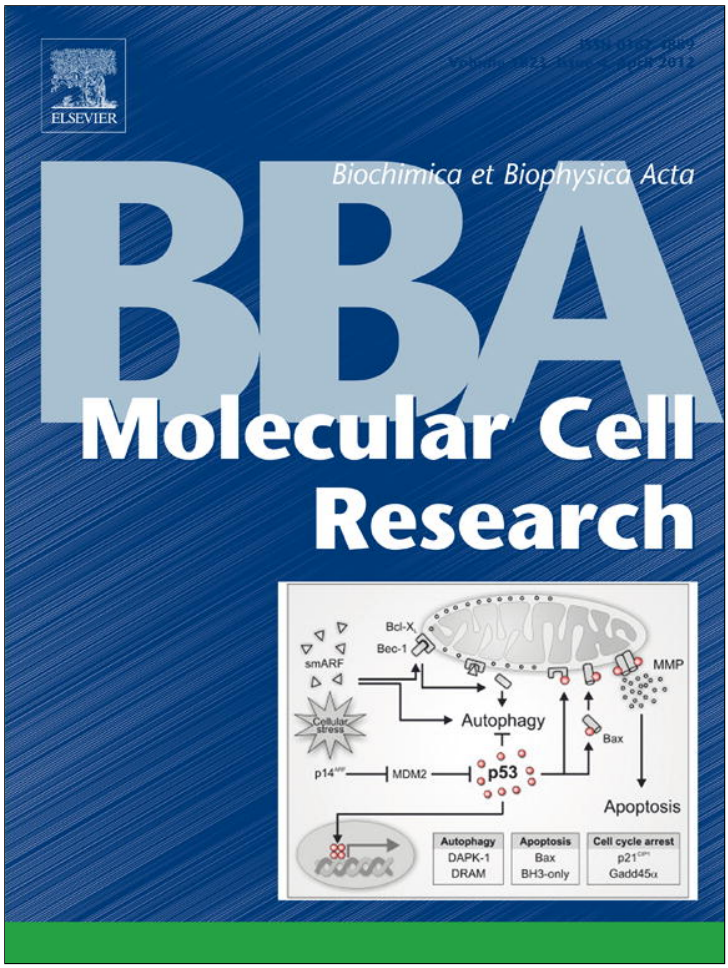

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NDM29, a RNA polymerase III-dependent non coding RNA, promotes amyloidogenic processing of APP and amyloid β secretion

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ABSTRACT

Neuroblastoma Differentiation Marker 29 (NDM29) is a RNA polymerase (pol) III-transcribed non-coding (nc) RNA whose synthesis drives neuroblastoma (NB) cell differentiation to a nonmalignant neuron-like phenotype. Since in this process a complex pattern of molecular changes is associated to plasma membrane protein repertoire we hypothesized that the expression of NDM29 might influence also key players of neurodegenerative pathways. In this work we show that the NDM29-dependent cell maturation induces amyloid precursor protein (APP) synthesis, leading to the increase of amyloid β peptide (Aβ) secretion and the concomitant increment of Aβ x-42/Aβ x-40 ratio. We also demonstrate that the expression of NDM29 RNA, and the consequent increase of Aβ formation, can be promoted by inflammatory stimuli (and repressed by anti-inflammatory drugs). Moreover, NDM29 expression was detected in normal human brains although an abnormal increased synthesis of this ncRNA is induced in patients affected by neurodegenerative diseases. Therefore, the complex of events triggered by NDM29 expression induces a condition that favors the formation of Aβ peptides in the extracellular space, as it may occur in Alzheimer's Disease (AD). In addition, these data unexpectedly show that a pol III-dependent small RNA can act as key regulator of brain physiology and/or pathology suggesting that a better knowledge of this portion of the human transcriptome might provide hints for neurodegeneration studies.

1. Introduction

Soluble and insoluble aggregates of Aβ or intracellular tangles of hyperphosphorylated Tau protein have been alternatively regarded as the most likely causes of neurodegeneration in AD (reviewed in [1,2]). In particular, the "amyloid hypothesis" originates by the observation that post mortem analysis of AD patients' cerebral cortices revealed amyloid plaques within the brain parenchyma [3]. This phenomenon is mainly due to an increased secretion of Aβ (the main component of amyloid plaques) in the extracellular space. In particular, an enhanced ratio between the two Aβ molecular variants (Aβ x-42 and Aβ x-40), favoring the formation of the less soluble Aβ x-42 variant, was observed. As a consequence, high amounts of a mixture of neurotoxic Aβ peptides accumulate in the brain. However, although increasing body of evidence supports a primary role of Aβ in the etiology of AD, little is known about the upstream events that trigger the amyloid cascade, so that the first disease-causing events still remain unknown. In this context several research approaches have been developed to investigate different possible pathogenetic events for AD. Among these, prolonged inflammatory stimuli are thought to be part of the complex pattern of events that ultimately lead to the onset of the disease-associated conditions, such as increased secretion and aggregation of Aβ peptides synaptic dysfunction, reactive gliosis and the generation of neurofibrillary tangles of hyperphosphorylated Tau [4–9].

Besides this working hypothesis, emerging evidence supports the possibility of incomplete, defective attempt to cell cycle re-entry as a possible cause of neuron death in AD [10,11]. In this view aberrant mitotic stimuli might be generated in neurons of AD patients and, as a consequence lead post-mitotic neurons to apoptosis [12]. This hypothesis is currently supported by experimental observations, showing an association between high aneuploidy rate, chromosomal missegregation and AD [13]. Interestingly, a link between the control of cell cycle and AD has been also recently proposed by epidemiological observations. Indeed, a diminished risk of AD in cancer patients and a highly reduced risk of cancer associated to AD have been documented [14].

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In a recent work we described Neuroblastoma Differentiation Marker 29 (NDM29), a RNA pol III-transcribed ncRNA whose synthesis is lead by an extragenic type 3 promoter composed by a TATA box, a Proximal Sequence Element (PSE) and a Distal Sequence Element (DSE) [15]. A preliminary expression analysis showed that NDM29 is actively transcribed in cells of the central nervous system, whereas cells characterized by epithelial-like phenotype synthesize a modest amount of this ncRNA. In order to investigate the functions of NDM29 we generated two genetically engineered SKNBE2 neuroblastoma (NB) cell lines that overexpress NDM29 [16,17]. Interestingly, we found a direct relationship between the expression levels of this ncRNA and cell differentiation toward a neuron-like phenotype. Indeed, the expression of NDM29 strongly restricts the malignant potential of NB cells leading to their maturation to a non-malignant neuron-like phenotype [16,17]. In particular, we demonstrated that NDM29 overexpression caused neuronal differentiation of S1.1 cells in terms of molecular markers (high increase in the transcription of GD2 synthase and NF68, while modest increase in glial markers) and electrophysiological behavior (we detected high levels of fast inactivating Na^+ current, able to generate mature action potentials and induction of the expression of functional GABA-A receptors on their membrane) [17]. These indicators show that NDM29 expression in S1.1 cells is accompanied by a well-coordinated differentiation process toward the neuron-like phenotype [17].

In this context, the aim of this study was to evaluate whether the expression of NDM29 might also influence the processing of APP and/or the amyloid cascade whose perturbation is involved in AD development.

We show that a sustained overexpression of NDM29 actively promotes the molecular processing and the secretion of Aβ peptides, thus influencing the main secretory pathway thought to be involved in AD development. High level of transcription of NDM29 increases APP synthesis and leading to the increase of Aβ secretion. Notably, the increase in Aβ production is mainly ascribed to the toxic insoluble form Aβ x-42, considered of primary relevance for AD etiology [18]. Finally, we demonstrate that the synthesis of NDM29 RNA can be promoted by inflammatory stimuli (and repressed by antiinflammatory drugs) and that this ncRNA, actually detected in human brains, is abnormally expressed in AD patient brains.

2. Materials and methods

2.1. Cell culture and transfection

Different cell lines were used: S1.1, S6, S7, M and M2 cells are SKNBE2 neuroblastoma cells (provided by the cell bank of the National Institute of Cancer Research (IST) Genoa, Italy and obtained from ECACC, [19]) stably transfected with pNDM29-EGFPN1 or pEGFPN1 empty vector respectively as described in [20] (both grown in RPMI supplemented with 10% FBS, 1% glutamine and 200 μg/mL G418) and transiently transfected with pNDM29-EGFPN1, pAntiNDM29-EGFPN1 (pS1.1/A) and pEGFPN1 (grown in DMEM supplemented with 10% FBS, 1% glutamine). HEK293-APP cells were a kind gift of Prof. Luciano D'Adamio (Albert Einstein College of Medicine, NY, USA). Cells were transfected using PEI (polyethylenimine) (SIGMA P3143).

2.2. Real time quantitative RT-PCR analysis

Total RNAs from samples were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, DNAseI-digested and subjected to reverse transcription by Transcriptor High Fidelity cDNA Synthesis Kit (Roche 05081955001) as described in [21]. The total RNA from the samples was measured by real-time quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) following manufacturer's instructions. The sequences of forward and reverse primers were: c-kit: 5′-GCAAGTCAGTGCTGTCGGAA and 5′-AAGATAGCTTGCTTTGGACACA-GA-3′; mmp-9: 5′-CACTCGCGTGTACAGCCG-3′ and 5′-TCGAAG-GGATACCCGTCTCC-3′; NF-68: 5′-CAAGGACGAGGTGTCCGAG-3′ and 5′-CCCGGCATGCTTCGA-3′; APP: 5′-TGGCCCTGGAGAACTACATCA3′ and 5′-CGCGGACATACTTCTTTAGCATATT-3′; NDM29: 5′-GGCAGG-CGGGTTCGTT-3′ and 5′-CCACGCCTGGCTAAGTTTTG-3′. For endogenous control the expression of Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) gene was examined. The sequences for human G3PDH primers were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′- GAAGATGG-TGATGGGATTTC-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.

2.3. Immunofluorescence detection

S1.1 and Mock SKNBE2 cells grown on slides, were fixed with methanol, washed 3 times with PBS and incubated with the different primary antibodies in PBS plus 1% NGS for 1 h, and finally incubated with antigen-specific secondary antibodies (Alexa 488 or 568-conjugated, and AMCA from Invitrogen Corporation, Carlsbad, CA, USA). Cells were then mounted by Mowiol and analyzed on BioRad-MRC 1024 ES confocal microscope, equipped with a Nikon Eclipse TE 300 inverted microscope with a 60× objective lens as reported in [22]. Antibody for mouse GM130 (BD USA) was used at the dilution 1:100, mouse α-tubulin (Sigma) 1:1000 and Rabbit N-terminal APP (Sigma) 1:100; Alexa Fluor® 568 and Alexa Fluor® 488 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) were used 1:200.

2.4. ELISA Aβ detection

The amount of secreted Aβ x-40 and Aβ x-42 were evaluated by Human Amyloid (1–40) Assay Kit (IBL 27713) and Human Amyloid (1–42) Assay Kit (IBL 27711) according to the manufacturer's protocol.

2.5. Western blots

Proteins were quantified using a commercial protein quantification kit (Protein Assay, Bio-Rad 500–0006) as described in [23]. The samples were subsequently analyzed by 10% SDS PolyAcrylamide Gel Electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Whatman, Inc.). The membranes were initially blocked by an incubation of 2 h 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: rabbit polyclonal anti-APP c-terminal (A 8717 Sigma) (1:1000); rabbit polyclonal anti-Presenilin1 (S182 Sigma) (1:2000); rabbit polyclonal anti-Presenilin2 (sc-7861 Santa Cruz Biotechnology) (1:200); rabbit polyclonal antibody anti-BACE1 (pc529, Calbiochem) (1:300). After washing with TBST membranes were incubated with peroxidase-conjugated secondary antibodies [anti-rabbit IgGs (A 0545, Sigma)(1:16000)] for 1 h at room temperature. After washing the reactive bands were revealed with ECL Plus Western Blotting Detection Reagents (Amersham RPN2132). In order to normalize the protein levels, western blot membranes were stripped with "Restore" (Pierce 21059), a western blot stripping reagent, then probed with a monoclonal antibody against-α-tubulin (T 5168 Sigma) (1:2000). Amyloid C-Terminal Fragment western blot analysis was performed by running protein samples on 14% Tris-Tricine gel. The membranes were blocked by an incubation of 1 h in Phosphate-buffered saline Tween 20 containing 5% non-fat dried milk. The blot was incubated overnight with the primary antibody, rabbit polyclonal anti-APP, c-terminal (A 8717 Sigma) (1:1000), diluted in 0.1% NaN_3 in PBS. The

densitometric analysis of protein bands was performed taking advantage of ImageJ software system.

2.6. Human brain samples

Frontal and temporal cortices from AD (diagnosis was made after evaluating clinical history of disease and pathological diagnosis according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria) and control cases (AD excluded by clinical history and by immunohistochemical analysis) derive from: 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. Small pieces (50–100 mg) of gray matter from frontal and temporal cortices were excised under sterile conditions; tissue RNA and proteins were extracted following standard procedures.

The frozen brain samples collected at the Brain Bank indicated in Section 3.5 were collected in different years under the approval of the local ethics committees for the routine activity proper of the Brain Bank. The agreements between Brain Banks and Universities normally only require that the research activities with these tissues will be conducted within the aim of non profit scientific research. In fact the specific agreement between the Brain Banks and CR states that the research should be made under non profit conditions and that the Bank will be acknowledged (see Section 3.5).

2.7. Pro- and anti-inflammatory stimuli

Cells treated with TNF-α (tumor necrosis factor α), LPS (bacterial lipopolysaccharides) and IL-1α (pro-inflammatory stimuli) or diclofenac 20 μM (anti-inflammatory stimuli) for 16 h were processed for the extraction of RNA to be analyzed by RT-PCR as described in Section 2.2 while the conditioned media were collected for Aβ x-40 and Aβ x-42 detection.

3. Results

3.1. NDM29 RNA expression leads to the neuron-like differentiation of SKNBE neuroblastoma cells

We recently challenged the human genome in order to identify novel RNA pol III transcription units [15]. We found that one transcription unit of the newly obtained collection maps in 11p15.3, in the first intron of the Achaete Scute-Like 3 gene (Acc. No. AJ400877), an Helix-Loop-Helix transcription factor involved in the development of the nervous system in drosophila [24]. Since its synthesis was increased in the advanced stages of NB cell maturation we named this small RNA (NDM29) [16]. In previous studies on NDM29 function, we showed that its overexpression confers a neuron-like phenotype to NB cells, including membrane ion currents usually observed in cells endowed with functional synapses [17]. Interestingly, the synthesis of NDM29 strongly restricts NB malignant potential and makes cells susceptible to the action of some antiblastic drugs inhibiting the synthesis of the multidrug resistant (MDR) 1 detoxification pump [16].

In order to investigate in detail the function of NDM29, we generated a NDM29-overexpressing cell line by the permanent transfection of a pNDM29-EGFPN1 plasmid [harboring NDM29 transcriptional unit driven by its endogenous promoter and a Green Fluorescent Protein (GFP) cassette] in SKNBE2 NB cells. After selection for antibiotic resistance, we observed that cells that integrated extra copies of NDM29 transcription unit exhibit a population doubling time (PD) of 48 h (whereas their Mock counterpart have a PD of 30 h) and are characterized by a dramatic reduction of the malignant potential. A subclone of this cell population (hereafter referred to as S1.1) was further characterized for its differentiation potential. As shown in Fig. 1A, S1.1 cells show a marked neuron-like phenotype characterized by a big cell body and well organized neuritic processes. Real time RT-PCR analysis of these cells evidenced a 2.5-fold increased expression of NF68 [Neurofilament 68, a protein specific of neurofilaments (NP_006149.2)] (Fig. 1B), while a 3.9-fold increase of positivity to HuD [Hu-antigen D, a neuron-specific marker (NP_001138246.1)] was demonstrated by immunofluorescence detection (Fig. 1C). In these experiments the neuron-like maturation stage of S1.1 and Mock cells was assessed by measuring with Real Time RT-PCR the expression of neuron-specific molecular markers such as MMP9 [Matrix Metalloprotease 9], a marker of differentiation toward neuron (NP_004985.2) [25], c-Kit [a marker of stemness highly expressed by undifferentiated neuroblastoma cells (NP_000213.1)] and NDM29 RNA. Results, normalized to Mock controls, show 8.4-fold and 5.8-fold increased expression of NDM29 and MMP9, respectively, in S1.1 cells as expected in a well-differentiated stage of maturation. Accordingly, in these cells the synthesis of c-Kit is strongly inhibited, corroborating the commitment toward a neuron-like differentiation acquired by S1.1 cells (see Supporting Information File Figure S1). Altogether with previous experiments described elsewhere [16], these results demonstrate that the phenotype of S1.1 NB cells recalls that of neuron-like cell and might represent a useful in vitro model to assess the role of NDM29 in neurological syndromes.

3.2. The expression of NDM29 promotes Aβ secretion from SKNBE2 neuroblastoma cells

New emerging evidences suggesting that ncRNAs mediate several brain regulatory events [26-28] along with the observation that NDM29 actively leads NB cells to a neuron-like differentiated stage, allowed us to postulate the possible involvement of this ncRNA in the physiology and/or pathology of neurons. To test this hypothesis, we investigated the possible correlation between the expression of NDM29 and Aβ formation. To this aim we measured using ELISA the amount of Aβ x-40 and Aβ x-42 in the cell culture medium conditioned for 2 days by Mock (M) or S1.1 cells. Results show that the concentration of total Aβ formed by S1.1 cells was increased up to 1179 pg/l with respect to the original level of Mock control (758.6 pg/l) (Fig. 2A). Indeed, in S1.1 cells, harboring extra copies of NDM29 transcription unit, we observed 1.5- and 2.0-fold increases in the amount of Aβ x-40 and Aβ x-42, respectively, as compared to Mock control cells. Next, to assess a direct correlation between NDM29 synthesis with Aβ secretion we re-overexpressed NDM29 RNA (transfecting transiently NDM29 plasmid in S1.1 cells) and/or we restored the basal level of NDM29 expression (transfecting a plasmid encoding antiNDM29, a microRNA directed against NDM29 RNA able to decrease its amount in the cell) and measured the correspondent amount of Aβ secreted. As expected, we found a significant increase of both Aβ x-40 and Aβ x-42 in S1.1 cells transiently transfected with NDM29 plasmid whereas downregulation of this phenomenon was observed in cells transfected with Anti-NDM29 construct (Fig. 2B,C).

Therefore, NDM29 synthesis provokes a general increase of the total amyloid secreted and an enhanced Aβ x-42 vs Aβ x-40 ratio.

3.3. NDM29 expression promotes the amyloidogenic processing of the amyloid precursor protein

To gain insights into the molecular mechanism by which NDM29 triggers Aβ secretion, we evaluated the possible modulation of the synthesis of key proteins involved in Aβ formation induced by this ncRNA. To this aim we measured the expression of APP (Amyloid Precursor Protein), PS1 (Presenilin 1, Acc. No. NP_000014.8), PS2 (Presenilin 2, Acc. No. NC_000001.10) and BACE (Beta Site APP Cleaving Enzyme, Acc. No. NC_000011.9) in S1.1 and Mock cells. The results obtained demonstrate that in S1.1 cells the synthesis of APP is 3.0- and 3.4-fold increased at mRNA

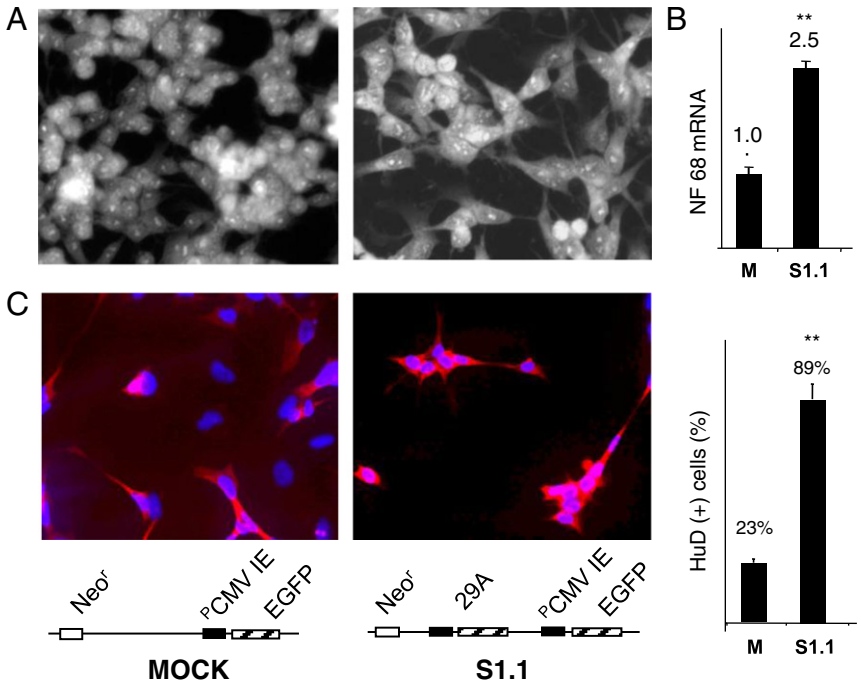

Fig. 1. Differentiated phenotype of NDM29-overexpressing cells. A. Mock (left panel, M) and S1.1 (right panel) cells. B. Real Time RT-PCR analysis of NF68 expression; results are normalized to M cells ($p = 0.0007$). C. Increased positivity to HuD antigen in S1.1 (right panel) with respect to Mock (left panel) cells. The quantitative determination is reported as resulting by the averaged percentage of positive cells in 10 randomly chosen microscope fields ($p = 0.0001$).

and protein level, respectively (Fig. 3A). To verify the specificity of this finding we measured the amount of NDM29 RNA and APP (mRNA and protein) in Mock, S1.1 and S1.1 transfected with a plasmid encoding antiNDM29. As expected, the results showed that also in transient conditions the up- and down-regulation of NDM29 RNA is accompanied by the correspondent modulation of APP synthesis (Fig. 3A′). Last, we excluded that the observed effects were due to clonal selection of the NDM29 expressing cells, measuring the amount of NDM29 and APP (protein and mRNA) in three additional S1.1 cell lines (a novel Mock and two independent NDM29-overexpressing clones) evidencing that also in different cell clones the upregulation of NDM29 RNA drives the increase of APP synthesis (Fig.3A″).

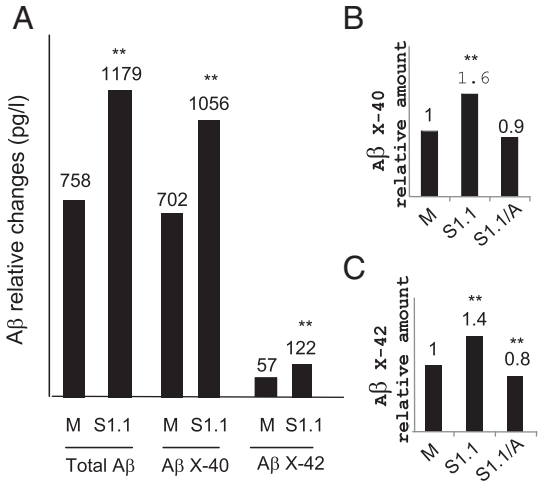

Fig. 2. Aβ secretion. A. Average of three independent determinations of Aβ secretion by M- and S1.1-conditioned media as a result of using Elisa assay (S.D. is not detectable) ($Aβ_{TOT}$ $P = 0.0004$; $Aβ_{40}$ $P = 0.0005$ $Aβ_{42}$ $P = 0.0006$; ratio Aβ 42/Aβ x40 $P = 0.0006$). B,C. Aβ x40 (B) and Aβ x42 (B) determinations in media conditioned by S1.1 cell re-transfected transiently with pNDM29 (S1.1) or pAnti-NDM29 (pS1.1/A) plasmids.

In the same cells, BACE protein level is unaltered (Fig. 3B). Similarly, the expression of PS1 and PS2 modulations of protein expression, as well as of their N-terminal fragments, is only barely detectable and statistically not significant (Fig. 3C,D). These data indicate that the NDM29-dependent induction of APP synthesis might play a role in the increase of Aβ secretion with a mechanism that does not involve the up-regulation of the components of the β- (BACE) or γ-secretase (PS1 and PS2) complexes (Fig. 3A–D).

In order to assess the specificity of the effects elicited by NDM29 on APP synthesis, we verified, by Western blotting, the expression of two unrelated neuronal proteins involved in neurodegenerative disorders, prion protein (PrP) and calsenilin (potassium channel interacting protein 3 or KChIP3). PrP is a small glycoprotein responsible of transmissible spongiform encephalopathies such as scrapie and bovine spongiform encephalopathy in animals and Kuru, Creutzfeldt-Jacob disease, Gerstmann–Straussler syndrome and fatal familial insomnia in humans [29]. Calsenilin is a neuronal calcium-binding protein, that has been shown to have multiple functions including the regulation of calcium signaling, cell death presenilin processing, repression of transcription and modulation of A-type potassium channels [30].

No significant differences in the expression of these two proteins in the S1.1 with respect to M cells were observed (Fig. 3E), supporting the notion that the NDM29-dependent effects here documented are associated to the overexpression of this ncRNA and not to a general/aspecific increase of protein synthesis.

To verify whether the increase in Aβ secretion was the resultant of alterations in APP subcellular localization, we analyzed changes in APP intracellular localization in S1.1 and Mock cells, by confocal microscopy. Specific primary antibodies were used to label APP at the N-terminal domain followed by secondary antibodies carrying fluorophores (Alexa 568). First, we quantified the fluorescence intensity induced by APP immunolabeling (measured by Laserpix software, Bio-Rad Microscience Ltd) showing a 2.45 fold increased signal of APP in S1.1 cells with respect to Mock control, confirming that in NDM29-overexpressing cells the amount of APP suitable for Aβ processing is significantly enhanced. Next, we tested the possible alterations of

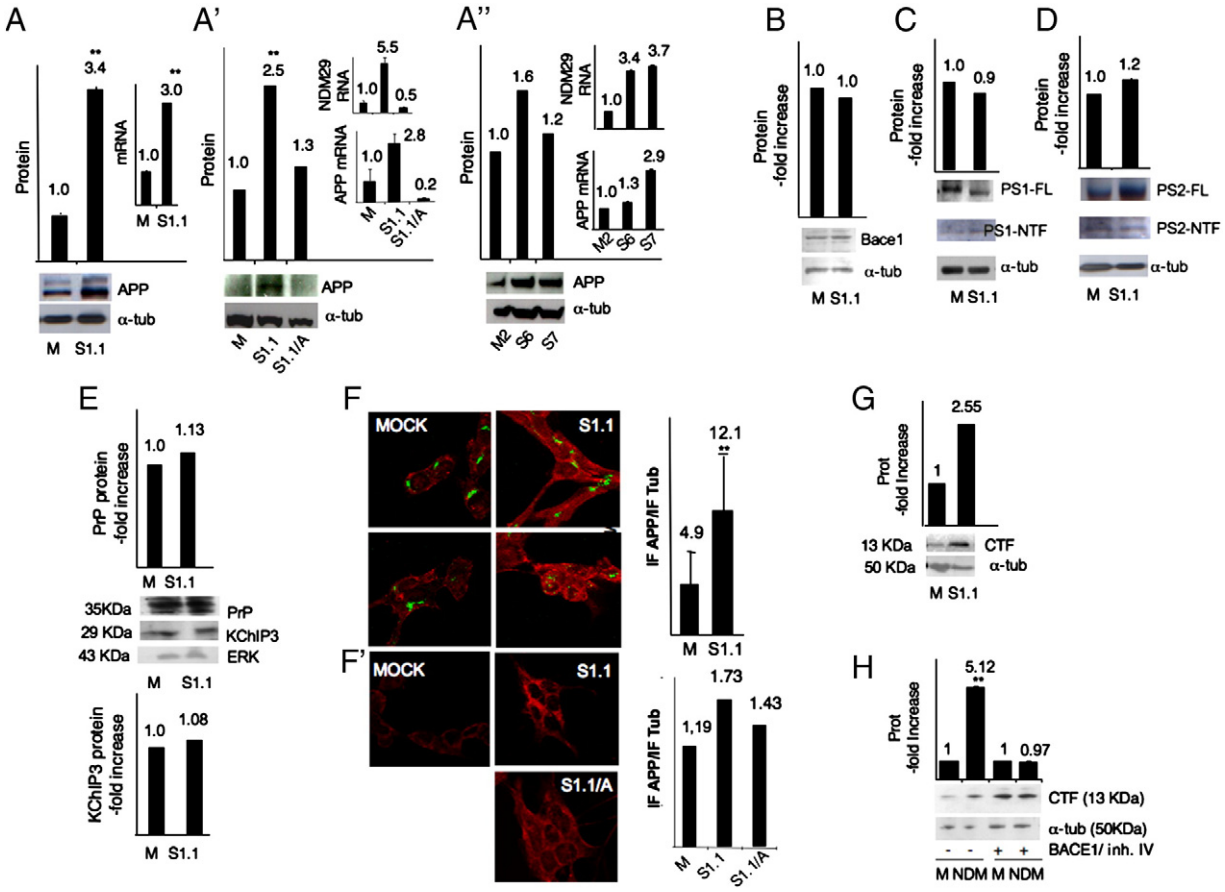

Fig. 3. NDM29-driven increase of APP processing. A–D. APP, BACE, Presenilin 1 and Presenilin 2 expressions increase in M or S1.1 cells either at protein or at mRNA level as a result by western blotting and Real Time RT-PCR respectively (APP protein $p = 0.0003$, APP RNA $p = 0.0007$; BACE protein $p = 0.0003$). X axis, cell type; Y axis, -fold increase. A′. APP mRNA, APP protein and NDM29 expressions in Mock, S1.1-transfected and/or S1.1 silenced cells. A″. APP mRNA, APP protein and NDM29 expressions in additional NDM29-overexpressing cell lines. E. Prion Protein and KChIP3 western blotting quantitation; ERK, protein loading control; quantitative determination of APP fluorescence intensity by confocal microscopy in F: Mock and S1.1 cells ($p = 0.0012$). Red (Alexa 568), APP; green (Alexa 488), GM130. F′: Mock, S1.1-transfected (S1.1) ($p = 0.00052$) and/or S1.1-silenced (S1.1/A) cells ($p = 0.0051$). Red (Alexa 568), APP; green (Alexa 488), α-tubulin. G. C-terminal fragments western blotting analysis in M and S1.1 cells. H. C-terminal fragment western blotting analysis in M and S1.1 cells in BACE Inhibitor IV-treated and/or untreated HEK293 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

APP subcellular localization in NDM29-overexpressing cells. To this aim we labeled cells with primary antibodies directed against APP and GM130 (Golgi matrix protein of 130 kDa, a Golgi apparatus associated protein), followed by secondary antibody carrying Alexa 568 for APP, and Alexa 488 for GM130. Confocal analysis showed extensive perinuclear, endoplasmic reticulum, and spots of co-localization between APP and GM130 in Golgi demonstrating that, besides the alteration of APP content, no differences in terms of subcellular localization were detected between S1.1 and Mock cells (Fig. 3F). In summary, confocal analysis showed an equivalent APP subcellular localization between S1.1 and Mock cells but a significant increase of APP immunolabeling in S1.1 cells, thus confirming that NDM29 promotes Aβ formation increasing the amount of APP suitable for processing. Next, in order to strengthen the above result we repeated the same fluorescent determinations in an experimental setting of transient up-/down-regulation of NDM29 synthesis. Again, results showed that the expression level of APP is directly related to the synthesis of NDM29 RNA corroborating further the results obtained previously (Fig. 3F′).

Then, we investigated whether NDM29-dependent increased secretion of Aβ is only caused by augmented APP synthesis or is also favored by enhanced cleavage by β- and/or γ-secretase complexes. Notably, we observe a strong increase of APP C-terminal fragments detected in S1.1 cells by SDS PAGE/immunoblotting analysis, suggesting that the expression of NDM29 specifically promotes the increase of BACE cleavage (Fig. 3G). To clarify whether the increase of CTFs observed in NDM29-overexpressing cells was due to an enhanced cleavage by β-secretase, we took advantage of a BACE-specific inhibitor (565788 β-Secretase Inhibitor IV, Calbiochem) [31]. To overcome the low production of CTFs of wt NB cells during the limited time of treatment, we incubated overnight HEK293 cells with 5nM β-Secretase Inhibitor IV followed by a transfection with pNDM29- (NDM) or pMock- (M) plasmid construct. As shown in Fig. 3H, in the presence of the BACE inhibitor, the increase in C-terminal fragments produced by untreated pNDM29-transfected cells was abolished. This indicates that, as hypothesized, the increase of CTFs is a consequence of an increased cleavage activity of BACE.

Altogether the above experiments demonstrate that the overexpression of NDM29 ncRNA leads to an increased cleavage of APP by β-secretase. Therefore, NDM29 ncRNA expression promotes Aβ secretion via a complex mechanism that involves the increased synthesis of Aβ precursor and the improvement of its processing by BACE that, in turn, generates an enhanced amount of APP C-terminal fragments suitable for further processing by the γ-secretase cleavage complex.

3.4. NDM29 expression is triggered by inflammatory stimuli

Because the above findings suggest a possible role of NDM29 in AD, we focused the investigation on the mechanisms regulating the pol III-dependent synthesis of NDM29 in neurodegenerative disorders. In light of the several reports supporting the hypothesis of a

relevant contribution of prolonged inflammatory stimuli to AD onset, we tested the effect of a widely used pro-inflammatory molecule, interleukin 1α (IL-1α) on NDM29 transcription. We found that 12 h after treatment of SKNBE *wt* cells with IL-1α (0.5 ng/ml) the expression of NDM29 is significantly increased, whereas in samples pretreated with diclofenac, a potent non-steroidal antiinflammatory drug, basal transcription of NDM29 is inhibited (Fig. 4A). Interestingly, IL-1α-dependent induction of NDM29 was correlated with a significant increase in the formation of total β-amyloid and Aβ x-42/Aβ x-40 ratio that was exclusively associated with enhanced amount of the Aβ x-42 molecular species (Fig. 4B). In order to extend the possible induction of NDM29 synthesis by other inflammatory agents we treated S1.1 cell with different dosages of TNFα (tumor necrosis factor α) and/or LPS (bacterial lipopolysaccharide) and measured possible variations of NDM29 RNA synthesis. Results showed that, with the exception of one dose of LPS (0.5 μg/ml), NDM29 synthesis was induced by the treatment in a dose-dependent manner.

Altogether the above results demonstrate that NDM29 synthesis (and its detrimental consequences on Aβ formation) can be driven by inflammatory stimuli.

3.5. NDM29 is overexpressed in AD post mortem cerebral cortex samples

Since the overexpression of NDM29 generates a cascade of reactions associated to neurodegenerative conditions, we tested the possible overexpression of NDM29 in post-mortem cerebral cortices comparing AD patients and non-diseased control individuals (see Supporting Information File Table S1) by Real Time RT-PCR. The analysis of the expression of NDM29 in 10 samples of non-demented individuals and 13 AD cases showed that the averaged expression level of the AD samples was 6-fold higher than that of non-diseased controls (Fig. 5). However, a high individual variability was observed among diseased and control samples. We thus crossed individual expression level of NDM29 with different pathological characteristics available for the patients. We found that, despite on average NDM29 is unequivocally upregulated in AD cerebral cortices, its expression is not directly associated to any of the specific conditions analyzed, thus suggesting that there might be a different factor that is able to mediate the expression of NDM29 in AD (see Supporting Information File Table S1). In any case, altogether these data suggest that in vivo NDM29 synthesis occurs in normal human brain and that it is enhanced in the brains of AD patients, possibly contributing to increased Aβ secretion.

4. Discussion

We recently published the discovery of NDM29, a pol III-transcribed ncRNA whose synthesis strongly restricts NB development. Indeed, the increased expression of NDM29 in neuroblastoma cells activates a cascade of molecular changes that, strongly restricts the malignant potential of NB cells and, leads to the acquisition of a neuron-like phenotype [16,17].

In this study, we hypothesized that the expression of NDM29 might also influence the processing of APP and/or the amyloid cascade whose perturbation are involved in AD development.

We demonstrate that the complex reorganization of cell membrane protein repertoire that attends NDM29-dependent differentiation also affects the expression and/or the enzymatic activity of key players of neurodegenerative pathways. Indeed, a high level of expression of NDM29 leads to (1) the overexpression of APP, (2) the increase of the β-secretase cleavage, (3) the consequent increase of Aβ formation and (4) the concomitant increment of Aβ x-42/Aβ x-40 ratio. Altogether, these events generate a condition previously hypothesized to favor the toxic cascade associated to the development of AD. Therefore, these findings suggest that the complex pattern of molecular changes driven by NDM29 overexpression might contribute actively to the formation of amyloid in the extracellular space favoring neurodegeneration.

Increased APP level is per se sufficient to induce AD phenotype in familial patients either bearing mutations in the promoter region of APP [32,33] or extra copies of APP as in Down's syndrome subjects. In sporadic AD cases it is hypothesized that mosaicism and local defective chromosomal instability might lead to enhanced APP levels [34,35].

In this paper we provide evidence for a further mechanism by which APP levels and processing may be modulated, related to the expression of a novel a non-coding RNA, whose levels of transcription are upregulated by inflammatory stimuli (a schematic description of the proposed model is reported in Fig. 6). Interestingly, while we identify in NDM29 overexpressing cells increased β-secretase activity, the protein content of the main components of the β-secretase complex, PS1 and PS2, is unchanged. We propose that, the increase of substrate availability can be translated in higher amount of cleavage products (see Fig. 3G).

A further demonstration of the possible role of NDM29 in the regulation of Aβ secretion in vivo was obtained by the analysis of the expression of this ncRNA in the cortex of post mortem human brain

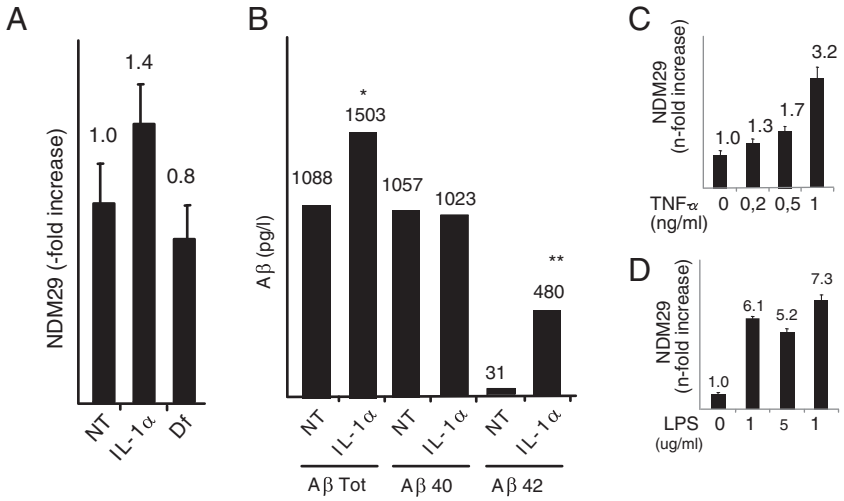

Fig. 4. Pro- and anti-inflammatory stimuli .A. Modulation of NDM29 synthesis by inflammatory and antiinflammatory agents in SKNBE *wt* cells; induction by IL-1α treatment and inhibition by Diclofenac of NDM29 expression as resulting by Real Time RT-PCR analysis. NT, untreated sample; IL-1α, Interleukin 1α; Df, Diclofenac. B. Perturbation of Aβ secretion by IL-1α treatment ($A\beta_{TOT}$ $P = 0.002$, $A\beta_{42}$ $P = 0.002$). S1.1 cell treated with different dosages of TNF-α C or LPS D.

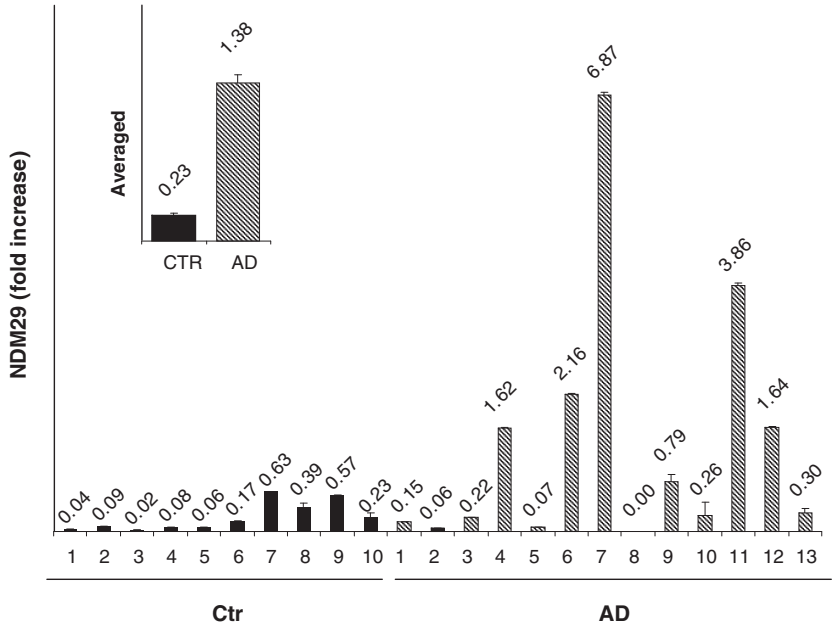

Fig. 5. NDM29 expression in brain samples .AD cases (striped bars) and non-AD control (full bars) individuals as determined by Real-Time RT-PCR. The averaged result is also reported in the inset.

samples. It is important to underline the fact that NDM29 is actually expressed in the human brain to a basal low level (see data in non-demented control brains) and that in particular, notwithstanding significant inter-individual differences, NDM29 is overexpressed in AD brains, in comparison to non-AD control samples. At the present state further experiments are needed to define the cause of the high individual variation and possibly its correlation with clinico-pathological conditions, although we can hypothesize a relationship with the individual brain inflammatory conditions. In any case, these data suggest that the overexpression of NDM29 might be directly involved in, or contribute to Aβ production in AD patients, promoting a condition that favors the onset of neurodegenerative processes. Importantly, the observation that NDM29 expression can be promoted by inflammatory mediators and, possibly, reduced by non-steroidal antiinflammatory drugs keeps in line with the hypothesis of prolonged inflammation as one of the possible causes of AD onset, a view also supported by epidemiological data. As far as IL-1α regulation of Aβ production is concerned, it is worth to note that a different pattern of APP fragments was generated (a high increase in Aβ x42 and no change in Aβ x-40) as compared to S1.1 cells (in which both fragments were induced although with a higher effect on Aβ x-42). However, we have to point out that (1) in both conditions we have the same final effect (increase of Aβ x-42/Aβx-40 ratio) correlated with high levels of NDM29; (2) the different response may reflect other IL-1α effects independent of NDM29; (3) differently from the cells treated with the cytokine, S1.1 cells are stably expressing NDM29 and thus, in the latter case, some adaptive mechanisms may be generated that somehow partially modify the ncRNA activity.

Last, the fact that a regulatory RNA that strongly restricts the malignant potential of cancer cells can promote amyloidogenesis is particularly intriguing in the light of recent data evidencing that cancer is associated to a significantly reduced risk of AD whereas AD is accompanied by a strongly decreased risk to develop cancer [14,36]. In this context NDM29 and the modulation of its synthesis might provide a molecular link between these two pathological manifestations.

In conclusion, the results presented here describe the active role played by a pol III-transcribed ncRNA in the regulation of amyloid processing (Fig. 6), providing a novel sight on AD-related processes and, possibly, its link with tumor malignant potential. In addition, these data unexpectedly show that a pol III-dependent small RNA can act as key regulator of brain physiology and/or pathology

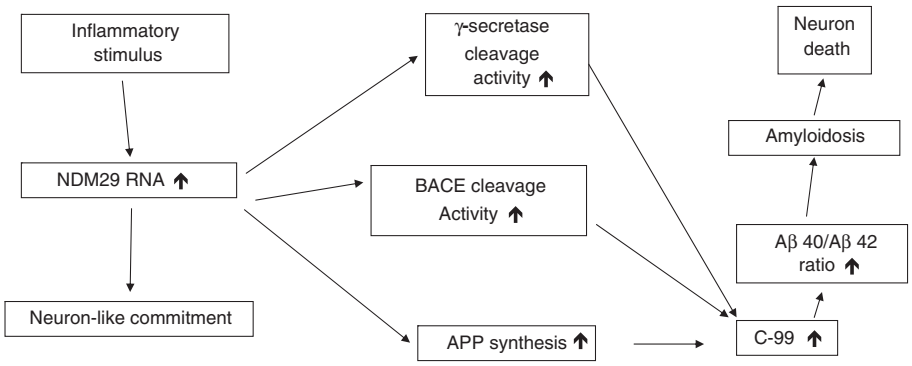

Fig. 6. Schematic model of the possible contribution of NDM29 to amyloid secretion.

suggesting that further investigation of this portion of the human transcriptome and its expression regulation is needed as it might provide further hints for neurodegeneration studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.05.001.

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