An intronic ncRNA-dependent regulation of SORL1 expression affecting Aβ formation is upregulated in post-mortem Alzheimer’s disease brain samples

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About Author contributions: Sara Massone and Eleonora Ciarlo, performed the most of the experiments. Ilaria Penna and Arianna Gigoni performed RT-PCR analysis. Mario Nizzari performed the fluorescence microscopy analysis. Claudio Russo performed the analysis of APP C-terminal fragments. Tullio Florio, Giogio Dieci and Ranieri Cancedda participated to the interpretation of results and critically read the manuscript. Aldo Pagano leaded the project, participated to the experiments and wrote the paper.
SUMMARY

Recent studies indicated sortilin-related receptor 1 (SORL1) to be a risk-gene for late-onset Alzheimer’s Disease (AD), although its role in the aetiology and/or progression of this disorder is not fully understood. Here, we report the finding of a novel non-coding (nc) RNA (hereafter referred to as 51A) that maps in antisense (AS) configuration in intron 1 of SORL1 gene. 51A expression drives a splicing shift of SORL1 from the synthesis of the canonical long protein variant 1 to an alternatively spliced protein form. This process, resulting in a decreased synthesis of SORL1 variant 1, is associated with an impaired processing of APP, leading to increase of Aβ formation. Interestingly, we found that 51A is expressed in human brains, being frequently up-regulated in cerebral cortices from Alzheimer’s disease patients. Altogether these findings document a novel ncRNA-dependent regulatory pathway that might have relevant implications in neurodegeneration.
INTRODUCTION

Sorting protein-related receptor 1 (SORL1), also known as LR11, is a 250-kDa type-1 membrane protein of unknown function that is expressed in neurons of the central and peripheral nervous system (Jacobsen et al., 1996; Yamazaki et al., 1996; Hermans-Borgmeyer et al., 1998; Motoi et al., 1999). Although its function is still poorly understood, SORL1 is a member of a family of neuronal receptors that share structural similarity with the vacuolar protein sorting 10 protein (Vps10p), a sorting-specific yeast polypeptide that transports carboxypeptidase Y from the Golgi to vacuoles (Marcusson et al., 1994). Interestingly, besides its potential role in certain cancer pathways (Akil et al., 2011; Demont et al., 2012), SORL1 expression is reduced in brain tissues from Alzheimer’s Disease (AD) patients, suggesting a potential role in AD pathogenesis (Rogaeva et al., 2007; Reitz et al., 2011). The link between SORL1 and AD has been further strengthened by the recent demonstration that reduction of SORL1 expression promotes an increase of Aβ formation by a mechanism that has been only partially elucidated (Andersen et al., 2005; Small et al., 2005; Offe et al., 2006; Wang et al., 2007). Indeed, the initial processing of APP by α- and β-secretases is intimately associated with post-Golgi compartments and requires efficient transition of the precursor through these organelles (Haass et al., 1993; Yamazaki et al., 1995). In this context SORL1 interacts with APP and affects its trafficking and proteolytic processing in the brain, acting as a sorting receptor for APP holoprotein. On the contrary, the absence or downregulation of SORL1 expression shifts APP holoprotein from the retromer recycling pathway to the β-secretase cleavage pathway, increasing sAPPβ production and, subsequently, Aβ formation (Peraus et al., 1997; Khvotchev and Sudhof, 2004).

The link between SORL1 and AD was also supported by the identification of AD-associated allelic variants in distinct regions of SORL1 gene, in different populations. These results also suggested that these variants might map in still unknown intronic regulatory regions that might govern cell type- or tissue-specific expression of SORL1 (Bruni et al., 2007; Hinerfeld et al., 2007;
Klein et al., 2007; Lee et al., 2007; Matsui et al., 2007; Rogaeva et al., 2007; Shibata et al., 2007; Lee et al., 2008; Xiao et al., 2008; Massone et al., 2012). Thus, expression of these variants might affect AD risk altering the physiological role of SORL1 in the processing of APP holoprotein (Schmidt et al., 2012).

In recent works, we have documented pivotal roles of pol III-transcribed ncRNAs in gene expression regulation and, in particular, in the regulation of alternative splicing (Dieci et al., 2007; Pagano et al., 2007; Castelnuovo et al., 2010; Massone et al., 2011a, b; Vella et al., 2012). A still uncharacterized transcription unit of our collection (hereafter referred to as 51A) maps in intron 1 of SORL1 gene (a genomic portion subjected to alternative splicing events) in antisense configuration. Thus, we hypothesized a possible control of SORL1 pre-mRNA maturation mediated by 51A ncRNA expression. According to this working hypothesis, the synthesis of this ncRNA and its possible RNA:RNA pairing with SORL1 pre-mRNA would mask canonical splicing sites leading to alternative splicing events. By addressing such hypothesis, in this work we demonstrate that: i) 51A is a novel ncRNA whose synthesis promotes the expression of SORL1 alternatively spliced protein variants to the detriment of the canonical SORL1 splice variant A; ii) this event triggers an altered processing of APP that follows to its impaired internalization; iii) this process ultimately leads to an increased amyloid secretion; and iv) 51A is up-regulated in post-mortem cerebral cortices from AD patients.

METHODS

Genomic and cDNA clones

The genomic clone herein analyzed was generated following molecular biology procedures previously reported (Pagano et al., 2003). The oligos used to generate the insert were 5’-ATGCATTAATTTAAGAGCAAGGACCTTGAT-3’ and 5’-
ATGCATTAATTAGTGTATCATCAGTGGCA-3’ and span a region containing the PSE/TATA pol III type 3 promoter together with the transcribed portion of 51A.

**Human brain samples**

Frontal and temporal cortices from AD [clinical history of disease; pathological diagnosis according to the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) criteria] and control cases (AD excluded by clinical neurological assessment and immunohistochemical analysis), as described in Table 1, derive from two different sources: the Brain Bank at Case Western Reserve University, Cleveland, OH, and the Joseph and Kathleen Bryan Alzheimer's Disease Research Center (Bryan ADRC) at Duke University Medical Center, Durham, NC. Twenty-three controls and 23 AD samples were analyzed. Average age was 71 years for controls and 78 for AD brains.

**Cell culture, transfection and luciferase assay.**

Different cells lines were used: HEK-293, NCTC-2544, HepG2 and HeLa cells were maintained on Dulbecco’s modified Eagles medium (DMEM ECB7501L, EuroClone, Milan, Italy), 10% FBS (LONZA DE14-801F), L-Glutamine (2 mM; EuroClone, Milan, Italy), and penicillin–streptomycin (100 U/ml/ 100 ug/ml; EuroClone, Milan, Italy); Neuroblastoma cell lines (SKNBE2 and SHSY5Y) were maintained on RPMI 1640 medium (ECB9006L EuroClone, Milan, Italy), 10% FBS (LONZA DE14-801F), L-glutamine (2 mM; EuroClone, Milan, Italy), penicillin–streptomycin (100 U/ml/ 100 ug/ml; EuroClone, Milan, Italy) (standard medium); LoVo cells were maintained on F-12K medium (LONZA BE12-615F), 10% FBS (LONZA DE14-801F), L-glutamine (2 mM; EuroClone, Milan, Italy), penicillin–streptomycin (100 U/ml/ 100 ug/ml; EuroClone, Milan, Italy). SKNBE cells were transfected using Polyethylenimine (PEI) (P3143 SIGMA, St. Louis, MO, USA) (1µg DNA:2,5µl PEI 10mM) with pEGFP-N1 as control (referred in the text to as Mock) or pEGFP-N1-51A (referred to as 51A). G418 (Geneticin) was used in culture medium as mean of selection up to 1000µg/mL, until resistant clones were identified. After selection the clones were
preserved in 200µg/mL G-418 in standard culture conditions. Luciferase-based promoter activity assay was performed 48 hours after transfection by firefly luciferase activity determination with the dual-luciferase reporter-assay system (Promega) according to the manufacturer’s protocol.

**Real Time Quantitative RT PCR analysis.**

Total RNAs from samples were extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol and subjected to reverse transcription by Omniscript RT Kit (Qiagen, Valencia, CA, USA) as previously described elsewhere (Pagano et al., 2004). The total RNA from samples was measured by real-time quantitative RT-PCR using PE ABI PRISM® 7700 Sequence Detection System (Perkin Elmer, Wellesley, MA, USA) and Sybr Green method following manufacturer's instructions. The sequences of 51A forward and reverse primers were 5'-TGGGAGAGTCAGCATCTTGAAAG-3' and 5'-TGTACAGTCAGACAAGAGGTGTGTAT-3'. The sequences of SORL1 (Var A) forward and reverse primers were 5'-AGCCCGAGCCCATCAAG-3' and 5'-AATCAGATGGTGTGCACTGGG-3'. The sequences of SORL1 (Var B) forward and reverse primers were 5'-TTGGTTCTCGGCAGGTGAA-3' and 5'-ATCTGACAGCTCATACATCATGAT-3'. The sequences of SORL1 (Var F) forward and reverse primers were 5'-TCCTAGCATTTATTATTACTTTTCTC-3' and 5'-GTAGCTAATCCAGATGGCGACTT-3'. For endogenous control the expression of human Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) gene was examined. The sequences for human G3PDH primers were 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. Relative transcript levels were determined from the relative standard curve constructed from stock cDNA dilutions, and divided by the target quantity of the calibrator following manufacturer's instructions. For the determination of 51A RNA expression in human *post-mortem* brain samples we selected the appropriate housekeeping gene [Topoisomerase (DNA) I, NM_003286.2] taking advantage of geNorm Housekeeping Gene Selection Kit as described elsewhere (Penna et al., 2011).
**In vitro transcription analysis**

*In vitro* transcription reactions and primer extension experiments were carried out as previously described (Pagano et al., 2003; Pagano et al., 2007). To construct in vitro templates, a 51A-containing fragment was amplified from human genomic DNA and cloned into pGEM-Teasy vector (Promega) by using the primers 5’-ACAAACTCCATCTGCAATTCCTCG-3’ and 5’-CAGGTATAGGGGTTGCAGC-3’.

**Immunofluorescence detection.**

SKNBE2 cells were grown overnight on culture slides and then transfected with pEGFPN1-51A (or pEGFPN1 as control) using PEI (Sigma). 48 hours after transfection the cells were washed in PBS, fixed for 10 min with 10% buffered formalin and blocked for 15 min with 3% bovine serum albumin in PBS. Cells were subsequently incubated with primary antibody overnight at 4°C in 0.5% BSA in PBS. The day after the cells were labeled with secondary antibodies for 45 min in 0.5% BSA/PBS solution. Cells were then incubated with DAPI for 5 min and mounted with Mowiol (Invitrogen) as described elsewhere (Thellung et al., 2011). Immunostained cells were observed with the appropriate filters on Axiovert 200 M (Zeiss, Jena, Germany) microscope and captured at the same adjustments of laser intensity and photomultiplier sensitivity using Axio Vision software. Primary Antibody: anti-SorLA (H-300), sc-33822 rabbit polyclonal antibody raised against amino acids 86-385 mapping within an N-terminal extracellular domain of SorLA of human origin (Santa Cruz). Secondary antibody: anti-rabbit Rhodamine-TRITC (1:200) (Jackson ImmunoResearch)

**Aβ ELISA**

The amount of secreted Aβ x-40 and Aβ x-42 were evaluated by sandwich ELISA (IBL, Gumna, Japan) following the procedure here described. Media of 51A permanently transfected SKNBE2 cells were diluted in EIA buffer and processed using a kit specific for both Aβ species, following
the indications of the manufacturer. The kits are solid phase sandwich ELISA using plates pre-coated with the specific polyclonal anti-human Aβ antibody (raised against residues 38-42 or 35-40). An HRP-conjugated monoclonal human anti-Aβ antibody (11-28) was also supplied. Both assays show a linear reactivity within the range of concentration 7-1000 pg/ml for both Aβ species. Aβ concentration was determined using Benchmark Microplate Reader and evaluated by Microplate Manager Version 5.1 Software (Biorad, Hercules, CA, USA).

**Western Blot**

The proteins were quantified using a commercial protein quantification kit (Protein Assay, Bio-Rad) following the manufacturer’s instructions. The samples were subsequently analyzed by 10% SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Whatman, Inc. GE Healthcare, New York, NY) as previously described (Zerega et al., 2004). In detail, the membranes were initially blocked by an incubation of 2 hours in Tris-buffered saline Tween 20 (TBST; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.05% Tween 20) containing 5% non-fat dried milk. The blots were incubated for 1 h with the appropriate primary antibodies: SorLA N-terminal (H-300, sc-33822 Santa Cruz) and Anti-SORL1 C-terminal (Sigma-Aldrich S9200). In order to normalize the protein levels of SORL1 Western blot membranes were stripped with the stripping reagent “Restore” (Pierce), then probed with a monoclonal antibody against-α-tubulin (clone B-5-1-2)(T 5168 Sigma) (1:2000). All primary antibody were then diluted in TBS containing 0.1% NaN₃ and 1% BSA. After washing with TBST membranes were incubated with peroxidase-conjugated secondary antibodies [anti-mouse IgGs (A 0168, Sigma) (1:12000); anti-rabbit IgGs (A 0545, Sigma)(1:20000)] for 1 h at room temperature. After washing the reactive bands were revealed by ECL (Amersham Biosciences, GE Healthcare). Densitometric analysis of protein bands was performed using the ImageJ software system.

**Statistics**
Experiments were performed in triplicate and repeated three times. Data are reported as mean value ± standard deviation, and statistical significance was examined using the unpaired Student's t test. For analysis of 51A expression in control and AD brains the non-parametric test Mann-Whitney-U test was used. P values less than 0.05 were considered statistically significant.

In the figures, * and ** indicate statistical significance at p values < of 0.05 and 0.01, respectively.

RESULTS

51A is a novel PSE/DSE-dependent transcriptional unit.

Alternative splicing variants of SORL1 with distinct biochemical properties have been reported (see supplementary Table S1), although the regulation of this phenomenon is still obscure. We have recently identified in silico 51A, a predicted transcriptional unit that maps in the intron 1 of SORL1 gene in antisense configuration. (Dieci et al., 2007; Pagano et al., 2007). Since intronic pol III-transcribed RNAs might regulate the splicing of AD-involved protein-coding genes (Massone et al., 2011), we hypothesized that the transcription of 51A might interfere with the maturation of SORL1 pre-mRNA leading to the occurrence of alternative splicing events potentially interesting for AD studies.

To test this hypothesis we first established the transcriptional activity of 51A promoter by co-transfecting SKNBE2 neuroblastoma cells with a plasmid construct expressing a luciferase-silencing hairpin driven by 51A promoter (hereafter referred to as pSHAG-51A) together with a plasmid expressing luciferase (pGL3). In this condition, if 51A promoter is active, the transcription of the hairpin drives the post-transcriptional silencing of a co-transfected luciferase cDNA and the decrease of its signal; on the contrary, an unaltered luminescent signal indicates that luciferase is not silenced because 51A promoter is not active.

A reduced luminescence signal was detected in pSHAG-51A-transfected cells 48h after transfection (0.4 ± 0.008 as normalized to cells transfected with pGL3 alone) as a consequence of
efficient transcription of the silencing hairpin driven by 51A promoter. In the same experiment, two well assessed pol III type 3 promoters (U6 and H1 snRNA promoters) were used as positive controls driving the active transcription of the silencing hairpin, whereas a promoterless construct (named No Promoter, NP) was referred to as negative control since, as expected, it did not influence luciferase expression (Fig. 1A, B). Altogether, these experiments demonstrate the transcriptional activity of 51A pol III type 3 promoter.

Next, to more directly test 51A RNA synthesis, we performed in vitro transcription of the 51A template, using a nuclear extract from SKNBE2 cells. As shown in Figure 1C, two main 51A-specific transcripts were produced. The shorter approximately co-migrated with a 300-nt RNA size marker. Such a transcript size was compatible with transcription initiating downstream of the PSE (but upstream of the putative TATA-like element) and terminating at a run of 4 Ts located ~300 bp downstream (evidenced in Figure 1B). The predominant 51A transcription product, however, was a longer transcript, whose size could be explained by Pol III read-through at the T4 signal followed by termination at a downstream located termination signal (Fig. 1B). The transcription start site of 51A was also investigated by primer extension analysis conducted on in vitro produced, unlabeled transcripts. In both SKNBE2 (Fig. 1D) and HeLa cells (not shown) nuclear extracts, a single predominant extension product, corresponding to transcription initiation at an A residue located 33 bp downstream of the start of the PSE, was observed. Such short distance between PSE and the TSS is unusual, suggesting the possibility that other, still uncharacterized cis-acting elements might influence 51A TSS selection.

**51A transcription drives SORL1 alternative splicing**

In order to test the possible influence of 51A synthesis on SORL1 pre-mRNA splicing, we measured by Real Time RT-PCR the expression of 51A RNA and SORL1 splicing variants in different cell lines in order to identify an in vitro cellular model expressing both 51A RNA and SORL1 variants. Results showed that, with the exception of HeLa cells and NCTC-2544 (Human Disease Models & Mechanisms: DMM: Accepted manuscript).
Skin Keratinocyte), all examined cells express 51A RNA (Fig 2A). Similarly, SORL1 Variant A showed a rather ubiquitous expression profile, whereas the two alternatively spliced forms of SORL1 [Variants B and F (see S1 for details)] are specifically expressed in SKNBE2 cells and undetectable in all the other cell lines (Fig. 2 B-D). Therefore, since only SKNBE2 cell line express simultaneously A, B and F protein variants we used this cell line as model to test possible variations of their relative amounts (by the means of protein variants ratio) induced by the overexpression of 51A ncRNA.

Next, we tested whether the synthesis of 51A RNA might drive the splicing shift of SORL1. To this aim we transiently transfected SKNBE2 cells with a plasmid construct harbouring the whole 51A transcriptional unit (p51A-EGFPN1) and measured by Real Time RT-PCR the amount of the individual SORL1 splicing products (A, B and F). We found that 48 hours after transfection, the increased synthesis of 51A RNA is accompanied by a strong decrease of the SORL1 splice variant A mRNA content [up to 5% of the original level (p = 0.0002)] (Fig. 2 E,F).

Next, taking advantage of a SORL1-specific antiserum raised against the C-terminal portion of the protein (that potentially recognizes all the protein variants) we tested by Western blotting whether the decrease of SORL1 Variant A transcription, corresponds to a reduced amount of SORL1 protein. Results showed that an immunoreactive band of about 270 KDa (a size corresponding to the long canonical Variant A) is significantly reduced (up to 14% of the original level) in 51A-overexpressing cells confirming, at protein level, that the expression of 51A ncRNA favours the down-regulation of the synthesis of SORL1 protein form A. Interestingly, in the same protein extract a SORL1 immunoreactive band at about 110 KDa is up-regulated in 51A-overexpressing cells although at the present state it is not possible to ascribe its signal to variant B or rather to variant F (Fig. 2G).

Next, to further prove that the down-regulation of SORL1 Variant A is specifically induced by the synthesis of 51A RNA we took advantage of a different SORL1-specific polyclonal antiserum raised against the N-terminal portion of the splice variant A, thus ineffective to recognize the
alternative protein forms B and F. By immunofluorescence microscopy we detected a marked signal
of SORL1 in pMock-transfected cells; in this condition 51A RNA is not overexpressed and the
SORL1 variant A is predominantly synthesized and recognized by this antiserum (Fig. 2H). On the
contrary, a strongly decreased signal was detected in cells overexpressing 51A RNA (Fig. 2I). In
this case, although we were unable to detect the increased synthesis of the alternative protein forms
B and F (they harbor a different N-terminal portion and are thus not recognized by the antiserum),
we found that the overexpression of 51A RNA, leads to a decreased amount of SORL1 Variant A
that, as a consequence, is only barely detectable. In the same microscope fields untransfected cells,
that do not express GFP and 51A, are positive for SORL1 according with a 51A-dependent change
in the protein biochemical variant.

Altogether, the above results demonstrate that the synthesis of 51A RNA leads to the
splicing shift of SORL1.

The 51A-dependent alternative splicing of SORL1 leads to the impairment of Aβ secretion

Since it has been demonstrated that the down-regulation of SORL1 leads to the increase of
Aβ formation we hypothesized that the 51A ability to specifically limit the synthesis of SORL1
variant A might also represent an upstream control of Aβ secretion. To address this hypothesis
without the technical limitations imposed by the transient transfection procedure, we generated a
transgenic SKNBE2 cell line permanently transfected with 51A expression plasmid (hereafter
referred to as SKNBE2-51A). As a negative control we generated a SKNBE2 cell line permanently
transfected with the empty vector (SKNBE2-Mock). In order to preliminarily characterize this
experimental model we measured, by Real Time RT-PCR, the expression of 51A RNA and by
Western blotting the extent of SORL1 protein variant A down-regulation. SKNBE-51A cells
expressed 51A RNA at a 6.3-fold higher level than SKNBE2-Mock and showed a parallel
significant decrease in SORL1 protein content (p = 0.0027) (Fig. 3A, B). To evaluate the effects of
51A expression on the different SORL1 isoforms, that cannot be discriminated by the antibody used in Western blot experiments, we analyzed by Real-Time RT-PCR SORL1 mRNA variants content in 51A-overexpressing cells. We found a decrease of SORL1 variant A mRNA (p = 0.0017) and a concomitant increase of variants B (p = 0.003) and F (p = 0.001) mRNAs in SKNBE2-51A cells as compared to SKNBE2-Mock, thus demonstrating that a prolonged overexpression of 51A RNA leads to the stable shift of SORL1 splicing from the long form A to the alternative forms B and F (Fig. 3C).

Considering that proteolytic processing of APP plays a central role in AD etiology through the formation of neurotoxic ß-amyloid peptides (Aß) (Johnson et al., 1990; Selkoe, 1990) and that this processing is influenced by the down-regulation of SORL1, we investigated whether the stable overexpression of 51A in SKNBE2 cells would affect the formation of Aß. Hence, we measured by ELISA the relative amount of Aß x-42 and Aß x-40 molecular species in the culture medium conditioned for 48 hours by SKNBE2-Mock or SKNBE2-51A cells. We found that the amount of both Aß x-42 and Aß x-40 is increased in SKNBE2-51A cells as compared to mock-transfected cells (1.35-fold, p = 0.004 and 2.24-fold increase, p = 0.001, respectively) (Fig. 3D). Therefore, these results demonstrate that, as expected, the overexpression of 51A and the consequent down-regulation of SORL1 protein isoform, cause a significant overproduction of Aß.

51A RNA is overexpressed in AD post mortem samples

In consideration of the role of 51A RNA in the regulation of amyloid formation in the in vitro model, we tested whether its expression may contribute to AD generation in ex-vivo human brain samples. To this aim, we measured by Real Time RT-PCR, the amount of 51A RNA in cerebral cortices from 23 AD patients and 23 non-demented control samples. Interestingly, we found that 51A is actually expressed in human brains and that, although with significant individual variations, the average of 51A expression is up-regulated in AD samples (mean values: control=
1.13; \(AD= 2.14; \ p = 0.049\) (Fig. 5A). To verify whether the increase in 51A expression was specific for AD, we measured its expression in 5 samples of Parkinson disease. The average level of expression (using Top1 to normalize) was slightly higher than controls (1.13 vs. 1.42) but much lower than observed in AD patients (1.42 vs. 2.14). These results, while suggesting a possible specificity of 51A overexpression in AD in comparison to other neurodegenerative diseases, due to the small number of samples available did not allow us to any definitive conclusion, and larger studies will be required to assess this issue. Importantly, the differences in 51A expression observed in control vs. AD patients were not related to the time of the sampling, since we observed the lack of any relationship between the post-mortem delay of brain sampling and 51A expression (data not shown).

Therefore, in light of this result and that: 1) the SORL1 Variant A is down-regulated in AD (Scherzer et al., 2004); 2) the functional correlation between the expression of 51A and SORL1 splicing (that leads to a decreased amount of the canonical variant A), these data clearly suggest that 51A RNA might play an active role in altering SORL1 expression in AD patients leading to increased amyloid production that ultimately may induce neurodegeneration.

**DISCUSSION**

In previous papers, we documented the relevant role of pol III-transcribed ncRNAs, in particular in the regulation of alternative splicing (Dieci et al., 2007; Pagano et al., 2007; Massone et al., 2011), and in the molecular events leading to neuron differentiation (Dieci et al., 2007; Pagano et al., 2007; Castelnuovo et al., 2010; Gavazzo et al., 2011; Massone et al., 2011).

In this work we report the characterization of a novel transcriptional unit, named 51A, that maps in the intron 1 of SORL1 gene, and from which a novel regulatory ncRNA is synthesized. The expression of 51A leads to a splicing shift of SORL1 mRNA determining the maturation of alternative protein forms instead of the canonical protein variant A. This event ultimately brings to a significant down-regulation of the canonical form of SORL1. Reduction of SORL1 expression was
shown to represent a condition associated to detrimental pathological consequences. In particular, SORL1 down-regulation was reported to induce increase of Aβ production (Andersen et al., 2005; Small et al., 2005; Offe et al., 2006; Wang et al., 2007). Thus, due to its influence on SORL1 function, this ncRNA might be of particular relevance as determinant of induction of Aβ-dependent neurodegeneration. We propose that triggering of 51A RNA overexpression may represent *per se* one of the upstream regulatory events of Aβ generation.

Therefore, the results here reported support previous studies demonstrating that down-regulation of SORL1 drives the over-formation of Aβ and disclose a possible role of 51A as novel regulatory element, acting upstream to Aβ formation *via* SORL1 variant A down-regulation. In this context, the observation that in 51A-overexpressing cells a significant Aβ increase is observed, whereas in SORL1 KO mice where Aβ changes are more modest (Andersen et al., 2005) may plausibly due to the different intensity of SORL1 down-regulation observed in the two experimental systems, with the activation of compensatory pathways in KO animals that are not present in our transiently transfected cells.

It is also important to underline that 51A is expressed in non-AD human brains although at low levels, indicating a potential, still not identified physiological role for this ncRNA. Interestingly, the expression of 51A is significantly increased in AD brains (although with individual variations) suggesting that it might be involved in the Aβ generation in these patients, through the inhibition of SORL1 expression. At the present state further experiments are needed to define the cause of the individual variation in 51A expression in post mortem AD brains and possibly its correlation with clinical-pathological conditions as individual brain inflammatory conditions. In any case, the reduction of Aβ production by 51A overexpression is associated to a reduction of SORL1 variant A content that could, in turn, affect Aβ production. Further studies are required to show a causative involvement of the 51A-dependent SORL1 modulation in the regulation of APP processing.
In conclusion, our results identify an active role played by a pol III-transcribed ncRNA, acting upstream to Aβ formation via SORL1 variant A down-regulation, in the control of amyloid processing, providing a novel sight on AD-related processes.

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TRANSLATIONAL IMPACT

Background
The mechanisms that cause Alzheimer’s disease (AD) are still unclear. While rare familial forms are clearly linked to mutations on a few genes involved in amyloidosis, such as APP and presenilins, the genesis of sporadic forms is still obscure. In this scenario there is a patent need for information on molecular mechanisms underlying the disease. To this end there is increasing interest in a deeper comprehension of tightly regulated molecular events, such as gene expression and protein regulation, whose dysfunction can be associated to neuronal death in AD. The discovery that peculiar non-coding RNA (ncRNA) sequences can regulate protein expression and alternative splicing events, paved the way for specific investigations on AD-related genes potentially involved in modulating the onset of the disease, most likely as risk factors. In fact recent data indicate that newly identified
ncRNAs are produced in human brain, are overexpressed in AD subjects, modulating alternative splicing events that ultimately regulate amyloid formation. In search for new candidate genes, bearing ncRNA potentially involved in AD, the authors investigated SORL1: a receptor for apolipoprotein E (a well known risk factor for late-onset AD) genetically associated to AD, and likely involved in amyloid formation as well. Indeed, allelic variants in distinct regions of SORL1 gene have been recently associated to AD, and preliminary results suggest that in AD there is a reduced function of SORL1.

Results

Here the authors describe a new ncRNA (named 51A) mapping in antisense orientation into intron 1 of SORL1 gene, and whose synthesis promotes the expression of SORL1 alternatively spliced protein variants. The expression of 51A was detected in normal human brain samples and significantly overexpressed in AD brain. Analyzing the molecular mechanisms triggered by 51A, the authors discovered that in vitro 51A reduces the expression of SORL1 isoform A while enhancing alternative. Indeed, the expression of 51A induces a significant increment on amyloid formation in cultured cells. Altogether authors hypothesize that abnormal increments on this specific ncRNA on SORL1, acting upstream to amyloid formation via SORL1 variant A down-regulation, might be correlated to an AD-related phenotype.

Implications and future directions

Considering the importance of SORL1 as AD-linked risk factor (even indirectly as Apolipoprotein E receptor), and considering the increasingly relevance of ncRNA as specific mechanisms for alternative splicing, this work indicates that new, alternative and unconventional mechanisms might be likely involved in amyloid formation and neurodegeneration. Considering the novelty and the limited knowledge that we have to-date on ncRNA’s world, further studies are needed to deepen our knowledge on the link between ncRNAs and AD. At the same time it is evident that, if confirmed,
these studies will potentially lead to the identification of new therapeutic targets, to design new drugs and to identify validated and reliable markers for the prevention or for the cure of the disease.

References


TABLE 1: Patient’s characteristics (blank cells are representative of not available data)

A) Control cases

<table>
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<tr>
<th>Case Number</th>
<th>Sex</th>
<th>Mental status</th>
<th>Age (y.o.)</th>
<th>Post-mortem</th>
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<th>Angiopathy</th>
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Figure Legend

Figure 1. 51A transcription unit is actively transcribed in SKNBE2 cell nuclear extracts. (A) Promoter activity transfection assay in human SKNBE2 cells. A specific luciferase-silencing hairpin is transcribed by 51A PSE/DSE-dependent promoter. The promoter region encompasses the putative pol III type 3 regulatory regions (TATA box, PSE, and DSE). pGL3/pRL, negative control; pShag-NP, pShag-No promoter negative control; pShag-U6, positive control; pShag-H1, positive control; pShag-51A, sample of interest. (B) Sequence of the transcription unit. The PSE is in bold red and underlined, as is the putative T4 termination signal. A TATA-like element is underlined. (C) In vitro transcription of plasmid-borne 51A (lane 3) or empty pNEB193 plasmid (lane 2) was carried out in a nuclear extract from SKNBE2 cells. The main 51A-specific transcripts are indicated by arrowheads on the right. The migration position of a 300-nt RNA size marker is indicated on the left. Lane 1, no DNA. (D) Primer extension analysis was conducted on unlabeled RNA products of in vitro transcription reactions programmed with no DNA (lane 1), empty pNEB193 plasmid (lane 2) or pNEB193-51A (lane 3). Shown in lanes 4-7 are the results of sequencing reactions conducted with the same 5’-labeled oligonucleotide utilized for primer extension. The sequence of the non-transcribed DNA strand around the TSS (+1) is indicated on the right. The position of the main, 51A-specific primer extension product is indicated by an arrowhead on the left.

Figure 2: The overexpression of 51A ncRNA leads to SORL1 alternative maturation. (A) 51A, (B) SORL1 Variant A, (C) SORL1 Variant B, (D) SORL1 Variant F RT-PCR expression profile in different cell lines. (E) RT-PCR expression analysis of 51A ncRNA and (F) SORL1 variant A in 51A-transfected SKNBE2 cells and/or pMock controls; ** indicate statistical significance at \( p \) values \( \leq 0.01 \). (G) Western blot expression analysis of SORL1 Variant A and B (or F) in 51A-transfected SKNBE2 cells. (H-I) Immunofluorescence detection of SORL1 alternatively spliced...
protein products. (Panel H) Antibodies raised against SORL1 N-terminus of the protein form A show a clearly detectable signal in GFP-expressing cells only in the absence of a concomitant 51A overexpression demonstrating the synthesis in this condition of the canonical protein form (1, GFP; 2, DAPI, 3, Tritch; 4, Merge); three independent microscope fields are reported. (Panel I) SORL1 Variant A signal is absent or very weak in cells transfected with a construct co-expressing 51A and GFP: in this case the transient overexpression of the ncRNA leads to the synthesis of the alternative forms of SORL1 endowed with a peculiar N-terminal portion not recognized by the same IgGs (1, GFP; 2, DAPI, 3, Tritch; 4, Merge); three independent microscope fields are reported. Untransfected cells, that do not express GFP and 51A, are positive for SORL1 according with a 51A-dependent change in the protein variant.

**Figure 3.** 51A ncRNA overexpression and the consequent alternative splicing of SORL1 drive to an impairment of amyloid release. (A) RT-PCR analysis of 51A ncRNA expression in 51A-permanently transfected SKNBE cells. (B) SDS-PAGE expression analysis of SORL1 Variant A in 51A-permanently transfected SKNBE cells. RT-PCR analysis of SORL1 Variant A (C), Variant B (D) and Variant F (E) in 51A-permanently transfected SKNBE cells. Increased secretion of total amyloid β (F), Aβ x-40 (G) and Aβ x-42 (H) in 51A-overexpressing SKNBE2 cells. X axis: transfected plasmids. Y axis: quantitative determination of Aβ (pg/ml) secreted in the medium 48 hours after medium replacement as determined by sandwich ELISA (results were normalized to the pMock-transfected cell line).

**Figure 4.** The expression of 51A is significantly increased in AD brains. (A) 51A expression in AD cases (black columns) and non-AD control individuals (grey columns) as determined by Real-Time RT-PCR of post mortem cerebral cortex samples. (B) Box plot and median values are reported. Statistical analysis by Mann-Whitney U test demonstrated a significant difference between groups (p= 0.049).
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Schematic view of SORL1 protein variants

Gene SORL1 5' → 3' encoded on plus strand of chromosome 11 from 120828122 to 121009681

Diagram of SORL1 protein variants with annotations for exons and introns.