The Down syndrome critical region protein TTC3 inhibits neuronal differentiation via RhoA and Citron kinase

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Summary

The Down syndrome critical region (DSCR) on Chromosome 21 contains many genes whose duplication may lead to the major phenotypic features of Down syndrome and especially the associated mental retardation. However, the functions of DSCR genes are mostly unknown and their possible involvement in key brain developmental events still largely unexplored. In this report we show that the protein TTC3, encoded by one of the main DSCR candidate genes, physically interacts with Citron kinase (CIT-K) and Citron N (CIT-N), two effectors of the RhoA small GTPase that have previously been involved in neuronal proliferation and differentiation. More importantly, we found that TTC3 levels can strongly affect the NGF-induced differentiation of PC12 cells, by a CIT-K-dependent mechanism. Indeed, TTC3 overexpression leads to strong inhibition of neurite extension, which can be reverted by CIT-K RNAi. Conversely, TTC3 knockdown stimulates neurite extension in the same cells. Finally, we find that Rho, but not Rho kinase, is required for TTC3 differentiation-inhibiting activity. Our results suggest that the TTC3–RhoA–CIT-K pathway could be a crucial determinant of in vivo neuronal development, whose hyperactivity may result in detrimental effects on the normal differentiation program.

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Key words: Citron kinase, Neuronal differentiation, Rho, TTC3

Introduction

The transition of neuronal precursors from a proliferating state to a completely differentiated phenotype represents one of the most crucial events that characterize the central nervous system (CNS) developmental program. In a relatively short time, neuroblasts permanently lose their ability to re-enter the cell cycle, migrate to their definitive positions and undergo major morphological changes, which result in the establishment of an astonishingly complex neuronal network. The dynamic reorganization of the actin cytoskeleton plays a crucial role during every stage of this process (Da Silva and Dotti, 2002; Luo, 2002). In particular, actin filaments in proliferating neuronal precursors are mainly involved in the assembly of the contractile ring, a prominent structure that separates the two daughter cells during cytokinesis (Glotzer, 2005). On the other hand, in differentiating neuroblasts, actin rearrangements drive the extension and the pathfinding of axons and dendrites and contribute to the establishment of synaptic contacts (Smith, 1988; Da Silva and Dotti, 2002; Luo, 2002; Dillon and Goda, 2005). Actin filaments are strongly enriched at these latter sites, especially in the post synaptic density (PSD), a highly structured electron-dense apparatus attached to the cytosolic surface of the postsynaptic membrane, which concentrates and organizes neurotransmitter receptors, ion channels and signal transduction proteins (Dillon and Goda, 2005). Therefore, understanding the molecular basis of actin cytoskeleton rearrangements in CNS development represents one of the major goals of modern neurobiology. Studies conducted in the last decade in several experimental models have shown that small GTPases of the Rho family are master regulators of the actin cytoskeleton in every cell type (Hall, 2005). Not surprisingly, these molecules have been shown to play crucial roles in several aspects of the neuronal differentiation program (Govek et al., 2005) and have been involved in many neurological syndromes characterized by mental retardation (Newey et al., 2005; van Galen and Ramakers, 2005). Rho GTPases are molecular switches that cycle from an active GTP-bound state to an inactive GDP-bound conformation and exert their functions through a complex network of effector proteins, which physically interact with the GTP-bound form and change their activity upon binding (Bishop and Hall, 2000). Among the different effectors of Rho GTPases, Citron kinase (CIT-K) and Citron N (CIT-N) play essential roles in different aspects of neuronal development. These two multi-domain proteins are produced by the same gene and differ only in the presence or absence, respectively, of an N-terminal Ser/Thr kinase domain (Di Cunto et al., 1998; Madaule et al., 1998). CIT-K is a cytokinesis regulator, expressed

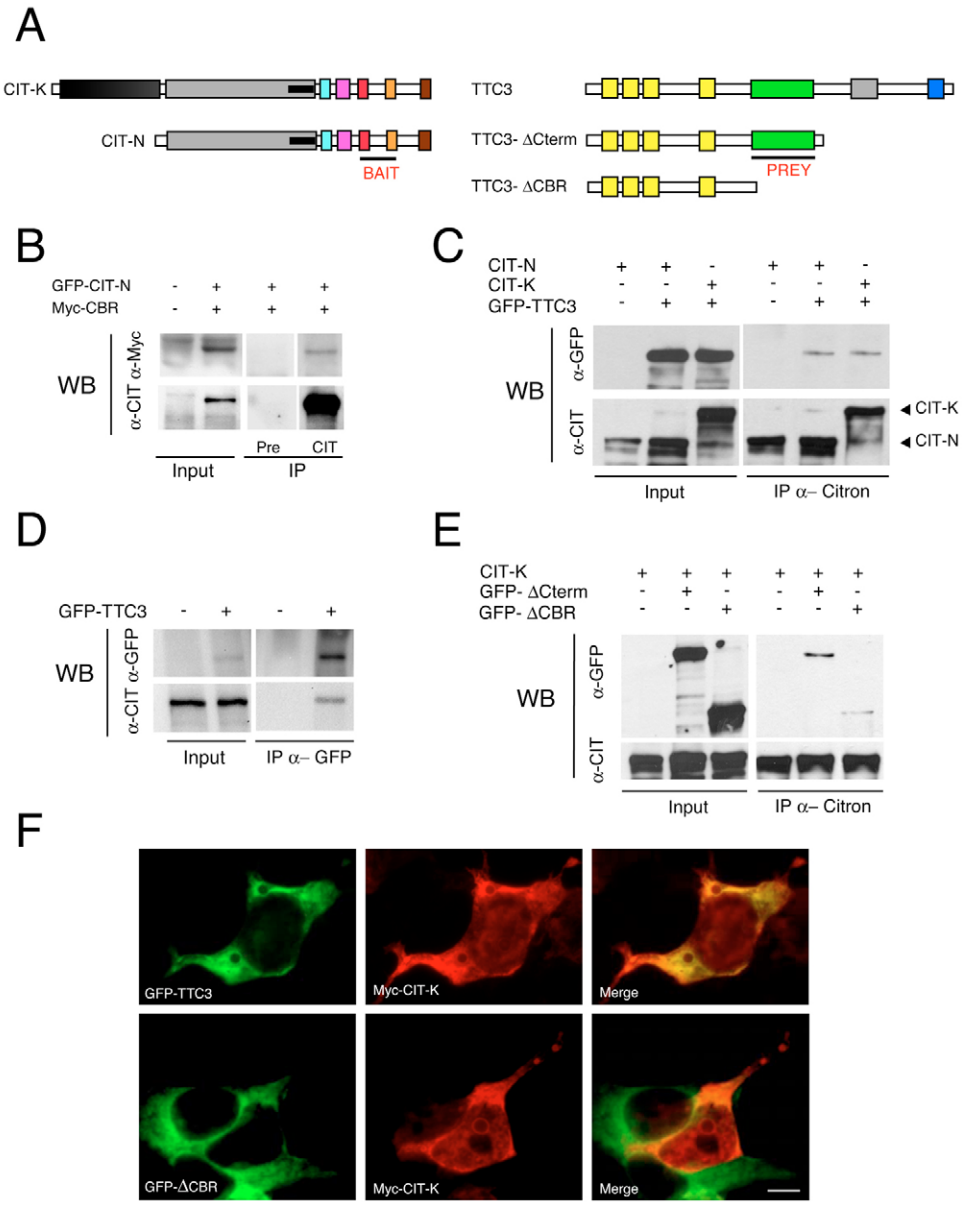

Fig. 1. Interaction between Citron proteins and TTC3. (A) Domain structure of Citron proteins, TTC3 and the described mutants. Kinase domain (black), Rho-binding domain (black-box), coiled coil region (grey), C1 domain (cyan), PH domain (pink), CNH domain (red), Proline-rich region (orange), PDZ-binding domain (brown), TPR motifs (yellow), Citron binding region CBR (green) and ring finger domain (blue) are indicated. The sequences used as bait, and found as prey are indicated. (B) Total cell lysates from HEK293 cells co-transfected with GFP-CIT-N and Myc-CBR were immunoprecipitated with anti-Citron antibodies and analyzed by western blotting as indicated. (C) Total cell lysates from HEK293 cells co-transfected with GFP-TTC3 and CIT-N or CIT-K were immunoprecipitated with anti-Citron antibodies and analyzed by western blotting as indicated. (D) PC12 cells were co-transfected with GFP-TTC3 and allowed to differentiate by NGF treatment for 2 days after transfection. Total cell lysates were then immunoprecipitated with anti-GFP antibodies and endogenous CIT-K was revealed by western blotting. (E) The TTC3 mutants ΔCterm and ΔCBR were co-transfected with CIT-K and analyzed as in C. (F) Myc-CIT-K was co-transfected in HEK293 cells with GFP-TTC3 (upper panel) or with GFP-ΔCBR (lower panel). Bar, 10 μm.

ubiquitously, but specifically required in proliferating neuronal precursors. Indeed, mice lacking CIT-K display severe microcephaly (Di Cunto et al., 2000), caused by cytokinesis failure and apoptosis of the mentioned cell types. Moreover, CIT-K regulates the extension of neurites and dendrites, both in cultured neuroblastoma cells and in vivo (Di Cunto et al., 2002).

On the other hand, CIT-N is specifically expressed by differentiating neuroblasts and differentiated neurons. In these cells, at early stages of differentiation, it colocalizes with the Golgi apparatus, regulating its structural organization via an actin-dependent mechanism (Camera et al., 2003). At later stages, it is also localized to dendritic spines and PSDs (Furuyashiki et al., 1999; Zhang et al., 1999), where its functional role remains to be established. Interestingly, recent studies have shown that the human *CIT* locus displays a positive linkage with some forms of familial bipolar disorders, and that Citron proteins may interact with DISC1 (Lyons-Warren et al., 2005), which was previously implicated in schizophrenia and in bipolar disorders (Hayden and Nurnberger, 2006).

In this study we have found that CIT-K and CIT-N interact with TTC3, a protein of unknown function encoded by a gene mapped to the Down syndrome critical region (DSCR) (Ohira et al., 1996; Tsukahara et al., 1996). More importantly, we show that TTC3 may control the differentiation program of neuronal cells via a Rho-dependent mechanism that requires functional cooperation with CIT-K.

Results

Citron proteins interact with TTC3

To identify new molecular partners of Citron proteins we used a two-hybrid (TH) assay to screen a human fetal brain cDNA library, with a bait comprising the CNH domain and the proline-rich portion of Citron, common to both isoforms (Fig. 1A). Indeed, no interactions have been so far described for this

region, although it is known that the coiled-coil domain mediates multimerization (Zhang and Benson, 2006) and that the C-terminal PDZ-binding domain associates with MAGUK proteins such as PSD-95 (Furuyashiki et al., 1999; Zhang et al., 1999).

Interestingly, one of the clones strongly positive for all the reporter genes (data not shown), contained a fragment of the TTC3 cDNA. The corresponding gene was previously mapped to the DCR on chromosome 21q22.2 (Ohira et al., 1996; Tsukahara et al., 1996) and encodes a 2025-residue protein, described to contain three tetratricopeptide repeat (TPR) motifs in its N-terminal region (Sikorski et al., 1990; Tsukahara et al., 1996). However, by performing a new bioinformatic scan, we found that, in addition, it also contains a fourth N-terminal TPR motif, probably a coiled-coil region (Lupas et al., 1991) and a C-terminal ring finger domain (Freemont, 1993) (Fig. 1A). The fragment identified in our screening encodes aa 733-1182, which lie between the fourth TPR repeat and the coiled-coil region (Fig. 1A).

To confirm that the interaction detected by TH assay can occur even in mammalian cells, we fused the TTC3 pray fragment (referred as the Citron-binding region or CBR) with a MYC tag and co-expressed this construct with full-length CIT-N. Under these conditions, the MYC-CBR was co-precipitated by a specific polyclonal anti-Citron antibody (Fig. 1B).

To address whether full-length TTC3 may interact with both Citron isoforms, a GFP expression construct (GFP-TTC3) was co-transfected in HEK293 cells with CIT-N or CIT-K plasmids. Interestingly, TTC3 was co-precipitated in both cases by anti-Citron antibodies (Fig. 1C). Similar results were obtained by a reciprocal immunoprecipitation assay (data not shown).

Since specific antibodies against TTC3 were unavailable for this study, we could not test directly whether the endogenous proteins form a complex in vivo. However we found that endogenous CIT-K can be co-immunoprecipitated with TTC3 overexpressed in HEK293 cells (data not shown) and in the rat pheochromocytoma PC12 line (Fig. 1D).

To confirm that the CBR is the main determinant of the interaction with Citron proteins, we produced two TTC3 truncation mutants, lacking the C-terminal portion distal to the CBR (GFP-ΔCterm) or even the CBR (GFP-ΔCBR), respectively (Fig. 1A). Interestingly, the GFP-ΔCterm mutant protein was co-precipitated with CIT-K as efficiently as the FL-TTC3 (supplementary material Fig. S1A). By contrast, deletion of the CBR strongly reduced the association (Fig. 1E and supplementary material Fig. S1B).

Finally, we determined whether Citron proteins and TTC3 co-localized. Interestingly, upon co-transfection in HEK293 cells, the signals of GFP-TTC3 and CIT-K appear to overlap extensively (Fig. 1F, upper panel), and TTC3 is enriched in the vacuolar structures that are characteristically induced by CIT-K overexpression (Eda et al., 2001). Similar results were obtained with the TTC3-ΔCterm construct (data not shown). By contrast, the TTC3-ΔCBR protein displayed a lower degree of co-localization, and was recruited to CIT-K structures much less efficiently than the full-length protein (Fig. 1F, lower panel).

Taken together, these observations indicate that TTC3 may physically interact with Citron proteins and that the CBR region is sufficient, although not completely necessary, for this interaction.

TTC3 inhibits the morphologic differentiation induced by NGF in PC12 cells

The function of TTC3 has not been so far addressed, although it was reported that its mRNA is enriched in the developing CNS (Lopes et al., 1999; Rachidi et al., 2000). Since we previously showed that CIT-K inhibits neurite outgrowth both in vitro and in vivo (Di Cunto et al., 2002), the interaction that we detected led us to investigate whether TTC3 might also be involved in this process. We overexpressed GFP-TTC3 constructs in PC12 cells, which were induced to differentiate into a neuronal-like phenotype after stimulation with the neurotrophin NGF (Vaudry et al., 2002).

After 3 days of NGF treatment, ~90% of PC12 cells transfected with an empty GFP control vector showed neurites longer than the cell body. By contrast, most of the cells overexpressing GFP-TTC3 displayed a round morphology, with neurites barely detectable at all (Fig. 2). This effect was still evident 5 days after NGF stimulation, and was not dependent on the particular construct, since similar results were obtained with RFP and MYC fusions (data not shown).

A possible explanation for the round morphology of TTC3-positive cells could be a non-specific toxic effect of the overexpressed protein, leading to the activation of programmed cell death. However, nuclei of GFP-TTC3-overexpressing cells were clearly non-apoptotic as revealed by an anti-activated Caspase-3 immunostaining (supplementary material Fig. S2).

To analyze which regions of TTC3 are involved in inhibiting neurite extension and whether the CBR is necessary, we also tested the activity of the TTC3-ΔCterm and TTC3-ΔCBR constructs. Interestingly, both mutants were much less effective inhibitors than the full-length protein and did not give significantly different results (Fig. 2B), thus suggesting that the N-terminal and C-terminal portions of the molecule are both necessary for full activity, while the CBR is not required.

The above results suggest that TTC3 levels can negatively modulate the response of PC12 cells to differentiating stimuli. If this was the case, TTC3 knockdown would be expected to enhance NGF-induced differentiation. To test this prediction we developed different TTC3 RNAi vectors. One of them was based on the p-DECAP technology (Shinagawa and Ishii, 2003), whereas the other two constructs were based on the sh-mir (short hairpin microRNA) design, which has recently been shown to work much more efficiently than traditional shRNAs (Silva et al., 2005). Compared with the corresponding controls, these RNAi vectors were able to specifically decrease the levels of TTC3 overexpressed in HEK293 cells (Fig. 3A,B). Moreover, RNAi vectors efficiently reduced the levels of endogenous TTC3 mRNA upon transient transfection in PC12 cells (Fig. 3C).

We then analyzed the morphology of PC12 cells transfected with these constructs after 3 days of NGF treatment. Under these conditions, the percentage of neurite-bearing cells was not significantly different between RNAi and control plasmids (data not shown). By contrast, quantification of neurite length in the same experiments showed that this was significantly increased by all the RNAi constructs (Fig. 3D,E). Taken together, these observations indicate that TTC3 may act as an inhibitor of neuronal cell differentiation.

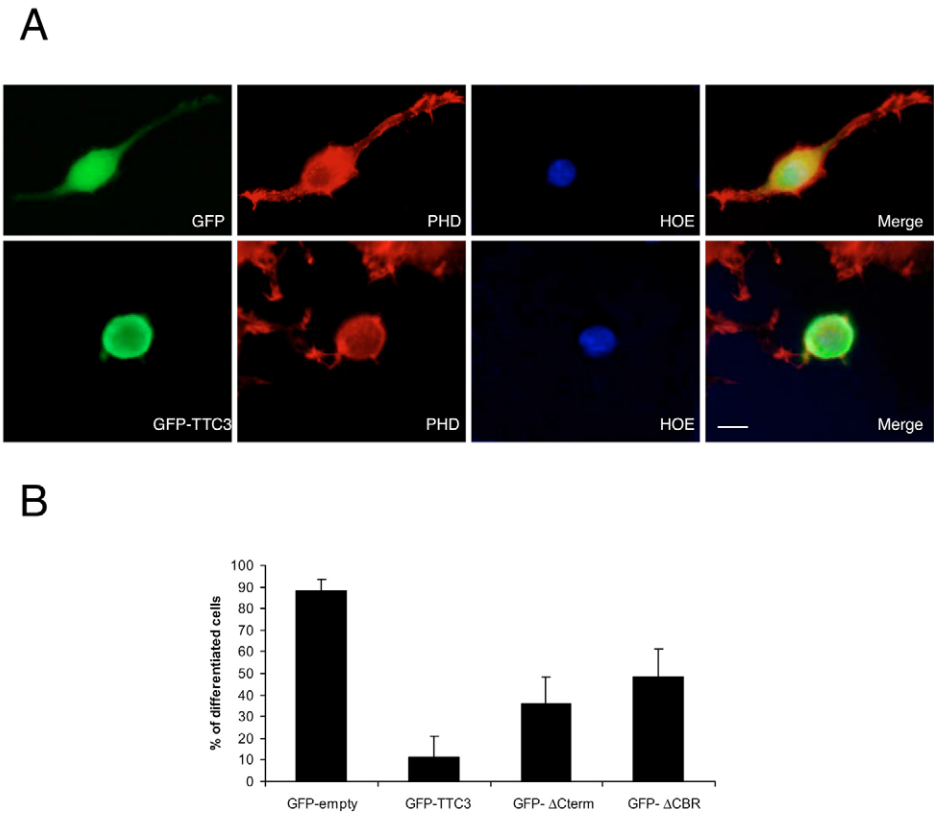

Fig. 2. Inhibition of neurite extension by TTC3 overexpression. PC12 cells were transfected with GFP-empty, GFP-TTC3 and GFP-TTC3 mutants. The cells were treated with NGF 12 hours after transfection, allowed to differentiate for 3 days and analyzed by immunofluorescence microscopy. (A) Cells transfected with TTC3 showed a dramatic reduction of neurite sprouting, when compared to control cells. PHD, phalloidin; HOE, Hoechst 33258. Bar, 10 μm. (B) The percentage of differentiated cells, defined as those bearing at least one neurite longer than the main cell body axis, was quantitatively analyzed in the indicated transfections. The histograms represent the average of three independent experiments. All the differences were statistically significant ($P<0.001$, χ-square test), except the one detected between the ΔCterm and ΔCBR mutants. Error bars represent s.d.

Functional cooperation between TTC3 and CIT-K in PC12 differentiation

In light of the similar activity displayed by CIT-K and TTC3, and of the physical interaction between the two proteins, we reasoned that they might modulate neuronal cell differentiation by acting in the same pathway. To directly address this possibility, we performed combined overexpression and knockdown studies for TTC3 and CIT-K in differentiating PC12 cells. Therefore, in addition to the constructs already described, we produced a pDECAP RNAi plasmid, which significantly reduced the expression of endogenous CIT-K (supplementary material Fig. S2), the only Citron isoform produced by PC12 cells (data not shown). Moreover, to support the specificity of this construct, we also used a previously validated shRNA (Gruneberg et al., 2006). In good agreement with previous findings (Di Cunto et al., 2000; Gruneberg et al., 2006), PC12 cells transfected with these vectors become binucleated very frequently owing to cytokinesis failure (data not shown). Moreover, as expected, CIT-K RNAi consistently increased neurite length of transfected and differentiated PC12 cells (supplementary material Fig. S3).

To investigate the epistatic relationship between CIT-K and TTC3, we first analyzed the percentage of differentiated PC12 cells after co-transfection with the GFP-TTC3 and CIT-K RNAi constructs. As shown in Fig. 4A,B, CIT-K RNAi consistently reverted the TTC3 overexpression phenotype, as the percentage of neurite-bearing cells was similar to that of cells co-transfected with control plasmids.

By contrast, the pDECAP-TTC3 construct was unable to counteract the differentiation-inhibiting activity of overexpressed CIT-K (Fig. 4C). These results indicate that in differentiating PC12 cells, the two proteins may act in a linear cascade, with CIT-K downstream of TTC3.

The differentiation-inhibiting activity of TTC3 requires Rho but not ROKs

Rho small GTPases are crucial mediators of neurite extension or retraction induced by extracellular stimuli. In particular, it has been shown that the activity of RhoA is necessary for neurite retraction in many neuronal differentiation models, including NGF-treated PC12 cells (Govek et al., 2005). Moreover, among the different effectors of RhoA, Rho kinases (ROKs) are believed to play a particularly important role in transducing neurite-inhibitory signals (Hirose et al., 1998; Da Silva et al., 2003). Therefore, to gain further mechanistic insight into the intracellular signaling cascade activated in differentiating PC12 cells by TTC3 overexpression, we asked whether it requires the RhoA-ROKs pathway.

To this aim, we first evaluated whether treatment with a specific inhibitor of ROKs (Y27632) could reverse the effects of increased TTC3 levels. As expected, in PC12 cells transfected with a control vector and treated with NGF, Y27632 significantly increased the percentage of differentiated cells and strongly enhanced their neurite length (Fig. 5). By contrast, Y27632 had a much smaller effect in TTC3-transfected cells. Indeed, even though an increase of differentiated cells and neurite length was observed, these parameters remained far below the basal levels of mock-transfected cells (Fig. 5). This result indicates that TTC3 action is largely independent of ROKs activity.

When PC12 cells were co-transfected with GFP-TTC3 and

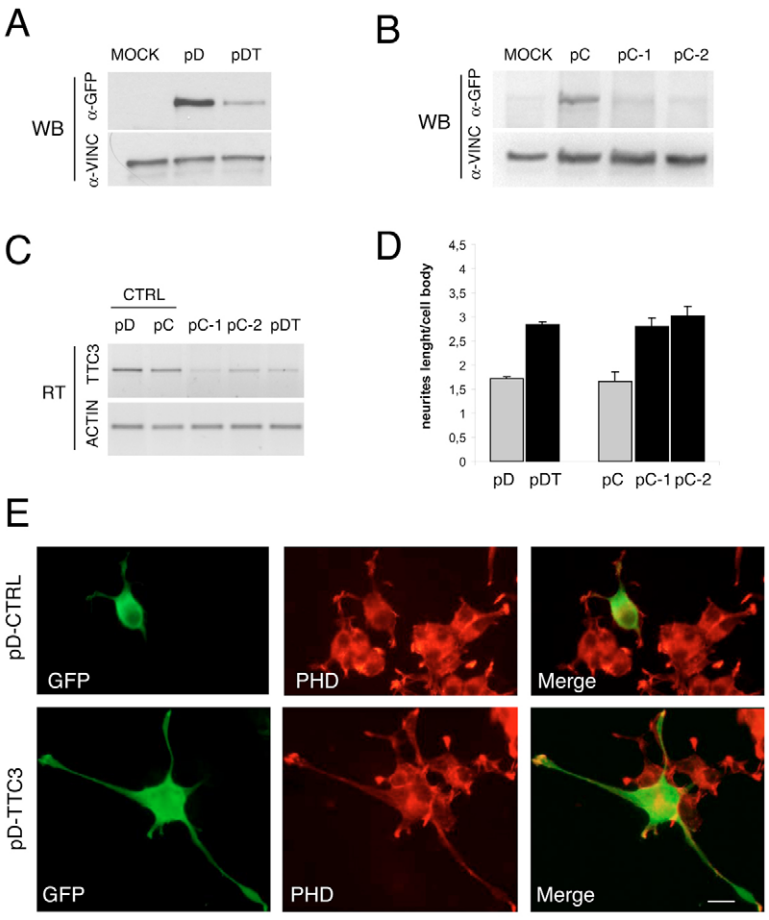

Fig. 3. Knockdown of TTC3 in differentiated PC12 cells. (A) HEK293 cells were co-transfected with GFP-TTC3 and unrelated pDECAP control (pD) or pDECAP-TTC3 (pDT) plasmids. The expression of GFP-TTC3 and that of an internal loading control (Vinculin) were then evaluated by western blot 48 hours after transfection. (B) HEK293 cells were co-transfected with GFP-TTC3 and pCMV-GIN-ZEO sh-mir plasmids, expressing a double mismatch (pC) and two perfect match (pC-1 and pC-2) interfering RNAs. The reduction of GFP-TTC3 was evaluated as in A. (C) PC12 cells were transfected with the indicated control and RNAi plasmids. The levels of endogenous TTC3 mRNA were evaluated 48 hours after transfection by semi-quantitative RT-PCR, using internal control β-actin primers. (D,E) PC12 cells were transfected with the indicated control and RNAi plasmids and treated with NGF for 3 days. Cells were then fixed, stained with phalloidin and analyzed by IF. The ratio between length of the main neurite and principal diameter of cell body was determined for at least 200 cells. Histograms represent the average of three independent experiments (D). The differences between control (gray bars) and RNAi (black bars) samples were statistically significant, as determined by the Student's *t*-test ($P<0.0001$). Error bars represent s.d. Bar, 10 μm.

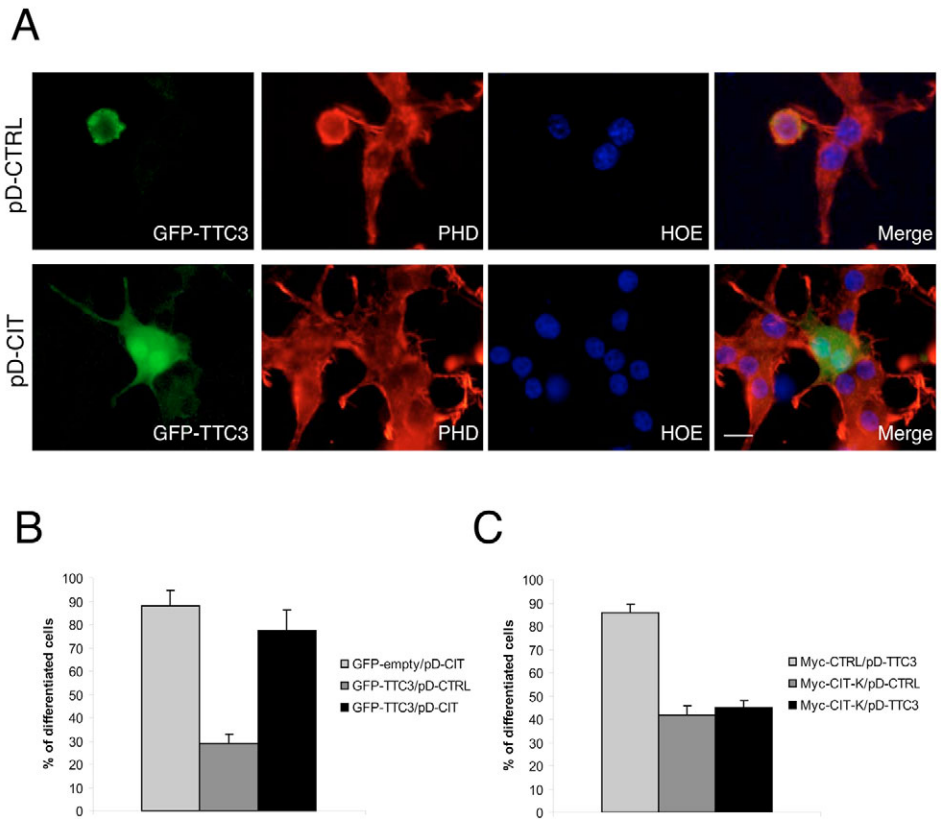

Fig. 4. Epistatic analysis of TTC3 and CIT-K in differentiating PC12 cells. (A) PC12 cells were co-transfected with GFP-TTC3 and pDECAP control (pD-CTRL) or pDECAP CIT-K (pD-CIT), allowed to differentiate for 3 days and analyzed as in Figs 2 and 3. The image shown in the lower panel corresponds to an exemplar binucleated cells, bearing long neurites. Bar, 10 μm. (B) Quantitative analysis of differentiated cells in the experiment described in A. The first bar represents the result of a parallel control sample co-transfected with a GFP empty vector and pD-CIT. (C) PC12 cells were co-transfected with the indicated combinations of Myc-CIT-K, unrelated Myc expression plasmid (Myc-CTRL), pD-CTRL and pDECAP-TTC3 (pD-TTC3). Slides were analyzed as in A and B, with the exception that transfected cells were identified by staining with anti-Myc antibodies. Error bars represent s.d.

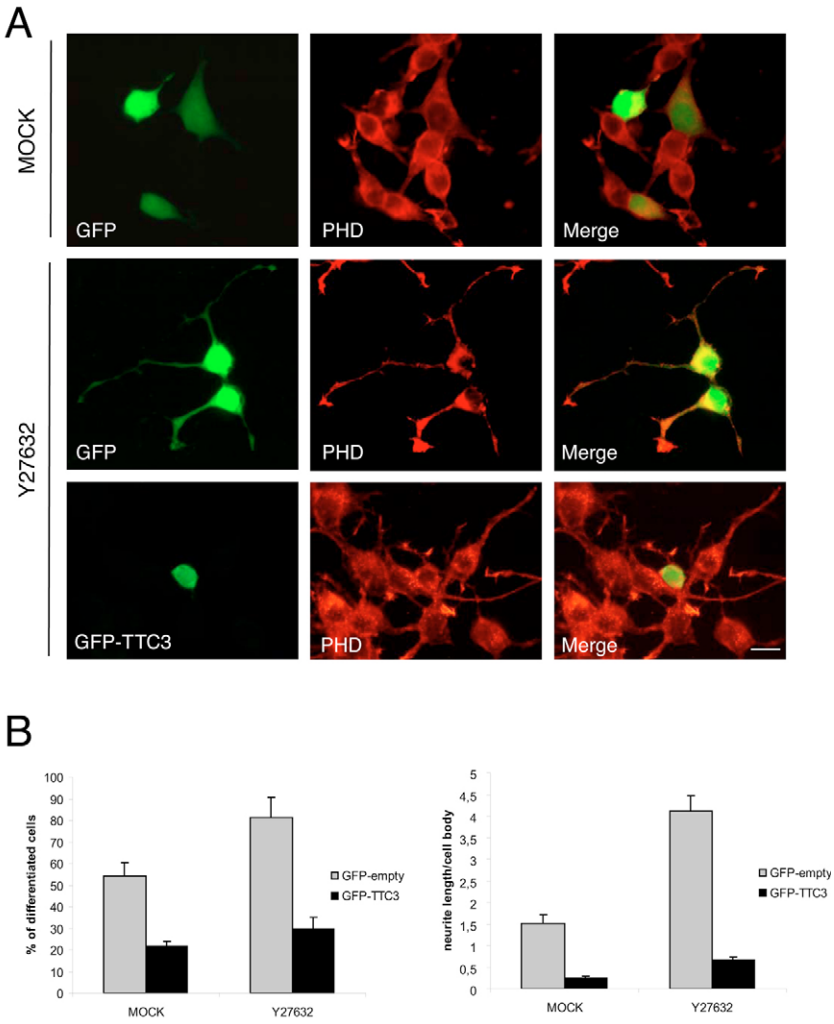

Fig. 5. Rho kinase inhibition is unable to revert the TTC3 overexpression phenotype in differentiated PC12 cells. (A) PC12 cells were transfected with an empty GFP or GFP-TTC3 construct, treated with NGF after 12 hours and allowed to differentiate for 2 days. Afterwards, the culture medium was changed and vehicle alone (MOCK) or Y27632 were added. The cells were kept in culture for additional 18 hours and then processed for immunofluorescence with anti GFP antibodies (green) and phalloidin (red). Bar, 10 μm. (B) Quantitative analysis of the percentage of differentiated cells (left panel) and of neurite length (right panel) in the above experiments, performed as in Figs 2 and 3, respectively.

the Rho inhibitor C3-transferase (Reshkin and Murer, 1992) and allowed to differentiate, the percentage of neurite-bearing cells was restored to control values (Fig. 6A,B). Moreover, many of the TTC3/C3-expressing cells possessed long neurites, comparable with those of cells transfected with GFP alone or co-transfected with GFP and C3 (Fig. 6A). This result indicates that Rho activity is required by TTC3 to prevent neurite outgrowth, suggesting that RhoA may act downstream of TTC3. In this case, we would expect that TTC3 protein levels specifically affect the activation state of RhoA. Accordingly, a RhoA pull-down assay showed that TTC3 overexpression strongly increases the levels of active RhoA in transiently transfected HEK293 cells and in PC12 cells (Fig. 6C). Taken together, these results strongly suggest that TTC3 may act in neuronal differentiation upstream of RhoA but independently of ROKs.

Discussion

The differentiation program of neuronal cells requires a very complex series of intracellular events, leading to a drastic reorganization of cytoskeleton and membrane trafficking. In the last few years, studies performed in many model systems have clearly elucidated that different members of the Rho small GTPase family are critical switches of the master circuit. In particular, the commonly accepted view is that neuronal differentiation depends on a tightly regulated balance between the antagonistic activities of the RhoA and Rac1/Cdc42 pathways (Govek et al., 2005). The rat PC12 line represents one of the principal model systems that has been used to dissect the molecular details of neuronal differentiation. In these cells, stimulation of the TrkA tyrosine kinase receptor by NGF, leads to the PI3K-dependent activation of Rac1 and Cdc42 and causes at the same time relocalization and inactivation of RhoA (Nusser et al., 2002). On the other hand, RhoA activation antagonizes these effects, causing neurite retraction (Katoh et al., 1998).

Among the different downstream effectors of RhoA, ROKs are known to play a crucial role in inhibiting neurite extension. In particular, in PC12 cells treated with NGF, it was shown that the inactivation of RhoA is accompanied by its simultaneous dissociation from ROK-alpha (Nusser et al., 2002), while treatment with the ROKs inhibitor Y27632 leads to increased neurite extension (Lambrechts et al., 2006).

CIT-K and CIT-N are two ROK-related effectors of RhoA, produced by the same gene, which play crucial roles during the transition between proliferation and differentiation. In vivo, CIT-K is expressed at high levels in proliferating neuronal precursors and at lower levels in differentiating and differentiated neural cells (Di Cunto et al., 2002). On the other hand, CIT-N is mainly expressed by differentiating and differentiated nerve cells, where it has been associated to the Golgi (Camera et al., 2003) and to post synaptic densities (Furuyashiki et al., 1999; Zhang et al., 1999). By specifically knocking out CIT-K in mice, we previously showed that it is mainly required by proliferating precursors to complete cytokinesis (Di Cunto et al., 2000). However, by analyzing the same mouse line and N1E-115 neuroblastoma cells overexpressing wild-type and mutated CIT-K constructs, we also found that it may inhibit neuronal differentiation similarly to ROKs (Di Cunto et al., 2002). Here, we have found that this is also true in differentiating PC12 cells (supplementary material Fig. S2), where CIT-K is the only expressed isoform (not shown).

Under this general perspective, the finding of TTC3 as a specific interactor of Citron proteins seemed to us very

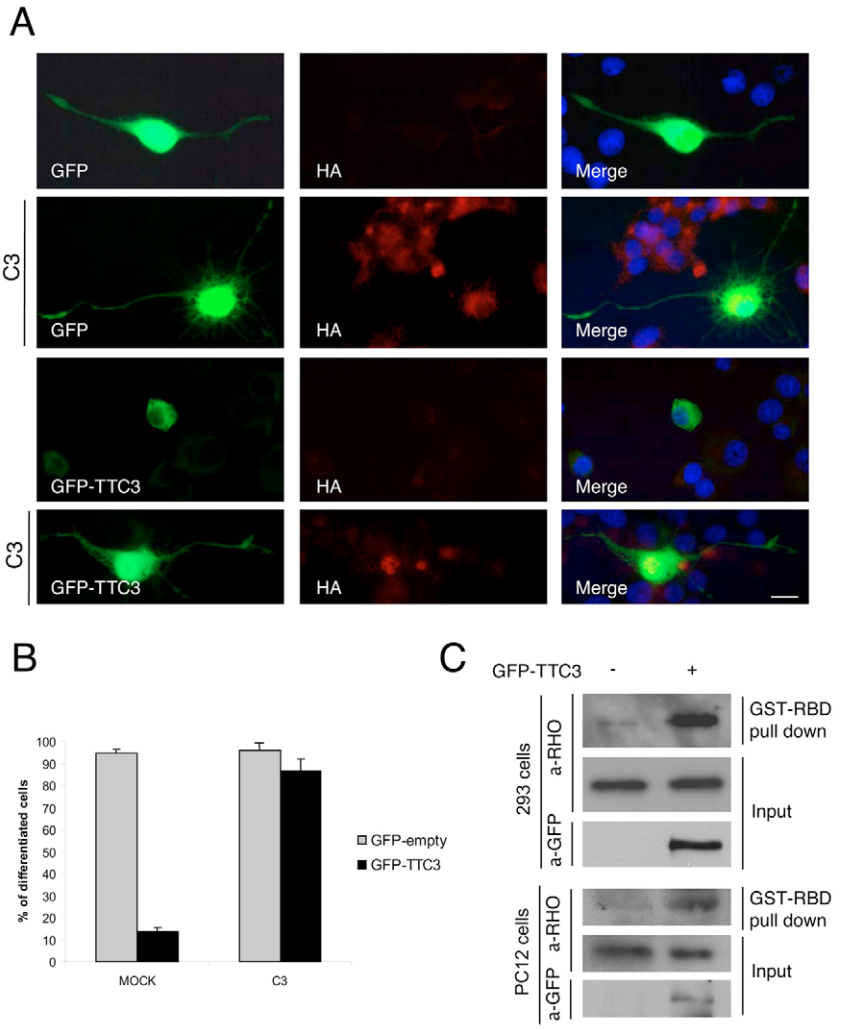

Fig. 6. Rho inhibition reverts the phenotype induced by TTC3 overexpression in PC12 cells. (A) PC12 cells were transfected with empty GFP or GFP-TTC3 constructs, either alone or in combination with HA-C3 expression plasmid. Cultures were treated with NGF after 12 hours, allowed to differentiate for 3 days and processed for IF. (B) Quantitative analysis of differentiated cells in the above experiment, performed as in Fig. 2. (C) HEK293 cells (upper panel) or PC12 cells (lower panel) were transfected with empty GFP or GFP-TTC3 expression vectors and analyzed after 48 hours by Rho pull-down assay, using recombinant Rhotekin Rho-binding domain (GST-RBD).

intriguing. Indeed, TTC3 represents one of the products of the DCR locus that is very likely to significantly contribute to mental retardation in Down syndrome. In particular, it was previously shown that a 700 kb human DCR fragment, containing only the TTC3 and Minibrain genes, is sufficient to cause learning and memory defects when transgenically introduced in the mouse genome (Smith et al., 1997). Moreover, it was observed that, during development, TTC3 mRNA is progressively enriched in post-mitotic regions of the central nervous system (CNS) (Lopes et al., 1999; Rachidi et al., 2000), thus suggesting its possible involvement in neuronal differentiation. Accordingly, we have found that TTC3 overexpression in PC12 cells strongly inhibits the neurite extension induced by NGF, whereas TTC3 RNAi consistently increases neurite length.

Therefore, considering their physical interaction and their common overexpression phenotype, we determined whether CIT-K and TTC3 functionally cooperate in modulating NGF-induced differentiation of PC12 cells. Importantly, we found that the differentiation-inhibitory activity of TTC3 is substantially reverted by the knockdown of CIT-K, whereas TTC3 RNAi is unable to modify the effects of CIT-K overexpression, indicating that the two molecules are in the same pathway, and that CIT-K acts downstream of TTC3. In light of the established role of RhoA and ROKs in this model, we also investigated their relationships to TTC3. Interestingly, we found that TTC3 strongly increases the levels of active RhoA and that its neurite inhibitory activity is reverted by Rho inactivation, whereas it is insensitive to ROK inhibition.

Thus, our results suggest that in PC12 cells, the neurite extension induced by TrkA signaling can be counteracted by at least two pathways, both requiring RhoA activation, but specifically involving TTC3 and CIT-K or ROKs, respectively. The direct interaction between CIT-K and TTC3 would seem an attractive mechanism to explain this specificity. However, this is probably not the case, since the TTC3-ΔCterm and TTC3-ΔCBR mutants were equally effective inhibitors of PC12 differentiation (although much less active than the wild type protein), suggesting that CIT-K binding to the CBR is not crucial for TTC3 activity. At the moment, the most likely alternative explanation appears that TTC3 may modulate CIT-K indirectly through RhoA activation. Accordingly, we found that TTC3 overexpression potently activates RhoA in both non-neuronal and neuronal cells, suggesting that TTC3 could be part of a complex containing both Rho modulators and the Rho effector CIT-K. If this is the case, RhoA is probably only a transient interactor of the complex, as we were not able to co-immunoprecipitate it with TTC3 from transfected neuronal and non-neuronal cells (data not shown). The identification of other molecules that interact with CIT-K and/or TTC3 will shed more light on this issue. Considering that CIT-K and CIT-N are equally effective in binding to TTC3, another important question is whether TTC3 may also cooperate with CIT-N at later stages of neuronal differentiation.

In conclusion, we have characterized TTC3 protein function and suggest that dosage imbalance of the TTC3 locus contributes to the pathogenesis of mental retardation in Down syndrome by specifically altering the neuronal differentiation program.

Materials and Methods

Plasmids

The bait plasmid used for the TH screen was made by cloning a PCR fragment of the mouse CIT-K sequence corresponding to amino acids 1608-2017 (96% identity

with human sequence) into the pGBKT7 (Clontech), to produce a fusion with the Gal-4-binding domain. CIT-K and CIT-N plasmids have been previously described (Camera et al., 2003; Di Cunto et al., 2002). The MYC-CBR construct was produced by PCR and cloning into the pcDNA3 vector (Invitrogen). pEGFP-TTC3 was obtained by merging the coding sequences of mouse EST clones BC057207 and CD349529, and cloned into the pEGFP-C1 (Clontech). The ΔCterm and ΔCBR mutants were obtained from pEGFP-TTC3 by restriction digestions. The pDECAP vector was obtained by Shunesuke Ishii. The pDECAP-CIT and pDECAP-TTC3 were produced by cloning nucleotides 2503-3087 of the mouse CIT-K, and nucleotides 2173-2672 of the mouse TTC3 sequences, respectively, in the pDECAP vector. The sh-mir RNAi constructs were based on the pCMV-GIN(Zeo) lentiviral vector. The control (pC) corresponds to nucleotides 1540-1562 of the human RefSeq TTC3 sequence (GGGTGTGCAATATAAAGATTATA) and shows two mismatches to the mouse and rat sequences. The perfect match plasmids correspond to nucleotides 2164-2186 (pC-1; AGCCATTGAATATAGACCTGAAA) and nucleotides 2162-2184 (pC-2; AGAGCCATTGAATATAGACCTGA) of the human RefSeq sequence, that are shared with mouse and rat cDNAs. The pRc/HA-C3 was a gift from Jeffry Settleman.

Yeast two-hybrid assay

The bait plasmid was transfected in the Y187 strain, checked for auto-activation and used to screen by mating 2×10^7 clones of a pre-transformed Matchmaker human cDNA fetal brain library (Clontech) according to the manufacturer's specifications.

Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS). PC12 cells (a gift from Emanuele Cocucci, University of Milan, Milan, Italy) were grown in DMEM supplemented with 10% horse serum and 5% FCIII on poly-D-lysine-coated plates. Differentiation was induced for the indicated times with medium containing 10 μg/ml NGF (Alomone). HEK293 and PC12 cells were transfected using Effectene (Qiagen) or Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's specifications.

Antibodies and inhibitors

The following antibodies were used: rabbit polyclonal anti-GFP (Abcam); mouse monoclonal anti-MYC (Sigma); rabbit polyclonal anti-Citron (Di Cunto et al., 2000); mouse monoclonal anti-vinculin (Sigma); mouse monoclonal anti-ROK-α (BD-Transduction Laboratories) and mouse monoclonal anti-RhoA (Santa Cruz). Y-27632 was used at 37 nmol/ml for 18 hours (BIOMOL research laboratories); rabbit polyclonal anti-Caspase 3 (Cell Signaling).

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, and permeabilized with 0.1% Triton X-100 for 5 minutes. Immunofluorescence microscopy (IF) was performed using the described antibodies, followed by incubation with appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Polymeric F actin was detected with phalloidin TRITC (Sigma), and nuclei were stained with Hoechst (Sigma).

Immunoprecipitation and immunoblotting

For immunoprecipitations, cells were extracted with lysis buffer (1% Triton X-100, 120 mM sodium chloride, 50 mM Tris-HCl (pH 7.5), protease inhibitors (Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were then clarified by centrifugation. Antibodies and protein-G-Sepharose beads (Amersham, Uppsala, Sweden) were added to lysates and incubated overnight at 4°C. Pellets were washed six times with lysis buffer and analyzed by SDS-PAGE.

For immunoblots, immunoprecipitates or equal amounts of proteins from total cell lysates were resolved by reducing SDS-PAGE and transferred to nitrocellulose filters, which were then incubated with the indicated antibodies and developed using the ECL system (Amersham Biosciences).

Rho pull-down assay

Cells were washed twice with cold PBS and lysed in 50 mM Tris-HCl (pH 7.5), 1% NP-40, 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4 and protease inhibitors (Roche, Mannheim, Germany). Lysates were clarified by centrifugation, and equal amounts of protein were incubated with 20 μg of purified GST-RBD, immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). After 30 minutes of rocking at 4°C, the beads were washed twice in lysis buffer and bound Rho-GTP was detected by western blotting using a mAb against Rho (Santa Cruz, CA).

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