Metabolic Checkpoints in Cancer Cell Cycle

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METABOLIC CHECKPOINTS IN CANCER CELL CYCLE

By

Mahesh Saqcena

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York
2014
This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

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by

Mahesh Saqcena

Advisor: Dr. David A. Foster

Growth factors (GFs) as well as nutrient sufficiency regulate cell division in metazoans. The vast majority of mutations that contribute to cancer are in genes that regulate progression through the G1 phase of the cell cycle. A key regulatory site in G1 is the growth factor-dependent Restriction Point (R), where cells get permissive signals to divide. In the absence of GF instructions, cells enter the quiescent G0 state. Despite fundamental differences between GF signaling and nutrient sensing, they both have been confusingly referred to as R and therefore by definition considered to be a singular event in G1. Autonomy from GF signaling is one of the hallmarks in cancer; however, cancer cells also have metabolic rewiring enabling them to engage in anabolic biosynthetic pathways. In the absence of GF instructions and nutrients, cells commonly undergo apoptotic cell death. Thus, it is of importance to elucidate the differences between GF and nutrient deregulation in cancer to develop novel strategies in targeting tumor cell proliferation and survival.

Here, we report that the GF-mediated mid-G1 restriction point (R) is distinct and distinguishable from a series of late-G1 metabolic checkpoints mediated by essential amino acids, conditionally essential amino acid – glutamine, and mTOR – the mammalian target of rapamycin. Our data indicate that the arrest sites mediated by various blocking conditions are in the order of
GF → EAA → Q → mTOR. We temporally mapped the EAA and glutamine checkpoints at 12 hr from G0 and mTOR mediated arrest occurring at 16 hr from G0. Distinct profiles for cell cycle regulator expression and phosphorylation was observed when released from restriction point relative to the metabolic checkpoints. These data are consistent with a mid-G1 R where cells decide whether they should divide, followed by late-G1 metabolic checkpoints where cells determine whether they have sufficient nutrients to divide. Since mTOR inhibition using rapamycin or Torin1 arrested the cells latest in G1, mTOR may serve as the final arbiter for nutrient sufficiency prior to replicating the genome. Significantly we also observed that in addition to GF autonomy, several cancer cells also have dysregulated nutritional sensing, and arrest in S- and G2/M phase upon essential amino acid and glutamine deprivation. We identified K-Ras mutation as the underlying genetic cause for this phenomenon. We found that treating cancer cells harboring K-Ras mutation with aminooxyacetate (AOA) – drug that interferes with glutamine utilization – causes them to arrest in S- and G2/M-phase, where synthetic lethality could be created to phase-specific cytotoxic drugs. Thus, besides addressing the long standing assumption of GF and nutrients regulating G1 cell cycle progression, our work provides rationale and proof of principle for targeting metabolic deregulations in cancer cells.
Acknowledgements

I shall ever remain indebted to my parents for their unconditional love and support. My brother, Durgesh, has in no less part been a person responsible for my interest in science. I still vividly remember our mini-experiments at home with water, salts, magnetism, light, and electricity during my high school years – often times much to the annoyance of my mother!

I am extremely thankful to my thesis advisor, Dr. David Foster, who introduced me to “What this mean?” philosophy and to looking at the positive side of experiments, i.e., looking at what we have instead of what we don’t. I feel very proud belonging to such a rich scientific lineage (Dr. Renato Dulbecco > Dr. Harry Rubin > Dr. Hidesaburo Hanafusa > Dr. David Foster > myself), glimpses of which I obtained both during Hanafusa memorial symposium and David’s 25th Anniversary Alumni Symposium.

I have been blessed with great science teachers who challenged, encouraged and mentored me right from my school years: J.V. sir (6th – 8th grade), G.L. teacher (8th – 10th grade), Mr. S. Adhiappan (10th – 12th grade), Dr. S. B. Namdas (Master’s), Dr. Viswas Sarangdhar (Master’s), Dr. M.E. Subramaniam (Advy Chemical), and Dr. David Foster (Ph.D.). They deserve special mention and forever have my greatest respects, and I’ll never be able to repay their gratitude. Dr. Rudolph Spangler (dec.) will always be missed; he was a great friend and guide who always encouraged me to take the next logical step.

I am also thankful to Dr. Wilma Saffran (Queens College), Dr. Susan Rotenberg (Queens College), and Dr. Paola Bellosta (City College) for allowing me to be a part of their lab during my lab rotations; to Dr. Jill Bargonetti and Brian Herbert for their experienced insights in teaching the
lab course; to Dr. Lesley Davenport, Dr. Edward Kennelly, and Judy Li of Biochemistry Ph.D. program, who provided valuable guidance to navigate through my Ph.D.

I am also thankful to my colleagues with whom I have had fruitful collaborations: Arwa, Deepak, Deven, Suman, and Victor. Many thanks also to my lab colleagues, most notably Amrita, Darin, Maria, and Matthew for scientific discussions, intellectual stimulation, and conducive atmosphere.

I am thankful to my committee members: Dr. Mitch Goldfarb and Dr. Paul Feinstein who were always available and provided great suggestions, Dr. Richard Kolesnick and Dr. Neal Rosen for their invaluable time and constructive criticisms during my second level research proposal. I am also thankful to Dr. Frederick Cross (also of Hanafusa’s legacy) for accepting to be in my Ph.D. defense committee at such a short notice, and for his expert comments.

Lastly, my appreciation and thanks go out to my wife, Chaitali, and son, Vedant, for always being there through my ups and downs of Ph.D., and for bringing love and joy in my life.
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4EBP1  Eukaryotic initiation factor 4E Binding Protein 1
AOA  Aminooxyacetate
CDK  Cyclin Dependent Kinase
CM  Complete Medium
EAA  Essential Amino Acids
EGCG  Epigallocatechin gallate
ERK  Extracellular-signal Regulated Kinase
FACS  Fluorescence-Activated Cell Sorting
GF  Growth Factor
MAPK  Mitogen-Activated Protein Kinase
mTOR  Mammalian Target of Rapamycin
Mut  Mutant
Q  Glutamine
Rapa.  Rapamycin
R  Restriction Point
Rb  Retinoblastoma protein
S6K  Ribosomal protein S6 Kinase
T  Torin1
TdR  Thymine Deoxyribose
TGFβ  Transforming Growth Factor β
WT  Wild Type
Chapter 1: Introduction
Mammalian Cell Cycle

The mammalian cell cycle has been divided into four distinct phases, namely, G1 (for gap 1), S (for synthesis of DNA), G2 (for gap 2), and M (for mitotic phase). The vast majority of mutations that contribute to cancer are in genes that regulate progression through the G1 phase of the cell cycle (1). A key regulatory site in G1 is the growth factor-dependent Restriction Point (R), originally described by Pardee (2), where cells get permissive signals to progress through G1 and divide. Alternatively, in the absence of growth factor (GF), cells enter a quiescent G0 state. This GF-dependent R has been mapped to a site about 3 to 4 hr post-mitosis (3). In addition to GF instructions, it is widely acknowledged that nutrient availability and mTOR activity also impact on G1 cell cycle progression (4, 5). It has been suggested that R in mammalian cells is analogous to START in yeast cell cycle. However, yeasts are single celled and it is known that TOR-regulated START in yeast is a sensor of nutrient availability (6, 7). We have hypothesized the existence of a distinct Cell Growth checkpoint(s) in late G1, where cells ensure the availability of adequate raw materials (essential amino acids, glutamine) before committing for proliferation (8). Thus, START in yeast is more likely evolutionally related to the proposed Cell Growth checkpoint rather than the GF-mediated R.

Despite fundamental differences between GF signaling and nutrient sensing, they both have been confusingly referred to as R and therefore by definition considered to be a singular event in G1. Some of the reasons that suggest that R and Cell Growth checkpoints are distinct are: (i) there are mechanistic differences between sensing mechanisms for GF and nutrient sufficiency, (ii) normal non-transformed cells respond differently to GF and nutrient deprivation – for example arresting in a quiescent G0 state vs. induction of autophagy, respectively, (iii) cancer cells respond differently to GF and nutrient deprivation – for example most cancer cells exhibit autonomy from
growth factor signaling but are exquisitely sensitive to nutritional deprivation triggering apoptosis, and (iv) complementing genetic changes required for transformation are in genes that override restriction point and in genes that override the nutritional sensing (8).

Complementing Genetic Mutations Required for Transformation

Weinberg et al. have shown that in addition to telomerase, Ras along with SV40 large and small T antigens are required to transform normal human cells (9, 10). The molecular and cell cycle targets of Ras and large and small T antigen implicated in G1 cell cycle progression is shown in Table 1. While the telomerase requirement indicated a need to acquire immortality and avoid cell senescence, all of the other genes have been implicated in progression through G1 (Table 1).

Table 1. Genetic requirements for the transformation of Human Cells.

[From Foster et al. (8)]

| Genetic Requirements for the Transformation of Human Cells with Viral Genes |
|-----------------------------|-----------------------------|-----------------------------|
| Gene                        | Molecular Target            | Cell Cycle Target           |
| Ras                         | Growth factor signals       | Restriction point           |
| SV40 large T                | p53                         | G1/S checkpoint(s)          |
|                             | Rb                          | All G1 checkpoints          |
| SV40 small t                | PP2A                        | Cell growth checkpoint      |

| Genetic Requirements for the Transformation of Human Cells without Viral Genes |
|-----------------------------|-----------------------------|-----------------------------|
| Gene                        | Molecular Target            | Cell Cycle Target           |
| Ras                         | Growth factor signals       | Restriction point           |
| P53 null                    | p53                         | G1/S checkpoint(s)          |
| Rb null                     | Rb                          | All G1 checkpoints          |
| Myc                         | Gene expression             | Cell growth checkpoint      |
| PTEN loss                   | Akt/mTOR                    | Cell growth checkpoint      |

Note: The genetic requirements for transforming human cells with (upper section) and without viral genes (lower section) are presented along with the molecular target of the genetic alteration and where in the cell cycle the genetic alteration impacts. Telomerase expression, which is required in both cases, is not included because it does not impact directly on G1 cell cycle progression. These data are mostly from the work of Hahn and Weinberg (9, 10, 11).
Ras pathway is activated in response growth factor signaling and mutation in the pathway is known to confer autonomy from GFs. Both SV40 large and small T antigens inhibit proteins which are involved in G1 cell cycle progression. Thus, this suggests that dysregulating signals involved in progression through G1 phase of cell cycle is sufficient for transformation. Attempting transformation without viral genes, Hahn et al. showed that human cells can be transformed using a combination of Ras, suppression of p53 and Rb, increased expression of Myc and suppression of PTEN expression, in addition to telomerase (11). Thus, activation of both Ras and PI3K/mTOR pathway (Fig. 1) is required for transformation of normal human cells.
Figure 1. Complementary signaling pathways activated in human cancer cells.

Two signaling pathways are shown that are commonly activated in cancer cells. It is proposed that activation of these two signaling pathways promotes progression through different regulatory points in G1 of the cell cycle.  

A) The Ras pathway involves the activation of a kinase cascade consisting of Raf, Mek and MAP kinase (MAPK), leading to increased expression of cyclin D and passage through R. This pathway is ordinarily activated by growth factors that prevent G1 cell cycle exit to quiescence.  

B) The mTOR pathway is complicated and has many inputs. However, a common theme in this complex signaling network is that it is highly sensitive to the presence of the energy and nutrients needed for cell growth and leads to the activation of cyclin E and its partner kinase CDK2 [From Foster et al. (8)].
mTOR is a key sensor of nutrient availability

In addition to the GF-mediated restriction point (R), non-transformed cells also have nutrient sensing checkpoints and p53-mediated G1/S checkpoint for assessing genomic integrity (Fig. 2). mTOR is a key sensor of nutrient availability and in addition to GF signaling is also responsive to energy status, glucose, amino acids, and lipid levels. mTOR regulates the proposed cell growth checkpoint by suppressing TGF-β signaling, which stimulates the expression cell cycle inhibitor p27. Based on the complementing genetic mutations required for tumorigenesis, there is an apparent need to deregulate all of these checkpoints.
Figure 2. Proposed G1 Cell Cycle Checkpoints.

Two major cell cycle checkpoints in G1 are proposed: 1) The Restriction Point that monitors growth factor instructions and is regulated in part by cyclin D-CDK4/6; and 2) A checkpoint mediated by mTOR and TGF-β that monitors nutritional sufficiency and that is equivalent to START in yeast. This site is regulated in part by cyclin E-CDK2 [From Foster et al. (8)].
Metabolic Reprogramming – The Seventh Hallmark in Cancer

Metabolic dysregulation is an emerging hallmark in cancer (12). Cancer cells exhibit a marked propensity to ferment glucose into lactate even in the presence of adequate oxygen. This metabolic transformation was first described by Otto Warburg in the early 1920s – and named Warburg effect (13), who postulated that defects in mitochondrial activity accounts for tumorigenesis. Since then, it has become apparent that metabolic transformation is not a cause but rather a consequence of tumorigenesis and that cancer cells do have a functional mitochondria. Nonetheless, cancer cell’s “sweet tooth” (14) for glucose uptake has been exploited in FDG-PET scanning, a diagnostic procedure that can localize sites of tumor invasion and metastases. Several oncogenes and survival signals have been shown to directly upregulate glycolytic enzymes and the metabolic reprogramming (15-17). For instance, in many cancers the constitutively expressed transcriptional regulator hypoxia inducible factor (HIF-1) has been shown to upregulate glycolysis (18, 19). Similarly, activated Akt has been shown to stimulate aerobic glycolysis in cancer cells (20). The Myc oncogene can transcriptionally upregulate lactate dehydrogenase and pyruvate dehydrogenase kinase, both of which can shift the metabolic flux to increased lactate production and decreased mitochondrial respiration (21, 22). Mutant p53 has been shown to transcriptionally upregulate hexokinase II and trigger the Warburg effect (23, 24). Christofk et al. have found that the M2 spliced variant of pyruvate kinase (PKM2) can by itself lead to metabolic switch (25, 26). In addition to PKM2 isoform having lower activity than PKM1, which would redistribute the glucose to anabolic pathways, it was hypothesized that PKM2 promotes tumor growth and aerobic glycolysis by virtue of its unique interaction, amongst all PK isoforms, with tyrosine-phosphorylated proteins, leading to its inhibition. The “Warburged state” was reversed upon switching the pyruvate kinase expression to the adult, M1 isoform. Mutations in several of the
citric acid cycle enzymes such as isocitrate dehydrogenase, succinate dehydrogenase, and fumarate hydratase have been linked to oncogenesis and/or familial paragangliomas/leiomyomas (27-31).

**Glutamine in Cancer Metabolism**

In the absence of glucose-derived acetyl CoA to fuel the mitochondrial citric acid cycle owing to aerobic glycolysis, glutamine acts as the anaplerotic precursor to generate alpha-ketoglutarate, an intermediate in the TCA cycle. It was reported that cancer cells convert 90% of glucose consumed into lactate and about 60% of glutamine consumed is also metabolized to lactate via malic enzyme (32). It has also been shown that oncogene Myc can upregulate glutaminase activity, leading to glutamine addiction in cancer cells (33, 34). Nicklin et al. have reported that glutamine uptake is followed by its rapid efflux from the cells in exchange for essential amino acids, and either the blockage of this exchange or glutamine withdrawal can induce autophagy (35). Thus, there is mounting evidence that underline the importance of glutamine in cancer cell metabolism. It is hypothesized that glutamine favors proliferation by replenishing citric acid cycle intermediates. Glutamine has also been implicated as a regulator of DNA and protein biosynthesis in solid human cancer cell lines (36). The response of cancer cells to glutamine deprivation seems to depend on mutations they harbor. For example, glutamine deprivation has been reported to cause apoptosis in Myc-dependent manner (37). On the other hand, in K-Ras transformed fibroblasts, glutamine deprivation has been shown to induce abortive S-phase (38). While glutamine has been reported to play pleiotropic roles in tumor proliferation (39, 40), the impact of glutamine deprivation on cancer cell cycle is less well known.
Scope of Work

In Chapter 2, we provide evidence indicating that R and nutritional checkpoints are distinct and temporally distinguishable. We show that GF and nutritional deprivation cause G1 cell cycle arrest utilizing different cell cycle regulators. Importantly, we demonstrate that in addition to GF autonomy, nutritional sensing is dysregulated in most cancer cells leading to override of G1 cell cycle arrest upon nutrient starvation. In addition to underscoring critical differences between R and nutritional checkpoints, our data also suggest that metabolic deregulation in cancer provides novel molecular targets for therapeutic intervention.

Extending our observation that some cancer cells have dysregulated amino acid sensing, in Chapter 3 we show that in cancer cells oncogenic K-Ras mutations provide G1 arrest override upon glutamine deprivation. We also demonstrate that the cyclin profiles in K-Ras mutant cells that arrest in S- and G2/M-phase correlates with the cell cycle arrest seen in flow cytometry. We find that dual inhibition of K-Ras/MAPK and PI3K/mTOR signaling pathways is required to restore G1 arrest upon glutamine deprivation, suggesting that cross-talk and compensation between these two pathways provide the G1 arrest override in K-Ras mutant cancer cell lines. Importantly, we also demonstrate that this differential sensitivity to glutamine can be exploited in specifically targeting K-Ras mutant cancer cells with cell cycle phase-specific chemotherapeutic drugs.
Chapter 2: Amino acids and mTOR Mediate Distinct Metabolic Checkpoints in Mammalian G1 Cell Cycle

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Published in PLOS One 2013; 8: e74157
Abstract

In multicellular organisms, cell division is regulated by growth factor signaling. In the absence of growth factors, cells exit the cell cycle at a site in G1 referred to as the restriction point (R) and enter a state of quiescence known as G0. Additionally, nutrient availability also regulates G1 cell cycle progression. While there is a vast literature on the regulation of G1 cell cycle progression, there remains confusion as to the nature of G1 cell cycle checkpoints – especially regarding the temporal location of R and nutritional sensing. In this report, using sequential blocking experiments we show that the GF-mediated R can be distinguished from G1 cell cycle checkpoints mediated by essential amino acids, glutamine, and mTOR. We demonstrate different profiles for cell cycle regulators when released from restriction point relative to the metabolic checkpoints. Significantly we also show that in addition to GF autonomy, cancer cells also have dysregulated nutritional sensing. These data are consistent with a mid-G1 R where cells decide whether they should divide, followed by late-G1 metabolic checkpoints where cells determine whether they have sufficient nutrients to divide. Since rapamycin and Torin1 arrest cells the latest in G1, mTOR may serve as the final arbiter for nutrient sufficiency prior to replicating the genome.
Materials and Methods

Cells and cell culture conditions. The BJ hTERT, MCF7, MDA-MB-231, and Panc-1 cells used in this study were obtained from the American Tissue Type Culture Collection. All the cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Sigma).

Materials. Reagents were obtained from the following sources: Antibodies against Akt, phospho-Akt (T308 and S473), S6K, phospho-S6K (T389), 4EBP1, phospho-4EBP1, LC3-II, Rb, phospho-Rb (T807/811), cyclin E, and actin were obtained from Cell Signaling; antibodies against p21 and p27 were obtained from Santa Cruz Biotechnology; antibody against cyclin D was obtained from BD Biosciences; and anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Promega. DMEM (D6429), DMEM lacking Gln (D5546), DMEM lacking Arg, Leu and Lys (D9443), dialyzed fetal bovine serum (F0392), and glutamine (G7513) were obtained from Sigma. Rapamycin was obtained from LC Laboratories, and Torin1 was obtained from Tocris. Ultima Gold scintillation fluid (6013681) and [3H]-thymidine (NET-027E) were obtained from Perkin Elmer.

Western Blot Analysis. Proteins were extracted from cultured cells in M-PER (Thermo Scientific, 78501). Equal amounts of proteins were subjected to SDS-PAGE on poly-acrylamide separating gels. Electrophoresed proteins were then transferred to nitrocellulose membrane. After transfer, membranes were blocked in an isotonic solution containing 5% non-fat dry milk in PBS. Membranes were then incubated with primary antibodies as described in the text. Depending on the
origin of the primary antibody, either anti-mouse or anti-rabbit HRP conjugated IgG was used for detection using ECL system (Pierce).

**Thymidine Incorporation Assay.** To determine the progression from G1 to S-phase, cells were labeled with 1µCi/ml [³H]-thymidine. At indicated times, cells were washed twice with 1ml PBS, then precipitated twice with 1ml 10% TCA. The precipitates were solubilized in 0.5 ml of 0.5% SDS/0.5M NaOH solution, and the extent of thymidine incorporation was quantified using 75 µl of sample and 3 ml of scintillation fluid.

**Flow cytometric analysis.** Cultured cells were washed and trypsinized. Cell suspensions were recovered and resuspended in the following fixing solution: 7ml 1X phosphate buffered saline, 2% bovine serum albumin, 5mM EDTA, 0.1% NaN₃. 3ml of 100% ethanol was added drop wise. Fixed cells were centrifuged, washed, and then resuspended in 500µl sorting buffer: 1X phosphate buffered saline, 0.1% Triton-X 100, 2% bovine serum albumin, 5mM EDTA, 40µg/ml propidium iodide, 100µg/ml RNAse A, and incubated at 37C for 30 min. The cells were filtered through 70-µm mesh to remove cell aggregates. The DNA content was analyzed by flow cytometry (FACSCalibur; Becton Dickinson), and percentages of cells within each phase of the cell cycle were determined using WinCycle software (Phoenix Flow Systems).
Results

Growth Factor and amino acids deprivation, as well as mTOR inhibition induce G1 cell cycle arrest.

It has been reported that many cancer cell lines do not arrest in G1 upon GF deprivation, and indeed autonomy from GF signaling is one of the hallmarks of cancer (41). We therefore used the human foreskin fibroblast BJ hTERT cells, which are immortalized by introduction of telomerase to prevent replicative senescence (42), to characterize the G1 arrest upon GF and nutrient deprivation or mTOR inhibition. BJ hTERT cells were shifted to medium lacking GF, EAA, Q, or complete medium containing 20 µM rapamycin or 250 nM Torin1 for 24 or 48 hr. High dose (20 µM) rapamycin is required to get complete G1 arrest by virtue of being able to suppress phosphorylation of the mTORC1 substrate eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) (43). To monitor progression into S-phase, the cells were labeled using [³H]-thymidine for the final 24 hr of treatment. As shown in Fig. 3A, GF, EAA, or Q deprivation causes 50-70% decrease in [³H]-thymidine incorporation in initial 24 hr. However, by 48 hr, thymidine incorporation reduced to less than 5% of control, indicating complete cell cycle arrest under these conditions. Rapamycin caused complete arrest at both 24 and 48 hr of treatment.

We next examined cell cycle distribution in BJ cells by measuring DNA content using flow cytometry. The cells were placed in various blocking conditions for 48 hr, fixed and stained using propidium iodide and analyzed by FACS. The cells had a marked increase in G1 cell population at the expense of S- and G2/M-phase cells upon serum deprivation and to a lesser extent with rapamycin (Fig. 3B). However upon EAA and Q deprivation, BJ cells maintained their S-phase DNA content despite the observation in Fig. 3A that there was no DNA synthesis after 48 hr of
deprivation. This would indicate that for cells in S-phase, the lack of either EAA or Q prevents cells from progressing out of S-phase (Fig. 3B).

To determine whether cells are capable of re-entry into cell cycle, we measured the kinetics for progression into S-phase upon release from various arrested states. In brief, cells were placed in various blocking conditions for 48 hr. Cells were then released from the block by replacing with complete medium, and pulsed with $[^3\text{H}]$-thymidine for 1 hr at indicated time points (0 to 28 hr). As shown in Fig. 3C, cells starting from G0 (GF deprivation) took approximately 16 hr to enter the S-phase. Cells starting from rapamycin block also entered the S-phase after 16 hr. Surprisingly, cells starting from EAA or Q block began synthesizing DNA with a longer lag phase of 18 to 20 hr. While there are a small fraction of cells in S-phase (5 – 15%) with various blocking conditions as seen by flow cytometry (Fig. 3B), the prolonged lag phase along with low baseline and sharp transition indicates that thymidine incorporation occurs predominantly from G1-phase cells released from blocking conditions into S-phase (Fig. 3C). Our observation for the time required to traverse from G0 to S-phase is similar to what has been described previously (44-46). Thus, the kinetic analysis shows that cells are able re-enter the cell cycle upon release from various blocking conditions. These data also reveal differences in recovery times after being subjected to EAA, Q and GF deprivation; however they do not provide insight as to the temporal relationships of the different blocking mechanisms.
GF, EAA, Q, and rapamycin mediated G1 cell cycle arrests are distinct and distinguishable

In order to distinguish G1 cell cycle arrest caused by different blocking conditions, we performed a series of sequential blocking experiments. In brief, cells were exposed to various blocking conditions for 48 hr to cause complete arrest. At this point, the first block was removed and a second block was applied along with [$^3$H]-thymidine for 24 hr. If the second block applied is either at the same point or downstream of the first block, then [$^3$H]-thymidine incorporation should not occur. However, if the second block site is upstream of the first block, then the cells should progress into S-phase and incorporate the label. The extent of [$^3$H]-thymidine incorporated by cells released into complete medium after various first blocks was considered to be 100%. As shown in Fig. 4A, when GF deprivation was applied as the first block and when either EAA or Q deprivation, or rapamycin treatment were applied as second blocks, there was very little [$^3$H]-thymidine incorporation – indicating that the GF arrest site is either upstream or at the same site as other blocking conditions. When EAA deprivation was applied as the first block (Fig. 4B), followed by a second block of GF deprivation, increased [$^3$H]-thymidine incorporation was seen. However, with either Q deprivation or rapamycin treatment as the second block, no significant [$^3$H]-thymidine incorporation was observed. When Q deprivation was applied as the first block (Fig. 4C), only rapamycin treatment as the second block prevented progression into the S-phase, whereas a second block of GF or EAA deprivation failed to arrest the cells. Lastly, when rapamycin treatment was applied as a first block followed by GF, EAA, or Q deprivation as the second block, there was an increase in [$^3$H]-thymidine incorporation in all the cases, indicating that all the blocks are upstream of rapamycin arrest site (Fig. 4D). Taken together, the data indicates that the arrest sites mediated by various blocking conditions are distinguishable, in the order of GF → EAA → Q → mTOR (Fig. 4E).
Temporal mapping of the G1 cell cycle checkpoints

To better understand the temporal map shown in Fig. 4E, we examined the ability of EAA, Q, and rapamycin to block G1 cell cycle progression after release from G0. Cells were synchronized in G0 using serum deprivation for 48 hr. The cells were reinitiated into cell cycle by providing complete medium and [3H]-thymidine. At indicated time points, cells were shifted to various blocking conditions to determine the point when blocking no longer prevented entry into S-phase (schematic shown in Fig. 5A). As shown in Fig. 5B, starting from G0, EAA or Q withdrawal until 12 hr caused the cells to arrest in G1, after which their withdrawal did not arrest the cells as evidenced by increased thymidine incorporation. Addition of rapamycin continued to suppress thymidine incorporation until 16 hr after release from G0, as did a catalytic inhibitor of mTOR – Torin1. This suggests that EAA and Q checkpoints are 12 hr from G0 and are upstream from rapamycin-mediated arrest, which apparently is very close to the G1/S border since it takes 16 hr from the time of restoring GF to increased thymidine incorporation (Fig. 3C).

Restriction point and metabolic checkpoint arrest lead to differential patterns of cell cycle regulator expression and phosphorylation

The data in Figs. 4 and 5 indicate a temporal difference in the ability of GF, EAA, and Q deprivation, and rapamycin to arrest cells in G1. To further establish that the cell cycle checkpoints are distinct – especially between the EAA and Q checkpoints, which apparently are temporally very close to each other – we examined the impact on cell cycle regulatory signals. A key cell cycle regulatory signaling pathway is mediated by phosphatidylinositol-3-kinase (PI3K) and Akt signals that impact on mTOR (47). Akt is phosphorylated at Thr308 in response to GF stimulation of PI3K activation (48). As expected, GF deprivation led to a decrease in Akt phosphorylation at Thr308
(Figs. 6A and 6B). There was also a decrease in Akt phosphorylation at Ser473 - a downstream target of mTORC2 (49). There was a marked decrease in phosphorylation of p70-S6 kinase (p70S6K) and a smaller decrease in the phosphorylation of 4EBP1 upon GF deprivation. In contrast, EAA and Q deprivation had no effect on Akt phosphorylation at either Thr308 or Ser473, indicating no effect on PI3K or mTORC2 activity (Figs. 6A and 6B). However, both EAA and Q deprivation did suppress p70S6K phosphorylation. Interestingly, EAA deprivation suppressed 4E-BP1 phosphorylation, whereas Q deprivation did not – revealing a differential impact on mTORC1 in response to EAA and Q deprivation (Figs. 6A and 6B). Treatment of cells with rapamycin led to no noticeable change in Akt phosphorylation at either Thr308 or Ser473. As expected, rapamycin suppressed the phosphorylation of both mTORC1 substrates p70S6K and 4EBP1. We also examined the impact of nutrient and GF deprivation on autophagy by looking at increased levels of the autophagy marker LC3-II. Significantly, EAA, but not Q deprivation increased LC3-II levels. As expected, rapamycin treatment, which is known to induce autophagy (50), also led to increased levels of LC3-II (Figs. 6A and 6B). Thus, although all of the conditions used here cause G1 cell cycle arrest, they impact differentially on PI3K and mTOR kinase activity supporting the hypothesis that the checkpoints identified represent distinct sites in G1 – especially between the two amino acid sites.

We next examined the impact of different blocking conditions on known G1 cell cycle regulators. For this approach, cells were placed under various blocking conditions for 48 hr and subsequently released by restoring complete medium. Cell lysates were collected at indicated time points and analyzed for phosphorylated-Rb (P-Rb), Rb, cyclin D, and p21 using Western blot analysis. For cells arrested by GF deprivation, we see much less Rb protein and P-Rb Ser807/811 at time 0, but there was a dramatic increase in both Rb protein and P-Rb Ser807/811 levels from 11 hr
onwards (Figs.6C and 6D). A similar Rb profile was seen with cells starting from a Q deprived state. However, for cells starting from the EAA deprived state, there were high levels of Rb at time 0 that did not change much after restoring the EAA. This effect was clearly distinct from that seen with Q deprivation, where there were much lower levels of both Rb and P-Rb. Rapamycin treatment did not significantly reduce the levels of Rb protein or P-Rb - indicating that cells had arrested in late-G1 where Rb is already hyperphosphorylated. Passage through R correlates with an increase in cyclin D levels (51). Upon restoration of complete medium to GF-deprived cells, there was a significant increase in the level of cyclin D1 between 5 and 14 hr (Fig. 6E). In contrast, cells starting from EAA, Q, or rapamycin blocking conditions showed lesser changes in cyclin D1 levels – indicating a clear distinction between the GF-dependent R and the later nutrient-dependent metabolic checkpoints. Cells starting from all blocking conditions showed very similar cyclin E profiles, with cyclin E levels increasing from 11 hr onwards (data not shown). The CDK inhibitor p21 plays complex roles in controlling G1 cell cycle progression. For cells starting from GF-deprived state, there was very little p21 at time 0 but its level increased significantly by 2 hr and then dropped after 7 hr (Fig. 6F). The drop in p21 levels coincided with the increase in cyclin D levels and Rb phosphorylation at S807/811 (Figs. 6C and 6E). With EAA and Q deprivation, there were very low levels of p21 at time 0 for EAA and high levels with Q – again clearly distinguishing these two checkpoints. With rapamycin block there was very little p21 at time 0 that was maintained over the 20 hr time course. Collectively, the data in Fig. 6 reveal differential impact of various blocking conditions on the expression and phosphorylation of cell cycle regulatory proteins. While the data do not provide mechanistic insight into cell cycle arrests mediated by different blocking conditions, they clearly establish that the cell cycle arrest caused by various blocking conditions represent unique cell cycle checkpoints.
**Metabolic checkpoints are dysregulated in cancer cells**

Complementing genetic mutations required for transformation suggest that in addition to GF autonomy, cancer cells may also have dysregulated metabolic checkpoints to allow progression through both R and the late-G1 metabolic cell growth checkpoints. To further characterize the impact of GF and nutritional inputs in cancer cells, we examined cell cycle distribution in three human cancer cell lines. Cells were placed in various blocking conditions for 48 hr and analyzed by flow cytometry. MCF7 breast cancer cells had a marked increase in G1 cell population at the expense of S-phase cells with serum or amino acid deprivation and rapamycin treatment (Figs. 7A and 7D top panel). The G2/M-phase cells remained constant indicating a G2/M-phase arrest of cells as was observed for BJ cells deprived of EAAs in Fig. 3B. The BJ cells deprived of Q displayed an S-phase and G1 phase arrest, but not a G2/M arrest (Fig. 3B) indicating a differential sensitivity to EAA and Q for the MCF7 breast cancer cells. In stark contrast to both the BJ cells and the MCF7 cells, MDA-MB-231 breast and Panc-1 pancreatic cancer cell lines displayed a dramatic loss of any G1-phase arrest in response to both EAA and Q deprivation (Figs. 7B, 7C, and 7D middle and lower panel). Both of these cell lines retained a G1 arrest in response to serum withdrawal. This observation supports the hypothesis that the cells have a mechanism for arresting in S- and G2/M-phase upon EAA and Q deprivation. Importantly, it also demonstrates that the ability of EAA and Q to arrest in G1 has been lost in the MDA-MB-231 and Panc-1 cells. Rapamycin caused an increase in the G1 cell population in all the cell lines tested, indicating that inhibition of mTORC1 activity is sufficient to cause G1 cell cycle arrest. These observations indicate that EAA and Q sensing acts through separate mechanism than rapamycin treatment or GF
sensing to cause G1 arrest, and that metabolic deregulation in these cancers cause override of G1 cell cycle arrest upon amino acid deprivation.

Discussion

In this report, we have provided evidence that the GF-mediated R and nutrient-mediated metabolic checkpoints are distinct and distinguishable. Sequential blocking experiments show that R is upstream of two amino acid checkpoints that are upstream from a checkpoint mediated by mTOR. Although the checkpoints mediated by EAA and Q were temporally close, they could be distinguished by sequential blocking and by distinct profiles of cell cycle regulatory protein expression and phosphorylation. Suppression of mTOR with rapamycin blocked cell cycle progression significantly later in G1 than amino acid deprivation. Collectively, this study distinguishes the GF-dependent R, which assesses whether it is appropriate for the cell to divide, from a series of metabolic checkpoints late in G1 that determine whether division is feasible. In addition to mediating unique late-G1 checkpoints, our data also reveals novel sensing requirements for EAA and Q in S- and G2/M-phase of the cell cycle (Fig. 8A). Using Swiss 3T3 cells, Yen and Pardee had previously found that GF deprivation led to mid-G1 arrest whereas isoleucine deprivation caused late-G1 and S-phase arrests (52). This result more closely approximates the findings reported here with the human BJ fibroblasts.

It was somewhat surprising that inhibiting mTOR blocked cell cycle progression downstream of EAA. It is well established that mTORC1 is responsive to EAA (53). Thus, it was anticipated that the absence of EAA would block cell cycle progression at the same place as rapamycin. This was clearly not the case – there was a two-hour difference in the time it took for EAA deprivation to no longer prevent progression to S-phase relative to rapamycin (Fig. 5B).
However, mTORC1 is also responsive to glucose (54), ATP levels (55), and phosphatidic acid (56), a critical intermediate in the synthesis of membrane lipids (57). Thus, mTOR may not be fully active until it has sensed sufficient glucose, ATP, lipids, in addition to EAA. Thus mTOR likely serves as a master regulator that senses complete nutritional sufficiency before committing to replicating the genome.

An important conclusion from this study is the distinguishing of R in mid-G1 from the late-G1 metabolic checkpoints that control entry into S-phase. The point in G1 where the cells are no longer sensitive to the withdrawal of growth factors (R) has been mapped by Zetterburg and colleagues to about 3.5 hr in virtually all mammalian cells tested (3). The metabolic checkpoints downstream from R in this report are similar to a series of checkpoints in yeast collectively known as START (7, 58), where nutritional sufficiency is evaluated in a TOR-dependent manner in yeast (6, 59). R has commonly been referred to as the mammalian equivalent of START, but as shown here, the metabolic checkpoints that correspond with START are clearly distinguishable from R. It is likely that R evolved much later than START as a means for multicellular organisms to regulate proliferation through intercellular communication.

Part of the controversy over the location of a growth factor-dependent R is that different groups have reported responsiveness to growth factors later in G1 than described by Zetterberg (3). Notably Pledger and Stiles reported that PDGF could stimulate quiescent cells to “competence” with a short duration of treatment (60). These competent cells could then be induced to progress through the remainder of G1 by “progression” factors like insulin-like growth factor-1 (IGF1) (60). Similar studies with hepatocytes induced from quiescence showed a growth factor dependence that likely was extended into later stages of G1 (61, 62). The major distinction between these studies and Zetterberg’s work was that the Zetterberg study followed cells from mitosis, whereas the other
studies looked at cells leaving quiescence. Thus, cells starting from quiescence or G0 and cells starting from mitosis apparently have different needs for progression to S-phase. What was clear from the Zetterberg study was that after approximately 3.5 hr post mitosis, if serum growth factors were removed, cells did not enter quiescence and proceeded through S-phase to mitosis without any additional growth factor stimulation. In our double block experiments, the cells arrested by amino acid depletion or rapamycin could proceed to S-phase in the absence of growth factors upon restoration of amino acids or removal rapamycin. This is especially relevant for the mTOR checkpoint, since mTOR is activated in response to IGF1 and other growth factors. Importantly, the cells that arrested in G1 in response to amino acid deprivation and rapamycin, like in the Zetterberg study, were coming from mitosis, not quiescence, and therefore did not need growth factors to proceed to S-phase. This would indicate that under conditions where cells have passed through mitosis and avoided quiescence, mTOR does not need additional growth factor stimulation. However, this is apparently not the case when cells are coming out of quiescence where further stimulation of mTOR by IGF1 may be required.

GF autonomy is one of the more significant hallmarks in cancer (41). However, it has been suggested that mutations leading to elevated mTOR kinase activity are the most common mutations in observed human cancer (63, 64). Moreover, dysregulation of cellular metabolism is considered as an emerging hallmark of cancer (12). Several oncogenes and survival signals have been shown to directly upregulate glycolytic enzymes and induce metabolic reprogramming (15-17). Consistent with this emerging role for metabolism in cancer cells, we have demonstrated here that nutrient sensing metabolic checkpoints are dysregulated in cancer cells. Surprisingly, MDA-MB-231 breast cancer cells and Panc-1 pancreatic cancer cells deprived of EAA and Q arrested in S and/or G2/M-phase – indicating an override of the G1 arrest observed in normal BJ fibroblasts and MCF7 breast
cancer cells. Thus, the late-G1 metabolic checkpoints, like R, are apparently dysregulated as well. Of interest was the apparent “freeze” in cell cycle progression in the BJ cells in response to amino acid deprivation – indicating that collection of cells in S- and G2/M-phase in cancer cells is a property of the normal cells. It is also possible that cells could synthesize glutamine and also obtain other essential amino acids through autophagy causing reversibility of the checkpoints, enabling them to tide over the late-G1 metabolic checkpoints and thereby collect in other phases of cell cycle. As shown in our model (Fig. 8B), in addition to genetic defects that confer autonomy to GF signaling, we hypothesize that specific genetic mutations override the late-G1 nutritional checkpoints causing them to arrest in S- and G2/M-phase of the cell cycle, where we have shown that additional amino acid sensing occurs through as yet unknown mechanisms. Cancer cells arrested in S- and G2/M-phase are uniquely sensitive to the apoptotic insult of DNA damaging agents. Thus, synthetic lethality created by interfering with Q utilization and phase-specific cytotoxic drugs could provide novel therapeutic opportunities that kill cells arrested in S- and G2/M-phase.
Figure 3. Growth factor and amino acid deprivation, as well as mTOR inhibition induce G1 cell cycle arrest.

(A) BJ hTERT cells were plated at 20% confluence in DMEM containing 10% FBS for 24 hr at which time they were shifted to complete medium (CM) or various blocking conditions [-GF, -EAA, -Q, +Rapamycin (20 μM)] for 24 or 48 hr. The blocking conditions for Q used DMEM lacking Q; and for EAA, DMEM lacking Leu, Lys, and Arg as described in Material and Methods. The CM contained 10% DFBS (dialyzed FBS) instead of 10% FBS. Cells were labeled with [³H]-thymidine (TdR) for the initial or final 24 hr of treatment, after which the cells were collected and the incorporated label was determined by scintillation counting as described in Materials and Methods. Error bars represent the standard error for the experiment repeated at least four times. (B) BJ cells were plated and shifted to CM or various blocking conditions for 48 hr as in (A), after which the cells were harvested and analyzed for cell cycle distribution by measuring DNA content/cell as described in Materials and Methods. Error bars represent the standard error from independent experiments repeated four times. (C) To investigate the kinetics for progression into S-phase, BJ cells were plated and shifted to blocking conditions for 48 hr as in (A). Cells were subsequently released by shifting to complete medium, and pulsed with [³H]-thymidine (TdR) at indicated time points for 1 hr after which the cells were collected and the incorporated label was determined. Error bars represent the standard error of mean for experiments repeated three times.
Figure 3

A

B

C

<table>
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<th>G1</th>
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<tr>
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</tr>
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<td>+Rapa.</td>
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<td>11.7 ± 1.4</td>
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Figure 4. GF, EAA, Q, and rapamycin mediated G1 cell cycle arrests are distinct and distinguishable.

(A-D) BJ hTERT cells were plated and shifted to various first blocking conditions for 48 hr as in Fig. 3A. The cells were subsequently shifted to CM or different second block conditions containing \[^3\text{H}\]-thymidine (TdR) for 24 hr, after which the cells were collected and the incorporated label was determined. Error bars represent the standard error for the experiment repeated at least four times.

(E) Schematic model showing relative positions of different metabolic checkpoints relative to R (not drawn to represent precise time scales). G1-pm is post-mitotic phase in G1, G1-ps is pre-S phase of G1.
Figure 4

A

First Block: -GF

\[ 3^{\text{H}}\text{-TdR Inc. (% Ctrl)} \]

CM -GF -EAA -Q +Rapa
Second Block Conditions

B

First Block: -EAA

\[ 3^{\text{H}}\text{-TdR Inc. (% Ctrl)} \]

CM -GF -EAA -Q +Rapa
Second Block Conditions

C

First Block: -Q

\[ 3^{\text{H}}\text{-TdR Inc. (% Ctrl)} \]

CM -GF -EAA -Q +Rapa
Second Block Conditions

D

First Block: +Rapa.

\[ 3^{\text{H}}\text{-TdR Inc. (% Ctrl)} \]

CM -GF -EAA -Q +Rapa
Second Block Conditions

E

G0

G1-pm G1-ps

GF EAA Q Rapa.
Figure 5. Temporal mapping of the metabolic checkpoints from G0.

(A) Schematic representation of the experiment shown in (B). (B) BJ cells were plated as in Fig. 3A for 24 hr. Cells were synchronized in G0 by shifting to DMEM+1mM Q lacking GF for 48 hr. The cells were released from G0 by shifting to CM containing DMEM (1mM Q) and 1 µCi/ml [3H]-thymidine. Various blocking conditions along with [3H]-thymidine were applied at indicated time points. After 36 hr from the release from G0, cells were collected and the incorporated label was determined. This experiment utilized DMEM with reduced Q (1 mM vs. 4 mM) because Q withdrawal following DMEM with high Q did not give strong G1 arrest. Error bars represent the standard error for experiments repeated three times.
Figure 5

A

B
Figure 6. Restriction point and metabolic checkpoint arrest lead to differential patterns of cell cycle regulator expression and phosphorylation.

(A) Cells were plated at 30% confluence in 10-cm plates in DMEM containing 10% FBS. After 24 hr, the cells were shifted to CM or blocking conditions for 4 hr, at which time the cells were harvested and the levels of the indicated protein or phosphoprotein was determined by Western blot analysis. The data shown are representative of experiments repeated at least two times. (B) Quantitative analysis of relative protein levels for Western blots shown in (A) using ImageJ software. (C-F) BJ cells were plated and shifted to various blocking conditions for 48 hr as in Fig. 3A. The cells were subsequently released by shifting to CM, and the cells were harvested and lysates collected at indicated time points. The levels of the indicated protein or phosphoprotein were determined by Western blot analysis. The data shown are representative of experiments repeated at least two times. Also shown in the line graphs are the kinetic analyses of relative protein levels normalized to actin and quantitated using ImageJ.
Figure 6

A

B

C

D

E

F

33
Figure 7. Metabolic checkpoints are dysregulated in cancer cells.

MCF7 (A), MDA-MB-231 (B), and Panc-1 (C) cells were plated at 20% confluence in 10-cm plates in DMEM containing 10% FBS. After 24 hr, the cells were shifted to CM or various blocking conditions for 48 hr, at which time the cells were harvested, fixed, stained with propidium iodide, and analyzed for distribution in different phases of cell cycle by measuring DNA content/cell as described in Materials and Methods. Error bars represent the standard error from independent experiments repeated four times. Table with the mean and standard error for the graphs is also shown. (D) Representative flow histograms showing increases in S- and G2/M-phase cell population upon EAA and Q deprivation in MDA-MB-231 and Panc-1 cells.
Figure 8. Restriction point and metabolic checkpoints are distinct, and dysregulated in cancer cells.

(A) In non-transformed primary cell, intact GF-mediated mid-G1 R and amino acid and mTOR mediated late-G1 “Cell Growth Checkpoint” is shown. The proposed cell growth checkpoint, but not R, is likely evolutionally related to START in yeast. We present evidence here suggesting that G1 checkpoints are ordered as \( \text{GF} \rightarrow \text{EAA} \rightarrow \text{Q} \rightarrow \text{mTOR} \), such that mTOR acts as a final arbiter before committing to DNA replication. Besides mediating unique late-G1 checkpoints, novel sensing requirements for EAA and Q in S- and G2/M-phase of the cell cycle is also shown. (B) In cancer cell, autonomy from GF signaling and deregulation of metabolic checkpoint is modeled. mTOR serves as a master regulator coupling cell growth to nutritional sufficiency, and its sole inhibition was found to be sufficient to cause G1 arrest in all the cell lines used in this study. Additional EAA and Q sensing is also shown in S- and G2/M-phase of the cancer cell cycle, such that in the event of late-G1 metabolic checkpoint override, cancer cells arrest in S- and G2/M-phase of cell cycle.
Figure 8
Chapter 3: Blocking anaplerotic entry of glutamine to TCA cycle sensitizes K-Ras mutant cancer cells to cytotoxic drugs

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Manuscript submitted (under review).
Abstract

Cancer cells engage in aerobic glycolysis to meet their increased anabolic demands. In the absence of glucose-generated acetyl CoA, cancer cells rely on glutamine to support the anaplerotic citric acid cycle. We report here that glutamine depletion causes K-Ras mutant cancer cells to arrest in S- and G2/M-phase of the cell cycle, whereas cancer cells with WT K-Ras arrest in G1 phase. Inhibition of K-Ras pathway using MEK inhibitor U0126 or mTOR pathway using catalytic inhibitor Torin1 does not cause reversal to G1 arrest upon glutamine starvation. However, dual inhibition of MEK and mTOR pathway restores G1 cell cycle arrest in K-Ras mutant cancer cells indicating that cross-talk between K-Ras and PI3K/mTOR pathway is required to override the glutamine mediated G1 arrest. Treatment with aminooxyacetate (AOA), a glutamate oxaloacetate transaminase (GOT) inhibitor, mimics glutamine deprivation, causing S- and G2/M-phase arrest in K-Ras mutant cancer cells. Significantly, glutamine deprivation or AOA treatment causes enhanced sensitivity to phase-specific cytotoxic drugs, Capecitabine and Paclitaxel. Besides providing novel strategies for therapeutic intervention, our data suggest that cancers with specific oncogenic drivers and atypical metabolic addictions could offer an increased therapeutic index to currently existing chemotherapeutic regimens.
Materials and Methods

Cells and cell culture conditions. The Calu-1, DU-145, LNCaP, MCF7, MDA-MB-231, and Panc-1 cells used in this study were obtained from the American Tissue Type Culture Collection. LNCaP cells were maintained in RPMI-1640, and all the other cells were maintained in DMEM supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Sigma).

Materials. Reagents were obtained from the following sources: Antibodies against phospho-Akt (T308 and S473), phospho-S6K (T389), phospho-4EBP1 (T37/46), phospho-ERK1/2 (T202/Y204), phospho-Rb (S807/811), cyclin E, cleaved PARP, and actin were obtained from Cell Signaling; antibody against p27 was obtained from Santa Cruz Biotechnology; antibodies against cyclin A, cyclin B, and cyclin D were obtained from BD Biosciences; and anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Promega. DMEM (D6429), DMEM lacking Gln (D5546), RPMI-1640 (R8758), RPMI-1640 lacking Gln (R0883), dialyzed fetal bovine serum (F0392), and glutamine (G7513), and aminooxyacetic acid hemihydrochloride (AOA) (C13408), were obtained from Sigma. U0126 (9903) was obtained from Cell Signaling, Torin1 (4247) was obtained from Tocris. Paclitaxel (P-9600) and Capecitabine (C-2799) were obtained from LC Laboratories.

Flow cytometric analysis. Cells were washed twice in phosphate buffered saline (PBS) and harvested. Cell suspensions were resuspended in the following fixing solution: 7ml PBS, 2% bovine serum albumin (BSA), 5mM EDTA, 0.1% NaN₃; and 3ml of 100% ethanol was added dropwise. Fixed cells were centrifuged, washed using PBS, and then resuspended in 500µl sorting buffer: PBS, 0.1% Triton-X 100, 2% BSA, 5mM EDTA, 40µg/ml propidium iodide, 100µg/ml
RNAse A, and incubated at 37C for 30 min. The cells were filtered through 70-µm mesh to remove cell aggregates. The DNA content was analyzed by flow cytometry (FACSCalibur; Becton Dickinson), and percentages of cells within each phase of the cell cycle were determined using WinCycle software (Phoenix Flow Systems).

**Cell proliferation assay.** At indicated times, cells in six-well plates were washed once with PBS, trypsinized with 500 µl trypsin, resuspended in 500 µl complete medium. The cells were stained using 0.35% crystal violet solution (Millipore), and then counted twice using hemocytometer.

**Western Blot Analysis.** Cell lysates were collected using M-PER (Thermo Scientific, 78501), and proteins were separated on denaturing SDS-PAGE gels. Electrophoresed proteins were transferred to nitrocellulose membrane. After transfer, membranes were blocked in an isotonic solution containing 5% non-fat dry milk in PBS. Membranes were incubated with primary antibodies as described in the text, and depending on the origin of the primary antibody, either anti-mouse or anti-rabbit HRP conjugated IgG was used for detection using ECL system (Pierce).

**Trypan blue exclusion cell viability assay.** At indicated time points post-treatment, floating and adherent cells were collected and pelleted by centrifugation. The cell pellet was resuspended in 500 µl PBS, stained using 500 µl 0.4% trypan blue dye (Sigma), and incubated at room temperature for 10 min. Both dead (stained) and live (unstained) cells were counted twice using hemocytometer, and percent non-viable cells was determined from dividing dead cells by total number of cells.
Results


Glutamine deprivation is known to cause G1 cell cycle arrest in nontransformed primary cells (2, 65). We have previously shown that MDA-MB-231 breast and Panc-1 pancreatic cancer cell lines fail to arrest in G1 upon glutamine or essential amino acid deprivation (Fig. 7). We therefore screened several cancer cell lines to identify the underlying genetic mutations that override the amino acid mediated G1 checkpoint. As seen in figure 9A, glutamine deprivation for 48 hr caused significant accumulation of cells in G1 phase at the expense of S- and G2/M-phase cells in MCF7 breast, and DU-145 and LNCaP prostate cancer cell lines. On the other hand, glutamine deprivation caused accumulation of S- and G2/M-phase cells along with a reduction in G1 cell population in MDA-MB-231 breast, PANC-1 pancreatic, and Calu-1 lung cancer cells. The cell lines that failed to arrest in G1 upon glutamine deprivation were found to have oncogenic K-Ras mutation (Fig. 9B). However, failure to arrest in G1 upon glutamine deprivation in these K-Ras mutant cancer cell lines was neither tissue specific nor K-Ras mutation site specific. In all the cell lines, irrespective of cell type and K-Ras status, cells appeared significantly smaller in size and rounded upon glutamine deprivation (Fig. 9C) suggesting that glutamine deprivation causes alterations in actin cytoskeleton. There were also fewer cells in glutamine deprived condition. To further establish that glutamine deprivation was not merely prolonging S phase and thereby causing increased accumulation in S-phase, we performed cell proliferation assay. In all the cell lines tested, there was a significant loss of cell proliferation upon glutamine deprivation (Fig. 9D). Taken together, the data shows that glutamine deprivation causes G1 cell cycle arrest in K-Ras WT cells, and S- and G2/M-phase arrest in K-Ras mutant cancer cells.
Glutamine deprivation induces loss of cyclin D/cyclin E and p27 protein expression in K-Ras mutant cancer cells.

To further elucidate the differences between K-Ras mutant and WT cells, we next compared the effects of glutamine deprivation on PI3K/mTOR kinase activity. Glutamine deprivation caused decrease in S6K phosphorylation but not 4EBP1 phosphorylation in both K-Ras WT and mutant cancer cells (Fig. 10A). This was similar to the response seen in non-transformed BJ fibroblasts (Fig. 6A and 6B). Release of feedback inhibition upon suppression of S6K phosphorylation led to increase in Akt-S473 phosphorylation in K-Ras mutant cells, but not in K-Ras WT cells. There was also an increase in Akt-T308 phosphorylation upon glutamine deprivation.

We next analyzed cell cycle regulator expression and phosphorylation profile to further assess the impact of glutamine deprivation on K-Ras WT and K-Ras mutant cancer cells. K-Ras WT cells showed a decreased in cyclin B protein levels, indicating that there were fewer cells in the G2/M-phase upon glutamine deprivation (Fig. 10B). This is consistent with the flow cytometry data showing an increase in G1 cell population (Fig. 9A). However, cyclin D, cyclin E, and cyclin A levels were unchanged upon glutamine deprivation in K-Ras WT cells. It is possible that glutamine deprivation causes G1 arrest in these cells by influencing the association of these cyclins with the CDKs and not on the protein levels itself. However, in the case of K-Ras mutant cancer cell lines, glutamine deprivation caused loss of cyclin D, cyclin E, and p27 protein levels and an increase in cyclin A protein expression (Fig. 10B), which is also consistent with the flow cytometric data shown in Fig. 9B, wherein glutamine deprivation caused K-Ras mutant cancer cells to arrest in S-phase.
Inhibition of K-Ras effector pathways restores G1 arrest upon Q deprivation.

The data in Figure 9 reveal a correlation between K-Ras mutation and dysregulated G1 cell cycle progression through a Q-dependent checkpoint. To investigate whether mutant K-Ras is sufficient to override the Q-dependent G1 checkpoint, we introduced a vector that expresses mutant K-Ras into the immortalized human diploid fibroblast cell line BJ-hTERT (42) and evaluated the impact of Q deprivation on cell cycle progression. As shown in Figure 11A, mutant K-Ras, by itself, failed to override G1 cell cycle arrest – indicating that additional genetic alterations in the cancer cells are required for dysregulating the Q-dependent G1 checkpoint.

Based on elegant studies by Weinberg and colleagues on the minimal genetic requirements for the transformation of human cells (9, 10), we have proposed that the cooperating genetic mutations in human cancer cells impact on signaling pathways that lead to passage through two major G1 cell cycle checkpoints (8) that have both been referred to as the restriction point (2). The first checkpoint is in mid-G1 and is dependent on growth factors and facilitates passage through a checkpoint regulated by cyclin D and ERK; the second checkpoint is dependent on nutrients (including Q) and is regulated by cyclin E and mTOR (66). Interestingly, both of these pathways can be activated by mutant K-Ras (67, 68). To investigate if either or both of these key regulatory pathways are critical for overriding the Q-dependent G1 checkpoint, we investigated whether we could restore G1 arrest in response to Q deprivation by pharmacological suppression of the Raf/Mek/ERK and the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) pathways (Figure 11B). We first evaluated the effect of U0126 (Mek inhibitor) and Torin1 (mTOR inhibitor) on Q-induced cell cycle arrest in MDA-MB-231 cells. As shown in Figure 11C, in the absence of Q there was the S-phase arrest seen in Figure 9. By themself, neither U0126 nor Torin1 reverted the cells to G1 arrest upon Q deprivation. However, treatment with
U0126 and Torin1 together did revert the cells to G1 arrest in the absence of glutamine (Figure 11C). As shown in Figure 11D, U0126 and Torin1 suppressed the phosphorylation of the Mek and mTOR substrates ERK and S6 kinase respectively. We also evaluated the impact of U0126 and Torin1 on Q-induced cell cycle arrest in the Panc-1 cells. Unlike the MDA-MB-231 cells, the Panc-1 cells were largely reverted to G1 arrest with only Torin1 – U0126 did not do much by itself and marginally improved G1 arrest when combined with Torin1 (Figure 11E). Neither drug alone nor a combination of both, revert the G2/M arrest observed in the Panc-1 cells (Figure 11E).

Lastly, we examined the MCF7 cells, which do not have a K-Ras mutation and arrest in G1 in response to glutamine deprivation. These cells were treated with the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) to stimulate the Raf/Mek/ERK pathway, which is commonly activated by mutant K-Ras. As shown in Figure 11F, TPA treatment caused a shift from G1 to S-phase arrest in response to Q deprivation. This change in arrest pattern could be reverted to G1 arrest with U0126, but not with Torin1. Collectively, the data in Figure 11 reveal that multiple genetic alterations contribute to override of the Q-dependent G1 cell cycle checkpoint and that while K-Ras mutations in human cancer cell lines correlate with override of the Q-mediated G1 checkpoint, mutant K-Ras by itself is not sufficient to promote passage in the absence of Q. These data also reveal that K-Ras is not required for override of the Q-dependent G1 as revealed by TPA treatment of the MCF7 cells.
Glutamine deprivation sensitizes K-Ras mutated cancer cells to phase-specific cytotoxic drugs.

We next examined whether differential cell cycle arrest upon glutamine deprivation could create synthetic lethality to phase specific cytotoxic drugs in K-Ras mutant cancer cells. To specifically target the S-phase arrested cells, we used capecitabine, a prodrug that is converted enzymatically to 5-fluorouracil (5-FU) and elicits DNA damage response leading to apoptosis. In order to target the G2/M-phase arrested cells upon glutamine deprivation, we used paclitaxel which stabilizes microtubules and induces apoptotic cell death in the mitotic phase. As seen in figure 12A and B, glutamine deprivation alone led to a modest increase in nonviable cells in both K-Ras WT and K-Ras mutant cancer cell lines. In K-Ras WT cell lines, addition of capecitabine or paclitaxel to glutamine deprived cells did not cause significant increases in dead cells compared to the drugs added to CM control cells. However, in MDA-MB-231 and PANC-1 K-Ras mutant cells, glutamine deprivation created synthetic lethality to the cytotoxic drugs, and there was a four-fold increase in the number of nonviable cells.

Pharmacological inhibition of Q anaplerosis sensitizes K-Ras mutant cancer cells to cytotoxic drugs.

Glutamine, via anaplerotic entry to the TCA cycle, replenishes the intermediates lost by the exit of citrate from the mitochondria for fatty acid and cholesterol biosynthesis (69). Glutaminase catalyzes the deamination of Q to generate glutamate. Glutamate then acts as an amide donor to oxaloacetate in the transamination reaction catalyzed by glutamate oxaloacetate transaminase (GOT) to generate aspartate and α-ketoglutarate – a key TCA cycle intermediate (schematic shown in Figure 13A). Anaplerotic entry of Q into the TCA cycle can be inhibited by aminooxyacetate
(AOA), which inhibits GOT and consequently the entry of glutamine into the TCA cycle (70, 71). Treatment of both MCF-7 and MDA-MB-231 cells with AOA for 48 hr led to morphological changes similar to that observed with Q deprivation (Figure 13B) – indicating that AOA mimics Q deprivation in both cell types. As seen in Figure 13C, AOA treatment caused G1 arrest in the MCF-7 cells and S- and G2/M-phase arrest in the MDA-MB-231 cells – as was observed with Q deprivation in Figure 9. We therefore investigated whether AOA treatment would mimic Q deprivation to create the synthetic lethality observed with cell cycle phase-specific cytotoxic drugs in K-Ras mutant cancer cells. AOA treatment by itself led to minimal increase in nonviable cells in both MCF-7 and MDA-MB-231 cells (Figure 13D). AOA treatment alone also did not induce significant increases in cleaved PARP (Figure 13D), an indicator of apoptosis – suggesting that AOA has low cytotoxicity. Similar to what was observed with Q deprivation, the combination of AOA and the cytotoxic drugs did not increase the percentage of nonviable cells and cleaved PARP levels in MCF-7 cells (Figure 13D). However, the combination of AOA and the cytotoxic drugs caused a significant increase in nonviable cells and cleaved PARP levels in MDA-MB-231 cells (Figure 13D). These data indicate that in K-Ras mutant cancer cells, pharmacological inhibition of anaplerotic entry of Q into the TCA cycle mimics Q deprivation to produce aberrant cell cycle arrest creating synthetic lethality to cell cycle phase specific cytotoxic drugs.
Discussion

Metabolic transformation is an emerging hallmark in cancer biology. We have previously shown that some cancer cells arrest in S- and G2/M-phase of the cell cycle upon glutamine and essential amino acid deprivation. In this report, we provide evidence showing that cancer cell lines harboring K-Ras mutation override the glutamine-mediated G1 cell cycle arrest, and instead cause S- and G2/M-phase arrest (Fig. 9A). Our findings suggest that there are additional amino acid sensing mechanisms in S- and G2/M-phase of the cell cycle. It is possible that glutamine plays different roles in different phases of the cell cycle. There was a profound proliferative defect upon glutamine deprivation in all of the cell lines irrespective of the cell type or K-Ras status, and the cells also appeared smaller and rounded in morphology. Consistent with a non-G1 arrest in K-Ras mutant cancer cells, glutamine deprivation caused loss of cyclin D/E and p27 protein levels which are markers of G1 phase cells (Fig. 10B).

While there was a correlation between cancer cells harboring K-Ras mutations and override of the Q-dependent G1 cell cycle checkpoint, K-Ras, by itself, was not sufficient to induce override of the Q-dependent G1 checkpoint. In addition, we could also induce override of the Q-dependent G1 cell cycle checkpoint by treating MCF-7 cells with TPA – indicating it is not strictly a K-Ras-dependent phenomenon. However, we have found that other human cancer cell lines harboring K-Ras mutants display the override of G1 cell cycle arrest caused by Q depletion.

We found that combining inhibition of both ERK and mTOR could revert the dysregulated Q-dependent G1 cell cycle checkpoint in MDA-MB-231 cells. Panc-1 cells – a human pancreatic cancer cell line harboring a K-Ras mutation – that arrested in S and G2/M phase with Q deprivation could be largely reverted to G1 arrest with only mTOR suppression. Thus, it is clear that the
override of the Q-mediated G1 checkpoint is complex involving more than one signaling pathway. Combined inhibition of two key regulators of G1 cell cycle progression – ERK and mTOR – are able to revert the override of the Q-mediated G1 checkpoint, indicating that override is dependent on two key signaling nodes implicated at two distinct regulatory G1 checkpoints that have both been referred to as the restriction point (66). There may be other means to accomplish this without K-Ras, as observed with TPA-treated MCF-7 cells, but there is a strong correlation with cancer cells harboring K-Ras mutations.

Glutamine deprivation has been shown to be sufficient to cause apoptotic cell death in cancer cells with Myc mutation (37). It has also been reported that glutamine deprivation sensitizes cancer cells to redox stress, and drugs such as fenretinide and hydrogen peroxide cause cell death upon glutamine deprivation (72, 73). Based on our finding that glutamine deprivation caused S- and G2/M-phase arrest in K-Ras mutant cancer cells and because many cytotoxic drugs used in chemotherapy are cell cycle phase-specific, we investigated whether glutamine deprivation could create a synthetic lethality to the phase-specific cytotoxic drugs. Indeed glutamine deprivation caused increased sensitivity to both capecitabine and paclitaxel in K-Ras mutant cancer cells, but not in K-Ras WT cells (Fig. 12). To mimic glutamine deprivation, we used AOA which prevents anaplerotic glutamine utilization by inhibiting GOT activity. Flow cytometric analyses showed that AOA treatment caused S- and G2/M-phase arrest in MDA-MB-231 K-Ras mutant cancer cells (Fig. 13). We also found that similar to glutamine deprivation, AOA treatment also sensitized MDA-MB-231 cells to phase-specific cytotoxic drugs.

Glutamine is a conditionally essential amino acid and has been shown to be required for cell proliferation and survival. Consistent with several reports underscoring the importance of glutamine in vitro, it has been reported that tumors in vivo also have increased glutamine uptake
To mimic glutamine deprivation in vivo, glutamine analogs DON (6-diazo-5-oxo-L-norleucine) and acivicin have been used successfully in animal models of cancer. However, they were proven to be unacceptably toxic to humans in clinical trials (37, 75). The difference could be due to cells from different organisms respond differently to amino acid deprivation. Consistent with our findings reported here with human cancer cell lines, K-Ras overexpression has been shown to cause abortive S-phase in mouse embryonic fibroblasts (38).

Ras is mutated in approximately 30% of all cancers and occurs in over 90% of the pancreatic cancers (76), which has a 5-year survival rate of less than 5% (77). However, it is considered “therapeutically undruggable” owing in part to its extremely high affinity with GTP (in the picomolar range) (78). We have previously shown that some cancer cell lines arrest outside of G1 upon glutamine or EAA deprivation and in this report we show that K-Ras mutation causes G1 override upon glutamine deprivation. This aberrant response to amino acid deprivation in general could prove to be an Achilles heel in K-Ras mutant cancer cells, especially pancreatic cancers.
Figure 9. Glutamine deprivation causes S- and G2/M-phase arrest in K-Ras mutant cancer cells.

(A-B) Cells were plated at 30% confluence in 10-cm plates in complete media containing 10% serum. After 24 hr, the cells were shifted to complete medium (CM) or medium lacking Q for 48 hr. Both CM and –Q medium contained 10% dialyzed FBS (DFBS). After 48 hr, the cells were harvested, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by measuring DNA content/cell as described in Experimental Procedures. The error bars represent standard error of mean for experiments repeated four times. The mutations present in the cancer cell lines were obtained from the Sanger Institute COSMIC database. (C) Cells were plated and treated as described above and after 48 hr cell morphology was examined using phase-contrast microscopy. (D) Cells were plated at 20% confluence in six-well plates in complete media containing 10% serum. After 24 hr (day 1), cells were shifted to CM or medium lacking Q. Cells were harvested at indicated time points, stained using crystal violet, and quantified by light microscopy as described in Experimental Procedures. Error bars represent the standard error for the experiment repeated three times.
Figure 9

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(C)
Figure 10. Cyclin profile correlates with differential cell cycle arrest pattern upon glutamine starvation.

(A) Cells were plated at 30% confluence in 10-cm plates in complete media containing 10% serum for 24 hr, at which time they were shifted to CM or medium lacking Q for 4 hr. The cells were subsequently harvested and cell lysates were collected. The indicated protein or phosphoprotein levels were determined by Western blot analysis. (B) The cells were plated and treated as in (A) for 48 hr, at which time cells were harvested, lysed, and analyzed for protein levels using Western blot. The data shown are representative of experiments repeated two times.
### Figure 10

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Figure 11. Dual inhibition of K-Ras and PI3K/mTOR pathway restores G1 arrest in K-Ras mutant cancer cells.

(A) BJ-hTERT human diploid fibroblasts were transfected with either mock control or a vector expressing mutant K-Ras (G12V; obtained from Missouri S&T cDNA Resource Center, Rolla, MO, USA) using PolyFect transfection reagent (Qiagen, Germantown, MD, USA) per manufacturer’s recommendations. The cells were then put in either complete medium (CM) or in medium lacking Q for 48 hr, at which time cell cycle status was determined as in Figure 9A. (B) Schematic diagram of the Raf/Mek/ERK and PI3K/Akt/mTOR signaling pathways that regulate distinct G1 cell cycle checkpoints (66). (C) MDA-MB-231 cells were plated at 30% confluence in 10-cm plates in CM containing 10% serum. After 24 hr, the cells were shifted to CM or medium lacking Q and treated with 10 µM U0126 and/or 250 nM Torin1 for 48 hr. The cells were analyzed by flow cytometry as in Figure 9A. Error bars represent standard error of mean for the experiment repeated four times. (D) The MDA-MB-231 cells were similarly treated as in (C) for 4 hr at which time cell lysates were prepared and used for Western blot analysis of the levels of phospho-Akt (S473), phospho-S6 kinase (S6K) (T389), phospho-ERK1/2 (T202/Y204), Akt, S6K, ERK1/2, and actin. The data shown is representative of experiments repeated at least two times. (E) Panc-1 cells were prepared and subjected to the same analysis used in (D) for the MDA-MB-231 cells. (F) MCF7 cells were prepared as in (D) and (E) and evaluated similarly as for the MDA-MB-231 and Panc-1 cells except that cells were also treated with 100 nM TPA concomitantly with U0126 or Torin1.
Figure 11

A

B

C

D

E

F

Figure 11
Figure 12. Glutamine deprivation sensitizes K-Ras mutated cancer cells to phase-specific cytotoxic drugs.

(A-D) Cells were plated at 20% confluence in six-well plates in complete media containing 10% serum for 24 hr, after which the cells were shifted to CM or medium lacking Q for 48 hr. After 48 hr, the cells were additionally treated with 50 nM Paclitaxel or 1 µg/ml Capecitabine for 24 hr. Percent cell viability was determined using trypan blue dye exclusion assay as described in Experimental Procedures. The error bars represent the standard error of mean for experiments repeated three times.
**Figure 12**

**WT K-Ras**

(A) MCF 7

(B) DU-145

**Mutant K-Ras**

(C) MDA-MB-231

(D) PANC-1

% Non-viable Cells

Figure 13. Pharmacological inhibition of Q anaplerosis sensitizes K-Ras mutant cancer cells to cytotoxic drugs.

(A) Schematic overview of anaplerotic Q utilization. Q is deaminated to glutamate by glutaminase (GLS). Glutamate is then converted to α-ketoglutarate by transamination catalyzed by GOT, which uses oxaloacetate as the amino group acceptor to generate aspartate, which can reenter the TCA cycle via conversion to fumarate in the urea cycle. AOA blocks the GOT and thusly, the generation of α-ketoglutarate from Q-derived glutamate. (B) Cells were plated at 20% confluence in 10-cm plates in complete medium (CM) containing 10% serum. After 24 hr, cells were shifted to CM, or medium lacking Q, or CM containing 0.5 mM AOA for 48 hr – at which time the cells were observed using phase-contrast microscopy. (C) MCF7 and MDA-MB-231 cells were plated and treated as in (B) for 48 hr, at which time cells were analyzed for cell cycle distribution as in Figure 9. Error bars represent standard error of mean for experiments repeated three times. (D) MCF7 and MDA-MB-231 cells were plated as in (A) and shifted to CM and treated with 0.5 mM AOA for 48 hr. The cells were additionally treated with 50 nM Paclitaxel or 1 µg/ml Capecitabine for 24 hr, at which time the percentage non-viable cells were determined using trypan blue exclusion assay. Error bars represent the standard error of mean for experiments repeated three times. Cell lysates were also collected, and the levels of cleaved PARP (antibody from Cell Signaling) were determined by Western blot analysis. Data shown are representative of experiments repeated two times. (E) Model depicting that AOA treatment mimics Q deprivation causing G1 cell cycle arrest in K-Ras wild type cells and S- and G2/M-phase arrest in K-Ras mutant human cancer cell lines, which creates synthetic lethality to cell cycle phase-specific cytotoxic drugs causing apoptotic cell death.
Figure 13

A

B

C

D

E
Chapter 4: Concluding Remarks and Future Directions

Based on the rationale presented in chapter 1 regarding the genetic and temporal distinctions between GF-mediated restriction point (R) and nutrient-mediated proposed Cell Growth Checkpoint, and also based on my preliminary findings that various cancer cells exhibit complex cell cycle arrest pattern upon glutamine deprivation as evidenced by FACS profile, major questions that we sought to address were (i) Is there a distinct G1 checkpoint mediated by amino acids different from GF-dependent R and mTOR-mediated cell growth checkpoint? (ii) Are there novel S- and G2/M phase checkpoints mediated by glutamine? Using several approaches detailed in Chapter 2, we show that GF-mediated R, amino acid-mediated checkpoints, and mTOR-mediated late-G1 checkpoint are indeed distinct and distinguishable. Importantly, we also show that amino acid sensing is dysregulated in some cancer cells, whereby they arrest in S- and G2/M-phase of the cell cycle.

In chapter 3, we identify that K-Ras mutation is required for overriding the amino-acid mediated G1 cell cycle arrest. We also show that this aberrant response to amino acid deprivation could create synthetic lethality to phase-specific cytotoxic drugs and prove to be an Achilles heel in K-Ras mutant cancer cells.

The work described here raises a few interesting questions:

(i) What is the mechanism through which nutrient amino acid deprivation mediates G1 arrest in non-transformed cells? From our data in cancer cells, where rapamycin/Torin1 cause G1 arrest but amino acid deprivation does not, the G1 arrest upon amino acid deprivation is likely independent of mTOR pathway or perhaps there are other pathways involved in addition to the mTOR pathway to mediate the arrest. K-Ras mutant cancer
cells override the amino acid-mediated G1 checkpoint, indicating that cross-talk and compensation between the K-Ras/MAPK and PI3K/mTOR pathway is involved in mediating G1 cell cycle arrest.

(ii) Are there novel S- and G2/M-phase checkpoints? With the cell cycle freeze in BJ cells upon amino acid deprivation and non-G1 arrest in cancer cells, it is evident that there is additional amino acid sensing in S- and G2/M-phase of the cell cycle. The need and the mechanism for amino acid sensing warrants further investigation.

(iii) It has been suggested that glutamine favors proliferation by replenishing citric acid cycle intermediates. However, other important functions of glutamine in intermediary metabolism – such as in nucleotide biosynthesis and participating in overall nitrogen balance – remain largely ignored. It is also likely that glutamine plays different roles in different phases of the cell cycle.

Given the extensive metabolic deregulation in cancer, exploiting the metabolic vulnerabilities could offer novel therapeutic windows in developing innovative therapeutic strategies.
References


