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Overexpressed Down Syndrome Cell Adhesion Molecule (DSCAM) Deregulates P21-Activated Kinase (PAK) Activity in an In Vitro Neuronal Model of Down Syndrome: Consequences on Cell Process Formation and Extension

Ramón Pérez-Núñez¹ · Natalia Barraza¹ · Arlek Gonzalez-Jamett² · Ana Maria Cárdenas² · Jean-Vianney Barnier³ · Pablo Caviedes¹

Abstract In humans, Down syndrome (DS) is caused by the presence of an extra copy of autosome 21. The most striking finding in DS patients is intellectual disability and the onset of Alzheimer's disease (AD)-like neuropathology in adulthood. Gene overdose is most likely to underlie both developmental impairments, as well as altered neuronal function in DS. Lately, the disruption of cellular signaling and regulatory pathways has been implicated in DS pathophysiology, and many of such pathways may represent common targets for diverse DS-related genes, which could in turn represent attractive therapeutical targets. In this regard, one DS-related gene Down Syndrome Cell Adhesion Molecule (DSCAM), has important functions in neuronal proliferation, maturation, and synaptogenesis. p21-associated kinases (PAKs) appear as a most interesting possibility for study, as DSCAM is known to regulate the PAKs pathway. Hence, in DS, overexpressed DSCAM could deregulate PAKs activity and affect signaling pathways that regulate synaptic plasticity such as dendritic spine dynamics and axon guidance and growth. In the present work, we used an immortalized cell line derived from the cerebral cortex of an animal model of DS such as the trisomy 16 (Ts16) fetal mouse (named CTb), and a similar cell line established from a normal littermate (named CNh), to study the effect of DSCAM in the PAKs

pathway. The present study shows that DSCAM is overexpressed in CTb cells by approximately twofold, compared to CNh cells. Congruently, PAK1, as well as its downstream effectors LIMK and cofilin, stay phosphorylated for longer periods after DSCAM activation in the CTb cells, leading to an altered actin dynamics, expressed as an increased basal F/G ratio and reduced neurite growth, in the trisomic condition. The present work presents the correlation between DSCAM gene overexpression and a dysregulation of the PAK pathway, resulting in altered morphological parameters of neuronal plasticity in the trisomic cell line, namely decreased number and length of processes.

Keywords P21-activated kinases · Trisomy 21 · Down syndrome · DSCAM

Introduction

Down syndrome (DS) is caused by the presence of an extra copy of autosome 21 (Epstein 1986a, b), and it represents the aneuploidy that most frequently survives gestation. Currently, DS has an incidence of 1/700–1000 live births (Gardiner 2014), and has risen steadily over the last two decades. DS results in various anomalies such as muscular hypotonia, cardiovascular malformations, immunodeficiencies, and increased incidence of leukemia. At present, many of these ailments can be treated to various extents. Yet, the most striking features, which are mental retardation and an early onset of neuropathology similar to that of Alzheimer's disease (AD) (Head et al. 2016; Pulsifer 1996; Oster-Granite 1986), have yet no solution in sight. Further, as the life expectancy of DS patients has increased, the potential effects of the aforementioned AD-related

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pathology have added more concerns to the prognosis in these patients.

Human chromosome 21 was the first human autosome to be sequenced, allowing the identification of 127 genes and 98 novel genes (Delabar et al. 1993; Hattori et al. 2000), where those mapped to the so-called critical region of autosome 21 (DSCR, q22.1 to qter) have been deemed particularly relevant (Rapoport 1988; Epstein et al. 1991). In mouse models, Belichenko et al. (2009) confirmed that this region suffices to generate characteristic behavioral and neurophysiological anomalies of DS. However, the sole triplication of this region fails to reproduce characteristic craniofacial alterations of DS (Olson et al. 2004), and the predominant heart septal defects in DS have been linked to a locus outside the critical region (Wilson et al. 1993). Although this evidence has prompted discussion on the role of the DCSR in all the known dysfunctions, its role in the neuronal pathophysiology is still supported. Further, and in spite of the accumulated knowledge in the past two decades, the specific implications of the overexpression of DS-related gene products and their most complex interactions to alterations in specific cell functions remain poorly understood (Jiang et al. 2015; Saud et al. 2006).

Among the structural neuronal abnormalities reported in DS, brains are an anomalous development of the superior temporal neocortex, in particular in relation to lamination (Golden and Hyman 1994). The authors suggest that this phenomenon may reflect an abnormality in axonal and dendritic arborization, yet the mechanisms underlying the latter neuronal alterations are still unclear. In light of the aforementioned evidence, the search and study of DS-related genes that regulate development, in particular in the CNS, as well as those that affect neuronal function and eventually AD-like degeneration in adulthood, has become very important. In this regard, one interesting candidate is the gene encoding for Down syndrome cell adhesion molecule (Dscam), which has important functions in neuronal generation, maturation, and synaptogenesis (Jia et al. 2011). The DSCAM protein functions as an adhesion molecule, and it is expressed in the developing nervous system, where it intervenes in various stages of neuronal development (Jia et al. 2011). Such effects range from functions in early development (generation, migration, differentiation), to plasticity (Hattori et al. 2008), and formation of neuronal networks (Chen et al. 2006; Millard and Zipursky 2008). Therefore, the overexpression of this protein in DS patients may be related to cellular dysfunctions that impact the development of the CNS, and/or favor AD-related dementia in adulthood.

One known target for DSCAM are p21-associated kinases (PAKs), as the former has been shown to regulate the function of the latter as described in drosophila (Rao 2005) and mice (Liu et al. 2009). Group I PAKs are a

family of three (PAK1-3) serine/threonine protein kinases, which are activated by binding to the small (p21) GTP-binding proteins Cdc42 and Rac (Bokoch 2003; Rousseau et al. 2003). Physiologically, PAKs play important roles in two series of events, both of which are impaired in DS neurons. Firstly, neuronal PAKs regulate cytoskeletal organization, cellular morphogenesis, and survival. Secondly, PAKs play an important role in synaptic plasticity (Kreis and Barnier 2009). Indeed, PAKs modulate actin dynamics through the regulation of the Lim kinases which in turn regulate the actin severing/depolymerizing function of cofilin (Bokoch 2003), thus regulating the actin cytoskeleton. This pathway controls cell movement and dendritic function such as dendritic spine formation and maturation (Ma et al. 2008; Kreis et al. 2007; Dubos et al. 2012).

In relation to DS, previous studies have demonstrated that DSCAM is overexpressed in these patients. Bahn et al. (2002), demonstrated a 20 % increase in DSCAM expression in DS fetuses, compared to euploid controls. Interestingly, DSCAM labeling in DS brain neurons remains in higher levels of expression in spite of age, whereas in normal brains such labeling tends to diminish (Saito et al. 2000). In the present work, we also observed DSCAM overexpression in the CTb cell line, derived from the cerebral cortex of a fetal trisomy 16 (Ts16) mouse (an animal model of human DS. Reeves et al. 1986), compared to the CNh line, derived from the same tissue of a euploid littermate. In agreement with such finding, PAK1 as well as its downstream effectors LIMK and cofilin, stay phosphorylated for longer periods after DSCAM stimulation in the CTb cells, explaining the altered actin dynamics and neurite growth in the trisomic condition. Consequently, CTb cells exhibited a reduced capability to extend neurite processes and branching when submitted to differentiation conditions. Our studies suggest that the deregulation of the PAKs pathway may be important in neuronal connectivity dysfunction in DS.

Materials and Methods

Cell Lines

The cells were grown in DME/HAMF12 nutrient mixture (1:1) (Sigma Co., St. Louis, MO) supplemented with 6 g/L glucose, 1 g/L bicarbonate, 10 % (v/v) adult bovine serum and 2.5 % fetal bovine serum, and 40 µg/mL gentamycin, at 37° C, 100 % humidity, and an atmosphere of 5 % CO₂. The media were renewed twice per week and passages were carried out by detaching the cells with 0.1 % trypsin (Invitrogen, Grand Island, NY). For differentiation, cells were cultured with a medium composed of Neurobasal/B-

27[®] (Gibco, NYSE-TMO), forskolin (10 μ M), bFGF 100 ng/mL (ProSpec-Tany, Ness-Ziona, Israel), as previously described (Jang et al. 2010).

Reagents

For Western blotting the primary antibodies used were (Abcam, Cambridge, MA, USA): polyclonal to DSCAM (ab85362), monoclonal to PAK1 (ab40852), PAK2 (ab76293), PAK3 (ab40808), polyclonal to phosphor-PAK1 S204 (ab79503), monoclonal to LIMK1 (ab117623), polyclonal to phosphor-LIMK1 T508 (ab38508), polyclonal to Cofilin (ab42824), phospho S3 Cofilin (ab12866), monoclonal to beta Tubulin (ab11308), and secondary antibodies HRP conjugated (ab6802 and ab6820). For DSCAM stimulation, 500 ng/mL Mouse Netrin 1 peptide (ab91607, Abcam) was used.

Analysis of Protein Expression

Protein expression was studied by Western blot (WB) and flow cytometry (FACS). For WB, proteins from CNh and CTb cells were extracted with a lysis buffer composed of (in mM): 20 Tris-HCl, pH 7.5, 2 EDTA, 150 NaCl, and 0.5 % Triton X-100, protease and phosphatase inhibitors tablets (100 nM sodium orthovanadate, sodium pyrophosphate, and sodium fluoride) (Roche[®]). Samples were then homogenized with a Microson stem sonicator at 14 Watts potency for 10 s. The protein concentration was determined by the Bradford method, as specified by the manufacturer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the protein extract, was mixed with a 3 \times sample buffer containing 20 % SDS and 1.5 mL β -mercaptoethanol, and denatured by heating (5 min at 100 $^{\circ}$ C). Subsequently, the proteins (40 μ g) were separated in a 12 % SDS polyacrylamide gel (SDS-PAGE), and transferred to a nitrocellulose membrane. Membranes were then washed with a TBS-0.1 % Tween-20 solution (140 mM NaCl, 25 mM Tris/HCl pH 7.5, and 0.1 % Tween-20), blocked overnight with 5 % bovine serum albumin (BSA) in TBS-Tween-20 0.1 %, and incubated with primary and secondary antibodies HRP conjugated. Finally, WBs were developed with an ECL chemo-luminescent reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis of the immunoreactive bands was performed with the Gel Pro Analyzer 4.0 software Media Cybernetics, Inc.

For flow cytometry, 1×10^5 cells were used, which were recovered from culture dishes with a solution of 25 mM EDTA (ethylenediaminetetraacetic acid). The cells were washed twice with PBS and later incubated for 30 min with PBS-BSA 2 %, in order to block nonspecific binding sites. The cell were then washed with PBS and

incubated for 1 h with the primary antibody (Abcam, ab85362) diluted in PBS-BSA 2 %, then washed twice with PBS, and incubated for 30 min with the secondary antibody. Afterward, cells were again washed twice with PBS, and fixated in 2 % para-formaldehyde diluted in PBS. Samples were acquired on a FACSsort (Becton-Dickinson) flow cytometer and analyzed using the WinMD program, version 2.9. Analyses were performed on at least 10,000 events from the total population, and the percentage of cells expressing the target protein within this population was determined. All the data were corrected with autofluorescence controls and nonspecific antibody binding.

Quantification of F-actin/G-actin

To evaluate the relative amounts of filamentous (F-) and monomeric (G-actin), we used an F-actin/G-actin kit from Cytoskeleton, Inc (Denver, CO, USA. Cat #BR037) according to instructions by the manufacturer. Firstly, a LAS2 lysis buffer provided by the kit was prepared, composed of 1 mL LAS01 (Lysis and F-actin stabilization buffer), 10 μ L BSA04 (100 mM ATP stock solution), and 10 μ L PIC02 (100 \times Protease inhibitor cocktail stock). This LAS2 buffer was heated to 37 $^{\circ}$ C for 30 min prior to the assay. In parallel, cells were detached with 0.2 % trypsin in PBS, centrifuged at 2000 rpm for 5 min, and washed with PBS 1 \times . Later, 600 μ L of warm LAS2 were added to the resulting cell pellets, followed by incubation 37 $^{\circ}$ C for 10 min. Samples were then again centrifuged at 2000 rpm for 2 min to pellet unbroken cells and tissue debris. Supernatants were then collected and ultracentrifuged at 100,000 \times g, 37 $^{\circ}$ C for 1 h to pellet F-actin, whereas G-actin remained in the supernatant. Supernatants were removed and placed in fresh tubes designated as G-actin samples. For the pellet, 600 μ L of F-actin depolymerization buffer were added, followed by incubation in ice for 1 h to allow actin depolymerization to occur. The pellet was then resuspended by repeated pipetting every 15 min. Finally, 25 μ L of 5 \times SDS sample buffer were added to the samples, and Western blots were performed as previously described to quantify actin and calculate F-actin/G-actin ratio. Experiments were conducted in paired samples.

Morphological Analysis

For morphometric analysis, the criterion of cell differentiation was adapted from (Yu et al. 2008). Namely, a cell was deemed differentiated when it presented at least two processes, which exceeded a distance equivalent to one radius of the soma from the outer surface. The number and length of processes were studied under phase contrast microscopy, using a Lieder microscope (Model: MI-535).

Quantification was carried out in different fields per dish and condition, with a distribution of H or Z. At least seven of these fields were analyzed with an array or template of superimposed concentric rings (Sholl analysis) in increasing distances with respect to the cell soma, wherein the first ring was placed at a distance corresponding to the radius of the soma, previously measured for each cell and the following concentric rings 5 μm of distance between them. For analysis of cellular processes length, NeuronJ (ImageJ plugin) with pseudoimages acquired using bright field to improve contrast, designed by the ImageJ program, was used .

Statistical Analysis

Statistical significance between datasets was determined by ANOVA, followed by a post hoc test (*t* test, Tukey, Mann–Whitney), for *p* levels <0.05.

Results

We used neuronal cell lines derived from normal and Ts16 mice. The cell lines were established from the cerebral cortex of a normal (named CNh) and trisomic (named CTb) mouse fetuses. These cell lines retain neuronal markers, functional neurotransmitters receptors, and express cholinergic function. The trisomic-derived lines reproduce glutamatergic and cholinergic dysfunction of Ts16 neurons, and possess altered voltage-activated Ca^{2+} currents and intracellular Ca^{2+} signaling (Cárdenas et al. 1999; Allen et al. 2000; Acuña et al. 2012).

Figure 1 presents morphometric analyses carried out in CNh and CTb cells cultured in differentiation media. Figure 1a shows typical micrographs of cells kept 72 h in the differentiation media, as well as examples of pseudoimages and Sholl analysis of the length of the neuritic processes. The length of the longest process studied did not differ between the two cell lines (Fig. 1b). Cell differentiation, as depicted by our criteria (cells with two neurite processes with of at least one radius in length from the cell surface) did not differ significantly between both cell types at all times studied (Fig. 1b). According to our morphometric analysis, after 72 h in differentiation media around 70 % of CNh and CTb cells display at least two processes, hence we chose this period to perform the Sholl analysis and analyze the number of neurite crossings with concentric circles, that quantifies number and arborization of the processes. Our results indicate that the CTb cell line exhibits a reduction in this parameter, compared to CNh cells (Fig. 1c), which becomes significant for neurite processes longer than

35 μm . Whereas the length of the longest process did not differ between the two cell lines, the total neurite length in the CTb cells is approximately 15 % smaller than that in CNh cells (Fig. 1d). These results strongly suggest a reduced capability of CTb cells to extend processes.

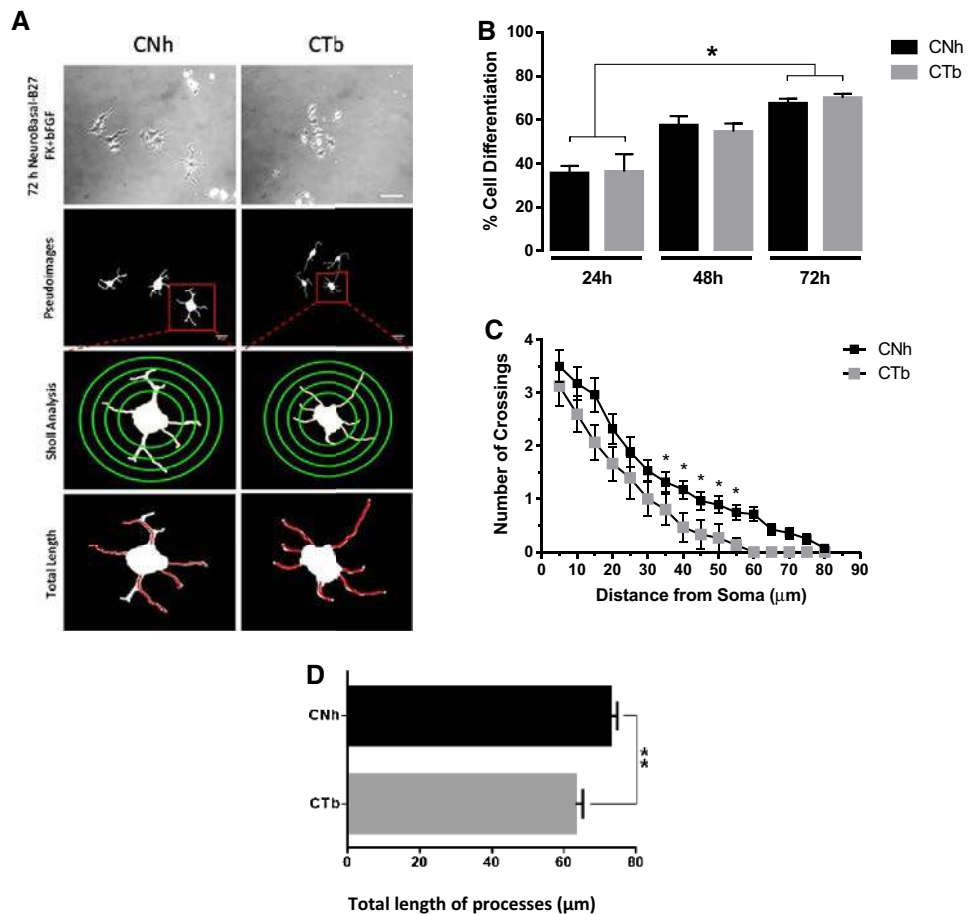
Considering that DSCAM is overexpressed in Down syndrome (Saito et al. 2000) and that it also plays a role governing neurite arborization (Fuerst et al. 2008), by mechanisms that probably include PAK-mediated pathways (Kreis and Barnier 2009), we then compared the expression of DSCAM and the activation of the PAK signaling pathway in CNh and CTb cells.

Figure 2A shows the expression of DSCAM in CNh and CTb cells evaluated by WB assays. The respective densitometric analysis indicates that DSCAM expression is twofold higher in the trisomic CTb cell line, compared to CNh cells (Fig. 2b). Similar results were obtained using FACS analyses in nonpermeabilized cells, which show that DSCAM is also overexpressed at the membrane of CTb cells (Fig. 2c, d). Further, unlike to that observed in CNh cells, DSCAM remains overexpressed in CTb cells after 24 and 48 h in differentiation medium (Fig. 2e, f).

We then studied the expression of neuronal PAKs in both, CNh and CTb cells. Figure 3a shows that PAK1, 2, and 3 are all expressed in both cell lines, although PAK1 is the most abundant. This result is in agreement with the higher expression of PAK1 in neuronal tissue (Arias-Romero and Chernoff 2008). Therefore, we decided to focus our study in this kinase. As shown in Fig. 3, we found no difference in PAK1 expression in CNh and CTb cells, even after 24–72 h in differentiation medium (Fig. 4a, b). We also found no difference in the basal activation of this PAK, as evidenced by the abundance of its form phosphorylated at residue S204, which is a main PAK1 specific site of autophosphorylation (Chong et al. 2001) (Fig. 4a, b).

Since DSCAM related processes are essentially phasic in nature (Schmucker and Chen 2009), we decided to explore the sensitivity of the PAK1 pathway by stimulating the cells with a known DSCAM agonist, such as netrin1 (Palmesino et al. 2012). After 5 min of stimulation with this agonist, the expression of PAK1 phosphorylated at residue S204 increased about 50 % over basal levels in both cell types (Fig. 4c, d). However, after 10 min post-stimulation, PAK1 remains phosphorylated in CTb cells, whereas in CNh cells, this kinase returns to phosphorylation basal levels. These observations suggest that the overexpression of DSCAM in CTb cells induced a prolonged activation of PAK1, therefore, we studied two downstream effectors of the PAK pathway, namely LIMK1 and cofilin. Figure 5 shows the phosphorylation state of

Fig. 1 Morphometric studies of processes in neuronal cell lines. **a** Representative phase contrast images of CNh and CTb cells cultured in neuronal differentiation medium for 72 h (upper panels). Pseudoimages of the latter images are also presented, as well as the corresponding Sholl ring insertion (green), and measurement of process extension (red). Scale bar 50 μm . **b** Cell differentiation index in function of the presence of processes extending on radius length from the cell soma (5 different cultures of each cell line). **c** Sholl analysis of CNh and CTb kept 72 h in differentiation media. n CNh = 65, CTb = 55 from three different cultures. **d** Total length of neurite processes in CNh and CTb cells in differentiation media for 72 h. n = 176 processes per cell line. Values represent mean \pm SEM. *: Significant for $p < 0.05$ and ** for $p < 0.001$, Tukey test (Color figure online)



these two latter proteins in response to netrin1 stimulation, in CNh and CTb cells. Similarly to what we observed with PAK1, a maximal phosphorylation of LIMK1 at the residue T508 was observed 5 min after stimulation with netrin1 in both cell types. However, unlike in CNh cells, such phosphorylation remains higher in CTb cells 10 min after stimulation, returning later to the basal phosphorylation state (Fig. 5a).

Cofilin, the direct target of LIM kinases, also shows a similar prolonged phosphorylated phase in CTb cells, as evidenced by the longer presence of its phosphorylated form at the residue S3, compared to CNh cells. This form remains elevated for up to 20 min post netrin1 stimulation, whereas CNh cells returned to basal phosphorylated levels after 10 min of induction with netrin1 (Fig. 5b).

To assess the possible downstream effect of this observed prolonged cofilin phosphorylation in the trisomic condition, we studied F-actin/G-actin ratio in our cell lines (Fig. 5c). Interestingly, and in agreement with the prolonged presence of phosphorylated cofilin, CTb cells expressed an approximate twofold increase in this ratio compared to CNh cells in basal conditions (CNh: 0.88 ± 0.55 ; CTb: 1.68 ± 0.85), suggesting in CTb cells

than in CNh cells, hindering their capability to be remodeled.

Discussion

As previously stated, DS constitutes a developmental disease as well as a condition that alters cell function (Saud et al. 2006), and although the former constitutes a much more complex process, our goal in this work was to address altered cell function with in vitro models that may shed light on both aspects of DS pathology. Also, no clear association exists between DS and specific neuronal structural abnormalities (Purpura 1974; Marin-Padilla 1972, 1976; Takashima et al. 1981; Wisniewski et al. 1985; Schapiro et al. 1987, 1989; Schmidt-Sidor et al. 1990). It thus appears that the pathological symptoms are more related, at least initially, to neuronal dysfunction rather than to structural abnormalities (Takashima et al. 1981; Schmidt-Sidor et al. 1990). This is consistent with our previous work and that of other groups, which have reported specific functional defects at the cellular level in trisomic neurons. For example, electrophysiological studies

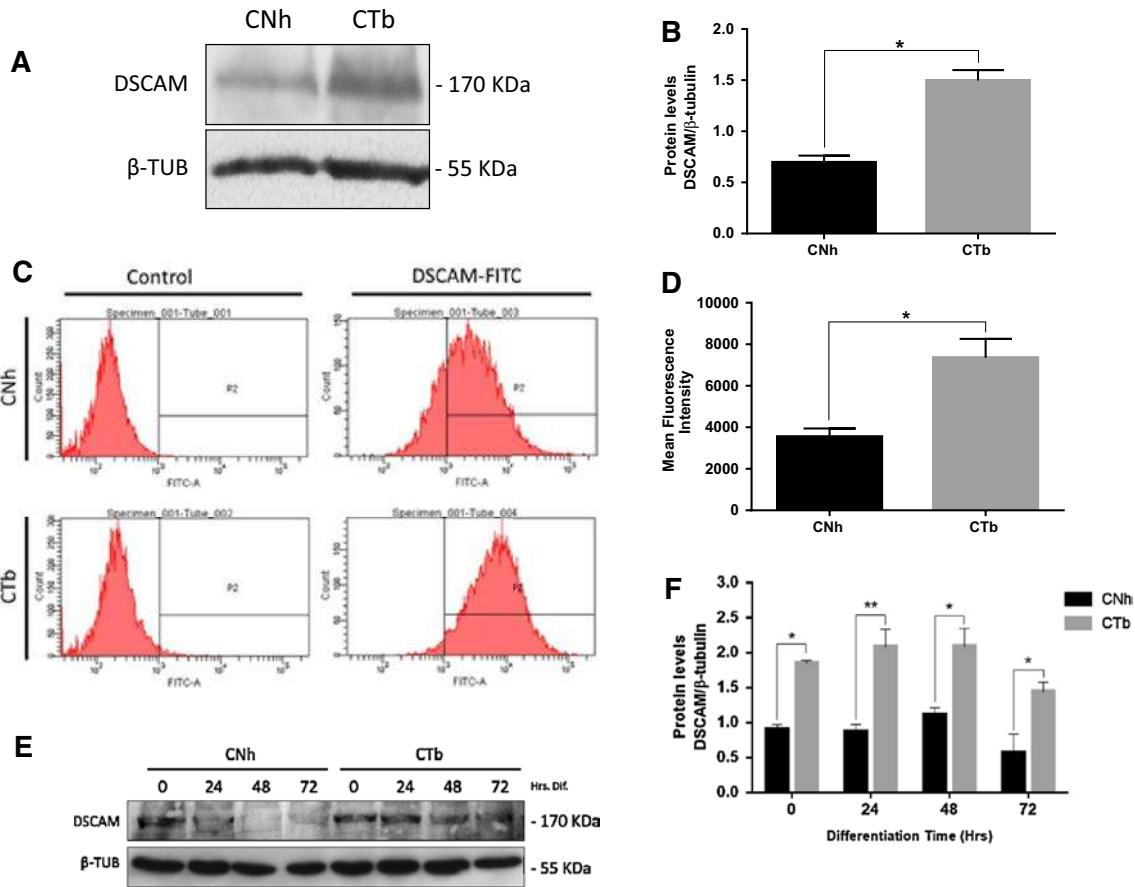


Fig. 2 DSCAM is overexpressed in CTb cells. **a** Representative image of DSCAM protein expression analyzed by Western blot in CNh and CTb cells, with the corresponding load control β -tubulin. **b** Analysis of DSCAM expression abundance. Values are mean \pm SEM from 3 separate experiments. $*p < 0.05$ (Mann-Whitney Test). **c** Representative experiments using FACS, showing the expression levels of DSCAM at the cell surface in nonpermeabilized CNh and CTb cells. Control experiments were carried out in the absence of the primary antibody. Results correspond to 10,000 events per sample.

Plots represent mean fluorescence intensity. **d** Analysis of DSCAM abundance in the cell surface. Values are mean \pm SEM of 4 separate experiments per condition. $*p < 0.05$ (Mann-Whitney Test). **e** Representative Western blot of DSCAM abundance at different times of differentiation in culture. **f** Quantification of DSCAM expression. Note that DSCAM abundance in CTb cells remains elevated at all times studied, compared to CNh. Values are mean \pm SEM of 3 independent experiments. $*$: Significant for $p < 0.05$ and $**$ for $p < 0.01$ (ANOVA, followed by Tukey's test)

in cultured dorsal root ganglia neurons of human DS and murine Ts16, an animal model of DS, have shown that the duration of the action potential is greatly reduced in trisomic cells, compared to normal neurons (Nieminen et al. 1988; Caviedes et al. 1990), probably due to the existence of altered kinetics and density of sodium and potassium currents (Orozco et al. 1988; Nieminen et al. 1988; Caviedes et al. 1990; Ault et al. 1989). Further, our group has also shown a reduction in the amplitude of voltage dependent Ca^{2+} currents in Ts16 dorsal root ganglion neurons (Caviedes et al. 2006). Yet, in central neurons, such currents exhibited increased amplitude (Galdzicki et al. 1998), a situation that was also reported in the CTb cell line (Acuña et al. 2012). DS also compromises neurotransmitter metabolism in the brain, expressed in significant reduction of noradrenaline in the hypothalamus

and reduced acetylcholine transferase (ChAT) activity throughout the brain (Yates et al. 1983). In this regard, our group and others have reported similar cholinergic dysfunction in cultured TS16 neurons (Fiedler et al. 1994; Nelson et al. 1997), as well as in our cell line model (Allen et al. 2000; Opazo et al. 2006).

In light of the aforementioned evidence, the search and study of DS-related genes that regulate development, in particular in the CNS, as well as those that affect neuronal function, has become very important. Lately, the disruption of genetic regulatory circuits (GRCs) has been implicated in DS pathophysiology (Arron et al. 2006). GRCs carry out critical tasks in development, and respond to environmental queues and various feedback loops in the cell. Such loops can be greatly impaired by conditions such as DS, where increased gene dosage could affect them decidedly, and

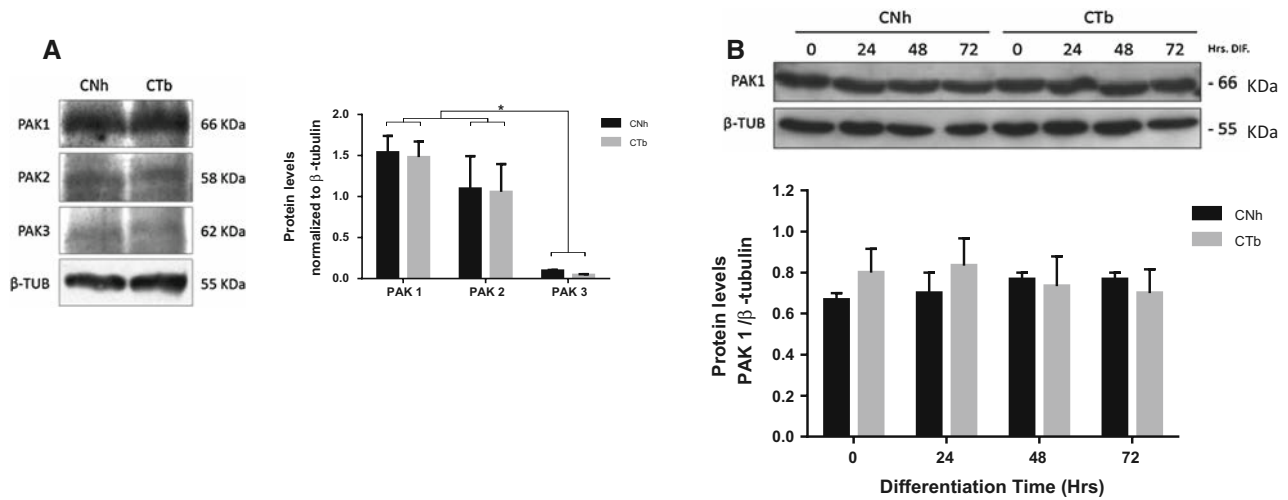


Fig. 3 PAK expression in CNh and CTb cells. **a** Representative Western blot of PAK1, PAK2, and PAK3 in CNh and CTb cells, and their relative quantitation in terms of pixel density by area normalized by β -tubulin expression. Values are mean \pm SEM from of 3 independent experiments; * $p < 0.05$ (ANOVA followed by Tukey's test). No differences were evident among different isoform

expressions between the two cell lines, although a greater abundance of PAK1 is evident. **b** Representative Western blot and relative quantitation of PAK1 abundance does not differ between the two cell types in response to differentiation. Values represent mean \pm SEM ($n = 3$)

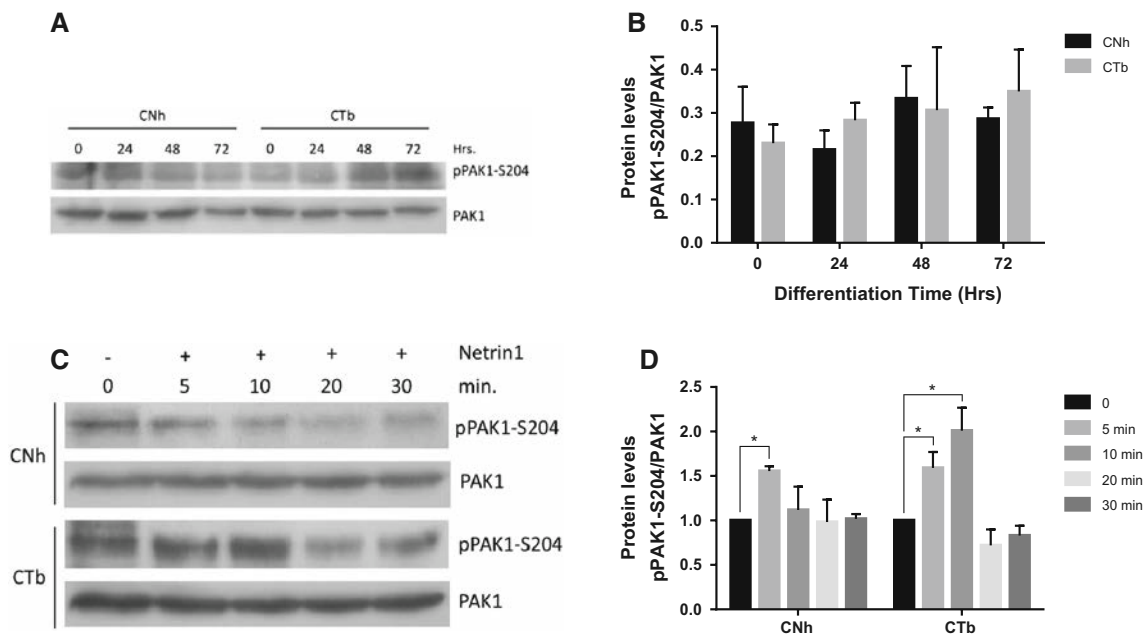


Fig. 4 PAK1 phosphorylation is increased in both neuronal cell lines after stimulation with netrin1. S204-phosphorylated forms of PAK1 are present in comparable quantities in both cell types at different times in differentiation conditions, as shown in the representative Western blot (**a**) and relative quantitation (**b**) ($n = 3$). A substantial

increase in S204 phosphorylation is observed in both cell types after 5 min of stimulation with the DSCAM agonist netrin1 (**c**, **d**). Yet, note that CTb cells present increased PAK1 phosphorylation up to 10 min post-stimulation. Values represent mean \pm SEM ($n = 3$). *Significant for $p < 0.05$ (Mann-Whitney test)

even have cooperative effects in destabilizing. One notable example in this regard is the reported convergence of two DS-related gene products, such as the Regulator of calcineurin-1 (DSCR1/Rcan1) and the dual specificity tyrosine kinase 1A (Dyrk1A), in the function of NFATc4

pathway (Gwack et al. 2006). Indeed, when both gene products are overexpressed, they cooperatively hyperphosphorylate NFATc4 (Arron et al. 2006), a transcription factor critical for neuronal development and function (Epstein 2006). This evidence has prompted for the search

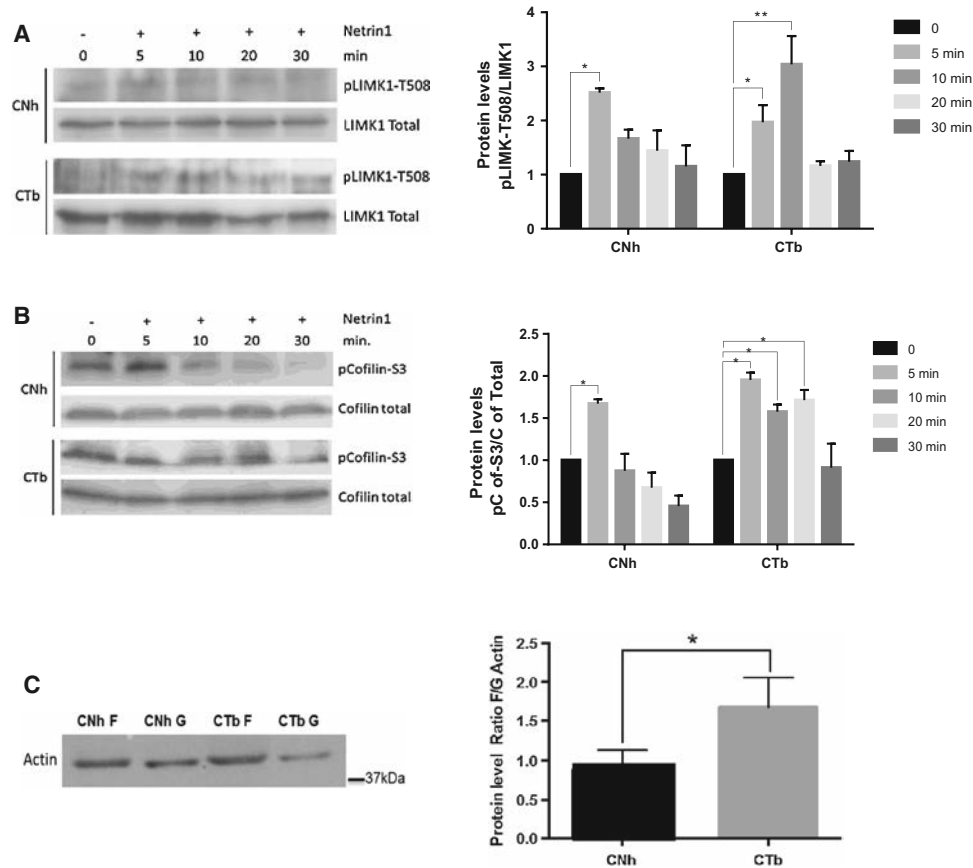


Fig. 5 Downstream effectors of PAK1, namely LIMK1 and cofilin, also exhibit increased phosphorylation in response to netrin1 stimulation, resulting in an increased F-actin/G-actin ratio. **a** Representative Western blot and relative quantitation of pT508-LIMK. Note the increase in LIMK phosphorylation in CNh cells after 5 min of stimulation. In CTb, this effect persists up to 10 min ($n = 3$). **b** Representative Western blots and relative quantitation of pS3-cofilin. Note the persistence of increased levels of the phosphorylated

form in CTb cells compared to CNh ($n = 3$). Values represent mean \pm SEM. *Significant for $p < 0.05$ (Mann-Whitney test). **c** Representative Western blot of F- and G-Actin and relative quantification expressed as a ratio between the two proteins, studied in CNh and CTb cells in basal conditions. Note the increased ratio in the trisomic condition. Values are mean \pm SEM, $n = 5$ paired independent experiments. *Significant for $p < 0.05$

of checkpoints in such pathways that can be destabilized by more than one overexpressed DS-related gene product, and that could in turn represent attractive therapeutic targets. One of such genes is Down Syndrome Cell Adhesion Molecule (DSCAM), which has important functions in neuronal generation, synaptogenesis, axonal outgrowth, and neurite arborization (Fuerst et al. 2008; Montesinos 2014), all of which could be severely affected when DSCAM is overexpressed. Yet, what GRCs could be affected in this situation? In this regard, PAKs appear as one of the most interesting candidates, as they are known to be regulated by DSCAM, and they also play a role in neurite and axon growth and remodeling (Liu et al. 2009; Kreis and Barnier 2009). Hence, in this study, we decided to use the trisomic CTb cell line to study DSCAM expression, PAKs activity and process extension in response to differentiation stimuli. Our results presented in Fig. 1 show that CTb cells exhibit a reduction in the

number of neurite crossings per ring, compared to normal CNh cells. This reduction becomes significant from a distance of 35 μ m from the soma. Further, the total length of the processes is shorter in the trisomic condition. This situation suggests an impediment in the progression of process extension. In this regard, long-term overexpression of LIMK1 in cultured rat hippocampal neurons, reportedly results in growth cone collapse, axon retraction, and a significant reduction in the number of collateral branches (Rosso et al. 2004). Further, these authors also reported a dramatic increase in phosphorylated cofilin in the transfected condition. Indeed, phosphorylation by LIMK1 inactivates cofilin, leading to accumulation of actin filaments (Arber et al. 1998), which in turn can result in an altered actin dynamics that disturbs neurite growth and axonal integrity (Cook et al. 2014).

As previously stated, PAKs can be activated by DSCAM. Hence, in a situation where the latter is present in

excess, the entire PAKs pathway could be deregulated, in turn promoting the morphological alterations described herein. In light of the above, we decided to pursue the effect of DSCAM regulation in our trisomic model. Firstly, we confirmed the overexpression of DSCAM in cell extracts by WB (Fig. 2a) and, most relevantly, at the cell membrane level with FACS (Fig. 2b). Regarding group I PAKs, we found that PAK1 was the most abundant form present in both cell lines (Fig. 3), which is in agreement with the high expression of this protein in neural tissue (Kreis and Barnier 2009). Most importantly, PAK1 was equally expressed in both CNh and CTb cells, either in undifferentiated or differentiated culture conditions. PAK1 activity, expressed as the ratio of activated phosphorylated PAK1 versus total PAK1 (phosphoPAK1), was similar between both cell types at basal conditions. This could be explained by the fact that DSCAM activity is more phasic in nature, rather than tonic (Palmesino et al. 2012). We therefore decided to stimulate DSCAM with a known specific agonist, such as netrin (Palmesino et al. 2012). Under these conditions, we observed a substantial increase in phosphoPAK1 in either CNh or cells at 5 min post-stimulation (Fig. 4). This effect was still evident after 10 min of stimulation with netrin in CTb cells, but not in CNh cells, where the phosphorylated form of the protein had returned to basal levels (Fig. 3). A similar pattern was evident in LIM Kinase 1 and cofilin phosphorylation, which remained in such phosphorylated state for more prolonged time in the trisomic cell line (Figs. 4, 5). Reportedly, increased phosphorylation of cofilin leads to an accumulation of filamentous actin (Arber et al. 1998), which in turn disturbs actin dynamics and consequently neuronal extension (Cook et al. 2014), which agrees with our finding of an increased F/G-actin ratio in CTb cells. Interestingly, such a mechanism could also underlie other neuronal diseases that result in impaired neurite extension. Indeed, in neurons from familial amyotrophic lateral sclerosis models, the deregulation of the F/G-actin ratio, in particular in areas of high actin dynamics such as growth cones, results in a significant reduction in their size as well as in altered morphology (Wu et al. 2012). Also, in Spinal muscular atrophy (SMA), hyperphosphorylation of cofilin has been linked to an increased F/G-actin ratio, in turn resulting in decreased neural process extension (Nölle et al. 2011). It is also noteworthy that an abnormal F-actin accumulation constitutes a substrate for AD-linked neuronal degeneration (Fulga et al. 2007). In this regard, a hyperactivated PAK pathway, in this case due to an overexpressed DSCAM, could introduce an imbalance in actin dynamics in the trisomic condition, favoring the accumulation of actin in the F-form. In this regard, we propose a model of the status of the induced deregulation of the PAK pathway (see Fig. 6). Due to DSCAM overexpression in

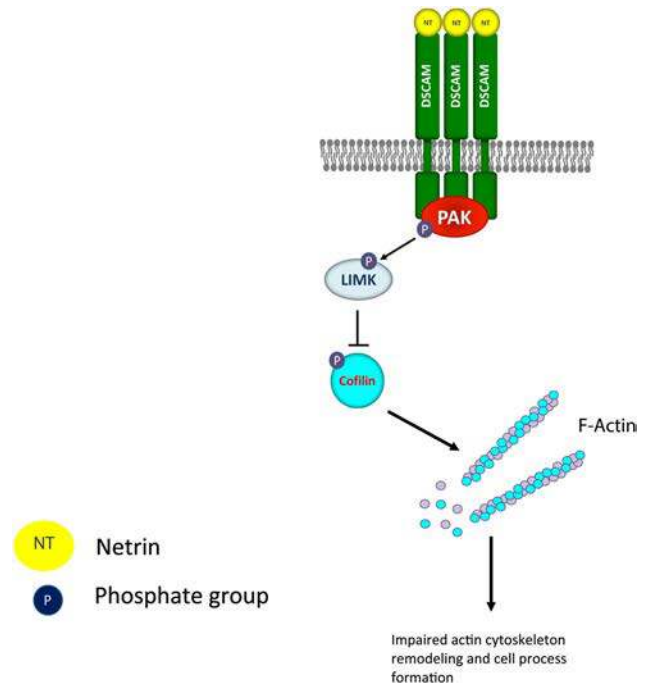


Fig. 6 Model of the impact of DSCAM overexpression on the PAK pathway in CTb cells. Overexpressed DSCAM could result in an overactivation of the PAK pathway upon stimulation with the agonist. In the trisomic condition, netrin stimulation produces abnormally increased PAK phosphorylation, thus activating this protein for longer periods of time. In turn, PAK phosphorylates LIMK, resulting in a long-lasting phosphorylation and activation of LIMK, and a prolonged phosphorylation and inactivation of its effector, the actin-depolymerizing factor cofilin. An inactive cofilin fails to depolymerize actin, which tends to remain in the F-state. The latter results in impairment of neurite formation and/or extension in the trisomic cell line

the trisomic condition, the activation of this pathway with a ligand would overactivate PAK1, and in turn LIMK1 and cofilin (Fig. 6). Hence, this deregulated pathway could induce accumulation of the F-form of actin, in turn impairing the capability of the trisomic cells to remodel F-actin and extend processes.

We have to note that PAKs can also affect actin dynamics via other downstream effector pathways, namely Raf/MEK/ERK, p38, and JNK (Bagrodia et al. 1995; Purohit et al. 2012; Qu et al. 2013; Wang et al. 2013). Nevertheless, these latter such pathways have more delayed effects, since they depend on transcriptional regulations (Dubos et al. 2012). Also, in DS, PAKs could also be deregulated due to overexpressed amyloid precursor protein APP (Epstein 1986a, Pozueta et al. 2013), a situation that reportedly exists in our trisomic cell line (Opazo et al. 2006). Further, the C31 peptide, which derives from caspase-induced cleavage of APP, can interact and affect PAK activity (Nguyen et al. 2008). Hence, in DS, as well as our CTb cell line, cleavage of APP and release of

peptide C31 could be elevated, further contributing to the increased activity of the PAK pathway. More studies are needed to confirm if this alternative route could also be affecting the dynamics of the actin cytoskeleton and consequently neurite guidance and growth. On the other hand, netrin is known to activate Deleted in Colon Carcinoma (DCC), which also mediates netrin effects on axon outgrowth (Keino-Masu et al. 1996). Yet, DCC is mapped to autosome 18 in both humans and mice, hence a gene dosage hypersensitivity related solely to DCC is unlikely in our trisomic cell line. However, we acknowledge that definite proof of the association of DSCAM with the hypersensitivity of the PAK pathway is needed, and in pursuit of this goal, we are currently restudying these mechanisms after knocking down DSCAM in CTb cells with siRNAs, in order to reduce expression of this protein to levels comparable to those of CNh. Interestingly, DCC and DSCAM interact in a collaborative fashion to regulate actin and microtubule dynamics, via a mechanism related to binding with TUBB3, a very dynamic β -tubulin isoform in neurons (Huang et al. 2015). It is tempting to speculate that such mechanisms may be potentiating the effects herein described on the PAK pathway, a possibility that certainly merits exploration.

Conclusions

Down Syndrome being a disease based on excess gene dosage, a gene therapy approach is largely impractical due to the great number of genes involved. Further, the multiple interactions between gene products that arise in the condition introduce further complications in gene targeting therapy. Hence, the identification of regulatory circuits where several DS-related gene products converge to destabilize their function appears as a most desirable approach. In this regard, the PAK pathway presents several potential and specific therapeutical targets. Further, PAK inhibitors do exist (IPA-3, FRAX486) which could constitute lead compounds to generate pharmacological agents that could revert the imbalance herein reported, and correct a critical neuronal function such as process extension.

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