T-type Ca 2+ channels elicit pro-proliferative and anti-apoptotic responses through impaired PP2A/Akt1 signaling in PASMCs from patients with pulmonary arterial hypertension

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ABSTRACT

Idiopathic pulmonary arterial hypertension (iPAH) is characterized by obstructive hyperproliferation and apoptosis resistance of distal pulmonary artery smooth muscle cells (PASMCs). T-type Ca^{2+} channel blockers have been shown to reduce experimental pulmonary hypertension, although the impact of T-type channel inhibition remains unexplored in PASMCs from iPAH patients. Here we show that T-type channels Cav3.1 and Cav3.2 are present in the lung and PASMCs from iPAH patients and control subjects. The blockade of T-type channels by the specific blocker, TTA-A2, prevents cell cycle progression and PASMCs growth. In iPAH cells, T-type channel signaling fails to activate phosphatase PP2A, leading to an increase in ERK1/2, P38 activation. Moreover, T-type channel signaling is redirected towards the activation of the kinase Akt1, leading to increased expression of the anti-apoptotic protein survivin, and a decrease in the pro-apoptotic mediator FoxO3A. Finally, in iPAH cells, Akt1 is no longer able to regulate caspase 9 activation, whereas T-type channel overexpression reverses PP2A defect in iPAH cells but reinforces the deleterious effects of Akt1 activation. Altogether, these data highlight T-type channel signaling as a strong trigger of the pathological phenotype of PASMCs from iPAH patients (hyper-proliferation/cells survival and apoptosis resistance), suggesting that both T-type channels and PP2A may be promising therapeutic targets for pulmonary hypertension.

1. Introduction

Pulmonary arterial hypertension (PAH) is a severe disease caused by vasoconstriction and vascular remodeling of small arteries leading to right heart failure and premature death. Currently approved PAH therapies attempt to restore the balance between vasodilator and vasoconstrictor mediators [1,2]. However, the therapeutic options are limited and are unable to reverse or cure PAH that remains associated with poor prognosis. Hypertrophy, hyperplasia and apoptosis resistance of pulmonary artery smooth muscle cells (PASMCs) are crucial components of the remodeling process in PAH [3]. Several growth factors and associated signaling pathways have already been identified to promote PASMC proliferation during PAH [4–7]. Elevated level of serotonin [4], endothelin 1 [8], PDGF [9], angiotensin II and aldosterone [10] are reported to be key mediators of PAH pathobiology. Recent studies in experimental models of PAH demonstrated a crucial role of the Akt/mammalian target of the rapamycin (mTOR) pathway in PAH progression [11–13]. Several phosphatases regulate Akt1

Abbreviations: CDK4/6, cyclin-dependent kinase 4 and 6; CTL, control; CycD1, cyclin D1; FoxO3A, forkhead transcription factor; I_{CaT}, T-type Ca^{2+} current; OA, okadaïc acid; PAH, pulmonary arterial hypertension; iPAH, idiopathic PAH; PASMC, pulmonary arterial smooth cell; PFA, paraformaldehyde; PP2A, protein phosphatase 2A; RCaG, vector overexpressing Cav3.1; RC, empty vector

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activation. Among them, protein phosphatase 2A (PP2A) and phosphatase and tensin homolog (PTEN) are both considered to be tumor suppressors [14,15].

Previously, we have shown that the T-type voltage-gated Ca^{2+} current (I_{CaT}) generates signaling that participates in PP2A activation, inducing apoptosis in rat cardiomyocytes [16,17]. Interestingly, mediators relevant to PAH are shown to increase I_{CaT} channel expression and current density. Indeed, we have shown that the endothelin-1 and the renin-angiotensin II-aldosterone systems increase I_{CaT} density in rat cardiomyocytes [16–18]. Moreover, in rat glomerulosa cells, activation of the serotonin receptors increases Ca^{2+} influx though I_{CaT} [19], and serotonin receptor blockers, such as fluoxetine, blocks I_{CaT} channel activity [20]. Conversely, the vasodilator nitric oxide (whose production is deficient in PAH) suppressed I_{CaT} in cerebral arterial smooth cells [21].

Few studies assessed the role of I_{CaT} in PAH progression using experimental models. In mice with carotid injury, I_{CaT} is required for neointimal formation [22]. Monocrotaline-induced PAH resulted in I_{CaT}-induced electrical remodeling that altered excitation-contraction coupling in the right ventricular and atrial cells [23,24]. Hypoxia and monocrotaline-induced PAH were associated with a depolarization of PASMC mainly explained by a decrease in voltage-gated K^+ currents (Kv1.5) and two pore K^+ channels (KCNK3) [25,26]. T-type Ca^{2+} channels Cav3.1 and Cav3.2 are low-voltage activated Ca^{2+} channels that open during membrane depolarization, so the decrease of expression/function of Kv and KCNK3 channels should be associated with an elevation in cytosolic Ca^{2+} concentration through I_{CaT} [27,28]. In contrast to wild type mice, mice lacking the Cav3.1 encoding gene do not develop pulmonary arterial wall remodeling when exposed to chronic hypoxia [29].

To our knowledge, only one study has reported the presence of I_{CaT} in human PASMCs that was mediated through Cav3.1 activity [30]. Thus, in the present study, using specific pharmacological and overexpression approaches, we investigated whether I_{CaT} signaling contributes to pathological dysfunction in PASMCs from patients with idiopathic PAH (iPAH).

2. Materials and methods

2.1. Culture conditions

This study was approved by the local ethics committee (CPP Ile-de-France, Le Kremlin-Bicêtre, France). The patients gave informed consent before participating in the study. Pulmonary arteries and frozen lung tissue were sampled at the time of lung transplantation in 12 iPAH patients (7 men aged 34 ± 10 years and 5 women aged 39 ± 15 years). All patients were in New York Heart Association functional class III-IV at the time of lung transplantation and displayed severe pre-capillary pulmonary hypertension. Control pulmonary arteries (CTL) were obtained from 18 patients (12 men aged 61 ± 5 years and 6 women aged 50 ± 14 years) undergoing surgery for localized lung cancer; the control samples used for the study were taken at a distance from the tumor site.

Culture of human PA-SMCs: PA-SMCs were cultured from arteries as already described [4] with minor modifications. Arteries (diameter: 5–10 mm) were kept in DMEM at 4 °C before their intimal cell layer and residual adventitial tissue were stripped off using forceps. The dissected media of the vessels was then cut into small pieces (3–5 mm), which were transferred into 6 well plate (one piece per well). The explants adhered to the support and to allow the PA-SMCs to grow out, the explants were incubated in DMEM supplemented with 20% FCS, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin, 10 U/ml EGF, insuline). After 2 weeks of incubation, the PA-SMCs collected in the culture medium and the vessel tissues were transferred into new cell-culture flasks. Cultured PA-SMCs were used between passages 3 and 7. Explants from the same pulmonary artery were usually transferred several times.

Characterization of cultured PA-SMCs was assessed by positive staining with antibodies against smooth muscle cell α-actin, desmin, and vinculin as described elsewhere [4].

PASMCs were synchronized by serum starvation for 48 h and thereafter stimulated with 10% serum. For western blots analysis, we used 200 nM TTA-A2 (Alomone labs) to block I_{CaT} channels activities and 10 nM okadaïc acid (OA) to block PP2A activity. Cells were treated with blockers for 24 h.

2.2. Cell growth and flow cytometry

Cell growth was assessed by counting the number of cells excluding trypan blue dye. PASMCs were treated with or without TTA-A2 for 8 days with a change in culture medium every 48 h.

Cell cycle phase was by flow cytometry in propidium iodide-stained PASMCs, as previously described [16]. PASMCs were treated with or without TTA-A2 for 48 h.

2.3. Immunohistochemistry and immunocytochemistry

Frozen tissue blocks were prepared and immunostained as previously described [31]. Primary antibodies were rabbit anti-I_{CaT} channel Cav3.1 (ACC-021, 1/100, Alomone) and Cav3.2 (ACC-025, 1/100, Alomone) and mouse anti-α-smooth actin (clone 1A4, 1/200, Sigma Aldrich). After 4% PFA fixation and permeabilization, we used the same procedure to stain cultured cell by immunocytochemistry. Sections were viewed under a LSM 700 microscope (Carl Zeiss, Le Pecq, France). Images were recorded and analyzed with ZEN software (Carl Zeiss).

2.4. Intracellular calcium measurements

Human PASMCs were plated on 18-mm glass coverslips and loaded with 2 μM fura-2 AM dissolved in DMSO plus 20% Pluronic acid (Life Technologies), and then incubated at 37 °C for 30 min in darkness in Krebs solution (in mM: 135 NaCl, 5.9 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, pH 7.4).

Loaded cells were washed twice with the physiological solution before imaging. Ratiometric Fura-2 images of Ca^{2+} signal were obtained as previously described [32]. Experiments were performed at room temperature and PASMCs were perfused with high K^+ Krebs solution (in mM: 82 NaCl, 59 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, pH 7.4). The recording protocol consisted of 1 min perfusion with normal Krebs (to record the baseline calcium), 30-s high K^+ Krebs (to estimate the response to depolarizing pulses) and wash with Krebs for 2 min.

Image acquisition in selected cells and analysis were performed with Metafluor 7.8 imaging software (Molecular Devices).

2.5. Preparation of total, nuclear and cytosolic extracts

Cells were washed twice with phosphate buffered saline (PBS), scraped with trypsin (Gibco-BRL) following 2 min incubation at 37 °C. Protocol of proteins extraction was based on the procedure originally described by Dignam et al. [33]. Briefly, after cold centrifugation at 3000 g for 5 min and washing once with PBS, the cell pellet was re-suspended in ice-cold hypotonic buffer A containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM Phenylmethylsulfonyl fluoride (PMSF). After a cold centrifugation at 4000 g for 15 min, the supernatant of cytosolic proteins was collected and the cell pellet was re-suspended in ice-cold hypertonic buffer B containing 20 mM HEPES pH 7.9; 25% glycerol, 550 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT and a protease inhibitor cocktail (Roche). After 30 min incubation on ice and a subsequent centrifugation at 20000 g, the

Table 1
Characteristics of primary antibodies used in Western blots studies.

Target	Host specie	Supplier	Reference	Concentration
Akt1	Goat	Santa Cruz	1618	1/2000
phospho-AKT1 (Thr308)	Rabbit	Cell signaling	4056	1/2000
b-actin	Mouse	Sigma	A5316	1/80000
Caspase 9	Mouse	Cell signaling	9508	1/2000
Cav3.1	Rabbit	Alomone	ACC-021	1/200
Cav3.2	Rabbit	Alomone	ACC-025	1/200
CDK4	Mouse	Cell signaling	2906	1/2000
CDK6	Mouse	Cell signaling	3136	1/2000
CycD1	Mouse	Cell signaling	2926	1/2000
FoxO3a	Rabbit	Cell signaling	12,829	1/1000
phospho-FoxO3a Ser253	Rabbit	Cell signaling	13,129	1/1000
ERK1/2 (p44/42)	Rabbit	Cell signaling	9102	1/4000
phospho-ERK1/2 (Thr202/Tyr204)	Rabbit	Cell signaling	9101	1/4000
P21 Waf1/Cip1	Rabbit	Cell signaling	2947	1/2000
PP2AC	Rabbit	Cell signaling	2259	1/2000
P38	Rabbit	Cell signaling	9212	1/2000
phospho-P38 (Thr180/Tyr182)	Rabbit	Cell signaling	9211	1/2000
Survivin	Rabbit	Cell signaling	2808	1/2000

supernatant of the nuclear proteins was collected. Total proteins were extracted using buffer B in the same conditions as nuclear proteins. The concentrations of proteins were determined by Bradford protein assay (Biorad).

2.6. Western blot analysis and phosphatase assay

Proteins were separated by 8% sodium dodecyl sulphate (SDS)-polyacrylamide gels electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoridenitrocellulose (PVDF) membranes (Millipore Corporation). After blocking in 5% bovine serum albumin (BSA, sigma), membranes were incubated with the appropriate primary antibody in 5% BSA/0.05% Tween-20/Tris Buffered Saline for 1 h at room temperature or overnight at 4 °C. Subsequently, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (cell signaling) for 1 h at room temperature. Blots were re-probed with an antibody against β-actin for normalisation. Protein bands were visualised with the Lumi-lightplus blotting Kit (Roche) and analysed with Gene Tools from Syngen (Cambridge, UK). The antibodies used for western blots were listed in Table 1.

The PP2A/PP1 phosphatase activity was measured using a serine/threonine phosphatase assay system (Promega, Madison, WI) as previously described [16]. Phosphatase activity was calculated as the rate of Pi released from a pre-phosphorylated peptide and normalized with respect to basal levels (sample without phosphopeptide).

2.7. Transfection

The human Cav3.1 (RCaG) and Cav3.2 (RCaH) cDNA-containing plasmid were purchased from OriGene (CACNA1G:RC214754, and CACNA1H:RC212772) and transferred into PASMC using lipofectamine LTX-plus™ (Invitrogen). The empty vector was used to control for transfection (noted as RC hereafter).

2.8. Statistical analysis

The data are expressed as the mean ± SE ($n \geq 5$). Two-way ANOVA followed by the Bonferroni test was used to compare the time dependent changes in cell number (Fig. 1A and B).

Statistical significance of differences between control and PAH groups was estimated using one-way ANOVA followed by Dunnett's test. Statistical significance of differences between untreated and TTA- or OA-treated cells was performed using two-tailed Student's *t*-test.

Differences were considered significant if $P < 0.05$.

3. Results

3.1. Detection of the T-type Ca^{2+} channels in human lung tissues and in PASMCs

In cardiovascular system, there are 2 known types of α subunit of T-type Ca^{2+} channels; Cav3.1 and Cav3.2. By immunostaining and confocal imaging, we revealed the presence of both the Cav3.1 and Cav3.2 isoform in human lung tissue and cultured PASMCs from CTL and iPAH patients (Fig. 1). Consistent with previous results obtained with human PASMCs [30], Cav3.1 labeling was found in the media of large and small pulmonary arteries from CTL patients (Fig. 1A).

We also detected expression of Cav3.2 that has not been previously observed. Lung tissue from iPAH patients displayed remodeled pulmonary arteries strongly expressing I_{CaT} channels. The expression of Cav3.1 and Cav3.2 was observed in cultured PASMCs from CTL and iPAH patients (Fig. 1B). Cav3.1 labeling was mainly present in the cytoplasm, whereas Cav3.2 labeling was strongly localized in the nucleus. Again, immunostaining was stronger in iPAH cells compared to CTL cells. Finally, we showed strong immunostaining for Cav3.1 in PASMCs transfected with a vector overexpressing Cav3.1 (RCaG) compared to cells transfected with an empty vector (RC) (Fig. 1C), validating the antibody and over-expressing strategy.

To evaluate the involvement of T-type Ca^{2+} channels to Ca^{2+} homeostasis in human PASMC from control and iPAH patients, we evaluated the intracellular Ca^{2+} signal to a pulse of high K^+ Krebs solution (60 mM to depolarize PASMC) in presence or in absence of T-type Ca^{2+} channels blocker TTA-A2 (200 nM) (Fig. 1D).

In Fura-2 loaded cells, the cytosolic Ca^{2+} measures were expressed as the ratio of Fura-2340/380 nm (Fig. 1D). The basal cytosolic Ca^{2+} concentration was higher in iPAH cells than in control cells (Fig. 1E, red vs black), and both was significantly reduced in presence of TTA-A2, suggesting that T-type Ca^{2+} channels activities contribute to resting intracellular Ca^{2+} concentration. The excess of cytosolic Ca^{2+} concentration seen in iPAH cells vs control resulted from T-type Ca^{2+} channels activity which was abolished by TTA-A2 application.

The elevation of intracellular Ca^{2+} concentration induced by plasma membrane depolarization with high K^+ medium was quantified by measuring the area under the curve (Fig. 1E). This Ca^{2+} elevation was partly reduced in cells treated with TTA-A2 (Fig. 5DF). However in high K + stimulated PASMCs the contribution of T-type Ca^{2+} channels was not significantly different between control and iPAH cells.

Lastly, the amount of the T-type Ca^{2+} channels Cav3.1 and Cav3.2 were evaluated in pulmonary arteries by western blots. Interestingly, we found an increase in the amount of Cav3.1 in iPAH (Fig. 5G). Also, the amount of Cav3.2 tends to increase in iPAH but the differences between iPAH and control were not significant.

3.2. I_{CaT} channel signaling increased iPAH PASMCs proliferation

As expected, we confirm that iPAH PASMCs have a faster growth compared to CTL cells (Fig. 2A). Indeed, cultures of iPAH cells were confluent and exhibited contact inhibition 48 h before those of CTL (Fig. 2A, left panel). Consistently, the cell cycle analyzed by flow cytometry showed that the percentage of iPAH PASMCs in the G0/G1 cell cycle phase was significantly reduced compared to CTL cells, whereas the percentages in the S and G2 phases were significantly increased reducing cell cycle duration (Fig. 2A, right panel). Thus, the hyperproliferative phenotype of PASMCs from iPAH patients results from an altered control mechanism of both G1/S and G2/M checkpoints.

Specific pharmacological blockade of the I_{CaT} channel by 200 nM TTA-A2 reduced cell proliferation in both CTL and iPAH, with a marked effect in iPAH cells visible from the start of treatment (Fig. 2B). Flow

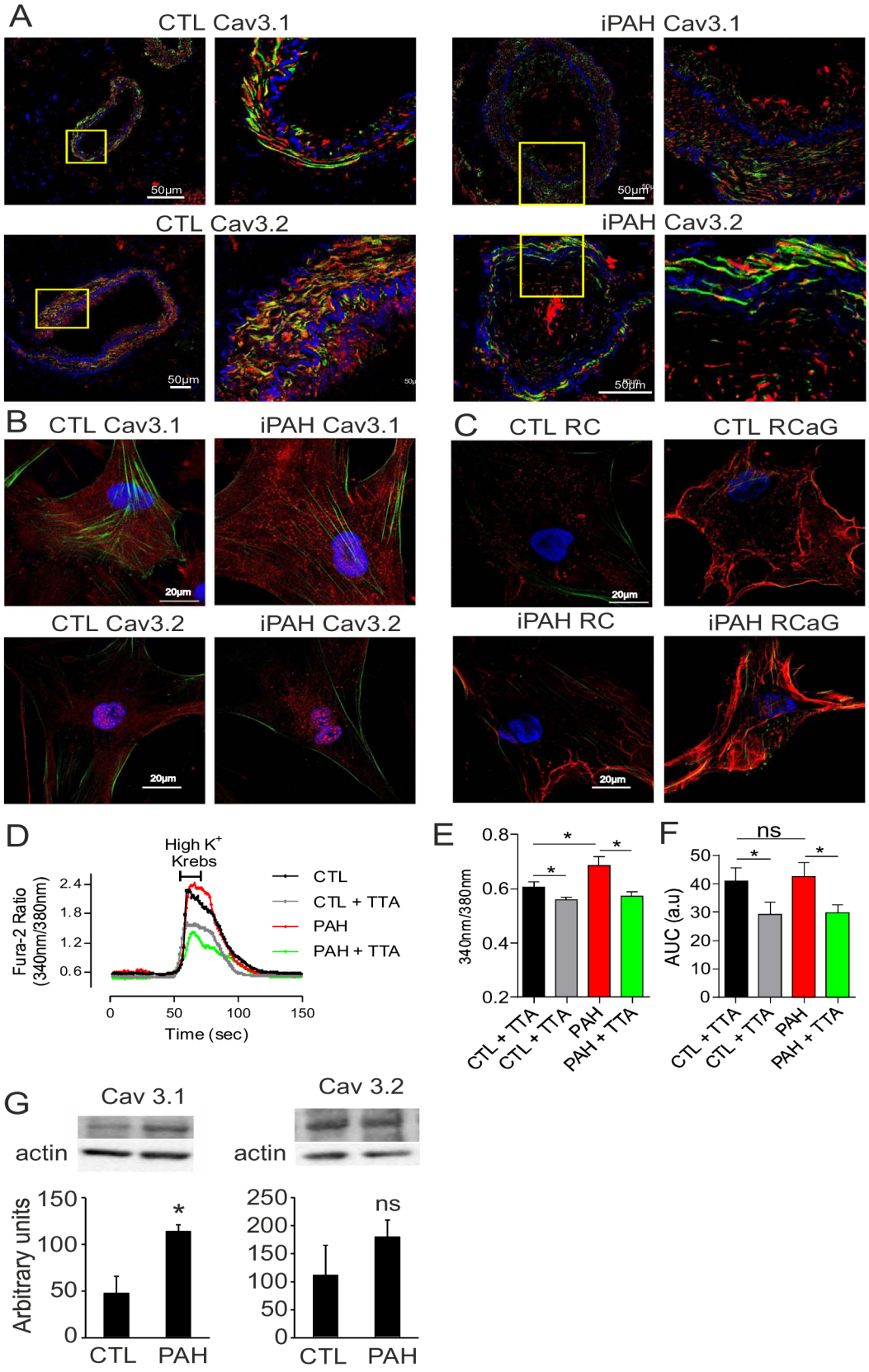

Fig. 1. Expression of I_{CaT} channels in human lungs and in PASMCs from control and iPAH patients. Confocal images of immunostaining for Cav3.1 and Cav3.2 (A) in lung tissues from control (CTL) and iPAH (PAH) patients: 40 × objective, (B) in PASMCs from CTL and iPAH patients and (C) in PASMCs from CTL and PAH cells transfected with empty vector (RC) or cDNA overexpressing Cav3.1 channel vector (RCaG). B and C: 63 × objective. Red fluorescence for I_{CaT} channels, green for α-actin and blue DAPI for nuclei. D) Intracellular Ca^{2+} levels measured with fura-2 AM in both CTL and iPAH PAMCs expressed as ratio of fluorescence intensity at 340 and 380 nm. Representative traces are for resting levels of Ca^{2+} and the response to high K + (60 mM) Krebs in presence or not of 200 nM TTA-A2. E) Quantification of resting baseline intracellular Ca^{2+} levels expressed as fluorescence ration 340 nM/380 nM and F) quantification of the area under the curve (AUC) expressed as arbitrary units (a.u) (26-45 cells). G) Western blots showing the relative amount of Cav3.1 and Cav3.2 channels in pulmonary arteries from CTL (n = 5) and iPAH patients (n = 5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this

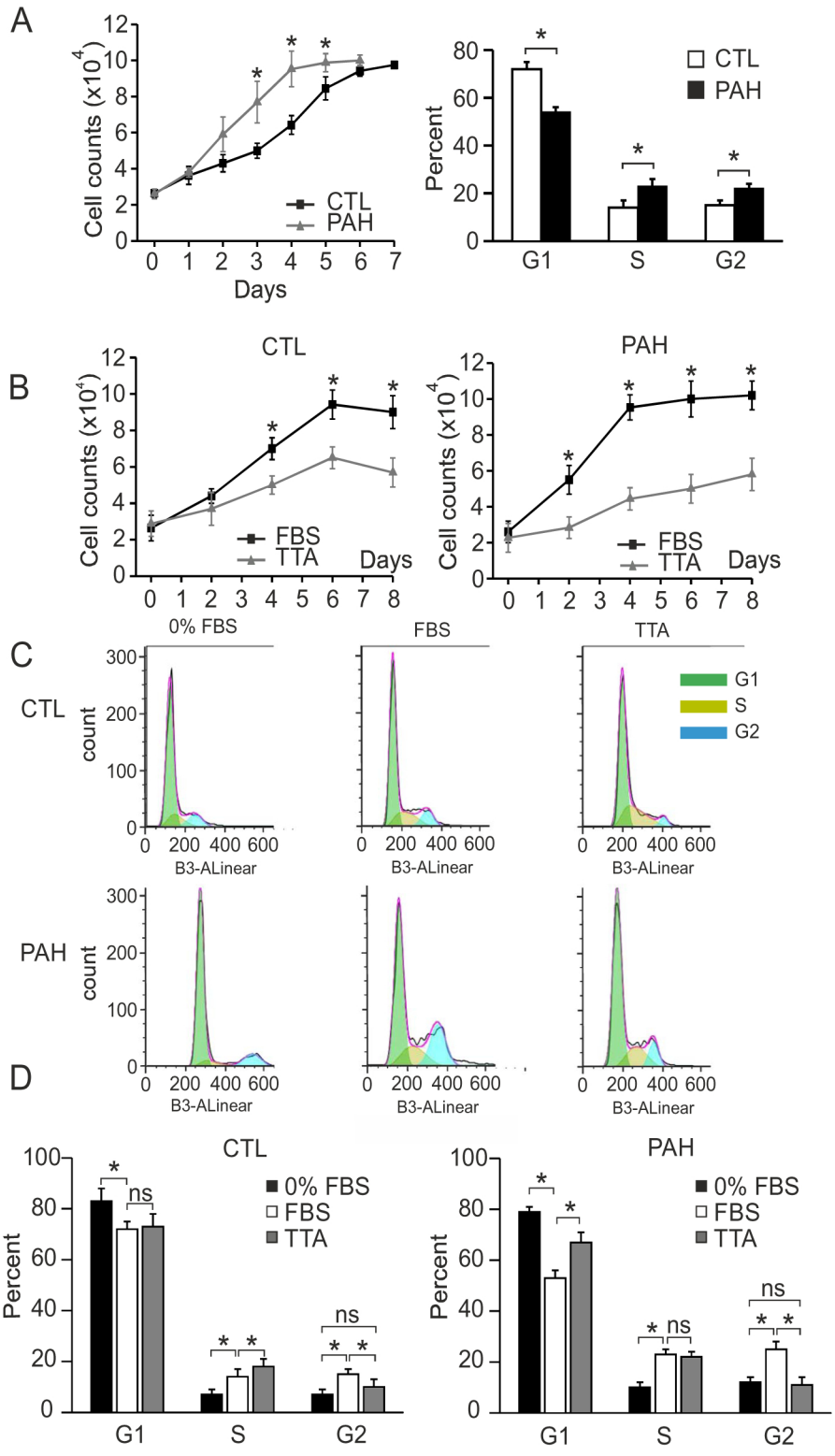

Fig. 2. I_{CaT} impacts an unexpected phase of the cell cycle during PAH. A) Hyperproliferative phenotype of PASMCs from iPAH patients. Left, growth curves from CTL and iPAH cells. The curves show the hyperproliferative phenotype of cells from patients with iPAH compared to CTL. $n = 6$ for CTL and $n = 7$ for iPAH. Right, cell cycle analysis by flow cytometry. The histogram represents the percentage of cells present in the different cell cycle phases. B) Effect of I_{CaT} channels blocker on cell growth. PASMCs were maintained in growth medium (FBS) with or without TTA-A2 (TTA), $n = 6$ for CTL and $n = 7$ for iPAH. C) Effect of I_{CaT} channels blocker on cells cycle phases. The cells were maintained in a serum-free (0% FBS) or FBS with or without 200 nM TTA-A2 for 48 h. Examples of MACS analysis (above). Percentage of PASMCs in G0/G1, S and G2/M (below). The blockade of I_{CaT} channels reduces S/G2 transition in CTL cells and the G1/S plus S/G2 transition in iPAH cells. $n = 10$ for CTL and $n = 8$ iPAH.

cytometric analysis showed that TTA-A2 treatment affected the transition between S and G2 phases in CTL cells (Fig. 2C, left). Interestingly, in iPAH cells, T-type Ca^{2+} channel blockade mainly altered the transition to G1/S, in addition to delaying the transition to S/G2 (Fig. 2C, right). Therefore, I_{CaT} generated Ca^{2+} signaling promotes cell proliferation through specific cell cycle regulation in PAH PASMCs.

3.3. I_{CaT} channel signaling failed to regulate $P21^{CIP/Waf1}$ in iPAH cells

Because the G1/S transition was altered in PAH PASMCs, we looked for the relative amount of proteins involved in the G1/S transition of the cell cycle (Fig. 3). We tested the cyclin-dependent kinases 4 and 6 (CDK4/6), the cyclin D1 (CycD1) and the cell cycle inhibitor protein $P21^{CIP1/WAF1}$ (P21) (Fig. 3A). The levels of CDK4/6 and CycD1 remained unchanged between CTL and iPAH, whereas the level of P21

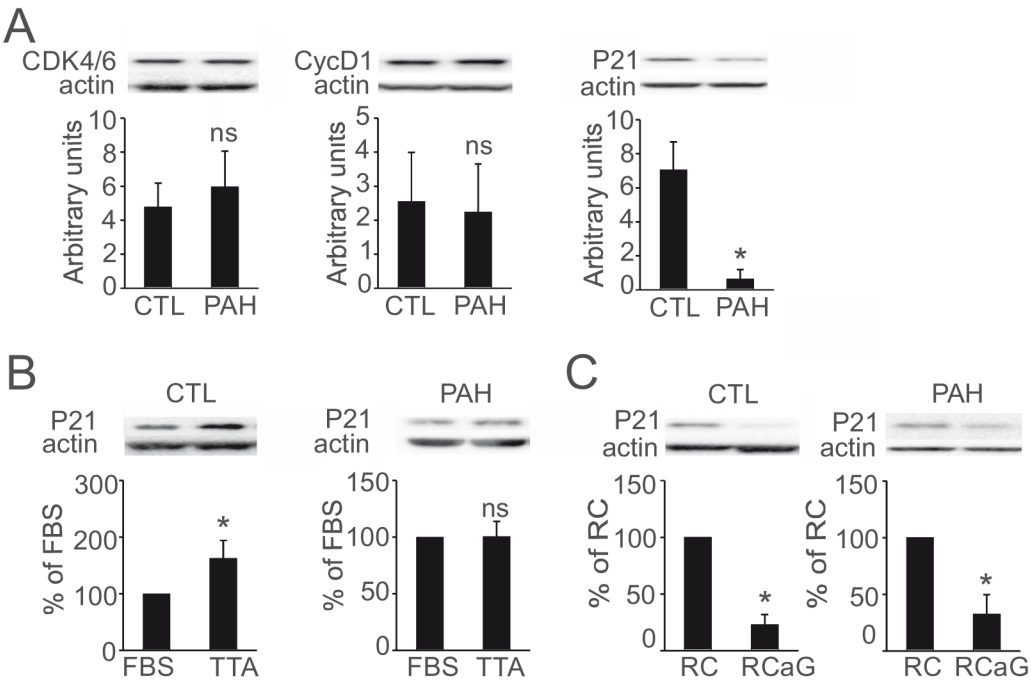

Fig. 3. I_{CaT} channel signaling fails to downregulate the amount of P21 in iPAH cells. A) Western blots showing the relative amounts of CDK4/6, CycD1 and P21 in CTL and iPAH cells. $n = 8$ for CTL and iPAH. B) Effect of I_{CaT} channels blocker on the amount of P21. $n = 10$ for CTL and $n = 7$ for PAH. C) Effect of the overexpression of Cav3.1 on P21 amount. $n = 6$ for CTL and iPAH.

was dramatically lowered in iPAH cells.

The blockade of T-type Ca^{2+} channels induced a significant increase in the P21 steady-state level in CTL but not in iPAH cells (Fig. 3B). However, in cells overexpressing the Cav3.1 channels, the amount of P21 decreased in both CTL and iPAH cells (Fig. 3C). Hence, I_{CaT}-induced downregulation of P21 expression that occurs in CTL was abolished in the iPAH cells, but could be restored by an overexpression of Cav3.1.

3.4. I_{CaT} channel signaling fails to activate PP2A in iPAH cells

Because PP2A is a critical regulator for cell cycle progression and because I_{CaT} may influence PP2A activation [16], the level of PP2A activity was assessed in CTL and iPAH cells, as well as the putative effect of TTA-A2 (Fig. 4). We found that iPAH cells reduced phosphatase activity by 50%. Almost no phosphatase activity could be measured after the use of 12 nM okadaïc acid, which is required for PP2A inhibition, assuming that the measured phosphatase activity was mostly due to PP2A activity (Fig. 4A).

The variation in PP2A activity between CTL and iPAH cells was not due to variation in the amount of PP2A (Fig. 4B). Moreover, TTA-A2 halved the PP2A activity observed in CTL cells that was not associated with a variation in the steady state level of PP2A (Fig. 4C). In contrast, in iPAH cells, the remaining PP2A activity was insensitive to TTA-A2 (Fig. 4A), such that I_{CaT} no longer activated PP2A. Finally, cells overexpressing Cav3.1 exhibited an increase in PP2A activity in both CTL and iPAH cells (Fig. 4D) that can be partly reversed in presence of TTA-A2 (data not shown). Hence, iPAH cells displayed a deficiency in PP2A activity, resulting from a decoupled signaling pathway between I_{CaT} and PP2A that could be restored by overexpression of Cav3.1.

3.5. I_{CaT} channel signaling is redirected from ERK1/2 inactivation towards Akt1 activation iPAH cells

ERK1/2, P38 and Akt1 signaling are important regulators of cell proliferation, survival and apoptosis. PASMCs from iPAH patients exhibited an increase in the activation of ERK1/2, P38 and Akt1. Except for P38 whose synthesis was also increased in iPAH cells, the increase in pERK1/2 and pAkt1 was associated with a kinase/phosphatase imbalance rather than an increase in gene expression (Fig. 5A).

The use of a T-type channel blocker or PP2A inhibitor had no impact on ERK1/2, P38 and Akt1 protein synthesis (supplement data 1AB), while both dysregulated ERK1/2, P38 and Akt1 phosphorylation (Fig. 5BC). In CTL cells, the blockade of I_{CaT} or PP2A induced an increase in the phosphorylation of ERK1/2 and Akt1 (Fig. 5B). In contrast, the phosphorylation of P38 was regulated by PP2A through a mechanism independent of I_{CaT}-induced PP2A activation. In iPAH cells, PP2A blockade had the same effect as in CTL cells by increasing kinase phosphorylation (Fig. 5C). As expected from the decoupled signaling between I_{CaT} and PP2A in iPAH cells, T-Type channel inhibition by TTA A2 had no effect on ERK1/2 phosphorylation in these cells. Unexpectedly, TTA-A2 provoked a decrease in Akt1 phosphorylation suggesting that I_{CaT} signaling was redirected to Akt1 activation in iPAH cells. This aberrant I_{CaT} signaling was also observed in Cav3.1-transfected cells in which the overexpression of Cav3.1 induced an increase in Akt1 activation, without an associated increase in protein synthesis (Fig. 5D and supplement data 1C).

The consequences of the overexpression of Cav3.1 (or Cav3.2, supplement data 2) on CTL and iPAH cell cycles were similar, decreasing the number of cells in G1 phase (Fig. 5E). As expected in cells exhibiting hyper-activation of PP2A [34], overexpression of Cav3.1-induced PP2A activation results in G2 cell cycle arrest in both CTL and iPAH cells.

3.6. I_{CaT} channel signaling promotes survival and anti-apoptosis phenotype in iPAH cells

We further investigated whether I_{CaT}-induced Akt1 activation contributed to cell survival and resistance to apoptosis in iPAH cells. We assessed the relative amount of caspase 9, an inhibitor of apoptosis, the protein survivin and the pro-apoptotic transcription factor FoxO3A (Fig. 6A). Compared to CTL cells, the iPAH cells presented a deficit in pro-caspase 9 (without change in the amount of cleaved caspase 9, data not shown), an excess of survivin and an over-inactivation of FoxO3A (increase in phosphorylated forms). These were characteristic hallmarks of the apoptotic resistance phenotype described in PASMCs from iPAH patients. The blockade of I_{CaT}/ PP2A signaling pathway in CTL cells reduced the activation of caspase 9 (Fig. 6B) and increased the amount of survivin protein (Fig. 6C). Unexpectedly, in iPAH cells, neither I_{CaT} nor PP2A signaling was able to regulate caspase cascade

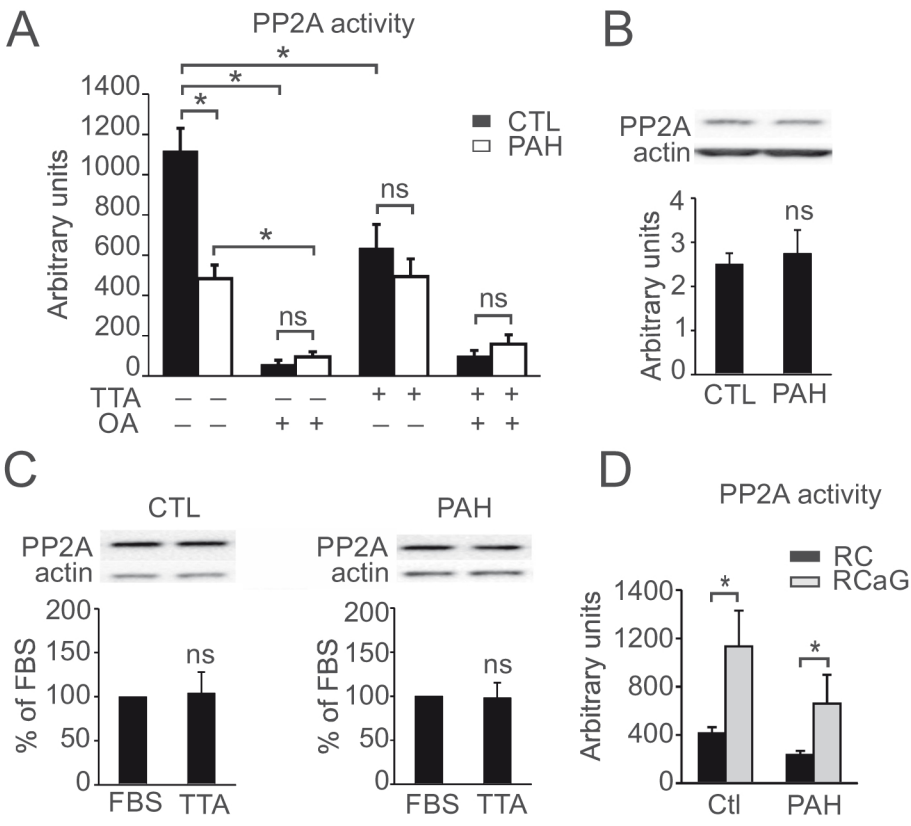

Fig. 4. I_{CaT} channel signaling fails to activate PP2A in iPAH cells. A) Quantification of PP2A/PP1 activity. Cells were cultured with or without TTA and the reactions were performed with or without a PPA2 inhibitor, 12 nM okadaïc acid (OA). iPAH cells presents only half of the PP2A/PP1 activities observed in CTL cells. I_{CaT} blockade reduces by 50% PP2A activity in CTL cells but not in iPAH cells. I_{CaT}–induced PP2A activation is lacking in iPAH cells. $n = 8$ for CTL and PAH. B) Western blots showing no change in the amount of PP2A in CTL and iPAH cells. $n = 7$ for CTL and iPAH. C) I_{CaT} blockade has no effect on the amount of PP2A. $n = 7$ for CTL and $n = 6$ for iPAH. D) PP2A activity increased in cells overexpressing Cav3.1. $n = 6$ for CTL and iPAH.

through caspase 9 (Fig. 6D).

In contrast to CTL cells, in iPAH cells the amount of survivin decreased after I_{CaT} channel blockade, but this was insensitive to PP2A inhibition (Fig. 6E). Therefore, in iPAH cells, I_{CaT}-induced Akt1 activation up-regulated survivin, but PP2A-induced Akt1 inactivation failed to reduce survivin.

Because Akt1-induced FoxO phosphorylation triggers FoxO3A cytoplasmic localization and degradation, we assessed the amount and the phosphorylated status of FoxO3A in the cytoplasmic (Cyt) (Fig. 6F) and nuclear (Nuc) (supplement data 3) fraction of the PASMCs. In CTL cells, the blockade of I_{CaT}/PP2A signaling elicited FoxO3A phosphorylation and degradation consistently with an increase in Akt1 activation in this context (Fig. 6E and supplemental data 3 AB). In iPAH cells, the blockade of I_{CaT}-induced Akt1 activation reduced FoxO3A phosphorylation promoting its nuclear transcriptional function (Fig. 6E and supplemental data 3 AC). Finally, the impact of PP2A signaling on FoxO3A regulation was similar in iPAH and in CTL cells (Fig. 6E and supplement data 3).

In PASMCs overexpressing Cav3.1 channel, the amount of survivin and pFoxO increased in both CTL and iPAH transfected cells (supplement data 4). Therefore, overexpression of I_{CaT} channel induced an apoptosis resistance phenotype in CTL and reinforced the pathological phenotype in iPAH cells.

4. Discussion

The combination of I_{CaT} channel blockade and I_{CaT} channel overexpression strategies has demonstrated the critical impact of impaired I_{CaT} signaling on proliferation, survival and apoptosis resistance of PASMCs from iPAH patients (Fig. 7 and supplement data 5).

In the present study we show that i) I_{CaT}-induced PP2A activation regulates ERK1/2 and Akt1 activation in CTL cells; ii) I_{CaT} signaling is disrupted in iPAH, decreasing PP2A activity by half; iii) I_{CaT} signaling is readdressed to Akt1 activation in iPAH cells; iv) I_{CaT}-induced Akt1 activation increases survivin amount and FoxO3A inactivation; v) The blockade of I_{CaT} channels alters cell cycle and proliferation in iPAH cells and reverses the pathological phenotype of PAH cells through arrest of I_{CaT}-inducing Akt1 activation; and vi) the overexpression of T-type channels restores the signaling between I_{CaT} and PP2A in iPAH cells, but exacerbates Akt1 signaling.

For the first time, we show that in addition to Cav3.1, human PASMCs express the Cav3.2 channel. Moreover, immunostaining and western blots from pulmonary arteries suggests an increase in I_{CaT} channel (Cav3.1 and Cav3.2 isoform) expression in large and small remodeled arteries in the lungs of iPAH patients and also in subsequent PASMC cultures. At least, T-type Ca^{2+} channels were involved in the resting membrane potential of PASMCs. In experimental PH induced by chronic hypoxia exposure, I_{CaT} was previously shown to be enhanced [35] and implicated in the development of hypoxic PAH [29]. Moreover, the use of an I_{CaT} channel blocker or the deletion of Cav3.1 encoding gene protects animals against the development of chronic hypoxic PH [29].

In studies of systemic vascular smooth muscle cells, multiple functions have been assigned to I_{CaT} channels, including cell proliferation [30,36]. Indeed, in iPAH cells we found that the blockade of I_{CaT} channels induces a slower G1/S transition and has a drastic effect on cell growth rate. This effect is not mediated through a regulation of P21 expression in iPAH cells –Fig. 7), as blockade of I_{CaT} fails to decrease the amount of P21.

P21 plays a critical and versatile role in cell growth. A high concentration of P21 acts negatively on cyclin-mediated cell cycle progression, whereas a low concentration acts positively stabilizing cyclin D/CDK complexes. In vascular smooth cells, P21 promotes the functional interaction between CycD1 and CDK4, inducing cell proliferation, whereas overexpression of P21 results in growth suppression [37]. The level of P21 is dramatically lowered in iPAH cells compared to CTL cells; this likely increases the rate of proliferation of PASMCs in iPAH.

Furthermore, the impact of I_{CaT} on iPAH cells proliferation is not driven by classic targets of PP2A such as MAPkinase signaling, since in this context I_{CaT} is no more able to activate PP2A (Fig. 7). Indeed, one of

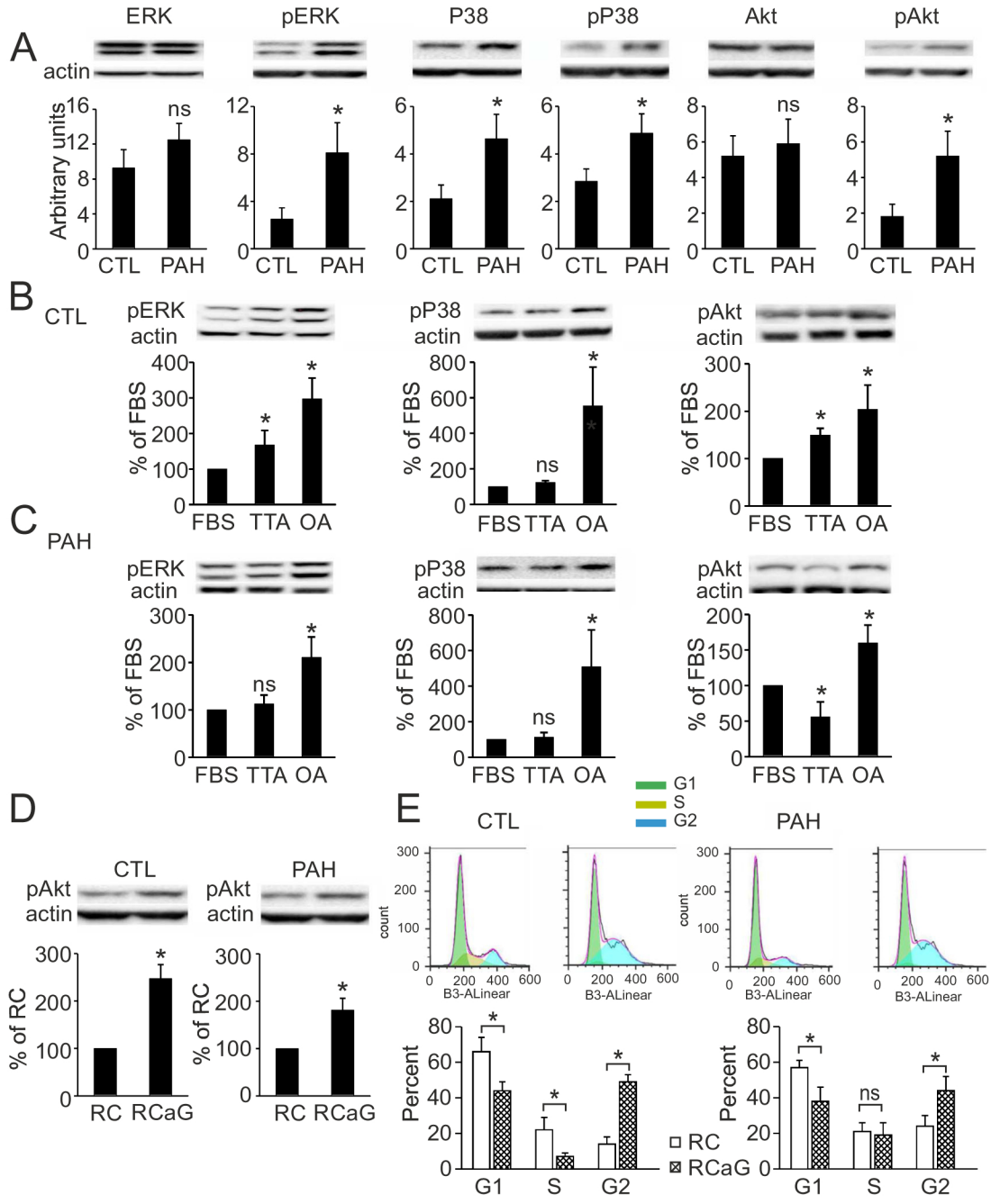

Fig. 5. I_{CaT} channel signaling is redirected from ERK1/2 inactivation towards Akt1 activation in iPAH cells. A) Relative amount of total and phosphorylated ERK1/2, P38 and Akt1 in CTL and in iPAH cells. $n = 8$ for CTL and iPAH. Effect of TTA and OA on ERK1/2, P38 and Akt1 phosphorylation in CTL cells (B) and in iPAH cells (C). $n = 8$ for CTL and $n = 7$ for iPAH. Effect of the overexpression of Cav3.1 on Akt1 phosphorylation (D) and cells cycle phases (E). $n = 6$ for CTL and iPAH.

the main findings in this study was demonstrating that the defect in PP2A activity in iPAH cells was mainly explained by the disruption of I_{CaT}-inducing PP2A activation. Following this, the defect in PP2A activity results in over-activation of MAP kinases (ERK1/2 and P38) and Akt1 signaling (Fig. 7). These perturbations that promote cell proliferation and anti-apoptotic responses [38] have also been observed in experimental models and patients with PAH of various etiologies [10,39]. We found that the decrease in PP2A activity is not due to a defect in PP2A catalytic subunit synthesis, so multiple origins can be considered. PP2A is a trimeric holoenzyme that forms complexes with a plethora of regulatory subunits, and mutation in the structural subunit has been associated with human cancer [40]. Moreover, alterations in posttranslational modification, such as phosphorylation and methylation or dysregulation of the intracellular inhibitors I_1^{PP2A} and I_2^{PP2A} may account for the observed deficiency in PP2A activity in PAH PASMCs. Further studies will be needed to elucidate the mechanism by which I_{CaT}-generated Ca^{2+} signaling becomes inefficient enough to activate PP2A during PAH.

The impact of I_{CaT} on iPAH cell proliferation is likely to be due to an over-activation of Akt1 signaling that is the second major message of this study (Fig. 7). Indeed we showed that I_{CaT} signaling is redirected toward Akt1 activation in PAH PASMCs. Recent studies showed the deleterious role of Akt1 in PAH progression. In hypoxic pulmonary hypertension, the Akt1-S473/ mTORC1 signaling pathway is associated with PASMC proliferation [12,41], and mice with an invalidated Akt1 encoding gene are protected against pulmonary hypertension [13]. The relationship between I_{CaT} and Akt1-T450/mTORC2 is seen in brain tumor cells eliciting apoptosis resistance [42], where blockade of I_{CaT}

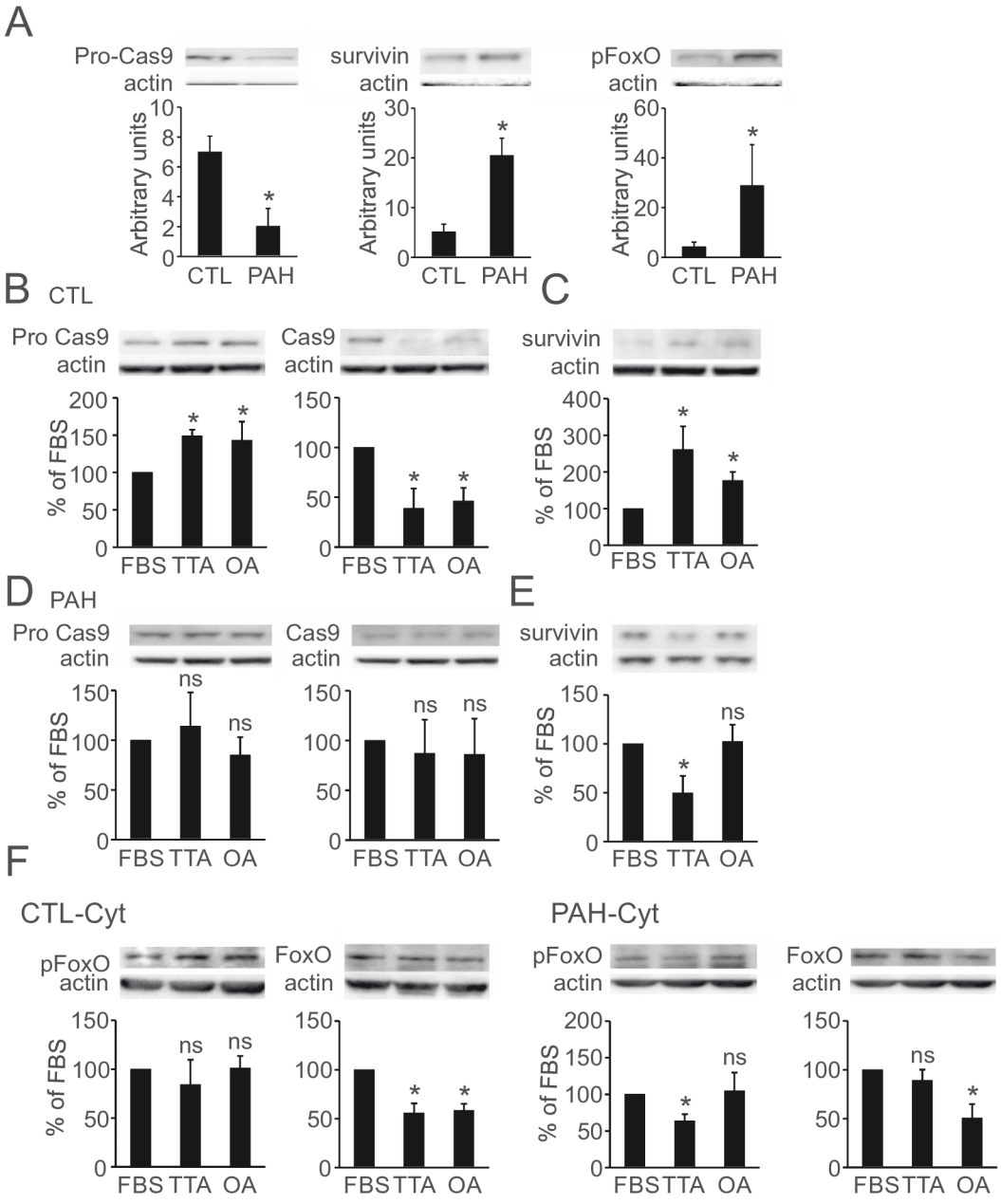

Fig. 6. I_{CaT} channel signaling impairs apoptosis and survival signals in iPAH cells. A) Relative amount of Pro-caspase 9, survivin and phosphorylated FoxO3A in CTL and iPAH cells. $n = 6$ for CTL and PAH cells. Effect of TTA and OA on Caspase 9 activation B) and survivin C) steady state level in CTL cells $n = 6$ and in PAH cells $n = 8$ (D-E). F) Effect of TTA and OA on the amount of phosphorylated FoxO3A localized in the cytoplasmic fraction of CTL (CTL-Cyt) and PAH (PAH-Cyt) cells. $n = 6$ for each condition.

inhibited mTorC2-induced Akt1-S473 phosphorylation and, to a lesser extent, PDK1-induced Akt1-T308 phosphorylation. Here, we show for the first time an increase in PDK1-induced Akt1-T308 phosphorylation in PASMCs from iPAH patients that result in part from a deficiency in PP2A, and also from activation through I_{CaT}-generated Ca^{2+} signaling. Consequently, anti-apoptotic markers such as survivin are increased and pro-apoptotic markers such as FoxO3A are decreased. These data are consistent with previous reports from experimental PAH showing a beneficial effect of a lowered expression of survivin [43,44] and the deleterious consequence of an inactivation of FoxO [45]. Unexpectedly, regulation of the caspase cascade by the PP2A/Akt1 axis seen in control PASMCs is absent in iPAH cells.

We attempted to reconnect I_{CaT}/PP2A signaling in PAH cells by overexpressing T-type channels. Indeed we found that Cav3.1 overexpression restores I_{CaT}/PP2A signaling in iPAH cells thus reduces ERK1/2 activation. Unfortunately, T-type channel overexpression also exacerbates the PAH phenotype through an upregulation of Akt1 and an increase in survivin and inactivated FoxO3A. Consistent with the fact that increases in I_{CaT} channel expression provokes aberrant signaling, overexpression of Cav3.1 in CTL cells mimics, in some respects, PAH features.

In conclusion, impaired signaling between I_{CaT}, PP2A and Akt1 is observed in PASMCs from iPAH patients, leading to a decrease in PP2A activity and hyper-activation of MAP kinase and Akt1 signaling, promoting the hyper-proliferative and apoptosis-resistant PAH phenotype. Neither I_{CaT} blockade nor I_{CaT} channel overexpression fully reversed these features. The tyrosine kinase inhibitor, imatinib, was shown to increase PP2A activity [46] and inhibit the proliferation of PASMCs induced by PAH mediators [9]. However, the use of imatinib as a therapeutic agent for PAH has been abandoned due to significant side effects, resulting in an unfavorable benefit/risk ratio [47]. Hence, it seems essential to further determine the composition and regulation of

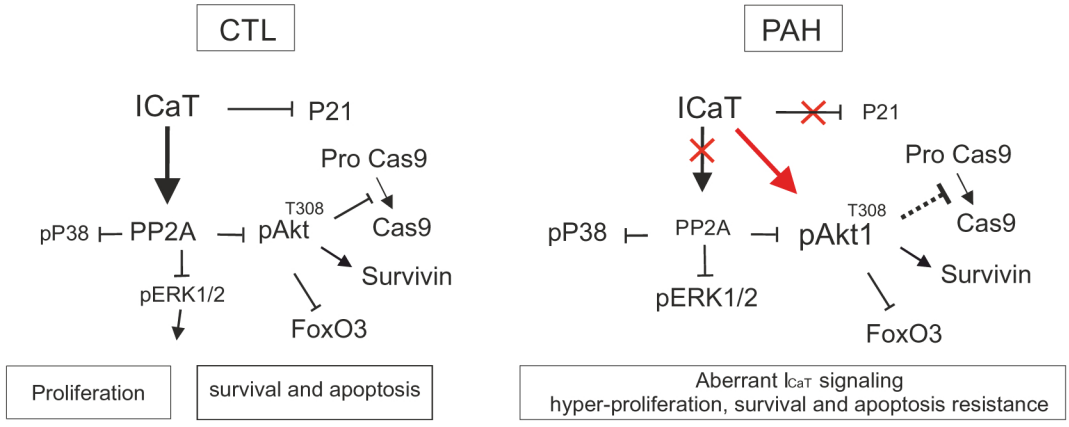

Fig. 7. Scheme of altered I_{CaT} signaling pathway in PASMCs from iPAH patients. The schematic integrative pathways is focused on the molecules and signaling pathways affected by a change in I_{CaT} signaling. I_{CaT} activates half of PP2A activity and inhibits P21 synthesis in CTL cells. A cross in PAH cells indicates the absence of these signaling pathways. In CTL cell, the amount of P21, PP2A and Cas9 are higher vs iPAH cells whereas in iPAH cells the phosphorylated state of P38, ERK1/2 and Akt1 are higher than in CTL. The size of typescript indicates the overabundant protein forms between CTL and iPAH cells. In iPAH cells, the signaling pathway between I_{CaT} and PP2A is directed toward Akt1 activation (red arrow). The putative regulation of caspase 9 activation by Akt1 seems inefficient in iPAH cells (dotted line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the PP2A holoenzyme in PAH to develop PASMC-specific therapeutic targets, considering that both an increase in PP2A activity and a decrease in I_{CaT}-induced Akt1 activity might have a beneficial effect on the onset of PAH and disease progression.

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Conflict of interest/disclosures

M.H. has relationships with drug companies including Actelion, Bayer, GSK, Novartis, and Pfizer. In addition to being investigators in trials involving these companies, other relationships include consultancy services and memberships of scientific advisory boards. Other authors report no conflicts of interest.

Author contribution

S.S and S.M. contributed equally to this work, designed and performed experiments, analyzed and interpreted data. F. A., Y. R., F. L. and J. S. performed experiments, analyzed and interpreted data. L.P. helped write the manuscript. J.A.A. and S.B. helped with pulmonary arterial smooth muscles cells harvest and culture. E.F. harvested human organ. P.D. helped with immunohistochemistry, analyzed and interpreted data. S.E, M. H. and F.P. provided conceptual and editing input. V.C. oversaw design and analysis of all experiments and wrote the manuscript. All authors were involved in the reading and critically revising the manuscript.

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