FOXO1 Controls Thyroid Cell Proliferation in Response to TSH and IGF-I and Is Involved in Thyroid Tumorigenesis

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TSH and insulin/IGF-I synergistically induce the proliferation of thyroid cells mainly through the cAMP and phosphatidylinositol 3-kinase (PI3K) pathways. However, the events involved in this cooperative induction remain unknown, and molecules that are potentially controlled by both TSH and IGF-I are interesting candidates as integrators of both stimuli. The finding that the PI3K pathway is frequently activated in thyroid malignancies has attracted attention to this pathway in the thyroid field. One of the targets of PI3K is Forkhead box O (FoxO)-1, a widely expressed transcription factor involved in a variety of cellular processes such as differentiation, proliferation, and apoptosis. Here we show that FoxO1 is highly expressed in differentiated rat thyroid cells and human thyroid tissue compared with human thyroid tumor-derived cells and surgically removed thyroid tumors, in which its expression is reduced. In differentiated cells, TSH/cAMP treatment decreases *FoxO1* mRNA and protein levels through proteasome activation, whereas both TSH and IGF-I control FoxO1 localization by promoting a rapid exclusion from the nucleus in an Akt-dependent manner. FoxO1 can control $p27^{KIP1}$ expression in differentiated and tumor cells of the thyroid. Furthermore, FoxO1 reexpression in tumor cells promotes a decrease in their proliferation rate, whereas FoxO1 interference in differentiated cells increases their proliferation. These data point to an important role of FoxO1 in mediating the effects of TSH and IGF-I on thyroid cell proliferation and provide a link between loss of FoxO1 expression and the uncontrolled proliferation of thyroid tumor cells. **(*Molecular Endocrinology* 27: 50–62, 2013)**

Thyroid epithelial cells present the peculiarity that proliferation and differentiation are controlled by the same stimuli. Thyrotropin and IGF-I increase proliferation synergistically in a variety of cell systems (1, 2) and stimulate genes involved in differentiation in an additive manner (3, 4). The sodium/iodide symporter gene is an exception because its expression is increased by thyrotropin, whereas its induction is counteracted by IGF-I (5). Both factors activate several transduction pathways. Thyrotropin activates the cAMP-protein kinase A (PKA) pathway, which is considered the main mediator of the thyrotropin effects on the thyroid cell (6). IGF-I/insulin action is mainly mediated by the phosphatidylinositol 3-kinase (PI3K) pathway, whereas activation of this pathway by thyrotropin is controversial. It was recently reported that thyrotropin-induced phosphorylation of Akt is independent of PI3K and does not promote Akt activation (7), although other connections between thyrotropin and the PI3K pathway have also been reported in the thyroid (8–10). Another common effector of both hormones is mammalian target of rapamycin (mTOR) (7) that regulates and is regulated by Akt (11, 12). Despite the many data regarding thyrotropin and IGF-I control of the different cellular processes of the thyrocyte (13–15), the mechanism of synergism between both factors is far from being understood. For this reason, the study of molecules that can be controlled by both stimuli becomes a major point of interest because this may provide information about possible integrators of their effects on the thyrocytes.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; CTX, cholera toxin; DAPI, 4′,6′-diamino-2-phenylindole; FoxO, Forkhead box O; GFP, green fluorescent protein; 6H, six-hormone mixture; MEK, MAPK kinase; mTor, mammalian target of rapamycin; NT, nontargeting; p, phosphorylated; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; Sp1, specificity protein-1.

From this point of view, the proteins of the Forkhead box O (FoxO) family are promising candidates. FoxO forkhead transcription factors have been described to participate in a wide range of cellular processes such as proliferation, differentiation, oxidative stress response, apoptosis, and control of metabolism, depending on the tissue (16). This broad control of cell functions and the demonstration of loss of function of FoxO family members in different tumor types in humans (17) have led to the proposal that FoxO proteins are tumor suppressors. The FoxO family of proteins consists of four members: FoxO1, FoxO3a, FoxO4, and FoxO6. With the exception of FoxO6, whose expression is restricted to the nervous system, FoxO proteins present a wide expression pattern and affect most cellular processes, in many cases with overlapping functions, because they can recognize the same DNA binding site and can potentially control the expression of the same target genes (18). Despite the supposed similarity between FoxO proteins regarding their function, studies in knockout mice indicate that only *FoxO1* null mice die before birth (19), suggesting functional differences between FoxOs.

The PI3K pathway, through posttranslational modifications that determine the subcellular localization of FoxO proteins, is the main regulator of these forkhead transcription factors. Besides control of its localization, FoxO1 can also be degraded by the proteasome machinery in an Akt-dependent manner (20). Interestingly, the PI3K pathway is frequently constitutively activated during thyroid tumorigenesis (21–23).

A recent study suggests that FoxO3a deregulation is involved in the escape from apoptosis of thyroid tumor cells (24), and mice haploinsufficient for *Pten* show decreased FoxO3a activity and decreased apoptosis (22). However, to our knowledge there is no information regarding FoxO1 in thyroid cells. We studied FoxO1 from its expression to its control and function in both differentiated and tumor cells. We show that FoxO1 is expressed in differentiated rat thyroid cells, in which its expression and subcellular localization is negatively regulated by thyrotropin and IGF-I. In contrast, FoxO1 expression is severely reduced in human thyroid tumor-derived cells, and it is also decreased in surgically removed human papillary thyroid tumors. Importantly, FoxO1 controls the expression of the cell cycle inhibitor $p27^{KIP1}$ and participates in the proliferation process both in differentiated and tumoral thyroid cells. Our data point to FoxO1 as an effective tumor suppressor in thyroid cancer and highlight the role of FoxO1 in the control of the proliferation and potentially other processes elicited by thyrotropin and IGF-I in the thyroid cell.

Materials and Methods

Cell culture

PCCl3 (25) and FRTL5 (26) cells are continuous lines of thyroid follicular cells derived from Fischer rats. These cells constitute a model system for the study of differentiation and growth regulation in a thyroid epithelial cell setting (27). Cells were cultured in Coon's modified Ham's F12 medium supplemented with a six-hormone mixture (6H; 1 nM thyrotropin, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 nM hydrocortisone, and 10 ng/ml glycyl-L-histidyl-L-lysine acetate), all from Sigma (St. Louis, MO), and 5% donor calf serum from Life Technologies, Inc. (Gaithersburg, MD). The effect of the hormones and growth factors was studied by starving near-confluent cells for thyrotropin and insulin in the presence of 0.2% BSA [starvation medium, indicated as (−) in the figures] for 2–5 d, depending on the requirements of the assay.

K- and H-Ras FRTL5 cells (25) are stably transformed cell lines that constitutively express the indicated oncogene. $BRAF^{V600E}$ PCCl3 (28) is a doxycycline-inducible cell line that expresses the $BRAF^{V600E}$ oncogene in the presence of the antibiotic.

All tumor cell lines used in this study are derived from human thyroid tumors from diverse origins (29). TPC1 cells are derived from a papillary carcinoma; WRO cells are derived from a follicular carcinoma; and FRO cells and SW1736 cells are derived from two different anaplastic carcinomas. The tumor cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, both from Life Technologies, Inc.

The following agents were added to the cells where indicated. Forskolin (Sigma) was added at a final concentration of 10 μM; wortmannin (Calbiochem, La Jolla, CA) at a final concentration of 250 nM; and Akt-i-VIII (Calbiochem) at a final concentration of 10 μM. Rapamycin and bortezomib were from LC Labs (Woburn, MA) and were added at final concentrations of 100 nM and 10 nM, respectively. IGF-I was used at a final concentration of 100 ng/ml and was from Peprotech (Rocky Hill, NJ). Cholera toxin (CTX) was used at a final concentration of 200 ng/ml.

Human tissue

Human thyroid tumor samples were kindly provided by the IdiPAZ Biobank integrated in the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es). Samples were registered, processed following current procedures, and frozen immediately upon reception. All patients participating in the study gave their informed consent and protocols were approved by institutional ethics committees.

Constructs and plasmids

pRL-CMV, which contains a cDNA coding for Renilla, was used to monitor transfection efficiency. The human $p27^{KIP1}$-promoter-Luc, is a luciferase expression vector under the control of the human $p27^{KIP1}$ promoter. The expression vectors used were: the green fluorescent protein (GFP)-FoxO1 that encodes a chimeric GFP/mouse FoxO1 protein and the FoxO1ADA expression vector that encodes a mouse FoxO1 with a triple mutation in the T24A, T253D, and S316A residues, impairing the phosphorylation of Akt that promotes FoxO1 nuclear exclusion.

Promoter activity assays

Cells were plated at a density of 6×10^5 per 60-mm-diameter tissue culture dish 48 h before transfection. Transfections were performed with JetPai transfection reagent from Polyplus-Transfection Inc. (New York, NY). For the PCCl3 cells, culture medium was changed to starvation medium 24 h after transfection and cells were maintained in this medium for 48–72 h. After this time, cells were treated with the different hormones for the times indicated in each experiment. Cells were transfected with 1 μg of the *p27-^{KIP1}*-LUC expression vector. To correct for transfection efficiency, 0.2 μg of the Renilla-encoding pRL-CMV vector was included in all samples. The tumor cells were additionally transfected with 3 μg of the FoxO1ADA expression vector or an empty vector. After 48 h cells were harvested, lysed, and analyzed for luciferase and Renilla activities. The promoter activity in cells transfected with the expression vector was determined as the ratio between luciferase and Renilla, relative to the ratio obtained in cells transfected with the corresponding control vector. The results shown are the average ± SD of three different experiments performed in triplicate, and data were analyzed with GraphPad Prism software (Intuitive Software for Science, San Diego, CA). Statistical significance was determined by *t* test analysis (two tailed), and differences were considered significant at $P < 0.05$.

Protein extraction and Western blot assay

Total protein extracts were obtained by scraping the cells in radioimmunoprecipitation assay buffer [1% (wt/vol) PBS, 0.5% (wt/vol) Nonidet P-40, 0.1% (wt/vol) sodium deoxycholate, 0.1% sodium dodecyl sulfate]. For assays involving fractionated proteins, the cells were scraped in a buffer containing 20 mM HEPES (pH 8.0), 10 mM KCl, 0.15 mM EDTA (pH 8.0), and 0.15 mM EGTA (pH 8.0), centrifuged for 5 min at 13,000 × *g*, and the supernatant containing the cytoplasmic fraction was collected; the nuclear fraction was extracted by resuspending the pellet in a buffer containing 20 mM HEPES (pH 8.0), 450 mM NaCl, 25% glycerol, 0.25 mM EDTA (pH 8.0), 0.15 mM EGTA, and 1.5 mM $MgCl_2$, followed by centrifugation as above. Protein extracts (20–30 μg) were separated on 6–12% sodium dodecyl sulfate-polyacrylamide gels and immunodetected after Western blotting. Tubulin, Akt, specificity protein-1 (Sp1), ERK, and $p27^{KIP1}$ antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), the p^{ser473}Akt antibody was from Pharmingen (San Diego, CA), the p70S6K and FoxO1 antibodies were from Cell Signaling Technology (Beverly, MA), the cAMP response element-binding protein antibody was from Calbiochem, and the phosphorylated (p) ERK1/2 antibody was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Equal protein loading was first monitored by the Bradford method (30) and after transfer by detection of the nonregulated proteins, tubulin, total Akt, or total ERK. Immunoreactive bands were visualized with enhanced chemiluminescence Western blotting substrate from Thermo Fisher Scientific (Rockford, IL) and detected by autoradiography. Protein expression levels were quantified using ImageQuant software by Molecular Dynamics Inc. (Sunnyvale, CA). The protein of interest was quantified and normalized in all cases to its loading control. The graph accompanying each Western blot assay shows the average of at least three different experiments. Data were analyzed with GraphPad Prism by Intuitive Software for Science (San Diego, CA). Relative protein expression is expressed as the mean ± SD. Statistical significance was determined by *t* test analysis (two tailed), and differences were considered significant at $P < 0.05$.

Epifluorescence and immunofluorescence assays

For epifluorescence assays, cells were seeded on cover slips and transfected with 2 μg of the GFP-FoxO1 expression vector using JetPai transfection reagent from Polyplus-Transfection Inc. After a 2-d starvation period, cells were stimulated with thyrotropin or IGF-I for 30 min. The coverslips were washed three times and fixed in 70% methanol at −20 C for 10 min, washed again, blocked with PBS containing 5% donor calf serum and 0.05% Tween-20 for 1 h at room temperature, washed twice in PBS-Tween-20 for 5 min, once in PBS-Tween-20 containing 0.5 μg/ml 4′,6′-diamino-2-phenylindole (DAPI) for 5 min, and mounted on Prolong. Epifluorescence of the cells expressing GFP-FoxO1 was observed under a Nikon E600 optical microscope (Tokyo, Japan) with a ×63 oil immersion objective.

For the immunofluorescence assay, cells were seeded on coverslips, and after a 2-d starvation period cells, were stimulated with thyrotropin for 30 min. The cover slips were washed three times and fixed in 70% methanol at −20 C for 10 min, washed again, blocked with PBS containing 5% donor calf serum and 0.05% Tween-20 for 1 h at room temperature, incubated with anti-FoxO1 antibody for 1 h at room temperature, washed three times in PBS-Tween-20 for 5 min, incubated for 1 h at room temperature with the secondary antibody Alexa 488 from Invitrogen (Carlsbad, CA), washed three times with PBS-Tween 20, the last time in buffer containing DAPI, and mounted on Prolong. Cells were observed under a confocal microscope with an ×63 oil immersion objective (Leica Corp., Deerfield, IL).

Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells following the standard TRIzol reagent method (Invitrogen) including a deoxyribonuclease treatment. RNA was reverse transcribed using random primers. Quantitative PCR was performed using the SYBRVR Green PCR kit (Stratagene, La Jolla, CA). Samples were subjected to 40 cycles of amplification in a Stratagene Mx 3000P System for 10 min at 95 C, 30 sec at 95 C, 1 min at 58 C, 30 sec at 72 C, 1 min at 95 C, 30 sec at 60 C, and 30 sec at 95 C. The reactions were normalized using β-glucuronidase as a standard and 5-carboxy-X-rhodamine as reference dye. The primers were: FoxO1, forward, GGTGAAGAGTGTGCCCTACTT, reverse, CTTGCCTCCC TCTGGATTGA; FoxO3a, forward, CGGCTCACTTTGTC-CCAGAT, reverse, CTTCCCACTCTTTCCCCCAT; and FoxO4, forward, TTCAAGGACAAGGGTGACAG, reverse, AACTGCTT CGTGGACGGAA.

Proliferation assays

PCCl3 cells were seeded in 96-well plates and transfected with a FoxO1 small interfering RNA (siRNA) or a nontargeting siRNA at a 10 nM final concentration, from Thermo Fisher Dharmacon (Rockford, IL); 24 h later the cells were left for another 24 h in 6H medium or cultured in starvation medium for 48 h. Tumor cells were seeded in 96-well plates and transfected with 1.2 μg of the FoxO1ADA expression vector or the empty vector. The cells were collected after 24, 48, and 72 h for 5-bromo-2′-deoxyuridine (BrdU) incorporation measurement.

Cells were treated for BrdU labeling and measurement with the cell proliferation ELISA, BrdU kit from Roche (Basel, Switzerland), following the instructions of the manufacturer. The results shown are the average ± SD of three independent experiments performed in triplicate and were analyzed with GraphPad Prism by Intuitive Software for Science. Relative BrdU incorporation is shown as the mean ± SD. Statistical significance was determined by *t* test analysis (two tailed), and differences were considered significant at $P < 0.05$.

Results

Thyrotropin decreases FoxO1 protein levels in a cAMP-mediated manner by activating the proteasome machinery

To our knowledge, there are no data on FoxO1 expression in the thyroid, so we first confirmed that differentiated rat thyroid PCCl3 cells express *FoxO1* mRNA (Fig. 1A). Treatment with thyrotropin for 24 h significantly (40%) decreased *FoxO1* mRNA levels, whereas IGF-I had no significant effect. Furthermore, we confirmed that *FoxO3a* and *FoxO4*, two other members of the family, were also expressed in these cells and that they were differently regulated by thyrotropin and IGF-I. We observed that *FoxO3a* mRNA levels were increased 2.5-fold by thyrotropin treatment (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), whereas *FoxO4* mRNA levels were controlled by thyrotropin and IGF-I in a manner similar to FoxO1 (Supplemental Fig. 1B). These data show a different control of the mRNA levels of the FoxO family members in thyroid cells and suggest different functions of FoxO proteins in mediating the effects of thyrotropin and IGF-I.

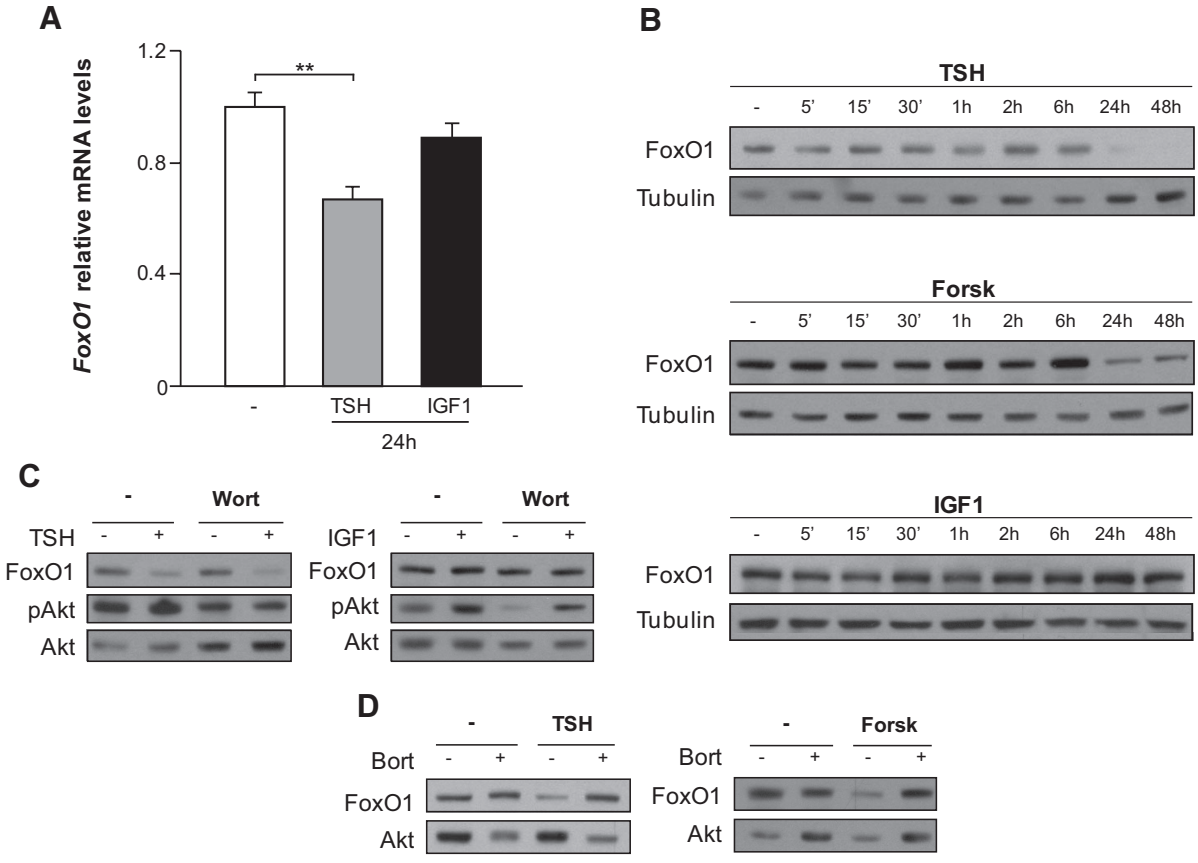

FIG. 1. Thyrotropin/cAMP decrease FoxO1 protein levels by activating the proteasome machinery. PCCl3 cells were cultured for 5 d under serum-free conditions in the absence of thyrotropin and IGF-I. A, Cells were left untreated (−) or treated for 24 h with thyrotropin or IGF-I. Total RNA was extracted and a qRT-PCR assay was performed to detect *FoxO1* mRNA levels. Results are the mean ± SD of three independent experiments performed in triplicate and changes with respect to the mRNA levels of untreated cells were considered significant at $P < 0.05$. **, $P = 0.01–0.001$ (two tailed *t* test). B, Cells were treated for increasing times with thyrotropin, forskolin (Forsk), or IGF-I. Total protein extracts were prepared and analyzed by Western blot using specific anti-FoxO1 and antitubulin antibodies; tubulin was used as a loading control. Western blots representative of three independent experiments are shown. C, Cells were treated with the PI3K inhibitor wortmannin (Wort) or with vehicle (−) for 2 h and 30 min; next, cells were treated with thyrotropin and IGF-I for 24 h or left untreated (−). Protein extracts were collected and submitted to a Western blot assay visualizing FoxO1, p^{S473}Akt to assess the efficiency of the inhibitor and total Akt as a loading control. Representative Western blots of three independent experiments are shown. D, Cells were treated with the proteasome inhibitor bortezomib (Bort) for 24 h or with vehicle; next, cells were left untreated (−) or were treated with thyrotropin or forskolin for 24 h. Protein extracts were collected and submitted to a Western blot assay to detect FoxO1 and total Akt as loading control. Representative Western blots of three independent experiments are shown.

FoxO1 protein expression was also confirmed, and its levels were greatly decreased by thyrotropin treatment for 24–48 h (Fig. 1B). Addition of forskolin, an agent that constitutively activates adenylyl cyclase, reproduced the effect of thyrotropin on FoxO1 protein levels, whereas IGF-I had no effect (Fig. 1B).

These data clearly point to the cAMP pathway as the mediator of the thyrotropin effects on FoxO1 protein levels. However, in other tissues FoxO1 degradation has been exclusively related to the activation of the PI3K pathway and the proteasome machinery (20). To address this point, FoxO1 protein levels were measured in PCCl3 cells treated for 24 h with thyrotropin or IGF-I in the presence or absence of the PI3K inhibitor wortmannin. Contrary to what has been described in other tissues (31), blocking this pathway did not impair FoxO1 protein decrease in response to thyrotropin (Fig. 1C, *left panel*). Wortmannin in combination with IGF-I did not affect FoxO1 protein levels (Fig. 1C, *right panel*). The long times required for thyrotropin to decrease FoxO1 levels did not allow us to observe clear concomitant effects of wortmannin, thyrotropin, or IGF-I on the levels of pAkt, which after 24 h have returned to nearly basal levels. However, the efficiency of the different treatments was clearly visible at a shorter time (30 min) of treatment (data not shown). Treatment with bortezomib, a proteasome inhibitor, impaired FoxO1 protein decrease after 24 h of thyrotropin (Fig. 1D, *left panel*) or forskolin treatment (Fig. 1D, *right panel*), whereas the levels of the protein in cells treated with IGF-I were unaffected (data not shown).

Taken together, these data indicate that thyrotropin controls both *FoxO1* mRNA and protein levels. The decrease of FoxO1 protein levels involves degradation by the proteasome in a cAMP-dependent manner. IGF-I has no significant effects on *FoxO1* mRNA and protein levels.

Thyrotropin and IGF-I promote a rapid exit of FoxO1 from the nucleus

FoxO1 function is mainly controlled through the regulation of its subcellular localization in response to different stimuli (18). In general, growth factors that activate the PI3K pathway promote the cytoplasmic localization of the protein. Given the relation of thyrotropin and IGF-I with this pathway, their actions on FoxO1 localization were analyzed in PCCl3 cells. Fractionated protein extracts were separated on a gel, and Western blot assays showed that under conditions of starvation (*i.e.* in the absence of thyrotropin and IGF-I), FoxO1 was located both in the nucleus and the cytoplasm; upon thyrotropin treatment, FoxO1 became mainly cytoplasmic (Fig. 2A). This effect was very rapid, clearly observed after only 5 min of treatment, suggesting that FoxO1 was being actively excluded from the nucleus. This result was confirmed in an immunofluorescence assay showing that in starvation medium FoxO1 presented a diffuse distribution over the entire cell and became clearly cytoplasmic after 30 min of thyrotropin treatment (Fig. 2B). IGF-I addition to the cells promoted a similar effect, although FoxO1 exits from the nucleus reached a higher level (Fig. 2C). To further confirm these results, PCCl3 cells were transfected with an expression vector encoding a GFP-FoxO1 fusion protein. The exogenous GFP-FoxO1 protein showed a predominantly nuclear and perinuclear localization in starved cells and moved to the cytoplasm after 30 min of treatment with thyrotropin or IGF-I (Fig. 2D).

We observed that FoxO1 is driven very quickly from the nucleus to the cytoplasm upon thyrotropin or IGF-I treatment. To analyze the kinetics of the opposite process, FoxO1 moving from the cytoplasm to the nucleus, a complementary experiment was performed with PCCl3 cells growing in the presence of thyrotropin and IGF-I followed by removal of both factors for increasing times. Western blots of the fractionated protein extracts showed that FoxO1 did not accumulate in the cytoplasm, but an increase in protein levels was observed in the nucleus after 24 h of thyrotropin and IGF-I withdrawal, and nuclear accumulation continued until at least 72 h after withdrawal (Fig. 2E). As shown in Fig. 1, FoxO1 is degraded by prolonged (24–48 h) thyrotropin treatment, suggesting that upon thyrotropin and IGF-I withdrawal FoxO1 is being synthesized *de novo* and enters into the nucleus, presumably allowing the expression of its target genes.

Thyrotropin- and IGF-I-induced exit of FoxO1 from the nucleus is decreased by Akt1/2 inhibition

Thyrotropin and IGF-I promote the activation of several signal transduction pathways that control different aspects of the thyroid cell. thyrotropin effects have been mainly related to the cAMP-PKA pathway. To analyze the involvement of this pathway in the control of FoxO1 localization, we stimulated PCCl3 cells with CTX; this drug constitutively activates Gs (cAMP stimulating) proteins and was used to mimic the cAMP branch of the thyrotropin signaling pathway. FoxO1 localization was barely affected by the increased cAMP levels elicited by CTX (Fig. 3A). Addition of forskolin was also unable to promote FoxO1 relocalization to the cytoplasm (data not shown).

In most tissues the control of FoxO1 localization has been associated with the PI3K-Akt cascade. To explore this possibility in thyroid cells, we treated PCCl3 cells with a specific and potent Akt1/2 inhibitor (Akt-i-VIII)

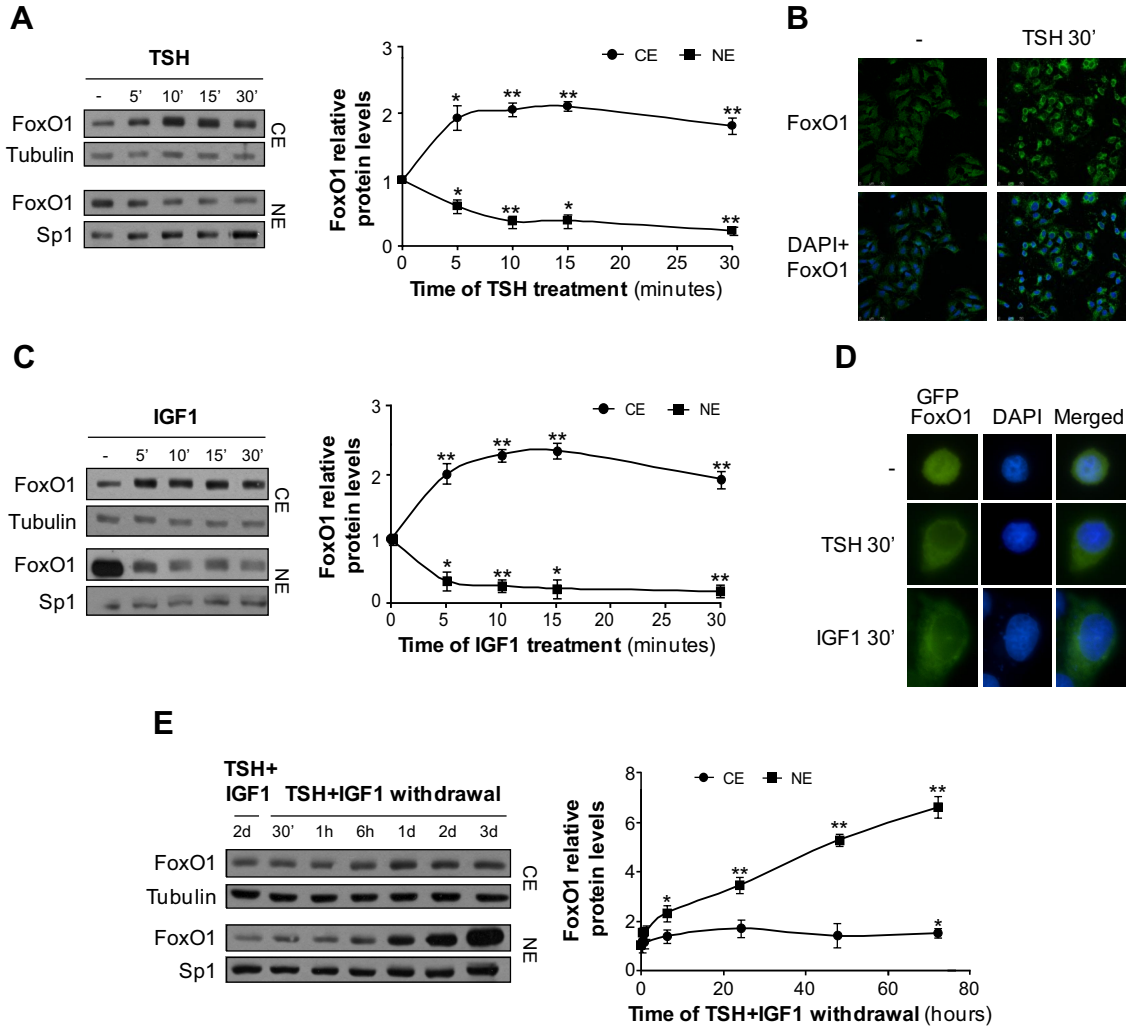

FIG. 2. Thyrotropin and IGF-I promote a rapid exit of FoxO1 from the nucleus. A and C, PCCl3 cells were cultured for 5 d in serum-free medium in the absence of thyrotropin and IGF-I; the cells were then treated with thyrotropin (A) or IGF-I (C) for 5–30 min or left untreated (−). Cells were collected and nuclear (NE) and cytoplasmic (CE) protein extracts were submitted to Western blot analysis to detect FoxO1; tubulin and Sp1 were used as cytoplasmic and nuclear loading controls, respectively. The *graph to the right* of each Western blot shows the mean ± SD of FoxO1 levels relative to their respective loading controls. *, Changes with respect to the FoxO1 levels of untreated cells were considered significant when $P = 0.05–0.01$; **, $P = 0.01–0.001$ (two tailed *t* test). B, PCCl3 cells were seeded on coverslips and cultured in serum-free medium for 5 d in the absence of thyrotropin and IGF-I. After that cells were left untreated (−) or cells were treated with thyrotropin for 30 min. Cells were then methanol fixed, and FoxO1 expression was observed by confocal immunofluorescence. FoxO1 is observed in *green*, and nuclear staining with DAPI is shown in *blue*. D, PCCl3 cells were seeded on coverslips and transfected with 1 μg of a GFP-FoxO1 expression vector. After 2 d of starvation, cells were treated with thyrotropin or IGF-I for 30 min. Then cells were methanol fixed, stained with DAPI, and observed under an epifluorescence microscope to detect GFP-FoxO1 expression. E, PCCl3 cells were grown in a thyrotropin and IGF-I containing medium for 2 d, after which both hormones were removed for increasing periods of time. Nuclear and cytoplasmic protein extracts were analyzed by Western blot to detect the protein levels of FoxO1; tubulin and Sp1 served as cytoplasmic and nuclear loading controls, respectively. The *graph to the right* of the Western blot shows FoxO1 levels relative to their respective loading controls (mean ± SD). *, $P = 0.05–0.01$; **, $P = 0.01–0.001$ (two tailed *t* test).

before thyrotropin or IGF-I addition. Akt inhibition largely impaired thyrotropin or IGF-I induced FoxO1 cytoplasmic accumulation and nuclear exclusion (Fig. 3B). Furthermore, PI3K inhibition by wortmannin or LY290042, which in our hands was less efficient in inhibiting Akt phosphorylation and also decreased the FoxO1 exit from the nucleus, albeit to a lesser extent (data not shown). In the absence of both hormones, Akt inhibition resulted in a small decrease in the levels of FoxO1 in the cytoplasm, suggesting that a basal Akt activation exists in the absence of thyrotropin and IGF-I. This small change is not observed in the nuclear levels of FoxO1 and is probably due to the high levels of the protein present in the nucleus.

The MAPK pathway can also be activated by thyrotropin (32) and IGF-I (1), and in certain tissues this signal cascade is involved in the control of FoxO1 localization (33). However, MAPK inhibition in PCCl3 cells did not impair FoxO1 exit from the nucleus in response to thyrotropin (Supplemental Fig. 2A) or IGF-I (Supplemental Fig. 2B).

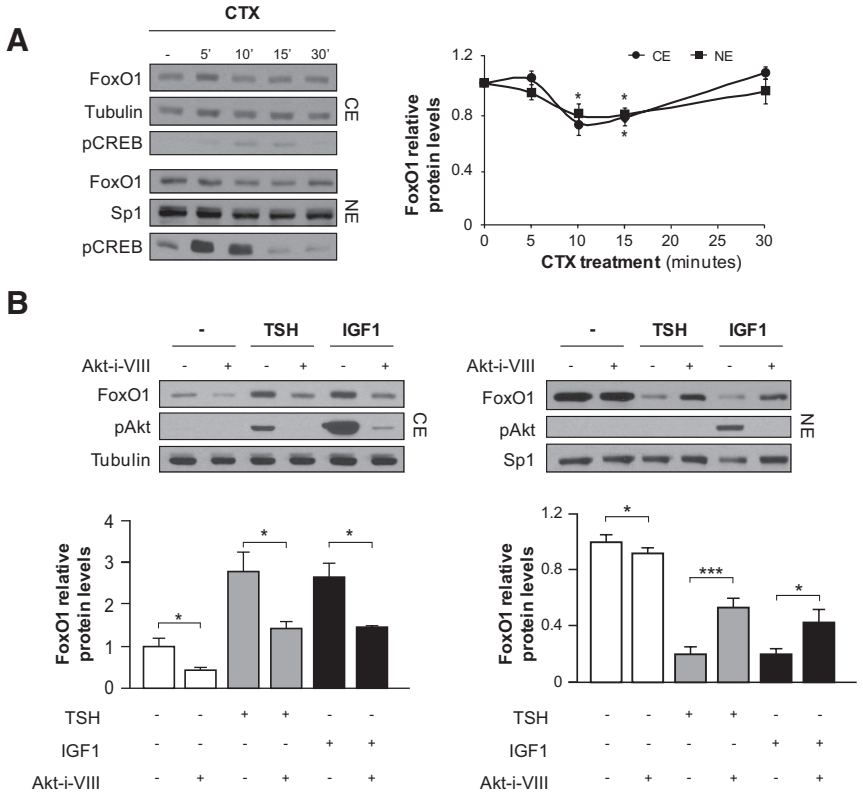

FIG. 3. Thyrotropin- and IGF-I-induced exit of FoxO1 from the nucleus is mediated by Akt. Cells were cultured for 5 d in the absence of thyrotropin, IGF-I, and serum before stimulation with the indicated factors. A, Cells were treated with CTX for the times indicated. Then nuclear and cytoplasmic extracts were collected and submitted to a Western blot assay to detect FoxO1 and phosphorylated cAMP response element-binding protein (pCREB) as a readout of cAMP/PKA activation. Tubulin and Sp1 were used as cytoplasmic and nuclear loading controls, respectively. The *graph to the right* shows FoxO1 levels relative to their respective loading controls (mean ± SD). *, $P = 0.05–0.01$ (two tailed *t* test). B, Cells were pretreated with the Akt 1/2 inhibitor Akt-i-VIII for 1 h. Cells were then left untreated (−) or treated with thyrotropin or IGF-I for 30 min, and nuclear (*right panel*) and cytoplasmic (*left panel*) extracts were collected and submitted to a Western blot assay to detect the expression of FoxO1, p^{S473}Akt to assess the efficiency of the inhibitor, tubulin as cytoplasmic loading control, and Sp1 as nuclear loading control. The *graph below each western blot* shows FoxO1 levels relative to their respective loading controls (mean ± SD). *, $P = 0.05–0.01$; ***, $P < 0.001$ (two tailed *t* test).

mTOR is another effector of thyrotropin (7) and IGF-I and reciprocally regulates Akt (11). The possible involvement of mTOR in FoxO1 localization was analyzed using the mTOR inhibitor rapamycin. Although p70S6K-induced phosphorylation by mTOR in response to thyrotropin or IGF-I was impaired by rapamycin treatment, FoxO1 exit from the nucleus was unaffected (Supplemental Fig. 2C), showing that at least the rapamycin-sensitive mTOR complex is not involved in the control of FoxO1 localization.

Altogether these results show that thyrotropin and IGF-I can induce a rapid FoxO1 exit from the nucleus in an Akt-dependent manner. Thyrotropin controls FoxO1 at several levels: it decreases the rate of transcription of the gene, promotes degradation of the protein by the proteasome, and induces FoxO1 exit from the nucleus. IGF-I does not decrease FoxO1 protein levels, but due to its higher potency in activating the PI3K pathway, the effect of IGF-I on FoxO1 localization was greater than that of thyrotropin. This may explain why thyrotropin and IGF-I present cooperative effects in thyroid cell processes such as proliferation.

FoxO1 controls $p27^{KIP1}$ expression and PCCl3 cell proliferation

We next studied whether FoxO1 was able to control the expression of target genes in thyroid cells. To this end a clearly defined FoxO1 target gene like the $p27^{KIP1}$ cell cycle inhibitor (34) was chosen, which could also give some clues regarding a possible control of thyroid cell proliferation by FoxO1. To address this point, we first measured whether thyrotropin and IGF-I were able to control the activity of a luciferase expression vector driven by the $p27^{KIP1}$ human promoter in PCCl3 thyroid cells. The activity of the promoter was significantly decreased by 24 h of thyrotropin or IGF-I treatment, and the addition of both factors together (6H) promoted a further decrease in the activity of the $p27^{KIP1}$ promoter (Fig. 4A).

To study whether the $p27^{KIP1}$ promoter is directly controlled by FoxO1 in thyroid cells, PCCl3 cells growing in complete medium in the presence of thyrotropin and IGF-I were cotransfected with an expression vector encoding a constitutively nuclear form of FoxO1 (FoxO1ADA), which contains a triple mutation in the residues phosphorylated by Akt responsible for the nuclear exclusion of FoxO1 (35), together with the already described luciferase expression vector driven by the human $p27^{KIP1}$ promoter. The results showed that FoxO1ADA expression significantly increased the activity of the $p27^{KIP1}$ promoter 24 h after transfection (Fig. 4B, *left panel*). When thyrotropin alone was added to the cells, expression of FoxO1ADA also promoted an increase in the activity of the $p27^{KIP1}$ promoter (data not shown), indicating that the thyrotropin-induced decrease of FoxO1 action at least partially depends on Akt-mediated signals. Consistent with this result, the levels of $p27^{KIP1}$ protein were also increased upon FoxO1ADA

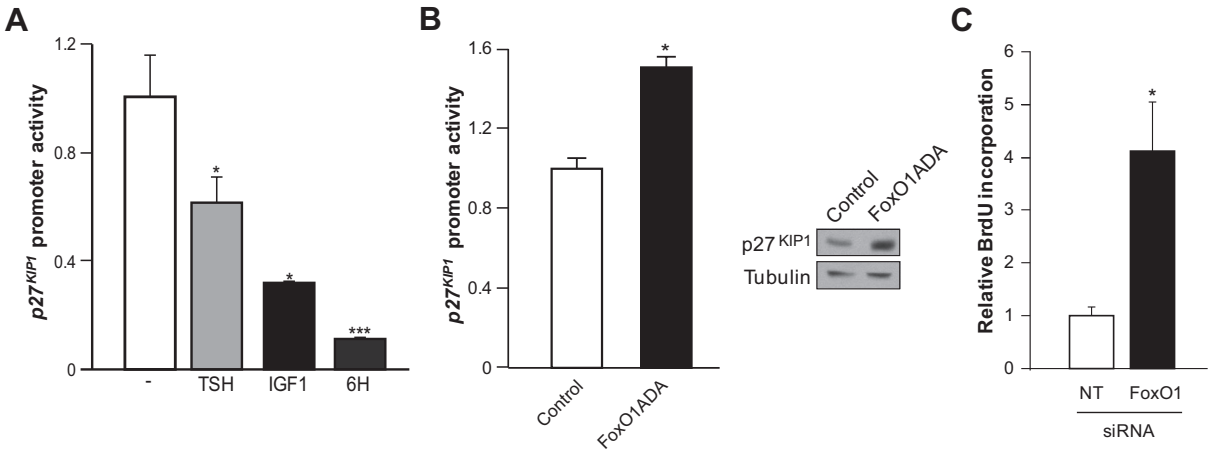

FIG. 4. FoxO1 controls p27^{KIP1} expression and PCCl3 cells proliferation. A, PCCl3 cells were transfected with 1 μg of a luciferase expression vector driven by the *p27^{KIP1}* promoter plus 0.2 μg of the CMV-Renilla vector; 24 h later the medium was replaced by starvation medium for 2 d, and cells were treated with thyrotropin, IGF-I, or both factors (6H) plus serum for 24 h. The cells were then collected to measure luciferase activity. Results are the mean ± SD of the luciferase levels relative to the nonregulated Renilla levels of three independent experiments. B, *Left panel*, PCCl3 cells growing in complete medium were transfected with 3 μg of a FoxO1ADA expression vector or the empty vector plus 1 μg and 0.2 μg of the p27^{KIP1}-Luc and CMV-Renilla constructs, respectively. Twenty-four hours after transfection, cells were collected and luciferase and Renilla levels were measured. Results are shown as the mean ± SD of the luciferase levels relative to the nonregulated Renilla levels of three independent experiments. *, $P = 0.05$ to 0.01; ***, $P < 0.001$ (two tailed *t* test). *Right panel*, PCCl3 cells growing in the presence of thyrotropin, IGF-I, and serum were transfected with 3 μg of the FoxO1ADA expression vector and 48 h later protein extracts were collected and submitted to a Western blot assay to detect the protein levels of p27^{KIP1} and tubulin as a loading control. A representative Western blot of three independent experiments is shown. C, PCCl3 cells growing in complete medium were transfected with specific FoxO1 or NT siRNA and left in the presence of thyrotropin and IGF-I for 24 h. Then the cells were fixed and treated for the measurement of BrdU incorporation. The results show the mean ± SD of BrdU incorporation of FoxO1-depleted cells relative to the incorporation in cells transfected with the NT siRNA from three independent experiments. *, $P = 0.05$–0.01 (two tailed *t* test).

expression in PCCl3 cells growing in full medium (Fig. 4B, *right panel*).

As we already mentioned, thyrotropin and IGF-I promote the proliferation of thyroid cells, and we have shown that both factors negatively regulate FoxO1 at different levels and that FoxO1 increases the expression of the cell cycle inhibitor p27^{KIP1}. These results suggest that FoxO1 could be involved in the control of the proliferation of thyroid cells in response to thyrotropin and IGF-I. To address this point, FoxO1 expression was interfered using a specific FoxO1 siRNA in PCCl3 cells. Cell proliferation was analyzed by BrdU incorporation. In cells transfected with FoxO1 siRNA in the presence of thyrotropin and IGF-I, proliferation was increased by 4-fold compared with cells transfected with a nontargeting (NT) siRNA (Fig. 4C). In the absence of thyrotropin and IGF-I, FoxO1 siRNA had no significant effect on cell proliferation (data not shown).

Altogether these data indicate that thyrotropin and IGF-I induce PCCl3 thyroid cell proliferation, at least in part, by impairing FoxO1 function at different levels and therefore controlling the expression of FoxO1 target genes like *p27^{KIP1}*.

FoxO1 expression is lost in human thyroid tumor derived cells and decreased in surgically removed papillary thyroid tumors

The importance of the PI3K pathway in thyroid tumor progression has been clearly established (21–23) and suggests the implication of this pathway in the uncontrolled proliferation of these cells. Because oncogenic Ras can activate that pathway, protein levels of FoxO1 were measured in H- and K-Ras transformed FRTL5 cells. FRTL5 is a rat thyroid-derived cell line that has similar properties as the PCCl3 cell line. In both oncogene-transformed cell lines, protein levels of FoxO1 were highly reduced (Fig. 5A). Interestingly, the K-Ras transformed cells presented a more aggressive phenotype and had almost undetectable levels of FoxO1. Ras is also able to activate the MAPK kinase (MEK) pathway, which is also frequently activated in thyroid malignancies.

To test the possible involvement of the MEK pathway in the control of FoxO1, we used a doxycycline-inducible PCCl3 cell line that conditionally expresses oncogenic BRAF^{V600E}. Induction of BRAF^{V600E} expression can be observed by the increased phosphorylation of ERK, which is two steps downstream in the cascade. As can be observed in Fig. 5B, BRAF^{V600E} did not decrease FoxO1 protein levels in the absence of hormones, indicating that the MEK pathway is not involved in the control of FoxO1.

Given that FoxO proteins are considered as possible tumor suppressors, we next analyzed the expression of the different FoxO family members in human thyroid tumor cells. To this end we performed quantitative RT-PCR (qRT-PCR) with mRNAs isolated from a panel of four cell lines derived from human thyroid tumors of dif-

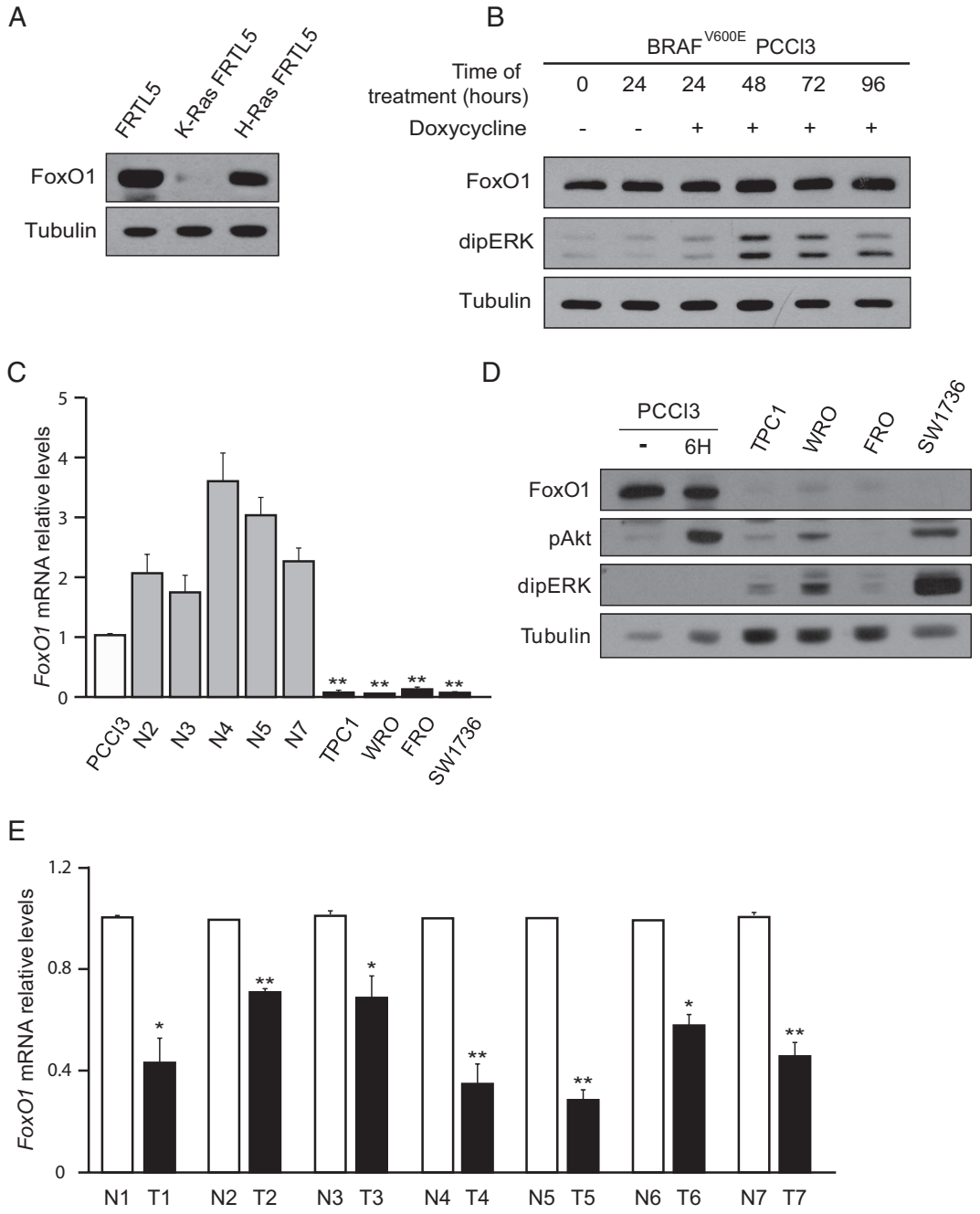

FIG. 5. FoxO1 expression is decreased in Ras-transformed FRTL5 cells, human thyroid tumor cell lines, and human thyroid tumors. A, Cells were cultured for 2 d in complete medium including thyrotropin and IGF-I, and total protein extracts were recovered and submitted to a Western blot assay to detect FoxO1 and tubulin levels as loading control. A Western blot representative of three independent experiments is shown. B, $BRAF^{V600E}$ PCCl3 cells were grown in 6H medium for 2 d and then cultured in the absence of thyrotropin, IGF-I, and serum for 2 more days. Then cells were treated (+) or left untreated (−) with doxycycline for increasing times. After protein extraction, FoxO1 levels were detected by Western blot. diP-ERK is shown to confirm doxycycline-induced $BRAF^{V600E}$ expression, and tubulin is shown as loading control. C, Total RNA from PCCl3 cells growing in the presence of thyrotropin and IGF-I from surgically removed normal thyroid tissue (N2, N3, N4, N5 and N7) and from TPC1, WRO, FRO, and SW1736 human thyroid tumor-derived cell lines was extracted, and a qRT-PCR assay was performed to detect *FoxO1* mRNA. Results are the mean ± SD of *FoxO1* mRNA levels relative to the β-glucoronidase (*GUS*) levels from three independent experiments performed in triplicate and changes respective to the levels of the differentiated cells were considered significant at $P < 0.05$. **, $P = 0.01–0.001$ (two tailed *t* test). D, PCCl3 cells were cultured in the absence (−) or presence (6H) of thyrotropin, IGF-I, and serum for 2 d. Tumor cells were seeded and cultured for 2 d in complete medium; next, total protein extracts were recovered and submitted to a Western blot assay to detect the levels of FoxO1, p^{Ser473}Akt (pAkt), phospho-p44/42 Erk1/2 (dipERK), and tubulin as loading control. A Western blot representative of three independent experiments is shown. E, Tumoral (T) and normal (N) human thyroid tissue from the corresponding unaffected lobe was obtained from seven different patients. Total RNA was extracted and qRT-PCR was performed to detect *FoxO1* mRNA levels. Results are the mean ± SD of three independent experiments performed in triplicate and changes with respect to the mRNA levels of normal cells were considered significant at $P < 0.05$. *, $P = 0.05–0.01$; **, $P = 0.01–0.001$ (two tailed *t* test).

ferent origin: TPC1, derived from a papillary carcinoma; WRO, derived from a follicular carcinoma; and FRO and SW1736 derived from two different anaplastic carcinomas. As positive controls, we used mRNA from PCCl3 cells or from surgically removed human normal thyroid tissue. FoxO1 expression in human thyroid samples is 2- to 4-fold higher than in PCCl3 cells. Due to the small amounts of human tissue available, in the following experiments, PCCl3 cells were used as a positive control of FoxO1 expression. We found that *FoxO1* mRNA levels were greatly reduced in all cell lines analyzed (Fig. 5C) compared with the levels of PCCl3 cells and normal human thyroid tissue. *FoxO3a* mRNA levels were also almost undetectable in these cell lines (Supplemental Fig. 3A) and *FoxO4* mRNA levels presented an approximate 60% reduction in all tumor cell lines compared with the levels of a differentiated thyroid cell line (Supplemental Fig. 3B).

To confirm this result, FoxO1 protein levels were measured in these cell lines. We found that FoxO1 protein levels were strongly reduced in TPC1, WRO, FRO, and SW1736 cell lines (Fig. 5D). It is also interesting to note that PI3K activation measured as pAkt levels reached the highest level in the SW1736 and WRO cell lines, suggesting that this pathway could be more strongly affecting FoxO1 function in these cells.

In these tumor cell lines, PI3K activation may be responsible for FoxO1 repression. To address this, TPC1, WRO, FRO, and SW1736 cells were treated with the Akt inhibitor Akt-i-VIII for 24 h. This resulted in a slight increase in FoxO1 protein levels (Supplemental Fig. 3C), indicating that PI3K activation in thyroid tumor cells decreases FoxO1 protein levels, although the activation never reaches the levels seen in differentiated cells (compare Fig. 5, C and D). By contrast, inhibition of the MEK/ERK pathway, another important signaling cascade that is frequently activated in thyroid tumors (36), did not increase FoxO1 protein levels (Supplemental Fig. 3D). Note that the effect of the inhibitor was lost after 24 h in WRO cells. This could reflect a faster activation of this pathway in these cells compared with the other cells. In fact, WRO cells express mutant $BRAF^{V600E}$ and present a constitutive activation of the MAPK pathway (29).

Finally, FoxO1 mRNA levels were measured in surgically removed human thyroid tumors with a papillary phenotype. Unfortunately, due to the limited availability of samples from the most aggressive tumors, we had only access to samples from papillary tumors that represent the less aggressive kind of thyroid tumors. However, the levels of FoxO1 were significantly reduced in all tumor samples compared with the levels in tissue extracted from the unaffected contralateral lobe (Fig. 5E). These results highlight the role of FoxO1 in thyroid tumorigenesis, and suggest a role for FoxO1 as a tumor suppressor in the thyroid.

FoxO1 reexpression increases $p27^{KIP1}$ promoter activity and decreases proliferation of WRO and SW1736 human thyroid tumor cells

Loss of FoxO1 expression in thyroid tumor cells may represent an event secondary to transformation, but it may also be a direct cause of this process. To address this issue, we analyzed the effect of FoxO1 expression on the activity of the $p27^{KIP1}$ promoter in the WRO and SW1736 cell lines, which had shown the highest activation of the PI3K pathway (Fig. 5D). Cells were cotransfected with the FoxO1ADA expression vector and the luciferase expression vector driven by the $p27^{KIP1}$ promoter, and 24 h later the cells were lysed and luciferase levels were measured. The results show a significant 2- to 3-fold increase in the activity of the $p27^{KIP1}$ promoter when FoxO1ADA is expressed in both cell lines (Fig. 6A).

The effect of FoxO1 on WRO and SW1736 tumor cell proliferation was next analyzed. To this end, cells were transfected with the expression vector encoding the FoxO1ADA nuclear mutant and BrdU incorporation was measured after 24, 48, and 72 h. The rate of BrdU incorporation was remarkably decreased in WRO and SW1736 cells expressing the constitutively active FoxO1 mutant (Fig. 6B).

These results show that lack of FoxO1 expression is a requirement for these human thyroid tumor cells to proliferate because FoxO1 reexpression largely impaired the proliferation of these cells. We already observed that *FoxO3a* and *FoxO4* expression is also decreased to different extents in human thyroid tumor cells, suggesting an important role of the FoxO family of proteins in the control of proliferation of thyroid cells.

Discussion

FoxO1 is a multifunctional protein involved in many different cellular processes, such as control of proliferation, stress response, control of metabolism, and differentiation (37–40). Furthermore, the overlapping functions of the different members of the family in mammals highlight their importance in these processes. However, despite these overlapping functions, specialized roles of FoxO proteins are beginning to be delineated, as can be concluded from the fact that only the FoxO1 deficient mouse is not viable (19). Supporting these data, we found a different regulation of FoxO1 and FoxO3a by thyrotropin: *FoxO1* mRNA levels are decreased, but *FoxO3a* mRNA

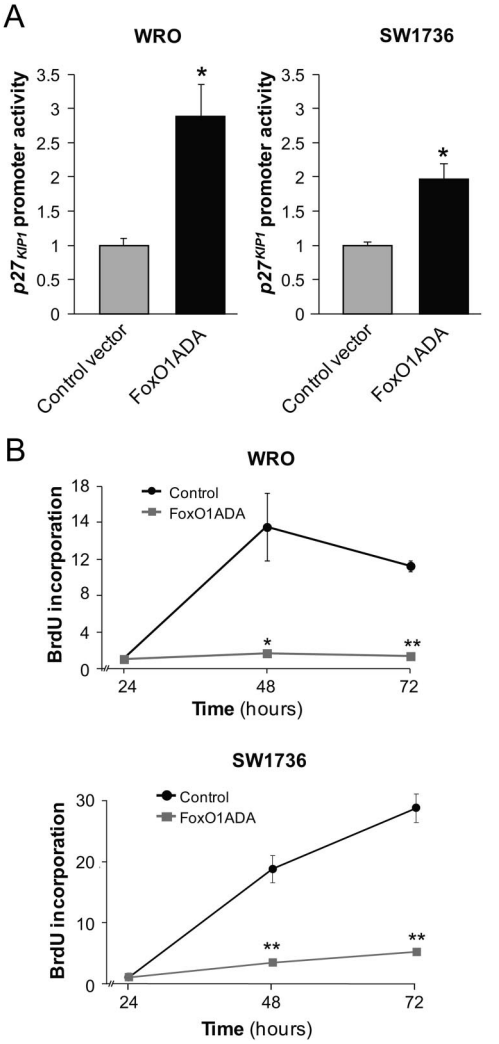

FIG. 6. FoxO1 reexpression increases $p27^{KIP1}$ promoter activity and decreases proliferation of WRO and SW1736 human thyroid tumor cells. A, WRO and SW1736 human thyroid tumor cell lines were transfected with 3 μg of the FoxO1ADA nuclear mutant or vehicle/control vector, together with 1 μg of the $p27^{KIP1}$-Luc and 0.2 μg of the CMV-Renilla expression vectors to measure the activity of the $p27^{KIP1}$ promoter. After 24 h transfection, cells were collected for the determination of luciferase and Renilla levels. Results are shown as the mean ± SD of the luciferase levels relative to the nonregulated Renilla levels of three independent experiments. The changes in the activity of the $p27^{KIP1}$ promoter in cells transfected with the FoxO1ADA expression vector relative to cells transfected with vehicle vector were considered significant at $P < 0.05$. *, $P = 0.05–0.01$ (two tailed *t* test). B, WRO and SW1736 cells were transfected with 1.2 μg of the FoxO1ADA constitutive nuclear mutant or vehicle vector (control). Cells were fixed 24, 48, and 72 h later and treated for the measurement of BrdU incorporation. The results show the mean ± SD of BrdU incorporation of FoxO1ADA-expressing cells relative to the incorporation of cells transfected with the vehicle vector from three independent experiments. *, $P = 0.05–0.01$; **, $P = 0.01–0.001$ (two tailed *t* test).

levels are increased. In thyroid tumor cells, *FoxO4* mRNA levels are less reduced than the levels of FoxO1 and FoxO3a, suggesting that FoxO4 depletion is less important for the progress of thyroid tumorigenesis. These initial results on the several members of the FoxO family in thyroid cells indicate that they may have diverse functions and suggest a different role for each of the FoxO family members in the progression of thyroid tumorigenesis. It is possible that FoxO3a participates in thyroid differentiation and proliferation because its expression is increased by thyrotropin, the main regulator of thyroid differentiation, and is lost in tumor cells. These data are in agreement with data recently published describing the role of FoxO3a in thyroid tumorigenesis and its involvement in the expression of $p27^{KIP1}$ (24).

We observed that Akt is involved in the control of FoxO1 localization in differentiated thyroid cells. However, the mechanism by which thyrotropin activates Akt which then promotes FoxO1 cytoplasmic localization remains to be fully elucidated. In other tissues FoxO1 is phosphorylated by Akt in the nucleus, but pAkt seems to be mostly located in the cytoplasm in the presence of thyrotropin in thyroid cells. In the absence of hormones FoxO1 is located both in the nucleus and the cytoplasm and becomes mainly cytoplasmic upon thyrotropin or IGF-I addition. It therefore seems possible that FoxO1 is phosphorylated by pAkt in the cytoplasm upon hormone treatment, impairing its nuclear localization. By contrast, the control of mRNA and protein levels is under the control of the cAMP pathway. To our knowledge, this is the first study that reports a control of FoxO1 levels by the cAMP pathway. Treatment of differentiated cells with a proteasome inhibitor impairs the thyrotropin-induced FoxO1 decrease at the protein level. This indicates that thyrotropin can activate the proteasome machinery, which could represent a new model of regulation by thyrotropin of processes like proliferation, differentiation and survival.

A different situation seems to exist in human thyroid tumor cells where the PI3K pathway is frequently activated (22), and we found greatly decreased levels of FoxO1. Furthermore, PI3K inhibition in these cells only slightly increased FoxO1 protein levels, whereas inhibition of the MAPK pathway was unable to increase FoxO1 levels. Inhibition of the proteasome did not markedly alter FoxO1 protein levels in tumor cells. We were not able to restore FoxO1 levels by the inhibition of the main signaling mediators of the tumorigenic process in thyroid cells or by the inhibition of the proteasome that is often involved in the degradation of FoxO1 in other tissues. It is therefore possible that FoxO1 is affected at several levels in tumor cells and probably in an irreversible way.

Regarding FoxO1 targets in thyroid cells, we focused on $p27^{KIP1}$ because this is a well-described FoxO1 target (41) and it plays an important role in proliferation. Given the broad range of target genes described for FoxO1 (42),

other proteins besides $p27^{KIP1}$ may very well be involved in the control of proliferation of the cells described in the present study. Our data indicate that this is not the case for cyclin D1; although this is a FoxO1 target (43), its levels were not affected by FoxO1 expression in tumor cells (data not shown).

The described control and repression of FoxO1 is compatible with FoxO1 having a role in thyroid proliferation. The rapid exit of FoxO1 from the nucleus after thyrotropin or IGF-I addition allows the cell cycle to begin, and in tumor cells, which escape the control of growth factors, FoxO1 is not expressed. When the signals elicited by thyrotropin or IGF-I are turned off, FoxO1 is reexpressed and enters into the nucleus promoting the expression or repression of genes that stop proliferation until thyrotropin and IGF-I reinitiate the process. Furthermore, thyrotropin and IGF-I control FoxO1 at several levels and in different ways. thyrotropin decreases FoxO1 mRNA and protein levels, but IGF-I more strongly induces the exit of FoxO1 from the nucleus. This can explain the synergistic effects of thyrotropin and IGF-I on the proliferation process. IGF-I increases the speed of the process by promoting a fast and potent FoxO1 nuclear exclusion, whereas thyrotropin ensures the duration of the process by degrading FoxO1 mRNA and protein.

Furthermore, preliminary data from our laboratory show that FoxO1, as was demonstrated for FoxO3a (24), may be involved in the oxidative stress response of thyroid cells because treatment of PCCl3 cells with H_2O_2 promotes nuclear localization of FoxO1 despite the presence of thyrotropin and IGF-I.

The results presented in this work show that FoxO1 is a critical mediator of the control of thyroid cells by thyrotropin and IGF-I, allowing the regulation of proliferation by both factors in a finely tuned process that involves the reversible regulation of FoxO1 at different levels. This contrasts with the scenario in thyroid tumor cells, in which the uncontrolled proliferation of the cells is accompanied by an irreversible loss of expression of FoxO1; importantly, decreased FoxO1 levels are also observed in surgically removed human thyroid tumors. These data indicate that FoxO1 is a candidate tumor suppressor for thyroid malignancies.

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