A signaling loop of REST, TSC2 and β-catenin governs proliferation and function of PC12 neural cells

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Summary

The RE-1-specific silencing transcription factor (REST or NRSF) is a transcription repressor that orchestrates differentiation and also operates in differentiated neurons and neurosecretory cells (neural cells). Its role in proliferation has been investigated so far only in rapidly growing tumors, with conflicting results: suppression in non-neural tumors, stimulation in medulloblastomas. Working with two clones of chromaffin–neuronal PC12 cells, which express different levels of REST, and using genetic complementation and knockdown approaches, we show that REST also promotes proliferation in differentiated neural cells. Mechanistically, this occurs by a signaling pathway involving REST, the GTPase-activating protein tuberin (TSC2) and the transcription co-factor β-catenin. In PC12 cells, raised expression of REST correlates with reduced TSC2 levels, nuclear accumulation and co-transcriptional activation of β-catenin, and increased expression of its target oncogenes *Myc* and *Ccnd1*, which might account for the proliferation advantage and the distinct morphology. *Rest* transcription is also increased, unveiling the existence of a self-sustaining, feed-forward REST–TSC2–β-catenin signaling loop that is also operative in another neural cell model, NT2/D1 cells. Transfection of REST, knockdown of TSC2 or forced expression of active β-catenin recapitulated the biochemical, functional and morphological properties of the high-expressing REST clone in wild-type PC12 cells. Upregulation of REST promoted proliferation and phenotypic changes, thus hindering neurosecretion. The new REST–TSC2–β-catenin signaling paradigm might have an important role in various aspects of neural cell physiology and pathology, including the regulation of proliferation and neurosecretion.

Key words: PC12 cells, NT2/D1 cells, Neural cell differentiation, Neurosecretion, Gene expression

Introduction

The RE-1-specific silencing transcription factor (REST, also referred to as NRSF) is a well-known repressor of hundreds of genes that contain the specific binding sequence RE-1 in their promoter (or other regulatory) region (Bruce et al., 2004; Wu and Xie, 2006; Otto et al., 2007). Many such genes are specific for neurons and neurosecretory cells (together known as neural cells). High levels of REST are typical of pluripotent stem cells and also of mature non-neural cells where REST precludes the expression of its target genes. In neural progenitor cells, a rapid downregulation of REST orchestrates the expression of a variety of neural-specific genes, including those encoding channels, receptors, enzymes and intracellular regulatory proteins (Ballas and Mandel, 2005; Johnson et al., 2007; Ooi and Wood, 2007). Additional genes, which are not direct targets of REST, are regulated indirectly, for example by the repression of transcription factors such as Isl1, Phox2a and Myt1 (Bruce et al., 2004; Otto et al., 2007; Quareshi and Mehler, 2009). Finally, REST has been found to repress the expression of non-coding RNAs such as miRNAs and maRNAs, acting at various post-transcriptional

levels including RNA processing, editing and trafficking (Conaco et al., 2006; Wu and Xie, 2006; Johnson et al., 2009; Quareshi and Mehler, 2009). The role of REST is important not only during development but also in mature neural cells, where its levels remain low, but are modulated in various physiological and pathological conditions. REST, therefore, is not only a master regulator of cell differentiation, but is also instrumental in the regulation of mature neural cell homeostasis and plasticity.

In addition to single genes, REST also governs the expression of comprehensive processes. Neurosecretion, a process typical of mature neural cells, takes place only in low-expressing ('low') REST cells inasmuch as most genes encoding for the proteins involved are targets of the repressor (Bruce et al., 2006; D'Alessandro et al., 2008). Moreover, the plasma membrane expansion necessary for neurite outgrowth can be sustained by the exocytosis of vesicles, the enlargeosomes, expressed under the control of REST (Racchetti et al., 2010; Schulte et al., 2010). Whether REST also has a role in the proliferation of competent neural cells remains unclear. In a rapidly growing, undifferentiated neural cell tumor, the medulloblastoma, high levels of the

repressor were reported to maintain the stemness of the cells (Su et al., 2006; Kagalwala et al., 2008). This mechanism, however, should not work in proliferating neural cells characterized by a more differentiated phenotype.

In the present study, carried out in PC12 cell pheochromocytoma line, a differentiated neural cell model that acquires a neuron-like phenotype upon treatment with NGF, we show in contrast that upregulation of REST promotes proliferation. This regulatory role of REST occurs through its functional interaction, never reported before, with two key ubiquitous signaling proteins known to promote proliferation: the

GTPase-activating protein tuberin (also known as tuberous sclerosis complex 2, TSC2) and the multifunctional protein β-catenin, which acts as a co-transcription factor. Gain of REST expression lowers TSC2 levels, which favors nuclear accumulation of β-catenin, ultimately enabling slowly proliferating cells with a higher rate of cell division. Of note, downregulation of TSC2 and β-catenin co-transcription not only promote PC12 cell proliferation, but also reinforces the expression of REST, which in turn induces the coordinated repression of neurosecretion. These results were duplicated in another type of neural cell, NT2/D1 cells (Andrews, 1984), investigated before and after differentiation by treatment with retinoic acid and transfection with REST. The self-sustaining, feed-forward signaling loop of REST, TSC2 and β-catenin appears therefore to be a signaling paradigm that participates in the regulation of functions that were until now believed to be controlled independently. It might therefore have a major role in the cell biology and physiology of neural cells.

Results

High-REST PC12-27 cells reveal a proliferation advantage when compared with low-REST wtPC12 cells

To investigate the role of REST in proliferation we took advantage of two PC12 clones extensively characterized in our laboratory: wild-type PC12 (wtPC12) and PC12-27 (Malosio et al., 1999; Grundschober et al., 2002). Whereas the first expresses the very low levels of REST typical of mature neural cells, the second spontaneously expresses levels of REST ~50-fold higher (D'Alessandro et al., 2008). The low and high REST levels accounted for full or defective competence for neurosecretion, respectively (D'Alessandro et al., 2008). In addition to their distinct neurosecretory competence, the two clones revealed clear differences in shape, size and cytoskeletal organization (Fig. 1A). wtPC12 cells exhibited the quasi-spherical phenotype typical of the cell line during growth (Greene and Tischler, 1976), whereas PC12-27 cells appeared larger, spread out and strictly adherent to the culture surface (Fig. 1A). The distribution of actin, which was ordered and concentrated in the cortical cytoplasm of wtPC12, was by contrast, mostly spread in thick fibers running through the
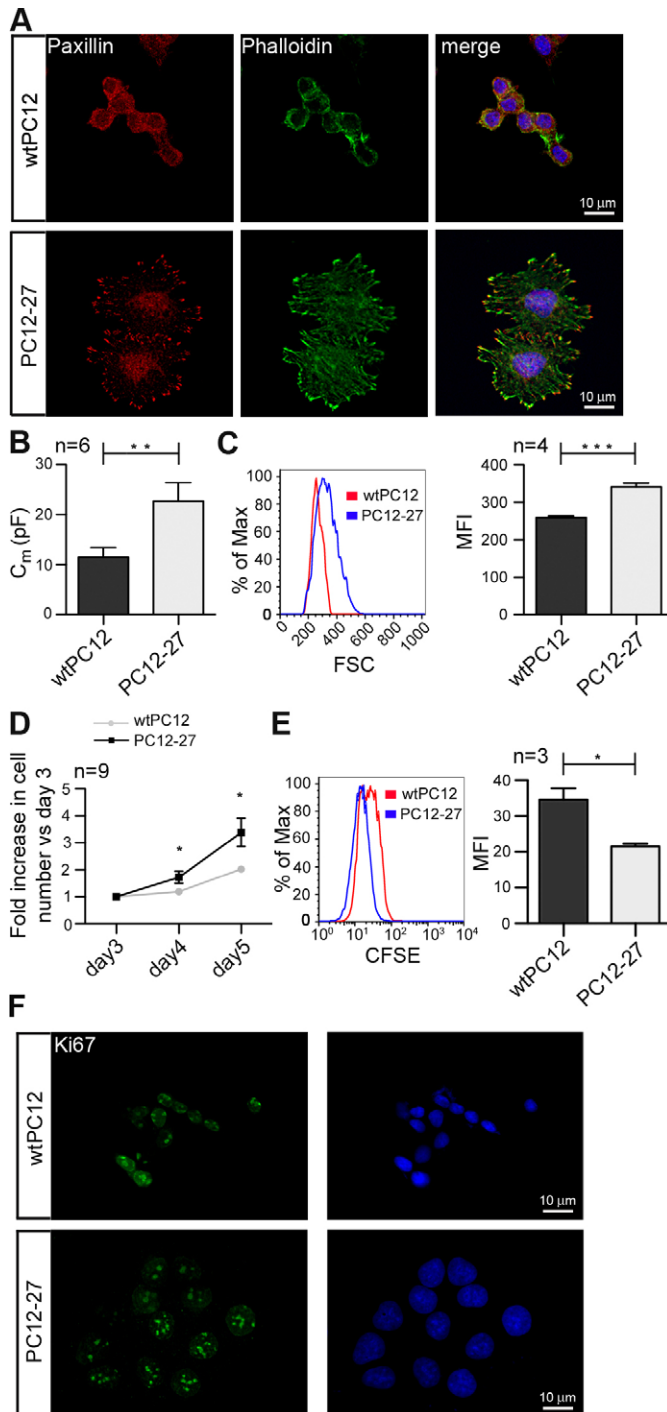
Fig. 1. PC12-27 cells reveal larger size and surface and proliferative advantage when compared with wtPC12 cells. (**A**) wtPC12 and PC12-27 cells, fixed and dually stained with antibodies against paxillin (left) and phalloidin–FITC conjugate (middle), were analyzed by confocal microscopy and image deconvolution. Nuclei were stained with DAPI. Images on the right were obtained by merging the three individual channels. (**B**) The surface area of attached wtPC12 and PC12-27 cells was evaluated by whole-cell patch-clamp capacitance (C_m) assays. Data shown, expressed in pF, are means ± s.e.m. of the results in 13 wtPC12 and 6 PC12-27 cells. (**C**) The volume of wtPC12 and PC12-27 cells was evaluated by cytofluorimetry. Representative histograms of the forward scatter (FSC) (left) and means ± s.e.m. of quantified results of four independent experiments (right) are shown. MFI, mean fluorescence intensity. (**D**) wtPC12 and PC12-27 cell proliferation was measured daily by viable Trypan-Blue-assisted counting of triplicate wells. The data shown are means ± s.e.m. of nine independent experiments expressed as fold increases in cell number versus day 3. Proliferation was revealed also by two additional techniques. (**E**) Flow cytometry of wtPC12 and PC12-27 cells labeled with the CFSE fluorescent dye: a representative day 5 histogram (left) and means ± s.e.m. of quantified results in three experiments with replicate wells (right) are shown. (**F**) Immunofluorescence confocal microscopy of the same cells dually stained in the nucleus with anti-Ki67 pAb and DAPI. ***$P<0.001$; **$P<0.01$; *$P<0.05$. Scale bars: 10 μm.

whole cytoplasm in PC12-27 cells. Paxillin, an adaptor protein of the complex linking the actin cytoskeleton to plasma membrane integrins, was also differently distributed in the clones, being concentrated in many discrete, small and closely adjacent puncta over the basal plasmalemma in wtPC12 cells, and in fewer, much larger structures, prominent especially in finger-like protrusions surrounding the cell profile of PC12-27 cells (Fig. 1A). The differences in surface area of attached wtPC12 and PC12-27 cells were quantified by patch-clamp cell capacitance assay (Racchetti et al., 2010). When compared with wtPC12, PC12-27 cells showed almost double capacitance values (22.7 ± 3.7 versus 11.4 ± 2.0 pF, corresponding to 1634 versus 892 μm^2) (Fig. 1B).

Likewise, when the cells were analyzed by flow cytometry, PC12-27 cells reproducibly showed a significantly larger forward scatter (FSC), which is proportional to cell size (Fig. 1C). We also noticed that PC12-27 cells reproducibly reached confluence faster than wtPC12 cells. To investigate the possibility that high levels of REST confer a proliferative advantage, we analyzed the two clones by daily, viable Trypan-Blue-assisted counting and established their single cell division rates by the 5-(6)-carboxyfluorescein succinimidylester (CFSE) dilution assay. Compared with wtPC12 cells, we found that, starting by day 4 after seeding, PC12-27 cells accumulated to higher numbers (Fig. 1D), and this was due to a faster rate of cell division on a per cell basis (Fig. 1D,E). To exclude the possibility that PC12 cells entering senescence might contribute to the observed differences, expression of Ki67, a indicator of active proliferation, was investigated. Fig. 1F shows that Ki67 immunolabeling was intense and followed the distribution of chromatin in the nuclei of both wtPC12 and PC12-27 cells, characterized by small and large areas, respectively. This result supports the notion that the growth advantage of PC12-27 over PC12 cells (depicted in Fig. 1D) is caused by increased proliferation of the former, rather than premature senescence of the latter. Thus, low and high REST-expressing PC12 cells show differences not only in neurosecretion (D'Alessandro et al., 2008), but also in cell size, shape and proliferation rate.

Faster proliferation rate of high-REST PC12-27 cells reflects downregulation of TSC2 and increased β-catenin co-transcriptional activity

The general phenotype of PC12-27 cells, combined with their faster rate of proliferation, was reminiscent of the phenotype previously reported for HeLa cells defective of rictor (Sarbassov et al., 2004). Rictor is a member of the mammalian target of rapamycin protein kinase complex 2 (mTORC2). Knockdown of rictor results in a defect of mTORC2 accompanied by overstimulation of mTORC1 with ensuing inhibition and activation, respectively, of the signaling cascades governed by the two mTOR complexes (Sarbassov et al., 2004). To investigate whether mTORCs have a role in wtPC12 and PC12-27 cells, we assayed the phosphorylation of target proteins downstream of the two complexes. S6 and 4E-BP1, commonly used as readouts of mTORC1 activity, were phosphorylated in both wtPC12 and PC12-27 cells, however, to a higher extent in the latter. By contrast, phosphorylation of Akt at S473, a readout of mTORC2 activity, and of the Akt substrate, glycogen synthase kinase 3β (GSK3β) at S9, was lower in the high-REST PC12-27 cells when compared with the low-REST wtPC12 cells (supplementary material Fig. S1A). Thus, in PC12-27 cells, the signaling of both mTORC1 and mTORC2 appears to be deregulated.

To establish whether the proliferative advantage of PC12-27 cells was dependent on the increased activity of mTORC1, we investigated the effect of the inhibitory drug rapamycin. In spite of the marked inhibition of the mTORC1 phosphorylation (see supplementary material Fig. S1B), the proliferation of high-REST PC12-27 cells was largely insensitive to the drug (supplementary material Fig. S1C). Because of the well-known inhibitory action of rapamycin on mTORC1, and in spite of the caveats associated with the use of pharmacological tools, these results strongly suggest that the faster proliferation of PC12-27 cells depends only to a minor extent on the kinase. Because of this, mTORC1 was not investigated any further.

We therefore turned our attention to the regulatory steps upstream of mTORC1. A main controller of the latter kinase is the tuberous sclerosis (TSC) complex, which is composed of two proteins, TSC1 and TSC2. Although the complex, by its binding to the small GTPase Rheb, inhibits mTORC1, it promotes mTORC2 signaling (Huang et al., 2008; Huang and Manning, 2009; Laplante and Sabatini, 2009). In addition, the TSC1–TSC2 complex can have an impact on cell proliferation through its positive control of the turnover of β-catenin (Mak et al., 2003; Jozwiak and Wlodarski, 2006; Barnes et al., 2010). We thus investigated the expression of the TSC complex and β-catenin in wtPC12 and PC12-27 cells. Although TSC1 levels did not differ to a significant extent (supplementary material Fig. S2A), TSC2 levels were consistently and significantly lower (–45%) in PC12-27 compared with wtPC12 cells (Fig. 2A). This was due to post-transcriptional event(s), because Tsc2 mRNA levels were similar in wtPC12 and PC12-27 cells (supplementary material Fig. S2B).

In line with reduced TSC2 levels, expression of β-catenin was higher in PC12-27 cells when compared with wtPC12 cells, with significantly higher representation in the nucleus (Fig. 2B). This correlated with a higher β-catenin-dependent transcriptional activity (revealed by a luciferase reporter assay, Fig. 2C) and with the higher expression of known β-catenin–TCF-LEF target genes Myc, Rest (Willert et al., 2002; Nishihara et al., 2003) and (to a lower extent) also Ccnd1 (Fig. 2D).

To investigate whether β-catenin transcription activity was indeed responsible for the higher target gene expression and the proliferation advantage of PC12-27 cells, we adopted a pharmacological approach using two drugs known to operate by different mechanisms. We took advantage of quercetin, a blocker of the β-catenin–TCF-LEF transcription (Park et al., 2005) and of endo-IWR1, which favors β-catenin degradation (Chen et al., 2009). In PC12-27 cells, both drugs inhibited to a significant extent the β-catenin-dependent reporter gene expression (Fig. 2E) and the expression of Myc and Rest (Fig. 2F), whereas in wtPC12, these effects were smaller and non-significant (not shown). Moreover, the two drugs abrogated the proliferation advantage of high-REST PC12-27 cells (Fig. 2G). Taken together, results obtained by the comparison of wtPC12 and PC12-27 cells link REST levels to TSC2 levels and to β-catenin nuclear activity, which is critical for cell proliferation.

REST, TSC2 and β-catenin, interconnected in a feed-forward loop, control PC12 cell proliferation

The results reported so far (Figs 1 and 2), which reveal differences in structure, signaling and proliferation between the two PC12 clones, the low-REST wtPC12 and the high-REST PC12-27, suggested that REST, TSC2 and β-catenin might be interconnected in a signaling loop controlling proliferation of
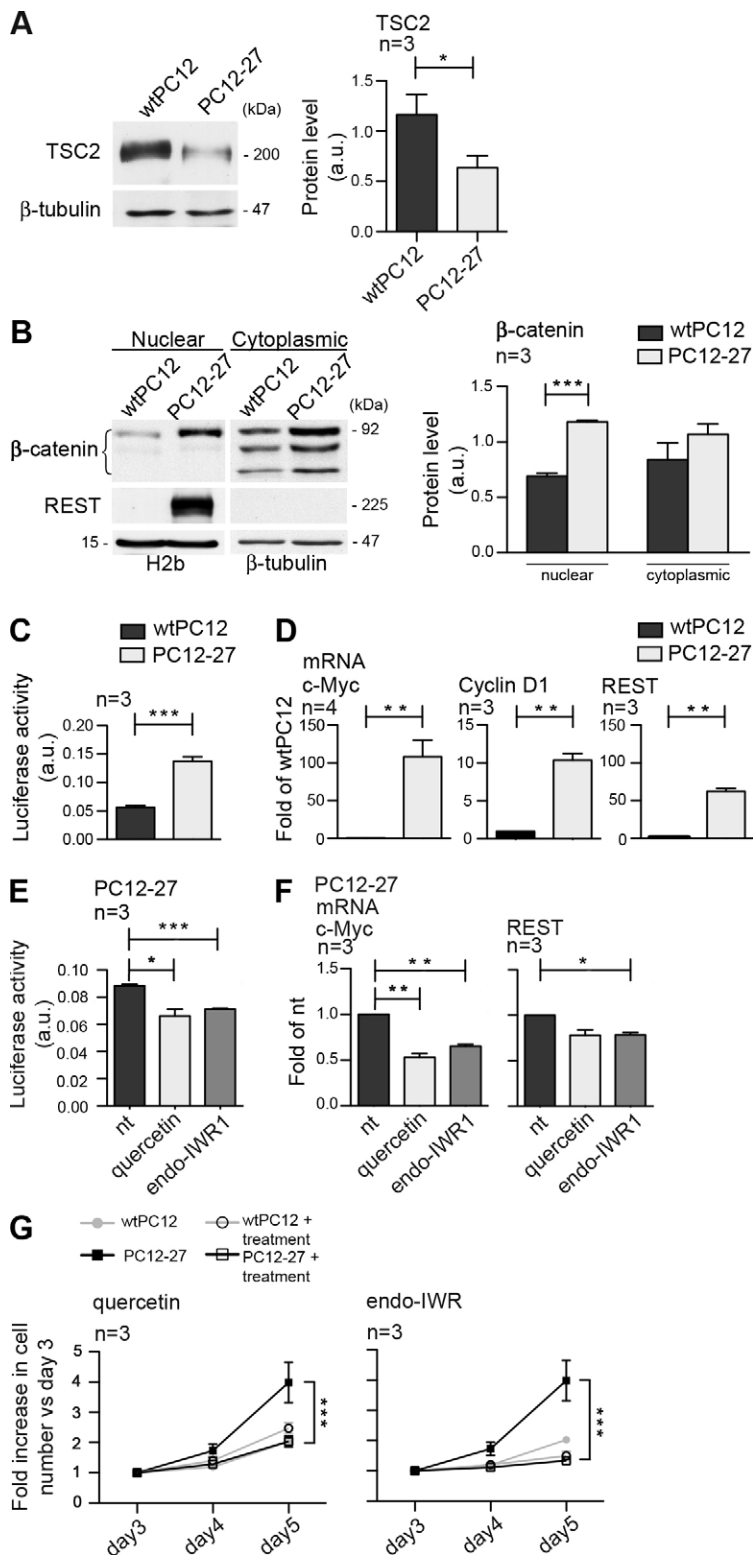
Fig. 2. In high-REST PC12-27 cells, reduced TSC2 levels correlate with β-catenin nuclear accumulation and with increased β-catenin-dependent transcription and proliferation. (**A**) Lysates and (**B**) nuclear and cytosolic fractions of wtPC12 and PC12-27 cells were analysed by SDS-PAGE and western blotting. β-tubulin and the histone H2b were used for normalization. Representative western blots, with molecular size markers (indicated here and in the following figures in kDa), are shown to the left; means ± s.e.m. of the results of three independent experiments quantified by densitometry, on the right. a.u., arbitrary units. (**C**) β-catenin co-transcriptional activity was evaluated by a luciferase reporter assay. wtPC12 and PC12-27 cells were transfected, the luciferase activity was measured 24 hours later. Results shown are means ± s.e.m. from three independent experiments. (**D**) Expression of β-catenin target genes in wtPC12 and PC12-27 cells. mRNA encoding Myc, cyclin D1 and REST was assayed by RT-PCR and values were normalized to the *GAPDH* housekeeping gene and then expressed relative to control wtPC12 cells. Means ± s.e.m. of the results from three independent experiments are shown. (**E**) β-catenin co-transcriptional activity evaluated by the luciferase reporter assay: effects of quercetin (a blocker of β-catenin-dependent transcription, 100 μM) and endo-IWR1 (that favors β-catenin degradation, 10 μM) administered from the fourth and fifth day of culture. Nt, untreated cells. Results shown are means ± s.e.m. from three independent experiments. (**F**) Expression of *Myc* and *Rest* genes in PC12-27 cells, effects of quercetin and endo-IWR1, conditions and processing and presentation of the results as in D. Results shown are means ± s.e.m. from three independent experiments. (**G**) wtPC12 and PC12-27 cell proliferation measured daily by viable Trypan-Blue-assisted counting of triplicate wells as in Fig. 1D. nt, PC12-27 cells untreated with the drugs. Treatment with quercetin and endo-IWR1 as in E. Results shown are means ± s.e.m. from three independent experiments. Significance shown between PC12-27 cells without and with drug. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

PC12 cells. In view of the considerable differences in gene expression existing between the two clones (Grundschober et al., 2002), however, the possibility of the results to be correlative, rather than conclusive, could not be excluded. To directly prove the link of REST, TSC2 and β-catenin we carried out gene complementation and downregulation experiments.

First, we investigated the impact of REST downregulation and TSC2 overexpression in PC12-27 cells. Transient transfection of a dominant-negative construct of REST (D'Alessandro et al., 2008) correlated with significant increases of the TSC2 levels (supplementary material Fig. S3A), suggesting that the latter is under the inhibitory control of the repressor. Likewise, transfection

of PC12-27 cells with a full-length construct of TSC2 caused a significant decrease of *Myc* and *Rest* gene expression (supplementary material Fig. S3B), together with a gain of the β-catenin and REST proteins. These initial data suggest therefore that the expression of REST, TSC2 and β-catenin is linked.

Then we turned to wtPC12 cells. First, the cells were infected with lentiviral vectors encoding GFP (control cells) or for GFP–Myc-tagged REST, and then sorted by FACS. Forced REST upregulation (Fig. 3A,B) caused a downregulation of TSC2 with a gain of β-catenin levels (Fig. 3C) and activity (Fig. 3D,E). These changes were accompanied by changes of protein phosphorylation expected in low-TSC2 cells, i.e. increased phosphorylation of the mTORC1 targets, S6 and 4E-BP1, and decreased phosphorylation of the mTORC2 target Akt and of GSK3β (supplementary material Fig. S4). REST-infected PC12 cells also revealed a proliferation advantage when compared with control-infected cells (Fig. 3F).

We next investigated the effects of TSC2 knockdown by shRNA. TSC2 levels were reduced when compared with the wtPC12 cells transfected with a control shRNA (Fig. 4A). TSC2 downregulation correlated with increased accumulation of β-catenin (Fig. 4A) that proved to be transcriptionally active (Fig. 4B), and with large increases of *Myc* and *Ccnd1* gene expression (Fig. 4C). In addition to β-catenin, also REST levels and gene expression were found to increase following TSC2 downregulation (Fig. 4A,C), and TSC2-knockdown cells accumulated to greater numbers compared with control wtPC12 and similarly to PC12-27 cells (Fig. 4D). Of note, this proliferation advantage was abrogated by quercetin and endo-IWR1, drugs that affect β-catenin transcription and promote its degradation, respectively (Fig. 4E).

Last, we evaluated the impact of forcing β-catenin nuclear accumulation and transcriptional activity. PC12 cells were stably transfected with a β-catenin construct truncated of the N-terminal
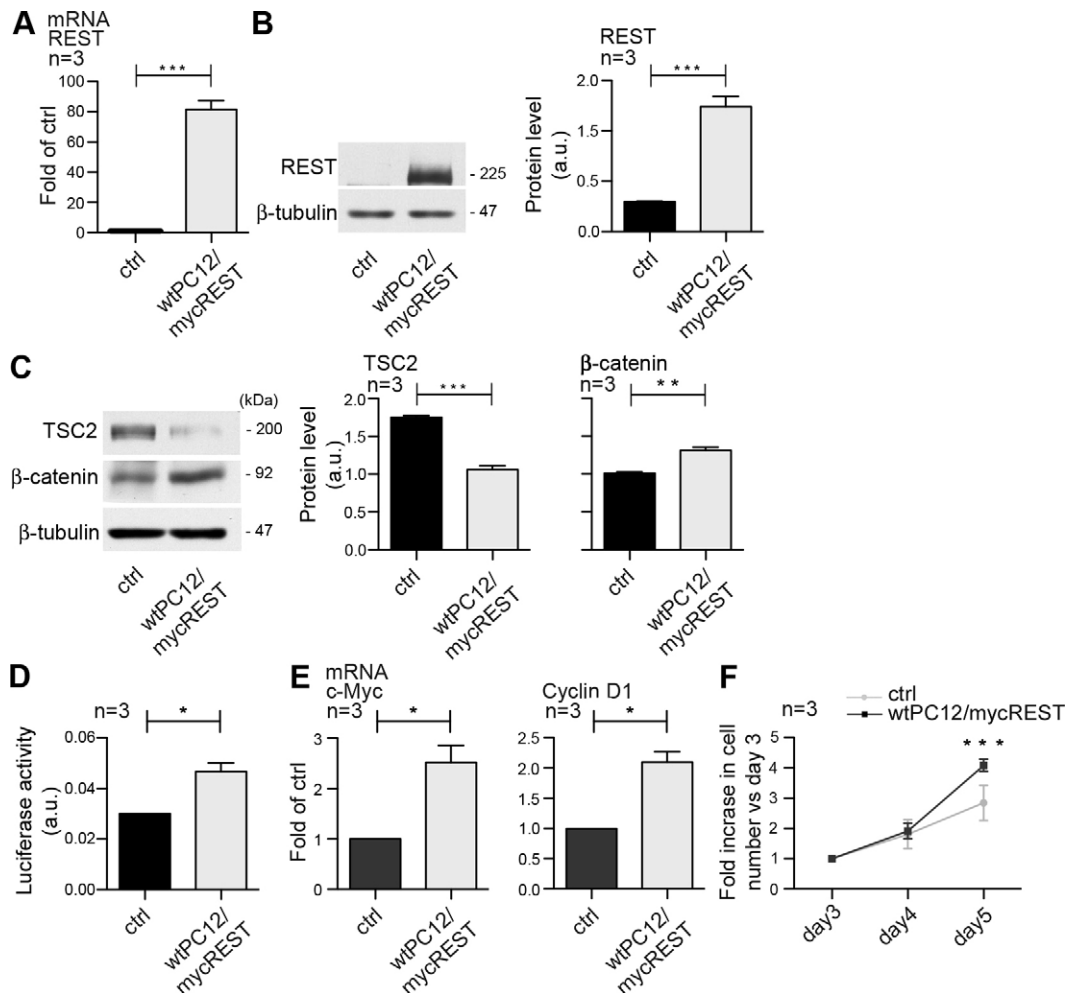
Fig. 3. REST regulates the levels of TSC2 and β-catenin and cell proliferation in wtPC12. wtPC12 cells were infected with GFP (ctrl) or GFP–Myc-tagged REST (mycREST) lentiviral vectors. (**A**) Expression of *Rest* mRNA. (**B**) Levels of REST in the two infected cell populations, representative western blots also showing β-tubulin used for normalization (left); and means ± s.e.m. of the results of three independent experiments quantified by densitometry (right). (**C**) TSC2 and β-catenin in the two infected populations, presentation as in A. (**D**) β-catenin co-transcriptional activity evaluated by the luciferase reporter assay in the two infected cell populations. Conditions and processing and presentation of the results (from three independent experiments) as in Fig. 2C. (**E**) Expression of β-catenin target genes, conditions and processing and presentation of the data (from three independent experiments) as in Fig. 2D. (**F**) Proliferation of the two infected cell populations measured daily by viable Trypan-Blue-assisted counting of triplicate wells; means ± s.e.m. from three independent experiments as in Fig. 1D. ***$P<0.001$; **$P<0.01$; *$P<0.05$.
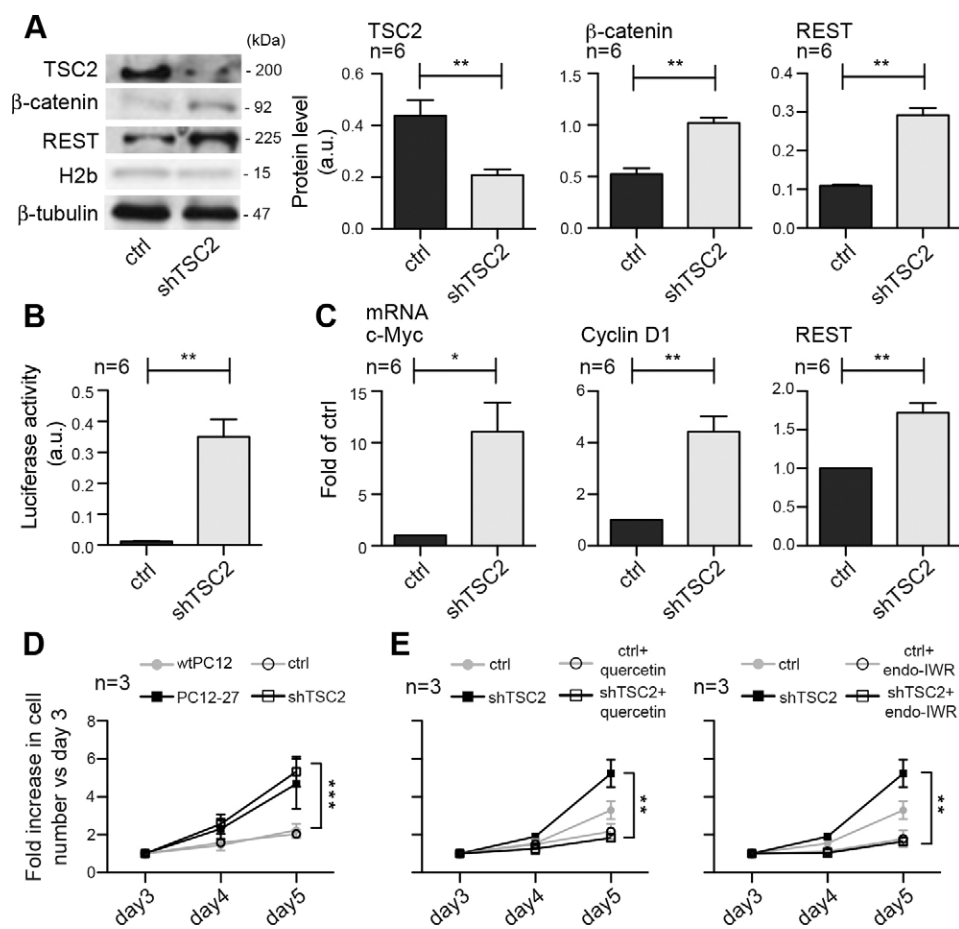
Fig. 4. TSC2 regulates β-catenin levels and cell proliferation in wtPC12. wtPC12 cells were stably transfected with the control construct (ctrl) or the shRNA TSC2 construct (shTSC2). (**A**) Representative western blots of cells infected with the two constructs showing the levels of TSC2, β-catenin and REST together with H2b and β-tubulin used for normalization (left); and means ± s.e.m. of the results of six independent experiments quantified by densitometry (right). (**B**) β-catenin co-transcriptional activity evaluated in the control and shTSC2-transfected cells by the luciferase reporter assay. Conditions and processing/presentation of the results (from six independent experiments) as in Fig. 2C. (**C**) Expression of the *Myc*, *Ccnd1* and *Rest* genes in the control and shTSC2-transfected cells. Conditions and processing and presentation of the data from six experiments as in Fig. 2D. (**D**) wtPC12, PC12-27, control and shTSC2 cells proliferation measured daily by viable, Trypan-Blue-assisted counts of triplicate wells; means ± s.e.m. from three independent experiments as in Fig. 1D. Significance shown between control and shTSC2-transfected cell populations. (**E**) Control and shTSC2 cell proliferation, effects of quercetin (middle) and endo-IWR (right), presented as in D. Significance shown between shTSC2 and shTSC2+drug cell populations. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

90 amino acid residues (Δ90βCat-GFP). Loss of these residues increases β-catenin stability (Chenn and Walsh, 2002). Expression of Δ90βCat (Fig. 5A) was reflected by gained β-catenin co-transcriptional activity (Fig. 5B) and upregulation of *Myc* and *Rest* gene expression (Fig. 5C). Expression of the *Ccnd1* gene was also increased, albeit to a lower extent (Fig. 5C). The levels of the REST protein were increased in Δ90βCat-transfected wtPC12 cells, whereas TSC2 levels were decreased (Fig. 5D). Δ90βCat-transfected cells also revealed a proliferation advantage that was similar to that of PC12-27 cells when compared with parental and control cells (Fig. 5E). Interestingly, this advantage was largely abrogated by quercetin (Fig. 5F, left panel), but not by endo-IWR (Fig. 5F, right panel). This latter result was expected because endo-IWR is largely ineffective on the degradation-resistant Δ90βCat construct.

Thus, forcing upregulation of REST, downregulation of TSC2 or increased transcriptional activity of β-catenin promote reciprocal changes in their relative expression levels, and have an impact on wtPC12 cell proliferation. These results, which recapitulate in wtPC12 the properties of PC12-27 cells, directly link REST, TSC2 and β-catenin in a feed-forward loop favoring PC12 cell proliferation.

Interfering with the level or the activity of REST, TSC2 or β-catenin co-ordinately impacts the structure and neurosecretion of wtPC12 cells

In addition to the differences in proliferation, the comparative study of the low- and high-REST PC12 clones, wtPC12 and PC12-

27, revealed marked differences in their shape and cytoskeleton organization (Fig. 1A–C). The question now was whether the changes of individual components of the REST–TSC2–β-catenin signaling loop, described in Figs 3–5 and supplementary material Fig. S3, were also able to impact the general structure of wtPC12 cells, making them similar to the PC12-27 cells. Fig. 6 demonstrates that this was indeed the case. Although the changes observed were not identical, the wtPC12 cells infected with REST (Fig. 6A), knocked down for TSC2 (Fig. 6B) or transfected for Δ90βCat (Fig. 6C) appeared all larger, flatter and more strictly attached to the culture surface when compared with the various controls, transfected with the empty plasmids, that appeared largely analogous to the parental wtPC12 cells. The actin cytoskeleton was reorganized in fibers running through the whole cytoplasm (evident especially in the REST-infected cells, Fig. 6A) and in discrete structures (prominent in the Δ90βCat cells, Fig. 6C), whereas paxillin was also redistributed with some accumulation in the peripheral cytoplasmic protrusions (prominent in the REST-infected cells, Fig. 6A).

In various cell types, the acquisition of a proliferative advantage is accompanied by defects of cell-specific functions. Because REST is a repressor of many neural cell-specific genes, in particular of those encoding proteins involved in neurosecretion (Bruce et al., 2006; D'Alessandro et al., 2008), we investigated whether decreasing TSC2 levels and favoring β-catenin transcription activity might also have an impact on the expression of neurosecretory genes. To this aim, we analyzed the expression of three specific genes known to be REST targets
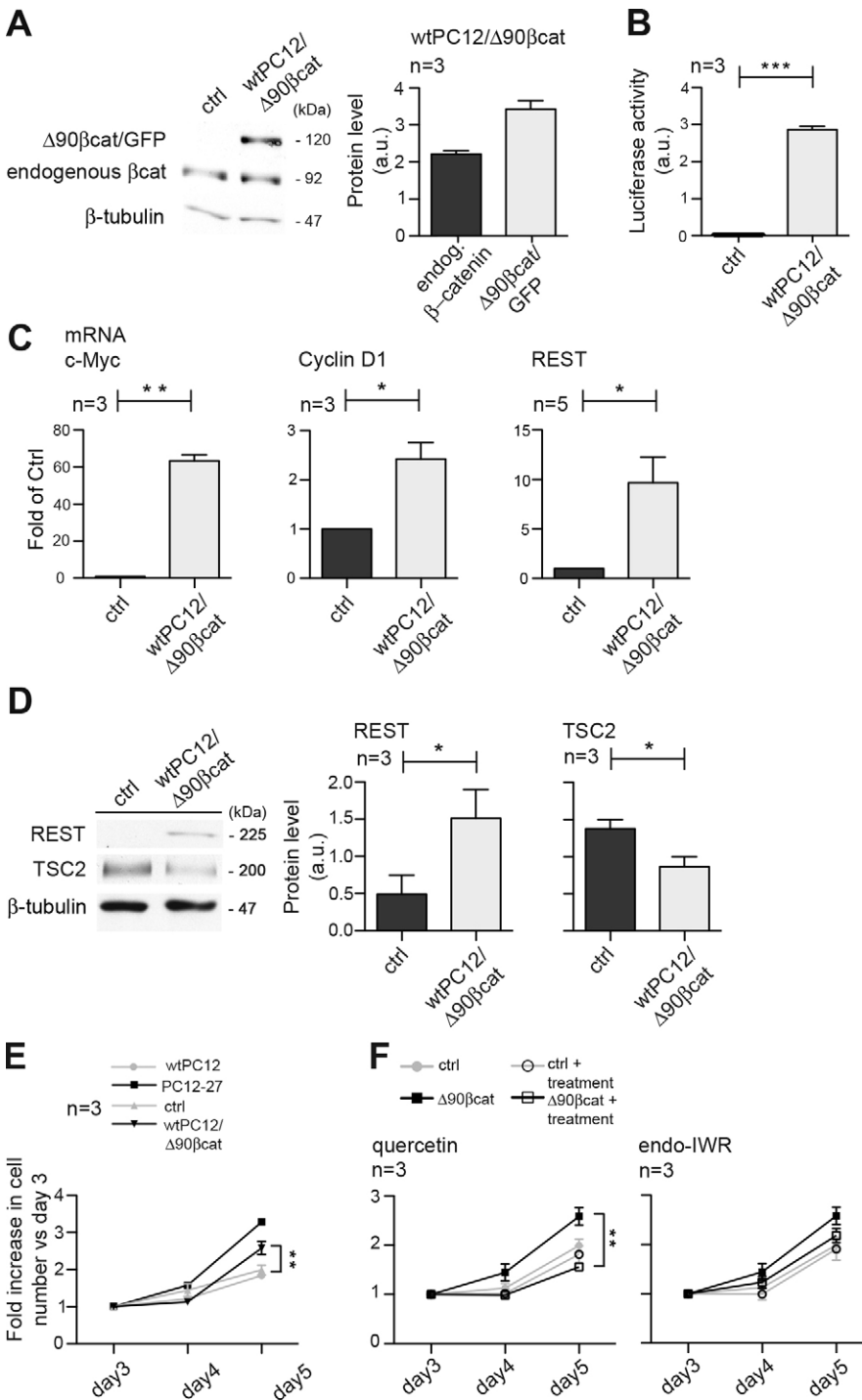
Fig. 5. β-catenin regulates REST and TSC2 levels and cell proliferation in wtPC12. wtPC12 cells were stably transfected with either an empty construct (ctrl) or a construct encoding an N-terminal-truncated form of β-catenin (Δ90βcat). (**A**) Representative western blots of cells infected with the control and Δ90βcat constructs showing the levels of Δ90βcat-GFP and endogenous β-catenin together with β-tubulin used for normalization (left); and comparison of the levels (means ± s.e.m.) of the two forms of β-catenin, results of three independent experiments quantified by densitometry (right). (**B**) β-catenin co-transcriptional activity evaluated by the luciferase reporter assay in the control- and Δ90βcat-transfected cells. Conditions and processing/presentation of the results (from three independent experiments) as in Fig. 2C.
(**C**) Expression of the *Myc*, *Ccnd1* and *Rest* genes in the control and Δ90βcat-transfected cells. Conditions and processing and presentation of the data from three to five experiments as in Fig. 2D. (**D**) Representative western blots of cells infected with the control and the Δ90βcat constructs showing the levels of REST and TSC2 together with β-tubulin used for normalization (left); and means ± s.e.m. of the results of three independent experiments about the REST and TSC2 levels quantified by densitometry (right). (**E**) Control and Δ90βcat-transfected cell proliferation measured daily by viable Trypan-Blue-assisted counts of triplicate wells. Comparison with non-transfected wtPC12 and PC12-27 (left), Means ± s.e.m. from three independent experiments as in Fig. 1D. Significance shown between control and Δ90βcat. (**F**) Control and Δ90βcat populations, effects of quercetin (middle) and endo-IWR (right). Means ± s.e.m. from three independent experiments as in Fig. 1D. Significance shown between Δ90βcat and Δ90βcat+drug cell populations. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

(Bruce et al., 2006; D'Alessandro et al., 2008): the dense-core vesicle secretory granin, chromogranin B, the vesicle Ca^{2+} sensor synaptotagmin-1 and the plasma membrane SNARE SNAP25. In the wtPC12 cells infected with REST (Fig. 7A), knocked-down for TSC2 (Fig. 7B) or transfected with Δ90βCat (Fig. 7C), the proteins encoded by these neurosecretion genes were decreased, to significant degrees except for synaptotagmin-1 in the Δ90βCat-transfected cells. Thus, changes in either REST, TSC2 or β-catenin affect not only proliferation, but also the shape, cytoskeleton and neurosecretory function of PC12 cells.

The REST–TSC2–β-catenin loop operates also in NT2/D1 cells

To establish whether the results reported for PC12 cells might be valid also for other neural cells, we investigated the human NT2/D1 neural cell model. NT2/D1 cells grown in culture exhibit an undifferentiated phenotype that is converted irreversibly into neuron-like during long-term treatment with retinoic acid (Andrews, 1984). Fig. 8A shows that expression of *Myc* and *Rest* was very high in undifferentiated NT2/D1 cells, and greatly reduced upon 4 weeks of treatment with retinoic acid (10 µM).
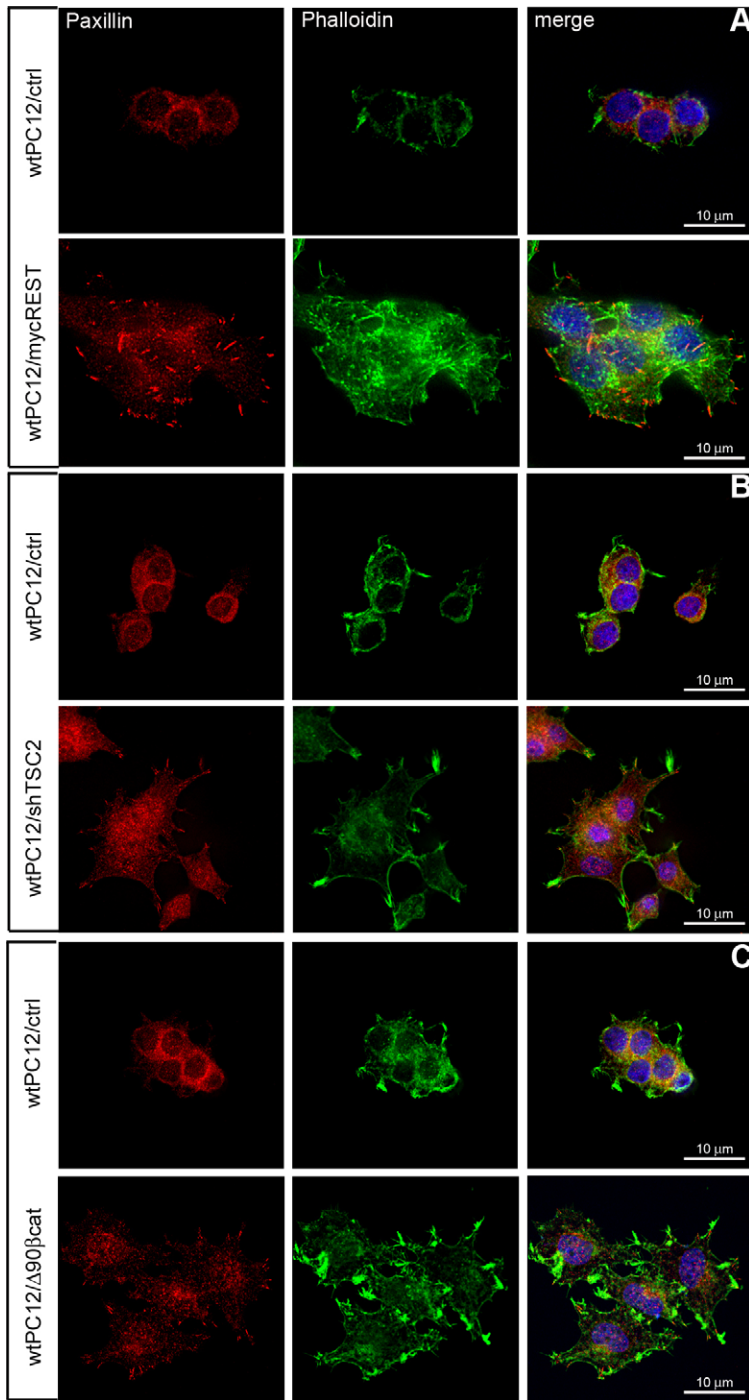
**Fig. 6. wtPC12 cell size, shape and cytoskeleton before and after infection or transfection with REST, TSC2 or β-catenin.** Representative images of wtPC12 infected or transfected with the control constructs (ctrl) of Figs 3, 4 and 5 (top panels of **A**, **B** and **C**) and the corresponding cells infected or transfected with mycREST, shTSC2 and Δ90bcat–GFP (bottom panels), labeled by dual immunofluorescence with anti-paxillin antibodies (left) and FITC-conjugated phalloidin (middle). Nuclei were stained with DAPI. Merged images are on the right. Scale bars: 10 μm.

This was accompanied by a significant gain of TSC2 levels, by the downregulation of REST and β-catenin (Fig. 8B) and by significant increases of neurosecretion markers, synaptotagmin-1 and SNAP25 (Fig. 8C). Infection of the retinoic-acid-differentiated NT2/D1 cells (NT2-N in Fig. 8D–F) with REST induced a reversal of these changes: expression of *Myc* and *Rest* was re-established (Fig. 8D), TSC2 levels were greatly reduced, whereas β-catenin levels were increased (Fig. 8E). These changes were accompanied by the attenuation of the neurosecretion markers, synaptotagmin-1 and SNAP25 (Fig. 8F). The link between REST, TSC2 and β-catenin appears therefore to operate in NT2/D1 cells analogously to PC12 cells.

Discussion

Previous studies on the role of REST in cell proliferation have been carried out in rapidly growing tumors. The results indicated that REST could function both as a tumor suppressor and as an oncogene, depending upon the cellular context (Coulson, 2005; Majumder, 2006). Aggressive tumors of non-nerve origin (of the lung, breast, colorectum and prostate) were found to have lost (or greatly reduced) the otherwise high REST levels of their cells of origin (Coulson, 2005; Majumder, 2006; Wagoner et al., 2010; Lv et al., 2010). This suggested that REST functions in these cells as a tumor suppressor (Westbrook et al., 2005) by repressing transcription of the proto-oncogene TrkC (Mulligan et al., 2008)
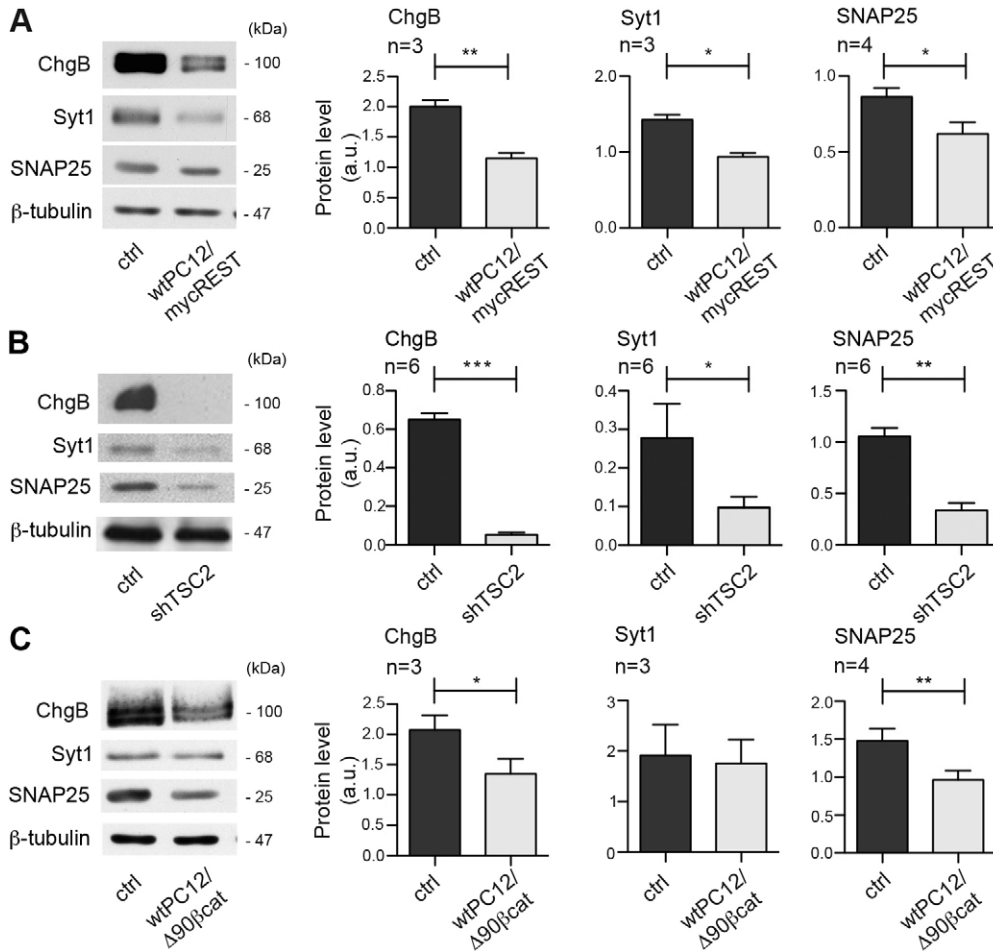
Fig. 7. Changes in TSC2, β-catenin or REST expression levels affect the wtPC12 cell neurosecretory machinery. (A–C) Representative western blots of three proteins involved in neurosecretion, the secretory protein chromogranin B (ChgB), the Ca^{2+} sensor synaptotagmin-1 (Syt1) and the pSNARE SNAP25, in the transfected wtPC12 cells of Figs 3, 4 and 5, i.e. the three controls (ctrl) and the cells transfected with Myc–REST (A), shTSC2 (B) and Δ90βcat (C). The corresponding means ± s.e.m. of the results of three to six independent experiments quantified by densitometry are shown on the right. ***$P<0.001$; **$P<0.01$; *$P<0.05$.

and/or of the kinase Akt2 (Kreisler et al., 2010). By contrast, studies on several rapidly growing medulloblastomas and medulloblastoma cell lines provided clear hints for an oncogenic role for REST. In these cells, REST expression was found to be aberrant and much higher than that found in differentiated neural cells (Lawinger et al., 2000). Moreover, forced expression of a dominant-negative construct reduced the tumorigenic potential of these cells (Fuller et al., 2005). High levels of REST alone were, however, insufficient for tumorigenesis and cooperation with high levels of Myc in this process was needed (Su et al., 2006). These findings raised questions regarding the mechanism by which high REST promotes its tumorigenic effects. The mechanism proposed, maintenance of cell stemness (Su et al., 2006; Kagalwala et al., 2008), could be appropriate for rapidly growing tumors, but not for differentiated neural cells.

Our present work in the PC12 pheochromocytoma cell line extends the study to a new area. Indeed, PC12 cells profoundly differ from poorly differentiated, rapidly growing medulloblastomas because they are more slowly proliferating, chromaffin-like cells, which, upon exposure to NGF, stop growing and are converted into neuronal-like cells (Greene and Tischler, 1976). For these reasons, they are the most widely used to model differentiated neural cells. By the comparative analysis of two clones of PC12, chosen because of their high (PC12-27) and low (wtPC12) expression levels of REST, and by genetic complementation results, we demonstrate that high levels of

REST confer a selective proliferation advantage in PC12 cells. Mechanistically, we found that this is due to the ability of REST to lower TSC2 levels by a post-transcriptional process that remains to be identified, and consequently allow β-catenin co-transcriptional activity. Initially, we considered the possibility that the proliferation advantage was due to hyperactivation of mTORC1, which indeed was evident in PC12-27 cells. However, the proliferation advantage of the latter cells was mostly insensitive to the classical blocker of mTORC1, rapamycin. This finding, which is consistent with a previous report of PC12 cells treated with growth factors (Powers et al., 1999), excludes a main role for mTORC1 in PC12 cell proliferation, which therefore was not investigated any further.

Low levels of TSC2 in PC12-27 cells were also found to be accompanied by the gain and the ensuing nuclear translocation of β-catenin, a co-transcription factor known to control cell proliferation. The cellular levels of β-catenin are controlled by its turnover, which is governed by the GSK3β complex. A number of signaling pathways, including the Wnt pathway, converge to repress the activity of this complex. In PC12-27 cells, however, a major role for GSK3β phosphorylation by kinases such as Akt appears unlikely, because its target site, the S9 residue of GSK3β (Zhang et al., 2006), was phosphorylated to a much lower extent in PC12-27 cells when compared with wtPC12 (supplementary material Fig. S1A). Furthermore, previous studies in PC12 cells reported the ability of Wnt to increase β-catenin levels and cell proliferation only upon

application of the ligand or overexpression of Wnt, its frizzled receptors or the intracellular effector dishevelled (Chou et al., 2000; Kaliwara et al., 2008; Chacòn et al., 2008; Spinsanti et al., 2008), which is not the case in our study. Thus, our data suggest that, as previously reported in other cell systems (Mak et al., 2003; Jozwiak and Wlodarski, 2006; Barnes et al., 2010), a decrease of TSC2 hinders the stability of the GSK3β complex and hence favors the decreased degradation and nuclear

translocation of β-catenin, with ensuing strengthening of its transcriptional activity.

In addition to proliferation, which is probably caused by the increased transcription of oncogenes such as *Myc* and *Ccnd1*, the gain and nuclear translocation of β-catenin was found to increase transcription of *Rest*. Although the REST protein has a short half-life, and its levels are believed to depend primarily on its rapid turnover (Ballas and Mandel, 2005; Guardavaccaro et al., 2008; Westbrook et al., 2008), a β-catenin control of *Rest* gene transcription has been reported in teratocarcinoma (Willert et al., 2002) and neural stem (Nishihara et al., 2003) cells. Thus, the TSC2-controlled gain of β-catenin and the β-catenin–TCF-LEF transcription of the *Rest* gene has already been reported; however, the connection between these pathways was previously unrecognized. The notion that changes of REST protein levels might also be ascribed to transcriptional regulation in PC12 cells was underlined by the inhibitory effect of quercetin, a drug that blocks the β-catenin–TCF-LEF transcription system. Our results uncovered the existence of a loop, and more specifically, the ability of REST to set the level of TSC2. Although the mechanism of the latter process remains to be clarified, the identification of the link has been crucial to reveal the signaling architecture of the loop, which appears not to be restricted to PC12 cells, but is also present in retinoic-acid-treated NT2/D1 cells.

Interfering with REST, TSC2 or β-catenin was found to impact not only the proliferation but also the size, shape, cytoskeletal organization and even neurosecretory function of PC12 cells. The role of TSC2 in the manipulation of the actin cytoskeleton and focal adhesions, taking place through the small GTPase Rac1, was known (Goncharova et al., 2004). Our results demonstrate for the first time the similar effects induced by manipulations of the other two factors, possibly mediated by their connection with TSC2. The inhibition of neurosecretion provides a mechanistic interpretation to the finding that rapidly proliferating neural cells are poor or defective in neurosecretion, whereas differentiated neural cells have lower or no proliferating capacities and proper neurosecretory functions, thus unveiling a new pathway that concomitantly governs the two processes (see model in supplementary material Fig. S5).

In conclusion, our results demonstrate that REST, TSC2 and β-catenin are linked in an integrated, self-sustaining feed-forward loop that is crucial for PC12 cell proliferation and neurosecretory functions. This loop, which could crosstalk with the growth factor
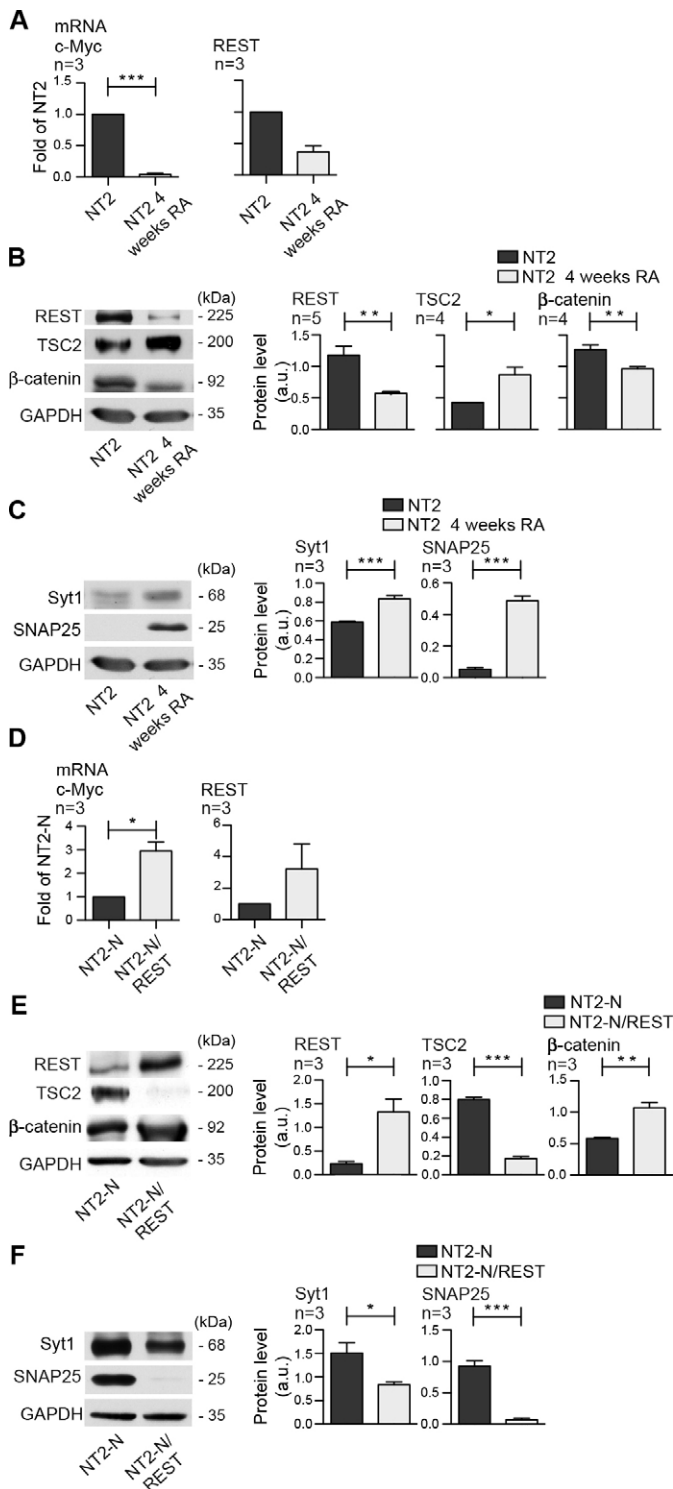
Fig. 8 The REST, TSC2 and β-catenin signaling loop also operates in NT2/D1 neural cells. (A–C) Results with NT2/D1 cells before and after differentiation with retinoic acid (10 μM, 4 weeks). (A) Expression of the *Myc* and *Rest* genes, means ± s.e.m. of three experiments. (B) Representative western blots showing the levels of REST, TSC2 and β-catenin together with GAPDH used for normalization (left); and means ± s.e.m. of the results of four to five independent experiments quantified by densitometry (right). (C) Representative western blots showing the levels of two proteins involved in neurosecretion, synaptotagmin-1 (Syt1) and SNAP25 together with GAPDH (right) and means ± s.e.m. of the results of three independent experiments quantitated by densitometry (right). (D–F) The corresponding results obtained with retinoic-acid-differentiated NT2/D1 cells (NT2-N) infected with control or REST vector. (D) *Myc* and *Rest* gene expression, means ± s.e.m. of three experiments. (E) Levels of REST, TSC2 and β-catenin, representative western blots to the left and means ± s.e.m. of three experiments on the right. (F) Levels of synaptotagmin-1 (Syt1) and SNAP25; representative western blots (left) and means ± s.e.m. of the results of three independent experiments (right).

and the canonical Wnt pathways (Kwiatkowski and Manning, 2005; Laplante and Sabatini, 2009; Huang and Manning, 2009; MacDonald et al., 2009), might have a role in other neural cell types (as suggested by the results already obtained in NT2/D1 cells) and might also be active in neurons. Although neurons do not proliferate, they remain sensitive to TSC2 and β-catenin regulation of important processes (Tavazoie et al., 2005; Abe and Takeichi, 2007; Wisniewska et al., 2010; Nie et al., 2010). In this light, it is tempting to speculate that the REST–TSC2–β-catenin signaling paradigm could be targeted for the development of combined drug therapies targeting the TSC–mTOR and the Wnt–β-catenin pathways, now being investigated separately for the prevention of neurodegeneration (Pei and Hugon, 2008; Cerpa et al., 2009) and the treatment of medulloblastomas and other neural cell tumors (Klesse and Bowers, 2010; Schmidt et al., 2010).

Materials and Methods

Antibodies and reagents
The IgG2a, rat-specific anti-chromograninB (ChgB) monoclonal antibody (mAb), generated in our laboratory, was used as described previously (D'Alessandro et al., 2008). Other antibodies were from commercial sources: anti-REST and anti-H2B polyclonal antibodies (pAbs) (Upstate); anti-synaptotagmin-1 and anti-β-tubulin mAbs (Synaptic Systems); anti-SNAP25 mAb (Sternberger Monoclonals); anti-GFP mAb (Roche); anti-β-catenin and anti-paxillin mAbs (BD Transduction); rabbit pAbs anti-Akt, anti-P(S473)-Akt, anti-4E-BP1, anti-TSC2, anti-TSC1, anti-GSK3β, anti-P(S9)-GSK 3β, anti-P(Ser235/236)-S6 and anti-S6 mAb (Cell Signaling); anti-Ki67 pAb (Millipore); anti-actin goat pAb (Santa Cruz); phalloidin-FITC conjugate and the CellTrace CFSE cell proliferation kit (Molecular Probes); FITC-conjugated and TRITC-conjugated goat anti-rabbit pAbs, and goat anti-mouse IgG subclasses (Southern Biotech); horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit pAbs (Bio-Rad). The BCA Protein Assay Kit was from Pierce; rapamycin and G418 were from Calbiochem; endo-IWR1 was from Tocris and phosSTOP was from Roche. The fluorescent DNA-binding probe DAPI, quercetin, all-trans retinoic acid and other chemicals were from Sigma.

Cells and cell clones.
The PC12 clones, wtPC12 and PC12-27, and the PC12-27 subclone PC12-27/DBD5, stably transfected with the REST DNA-binding domain, were as described previously (D'Alessandro et al., 2008). NT2/D1 cells were as used previously (Nikcevic et al., 2008). The other subclones were generated in this work. Cells, clones and their subclones were grown and incubated at 37°C.

Transient and stable transfections and infections
Transient and stable transfections were carried out as reported previously (D'Alessandro et al., 2008) using lipofectamine 2000TM (Invitrogen) in all cases. The pcDNA3-FLAG-TSC2 cDNA or the corresponding empty vector (gift from Brendan Manning, Dept. of Genetics, Harvard School of Public Health, Boston, MA) were transiently transfected into PC12–27 cells that were processed 48 hours thereafter. The nt5iD90βcat/GFP construct (gift from Anjen Chenn and Christopher Walsh, Dept. Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA) or the vector backbone pEGFP-1 were used for stably transfecting experimental and control wtPC12 cells. Subclones, indicated as wtPC12/Δ90βcat, were grown in complete medium supplemented with 450 μg/ml of G418. To generate cells with stable TSC2 knockdown, we transfected wtPC12 cells with TRC1 lentiviral plasmid vector pLKO.1-puro (Sigma) expressing control or TSC2 shRNAs. Forty-eight hours after transfection, we added 2.5 μg/ml puromycin and selected clones. Knockdown of TSC2 was confirmed by qPCR and immunoblotting. Clones were then cultured in the presence of 2.5 μg/ml puromycin. For infection of 1.5×10^6 wtPC12 or NT2/D1 cells we used a lentiviral vector (#945.PCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK. deltaLNGFR.Wpre, 3×10^8 particles/10 μl) including or not (controls) the full-length REST tagged with Myc. After 24 hours of incubation, the cells were washed and cultured for 6 days after which they were sorted by FACS. The fraction with high GFP fluorescence was collected and used for the assays.

Cell proliferation, cell volume and cell surface area assays
Competence of cells for proliferation was investigated by Ki67 immunofluorescence. wtPC12, PC12-27 and PC12-27/DBD5 cells were plated at 1×10^4/well in a 24-well dish. Medium was replaced every 48 hours. Upon 3, 4 and 5 days in culture, cells were trypsinized and counted after Trypan Blue exclusion. Differences among the various samples became evident after 4 days, therefore significance was calculated at the fifth day. 0.1 μM rapamycin, 100 μM quercetin and 10 μM endo-IWR1 were administered for 24 hours on the fifth day of culture. The vital CFSE dye dilution assay was carried out according to the manufacturer's instructions. Briefly, trypsinized cells, washed and resuspended in PBS at a final density of 20×10^6/ml, were mixed with the same volume of CFSE fluorescent dye in PBS (final concentration 1 μM) and incubated for 8 minutes at room temperature. Deacetylated CFSE was quenched by the addition of FBS. Cells were then washed with complete medium and seeded in 24-well dishes. CFSE dilution as a result of cell division was evaluated in a BD Biosciences FACSCalibur dual-laser cytometer using Flowjow acquisition analysis software. Where indicated, the forward scatter (FSC) of the cells was analyzed as an indicator of cell size.

Cell surface area was estimated in patched-clamped wtPC12 and PC12-27 by membrane capacitance electrophysiological assays. Experiments were carried out and results were corrected as described previously (Racchetti et al., 2010).

q-PCR
Total RNA was extracted using RNeasy mini columns (Qiagen, Valencia, CA), following the manufacturer's instructions, and its concentration was determined by spectrophotometry. 1–2 μg of total RNA were used to generate cDNA templates for RT-PCR, using Oligo dT, dNTPs and RNAsin RNAse (from Promega). Retrotranscriptase M-MLV was from Invitrogen. q-PCR was performed using Real Time Taqman universal master mix (Applied Biosystems) according to a standard protocol, using 50 ng template cDNA. All primers were used at the final concentration of 900 nM. Values were normalized to the concentration of Gapdh mRNA. q-PCR was performed using an ABI PRISM 7700 Sequence Detection System. Values are expressed as either fold of wt or control PC12 or as a percentage of untreated cells.

Western blotting
Total cell extracts were obtained by suspending cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and protease and phosphatase inhibitors (Xu et al., 2003). The nuclear fraction was separated from the first, low-speed pellet by high-speed sedimentation through a dense (1.5 M) sucrose cushion; the cytoplasmic fraction was the supernatant of the low-speed centrifugation. Proteins were quantified by BCA assay and appropriate amounts (most often 50 μg) were separated by SDS-PAGE. After transfer to nitrocellulose filters, they were immunolabeled as described (Dignam et al., 1983). Photographic development was by chemiluminescence (ECL, Amersham Bioscience or Immobilon substrate, Millipore). Western blot bands were quantified by the ImageJ program (NIH), normalized to markers (β-tubulin, actin, GAPDH or histone 2b) immunolabeled in parallel. Data are expressed as either a percentage of the corresponding values in wt or control PC12 cells, or as arbitrary units (a.u.).

Luciferase assay
β-Catenin transcription assay was performed using the Dual-luciferase reporter assay kit (Promega). The $16 \times$ TOPFLASH reporter plasmid (1 μg) (a gift from Randall Moon) (DasGupta et al., 2005) and 100 ng of SV40-Renilla-luc were cotransfected using lipofectamine 2000TM, and luciferase activity was measured 24 hours later, using a luminometer (GloMax Multi Detectionm System of Promega). Data are expressed as a.u.

Immunofluorescence
Experiments were performed as described previously (D'Alessandro et al., 2008). Specifically, cell monolayers on coverslips were fixed with 4% formaldehyde for 10 minutes at room temperature and quenched in 0.1 M glycine, then permeabilized for 20 minutes in PBS containing 0.2% Triton X-100 and 1% BSA, and immunolabeled for 1 hour with anti-paxillin mAb, anti-Ki67 pAb or phalloidin–FITC conjugate diluted in PBS with 1% BSA. The bound antibodies were stained with FITC-conjugated and TRITC-conjugated goat anti-rabbit pAbs, or goat anti-mouse IgG subclasses. Nuclei were stained with DAPI. Samples were studied in a Perkin-Elmer Ultraview ERS confocal microscope. Image deconvolution was performed in a wide-field microscope of the Delta Vision system.

Statistical analyses
The significance of the data was assessed using the two-tailed unpaired t-test and the ANOVA test. Data shown are means ± s.e.m. The number of experiments is specified in the figures or legends. $P<0.05$ is considered significantly different. In the figures, ***$P<0.001$; **$P<0.01$; *$P<0.05$.

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