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Abstract

Adrenocortical tumors (ACTs) frequently cause steroid excess and present cell-cycle dysregulation. cAMP/PKA signaling is involved in steroid synthesis and play a role in cell-cycle regulation. We investigated, by cell synchronization in the different phases of the cell-cycle, the control of steroidogenesis and the contribution of PKA in adrenocortical cells (H295R and culture of primary pigmented nodular adrenocortical disease cells). Cells showed increased steroidogenesis and a maximal PKA activity at G2 phase, and a reduction at G1 phase. *PRKACA* overexpression, or cAMP stimulation, enhanced PKA activity and induced steroidogenesis in all synchronized groups but is not sufficient to drive cell-cycle progression. *PRKAR1A* inactivation enhanced PKA activity and induced *STAR* gene expression, only in cells in G1, and triggered cell-cycle progression in all groups.

These findings provide evidence for a tight association between steroidogenesis and cell-cycle in ACTs. Moreover, *PRKAR1A* is essential for mediating the function of PKA activity on both steroidogenesis and cell-cycle progression in adrenocortical cells.

1. Introduction

Adrenocortical tumors (ACTs) are often associated with increased steroid secretion, which contributes to morbidity. This has been observed for benign tumors, such as those of primary pigmented nodular adrenocortical disease (PPNAD) (Espiard et al., 2014), macronodular hyperplasia (Louiset et al., 2013), adrenocortical adenomas (Bassett et al., 2005), and malignant adrenal cortical carcinoma (ACC) (Nakamura et al., 2015;Abiven et al., 2006). Even ACCs, which are classified as endocrine-inactive based on routine hormonal assessment, are often responsible for abnormal steroid secretion, as demonstrated by mass spectrometry analysis (Arlt et al., 2011). ACT cells also frequently display cell-cycle control defects (Ragazzon et al., 2011;Stratakis, 2009;Assie et al., 2014). Consistent with this observation, amplifications and deletions of genes encoding key regulators of the cell cycle (CDK4, CCNE1, RB1, CDKN2A) have been described in several studies and are more frequent in ACCs (Assie et al., 2014;De Martino et al., 2013;Ross et al., 2014;Pereira et al., 2018).

It is well established that the effects of steroid hormones, such as glucocorticoids, androgens, and estrogens, on cell proliferation and differentiation are mediated by the regulation of key events in the control of cell-cycle progression or arrest (Kullmann et al., 2013;Heisler et al., 1997;Amanatullah et al., 2002), but little is known concerning steroid-hormone secretion during the cell cycle in the adrenal gland. The adrenolytic drug mitotane (op'DDD), used in ACC treatment, decreases cortisol secretion and induces the destruction of the zona fasciculata and zona reticularis of the adrenal cortex (Cerquetti et al., 2008). Its use has been shown to be associated with cell-cycle arrest at the G2/M phase and apoptosis (Doghman et al., 2013;Hescot et al., 2013).

Genetic alterations leading to constitutive activation of the cAMP/PKA pathway in various types of ACTs, such as cortisol secreting adenomas (ACA) or PPNAD, result in steroid over-secretion (Lodish et al., 2016). The best examples are inactivating mutations of the regulatory subunit R1A of PKA (PRKAR1A), which is mutated in the germline DNA of patients with PPNAD and Carney complex or tumor DNA in a subset of secreting ACAs and ACCs (Kirschner et al., 2000;Bertherat et al., 2003;Zheng et al., 2016). In benign adrenal tumors associated with Cushing syndrome, *PRKACA* somatic mutations also result in constitutive PKA activity by disrupting the interaction between *PRKACA* and the regulatory subunits of PKA, including *PRKAR1A* (Beuschlein et al., 2014;Goh et al., 2014;Calebiro et al., 2014;Cao et al., 2014;Sato et al., 2014). Changes in cAMP signal transduction also play a role in regulating the cell cycle (Massimi et al., 2017;Gupta et al., 2017;Sun et al., 2009). Activation of the cAMP/PKA pathway in many cell types inhibits proliferation by preventing cells from entering the S phase and arresting cells in G1 (Duncan et al., 2006;Hewer et al., 2011). In contrast, stimulation of the cAMP/PKA pathway may facilitate cell proliferation, such as in thyroid and pituitary tumors (Medri et al., 1994;Spada et al., 1992).

Cyclic AMP exerts its actions primarily through the activation of protein kinase A (PKA). PKA is composed of two types of regulatory and four types of catalytic subunits. The PKA regulatory subunits have been studied for their role in tumor formation (Taylor et al., 2013). *In vivo* and *in vitro* studies have shown that inactivating *PRKAR1A* leads to tumorigenesis (Kirschner et al., 2005;Nadella et al., 2005). These studies showed that the lack of Prkar1a protein in primary mouse cells i*n vitro* causes constitutive PKA activation and immortalization of primary mouse embryonic fibroblasts (MEFs). At the molecular level, knocking out *Prkar1a* led to the upregulation of D-type cyclins (Kirschner et al., 2005). Similarly, we showed that inactivation of *PRKAR1A* confers resistance to apoptosis and enhances proliferation in the adrenocortical cell line H295R (Basso et al., 2014). *PRKAR1A*

inactivation disrupted the cell-cycle checkpoint and cells accumulated at the G2 phase with an increase in cyclin D and PKA CA activity. These results suggested that there may be a link between the increase in steroid secretion and cell-cycle dysregulation, which are concomitantly observed in adrenal tumors.

Here, we investigated the cell-cycle-dependent control of adrenal-steroid oversecretion and the contribution of cAMP/PKA in two cell lines synchronized by pharmacological drugs: H295R, a human adrenal cortex cell line derived from an ACC (Gazdar et al., 1990) that does not harbor any mutations in the cAMP/PKA pathway, and primary cultured PPNAD cells, with inactivating mutations of *PKAR1A*. We mimicked the constitutive activation of PKA that is observed in adrenal tumors with *PRKAR1A* inactivating (Kirschner et al., 2000;Bertherat et al., 2003) or *PRKACA* activating (Beuschlein et al., 2014;Calebiro et al., 2014;Weigand et al., 2017) mutations using either cAMP treatment, transient silencing of *PRKAR1A* by transfection with an siRNA, or overexpression of one of the catalytic subunits of PKA, concomitant with the pharmacological drugs.

Overall, our data show that the hypersecretion of steroids by the H295R ACC cell line and PPNAD is controlled by the phase of the cell cycle and that PKA activity plays a role in such coordination.

2. **Materials and methods**

2.1 Cell culture

Human H295R adrenocortical carcinoma cells, purchased from the ATCC, were grown as previously described (Bouizar et al., 2010;Ragazzon et al., 2009;Groussin et al., 2000), in DMEM Ham/F12 medium (Sigma, St. Quentin Fallavier France) supplemented with 50 units/ml penicillin, 50 mg/ml streptomycin, 2 mmol/L glutamine, 2% Ultroser G2 (Biosepra, Fremont, CA), and ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) (Sigma) at 37°C in an atmosphere of 5% carbon dioxide/95% air. Cells were allowed to attach for 24 h before use.

2.2 Primary cell culture of PPNAD

Human adrenals were obtained after informed consent from two CNC patients undergoing surgery for PPNAD. Adrenal tissue collection was approved by the ethics committee of Cochin Hospital. PPNAD adrenal tissues were obtained during surgery and immediately immersed in culture medium. Briefly, adrenocortical explants were enzymatically dispersed as previously described (Cazabat et al., 2014;Bram et al., 2016). Adrenocortical cells were cultured at 37°C in 5% CO_2 and allowed to attach for four days before use. Cells were incubated with various pharmacological inhibitors to synchronize the cell-cycle for 24 h with fresh DMEM medium, as for the H295R cell line.

2.3 Western blotting

Whole-cell protein lysates were obtained by extraction with RIPA lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% v/v Nonidet P-40 (NP40), anti-phosphatase, and anti-protease (Roche, Boulogne Billancourt, France) as previously described (Basso et al., 2014). Western blotting was performed with primary mouse antibodies for R1A (1/500, BD Laboratories, Rungis, France), R2B (1/1000), PKA catalytic subunits (1/1000)

(Santa Cruz Biotechnology, Nanterre, France), and Star (1/1000) (Cell Signaling, St Quentin en Yvelines, France).We used anti-□-actin (1/2500) (Sigma) or GAPDH antibodies (1/1000) (Santa Cruz Biotechnology) as loading controls. The antigen-antibody complexes were visualized with appropriate secondary antibodies (Santa Cruz Biotechnology) by chemiluminescence detection (ECL^{TM} kit, Thermo scientific, Saint Herblan France). The signals were digitized with the Syngene/Gene Tool analysis system. (Ozyme, Montigny Le Bretonneux, France)

2.4 Cell-cycle distribution

Cell cultures were harvested, rinsed with PBS, fixed in 500 µl 70% ethanol, and frozen (-20°C). Fixed cells were centrifuged, rinsed in PBS, suspended in 500 µl PBS containing 50 µg/ml propidium iodide (Sigma) and 100 µg/ml RNase A (Sigma), and examined by flow cytometry (FACscan, EPICS XL Coulter, Villepinte France). Data were analyzed using Multicycle software. All experiments were carried out three times. Each histogram was constructed from at least 10,000 cells (Rizk-Rabin et al., 2008).

2.5 Cell-cycle synchronization

We used three different inhibitors, each specific for a cell-cycle check point: L mimosine for G1 (Vackova et al., 2003), aphidicolin for G1/S (Sorscher et al., 1991), and nocodazole for G2 (Ho et al., 2001) (Sigma). The cell-cycle phases of H295R cells arrested at a specific point in the cell cycle were well defined in a previous report (Bouizar et al., 2010). Cells were cultured in 12-well plates at a density of 150 x 10^3 cells/well and then treated for 24 h with L-mimosine (400 µM) or aphidicolin (10 µM) or for 24 h or 48 h with nocodazole (10 µM). For cell-cycle release of the H295R cells, following 24 or 48 h of synchronization, cells were washed three times with PBS and cultured in medium without drugs for 6, 12, 24, 36, or 48 h. For PKA activation or inhibition, cells were transiently transfected with an siRNA for

PRKAR1A, the control scramble RNA (siS), *PKACA* (in the PCDNA3+ vector, a gift from Dr D. Calebiro), or the empty vector, or incubated with the PKA inhibitor PKI 14-22 (Calbiochem, Fontenay sous-Bois France) and cultured with the different drugs for 24 h.

2.6 Transfection and co-transfection

H295R cells cultured in six-well plates were transfected with 100 pmol siRNA *PRKAR1A* (UGAAUGGGCAACCAGUGUUdTdT), the siRNA negative control (siS) (CAGUCGCGUUUGCGACUGGdTdT), 500 ng *PKACA* or PCDNA3+, as empty vector, using the Effectene reagent (Promega, Charbonnières les Bains, France). Cells were analyzed 24 h after transfection. For luciferase assays, 24-well plates were used. Cells were co-transfected with the siRNAs or vectors and a luciferase reporter gene driven by Star promoters. Firefly and Renilla luciferase activities (Promega) were measured as previously described (Basso et al., 2014;Ragazzon et al., 2009).

2.7 Steroid measurement.

Cortisol and aldosterone levels in cell medium were measured by competitive immunoassays and revealed by chemiluminescence assays (Laboratory of Hormonology at Cochin Hospital, France).

2.8 Protein kinase A assay

The PepTag nonradioactive protein kinase assay kit (Promega) was used to measure PKA activity, according to the manufacturer's instructions, as described in (Rizk-Rabin et al., 2008)

2.9 Analysis of RNA by quantitative PCR

Total RNA, extracted from the cell lines, was treated with DNase and further purified using the RNeasy Mini kit and RNase-free DNase Set (Promega), according to the manufacturer's instructions. Purified RNA was reverse transcribed with high-capacity cDNA reverse

transcriptase (Applied Biosystems, Illkirch France) and expression levels of the target genes analyzed by quantitative PCR using a LightCycler Fast Start SyberGreen kit (Roche Diagnostics, Meylan France), according to the manufacturer's instructions. The PCR conditions for all target steroidogenic and cyclin genes are shown in Table 1. Relative quantification of target cDNA was determined by calculating the difference in cross-threshold (CT) values after normalization to PPIA (CYCLO) signals (DDCT method).

2.10 Statistical analysis

Data were analysed by ANOVA, Student's t-test, o regression analysis with Fisher's projected least significant difference or Scheffe's F test for comparison of the means (StatView 5.0, SAS Institute, Cary, NC, USA). Data are expressed as the means ± S.E.M. Values of $P = 0.05$ were considered significant. The number of experiments is indicated in each figure legend. Results of all data for which statistical analysis was performed represent the quantification of at least three duplicate experiments. Control conditions were set as 1. Significance was set at $p < 0.05$(*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

3.1 Cell-cycle synchronization of H295R and PPNAD cells

We synchronized the cells by treatment with L-mimosine, aphidicolin, or nocodazole, assessed by propidium iodide staining and cytometry analysis (Figure 1). We obtained similar results for both cell types. Asynchronous cells treated with vehicle (DMSO) displayed a typical profile, with a higher percentage of cells in G1 (Figure 1A I,II) . Treatment of the cells with L-mimosine resulted in the accumulation of cells in G1 and decreased the percentage of cells in G2 (Figure 1B I,II). Aphidicolin treatment resulted in the accumulation of cells in late G1, with fewer cells in S and G2 than for the control (Figure 1C I,II) as demonstrated in (Bouizar et al., 2010). Nocodazole treatment resulted in a decrease in the number of cells in G1 and the accumulation of cells in G2 (Figure1D I,II) as assessed in (Bouizar et al., 2010). All phases of the cell cycle were verified by analyzing the expression of various cyclins to distinguish between the different check points of the cell cycle (Supplemental Figure 1). Cyclin D1 mRNA levels increase substantially in cells blocked with L-mimosine and this increase serves as a marker of initiation of the G1 phase, the level of Cyclin E increases in Aphidicolin-treated cells and serves as a marker of late G1/S, and increases in cyclin B and D levels serve as markers of the G2 Phase (Pereira et al., 2018).

3.2 Steroidogenesis is higher in the G2 phase in synchronized H295R and PPNAD cells

H295R cells secrete both glucocorticoids and mineralocorticoids, whereas PPNAD secretes only glucocorticoids. The analysis of steroid secretion showed significantly greater cortisol secretion by both H295R and PPNAD cells at G2 (Figure 1D III, IV). In H295R cells, aldosterone secretion was also higher at G2 (Figure 1D III). In contrast, cortisol and aldosterone secretion was lower in H295R at G1 ($p < 0.01$), whereas there was no difference for PPNAD (Figure 1A III, IV). Finally, we observed no differences for either cell type at G1/S (Figure 1C III, IV).

We analyzed the impact of cell-cycle synchronization on the expression of genes responsible for steroidogenesis by real-time RT-PCR. Cells synchronized with nocodazole accumulated at G2 and showed a significant increase in mRNA levels for all studied steroidogenic enzyme genes, both in H295R and PPNAD, except for *CYP11A1* in H295R and *CYP17A* in PPNAD (Figure 2C I, II). In contrast, we only observed a significant increase in *CYP17A* ($p < 0.05$) and decrease in *CYP21A2* mRNA levels in H295R in cells synchronized in G1 (Figure 2B I), whereas we observed a decrease in *CYP11A1* and *CYP21A2* mRNA levels in PPNAD cells (Figure 2A II). H295R cells at the early G1/S transition also showed a significant decrease in *CYP11A1* mRNA levels (Figure 2B I). Star protein expression only increased in cells at G2 in H295R (Figure 2 III). The increase in Star protein levels at G2 correlated with an increase in mRNA expression in cultured the H295R and PPNAD cells (Supplemental Figure 2).

We verified that the changes in steroidogenesis during the cell cycle resulted from physiological regulation and were not due to side effects of the drugs by performing similar analyses on H295R cells that were released from synchronization and cultured in normal medium to follow their progression through the cell cycle from 0 to 6, 12, 24, 36, and 48 h after release. The release of cells synchronized at G1 (53.2% G1, 37% S, and 9.7% G2) by L-mimosine treatment, resulted in an increase in the percentage of cells in S at 12 h after release, followed by an increase in cells in G2 at 36 and 48 h (Figure 3A I). The expression of steroidogenic enzyme genes by RT/PCR at various times after release is presented as the fold change with respect to asynchronous cells at time 0, set to one. There was a progressive increase of *CYP11B1*, *CYP11B2*, *CYP11A1*, *NR5A1* (*SF1*), and *STAR* mRNA levels from 6 to 12 h, followed by a peak of expression after 36 h, concomitant with entry into G2 (Figure 3B I and C I).

The release of cells blocked in G2 (11.7% G1, 16.8% S, and 71.5% G2) with nocodazole (48 h of treatment) resulted in their progression through the cell cycle (Figure 3A, right). They then re-entered G2 phase from 36 to 48 h after release (Figure 3A II). There was a progressive decrease in *CYP11B2* mRNA levels from 0 to 24 h, coinciding with the decrease in the percentage of cells in G2, followed by a distinct increase from 36 h that reached the initial level observed at time 0 of the release (Figure 3B II). The expression of *CYP11B1* and *CYP11A1* mRNA levels did not change from 0 to 24 h and then increased (Figure 3B II). *NR5A1* (*SF1*) and *STAR* mRNA levels increased at 36 h (Figure 3C II).

These data confirm that steroidogenic enzyme mRNA levels change during cell-cycle progression.

3.3 PKA and MAP kinase activity in synchronized H295R cells

Cell-cycle progression is often associated with the modulation of PKA and MAP kinase activity. Thus, we next assessed PKA and MAP kinase activity in synchronized cells. ERK1/2 phosphorylation, reflecting MAP kinase pathway activity, was significantly higher in all synchronized groups than in asynchronous cells (Figure 4A-C I and Supplemental Figure 3). MEK phosphorylation was also significantly higher in cells in G2 (Figure 4C I). Moreover, there was significantly less PKA activity in cells in G1 (Figure 4A II), whereas it was significantly higher in cells in G2 (Figure 4C II) than in asynchronous cells.

Under the same conditions, PKA R1A protein levels were markedly lower in cells at G2 (Figure 4C III), whereas those of PKA R2B and PKA CA were not significantly altered in any of the studied groups (Figure 4A-C).

3.4 The effect of PKA activation on the control of the cell cycle and steroidogenesis in synchronized H295R cells

The overexpression of steroidogenic enzyme genes at G2 is associated with an increase in the level of PKA activity. We next investigated whether increased PKA activity effects the phases of the cell cycle and steroidogenesis in synchronized cells. We thus increased PKA activity by overexpressing *PRKACA* (Ca) or silencing *PRKAR1*A (Figure 5 I-II). PKA Ca was overexpressed in transfected cells of the asynchronous control and all synchronized groups. However, it was less highly overexpressed in the nocodazole group, for which the basal level was much higher than that of the control asynchronous cells (Supplemental Figure 4A). The expression of the PKA RI protein was reduced in all depleted *PRKAR1A* synchronized groups (Supplemental Figure 4 B). However, the magnitude of the increase in Ca protein levels and decrease in RIa protein levels depended on the phase of arrest.

Overexpression of *PRKACA (Ca)* for 24h in H295R cells had no effect on the cell cycle (Figure 5A I) but increased both the activity of PKA and a luciferase reporter construct driven by the *STAR* promoter (*STAR*-Luc) in all groups, independently of the phase of cell-cycle arrest (Figure 5B and C I). *STAR*-Luc activity was the highest in cells in G2 (Figure 5C I). Similarly, PKA activation by cAMP had no effect on the cell cycle (Supplemental Figure 5).

Transient depletion of *PRKAR1A* for 24 h was sufficient to increase the percentage/number of cells in G2 in the asynchronous group and decreased the percentage of cells in G1 relative to the control (siS, asynchronous cells; Figure 5A II). In L-mimosine treated cells, the invalidation of *PRKAR1A* shifted the cells toward the S and G2 phases and decreased the percentage of cells in G1 ($p \leq 0.02$ siR1A *vs* siS). Simultaneous transfection with siR1A and aphidicolin treatment for 24 h decreased the percentage/number of cells in G1 ($p < 0.001$ siR1A *vs* siS) and subsequently increased those in S ($p < 0.05$ siR1A *vs* siS). Simultaneous nocodazole treatment and R1A silencing increased the percentage of cells in S and decreased the percentage of those in G2 ($p \leq 0.05$) (Figure 5A II). As expected, *PRKAR1A* silencing

enhanced PKA activity in all groups (Figure 5B II). Under the same conditions, *PRKAR1A* silencing led to increased activity of the *STAR*-Luc reporter in the asynchronous group ($p < 0.05$) and L-mimosine treated cells ($p < 0.05$), whereas its activity remained unchanged in the aphidicolin and nocodazole-treated cells (Figure 5C II).

3.5 Effect of PKA inhibition on control of the cell cycle in synchronized H295R cells

We confirmed the above data with the pharmacological inhibition of PKA activity by treatment with the cell-permeable specific inhibitor Myr-PKI (14-22), which contains a PKA substrate consensus sequence with the serine replaced by alanine (Wiley et al., 1999). The major effect of PKA inhibition was observed in nocodazole treated cells (G2), for which there was a decrease in the percentage of cells in G2 ($p < 0.001$) and an increase in the percentage of those in S ($p < 0.02$) (Figure 6A). This effect was paralleled by a marked and significant decrease in *STAR*-Luc activity in the nocodazole-treated cells ($p < 0.001$) (Figure 6B). Overall, our results show that the PKA R1A subunit mediates the effect of PKA activity on cell-cycle progression and the activation of steroidogenesis, depending on the phase of the cell cycle.

4. Discussion.

Excess adrenal-steroid secretion can be explained by mutations of various key components of the cAMP pathway that are also likely to be involved in adrenal tumorigenesis (Bertherat et al., 2003;Beuschlein et al., 2014;Calebiro et al., 2014;Horvath et al., 2006;Horvath et al., 2008). Here, we demonstrate that steroidogenesis is modulated according to specific cell-cycle phases in both an established human adrenal cortical carcinoma cell line, H295R, and a primary cell culture of PPNAD (harboring *PKARIA* inactivating mutations) from patients with cortisol excess.

Major actors of steroidogenesis (NR5A, Star, and enzymes) were preferentially induced during G2 (Figures 1, 2 and 3), leading to peak steroidogenesis during the same phase.

It is well known that cAMP/PKA and the MAP kinase activity vary during the cell cycle and that MAP kinase activity is higher during the G2 phase in most cells (Philipova et al., 1998). However, in most cells, cAMP levels and PKA activity are low during G2 and mitosis and then become high in G1 (Bolte et al., 2003;Kurokawa et al., 1998;Yu et al., 2008). This variation is cell-type specific, as the elevation of intracellular cyclic AMP (cAMP) is considered to be a potent mitogenic signal for a number of cell types, including Swiss 3T3 cells, thyroid epithelial cells, and somatotroph cells of the anterior pituitary (Withers, 1997).

Here, we demonstrated that only the level of PKA activity varied notably according to the stage of the cell cycle in the adrenocortical cell line H295R. PKA activity was high in G2 (Figure 4C II) and lower in G1 (Figure 4A II). Thus, both PKA and MAP kinase were activated in G2 (Figure 4C, I, II). The change of PKA activity in H295R cells at G2 is a molecular mechanism in which the cAMP/PKA pathway is specifically involved in the control of mitosis, as it phosphorylates several substrates involved in its progression. The in vivo dynamics of PKA activity during mitosis was highlighted by the study of Vandame P et

al. (Vandame et al., 2014) using FRET-based biosensors in single living Hela cells. They showed that PKA activity is required for the maintenance of chromosome condensation and that it may also be required during mitosis, because it phosphorylates histone H1.4, resulting in the dissociation of this protein from the chromatin during mitosis. Moreover, Aurora A kinase, a key protein for the progression of mitosis, is also a target of PKA. The high PKA activity in G2 corresponded with a decrease in the level of the regulatory subunit R1A of PKA (Figure 4C III). One explanation for the decrease in the level of PKA RIA protein is that the increase in PKA activity at G2 liberates the PKA R1A protein from PKA CA, making it more accessible to degradation. Another possibility is the consequence of the association of PKA R1A with the cytoskeleton or microtubules through either low-affinity or highly dynamic interactions, as shown in BW1J hepatoma cells (Imaizumi-Scherrer et al., 2001).

Mutations of PKA signaling components (*GNAS*, *PRKAR1A*, *PRKACA*, and phosphodiesterase genes) in adrenal tumors activate the pathway (Nakajima et al., 2016;Mete et al., 2018;Drougat et al., 2015). Although these mutations are associated with the pathogenesis of benign tumors, we tested whether PKA may regulate the cell cycle and steroidogenesis in a coordinated fashion by activating PKA in the adrenocortical cell line H295R through overexpression of the PKA catalytic subunit alpha, stimulating the cells with Bu2cAMP, or silencing *PRKAR1A* in combination with cell synchronization. The responses of the cells to the activation of PKA were different depending on the PKA subunits used. *PRKACA* overexpression stimulated the activity of a PKA-dependent construct (*STAR*-Luc) in all groups but did not alter the phase of the cell cycle (Figure 5 A and C, I). The activation of PKA by wild type *PRKACA* overexpression may not mimic the effect of *PRKACA* mutations, as Bathon et al (Bathon et al., 2019) recently showed that transfection of HEK293 cells with various PRKACA mutant constructs for 48 h highly increased PKA activity over that of wild type levels and increased the phosphorylation of the specific PKA substrate histone H1.4,

involved in proliferation, relative to that by wild type *PRKACA*. Our result represents the early effect of PKA activation (24-h transfection of H295R) by wild type *PRKACA*, which indiscriminately activates substrates and not selected ones. Similarly, 24-h activation of PKA by Bu2 cAMP did not alter the cell cycle profile, neither of asynchronous nor synchronized cells. Our results are consistent with those of the study of Liu et al (Liu et al., 2004), in which the effect of Bu2cAMP on proliferation and apoptosis was not observed at an early time point, one to two days of incubation, but later, after three days to one week of incubation.

As expected and described by Basso *et al.* (Basso et al., 2014), inactivation of *PRKAR1A* in asynchronous cells for 24 h increased both PKA and *STAR*-Luc reporter activity (Figure 5B and C II) and led to the accumulation of the cells in G2 and a decrease in the number of cells in G1 (Figure 5A II). In synchronized cells, PKA activity increased after *PRKAR1A* depletion. The increase was greater in cells treated for G1 arrest than those treated for G1/S and G2 phase arrest. In contrast, the effects of the increase in PKA activity on steroidogenesis were restricted to the cells treated for G1 arrest. In addition, the depletion of *PRKAR1A* induced cell-cycle progression in all synchronized groups, as demonstrated by a significant decrease in the percentage/number of cells in G1 reflecting progression to the S and then G2 phases (Figure 5A II).

Moreover, PKA controlled progression of the cells through the cell cycle, as the inhibition of PKA activity by PKI slowed the progression of cells with a high level of PKA activation and those in G2 (Figure 6).

Thus, in our model, the essential and the early determinant of PKA activity as a regulator of cell-cycle progression is the level of PKA R1A. However, our findings on malignant adrenocortical cells that harbor different mutations (Tissier et al., 2005;Ragazzon et al., 2010)

might not apply to normal adrenocortical cells or benign adrenocortical tumors carrying alterations in PKA signaling (Bathon et al., 2019).

PKA R1A can also interact directly with other specific proteins and modulates a number of other signaling pathways. It has been demonstrated that PKA and PKA R1A are important for regulation of the meiotic spindle in mammalian oocytes, in which increased PKA activity is responsible for maintaining meiotic arrest. On the other hand, the knockdown of *PRKAR1A*, as expected, increased PKA activity but the meiotic arrest of mouse oocytes was not maintained (Duncan et al., 2006). PKA R1A is also associated with the second subunit of replication factor C (RFC40) and functions as a nuclear transporter for RFC40, causing G1 arrest of MCF7 cells (Gupte et al., 2005). In the absence of PKA R1A, RFC40 may remain in the cytoplasm and cells progress toward the G2 and M phases.

The role of PKA R1A on the control of cell-cycle progression can be explained by the spatial regulation of PKA activity by A-kinase anchor proteins (AKAPs). AKAPs serve as scaffold proteins to tether PKA in close proximity to its substrate, thereby locally regulating its activity and PKA subunit specificity (Pidoux et al., 2010;Day et al., 2011). Recently, R1A was shown to be associated with AKAPs, which form a complex with the ribosomal S6 protein kinase RSK1 kinase (Chaturvedi et al., 2009;Chaturvedi et al., 2006). *Prkar1a* silencing in mouse lung fibroblasts (B82L) decreased the nuclear accumulation of active RSK1 and increased its cytoplasmic localization. The resulting increase in active cytosolic RSK1 levels is accompanied by increased phosphorylation of its cytosolic substrate tuberous sclerosis complex 2 (TSC-2) and the anti-apoptotic factor BAD. As RSK1 shares common phosphorylation sites with PKA on certain substrates, such as CREB, BAD, and Nur77, part of the cell response in the absence of PKA R1A may not be due to increased PKA activity (Chaturvedi et al., 2009)

In conclusion, we have provided evidence of a tight association between the phase of the cell cycle and steroidogenesis in *two* adrenal tumors, *PPNAD and the malignant H295R cell line.* Enhancing PKA activity clearly appears to be critical for steroid excess, whereas the PKA R1A subunit is essential for triggering the cell cycle.

Declaration of interest: there is no conflict of interest that could be perceived to affect the impartiality of the research reported. All authors declare that there is no conflict of interest.

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Figure legends

Figure 1. The effect of cell-cycle synchronization of the H295R cell line and primary PPNAD cell culture for 24 h on the cell-cycle profile and steroid secretion.

Flowcytometry analysis of DNA content by propidium iodide incorporation in synchronized H295R cells and PPNAD primary culture for **A)** control cultures without treatment and cultures treated with **B)** L-mimosine, **C)** aphidicolin, or **D)** nocodazole. Histograms of cortisol and aldosterone content secreted into the cell medium after cell-cycle synchronization are shown to the right. The results are presented as the means ± SEM of three duplicate independent experiments for H295R and one representative example of two duplicate experiments for PPNAD. *$p < 0.05$, ** $p< 0.01$, ***$p < 0.001$.

Figure 2. The effect of cell synchronization on steroidogenic enzyme and Star protein expression, mRNA expression by RT/PCR of steroidogenic enzymes in H295R cells and PPNAD primary cultures in the **A)** G1, **B)** G1/S, and **C)** G2 phases of the cell cycle. Each studied steroidogneic enzyme is normalized to the corresponding results in the control asynchronous cells, which is set to one. For Star protein expression, the results are normalized against those of control cells and a histogram of the western blot in synchronized H295R cells and PPNAD primary cultures are shown on the right. The results are presented as the means ± SEM of three duplicate independent experiments for H295R and one representative example of two duplicate experiments for PPNAD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 3. Cell-cycle profile and the kinetics of steroidogenic enzyme expression after cell-cycle arrest and release of H295R cells. 0 h represents the profile at the end of synchronization after 24 h of treatment with L-mimosine (I) or 48 h with nocodazole (II). Each time point is normalized to asynchronous control cells at 0 h of release. **A)** Histogram showing the percentage of cells in each phase of the cell cycle at each time point, analyzed by

flow cytometry, showing the progression in the cell cycle. RT/PCR analysis of **B)** steroidogenic enzyme and **C)** SF-1 and Star mRNA levels for each time point. The results are presented as the means ± SEM of three duplicate independent experiments. The level of significance is indicated at the top of each figure.

Figure 4. Effect of the different drugs on cell signaling pathways and PKA subunit regulation in H295R cells. The effects are shown for **A)** L-mimosine, **B)** aphidicolin, and **C)** nocodazole. MAP Kinase activity is shown by histograms of western blots of pERK/total ERK, and pMEK/total MEK protein levels normalized to the control, set to one. We spliced the original full gel images of ERK and MEK, presented in Supplementary Figure 3, to re-order the samples in a more logical manner. PKA activity was measured by a nonradioactive PKA assay and is presented as the ratio of free PKA/total PKA. Protein levels of the PKA subunits R1A, R2B, and Ca in cells treated with the different drugs are shown by histograms of western blots. The results of the histograms are expressed as the percentage relative band density/the band density of GaPDH, normalized to the corresponding controls (arbitrarily set to one) in H295R-treated cells. The results are presented as the means ± SEM of three duplicate independent experiments. *$p < 0.05$, **$p < 0.01$.

Figure 5. Effect of PKA activation, by the over expression of *PRKACA* or *PRKAR1A* depletion, on the regulation of the cell cycle, PKA activity, and steroidogenic response (Star/luc-reporter gene activity) simultaneously with the synchronization of H295R cells. **A)** Cell-cycle distribution analyzed by flow cytometry. **B)** PKA activity measured by a non-radioactive assay and presented as the ratio of free phosphorylated PKA/total PKA. **C)** *STAR* reporter gene activity measured by Luciferase/Renilla. The results are presented as the means ± SEM of three duplicate independent experiments normalized to the control of each group,

757 set to one. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 for the over expression of
758 PKA CA.

759 Figure 6. Inhibition of PKA activity by the PKA inhibitor PKI (5 µM) throughout cell cycle
760 synchronization (24 h) showing that mostly the nocodazole-treated cells were affected. **A)**
761 Cell-cycle phase distribution. **B)** Star/luc reporter gene activity. The measurement of the PKA
762 activity is not presented because PKA activity was inhibited and thus no PKA
763 phosphorylation. The results are presented as the means ± SEM of three duplicate
764 independent experiments. * p < 0.05, **p < 0.01, ***p < 0.001.
765
766 Supplementary Figures:
767 Supplementary Figure 1. Distribution of cyclin mRNA expression in in H295R cells arrested
768 at various phases of the cell cycle. **A**) Arrested in early G1 by L-mimosine treatment. **B**)
769 Arrested in late G1/S by aphidicolin treatment. **C**) Arrested in G2 by nocodazole treatment.
770 The results are presented as the means ± SEM of three duplicate independent experiments
771 normalized to the control of each group, set to one. *p < 0.05, **p < 0.01, ***p < 0.001.

772 Supplementary Figure 2. Star mRNA expression by RT/PCR in H295R cells and PPNAD
773 primary cultures in the G1, G1/S, and G2 phases of the cell cycle. The results are presented as
774 the means ± SEM of three duplicate independent experiments for H295R and one
775 representative example of two duplicate experiments for PPNAD. *p < 0.05, **p < 0.01,***p
776 < 0.001.
777 Supplementary Figure 3. Image of the full western blot of MAPKinase cell signaling used for
778 Figure 4. **A**) Image of the full western blot**. B**) The corresponding Histogram, as in Figure 4.
779

Supplementary Figure 4. PKA CA and PKA R1A protein expression in simultaneous **A)** overexpression of wildtype *PRKACA or* **B)** depleted *PRKAR1A* and cell-cycle synchronization. Results are normalized to the control vector or sis, set to one. The comparison is inter group (Vector *vs* Ca or SiS *vs* siRI) for each group. The results are presented as the means ± SEM of three duplicate independent experiments normalized to the control of each group, set to one. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Figure 5. Effects of cAMP stimulation on cell-cycle synchronization. The distribution of the phases resulting from cell-cycle arrest were not affected by stimulation with the cAMP analogue (dibutyryl adenosine 3'-5' cyclic monophosphate). The measurement of PKA activity and Star Luc reporter gene activity are not presented because they are equally fullyl saturated in all of the synchronized groups.

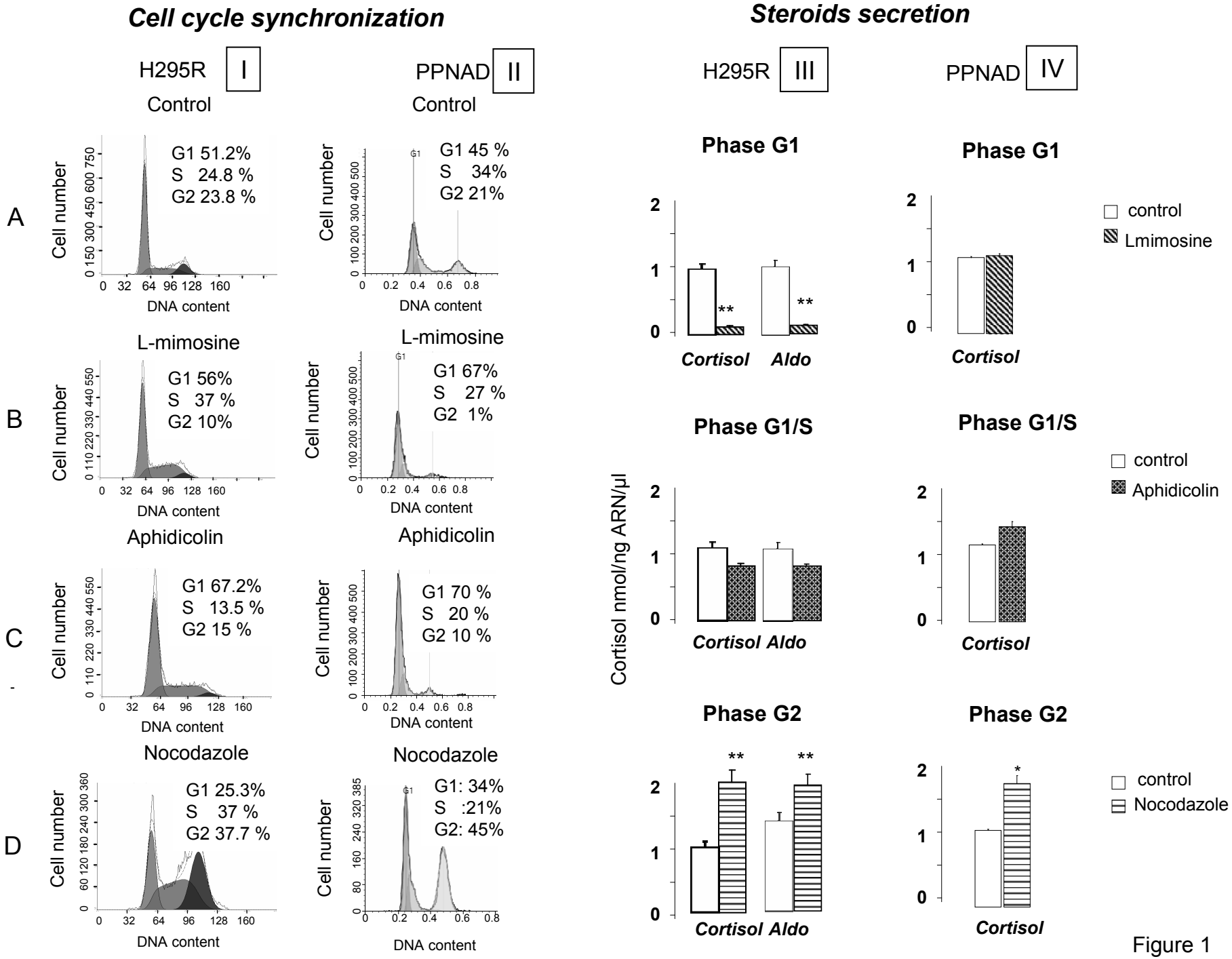


Figure 1

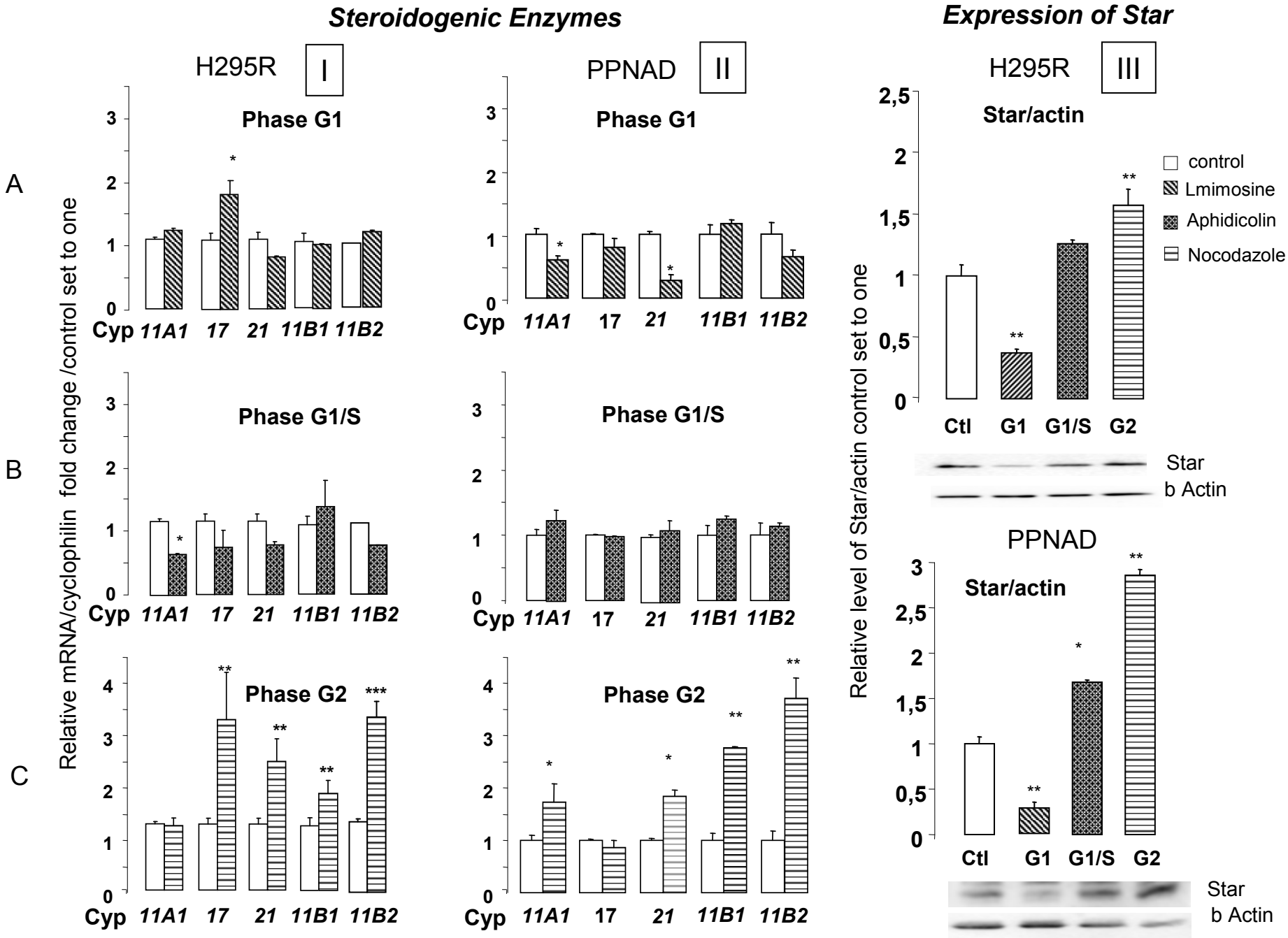


Figure 2

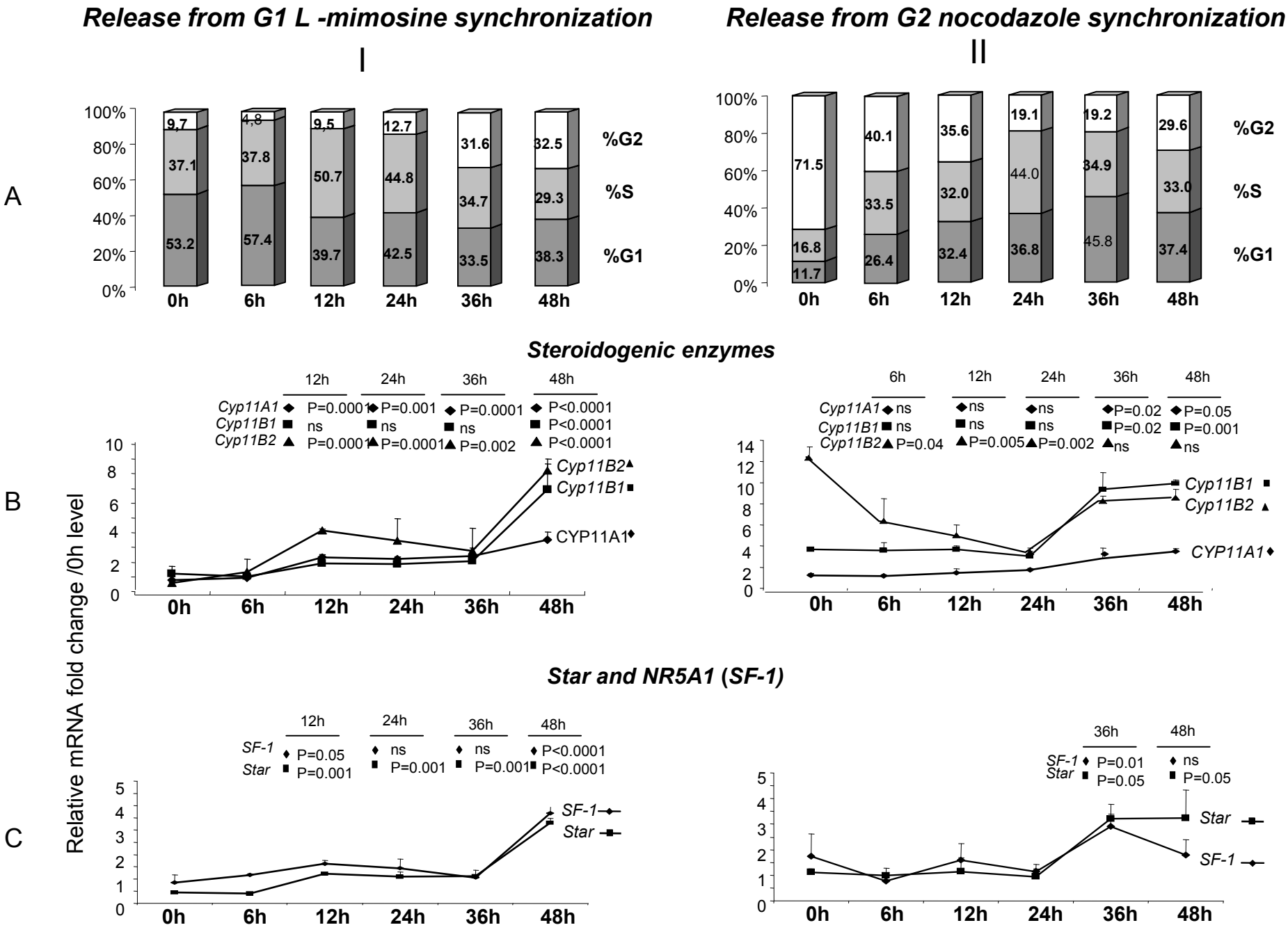


Figure 3

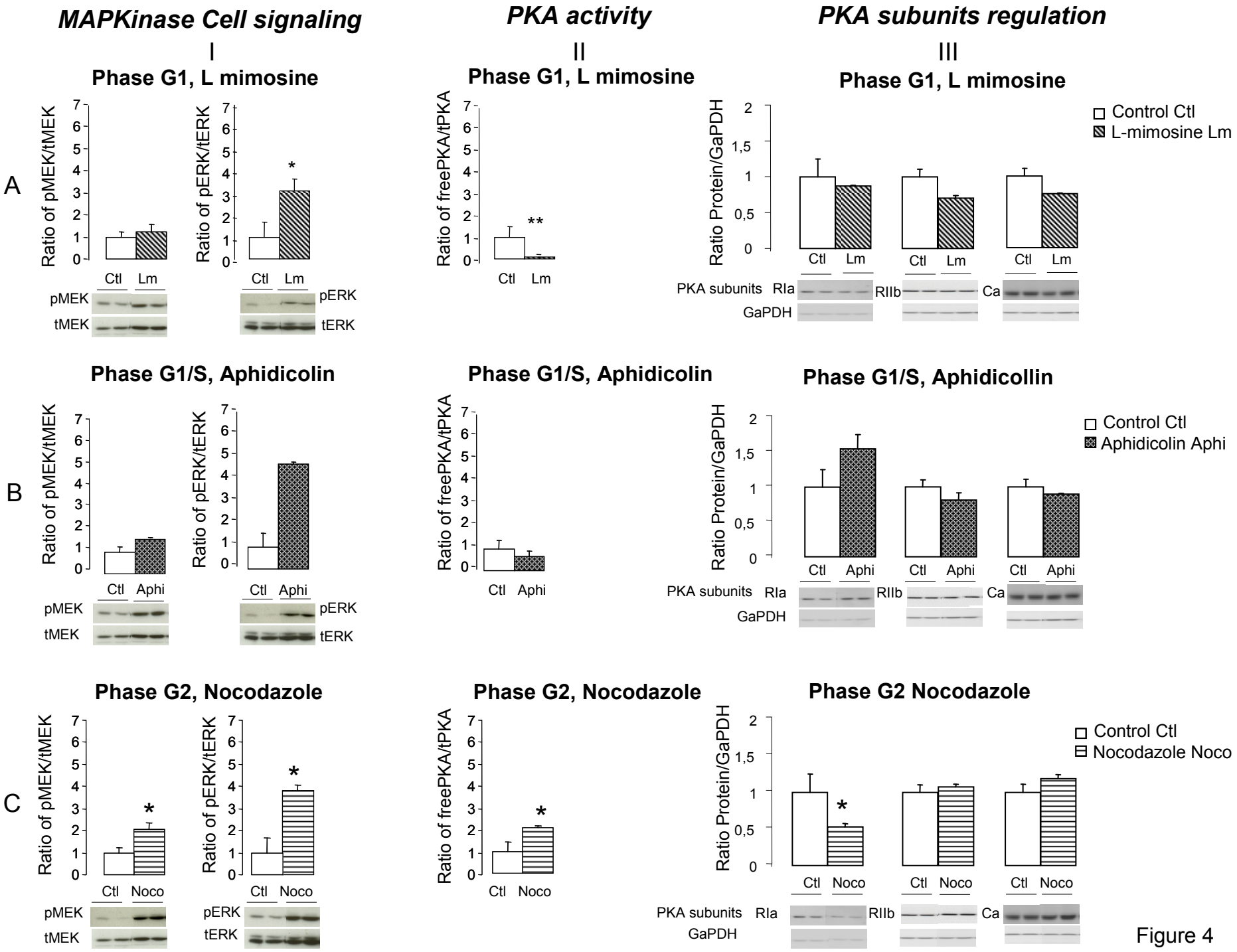


Figure 4

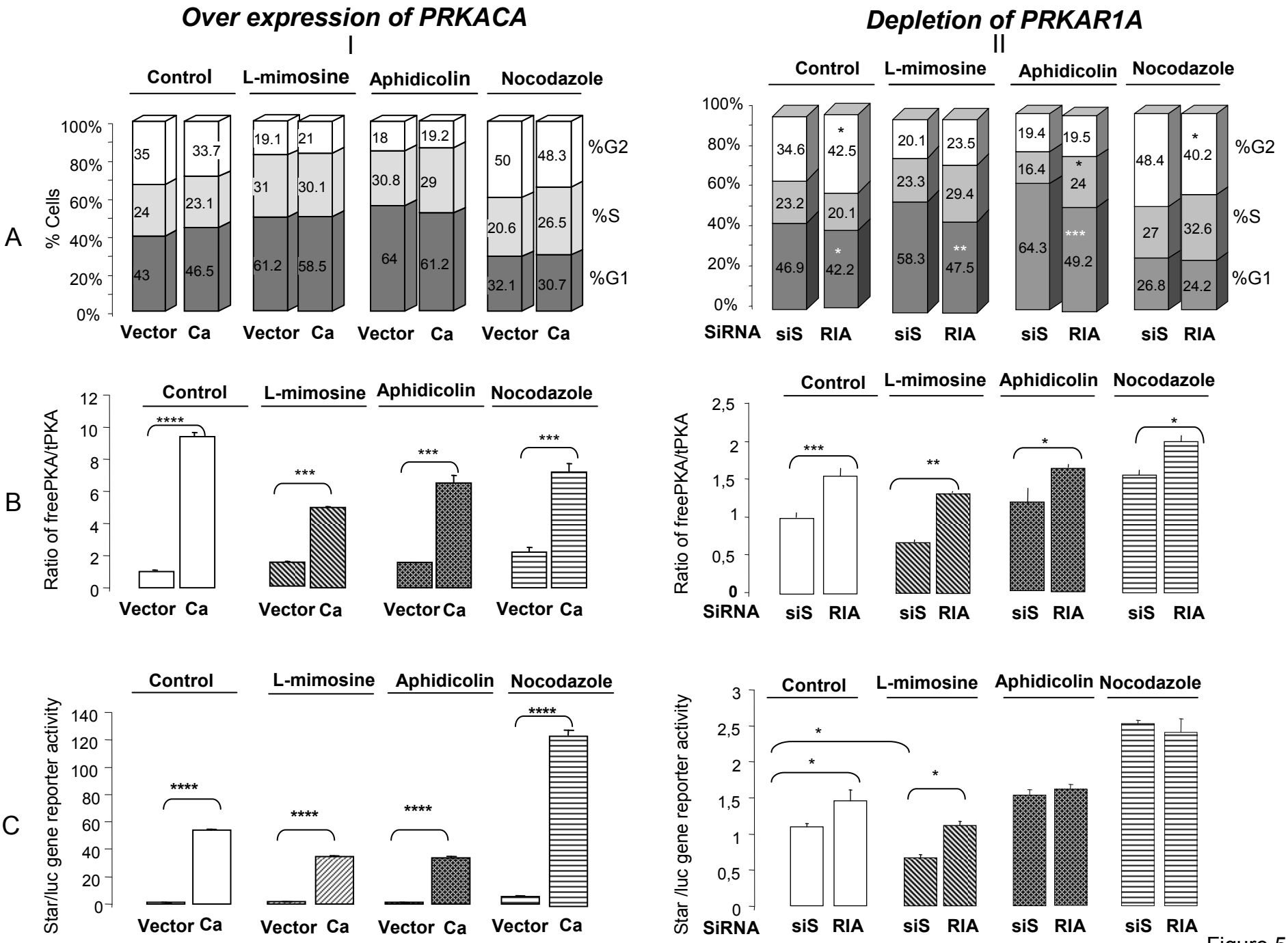


Figure 5

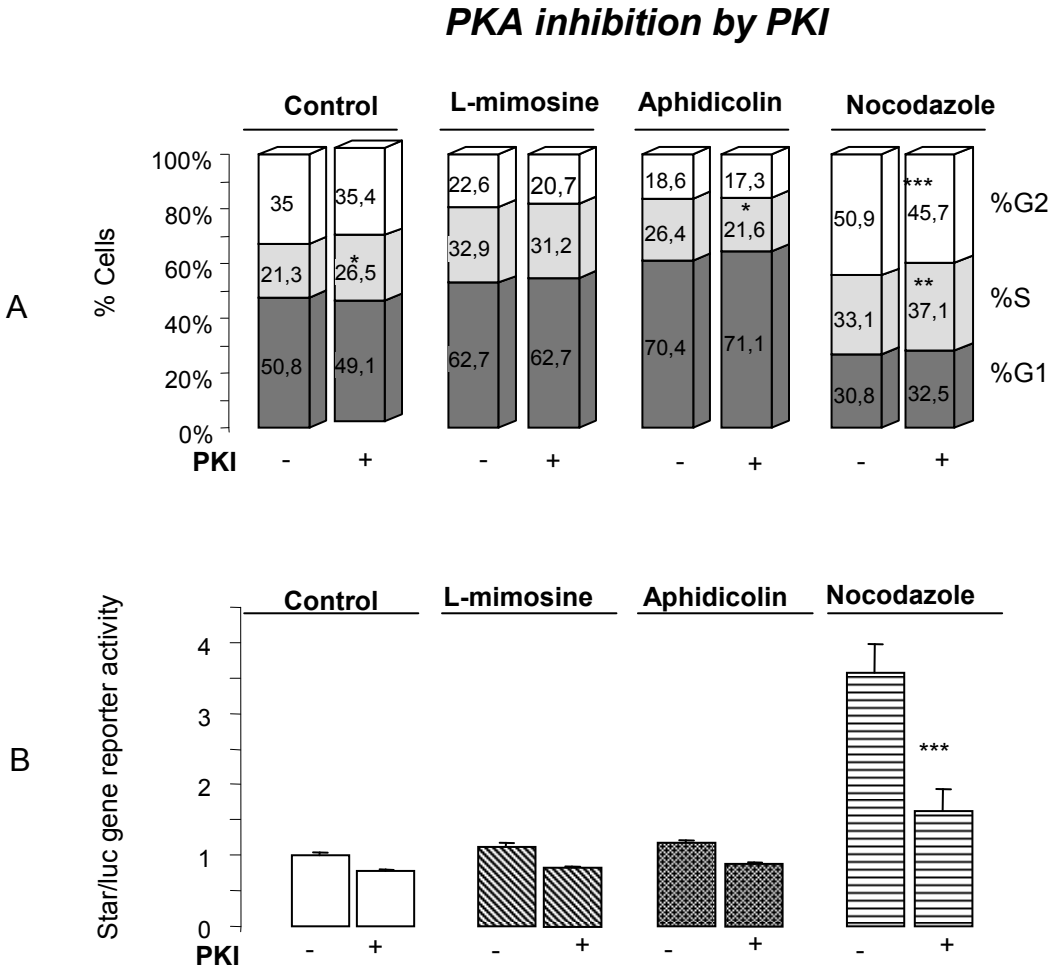
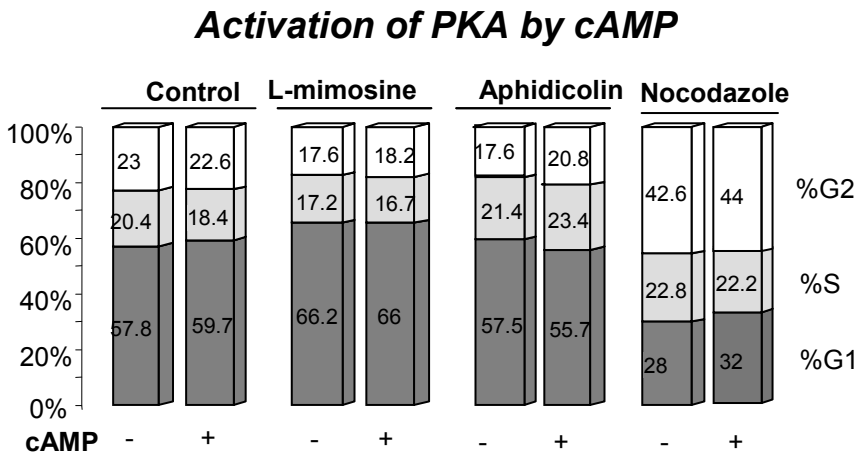
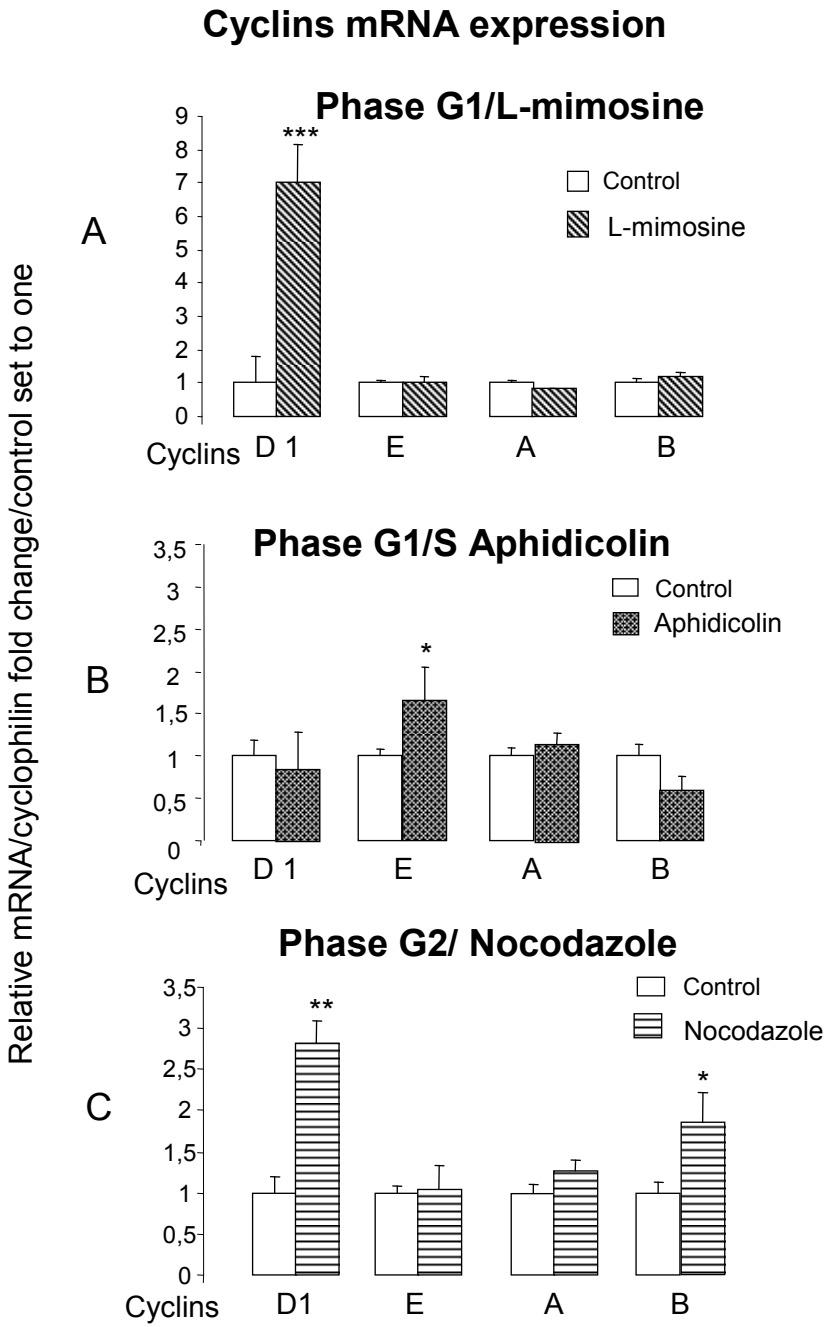


Figure 6



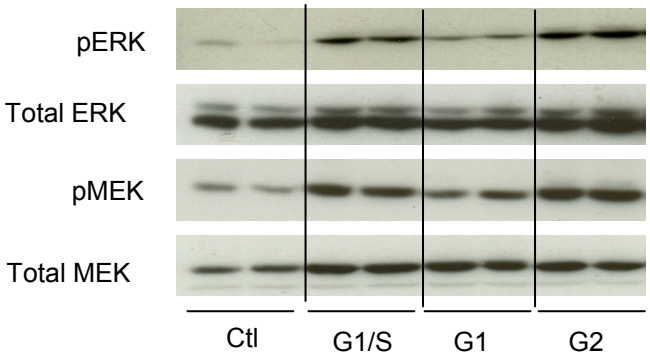
Supplementary Figure 5



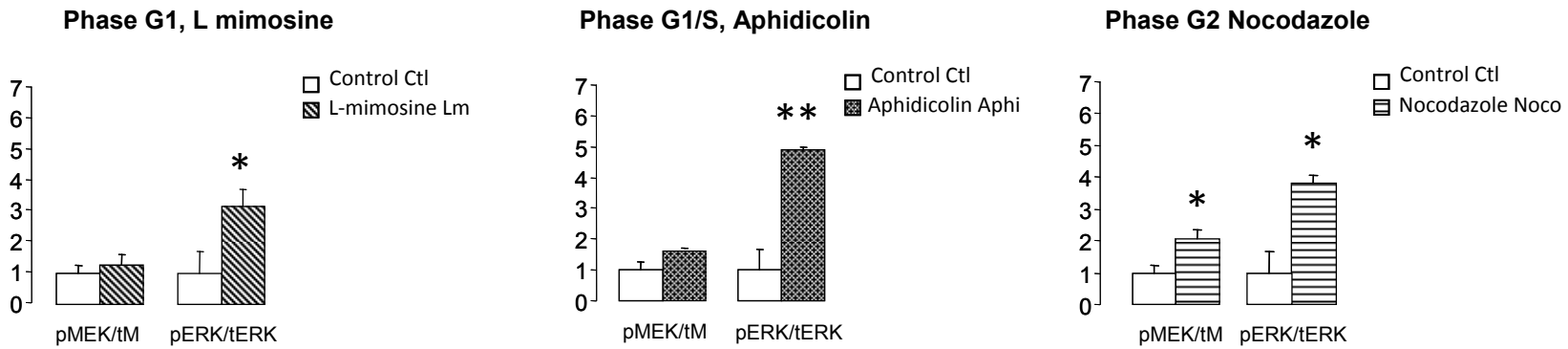
Supplementary Figure 1

MAPKinase Cell signaling: full western blot image

A

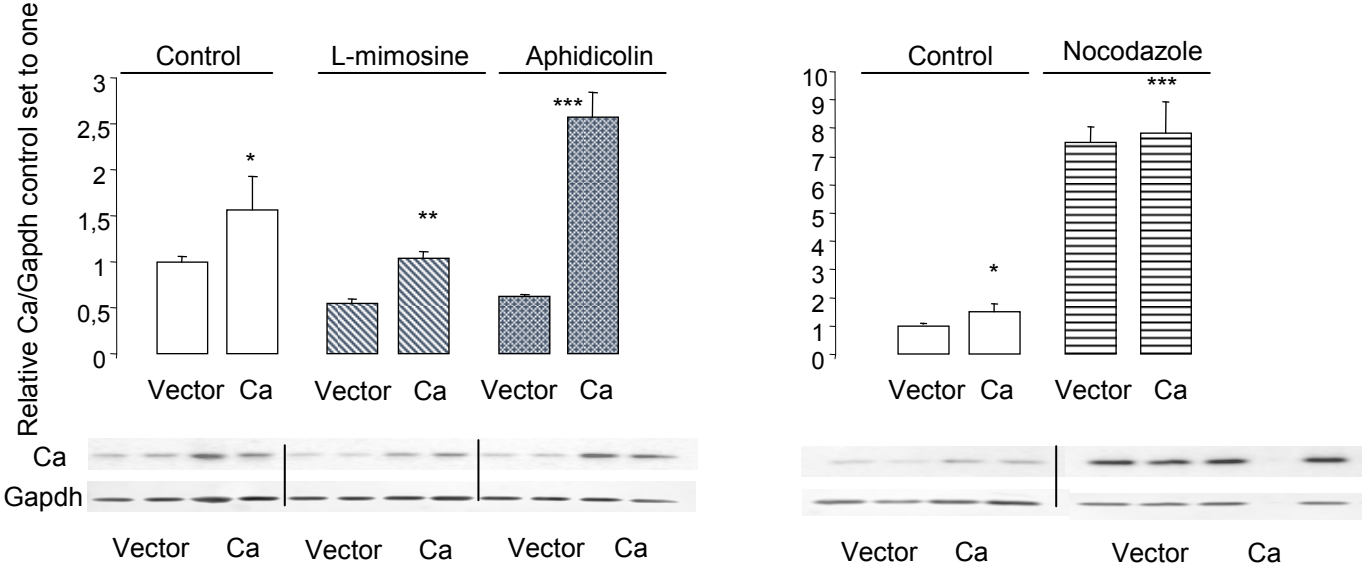


B



Supplementary Figure

A



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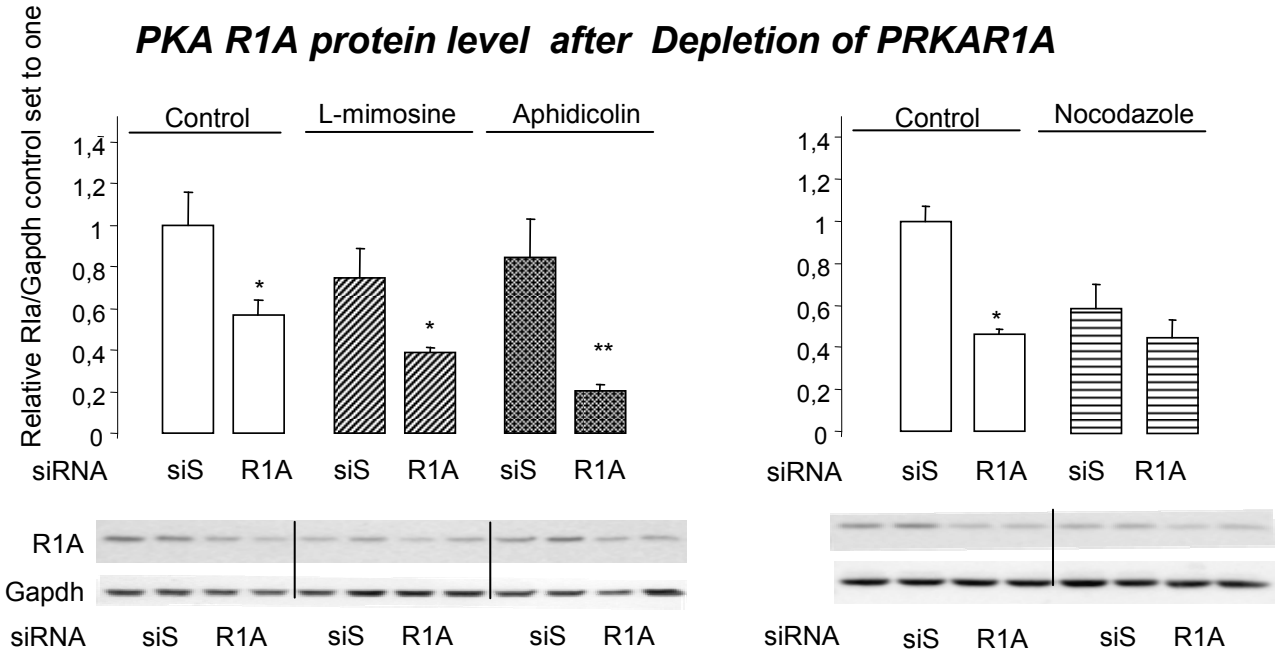
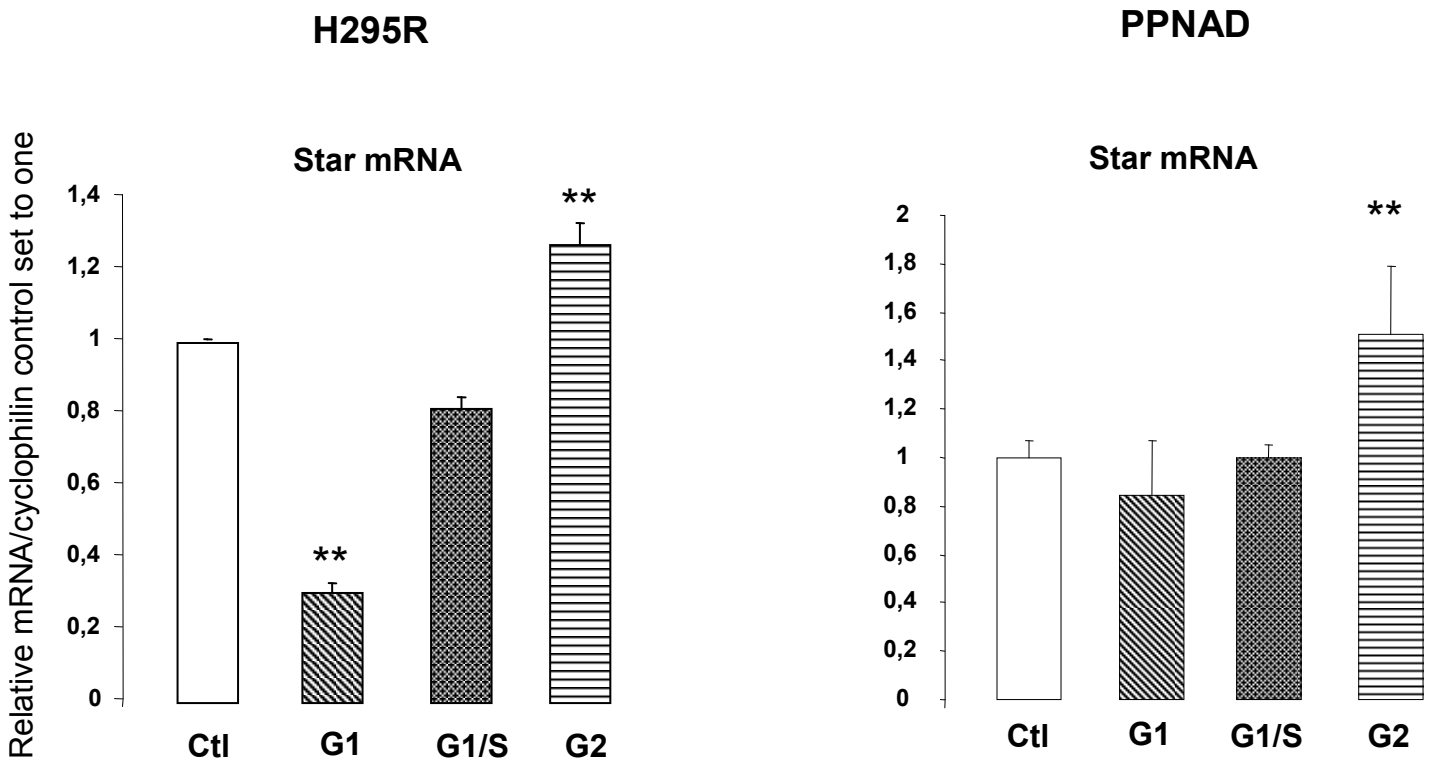


Expression of Star mRNA in synchronized cells



Supplementary Figure 2

Link between steroidogenesis, the cell cycle, and PKA in adrenocortical tumor cells

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Declaration of interest: there is no conflict of interest that could be perceived to affect the Impartiality of the research reported. All authors declare that there is no conflict of interest.

Highlights

Modulation of steroidogenesis by specific cell cycle phases in adrenal cell tumors.

PKA activity varied notably according to the stage of cell cycle in the H295R cells.

Early effect of enhancing PKA activity appears to be critical for steroid excess.

PRKAR1A mediated the function of PKA activity on both steroidogenesis and cell cycle.