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Effects of RhoA and RhoC upon the sensitivity of prostate cancer cells to glutamine deprivation

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Abstract

RhoA and RhoC contribute to the regulation of glutamine metabolism, which is a crucial determinant of cell growth in some types of cancer. Here we investigated the participation of RhoA and RhoC in the response of prostate cancer cells to glutamine deprivation. We found that RhoA and RhoC activities were up- or downregulated by glutamine reduction in PC3 and LNCaP cells, which was concomitant to a reduction in cell number and proliferation. Stable overexpression of wild type RhoA or RhoC did not alter the sensitivity to glutamine deprivation. However, PC3 cells expressing dominant negative $RhoA^{N19}$ or $RhoC^{N19}$ mutants were more resistant to glutamine deprivation. Our results indicate that RhoA and RhoC activities could affect cancer treatments targeting the glutamine pathway.

Introduction

In cancer cells, a substantial amount of energy is produced by aerobic glycolysis and most of the incoming glucose is converted to lactate (Warburg effect) [1]. The lower rates of ATP generated by aerobic glycolysis are partially compensated by glutaminolysis. In this process, glutamine is converted to glutamate by glutaminase and further metabolized to α-ketoglutarate, which feeds into the tricarboxylic acid (TCA) cycle [2]. Glutamine also participates in the prevention of oxidative damage, and in the biosynthesis of nonessential amino acids, nucleotide and fatty acids [3]. Therefore, many tumor cells rely on glutamine to maximize their growth [4] and targeting the glutamine pathway is considered a promising strategy for treating cancer.

RhoA and RhoC are highly homologous GTPases (approximately 92% amino acid identity) that have each been implicated in glutamine metabolism [5-7]. RhoA has been better characterized than RhoC, however several studies have demonstrated that they present distinct functions in cancer. RhoA is frequently involved in cell cycle progression and migration, whereas RhoC is linked to metastasis and reduced cell survival [8, 9].

RhoA inhibition in an epithelial mammalian cell model was synthetic lethal with Myc, a master regulator of glutamine metabolism [7]. Inhibition of RhoA induced apoptosis in Myc-transformed cells, which was reversed by exogenous α-ketoglutarate treatment. In addition, Myc was unable to elevate glutamate levels when the Rho subfamily was inhibited with C3 transferase Rho inhibitor, suggesting that Myc is

dependent on the signaling mediated by Rho to supply the glutamine demands of transformation [7]. In NIH3T3 cells, the pharmacological inhibition of a specific splice variant of mitochondrial glutaminase (GLS1) was capable of reversing the transformation caused by the RhoGEF Dbl oncogenic protein [5]. Dbl has been shown to activate several Rho GTPases, including RhoA [10]. The colony forming ability of NIH3T3 cells transformed by overexpression of RhoC, Rac1 or Cdc42 was also blocked by GLS1 knockdown, indicating that inactivation of glutaminolysis was sufficient to prevent Rho GTPase-induced transformation [5]. Silencing of RhoC in inflammatory breast cancer cells markedly decreased glutamine uptake, without altering glucose uptake or lactate production. Regardless of the lower glutamine uptake, the cells remained dependent on glutamine. RhoC knockdown also increased glutamine synthetase, but did not alter GLS1 [6].

Despite these recent studies, the relative contributions of RhoA and RhoC in glutamine metabolism remain unclear. Here, we compare the roles of both proteins in glutamine dependency of prostate cancer cells by evaluating the effects of RhoA or RhoC activation and inactivation on the survival of prostate cancer cell lines cultured under glutamine deprivation.

Results

To measure the sensitivity of prostate cancer cells to glutamine deprivation, we evaluated the MTT staining (which measures cell metabolic activity and reflects in part the number of viable cells), number, proliferation, cell cycle, apoptosis and autophagy of PC3 and LNCaP cell lines cultured under different concentrations of glutamine for 72 hours. MTT staining of both cell lines was gradually reduced with decreasing glutamine, showing an approximately 40% decrease in the complete absence of glutamine ($p<0.05$) (Figure 1A-B). Glucose deprivation in the presence of normal glutamine concentration (300 mg/L) also decreased MTT staining (Figure 1C-D), indicating that glutamine is necessary though insufficient to maintain high cellular metabolic rates. PC3 and LNCaP cell number and relative proliferation decreased when cultured under an intermediate concentration (37.5 mg/mL) or absence of glutamine (Figure 1E-H). PC3 cell cycle is not statistically changed by the reduction or absence of glutamine (Figure 1I). In LNCaP cells, glutamine reduction induced an accumulation in S and G_2/M cell cycle phases, whereas the complete absence of glutamine decreased the

percentage of cells in G_2/M phases (Figure 1J). Surprisingly, a significant protective effect on apoptosis was observed by reduction or absence of glutamine in LNCaP cells but not PC3 cells (Figure 1K-L). Autophagy was not induced by glutamine reduction in either PC3 or LNCaP cells, as indicated by the ratio of LC3II/I (Figure M-N).

We next sought to investigate the activities of RhoA and RhoC in prostate cancer cells cultured under glutamine deprivation. PC3 cells cultured under reduction or absence of glutamine showed a trend of increase in RhoA and RhoC activities, compared to normal glutamine concentration (Figure 2A-B). In LNCaP cells, RhoA and RhoC activity showed a trend of a biphasic response to glutamine levels: it was increased at the intermediate dose and slightly reduced in the absence of glutamine when compared to normal glutamine concentration (Figure 2C-D).

We then investigated the effects of RhoA and RhoC on glutamine dependency by stably expressing GFP-tagged wild type and dominant negative mutants of RhoA ($RhoA^{N19}$) and RhoC ($RhoC^{N19}$) in PC3 cells. Transfection efficiency was confirmed by GFP detection using flow cytometry (Figure 3A) and western blotting (Figure 3B-C). More than 60% of GFP positive cells were obtained in all conditions, with the exception of $RhoC^{wt}$ expression, which resulted in a strong reduction in cell proliferation and consequent low transfection efficiency with approximately 10% of GFP-positive cells after sorting (Figure 3A). Cell morphology was visualized by fluorescence microscopy to detect GFP (Figure 3D) and cell area and circularity were analyzed. $RhoA^{wt}$ [median 405 (range 134 – 1757 μm^2)], $RhoA^{N19}$ [788 (164 – 1907 μm^2)], $RhoC^{wt}$ [930 (291 – 1652 μm^2)] and $RhoC^{N19}$ [547 (198 – 2104 μm^2)] expressing cells had decreased cell area in comparison with control cells (empty vector) [1072 (269 – 3365 μm^2)], all $P<0.05$ (Figure 3E). Circularity was decreased in $RhoA^{N19}$ [median 0.36 (range 0.06 – 0.94)] and increased in $RhoC^{N19}$ [0.72 (0.32 – 0.93)] expressing cells, compared to control [0.52 (0.12 – 0.84)], all $P<0.05$ (Figure 3F); ANOVA with Dunnett´s post-test. It was not possible to study the responses of $RhoC^{wt}$-expressing cells to glutamine deprivation due to the low transfection efficiency (data not shown). Expression of $RhoA^{wt}$ and $RhoA^{N19}$ decreased MTT cell labelling under normal glutamine concentration (300 mg/L). PC3 cells expressing $RhoA^{wt}$ were still sensitive to glutamine deprivation (Figure 3G). Conversely, PC3 cells expressing $RhoA^{N19}$ or $RhoC^{N19}$ were more resistant to glutamine deprivation, since their MTT labelling was not significantly affected by glutamine

withdrawal (Figure 3G-H). Apoptosis was not significantly altered by $RhoA^{wt}$, $RhoA^{N19}$ or $RhoC^{N19}$ in any of the tested conditions (Figure 3I-J).

Discussion

Reliance on glutamine varies substantially between different cancer cells. Some types of cells are highly sensitive to glutamine deprivation, while others do not require an exogenous source of this amino acid to survive [4]. Here we show that PC3 and LNCaP prostate cancer cells respond to glutamine reduction or deprivation mainly by decreased cell proliferation. However, the effects of glutamine deprivation on the cell cycle and apoptosis varied between these cell lines. PC3 and LNCaP cells were established from very different prostate tumors [11, 12] and diverge in their metastatic potential, gene expression profile and metabolism control[13, 14]. Therefore, our data are in accordance with other studies that showed that glutamine sensitivity is related to aggressiveness [15], modulation of glucose metabolism and the status of other genes [16].

It is interesting that PC3 cells and LNCaP cells show different patterns of RhoA/RhoC activity depending on glutamine levels. Whereas both RhoA and RhoC activity increased in PC3 cells in response to glutamine reduction or deprivation, in LNCaP cells, RhoA and RhoC activity was increased only under glutamine reduction but not deprivation. Rho GTPases play a part in G_1 progression in many cell types [17] and the biphasic response in RhoA/RhoC activity is similar to the decrease in G_1 cell cycle phase in LNCaP cells cultured in reduced glutamine levels. Thus, it is possible that increased RhoA/RhoC activity stimulates G_1/S phase transition in these cells. In addition, our previous study showed that PC3 cells are more responsive to the alteration of RhoA and RhoC pathways compared to LNCaP cells [18], which may also explain the difference induced by glutamine deprivation in RhoA/C activities between the cell lines.

Stable overexpression of wild type or dominant negative mutants of RhoA or RhoC produced substantial alterations in the morphology and MTT staining of PC3 cells even in normal glutamine concentration. Cell circularity was increased by $RhoA^{N19}$ and decreased by $RhoC^{N19}$, highlighting the different functions of these two highly homologous proteins. Distinct effects of RhoA and RhoC have been previously described in the morphology of prostate cancer cells [9, 19]. In addition, $RhoC^{wt}$ effects on the reduction of MTT staining were stronger than $RhoA^{wt}$. RhoA or RhoC have been described to increase cell growth [18, 20, 21]. However, their stable overexpression may also

lead to a decrease in cell growth caused by the profound changes in the cytoskeleton [22, 23], which is accordance with our results.

RhoA and RhoC can have both pro- and anti-oncogenic activities depending on the context [24-26]. In addition, RhoA gain and loss-of-function mutations have been identified in cancer patients [27, 28]. We observed that dominant negative mutant forms of RhoA or RhoC prevented the decrease in MTT staining induced by glutamine deprivation, implying that RhoA and RhoC promote growth inhibition under these conditions. Inflammatory breast cancer cells depleted for RhoC presented decreased glutamine uptake, but remained dependent on glutamine [6]. However, this is the first time that $RhoA^{N19}$ and $RhoC^{N19}$ effects on glutamine sensitivity are evaluated. All together, these results indicate that determining RhoA/C activity in cancer cells is relevant when considering targeting the glutamine pathway therapeutically.

Further studies are important to elucidate the roles of RhoA and RhoC in glutamine metabolism. Our findings add new insights regarding the participation of these two proteins in the sensitivity of prostate cancer cells to glutamine deprivation.

Materials and Methods

Cell culture

The human prostate cancer cell lines LNCaP and PC3 were acquired from ATCC (American Type Culture Collection, Philadelphia, USA). Cells were cultured in RPMI medium (Gibco) with 10% FBS and maintained in a humidified incubator at 37°C and 5% CO_2. To evaluate the cellular response to glutamine deprivation, cells were grown in culture medium containing 10% FBS and different glutamine concentrations.

MTT assays and determining cell number

For Methylthiazoletetrazolium (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Sigma) staining, 9×10^3 cells per well were cultured in 96-well plates in RPMI with 10% FBS for 72 hours. After this period, 10 µL of 5 mg/mL MTT solution were added to each well and the cells were incubated at 37°C for 4 hours. The reaction was stopped by addition of 100 µl of 0.1 N HCl in isopropanol and the optical densities were measured with a spectrophotometer at 595 nm absorbance. Cell number was calculated by counting the cells cultured under different conditions using a Neubauer chamber.

CFSE cell labelling

Cell proliferation was evaluated by CellTrace™ CFSE Cell Proliferation Kit Protocol (Thermofisher) according manufacture instructions. In brief, 2×10^5 cells were staining with 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE) for 20 minutes in a 37°C water bath, washed and returned cell culture in the presence or absence glutamine. After 0 (initial time) and 72 (proliferation time) hours, CFSE fluorescence intensity analyzed in a FACSCalibur (BD Biosciences). The proliferation indexes were determined using the ModFit Software (BD Biosciences). Ten thousand events were acquired for each sample.

Cell cycle analysis

Cell cycle was evaluated by flow cytometry, as previously described [29]. Briefly, the cells were collected, fixed in 70% ethanol and stored at 4°C for at least 4 hours. After washing in PBS, the samples were suspended in cell cycle buffer (0.1% Triton X-100, 10 mg/mL propidium iodide, 0.1 mg/mL RNase A) and incubated at 37°C for 30 minutes. Fluorescence was detected with a FACSCalibur (BD Biosciences) and ModFit Software (BD Biosciences) was used to determine the proportions of cells in each cell cycle phase. Ten thousand events were acquired for each sample.

Rho GTPase activity assays

RhoA, Cdc42 and RhoC activities were determined by affinity precipitation assay, as previously described [18]. Briefly, cell lysates (25mM Hepes, pH 7.5, 150mM NaCl, 1% Nonidet P-40, 10mM $MgCl_2$, 1mM EDTA, 25mM NaF, 1mM Na3VaO4, 10μg/mL aprotinin, 100μM PMSF, and 10% glycerol) were incubated with the GST-RBD at 4°C with rotation for 4 hours. After four washes, the pull down samples and total protein extracts were subjected to SDS-PAGE and western blotting analysis with specific antibodies.

Western blotting

Cells were lysed with protein extraction buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg Aprotinin, PMSF, 35 mg/mL, 10 mM Na3VO4, 100 mM NaF, 10 mM Na4P2O7 and 4 mM EDTA. The protein extracts were subjected to SDS-PAGE and western blotting analysis with specific primary antibodies: anti-LC3

(ab128025) from abcam, Anti-RhoA (2117S) and anti-RhoC (3430S) from Cell Signaling Technology and anti-GFP (sc-9996) from Santa Cruz Technologies. The ECLTM Western Blotting Analysis System kit (Amersham Pharmacia Biotech) was used for developing. Anti-actin (sc-1616) or anti-GAPDH (sc-32233) from Santa Cruz Biotechnology were used as loading control and band quantification was performed with UN-SCAN-IT gel densitometry software.

Stable transfection

GFP-fusions of wild type forms of RhoA ($RhoA^{wt}$) and RhoC ($RhoC^{wt}$) and dominant negative mutants of RhoA ($RhoA^{N19}$) and RhoC ($RhoC^{N19}$) (in EGFP) were transfected into PC3 cells. Briefly, the cells were transfected using jetPEI (Polyplus transfection), according to the manufacturer´s instructions. Cells expressing eGFP were used as control cells. Cells were then selected with 700µg/mL G418 (geneticin) for at least ten days then sorted for GFP using a FACsAria Fusion (Becton–Dickinson Biosciences).

Analysis of cellular morphology

Cell morphology was observed by fluorescence microscope through GFP detection. Image J software (National Institutes of Health) was used to measure the cell area and circularity. Circularity was calculated as previously described [30] and values ranged from 0.0 to 1.0, where a value of 1.0 designates a perfect circular shape and a value of 0.0 indicates an elongated polygon. At least 30 cells were analyzed for each condition.

Assessment of apoptosis

Apoptosis was determined with Annexin V Apoptosis Detection Kit (BD Biosciences), according to the manufacturer's instructions. All specimens were analyzed by a FACSCalibur (BD Biosciences) and at least ten thousand events were acquired for each sample.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). For comparisons, ANOVA test and Dunnett´s post-test were used. A P value <0.05 was considered as statistically significant.

Figure Legends

Figure 1. Effects of glutamine reduction on prostate cancer cell MTT staining, cell number, CFSE cell labelling, cell cycle, apoptosis and autophagy. PC3 and LNCaP cells were cultured for 72 hours under the indicated glutamine concentrations. (A-B) PC3 and LNCaP cell growth are decreased by reduced glutamine in the presence of glucose and (C-D) by reduced glucose in the presence of glutamine. (E-H) Decreased LNCaP and PC3 cell number and proliferation under reduced glutamine conditions. (I) PC3 cell cycle is not statistically changed by the reduction or absence of glutamine. (J) In LNCaP cells, reduced glutamine induces an accumulation in the S and G_2-M phases of the cell cycle, whereas the absence of glutamine leads to the reduction of G_2/M phases of the cell cycle. (K-L) The percentage of annexin V-positive cells was decreased upon glutamine reduction in LNCaP cells, but not in PC3 cells. (M-N) Glutamine reduction did not alter the ratio of LC-3II/LC3I in PC3 and LNCaP cells. Actin (42 kDa) was used to determine sample loading; the antibodies used for immunoblotting (IB) are indicated. Densitometry was performed and the ratio of target protein versus actin compared with the normalized value of control is shown. Analysis of variance (ANOVA) followed by a Dunnett´s test was used to determine significance. **P*<0.05, ***P*<0.01, ****P*<0.01. NS = not significant. Data shown are presented as mean ± standard deviation from at least four independent experiments.

Figure 2. Effects of glutamine reduction on the activities of RhoA and RhoC in PC3 and LNCaP cells. (A-B) RhoA and RhoC activities trend to increase by glutamine reduction or deprivation in PC3 cells. (C-D) LNCaP cells cultured under the intermediate glutamine concentration (37.5 mg/L) presented a trend of increase in RhoA and RhoC activities, whereas a trend of decrease in RhoA and RhoC activities was observed upon glutamine deprivation. (Upper panel) RhoA and RhoC activities were determined by pool down assays; actin (42 kDa) or GAPDH (37 kDa) were used to determine sample loading; the antibodies used for immunoblotting (IB) are indicated. (Lower panel) Bar graphs represent relative densitometry ratios of RhoA-GTP/RhoA (total) and RhoC-GTP/RhoC (total) of at least three independent experiments.

Figure 3. Effects of the overexpression of wild type and dominant negative mutant RhoA/C in PC3 and LNCaP cells cultured under graded reduction of glutamine.

(A) PC3 cells were stably transfected with wild type ($RhoA^{wt}$ or $RhoC^{wt}$) and dominant negative mutant RhoA/C ($RhoA^{N19}$ or $RhoC^{N19}$) fused to green fluorescent protein (GFP) and transfection efficiency was evaluated by flow cytometry. PC3 cells expressing eGFP were used as a control. (B-C) Transfection efficiency was also assessed by western blotting using anti-RhoA, anti-RhoC (upper blots) and anti-GFP antibodies (middle blots). Actin (42 kDa) or GAPDH (36 kDa) were used to determine sample loading. (D) Morphology of PC3 cells expressing $RhoA^{wt}$, $RhoC^{wt}$, $RhoA^{N19}$ and $RhoC^{N19}$ was observed by fluorescence microscopy through GFP detection. Scale bar = 50μm. (E-F) Area and circularity of PC3 cells were quantified with Image J Software. ANOVA followed by a Dunnett´s test was used to determine significance. *P<0.05, ***P<0.01. (G-H) Relative MTT staining of PC3 cells expressing $RhoA^{wt}$, $RhoA^{N19}$ and $RhoC^{N19}$. Expression of $RhoA^{wt}$ and $RhoA^{N19}$ decreased MTT staining under normal glutamine concentration (300mg/L) (both $^{***}P \leq 0.001$). Glutamine deprivation decreases the MTT staining in control cells (empty vector) and in $RhoA^{wt}$ expressing cells ($^{\#\#\#}P \leq 0.001$), but not in $RhoA^{N19}$ or $RhoC^{N19}$ expressing cells (NS=not significant). (I-J) Expression of $RhoA^{wt}$, $RhoA^{N19}$ and $RhoC^{N19}$ did not alter the apoptosis of PC3 cells cultured in the presence or absence of glutamine. ANOVA followed by a Bonferroni test was used to determine significance.

Disclosure of interest

The authors report no conflicts of interest.

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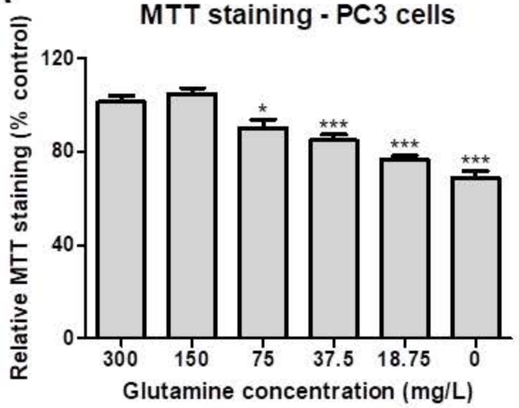

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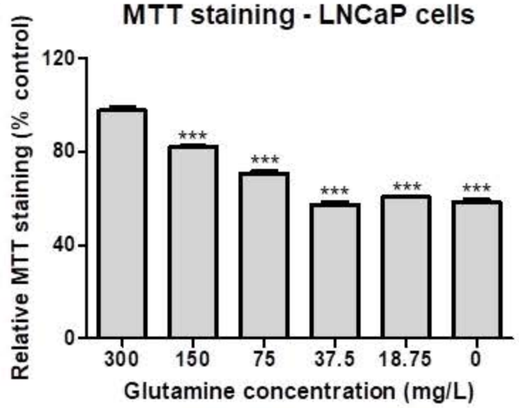

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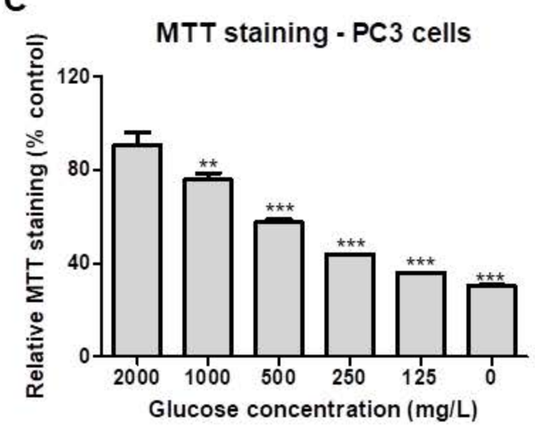

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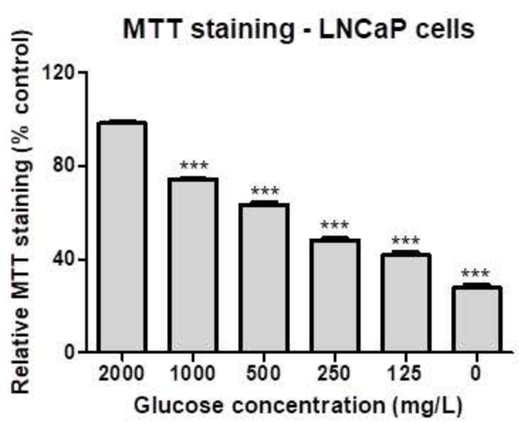

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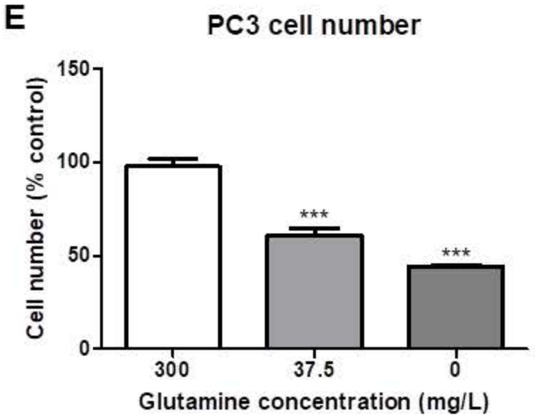

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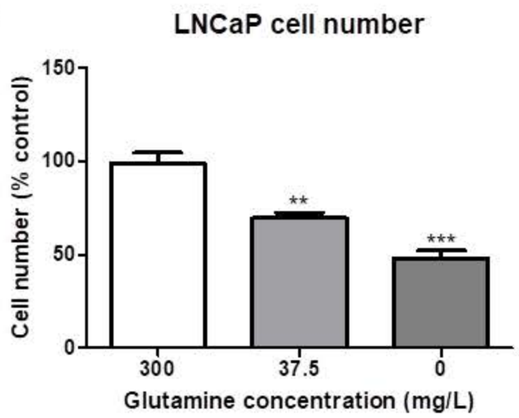
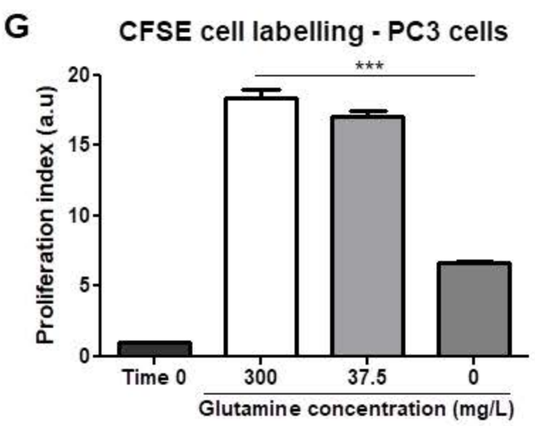
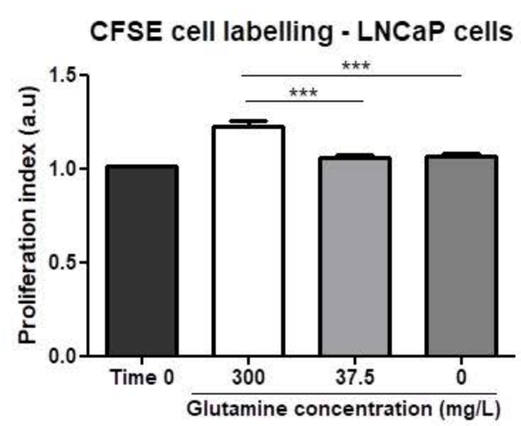


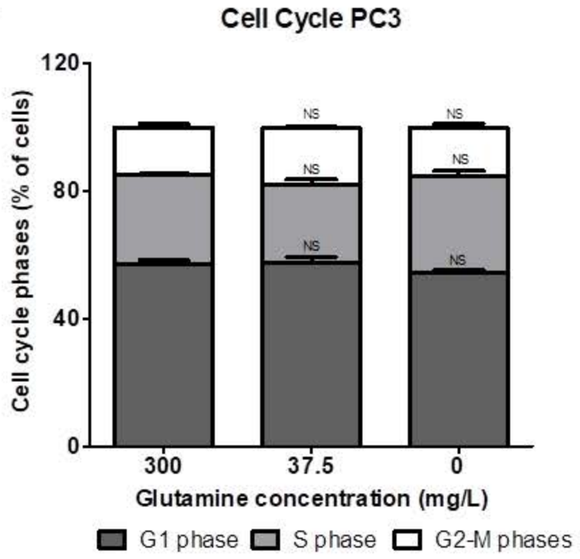
G



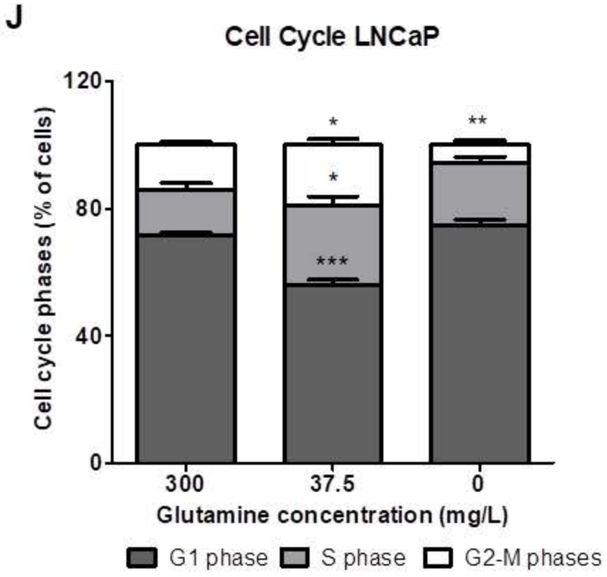
H



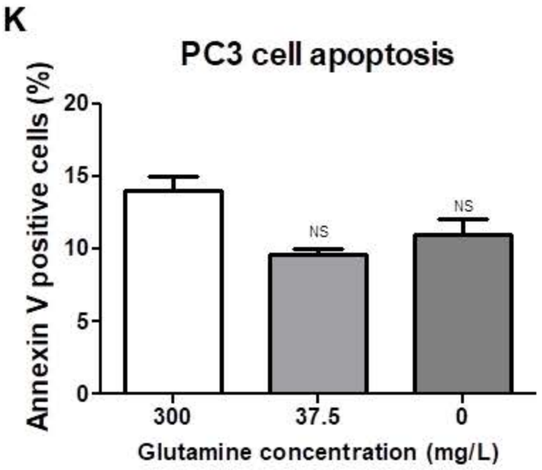
I



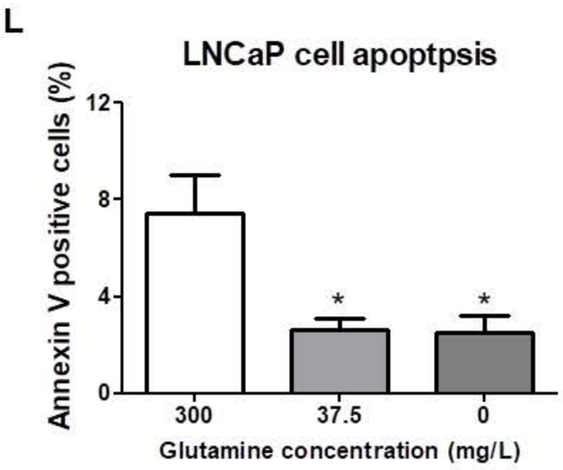
J



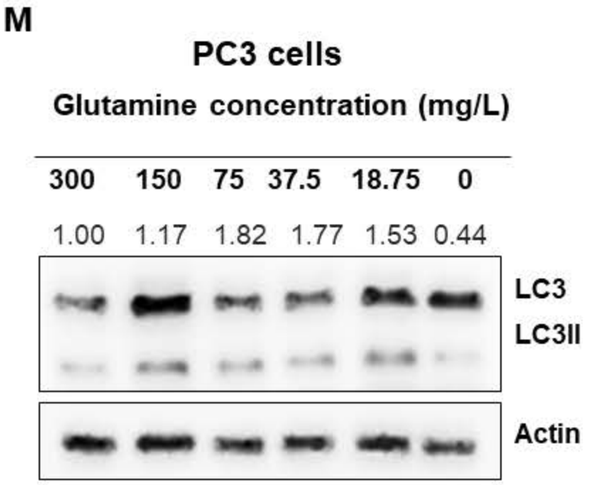
K



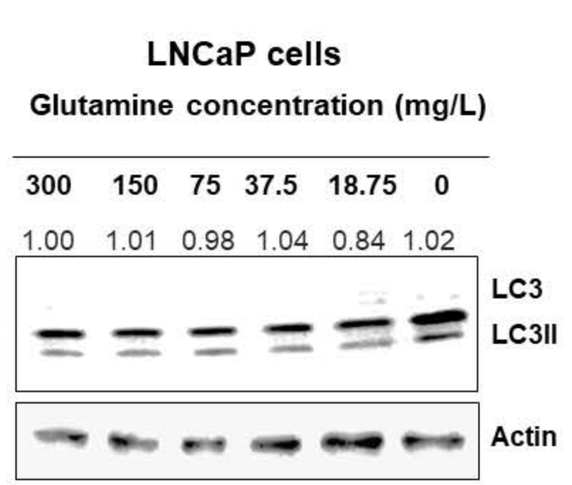
L

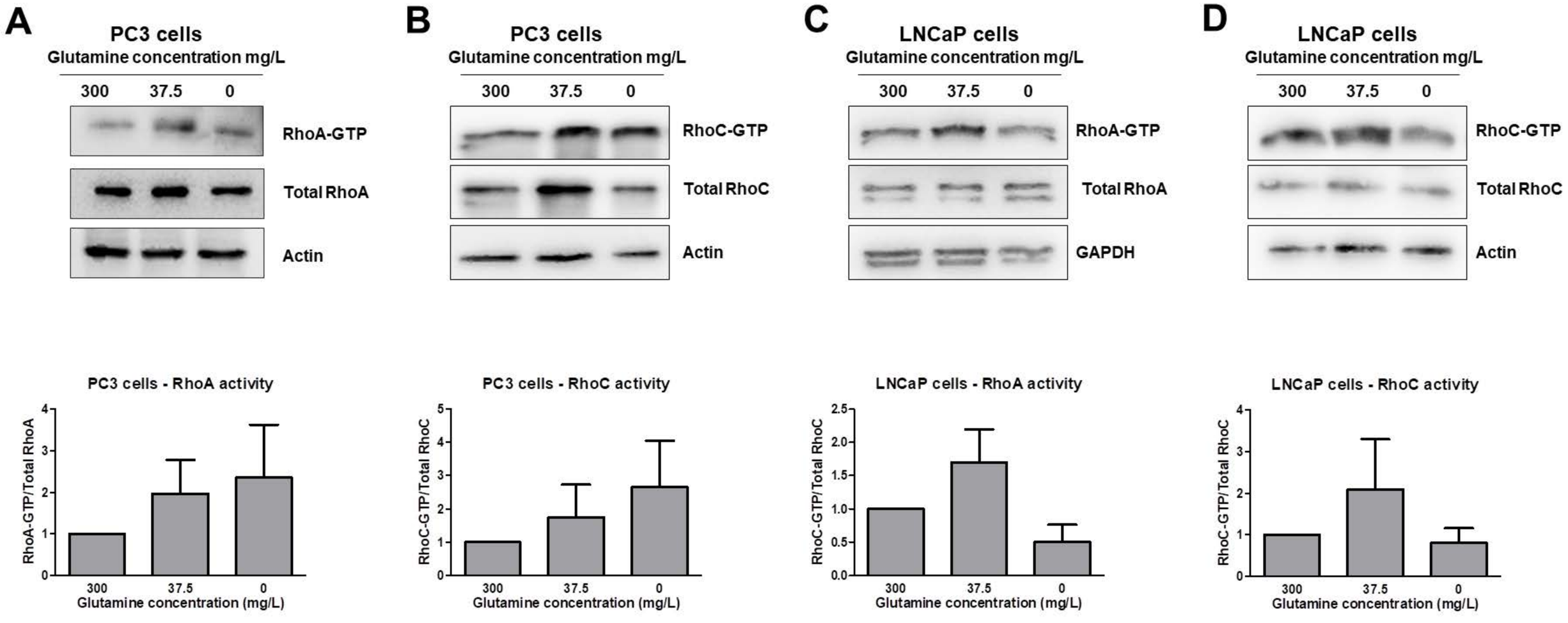


M

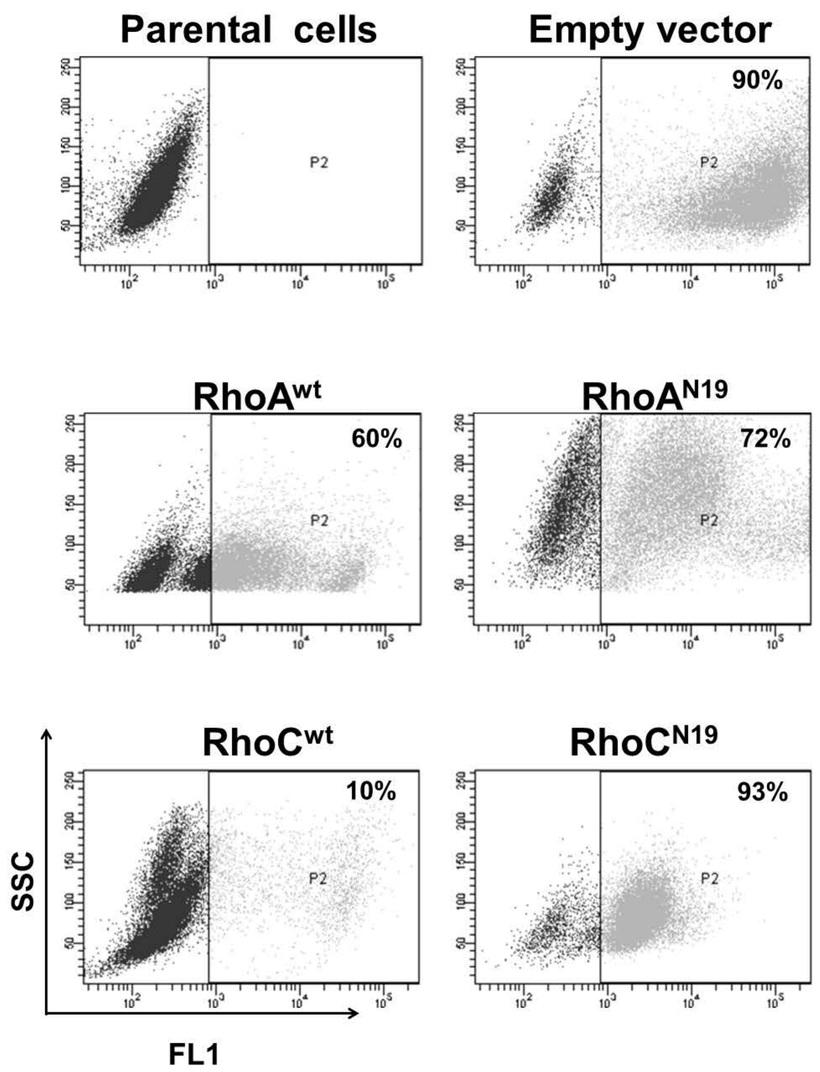


N

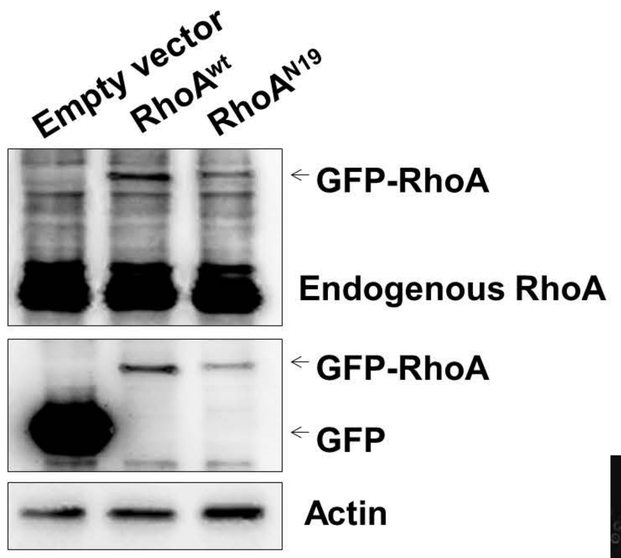



A
PC3 cells
Glutamine concentration mg/L
300
37.5
0
RhoA-GTP
Total RhoA
Actin
B
PC3 cells
Glutamine concentration mg/L
300
37.5
0
RhoC-GTP
Total RhoC
Actin
C
LNCaP cells
Glutamine concentration mg/L
300
37.5
0
RhoA-GTP
Total RhoA
GAPDH
D
LNCaP cells
Glutamine concentration mg/L
300
37.5
0
RhoC-GTP
Total RhoC
Actin
PC3 cells - RhoA activity
RhoA-GTP/Total RhoA
Glutamine concentration (mg/L)
PC3 cells - RhoC activity
RhoC-GTP/Total RhoC
Glutamine concentration (mg/L)
LNCaP cells - RhoA activity
RhoC-GTP/Total RhoC
Glutamine concentration (mg/L)
LNCaP cells - RhoC activity
RhoC-GTP/Total RhoC
Glutamine concentration (mg/L)

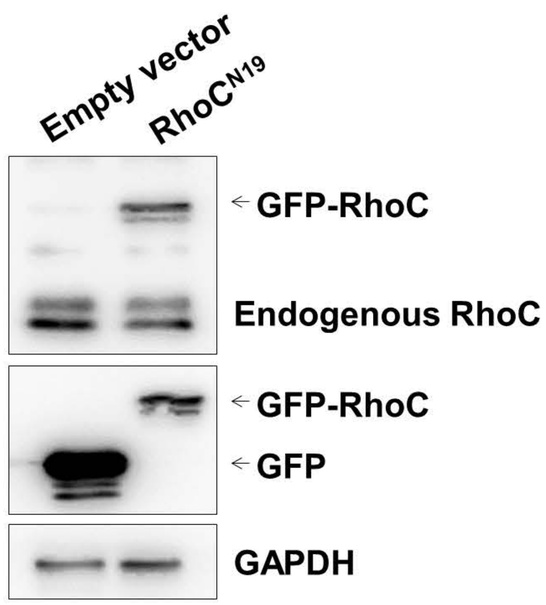
A


Parental cells
Empty vector
90%
RhoA^wt
60%
RhoA^N19
72%
RhoC^wt
10%
RhoC^N19
93%
P2
SSC
FL1

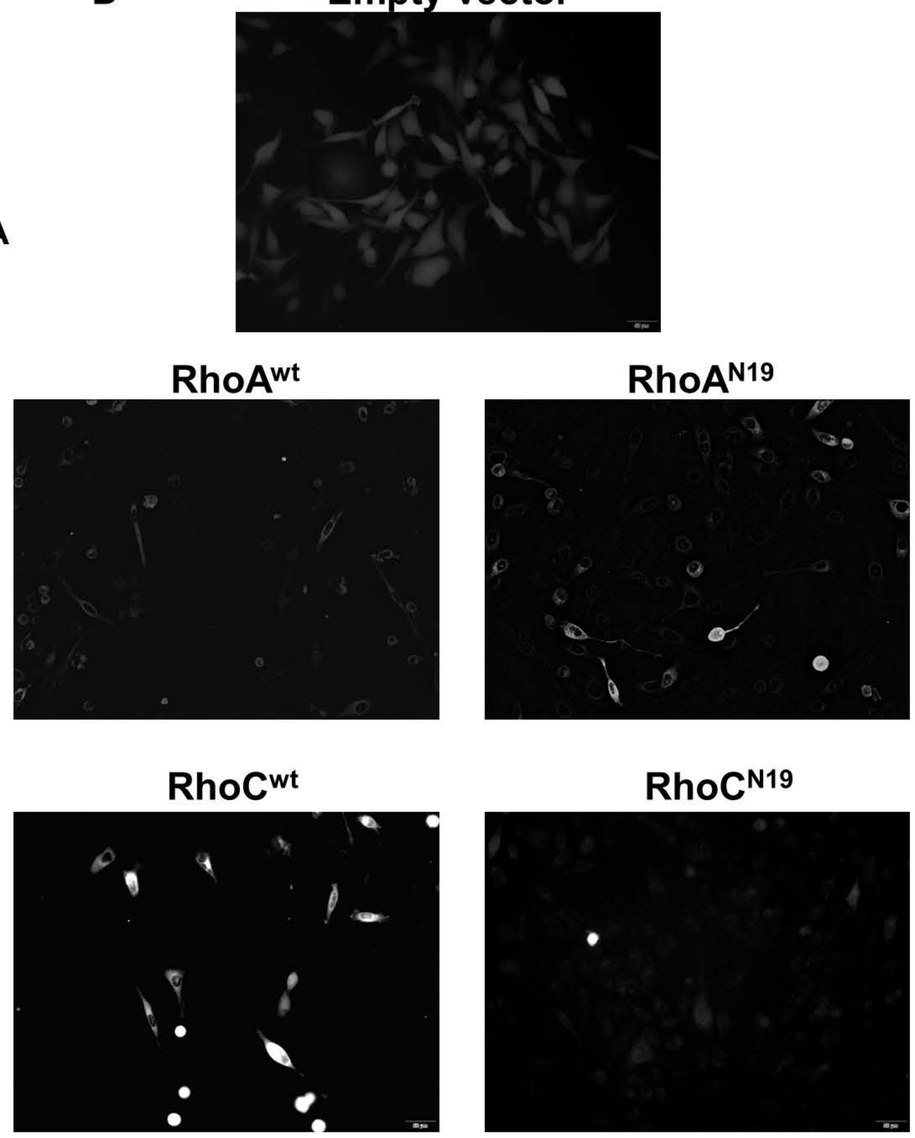
B


Empty vector
RhoA^wt
RhoA^N19
GFP-RhoA
Endogenous RhoA
GFP-RhoA
GFP
Actin

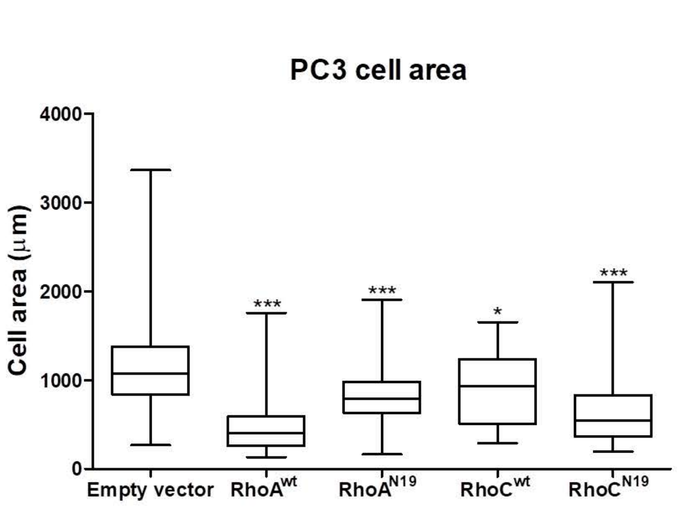
C


Empty vector
RhoC^N19
GFP-RhoC
Endogenous RhoC
GFP-RhoC
GFP
GAPDH

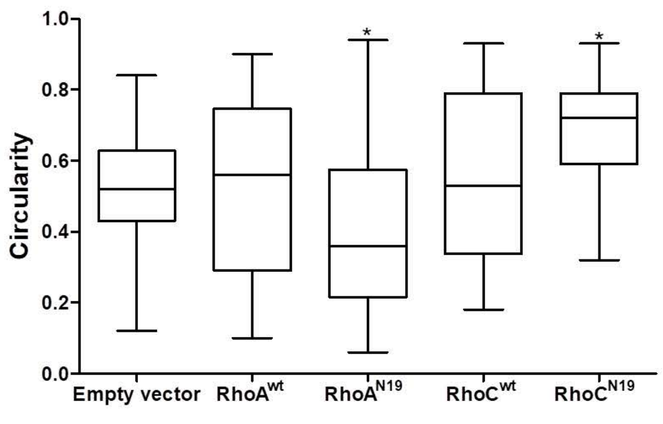
D


Empty vector
RhoA^wt
RhoA^N19
RhoC^wt
RhoC^N19

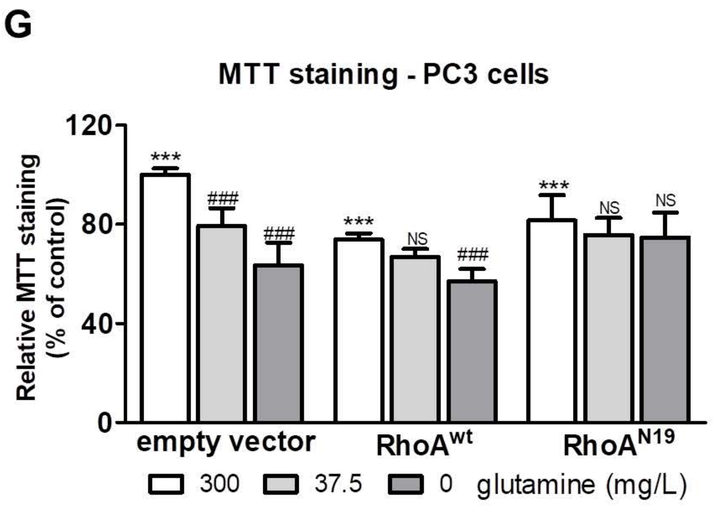
E


PC3 cell area
Cell area (μm)
Empty vector
RhoA^wt
RhoA^N19
RhoC^wt
RhoC^N19

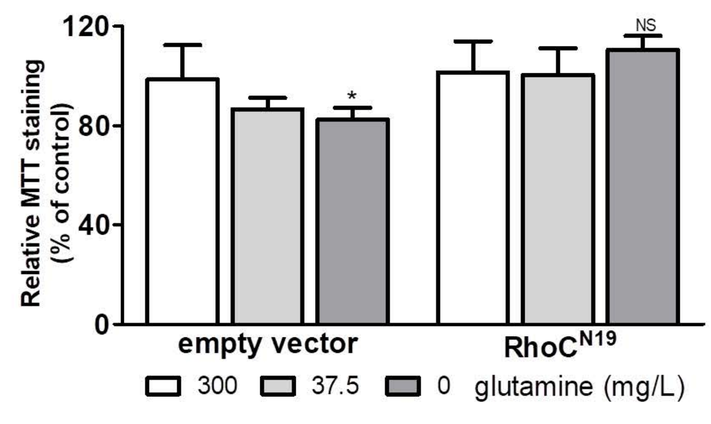
F


PC3 cell circularity
Circularity
Empty vector
RhoA^wt
RhoA^N19
RhoC^wt
RhoC^N19

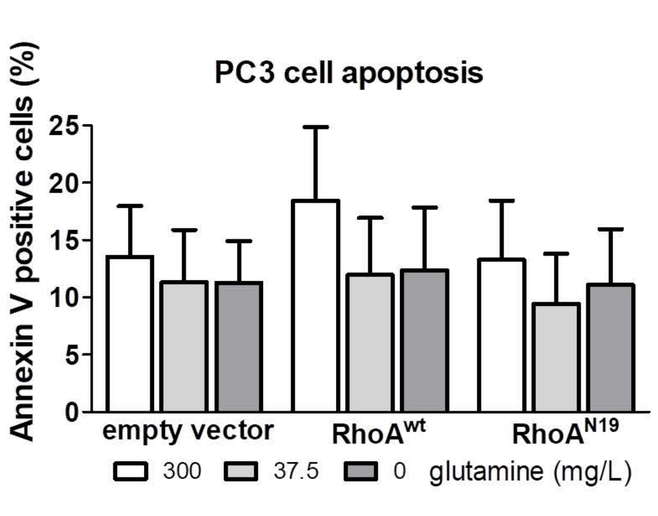
G


MTT staining - PC3 cells
Relative MTT staining (% of control)
empty vector
RhoA^wt
RhoA^N19
300
37.5
0
glutamine (mg/L)

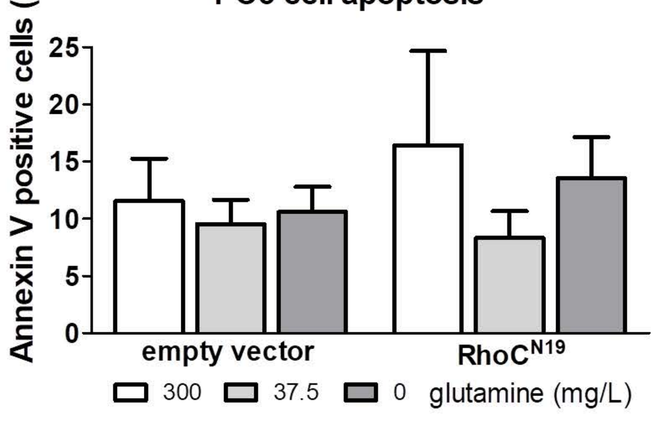
H


MTT staining - PC3 cells
Relative MTT staining (% of control)
empty vector
RhoC^N19
300
37.5
0
glutamine (mg/L)

I


PC3 cell apoptosis
Annexin V positive cells (%)
empty vector
RhoA^wt
RhoA^N19
300
37.5
0
glutamine (mg/L)

J


PC3 cell apoptosis
Annexin V positive cells (%)
empty vector
RhoC^N19
300
37.5
0
glutamine (mg/L)