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The ATPase activity of Reptin is required for its effects on tumor cell growth and viability in hepatocellular carcinoma

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Abstract

Reptin is overexpressed in most human hepatocellular carcinomas (HCC). Reptin is involved in chromatin remodeling, transcription regulation or supramolecular complexes assembly. Its silencing leads to growth arrest and apoptosis in cultured HCC cells and stops HCC progression in xenografts. Reptin has an ATPase activity linked to Walker A and B domains. It is unclear whether every Reptin function depends on its ATPase activity. Here, we expressed Walker B ATPase-dead mutants (D299N or E300G) in HCC cells in the presence of endogenous Reptin. Then we silenced endogenous Reptin and substituted it with siRNA-resistant wild type or Flag-Reptin mutants. There was a significant decrease in cell growth when expressing either mutant in the presence of endogenous Reptin, revealing a dominant negative effect of the ATPase dead mutants on HCC cell growth. Substitution of endogenous Reptin by wild type Flag-Reptin rescued cell growth of HuH7. On the other hand, substitution by Flag-Reptin D299N or E300G led to cell growth arrest. Similar results were seen with Hep3B cells. Reptin silencing in HuH7 cells led to an increased apoptotic cell death, which was prevented by wild type Flag-Reptin but not by the D299N mutant. These data show that Reptin functions relevant for cancer are dependent on its ATPase activity, and suggest that Reptin ATPase activity antagonists may be useful as anti-cancer agents.

Introduction

Reptin (RUVBL2, TIP48) is a member of the AAA+ (ATPases Associated with various cellular Activities) family (1). Reptin and its homolog and partner protein Pontin (RUVBL1, TIP49a) are essential for many cellular functions. They are involved in the remodeling of chromatin, the regulation of transcription, and in DNA damage sensing and repair (2, 3). They function as chaperones and are required for the assembly of ribonucleoprotein complexes such as telomerase (4) or snoRNPs (5), or, as part of the R2TP complex, for the assembly and/or stabilization of the RNA polymerase II complex (6) and of all members of the PIKK family including ATM, ATR, DNA-PKcs, SMG-1, TRRAP and mTOR (7).

We have previously demonstrated an overexpression of Reptin in hepatocellular carcinoma (HCC) (8) and shown that Reptin silencing reduced tumor cell growth and viability *in vitro* (8, 9), a finding confirmed by others in a number of cell lines of different origins (10-12). Remarkably, Reptin silencing *in vivo* led to regression of HCC tumor xenografts, in association with the induction of tumor cell senescence (9). These results, which are in agreement with the reported functions of Reptin, suggest that it could be an interesting target for cancer therapy. We have recently reported, as a proof of principle, that it is possible to inhibit the ATPase activity of the related protein Pontin with small molecules (13). The same applies to Reptin (Lestienne P, unpublished data) and it is tempting to speculate that drugs inhibiting Reptin ATPase activity could have anti-tumor effects.

The ATPase activity of AAA+ proteins relies on the presence of the conserved Walker A and B domains, responsible for ATP binding, and hydrolysis, respectively. Several studies have used mutants of these domains, designed according to the homology of Reptin and Pontin with the bacterial ATPase and DNA helicase RuvB (14), in order to probe the role of their ATPase activity. Studies in mammalian cells are scarce and have been mainly performed for Pontin.

Overexpression of the Walker B mutant Pontin D302N in the presence of endogenous Pontin thus inhibited cell transformation by several oncogenes such as E1A (15), c-myc (16), or β -catenin (17). Regarding Reptin, the Walker B mutant D299N was not able to complement the effect of the loss of endogenous Reptin on the amount of PIKK proteins in HEK293 cells (7), suggesting that the ATPase activity of Reptin is indeed required for the regulation of PIKK levels. However, the same mutant was as efficient as wild type Reptin for the repression of the influenza A virus polymerase (18) or of the transcriptional activity of ATF2 (19). In the latter case, the C-terminus part of Reptin lacking both Walker domains was almost as efficient as the complete protein. Altogether, these data suggest that some functions of Reptin are independent of its ATPase activity, and may rather depend on protein-protein interactions. Particularly, it remains to be demonstrated that the effects of Reptin underlying its role in tumor cell growth and viability are dependent on its ATPase activity.

Thus, in this study, we have used Reptin Walker B mutants to systematically probe the role of the ATPase activity on phenotypic properties of HCC cells relevant for carcinogenesis.

Material and methods

Cell culture and siRNA transfection

The human HCC cell lines HuH7 and Hep3B were grown in DMEM supplemented with 10% fetal calf serum in a 5% CO₂ atmosphere at 37°C. Cells were authenticated using short tandem repeat analysis and tested for mycoplasma contamination on a regular basis.

The siR2 siRNA targeting Reptin mRNA and the control siGL2 siRNA were previously described (8). They were bought from Eurofins MWG Operon (Ebersberg Germany) and

transfected by reverse transfection using Lipofectamine™ RNAiMAX (Invitrogen, Cergy Pontoise, France) at a final concentration of 16 nM.

Constructs and lentiviral transduction

The D299N mutant Flag-Reptin cDNA was a gift from M Cole (Lebanon, USA). The E300G mutant was constructed with the QuikChange site-directed mutagenesis kit from Agilent Technologies (Massy, France) and verified by sequencing. Mutants and wild type Flag-Reptin were inserted in the previously described lentiviral vector pRRLSIN-MND-MCS-WPRE (8). Titers were determined by the transduction of HEK 293T cells through the serial dilution of the lentiviral supernatant and were analyzed for p24 protein expression 5 days later.

Cell Proliferation

Cells were seeded at a density of 50,000 per well in 12-well plates. Adherent cells were counted at various times with a Coulter counter (Beckman Coulter, Villepinte, France) in triplicate wells.

Propidium iodide staining and flow cytometry

For cell cycle analysis, cells were collected by trypsinization and fixed in 70% ethanol at 4°C for a minimum of 30 minutes. After fixation, cells were washed with PBS, and the DNA was stained with propidium iodide at a final concentration of 0.05mg/ml in 0.1 % NaCitrate-0.2% Triton X-100. The samples were analyzed by flow cytometry using a BD LSRFortessa™ flow cytometer (BD Biosciences).

Caspase 3 activity assay

Cells were transduced as described above. After 6 days, 2. 10⁶ cells were solubilized in 100 µl of buffer and assayed with the caspase-3 colorimetric activity assay from Chemicon international (Millipore, Billerica, MA) according to the instructions from the manufacturer.

Western Blot and immunoprecipitation

Western blot was done as described (20). All blots were analyzed with the Odyssey system (Li-Cor Biosciences, Lincoln, NE). We used anti-Reptin mouse monoclonal (BD Biosciences, Pharmingen, Le Pont de Caix, France), anti-Pontin rabbit polyclonal (ProteinTech, Chicago, IL), anti-FLAG-M2 and anti- β -actin mouse monoclonal (Sigma-Aldrich, Saint-Quentin Fallavier, France) antibodies.

For immunoprecipitation, cells were washed in PBS. Lysis buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 1mM NaVO₃, 100mM NaF) supplemented with protease inhibitor cocktail (Roche, Meylan, France) was added to the pellet. After 20 min of incubation of ice, pellet was vortexed and centrifuged at 15000 g for 5 min. Cell extracts were incubated overnight at 4°C with monoclonal anti-Flag M2 beads (Sigma-Aldrich, Saint-Quentin Fallavier, France). The beads were washed 5 times with lysis buffer and eluted with Laemmli sample buffer. Eluates were separated on a 10% SDS-PAGE for Western Blotting.

Senescence assay

Senescence-Associated Heterochromatin Foci (SAHF) (21) were visualized with DAPI staining (Sigma, 0.5 μ g/ml) and quantified by counting using a Zeiss Axioplan microscope. Representative pictures were obtained with a Leica SP5 scanning laser microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Results

Expression of a Reptin ATPase-dead mutant impairs HCC cell growth.

Following the observation that the D113N mutation in bacterial RuvB impairs ATP hydrolysis, it was shown that the homologous D299N point mutation in the Walker B domain of human Reptin led to the loss of the ATPase activity of Reptin-Pontin complexes (22). We thus used this point mutant as a tool to investigate the role of Reptin ATPase activity in HCC cells. We first made sure that this mutation did not affect key properties of Reptin, such as its subcellular localization and its ability to interact with Pontin. Nuclear and cytoplasmic repartition of wild type Flag-Reptin or Flag tagged mutants were examined by Western Blot after cell fractionation. As observed for wild type Flag-Reptin, the D299N and the E300G mutants were both expressed in the nucleus as well as in the cytoplasm (Supplementary Fig. S1). We also examined the capacity of the D299N mutant to interact with Pontin, which is the major protein partner of Reptin. As shown on Fig. 1A, Pontin was co-immunoprecipitated with both Flag-Reptin and Flag-Reptin D299N. Furthermore, Flag-Reptin D299N was also able to interact with endogenous Reptin as observed for wild type Flag-Reptin.

Altogether, these results show that the D299N mutation does not alter Reptin localisation or its interaction with Pontin. These experiments also showed that the D299N mutant was counter selected over time (in Fig. 1A, compare Input of wild type Flag-Reptin and D299N Flag-Reptin). For all following experiments, we thus carefully selected a time window where mutant and wild type Reptin were expressed at similar levels.

We previously showed that silencing of endogenous Reptin through RNA interference induced a decrease in growth of HCC cells (8, 9). With the aim of determining the role of Reptin ATPase activity in HCC cell growth, we first expressed the ATPase dead mutant D299N in the presence of endogenous Reptin, using lentiviral vectors. In agreement with our previous results showing a tight post-translational control of Reptin expression (20), this strategy did not allow for an overexpression of Flag-tagged Reptin, either wild type, or mutated. Indeed, the total amount of Reptin was similar between the 3 conditions, with Flag-tagged Reptin representing about 50% of

the total amount in transduced cells ([Fig. 1B and C](#)). Cells expressing wild type Flag-Reptin had a normal growth pattern as compared to non-transduced cells, emphasizing that the Flag tag did not have an impact on Reptin function. On the contrary, we observed a significant diminution of cell growth in HuH7 cells expressing the D299N Reptin mutant ([Fig. 1D](#)). This reveals a dominant negative effect of the ATPase dead mutant on HCC cell growth.

In order to determine the effects of Reptin ATPase dead mutant in the absence of endogenous Reptin, we used a substitution strategy as previously described (9). Briefly, we previously generated a Flag-tagged Reptin cDNA harbouring silent mutations that made the mRNA insensitive to the anti-Reptin siR2 siRNA. By directed mutagenesis, we then inserted the D299N point mutation in this sequence. HCC cells were first transduced with siR2-resistant Flag-Reptin (FRrR2) or Flag-Reptin D299N (FRD299NrR2) and then transfected with the siR2 siRNA. As shown on [Fig. 2](#), this led to the quantitative replacement of endogenous Reptin by resistant wild type or D299N mutant Flag-tagged Reptin, respectively ([Fig. 2A and B](#)). We previously showed that Reptin silencing led to a co-depletion of Pontin through a post-translational mechanism (20). Here we show that substitution of endogenous Reptin with siRNA-resistant Flag-tagged Reptin preserved Pontin expression, whether Reptin was wild type or D299N ([Fig. 2A](#)). When we carried out quantitative substitution of Reptin by wild type Flag-Reptin, cell growth of HuH7 was normal ([Fig 2C](#)). On the other hand, quantitative substitution of Reptin by Flag-Reptin D299N led to growth arrest in HuH7 cells from 3 days after transfection by siR2 ([Fig. 2C](#)). Similar effects were seen in the Hep3B cell line ([Supplementary Fig. S2](#)).

We then tested the effects of Reptin on cell cycle progression using propidium iodide staining and flow cytometry. The most striking effect was a large accumulation of cells in G2/M phase upon Reptin silencing, which was rescued by complementation with siRNA-resistant Flag-tagged Reptin, but not with the D299N mutant ([Supplementary Fig. S3](#)).

In order to rule out that our results were linked to the specific mutation used, we checked the effects of another point mutation in the Walker B domain of human Reptin. The mutation was

based on the yeast Reptin E297G ATPase dead mutant previously used to demonstrate the requirement of the Walker B domain for yeast viability (23). As with the D299N mutant, we were able to achieve a quantitative substitution of endogenous Reptin, and to preserve Pontin levels upon silencing of endogenous Reptin (Fig. 3A and B). As shown on Fig. 3C, the E300G Reptin mutant imposed a dominant negative effect on cell growth in the presence of endogenous Reptin. Furthermore, and as observed for the D299N mutant, quantitative substitution of endogenous Reptin by Flag-Reptin E300G led to cell growth arrest in HuH7.

Role of Reptin ATPase activity on HCC cells viability and senescence.

We previously showed that Reptin silencing resulted in increased apoptotic cell death (8). We thus examined whether an intact Reptin ATPase activity was required to prevent cells from apoptotic death. As a marker of apoptotic death, we quantified cells with less than 2n DNA using propidium iodide staining and flow cytometry. The increased cell death consequent to Reptin silencing was completely prevented in HuH7 cells expressing wild type Flag-Reptin resistant to the siRNA. Interestingly, the simple expression of Flag-Reptin D299N in the presence of endogenous Reptin led to a 2-fold increase in cell death as compared to non-transduced HuH7 (Fig. 4). Moreover, quantitative substitution of Reptin by the D299N mutant dramatically increased cell death. Similar results were obtained when cell death was assayed through the measurement of caspase 3 activity (Supplementary Fig. S4).

We have reported the induction of senescence following Reptin silencing in HCC cells (9). In order to test the requirement of Reptin ATPase activity for the prevention of senescence, we quantified senescence-associated heterochromatin foci (SAHF) as a marker (21). As expected, Reptin silencing induced a large increase in SAHF formation that was rescued when expressing the siRNA-resistant Reptin. However, silencing of endogenous Reptin in cells expressing one or the other mutant did not induce a significant increase in SAHF. Similar results were obtained at either 5 days (Fig. 5), or 6 days after transduction (Supplemental Figure S5).

Discussion

Previous studies have suggested that Reptin was an attractive target for cancer therapy. In this respect, antagonizing its ATPase activity appears as an attractive option and could be feasible since we have been recently able to discover small molecule antagonizing the ATPase activity of the Reptin homolog, Pontin (13). However, because it is uncertain that every Reptin function is dependent on its ATPase activity, we investigated in depth this key issue.

Here, we show that expression of two different Walker B mutants in the presence of endogenous Reptin, or substitution of endogenous Reptin by those mutants, had effects reminiscent of those evoked by Reptin silencing with respect to cell growth and viability. Identical effects were observed in two different HCC cell lines. Reptin mutants retained the same subcellular distribution as wild type Reptin, and also their ability to partner with Pontin, suggesting that they had no gross defects besides the loss of ATPase activity that could explain their phenotypic effects. We and others have previously shown that Reptin silencing induced the co-depletion of Pontin, via a post-translational mechanism (4, 7, 20). However, we show here that cells reconstituted with the Reptin Walker B mutants expressed normal levels of Pontin like those reconstituted with wild type siRNA-resistant Reptin (20), thus ruling out that some of the effects seen with the mutants might be due to the loss of Pontin. Although the issue is not completely settled, the most recent evidence suggests that human Reptin and Pontin are organized in heterohexamers (24-26). It is thus likely that, when expressed in the presence of endogenous Reptin and Pontin, Walker B mutants will replace wild type Reptin within hexamers and can thus exert a dominant negative effect. This was demonstrated in the case of bacterial RuvB where the D113N mutant (homologous to the D299N Reptin mutant) associated in heterohexamers with wild type RuvB (14). This resulted in a dose-dependent loss of ATPase

activity of the hexamers (14). Similarly, Puri et al. showed that the ATPase activity of Reptin-Pontin complexes was lost when a Reptin Walker B mutant was associated with wild type Pontin (22). In our experiments, immunoprecipitation of the mutants using the Flag epitope pulled down together endogenous Pontin and Reptin, a finding compatible with the incorporation of mutant Reptin in mixed heterohexamers.

Unexpectedly, cells expressing the D299N or the E300G mutant were apparently protected against senescence induced by silencing of endogenous Reptin almost as efficiently as cells expressing wild type siRNA-resistant Reptin. We propose that the mutants exhibit *per se* a toxic activity that drives cells towards death, preventing them to enter senescence. This is supported by our observations showing that mutant-expressing cells exhibit a larger reduction in growth (see Figs 2C and 3C) and a higher rate of death (Fig. 4) as compared to cells only silenced for Reptin. Thus, even though those cells exhibit somehow less senescence, the net effect of expressing the mutants is still reduced cell growth and increased cell death.

Altogether, our data show that the ATPase activity of Reptin is required for its effects on the growth and viability of HCC cells. Several known functions of Reptin may explain why its silencing would reduce cell growth and increase cell death. Indeed, together with Pontin, Reptin is involved in the assembly of telomerase and their silencing leads to a decreased telomerase activity (4, 9). It also controls the levels of all members of the phosphatidylinositol 3-kinase-related protein kinases family, notably mTOR, with Reptin silencing leading to decreased mTOR level and a consequent defect in signaling through S6 kinase (7).

Our data also lead us to conclude accordingly that antagonists of Reptin ATPase activity may be of benefit for the therapy of HCC. Besides HCC, an overexpression of Reptin has been demonstrated in several types of tumors (2, 11, 12, 27), and Reptin silencing also affects growth and viability of a variety of tumor cells (10-12). We thus propose that targeting Reptin ATPase activity may have broad applications in oncology.

Authors' Contributions

Conception and design: A. Grigoletto, J. Rosenbaum

Development of methodology: A. Grigoletto

Acquisition of data: V. Neaud, N. Allain-Courtois, A. Grigoletto

Analysis and interpretation of data: A. Grigoletto, P. Lestienne, J. Rosenbaum

Writing, review, and/or revision of manuscript: A. Grigoletto, J. Rosenbaum

Study supervision: J. Rosenbaum

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Figure legends

Figure 1: Expression of Flag-Reptin D299N and its effect on cell growth in HuH7 cells. A. Reptin D299N mutant interacts with Pontin and endogenous Reptin. HuH7 were left either non transduced (Ctl), or were transduced with Flag-Reptin or Flag-Reptin D299N lentiviral vectors. Twenty days after transduction, whole cell extracts were used for anti-Flag immunoprecipitation. Reptin and Pontin were detected by Western Blot. The lower amount of D299N Reptin as compared to wild type Reptin in the input is due to a counter selection of cells at the time of the experiments. B. HuH7 cells were transduced with lentiviral vectors coding Flag-Reptin or Flag-Reptin D299N, or left non-transduced (NT). Six days later, they were plated at an initial density of 50,000/well. Expression of wild type or D299N Flag-Reptin was controlled by Western Blot on whole cell extracts 9 days after lentiviral transduction. C. Quantification of endogenous Reptin and Flag-Reptin expression. The graph shows the mean \pm SD of 3 independent experiments. Total Reptin amount was not significantly changed in any condition. Note that Flag-Reptin D299N systematically migrates slightly slower than wild type Flag-Reptin. D. Adherent cells numbers were monitored at the indicated time points with a Coulter counter. (n=3; *** p<0.001 for day 4 and day 7 by Two-way ANOVA followed by Bonferroni test). Note that standard deviations are not always visible owing to their small size.

Figure 2: Substitution of endogenous Reptin with D299N mutant leads to HuH7 cell growth arrest. Seven days after lentiviral transduction by siR2-resistant Flag-Reptin (FRrR2) or siR2-resistant Flag-Reptin D299N (FRD299NrR2), HuH7 cells were transfected with a control (siGL2) or an anti-Reptin siRNA (siR2). A. Quantitative substitution of endogenous Reptin by Flag-Reptin or Flag-Reptin D299N was controlled by Western Blot on whole cell extracts 3 days after siRNA transfection. B. Quantification of endogenous Reptin and Flag-Reptin expression. The graph shows the mean \pm SD of 3 independent experiments. Total Reptin or Pontin amount

was not significantly changed in any condition. C. Adherent cells numbers were monitored at indicated time points with a Coulter counter. The graph shows the mean \pm SD of 3 independent experiments (n = 3; *** p<0.001 for day 3 and day 6 by Two-way ANOVA followed by Bonferroni test). Note that standard deviations are not always visible owing to their small size.

Figure 3: Effect of Reptin E300G mutant expression and endogenous Reptin replacement on HuH7 cell growth. Seven days after lentiviral transduction by siR2-resistant Flag-Reptin (FRrR2) or siR2-resistant Flag-Reptin E300G (FRE300GrR2), HuH7 were transfected with a control (siGL2) or anti-Reptin siRNA (siR2). A. Expression of Flag-Reptin (wild type or E300G) and quantitative substitution of Reptin was controlled by Western Blot on whole cell extracts 3 days after transfection. B. The graph shows the mean \pm SD of 3 independent experiments. Total Reptin or Pontin amount was not significantly changed in any condition except when endogenous Reptin was silenced without being replaced. C. Adherent cells numbers were monitored at indicated time points with a Coulter counter (n=3; *** p<0.0001 by Two-way ANOVA followed by Bonferroni test).

Figure 4: Measurement of cell death by propidium iodide staining. HuH7 non transduced or transduced by siR2-resistant Flag-Reptin (FRrR2) or Flag-Reptin D299N (FRD299NrR2) were transfected with control (siGL2) or anti-Reptin (siR2) siRNA. Five days after siRNA transfection, cells were stained with propidium iodide and the percentage of subG1 cells was quantified with flow cytometry. The upper panels show graphs from a representative experiment and the graph shows the mean of 3 experiments (*** p<0.0001 by One-way ANOVA followed by Bonferroni test).

Figure 5: Measurement of senescence by counting Senescence-Associated Heterochromatin Foci (SAHF). HuH7 non transduced (NT) or transduced by siR2-resistant Flag-Reptin (FRrR2) or

Flag-Reptin D299N (FRD299NrR2) were transfected with a control (siGL2) or an anti-Reptin (siR2) siRNA. Five days after siRNA transfection, cells were fixed and nuclei were stained with DAPI. The upper panel shows representative pictures for each condition obtained using confocal microscopy. The percentage of cells with SAHF was quantified by counting (n=4; *** p<0.0001 by One-way ANOVA followed by Bonferroni test).

Figure 1

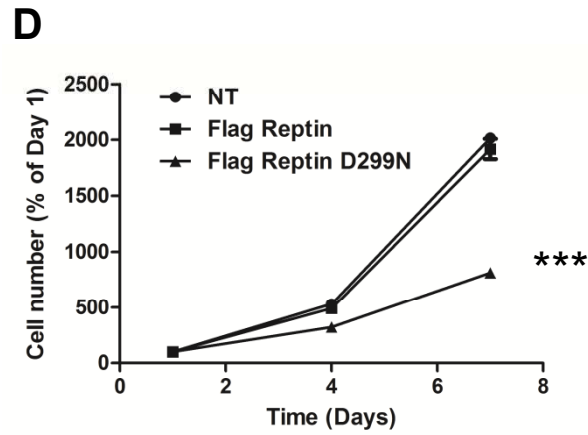
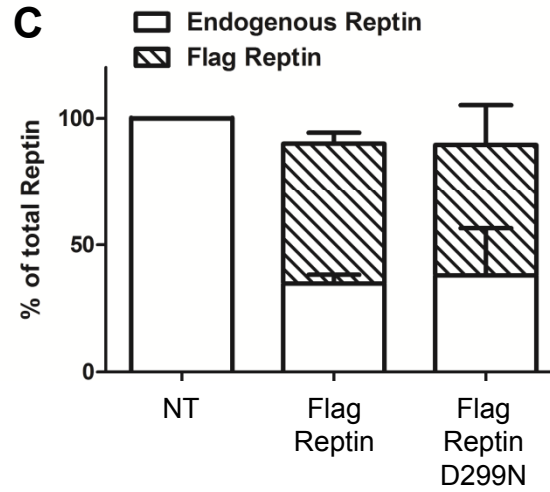
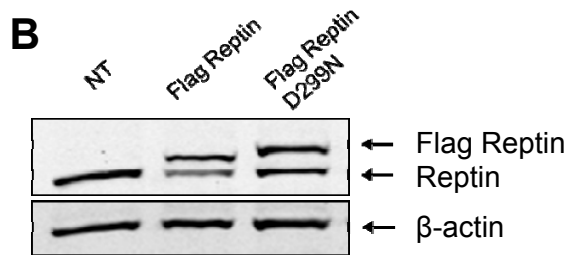
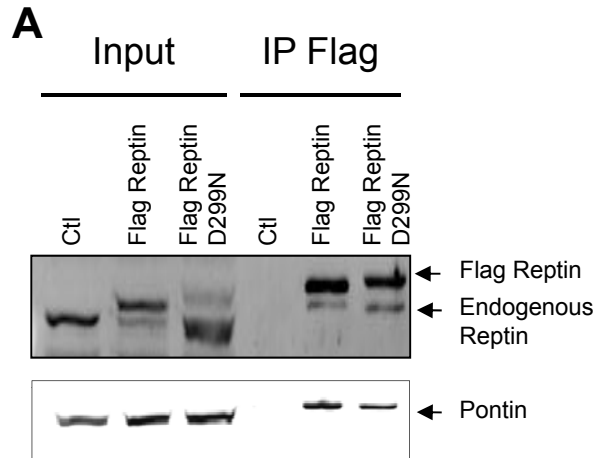
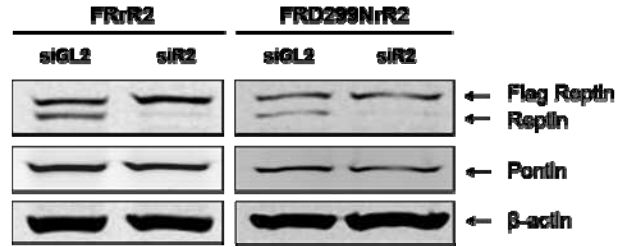
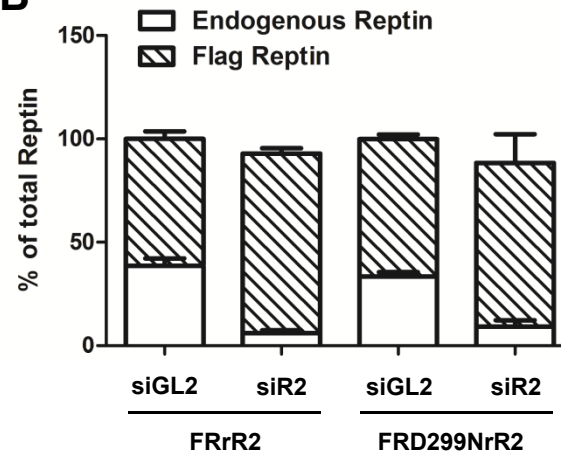


Figure 2

A



B



C

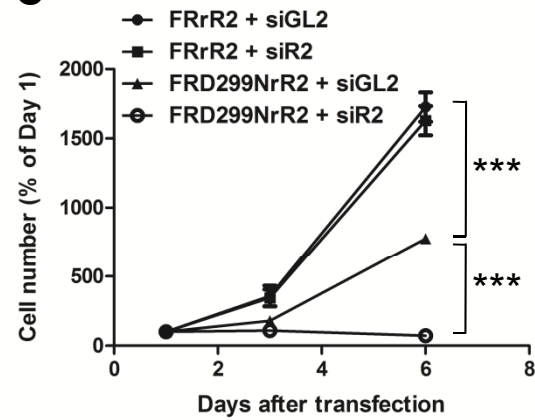


Figure 3

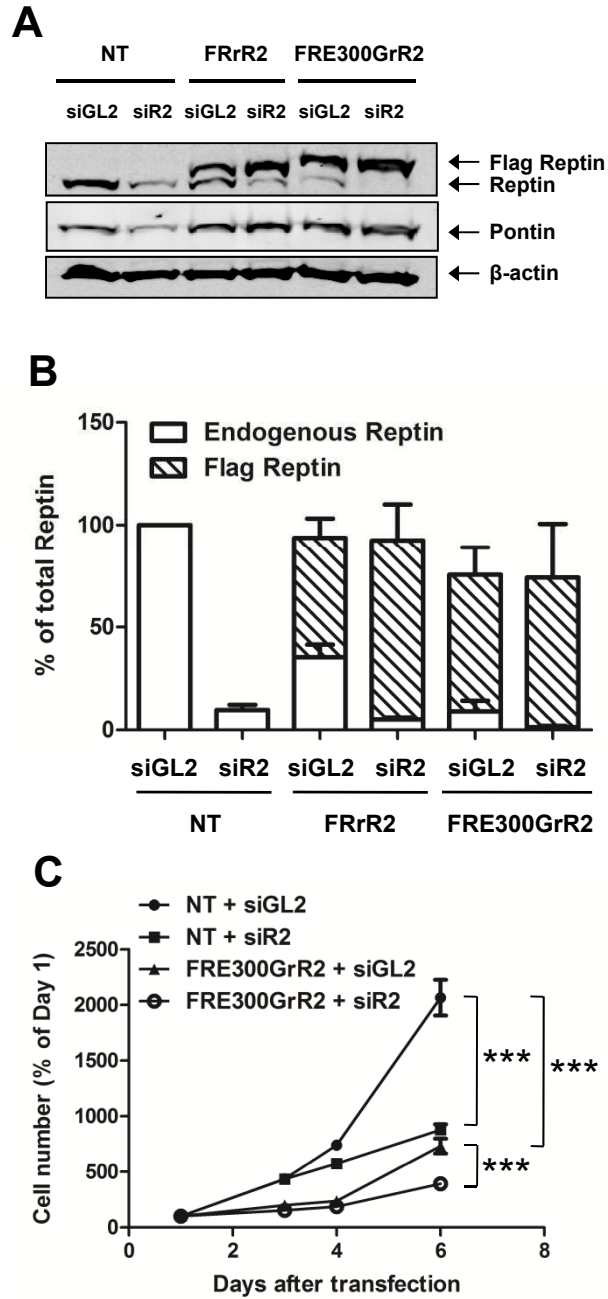


Figure 4

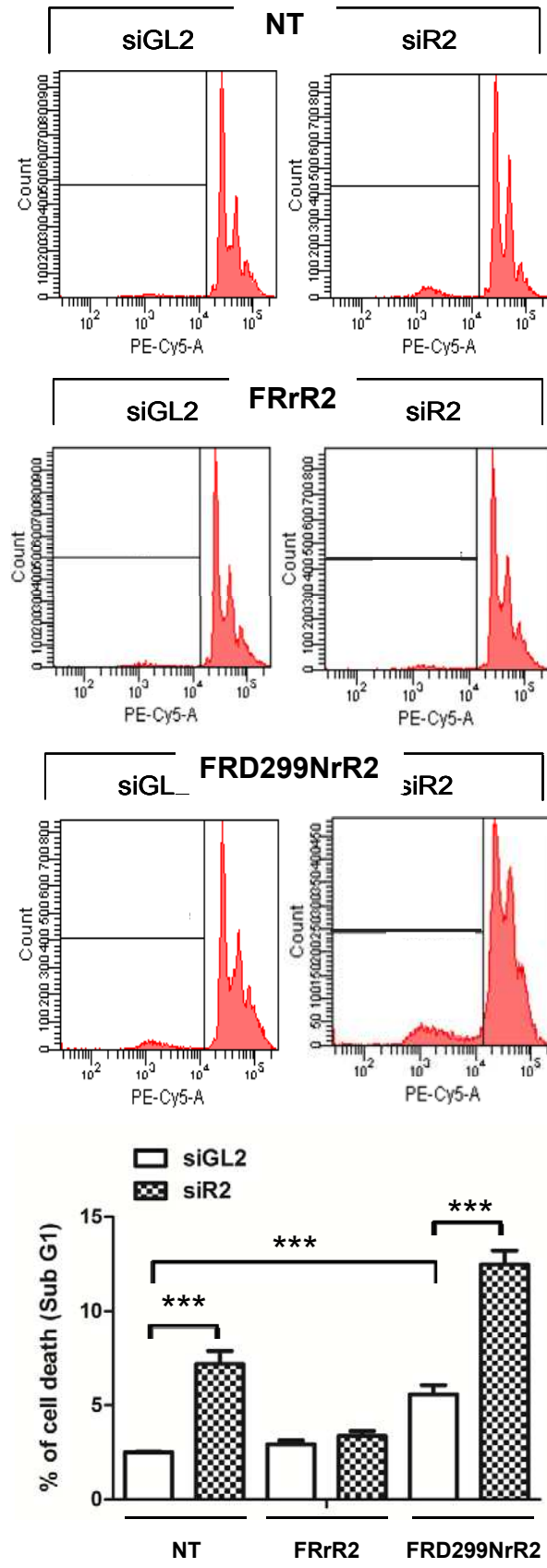


Figure 5

