
<td>13.5, 19.1</td>
<td>8.6</td>
<td>7.6, 9.6</td>
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<tr>
<td>AcCoAmit+OAA→Cit</td>
<td>16.1</td>
<td>13.5, 19.1</td>
<td>8.6</td>
<td>7.6, 9.6</td>
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<tr>
<td>Cit→AKG+CO2</td>
<td>10.1</td>
<td>9.1, 11.2</td>
<td>5.4</td>
<td>4.9, 6.0</td>
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<td>Cit→AKG+CO2</td>
<td>3.3</td>
<td>2.7, 3.9</td>
<td>3.7</td>
<td>3.2, 4.2</td>
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<tr>
<td>AKG→Suc+CO2</td>
<td>12.1</td>
<td>10.6, 13.7</td>
<td>7.4</td>
<td>6.1, 8.7</td>
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<tr>
<td>Suc→Fum</td>
<td>12.1</td>
<td>10.6, 13.7</td>
<td>7.4</td>
<td>6.1, 8.7</td>
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<td>Suc→Fum</td>
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<td>0</td>
<td>0.0, 1.5</td>
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<td>Fum→Mal</td>
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<td>6.1, 8.7</td>
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<td>0.0, Inf</td>
<td>42.250</td>
<td>1.5, Inf</td>
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<td>Mal→OAA</td>
<td>10.3</td>
<td>6.7, 11.3</td>
<td>5.6</td>
<td>3.5, 6.1</td>
</tr>
<tr>
<td>Mal→OAA</td>
<td>36.2</td>
<td>0.0, Inf</td>
<td>57.630</td>
<td>3.2, Inf</td>
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<tr>
<td>Pyr→Pymit</td>
<td>14</td>
<td>12.0, 17.0</td>
<td>7</td>
<td>6.0, 8.0</td>
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<tr>
<td>Pyr→Pymit</td>
<td>74</td>
<td>39.0, 133</td>
<td>276</td>
<td>87.0, 4566</td>
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<td><strong>Glutamine metabolism</strong></td>
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<tr>
<td>Glute→Glu</td>
<td>20</td>
<td>19.0, 22.0</td>
<td>16</td>
<td>15.0, 17.0</td>
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<td>Glu→Gluext</td>
<td>20</td>
<td>19.0, 21.0</td>
<td>16</td>
<td>15.0, 17.0</td>
</tr>
<tr>
<td>Glu→Gluext</td>
<td>18</td>
<td>16.0, 19.0</td>
<td>14</td>
<td>12.0, 15.0</td>
</tr>
<tr>
<td>Glu→AKG</td>
<td>2</td>
<td>1.0, 3.0</td>
<td>2</td>
<td>1.0, 3.0</td>
</tr>
<tr>
<td>Glu→AKG</td>
<td>168</td>
<td>115, 341</td>
<td>202</td>
<td>106, 1995</td>
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<tr>
<td>Pyrmit→Glu→Ala+AKG</td>
<td>0.15</td>
<td>0.13, 0.16</td>
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<td>0.14, 0.17</td>
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<tr>
<td>OAA+Glu→Asp+AKG</td>
<td>0.18</td>
<td>0.16, 0.20</td>
<td>0.18</td>
<td>0.16, 0.20</td>
</tr>
</tbody>
</table>

Detailed description, assumptions, complete results, and data are available in Supplementary information. Net flux: (uF/C0uR) and Exchange flux: min (uF, uR)(2). Some scaling and undetermined exchange fluxes (i.e., confidence intervals of infinity, Inf) are omitted for brevity. Comparison of fluxes (Transformed versus Normal) is described as U=up, D=down, NC=no change, and ND=no detectable. Fluxes are listed × 10^14 mol/(cell × h).
These data, represented by heat maps in Supplementary Figure S5, indicate that several genes of the investment phase of glycolysis (Hk1 and Pfkb) as well as Pfkb2, 3, and 4, which through the regulation of fructose-2,6-bisphosphate level allosterically control 6-phosphofructo-1-kinase, were consistently expressed at lower levels in T cells as compared with N cells (Supplementary Figure S5A) (green color). In addition, five genes encoding glycolytic enzymes (Gpi1, Pfkl, Pfkm, Ado4, and Tpi1) were highly expressed, particularly at 48 h, in T cells as compared with N cells (red color) (Supplementary Figure S5A). Moreover, a high expression for genes involved in the pay-off phase of glycolysis, where ATP is generated, was observed. Indeed, almost all the genes of this phase, at both time points 48 and 72 h, were expressed at a higher level in T cells as compared with N ones (Gapdh, Pfk1, Eno1, Eno2, Pkm2, Ldhα and Ldhβ).

Consistent with the reduction of TCA flux that we observed, three genes encoding kinase that inhibit the PDH enzyme complex activity (Pdk1, Pdk3, and Pdk4) were significantly expressed in T cells (Supplementary Figure S5B). In addition, expression of Ogdh, which encodes one subunit of the oxoglutarate dehydrogenase complex, another key control point in the TCA cycle, was decreased in T cells (Supplementary Figure S5B). On the contrary, few genes encoding for positive regulators of TCA cycle, as Aco2, Suc2a2 and Sucg2a2, as well as Idh2, which is able to catalyze the reductive carboxylation of AKG, were expressed at higher levels in T cells throughout the time window of the analysis as compared with N ones (Supplementary Figure S5B). Taken together, these results indicate that numerous genes regulated by oncogenic K-Ras may drive the observed metabolic reprogramming, although we cannot exclude the possibility that other mechanisms (e.g., post-translational modifications, mass action, and allosteric enzyme regulation) also participate in the observed metabolic alterations of transformed cells.

Transcriptional analysis of glutamine entry into TCA cycle

Previous experiments (Figure 1) indicated that Gln contributes to precursors of cellular biomass (e.g., aspartate) to a greater extent in T cells as compared with N cells, despite the observed small decrease in uptake. These findings are consistent with other reports describing Myc-driven increases of Gln usage through glutaminolysis, mediated in part via expression of genes involved in Gln uptake and metabolism (Wise et al., 2008; Gao et al., 2009). To investigate this feature, we have analyzed the expression of genes encoding enzymes involved in Gln uptake and metabolism, with particular attention to those involved in metabolic pathways able to generate Asp. As shown in Figure 1E, Asp can be produced from Gln both in cytoplasm and mitochondria through AKG by oxidative decarboxylation and reductive carboxylation. Gln enters into the cells by high-affinity glutamine importer Slc1a5 and is metabolized to Glu by glutaminase (Gls) (Figure 1E). Subsequently, Glu may be converted to AKG, either via transaminase activity of cytoplasmic and mitochondrial aspartate and alanine aminotransferases (Got1 and Got2 and Gpt1 and Gpt2, respectively) or via glutamate dehydrogenase-dependent oxidative deamination (Gld1) (Figure 1E). As shown in Figure 1F, Slc1a5, Gls1, and Gld1 were consistently expressed at lower levels in T cells (about two-fold) relative to N cells. On the contrary, Got1 and 2, showed an opposite behavior, with both genes slightly upregulated in T cells compared with N cells. These transcriptional changes may partially justify the slight reduction in Gln uptake (e.g., downregulation of Slc1a5 in transformed cells) shown in Figure 1A and suggest, by analysis of the metabolic routes depicted in Figure 1E, a relevant role of AKG for the generation of labeled M4 Asp through oxidative metabolism (see also transcriptional data in Figure 1F and Supplementary Figure S5). In addition, in T cells, higher expression of Idh2 (Supplementary Figure S5) and in particular of Acy, whose level was at least two-fold higher than N cells (Figure 1F), is coherent with the increased M3 Asp labeling previously shown. However, while transcriptional data are for the most part consistent with the reduction of glutamine uptake and its use for biosynthesis, not all metabolic fluxes are expected to be only regulated at the transcriptional level.

Transformed cells increase their use of glutamine nitrogen for biosynthetic processes

To further investigate the role of Gln in cellular biosynthesis, we performed a non-targeted tracer fate detection (NTFD) analysis using amino-labeled glutamine ([15N]glutamine) to identify and quantify metabolites containing glutamine-derived nitrogen. This method enables the detection and quantification of label in all observable metabolites downstream of the tracer substrate (Hiller et al., 2010). Cells require nitrogen atoms to build molecules such as nucleotides, amino acids, amino sugars with Gln and Glu representing the primary cellular reservoir of nitrogen. The amino nitrogen of Gln is retained on Glu and used as a nitrogen donor in transaminase reactions. As expected, the NTFD algorithm identified many amino acids and associated compounds that were highly labeled from [15N]glutamine tracer, including Glu, Ala, Asp, and 5-oxoproline (5-oxo) (Figure 2A). With the exception of Asp, each of these metabolites was more significantly labeled in T cells as compared with N cells. In addition, we observed that M1 and M2 labeling on the nucleobase adenine, generated from [15N]glutamine-derived nitrogen atoms from Asp, was over 2–3-fold higher in T cells compared with N control cells (Figure 2B).

Transcriptional data support results from NTFD analysis with [15N]glutamine. Absolute gene expression values of N and T cells and their ratio (T/N ratio) as well, shown in Figure 2C, Supplementary Table S1, highlight the elevated expression of genes closely related to Gln utilization in T cells. In particular, the expression of genes involved in glutathione metabolism (e.g., Gss, Gclm), hexosamine metabolism (e.g., Gfpt1), and nucleotide biosynthesis (e.g., Cad, Add1, and Add2) was increased in T cells as compared with N cells. Such transcriptional changes suggest increased activity of glutathione pathway and the enhancement of nucleotide synthesis, as indicated respectively by downstream metabolite 5-oxo labeling (Figure 2A) and M1 and M2 labeling on the nucleobase adenine (Figure 2B). In this regard, detailed
analysis of genes specifically involved in purine metabolism (Figure 2D) have indicated a more sustained expression in T cells as compared with N cells. Altogether, these findings further suggest the main role of Gln in supporting anabolic processes of T cells as compared with N cells.

K-Ras transformed human cells show metabolic alterations similar to those observed in mouse transformed cells

To test whether human cancer cells exhibit similar metabolic alterations to the previously described mouse model of transformation, we next sought to expand our metabolic and transcriptional analyses to the MDA-MB-231 human breast carcinoma cell line, which express an activated K-Ras protein (G13D). To assess the glycolytic phenotype of MDA-MB-231 cells, asynchronous cells grown in high and low Glc (25 and 1 mM, respectively), were followed in a time course of 96 h. As shown in Figure 3A, MDA-MB-231 cells grown in 25 mM Glc continued to proliferate and reached a much higher cell density compared with those in 1 mM Glc. In fact, in low Glc cells completely lost their proliferation ability and showed evident signs of cell death at 48 h, as revealed by proliferation curves (Figure 3A) and by morphological analysis (data not shown). In addition, MDA-MB-231 cells are dependent upon
Gln for proliferation; even low levels of Gln (0.5 mM) significantly limited growth compared with normal culture (Figure 3A). Since the effects of Glc and Gln shortage on cell viability and proliferation of MDA-MB-231 cells were very similar to those observed in the mouse model of K-Ras-dependent transformation, we analyzed the metabolic phenotype of these human cancer cells in greater detail. Extracellular flux quantifications indicated that MDA-MB-231 cells convert Glc to Lac at approximately stoichiometric levels (i.e., 2:1; Figure 3B). Indeed GC/MS analysis of cells cultured with a 1:1 mixture of [1-13C]glucose and [U-13C6]glucose have demonstrated that at least 90% of Lac was derived from Glc (i.e., M3 Lac abundance was measured at 44.6 ± 0.1 %). Compared with murine N and T cells, MDA-MB-231 cells consume much higher amounts of Glc relative to Gln (Figure 3C). However, direct oxidative conversion of Glc still accounted for as much as 40% of the Fum, Mal, and Asp pools in cells cultured with [U-13C5]glutamine (see M4 mass isotopomer abundances; Figure 3D). Taken together, these data indicate that, much like transformed mouse cells, MDA-MB-231 cells divert most Glc to Lac and rely on Gln carbon for anaplerosis.

Using extracellular flux data and MIDs obtained from MDA-MB-231 cells cultured for 54 h with [U-13C5]glutamine, we performed MFA to determine intracellular fluxes throughout central carbon metabolism. The results of this analysis (Supplementary Figure S6; Supplementary Table S4) indicated that, in a similar way to mouse T cells, MDA-MB-231 cells exhibit high glycolytic flux and significantly lower TCA cycle activity. This comparison is best demonstrated by plotting the ratio of PDH flux to Glc consumption (Figure 3E). While Pyr oxidation flux is ~25% of Glc uptake in control N cells, cells with oncogenic K-Ras exhibit a PDH flux ≤10% of Glc consumption. Therefore, oncogenic K-Ras consistently drives the decoupling of central carbon metabolism, increasing the glycolytic flux to Lac and decreasing oxidative TCA flux in cells with different genetic backgrounds (Figures 1 and 3).

Importantly, MDA-MB-231 cells carry a mutant p53 (Olivier et al., 2002), whereas K-Ras-NIH3T3 cells have wild-type p53 (Wadhwa et al., 1999). Given the fact that recent literature results have implicated the p53 tumor suppressor protein as a regulator of mitochondrial respiration and the Warburg effect (Matoba et al., 2006), we tested the effect of p53 status on these metabolic changes to ensure our results were specific to oncogenic K-Ras. Comparing p53+/+ wild-type (HCT116 p53+/+) or a p53−/− KO (HCT116 p53−/−) colon carcinoma cells, which express oncogenic K-Ras (Bunz et al., 1998), we observed no significant differences in proliferation under nutrient deprivation (Supplementary Figure S7A and B) or intracellular metabolite abundances (Supplementary Figure S7C–F). These data indicate that oncogenic K-Ras, at least in these cellular models, contributes to cancer-specific metabolic changes in a way that is not dependent on the presence of wild-type p53.

In order to analyze the relationship between our metabolic analysis and transcriptional profiling, microarray datasets of MDA-MB-231 cells and normal breast tissues were recovered from publicly accessible gene expression profile dataset (GEO and CellMiner). Data were analyzed as previously described (Balestrieri et al., 2009), and results indicated that several glycolytic genes (i.e., Hk1, Pfkp, Eno1, and Ldha) were expressed at a higher level in cancer cells as compared with normal breast tissue (Supplementary Figure S8A), in agree-
ment with the more active glycolysis shown in Figure 3B. Differences in gene expression were also observed for several TCA cycle genes (Supplementary Figure S8B).

These findings, collectively, suggest that oncogenic K-Ras transformation induces cell-specific transcriptional programs that have a role in the metabolic alterations we observed (i.e., increased glycolytic activity) and highlight the utility of systems-level approaches. Figure 4 depicts a schematic view of metabolism, indicating the changes we observed upon K-Ras transformation using $^{13}$C MFA and transcriptional profiling. In particular, it can be observed that both K-Ras transformed mouse and human cells, as shown by previous metabolic analyses, divert most part of consumed Glc to Lac (Figure 4A, red arrows). Such a preferential metabolic flux is

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**Figure 4** Schematic integrative representation of metabolic fluxes and transcriptional data in normal and mouse K-Ras transformed cells. (A, B) Integration maps of metabolic routes identified in T cells as compared with N cells by using flux analysis and transcriptional data. The ratio between T and N cells of both metabolic fluxes and gene expression values are represented by a color code (as indicated in the upper right legends) as follow; black (no change value), red (increased value), and green (decreased value) for flux analysis and Up—red color—T/N ratio $>1.1$, down—green color—T/N ratio $<0.9$ and no change—yellow color—T/N ratio between 0.9 and 1.1 for transcriptional data. The list of gene abbreviations used in the figure can be found in ‘Supplementary information’. Metabolic flux analysis of central carbon metabolism was obtained by using the data derived from [$U-^{13}$C$_5$] glutamine, a mixture of [$U-^{13}$C$_6$] glucose and 1-$^{13}$C glucose (A) and [$\alpha-^{15}$N]glutamine (B) as specific isotopic tracers. The net and exchange fluxes were calculated as described under ‘Materials and methods’. Transcriptional data of N and T regulated genes were obtained as described in ‘Materials and methods’.

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supported by the observed high level of expression of significant glycolytic genes in both cell lines (i.e., Adpgk, Gadph, Pkg, En0 1 and 2, Pkm2 and Ldha; Figure 4A, right box). In addition, the reduced flux of pyruvate entering into mitochondria (Figure 4A, green arrow) and the deregulation of several genes encoding TCA cycle enzymes (Figure 4A) in part provide an explanation of the decreased TCA cycle flux observed in both transformed cells lines (Figure 4A, green arrows). At the same time, our metabolic data indicate that such a reduced TCA cycle flux in transformed cells has an important anabolic role and is essentially fueled by Gln, as suggested by the identification of labeled Asp, a product of Gln-derived TCA cycle intermediates (Figure 4A). Furthermore, the important role of Gln in supporting anabolic processes of cancer cells (Figure 4B, red arrows) is suggested by the identification of labeled metabolites containing Gln-derived nitrogen and associated increases in the expression of genes involved in glutathione metabolism (Gctm, Gss, and Gpt1), hexosamine metabolism (Gfpt1), and nucleotide biosynthesis (Cad, Adss, and Adsl) (Figure 4B). Taken together, these findings indicate a major alteration in cancer cell metabolism with a decoupling between Glc and Gln fates.

To further test whether Gln metabolism induced in T cells was dependent on the K-Ras oncogene, we treated N, T, and R cell lines with aminoxyacetate (AOA), an inhibitor of aminotransferase activity (Figure 5E) and epigallocatechin gallate (EGCG), an inhibitor of glutamate dehydrogenase (GDH) activity (Figure 5F). As shown in Figure 5E, only T-cell proliferation was inhibited by AOA treatment (Figure 5E, compare middle panel versus left panel:N cells and right panel: R cells) and rescued by DMD addition and not by DMK addition (Figure 5E). These data suggest that aminotransferase activity is necessary to sustain cancer cell growth and Gln-derived Asp is an important anabolic precursor for cancer cell growth.

Analogous proliferation experiments were performed in N, T, and R cell lines upon treatment with EGCG. As shown in Figure 5F, the treatment induced cell death in all cell lines. Nevertheless, the reduction of cell number was markedly greater in T cells compared with that observed in N and R cells. Importantly, addition of DMK completely rescued viability in all the three cell lines (Figure 5F). Given the significant level of cell death observed in all cells at 24 h, we investigated the effects of EGCG on cell survival at earlier time points (between 0 and 8 h) to better understand the mechanism of cytotoxicity. EGCG reduced viability in all three cell lines in a time-dependent manner, with T cells being the most sensitive to EGCG treatment (Figure 6A, light gray bars). Interestingly, the antioxidant N-acetyl-l-cysteine (NAC) protected only T cells from EGCG-mediated cytotoxicity (Figure 6A), suggesting a specific role of ROS accumulation for cell death. To ascertain whether EGCG induced a different type of cell death in T cells as compared with N and R cells, cells were treated for 8 h with EGCG and stained with Annexin V (AV) and propidium iodide (PI), indicators of late apoptosis and of plasma membrane permeability, respectively. Untreated cells exhibited an AV-negative/PI-negative pattern showing viable cells (Figure 6B, upper panels), whereas N and R cells treated with EGCG for 8 h exhibited an AV-negative/PI-positive pattern, showing a dead population with loss of plasma membrane integrity, typical of necrosis (Figure 6B, bottom panels). T cells treated with EGCG contained a population of AV-positive/PI-positive pattern, showing a typically apoptotic population with an increased AV signal. These results were confirmed also by cell morphology analysis after EGCG treatment. While EGCG induced flattened and attached cell remnants in N and R cells (almost all PI-positive cells), EGCG-treated T cells were detached and rounded cells (data not shown).

Given the fact that ATP depletion is more likely to induce necrosis, while ROS accumulation is more typically associated with apoptosis, we measured intracellular ATP and ROS levels upon 3 h of EGCG treatment in each cell line. As shown in Figure 6C, EGCG caused a significant decrease of intracellular ATP levels in N and R cells, which could not be rescued by NAC treatment. On the contrary, ROS levels were high and almost constant in T cells regardless of EGCG treatment, whereas N and R cells experienced a decrease in ROS levels upon EGCG treatment (Figure 6D). Absolute levels of intracellular ROS in untreated T cells at 3 h were about two-fold higher as compared with N and R cells (e.g., N: 193.5; T: 317.2; R: 141.7), and addition of NAC reduced ROS levels in all cells (Figure 6D). Altogether, these findings suggest that GDH activity, through...
different mechanisms is necessary for the survival of all three cell lines, and its inhibition may cause in N and R cells an energy derangement followed by necrosis, due to ATP depletion, and in T cells an antioxidant depletion that ultimately may bring to apoptosis. In conclusion, these results suggest a role of glutamine in endogenous antioxidant production, for instance glutathione synthesis, further confirming the anabolic role of glutamine in T cells.

Figure 5  Relative metabolites concentrations in N, T, and R cell lines and effect of aminooxyacetate (AOA) and epigallocatechin gallate (EGCG) treatments on their proliferation. Evaluation of Cit (A), Mal (B), Glu (C), and Asp (D) intracellular concentrations was carried out after 54 h of grown in normal medium by using enzymatic assays. Analysis of AOA (E) and EGCG (F) treatments on N, T, and R cell lines proliferation. Cells, seeded at density of 3000 cells/cm², after 18 h and following a complete medium replacement, were treated with 100 μM AOA, or 1 mM dimethyl aspartate (DMD), or 2 mM dimethyl α-ketoglutarate (DMK) or 20 μM EGCG and counted at indicated time points. Error bars indicate s.e.m. (n=3).
Discussion

Cancer is a complex disease that arises from numerous molecular changes on a various cellular pathways (Mazurek et al., 1998; Hanahan and Weinberg, 2000). Among these alterations is the reprogramming of metabolic pathways associated with bioenergetics and cellular biosyntheses, and metabolic interventions are now emerging as potential therapeutic targets (Michelakis et al., 2010; Vander Heiden et al., 2010; Wise and Thompson, 2010). In this paper, we used 13C MFA, NTFD analysis, and transcriptional profiling to provide a comprehensive analysis of cancer cell metabolism and of its regulation by the K-ras oncogene. The results, summarized in Figure 7, highlight the reprogramming of metabolic pathways that occurs upon introduction of mutant forms of K-Ras. In cancer cells, most consumed Glc is diverted to Lac (Figure 7, red arrows), while the oxidation of Pyr to AcCoA via PDH is consistently decreased (Figure 7, green arrows). This decrease results in a marked reduction of TCA cycle flux (Figure 7, green circle arrow), presumably caused by expression of Pdk genes. It is intriguing to note that both mouse and human transformed cells herein analyzed, present a substantially lower mitochondrial Complex I activity (Ishikawa et al., 2008; Baracca et al., 2010). Consistent with such a decrease in glucose utilization through the TCA cycle, in cancer cells Gln becomes the more relevant source of both carbon and nitrogen for cellular biosynthesis (Figure 7, black, blue, and sky-blue arrows), through aminotransferase...
At 37°C in a humidified atmosphere of 5% CO₂, HCT116 p53⁻/⁻ and HCT116 p53⁻/⁻ cells, obtained from the European Institute of Oncology (Milano, Italy), were cultured with complete DMEM plus 10% FBS. Cells were passed using trypsin-EDTA (Invitrogen) and maintained in culture for 48 h before manipulation.

**Cell proliferation analysis and cell treatments**

For long-term studies, cells were plated at the density of 3000 cells/cm² in complete growth medium. After 18 h, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in a media with different glucose concentrations (25 and 1 mM glucose) or different glutamine concentrations (4 and 0.5 mM glutamine) (time 0). When indicated, cells were treated with 100 μM AOAA (O-[carboxymethyl]hydroxylamine hemihydrochloride), or 20 μM 1-EGCC, or 2 mM dimethyl 2-Oxoglutarate -DMK- or 1 mM L-Aspartic acid β-methyl ester hydrochloride -DMD- (Sigma-Aldrich Inc.). For short-term studies, cells were plated and grown as above and then treated with 20 μM EGCG or 5 mM NAC (Sigma-Aldrich Inc.). To measure cell proliferation, harvested cells were counted by Burker chamber.

**Microarray data collection**

Microarray transcriptional profiles were extracted from two different datasets. The mouse time course data were obtained from a transcription profiles collection of NIH3T3 and NIH3T3 K-Ras mouse fibroblasts, generated in our laboratory, grown in 25 mM glucose and 4 mM glutamine, along a time course of 72 h. In particular, the cells used for the time course transcription analysis, were collected at 18 h from the initial seeding, time corresponding to the change of medium (indicated as T₀) and at 24, 48 and 72 h (NCBI GEO database accession from GSM741354 to GSM741361 and from GSM741368 to GSM741375 of NIH3T3 and NIH3T3 K-Ras, respectively). For each time points, labeled cRNA was generated by using the Affymetrix One-Cycle Target Labeling and Control Reagent kit (Affymetrix Inc., Santa Clara, CA, USA), following the manufacturer’s protocol and was hybridized using Affymetrix Genechips (Mouse Genome 430 2.0 Array) in order to determine the global gene expression patterns. Arrays were washed and scanned on the Affymetrix Complete GeneChip® Instrument System and processed into CEL files.

Human raw expression data for U133A arrays were obtained from publicly accessible gene expression profile database GEO (http://www.ncbi.nlm.nih.gov/geo/) or CellMiner (http://discover.nci.nih.gov/cellminer/home.do). In particular, we used the transcription profiles data of the human breast cancer cell line MDA-MB-231.
(CellMiner accession 134091hg13a21) and of the human normal breast tissue (NCBI GEO accession GSM44683). Both mouse and human transcriptional data were separately submitted on the same procedure of normalization and filtering of noise, in order to obtain a good degree of comparison across the different experiments. Briefly, the data files (CEL files) were imported into GeneSpring GX 11.0.2 software (Agilent Technologies Inc.), by using RMA method, were normalized and summarized as probe-level intensity measurements (Irizarry et al., 2003). In particular, the algorithm implemented in GeneSpring, starting from the perfect-match probe-level data of a set of arrays, performs the background correction, the normalization and finally summarize the raw data into a set of summary measures for each probe set. Subsequently, also ‘per-gene normalization’ was performed as described in Gene-Spring’s manual. Altogether, these procedures permitted to obtain the relative expression of each probe set in a log2 scale with the value 0 as center. This scale of measurement was used for the heat maps showed in the paper. From these two datasets (mouse and human) have been identified and gathered the expression levels for genes encoding proteins involved in glycolysis, TCA cycle and glutamine metabolism, at 48 and 72 h for mouse datasets and, at time indicated by the authors for both human tissue and cell line samples. Total selected mouse and human genes and their intensity expression levels have been reported in Supplementary Dataset.

**Metabolite extraction**

For labeling experiments and GC/MS-based metabolomics, asynchronous NIH3T3, NIH3T3 K-Ras, and MDA-MB-231 were plated at 3000 cells/cm² in 6-well plates with normal growth medium. In all, 18 h after seeding, cells were washed with PBS and incubated in different DMEM (Sigma) plus 10% dialyzed serum supplemented with unlabeled glucose or glutamine and either 4 mM [U-13C5]glutamine or a 25 mM 1:1 mixture of [U-13C5]glucose and [1-13C]glucose. After 54 h, spent medium was collected and analyzed for glucose, lactate, and glutamine consumption on a YSI7100 analyzer. Cells were quickly rinsed with PBS to minimize spent medium carryover and quenched with 0.4 ml ice-cold methanol. An equal volume of water containing 1 μg norvaline internal standard was added, and cells were collected by scraping with a pipette tip. Two volumes of chloroform were added, followed by 1 ml of cell suspension containing 104 cells was added to a 96-well plate. The emitted luminescence was collected after incubation at the wavelength of 560 nm using Cary Eclipse spectrofluorimeter (Varian). The samples were centrifuged at 15 000 g for 10 min to remove cell debris and the proteins were analyzed by using a perchoric acid/KOH protocol (BioVision). The samples were added into triplicate wells of a 96-well plate and the volume was added with 25 mM glucose and 4 mM glutamine. After 54 h, the cells were washed twice with PBS cold and collected using trypsin-EDTA (Invitrogen). The cells were counted and 100 000 cell aliquots were rapidly homogenized with appropriate assay buffer on ice. The samples were centrifuged at 15 000 g for 10 min to remove cell debris and deproteinized using a perchloric acid/KOH protocol (BioVision). The samples were added into triplicate wells of a 96-well plate and the volume was added to 0.1 ml of cell suspension containing 104 cells was added to an equal volume of CellTiter Glo Reagent in a single well of 96-well plate. The emitted luminescence was collected after incubation at the wavelength of 560 nm using Cary Eclipse spectrophotometer (Varian) and the luminescence values were converted in ATP quantities after the setting of a calibration curve.

**Non-targeted tracer fate detection**

The NTTFD analysis was performed to determine the metabolic fate of the α-amine nitrogen atom of glutamine (Hiller et al., 2010). Cells were incubated for 54 h as described above, but instead of unlabeled glutamine, a 1:1 mixture of unlabeled and [2-13N]glutamine (Cambridge Isotope Laboratories) was employed for each growth condition. In a total selected mouse and human cells, the NTTFD experiments were performed in triplicate and intracellular metabolites were extracted and measured as described above. The raw GC/MS data were used as input for the NTTFD software and all detected labeled compounds for all growth conditions were matched using algorithms of the Metabolite Detector software package (Hiller et al., 2009). Finally, the NIST reference library was employed for compound identification (Babushok et al., 2007).

**Metabolite assay**

For enzymatic assay quantitation of aspartate, citrate, malate, and glutamate, asynchronous NIH3T3, NIH3T3 K-Ras, and NIH-GEF-DN were plated at 3000 cells/cm² with normal growth medium. In all, 18 h after seeding, cells were washed with PBS and incubated in medium with 25 mM glucose and 4 mM glutamine. After 54 h, the cells were washed twice with PBS cold and collected using trypsin-EDTA (Invitrogen). The cells were counted and 500 000 cell aliquots were added into triplicate wells of a 96-well plate. The emitted luminescence was collected after incubation at the wavelength of 560 nm using Cary Eclipse spectrophotometer (Varian) and the luminescence values were converted in ATP quantities after the setting of a calibration curve.

**Intracellular ATP quantification**

Intracellular ATP levels were measured using CellTiter Glo® luciferin-luciferase assay (Promega) according to the manufacturer’s protocol. In particular, 100 μl of cell suspension containing 10⁵ cells was added to an equal volume of CellTiter Glo Reagent in a single well of 96-well plate. The emitted luminescence was collected after incubation at the wavelength of 560 nm using Cary Eclipse spectrophotometer (Varian) and the luminescence values were converted in ATP quantities after the setting of a calibration curve.

**ROS levels measurement**

Detection of ROS levels was carried out through flow cytometric analyses using a FACScan flow cytometer (Becton-Dickinson) with CellQuest software (BD Biosciences). ROS levels were measured through staining plated cells with 5 μM dichloro-dihydro-fluoresceine-diacetate (DCFH₂-DA; Molecular Probes, Invitrogen) for 30 min at 37°C. Then, the cell were trypsinized, collected in ice and acquired by FACScan. Analysis of flow cytometric data was carried out using WinMDI software.
AV/PI cell death assay

Cell death was estimated using the Apoptosis Detection Kit (Immunological Sciences). Cells were plated on coverslips previously treated with 2% gelatin. Cells on coverslips were stained by adding 50 µl of binding buffer, 5 µl of Annexin V-FITC, and 5 µl of PI. After 15 min of incubation at 37°C in the dark, cells were treated with 5 µg/ml Hoechst (Invitrogen) for 5 min at RT. The cover glasses, mounted in DABCO, were analyzed under a Nikon Eclipse 90i fluorescence microscope equipped with a b/w CCD camera (Hamamatsu-CoolSNAP, Hamamatsu Corporation Japan), using Plan Apo objective (× 60 oil; numerical aperture 0.75 and 1.4, respectively). The images were acquired using the imaging software Metamorph 7, and then processed in Adobe Photoshop CS3 with adjustments of brightness and contrast.

Supplementary information

Supplementary information is available at the Molecular Systems Biology website (www.nature.com/msb).

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Author contributions: LA and GS planned the project. DG, CMM, LA, GS, and FC conceived and designed the research. DG, CMM, LA, GS, and FC wrote the manuscript. FC and DG performed transcriptome experiments. CB performed transcriptomic data analysis. DG, CMM, and PAG performed metabolic analysis. CMM and KH performed statistical computations of metabolic analysis. DG and LSD performed cell biology experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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**K-Ras decouples glucose and glutamine metabolism**

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