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Blocking anaplerotic entry of glutamine to TCA cycle sensitizes K-Ras mutant cancer cells to cytotoxic drugs

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Abstract

Cancer cells undergo a metabolic transformation that allows for increased anabolic demands wherein glycolytic and TCA cycle intermediates are shunted away for the synthesis of biological molecules required for cell growth and division. One of the key shunts is the exit of citrate from the mitochondria and the TCA cycle for the generation of cytosolic acetyl-CoA that can be used for fatty acid and cholesterol biosynthesis. With the loss of mitochondrial citrate, cancer cells rely on the "conditionally essential" amino acid glutamine (Q) as an anaplerotic carbon source for TCA cycle intermediates. While Q deprivation causes G1 cell cycle arrest in non-transformed cells, its impact on the cancer cell cycle is not well characterized. We report here a correlation between bypass of the Q-dependent G1 checkpoint and cancer cells harboring K-Ras mutations. Instead of arresting in G1 in response to Q-deprivation, K-Ras driven cancer cells arrest in either S- or G2/M-phase. Inhibition of K-Ras effector pathways was able to revert cells to G1 arrest upon Q deprivation. Blocking anaplerotic utilization of Q mimicked Q deprivation – causing S- and G2/M-phase arrest in K-Ras mutant cancer cells. Significantly, Q deprivation or suppression of anaplerotic Q utilization created synthetic lethality to the cell cycle phase-specific cytotoxic drugs, capecitabine and paclitaxel. These data suggest that disabling of the G1 Q checkpoint could represent a novel vulnerability of cancer cells harboring K-Ras and possibly other mutations that disable the Q-dependent checkpoint.

Keywords

K-Ras; cell cycle; glutamine; synthetic lethality; anaplerosis

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Introduction

Metabolic dysregulation is an emerging hallmark in cancer.[1] Coupling oncogenesis with the needs of proliferative metabolism, several oncogenes that cause cellular transformation also upregulate glycolytic enzymes and promote metabolic reprogramming.[2, 3] In order to meet increased anabolic demand, cancer cells display elevated levels of glucose uptake. However, instead of complete oxidation of glucose through the tricarboxylic acid (TCA) cycle, most cancer cells convert glucose to lactate through a process known as aerobic glycolysis.[4] This metabolic transformation was first described by Otto Warburg in the early 1920s and named Warburg effect.[5] It has been proposed that less efficient utilization of glucose for ATP generation is overcome by a marked increase in glucose uptake.[6] Another metabolic shift is the utilization of the TCA cycle intermediate citrate for cytosolic generation of acetyl-CoA. After conversion of the glycolytic product pyruvate to acetyl-CoA in the mitochondria, there is a condensation reaction with oxaloacetate to generate citrate, which exits the mitochondria where it is converted back to oxaloacetate and acetyl-CoA, which can then be used for fatty acid synthesis. This creates a need for anaplerotic replenishment of TCA cycle intermediate that can regenerate oxaloacetate. The most common source for anaplerosis is glutamine (Q), which can be successively deaminated in two steps to produce α-ketoglutarate – allowing for the maintenance of TCA cycle function.[3] The Myc oncogene has been shown to upregulate glutaminolysis leading to Q addiction in cancer cells.[7, 8] While Q has been reported to play pleiotropic roles in tumor proliferation, the impact of Q deprivation on cancer cell cycle progression is less well characterized.[9, 10] This is further complicated by the differential response of cancer cells to Q deprivation, which likely depends on the mutations they harbor. For instance, cancer cells with Myc overexpression undergo apoptotic cell death in response to Q depletion.[11] On the other hand, in K-Ras overexpressing NIH 3T3 mouse fibroblasts, Q deprivation was shown to cause abortive S-phase.[12] Additionally, we recently reported that some cancer cell lines bypass a Q-dependent G1 cell cycle checkpoint and arrest in S- and G2/M-phase of the cell cycle upon Q deprivation.[13]

In this report, we demonstrate that cancer cells harboring K-Ras mutations arrest in S- and G2/M-phase of cell cycle rather than G1. Significantly we also show that this differential sensitivity to Q in K-Ras mutant cancer cells can be exploited using cell cycle phase specific cytotoxic drugs. Our study provides proof-of-principle that cancers with specific genetic defects and dysregulated metabolic cell cycle checkpoints can create a synthetic lethality to chemotherapeutic drugs and offer novel therapeutic options.

Results and Discussion

Glutamine deprivation causes S- and G2/M-phase arrest in K-Ras mutant cancer cells

Glutamine deprivation causes G1 cell cycle arrest in non-transformed primary cells.[14] We previously reported that MDA-MB-231 breast and Panc-1 pancreatic cancer cell lines fail to arrest in late-G1 upon Q or essential amino acid deprivation.[13] We therefore screened several cancer cell lines to identify underlying genetic mutations that override the Q-mediated G1 checkpoint. As seen in Figure 1a, Q 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, Q deprivation led to an increase primarily in S-phase cells and a reduction in G1-phase cells in MDA-MB-231 breast, PANC-1 pancreatic, and Calu-1 lung cancer cells. The cell lines that failed to arrest in G1 upon Q deprivation all harbor oncogenic K-Ras mutations (Figure 1a). The failure to arrest in G1 upon Q deprivation in these K-Ras mutant cancer cell lines was neither tissue specific nor K-Ras mutation site specific (Figure 1a). Since amino acid deprivation has been implicated in mTOR signaling, we also evaluated the impact of Q deprivation on mTOR substrate phosphorylation. While Q deprivation caused a modest reduction in S6 kinase phosphorylation, there were no significant differences between the cells that arrested in G1 and those that arrested in S- and G2/M-phase (Figure S1). However, there were elevated levels of Akt phosphorylation at Ser473 and Thr 308 observed with Q deprivation in the mutant K-Ras-driven cancer cells that is concomitant with non-G1 arrest. This observation is consistent with a recent report that phosphorylation of Akt occurs predominantly as cells progress into S-phase.[15] We also looked at the impact of Q deprivation on the cell cycle progression markers cyclins D, E, A, and B, phospho-Rb, and p27 protein levels. As shown in Figure S2, Q deprivation in the cancer cells with wild type K-Ras had very little impact on anything other than cyclin B, which was lower in the Q-deprived cells. In the cells with mutant Ras, there were subtle reductions in cyclins D and E, phospho-Rb, and p27 levels, with a concomitant increase in S-phase marker cyclin A. The reduction in G1 cell cycle markers in K-Ras mutant cancer cells is consistent with a non-G1 arrest observed by FACS analysis (Figure 1a); however, they do not provide insight into how K-Ras-driven cancer cells avoid G1 arrest. To establish that Q deprivation was not merely prolonging S-phase and thereby causing an increase in the percentage of cells in S-phase, we performed cell proliferation assay. In all the cell lines tested, there was a significant loss of cell proliferation upon Q deprivation (Figure 1b). The data in Figure 1 reveal a correlation between dysregulated Q-mediated G1 cell cycle arrest and K-Ras mutation.

Override of Q-dependent G1 checkpoint requires activation of multiple signaling pathways

Based on elegant studies by Weinberg and colleagues on the minimal genetic requirements for the transformation of human cells,[16, 17] we have proposed that cooperating genetic mutations in human cancer cells impact on signaling pathways that lead to passage through two major G1 cell cycle checkpoints[18] that have both been referred to as the restriction point.[14] 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.[13] Interestingly, both of these pathways can be activated by mutant K-Ras (Figure 2a).[19, 20] 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 2a). We evaluated the effect of U0126 (Mek inhibitor) and Torin1 (mTOR inhibitor) on Q-induced cell cycle arrest in MDA-MB-231 and Panc1 cells. As shown in Figure 2b, U0126 and Torin1 suppressed the phosphorylation of the Mek and mTOR substrates ERK and S6 kinase respectively in both the MDA-MB-231 and Panc1 cells. As

shown in Figure 2c, in the absence of Q, S-phase arrest was observed as in Figure 1 in the MDA-MB-231 cells. Neither U0126 nor Torin1, by itself, reverted the cells to G1 arrest upon Q deprivation. However, treatment with U0126 and Torin1 together did revert the MDA-MB-231 cells to G1 arrest in the absence of Q (Figure 2c). Similar results were obtained when we used the PI3K inhibitor wortmannin instead of Torin1 (Figure S3). We also evaluated the impact of U0126 and Torin1 on Q-induced cell cycle arrest in the Panc-1 cells, which arrested in both S- and G2/M phase. 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 2d). The Panc1 cells interestingly still remained arrested in G2/M when treated with Torin1 – indicating a differential mechanism for G2/M arrest for the MDA-MB-231 and Panc1 cells.

We next examined the MCF7 cells, which do not have a K-Ras mutation and arrest in G1 in response to Q deprivation. These cells were treated with the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which has been shown to stimulate the Raf/Mek/ERK pathway,[21] which is commonly activated by mutant K-Ras. As shown in Figure 2e, 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 – indicating that it was activation of the ERK pathway that was responsible.

The data in Figures 1 and 2e reveal a correlation between K-Ras mutation or activation of the Raf/Mek/ERK pathway and dysregulated G1 cell cycle progression through a Q-dependent checkpoint. We therefore investigated whether mutant K-Ras alone is sufficient to override the Q-dependent G1 checkpoint. We introduced a vector that expresses mutant K-Ras (G12V) into the immortalized human diploid fibroblast cell line BJ-hTERT[22] and evaluated the impact of Q deprivation on cell cycle progression. As shown in Figure 2f, 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. However, mutant Ras has also been reported to induce senescence in primary cells.[23] Weinberg and colleagues reported previously that mutant Ras in combination with TPA induces a transformed phenotype in primary cells.[24] We therefore investigated the effect of mutant K-Ras in combination with TPA on cell cycle progression when cells were depleted of Q. As shown in Figure 2f, the combination of mutant K-Ras and TPA resulted in S- and G2/M-phase arrest upon Q deprivation rather than G1. Collectively, the data in Figure 2 reveal that while mutant K-Ras, by itself, is not sufficient to cause override of the Q-dependent G1 cell cycle checkpoint, override can be achieved by cooperating genetic alterations or by activation of a cooperating signaling pathway.

Glutamine deprivation sensitizes K-Ras mutant cancer cells to phase-specific cytotoxic drugs

We next examined whether the differential cell cycle arrest observed with Q 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 pro-drug that is converted enzymatically to 5-fluorouracil and elicits a DNA damage response leading to apoptosis.[25] To target G2/M-phase arrested cells, we used paclitaxel, which stabilizes

microtubules and induces apoptotic cell death in the mitotic phase.[26] As seen in Figures 3a-d, Q deprivation alone led to a modest increase in nonviable cells in both K-Ras wild type and K-Ras mutant cancer cell lines. In the K-Ras wild type cell lines, addition of capecitabine or paclitaxel to Q-deprived cells did not cause significant increases in non-viable cells compared to controls (Figures 3a and 3b). However, in the K-Ras mutant MDA-MB-231 and PANC-1 cells, Q deprivation followed by treatment with either capecitabine or paclitaxel caused a significant increase in the nonviable cells (Figures 3c and 3d). Thus, in K-Ras mutant cancer cell lines, Q deprivation creates a synthetic lethality for the cell cycle phase specific cytotoxic drugs. There was a somewhat elevated background of cell death with Q deprivation in the MDA-MB-231 cells, but there are very stark differences between the sensitivity of the cells harboring K-Ras mutations with those that do not.

As shown in Figure 2, we were able to get MCF7 cells and BJ cells expressing mutant K-Ras to bypass the G1 Q-dependent cell cycle checkpoint and arrest in S- and/or G2/M phases when treated with TPA. We therefore examined whether these cells became sensitive to capecitabine and paclitaxel. As shown in Figure 3e and 3f, both MCF7 and BJ-K-Ras cells, upon Q depletion, became sensitive to capecitabine and paclitaxel when treated with TPA. Thus, it is the arrest in S and G2/M that sensitizes cells to capecitabine and paclitaxel.

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.[27] Glutaminase catalyzes the deamination of Q to generate glutamate. Glutamate can then be converted to α-ketoglutarate by either glutamate dehydrogenase or transaminase. Kimmelman and colleagues recently reported that K-Ras-driven pancreatic cancer cells preferentially utilize the transaminase pathway for anaplerotic glutamine utilization.[28] In the transaminase pathway, glutamate acts as an amino donor to oxaloacetate – a reaction catalyzed by glutamate oxaloacetate transaminase (GOT), which generates aspartate and α-ketoglutarate (schematic shown in Figure 4a). Anaplerotic entry of Q into the TCA cycle can be inhibited by aminooxyacetate (AOA) – a pan-transaminase inhibitor, which inhibits GOT and consequently the entry of glutamine into the TCA cycle.[29, 30] 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 4b). As seen in Figure 4c, AOA treatment caused G1 arrest in the MCF-7 cells and S- and G2/M-phase arrest in the MDA-MB-231 cells and also blocked proliferation (Figure 4d) as was observed with Q deprivation in Figure 1a and b – indicating that AOA mimics Q deprivation in both cell types. We also investigated whether the effect of AOA on cell cycle progression could be reversed by providing cell permeable analogues of α-ketoglutarate and aspartate – the products of the transamination reaction between glutamate and oxaloacetate. Dimethyl-α-ketoglutarate (DMKG) and β-methyl-aspartate (β-MD) were included along with AOA for the MDA-MB-231 cells. As shown in Figure 4e, neither compound by itself was able to completely reverse S- and G2/M arrest seen in the MDA-MB-231 cells, however the combination of both DMKG and β-MD did reverse the S- and G2/M arrest in these cells – indicating that generating both α-ketoglutarate and aspartate in the transaminase reaction is critical for passing through S- and

G2/M phases. This finding is similar to that observed by Kimmelman and colleagues who showed a requirement for both α-ketoglutarate and non-essential amino acids (which included aspartate) for colony formation by pancreatic cancer cells. The need for aspartate as well as α-ketoglutarate indicates that the aspartate generated by the transaminase reaction is important. Kimmelman and colleagues[28] demonstrated a critical need for conversion of aspartate → oxaloacetate, followed by conversion of oxaloacetate → malate, and then oxidative decarboxylation to pyruvate by malic enzyme in order to generate NADPH and maintain redox balance. However, a substantial amount of anaplerotic Q is converted to fatty acids.[4] Thus, the aspartate generated by the transaminase reaction between glutamate and oxaloacetate is likely destined to re-enter the TCA cycle via conversion to oxaloacetate followed by condensation with acetyl-CoA to form citrate (see Figure 4a).

We further 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 4f). AOA treatment alone also did not induce significant increases in cleaved PARP (Figure 4f), an indicator of apoptosis – suggesting that AOA has low cytotoxicity. This is important in that AOA also inhibits alanine transaminase as well.[28] 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 4d). 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 4d). 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.

Metabolic transformation is an emerging paradigm in cancer biology.[1] Glutamine has been suggested to play pleiotropic roles in tumor proliferation, and several oncogenes have been shown to promote aerobic glycolysis and Q addiction in cancer cells.[10, 31] We previously reported that while most cells arrest in late G1 in response to Q deprivation, some cancer cell lines arrested in S and G2/M.[13] An extension of this study revealed a correlation between G1 Q-checkpoint override in human cancer cell lines with K-Ras mutations. Upon Q deprivation, cancer cell lines harboring K-Ras mutations arrested in S and G2/M rather than G1. Whether there are actual Q-sensitive checkpoints in S- and G2/M phases is not clear from data presented here, however it is clear from Figure 1b that cell number is not increasing and therefore cells are stopping progression in S and G2/M phases and it is here that they are sensitive to cytotoxic drugs. Of significance, Q deprivation created a synthetic lethality for compounds that selectively target cells in S and G2/M phases of the cell cycle. Importantly, Q deprivation could be mimicked with AOA – a compound that interferes with the transaminase pathway for anaplerotic conversion of Q into the TCA cycle intermediate α-ketoglutarate. The ability of AOA to mimic Q deprivation is consistent with the recent report that K-Ras-driven pancreatic cells preferentially use the transaminase over the glutamate dehydrogenase pathway for generating α-ketoglutarate.[28] Thus, it is possible that a combination of drugs that block the generation of α-ketoglutarate via transamination and kill cells in S- and/or G2/M phase could be used to target K-Ras driven cancers (Figure 4g).

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 immortalized BJ cells. However, the inability to observe bypass of the Q-dependent G1 checkpoint was likely due to the induction of senescence by mutant K-Ras.[23] However, if TPA, which cooperates with mutant Ras to transform primary cells[24] was provided, the cells bypassed the G1 checkpoint and arrested in S- and G2/M phases and were now sensitive to capecitabine and paclitaxel. In addition, we observe override of the Q-dependent G1 cell cycle checkpoint by activating the ERK pathway in MCF-7 cells – indicating that bypass could be achieved by activating this K-Ras effector pathway. Panc-1 pancreatic cancer cells that harbor a K-Ras mutation arrested in S- and G2/M phase with Q deprivation, but could largely be reverted to G1 arrest with only mTOR suppression. Thus, it is clear that 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 – reverted 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.[13] Whether this phenomena is absolutely dependent on the K-Ras or K-Ras effector signals is not clear – nor is it clear that all cancer cells with K-Ras mutations will override the Q-dependent checkpoint. In fact the DU-145 prostate cancer cells used in this study harbors an unusual UBE2L3-KRas fusion protein, knockdown of which was shown to attenuate cell invasion and xenograft growth[32], but still arrested in G1 in response to Q deprivation as seen in Figure 1a. Thus, while the connection between activated K-Ras and override of the Q-dependent G1 checkpoint is not likely absolute, the correlation observed here with human cancer cell lines harboring K-Ras mutations and a synthetic lethal sensitivity to paclitaxel and capecitabine suggests novel strategies for therapeutic intervention in a class of cancers considered undruggable.

Oncogenic K-Ras mutations are common in many different cancer types and contribute to as many as 30% of all human cancers – including 90% of pancreatic cancers[33] - which have a poor prognosis with mortality usually within 6 months of detection and a five-year survivability of less than 5%.[34] K-Ras mutants have enhanced binding to GTP and are considered undruggable – due in part to the picomolar range for GTP binding.[35] Thus, targeting K-Ras-driven cancers has been problematic – especially for pancreatic cancer, which is likely the deadliest of human cancers. The observation reported here – that inhibition of anaplerotic utilization of Q in K-Ras-driven human cancer cell lines leads to cell cycle arrest in S- and G2/M rather than G1 – suggests a vulnerability for K-Ras-driven cancers that could be exploited therapeutically. We have demonstrated here an enhanced sensitivity to chemotherapeutic agents that target cells in S- and G2/M – providing proof-of-principle for the predicted synthetic lethality created by interfering with Q utilization.

An emerging pattern for Ras-driven tumorigenesis involves changes in nutrient utilization as reported here for glutamine and previously by Kimmelman and colleagues.[28] In addition, it was recently reported that Ras-driven cancer cells also have special needs for extracellular lipids[36, 37] and glutamine.[38] Significantly, the need for exogenously supplied lipids in Ras-driven cancer cells creates a synthetic lethality for rapamycin.[37] Thus, it is conceivable that

the altered nutritional and metabolic needs by Ras-driven cancers may actually prove to be an Achilles' heel for this deadly class of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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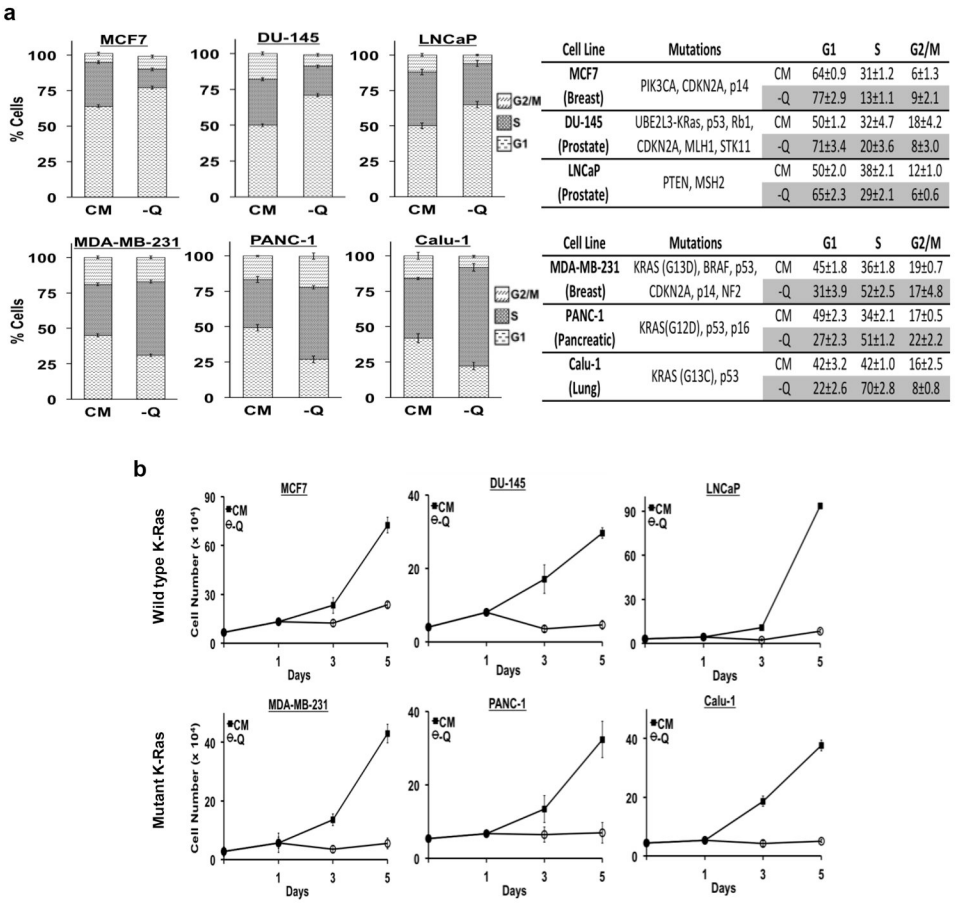

Cell Line	Mutations		G1	S	G2/M
MCF7 (Breast)	PIK3CA, CDKN2A, p14	CM	64±0.9	31±1.2	6±1.3
		-Q	77±2.9	13±1.1	9±2.1
DU-145 (Prostate)	UBE2L3-KRas, p53, Rb1, CDKN2A, MLH1, STK11	CM	50±1.2	32±4.7	18±4.2
		-Q	71±3.4	20±3.6	8±3.0
LNCaP (Prostate)	PTEN, MSH2	CM	50±2.0	38±2.1	12±1.0
		-Q	65±2.3	29±2.1	6±0.6

Cell Line	Mutations		G1	S	G2/M
MDA-MB-231 (Breast)	KRAS (G13D), BRAF, p53, CDKN2A, p14, NF2	CM	45±1.8	36±1.8	19±0.7
		-Q	31±3.9	52±2.5	17±4.8
PANC-1 (Pancreatic)	KRAS(G12D), p53, p16	CM	49±2.3	34±2.1	17±0.5
		-Q	27±2.3	51±1.2	22±2.2
Calu-1 (Lung)	KRAS (G13C), p53	CM	42±3.2	42±1.0	16±2.5
		-Q	22±2.6	70±2.8	8±0.8

Figure 1.

Glutamine deprivation causes S- and G2/M-phase arrest in K-Ras mutant cancer cells. (**a**) MCF7 breast, DU-145 prostate, LNCaP prostate, MDA-MB-231 breast Panc-1 pancreatic, and Calu-1 lung cancer cells (obtained from the American Type Culture Collection, Manassas, VA, USA) were plated at 30% confluence in 10-cm plates in complete media (CM) containing 10% serum. After 24 hr, the cells were shifted to CM or medium lacking Q for 48 hr. Both CM and –Q medium contained 10% dialyzed FBS (F0392, Sigma-Aldrich, St. Louis, MO, USA). After 48 hr, the cells were harvested and analyzed for cell cycle distribution by measuring DNA content/cell as described previously.[13] 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[39] and the Cancer Cell Line Encyclopedia.[40] (**b**) Cells were plated at 20% confluence in six-well plates in CM 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. Error bars represent the standard error of the mean for experiments repeated three times.

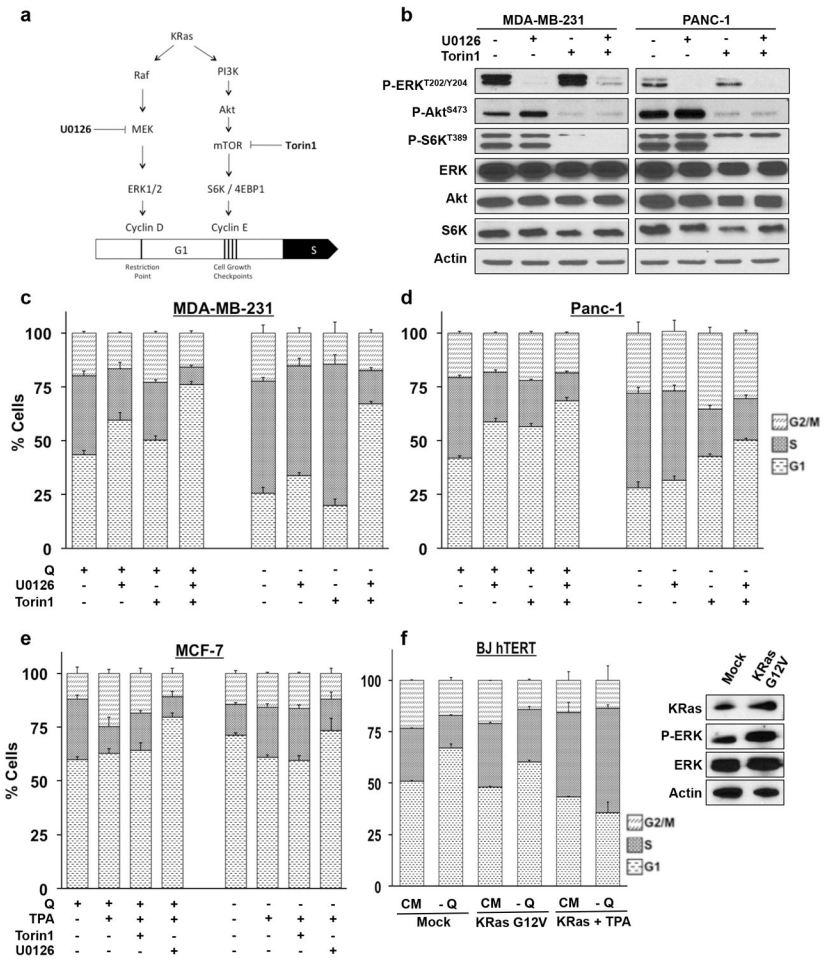

Figure 2.

Override of Q-dependent G1 checkpoint requires activation of multiple signaling pathways. (**a**) Schematic diagram of the Raf/Mek/ERK and PI3K/Akt/mTOR signaling pathways that regulate distinct G1 cell cycle checkpoints.[13] (**b**) MDA-MB-231 and Panc1 cells were plated at 30% confluence in 10-cm plates in CM containing 10% serum. After 24 hr, the cells were treated with 10 μM U0126 (Cell Signaling Technology, Danvers, MA, USA) and/or 250 nM Torin1 (Tocris Bioscience, Bristol, UK) as indicated 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 (antibodies all from Cell Signaling Technology). The data shown is representative of experiments repeated at least two times. (**c-d**) MDA-MB-231 and Panc1 cells were plated as in (**b**). 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 then analyzed by flow cytometry as in Figure 1. Error bars represent standard error of mean for the experiment repeated four times. (**e**) MCF7 cells were prepared as in (**c-d**) and evaluated similarly as for the MDA-MB-231 and Panc1 cells except that cells were also treated with 100 nM TPA (Cell Signaling Technology) as indicated concomitantly with U0126 or Torin1. (**f**) BJ-hTERT human diploid fibroblasts (obtained from ATCC) 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. K-Ras G12V overexpression and activation of downstream phospho-ERK was confirmed by Western blot analysis. 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 1a. TPA (100 nM) was added where indicated at the time of Q withdrawal. Error bars represent standard error of mean for experiments repeated three times.

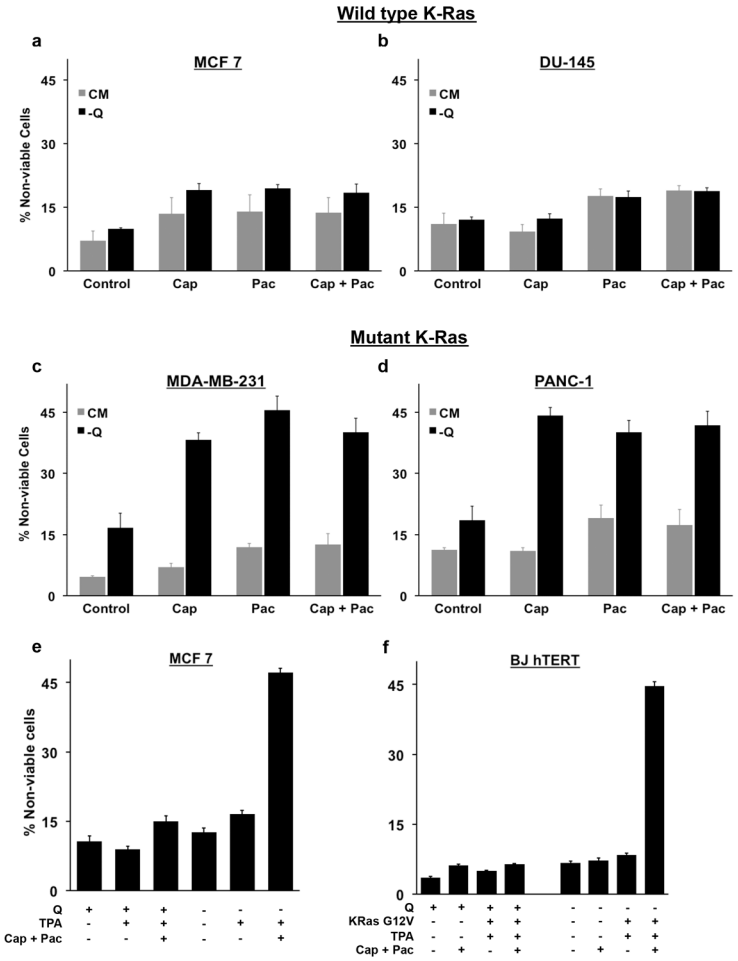

Figure 3.

Glutamine deprivation sensitizes K-Ras mutated cancer cells to phase-specific cytotoxic drugs. MCF7 (**a**), DU-145 (**b**), MDA-MB-231 (**c**), and Panc1 (**d**) cells were plated at 20% confluence in six-well plates in complete media (CM) 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 (Pac) or 1 μg/ml capecitabine (Cap) (both from Sigma-Aldrich) for 24 hr. Percent cell viability was determined using trypan blue dye exclusion assay as described previously.[41] MCF7 (**e**) and BJ-K-Ras (**f**) cells were placed in CM or medium lacking Q for 48 hr in the presence and absence of 100 nM TPA as indicated. After 48 hr, the cells were additionally treated with paclitaxel and capecitabine for 24 hr and cell viability was determined as in (**a-d**). Error bars represent the standard error of the mean for experiments repeated three times.

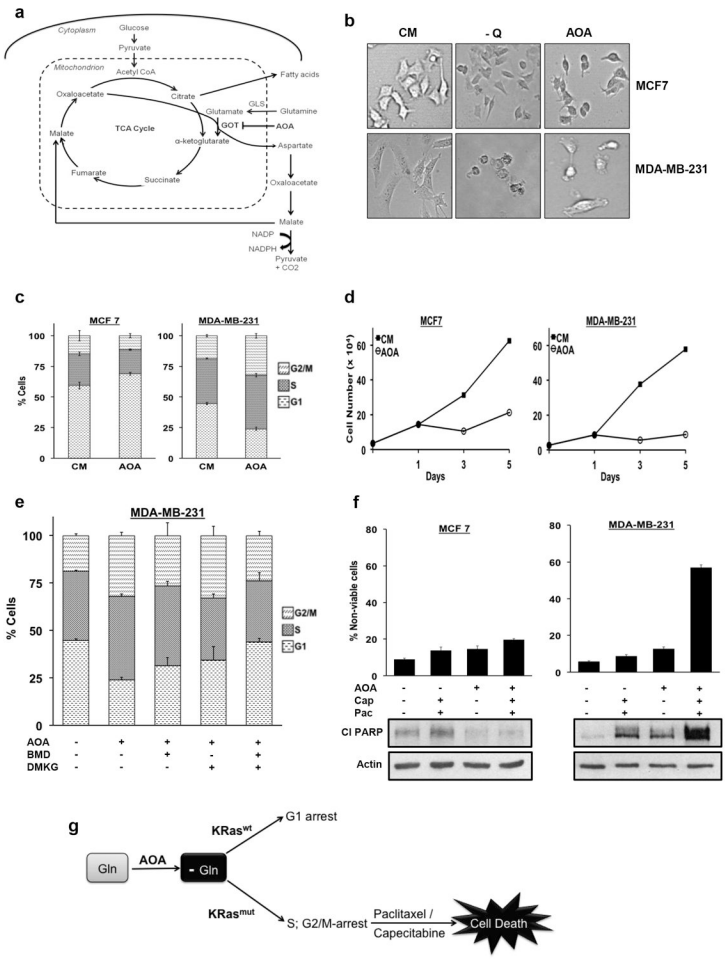

Figure 4.

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 via transamination catalyzed by GOT, which uses oxaloacetate as the amino group acceptor to generate aspartate. AOA inhibits GOT and therefore suppresses generation of α-ketoglutarate from Q-derived glutamate. Aspartate is critical for redox balance and NAPDH production and the generation of citrate for fatty acid synthesis. (**b**) Cells were plated at 20% confluence in 10-cm plates in complete medium (CM). After 24 hr, cells were shifted to CM, or medium lacking Q, or CM containing 0.5 mM AOA (C13408, Sigma-Aldrich) 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 1. In addition to AOA, the MDA-MB-231 cells were treated with cell permeable analogues of α-ketoglutarate (DMKG; 4 mM) (349631, Sigma-Aldrich) and aspartate (β-MD; 4 mM) (A8921, Sigma-Aldrich). Error bars represent standard error of mean for experiments repeated three times. (**d**) Cells were plated and treated as in (**b**), harvested at indicated time points, and scored after staining with crystal violet using light microscopy. Error bars represent the standard error of the mean for

experiments repeated three times. (**e**) MCF7 and MDA-MB-231 cells were plated as in (**b**) and shifted to CM or treated with 0.5 mM AOA for 48 hr. The cells were additionally treated with 50 nM Pac or 1 μg/ml Cap 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. (**f**) 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.