Received Date : 07-Sep-2018

Revised Date : 20-Sep-2018

Accepted Date : 22-Sep-2018

Article type : Research Letter

# SOX-11 regulates LINE-1 retrotransposon activity during neuronal differentiation

Andrés J Orqueda[1]*, Gatti Cintia R[1], Ogara María F[2], Falzone Tomás L[3, 4]

[1]Instituto de Medicina Traslacional e Ingeniería Biomédica (IMTIB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Hospital Italiano de Buenos Aires e Instituto Universitario del Hospital Italiano, Buenos AiresC1181ACH, Argentina.
[2] Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-CONICET-UBA), FCEN, UBA, Buenos Aires C1428EGA, Argentina.

[3]Instituto de Biología Celular y Neurociencias (IBCN-CONICET-UBA), Facultad de Medicina, UBA, Buenos Aires CP 1121, Argentina.

[4]Instituto de Biología y Medicina Experimental (IBYME-CONICET), Buenos Aires C1428ADN, Argentina.

* To whom correspondence should be addressed. Tel and fax: (5411)4959-0200; Email: andres.orqueda@hospitalitaliano.org.ar

## Abstract

Activity of the human long interspersed nuclear elements-1 (LINE-1) retrotransposon occurs mainly in early embryonic development and during hippocampal neurogenesis. SOX-11, a transcription factor relevant to neuronal development, has unknown functions in the control of LINE-1 retrotransposon activity during neuronal differentiation. To study the dependence of LINE-1 activity on SOX-11 during neuronal differentiation, we induced differentiation of human SH-SY5Y neuroblastoma cells and adult adipose mesenchymal stem cells (hASCs) to a

neuronal fate and found increased LINE-1 activity. We also show that SOX-11 protein binding to the LINE-1 promoter is higher in differentiating neuroblastoma cells, while knock-down of SOX-11 inhibits the induction of LINE-1 transcription in differentiating conditions. These results suggest that activation of LINE-1 retrotransposition during neuronal differentiation is mediated by SOX-11.

## 1. Introduction

The LINE-1 (long interspersed nuclear element 1) retrotransposon family is composed of repetitive elements present in mammalian genomes, capable of synthesizing DNA from self-RNA intermediary templates, and comprises17 % of the human genome. LINE-1 retrotransposons contain a 5´untranslated region and two sequences: one encoding a nucleic acid binding protein and the other an endonuclease with reverse transcriptase activity (Orf1p and Orf2p, respectively).They also contain an ORF0 sequence with anti-sense orientation, with a promoter capable of driving the synthesis of fusion proteins [1-4]. More than 80-100 transcriptionally active copies of LINE-1 have been described in the human genome [5, 6] and 3000 active copies in the mouse genome [7].

The process of LINE-1 retrotransposition occurs in cells with high pluripotency and during early stages of neural development, such as in mouse and human embryonic stem cells (ESC) [8, 9], human reprogrammed induced pluripotent stem cells (iPSC) [10, 11], differentiated neuroblastoma cells [12], and neural progenitor cells differentiating into neurons [13], among other types of cells.

Previous studies have demonstrated that LINE-1 elements can alter gene expression through mutations caused by insertions within genes, or by chromatin remodeling driven by insertions within or near genes [14]. Thus, it has been postulated that different numbers of LINE-1 copies (or quality of insertions) lead to cells with different gene expression patterns [15]. In the case of neurons, variation in the quantity and quality of LINE-1 insertions can lead to mosaicism and different neural identity [16].

The mechanisms that control LINE-1 expression are not completely understood, although DNA hypermethylation in CpG sequences in the 5´UTR has been reported as a process that downregulates LINE-1 retrotransposition [17]. Interestingly, MeCP2 was shown to repress LINE-1 activity by interacting with methylated sequences [18]. Yet, other mechanisms regulating LINE-1 activity have also been described: APOBEC3 cytidine deaminases were shown to restrict LINE-1 mobilization [19], while small interfering and piwi interacting RNAs are also believed to control expression [20]. Recently, it has been shown that mobilization of LINE-1 retrotransposons is restricted in mouse ESC by Tex19.1, a protein that directly interacts with ORF1p, stimulating its polyubiquitylation and later degradation [21].

The sex determining region Y-box 11(SRY-11) gene, also known as SOX-11, is a SOX-C gene family member that encodes a transcription factor involved in neurogenesis and tissue remodeling during embryogenesis, among other processes [22]. SOX-11 expression is necessary for neurite outgrowth and neuron survival [23]. Interestingly, two SOX-11 binding sites have been described in the LINE-1 promoter, as shown by electrophoretic mobility shift assay, while over-expression of exogenous SOX-11 increases LINE-1 promoter activity [24].

Due to the capacity forLINE-1 retrotransposition in neuronal progenitor cells, we investigated the role of SOX-11 in the activation of LINE-1 elements during neuronal differentiation. We chose as a well established and commonly used neuronal differentiation model the neuroblastoma cell line SH-SY5Y, and also human adipose-derived stem cells (hASCs), a multipotent cell type with promising potential in regenerative medicine due to its capacity to differentiate into a wide variety of tissues [25, 26].

## 2. Materials and methods

### *2.1. Cell culture and Neuronal differentiation*

SH-SY5Y cells were cultured in DMEM/F12 (Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Gibco). HeLa and 293T cells were cultured in DMEM supplemented with FBS and antibiotics. Differentiation ofSH-SY5Y cell line was

achieved with 10 μM retinoic acid (RA, Sigma) in DMEM/F12 containing 1 % FBS [27]. Neuronal differentiation was confirmed with neurite outgrowth assays with ImageJ 1.50i (Rasband, W., National Institutes of Health, USA), with positive cells showing a neurite/soma ratio of 1.5 or higher.

hASCs were obtained as described elsewhere [28] after informed consent and approval of the Ethics Committee of Research Protocols from Hospital Italiano de Buenos Aires, and cultured in DMEM with 20 % FBS, 100 U/ml penicillin, 100 μg/ml stremptomycin and 0.25 μg/ml amphotericin B. Neuronal differentiation of passages 3 to 5 subconfluent cells was achieved with a preinduction of 48 h with growth medium and 1 mM β-mercaptoethanol followed by induction with 100 μM butylated hydroxyanisole (BHA, Sigma), 1 μM RA, 10 ng/ml epidermal growth factor (EGF, Invitrogen, Brazil), and 10 ng/ml basic fibroblast growth factor (bFGF, Invitrogen) in DMEM during 14days, changing medium every 3 days [28].

### *2.2. RNA interference*

50 pmol short interfering RNA (siRNA) targeting SOX-11 (siRNA SOX11 pair #1 sense, GUCGCUGGUGGAUAAGGAUTT; anti-sense, AUCCUUAUCCACCAGCGACAG; siRNA SOX11 pair #2 sense, GACCUGAUGUUCGACCUGATT; anti-sense, UCAGGUCGAACAUCAGGUCGT; or scrambled siRNA (Invitrogen) were transfected with Lipofectamine 2000 (Invitrogen) in Optimem in SH-SY5Y cells for 24 h. After other 24 h incubation in growth medium, cells were treated with 10 μM RA in differentiation medium or with vehicle in growth medium for 48 h.

### *2.3. Transient transfections and luciferase assay*

100,000 SH-SY5Y cells plated in 12-well plates with antibiotic-free medium were transfected overnight with 4μg pL1.3-Luc plasmid (kindly provided by Dr G. Shumann), 4μg β-galactosidase control plasmid and 8μg polyethylenimine (Sigma). Next, cells were incubated in growth medium for 8 h and treated with RA. After 48 h,

luciferase activity was measured by chemoluminiscence (Promega) and normalized to β-galactosidase activity.

*2.4. DNA and RNA extraction*

Genomic DNA was isolated using a QIAmp DNA Blood mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions.

*2.5. Real time RT-qPCR*

RNA samples were first digested with RQ I DNase (Promega) according to manufacturer´s instructions. For reverse transcription, 1 µg of total RNA was used. Unless otherwise stated, the first cDNA strand was synthesized using ImProm-II Reverse Transcription System (Promega) and oligo(dT), according to manufacturer´s instructions. RNA was also retro-transcribed with random hexamers following manufacturer´s instructions. For the quantitative real-time RT-PCR (RT-qPCR), 1 µl cDNA (10-fold diluted) or 8 ng of genomic DNA were used. All reactions were conducted in a volume of 20 µl using LightCycler FastStart DNA Master SYBR Green I kit (Roche) in a LightCycler® 2.0 equipment (Roche). The oligonucleotides used for LINE-1 were reported by Coufal et al. (2009) [16], using the L1 database (http://l1base.charite.de). Sequences used were as follows: LINE-1-fw, TGCGGAGAAATAGGAACACTTTT; LINE-1-rev, TGAGGAATCGCCACACTGACT; SOX-11-fw, GGAGAGCTTGGAAGCGGAGA; SOX-11-rev, CAAGCCATGAATTCGCCCTC; RNA 5S-fw, CTCGTCTGATCTCGGAAGCTAAG; RNA 5S-rev, GCGGTCTCCCATCCAAGTAC; ACTIN-fw, CCCTTGCCATCCTAAAAGCC; ACTIN-rev, TGCTATCACCTCCCCTGTGT; LINE-1 3´ region-fw: CAAACACCGCATATTCTCACTCA; LINE-1 3´ region-rev: CTTCCTGTGTCCATGTGATCTCA; LINE-1 5´ region-fw: ACAGCTTTGAAGAGAGCAGTGGTT; LINE-1 5´ region-rev: AGTCTGCCCGTTCTCAGATCT.

Reactions were run for 40 cycles under the following conditions: 10 s at 94 °C, 10 s at 58-60 °C (according to the primers), and 10 s at 72 °C. The amplification of unique products in each reaction was verified by melting curve and ethidium bromide (Sigma)-stained agarose gel electrophoresis. The expression level of each gene was normalized to ACTIN or RNA 5S expression levels, using standard curve methods based on serial dilutions or known amounts of DNA, respectively.

### *2.6. Chromatin immunoprecipitation (ChIP)*

ChIP assays were performed as described previously [29, 30]. Briefly, chromatin was sonicated to an average of about 500 bp. Sonicated chromatin was then immunoprecipitated by using 2 µg of anti-SOX11 antibody (Santa Cruz Biotechnologies) and IgG as a control. The immunoprecipitate was collected by using protein A agarose beads (Millipore), which were washed repeatedly to remove nonspecific DNA binding. The chromatin was eluted from the beads, and cross-links were removed by incubation overnight at 65 °C. DNA was then purified and quantified by real-time PCR using Applied Biosystems StepOnePlus™ (Thermo Scientific). Primers used were: primer pair 1: GGCTTGCTTGGGTAAACAAA (forward) and CAGTCTGCCCGTTCTCAGAT (reverse); primer pair 2: TGCAGCTGGAGATCTGAGAA (forward) and GGGTTTTTGGTGTGGATGTC (reverse). The fold enrichment of target sequence in the immunoprecipitated (IP) compared to input (Ref) fractions was calculated using the comparative Ct (the number of cycles required to reach a threshold concentration) method with the equation 2Ct(Ref)-Ct(IP). Each of these values was referred to as relative abundance.

### *2.7. Statistical analysis*

Student's t-tests, difference of proportions test or ANOVA followed by Tukey's multiple comparisons test were used to detect significant differences among treatments. Normality and homogeneity of variances were tested with Shapiro-Wilks and Levene´s tests, respectively. Statistical analyses were performed with Infostat (Di Rienzo,J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo,

C.W. InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, http://www.infostat.com.ar). Differences were considered as significant if $p < 0.05$. Results are expressed as mean ± standard error of the mean (SEM) from at least three independent experiments.

## 3. Results

### *3.1. LINE-1 activity during neuronal differentiation*

The mechanisms that control LINE-1 activity during neuronal differentiation remain poorly understood, although SOX-2 and YY1 transcription factors have been shown to modulate LINE-1retrotransposition [31]. Thus, we studied the involvement of SOX-11 in the control of the activity of LINE-1 elements when neuroblastoma cell lines differentiate to polarized neuron-like cells.

Modulation of LINE-1 activity involves control of the transcription driving the synthesis of intermediary RNA molecules. To investigate this further, SH-SY5Y cell cultures were induced to differentiate into polarized neuronal cell type using10 μM RA. After 48h, LINE-1 activity was quantified by RT-qPCR using primers targeting the Orf-2 sequence (Fig. 1A) and the housekeeping gene ACTIN. Interestingly, LINE-1 RNA levels normalized to ACTIN mRNA were significantly higher (37 %) in RA-treated cells than in control cells (Fig.1B), suggesting that LINE-1 RNA expression is increased during neuronal differentiation.

Because aberrant LINE-1 transcription may occur [32], RNA levels are usually measured by RT-qPCR using cDNA primed with oligo(dT) and primers targeting the Orf-2 sequence near the 3'-end of the transcript. To also rule out the possibility of truncated or prematurely polyadenylated LINE-1 RNA affecting the qPCR-based quantification, LINE-1 RNA was retrotranscribed with random hexamers and subjected to real time PCR with primers targeting the Orf-2 sequence and also sequences 5´and 3' from Orf-2 (Fig. 1A). Not surprisingly, we found similar results, showing that RA treatment significantly increases LINE-1 RNA levels (Fig. 1 C)

Despite the significant difference, and due to the presence of many copies of the LINE-1 element, we determined whether transcript level variation corresponds to changes at the promoter level. Thus, neuroblastoma cells were transiently transfected with a reporter vector carrying a luciferase gene downstream of the active LINE-1 5´UTR sequence with promoter function [17], and then challenged with RA for 48 h. After differentiation cells were lysed and luciferase assayed. This experiment revealed a significant 3.7 folds increase in luciferase expression under control of the LINE-1 promoter after RA treatment in SH-SY5Y compared with control cells (Fig.1D). This result suggests that neuron-like differentiation induced by RA increases the promoter activity of LINE-1 elements.

After RNA transcription, LINE-1 elements are retrotranscribed and inserted into new genome sites; thus, if LINE-1 activity changes, the copy number of elements should vary. In order to determine changes in LINE-1 DNA during neuronal differentiation, as suggested by the changes in RNA levels and promoter activity, SH-SY5Y cells were treated with RA for 5 days (a longer period than that used for RNA synthesis to allow new insertions), DNA was extracted and the relative Orf-2 DNA content was analyzed by qPCR and normalized to the RNA5S gene, indicative of the relative copy number of LINE-1 elements. As shown in Fig. 1E, relative Orf-2 DNA content increased significantly (0.86 vs 1.53 relative Orf-2 DNA content, ≈1.78-fold increase) upon RA induction.

Adipose-derived mesenchymal stem cells have a great potential in regenerative medicine because of their capacity to differentiate into a vast number of cell types, including neuronal cells. Protocols for neuronal differentiation of hASCs have been exhaustively detailed in reports from our laboratory and others, and show that neuron-like cells derived from hASCs exhibit neurite outgrowth and expression of neuronal markers [33-35]. Thus, we decided to evaluate whether LINE-1 elements are activated in a model of neuronal differentiation of hASCs. Interestingly we found that neuron-like cells derived from hASCs contain higher levels of LINE-1 RNA as compared to control non-differentiated hASCs (0.60 vs 1.44 RNA levels, ≈2.4-fold increase), as shown in Fig. 2 A. We then analyzed relative Orf-2 DNA content, and observed that LINE-1 endogenous levels also increase in differentiating conditions (1.00 vs 1.24 relative Orf-2 DNA content), consistent with RNA levels (Fig. 2 B).

Thus, in neuron-like cells derived both from SH-SY5Y and hASCs, LINE-1 RNA levels are higher than in undifferentiated cells. To rule out the effects of RA on LINE-1 activity in other cell types, LINE-1 RNA levels were determined in HeLa and 293T cells treated with RA. In these cells LINE-1 activity remained unchanged under RA challenge (Fig 2. C).

### *3.2. SOX-11 expression increases during neuronal differentiation*

SOX-C transcription factors are relevant for the development of the nervous system, and two SOX-11 binding sites have been previously described in the 5´UTR of LINE-1 of 293 and NTera-2 cells. Thus, we decided to test whether variations in SOX-11 expression during neuroblastoma cells differentiation can modulate LINE-1 activity.

We first analyzed SOX-11 expression in SH-SY5Y cells undergoing differentiation with RA. SOX-11 mRNA levels quantified by RT-qPCR showed a significant increase (0.65 vs 1.75, ≈2.7-fold) in cells treated with RA for 24 h compared to control conditions (Fig. 3 A).This increase of SOX-11 RNA follows the process of SH-SY5Y differentiation, and mRNA levels remained high even at 72 h after incubation with RA (data not shown).

Next, we evaluated SOX-11 expression during differentiation of hASCs, measuring RNA levels in hASCs in control and differentiating conditions: Fig. 3 B shows that, similarly to our observations in SH-SY5Y cells, SOX-11 RNA levels are higher when cell are differentiated into neuron-like cells (1.9-fold). Thus, results obtained in hASCs strongly support that LINE-1 transcription is activated during neuronal differentiation, and that SOX-11 expression is induced.

### *3.3. SOX-11 protein binds to the 5´UTR sequence in LINE-1 during neuronal differentiation*

After showing that both LINE-1 and SOX-11 levels are increased under neuronal differentiation conditions, we tested whether SOX-11 binds to the 5´UTR of the human LINE-1 promoter. ChIP assays were performed using primers flanking two

SOX-11 binding sites observed in 3T3 and Ntera-2 cells [24], as illustrated in Fig. 4 A. Quantification of ChIP assays revealed that LINE-1 promoter sequences are enriched in SOX-11 proteins in SH-SY5Y cells treated with RA for 24 h compared with control. Primer pair 1, targeting the first SOX-11 binding site in the LINE-1 5´UTR, amplified a sequence enriched in SOX-11 proteins at higher levels in RA-treated cells compared to control (2.46 folds increase), as shown in Fig. 4 B. On the other hand, SOX-11 enrichment in the second SOX-11 binding site with primer pair 2 was also slightly higher in treated cells compared to control cells, although no significantly differences were observed. Specificity of this result is demonstrated with IgG control ChIP from RA-treated and control cells, with the latter showing levels similar to those of control cells (Fig. 4 B). Additionally, we amplified as a negative control a sequence located in the GREB1 gene known to not bind SOX-11. As expected, we did not observe recruitment of SOX-11 to this site neither in control cells nor in cells treated with RA. Moreover, the percentage of input was even lower than that obtained for the sites analyzed in the LINE-1 5'UTR (data not shown). The low percentage of input was probably due to the quantification method that compares input CT and immunoprecipitate CT: LINE-1 retrotransposons are over-represented in the genome, and the values were determined according to the formula described in material and methods, thus conducting to low input CT when compared to CT obtained for other genes. However, values of GREB1 negative control were even lower (data not shown).

These data show that SOX-11 transcription factor recruitment to binding sites in the LINE-1 promoter is increased when neuroblastoma cells differentiate into neuron-like cells.

### *3.4. SOX-11 expression regulates LINE-1 activity during neuronal differentiation*

In order to assess the relevance of SOX-11 in the rise of LINE-1 activity during neuronal differentiation, we tested 2 pairs of siRNA duplexes, pair #1 and pair #2, that interfere SOX-11 expression. RT-qPCR analysis revealed that SOX-11 was efficiently knocked down in SH-SY5Y cells with pair #2 when compared to scramble control (Fig. 5 A), although RA retained the capacity to slightly augment LINE-1 levels compared to siRNA-treated cells in non-differentiation conditions. Pair #1

yielded similar results with higher dispersion of values (data not shown). Interestingly, as shown in Fig. 5 B, the increase of LINE-1 RNA levels by RA, as observed by RT-qPCR, is lost when SOX-11 expression is inhibited (1.23 vs 0.41 LINE-1 RNA levels), suggesting that SOX-11 is key for RA-induced LINE-1 activation during neuronal differentiation.

In this context, we also analyzed neurites outgrowth as a parameter of neurogenesis. Results show that the number of cells with neuron phenotype under RA treatment remained the same in cells transfected with control scramble RNA or siRNA against SOX-11 (Fig. 5 C), suggesting that high SOX-11 mRNA levels are not needed for the induction of neuronal differentiation.

Because of the large number of cells required for ChIP assays in hASCs and the difficulties of transfection in this cell type, neither ChIP nor siRNA experiments could be performed. However, results presented here suggest that during neuronal differentiation the expression of SOX-11 increases, allowing its binding to the LINE-1 promoter and inducing LINE-1 retrotransposition.

## 4. Discussion

The LINE-1 family is composed of a great number of copies in the genome, mostly non-functional. However, about 80 copies are active in the human genome, mainly in the germline and in early embryo cells. Thus, LINE-1 activity has been widely associated with the early stages of embryonic development. Noteworthy, ORF1 expression has been observed in more than 40 % of human cancers [36], perhaps due to intrinsic endonuclease-driven tumorigenesis or mutagenic insertions [37, 38]. Importantly, repression of these elements in adult cells had been highlighted until LINE-1 retrotransposition was shown to occur in hippocampal neural progenitor cells [13]. However, mainly exogenous activity experiments have been addressed, as mainly reporter genes strategies were previously employed, while few reports combining both transfection assays and endogenous LINE-1 activity have been published such as the one describing LINE-1 retrotransposition in neural progenitor cells isolated from human fetal brain or derived from human embryonic stem cells [16].

In our study, we report that LINE-1 retrotransposition takes place when neuronal differentiation is induced with RA in neuroblastoma cells, based on the increase of LINE-1 RNA levels, Orf-2 DNA content, and LINE-1 promoter activity in RA-treated cells compared to basal conditions. Yet, we cannot rule out alterations in DNA content through mechanisms other than retrotransposition. The reason why LINE-1 RNA, promoter activity and DNA content vary differentially upon RA treatment remains elusive. Apart from differences among the times of RA treatment used for each assay, it is known that LINE-1 mobilization is controlled by promoter DNA methylation [16], APOBEC proteins [39], the exonuclease Trex1 [21] and Piwi proteins and Piwi-interacting RNAs [40], all mechanisms that affect LINE-1 activity at the transcriptional and post-transcriptional levels. Thus, kinetics studies, also using other housekeeping genes, *e.g.* SATA, due to the difference in the number of repeats among them, and extra studies of molecular mechanisms underlying retrotransposition could shed light on this.

The literature describes several protocols for the differentiation of human SH-SY5Y cells including phorbol esters such as 12-O-tetradecanoyl-phorbol-13 acetate or dibutyryl cyclic AMP, though RA has been widely used. These methods all trigger neurite outgrowth and expression of different neuronal markers in a time-dependent manner [41].

Apart from analyzing LINE-1 activity in commonly used model of neuronal differentiation of SH-SY5Y, we evaluated LINE-1 activity during neuronal differentiation of hASCs, and found a significant increase in RNA levels and a slight augmentation of relative LINE-1 DNA content in neuron-like cells derived from hASC compared to control cells. These results support the idea of LINE-1 activation in cells that are induced to differentiate into neuronal types *in vitro*. It has been recently shown that mesenchymal stem cell types -hASCs, bone marrow, and umbilical cord, isolated from adult sources- have very low levels of LINE-1 expression and retrotransposition [42].

Activity of LINE-1 was first described in proliferating early stages of development. Then, activity was observed in neuronal progenitors in the adult brain. Notably, LINE-1 retrotransposition has been recently demonstrated in mature non-dividing human neurons: using a LINE-1 cassette reporter, Macia et al. (2017) demonstrated that

LINE-1 mobilization is not restricted to somatic neuronal precursor cells in the human brain, but also takes place in mature neurons [42], suggesting that the impact of LINE-1 retrotransposition in somatic mosaicism in the human brain could be higher than previously thought.

LINE-1 retrotransposition requires an RNA intermediate that is retro-transcribed, after which the DNA copy is inserted into new genome locations. Differential LINE-1 activity in single neurons is believed to play a key role in brain mosaicism, with major effects on neuronal circuits and/or in determining neuron fate. However, in our study the identity of the LINE-1 elements retrotransposed and their localization in the genome remains to be established, a goal that could be achieved by the newly-developed technique Single-cell retrotransposon capture sequencing [43]. This technology has shed light on LINE-1 mosaicism at genomic loci expressed in hippocampal neurons: in a report by Upton et al. (2015), approximately 13.7 somatic LINE-1 insertions per hippocampal neuron were identified [44]. The authors demonstrate also that hippocampal neuron LINE-1 insertions are enriched in transcribed neuronal stem cell enhancers and hippocampus genes. Taking this into account, LINE-1 activation during hASC differentiation towards the neuronal lineage could resemble retrotransposition in hippocampal neuronal progenitor cells.

LINE-1 activity is controlled by several described mechanisms. After early embryogenesis, retrotransposition is restricted in most tissues. Not surprisingly, promoter methylation triggering LINE-1 inactivation has been deeply studied; the 5´UTR in the promoter contains a CpG island that is heavily methylated, and directs LINE-1 repression via binding of methyl-CpG-binding protein 2 (MeCP2) to methylated sequences [17]. Interestingly, a loss of function mutation affecting MeCP2 has been reported in Rett Syndrome, in which MeCP2-LINE-1 interaction is lost and high LINE-1 retrotransposition is observed [18].

Interestingly, the LINE-1 promoters in the mesenchymal stem cells were shown to be highly methylated [42]. Thus, basal low LINE-1 activity in hASCs could be explained by a high methylation status in its promoter, while the neuronal differentiation program might elicit a methylation rearrangement, including demethylation of LINE-1 promoter and its activation. Preliminary data show that LINE-1 RNA levels increase in neuron-like cells derived from hASCs treated with

DNA methyl transferases inhibitor 5-azacytidine (unpublished results); it would thus not be surprising if the RA receptor, similarly to other nuclear receptors, could modulate gene expression by recruiting chromatin remodeling complexes to specific DNA sequences.

Tumors are another case exhibiting LINE-1 activity deregulation. It has recently been proposed that LINE-1 protein expression is one key feature of many human cancers: almost half of all human high-grade malignant cancers contain high levels of LINE-1 ORF1p, which is rarely detected in early stages of tumorigenesis and is absent from normal somatic tissues [45]. Furthermore, the literature shows an association between cancer, high LINE-1 activity and LINE-1 promoter hypomethylation, even proposing LINE-1 hypomethylation as an epigenetic marker for cancer risk [46, 47]. Interestingly, a recent report shows that LINE-1 knock-down impairs telomerase activity and a consequent decreased telomere length in tumor cells [48].

SOX-11, a member of the SOX-C transcription factors family, is relevant to neuronal development and neurogenesis, being expressed mainly in neural cells that have already been committed to neuronal differentiation [49]. In the chick embryo, SOX-4 and SOX-11 activity is repressed by the transcriptional repressor protein REST/NRSF; upon differentiation SOX-4 and SOX-11 then function as transcriptional activators establishing pan-neuronal protein expression [50]. In the present study, we found that SOX-11 expression increases during neuronal differentiation of SH-SY5Y cells. Neuronal-like cells derived from hASCs also contain higher SOX-11 mRNA levels compared to control non-differentiated cells. Interestingly, we show that SOX-11 binds to LINE-1 promoter sequences in neuron-like cells derived from SH-SY5Y cells, and that the inhibition of SOX-11 expression in neuroblastoma cells blocked LINE-1 activity during neuronal differentiation. These results highlight the importance of SOX-11 in neuronal differentiation. Notably, members of the SOX-C family share functions, so SOX-11 may act redundantly with SOX-4 and -12 [51].

Our results show that, in spite of being necessary for LINE-1 transcription (as observed in SOX-11 knock-down assays), SOX-11 inhibition is not sufficient to repress neuronal differentiation of SH-SY5Y cells. It has been previously reported

that SOX-11 shares redundant functions with SOX-4 in neurogenesis in the mouse brain [52]. Thus, it seem possible that SOX-4 or -12 carry out functions in neuronal differentiation without affecting LINE-1 activity. However, we cannot exclude that SOX-4 and -12 bind LINE-1 sequences, or participate in the control of its retrotransposition.

Finally, SOX-11 and LINE-1 might be related to cancer. SOX-11 expression in chronic lymphocytic leukemia, among a number of human cancers, was shown to correlate with adverse prognostic markers such as Immunoglobulin heavy chain variable regionmutational status [53]. It seems possible that deregulation of SOX-11 expression has effects on abnormally high levels of LINE ORF1-encoded protein observed in different tumors. Thus, the study of LINE-1 retrotransposition mediated by SOX-11 will shed light both on brain neurogenesis and on tumor progression processes.

**Acknowledgement**

We thank Dr. G. Schumann and Dr. Pecci for plasmids pL1.3-Luc and beta-galactosidase, respectively. Dr. C. Brandi is acknowledged for fat tissue supply, and M. Ielpi for technical culture support.

## References

1- Martin, S.L. and Bushman, F.D. (2001) Nucleic acid chaperone activity of the ORF1 protein from the mouse LINE-1 retrotransposon. Mol. Cell. Biol., 21(2), 467-75.

2- Dombroski, B.A., Feng, Q., Mathias, S.L., Sassaman, D.M., Scott, A.F., Kazazian, H.H. Jr. and Boeke, J.D. (1994) An in vivo assay for the reverse transcriptase of human retrotransposon L1 in Saccharomyces cerevisiae. Mol. Cell. Biol., 14(7), 4485-92.

3- Feng, Q., Moran, J.V., Kazazian, H.H. Jr. and Boeke, J.D. (1996) Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition. Cell, 87(5), 905-16.

4- Denli, A.M., Narvaiza, I., Kerman, B.E., Pena, M., Benner, C., Marchetto, M.C., Diedrich, J.K., Aslanian, A., Ma, J., Moresco, et al. (2015) Primate-specific ORF0 contributes to retrotransposon-mediated diversity. Cell, 63(3), 583-93.

5- Brouha, B., Schustak, J., Badge, R.M., Lutz-Prigge, S., Farley, A.H., Moran, J.V. and Kazazian, H.H. Jr. (2003) Hot L1s account for the bulk of retrotransposition in the human population. Proc. Natl. Acad. Sci. USA, 100(9), 5280-5.

6- Szak, S.T, Pickeral, O.K., Makalowski, W., Boguski, M.S., Landsman, D. and Boeke, J.D. (2002) Molecular archeology of L1 insertions in the human genome. Genome Biol., 3(10), research0052.

7- Goodier, J.L, Ostertag, E.M., Du, K. and Kazazian, H.H Jr. (2001) A novel active L1 retrotransposon subfamily in the mouse. Genome Res., 11(10), 1677-85.

8- Packer, A.I., Manova, K. and Bachvarova, R.F. (1993). A discrete LINE-1 transcript in mouse blastocysts. Dev. Biol., 157(1), 281-283.

9- Garcia-Perez, J.L., Marchetto, M.C., Muotri, A.R., Coufal, N.G., Gage, F.H., O'Shea, K.S. and Moran, J.V. (2007) LINE-1 retrotransposition in human embryonic stem cells. Hum. Mol. Genet., 16(13), 1569-77.

10- Klawitter, S., Fuchs, N.V., Upton, K.R., Muñoz-Lopez, M., Shukla, R., Wang, J., Garcia-Cañadas, M., Lopez-Ruiz, C., Gerhardt, D.J., Sebe, A., et al. (2016) Reprogramming triggers endogenous L1 and Alu retrotransposition in human induced pluripotent stem cells. Nat. Commun., 7, 10286.

11- Wissing, S., Muñoz-Lopez, M., Macia, A., Yang, Z., Montano, M., Collins, W., Garcia-Perez, J.L., Moran, J.V. and Greene, W.C. (2012) Reprogramming somatic cells into iPS cells activates LINE-1 retroelement mobility. Hum. Mol. Genet., 21(1), 208-18.

12- Del Re, B., Marcantonio, P., Capri, M. and Giorgi, G. (2010) Evaluation of LINE-1 mobility in neuroblastoma cells by in vitro retrotransposition reporter assay: FACS analysis can detect only the tip of the iceberg of the inserted L1 elements. Exp. Cell. Res., 316(20), 3358-67.

13- Muotri, A.R., Chu, V.T., Marchetto, M.C., Deng, W., Moran, J.V. and Gage, F.H. (2005) Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature, 435(7044), 903-10.

14- Babushok, D.V. and Kazazian, H.H. Jr. (2007) Progress in understanding the biology of the human mutagen LINE-1. Hum. Mutat., 28(6), 527-39.

15- Thomas, C.A., Paquola, A.C. and Muotri, A.R. (2012) LINE-1 retrotransposition in the nervous system. Annu. Rev. Cell. Dev. Biol., 28, 555-573.

16- Coufal, N.G., Garcia-Perez, J.L., Peng, G.E., Yeo, G.W., Mu, Y., Lovci, M.T., Morel, l M., O'Shea, K.S., Moran, J.V. and Gage, F.H. (2009) L1 retrotransposition in human neural progenitor cells. Nature, 460(7259), 1127-31.

17- Yu, F., Zingler, N., Schumann, G. and Stratling, W.H. (2001) Methyl-CpG-binding protein 2 represses LINE-1 expression and retrotransposition but not Alu transcription. Nucleic Acids Res., 29, 4493-4501.

18- Muotri, A.R., Marchetto, M.C., Coufal, N.G., Oefner, R., Yeo, G., Nakashima, K. and Gage, F.H. (2010) L1 retrotransposition in neurons is modulated by MeCP2. Nature, 468(7322), 443-6.

19- Chiu, Y.L. and Greene, W.C. (2008) The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. Annu. Rev. Immunol.,26, 317-353.

20- Pezic, D., Manakov, S.A., Sachidanandam, R. and Aravin, A.A. (2014) piRNA pathway targets active LINE1 elements to establish the repressive H3K9me3 mark in germ cells. Genes Dev., 28(13), 1410-28.

21- MacLennan, M., García-Cañadas, M., Reichmann, J., Khazina, E., Wagner, G., Playfoot, C.J., Salvador-Palomeque, C., Mann, A.R., Peressini, P., Sanchez, L., et al. (2017) Mobilization of LINE-1 retrotransposons is restricted by Tex19.1 in mouse embryonic stem cells. Elife, 6. Pii, e26152.

22- Penzo-Méndez, A.I. (2010) Critical roles for SoxC transcription factors in development and cancer. Int. J. Biochem. Cell Biol., 42(3), 425-8.

23- Jankowski, M.P., Cornuet, P.K., McIlwrath, S., Koerber, H.R., Albers, K.M. (2006) SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth. Neuroscience, 143(2), 501-14.

24- Tchénio, T., Casella, J.F. and Heidmann, T. (2000) Members of the SRY family regulate the human LINE retrotransposons. Nucleic Acids Res., 28(2), 411-5.

25- Schaffler, A. and Buchler, C. (2007) Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. Stem cells, 25(4), 818-827.

26- Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P. and Hedrick, M.H. (2002) Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell, 13(12), 4279-4295.

27- Sharma, M., Sharma, P. and Pant, H.C. (1999) CDK-5-mediated neurofilament phosphorylation in SHSY5Y human neuroblastoma cells. J. Neurochem., 73(1), 79-86.

28- Cardozo, A., Ielpi, M., Gómez, D. and Argibay, A.P. (2010) Differential Expression of Shh and BMP Signaling in the Potential Conversion of Human Adipose Tissue Stem Cells Into Neuron-Like Cells In Vitro. Gene Expression, 14(6), 307-319.

29- Strutt, H. and Paro, R. (1999) Mapping DNA target sites of chromatin proteins in vivo by formaldehyde crosslinking. Methods Mol. Biol., 119, 455-467.

30- Vicent, G.P., Nacht, A.S., Zaurin, R., Font-Mateu, J., Soronellas, D., Le Dily, F., Reyes, D. and Beato, M. (2013) Unliganded progesterone receptor-mediated targeting of an RNA-containing repressive complex silences a subset of hormone-inducible genes. Genes Dev., 27(10), 1179-97.

31- Athanikar, J.N., Badge, R.M. and Moran, J.V. (2004) A YY1-binding site is required for accurate human LINE-1 transcription initiation. Nucleic Acids Res., 32(13), 3846-55.

32- Perepelitsa-Belancio, V. and Deininger, P. (2003) RNA truncation by premature polyadenylation attenuates human mobile element activity. Nat. Genet., 35(4), 363-6.

33- Mareschi, K., Novara, M., Rustichelli, D., Ferrero, .I, Guido, D., Carbone, E., Medico, E., Madon, E., Vercelli, A. and Fagioli, F. (2006) Neural differentiation of human mesenchymal stem cells: Evidence for expression of neural markers and eag $K^+$ channel types. Exp. Hematol., 34, 1563-1572.

34- Cardozo, A.J., Gomez, D.E. and Argibay, PF. (2011) Transcriptional characterization of Wnt and Notch signaling pathways in neuronal differentiation of human adipose tissue-derived stem cells. J. Mol. Neurosci., 44(3), 186-194.

35- Cardozo, A.J., Gómez, D.E. and Argibay, P.F. (2012) Neurogenic differentiation of human adipose-derived stem cells: relevance of different signaling molecules, transcription factors, and key marker genes. Gene, 511(2), 427-36.

36- Rodić, P., Kraguljac Kurtovic, N., Suvajdzic Vukovic, N., Djordjevic, M., Mitrovic, M., Sumarac, Z. and Janic, D. (2014) Flow cytometric assessment of lymphocyte subsets in Gaucher type 1 patients. Blood Cells Mol. Dis., 53(4), 169-70.

37- Lee, E., Iskow, R., Yang, L., Gokcumen, O., Haseley, P., Luquette, L.J. 3rd., Lohr, J.G., Harris, C.C., Ding, L., Wilson, et al. Cancer Genome Atlas Research Network. (2012) Landscape of somatic retrotransposition in human cancers. Science, 337(6097), 967-71.

38- Shukla, R., Upton, K.R., Muñoz-Lopez, M., Gerhardt, D.J., Fisher, M.E., Nguyen, T., Brennan, P.M., Baillie, J.K., Collino, A., Ghisletti, S., et al. (2013) Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. Cell, 153(1), 101-11.

39- Horn, A.V., Klawitter, S., Held, U., Berger, A., Vasudevan, A.A., Bock, A., Hofmann, H., Hanschmann, K.M., Trösemeier, J.H., Flory, E., et al. (2014) Human LINE-1 restriction by APOBEC3C is deaminase independent and mediated by an ORF1p interaction that affects LINE reverse transcriptase activity. Nucleic Acids Res., 42(1), 396-416.

40- Malone, C.D. and Hannon, G.J. (2009) Small RNAs as guardians of the genome. Cell, 136, 656-68.

41- Kovalevich, J. and Langford, D. (2013) Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol. Biol., 1078, 9-21.

42- Macia, A., Widmann, T.J., Heras, S.R., Ayllon, V., Sanchez, L., Benkaddour-Boumzaouad, M., Muñoz-Lopez, M., Rubio, A., Amador-Cubero, S., Blanco-Jimenez, E., et al. (2017) Engineered LINE-1 retrotransposition in non dividing human neurons. Genome Res., 27(3), 335-348.

43- Sanchez-Luque, F.J., Richardson, S.R. and Faulkner, G.J. (2016) Retrotransposon Capture Sequencing (RC-Seq): A Targeted, High-Throughput Approach to Resolve Somatic L1 Retrotransposition in Humans. Methods Mol. Biol., 1400, 47-77.

44- Upton, K.R., Gerhardt, D.J., Jesuadian, J.S., Richardson, S.R., Sánchez-Luque, F.J., Bodea, G.O., Ewing, A.D., Salvador-Palomeque, C., van der Knaap, M.S., Brennan, P.M., et al. (2015) Ubiquitous L1 mosaicism in hippocampal neurons. Cell, 161(2), 228-39.

45- Rodić, N., Sharma, R., Sharma, R., Zampella, J., Dai, L., Taylor, M.S., Hruban, R.H., Iacobuzio-Donahue, C.A., Maitra, A., Torbenson, M.S., et al. (2014) Long interspersed element-1 protein expression is a hallmark of many human cancers. Am. J. Pathol., 184(5), 1280-6.

46- Barchitta, M., Quattrocchi, A., Maugeri, A., Vinciguerra, M. and Agodi, A. (2014) LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: a systematic review and meta-analysis. PLoS One, 9(10), e109478.

47- Ardeljan, D., Taylor, M.S., Ting, D.T. and Burns, K.H. (2017) The Human Long Interspersed Element-1 Retrotransposon: An Emerging Biomarker of Neoplasia. Clin. Chem., 63(4), 816-822.

48- Aschacher, T., Wolf, B., Enzmann, F., Kienzl, P., Messner, B., Sampl, S., Svoboda, M., Mechtcheriakova, D., Holzmann, K. and Bergmann, M. (2016) LINE-1 induces hTERT and ensures telomere maintenance in tumour cell lines. Oncogene, 35(1), 94-104.

49- Uwanogho, D., Rex, M., Cartwright, E.J., Pearl, G., Healy, C., Scotting, P.J., and Sharpe, P.T. (1995) Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. Mech. Dev., 49, 23-36.

50- Bergsland, M., Werme, M., Malewicz, M., Perlmann, T. and Muhr, J. (2006) The establishment of neuronal properties is controlled by Sox4 and Sox11. Genes Dev., 20, 3475-3486.

51- Sock, E., Rettig, S.D., Enderich, J., Bosl, M.R., Tamm, E.R. and Wegner, M. (2004) Gene targeting reveals a widespread role for the high-mobility-group transcription factor Sox11 in tissue remodeling. Mol. Cell. Biol., 24, 6635-6644.

52- Feng, W., Khan, M.A., Bellvis, P., Zhu, Z., Bernhardt, O., Herold-Mende, C. and Liu, H.K. (2013) The chromatin remodeler CHD7 regulates adult neurogenesis via activation of SoxC transcription factors. Cell Stem Cell, 13(1), 62-72.

53- Roisman, A., Stanganelli, C., Nagore, V.P., Richardson, G.V., Scassa, M.E., Bezares, R.F., Cabrejo, M. and Slavutsky, I. (2015) SOX11 expression in chronic lymphocytic leukemia correlates with adverse prognostic markers. Tumour Biol., 36(6), 4433-40.

**Figure legends**

Figure 1. LINE-1 is activated during neuronal differentiation of SH-SY5H cells. **A.** Schematic sequence of LINE-1 showing the regions of annealing of the primers used in this study. 5´UTR is the 5´untranslated region, while ORF1 encodes a nucleic acid binding protein and ORF2 encodes an endonuclease with reverse transcriptase activity.**B.** RNA levels increase in neuroblastoma cells differentiating into neuron-like cells. SH-SY5H were grown in growth medium (CONTROL) or differentiation medium with retinoic acid (RA) for 48 h; RNA was collected and LINE-1 sequences quantified by RT-qPCR. Mean ± Standard error of the mean (SEM) values of LINE-1 relative to ACTIN levels with n = 3 are shown. **C.** Similarly to B, after treatment with RA for 48 h, RNA was retrotranscribed with random hexamers and 3 LINE-1 sequences were quantified by RT-qPCR: Orf-2 sequence, 5´ region and 3´ end. Mean ± SEM values of LINE-1 relative to ACTIN levels with n = 3 are shown. **D.** RA induces LINE-1 promoter activity. SH-SY5H cells were transiently transfected with a LINE-1 5´UTR reporter plasmid and β-galactosidase vector. Then cells were incubated with vehicle (CONTROL) or RA for 48 h (RA). Mean ± SEM values of luciferase activity normalized to β-gal with n = 3 are shown. **E.** SH-SY5H cells were stimulated with RA for 7 days; next, genomic DNA was collected and LINE-1 ORF2 sequences were quantified by qPCR. Mean ± SEM values of LINE-1 relative to RNA5S levels with n = 6 are shown. In all cases, Student's t-tests were used.

Figure 2. LINE-1 is activated during neuronal differentiation of hASCs. **A.** hASCs were grown in growth medium (CONTROL) or differentiation medium (DIF) for 14 days; RNA was collected and LINE-1 sequences were quantified by RT-qPCR. Mean ± SEM values of *LINE-1* relative to ACTIN levels with n = 3 are shown. **B.** Genomic DNA was collected from control (CONTROL) hASCs or cells differentiated for 14 days (DIF) and LINE-1 ORF2 sequences quantified qPCR. Mean ± SEM values of LINE-1 relative to RNA5S levels and normalized to control are shown, with * $p< 0.05$ and n = 4. **C.** RNA levels remain unchanged in 293T and HeLa cells treated with RA. Cells were grown in growth medium (CONTROL) or differentiation medium with RA for 48 h (RA); LINE-1 RNA sequences were quantified by RT-qPCR. Mean ± SEM values of LINE-1 relative to ACTIN levels with n = 3 are shown. In all cases, Student's t-tests were used.

Figure 3. SOX-11 expression increases in neuronal differentiation conditions. **A.** SH-SY5Y cells were challenged with RA (RA) or vehicle (CONTROL) for 24 h. Total RNA was collected and SOX-11 was quantified by RT-qPCR Mean ± SEM values of SOX-11 RNA levels normalized to ACTIN with n = 3 are shown. **B.** hASCs were cultured in growth medium (CONTROL) or in neuronal differentiation conditions (DIF) for 48 h. Then SOX-11 was quantified by RT-qPCR. Mean ± SEM values of SOX-11 RNA levels normalized to ACTIN and normalized to control are shown, with * $p< 0.05$ and n = 6. In all cases, Student's t-tests were used.

Figure 4. Binding of SOX-11 to the LINE-1 5´UTR during neuronal differentiation. **A.** Schematic sequence of the 5´UTR showing two consensus SOX-11 binding sites, the upstream ATG codon, and the binding sites of the 2 primer pairs used in the study. **B.** ChIP-qPCR for SOX-11 in SH-SY5Y cells treated with RA for 24 h, using primers for the two sequences in the LINE-1 5´UTR amplified by primer pairs 1 and 2, as described in **A**. Mean ± SEM values of % of input, with n = 3. In all cases, Student's t-tests were used.

Figure 5. SOX-11 is necessary for LINE-1 activation during neuronal differentiation. SH-SY5Y cells were transfected with control scrambled siRNA (SCRAMBLE) or siRNA against SOX-11 (siRNA SOX-11). Next, cells were treated with RA (RA) or vehicle (CONTROL) for 48 h. **A.** RNA was collected and SOX-11 was quantified by RT-qPCR. Mean ± SEM values of SOX-11 RNA levels normalized to ACTIN with n = 3 are shown. ANOVA followed by Tukey's multiple comparisons test was used. **B**. Similarly to A, mean ± SEM values of LINE-1 RNA levels normalized to ACTIN quantified by RT-qPCR are shown, with n = 3. ANOVA followed by Tukey's multiple comparisons test was used. **C**. The length of neurites and cell bodies of cells was measured and the percentage of cells exhibiting neurite outgrowth was estimated, considering cells as differentiated when the neurite/soma ratio was > 1.5. Mean ± SEM values of % of cells with neurite/soma ratio > 1.5 with n = 3 (including the averages of 3 fields in each case) are shown. * $p < 0.05$. Difference of proportions test was used.

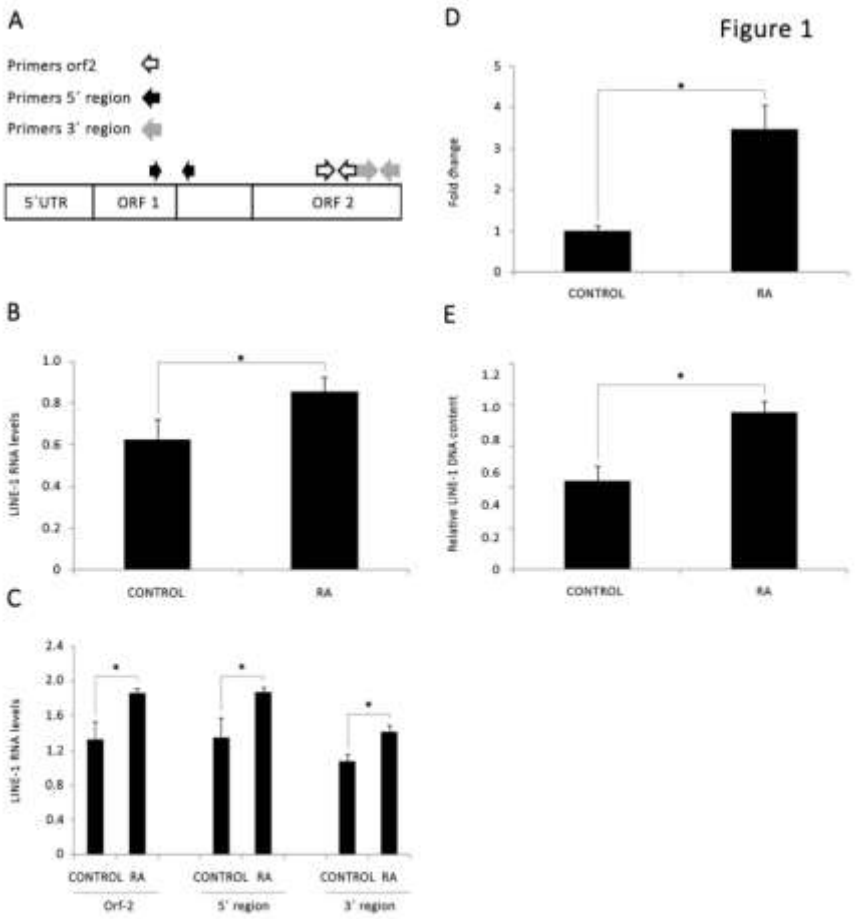
## Funding

This work was supported by the Instituto Universitario Grant (Instituto Universitario, Hospital Italiano de Buenos Aires) and PICT-2016-1719 (Agencia Nacional de Promoción Científica y Técnica).

## Conflict of interest

Conflict of interest statement: none declared.

Figure 1



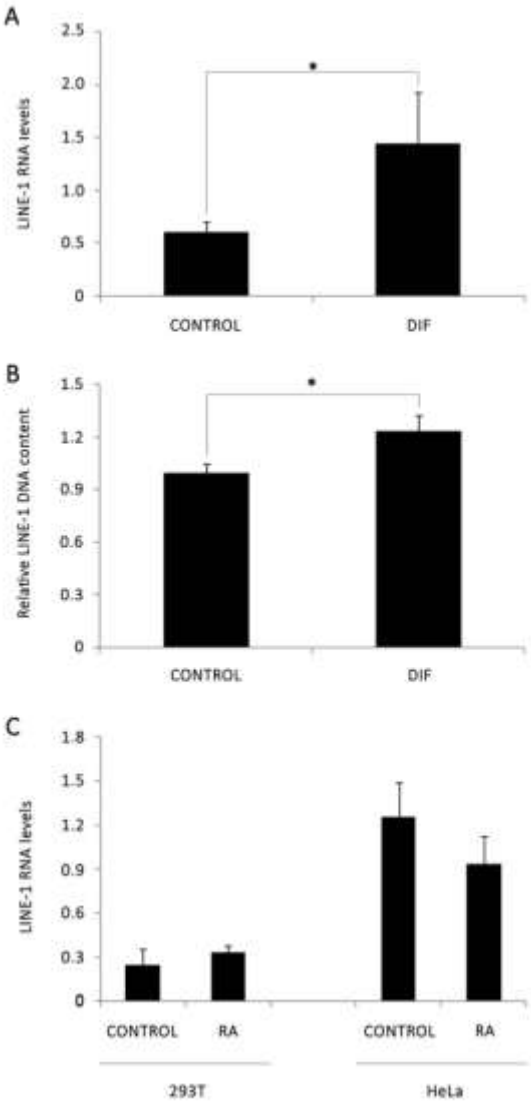



Figure 2

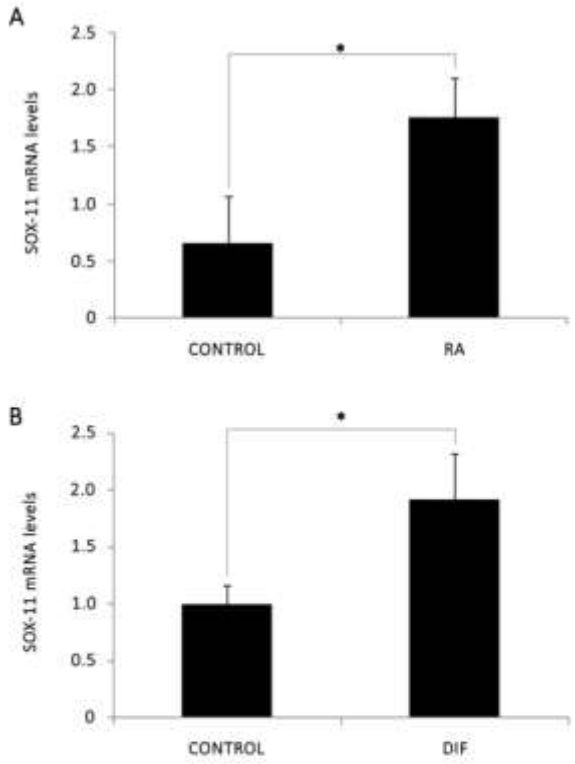
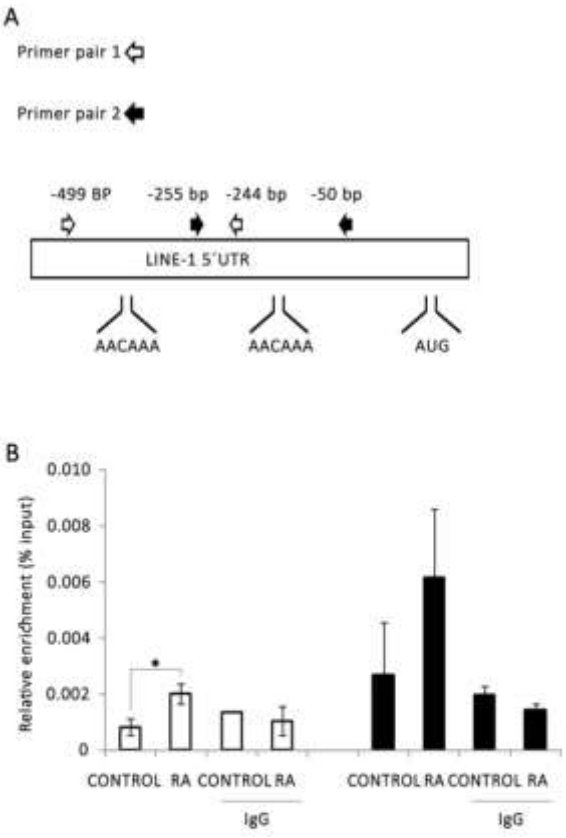



Figure 3

Figure 4



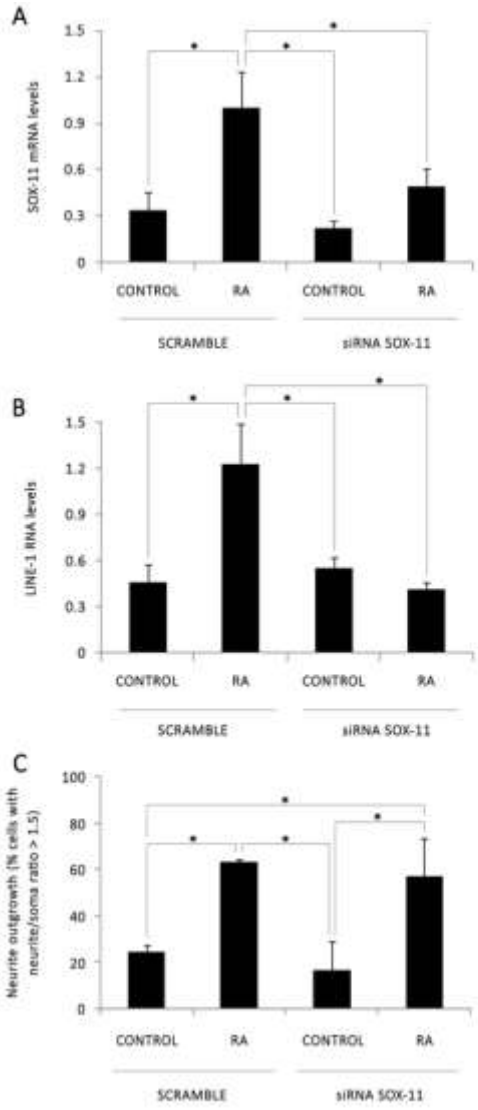



Figure 5