Scientific report

ESF/ENS@T Exchange visit grant
Fellowship recipient: Dr. Carmen Ruggiero

BACKGROUND

Steroidogenic Factor-1 (SF-1; NR5A1 in the standard nomenclature) is a transcription factor belonging to the nuclear receptor superfamily, which has a pivotal role in the development of adrenal glands and gonads (Lalli, 2010). It has been recently demonstrated by Dr. E. Lalli and co-workers that SF-1 overexpression, which is a hallmark of children adrenocortical tumours (Pianovski *et al*., 2006) and correlates with a poorer prognosis in adrenocortical cancer in adults (Sbiera *et al*., 2010), promotes adrenocortical tumour cell proliferation *in vitro* and triggers adrenocortical tumorigenesis in transgenic mice (Doghman *et al*., 2007a). Furthermore, SF-1 overexpression in adrenocortical tumour cells is sufficient to modify steroid hormone secretion and gene expression profile in a manner closely resembling the behaviour of adrenocortical tumours. For example, increased SF-1 dosage in the H295R adrenocortical tumour cell line causes decreased expression of the NOV/CCN3 gene, which encodes a multimodular secreted protein acting as a selective proapoptotic factor for adrenocortical tumour cells. Similarly, in adrenocortical tumours NOV/CCN3 expression is reduced compared to the normal adrenal cortex (Doghman *et al*., 2007 b).
The only role for SF-1 has been considered for a long time to be the regulation of steroidogenic gene expression. Indeed, studies from Dr. E. Lalli and co-workers revealed that SF-1, besides regulating genes involved in lipid and steroid metabolism, also controls in a dosage-dependent manner genes involved in apoptosis and cell cycle, cytoskeleton and adhesion to the extracellular matrix (Doghman *et al*., 2007a). The model used to investigate the effects of an increased SF-1 dosage on gene regulation is represented by the H295R cell line (the only available differentiated human adrenocortical cancer cell line existing), where SF-1 overexpression can be induced by doxycycline (Dox) treatment (Doghman *et al*., 2007a). Through a ChIP-sequencing (chromatin immunoprecipitation using an antibody specific for SF-1 followed by high-throughput sequencing to identify the DNA sites bound by this factor) approach, Dr. E. Lalli and co-workers have identified the *VAV2* gene as a novel dosage-dependent target for SF-1 in H295R cells. They have demonstrated that SF-1 binds to multiple intronic locations inside the *VAV2* gene only when overexpressed (unpublished data, see project proposal). This increased binding correlates with increased expression of *VAV2* mRNA and protein (unpublished data, see project proposal).
VAV2 encodes a multidomain guanine nucleotide exchange factor (GEF) for the Rho, Rac and Cdc42 families of GTPases. Through its GEF activity, it can regulate cytoskeleton dynamics in response to stimuli such as growth factor receptor activation, thus affecting adhesion, motility and proliferation of both normal and cancer cells (reviewed in Hornstein *et al*., 2004). It has been reported that in several cell lines Vav proteins function as potent proto-oncogenes that when mutated or overexpressed can induce cell transformation by activating Rho/Rac/Cdc42 GTPase function (Fernandez-Zapico *et al*., 2005; Bustelo *et al*., 1994). Growth factor stimulation induces the binding of Vav proteins to phosphotyrosine residues on activated growth factor receptors and their subsequent activation by phosphorylation and membrane translocation, where they activate Rho, Rac and Cdc42 through their GEF activity. Additionally, Vav proteins participate in the regulation of internalization and trafficking of the EGF receptor, through which they modulate growth factor signalling (Thalappilly *et al*., 2010; Támas *et al*., 2003).

PURPOSE OF THE VISIT

Dr. E. Lalli's laboratory is currently focused on the identification of genes which play a relevant role in the establishment of the proliferative and metastatic phenotype of adrenocortical cancer cells and might represent potential druggable targets. The discovery of the Vav2 GEF as a novel transcriptional target for SF-1 and the known oncogenic role for this gene in other tissues suggest that it should be one of them.
Indeed, on the basis of the literature evidence and the preliminary data obtained in Dr. E. Lalli's laboratory (see background) we formulated the following hypotheses:

- SF-1 might promote adrenocortical tumour cell migration, invasion and metastatic behaviour;
- Vav2, as a novel transcriptional target of SF-1, should play a relevant role in the establishment of the metastatic phenotype of adrenocortical cancer cells and might represent a druggable target for the therapy of adrenocortical tumours.

Thus, the main aims of the present project are the following ones:

- determine the role that an increased SF-1 dosage in human adrenocortical cancer cells exert on:
    - actin cytoskeleton remodelling;
    - Rho small GTPase activation;
    - Invasive ability.
- Investigate whether the possible effects observed on the remodelling of the actin cytoskeleton, Rho GTPase activation and invasion upon an increased SF-1 dosage are mediated by Vav2 and/or by other SF-1 targets.

During the reporting period I have mainly worked on the first aim obtaining promising results. A description of the work carried out and the results obtained is provided below.

WORK CARRIED OUT DURING THE VISIT AND MAIN RESULTS OBTAINED

Kinetic analysis of SF-1 and Vav2 induction

It has been described that upon stimulation by potent activators, like epidermal growth factor (EGF) or bradykinin for Cdc42, EGF or platelet-derived growth factor (PDGF) for Rac1, calpeptin or LPA (lysophosphatidic acid) for RhoA, Rho small GTPases are activated very rapidly and transiently (Kurokawa *et al.*, 2004; Ridley *et al.*, 1992; Kranenburg *et al.*, 1999; Schoenwaelder and Burridge, 1999). Maximal activation ranges from 30 seconds (s) to 30 minutes (min) and declines thereafter to basal levels.
A subclone of the human adrenocortical cancer cell line H295R (H295R TR/SF-1) where SF-1 overexpression can be induced in a Dox-dependent manner has been developed in Dr. E. Lalli's laboratory to dissect the cellular and molecular consequences of increased levels of SF-1 in human adrenal cells (Doghman *et al*., 2007a). SF-1 expression is induced in this system upon 72 hours (h) of Dox treatment. We thus asked whether under this condition it should be possible to detect any

changes in the actin cytoskeleton morphology or Rho small GTPase activation, considering the short temporal range of those biological responses (see above).
Therefore, as a first investigation step I performed time course experiments in order to establish the shortest time at which SF-1 and its novel identified target Vav2 were induced. Cells were treated with or without Dox and processed for immunoblotting at different time points (3h, 6h, 9h, 12 h, 24 h, 48 h). Western blotting analysis revealed that SF-1 starts to be induced after 6 h of treatment (Fig. 1), whereas the first increase in Vav2 protein expression (small, however statistically significant) was observed after 12 h (Fig.1). Higher SF-1 and Vav2 protein expression levels were detected upon longer induction times (Fig. 1).

Increased SF-1 dosage induces filopodia and lamellipodia-ruffles formation

Active reorganization of the actin cytoskeleton is an integral part of the cellular response to a variety of environmental signals. Rearrangement of the actin cytoskeleton is highly influenced by the activity of the Rho family GTPase member Cdc42, Rac1 and RhoA (Jaffe and Hall, 2005). Indeed, activation of Rac1, Cdc42 and RhoA has been shown to produce specific structural changes in the plasma membrane (PM) associated with cell movement (Heasman and Ridley, 2008). Tissue culture studies (carried out originally in fibroblasts, but later in other cell types) using constitutively active and dominant negative mutants, have shown that RhoA is responsible for the induction of stress fibres, Rac1 promotes the formation of lamellipodia and ruffles, whereas Cdc42 activation causes the formation of a third type of structures, which are named filopodia (Nobes and Hall, 1999). Stress fibres are long bundles of actin that traverse the cell and are linked to integrins at sites of focal adhesion (Pellegrin and Mellor, 2007). Their formation results in more contracted cells that exhibit enlarged focal adhesions. Lamellipodia are structures formed by a network of short, branched actin filaments. They have a major role in driving cell migration by attaching to the substrate and generating force to pull the cell body forward (Small *et al.*, 2002). Ruffles are waves arising at the leading edge of lamellipodia that moves centripetally toward the main cell body and form as a consequence of inefficient lamellipodia adhesion (Borm *et al.*, 2005). Finally, filopodia are long, finger-like projections at the edges of lamellipodia (Mattila and Lappalainen, 2008). They are proposed to sense external cues to set the direction of cell migration.
To evaluate possible morphological changes in the actin cytoskeleton upon an increased SF-1 dosage, H295R TR/SF-1 cells have been treated with or without Dox, fixed at different timepoints (6 h, 9 h, 12 h, 24 h, 48 h and 72 h) and processed for immunofluorescence. They have been stained with phalloidin for F-actin detection and with an antibody against SF-1 to appreciate SF-1 overexpression in Dox-treated cells respect to control untreated cells. The analysis of the images acquired revealed that an increased SF-1 dosage induces a remodelling of the H295R TR/SF-1 actin cytoskeleton. Indeed, it was possible to appreciate a small but statistically significant increase in the number of filopodia– forming cells respect to control cells starting from 12 h of treatment (Fig. 2). The increase was more evident after 24 h, 48 h and 72 h (Fig, 2). Moreover, a higher number of cells forming lamellipodia/ruffles was detected in Dox-treated cells respect to control cells starting from 24 h of treatment (Fig. 2). It was comparable to that measured after 48 h and 72 h (Fig. 2). Representative images of the effects described are reported (Fig. 3-6).
The above data indicate that an increased SF-1 dosage induces H295R actin cytoskeleton remodelling by stimulating the formation of filopodia and lamellipodia/ruffles at the leading edge of the cells.
The levels of SF-1 in Dox-treated cells are not homogeneous. Indeed, I could partition Dox-treated cells into three subpopulations on the basis of their SF-1 expression levels (Fig. 7). The subpopulations identified were the following ones:

- low SF-1 level – expressing cells, which account for about 30% of the total population (Fig. 7). The SF-1 levels expressed by these cells are comparable to those exhibited by untreated control cells;
- medium SF-1 level – expressing cells, which account for about 50% of the total population (Fig. 7);
- high SF-1 level – expressing cells, which account for about 15% of the total population (Fig. 7).

The analysis carried out on the three different subpopulations revealed that the cells displaying high SF-1 levels formed on the average 6 filopodia/cell, medium SF-1 level – expressing cells 4 filopodia/cell, whereas low SF-1 level – expressing cells exhibited 2 filopodia/cells, similarly to control cells (Fig. 7). These data indicate that a tight correlation exists between SF-1 expression levels and the number of filopodia formed.

SF-1 and Vav2 induction correlates with increased levels of active Cdc42 and Rac1

Filopodia formation is classically associated to Cdc42 activation, whereas lamellipodia/ruffles are linked to active Rac1 (see above). We thus decided to monitor the levels of active Cdc42 and Rac1 in Dox-treated cells versus control cells. The levels of active RhoA were also measured.
The activation levels of Rho small GTPases have been traditionally monitored through pull-down activation assays, wherein the GTP binding domain of a Rho small GTPase effector protein is coupled to agarose beads or to specific tags like GST or polyHis, allowing affinity based detection of the active GTPase in biological samples (Benard and Bokoch, 2002). However, these methods are time consuming, require large amount of sample, and tend to not be very consistent and easily reproducible. To measure active Cdc42, Rac1 and RhoA in our samples we thus exploited an assay developed by Cytoskeleton technology, which is based on the use of a 96-well plate coated with a binding domain of a Rho-family effector protein. The active GTP-bound protein in cell lysates binds to the wells while the inactive GDP-bound forms are removed during the washing steps. The bound active forms are then detected by incubation with a specific primary antibody followed by a secondary antibody conjugated to HRP. The signal is then developed by colorimetric or luminescence detection reagents. By this assay I was able to demonstrate that an induction in SF-1 and Vav2 levels after 12 h of Dox treatment correlated with increased levels of GTP-bound Cdc42 and GTP-bound Rac1 (Fig. 8). No changes in active RhoA levels were detected. The above data well correlated with the morphological phenotypes observed, as the activation of Cdc42 and Rac1 supports filopodia and lamellipodia/ruffles formation respectively (see above).

Increased SF-1 dosage promotes H295R invasiveness

Once established that an increased SF-1 dosage stimulates the remodelling of H295R actin cytoskeleton and induces the activation of Cdc42 and Rac1, we asked whether these phenomena were correlated to a functional phenotype. Indeed, the coordinated activation of Rho small GTPases via their GEFs, like Vav2, and the related active reorganization of the actin cytoskeleton are considered a possible mechanism underlying tumour cell motility and migration, an obvious prerequisite for invasion and metastasis. Determining the migratory and invasive capacity of tumour cells and clarifying the underlying mechanisms are relevant to unveil novel strategies for cancer therapies. Different assays have been developed throughout the years to study the migratory and invasive ability of cells (reviewed in Kramer *et al.*, 2013). One of the most popular is the so-called transwell invasion assay through Matrigel (Marshall J, 2011). The principle of the assay is based on two medium containing chambers separated by a porous membrane through which cell

transmigrate. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution (Matrigel). This basement membrane layer serves as a barrier to discriminate invasive cells from non-invasive ones. Invasive cells are able to degrade the matrix proteins in the layer and ultimately migrate through the pores of the membrane into the lower compartment, where medium containing an attractant or simply higher serum content is present. Finally, the cells are removed from the top of the membrane and the invading cells are stained and quantified. I subjected H295R TR/SF-1 cells treated or not with Dox to this kind of assay and I compared the invasive ability of Dox-treated cells to that of control cells. The quantification of this set of experiments revealed that Dox increased by around 2 fold the percentage of invading cells respect to control (Fig. 9). These data suggest that an increased SF-1 dosage stimulates the invasive ability of human adrenocortical carcinoma cells.

CONCLUSIONS

The results described in the present report indicate that an increased SF-1 dosage in human adrenocortical cancer cells:

- leads to increased levels of active Cdc42 and Rac1;
- promotes actin cytoskeleton remodelling through an increase in the number filopodia– and lamellipodia/ruffles– forming cells;
- stimulates cell invasiveness.

The results obtained during the granted period are very promising and suggest that SF-1 is involved in the acquisition of a motile and invasive phenotype by human adrenocortical cancer cells. Those data will be included in a publication that we are going to finalise by the end of 2013. ESF/ENS@T will be acknowledged for the crucial financial support provided.

MATERIALS AND METHODS

Cell culture

H295R cells overexpressing SF-1 in a Dox-inducible fashion (H295R TR/SF-1) were cultured in DMEM/F-12 supplemented with 2% NuSerum (Becton Dickinson), 1% ITS Plus (Becton Dickinson), and antibiotics, as described in (Doghman *et al.,* 2007a*)*.

Immunoblots

H295R/TR/SF-1 cells were treated for the indicated times with Dox (1 µg/ml) or with ethanol as a control. Protein extracts were prepared by harvesting cells in Laemmli buffer [50 mM Tris-HCl (pH 6.8), 50% glycerol, 2% sodium dodecyl sulphate, and 0.02% bromophenol blue] containing 5% β-mercaptoethanol. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Immunblot was performed using a chemiluminescence system for protein detection (ECL Plus; GE Healthcare Bio-Sciences Corp.). Primary antibodies used were: rabbit polyclonal anti SF-1 (1:1000 dilution; Upstate Biotechnology Inc.); rabbit monoclonal anti Vav2 (1:1000 dilution; Epitomics); and mouse monoclonal anti-β-tubulin (1:1000 dilution; Sigma-Aldrich). Densitometry was performed on scanned immunoblot images using the ImageJ gel analysis tool. The gel analysis tool was used to obtain the absolute intensity (AI) for each experimental SF-1 or Vav2 band and corresponding control β-tubulin band. Relative intensity (RI)

for each experimental band was calculated by normalizing the experimental AI to the corresponding control AI.

Immunofluorescence and filopodia, lamellipodia/ruffles detection

Cells were fixed (15 min at 22°C) in 4% paraformaldehyde in PBS and permeabilized by 2 treatments with 0,1% Triton X100 PBS (PT) for 10 min each. After blocking (30 min) in 2% BSA in PBS, cells were incubated o/n at 4°C with the rabbit polyclonal anti SF-1 antibody (1:1000 dilution, Upstate Biotechnology Inc.). Cells were washed 3 times with PT and incubated 1 hour at room temperature (rt) with Alexa 488- conjugated goat anti-rabbit secondary antibody (1:200; Invitrogen). To visualize F-actin for filopofia and lamellipodia/ruffles formation, cells were incubated 1 h with Alexa Fluor® 594 Phalloidin (1:400, Molecular Probes). Cells were washed 3 times again with PT and mounted in SlowFade Gold antifade reagent with 4′, 6-Diamidino-2-phenylindole (DAPI) (Invitrogen). Images were acquired with a Zeiss Axioplan 2 fluorescence microscope coupled to a digital charge-coupled device camera, processed and analyzed using ImageJ. About 250 cells were examined per condition per experiment for filopodia and lamellipodia/ruffles formation.

Cdc42, Rac1, RhoA activation assays

H295R TR/SF-1 were plated at 1 x 10^6 per well in a 6 well plate. After 24 h, they were treated with Dox (1 µg/ml) or with ethanol as a control for 12 h. Cell lysates were harvested on ice, snap-frozen in liquid nitrogen, and the total protein concentration of each lysate adjusted to 0,25 mg/ml for Cdc42-GTP detection and 0,5 mg/ml for Rac1-GTP and RhoA-GTP detection. Cdc42 activity was analyzed by G-LISA™ Cdc42 Activation Assay Biochem Kit™, colorimetric based (cat. BK127, Cytoskeleton); Rac1 activity was analyzed by G-LISA™ Rac1 Activation Assay Biochem Kit™ , luminescence based (cat. BK126, Cytoskeleton); RhoA activity was analyzed by G-LISA™ RhoA Activation Assay Biochem Kit™, luminescence based (cat. BK121, Cytoskeleton) according to manufacturer's instruction.

Cell invasion assay

The cell invasion assay was performed with a basement membrane-coated CytoSelect™ 24-well cell invasion assay kit according to the manufacturer's instructions (Cell Biolabs). Briefly, H295R TR/SF-1 cells (3 ×10^5 cells/well, complete culture medium) were plated in the upper chamber of the invasion plate. Complete culture medium was added to the lower chamber. After 24 h cells were treated with Dox (1 µg/ml in serum free medium) and DMEM/F-12 medium supplemented with 50% FBS was added to the lower chamber as chemoattractant. Ethanol was used as vehicle in untreated control cells. Cells were incubated for 72 h at 37°C in 5% CO_2 atmosphere. The media in the upper and lower chambers were then replaced by fresh culture medium +/- Dox and fresh medium containing 50% FCS respectively. Cells were incubated at 37°C in 5% CO_2 atmosphere for further 72 h. The non-invasive cells were then removed from the upper part of the basement membrane and the inserts were transferred to a clean well containing 400 µl of Cell Stain Solution and incubated for 10 min at room temperature. The inserts were then washed several times in a beaker of water, allowed to air dry and transferred to an empty well, were 200 µl of Extraction Solution was added per well. After 10 min incubation on an orbital shaker, 100 µl from each sample were transferred to a 96-well microtiter plate and the OD 560 nm was measured in a plate reader.

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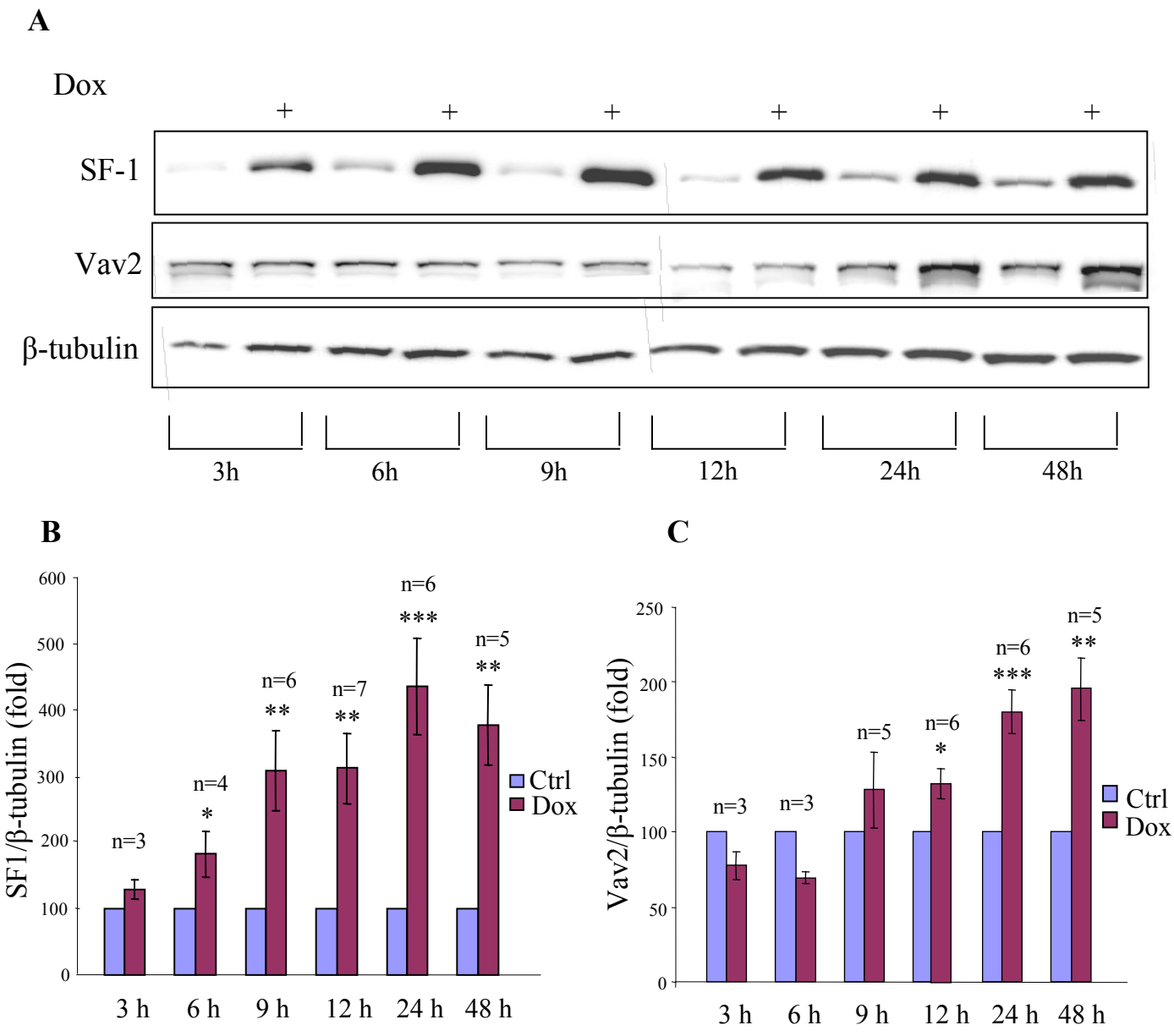

Figure 1. Kinetic analysis of SF-1 and Vav2 induction upon doxycycline treatment. **A**, SF-1 and Vav2 expression is induced in H295R TR/SF-1 cells by doxycycline (Dox) treatment (1 µg/ml) starting from 6 h and 12 h respectively. β-Tubulin expression is shown as a control. Immunoblots are representative of at least three different experiments. **B**, **C** Intensities of SF-1 and Vav2 were quantified by densitometric analysis, normalized to β-tubulin abundance and reported in **B** and **C** respectively as the mean +/- SEM of at least three independent experiments. * $p < 0,05$; ** $p < 0,01$, *** $p < 0,001$ (Student's t-test).

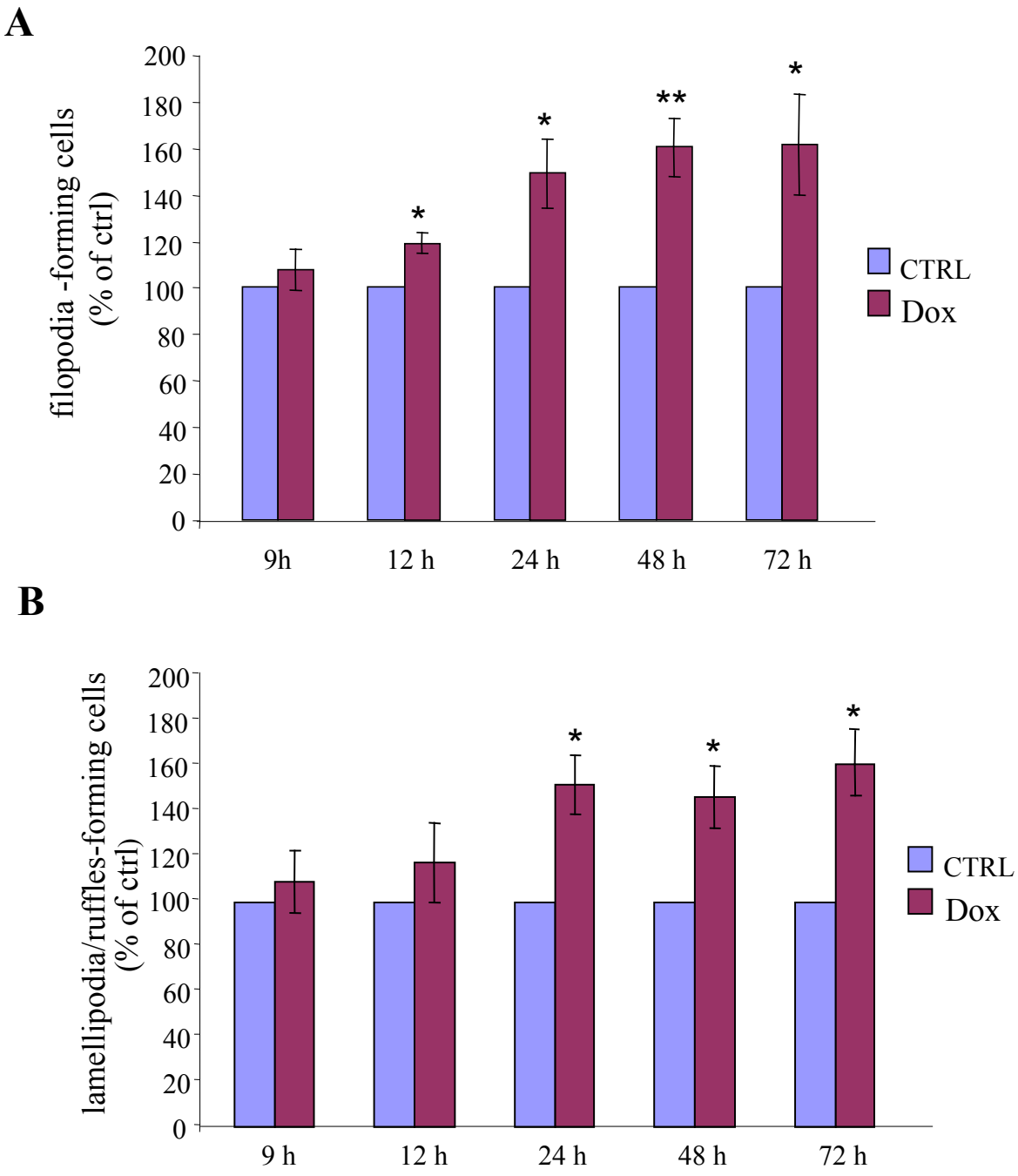

Figure 2. Increased SF-1 dosage promotes filopodia and lamellipodia/ruffles formation in human adrenocortical cancer cells. **A**, Quantification of the number of filopodia-forming cells as the mean +/- SEM of three independent experiments. ~ 250 cells have been quantified per condition per experiment (~750 cells/condition). Data are shown as percentage of control (ctrl) untreated cells. * $p < 0,05$; ** $p < 0,01$ (Student's t-test). **B**, Quantification of the number of lamellipodia/ruffles-forming cells as the mean +/- SEM of three independent experiments. ~ 250 cells have been quantified per condition per experiment (~750 cells/condition). Data are shown as percentage of ctrl untreated cells. * $p<0,05$ (Student's t-test).

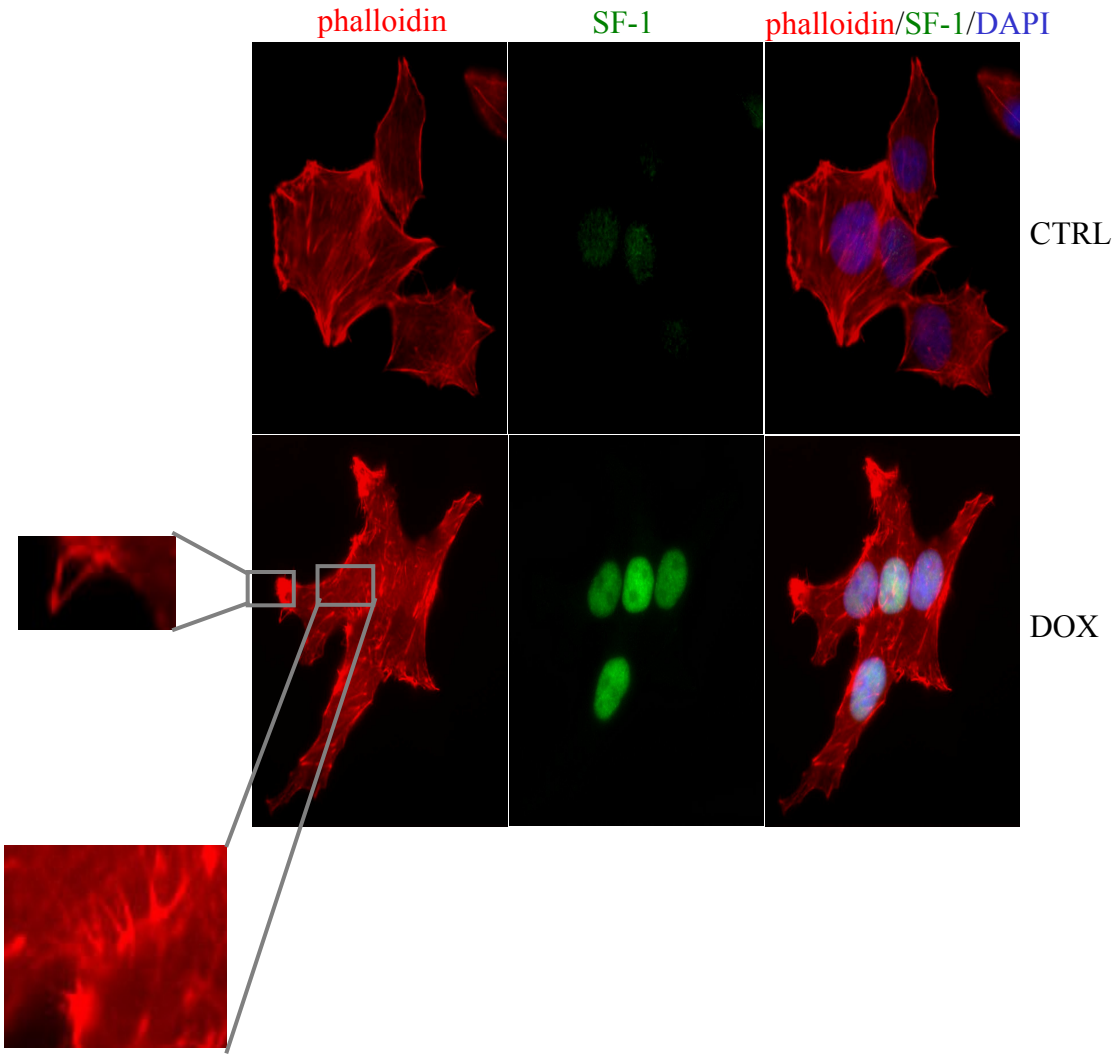

Figure 3. Increased number of filopodia-forming cells upon 12 h of doxycycline treatment. H295R TR/SF-1 cells treated with (lower panels) or without Dox (upper panels) were triple-stained for F-actin (phalloidin staining, red), SF-1 (green) and DAPI (blue). Images of red, green and blue signals are shown (phalloidin/SF-1/DAPI). The insets show higher magnification view of filopodia in Dox-treated cells. The images are representative of three different experiments.

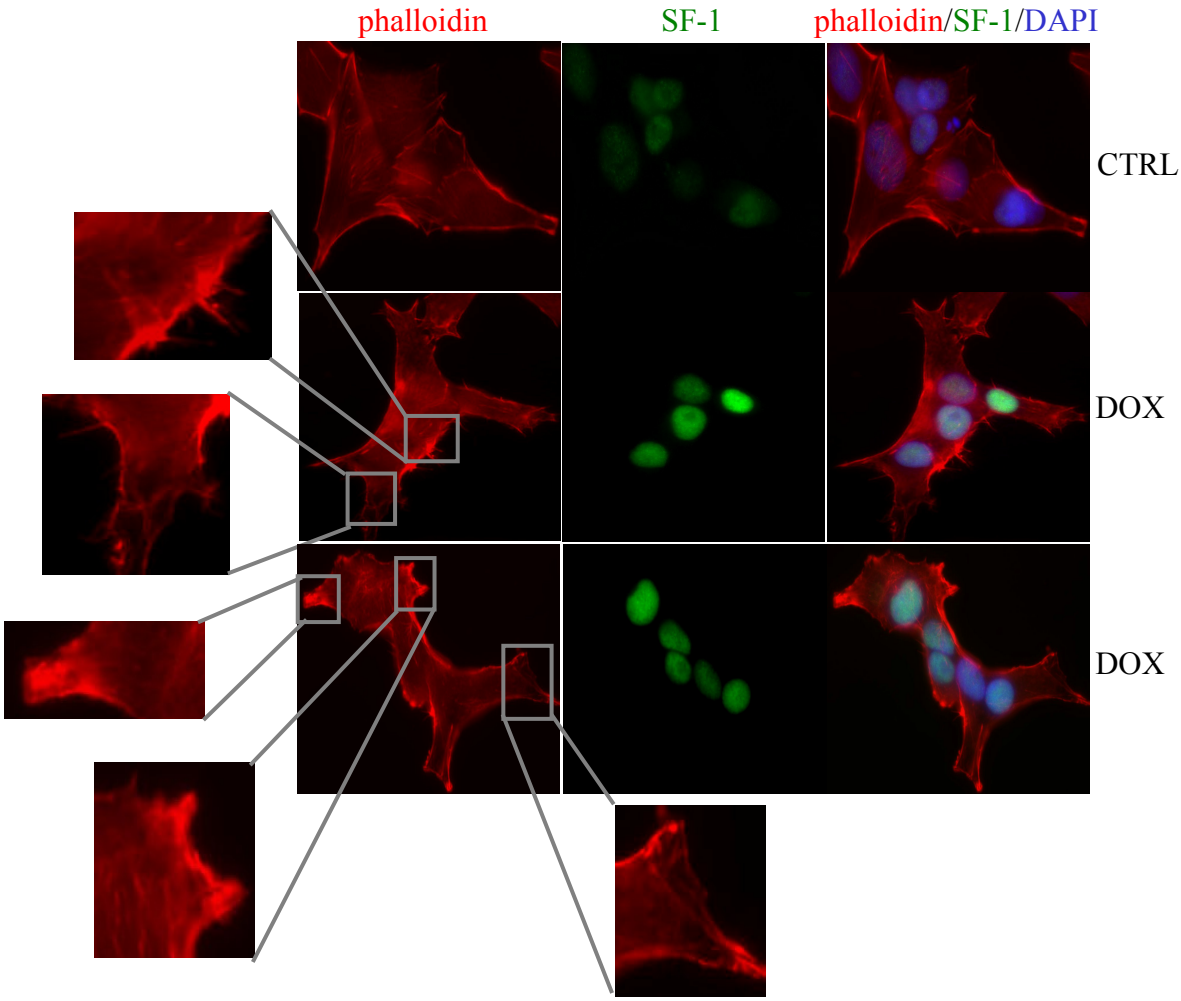

Figure 4. Increased number of filopodia- and lamellipodia/ruffles- forming cells upon 24 h of doxycycline treatment. H295R TR/SF-1 cells treated with (middle and lower panels) or without Dox (upper panels) were triple-stained for F-actin (phalloidin staining, red), SF-1 (green) and DAPI (blue). Images of red, green and blue signals are shown (phalloidin/SF-1/DAPI). The insets show higher magnification view of filopodia (middle panels) and lamellipodia/ruffles (lower panels) in Dox-treated cells. The images are representative of three different experiments.

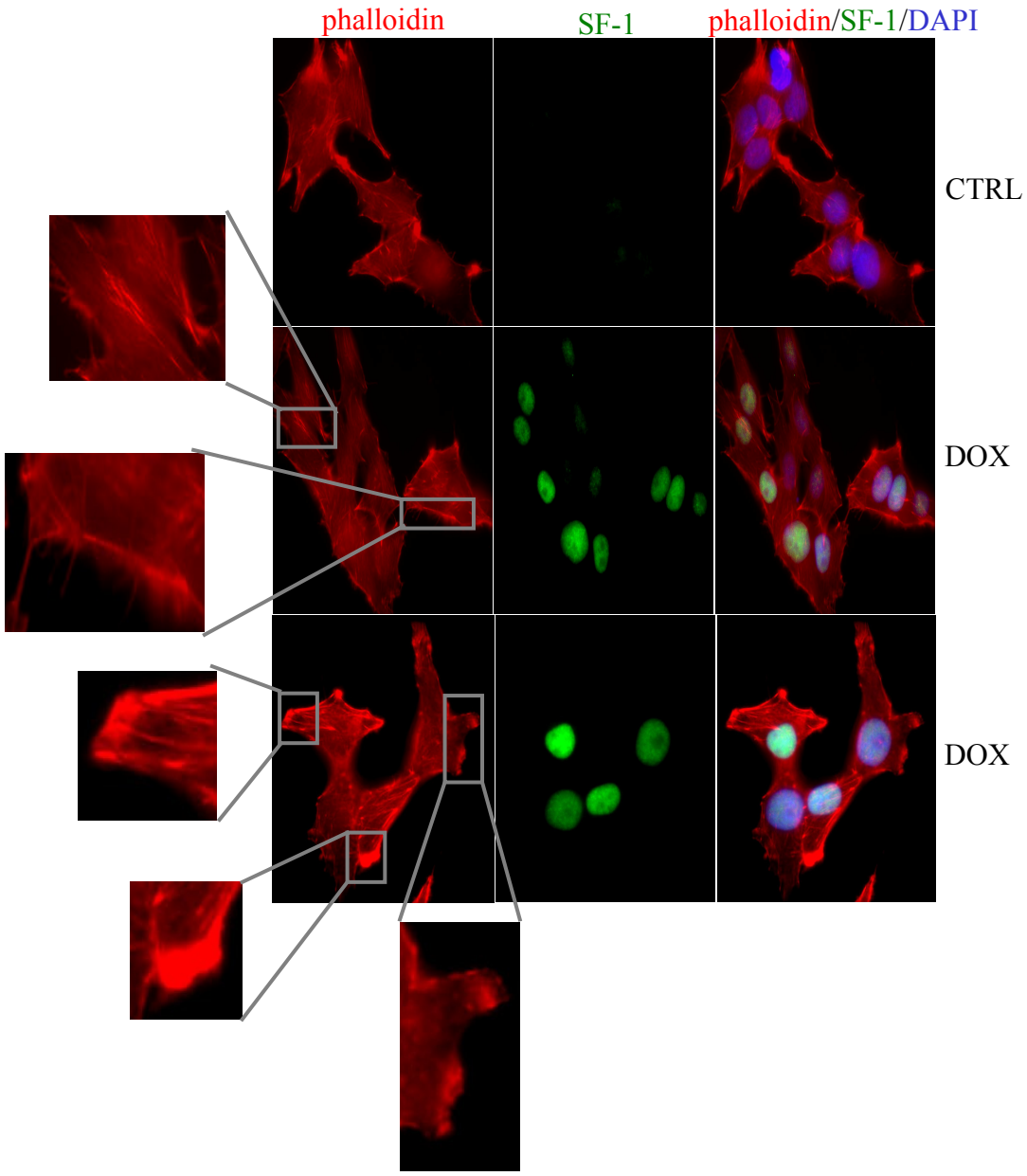

Figure 5. Increased number of filopodia- and lamellipodia/ruffles- forming cells upon 48 h of Dox treatment. H295R TR/SF-1 cells treated with (middle and lower panels) or without Dox (upper panels) were triple-stained for F-actin (phalloidin staining, red), SF-1 (green) and DAPI (blue). Images of red, green and blue signals are shown (phalloidin/SF-1/DAPI). The insets show higher magnification view of filopodia (middle panels) and lamellipodia/ruffles (lower panels) in Dox-treated cells. The images are representative of three different experiments.

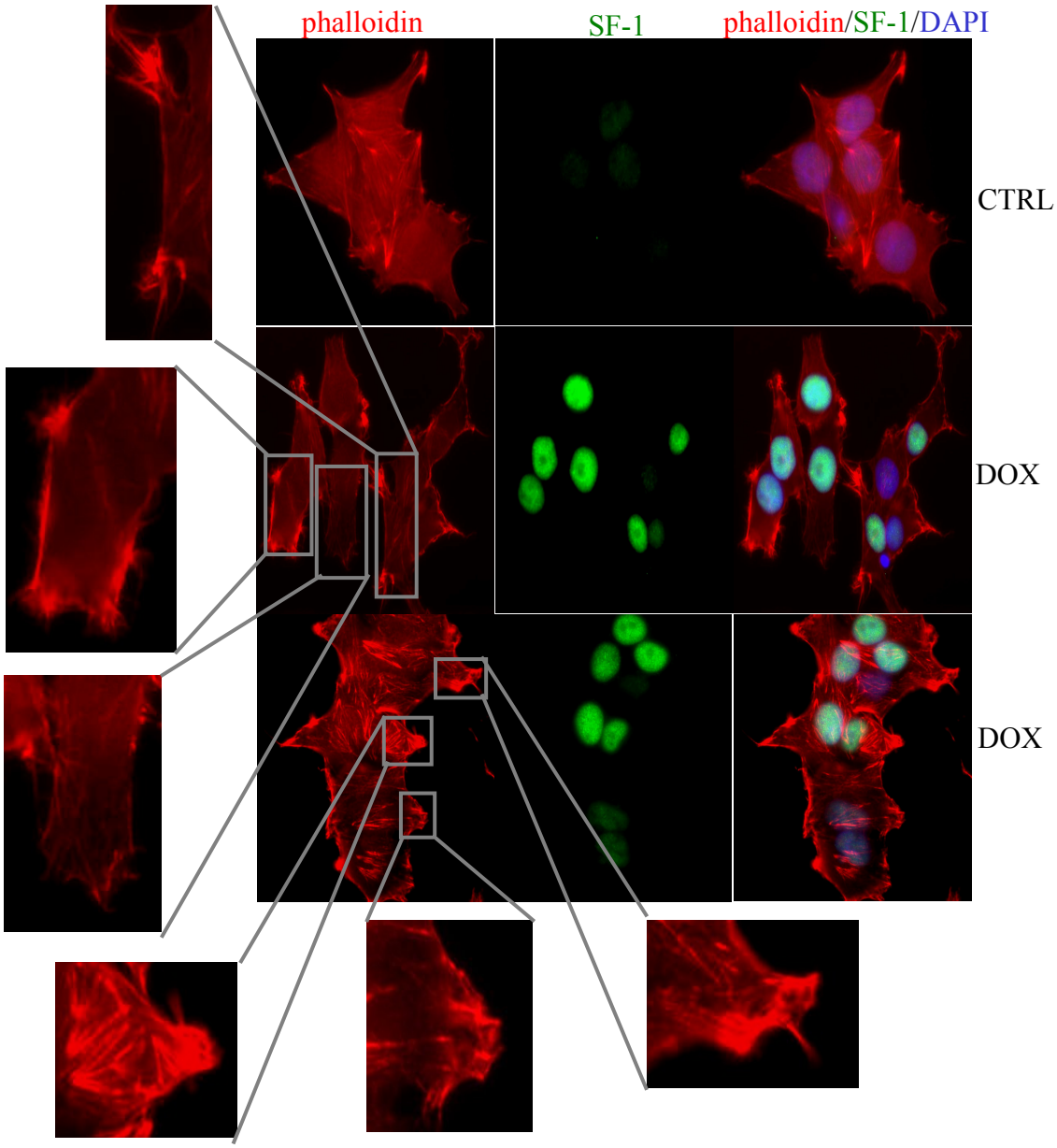

Figure 6. Increased number of filopodia– and lamellipodia/ruffles– forming cells upon 72 h of Dox treatment. H295R TR/SF-1 cells treated with (middle and lower panels) or without Dox (upper panels) were triple-stained for F-actin (phalloidin staining, red), SF-1 (green) and DAPI (blue). Images of red, green and blue signals are shown (phalloidin/SF-1/DAPI). The insets show higher magnification view of filopodia (middle panels) and lamellipodia/ruffles (lower panels) in Dox-treated cells. The images are representative of three different experiments.

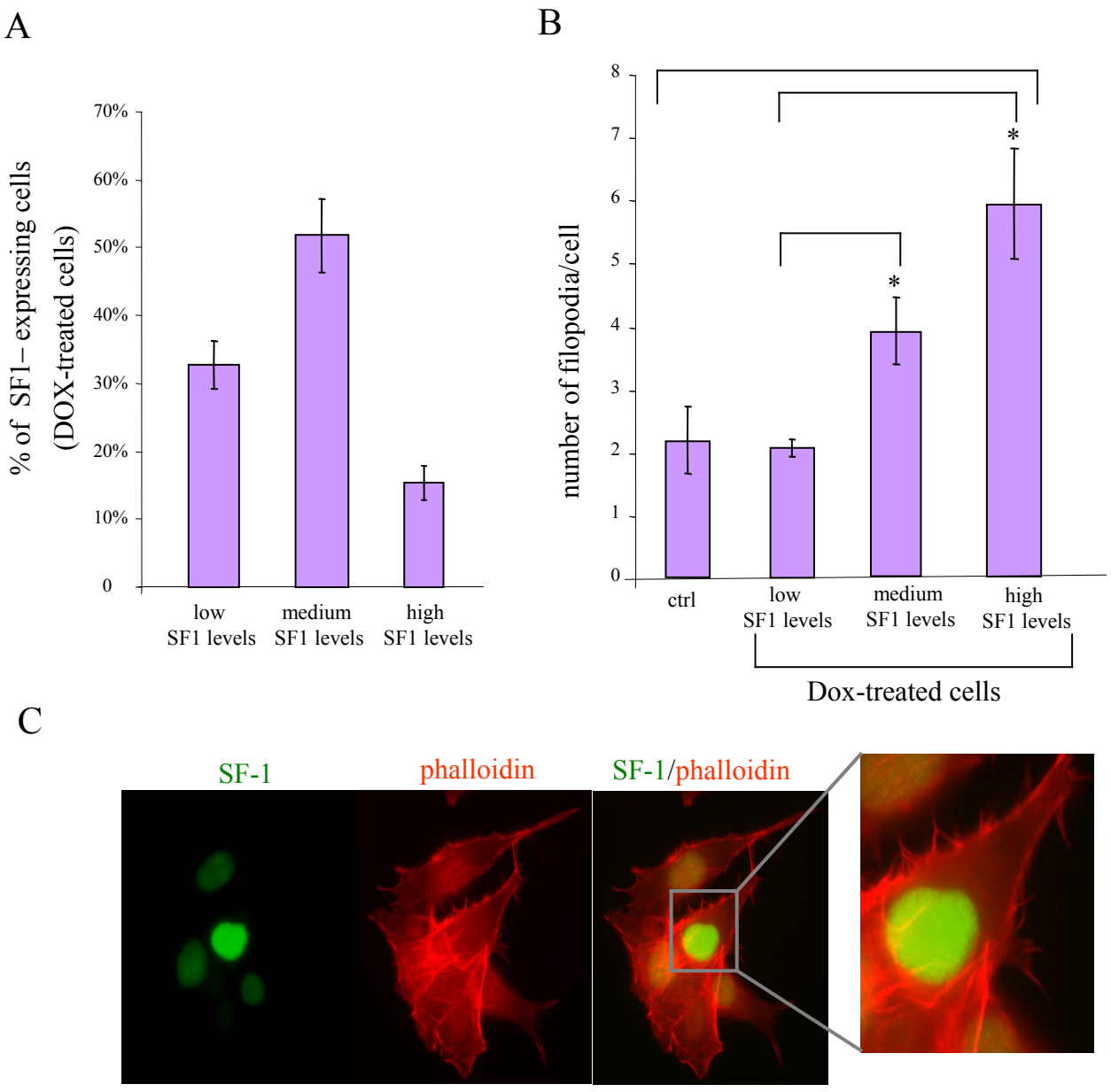

Figure 7. Tight correlation between filopodia number and SF-1 levels. **A**, Percentage of DOX-treated cells expressing low, medium and high SF-1 levels respectively. Data are expressed as mean +/- SEM of three different experiments. **B**, Number of filopodia/cells exhibited by control untreated cells (ctrl) and Dox-treated cells (3 subpopulations according to SF-1 expression levels) after 72 h of Dox treatment. Data are expressed as mean +/-SEM of three different experiments. *, p<0,05 high SF-1 level– expressing cells vs ctrl and low SF-1 level–expressing cells (Student's t-test); * p <0,05 medium SF-1 level– expressing cells vs low SF-1 level– expressing cells (Student's t-test). **C**, H295R TR/SF-1 treated with Dox for 72 h were double-stained for SF-1 (green) and F-actin (phalloidin staining, red). Images of green and red signals are shown (SF-1/phalloidin). The insets shows higher magnification view of the filopodia formed by the cell expressing the highest SF-1 levels. The images are representative of three different experiments.

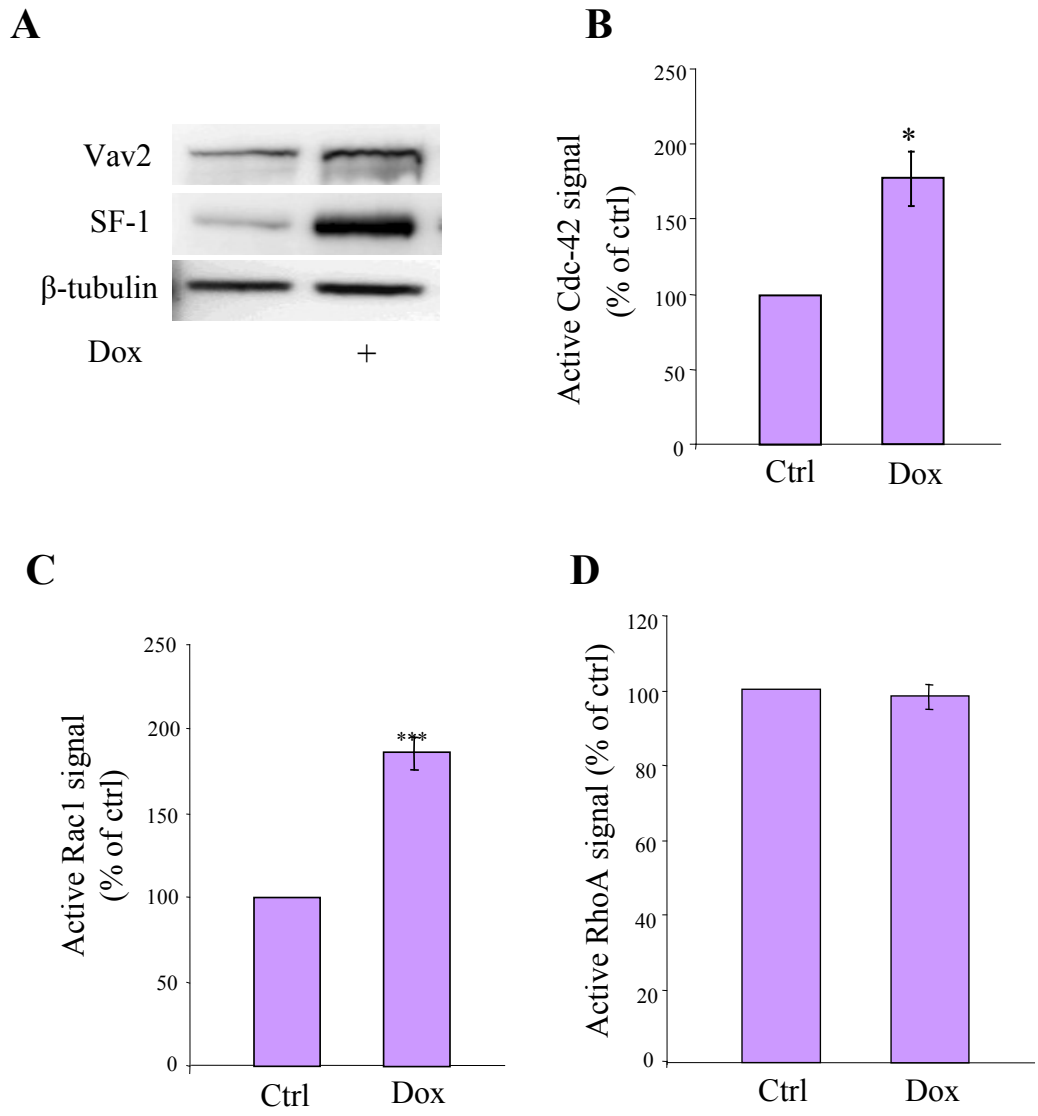

Figure 8. SF-1 and Vav2 induction correlates with increased levels of active Cdc42 and Rac1. **A**, SF-1 and Vav2 expression is induced in H295R TR/SF-1 after 12 h of Dox treatment (1 µg/ml). β-Tubulin expression is shown as a control. Immublots are representative of at least three different experiments. **B**, Active Cdc42 was assessed in H295R TR/SF-1 cells treated with or without Dox for 12 h by G-LISA® assay specific for Cdc42. Active Cdc42 signal is expressed as percentage of control (Ctrl) and reflects an average (+/- SEM) of three different experiments. * $p<0,05$ (Student's t-test). **C**, Active Rac1 was assessed in H295R TR/SF-1 cells treated with or without Dox for 12 h by G-LISA® assay specific for Rac1. Active Rac1 signal is expressed as percentage of Ctrl and reflects an average (+/- SEM) of three different experiments. ***$p<0,001$ (Student's t-test). **D**, Active RhoA was assessed in H295R TR/SF-1 cells treated with or without Dox for 12 h by G-LISA® specific for RhoA. Active RhoA signal is expressed as percentage of Ctrl and reflects an average (+/- SEM) of three different experiments.

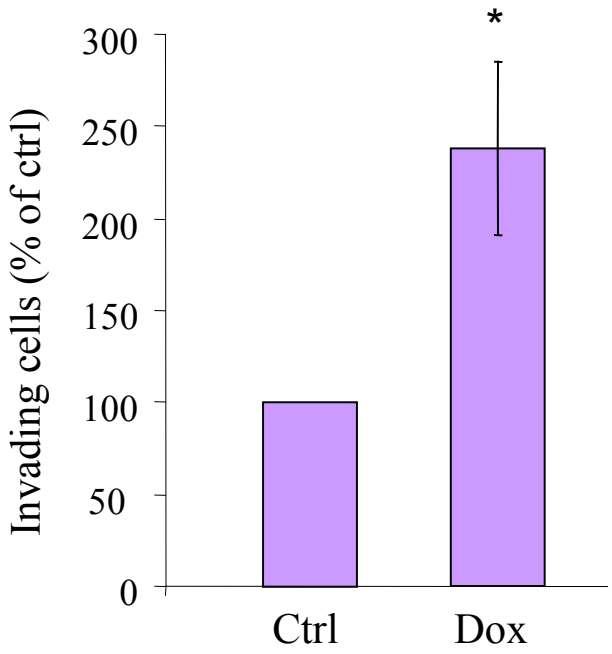

Figure 9. Increased SF-1 dosage promotes adrenocortical carcinoma cell invasion through Matrigel. H295R TR/SF-1 cells were allowed to invade toward 50% FBS for 6 days in the presence or absence of 1 µg/mL Dox. Invasive cells on the bottom of the invasion membrane were stained and quantified. They are expressed in the graph as percentage of control (Ctrl) untreated cells and reflect the mean +/- SEM of four different experiments. *p<0,05 (Student's t-test).