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**Role of Nutrient Availability on Proliferation and Cell
Cycle Execution of Immortalized and K-Ras
Transformed Mouse Fibroblasts.**

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2008 -2009

*Alla luce che mi ha seguito in
questo percorso, illuminandomi
tutte le volte che mi sono persa.*

Daniela

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SUMMARY

Mammalian cells proliferate, differentiate or die in response to extracellular signals as growth factors and nutrients. Cancer is essentially a disease in which cells have lost responsiveness to many of these signals and to normal checks on cell proliferation. Therefore, it may not be surprising that tumor cells, in order to meet the increased requirements of proliferation, often display fundamental changes in pathways of energy metabolism and nutrient uptake (Garber, 2006). In particular, several studies shown that have the process of tumorigenesis is often associated with altered metabolism of two major nutrients, glucose and glutamine (Mazurek et al, 1998; Chiaradonna et al., 2006; Gaglio et al., 2009). Moreover, these metabolic changes and cellular sensitivity to these nutrients can be induced or influenced by oncogenic transformation as i.e. *Ras* mutation (Chiaradonna et al., 2006; Gaglio et al., 2009) that has been found in 25% of human cancers.

Glucose and glutamine are involved in multiple pathways required for cell proliferation and survival both in normal and transformed cells. The role of these pathways in the survival of transformed cells is mostly based either on the fact that the pathways in question can be regulated by oncogenes, or that cell death following nutrients shortage is associated with changes in the activation state of these pathways. In particular, in this work the response of K-Ras transformed cells to glucose and glutamine availability has been studied.

Transformed cells have been shown to have a particular dependence on aerobic glycolysis compared to normal cells. Indeed, proliferation analysis of asynchronous K-Ras transformed fibroblasts as compared to normal cells, grown both in high and low glucose (25mM and 1mM), indicated that transformed cells showed a higher proliferation potential in 25mM glucose and lost completely this proliferative advantage in low glucose (1mM). Moreover, the strong reduction of glucose availability, observed at 72hrs in

cells grown in 1mM glucose, induced an enhanced apoptosis only in transformed cells. Indeed, the apoptotic process activated in normal cells was glucose independent and probably correlated to prolonged contact inhibition. This effect has been validated by both Annexin V/PI staining and caspases-3 activation. Since transformed cells were characterized by strong reduction of proliferation as well as apoptosis at low glucose concentration, the effects of Ras activation and glucose shortage on the cell cycle machinery has been analyzed, in particular during G₁/S transition in synchronized normal and transformed cells. These results indicated that the timing of G₁/S transition execution was glucose independent in both cell lines. Therefore, oncogenic *Ras* expression is able to induce a greater sensitivity to glucose shortage as compared to normal cells (decreased proliferation and enhanced apoptosis) only if the glucose shortage is persistent.

Another metabolic adaptation of cancer cells, that has been studied, is their propensity to exhibit increased glutamine consumption.

The results indicated that in asynchronous normal cells, contact inhibition, regardless of glutamine availability, brings to down-regulation of Akt that, together with AMPK up-regulation, observed at low glutamine, concurs to TOR pathway inactivation. As a result, the expression of cyclin D, E and A is down regulated, pRb phosphorylation is strongly reduced, p27^{kip1} level is increased and its localization becomes preferentially nuclear, establishing therefore a condition that bring to a G₁- cell cycle arrest. In synchronous normal cells, glutamine shortage slows the G₂/M transition, indicating a possible role of glutamine in such cell cycle phase.

In K-ras transformed cells, in which the level of activated Ras-GTP is very high (Nagase et al., 1995) and the contact inhibition is less efficient (Nagase et al., 1995), the deprivation of glutamine affects Akt and AMPK in a way opposite to that observed in normal cells, leaving the TOR pathway at least partially activated. This event allows sizable expression of cyclin D (at least until 72 hrs), E and A, sustained pRb phosphorylation, decreased p27^{kip1} and

its preferential cytoplasmic localization, conditions that, taken together, promote entrance into S phase. Surprisingly, in condition of glutamine shortage, transformed asynchronous cells accumulate in S phase. In synchronous transformed cells, glutamine shortage slows both the G₁ to S and the G₂/M transitions.

Since glutamine is an important intermediate in purine and pyrimidine biosynthesis, glutamine exhaustion could deplete intracellular nucleotide pools, bringing in turn to a failure in the execution of a normal cell cycle (Christofk et al., 2008; Martinez-Diez et al., 2006). This hypothesis has been confirmed by the fact that the proliferation defect of transformed cells is rescued by adding the four deoxyribonucleotides (precursors of DNA polymerization) to low glutamine medium. Moreover, experiments performed in synchronized transformed cells have shown that low-glutamine medium causes a 2 hrs delay in entering into S phase after serum re-addition. This effect on cell cycle timing is worsened by complete absence of glutamine, in which a 4 hrs delay in entering into S phase was observed. These data strongly indicated that the effect of glutamine limitation in transformed cells was first to slow down the S phase traverse, then, when a more severe limitation was established, to stick a large fraction of the cells population in S-phase. Indeed, addition of a mix of 10 μ M deoxyribonucleotides reverted completely S phase reaching.

Therefore in cells exhibiting high metabolic rates, such as rapidly dividing cancer cells grown in vitro, glutamine, being the most readily available amino acid used as energy source, may become the major source to sustain protein and nucleic acid synthesis (Ziegler et al., 2001), especially when glucose levels are low and energy demand is high. However, analysis of the levels of mRNA, proteins and above all of ATP in normal and transformed cells grown in high and low glutamine availability, did not show particular differences, suggesting an important role of glutamine for nucleotides synthesis in K-ras transformed cells.

In conclusion, glutamine shortage in K-ras transformed cells limits proliferation by inducing abortive S phase entrance, while glucose shortage in the same system enhanced cell death (Lopez-Rios et al., 2007; Mankoff et al., 2007). The differential effects of glutamine and glucose on cell viability are not a property of the transformed phenotype per se, but rather depend on the specific pathway being activated in transformation.

It has been previously shown that nutrient shortage influence cell proliferation and G₁/S transition of K-Ras transformed fibroblasts.

To understand how intracellular and extracellular signals are transmitted to the cell-cycle machinery and how the latter adjusts its frequencies accordingly is one of the major challenges in molecular biomedicine. To this aim, a computational model of the cell cycle based on experimental data has been developed. Indeed biochemical and genetic studies can be combined with bioinformatics and biosystems approaches in order to sketch a plan of the regulatory circuits governing cell cycle progression in normal cells, firstly, and then in transformed cells.

Taking in consideration that the trespassing of the Restriction Point influences the timing of the cell cycle execution and that such timing is influenced both by growth factors and nutrients availability, it has been initially identified the restriction point in normal mouse fibroblasts, synchronized in G₀ by serum starvation and stimulated to re-enter into S-phase by readdition of serum. During the time course of re-entering into cell cycle, from G₀ to G₁/S phase, and in agree with restriction point reaching, it has been observed a gradual increase of cyclin D and cyclin E, a constant expression of Cdk4 and Cdk2 and an abrupt decrease of p27^{Kip1}. Moreover, in quiescent cells, has been observed a completely nuclear localization of p27^{Kip1} and more cytoplasmic localization of Cdk4 and Cdk2. These data agreed with other results since, in most cases, the concentration of the kinase subunit is relatively constant, whereas the concentration of the cyclin subunit oscillates. This detailed study of G₁/S transition in normal fibroblasts

allowed a novel mathematical model to develop. Because tumor cells often display a reduced dependence on growth factors or an increased dependence on some nutrients, an understanding of the cell cycle and a dynamical computational model that include regulatory aspect might help explain the changes leading to tumor formation.

RIASSUNTO

Fattori di crescita e nutrienti sono i responsabili del controllo di molteplici meccanismi: quali proliferazione, differenziamento e morte cellulare. L'alterazione delle risposte cellulari ai fattori di crescita e ai nutrienti è alla base di molte patologie, tra le quali il cancro. Numerosi studi, infatti, hanno dimostrato una mancata responsività, di quest'ultimo, ai normali controlli della proliferazione cellulare. E' quindi lecito aspettarsi che le cellule tumorali mostrino cambiamenti sostanziali nel metabolismo energetico e nei processi di assorbimento dei nutrienti e che tali cambiamenti siano correlati ad aumentate esigenze proliferative (Garber, 2006). Diversi studi hanno mostrato che il processo di tumorigenesi è spesso associato con alterazioni nel metabolismo di due dei nutrienti principali, glucosio e glutammina (Mazurek et al., 1998; Chiaradonna et al., 2006; Gaglio et al., 2009). Tali alterazioni, e quindi la sensibilità della cellula a questi nutrienti, possono essere sia indotti che influenzati dalla trasformazione neoplastica, ad esempio mutazioni in Ras (Chiaradonna et al., 2006; Gaglio et al., 2009) che sono state individuate nel 25% dei tumori umani. Oltretutto, glucosio e glutammina sono coinvolti in molteplici vie metaboliche richieste per la proliferazione e la sopravvivenza cellulare, sebbene abbiano ruoli specifici in cellule normali e trasformate. Infatti, il ruolo di queste vie nella sopravvivenza di cellule trasformate è essenzialmente basato sul fatto che tali vie subiscono una regolazione da parte di oncogeni e che la morte cellulare indotta da deprivazione nutrizionale è associata a cambiamenti nello stato di attivazione di tali vie. In particolare, in questo lavoro è stata studiata la risposta di cellule trasformate con l'oncogene K-ras alla disponibilità di glucosio e glutammina nel mezzo di crescita.

Questo lavoro dimostra che le cellule trasformate possiedono una particolare dipendenza dalla glicolisi aerobica, contrariamente alle cellule normali. Infatti, le analisi di proliferazione di colture asincrone di fibroblasti

trasformati con l'oncogene K-ras in condizioni di alto o basso glucosio (25mM e 1mM), dimostrano che tali cellule mostrano un aumentato potenziale proliferativo in 25mM glucosio rispetto alla controparte normale. Tale vantaggio proliferativo viene però completamente perso in basso glucosio (1mM). Inoltre, la forte riduzione nella disponibilità di glucosio, osservata a 72 ore in cellule cresciute in 1mM glucosio, induce apoptosi correlata alla deprivazione da glucosio solo in cellule trasformate. Al contrario, il processo apoptotico attivato in cellule normali è indipendente dal glucosio e probabilmente correlato alla prolungata inibizione da contatto, come suggerito dalla letteratura. Questo effetto è stato validato attraverso colorazione AnnessinaV/PI e studi di attivazione di caspasi-3. Poiché le cellule trasformate sono caratterizzate da una notevole riduzione della proliferazione, così come da aumentati livelli di apoptosi in concentrazioni limitanti di glucosio, sono stati analizzati gli effetti dell'attivazione di Ras e della deprivazione di glucosio sul ciclo cellulare durante la transizione G₁/S in colture sincronizzate di fibroblasti normali e trasformati. I risultati indicano che la tempistica di esecuzione della transizione G₁/S è indipendente dal glucosio in entrambe le linee cellulari. Inoltre, l'espressione di Ras oncogenico induce una maggiore sensibilità alla deprivazione di glucosio determinata da minore proliferazione ed aumentata apoptosi solo nel caso in cui la deprivazione da glucosio sia persistente.

Un altro adattamento metabolico di cellule tumorali che è stato studiato è la loro propensione all'aumento del consumo di glutammina.

I risultati indicano che in cellule normali asincrone l'inibizione da contatto induce una regolazione negativa di Akt che, in concomitanza con la regolazione positiva di AMPK osservata in bassa glutammina, concorre all'inattivazione della via di TOR. Tale effetto è indipendente dalla disponibilità di glutammina. In queste condizioni, l'espressione delle cicline D, E ed A è regolata negativamente, la fosforilazione di pRb è fortemente ridotta, il livello di p27^{kip} è aumentato e la sua localizzazione diventa

preferenzialmente nucleare, stabilendo quindi una condizione che induce all'arresto del ciclo cellulare. In cellule normali sincronizzate, la deprivazione di glutammina rallenta la transizione G₂/M, indicando un possibile ruolo della glutammina in tale fase del ciclo cellulare.

In cellule trasformate, invece, in cui il livello di Ras-GTP attiva è molto alto (Nagase et al., 1995) e l'inibizione da contatto è meno efficiente (Nagase et al., 1995), la deprivazione di glutammina influenza Akt e AMPK in maniera opposta alle evidenze ottenute con cellule normali, anche se la via di mTOR risulta almeno parzialmente attiva. Ciò determina un'elevata espressione di ciclina D (almeno fino a 72 ore), E ed A, una sostenuta fosforilazione di pRb, diminuiti livelli di p27^{kip} e una sua localizzazione sostanzialmente citoplasmatica, ovvero condizioni che favoriscono l'ingresso in fase S. Sorprendentemente, in colture asincrone di cellule trasformate, la deprivazione di glutammina determina accumulo in fase S, mentre in nelle colture sincrone delle stesse cellule l'effetto della deprivazione risulta in un rallentamento sia la transizione G₁/S che la transizione G₂/M.

Poiche' la glutammina è un importante intermedio nella biosintesi delle purine e delle pirimidine, un suo esaurimento potrebbe ridurre le riserve intracellulari di nucleotidi, causando di conseguenza il fallimento del completamento del ciclo cellulare (Christofk et al., 2008; Martinez-Diez et al., 2006). Questa ipotesi è stata confermata dall'osservazione che il difetto proliferativo di cellule trasformate è revertito dall'aggiunta dei quattro deossiribonucleotidi (precursori della polimerizzazione del DNA) al terreno di crescita con bassa glutammina. Inoltre, esperimenti effettuati in cellule trasformate sincronizzate hanno mostrato che il mezzo con bassa glutammina provoca un ritardo di 2 ore nell'ingresso in fase S in seguito a rilascio in terreno contenente siero. Questo effetto sulla tempistica del ciclo cellulare è esacerbato dalla completa assenza di glutammina, in cui si evidenzia un ritardo di 4 ore nell'ingresso in fase S. Questi dati suggeriscono fortemente che la limitata disponibilità di glutammina in cellule trasformate

determina il rallentamento della progressione in fase S e in seguito, quando la limitazione è più severa, il blocco completo in fase S di una grande frazione della popolazione cellulare. Al contrario, l'aggiunta di una miscela di deossiribonucleotidi alla concentrazione di 10 μ M ristabilisce completamente la possibilità di giungere in fase S.

Quindi, in cellule che mostrano alte dinamiche metaboliche, come cellule cancerose in rapida divisione cresciute in vitro, la glutammina, essendo l'amminoacido più velocemente disponibile utilizzabile come fonte energetica, potrebbe diventare la fonte principale per sostenere la sintesi di proteine ed acidi nucleici (Ziegler et al., 2001), specialmente quando i livelli di glucosio sono bassi e la richiesta energetica alta. Tuttavia l'analisi dei livelli di mRNA, proteine e, al di sopra di tutto, di ATP in cellule normali e trasformate cresciute in alta e bassa disponibilità di glutammina, non hanno mostrato particolari differenze, suggerendo un ruolo importante della glutammina nella sintesi dei nucleotidi in cellule K-ras trasformate.

In conclusione, in fibroblasti trasformati con l'oncogene K-ras, la deprivazione di glutammina limita la proliferazione inducendo un ingresso abortivo in fase S, mentre la deprivazione di glucosio aumenta la morte cellulare (Lopez-Rios et al., 2007; Mankoff et al., 2007). I differenti effetti della glutammina e del glucosio sulla vitalità cellulare non sono delle proprietà del fenotipo trasformato per se, ma dipendono piuttosto dallo specifico pathway attivato nella trasformazione.

È stato mostrato precedentemente che la deprivazione nutrizionale influenza la proliferazione e la transizione G₁/S di fibroblasti K-ras trasformati.

Per capire come i segnali intra- ed extra-cellulari sono trasmessi al macchinario del ciclo cellulare e come quest'ultimo di conseguenza regoli le sue frequenze è una delle maggiori sfide della medicina molecolare. A questo scopo è stato sviluppato un modello computazionale del ciclo cellulare basato su dati sperimentali. Infatti, studi biochimici e genetici possono essere combinati con approcci bioinformatici e di biosistemistica al

fine di elaborare un piano dei circuiti regolativi che governano la progressione del ciclo cellulare inizialmente in cellule normali, e successivamente in cellule trasformate.

Considerando che la violazione del Restriction Point influenza la tempistica dell'esecuzione del ciclo cellulare e che tale tempistica è influenzata dalla disponibilità di fattori di crescita e nutrienti, è stato inizialmente identificato tale Restriction Point in fibroblasti murini normali, sincronizzati in G_0 attraverso deprivazione di siero e stimolati a rientrare in fase S per riaggiunta di siero. Durante la tempistica di rientro nel ciclo cellulare, dalla fase G_0 alla G_1/S , e in accordo con il raggiungimento del Restriction Point, è stato osservato un graduale aumento di ciclina D e ciclina E, una espressione costante di Cdk4 e Cdk2 ed una brusca diminuzione di $p27^{kip1}$. Inoltre, in cellule quiescenti, è stata osservata una localizzazione completamente nucleare di $p27^{kip1}$ ed una localizzazione più citoplasmatica di Cdk4 e Cdk2. Questi dati sono in accordo con dati di letteratura dato che, nella maggior parte dei casi, la concentrazione della subunità chinasi è relativamente costante, mentre la concentrazione della ciclina oscilla. Questo studio dettagliato della transizione G_1/S in fibroblasti normali ha permesso lo sviluppo di un nuovo modello matematico. Siccome le cellule tumorali spesso mostrano una ridotta dipendenza dai fattori di crescita o un' aumentata dipendenza da alcuni nutrienti, la comprensione del ciclo cellulare e un modello computazionale dinamico che include gli aspetti regolatori potrebbe aiutare a spiegare i cambiamenti che conducono alla formazione di un tumore.

Introductions

INTRODUCTION

Mammalian cells proliferate, differentiate or die in response to extracellular signals. Cancer is essentially a disease in which cells have lost responsiveness to many of these signals and to normal checks on cell proliferation. Therefore, it may not be surprising that tumor cells, in order to meet the increased requirements of proliferation, often display fundamental changes in pathways of energy metabolism and nutrient uptake [1]. In particular, several studies shown that the process of tumorigenesis is often associated with altered metabolism of two major nutrients, glucose and glutamine [2-4]. Moreover, this metabolic changes and cellular sensitivity to these nutrients can be induced or influenced by oncogenic transformation as i.e. *Ras* mutation [3, 4] that has been found in 25% of human cancers.

Glucose major fuel in cancer cells

The undisputed role of glucose as the main cellular energy source in cancer cells, have been discovered more than 70 years ago by Otto Warburg [5]. The scientist found that cancer cells consume more glucose than normal cells and proposed that the failure of mitochondrial oxidative phosphorylation is the cause of cancer [5].

Glucose is the preferred metabolic substrate of most mammalian cells and its breakdown in the cytoplasm and/or in mitochondria provides the energy and building blocks required for cellular maintenance, mass and/or proliferation. Glycolysis in the cytoplasm partially oxidizes the carbon skeleton of glucose into two molecules of pyruvate, yielding two moles of ATP and NADH per mol of glucose consumed. When the cells have a limited supply of oxygen or have a genetic impairment that impedes oxidation of pyruvate in mitochondria, glycolytic pyruvate is reduced to lactate in the cytoplasm by lactate dehydrogenase (LDH) with the purpose of regenerating the oxidized

form of NAD^+ for glycolysis to proceed. The excess of lactate produced in this situation is exported out of the cell and contributes to the acidification of the cellular environment. Normoxic cells, however, oxidize most of the pyruvate to CO_2 in the mitochondria by the sequential activities of pyruvate dehydrogenase and of the tricarboxylic acid (TCA) cycle enzymes. The electrons obtained by the oxidation of glucose in the cytoplasm and in mitochondria are shuttled into the complexes of the respiratory chain to generate the proton electrochemical gradient of mitochondria. The reentry of protons into the mitochondrial matrix through the H^+ -channel of the mitochondrial H^+ -ATP synthase will be used as driving force in oxidative phosphorylation for the synthesis of ATP out of ADP and P_i . The terminal oxidation of pyruvate in mitochondria yields almost a 20-fold higher amount of ATP than that provided by glycolysis (Figure 1).

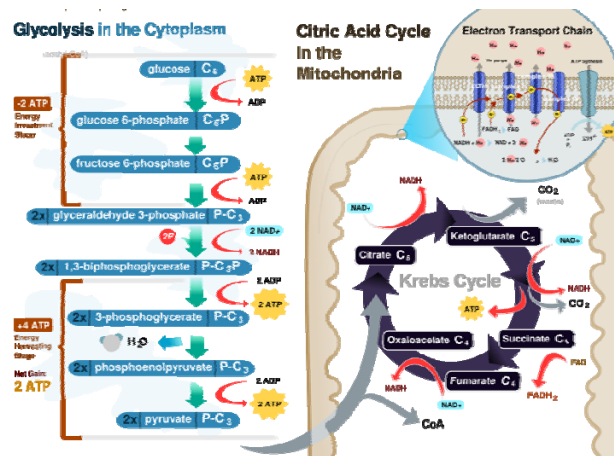


Figure 1: Relationship between glucose metabolism and TCA Cycle. The model shows some of the relevant aspect of the metabolism of glucose. After entering the cell by specific transporters, glucose can be utilized by aerobic glycolysis to generate pyruvate. In the cytoplasm, the pyruvate generated can be reduced to lactate or oxidized in the mitochondria by pyruvate dehydrogenase to generate acetyl-CoA, which is condensed with oxaloacetate in the TCA cycle.

Several studies have shown the “abnormal” glucose metabolism in cancer cells and tumor tissues. Different human tumors, implanted in rats, revealed that these carcinomas had an increased glucose consumption rate in spite of a low respiration and a concurrent high rate of lactate release when compared to normal tissues [6, 7]. Then, human renal cell carcinomas revealed an increase in the activity of the enzymes of glycolysis [8]. In addition, up-regulation of the glucose transporter isoform 1 (GLUT1) has been shown in several tumors (gastrointestinal carcinoma, breast carcinoma, squamous cell carcinoma of the head and neck, renal cell carcinoma) and it may represent a widespread mechanism of malignant cells to compensate the lack of energy (Figure 2) [9-12]. Proteomic approaches, valuable tools for the identification of cancer biomarkers and specific protein profiles of the disease (cancer signatures), have demonstrated that the increased expression of enzymes of the glycolytic pathway represents a hallmark of malignant cells [13, 14].

An enhanced glycolytic metabolism seems to be essential for Akt-mediated cell survival upon growth factor withdrawal [15] and other studies shown that Akt promotes a dose-dependent stimulation of glycolysis that correlates with tumor aggressiveness in vivo [16]. In addition, activation of Akt alone is sufficient to functionally drive glucose uptake and aerobic glycolysis and that tumor cells bearing activated Akt uniquely undergo rapid cell death following placement into low glucose [16]. Loss of ATP production, resulting from inhibition of glycolysis under low glucose conditions, has been shown to be responsible for the cell death and could be circumvented by activators of fatty acid oxidation, which create ATP by alternative means in the mitochondria [17]. The propensity of cells carrying active oncogenes (e.g. Ras, Her2, Akt) or even primary mouse cells lacking single tumor suppressors (e.g. TSC1/2, LKB1, p53) to preferentially undergo apoptosis under low glucose conditions is quite striking [3, 18-21]. Besides, over-expression of Akt in non-invasive radial-growth melanoma induces the expression of glycolytic markers, stimulates glycolysis, as well as

transformation of the tumor to an invasive vertical-growth phenotype (Figure 2) [22].

The high glycolytic activity has also been demonstrated in rat glioma *in vivo* by magnetic resonance imaging [23]. More recently, the development of positron emission tomography (PET) using the glucose analogue 2-deoxy-2-[¹⁸F]fluoro-D-glucose (FDG) as probe has conclusively demonstrated *in vivo* that most human carcinomas have an increased glucose demand when compared to the surrounding normal tissue [24]. Moreover, a high FDG uptake in thyroid, lung and breast carcinomas, sarcomas, lymphomas and gliomas is a significant predictor of a poor patient outcome [25, 26], strongly supporting that the “abnormal” metabolic phenotype of cancer plays a primary role in progression of the disease. Conversely, quantitative bioluminescence imaging of lactate content in various types of tumors also supports this statement [27].

Altogether, molecular and functional data indicate that tumors have a metabolic phenotype with enforced glycolysis which is very different from that of normal tissues. Several other studies have supported Warburg hypothesis. Indeed, it has been shown that cancer cells are often characterized by increased mtDNA mutations [28]. Besides, it has been proposed that the hypoxic environment, characterizing the developing tumors, promotes the stabilization of the hypoxia-inducible transcription factor (HIF1 α) which positively controls transcription of glycolytic enzymes as hexokinase (HK1 and HK2), lactate dehydrogenase A (LDHA) and lactate extruding enzyme monocarboxylate transporter 4 (MCT4) [29]. Of interest, metastatic breast cancer cell lines display a constitutive high expression of HIF1 α and increased glucose consumption compared to non-metastatic breast cancer cell lines (Figure 2) [30]. Moreover, a high glycolytic flux requires nicotinamide adenine NAD⁺ which may be efficiently generated from the conversion of pyruvate into lactate. This metabolic conversion

makes glycolysis self-sufficient as long as elevated glucose uptake is possible.

Glucose, surely, is considered to be the most important source of energy in transformed cells, but, in physiological processes the nutrient participates to the synthesis of several biosynthetic intermediates necessary to duplicate cell biomass and genome at each cell division. Indeed, glycolysis generates metabolic intermediates important for cell growth. Metabolism of glucose through the oxidative or non-oxidative arms of the pentose phosphate pathway (PPP) generates ribose-5-phosphate (R-5P), a key intermediates in nucleotide biosynthesis. The oxidative arm of the PPP can also produce NADPH, which supplies a pool of reducing equivalents for both nucleotide and fatty acid biosynthesis. NADPH also participates in the anti-oxidant defenses of tumor cells (Figure 2). Recent works suggest that pyruvate kinase-M2 (PK-M2), an isoform of the rate-limiting enzyme for pyruvate generation, preferentially expressed in tumor cells, regulates the flux of carbon into R-5P and nucleotides synthesis [31]. Interestingly, the enzymatic activity of PK-M2 is negatively regulated by phosphotyrosine signaling downstream growth factors receptors [32]. By regulating the amount of pyruvate generated by glycolysis, PK-M2 addresses the flow of carbon into nucleotide and fatty acid biosynthesis pathways necessary to support the proliferation of tumor cells. Glycolytic intermediates, including 3-phosphoglycerate (3-PG), serve as carbon source for amino acid and lipid synthesis. Finally, pyruvate can be imported into the mitochondria and converted into substrates for the production of additional amino acids or fatty acid synthesis, and used to maintain mitochondrial membrane potential. More recently, the tumor suppressor protein p53 has been also involved in the glycolytic shift of cancer cells. Indeed, has been shown that an inhibitor of fructose biphosphatase 2 (an enzymatic activity that competes with glycolysis) named TIGAR (TP53-induced glycolysis and apoptotic regulator) as well as the mitochondrial protein SCO2 (able to induce

decreased expression of two p53 targets: TP53-induced glycolysis and apoptosis regulator (TIGAR) and synthesis of cytochrome oxidase (SCO)2. This drives glycolysis by disfavoring oxidative phosphorylation and PPP. Hypoxia and/or oncogenes can activate hypoxia inducible factor (HIF1 α), which can drive transcription of nearly all glycolysis-related enzymes (most not depicted), including pyruvate kinase (PK) and lactate dehydrogenase (LDH). Some oncogenes can activate PK and LDH independently of hypoxia and HIF1 α .

Indeed, more recently, it has been documented that progression through the cell cycle is stringently controlled by the regulation of the metabolic pathways [36]. Essentially, during cellular proliferation the biosynthesis of DNA and of most of the mitochondrial components occurs during S/G₂/M phases of the cell cycle [37], in the so-called reductive phase of the “metabolic cycle”, which is supported by glycolysis [36]. In fact, cyclin D1, which marks the entrance of the cells into the S phase, inhibits mitochondrial function [38]. Consistently, proliferating lymphocytes [39] and thymocytes [40] switch to glycolysis as the main energy producing pathway by down-regulating mitochondrial activity. It has been suggested that the repression of mitochondrial bioenergetics function during cellular proliferation is needed to minimize the production of reactive oxygen species (ROS) that could damage cellular constituents at a stage when they are most vulnerable, i.e., nude DNA during replication [36, 40]. In this regard, the ROS scavenging activity of pyruvate [40] might represent another additional argument for the selection of glycolysis as the pathway for cellular proliferation.

All these findings may justify the observed increase of glucose consumption from cancer cells.

Role of glutamine in physiological process and in cancer

Another metabolic adaptation of cancer cells, that has long been established, is their propensity to exhibit increased glutamine consumption, although the effects induced by glutamine deprivation on cancer cells are still controversial, mostly because of the diverse cellular processes in which glutamine is involved.

Glutamine has traditionally been viewed as a nonessential amino acid whose primary functions are to store nitrogen in the muscle and to traffic it between organs. Although it contributes only 4% of the amino acid in muscle protein, glutamine accounts for more than 20% of the free amino acid pool in plasma and more than 40% in muscle [41, 42]. Mammals can synthesize glutamine in most tissues, but during periods of rapid growth or illness, the cellular demand for glutamine outstrips its supply and glutamine becomes essential (hence its designation as a “conditionally” essential amino acid). Proliferating cells show an intense appetite for glutamine, reflecting its incredible versatility as a nutrient and mediator of other processes (Figure 3) [43-45].

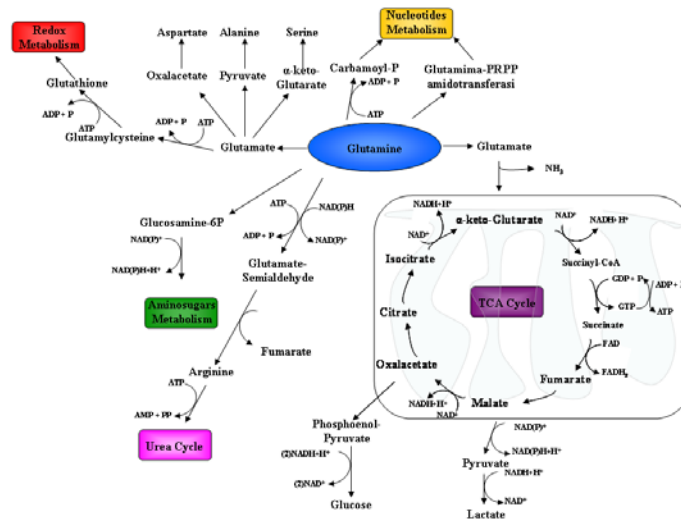


Figure 3: Glutamine metabolism. Schematic representation of metabolic pathways in which are involved glutamine, the most abundant free amino acid in the human body and glutamate, the mainly product of glutamine metabolism. Glutamine acts as a nitrogen donor for purine and pyrimidine nucleotide synthesis, for urea cycle, for synthesis of amino acids, for synthesis of carbamoylphosphate, for synthesis of amino sugars, and other metabolites. Moreover, via glutamate, is also converted to α -ketoglutarate, an integral component of the citric acid cycle and two other amino acids alanine, which is derived from pyruvate, and aspartate, which is derived from

oxaloacetate. Glutamine is also involved in the production of hexosamines and glutathione.

The metabolic fates of glutamine can roughly be divided into reactions that use glutamine for its γ -nitrogen (i.e. nucleotide synthesis and hexosamine synthesis) and those that use either the α -nitrogen or the carbon skeleton (i.e. TCA cycle) (Figure 4).

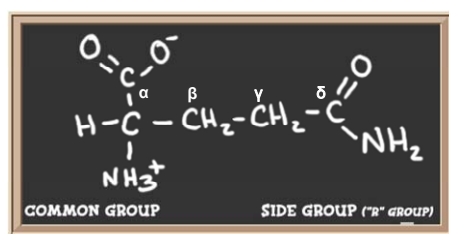


Figure 4: Chemical structure of glutamine. The metabolic fates of glutamine can roughly be divided into reactions that use glutamine for its γ -nitrogen (i.e. nucleotide synthesis and hexosamine synthesis) and those that use either the α -nitrogen or the carbon skeleton, which use glutamate and not glutamine as the substrate.

The latter reactions use glutamate, not glutamine, as substrate. Although tumor cells tend to have large intracellular pools of glutamate, maintained by the ability of the cells to convert glutamine into glutamate by a phosphate-dependent glutaminase (GLS), a mitochondrial enzyme that has been found highly expressed in tumors and tumor cell lines. Several studies have shown that GLS activity correlates with tumor growth rates in vivo [46, 47], and experimental models to limit GLS activity resulted in decreased growth rates of tumor cells and xenografts tumors [48, 49]. Thus, although not all the metabolic fates of glutamine require GLS activity, this enzyme is essential for the metabolic phenotype of many tumors.

Because of the high expression of GLS, tumor cells are poised to produce glutamate rapidly from glutamine, and a sizable fraction of their glutamate pool carries glutamine is a (amino) nitrogen. This nitrogen is then dispersed

into various pools of nonessential amino acids through the activity of transaminases, particularly alanine aminotransferase and aspartate aminotransferase. These enzymes catalyze the reversible transfer of amino groups between glutamate and alanine or aspartate, respectively. Alanine is used in protein synthesis, but is also avidly secreted by tumor cells, carrying some of the excess carbon from glycolysis. Aspartate, in contrast, remains inside the cell and contributes to the synthesis of proteins and nucleotides and to electron transfer reactions through the malate-aspartate shuttle. In this shuttle, aspartate goes out from the mitochondria and is converted to oxaloacetate (OAA) by aspartate aminotransferase. Oxaloacetate is then reduced to malate, using electrons donated by NADH generated in glycolysis. The malate then enters the mitochondria and donates the electrons to Complex I of the electron transport chain. Thus, the shuttle facilitates ongoing ATP production in both the mitochondria (through oxidative phosphorylation) and the cytosol (by resupplying NAD^+ for glycolysis) (Figure 5).

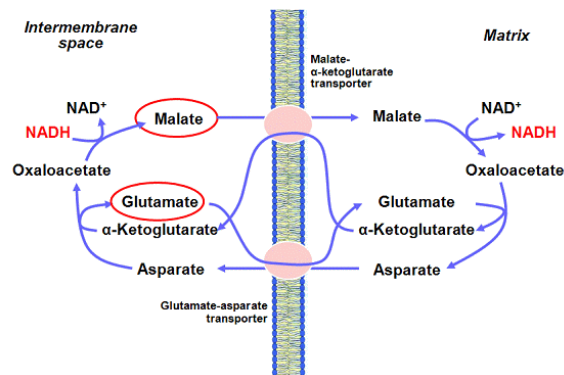


Figure 5: Malate/Aspartate reducing equivalent shuttle. Schematic of reactions involved in the malate-aspartate shuttle, which transfers an electron across the inner mitochondrial membrane resulting in the net transfer of NADH from the cytoplasm into the mitochondria. Glutamine metabolism can increase the activity of the malate/aspartate shuttle by increasing the concentration of aspartate.

Oxidation of carbon backbone of glutamine in the mitochondria is a primary source of energy for proliferating cells, including lymphocytes, enterocytes, fibroblasts and some cancer cell lines [50-52]. This requires conversion of glutamine to α -ketoglutarate (AKG), through phosphate-dependent glutaminase (GLS) activity followed by conversion of glutamate to α -ketoglutarate by either transaminases or glutamate dehydrogenase. Complete oxidation of glutamine carbon involves exit from the tricarboxylic acid (TCA) cycle as malate, conversion to pyruvate and then acetyl-Coenzyme A (ACoA), and finally reentry into the cycle. It should be noted, however, that cautious studies of tumor cell glutamine metabolism, revealed that neither its nitrogen nor its carbon are used to completion in vitro. Indeed, a high fraction of both of glutamine is nitrogen that is secreted as ammonia, alanine and glutamate and at least half of its carbon is secreted as lactate [53]. This form of rapid glutamine utilization and secretion of glutamine by-products, similar to the Warburg effect in its apparent inefficiency, has been proposed to be an additional hallmark of tumor cell metabolism [54].

One of the benefits of converting glutamine to pyruvate and then in lactate, is the reduction of NADP^+ to NADPH by malic enzyme. NADPH is a required electron donor for reductive steps in lipid synthesis, in nucleotide metabolism and in maintaining GSH in its reduced state. Therefore, proliferating cells must produce a large supply of it. Although cells contain numerous potential sources of NADPH, has been observed that the malic enzyme flux was estimated to be high enough to supply all of the reductive power needed for lipid synthesis [53].

Glutamine metabolism provides precursors for the synthesis of glutathione (GSH) the major thiol-containing endogenous antioxidant and serves as a redox buffer against various sources of oxidative stress. In tumors, maintaining a supply of GSH is critical for cell survival because it allows cells to resist the oxidative stress associated with rapid metabolism, DNA-damaging agents, inflammation and other sources [55].

Glutamine is a required nitrogen donor for the *de novo* synthesis of both purines and pyrimidines and therefore is essential for the net production of nucleotides during cell proliferation. Such a role of glutamine nitrogen in nucleotide biosynthesis may explain because some transformed cells show delayed transit through S phase in low-glutamine availability [4]. However, the glutamine utilization rate exceeds nucleic acid synthesis by more than an order of magnitude in proliferating cells, and thus nitrogen donation to nucleotides accounts for only a small fraction of total glutamine consumption [56].

Glutamine plays an important role in gluconeogenesis in liver and kidney. It can function as a substrate [57] and also controls the expression and activity of phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme of gluconeogenesis [58].

Glutamine can stimulate expression of adenylosuccinate synthase (ADSS-1), which can regulate cell proliferation via activation of protein kinase A and mTOR in neonatal rat cardiomyocytes [59]. The latter is an important intracellular transducer of a growth-related signaling pathway, which is rapamycin sensitive and dependent on activation of a 70 kDa S6 kinase (p70S6K) [59]. The S6 phosphorylation is known to be required for the translation of the terminal oligo-pyrimidine family of RNAs that contain an oligo-pyrimidine tract upstream of their transcription-initiation site. These messengers encode proteins belonging to the protein-translation machinery [60].

In tumor cells glutamine can serve as an alternative substrate for the Krebs cycle and ATP production during aerobic glycolysis. It has been noted that the pancreatic cancer PANC-1 and MIA Paca-2 cell lines are glutamine dependent and susceptible to growth inhibition by glutamine-deprivation [61]. Likewise, the MDA-MB-453 breast cancer cell line, in contrast to MCF-7 cells, is glutamine-dependent [62]. Altogether these observations support the higher rate of glutamine transport in cancerous cells as compared

to normal cells [63] and underline the fundamental role of both glucose and glutamine in cancer cell metabolism.

Mitochondrial and TCA cycle dysfunction in cancer

To produce two viable daughter cells, a proliferating cell must replicate all of its cellular contents. This imposes a large requirement for nucleotides, amino acids and lipids to generate biomass as well as ATP production to support biochemical reactions.

Mitochondria are central to cell metabolism and energy production. High-energy electrons coming from the oxidation of different carbon sources such as glucose and fatty acids enter the mitochondrial electron transport chain as reduced equivalents and their energy is gradually converted into a proton gradient. Mitochondria use this gradient to synthesize ATP later used for biosynthetic reactions [63, 64]. Mitochondria also control decisions for life and death. Changes in mitochondrial membrane permeability lead to the release of proapoptotic mediators that can kill cells i.e. following DNA damage or activated oncogenes expression [65].

If the induction of glycolysis in cancer cells is nowadays out of the question, the impairment of mitochondrial function has been a subject of extensive debate until quite recently. Otto Warburg first noticed that in the presence of oxygen tumor cells consume larger amounts of glucose and produce higher amounts of lactate than normal cells [5, 66]. Since he observed that cancer tissues had a relative low respiratory rate compared to nonmalignant ones, Warburg ascribed the abnormal aerobic metabolism of cancer to an impaired bioenergetic function of mitochondria and suggested that this metabolic change is an important event in tumorigenesis [5].

Once the activities of respiration and oxidative phosphorylation were attributed to mitochondria, a large number of studies were aimed to establish the presumed impairment of mitochondrial metabolic and bioenergetic

functions in the cancer cell [67]. In this regard, it was shown that pyruvate dehydrogenase of tumor mitochondria kinetically resembled that of embryonic tissues [68] and promoted the abnormal non-oxidative decarboxylation of pyruvate to acetoin, a product that competitively inhibits the oxidation of pyruvate by the tumor dehydrogenase [69]. Shunting of pyruvate into TCA cycle generates a source ACoA for the biosynthesis of lipids and amino acids. ACoA condenses with OAA to generate citrate, which is exported from the mitochondrion to the cytosol and converted back to ACoA by ATP citrate lyase (ACL). Inhibition of ACL results in decreased glucose-dependent lipid synthesis and impaired cell proliferation [70]. This cytosolic pool of citrate derived by ACoA is essential for the synthesis of lipids. To this regard, it has been shown that prostate gland cells are characterized by high level of citrate as compared to prostate cancer cells, in which the levels are lower, probably due to a reduced mitochondrial metabolism of the cancer cells [71]. One risk of converting mitochondrial activity to biosynthesis is the loss of mitochondrial integrity due to depletion of TCA cycle intermediates. Indeed, it has been shown that TCA cycle in tumor mitochondria was shown to be partially truncated at the level of the conversion to citrate [72]. Consistent with this observation, a recent proteomic approach has reported a defective TCA cycle in colorectal cancer samples [14]. In this situation, glutamine primes with carbon skeletons the TCA cycle of the cancer cells [69, 73]. Deficiencies in enzyme complexes of the respiratory chain and oxidative phosphorylation in cancer cells have also been described, including the over-expression of IF1, which is the physiologic inhibitor of the mitochondrial ATPase [74, 75]. Consistent with some of the molecular and functional alterations described in mitochondria of cancer cells, mitochondria have less cristae in cancer than in normal cells [67] and, microscopic study of tumors revealed mitochondrial hyperplasia. Moreover, profound ultrastructural alterations have been reported in the organelle of human hepatocellular carcinomas [76]. Likewise, some cancer

cells have a diminished mitochondrial complement when compared to non-cancerous tissues [67, 76, 77]. In addition, in many different types of human carcinomas a vast array of mitochondrial DNA mutations have been described [78]. In this regard, mitochondrial respiratory chain complexes exhibit a diminished activity in cancers due to a decreased expression of the subunits encoded by the mitochondrial DNA [79]. Indeed, up-regulation of the expression of mitochondrial cytochrome oxidase II mRNA in malignant breast tissues has been observed [80]. Expression analysis in glioblastoma cells showed reduction of cytochrome C oxidase subunit II and III (COX II, COX III), ATP synthase subunit 6 (ATP 6), NADH dehydrogenase subunit 1 and 4 (ND1 and ND4) transcripts [81]. Likewise, mutations in nuclear genes involved in mitochondrial energy transduction, such as fumarase and succinate dehydrogenase, have also been reported to cause predisposition to some types of inherited neoplasia syndromes [82].

Recent studies indicated that mitochondrial fission is involved in the induction of programmed cell death, an important area reviewed extensively in [83, 84]. During apoptosis, Bcl2 family members Bax and Bak coalesce on the mitochondrial surface and cause outer membrane permeabilization, which allows cytochrome c sequestered in the mitochondrial intermembrane space to be released into the cytosol. Cytochrome c release is a key event leading to activation of caspases, cysteine proteases necessary for cell death. In many cases of cells undergoing apoptosis, mitochondrial fragmentation is an early event that precedes caspase activation [85]. This fragmentation occurs close in time to two other important events: the coalescence of Bax and Bak on the mitochondrial surface and cytochrome c release [84]. Mitochondrial fragmentation during apoptosis is dependent on the normal mitochondrial fission machinery, and inhibition of Drp1 or Fis1 prevents mitochondrial fragmentation, inhibits cytochrome c release, and can delay or reduce the extent of cell death [85-87]. Therefore, mitochondrial fusion may play a protective role in apoptosis. Inhibition of mitochondrial fusion

facilitates cell death in response to some apoptotic signals (Figure 6) [88, 89].

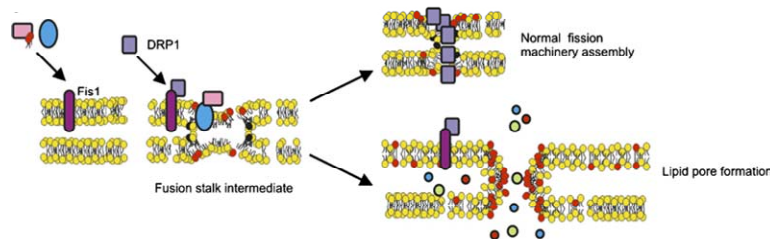


Figure 6: Mitochondrial model of fusion and fission processes. Proposed model for mitochondrial fusion and fission processes. Under normal conditions the fission machinery (Fis1, Drp1 and putative proteins implicated in membrane perturbation and lipids modifications) is recruited and assembled on local foci. Under physiological division, Drp1 assembles into a ring and constricts the mitochondria until it divides.

Beside these findings, ultimately it has been demonstrated a fascinating new role for mitochondria in the G_1/S transition [90]. Mitra and colleagues demonstrated a relationship between hyperfusion of the mitochondria and cell cycle control at the G_1/S boundary. In this study, by image analysis has been revealed that although growing cells appear to have a mixture of tubular and fragmented mitochondria, cells at the G_1/S border form a single, giant tubular network. This network appears to be unique, forming only at the G_1/S transition and is characterized by a syncytia of mitochondria that are both electrically coupled and unusually hyperpolarized [82]. These unusual electrical characteristics might relate to previous observations that described a peculiar increase in mitochondrial oxygen consumption during the G_1/S transition [91]. In an effort to understand how these mitochondrial events fit within the established paradigms of cell-cycle progression, Mitra and colleagues demonstrated that mitochondrial fusion could trigger expression of cyclin E. Moreover, additional experiments where the

mitochondrial membrane potential was purposely reduced, suggested that the unique mitochondrial structure, formed at the G₁/S transition, was required for the transition itself. Nonetheless, this mitochondrial mesh was a transient phenomenon, since if the tubular network was purposely maintained beyond the G₁/S boundary, cells were unable to proceed with normal cell cycle progression. Furthermore by using isogenic cells lines, this subsequent cell cycle arrest was shown to require the tumor suppressor p53 [90]. The correlation between cell cycle execution and energetic status of the cells has been shown also by Mandal et al [92]. Indeed, Mandal et al demonstrated the existence of a “mitochondrial checkpoint” in late G₁. Such checkpoint is triggered by low ATP that inducing the activation of an AMPK-p53-cyclin E-dependent pathway, presumably arrests energetically impaired cells, stopping them from making the presumably overtaxing commitment to cell division [92]. A similar result has been shown also in mouse embryonic fibroblasts (MEFs) grown upon glucose reduction. In this case, withdrawal of glucose has been shown to lead to an AMPK- and p53- dependent activation of a G₁/S checkpoint [21]. Activation of AMPK after energetic stress can also lead to phosphorylation of the mTOR [93]. These events lead to an inhibition of mTOR activity, which also regulates the G₁/S transition and represents another potentially distinct energetic checkpoint. Moreover, a recent study show that mitochondrial dysfunction can induce a G₁/S arrest by alternative means [94]. Indeed, a mutant in Complex I of the electron transport has been recently shown to induce cell-cycle arrest through a pathway involving mitochondrial reactive oxygen species (ROS) generation, followed by activation of the fly homologs of the c-Jun N-terminal kinase (JNK), the FOXO transcription factor, and the cell-cycle regulator p27 [94]. All of these studies suggest that mitochondrial function and energetic status are much more intimately connected to the cell cycle than originally believed.

Ras signaling in cancer

Cancer development is a multistep process through which cells accumulate genetic mutations [95] and *RAS* is one of the most frequently mutated oncogenes in human cancer [96].

The Ras proteins are members of a large superfamily of low molecular-weight GTP-binding proteins, which can be divided into several families according to the degree of sequence conservation. Three members of the *RAS* family -*H-RAS*, *K-RAS* and *N-RAS*- were found to be activated by mutation in human tumors [97]. These three members are very closely related, having 85% amino acid sequence identity and, although they function in very similar ways, some indications of subtle differences between them have recently come to light. The H-Ras, K-Ras and N-Ras proteins are widely expressed, in particular K-Ras being expressed in almost all cell types. Knockout studies have shown that H-ras and N-ras, either alone or in combination, are not required for normal development in the mouse, whereas K-ras is essential [98]. This might reflect different molecular functions of the three proteins, but is more likely to reflect the more ubiquitous expression of K-Ras.

The 20% of human tumors have activating point mutations in *RAS*, most frequently in *K-Ras* (about 85% of total), then *N-Ras* (about 15%), then *H-Ras* (less than 1%). These mutations all compromise the GTPase activity of Ras, preventing GAPs from promoting hydrolysis of GTP on Ras and therefore causing Ras to accumulate in the GTP-bound, active form. Almost all Ras activation in tumors is accounted for by mutations in codons 12, 13 and 61 [99].

Ras signaling pathways are commonly activated in tumors in which growth-factor-receptor tyrosine kinases have been over-expressed (Figure 7).

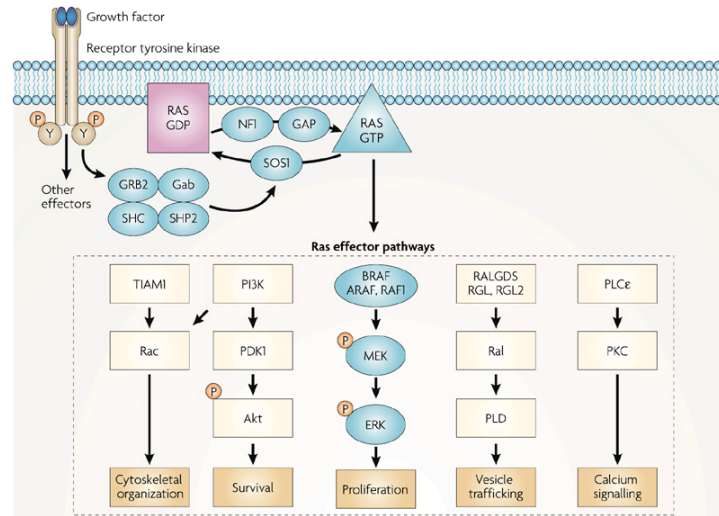


Figure 7: Ras signaling pathway. Ras signaling pathways are commonly activated in cancer. Growth factor binding to cell-surface receptors results in activated receptor complexes, which contain adaptors such as SHC (SH2-containing protein), GRB2 (growth-factor-receptor bound protein 2) and Gab (GRB2-associated binding) proteins. These proteins recruit SHP2 and SOS1, the latter increasing Ras-guanosine triphosphate (Ras-GTP) levels by catalysing nucleotide exchange on Ras. The GTPase-activating protein (GAP) neurofibromin (NF1) binds to Ras-GTP and accelerates the conversion of Ras-GTP to Ras-GDP (guanosine diphosphate), which terminates the signalling. Several Ras-GTP effector pathways have been described, and some of the key effectors are depicted above. The BRAF-mitogen-activated and extracellular-signal regulated kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) cascade. Ras also activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway that frequently determines cellular survival.

Indeed, it has been demonstrated that over-expression of EGF-R/erbB and HER2/neu in many types of cancer, including breast, ovarian and stomach carcinomas [100]. In addition, a mutation in the EGFR gene results in the expression of a truncated receptor that lacks part of the extracellular domain, and this mutated receptor was found to be over-activated in a significant proportion of glioblastomas and some other tumor types [101]. EGFR-family tyrosine kinases are also commonly activated by the autocrine production of EGF-like factors such as transforming growth factor- α (TGF- α) in tumors.

Recently, it has been shown that *B-RAF* was frequently activated in melanomas (~70%) and colon carcinoma (~15%) [102]. Mutations in *B-RAF* occur in a very limited number of residues in the kinase domain, all of which result in kinase activation [102].

Several recent papers have shown a direct association between oncogenic ras pathways activation and cancer cells metabolic alterations. A recent work has shown that in colorectal cancer cells lines, an increase in GLUT1 expression and glucose uptake was dependent on *K-Ras* and *B-Raf* mutations [103]. In other studies, have been shown that oncogenic *K-Ras* may regulate the expression of glycolytic enzymes through the activity of HIF1 α and other transcriptions factor [104]. Elstrom and colleagues demonstrated in human glioblastoma cells, that glucose transport and its metabolism are also increased by the serine/threonine kinase AKT [16]. Moreover, murine NIH3T3 fibroblasts expressing an oncogenic form of *K-Ras* and expressing a constitutively active AKT protein undergo apoptotic cell death upon glucose shortage. In addition, such a phenotype has been associated to strong metabolic alterations, including increased rate of glucose consumption, lactic acid accumulation and expression of glycolytic enzymes, as well as altered expression of mitochondrial genes, reduced mitochondrial activity, ATP production and increased ROS production [3, 105].

K-ras also stimulates signaling through the phosphatidylinositol 3-kinase (PI3K) pathway [106]. PI3K signaling through AKT can regulate glucose transporter expression, enhance glucose capture by hexokinase, and stimulate phosphofructokinase activity [52]. PI3K pathway activation renders cells dependent on high levels of glucose flux [17]. Moreover, PTEN, a negative regulator of PI3K, is also reported to be inactivated in some cancers [107]. Both PI3K and PTEN genetic alterations lead to chronic activation of Akt in tumor cells and consequently to an increase of mTOR activity that ultimately induces an increase of the expression of amino acid

transporters and regulates protein translation, thereby coordinating protein synthesis in proliferating tumor cells [108].

Several studies have shown that oncogenic *RAS* promotes tumor progression through genomic instability induction. Indeed, it has been demonstrated that expression of the human *H-Ras* oncogene in p53-null cells leads to premature entry of cells in S-phase. In addition Agapova et al, showed that expression of activated *H-Ras* bypass of G₂ DNA damage checkpoint in p53 mutant cells suggesting that genomic instability induced by oncogenic *RAS* may potentially be due to a relaxation of this checkpoint.

Through the combined action of these Ras-responsive signalling pathways, expression of activated mutant Ras in cells could promote several characteristic aspects of malignant transformation. These include increased proliferation due to induction of cell-cycle regulators such as cyclin D1, which leads to inactivation of the retinoblastoma (RB) pathway, and suppression of cell cycle inhibitors such as KIP1. Besides, cells become desensitized to apoptosis through AKT/PKB signaling and less well-defined mechanisms that are downstream of Raf. In addition to effects on cell proliferation and survival, Ras effector pathways also lead to the induction of angiogenesis, mainly by means of ERK-mediated transcriptional up-regulation of angiogenic factors, and to increased invasiveness, through both ERK-mediated expression of matrix metalloproteinases and Rac-mediated effects on the cytoskeleton. Targeting Ras and its effector pathways could therefore have a potential impact on several different aspects of malignancy.

G₁/S Transition and Restriction Point: Critical Steps in Cancer

Cell proliferation is an ordered, tightly regulated process involving multiple checkpoints that assess extracellular growth signals, cell size, and DNA integrity. The basic cell cycle is divided into an interphase, designated for cellular growth and DNA synthesis, and a mitotic phase, in which a single cell divides into two daughter cells. Interphase is further subdivided into two

gap phases (G_1 and G_2) separated by a phase of DNA synthesis (S phase). However, the vast majority of cells in the human body exist in a non-dividing, terminally differentiated state. When cells cease proliferation, either due to specific anti-mitogenic signals or to absence of proper mitogenic signaling, they exit the cycle and enter a non-dividing, quiescent state known as G_0 .

To ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering into a new phase until they have successfully completed the previous one [109]. The newly divided or quiescent cells could be influenced by external elements, such as the amount of availability nutrients or the intensity of the mitogenic information that cells receive at any given time.

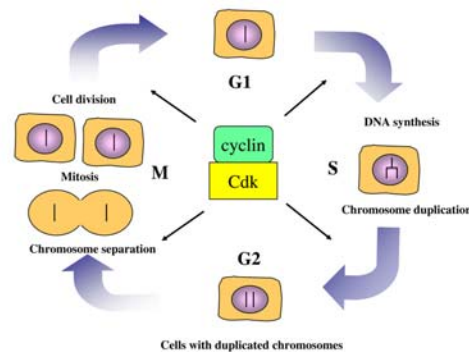


Figure 7: Mammalian cell cycle. Schematic representation of the mammalian cell cycle. In each cell division cycle, chromosomes are replicated once (DNA synthesis or S-phase) and segregated to create two genetically identical daughter cells (mitosis or M-phase). These events are spaced by intervals of growth and reorganization (gap phases G_1 and G_2). Cells can stop cycling after division, entering a state of quiescence (G_0) due to absence of nutrients. Progress through the cycle is accomplished in part by the regulated activity of numerous CDK/cyclin complexes.

The point between the early G₁ and late G₁ phase passage that represents an irreversible commitment to undergo one cell division is termed the 'restriction point' [110]. Importantly, the restriction point divides the cell cycle into a growth factor dependent early G₁ phase and growth factor independent phases from late G₁ through mitosis [110]. Growth factor signaling determines whether early G₁ phase cells transit the restriction point to undergo eventual cellular division or, because of insufficient signaling strength, exit the cell cycle, enter into G₀. Thus, overcoming growth factor signaling dependency is a major hurdle in the development of neoplastic disease. Indeed, molecular analysis of human tumors has shown that cell-cycle regulators were frequently mutated in neoplasias, which underscores how important the maintenance of cell cycle commitment was in the prevention of human cancer [111].

Major alterations include, over-expression of cyclins (mainly cyclin D1 and E) and CDKs (mainly CDK4 and CDK6), as well as loss of cyclin-cdk inhibitory (-CKi- mainly INK4, and KIP1) and retinoblastoma (Rb) expression.

It has been shown that cyclin D1 is over-expressed in many human cancers as a result of gene amplification or translocations targeting the D1 locus [112, 113]. In particular in esophageal, hepatic, and head and neck cancers, it has been shown a correlation between D1 amplification and cyclin D1 protein over-expression. Aberrant over-expression of cyclin D1 is also seen in sarcomas, colorectal tumors, and melanomas, even though D1 gene amplification frequencies are exceptionally low [113]. That cyclin D1 can directly contribute to oncogenesis was supported by studies with transgenic mice, in which targeted over-expression of D1 in mammary epithelial cells leads to ductal hyperproliferation and eventual tumor formation [114]. Conversely, mice nullizygous for D1 shown profound defects in mammary lobulo-alveolar development during pregnancy, indicating that cyclin D1 plays a critical, uncompensated role in the maturation of this tissue [115].

This special dependency of breast epithelial cells on cyclin D1, coupled with the ability of the same regulator to induce breast cancer, points toward a striking concordance between normal developmental controls and neoplastic processes. Reciprocally, mice lacking Cdk4 and Cdk6 exhibit late embryonic lethality and display similar phenotypes as triple cyclin D knockout animals [116, 117], implying overlapping roles of these proteins in development. However, in the absence of Cdk4 and Cdk6, Cdk2 binds to cyclin D to promote G₁-phase progression [117], indicating a cell intrinsic compensation mechanism. However, combined deletion of Cdk2/4/6 does not affect mouse organogenesis until midgestation, highlighting the critical role of Cdk1 in cell cycle progression during embryonic development and cell division [118]. Nevertheless, double knockout (DKO) of Cdk2 and Cdk4 affects mouse lethality and *in vivo* proliferation. These phenotypes have been correlated with heart defects and in mouse embryonic fibroblasts (MEFs) with impaired S-phase entry, reduced Cdk1 levels, and premature entry into senescence [119].

Recently, a new oncogenic role for cyclin D/CDK4/6 complexes has been proposed whereby cyclin D activity partially inactivates pRb function by disrupting complexes of pRb and histone deacetylase (HDAC) leading to de-repression of the cyclin E gene and consequential activation of cyclin E/CDK2 [120-122].

Loss or inactivation of pRb is also an important and frequent event in human tumors. Inactivation of *RB* itself is the *sine qua non* of retinoblastoma [123], but overall the gene is targeted more often in adult cancers [113]. *RB* KO mice die between mid and late gestation, owing to developmental defects in the placenta, the nervous system, and in erythropoiesis [124-127] even though proliferation rates of *RB* null MEFs are comparable to wild-type cells [128, 129]. In contrast, p107 or p130 KO mice are viable with different degrees of abnormalities depending on strain background [130, 131]. Several studies have shown that hypophosphorylated pRb in G₁ is the active form

that binds E2F transcription factors and represses expression of E2F target genes [132, 133]. In contrast, unphosphorylated pRb in G₀ does not associate with E2Fs and is considered inactive [132-134]. These data suggested that Rb is an important factor for cell survival and proliferation, which goes beyond its role in cell-cycle regulation [135].

Whereas cyclin–cdk complexes are key regulators of G₁ cell cycle progression, evolution has added yet another layer of G₁ cell cycle regulation in the form of CKI. At present, two classes of G₁ cyclin–CDK inhibitors, the INK4 (p15, p16, p18 and p19) that specifically bind monomeric CDK4/6 and Cip/Kip (p21, p27 and p57) that bind and inactivate heterodimeric cyclin–cdk2 complexes [136]. In particular, low expression of p27^{Kip1} in tumor samples has been correlated to poor prognoses in breast carcinoma, colon carcinoma, and other malignancies [136]. Moreover, cytosolic localization of p27^{Kip1} has been correlated with increased tumor aggressiveness [137]. However, as p27^{Kip1} is signaled for increased degradation [138, 139] and cytosolic localization in dividing cells, these correlations may have little to do with direct epigenetic selection for p27^{Kip1} loss of function during oncogenesis and may be consequence of increased degradation as a result of a higher fraction of dividing cells in aggressive tumors.

The most frequently mutated inhibitor of cell cycle in human cancer is p53 gene [140] and is an archetypal checkpoint regulator. Although it is not essential for normal mouse development [141], one of its roles is to ensure that, in response to genotoxic damage, cells arrest in G₁ and attempt to repair their DNA before it is replicated [142]. The p53 protein is a transcription factor that function in a complex signaling network to mediate cellular adaptation to stress [140]. In response to diverse cellular insults including DNA damage, hypoxia and oxidative stress, p53 becomes post-translationally modified, which promotes both its stabilization and activation, and activates the expression of genes that induce cell cycle arrest, senescence and apoptosis. A target of p53 is the gene encoding the CDK

inhibitor p21^{Cip1} [143, 144] and is at least partially responsible for p53-mediated G₁ arrest [145]. When treated with DNA-damaging drugs, cells lacking p21^{Cip1} appear to undergo repeated S phases, possibly reflecting aberrations in controls linking the completion of S phase with mitosis [146]. The loss of p53 predisposes cells to drug-induced gene amplification and decreases the fidelity of mitotic chromosome transmission [147]. Duplication of the centrosome normally begins at the G₁/S boundary, but in the absence of p53, multiple centrosomes appear to be generated in a single cell cycle, ultimately resulting in aberrant chromosomal segregation during mitosis [148].

Only recently has p53 been implicated in metabolic control, coordinating stress responses with changes in cellular metabolism and angiogenic potential. Activation of p53 by metabolic stress is driven by AMPK-dependent phosphorylation and influenced by mTOR [149]. In particular it has been shown that mTOR could be regulate cell cycle through control of cyclin D1 and p21^{Cip1} production [150, 151]. Indeed, it has been shown that the pathway PI3K/AKT/mTOR often could be regulated in cancer cells, resulting in the over-stimulation of growth promoting pathways [152]. In addition oncogenic activation of this pathway or inactivation of PTEN (PI3K inhibitor) inhibits apoptosis and further survival [152].

Systems Biology approach as new application in Drug Discovery.

Cell proliferation requires coordination and integration of different processes to modulate the activity of key cell-cycle regulators. They are controlled by numerous mechanisms that reflect the diversity of the signals they integrate and the central importance of their role in cell-cycle control. Proliferative disorders are a major challenge for human health, uncontrolled cell proliferation being the hallmark of cancer. In fact, in tumor cells, the balance between intra- and extracellular signals and the control of the cell cycle is

lost. To understand how intracellular and extracellular signals are transmitted to the cell-cycle machinery and how the latter adjusts its frequencies accordingly is one of the major challenges in molecular biomedicine. For many years research into the molecular basis of diseases focused on the products of individual genes. These were examined in parallel by different groups. Rarely were they studied in terms of the complete intracellular networks they are a part of. Neither the proper tools nor the data for such a network- based analysis were available. Indeed only recently has it become possible to analyze the expression of all genes in a pathway simultaneously. Furthermore, the genomics revolution has opened the way to other similar global approaches, such as proteomics and metabolomics. Considering the large amount of data emerging from these high-throughput techniques, only the development of new computer sciences and modeling methodologies will enable us to select the relevant from the irrelevant information and utilize it for health-care applications. In fact, molecular biology should begin to address the organization of the large network of molecules that determine cellular functions and their disturbance. In other words, molecular medicine has to be understood in terms of the functioning of modular networks of molecules. The result of this integrative/ interactive process is very relevant for two reasons. First, it will make it possible to analyze and understand the molecular basis of each disease. Thanks to the completeness of the information (genomics, proteomics, and metabolomics), all molecular determinants can in principle be identified, produced, and analyzed in terms of structure and manipulated to elucidate their function. Second, most diseases are multifactorial in their pathology, if not in their origin. Accordingly, understanding disease and the design of personalized therapy requires going beyond the product of a single gene, to all gene products with which that gene interacts, if not to the functioning of the entire system of interacting molecules. Furthermore, the interactive process of model construction and validation will enable identification of the robustness and

fragility of regulatory molecular circuits that are altered in a given pathological state. Thereby a strategy for the rational selection of specific molecular targets for drug discovery and development can be developed.

Materials and Methods

MATERIALS AND METHODS

Cell culture

Mouse embryonic fibroblast NIH3T3 cells (CRL-1658; American Type Culture Collection) and a K-Ras transformed NIH3T3-derived cell line, 226.4.1 [153], were routinely grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum, 4mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (normal growth medium) (all from Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂. Cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and maintained in culture for 48 h before manipulation.

Cell treatments

To verify cell response to glutamine, cells were plated at 3000 cells/cm² in normal growth medium (4mM Glutamine). 18 hrs after seeding, cells were washed 2x with phosphate-buffered saline (PBS) and incubated in media with different glucose (Glu -25mM and 1mM) or glutamine (GLN-4mM, 1mM and 0.5mM glutamine-) concentrations. When required, 2mM pyruvate (Sigma), 2mM malate (Sigma), 10µM dNTPs (Rovalab), and 20nM rapamycin (ALEXIS Biochemicals), were added as indicated in the text.

Cell synchronization

Cells were plated at 5500 cells/cm² in normal growth medium. 18 hrs after the seeding, cells were washed 2x with PBS and synchronized by 24 hrs of serum starvation with Dulbecco's modified Eagle's medium containing 4mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (Invitrogen), 6ng/ml sodium selenite and 6µg/ml transferrin (Sigma). Stimulation of quiescent cells was performed by adding 10% newborn calf serum in medium with the appropriate glucose or glutamine (and dNTPs,

when required) concentration. DNA was labelled with 33 μ M 5-Bromodeoxyuridine.

Flow cytometric analysis

The distribution of cells at specific cell cycle phases was evaluated by flow cytometry. Cells were trypsinized, washed with PBS and fixed in 75% ethanol at 4°C. The cells were washed in PBS for ethanol removal and incubated for 30 min in 0.25% Triton X-100 and HCl 2N. Subsequently, each sample was stained with an anti-BrdU primary antibody (Becton-Dickinson) for 1 h, and probed with Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes/Invitrogen) to identify S phase. In addition, the same samples were stained with propidium iodide (Sigma) and analyzed by FACS (FACScan, Becton-Dickinson), using the Cell Quest software (BD Bioscience). Data analysis was performed with WinMDI software.

Analysis of apoptosis was performed by using flow cytometry assay kit Annexin V/Propidium Iodide (EXBIO) and analyzed by FACS (FACScan, Becton-Dickinson), using the Cell Quest software (BD Bioscience). Data analysis was performed with WinMDI software.

Glutamine assay

Glutamine variation in supernatants of normal and transformed fibroblasts was determined by using a spectrophotometric glutamine/glutamate enzyme assay kit (Sigma) based on enzymatic deamination of L-glutamine and dehydrogenation of L-glutamate with conversion of NAD⁺ to NADH. The assay was performed as specified by manufacturer datasheet and it is specific for glutamine and does not cross-react with other amino acids or ammonia. To calculate the quantity of glutamine a linear regression analysis of the standard curve was performed.

Immunofluorescence microscopy

Cells were grown on coverslip previously treated with 0.2% gelatine. The cells were washed 3x with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Subsequently, were washed 3x with PBS, permeabilized with 0.1% Triton X-100 for 10 min, blocked with PBS + 10% goat serum for 30 min, probed with primary antibody p27kip1 (1:100) (Santa Cruz Biotechnology CA, USA) in PBS + 10% goat serum for 1 hrs at room temperature, washed 3x with PBS, probed with fluorescence-labeled secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (1:400) (Molecular Probes/Invitrogen) in PBS + 10% goat serum for 30 min at room temperature, washed 3x with PBS, stained with DAPI for 2 min (1:500) (Sigma-Aldrich Inc), and then mounted in DABCO (Sigma-Aldrich Inc). For BrdU (Sigma-Aldrich Inc.) labeling, cells were fixed, permeabilized and blocked as described above. After the cells were incubate with an anti-BrdU monoclonal antibody (1:10) (Becton-Dickinson), MgCl₂ (3 mM) and DNase I (Invitrogen) 100 U/ml for 1 h at RT. Subsequently, the cells were washed 3x with PBS and incubated with fluorescence-labeled secondary antibody Alexa Fluor 488 donkey anti-mouse IgG (1:100) (Molecular Probes/Invitrogen) in PBS + 10% goat serum for 30 min at room temperature, washed 3x with PBS, stained with DAPI for 2 min (1:500) and then mounted in DABCO.

Microscopy

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 (40x dry and 60x oil; numerical aperture 0.75 and 1.4 respectively). The images were acquired using the imaging software Metamorph 7, then processed in Adobe Photoshop 7.0.1 with adjustments of brightness and contrast. The quantitative analysis image was performed

using the imaging software Image J. The relative distribution of p27kip1 protein between the two compartments nucleo/cytoplasm, was calculated by measuring the pixel average signal both in the nucleus, cytoplasm and nucleo/cytoplasm compartments. At least 200 cells, together with negative controls (no primary antibody), were randomly selected. In order to exclude the background of staining and to select the positive stained parts of the cells for measurement, the images from the various samples were processed at the same threshold and then measured.

Immunoblot analysis

Cells were lysed in a buffer containing 150mM NaCl, 0.5% NP-40, 1% glycerol, 50mM HEPES (pH 7.5), 5mM ethyleneglycol tetracetate (EGTA), 1mM phenylmethylsulphonyl (PMSF), 50mM NaF and a cocktail of protease inhibitors (Roche). After incubation for 30 min on ice, the extracts were centrifuged at 13.200 r.p.m. for 20 min. Protein concentration of supernatant was measured by the Bradford procedure (Bio-Rad Laboratories, Richmond, CA, USA), using bovine serum albumin as a standard. These cellular extracts were electrophoresed in sodium dodecylsulfate (SDS) polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membrane by electroblotting and incubated with antibodies over night.

The antibodies used were monoclonal or polyclonal antibodies against cyclin D1, cyclin E, cyclin A, p27^{Kip1}, Cdk2, Cdk4 (all from Santa Cruz Biotechnology), phosphoRb-795, p70 S6 kinase, phospho-p70 S6 kinase and vinculine (all from Cell Signalling). Subsequently, the membranes were incubated with a peroxidase-coupled secondary antibody (Amersham, Othelfingen, CH) for 30 min at room temperature. The reaction was visualized with ECL (Amersham) followed by exposure to an x-ray film. Protein expression levels were quantified by densitometric evaluation of

antibody specific bands on scanned x-ray films by using the imaging software Image J.

RNA extraction and analysis

Total RNA was isolated from normal and transformed cell lines using TRIzol reagent (Invitrogen). RNA purity and integrity were checked by direct observation loading 1 μ g of total RNA on 1% agarose gel. Total RNA had a 28S:18S rRNA ratio of at least 2.0. Amount estimation was done by spectrophotometer analysis.

Results

RESULTS

Cell proliferation requires nutrients, energy and biosynthetic activity to duplicate all macromolecular components during each passage through the cell cycle. Cancer is essentially a disease in which cells have lost the normal cell proliferation checkpoints. Therefore, it may not be surprising that tumor cells, in order to meet the increased requirements of proliferation, often display fundamental changes in pathways of energy metabolism and nutrient uptake [1].

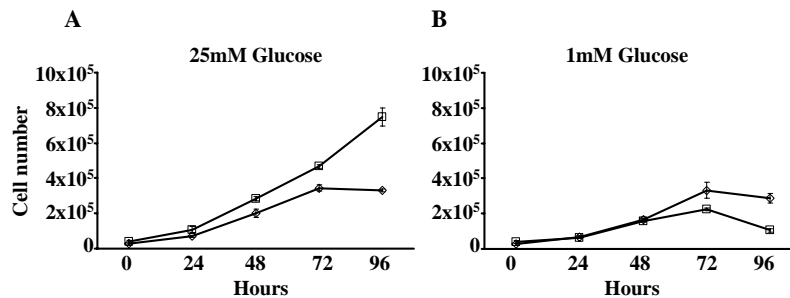
In this work, will be discuss how glucose and glutamine availability influence proliferation, survival and cell cycle in K-Ras transformed fibroblasts as compared to an immortalized counterpart.

Glucose depletion, in asynchronous Transformed fibroblasts, induces growth arrest and apoptosis but does not affect G₁/S transition

Cancer cells have been shown to have a particular dependence on aerobic glycolysis, "Warburg effect", a reduced oxidative phosphorylation, and a relative impairment of mitochondrial function. Results previously obtained by using our cellular model, given by NIH3T3 murine fibroblasts (normal) and NIH3T3 murine fibroblasts overexpressing an oncogenic form of K-ras (transformed) [154] subjected to nutritional perturbations (glucose deprivation) [155] have shown that K-ras transformed fibroblasts have a strong dependence on glycolysis, reduced oxidative phosphorylation ability, altered mitochondrial morphology and potential, decreased ATP content and a enhanced cell death towards glucose depletion as compared to normal counterparts [3, 155]. Moreover, detailed analysis of transformed cells transcriptional profiles, grown in high and low glucose (25 and 1mM), shows extensive changes in expression of genes encoding mitochondrial and glycolytic proteins (studied by Affymetrix).

Asynchronous normal and transformed cells, grown in high and low glucose, were followed in a time course of 24-96 hours (hrs), that is, from the moment of seeding to when they either reached confluence, started to grow in multi-strata or die. As shown in Figure 8 Normal cells stopped growth after 72 hrs, regardless of glucose concentrations (25mM and 1mM). At later time, cell number started to decrease (Figure 8A-B, \diamond symbol). Concurrently, apoptotic phenotypes (Figure 8C, upper panels) were observed in normal cells regardless of glucose concentration, possibly because of prolonged contact inhibition.

On the contrary, transformed cells grown in 25mM and 1mM glucose, continued to proliferate and reached a much higher cell density than normal cells in the first case, (Figure 8A, \square symbol) and completely lost the proliferation advantage in low glucose (Figure 8B, \square symbol). Moreover, the reduction of proliferation ability was followed by an enhanced cell death at 96 hrs, as confirmed by proliferation curves (Figure 1B, \square symbol) and by phase-contrast microscopy (Figure 1C, lower panels). These results confirmed that glucose is the preferred metabolic substrate of tumor cells [156].



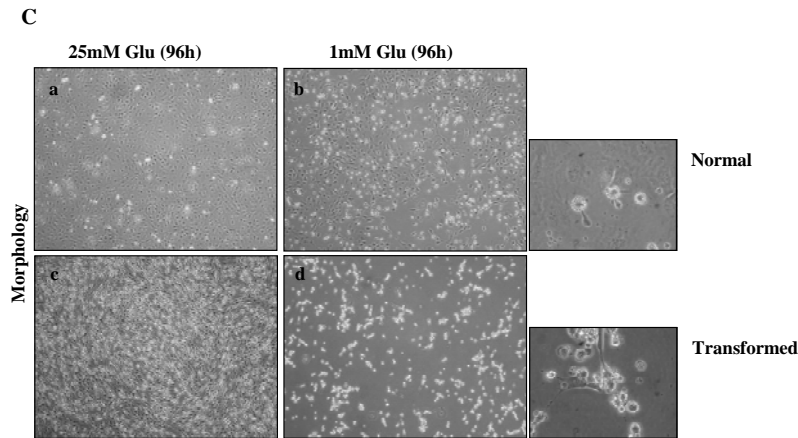
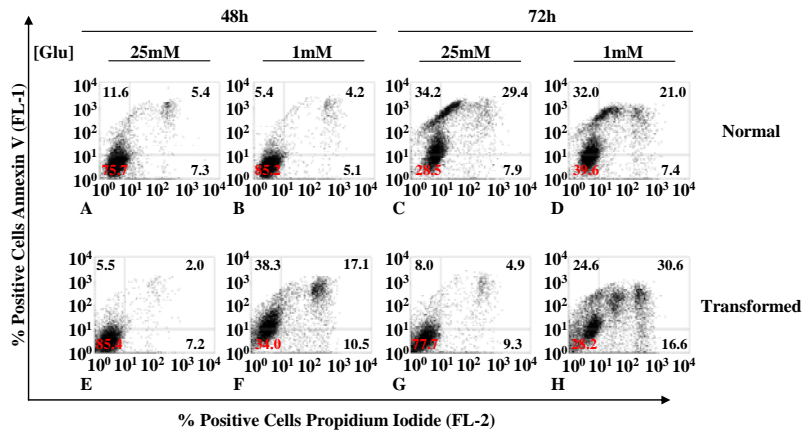


Figure 8: Glucose shortage induces cell proliferation arrest and enhanced cell death in oncogenic K-ras transformed fibroblasts. Normal (\diamond) and transformed (\square) cell lines were plated at 3000 cells/cm² in 6-well plates in normal medium. Culture medium was replaced after 24 hrs with different concentration of glucose -25mM (A), and 1mM (B)- and the cells were collected and counted at indicate time. (C) Morphological analysis of normal cells (upper images) and transformed cells (lower images) cultured in 25mM glucose (left images) and 1mM glucose (right images) at 96 hrs with evidence cell death in transformed cells. Cells were observed at phase-contrast microscopy.

To determine the apoptotic route activated in transformed cell upon low glucose availability, the cells were grown in 25mM and 1mM glucose for up to 96 hrs and stained with PI/Annexin V. Annexin-V is a Ca²⁺ dependent phospholipid-binding protein that has high affinity for phosphatidylserine (PS) which itself is translocated from the inner to the outer leaflet of the plasma membrane during the early phase of the apoptotic process. Apoptotic cells were identified by flow cytometry using Annexin-V conjugated to fluorescein isothiocyanate (FITC), in conjunction with propidium iodide (PI) to distinguish apoptotic cells (Annexin-V-FITC positive, PI negative; Annexin-V-FITC positive, PI positive) from necrotic cells (Annexin-V-FITC negative, PI positive). Using this method has been observed in transformed cells grown in 1mM glucose an earlier increase of cells positive for Annexin V staining at 48 hrs (Figure 9, panel F, -left upper quadrant-38.3 % and -

right upper quadrant-17.1 % compared to panel E, -left upper quadrant-5.5 % and -right upper quadrant-2.0 %) as compared to the same cells growth in 25mM glucose (Figure 9, panel E). Moreover, K-Ras transformed fibroblasts shown at 72 hrs in 1mM glucose both apoptotic and necrotic cells (Figure 9, panel H, -left upper quadrant-24.6 %, -right upper quadrant-30.6 % and -right lower quadrant-16.6 %, compared to panel G, -left upper quadrant-8.0 %, -right upper quadrant-4.9 % and -right lower quadrant- 9.3 %) that was not observed in 25mM glucose, confirming that the apoptotic event observed was glucose dependent. On the contrary in normal cells a glucose independent apoptotic process, and only at 72 hrs, was observed, (Figure 9, panel C, -left upper quadrant-34.2% and -right upper quadrant-29.4% compared to panel D, -left upper quadrant-32.0% and -right upper quadrant-21.0 %), suggesting a different mechanism of activation of apoptosis as compared to transformed cells grown in low glucose availability.



Normal									
h	25mM					1mM			
	0	24	48	72	96	24	48	72	96
UL(A)	1.0±0.37	1.8±0.2	5.3±2.9	33.6±0.9	44.9±12.5	2.4±0.1	6.9±2.1	21.0±4.7	22.3±0.9
UR(A/P)	2.3±0.8	1.5±0.6	3.0±0.9	24.9±6.4	20.3±8.8	2.2±0.0	3.7±0.7	11.9±6.3	23.8±0.0
LL(Un)	91.2±3.3	93.4±1.5	85.0±5.1	35.8±10.3	31.7±3.8	91.2±0.5	85.3±0.2	59.4±7.5	39.6±6.8
LR(PI)	5.2±2.1	3.3±1.2	6.7±1.4	5.7±3.1	3.1±0.1	4.2±0.4	4.0±1.5	7.6±3.5	14.4±2.3

Transformed									
h	25mM					1mM			
	0	24	48	72	96	24	48	72	96
UL(A)	2.1±0.4	1.8±0.6	4.9±2.8	9.5±1.8	21.2±0.3	11.1±0.1	42.5±4.5	38.9±2.1	30.3±0.5
UR(A/P)	2.6±0.5	1.8±0.2	1.4±0.2	19.4±1.2	15.0±6.2	1.9±0.5	14.3±3.5	16.2±9.1	29.9±5.4
LL(Un)	87.4±1.8	91.4±0.8	86.6±4.8	62.2±3.3	59.9±8.0	80.3±1.6	36.1±1.1	37.2±9.7	26.7±3.7
LR(PI)	8.0±0.9	4.9±1.6	7.2±2.3	8.8±3.7	4.0±2.0	6.7±2.0	7.1±4.1	7.7±4.1	13.1±0.7

Figure 9: Identification of apoptotic cell death mechanism in normal and transformed fibroblasts. Normal (upper panel) and transformed (lower panel) cells, grown in different glucose concentration (25mM and 1mM glucose) along time course 0-96 hrs, were analyzed by flow cytometry (FACScan) following staining with Annexin V/Propidium Iodide (apoptosis/necrosis). Red color in the plot represent the percentage of uncolored cells. Analysis was performed by using WinMDI 2.8 software. The data is representative of triplicate experiments.

Caspase-3 is an active cell-death protease involved in the execution phase of apoptosis. Further confirmation of the apoptotic pathway activation was obtained by analysis of caspase-3 activation by fluorescence microscopy in normal and transformed cells grown in high and low glucose. Indeed, in normal cells, caspase 3 activation was observed only at a later time point analyzed (96hrs) and in glucose-independent manner (Figure 10A, middle and right panels, 25 and 1mM, 96hrs). In contrast, transformed cells grown in low glucose (Figure 10A, middle and right panels, 1mM 96hrs), showed an earlier and stronger activation of caspase 3 as compared to transformed cells grown in high glucose (Figure 10A, middle and right panels, 25mM 96hrs) and to normal cells grown in both initial glucose concentrations (Figure 10A, left panels). These results were confirmed by quantitative analysis performed by using tools present in “Image J” program (Figure 10B).

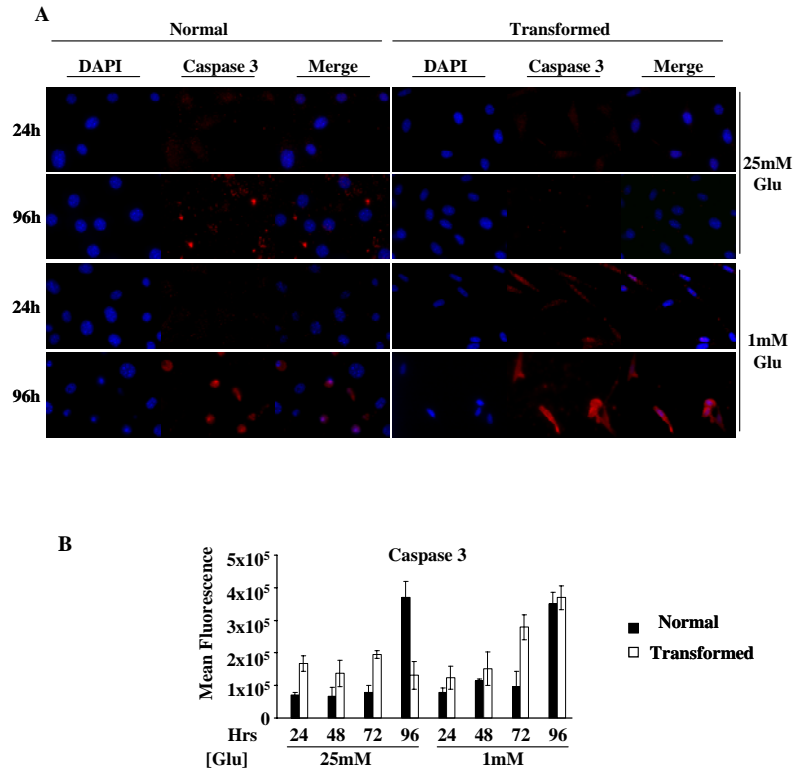
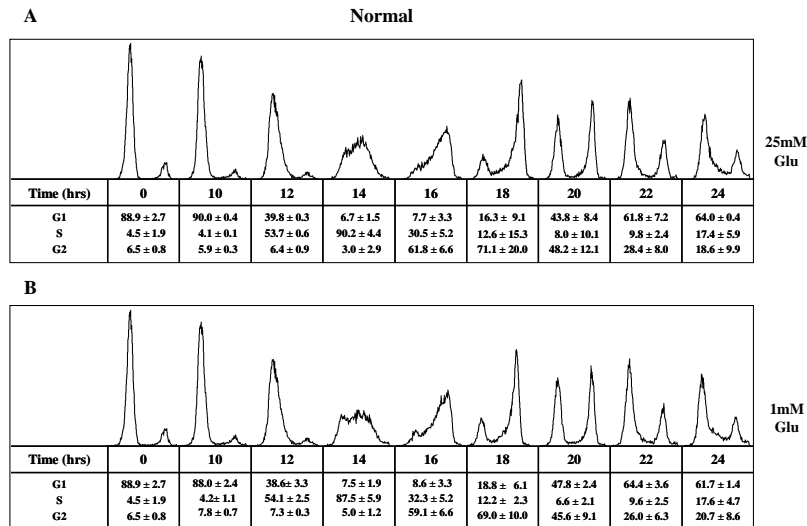


Figure 10: Caspase 3 activation in normal and transformed mouse fibroblasts. (A) Normal (left pictures) and transformed (right pictures) cells grown in 25mM and 1mM glucose, along a time course (24-96 hrs) were analyzed by immunofluorescence staining for Caspase 3 (red staining) using a specific antibody. Nuclei were visualized by DAPI staining (Blue staining). At least 200 cells were scored for each sample. The averages of three independent experiments are presented in B with errors bars indicating SD. The quantitative analysis were performed by using tools present in Image J program.

The results above described, showed that the higher proliferation potential of asynchronous transformed cells, is lost upon growth in media supplemented with 1mM initial glucose. Since glucose is an essential substrate for the

synthesis of several biosynthetic intermediates necessary to duplicate cell biomass and genome at each cell division, its deprivation could interfere also with the execution of G₁/S transition of synchronous transformed fibroblasts. To test this hypothesis, normal and transformed cells were synchronized by serum starvation for 24 hrs. The cell cycle block release was obtained by adding 10% serum in media containing 25mM (Figure 11A, C) and 1mM (Figure 11B, D) glucose. The samples were collected at indicated time points (10, 12, 14, 16, 18, 20, 22 and 24 hrs) and stained with propidium iodide. Normal (Figure 11A, B) and transformed (Figure 11C, D) cells, released in both glucose concentrations, showed a glucose-independent kinetics of re-entry into cell cycle. However, while normal cells reached the S phase peak at 14 hrs, transformed cells reached this peak at 12hrs (Figure 11A and B).



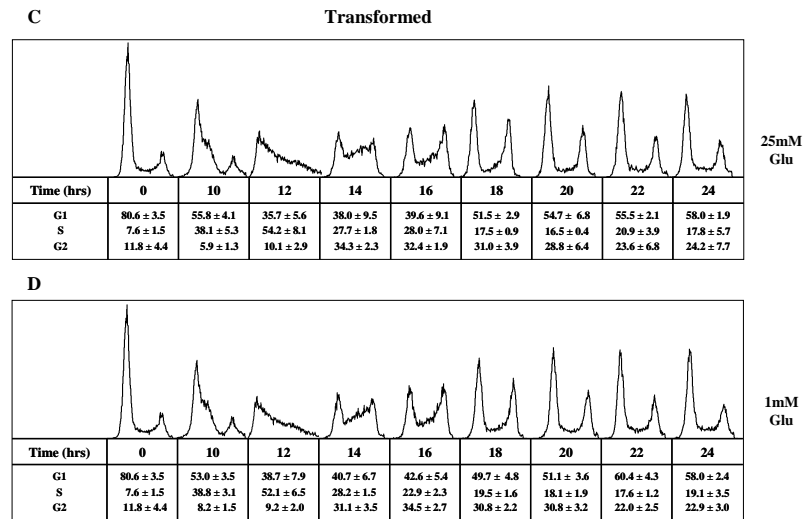


Figure 11: Cell cycle profiles of synchronized normal and transformed mouse fibroblasts in different glucose concentration. Normal (A, B) and transformed (C, D) cells were synchronized by serum starvation for 24 hrs. The cells were then released with 10% NCS and respectively 25mM (A and C) and 1mM (B and D) glucose, in order to determine the effect of glucose shortage during G₁/S transition. At indicate time points the cells were collected and stained with propidium iodide and analyzed by flow cytometry. The cell cycle analysis and the fraction of cells in each phase (G₁, S and G₂/M indicated as % cells) were determined using WinMDI 2.8 software. The data is representative of triplicate experiments.

Altogether these results indicated that the timing of the G₁/S transition execution, analyzed in synchronous cells, was glucose independent in both cell lines, and that transformed cells anticipated the entry in S phase of two hrs. Moreover, analysis of apoptotic process activation in asynchronous cells by different approaches, confirmed a high sensitivity of transformed cells to glucose deprivation. Indeed, while in normal cells apoptosis is observed in both glucose concentrations and only at a later time point (96hrs), probably due to prolonged contact inhibition, in transformed cells, instead, apoptosis is activated specifically by glucose deprivation.

Reduced proliferation of K-ras transformed fibroblasts in media containing low initial glutamine concentration is associated to an increased fraction of cells in S phase

Glutamine is an important substrate used in several cellular processes which use has been found increased in transformed cells. To dissect its role in the proliferation ability of transformed cells as compared to normal ones, it has been tested whether lowering initial glutamine concentration in culture medium elicited differential effects on the proliferation of transformed cells as compared to normal cells. Asynchronous normal and transformed cell lines were cultured in normal growth medium (4mM glutamine), in an intermediate medium (1mM glutamine) and in a low glutamine medium (0.5mM glutamine). These concentrations were chosen considering glutamine levels normally used in cell culture (between 4 and 2mM) as well as that determined in human plasma (0.6mM). All media were supplemented with 25mM glucose. Cells were followed for at least 144 hrs. Normal cells stopped growth after 72 hrs, regardless of glutamine concentration. At later time, cell number started to decrease (Figure 12A–C, ♦ symbol). Concurrently, apoptotic phenotypes - including the presence of floating, dead cells (Figure 12D, upper panels) - were observed in normal cells regardless of glutamine concentration, possibly because of prolonged contact inhibition. In normal and intermediate glutamine medium, transformed cells continued to proliferate and reached a much higher cell density than normal cells (Figure 12A-B, ■ symbol), but proliferation advantage of transformed cells was almost completely lost when they were grown in low glutamine medium as scored by both cell counting (Figure 12C, about 1,16106, 0,96106 and 0,46106 at 144 hrs respectively for cells grown in normal, intermediate and low glutamine concentration) and direct microscopic observation (Figure 12D, compare lower left and lower right bottom panels). Regardless of glutamine concentration, little, if any, floating, dead cells were observed in transformed cells (Figure 12D).

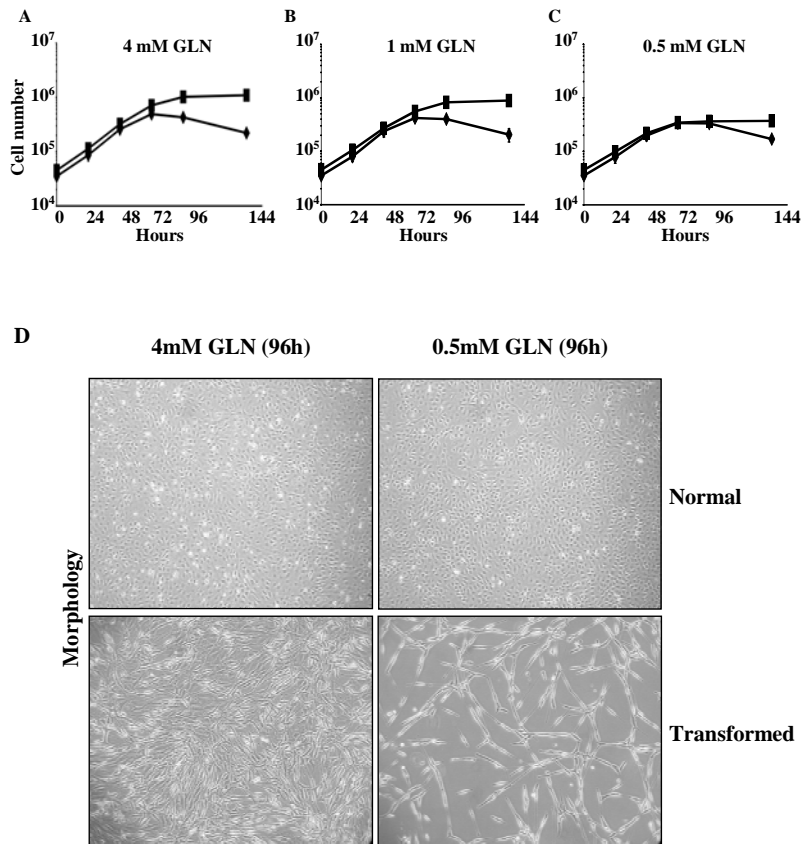


Figure 12: Glutamine shortage induces cell proliferation arrest in oncogenic K-ras transformed fibroblasts. Normal (◆) and transformed (■) cell lines were plated at 3000 cells/cm² in 6-well plates in normal medium. Culture medium was replaced after 24 hrs with a normal medium -4mM glutamine- (A), an intermediate medium -1 mM glutamine- (B) and a low medium -0.5mM glutamine- (C). The cells were collected and counted at indicate time. (D) Morphological analysis of normal cells (upper images) and transformed cells (lower images) cultured in 4mM glutamine (left images) and 0.5mM glutamine (right images) at 96 hrs. Cells were observed at phase-contrast microscopy.

As above shown, balanced exponential phase was very short, since despite exponential increase in cell number, the fraction of G₁ cells started to increase as early as at 48 hrs after the beginning of the experiment, the more

so for normal cells (Table 1). Remarkably, transformed cells grown in low glutamine medium retained quite a large fraction of S and G₂/M phase cells even at the 72 or 96 hrs time points, i.e., when the increase in cell number had stopped. Indeed, after 72 hrs of growth, transformed cells had a very similar fraction of cells in the S+G₂M phases regardless of glutamine concentration, while a significant difference was observed compared to normal cells (Tab.1).

[Glutamine]	4mM				0.5 mM				Cell line
	Time	24h	48h	72h	96h	24h	48h	72h	
phase									
G1	45 ± 5.0	60 ± 4.3	82 ± 7.7	85 ± 13.0	39 ± 3.0	60 ± 5.3	85 ± 3.7	85 ± 11.8	Normal
S	22 ± 1.0	17 ± 2.7	10 ± 6.4	10 ± 4.9	25 ± 3.5	17 ± 4.9	6 ± 0.8	7 ± 3.2	
G2+M	33 ± 5.2	23 ± 4.7	8 ± 1.7	5 ± 3.7	36 ± 4.2	23 ± 3.6	9 ± 2.9	8 ± 4.7	
G1	50 ± 4.1	59 ± 13	71 ± 8.5	81 ± 17.2	55 ± 4.4	64 ± 2.1	71 ± 2.9	75 ± 7.2	Transformed
S	27 ± 2.6	21 ± 2.4	18 ± 1.8	11 ± 2.3	26 ± 5.2	19 ± 8.4	15 ± 2.0	15 ± 1.4	
G2+M	23 ± 2.2	20 ± 2.4	11 ± 4.2	8 ± 2.0	19 ± 5.1	17 ± 7.5	14 ± 1.9	10 ± 0.9	

Table 1: Exponentially growing normal and transformed cells were incubated for increasing periods of time (24, 48, 72, 96 hrs) in medium containing 4mM and 0.5mM glutamine, then harvested, counted and subjected to FACS analysis after staining with propidium iodide. The cell cycle analysis and the fraction of cells in each phase (G₁, S and G₂/M indicated as % cells) were determined using WinMDI 2.8 software. Results are the mean of at least triplicate determinations with standard deviations indicated.

Quantification of the amount of BrdU incorporated into DNA is taken to be more precise than Propidium Iodide to evaluate S phase cells, therefore DNA synthesis was then analyzed by BrdU incorporation (30 min pulse) detected by immunofluorescence using a BrdU specific antibody and BrdU-positive cells were counted (Figure 13). While the fraction of normal BrdU-incorporating cells steadily decreased from 48 hrs, arriving to around 1% after 96–144 hrs of growth, the fraction of transformed BrdU-incorporating cells at 96–144 hrs of growth remained high, the more so in cells growing in media supplemented with 0.5mM glutamine. Compare for instance normal and transformed cells grown for 96 hrs in media supplemented with 0.5mM

glutamine: normal and transformed cells have reached the same, maximal density, but the fraction of BrdU-incorporating cells is almost 0% for normal cells and at least 15% in transformed cells. This value remains constant until the end of the experiment and is essentially the same for cells grown in 4mM or 0.5mM glutamine, despite the large difference in proliferation.

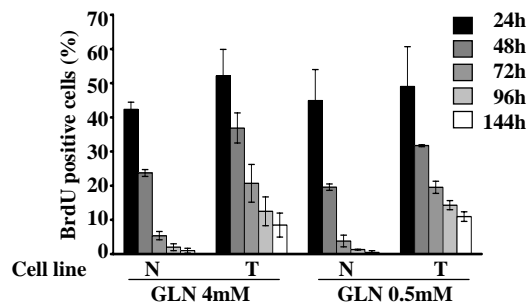


Figure 13: Glutamine shortage induces S-phase accumulation in oncogenic K-ras transformed fibroblasts. Exponentially growing normal and transformed cells were incubated for increasing periods of time (24, 48, 72, 96 and 144 hrs) in medium containing 4mM and 0.5mM glutamine, then harvested, counted and subjected to FACS analysis after staining with BrdU (pulsed 30 min.) and then analyzed by fluorescence microscopy using an anti-BrdU specific antibody. Results are the mean of at least triplicate determinations with standard deviations indicated. BrdU staining were analyzed at least 200 cells for each sample.

In order to identify glutamine uptake was indirectly measured by assaying residual glutamine in the medium (Figure 14A and B). At both glutamine availabilities, normal and transformed cells consumed glutamine at the same rate. Remarkably, glutamine uptake was much faster in cells grown at low initial glutamine concentration (Figure 14B). However, while in cells grown at 4mM initial glutamine concentration, at least 20% of initial glutamine (i.e. ca 0.8mM) was still present at 96 hrs, i.e. when normal cells have stopped to proliferate (Figure 14A), in cells grown in low initial glutamine concentration, no residual glutamine was present at 96 hrs. Transformed cells stopped growth afterwards, indicating that glutamine acts as a limiting nutrient under these conditions.

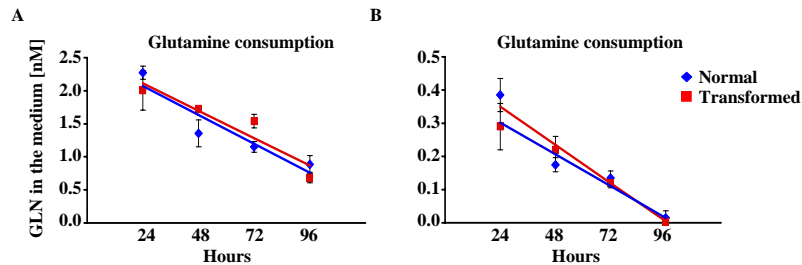


Figure 14: Glutamine consumption in Normal and Transformed fibroblasts. Analysis of glutamine concentration, along a time course of 96 hrs, of normal (◆) and transformed (■) cells plated in 4mM (A) and 0.5mM (B) initial glutamine availability. Medium was collected and analyzed for glutamine concentration by a quantitative, spectrophotometric assay. The slope of the data points are indicated in blue color for normal cells and in red color for transformed cells. Note the different scale used for 4mM and 0.5mM. The data points presented are the mean \pm SD of triplicate samples.

In transformed cells sustained S-phase in low glutamine is associated with prolonged expression of cyclin D, E and A, enhanced pRb phosphorylation, decreased level and cytoplasmic localization of p27^{Kip1}

Oncogenic Ras activation is known to promote the G₁ to S progression [3, 157, 158]. Results reported above indicate that transformed cells grown in low glutamine medium retain a large fraction of cells in S phase while proliferation is stopped suggesting that in transformed cells glutamine depletion does not efficiently shut-down the G₁ to S. It was therefore of interest to analyze both in normal and in transformed cells modulation by glutamine of relevant parameters, such as level, phosphorylation state and sub-cellular localization of proteins involved in the G₁ to S transition. Transcription of genes required for the onset of S-phase in mammalian cells is induced by the E2F/DP transcription factors, whose activity in early G₁ cells is down-regulated by the pRb protein. Release of inhibition by pRb requires its phosphorylation by upstream cyclin dependent kinase complexes, namely Cdk4/cyclin D and Cdk2/cyclin E [20]. Notably both

Cdk2 and Cdk4 kinase complexes phosphorylate, during the G₁ to S transition, pRb protein on Ser 795. Several Authors have considered this phosphorylation as a readout of Cdks activity. D-type and E-type cyclin/Cdk complexes are regulated by cyclin binding, by phosphorylation and by two families of Cdk inhibitors: the INK4 family, that acts specifically on Cdk4, and the KIP family, that comprises p21^{Cip1}, p27^{Kip1} and p57^{Kip2} acting both on Cdk4 and Cdk2 [159]. Besides, p21^{Cip1} and p27^{Kip1} also facilitate assembly and activation of cyclin D/Cdk4 in early G₁ [159, 160]. pRb phosphorylated on Ser795 was detected using a phosphospecific antibody raised against pRbSer795 and that does not cross react with unphosphorylated pRb.

In normal cells, and regardless of initial glutamine concentration, Ser795-phosphorylated pRb - i.e. the form that monitors the activity of both Cdk4 and Cdk2 kinases - decreases as cells approach and reach confluence (Figure 15, panels A and B4). Concurrently, drops in cyclin D1, E and A were observed, their level being at - or below - the detection limit at the 96 hrs time point (Figure 15, panels A through B3).

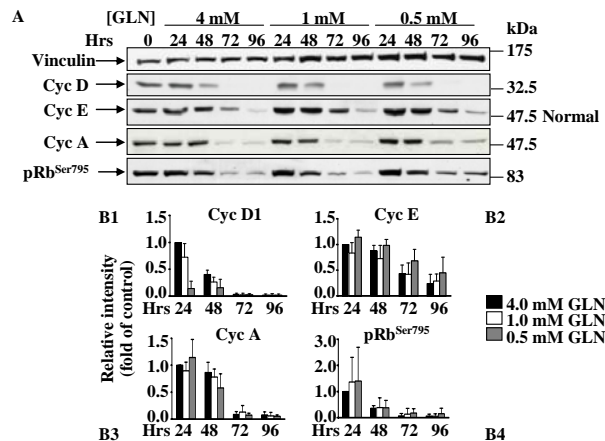


Figure 15: Expression of proteins involved in the G₁ to S transition in normal cells. For the protein expression analysis normal cells (A) grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine were collected at

appropriate time points and 40 μ g of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with appropriate antibodies. One of at least three independent experiments is shown. (B1-B4) Relative quantitative analysis of protein levels after Western blot film acquisition by scanner and densitometry analysis using Image J program. The densitometry values obtained for each protein were normalized by using the values of the corresponding vinculin and were plotted considering the densitometry value obtained for the sample 24 hrs/4mM glutamine as equal 1. Results are the mean of at least triplicate determinations with SD indicated.

A decrease in Cdk2 and a more permanent expression of Cdk4 was observed during the time course of the experiment (Figure 16).

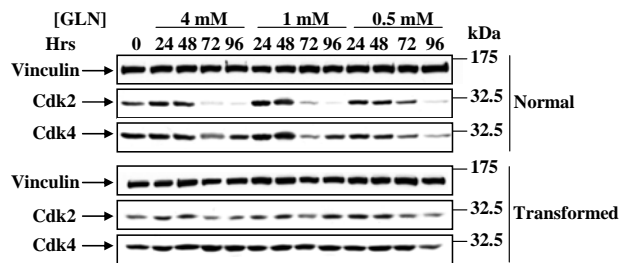


Figure 16: Cdk2 and Cdk4 proteins expression is normal and transformed cells. For the protein expression analysis normal and transformed cells grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine, were collected at appropriate time points and 40 μ g of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with specific Cdk2 and Cdk4 antibodies

Moreover, as shown in Figure 17A, B and C, in normal cells grown in media supplemented with the three initial glutamine concentrations, the expression of the p27^{Kip1} and p21^{Cip1} proteins showed a time-dependent increase for p27^{Kip1} and a decrease for p21^{Cip1} (independent of the initial glutamine concentration). The inhibitory function of p27^{Kip1} is regulated not only through synthesis and degradation, but also by sub-cellular localization. Indeed, cytoplasmic p27^{Kip1} has been detected in about 40% of primary human breast cancers in conjunction with Akt activation and is associated with poor patient prognosis and reduced Cdks inhibitory activity [161-163].

In normal cells, grown in 4 and 0.5mM GLN, associated with the time-dependent increase of p27^{Kip1}, a different cell localization was observed. Indeed, while at early time points (24–48 hrs - proliferating cells -, Figure 17, panels D, E, F and G) p27^{Kip1} staining remained diffuse, at later time points (72–96 hrs- arrested cells – Figure 17, panels D, E, F and G), p27^{Kip1} staining strongly accumulated in the nucleus . Increased p27^{Kip1} expression and nuclear localization well correlate with the decrease of S phase cells and of pRbSer795 phosphorylation observed during the time course of the experiment.

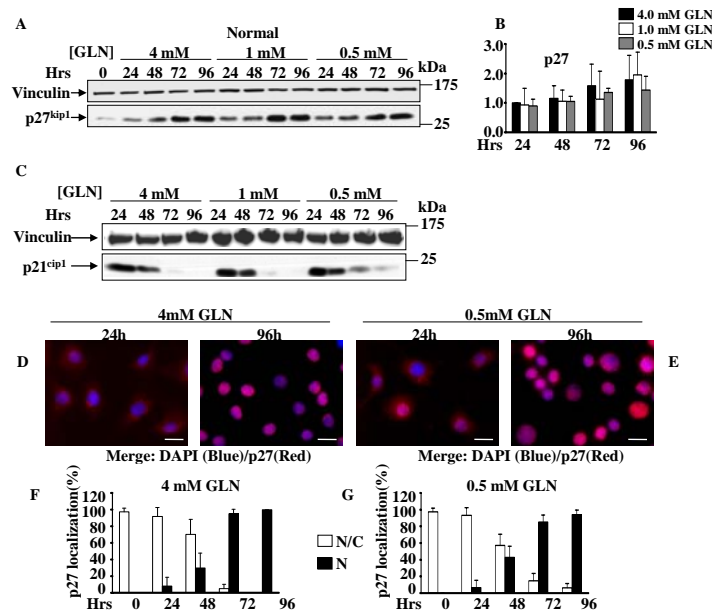


Figure 17: Expression and localization of p27^{Kip1} in normal cells. For p27^{Kip1} expression analysis normal cells (A) grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine were collected at appropriate time points and 40 μ g of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with p27^{Kip1} and p21^{cip1} specific antibodies. One of at least three independent experiments is shown. Relative quantitative analysis of p27^{Kip1} protein levels after Western blot film acquisition by scanner and densitometry analysis using Image J program, as described in Figure 15, in normal cells (B). p27^{Kip1} cellular localization in normal (D, E, F and G), cell lines was analyzed by

immunofluorescence staining for p27^{kip1} (red staining), after 24 and 96 hrs of growth in media supplemented with either 4mM glutamine (left pictures) or 0.5mM glutamine (right pictures). Nuclei were visualized by DAPI staining (Blue staining). The pictures are the result of a merge between the two single images acquired. At least 200 cells were scored for each sample. The averages of three independent experiments are presented in F and G with errors bars indicating SD.

For transformed cells the pattern was very different. In cells grown in media supplemented with 4mM glutamine, Cyclin D, A and E as well as phospho-Rb decreased only to about 50% of the level present at 24 hrs (Figure 18, panels A through B4), while Cyclin D1 decreased significantly more at intermediate and low glutamine concentrations.

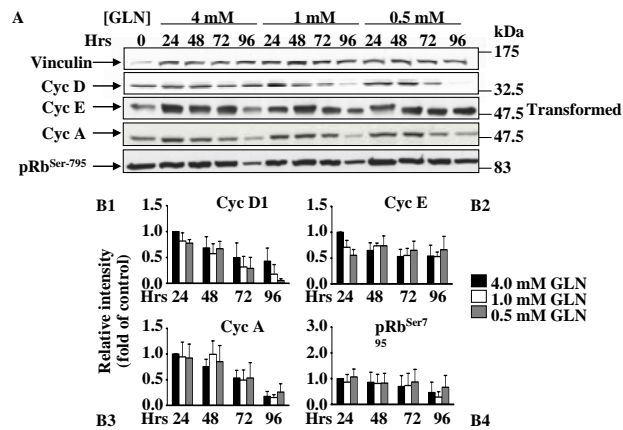


Figure 18: Expression of proteins involved in the G₁ to S transition in transformed cells. For the protein expression analysis transformed cells (A) grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine were collected at appropriate time points and 40µg of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with appropriate antibodies. One of at least three independent experiments is shown. (B1-B4) Relative quantitative analysis of protein levels after Western blot film acquisition by scanner and densitometry analysis using Image J program and densitometry analysis using Image J program, as described in Figure 15. Results are the mean of at least triplicate determinations with SD indicated.

Differently from the pattern observed in normal cells, transformed cells showed fairly constant Cdk4, Cdk2 and p21^{Cip1} levels during the time course

of the experiment (Figure 14 and 19C), and no increase in the level of p27^{Kip1} (Figure 19B): indeed p27^{Kip1} was barely detectable in cells grown in low glutamine, at the last time point (96 hrs) (Figure 19A and B). Immunofluorescence staining, in both low and high glutamine availability, revealed that in transformed cells a large fraction of p27^{Kip1} remained cytoplasmic, in contrast to the situation observed in normal cells where the majority of this Cki is nuclear at later time points (compare Figure 19D-G, for transformed cells with Figure 17D-G, for normal cells).

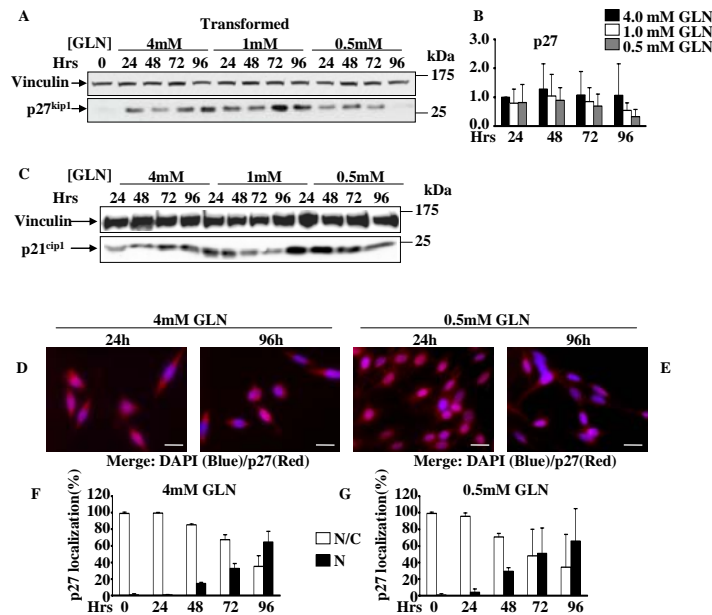


Figure 19: Expression and localization of p27^{kip1} in transformed cells. For p27^{kip1} expression analysis transformed cells (A) grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine were collected at appropriate time points and 40 μ g of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with p27^{kip1} and p21^{cip1} specific antibodies. One of at least three independent experiments is shown. Relative quantitative analysis of p27^{kip1} protein levels after Western blot film acquisition by scanner and densitometry analysis using Image J program, as described in Figure 15, in transformed cells (B). p27^{kip1} cellular localization in normal (D, E, F and G), cell lines was analyzed by immunofluorescence staining for p27^{kip1} (red staining), after 24 and 96 hrs of growth in media supplemented with either 4mM glutamine (left pictures) or 0.5mM

glutamine (right pictures). Nuclei were visualized by DAPI staining (Blue staining). The pictures are the result of a merge between the two single images acquired. At least 200 cells were scored for each sample. The averages of three independent experiments are presented in F and G with errors bars indicating SD.

Glutamine reduction does not substantially interfere with RNA synthesis, protein synthesis and energy metabolism

Amino acid deprivation leads to a fall in the rate of RNA and protein synthesis and consequently to a reduction of total cellular protein content [164, 165]. Such effect could be even more significant upon depletion of glutamine - the more abundant amino acid in tissues, body fluids and cell culture medium - because of its involvement in the biosynthesis of several amino acids (glutamate, aspartate, arginine, alanine) as well as in energy production [166-168] and nucleotide biosynthesis [169].

It has been first analyzed whether glutamine deprivation could affect total RNA and protein accumulation. To this end, the time course of average of RNA and protein content per cell was analyzed. The average RNA content of normal and transformed cells, regardless of glutamine availability, slightly decreased along the time course of the experiment. The time course decrease in RNA content of transformed cells growing in low glutamine, was somehow more sizeable as compared to that observed in normal cells suggesting that glutamine reduction may hamper biosynthesis of nucleotides and hence the synthesis of stable RNA (Figure 20A and B).

As cells approached and reached stationary phase, and regardless of glutamine concentration, a small decrease in protein content was observed in normal cells (Figure 20C and D). On the contrary, the protein content of transformed cells showed a substantial increase with time. These results are summarized in panels E and F, showing that in 4mM glutamine, time-dependent changes in the RNA/protein ratio for normal and transformed cells are very similar, while transformed cells show a more detectable decrease of this parameter when grown under limiting glutamine.

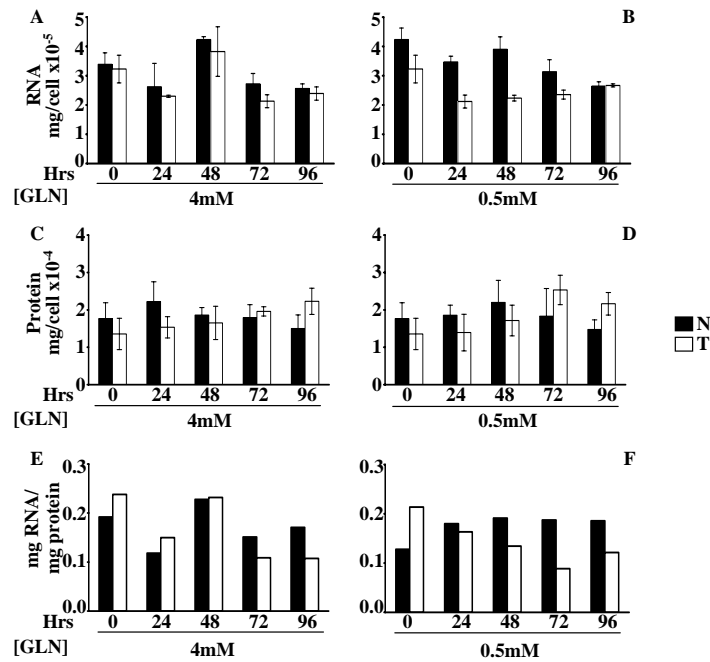


Figure 20: Analysis of some glutamine-dependent cellular processes in normal and transformed cells. Amount of the total RNA, proteins and ratio RNA/protein for single cell calculated after spectrophotometric determination of total RNA extracted by using TRIzol solution and different lysis buffer (protein) of normal and transformed cells grown in 4mM glutamine (A, C and E), and 0.5mM glutamine (B, D and F) along a time course. Results are the mean of at least triplicate determinations with SD indicated.

Glutamine is an oxidizable fuel, which enters the TCA cycle as α -ketoglutarate, by reactions catalyzed by aspartate/alanine amino transferase (AST) and by glutamate dehydrogenase [69, 170]. Accordingly it has been reported that glutamine depletion could cause a deficiency of this cycle [53, 171]. Supplementing the growth medium (containing 0.5mM glutamine) with either 2mM pyruvate, which can enter the TCA cycle mainly through its conversion in Acetyl-CoA or 2mM malate which can enter the TCA cycle by conversion into oxaloacetate and/or furnish reducing equivalents by malic enzyme activity, in the last steps of glutaminolysis, did not rescue the

proliferation defect caused by low glutamine in transformed cells (Figure 21A and B). Indeed the three growth curves, corresponding to 0.5mM glutamine alone or plus pyruvate or malate were completely superimposed for both cell lines.

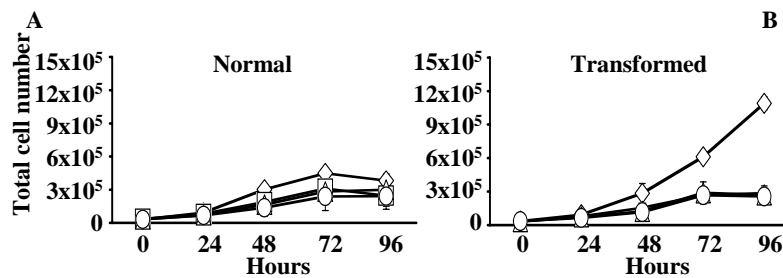


Figure 21: Proliferation analysis of normal and transformed cells adding in culture medium malate and pyruvate. Normal (A) and transformed (B) cell lines were plated at 3000 cells/cm² in 6-well plates in normal medium. Culture medium was replaced after 24 hours with a normal medium -4mM glutamine- (\diamond), a low medium -0.5mM glutamine- (\square), a medium with 0.5mM glutamine and 2mM pyruvate (Δ) and a medium with 0.5mM glutamine and 2mM malate (\circ) and then the cells were collected and counted at indicate time. Results are the mean of at least triplicate determinations with SD indicated.

The ATP level was measured in normal and transformed cells (24, 48, 72 and 96 hrs in 4, 1 and 0.5mM glutamine, Figure 22A, B and C). ATP decreased in both cell lines along the time-course of the experiment. In all cases substantial residual ATP was present even at the latest assayed time, however normal cells showed a more pronounced drop in ATP (20%, 41% and 38% for cells grown in media supplemented with 4mM, 1mM and 0.5mM initial glutamine, respectively) than transformed cells (5 %, 25% and 18% for cells grown in media supplemented with 4mM, 1mM and 0.5mM initial glutamine, respectively).

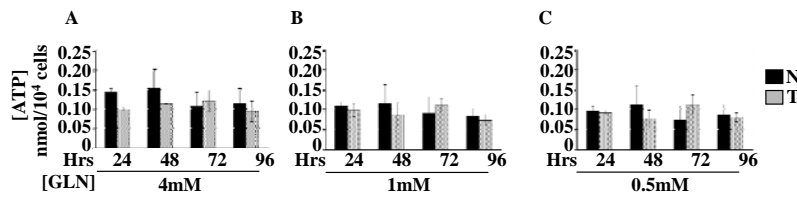


Figure 22: ATP production in normal and transformed fibroblasts in different glutamine concentration. ATP content in normal and transformed cells was measured using a luciferin-luciferase assay along the time course in 4mM (A), 1mM (B) and 0.5mM glutamine availability (C). ATP concentrations were calibrated against ATP standards and expressed as nanomoles per 10,000 cells. In all the experiments the averages of at least three independent experiments are presented with errors bars indicating SD.

Addition of deoxyribonucleotide triphosphates rescues the abortive S-phase entrance induced by low or full absence of glutamine in K-ras transformed fibroblasts

Results reported above indicate that in transformed cells energy metabolism under limiting glutamine concentration is not heavily unbalanced, since these cells have normal ATP and protein levels, and do not benefit from increasing fuel supply to the TCA cycle. On the other hand under limiting glutamine conditions, transformed fibroblasts fail to down-regulate the machinery responsible for the G₁ to S transition, as evidenced by sustained accumulation of S-phase cyclins, low levels and larger cytoplasmic localization of p27^{kip1} and ensuing sustained phosphorylation of pRb. Nevertheless transformed cells are unable to proceed with proliferation although maintaining a fairly high level of S phase cells. Since glutamine is an important intermediate in purine and pyrimidine biosynthesis, glutamine exhaustion could deplete intracellular nucleotide pools, bringing in turn to a failure in the execution of a normal cell cycle [169, 172]. To test this hypothesis, it has been examined whether adding deoxyribonucleotides (using a concentration that has been shown to not interfere with proliferation of several cell lines in vitro) [173] could affect proliferation of normal and

transformed cell lines. The presence of $10\mu\text{M}$ dNTPs (dATP, dGTP, dTTP and dCTP) had no effect on growth of both normal and transformed cells in media supplemented with 4mM glutamine (Figure 23, panels A and B) and on growth of normal cells in media supplemented with 0.5mM glutamine (Figure 24A).

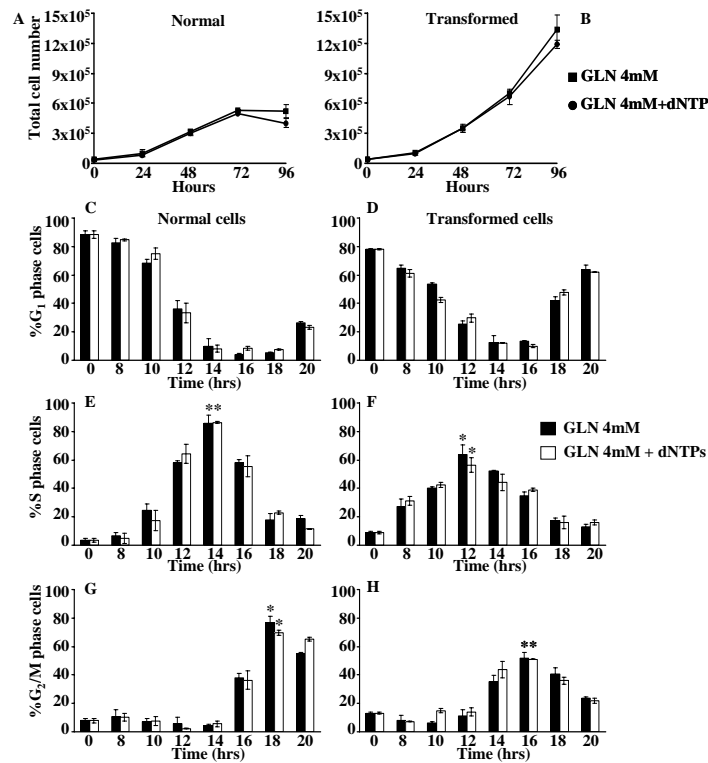


Figure 23: Proliferation ability and time of re-entry upon serum starvation and release in cells grown in 4mM glutamine. Normal (A) and transformed (B) cell lines were plated as previously described. Culture medium was replaced after 24 hrs with a normal medium -4mM glutamine- (■) and a medium with 4mM glutamine and $10\mu\text{M}$ dNTPs $-ATP, GTP, TTP$ and $CTP-$ (●). The cells were collected and counted at indicated time. The error bars correspond to standard deviations of duplicate analysis. (C, E and G) normal cells and (D, F and H) transformed cells, synchronized by 24 hrs serum starvation, were released in a medium containing 10% serum and 4mM glutamine or 4mM glutamine and $10\mu\text{M}$ dNTPs. S phase cells were scored by continuous labelling with BrdU, added directly in the releasing

medium. At indicated time points the cells were collected and stained with propidium iodide and an anti-BrdU specific antibody and analyzed by flow cytometry. The percentages of cells in G₁ (panels C and D), S (panels E and F) and G₂/M (panels G and H) phase of both cell lines, obtained by bi-parametric analysis, are represented. The asterisks indicate the S or G₂/M phase peaks identified in both glutamine availability. The error bars correspond to standard deviations of triplicate analysis.

On the contrary, addition of dNTPs sustained further growth of transformed cells, whether added once (at time 0 hrs) or twice (at time 0 and 48 hrs) (compare squares, triangles and circles in Figure 24B). These results indicate that sustained supply of deoxyribonucleotides relieves glutamine dependence of proliferation in transformed cells.

To better analyze the G₁ to S transition, cells grown in medium containing 4mM glutamine were synchronized by serum starvation for 24 hrs. As expected, transformed cells did not block in G₀/G₁ as effectively as normal cells (Figure 24C through F), being 89% for normal and 78% for transformed, the percentage of cells with prereplicative DNA content as determined by bi-parametric BrdU/PI staining. The cell cycle block was released by adding 10% serum in media containing either 4mM glutamine, 0.5mM glutamine or 0.5mM glutamine plus 10 μ M dNTPs, and collected at indicated time points (8, 10, 12, 14, 16, 18 and 20 hrs). The time-course of transit through the cell cycle phases was then followed. Normal cells showed equal kinetics of re-entry into cell cycle regardless of glutamine concentration. The fraction of G₀/G₁ cells reached a minimum 14-16 hrs after serum stimulation (panel E), while S-phase (panel G) and G₂/M phase (panel I) peaked at 14 and 18 hrs respectively. Transformed cells reproducibly anticipated their entry into S phase as compared to normal cells (8 vs. 10 hrs, panels G and H), regardless of glutamine concentration. The peak in S phase (panel H) and G₂/M phase (panel L) was also anticipated, but only in cells stimulated in media containing 4mM glutamine (black bars) or 0.5 glutamine plus 10 μ M dNTPs (white bars), but not in cells stimulated in media containing 0.5mM glutamine (grey bars).

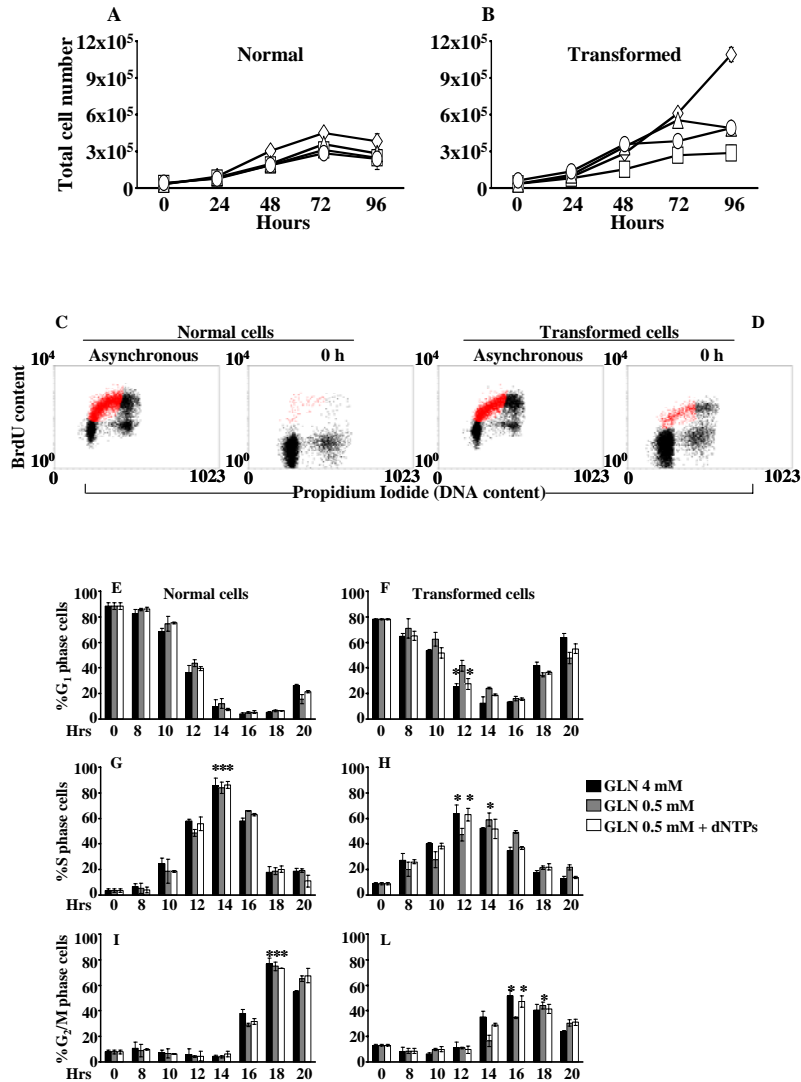


Figure 24: Proliferation analysis of normal and transformed cells adding in culture medium the four dNTPs.

Normal (A) and transformed (B) cell lines were plated as previously described. Culture medium was replaced after 24 hrs (indicated as 0 hrs) with a normal medium -4mM glutamine- (\diamond), a low medium -0.5mM glutamine- (\square), a medium with 0.5mM glutamine and 10 μ M dNTPs -ATP, GTP, TTP and CTP- (\triangle) and a medium with 0.5mM glutamine and 10 μ M dNTPs in which the dNTPs were added at 0 and

48 hrs (O), then the cells were collected and counted at indicate time. Normal (left panels) and transformed (right panels) cells, synchronized by 24 hrs serum starvation, were released in a medium containing 10% serum, 4mM or 0.5mM glutamine, dNTPs and BrdU for continuous DNA labeling. At indicate time points the cells were collected and stained with propidium iodide and anti-BrdU specific antibody and analyzed by flow cytometry. Representative cytograms, obtained for asynchronous and 0 hrs (time 0) time point samples respectively, plotting BrdU content (labeled cells) vs. PI staining (DNA content) (Figure 24C - normal cells - and D - transformed cells -). Red color in the plot represents the S-phase cells. The percentages of cells in G₁ (panels E and F), S (panels G and H) and G₂/M (panels I and L), phase of normal (left panels) and transformed (right panels) cells released in 4mM and 0.5mM glutamine alone or plus dNTPs obtained by bi-parametric analysis as shown above, are represented. The asterisks indicate the S or G₂/M phase peaks identified in both glutamine availability. The error bars correspond to standard deviations of triplicate analysis.

In order to evaluate the ability of dNTPs to substitute glutamine in the execution of G₁ to S transition, it has been investigated whether nucleotide addition was effective also on cells released from a serum starvation block in complete absence of glutamine using the same protocol used in Figure 24. Cell cycle re-entry of normal cells was unaffected by release in 0mM glutamine, regardless of the presence of nucleotides (Figure 25, panels A, C and E). In transformed cells, release in the absence of glutamine delayed S phase entrance of 2 hrs (Figure 25, panels B and D) and delayed reaching of the S-phase peak of further 2 hrs (Figure 25, panel D, asterisks). Addition of 10μM dNTPs to transformed cells released in 0 glutamine (white bars) rescued the delay in S phase entrance, the delay in reaching the S phase peak, but did not rescue the delay in traversing the G₂/M phase (panel F, compare black and white bars).

These data indicate that the major effect of glutamine limitation in transformed cells is to increase the length of S phase, the peak of S phase cells being reached 2 hrs later in low glutamine (Figure 24H, peaks indicated by asterisks) and 4 hrs later in full absence of glutamine (Figure 25D, peaks indicated by asterisks): this function of glutamine can be preserved by dNTPs. Complete glutamine depletion severely slows down transit through

G₂/M, regardless of dNTPs supply (Figure 25F), indicating a distinct role of glutamine in this cell cycle phase.

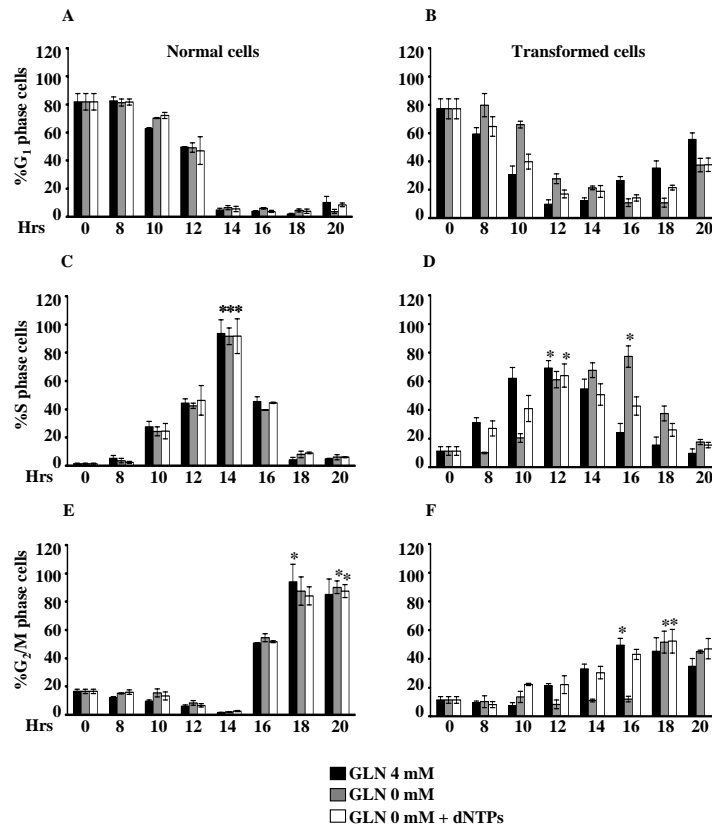


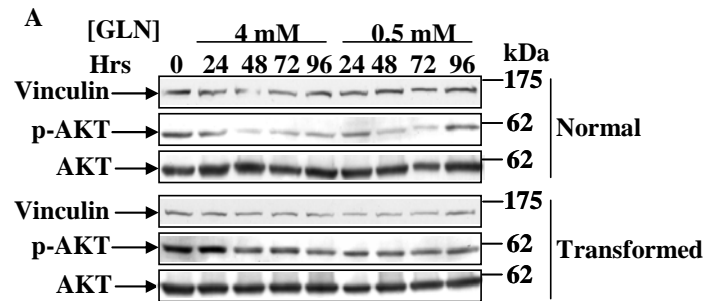
Figure 25: Cell cycle of synchronized normal and transformed cells released in 0mM glutamine ± dNTPs. Normal and transformed cells, synchronized by 24 hrs of serum starvation, were released in a medium containing 10% serum, 4mM or 0mM glutamine, dNTPs and BrdU for continuous DNA labeling. At indicate time points the cells were collected and stained with propidium iodide and anti-BrdU specific antibody and analyzed by flow cytometry. The percentages of cells in G₁ (panels A and B), S (panels C and D) and G₂/M (panels E and F) phase of normal (left panels) and transformed (right panels) cells released in 4mM and 0mM glutamine alone or plus dNTPs obtained by bi-parametric analysis are represented. The asterisks indicate the S or G₂/M phase peaks identified in both glutamine availability. The error bars correspond to standard deviations of triplicate analysis.

Response of selected signaling pathways to glutamine availability and effect of rapamycin treatment on proliferation ability in normal and transformed cells.

In different transformed cell lines glutamine or other amino acids shortage has been reported to induce accumulation of cells in G₀/G₁ and/or an increase in doubling time and a decrease in maximal reached cell density [172, 174, 175]. Such anti-proliferative effects, most likely mediated by the TOR pathway [176, 177], have been associated either to a general decrease of mRNA translation and/or protein biosynthesis or to a specific decrease/increase of expression of both positive and negative regulators of cell cycle, as observed in cells deprived of arginine (decrease of Cdk4) [178], in cells deprived of histidine (increase of p21^{waf1} and p27^{KIP1}) or methionine (increase of p21^{waf1}) [179, 180]. In non-transformed cells, upstream activators such as PI3K/Akt and Ras/Raf/Erk and upstream inhibitors such as the phosphates PTEN and the AMP kinase properly control activation of TOR pathway. In transformed cells, the TOR pathway is deregulated through several pathways, among which a primary role is assigned to oncogenic Ras signalling, that is able to activate both positive regulators of TOR pathways (PI3K and ERK) [181, 182].

To investigate the role of signal transduction in the response of normal and transformed cells to glutamine shortage, it has been analyzed the level of expression and activation of two main regulators of the TOR pathway, Akt and AMPK, as well as the level of expression and activation of a downstream TOR target p70 S6 kinase (S6K). As shown in Figure 26A, in normal cells, the activated form of Akt (probed with an anti-phosphoserine 473 antibody) decreases as a function of time and regardless initial glutamine concentration. In transformed cells the level of phosphorylated Akt is always very significant, being marginally higher at initial time points and not reduced as a function of time nor effected by glutamine concentration.

As previously reported, TOR protein is also controlled through AMPK, which is activated by a drop in the ATP/ADP ratio with ensuing AMP accumulation that leads to a signaling bringing to different cellular responses as well as to cell cycle arrest [183]. Western blot experiments to analyze the AMPK activation status in cells grown in different glutamine availability, showed that in correlation with a drop of ATP levels in both cell lines, only in normal cells, in which the ATP decrease was greater (see Figure 25A, B and C), a reliable and glutamine-dependent increase of AMPK activation was observed (Figure 26B, 1mM and 0.5mM glutamine samples). Analysis of the p70 S6K phosphorylation (probed with an anti-phosphothreonine 389, which closely correlated with p70S6K activity that in turn is widely used biological readout for TOR pathway activation) indicated a greater activity as well as expression in all transformed cell samples as compared to normal cell samples. These results well correlate with several observations from other Authors, highlighting the importance of both Akt and S6K in oncogenic ras signaling [184].



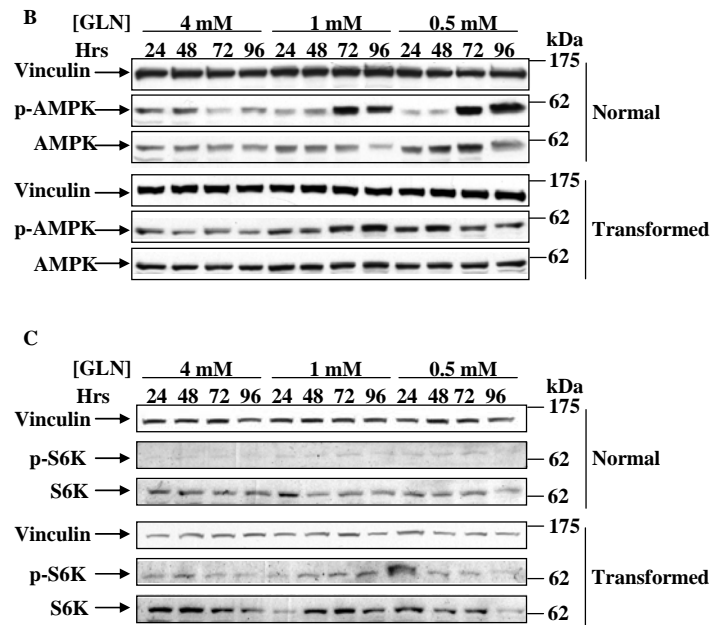


Figure 26: Activation of AKT, AMPK and TOR pathways in normal and transformed cells. (A) Time course expression and phosphorylation of the AKT protein in normal and transformed cell lines growing in media supplemented with different initial glutamine concentrations. For the protein expression analysis normal cells and transformed cells grown in media containing 4mM glutamine and 0.5mM glutamine, were collected at appropriate time points and 50µg of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with a specific anti-phosphorylated-Ser473 AKT (p-AKT) antibody and an anti-AKT (AKT) antibody. (B) Time course expression and phosphorylation of the AMPK α protein in normal and transformed cell lines growing in media supplemented with different initial glutamine concentrations. For the protein expression analysis normal cells and transformed cells grown in media containing 4mM glutamine, 1mM glutamine and 0.5mM glutamine, were collected at appropriate time points and 50µg of proteins from the total cellular extract were subjected to SDS-PAGE followed by Western blotting with a specific anti-phosphorylated-Thr-172 AMPK (p-AMPK) antibody and an anti-AMPK (AMPK) antibody. (C) Time course expression and phosphorylation of the S6K protein in normal and transformed cell lines grown as described in B. Western blotting analysis has been performed by using a specific anti-phosphorylated-Thr389 S6K (p-S6K) antibody and an anti-S6K (S6K) antibody. The panels are representative of a triplicate analysis (Akt and AMPK) or duplicate analysis (S6K).

To further explore the role of TOR pathway in the response of normal and transformed cells to glutamine shortage, the effects of rapamycin (known

pharmacologic inhibitor of TOR protein activity) on ability of synchronized cells to enter in S phase was investigated. It has been examined whether adding rapamycin to synchronized normal and transformed cells, grown both in 4mM or 0mM glutamine plus dNTPs, could interfere in particular with the execution of G₁ to S transition in transformed cells also in presence of the dNTPs. As shown in Figure 27A, C and E, normal cell samples, treated with rapamycin, showed a delay to exit from the G₁ arrest (panel A) and, thus a 4 hrs delay to reach the S phase peak (panel C, peaks indicated by asterisks) and consequently to execute the G₂/M transition that was not observed in the interval of time choose for this specific experiment (panel E). Transformed cells, released in 4mM glutamine plus rapamycin or 0mM glutamine, showed a slower exit from G₁ arrest and a 4 hrs delay to reach the S-phase and the G₂+M peaks (panel D and F, peaks indicated by asterisks) as compared to 4mM sample and 0mM plus dNTPs. Surprisingly, the sample released in 0mM glutamine plus dNTPs and treated with rapamycin, was almost unable to exit from the G₁ arrest and consequently to enter into S-phase. Indeed at later time point analyzed (18hrs), were scored 50% and 34% of cells respectively in G₁ and S phase.

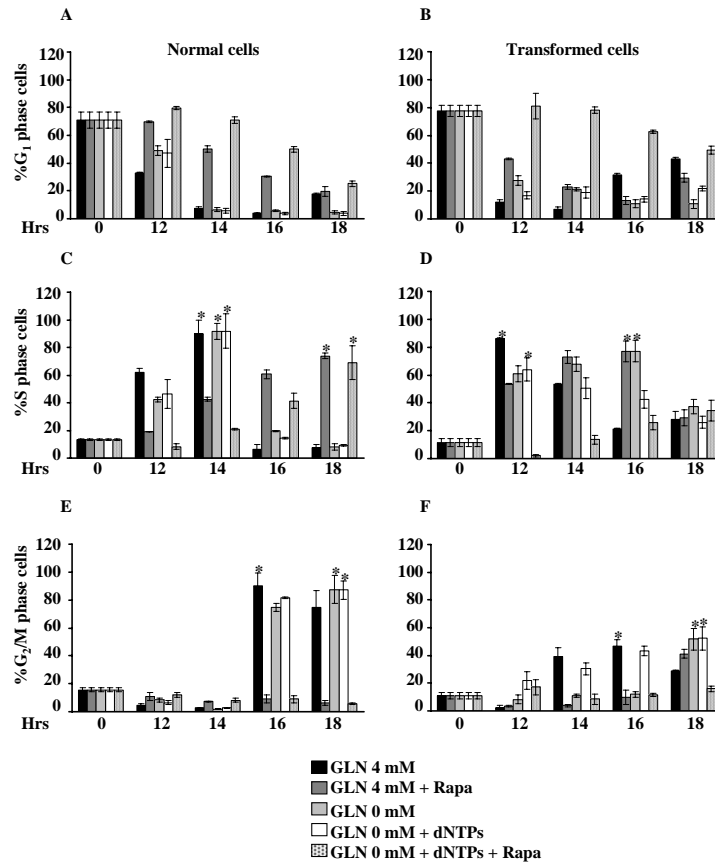


Figure 27: Analysis of cell cycle upon rapamycin treatment. Normal and transformed cells, synchronized by 24 hrs of serum starvation, were released in a medium containing 10% serum, 4mM glutamine \pm Rapamycin or 0mM glutamine \pm Rapamycin and \pm dNTPs and BrdU for continuous DNA labeling. At indicate time points the cells were collected and stained with propidium iodide and anti-BrdU specific antibody and analyzed by flow cytometry. The percentages of cells in G₁ (panels A and B), S (panels C and D) and G₂/M (panels E and F) phase of normal (left panels) and transformed (right panels) cells, released in the mediums indicated above, and obtained by bi-parametric analysis are represented. The asterisks indicate the S or G₂/M phase peaks identified in both glutamine availability. The error bars correspond to standard deviations of triplicate analysis.

These findings were further confirmed by western blot analysis of the expression of the G₁ to S inducing transition cyclin D1 protein. Indeed in both normal and transformed cells treated with rapamycin the level of

expression of cyclin D1 was lower than in untreated samples (data not shown).

Together these results indicate that in normal cells amino acid deprivation may participate in TOR signalling inhibition inducing a G₁ arrest, (i.e. by reducing mRNA translation, protein synthesis, ATP levels and expression of cell cycle regulators), in K-ras transformed cells such control is deranged by activation of Akt and by strong inhibition of AMPK, that, acting in a concerted fashion, maintain also at low or absent glutamine availability a sizable activity of the TOR pathway, able to promote entrance into S phase. Indeed, TOR signaling inhibition in synchronized cells completely block the ability of transformed cells to enter in S phase also in presence of dNTPs.

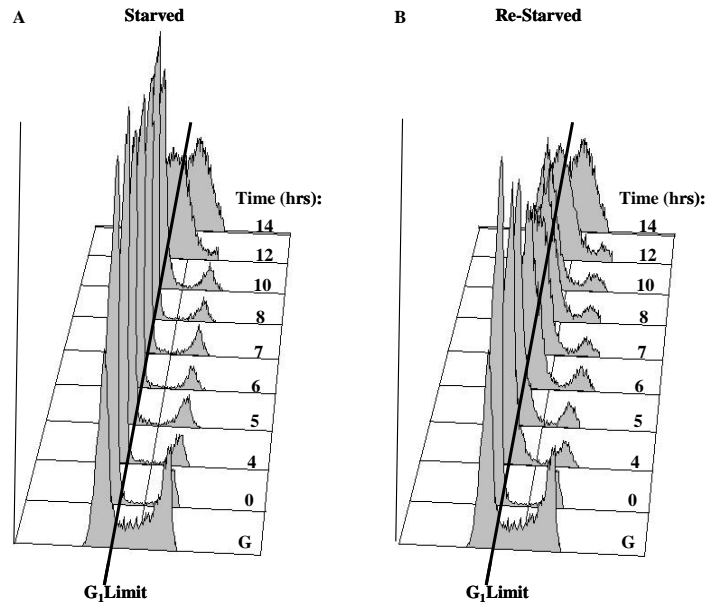
A systems biology approach of G₁/S transition in NIH3T3 normal fibroblasts to predict nutritional perturbation effects in normal and transformed cells

It has been above shown that nutrient shortage influence cell proliferation and G₁/S transition reaching in K-Ras transformed fibroblats.

Progression of quiescent G₀ into early G₁ and G₁/S transition are highly regulated processes. Nutrients and growth factors stimulation of G₀ quiescent cells drives them into the growth factor-dependent early G₁ phase of the cell cycle, followed by transition into the growth factor-independent late G₁ phase and progression into S phase [185, 186]. This irreversible commitment point, termed the restriction point [187], operates stringently in normal cells, but it is defective in cancer cells. In particular, tumor cells accumulate mutations resulting in constitutive mitogenic signaling and defective responses to anti-mitogenic signals that contribute to unscheduled proliferation [117, 188].

Therefore, the development in normal cells of a dynamic mathematical model that include regulatory aspect could explain the changes leading to tumor formation.

In order to better understand experimental data and to perform predictions regarding the ability of normal and possible of transformed cells to execute the G_1 to S transition, upon mitogenic factors or nutrients alterations, a computational analysis of the specific molecular mechanisms involved in the process has been done. With this aim, firstly the timing of the restriction point in normal cells was identified. To identify restriction point in normal fibroblasts, the cells were synchronized in early G_1 phase by serum starvation for 24 hrs. Afterwards, the cells were stimulated with serum for various times, and then deprived of serum again. The samples incubated with BrdU, were collected at indicated time points (4, 5, 6, 7, 8, 10, 12, 14) and stained with propidium iodide and anti-BrdU specific antibody and analyzed by flow cytometry. The cells able to continue through S phase in the absence of serum were those which had already passed the restriction point at the time of serum removal (Figure 28A and B). Figure 28A, show that normal cells were blocked in G_0/G_1 with a percentage of 89% and reached S phase at 12-14 hrs after serum stimulation. Approximately, 35% of the cells had passed the restriction point after 5 hrs of serum removal. However, even though around 90% of the cells were synchronized at moment of the release, they were unable to trespass in synchronous manner the restriction point. The heterogeneity with which cells overcome the R point and enter into S phase is most likely due to the limiting concentration of “competence” factors, like PDGF [189, 190], which are required to rescue the cells from the quiescent state stimulating them to grow and to activate the pathways needed to resume proliferation [191].



Time (Hrs)	% S Phase Starved (BrdU positive cells)	% S Phase Re-Starved (BrdU positive cells)
Grow	22.5±3.4	22.5± 3.4
0	4.0±1.6	4.03±1.6
4	4.5±1.4	8.2±4.0
5	2.9±0.71	15.6 ±3.0
6	4.1±1.9	35.8±1.8
7	2.6±0.2	46.3±3.6
8	3.3±0.9	54.8±9.8
10	6.7±3.8	65.3±1.6
12	65.0±13.7	67.5±17.7
14	83.6±17.2	83.6±17.2

Figure 28: Restriction point identification in NIH3T3 normal mouse fibroblasts. NIH3T3 cells, made quiescent by serum starvation for 24 hrs. The cells were then released with 10% NCS (A) or were serum starved again after different time periods (B) and BrdU for continuous DNA labeling. At indicate time points the cells were collected and stained with propidium iodide and anti-BrdU specific antibody and analyzed by flow cytometry. The cell cycle analysis and the fraction of cells in each phase (G₁, S and G₂/M indicated as % cells) were determined using WinMDI 2.8 software. The data is representative of triplicate experiments.

To further characterize the restriction point in normal cells, the expression and localization of regulatory proteins involved in the G₁/S transition at various time points following the release from G₀ block, were analyzed by Western blot and microscopic analysis (Figure 29A). Normal quiescent cells (at time 0 hrs) are characterized by very low levels of cyclin D and cyclin E and by sizable levels of Cdk4, Cdk2 and p27^{Kip1}. At 4-6 hrs the level of cyclin D substantially increased, while that of p27^{Kip1} starts to decrease (Figure 29A). At 6-8 hrs cyclin E starts to be detectable, while p27^{Kip1} disappears almost completely (Figure 29A). The localization analysis indicated that in quiescent cells the great majority of p27^{Kip1} is localized into the nucleus (Figure 29B, left panel, red color), while Cdk4 and Cdk2 are localized both in the nucleus and in the cytoplasm (Figure 29B, left panel both red color) followed by nucleus localization dots-like after 10 hrs (Figure 29B, right panel). In addition it has been observed, as partially expected, a nuclear localization of cyclin D and cyclin E at 10hrs (Figure 29B, right panel, both in green color).

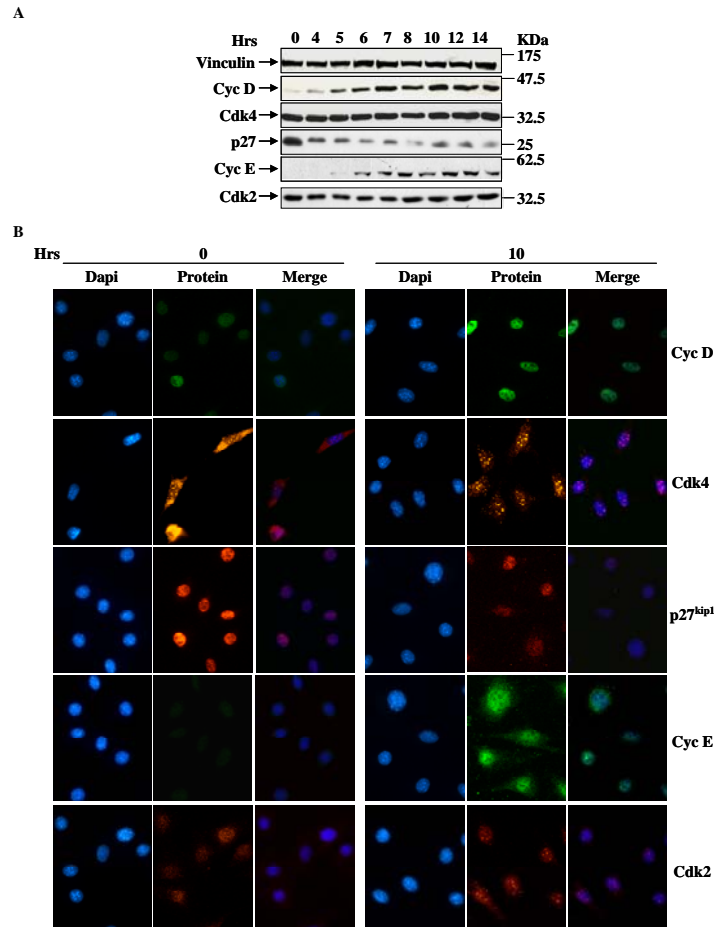


Figure 29: Expression and localization of cell cycle proteins in G_1 to S transition. (A) Time-courses of the expression of proteins involved in the control of G_1 to S transition. NIH3T3 cells, made quiescent by serum starvation, were stimulated with 10% serum and collected at appropriate time points and total cellular extracts were subjected to SDS-PAGE followed by Western blotting with appropriate antibodies. The Western blot is representative of at least three independent experiments. (B) Localization of proteins involved in the control of G_1 to S transition at time 0 and 10 hrs. NIH3T3 cells, after synchronization by serum starvation, were labeled with indicate antibodies (protein) and analyzed by fluorescence microscope. Nuclei were visualized by DAPI staining. The merged images are the result of a merge between the two single images acquired. At least 200 cells were scored for each sample and the images are representative of three independent experiments.

The quantitative experimental analysis of G₁/S transition allowed the development of a computational modeling of this cell cycle phase in normal cells.

In particular experimental data obtained in normal cells has been used to construct a mathematical model (Figure 30) of the molecular events that bring a normal quiescent mammalian cell to overcome the restriction point and to enter into S phase. The model allows to identify the molecular mechanism that underlies the R point, yields specific predictions and gives new insights on the role that the availability of inhibitors of cyclin dependent kinases (Cki) may have on the entrance into S phase.

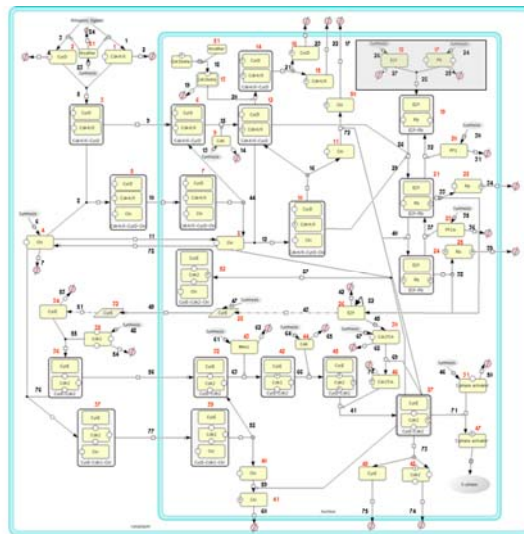


Figure 30: Processes Regulating the G₁/S transition in mammalian cells. Scheme of the G₁ to S transition of the mammalian cell cycle drawn with Cell Designer. Two compartments are considered, cytoplasm and nucleus. The scheme follows the systems biology graphical notation (SBGN); each component is associated with a red number and each reaction is associated with a black number. In the gray box a set of reactions which are not explicitly considered for the model.

In the model has been employed the quantitative analysis of cyclin D1, cyclin E, CDK2, CDK4 and p27^{Kip1} (Figure 31, panel A, B, and C) analyzed

by western blot and quantified using “Image J” software. In addition cyclin D, CDK4, p27^{Kip1} (Figure 31, panel E and F), cyclin E and CDK2 (data not show) localization was performed, as previously described, and analyzed by using quantitative tools of analysis present in “Methamorph 7” software.

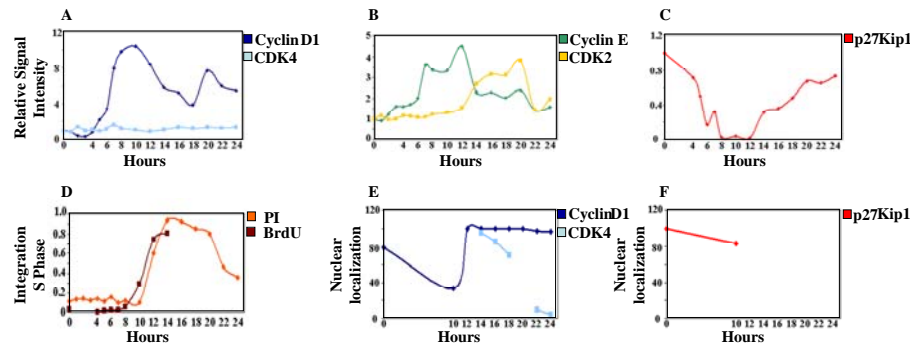


Figure 31: Analysis of expression and phosphorylation levels of some G_1 to S proteins. (A-C) Starved NIH3T3 cells were stimulated with serum and were collected at indicate times. Total cellular extract were subjected to SDS-PAGE followed by Western blotting with specific antibodies. Relative quantitative analysis of proteins levels after Western blot film acquisition by scanner and densitometry analysis using “Image J” program. (D) Identification of S phase entrance by PI staining and BrdU incorporation. Starved NIH3T3 cells were stimulated with serum as indicate above and analyzed by PI staining followed by FACS analysis or BrdU pulsed and analyzed by immunofluorescence microscope. (E-F) Protein localization by immunostaining with specific antibodies and fluorescence microscopy analysis (y axis indicates the percentage of total cell population showing nuclear localization of indicated protein).

Simulations of the model allows to recapitulate events happening from growth factor stimulation and shows successive building up of cyclin D, cyclin E and p27^{Kip1} with a timing consistent with the experimental ones (Figure 31, panel A, B, C, E and F). Active complexes are found in the nucleus at appropriate times and building-up of the phosphorylated S phase activator is also consistent with experimental data. Removal of the growth factor (that is simulated by turning off cyclin D synthesis and increasing cyclin D degradation and Cki synthesis) allows to construct a restriction

point curve that is similar to the one experimentally determined (Figure 31A).

Further development of the same computational model, could include a more sophisticated mathematical analysis for normal cells and used for to better explain the changes leading to tumor formation.

Discussion

DISCUSSION

Glucose and glutamine are involved in multiple pathways required for cell proliferation and survival. Indeed, the role of these pathways in the survival of transformed cells is mostly based either from the fact that the pathways in question can be regulated by oncogenes, or that cell death following nutrients shortage is associated with changes in the activation state of these pathways. In particular, in this work has been studied the response of K-Ras transformed cells to glucose and glutamine availability.

Transformed cells have been shown to have a particular dependence on aerobic glycolysis compared to normal cells. Indeed proliferation analysis of asynchronous K-Ras transformed fibroblasts as compared to normal cells, grown both in high and low glucose (25mM and 1mM), indicated that transformed cells enhanced their proliferation potential in 25mM glucose and lost completely this proliferative advantage in low glucose (1mM) (figure). In addition, the reduced ability to proliferate was followed by an enhanced cell death. Otherwise, normal cells stopped growth after 72 hrs, regardless of glucose concentration (25mM and 1mM) (figure). This glycolytic phenotype of transformed cells agrees with previous studies on cancer metabolism, showing that cancer cells preferentially employ glycolysis to fulfill their energetic requirements, releasing higher level of lactic acid in their environment [192-195]. Several reports have been shown how oncogenic K-Ras influences glucose uptake. Indeed K-ras, through the phosphatidylinositol 3-kinase (PI3K) pathway, can regulate glucose transporters expression, enhance glucose capture by hexokinase, and stimulate phosphofructokinase activity [52]. Beside, PI3K pathway activation renders cells dependent on high levels of glucose flux [17].

Interestingly, strong reduction of glucose availability, observed at 72hrs in cells grown in 1mM glucose, induced an enhanced cell death only in transformed cells. Indeed, the apoptotic process activated in normal cells was

glucose independent and probably correlated to prolonged contact inhibition. This effect has been validated by both Annexin V/PI staining and caspases-3 activation. Several studies have been shown that removal of glucose causes preferential cytotoxicity to human cancer cells compared to normal cells [196-198]. This response has been connected to a metabolic oxidative stress [199, 200] due to the overproduction of ROS such as superoxide and hydrogen peroxide.

Since transformed cells are characterized by strong reduction of proliferation as well as apoptosis at low glucose concentration, it was of interest to analyze the effects of Ras activation and glucose shortage on the cell cycle machinery, in particular during G₁/S transition in synchronized normal and transformed cells. These results indicated that the timing of G₁/S transition execution was glucose independent in both cell lines, and that the transformed cells anticipated the entry in S phase of two hrs. Therefore, oncogenic *Ras* expression is able to induce a greater sensitivity to glucose shortage as compared to normal cells (decreased proliferation and enhanced apoptosis) only if the glucose shortage is persistent.

Another metabolic adaptation of cancer cells, that has long been established, is their propensity to exhibit increased glutamine consumption, although the effects induced by glutamine deprivation on cancer cells are still controversial.

However the results indicated that in asynchronous normal cells, contact inhibition, regardless of glutamine availability, brings to down-regulation of Akt that together with AMPK up-regulation, observed at low glutamine, will concur to TOR pathway inactivation. As a result, the expression of cyclin D, E and A is down regulated, pRb phosphorylation is strongly reduced, p27^{kip1} level is increased and its localization becomes preferentially nuclear, establishing therefore a condition that bring to a G₁-cell cycle arrest. In synchronous normal cells, glutamine shortage slows the G₂/M transition, indicating a possible role of glutamine in such cell cycle phase.

In K-ras transformed cells, in which the level of activated Ras-GTP is very high [154] and the contact inhibition is less efficient [154], the deprivation of glutamine affects Akt and AMPK in a way opposite to that observed in normal cells, leaving the TOR pathway at least partially activated. This event allows sizable expression of cyclin D (at least until 72 hrs), E and A, sustained pRb phosphorylation, decreased p27^{kip1} and its preferential cytoplasmic localization, conditions that, taken together, promote entrance into S phase. Surprisingly, in condition of glutamine shortage, transformed asynchronous cells accumulate in S phase. In synchronous transformed cells, glutamine shortage slows both the G₁ to S transition or the G₂/M transition.

But why in low glutamine is the S phase of K-ras transformed cells abortive? Since glutamine is necessary for nucleotides synthesis, deprivation of glutamine could reduce also availability of nucleotides that leads to a small decrease in RNA accumulation compared to growth in high glutamine (at least until the 72 hrs) and a more dramatic effect on DNA synthesis. The notion that depletion of nucleotides is the major effect of glutamine deprivation that leads to reduced proliferation of transformed fibroblasts, is confirmed by the fact that the proliferation defect of transformed cells is rescued by adding the four deoxyribonucleotides (precursors of DNA polymerization) to low glutamine medium.

Therefore can be stated that when the low amount of glutamine is still compatible with cell cycle progression, as in serum starved cells substantially synchronized in G₁, in transformed cells low-glutamine medium causes a 2 hrs delay in entering into S phase after serum re-addition. The same delay is transmitted to the G₂/M transition, indicating that no major compensatory mechanism on cell cycle timing between completion of S phase and cell division are operative. This effect on cell cycle timing is worsened by complete absence of glutamine, in which 4 hrs delay in entering into S phase and in the execution of G₂/M transition have been observed. These data strongly indicate that the effect of glutamine limitation in

transformed cells is first to slow down the S-phase traverse, then, when a more severe limitation is established, to stuck a large fraction of the cells population in S phase.

Studies performed in several cellular models have shown that availability of dNTPs and therefore the regulation of their synthesis, plays a critical role in DNA replication [201]. Indeed expression of dNTP synthetic enzymes is cell cycle-regulated, with enhanced expression at the onset of S-phase [202, 203]. Additionally, dNTP levels vary within S phase of the cell cycle [201] thereby changing the rate of DNA replication during S phase [204]. Even subtle changes in the levels of dNTPs may have a sizable effect on DNA replication [205]. Nucleotide depletion, obtained through chemical inhibition of their biosynthesis, may induce either G₁ or S phase arrest or slow down the overall progression of S phase [206, 207]. Addition of a mix of 10 μ M deoxyribonucleotides reverted effects on both asynchronous and asynchronous cultures indicating that the earlier and major rate-limiting step dependent on glutamine-deprivation pathway is connected with nucleotide biosynthesis. Nevertheless, such connections appear to be lost both in asynchronous and synchronous cells when a complete limitation of glutamine in the culture medium occurs. Indeed at later time points of low glutamine proliferation curve of transformed cells (72-96 hrs), corresponding to almost a complete depletion of glutamine in the culture medium, dNTPs are not more able to restore the proliferation indicating that in such condition other cellular responses are activated by glutamine depletion. Furthermore in transformed synchronous cells, released in complete glutamine depleted medium, dNTPs are able to restore the S-phase transition but not the proper time of execution of G₂/M phase. Indeed such cell cycle phase was delayed as well as observed in the sample without glutamine and dNTPs. Other Authors have observed this result in different cell lines. For instance, Abcouwer et al. [208], showed that reduction of glutamine availability in the growth medium of several breast cancer cells might induce GADD45 and

GADD153 expression by mRNA stabilization. GADD45 overexpression has been shown to induce G₂/M cell cycle arrest [209] following several environmental stresses. Moreover, Drogat et al. [174], showed that in A549/8 carcinoma cells glutamine deprivation decreased the number of cells in the G₁ phase, transiently increased that of cells in the S phase, and induced a stable increase in cell number in the G₂/M phase. These findings may explain the delay to reach the G₂/M peak observed in both normal and transformed cells released in low or complete absence of glutamine.

In cells exhibiting high metabolic rates, such as rapidly dividing cancer cells grown in vitro, glutamine, being the most readily available amino acid used as energy source, may become the major source to sustain protein and nucleic acid synthesis [210], especially when glucose levels are low and energy demand is high. However, analysis of the levels of mRNA, proteins and above all of ATP in normal and transformed cells grown in high and low glutamine availability, did not show particular differences, suggesting an alternative use of glutamine in k-ras transformed cells.

A correlation between glutamine depletion and decreased level of Krebs cycle intermediates in myc-transformed cells has been identified [171].

Although, addition to culture medium of Krebs cycle intermediates, had no effect on K-ras transformed fibroblasts thereby suggesting that proliferation arrest may be independent on Krebs cycle intermediates depletion and energy shortage. On the contrary, ATP drop has been observed in the same cell lines, grown under limiting glucose availability. Such an effect is partially explained by the observations that transformed cells are unable to properly activate mitochondrial respiration [3, 105], eventually leading to cell death. Such partial derangement of mitochondrial function is further confirmed in the present work, since it has been shown that K-ras transformed cells grown in a medium in which glucose was completely replaced by 1mM pyruvate and 4mM glutamine, substrates used essentially for Krebs cycle and oxidative respiration, grow poorly and eventually die.

In conclusion, glutamine shortage in K-ras transformed cells limits proliferation by inducing abortive S phase entrance, while glucose shortage in the same system enhanced cell death [3, 105]. The differential effects of glutamine and glucose on cell viability are not a property of the transformed phenotype per se, but rather depend on the specific pathway being activated in transformation. For instance, myc-overexpressing cells die under complete glutamine depletion in a myc-dependent way and not under glucose depletion [171]. In the same experimental conditions, complete glutamine depletion, cell death of K-ras transformed cells die is marginal compared to that observed in myc-overexpressing cells (data not shown).

It has been previously shown that nutrient shortage influence cell proliferation and G₁/S transition of K-Ras transformed fibroblasts.

In fact, in tumor cells, the balance between intra- and extracellular signals and the control of the cell cycle is lost. To understand how intracellular and extracellular signals are transmitted to the cell-cycle machinery and how the latter adjusts its frequencies accordingly is one of the major challenges in molecular biomedicine. For this aim, is necessary to build a computational model of the cell cycle derived from experimental data. Indeed biochemical and genetic studies can be combined with bioinformatics and biosystems approaches in order to sketch a plan of the regulatory circuits governing cell cycle progression in normal cells, firstly, and then in transformed cells.

Taking in consideration that the trespassing of the Restriction Point influences cell cycle execution and that this point is influenced both by growth factors and nutrients availability, has been initially performed several experiments in order to identify such a restriction point in normal mouse fibroblasts synchronized by serum starvation and stimulated to re-enter into S phase by readdition of serum. During the time course of re-entering into cell cycle, from G₀ to G₁/S phase, it has been observed a gradual increase of cyclin D and cyclin E, starting from 4 and 6 hrs respectively and a constitutive expression of CDK4 and CDK2. On the contrary, high level of

p27^{kip1} in normal quiescent cells was observed, that disappeared completely after restriction point reaching. Interesting, in quiescent cells, while p27^{Kip1} localized completely into nucleus, Cdk4 and Cdk2 were strongly localized into cytoplasm. However, in association to the synthesis of cyclin D and cyclin E, both cyclins and CDKs localized especially into the nucleus with a peak at 10 hrs following restimulation with 10% serum.

These data agreed with other results since, in most cases, the concentration of the kinase subunit is relatively constant, whereas the concentration of the cyclin subunit oscillates. Indeed, the kinase is completely inactive without its cyclin partner, in addition to the binding of cyclin, activation of the holoenzyme requires the phosphorylation of a key residue in the activation loop of the kinase subunit [211]. Moreover, has been previously published that Cyclin D-, E-dependent kinases are negatively regulated by CDK inhibitors that include CIP1 and KIP1 [143, 144]. In particular KIP1 could be one of the the most important inhibitor regulating the trespassing of restriction point control. Indeed, in quiescent cells, p27^{KIP1} levels are high, but once cells enter the cycle, they fall [212].

Therefore, the cell cycle can be considered as a clock that has the properties to combine in a single central circuit the myriad of molecules involved in cell signaling. All positive and negative signals that can direct the cell toward growth, differentiation, quiescence, aging or death must relate with this circuit. Alteration in this circuit control systems and ensuing inability to coordinate entry into the cell cycle with mitogenic and nutritional signals leads to unregulated proliferation and ultimately to cancer [213].

This detailed study of G₁/S transition in normal fibroblasts allowed a novel mathematical model develop. Because tumor cells often display a reduced dependence on growth factors, an understanding of the cell cycle and a dynamical computational model that include regulatory aspect might help explain the changes leading to tumor formation.

Ringraziamenti

Eccoci qui alla fine di questo percorso duro e faticoso, ma incredibilmente affascinante.

I miei ringraziamenti sono rivolti a tutti quelli che hanno partecipato direttamente o indirettamente alla sua realizzazione.

L'orario al quale sto scrivendo mi porterà sicuramente a dimenticare qualcuno ma proverò a fare del mio meglio.

Il mio primo Grazie va a Ferdinando non soltanto perché Group Leader ma principalmente perché mio MENTORE e fonte inesauribile di conoscenza. Grazie per le lunghe ed intense chiacchierate scientifiche e per avermi reso la professionista che sono oggi.

A Lara, la mia piccola, grande e fedele compagna di lavoro. Grazie, 1000 volte grazie e ricorda: sono orgogliosa di te!

Alla piccola Roby, alla quale auguro di fare questo percorso duro (come negarlo!) ma allo stesso tempo ti accorgerai quanto è incredibile fare questo viaggio con Ferdinando.

A Marco per la sua disponibilità costante e per il suo aiuto prezioso in questo "tour de force".

A Chiara, non ultima del gruppo ma solo perché si dice "dulcis in fundo" e compagna di avventura del mio viaggio. Solo noi due delle volte possiamo capire! Grazie tesoro!

A Daniele per le colazioni, l'aiuto gentilmente concesso nei momenti disperati (vero Lara e Roby?!) e per tutte le cose speciali fatte per me.

A Fabri compagna di svaghi e di pause. Grazie per ascoltarmi tutte le volte che sono arrabbiata.

A Raffi "la mia sister americana", zio Sal e Giuseppe che hanno reso l'esperienza Boston indimenticabile.

Ai miei genitori che mi supportano sempre e costantemente nonostante la distanza.

A proposito di distanza, un grazie speciale a Dino e Nicola sempre li' pronti con i Kleenex in mano, anche quando sono lontana 10.000 km e a Lory per i suoi supporti psicologici. Grazie per la vostra presenza discreta e costante, di giorno, notte o in un altro continente voi siete sempre con me, Grazie!

Per concludere, un pensiero speciale va al piccolo frugoletto in arrivo (Dino e Lory). Ti auguro che tu possa godere nella pienezza che merita, questo incredibile e faticoso gioco che e' la vita. Cerchero' di essere una zia presente nonostante la distanza.

Ben Arrivato Gabry!

Grazie di cuore a tutti voi!

Daniela

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