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ORIGINAL ARTICLE

Dual REST-dependence of L1CAM: from gene expression to alternative splicing governed by Nova2 in neural cells

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Abstract

L1 cell adhesion molecule (L1CAM), an adhesion/signaling protein encoded by a gene target of the transcription repressor RE-1-Silencing Transcription factor (REST), is expressed in two alternatively spliced isoforms. The full-length isoform, typical of low-REST neural cells, plays key roles in survival/migration, outgrowth/fasciculation/regeneration of axons, synaptic plasticity; the isoform missing two mini-exons, abundant in a few high-REST non-neural cells, maintains some effect on migration and proliferation. To investigate whether and how L1CAM alternative splicing depends on REST we used neural cell models expressing low or high levels of REST (PC12, SH-SY5Y, differentiated NT2/D1 and primary neurons transduced or not with REST). The short isoform was found to rise when the low-REST levels of neural cells were experimentally increased, while the full-length isoform increased in high-REST cells when the repressor tone was attenuated. These results were due to Nova2, a neural cell-specific splicing factor shown here to be repressed by REST. REST control of L1CAM occurs therefore by two mechanisms, transcription and alternative splicing. The splicing mechanism, affecting not only L1CAM but all Nova2 targets (~7% of brain-specific splicing, including the mRNAs of other adhesion and synaptic proteins) is expected to be critical during development and important also for the structure and function of mature neural cells.

Keywords: β-catenin, NT2/D1 cells, PC12 cells, primary neurons, REST transfection/transduction, transcription.

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L1 cell adhesion molecule (L1CAM), composed of six extracellular immunoglobulin-like domains followed by five fibronectin type III repeats, a single-pass transmembrane sequence and a short cytoplasmic domain, is a multifunctional surface protein encoded by a gene located in the X chromosome. L1CAM was discovered in neurons and in various neurosecretory cell lines, including PC12 (Salton *et al.* 1983; Rathjen and Schachner 1984). Its binding to adhesion proteins and receptors, with ensuing activation of multiple signalling cascades (Panicker *et al.* 2003; Islam *et al.* 2004; Kulahin *et al.* 2008), plays pivotal roles in a variety of processes such as survival and migration of

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Abbreviations used: Δ90-βcat, β-catenin construct truncated of its N-terminal 90 aminoacids; ChIP, chromatin immunoprecipitation; ChIP-Seq, ChIP followed by ultra-high-throughput sequencing; FACS, fluorescence-activated cell sorter; fl-L1, full length isoforms of L1CAM; L1CAM, L1 cell adhesion molecule; mAb and pAb, monoclonal and polyclonal antibody; NFI-A, nuclear factor I-A; RA, retinoic acid; TSA, trichostatin A; sh-L1, short isoforms; REST, RE-1-Silencing Transcription factor.

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neurons; outgrowth, fasciculation and regeneration of axons; synaptic plasticity (recent reviews: Gerrow and El-Husseini 2006; Maness and Schachner 2007; Schmid and Maness 2008). The functional importance of L1CAM in the brain is documented also by its mutations, known as the cause of an intensively investigated brain genetic disease, the L1CAM syndrome or CRASH (reviews: Kamiguchi *et al.* 1998; De Angelis *et al.* 2002; Schäfer and Altevogt 2010).

L1CAM expression in neural cells depends on RE-1-Silencing Transcription factor (REST, also known as NRSF) (Kallunki *et al.* 1997), the master factor governing neural cell differentiation. High levels of REST, typical of stem cells, repress the expression of hundreds of neural-specific target genes. A rapid down-regulation, taking place during neural progenitor maturation, relieves the expression of these genes and orchestrates the acquisition of the mature phenotype (Ballas and Mandel 2005; Ooi and Wood 2007). Even in mature neural cells, however, the levels of REST can increase, although moderately, both in physiological and pathological conditions (Garriga-Canut *et al.* 2006; Spencer *et al.* 2006). Recently, another transcription repressor, nuclear factor I-A (NFI-A), was found to participate in the regulation of L1CAM expression in neural cells (Schneegans *et al.* 2009). Whether the role of NFI-A depends on REST, or whether it works independently, is unknown.

In many non-neural cells, where levels of REST are high, expression of L1CAM remains low. In others, however, such as Schwann cells, oligodendrocytes, epithelial cells and cells of the immune system, L1CAM is high, relevant for multiple, ubiquitous or specialized functions including myelination, lymph node architecture, T cell activation and cell migration (see, among others, Di Sciullo *et al.* 1998; Itoh *et al.* 2000; Balaian *et al.* 2000; Maddaluno *et al.* 2009). In addition, L1CAM is now recognized as a surface protein essential for stem cells self-renewal (Son *et al.* 2011) and as a marker of invasiveness and metastases of various types of human cancers (reviews: Raveh *et al.* 2009; Siesser and Maness 2009; Schäfer and Altevogt 2010). In at least some tumours expression of L1CAM is activated by the β-catenin/TCF transcription system (Gavert *et al.* 2005), operative in parallel to REST (Pfeiffer *et al.* 2010). Finally, in pancreatic adenocarcinoma expression of L1CAM was shown to depend on an additional transcription factor, SLUG (Pfeiffer *et al.* 2010; Geismann *et al.* 2011).

The L1CAM protein exists in two alternatively spliced isoforms. The full-length isoform (fl-L1), composed by 28 exons, is typical of neural cells; the short (sh-L1) or non-neural isoform, lacking two mini-exons (exon 2, extracellular, and exon 27, intracellular) is predominant in the other cell types. By their participation in L1CAM binding to extracellular (De Angelis *et al.* 2001) and cytoplasmic (Herron *et al.* 2009) proteins, respectively, the two spliced exons appear functionally important in neural cells and also in some tumours where fl-L1, and not sh-L1, promotes liver and lung metastases (Hauser *et al.* 2011). In a human breast cancer line, however, even over-expression of sh-L1 induced disruption of adherens junctions, increased β-catenin transcription, increased motility and cell scattering (Shtutman *et al.* 2006).

In spite of its recognized relevance in physiology and pathology, L1CAM splicing and its regulation had never been investigated in mechanistic terms. The present work was carried out by employing several cell models: two clones of the rat pheochromocytoma line PC12 (D'Alessandro *et al.* 2008), one (wtPC12), expressing the typical phenotype, with low REST and high fl-L1 levels, the other (PC12–27) expressing in contrast high REST and low sh-L1 levels; the high-REST neuroblastoma SH-SY5Y line; rat primary neurons and the human NT2/D1 cells differentiated with retinoic acid (RA), analyzed before and after REST transduction. Our results demonstrate that the low REST of neural cells governs not only the expression of L1CAM but also its specific splicing, induced through the expression of Nova2, a brain-specific splicing factor. The REST-dependence of splicing is likely a widely important process, not limited to L1CAM but extended to the whole panel of Nova2 targets [~7% of the brain-specific mRNAs spliced in the neocortex (Ule *et al.* 2005)].

Materials and methods

Reagents

Goat polyclonal antibodies (pAbs) against the C-terminal (sc-1508) and one N-terminal domains (sc-31032) of L1CAM were from Santa Cruz, a mouse monoclonal Ab (mAb) against the N-terminal (CD171) was from BD Biosci. Pharmingen (San Diego, CA, USA). The anti-REST rabbit pAb was from Millipore Corporation (07–579; Bedford, MA, USA); the anti-Nova2 and anti-NFI-A rabbit pAbs from MBL International (RN044PW; Woburn, MA, USA) and Abcam (Ab41851; Cambridge, UK), respectively. Horseradish peroxidase-conjugated pAbs anti-mouse, anti-rabbit and anti-goat immunoglobulins were from Bio-Rad Laboratories (Hercules, CA, USA); tetramethylrhodamine isothiocyanate-conjugated rabbit anti-goat pAb from SouthernBiotech (Birmingham, AL, USA); FITC-conjugated annexin V from Boehringer Mannheim Italia (Monza, Italy). The Fungene HD transfection factor was from Promega; lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA); the anti-β-tubulin mAb, DAPI, RA and the histone deacetylase inhibitor trichostatin A (TSA) from Sigma-Aldrich (St Louis, MO, USA); BSA from Roche Molecular Biochemicals (Indianapolis, IN, USA). The EZ-chromatin immunoprecipitation (ChIP)-A kit including the control rabbit IgGs was from Millipore; the BCA Protein Assay kit and the NE-PER® kit for nuclear protein extraction from Thermo-Scientific (Waltham, MA, USA).

Cells and cell clones

Rat pheochromocytoma wtPC12 and PC12–27 clones and the Myc-REST subclone stably transfected with REST were as in D'Alessandro *et al.* (2008); the wtPC12/Δ90βcat subclone and SH-SY5Y cells as in Tomasoni *et al.* (2011) and Racchetti *et al.* (2010), respectively. Rat P2 primary cortical neurons, grown in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) with 10%

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horse serum (Euroclone, Pero-Milano, Italy), 5% fetal clone III serum (Hyclone, Bonn, Germany), 2 mM ultraglutamine and 100 U/mL penicillin/streptomycin (Lonza) (Racchetti *et al.* 2010), were used after 6–10 days *in vitro*. NT2/D1 cells, described in Nikcević *et al.* (2008), were grown in Dulbecco's modified Eagle's medium with 10% fetal clone III serum, 2 mM ultraglutamine and 5 U/mL penicillin/streptomycin.

Transient and stable transfections and transductions. Scratch assay

Transient and stable transfections of PC12 clones, using lipofectamine 2000, were as in D'Alessandro *et al.* (2008). Subclones were grown in complete medium supplemented with G418 (450 μg/mL). For the scratch assay, PC12–27 cells were transiently co-transfected (Hauser *et al.* 2011) with *GFP* together with either *fl-L1* or *sh-L1* constructs (gifts of Ugo Cavallaro and Achim Krueger, respectively), grown for 1 day and then sorted. Fractions expressing similar levels of GFP were incubated to confluence (1 day) in 24-well dishes. Monolayers, wounded with a p200 pipette tip, were washed carefully and photographed in a phase-constrast microscope every 24 h for 3 days (Liang *et al.* 2007).

Three rat Nova2-targeting shRNAs were generated and inserted in the pGFP-V-RS vector (OriGene Technologies, Rockville, MD, USA). The HT120832D (ACGCAAGCCGCTCAATACCTCATCAGCCA) shRNA, showing the best activity and a non-effective scramble cassette in the same vector were used for Nova2-knockdown and as negative control. Fractions exhibiting high GFP fluorescence, sorted by fluorescence-activated cell sorter (FACS) 72 h after transfection with Fungene HD, were used for the assays. For transduction of REST in neuronal primary cultures and NT2/D1 cells, #945.PCCL.sin.cPPT.SV40ploy A.eGFP.minCMV.hPGK.deltaLNGFR.Wpre lentiviral vectors including GFP together with (or not, controls) the full length *REST* were employed. After 24 h incubation, the cells were washed, cultured for further 6 days and then sorted by FACS. Highly GFP-fluorescent fractions were used for the assays. For details, see Tomasoni *et al.* (2011).

Real-time polymerase chain reaction (RT-PCR)

Total RNAs, extracted using RNeasy mini columns (Qiagen, Valencia, CA, USA) and assayed by spectrophotometry, were used (1–2 μg) to generate cDNA templates for RT-PCR, using random primers, Rnase inhibitor and retrotranscriptase M-MLV (Applied Biosystems, Foster City, CA, USA). Assays of triplicate samples were carried out according to the SYBR Green procedure, using 50 ng template cDNA in the ABI 7900HT Sequence Detection System (Applied Biosystem). Results were normalized to the invariant mRNAs of calmodulin or GAPDH. PCR products in TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 7) were analyzed on 2% agarose gels for GAPDH and calmodulin, on 4% gels for L1CAM isoforms. Primers used in rat cells were: for calmodulin, fw: 5′-GAAGAAGAAATTAGAGAAGCGTTCC-3′; rev: 5′-GTAGTTTACCTGACCATCCCCAT-3′; for GAPDH, fw: 5′-CAAGGTCATCCATGACAACTTTG-3′; rev: 5′-GTCCACCACCCTGTTGCTGTAG-3′; for L1CAM-F2: 5′-ATGGTCATGATGCTGTGGTACGT-3′; for -R2: 5′-GGAACTCCACTTGGGGTCTGC-3′; for L1CAM-F27: 5′-AGGAGGACACTCAGGTAGATTCCG-3′; for -R27: 5′-GGTTTGATGTCTCCATTGAGAGATG-3′; for L1CAM-F2.1, 5′-CCTGATGAATATAAAGGACACC-3′; for L1CAM-F27.1: 5′-CGAGTACAGGTCCCTGGAGAGTG-3′; for L1CAM-Ex2, fw: 5′-CCCACCCCCGTCAGCAGGTA-3′; rev: 5′-ACCCTCAGCCACGAGCTGGA-3′; for L1CAM-Ex27, fw: 5′-CCGAGGGCTGGTTCATCGCC-3′; rev: 5′-CAGGTGTCTTGGCCCCTGGC-3′; for REST, fw: 5′-CTACATGGCACACCTGAAGCACCAC-3′; rev: 5′-GCGTAGTCACACACGGGGCAGTTGAAC-3′; for NFI-A, fw: 5′-AGCCCACATGCGACGCCATC-3′; rev: 5′-GGCATGGGCAGAGGCACAGG-3′; for Nova2, fw: 5′-CTCAATCATCGGCAAAGGTG-3′; rev: 5′-GGCTCTGTCGGGGTTCATC-3′. In human cells, primers used were: for hL1CAM-hF2: 5′-GGTCGTGGCGCTGCGGTACGTGTG-3′; -hR2: 5′-GCGGAACTGCACTTCGGGCTTGCC-3′; for hL1CAM-hF27: 5′-ACTCAGTGAAGGATAAGGAG-3′; -hR27: 5′-TTGAGCGATGGCTGGCTGCT-3′; hREST, fw: 5′-GAGGAGGAGGGCTGTTTACC-3′; rev: 5′-TCACAGCAGCTGCCATTTAC-3′; hNova2, fw: 5′-CATTGGCAAGGGCGGGCAGA-3′; rev: 5′-CACGCGCTCCTGCAGGTTGA-3′. SLUG expression was analyzed by a Taqman assay performed by the RT-PCR and DNA sequencing service of IFOM-IEO, Milan (assay IDs: Rn00709370_m1 and Hs00161904_m1).

Western blotting

Cell extracts in TD buffer containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF and protease inhibitors, were rocked (15 min) and then centrifuged (13 000 *g*, 4°C, 15 min). Protein samples, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters, were processed as in Cocucci *et al.* (2004). Photographic development was by chemiluminescence using ECL Western Blotting Detection reagent (Amersham Bioscience, Little Chalfont, UK) or Femto Signal (Pierce, Rockford, IL, USA). Signals were acquired by the Densitometer SI and Image Quant (Amersham Bioscience).

Immunofluorescence

For surface immunolabeling of L1CAM, PC12 and NT2/D1 cells on coverslips were fixed with 95% ethanol at −2°C (10 min) or 4% formaldehyde at 4°C (10 min), then washed and immunolabeled for 1 h with anti-L1CAM goat pAbs against the extracellular or the intracellular domains. The bound Ab was revealed by tetramethylrhodamine isothiocyanate-conjugated rabbit anti-goat pAb. Nuclei were stained with DAPI. Intracellular immunolabeling was revealed similarly, however upon Triton X100 permeabilization of the fixed cells. The surface appearance of phosphatidylserine was checked by labeling formaldehyde-fixed, non-permeabilized cells with FITC-annexin V (0.5 μg/mL, 15 min, 25 °C, in the presence of divalent cations). Samples were studied in a Perkin-Elmer Ultraview ERS confocal microscope. Image deconvolution was carried out in a wide field microscope of the Delta Vision system.

Chromatin immunoprecipitation

Chromatin of PC12–27 cells was extracted and immunoprecipitated using the EZ-ChIP-A kit, using the anti-REST pAb and control rabbit IgGs from Millipore. To reveal the REST binding sites, the rat *Nova2* gene nucleotide sequence was analyzed by the Genomatix Genome Analyzer. Immunoprecipitation of REST-associated DNA fragments was verified by RT-PCR using the following primers: for intron 2–3, fw: 5′-CCTGAGCAAGGGTTGTCCGGGA-3′; rev: 5′-

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CCACCTAGCCTGTCCCCATGCA-3′; for intron 4–5, fw: 5′-AGGAACTAGTAAGCTGTCATGGCG-3′; rev: 5′-TCCTTTAAG GGTCGGTCAGCAGGC-3′. ChIP-enriched DNAs were quantified by the qPCR-SYBR green method, using the ABI 7900HT Sequence Detection System (Applied Biosystem). ChIP samples were analyzed in triplicate by PCR. Relative enrichment = the ratio between the gene in the immunoprecipitates with specific and control Abs.

Statistical analyses

The significance of the data was assessed using the two-tailed unpaired *t*-test. Data shown are means ±SD. Statistical significance is indicated by asterisks: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

REST governs not only the expression but also the splicing of L1CAM

Expression of L1CAM was investigated in the wtPC12 and PC12–27 clones characterized by low and ~50-fold higher levels of REST mRNA and protein (Fig. 1; D'Alessandro *et al.* 2008). The mRNA levels of L1CAM were the opposite of those of REST, over 20-fold higher in the wtPC12 than in the PC12-27 clone (Fig. 1a and b). The differences of the L1CAM protein levels were analogous (over 15-fold higher in wtPC12, Fig. 1c). Immunofluorescence revealed a preferential distribution of L1CAM to the surface, accompanied by some labeling of cytoplasmic puncta evident in wtPC12 (Fig. 1d). The opposite levels of the L1CAM and REST mRNA and protein were confirmed also in other cells: the high-REST human neuroblastoma SH-SY5Y line (Racchetti *et al.* 2010) and a subclone of wtPC12 transfected with a full-length REST construct (Myc-REST: total level of REST ~4-fold higher than in wtPC12 cells, much lower than in PC12–27 cells, D'Alessandro *et al.* 2008) (Fig. 1a–c). In the high-REST PC12–27 and SH-SY5Y, the L1CAM mRNA and protein were increased when the REST tone was attenuated by TSA, a blocker of histone deacetylases, the main effectors of REST gene repression (D'Alessandro *et al.* 2008), employed at a concentration that fails to induce any surface increase of FITC-annexin V labeling, a marker of apoptosis (not shown; see D'Alessandro *et al.* 2008). In contrast, in the low-REST wtPC12 cells TSA induced no significant change (Figure S1A,B).

In addition to the difference in expression, the L1CAM protein of wtPC12 and PC12–27 clones differed also in apparent Mr (Fig. 1c). In western blots carried out in parallel with two pAbs and one mAb, over 90% of the wtPC12 protein migrated in the ~220 kDa band, close to a minor, faster migrating band which was the only band appreciable in PC12–27 and SH-SY5Y cells (Fig. 1c). In terms of mRNA, a duality of L1CAM was due to the alternatively spliced fl-L1

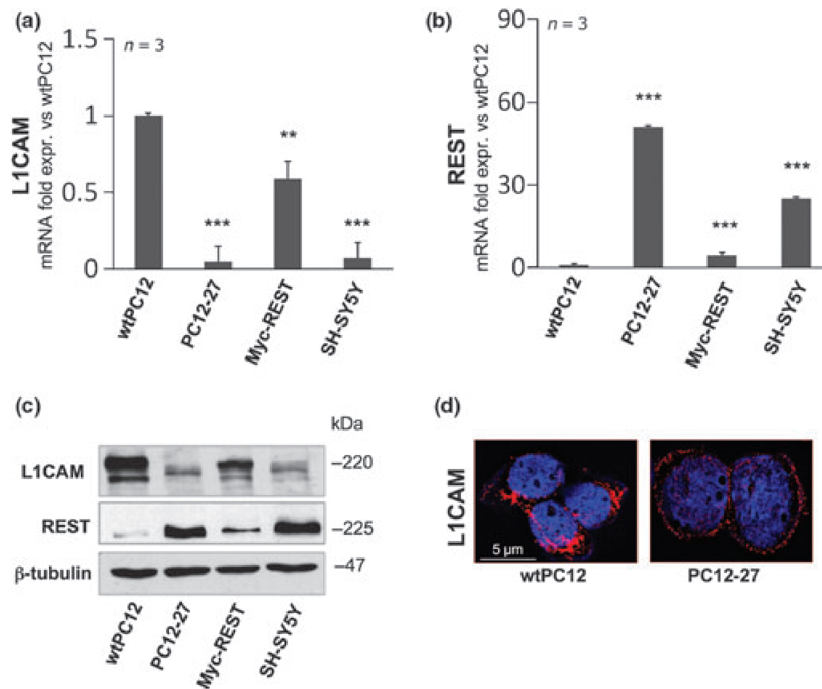

Fig. 1 Expression of L1CAM and REST in PC12 clones and SH-SY5Y cells. (a, b) mRNA levels of L1CAM and REST, respectively, in wtPC12, wtPC12 stably transfected with REST (Myc-REST), the PC12–27 clone and SH-SY5Y cells. In this and in the following figures, statistical significance is indicated by asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (c) Representative western blot of L1CAM and REST proteins in the same cells of panels (a) and (b); β-tubulin is a marker of loading. (d) Distribution of L1CAM (red) in permeabilized wtPC12 and PC12–27 cells. Nuclei are labelled by DAPI. The bar on the left, valid also for the right, is of 5 μm.

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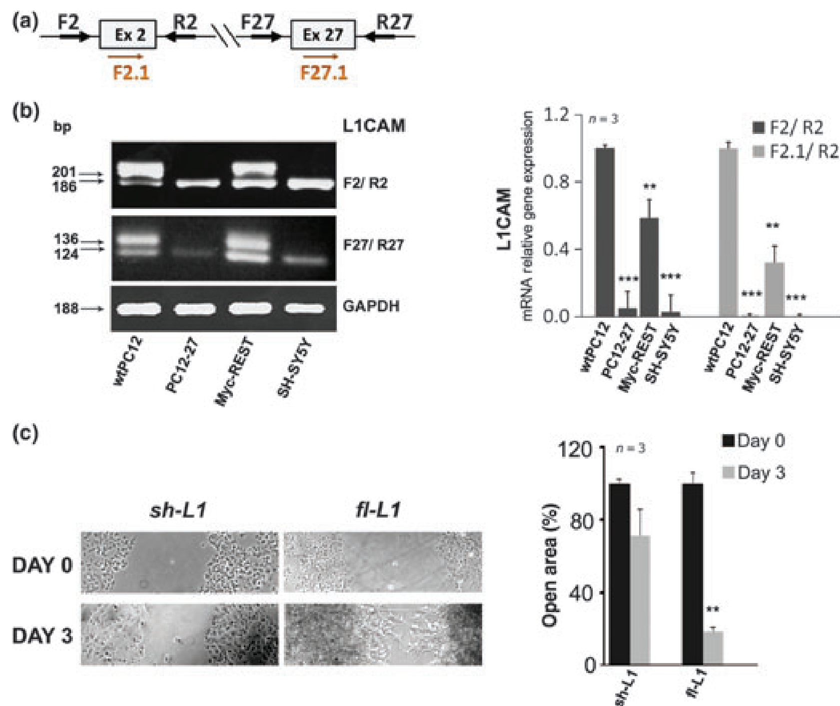

Fig. 2 Alternatively spliced isoforms of L1CAM, fl-L1 and sh-L1, in PC12 clones and SH-SY5Y cells; migration of PC12–27 cells transfected with fl-L1 and sh-L1. (a) The primers employed to investigate the expression of L1CAM exons 2 and 27. F2/R2 and F27/R27 include the flanking regions; F2.1 and F27.1 are strictly focused on the spliced exons. (b) Left: two spliced isoforms of L1CAM mRNA amplified with the F2/R2 and F27/R27 primers. Numbers indicate the size (bp) of each amplified band. In wt PC12, the higher band is prominent, accompanied by a minor lower band free of the 2 and 27 exons; in PC12–27 and SH-SY5Y cells only the lower band appears. Myc-REST is intermediate; right: L1CAM mRNAs (averages ±SD) amplified with the F2/R2 and the F2.1/R2 primers, expressed relative to the wtPC12 values. The PC12–27 and SH-SY5Y cell values, already low with F2/R2, reach 0 with F2.1/R2, confirming their lack of exon 2. (c) Scratch assay testing the migration of PC12–27 cells transfected with either the fl-L1 or the sh-L1 isoform; left: phase contrast images of the transfected cells before and after 3 days of migration; right: quantized data (averages ±SD). Statistical significance indicated by asterisks as in the Fig. 1a/b legend.

and sh-L1 isoforms, as revealed by RT-PCR results obtained with the primers F2/R2 and F27/R27 (see Fig. 2a). The differences between wtPC12 and Myc-REST versus PC12–27 and SH-SY5Y (Fig. 2b) were localized in areas close to the 5′ and 3′ ends including, respectively, the 2 and 27 exons. A more refined analysis, focused on the 15 bases of exon 2 and the 12 bases of exon 27 (F2.1 and F27.1, Fig. 2a) demonstrated their presence in the mRNAs of wtPC12 and Myc-REST, and their lack in those of PC12–27 and SH-SY5Y (Fig. 2b, right), as expected for the fl-L1 and sh-L1 isoforms, respectively. This identification was confirmed by the sequencing of the two L1CAM mRNA transcripts in wtPC12 and PC12–27 cells (not shown).

fl-L1 and sh-L1 isoforms affect differently PC12 cell migration

To extend the characterization of the two spliced L1CAM isoforms we carried out a functional test, taking advantage of the very low expression of the endogenous protein in PC12–27 cells (see Fig. 1a and c). The analysis of FACS-sorted, transiently transfected populations of PC12–27 could therefore be employed to investigate the separate effect of either isoform on migration. Figure 2c shows the results of a scratch assay. Compared with control PC12–27 cells, transfected with GFP only (not shown), the cells transfected with sh-L1 did not exhibit any increased migration. In contrast, the cells transfected with fl-L1 migrated distinctly faster, approaching the reestablishment of the contact across the scratch within 3 days, when the sh-L1-transfected cells were still far away. These results confirm the profound functional difference between the two L1CAM isoforms previously anticipated by others based on different experimental approaches (see Schäfer and Altevogt 2010; Hauser *et al.* 2011).

Role of NFI-A, SLUG and β-catenin in L1CAM expression/splicing in PC12

Three transcription factors, the repressor NFI-A and the stimulators SLUG and β-catenin, had been reported to play roles in the expression of the *L1CAM* gene in various tumours (Gavert *et al.* 2005; Schneegans *et al.* 2009; Pfeiffer *et al.* 2010; Schäfer and Altevogt 2010). Working with wtPC12, PC12–27 and Myc-REST clones we found the levels of the NFI-A mRNA and protein to vary analogously,

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but less extensively, to those of REST (compare Figure S2A to Fig. 1b, c). Interestingly, these NFI-A levels were not changed significantly by cell treatment with TSA (not shown), excluding the expression of NFI-A to be directly controlled by REST. Concerning SLUG, traces of its expression by RT-PCR were found neither in the rat wtPC12 and PC12–27 clones nor in the human SH-SY5Y cells (not shown).

To investigate the role of β-catenin we employed Δ90-βcat, encoding a truncated, slow-turnover protein active in the transcription of the β-catenin/TCF target genes (Chenn and Walsh 2002). No significant increase of L1CAM mRNA was observed in wtPC12 cells transfected with the construct (not shown). However in these cells the sh-L1 mRNA, with skipped exons 2 and 27, was increased to levels over 50% of the fl-L1 isoform (Figure S2C,D). This was most likely an indirect consequence of the increased transcription of the *REST* gene induced by the higher β-catenin tone (see Tomasoni *et al.* 2011).

Splicing of L1CAM in PC12 cells: involvement of the Nova2 factor

Alternative splicing of L1CAM in our low- and high-REST PC12 cells could be due to a splicing factor dependent on REST for its expression. We wondered whether any recognized splicing factor gene had been reported to include in its promoter the specific REST binding sequence, RE-1. Large-scale chromatin immunoprecipitation (ChIP-Seq) studies (Johnson *et al.* 2007) had predicted the sequence in the promoter of the gene encoding Nova2, a neural cell-specific factor known to govern the splicing of various channels and synaptic proteins (Ule *et al.* 2005; Yano *et al.* 2010; Allen *et al.* 2010). So far, however, the role of Nova2 had not been validated.

In wtPC12 and PC12–27 clones, the levels of Nova2 mRNA and protein were found to differ opposite to those of REST (compare Fig. 3a and b to Fig. 1b and c). This confirms the possibility of a negative control of Nova2 expression by the repressor. Moreover, treatment of PC12–27 with TSA induced a 15-fold increase of the Nova2 mRNA (Fig. 3c, left), similar to the increase in L1CAM mRNA revealed by the amplification with F2.1/R2 and F.27/R27. In contrast, in wtPC12 the TSA treatment was ineffective (Fig. 3c). To investigate whether the differential expression of the *Nova2* gene was due to the direct binding of REST or to an indirect mechanism, we carried out experiments of chromatin immunoprecipitation (ChIP). Fig. 3d shows that the *Nova2* gene of PC12–27 cells immunoprecipitated by the anti-REST Ab was enriched 3.5- to 4.5-fold with respect to a control Ab. This result confirms that expression of the *Nova2* gene is repressed by the direct binding of REST.

The role of Nova2 in wtPC12 was investigated also upon knock-down of the splicing factor to ~1/4th level of the control, induced by transient transfection of a specific shRNA plasmid (Fig. 3e). Analysis of the F2/R2 and F27/R27 mRNA fragments failed to reveal significant differences in L1CAM expression between knocked-down and control cells even if the ratio between upper and lower band was markedly decreased (Fig. 3f). However, the analysis of the two spliced mini-exons (F2.1/R2 and F27.1/R27 in Fig. 3a) revealed large (−70/75%) and significant decreases of their expression (Fig. 3f right). The results strongly suggest the two L1CAM isoforms, fl-L1 and sh-L1, to depend on the differential expression of the REST-dependent Nova2 splicing factor. Specifically, Nova2 promotes inclusion of the two mini-exons, 2 and 27, in the mRNA of L1CAM.

REST governs Nova2 expression and L1CAM splicing also in NT2/D1 cells and in primary neurons

These experiments were carried out in two additional cell models transduced with REST-encoding or scrambled retroviral vectors: human neuron-like cells differentiated from NT2/D1 cells by a 4 week treatment with RA and primary cultures of rat cortical neurons. The results of Figs 4 and 5 recapitulate in the two models the results obtained in the low- and high-REST PC12 clones. Untreated NT2/D1 cells, which express high REST levels (Fig. 4b; Tomasoni *et al.* 2011), exhibited very low and inappreciable levels of L1CAM mRNA and protein, respectively (Fig. 4a and b). During the RA treatment NT2/D1 cells, while developing a neural-type phenotype (Andrews 1984), exhibited a progressively decreasing REST expression, reaching very low levels in the fully differentiated, neuron-like cells (NT2-N, Fig. 4b). Concomitantly, expression of L1CAM increased (Fig. 4a and b), with distribution of the protein to the cell body and neurites (Fig. 4c). The Nova2 protein became clearly appreciable only in the NT2-N cells (Fig. 4b). Transduction of the low-REST NT2-N cells with the REST lentiviral vector, with ensuing large increase of the REST protein level (Fig. 4e), was followed by the drop of both L1CAM and Nova2 mRNA and protein, reaching levels 25% or lower of controls, and by the conversion of the predominant fl-L1 mRNA and protein into the sh-L1 isoforms (Fig. 4d and e).

In the primary cultures of neurons, which express very low levels of REST (Fig. 5a and c), Nova2 and L1CAM were high both at the mRNA and the protein level (Fig. 5b and c). Upon transduction with REST the levels of the transcription factor increased considerably (over 35- and 7-fold at the mRNA and protein level respectively, Fig. 5a and c). Concomitantly, Nova2 and L1CAM were significantly decreased (−70% or more) and L1CAM was switched from the fl-L1 to sh-L1 isoform (Fig. 5b and c), with disappearance of the mini-exons 2 and 27 (Fig. 5b).

In conclusion, both the differentiated, neuron-like NT2-N cells and the primary neurons, when transduced or not with REST, resembled PC12–27 and wtPC12 cells, respectively (compare Figs 4 and 5 to Figs 1 and 2). The REST-dependent, Nova2-mediated mechanism governing the gen-

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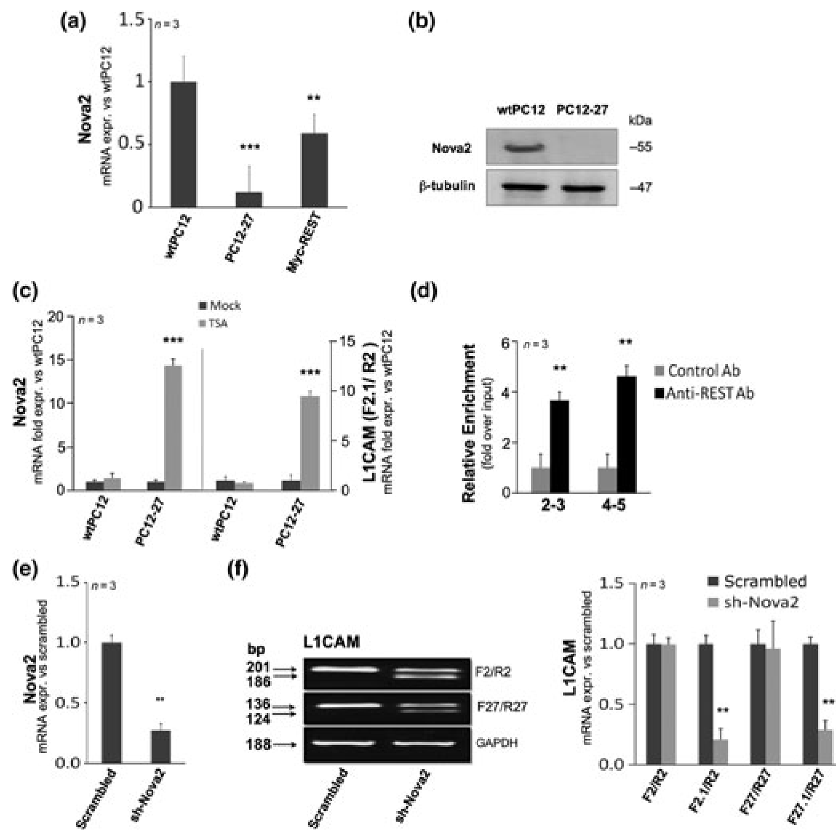

Fig. 3 Splicing of L1CAM is effected by Nova2 expressed under the control of REST. (a) Nova2 mRNA levels in the various PC12 clones (expressed relative to the wtPC12); (b) representative western blot of Nova2 in wtPC12 (distinct band) and PC12–27 (inappreciable). β-Tubulin was a loading normalizer. (c) Changes of Nova2 (left) and L1CAM (with F2.1/R2 primers, right) mRNA in wtPC12 and PC12–27 cells pretreated with TSA (1.5 μM, 18 h). Data expressed relative to the corresponding mock-treated clones. (d) ChIP enrichment of the PC12–27 chromatin fragments corresponding to the Nova2 introns 2–3 and 4–5, immunoprecipitated by the anti-REST pAb, with reference to control rabbit IgGs. (e) Decrease of Nova2 induced in wtPC12 cells by the specific shRNA, referred to the scrambled shRNA. (f) Left image: effects of the shRNAs on the L1CAM mRNA fragments of wtPC12 amplified by the F2/R2 and F27/R27 primers, with marked increase of the lower band induced by the specific Nova2 shRNA. Invariant GAPDH as loading normalizer. Right image: no quantitative difference appreciable between total levels of the L1CAM mRNA fragments amplified by the F2/R2 and F27/R27 primers upon transfection of the Nova2-specific and scrambled shRNAs. However, the values amplified by the fragments focused on exons, F2.1/R2 and F27.1/R27, are greatly (–75%) reduced. Data shown are referred to scrambled shRNA results. Panels (a) and (c–f) values are averages ±SD. Statistical significance shown by asterisks as in Fig. 1a/b legend.

eration of the two L1CAM isoforms, identified in the PC12 clones, appears therefore of wider, possibly general importance in neural cells.

Discussion

Expression of the *L1CAM* gene in two alternatively spliced isoforms, fl-L1 (neural) and sh-L1 (non-neural), had been described two decades ago (Reid and Hemperly 1992). So far, however, the mechanism of the two isoform generation had never been investigated. Our work, aimed at identifying this mechanism, was carried out first in clones of the rat pheochromocytoma PC12 line differing in the levels of REST, the master transcription factor of their differentiation. REST is low in wtPC12, as in the other neural cells, and 50- to 60-fold higher in PC12–27 cells (D'Alessandro *et al.* 2008).

In addition, the two PC12 clones exhibited differences also in L1CAM, abundant and prevalent of the fl-L1 isoform in wtPC12, scarce and represented only by sh-L1 in PC12–27 (and in the other high-REST line, SH-SY5Y). These properties were attenuated when the low-REST level of wtPC12 was increased, however of only 4-fold, by transfection of a full length construct, and when the high-REST tone of PC12–27 was weakened by TSA. Likewise, when NT2/D1 cells, differentiated to a neuronal-like phenotype by long-term treatment with RA, and primary cultures of rat neurons were transduced with REST, they decreased their L1CAM expression and switched large part of their L1CAM from the fl-L1 to the sh-L1 isoform. In the investigated neural cells,

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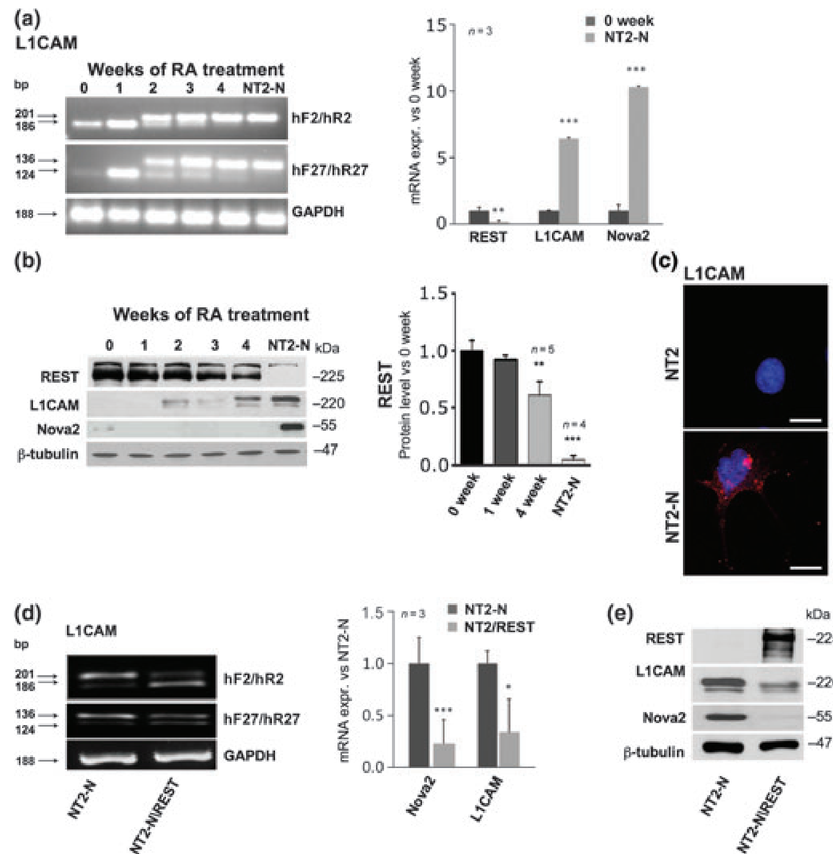

Fig. 4 Human NT2/D1 cells: expression/splicing of L1CAM and expression of Nova2 are governed by REST. (a) Left image: RA (10 μM)-induced differentiation of NT2/D1 cells in terms of L1CAM mRNA isoform expression. The F2/R2 and F27/R27-amplified RT-PCR products, low in untreated cells, appear as dual bands after 2 weeks, shifted predominantly to the upper, exon 2- and exon 27-containing bands after 3 and 4 weeks, becoming unique in the purified NT2-N. The invariant transcript of GAPDH as loading normalizer. (a) Right image: quantized RT-PCR data of L1CAM mRNA in untreated NT2/D1 and NT2-N, together with the corresponding data of REST and Nova2. Data referred to untreated cells. (b) Progressive decrease of the REST protein (quantitative data on the right) and increase of the L1CAM protein during RA treatment. Nova2 abundant only in the purified NT2-N cells. (c) Whole cell immunofluorescence of L1CAM, negative in untreated NT2/D1 cells and prominent in NT2-N, distributed both in the cell body and in the processes. (d) L1CAM mRNA isoform amplified by the F2/R2 and F27/R27 RT-PCR in NT2-N cells transduced with REST. The invariant transcript of GAPDH as loading normalizer. The marked decrease of the L1-CAM higher band, with partial switch to the lower bands (d, left and right images) was accompanied by a parallel, large decrease of Nova2 (d, right image, referred to NT2-N cells transduced with GFP only). (e) High REST in the transduced cells accompanied by marked decreases of the L1CAM (predominant sh-L1 isoform) and Nova2 proteins. The quantized data of panels a, b and d are averages ±SD. Statistical significance by asterisks as in Fig. 1a/b legend.

therefore, not only expression, but also splicing of L1CAM depends on REST. The different expression of the two L1CAM isoforms is important also functionally, with faster migration of PC12–27 cells transfected with fl-L1 with respect to those transfected with sh-L1, demonstrated by a scratch assay.

Additional factors previously reported to play a role in the transcription of the *L1CAM* gene do not seem as relevant as REST in neural cells. The repressor NFI-A appears to operate in parallel to, and possibly synergistically with REST, as previously hypothesized (Schneegans *et al.* 2009). β-Catenin, known as the stimulator of *L1CAM* gene transcription in various non-neural cancer cell types (Gavert *et al.* 2005; Pfeiffer *et al.* 2010), was investigated by transfection of its truncated construct, Δ90-βcat, known to stimulate transcription of target genes upon accumulation in the nucleus (Chenn and Walsh 2002). In spite of these properties, Δ90-βcat induced no significant change of the total L1CAM. A decrease of the fl-L1/sh-L1 ratio, observed in the Δ90-βcat-transfected cells, was most likely sustained by an increased expression of the REST gene (Nishihara *et al.* 2003) with ensuing 3- to 4-fold increase of the REST levels (Tomasoni *et al.* 2011). These results suggest that, in a low-REST neural cell such as wtPC12, the stimulatory role of β-catenin on L1CAM transcription is marginal, if any.

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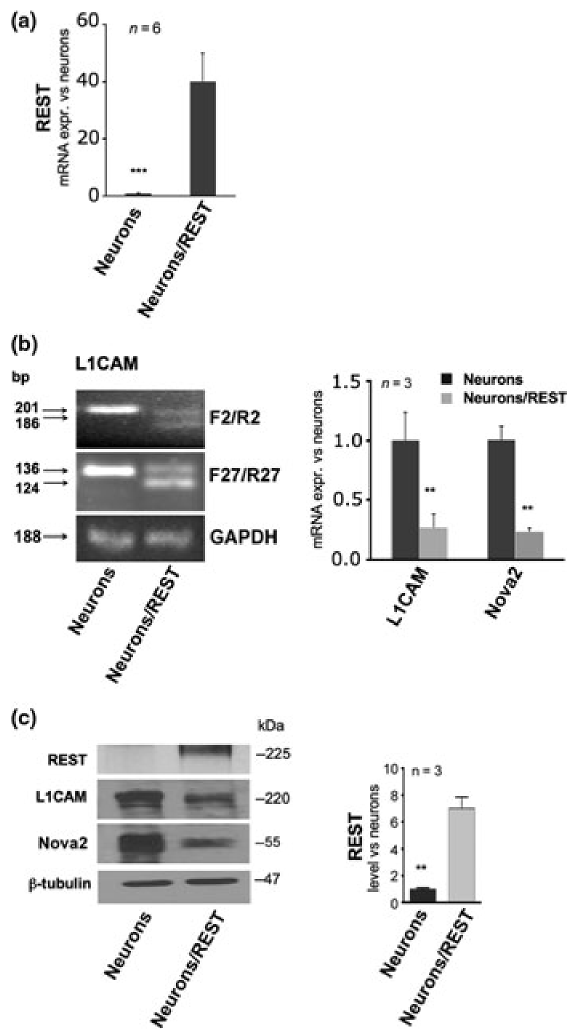

Fig. 5 Expression/splicing of L1CAM and expression of Nova2 are governed by REST also in primary neurons. (a) REST mRNA, very low in neurons, almost 40-fold higher after transduction of the transcription factor. (b) Increase of REST accompanied by marked decreases of L1CAM mRNA (amplified by F2/R2 and F27/R27), with partial switch from the higher to the lower bands, and large decrease of the Nova2 mRNA. Data refer to values in controls transduced with GFP only. (c) Increase of the REST protein in the transduced neurons, about 7-fold of the non-transduced cells (left and right images), accompanied by decrease of the Nova2 and L1CAM proteins, with partial switch of the latter from fl-L1 to sh-L1 isoform (left image). β-Tubulin as a loading normalizer. Statistical significance by asterisks as in Fig. 1a/b legend.

The most important result of this study was however the identification of Nova2, dependent on REST for its expression, as the splicing factor that governs the generation of the fl-L1 and sh-L1 isoforms of L1CAM. Together with Nova1, Nova2 was the first discovered mammalian splicing factor specific of neural cells. Its key relevance the brain was documented by a role in ~7% of the specific splicing of the neocortex, with preference for mRNAs encoding proteins active at synapses, including 7 adhesion proteins (Ule *et al.*

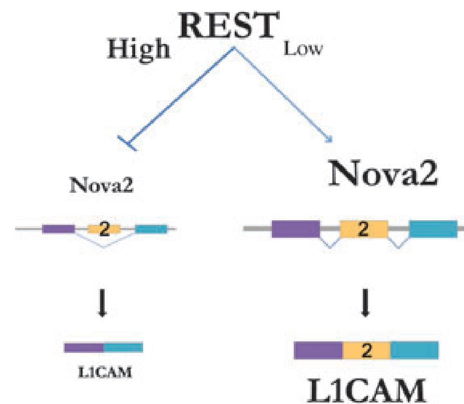

Fig. 6 REST control of L1CAM splicing via its repression of Nova2 transcription. In the cells with high REST (left) transcription repression of the REST target genes is high, therefore expression of Nova2 is low. Alternative splicing of L1CAM does not occur, exon2 (as well as exon 27, not shown) is skipped, expression of the adhesion protein is low, the sh-L1 isoform predominates. In contrast, when REST is low (right) Nova2 is high, splicing of L1CAM mRNA takes place with insertion of introns 2 and 27, the final protein is abundant and predominantly of the fl-L1 isoform.

2005). L1CAM, however, had not been identified as a Nova2 target. The REST dependence of Nova2 expression, envisaged by Johnson *et al.* (2007), was demonstrated here by both the ChIP of the *Nova2* gene by anti-REST Abs and the large switch of the fl-L1 to the sh-L1 isoform, observed in the low-REST wtPC12 cells knocked-down of Nova2. These results, which explain why the inclusion in the L1CAM mRNA of exons 2 and 27 by alternative splicing takes place in the low-REST cells, where Nova2 is expressed, and not in the high-REST cells, where Nova2 is low to inappreciable, were duplicated in the neuron-like NT2-N cells and in primary neuronal cultures of the rat brain cortex. The REST-dependence of the L1CAM alternative splicing appears therefore as a property of many, possibly all types of neural cells. A model illustrating the REST control of Nova2 expression and the ensuing consequences on L1CAM splicing is shown in Fig. 6.

In conclusion, our studies of neural cells: PC12, SH-SY5Y, NT2/D1 differentiated by RA and primary neurons, have shown that not only the expression but also the alternative splicing of L1CAM are governed by REST acting in two ways: directly by repressing the transcription of the gene, and indirectly by repressing the transcription of the *Nova2* gene. The first mechanism was known since long (Kallunki *et al.* 1997), the second is new, and its relevance appears already considerable. In fact, numerous important functions of L1CAM, including migration of neurons, outgrowth/fasciculation/regeneration of axons, and synaptic plasticity, depend specifically on fl-L1, the isoform spliced by Nova2. In addition, splicing by Nova2 extends to many other targets playing critical roles in brain-specific processes such as synapse assembly and axon guidance (Ule *et al.* 2005; Allen

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et al. 2010; Yano *et al.* 2010). The REST-dependence of the neural-specific alternative splicing, demonstrated here for Nova2 and by others for nSR100 in a paper (Raj *et al.* 2011) appeared during the revision of this paper, expand the role of the transcription factor to a new area. The gene targets affected by REST via this new way could be identified not by the occurrence in their promoter of RE-1, the classical REST-binding sequence, but by binding sites for REST-dependent splicing factors at critical sites within exons and/or introns of their encoded mRNA precursors. Changes of REST levels occur in neural cell during development (Ballas and Mandel 2005; Ooi and Wood 2007) and also upon maturation, when the transcription factor can moderately increase and fluctuate (Garriga-Canut *et al.* 2006; Spencer *et al.* 2006). The REST-dependent alternative splicing can therefore contribute to the array of effects governed by the transcription factor during the whole life of neural cells.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Effect of histone deacetylase inhibitor TSA on the expression of LICAM in PC12 clones and SH-SY5Y cells.

Figure S2. Expression of NFI-A and effects of truncated β-catenin construct on the expression of the two LICAM isoforms in PC12 clones.

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