UCHL1 PROTEIN SYNTHESIS UPON RAPAMYCIN TREATMENT INVOLVES ITS ANTISENSE RNA THROUGH EMBEDDED SINEB2 REPEAT.

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Declaration

The work described in this thesis was carried out at International School for Advanced Studies, Trieste, between October 2006 and September 2010 with exception of

-Polysome fractionation that was performed at Laboratory of Molecular Histology and Cell Growth, DIBIT, San Raffaele, Milano, under supervision of Prof. Stefano Biffo.

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Long non-coding antisense RNA controls UCHL1 translation through the 5' overlapping region and an embedded SINEB2 repeat.

Abbreviations:

lncRNAs, long non-coding RNAs

ncRNAs, non-coding RNAs

NATs, Natural Antisense Transcripts

DA, Dopaminergic neurons

PD, Parkinson's Disease

SN, *Substantia Nigra*

TH, Tyrosine Hydroxilase

LCM, Laser Capture Microdissection

D-FISH, Double Fluorescent *In-Situ* Hybridization

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1. INTRODUCTION

The initial description of genomes organization has consisted in the separation between regulatory and protein-coding DNA stretches.
This simple and elegant model has supported the “one region-one function” theory: a genome is a linear arrangement of functional elements interspersed with non-functional regions.
Recently the advances in transcriptomics technologies have shown that a genomic region can be used for different purposes and that functional elements can co-locate in the same region of the genome.
In-depth analysis of the transcriptional output of human-mouse and fly genome has revealed that the information content of the genome is growing exponentially along with the organism complexity (Taft et al. 2007).
Estimates made by the Encyclopedia of DNA elements (ENCODE) predicts that as much as 93% of the genomic sequences seems capable of being transcribed (Birney et al. 2007).
Pervasive transcription of the genome is mainly due to long non-coding RNAs (ncRNAs) and transcribed repetitive sequences, such as SINEs (short interspersed nuclear elements) and LINEs (long interspersed nuclear elements).

1.1 Non-coding RNAs

In the recent version of the genome annotation (Ensembl 60 release) the coding regions of the human genome are estimated to occupy ~ 34 Mb, which means 1.2% of the euchromatic genome. Furthermore, both cDNA libraries and genome tiling array studies proved that the portion of the expressed transcripts is underestimated (Bertone et al. 2004; Carninci et al. 2005; Cheng et al. 2005; Jongeneel et al. 2005; Kapranov et al. 2005). Large scale cDNA analysis of the mouse genome revealed that in front of 22287 protein coding transcripts, additional 34000 do not appear to encode for proteins.
By an integrated analysis of genome tiling arrays (Cheng et al. 2005) and massively parallel signature sequencing (MPPS) data (Jongeneel et al. 2005) many thousands of non-coding transcripts have been located to intronic sequences and over 37% of MPPS signatures have matched known loci outside of annotated exons, interestingly, 20% of them reside in the complementary strand of known transcripts. These data

suggest the transcription of at least 50000 different RNAs that have not been previously annotated in the human genome.
A large portion of these transcripts belongs to two RNA fractions that have not been previously characterized in details: poly A- RNAs and the nuclear-restricted poly A+ RNAs (Kiyosawa et al. 2005).
Until recently, most of the known non-coding RNAs fulfilled the relative generic function of being ‘infrastructural’ RNAs, such as rRNAs and tRNAs (involved in translation), spliceosomal uRNAs and small nuclear RNAs (snRNAs) (involved in splicing) and small nucleolar RNAs (snoRNAs) (involved in the modification of rRNAs).
Besides their structural role, some of these ncRNAs partecipates in regulatory processes. For examples, U1 RNA is an interactor of TFIIH and regulates RNA transcription (Kwek et al. 2002); small RNAs with similarity to box H/ACA sno RNA are components of telomerase and mutated in a genetic form of dyskeratosis congenital (Vulliamy et al. 2001); 7SL RNA is a component of the Signal Recognition Particle (SRP) that plays a key role in the delivery to the ER for proteins with a leader sequence (Nagai et al. 2003).

The world of ncRNA adds new members on almost a daily basis. Several types of classification have been proposed based on the length of the RNA species, their locations on the genome and their function.
Here I will divide ncRNAs in small and long RNAs and I will provide representative examples for each class.

1.1.1 Small nucleolar RNAs

Sno-RNAs generally range from 60 to 300 nucleotides and guide the site-spcific modification of nucleotides in target RNAs via short region of base-pairing. There are two classes of snoRNAs: the box C/D snoRNAs that guide the O’-ribose-methylation, and the H/ACA box which drives the pseudouridylation of target RNA. Initially it was thought that snoRNAs functions were restricted to rRNA modification in ribosome biogenesis, given their specific nucleolar locazation. Now it is evident that that they can target other cellular RNAs, as evidenced by the snoRNA involved in the

aberrant splicing of the serotonin receptor 5-HT-(2C)R gene in Prader-Willi syndrome patients (Kishore and Stamm 2006).
Although the snoRNA involved in ribosome biogenesis are located in the nucleolus, a subset of C/D snoRNAs is located into Cajal bodies (Meier 2005). Most of them come from intronic regions of protein-coding genes, but apparently some snoRNAs are independently transcribed as evidenced by the presence of methylated guanosine at their 5' end (Kiss 2002).

1.1.2 MicroRNAs and small interfering RNAs

miRNA and siRNA are 22 bps nucleotides long RNAs that derives from stem-loop or double-stranded RNA precursors. These miRNAs are transcribed by RNA polymerase II from introns of numerous protein-coding and non-coding genes (Lee et al. 2004; Rodriguez et al. 2004) as primary miRNA (pri-miRNA) transcripts. The hairpin structure formed in these primary miRNA transcripts are processed by RNAse-III enzyme Drosha into pre-miRNA and finally into mature miRNA by Dicer.
The mature miRNA is then incorporated into RISC complex and guides the RISC to the 3' UTR of target mRNA which results into either cleavage, or translational inhibition of the target mRNA (Bartel 2004). It is thought that the nature of base pairing determines whether the target RNA will be degraded or translation will be inhibited (Bartel 2004).
miRNA suppress translation via non-perfect base-pairing at the 3' UTR of target mRNAs or cause degradation of target RNA via activation of the RISC endonucleolitic complex in case of perfect complementarity whit the target site (RNA interference). miRNA derived from introns or exons of numerous protein-coding and non-coding genes (Lee et al. 2004; Rodriguez et al. 2004) as well as from retrotransposon sequences (Smalheiser and Torvik 2005). The expression of miRNA is a process tightly regulated and has been estimated central in several processes including cell proliferation (Brennecke et al. 2003), left-right patterning, neuronal gene expression (Klein et al. 2005), cell-fate, brain morphogenesis (Giraldez et al. 2005), muscle differentiation (Naguibneva et al. 2006) and stem-cell division (Croce and Calin 2005). miRNA have also a unequivocal role in human diseases, for example sequence variants in the binding site for *miR-189* in the SLITRK1 mRNA have been linked to Tourette's syndrome (Abelson et al. 2005). miRNA expression is clearly

disregulated in cancer cells (Iorio et al. 2005). As an other example of involvement in cancer, the proto-oncogene *c-myc* drives the expression of a cluster of six miRNAs on human chromosome 13; this cluster contains two miRNA (*miR17-5p* and *miR-20a*) that act as translational downregulator of the E2F1 factor that is a cell-cycle progressor (O'Donnell et al. 2005). Dysregulation of *miR17-5p* and *miR-20a* is observed in various cancer cell models.
Interestingly, miRNA were thought to interfere with translation of target mRNAs by incomplete base pairing at the 3'UTR. However, in 2004 two groups described that HOX gene suppression is dependent on a perfect match between the target mRNA and the miRNA which was causing post-transcriptional gene silencing via RNAi mechanism (Yekta et al. 2004).
Recent studies have shown that miRNA do not require evolutionary conservation and many newly discovered human RNA seem to be primate specific and drive 'higher'-mammals' specific fine tuning in gene regulation (Bentwich et al. 2005).

1.1.3 Piwi-interacting RNA (piRNA)

Piwi interacting RNAs were first identified from mouse testis as component of MILI ribonucleoprotein complex (Aravin et al. 2006). These RNAs are 24-30 nucleotides long and are generated by a Dicer independent mechanism. The mature piRNAs have 5' uridine monophosphate and a 2 -*O*-methylated 3' end (Aravin et al. 2006). Piwi family proteins are a subclass of argonaute proteins found in all multicellular organisms with a highly conserve expression pattern in the germ cells which indicates their importance in germ cell development (Aravin et al. 2007a).
Mutation in the Piwi proteins in *Drosophila* results in the activation of transposons and retrotransposons and loss of germline stem cells in both males and females (Cox et al. 1998). The *Drosophila* Piwi-associated small RNA are derived mostly from the repeat regions and retrotransposons and are therefore called repeat associated siRNA (rasiRNA) (Brennecke et al. 2007). However, in the case of mouse only a small subset of piRNA are derived from repeat regions and retrotransposons (Aravin et al. 2006). Interestingly, mutation of the Piwi homolog MIWI, MIWI2 and MILI leads to the degeneration of male germline (Aravin et al. 2007b).

1.1.4 Promoter-associated small RNAs (PASRs)

Promoter-associated small RNAs (PASRs) are broadly defined as short transcripts within a few hundreds bases of the transcription start site (TSS) of protein-coding and noncoding RNAs. They have been described in all major eukaryotic lineages. An hidden repertoire of TSS-proximal transcripts first emerged from gene expression studies after removal of specific exosome components in plants and human cells (Chekanova et al. 2007; Preker et al. 2008). Small RNAs of at least 70 nts long co-linear with the 5’ end of mRNAs have been then described in almost all genes in physiological conditions (Guenther et al. 2007).

At least three classes of these RNAs have been identified to date: 1) Promoter-associated small RNAs (PASRs) are generally 20–200 nt long, capped, with 5' ends that coincide with the TSSs (Fejes-Toth 2009; Kapranov et al. 2007a). They have been first detected using genome-wide tiling arrays of human cell lines and subsequently analyzed with high-throughput sequencing. Their biogenesis is not well understood, although it may result from transcription of independent, capped, short transcripts or from cleavage of larger capped (m)RNAs (Fejes-Toth 2009); 2) Transcription start site associated RNAs (TSSa-RNAs) are 20–90 nt long and localized within -250 to+50 of TSSs (Seila et al. 2008); 3) transcription initiation RNA (tiRNA) are predominantly 18 nt in length and found in human, mouse, chicken and Drosophila. Their highest density occurs just downstream of TSSs with positional conservation across species (Kapranov et al. 2007a; Taft et al. 2009).

All three classes are strongly associated with highly expressed genes and with regions of RNA Pol II binding. They are weakly expressed, showing a bidirectional distribution that mirrors RNA Pol II (Fejes-Toth 2009).

Interestingly, several studies have indicated that short 19-21 nts RNAs directed to promoter regions can be regulators of gene expression. In some cases gene silencing has been induced (Morris et al. 2004), whereas in others examples gene activation was surprisingly triggered (Janowski et al. 2007). Interestingly, analysis of PASRs-mimetics (20-250 nts) directed *in trans* against MYC and CTGF genes indicates that their levels correlate with decreased expression of the corresponding mRNAs (Fejes-Toth 2009).

The manipulation of PASRs may thus be gene-specific and leading to positive or negative regulation of target gene expression (Janowski et al. 2007).

1.1.5 Long non-coding RNAs (lncRNAs)

LncRNAs are arbitrarily considered as ncRNAs whose length range from 200 nt to several kilobases and include all those long ncRNAs implicated in dosage compensation and imprinting, i.e Xist and HOTAIT (Brown et al. 1992; Rinn et al. 2007).

These mRNAs can be grouped into a remarkably diverse set of transcripts. One classification has been based on their ability to be "*cis*-acting" or "*trans*-acting" lncRNAs.

The estimated number of long lncRNAs is 17,000 in the human and 10,000 in the mouse genome. The number is likely to be greatly underestimated since many primary transcripts are often processed into smaller ncRNAs.

In contrast to most mRNAs, which ultimately localize to the cytoplasm after processing, most long ncRNAs are localized in the nucleus at steady state. This is especially true for poly A- long ncRNAs that account for a large portion of the total transcribed sequences (Wu et al. 2008) and lncRNAs transcribed from intronic regions (Cheng et al. 2005). Just a small subset of lncRNAs is located in both the nucleus and the cytoplasm (Cheng et al. 2005; Imamura et al. 2004; Kapranov et al. 2007b; Wu et al. 2008) while some lncRNAs seem to be selectively localized in the cytoplasm (Louro et al. 2009).

A critical role of lncRNAs has been assessed in epigenetic regulation by driving chromatin-modifying factors/complexes to specific locations in the genome and in subnuclear sites (Chen et al. 2008b; Khalil et al. 2009; Rinn et al. 2007; Sanchez-Elsner et al. 2006; Zhao et al. 2008).

Recently, a genome-wide ChIP-RNA sequencing analysis found that up to 38% of the 3300 conserved large intergenic ncRNAs are associated with one of the following four chromatin-modifying factors: EZH2, SUZ12, CoREST, and JARID1C/SMCX (Khalil et al. 2009). LncRNAs may form RNA:RNA or RNA:DNA structures, which provide sequence specificity and serve as platforms to bind proteins that are not strictly sequence-specific, and to orient them to target sites (Mattick and Gagen 2001).

Tiling arrays covering all four human HOX clusters identified 231 novel ncRNAs, which are spatially expressed along developmental axes and show distinct histone methylation patterns (Rinn et al. 2007). Among these, a 2.2 kb lncRNA, HOTAIR,

transcribed from the boundary of two diametric chromatin domains in the HOXC locus was preferentially expressed in posterior and distal sites. HOTAIR recruits Polycomb repressive complex 2 (PRC2) complex in trans across 40kb of the HOXD locus on chromosome 2, it promotes histone H3K27 trimethylation leading to transcriptional repression of HOXD locus.

Despite the fact that most of the reported ncRNA-associated chromatin-modifying complexes are involved in gene repression, an equally large numbers of lncRNAs can recruit co-activator complexes, including components of the trithorax/COMPASS/MLL complex (Beisel et al. 2007; Schoeftner et al. 2006; Schuettengruber et al. 2007). As an example, a lncRNA Evf2 serves as a transcription coactivator in the developing mouse forebrain. Evf2 is a long, polyadenylated ncRNA transcribed from an ultraconserved intergenic enhancer region and its expression correlates with increased transcription of the Dlx-5/6 locus (Feng et al. 2006).

It is also possible that ncRNAs can alter their structure on "ligand" binding and function as "riboswitches" (St Laurent and Wahlestedt 2007; Wickiser et al. 2005). In this context, promoter-associated ncRNAs can function as ligands to mediate histone modifications as exemplified by the case of CCND1ncRNAs. The CCND1ncRNAs are upregulated in response to genotoxic stress, CCND1ncRNAs are capable to recruit TLS, a member of the TET RNA-binding protein family. In turn TLS proteins binds to two well-known histone acetyl transferases (HAT) CBP and p300 and cause a substrate-specific inhibition of its HAT enzymatic activity. In this way CCND1ncRNAs serve to establish the hypo-acetylation status of the chromatin and the consequent repression of the CCND1 mRNAexpression (Wang et al. 2008).

Analogous to the role of rRNAs in ribosome assembly, lncRNAs can exert functional roles in specific nuclear organelle assembly, exemplified by the actions of the lncRNA NEAT1 (MEN ε/β), which is functionally essential for structural integrity of nuclear paraspeckles (Sunwoo et al. 2009). Paraspeckles are subnuclear organels whose functions have been suggested in transcription, pre-mRNA splicing and nuclear retention of RNA (Fox et al. 2005).

LncRNAs also have been shown to modulate the activity of proteins by regulating their subcellular localization. As an example, the transcription factor NFAT (nuclear factor of activated T cells) localizes to the cytoplasm. When calcium-dependent signals occurs, NFAT is imported into the nucleus, where it activates transcription of target genes (Hogan et al. 2003). One of the key regulators of NFAT trafficking is a

lncRNA known as NRON (noncoding repressor of NFAT) that is alternatively spliced (0.8–3.7 kb) (Willingham et al. 2005). By binding to members of the nucleo-cytoplasmic trafficking machinery, NRON specifically inhibits the nuclear accumulation of NFAT, but not that of other transcription factors such as p53 and NFkB that also translocate from the cytoplasm to nucleus.

Recent genome-wide studies suggest that the function of a significant fraction of long unannotated transcripts may be to serve as precursors for small RNAs <200 nucleotides (nt) in length (Fejes-Toth 2009; Kapranov et al. 2007b). It has been proposed that many Promoter-Associated long ncRNAs (PALRs) may be post-transcriptionally processed to yield many promoter-associated small RNAs (PASRs) (Fejes-Toth 2009). Transfection of RNA mimetics to PASRs were found to reduce expression of the overlapping mRNA promoter, indicating that these newly identified small RNAs impact gene expression (Fejes-Toth 2009).

It is thus becoming clear that lncRNAs can have numerous molecular functions, including modulating transcriptional patterns, regulating protein activities, serving structural or organizational roles, altering RNA processing events, and serving as precursors to small RNAs. But it will be likely possible that newly discovered lncRNAs may serve to other functional paradigms.

A major current issue is to understand how the molecular functions of these lncRNAs affect the organism. It is already known that lncRNAs are implicated in numerous developmental events (Amaral and Mattick 2008), such as the formation of photoreceptors in the developing retina (Young et al. 2005) and the regulation of cell survival and cell cycle progression during mammary gland development (Ginger et al. 2006). The generation of specific knockout animal models will be a key tool to shed light on this issue and will likely definitively show hat many lncRNAs are not transcriptional ‘‘noise,’’ but are instead required for normal development.

LncRNAs are also misregulated in various human diseases, especially cancer (Prasanth and Spector 2007), and even thought the mechanisms by which these transcripts affect tumor initiation and/or progression are currently unknown, some are already used as specific markers of tumors (de Kok et al. 2002). Considering all these information it is reasonable that lncRNAs can also allow us to identify new therapeutic pathways and may be themselves therapeutic targets of molecular medicine.

1.2 Antisense transcription

Given the extend of the transcriptional overlap in the same genomic region, a large portion of the non-coding transcripts can be located in antisense direction to protein-coding loci.

It is estimated that 5880 human transcription clusters (22% of those analyzed) form sense–antisense pairs with most antisense transcripts being ncRNA (Chen et al. 2004), an arrangement that exhibits considerable evolutionary conservation between the human and pufferfish genomes (Dahary et al. 2005). A detailed analysis of the mouse transcriptome indicated that 43553 transcriptional units (72%) overlap with transcripts coming from opposite strand (Dahary et al. 2005)..

A large fraction of Natural Antisense Transcripts (NATs) are expressed in specific regions of the brain, supporting the idea that they're involved in sophisticated regulatory brain functions and may be involved in complex neurological diseases (Qureshi et al. 2010).

NATs appear to be involved in different cellular pathways, although it remains unclear whether they present any special sequence or structure-related feature as well as what are their protein partners.

As far as we know, NATs appear to be an heterogeneous group of regulatory RNAs with a variety of different biological roles and specific pattern of expression (tissues, cells, developmental stages).

1.2.1 Classification based on cis vs. trans NATs.

Many well-characterized regulatory ncRNAs acts in *cis* as receivers of other *trans* acting signals by forming secondary structures. Several examples of *cis* –acting RNAs are provided by the regulatory sequences found in the UTRs of mammalian genes that are known to be target of different processes: RNA editing, control of mRNA stability and/or translatability (Gebauer and Hentze 2004; Kuersten and Goodwin 2003; Moore 2005). Examples for *trans* –acting RNAs are the so-called riboswitches that are RNA molecules sensitive to several metabolic pathway, capable to bind to vitamins, lipids or small ligands and change their allosteric conformation in consequence of binding (Kubodera et al. 2003; Sudarsan et al. 2003).

Most NATs are *cis*-encoded antisense RNA (Kumar and Carmichael 1998; Vanhee-Brossollet and Vaquero 1998). By definition, *cis*-NATs are complementary RNA with

an overlapping transcriptional unit (TU) at the same chromosomal locus. *Trans*-NATs are complementary RNA transcribed from different chromosomal locations (Li et al. 2008b; Makalowska et al. 2005; Sioud and Rosok 2004).

1.2.1.1 Characteristics of Trans-NATs

A recent study on *Trans*-NAT showed that the abundance of these regulatory elements is much more than previously expected. Although the authors had applied very stringent criteria for selecting *trans*-NATs, eliminating all the NATs originating from repeat regions and pseudogenes, they reported that at least 4.13% of transcriptional units of various species are *trans*-NATs (Li et al. 2008b). Particularly, *trans*-NATs represented nearly 3,000 human TUs (or 2.89% of all human TUs) (Li et al. 2008a).

Trans-NATs often originate from pseudogenes or repeat regions. Repetitive sequences in genome and pseudogenes have long been considered to be non-functional artifacts of transposition pathways. However, an increasing number of reports point to the functional role for repetitive elements in post-transcriptional events (Peaston et al. 2004). Anti-sense transcription of pseudogenes may constitute a mechanism for controlling their cognate (parental) genes.

Such a regulatory role has been demonstrated for topoisomerase I, neural nitric oxide synthase, inducible nitric oxide synthase (NOS2A/anti-NOS2A) and fibroblast growth factor receptor-3 pseudogenes (Korneev et al. 2008; Korneev et al. 1999; Weil et al. 1997; Zhou et al. 1992). Importantly, recent reports proposed a role for a subset of mammalian pseudogenes in the production of endogenous siRNAs (endo-siRNA) through formation of double stranded RNA (Kawaji et al. 2008; Lavorgna et al. 2004; Tam et al. 2008; Watanabe et al. 2008).

1.2.1.1.1 Chimeric Trans-NATs

Chimeric NATs are RNAs with identity to more than one region of the genome (Lavorgna et al. 2004; Nigumann et al. 2002). They offer partial complementarity to more than one target transcript (Li et al. 2000), being capable of regulating many sense mRNA at the same time.

1.2.2 Characteristics of Cys-NATs

Cis-Antisense RNAs are widely distributed across the genome, althought they have a propensity for being located 5' and 3' to protein coding RNAs (Core et al. 2008).
Expression of the antisense transcript is not always linked to the expression of the sense protein-coding partner, thus suggesting the usage of alternative regulatory elements.
Antisense RNAs tend to undergo less splicing events and are often lower abundant than sense protein-coding partner. Knocking down antisense genes results in multiple outcomes, with the corresponding sense gene being either increased or decreased.
These results indicate that antisense transcription can operate through a variety of different mechanisms and are a heterogeneous group of regulatory RNAs.
Different models have been proposed for antisense –mediated regulation of sense gene.

1.2.2.1 NATs regulation of transcription

The act of transcription itself in the antisense direction modulates the transcription of the sense RNA, either for transcriptional collision or genomic rearrangements.
The first model is based on the assumption that RNA polymerase binds to the promoter of convergent genes on opposite strands and collide in the overlapping regions, blocking further transcription (**Figure 1a**).
Genomic rearrangements occurs in lymphocyte B through a recombination process in the variable region of immunoglobuline genes (somatic hypermutation) and in lymphocite T for T cell-receptor selection (class-switch recombination).
RNA transcription from antisense direction creates a transcriptional bulge of 17 ± 1 melted bases in the target DNA, making it accessible to the activation-induced cytidine deaminase enzyme (AID). This is involved in somatic recombination and requires at least 5 nucleotides in ssDNA for optimal cytosine deamination (**Figure 1b**).

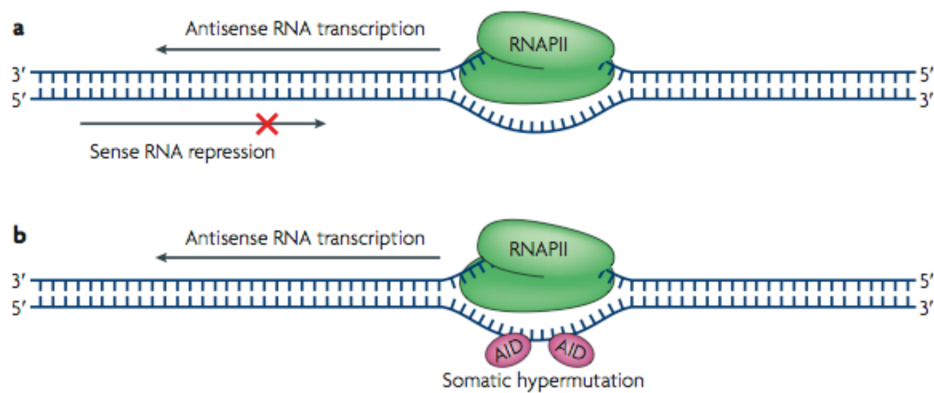


Figure 1: Transcription-related modulation a) Collision: In this model RNA transcription occurring in anti-sense direction halts sense transcription. In this model transcription occurs in only one direction at a given time. **b) Genomic rearrangements:** In B and T limphocytes natural antisense transcription opens a transcriptional bubble that exposes ssDNA to the activation-induced cytidine deaminase, conversion of deoxycytidine in deoxyuridine, a process that underlies somatic hypermutation

1.2.2.2 NATs-DNA interactions

NATs may also be involved in epigenetic regulation of transcription thought DNA methylation, chromatin modification and monoallelic expression.

Antisense RNA molecule itself can bind to complementary DNA target locus and triggers DNA methylation, DNA demethylation and chromatin modification of non-imprinted autosomal loci.

Some examples of antisense-mediated transcriptional silencing occurs at promoter regions of a-globin gene 2 (HBA2) site, p15, p21 and progesteron receptor (PR) by mean of methylation and heterochromatin formation (**figure 2a**). The proposed mechanism can explain functionality even when abundance of antisense RNA molecules is very low.

Pervasive antisense RNA transcription at promoter and termination region gives rise to promoter-associated small RNAs (PASRs) and termini associated RNAs (TASRs) or promoter-upstream transcripts (PROMPTs).

The local accumulation of those small RNAs along transcribed regions overlap with active chromatin domain marks such as trimethylation of Lys4 of histone3 (H3K4me3) (**Figure 2b**).

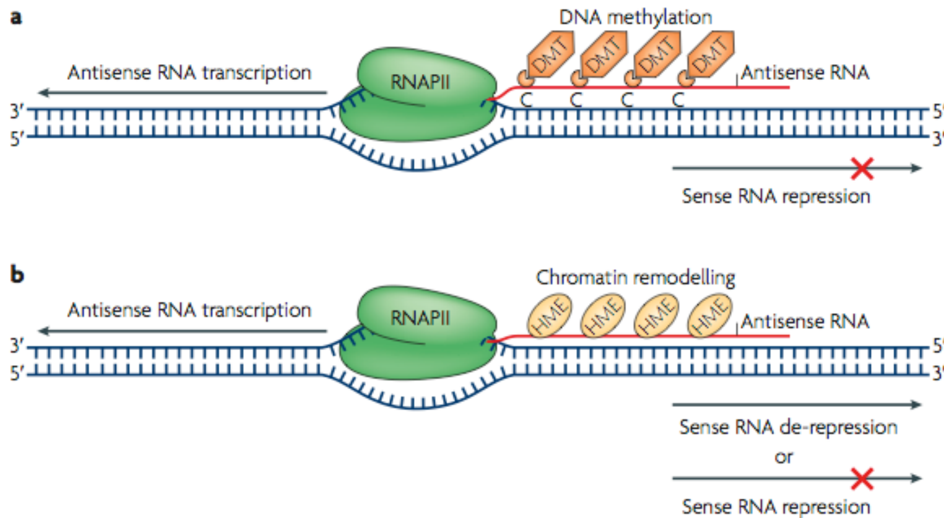


Figure 2 The RNA-DNA interaction model: a) DNA methylation The newly formed antisense RNA along with the template DNA can induce directly of indirectly the with DNA methyl tranferase (DMT), thus causing a repression of the sense RNA transcription. **b) Chromatin modification** Activation of the antisense RNA transcription results in a de-repression of the sense RNA, in particular prominent when the antisense RNA is located in or around the transcriptional start site (TSS) of the sense gene. Argonaute2 (AGO2) is thought to be involved in this process.

Additionally some of the antisense-mediated epigenetic changes might spread to neighboring region not complementary to the antisense transcript causing either random monoallelic exclusion or including a whole cluster of genes such as in genomic imprinting of the Kcnq1 locus. Finally, the expansion process occasionally involves the whole chromosome such as in the X-chromosome inactivation in females.

1.2.2.3 NATs and Nuclear RNA duplex formation

An alternative type of antisense-mediated gene regulation is based on the formation of hybrid RNA duplex in the nucleus that can be target of editing enzymes.

Alternative splicing and termination Antisense RNA can bind the sense mRNA, tuning the balance between different splicing forms, as it happens in the case of the thyroid hormone α-gene isoform variants TRα 1 and TRα 2. In a similar manner it can potentially cause alternative termination and polyadenylation of sense RNA (**Figure 3Ab**).

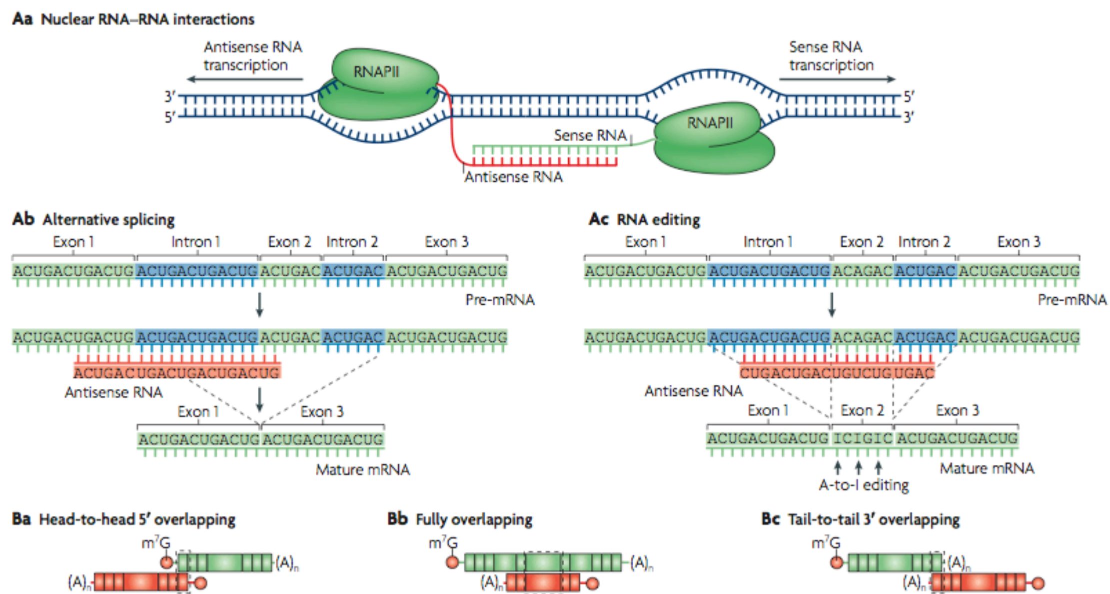


Figure 3. The sense RNA-antisense RNA pairing: Aa) Nuclear RNA pairing can occur locally after transcription interfering with A**b) RNA splicing** Natural antisense transcript can cover acceptor or donor splice sites in the sense pre-mRNA transcript influencing the balance between different splicing forms. A**c) RNA editing** Nuclear RNA duplex formation can recruit ADAR (Adenosine deaminase enzymes that act on RNA enzymes is thought to be involved in this process.

Transport, nuclear retention and editing: Duplex formation between sense and antisense RNAs can modulate nuclear export. Nuclear retention is commonly observed for non-coding RNAs. Several cell stressors can mobilizing antisense RNA molecules thus contributing to the nuclear shuffling of the protein-coding partner (**Figure 3Ac**).

In *Drosophila melanogaster* antisense transcription is also been related to RNA editing. Duplex RNA formation in the nucleus can recruit ADAR enzymes (adenosine deaminase that act on the RNA), which deaminates the target adenosine in inosine.

1.2.2.4 NATs and cytoplasmic RNA duplex formation

Cytoplasmic RNA hairpins can affect both RNA stability/ translation or cover miRNA binding sites on the sense mRNA.

RNA stability alteration: the expression of the sense and antisense pair in the same cell can result in the activation of the endogenous siRNA processing machinery, which mediates sequence-specific knock-down of targeted genes. However, co-expression of NATs with their sense counterparts, together with the frequently

observed concordant regulation of sense and antisense RNAs in many tissues and cell lines, provides evidences against endogenous siRNA being the sole mechanism of antisense-mediated gene regulation.
The overlapping RNA region might affect RNA stability by reducing mRNA decay, protecting the 5' of the sense RNA from exonucleolitic degradation by various RNAses. Antisense transcript for inducible NOS synthase (*INOS*) interacts with the AU-rich element (ARE) binding antigen R (HuR) suppressing iNOS degradation. On the contrary, antisense RNA of hypoxia inducible factor 1-α (HIF-1-α) exposes the ARE element in the HIF-a mRNA and reduces its stability. In the case of b-amyloid precursor protein cleaving enzyme (BACE), the expression of its antisense RNAs enhances the BACE mRNA half –life by increasing its stability.
Translational inhibition: in the case of PU.1 mRNA, its non-coding counterpart is a polyadenilated RNA with longer half-life than the protein-coding RNA. NATs/sense RNAs binding stalls PU.1 translation between initiation and elongation steps.

1.3 Repetitive elements

1.3.1 Evolution of distinct SINEs

Mammalian DNA typically contains hundreds of short interspersed elements called SINEs. They have been fixed in the mammalian genome since a single event of retrotransposition and thus have been a tremendous source of insertional mutagenesis through mammalian evolution. The human Alu is the most extensively studied SINE and it exemplifies most features of this unusual class of sequences.
There are at least 1 billion Alus per haploid genome (Schmid 1996). Individual Alus share a common consensus sequence of 283 bps which is typically followed by a 3'-A-rich region resembling poly(A) tail (**Figure 4**).
The Alu consensus sequence is a divergent tandem dimer in which the two monomers are separated by a short A-rich region that flanked the ancestral monomer. Except for 30 nt insertion in the right monomer, Alu monomers are homologous to SRP RNA, also known as 7SL RNA. Most Alus are flanked by short direct repeats which represent the duplicated insertion sites.

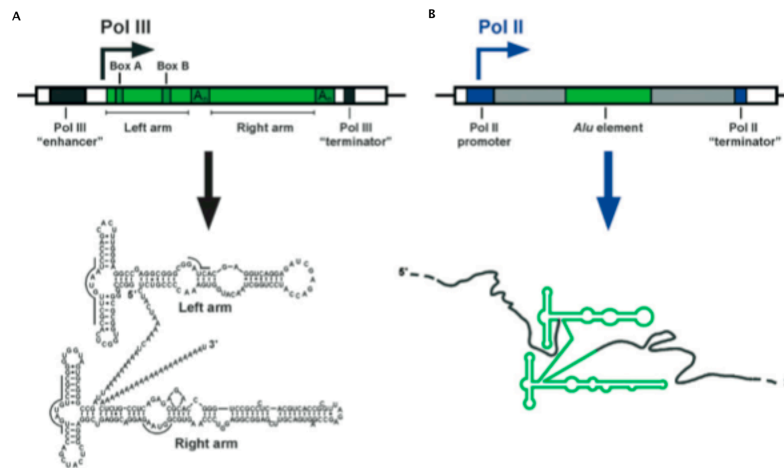


Figure 4. Alu RNA secondary structure. Secondary structure of a Pol.III transcribed Alu RNA drawn based on a previously determined secondary structure and adapted to the sequence of the Alu element of intron 4 of the a-Fetoprotein gene . Underlined blue letters and dots indicate the binding sites of SRP9/14 and the tertiary base pairing between the two loops, respectively, by analogy to SRP RNA.

Alu elements inherited the two highly conserved sequences A and B boxes from the 7SL RNA promoter gene. However these elements are not sufficient to drive the transcription *in vivo* and Alus depend on flanking sequences for their expression. RNA Pol. III-dependent transcripts of this class are referred to as "free" Alus. Alus transcribed in the context of larger transcriptional units of both protein coding and non-coding RNAs are called "embedded" Alus.

Non-mammals SINEs are unrelated to SRP RNA and believed to be derived from tRNA species. However, SINEs from mammals belong to either SRP- or tRNA-derived superfamilies. Rodents genomes contain both SRP RNAs (B1 elements) and tRNA superfamily (B2) SINEs with SINEB1 basically resembling the left human Alu monomer. Prosimiam species have both SINEs, B1 and B2, and full-length dimeric Alus. This intermediate composition suggests a transition between rodents and human. Sequence database analysis and hybridization experiment excluded the presence of tRNA SINEs in human genome. The reason why Alus flourished in higher primate genome while tRNA SINEs are undetectable are still unknown. It can be either that Alus may have established a state of complete neutrality with the human host or more probably they have compensate their hosts with selective advantages.

Several experimental evidences suggest that Alus may serve a variety of functions.

1.3.1.1 Free Alus and protein translation

Schmid C. W and collegues showed for the first time that free Alus have a function, when reporting that they stimulate translation of co-transfected reporter genes. Interestingly, synthetic Alu RNPs (composed of Alu RNA in complex with SRP14/9) and naked Alu RNAs have opposite effect toward protein translation in cell-free translation system. While Alu RNA are shown to stimulate translation of reporter mRNA both in cells and *in vivo* (Rubin et al. 2002), Alu RNP acts as general inhibitor of protein translation (Hasler and Strub 2006a). It is known from the structure of the SRP Alu domain that the binding of SRP9/14 induces strong conformational changes in the RNA. Alu RNAs are very unstable molecules, explaining their relative low accumulation in normal cells (Bovia and Strub 1996) and they might be stabilized by SRP9/14 binding. Moreover, free Alus can bind to SRP14/9 complex *in vitro* (Hasler and Strub 2006a). Given that there are free SRP14/9 molecules, the occurrence *in vivo* of Alu RNP seems therefore very likely to take place. Expression and function of Alu RNAs and AluRNPs are probably tightly controlled at spacial or temporal level, therefore be limited to certain physiological condition, such as stress (Liu et al. 1995), cancer (Tang et al. 2005) or by the tissue-specific control of gene expression. Alu RNA and Alu RNP should therefore be considered as part of complex system in which the concentration of SRP14/9 molecules available to bind free Alus shift the balance toward Alu formation and therefore toward a general inhibition of protein translation.

The exact mechanism by which Alu RNA and Alu RNP exert opposite effect on translation is still unraveled. Both of them seem to act at the level of translation initiation. The global inhibition of translation is usually exerted by controlling either the phosphorilation or the availability of initiation factors. In particular, under stress conditions, cellular protein kinase (Gebauer and Hentze 2004), when activated hyperhosphorilate eIF2 in its alpha subunit thus limiting the abundance of metionil tRNA molecules (Met-tRNA$_i^{Met}$) to be recruited at the cap structure. It was proposed that Alu-dependent control of translation involves PKR protein kinase which is actually known to bind in-vitro free Alus. However, it was then shown that free Alus stimulates translation of reporter genes in PKR knock out cells (Hasler and Strub 2006a).

Intriguingly, the established effect of Alu RNAs on the translation efficiency of cytoplasmic RNAs is not an overall effect but specific to certain mRNAs (Hasler and Strub 2006b). This activity of increased translation was reported only in regard to newly synthesized reporter mRNAs and not for all the endogenous mRNAs of the cell. Since it is established that the pool of cytoplasmic mRNA comprises variegated mRNAs with 5′- and 3′-untranslated regions (5′- and 3′-UTRs) of different length and structure, the stimulatory effect might only be directed to RNA species with specific structures.

Interestingly, other SINEs have been linked to translation. BC200 RNA and its putative murine functional analogue BC1 RNA showed protein translation inhibition *in vitro* and *in vivo*. BC1 RNA interferes with the formation of 48S pre-initiation complex via a direct interaction with eIF4A (Wang et al. 2002). Other studies have shown that the inhibitory effects of BC200 and BC1 RNAs were dependent on the poly(A) stretches of the RNA and could be competed by the addition of PABP (Kondrashov et al. 2005). These results suggest that the suppression is mediated through binding of PABP by BC1 and BC200 RNAs.

The same studies showed that Alu RNAs also inhibited translation, albeit at lower extent than BC200 RNA (Kondrashov et al. 2005). These results apparently contradicted the stimulatory effect previously observed (Hasler and Strub 2006a) although the stimulation was observed at concentrations ~5- to 20-fold lower and at relatively short incubation times (Kondrashov et al. 2005).

The low level of expression of repetitive elements at steady state condition might be the result of a selective pressure to prevent their accumulation and thereby their function in normal cells. The fact that Alu elements behave like cell stress genes with a transient overexpression during cell stress response and rapid decreases upon recovery (Fornace et al. 1989; Li et al. 1999) strongly suggests that these transcripts serve a function. Upon stress, regular cap-dependent translation of most proteins is greatly reduced (Patel et al. 2002) whereas the expression of a small group of proteins such as heat shock proteins is greatly enhanced (Rhoads and Lamphear 1995). The mechanisms that account for the selective translation of certain mRNAs when overall translation is momentaneously halted, are still incompletely understood. They may include the use of internal ribosome entry sites and ribosome shunting (Rubtsova et al. 2003).

The increased expression of Alu RNA under stress is consistent with a stimulatory role in the translation as previously proposed (Rubin et al. 2002). BC200 RNA is also of relatively low abundance and its presumed effect on protein translation in vivo is most plausibly explained by its accumulation at certain sites in neuronal cells (Tiedge et al. 1993). Likewise, the inhibitory effect of Alu RNPs may be spatially restricted to certain sites in normal cells or in cells with increased levels of Alu RNA.

1.3.1.2 Alus and alternative splicing

Altrnative splicing is the majour source for protein diversity accounting for the 30%-60% of human mRNA that become diversely spliced. One way to acquire altertative splice sites is when a gene harbors mutation in its pre-existing splice donor and/or at the acceptor site. The final outcome is the retention of a portion of the intron itself. This process is called exonization (**Figure 5**).

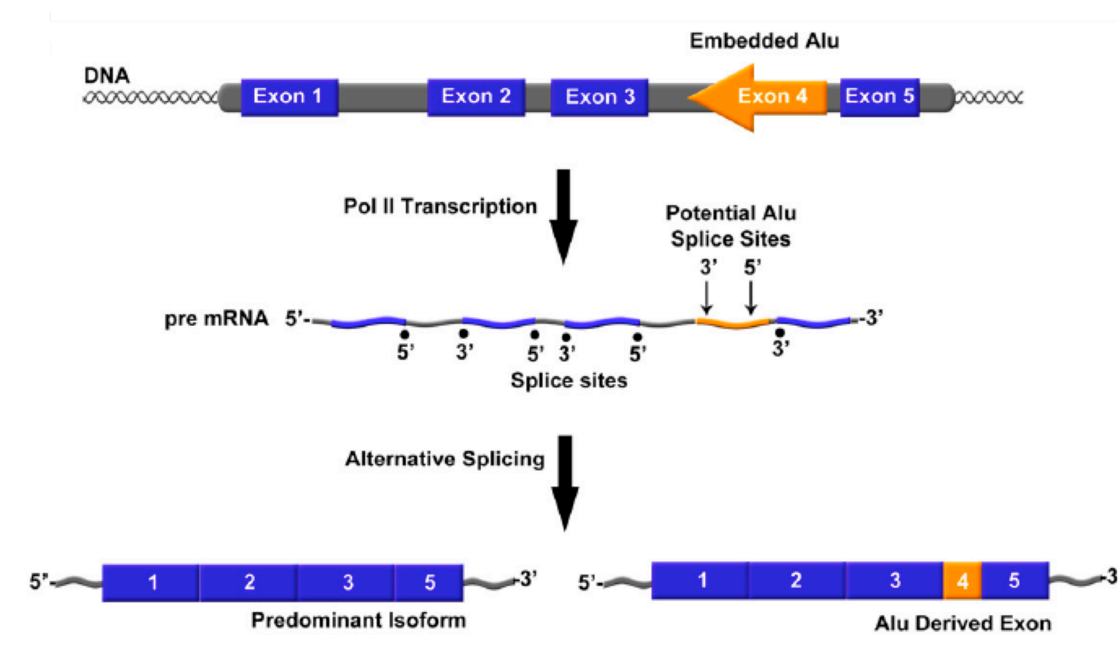


Figure 5. Alu RNA exonization promotes proteome diversity. Shown is an example in which embedded Alu RNA is exonized via alternative splicing into an mRNA.

When embedded in pre-mRNAs, Alu sequences become important mediators of gene expression diversity. The Alu consensus sequence contains 9 potential 5' splicing sites and 14 3' splicing sites, most of them in the minus orientation. It has been shown that Alus account for nearly the 6% of alternative splicing exons (Sorek et al. 2002). A synthetic minigene called ADAR2 containing embedded Alus in minus orientation alternatively at 5' and 3' of its pre-mRNA identified *in-vivo* the most favored position

used as splice sites (nucleotide 275 and 279 used as 3' splicing site and 158 as 5' slicing site) (Lev-Maor et al. 2003; Sorek et al. 2004). Alu RNAs embedded in intronic position can therefore constitute a large reservoir of protein diversity on one end, on the other end they can cause severe genetic disease like CCFDN (congenital cataracts, facial dysmorphism and neuropathy), a syndome caused by a mutation in intron 6 that creates an alternative spliced Alu exon (Varon et al. 2003).

1.3.1.3 Alus and A-to-I editing

RNA editing is a very well characterized post-transcriptional RNA modification. It consists in conversion of adenosine to inosine via the deaminase activity of a class of enzymes called ADARs (Adenosine Deaminase acting on RNA).

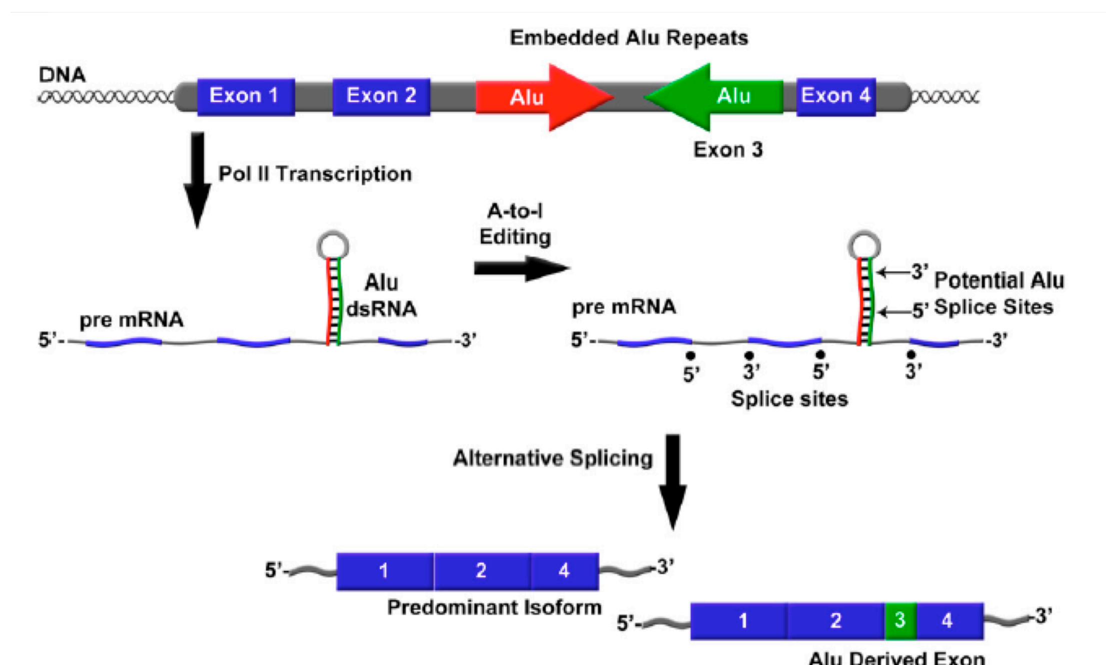


Figure 6. Alu RNA exonization promotes proteome diversity. Alu RNA indirect repeats embedded in an mRNA can become double stranded and undergo A-to-I editing, resulting in Alu exonization.

Alu account for the 90% of editing events in nuclear RNA (Nishikura 2006). ADAR enzymes preferentially edit adenosine present in double stranded RNA molecules and have no precise sequence specificity. Adenosine editing within an Alu sequence is favored when two Alus are present at a short distance in opposite orientation. This give rise to intramolecular base pairing events (**Figure 6**) as seen for NFkB1 and Cyclin M3 mRNAs editing (Kawahara and Nishikura 2006).

Furthermore, ds RNA formation has been related to nuclear retention and silencing of long non polyadenilated nuclear RNA (Chen et al. 2008a).

1.3.1.4 Alu embedded in mRNA

Untranslated regions (UTRs) in mature RNA have a crucial role in mRNA stabilization, cellular localization, export and ribosome loading. It is arguable that Alu insertion in 5' or 3' UTR portion of a mature RNA can exert a control on gene expression at different levels. Katerina Straub and collegues screened the human transcriptome to identify Alu elements contained in the transcribed UTRs of human cDNA library. They identified 299 Alus embedded in the 5' UTRs of 244 transcripts and 2142 Alus embedded in the 3' UTRs of 1548 transcripts (http://cms.unige.ch/sciences/biologie/bicel/Strub/researchAlu.html). For Alu RNAs embedded in 5' UTRs of specific mRNAs, a role in inhibiting translation has been proved. BRCA1 presents a transcript isoform specifically expressed in cancer tissue that contains an Alu sequence in its 5' UTR forming a stable secondary structure in its mRNA and causing a translational defect (Sobczak and Krzyzosiak 2002). Similarly, an antisense Alu element in the 5' UTR of the zinc finger protein ZNF177 (Landry et al. 2001), in the growth hormone receptor (Goodyer et al. 2001) and in the contactin mRNA isoform (Rome et al. 2006) decreased the translation efficency of relative mRNAs. Concerning Alu elements in the 3' UTR, it has been proposed that inverted Alus could generate adenine and uracile rich element called AREs, as for the LDL receptor transcript (Wilson et al. 1998).

1.3.1.5 Alus and miRNAs

Studing a cluster of miRNA on chromosome 19 (C19MC) which encodes 54 miRNAs, Bochert and colleagues have shown that transcription from the internal Pol. III promoter of Alu element can pass throught and transcribe the downstream miRNA precursors before encountering a Pol. III terminator (Borchert et al. 2006; Daskalova et al. 2006). Moreover, an *in-silico* analysis has shown that about 30 miRNA exibit a typical short-seed complementarity with specific sequence contained within sense Alus embedded in the 3' UTR of human RNA (Daskalova et al. 2006)

1.3.2 Mouse SINEs

Cellular RNA polymerase III can transcribe SINEs due to the presence of an internal promoter in their 5′ region, which is composed of A and B boxes spaced 30–40 nucleotides apart. SINE B1 family in mice originated from 7SL RNA, a component of SRP, involved in translation of secreted proteins in all eukaryotes. All these SINE families include sequences corresponding to the terminal regions of 7SL RNA with the central 144–182 nucleotides deleted. In contrast to human Alu, murine or rat B1 (~ 140 bp) is a monomer. However, it has an internal 29-bp duplication, which prompted Labuda et al. to consider B1 as a quasi-dimer (Labuda et al. 1991).

Mouse B2 RNA is encoded by short interspersed elements (SINEs), dispersed throughout the mouse genome, with ~350,000 copies per cell (Kramerov and Vassetzky 2005). SINEB2s are transcribed by RNA polymerase III to produce B2 RNAs that are ~180 nucleotides (nt) in length. The promoter elements (e.g., the A box and B box) that drive transcription are downstream from the transcription start site and therefore contained within the early transcribed region of SINEB2s. The 70 nt at the 5′ end of B2 RNAs are evolutionary related to tRNAs, and the very 3′ end of B2 RNAs contain an A-rich sequence conserved among all SINEs (Kramerov and Vassetzky 2005).

1.3.2.1 A SINEB2 serves as a chromatin boundary element

During the development of the pituitary gland, in specific cells, the murine growth hormone (GH) becomes transcriptionally active.

At embryonic stage 17.5 the promoter passes from a state in which H3 is trimethylated at K9 site (which is a mark for condensed heterochromatin) to a state in which H3 is dimethylated. The chromatin boundary where this transition starts has been localized to a region 10–14 kb upstream of the GH transcription start site. Intriguingly, this region contained a SINEB2 element.

To test the hypothesis of the boundary element, a 1.1 kb region containing the SINE B2 module has been tested for its enhancer blocking activity when placed between enhancer and the core promoter of a reporter gene and it resulted that the SINEB2 is necessary to block reporter gene expression. Strand specific PCR experiments showed that ongoing transcription through the Pol. III promoter (in sense direction) and

through the Pol. II (antisense direction) is important for enhancer blocking activity. These results suggest that a mouse SINEB2 serves as a boundary element and its bi-directional transcription causes a developmentally relevant change in chromatin structure (from heterochromatin to euchromatin), which establishes a permissive environment that allows transcription of the GH gene (Lunyak et al. 2007) (**Figure 7**).

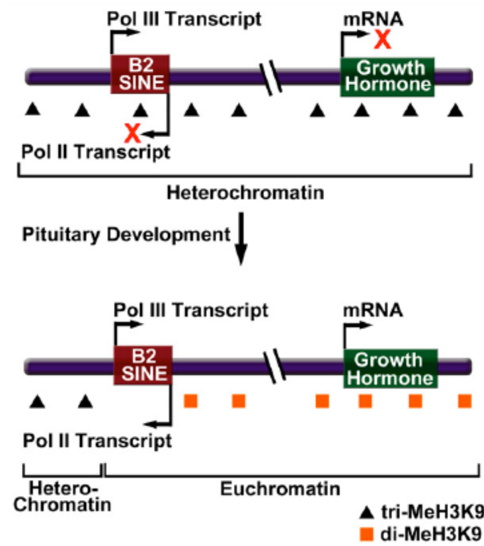


Figure 7. A B2 SINE serves as a boundary element to regulate transcription during pituitary development in mouse cells. Bidirectional transcription of a B2SINE upstream of the growth hormone locus facilitates a change in chromatin structure from a repressive heterochromatic state to a permissive euchromatic state.

Interestingly, in human cells over 9000 Alus are found within 1 kb upstream of transcription initiation sites, raising the possibility that some Alu SINEs might also affect mRNA transcription by serving as boundary elements (Dagan et al. 2004). tRNA genes have been found to serve as boundary elements in yeast, but the mechanism of function also appears to be somewhat different from that of the GH SINEB2 (Lunyak 2008). Although ongoing Pol III transcription of tRNA genes is required for boundary element function in yeast, there is no evidence of antisense Pol. II transcription through the tRNA genes

1.3.2.2 SINEB2 and Alus act as trans-regulators of mRNA transcription

SINEB2 and Alu RNAs may function as repressors of mRNA transcription during heat shock. As cells respond to heat shock, transcription of some genes is upregulated (e.g.,hsp70), while transcription of others is repressed (e.g., actin, and hexokinase II)

(Allen et al. 2004; Mariner et al. 2008). When Alu or SINEB2 RNAs are silenced , transcriptional repression at several genes upon heat shock is abrogated, indicating that SINEB2 and Alu RNAs act as inhibitor of transcription.

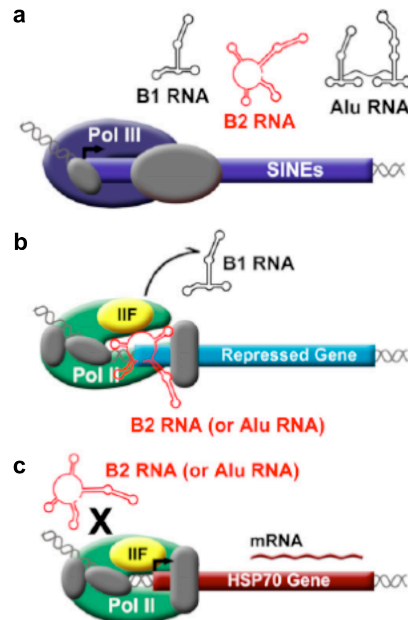


Figure 8. SINE RNAs control the heat shock response in mouse and human cells. **a,** Pol. III transcribed SINE RNAs increase upon heat shock. **b**, During heat shock, mouse B2 RNA or human Alu RNA enters complexes at thepromoters of repressed genes. TFIIF facilitates dissociation of B1 RNA from Pol. II. **c,** Heat shock activated genes are resistant to repression byB2 RNA and Alu RNA.

In an *in-vitro* transcription system, Alu RNA and SINE B2 RNAs were potent repressors of RNA Pol. II. Interestingly, physical binding of B2 RNA and Alu RNA to core Pol. II has been demonstrated. Consistent with this finding, ChIP assays have proved that in heat shocked mouse or human cells, SINEB2 RNA or Alu RNA co-localize with Pol. II at the promoters of transcriptionally repressed genes.

These observations led to the model where Alu RNA and SINEB2 RNA are upregulated upon heat shock, bind Pol. II to enter complexes at promoters, and finally block transcription (**Figure 8b**).

Deletion studies with SINEB2 RNA gave the first hints that these ncRNA share distinct functional domain. Nucleotides 81–130 of B2 RNA were fully functional for binding Pol. II and repressing transcription in vitro; however, further truncation to 99–130 yielded an RNA domain capable of binding Pol. II, but lacking the ability to repress transcription. Similarly, deletion analysis of Alu RNA demonstrated that it had two separable ‘Pol. II binding’ domains and two different ‘transcriptional

repression' domains (Mariner et al. 2008).

A series of in vitro studies were performed to determine the molecular mechanism by which Alu RNA and B2 RNA repress transcription. Given that these ncRNAs co-occupy promoters with Pol. II, they likely repress transcription after complexes bind DNA but before initiation, by preventing Pol. II from properly engaging the DNA after assembling into complexes with promoter-associated transcriptional factors (**Figure 8b**). B1 and B2 RNAs bind Pol. II competitively and with similarly high affinity, which raised the intriguing question of whether B1 RNA could block B2 RNA from binding Pol. II and repressing transcription. This possibility was investigated using an in vitro transcription system, and surprisingly, B2 RNA was found to repress transcription when B1 RNA had been pre-bound to Pol. II (Wagner et al. 2010). Further experiments elucidated that TFIIF facilitates the dissociation of B1 RNA from Pol. II (**Figure 8b**). Moreover, fusing a transcriptional repression domain from Alu RNA onto B1 RNA created a chimeric ncRNA that remained stably bound to Pol. II in the presence of TFIIF, showing that repression domains make Pol. II–ncRNA complexes resistant to the destabilizing effects of TFIIF.

1.3.2.3 SINEB2 and Alu RNAs act as trans-regulators of translation

Cell stress dramatically increases the abundance of human full length Alu RNAs (fl Alu RNAs) and other mammalian SINE RNAs (Fornace and Mitchell 1986; Liu et al. 1995). For example, heat shock causes a nearly 100-fold increase in mouse B1 RNA making this sparse transcript abundant (Fornace and Mitchell 1986). Mouse B2 and rabbit C RNAs show similar increases, indicating that the heat shock response is conserved by the SRP RNA and tRNA SINE superfamilies. In addition to heat shock, other classic cell stress treatments increase full length Alu RNAs (flAlu RNA) (Liu et al. 1995). Cell stress does not change the lifetime of flAlu RNA but probably increases Alu transcription (Liu et al. 1995). Viral infection or administering cycloheximide to cells also significantly increases the abundance of SINE RNA (Jang et al. 1992; Jang and Latchman 1992; Panning and Smiley 1993; Panning and Smiley 1995; Singh et al. 1985). The induction of Alu RNA by either cycloheximide or heat shock occurs <20 min after subjecting cells to these stresses (Liu et al. 1995). The rapidity of these responses suggests that they arise from the modification of existing factors and do not involve either DNA demethylation or *de novo* synthesis.

Do these increases in SINE RNA merely reflect an aberrant breakdown in regulation or are they a controlled response? Schmid has and colleagues have shown that after viral infection, along with a global block in protein synthesis, the cytoplasmic concentration of flAlu RNAs dramatically increases. The halt of translation initiation that we observe after exposure to viral-stress is usually due to a change in the activity of PKR (double-stranded RNA activated kinase). The PKR is an intracellular protein kinase whose function is to sense cell stresses. Upon exposure to double stranded viral RNA PKR increases its autophosphorylation activity and hyperphosporylate the alpha subunit of eiF2 complex. eIF2 is responsible for the transport of the metionil-tRNA at the site of translation initiation. Phosphorylation of eIF2a on Ser-51 inhibits the formation of the ternary complex with the met-tRNA and impairs general translation levels. Small highly structured RNAs (like Alu RNAs) sequester PKR as bound monomers and inhibit its autophosphorylation, thereby potentially increasing protein synthesis. Since flAlu RNAs increase protein synthesis after viral infection, it has been proposed that.this is mediated by PKR inhibition. However, translation stimulation is still present in knock-out cells (Rubin et al. 2002).

The absence of Alu RNAs in most mammals and tRNA SINEs in humans indicates that their sequence *per se* is not essential for function but their secondary structure is important. PKR binding primarily requires only a minimum number of base pairs within an RNA secondary structure so that several unrelated RNA sequences can functionally be Alu-substitutes as PKR inhibitors (Bhat and Thimmappaya 1983; Clemens 1987). As a classic example, when cells are infected and PKR activation blocks protein synthesis, the adenoviral VAI RNA gene inhibits virally induced PKR activation, restoring viral protein synthesis. Both protein synthesis and viral infectivity are impaired for VAI mutants. As an other example of PKR binding element, the gene for an entirely unrelated RNA, EBER1, rescues both infectivity and protein synthesis for VAI mutants (Bhat and Thimmappaya 1983).

According to this experiment, the cell stress-induced transcripts from the tRNA SINE superfamily could serve the same PKR regulatory role as Alu RNA. Yet a minimal RNA secondary structure alone cannot be sufficient to inhibit PKR *in vivo*. Otherwise, cellular RNAs, e.g. rRNA, would present an extraordinary number of PKR binding sites. Presumably, rRNA and other functional RNAs are unavailable for PKR binding because of their subcellular location or organization into RNP structures. Similarly, short-lived flAlu RNA (Chu et al. 1995) could be rapidly reduced to basal

levels when they are no longer required for PKR inhibition. As otherwise functionless RNAs, the RNP structure and subcellular location of SINE RNAs could promote their PKR accessibility. In support of the notion that Alu RNPs are accessible for PKR binding, the only other proteins known to form Alu RNPs are two small SRP 9/14 proteins and La, which transiently binds to the 3′-ends of nascent Pol. III transcripts.

1.4 PD

It is the second most common progressive neurodegenerative disorder, affecting 1-2% of all individuals above the age of 65. The main pathological hallmark of PD is the progressive loss of neuromelanin-containing dopaminergic neurons in the Substantia Nigra pars compacta (SNc) of the ventral midbrain and the presence of eosinophilic intraneuronal inclusions, called Lewy bodies (LBs), composed of specific cytoplasmic proteins like alpha-synuclein, parkin, synphilin, ubiquitin, and oxidized neurofilaments (Goldman et al. 1983).

The result of this cell loss is a severe dopamine depletion in the striatum, responsible for the motor symptoms associated with PD, especially bradykinesia, tremor at rest, rigidity, and loss of postural control (Bernheimer et al. 1973); (Ehringer and Hornykiewicz 1960). While the precise pathological mechanisms remain unclear,, the identification of several genes associated with rare, heritable forms of PD have highlighted potential pathogenic causes such as mitochondrial disfunction, oxidative and nitrosative stress and aberrant protein degradation.

	Locus	Inheritance	Gene	Onset	Functions	LB formation
PARK1/4	4q21.3-q22	Dominant	SNCA	Around 40	Membrane trafficking	+
PARK2	6q25.2-q27	Recessive	Parkin	<40	UPS, E3-ligase	±
PARK3	2p13	Dominant	Unknown	35–89		+
PARK5	4p13	Dominant	UCHL1	~50	UPS, Ubiquitin hydrolase	?
PARK6	1p36.12	Recessive	PINK1	32 ± 7	Mitochondria, kinase	?
PARK7	1p36	Recessive	DJ-1	27–40	Oxidative stress	?
PARK8	12q12	Dominant	LRRK2	~65	Membrane trafficking, Kinase	±
PARK9	1p36	Recessive	ATP13A2	11–16	Lysosome? Autophagy?	?
PARK10	1p	Dominant?	Unknown	65.8		?
PARK11?	2p37.1	Dominant?	GIGYF2	Late	IGF-1 signaling	?
PARK12	Xq21-q25	X-linked	Unknown	Late		?
PARK13?	2p13.1	Dominant	HTRA2/OMI	Late	Mitochondria, protease	?
PARK14	22q13.1	Recessive	PLA2G6	20–25	Phospholipase enzyme	?
PARK15	22q11.2-qter	Recessive	FBXO7	10–19	UPS, E3-ligase	+

Table 1. List of PD-associated loci.

About 5-10% of all cases of PD are familial. Two autosomal-dominant genes, (α-synuclein and LRRK2) and three autosomal recessive genes (parkin, DJ-1 and PINK1) have been repetitively found mutated in inherited PD (Bonifati et al. 2003;

Kitada et al. 1998; Paisan-Ruiz et al. 2004; Polymeropoulos et al. 1997; Valente et al. 2004; Zimprich et al. 2004).

In brief, α-synuclein was the first gene in which a mutation was identified to cause an autosomal-dominant form of Parkinsonism (Polymeropoulos et al. 1997). Furthermore, it was found to be the principal constituent of Lewy bodies (Spillantini et al. 1997). Its function is currently not known although it seems to be involved in fatty acid metabolism and synaptic transmission (Sharon et al. 2003).

LRRK2, whose function is also unknown, is a complex kinase for which it has been proposed that a simple gain of function could lead to toxicity (Greggio et al. 2006).

Parkin is an E3 ligase, whose functions in the cell may include targeting proteins for proteosomal degradation.

DJ-1 is an atypical peroxidase that protects from oxidative stress.

PINK 1 is a mitochondrial kinase.

1.4.1 Ubiquitin Carboxy Terminal Hydrolase-1 (PARK5)

Uchl1 is an abundant neuronal enzyme (1-5% of total brain protein) (Wilkinson et al. 1989). It possesses a well characterized de-ubiquitinating activity that catalyzes the hydrolysis of carboxyl-terminal esters and amides of Ubiquitin to generate free monomeric Ubiquitin (Larsen et al. 1996). Furthemore, Uchl1 associates with monoubiquitin and elevates the half-life of monoubiquitin in neurons, probably by preventing its degradation in lysosomes.

Interestingly, a natural Uchl1 null mutant is the Gad mouse (Gracile axonaly dystrophy), a mouse model of a recessively transmitted neurodegenerative disease characterized by progressive axonal degeneration. Loss of functional Uchl1 leads to a decrease of free Ubiquitin and subsequent inadequate ubiquitynilation of proteins. A decreased Ub-dependent degradation is clearly upstream the accumulation of non-degradated ub-proteins observed within the spheroid bodies in gad mice.

General involvement of UCHL1 in neurodegeneration has been assessed for Spinocerebellar ataxia (Fernandez-Funez et al. 2000) and Hungtinton's disease (Naze et al. 2002), while its role in the pathogenesis of PD is still debated. A missense mutation in the UCHL1 gene leading to a I93M substitution at the protein level has been reported in two affected siblings in a german family with an autosomal inherited form of PD. However, the existence of an unaffected carrier of the I93M mutation has

questioned the link between I93M mutation and PD. Intriguingly, UchL1^{I93M} transgenic mice exhibits a clear nigrostiatal degeneration and progressive dopaminergic cell loss (Setsuie and Wada 2007). Moreover, UCH-L1 ^{I93M} shows decreased solubility and display aberrant interaction with several cellular proteins, including tubulin whose polimerization is severely unbalanced in presence of mutant I93M or carbonyl-modified UCH-L1 (an oxidized form of UCH-L1 often found in PD *Post-mortem* brains) (Kabuta et al. 2008).

In search for additional I93M mutants, a previously unrecognized polymorphism in the UCHL1 gene (S18Y) was discovered and subsequently found to be linked to a decreased susceptibility to PD (Levecque et al. 2001). Protection of S18Y allele is dependent on the dosage, that is homozygotes have significantly lower risk than heterozygotes. The protection exerted by S18Y is not simply linked to UCH-L1 hydrolytic activity because S18Y variant showed similar hydrolase properties as the wild type. This fact suggested the existence of a distinct enzymatic activity that confers a gain-of-toxic-function for I93M mutant and confers protective properties to S18Y variant. This second activity was identified in the 2002 by the group of Peter Lansbury in a novel dimerization-dependent ubiquitin-ligase activity. UCH-L1 was reported to form K63 polyubiquitin chain *in vitro*, using α-synuclein as a model substrate (Liu et al. 2002). While I93M and wt UCH-L1 increased the α–synuclein levels in transfected cells inhibiting its degradation, S18Y did not.

Uchl1 ligase activity was found to be diminished for S18Y variant, which had indeed a dominant negative effect toward I93M mutant *in-vitro*, thus explaining the incomplete penetrance of I93M mutation.

Kabuta et al also showed an aberrant interaction between mutant UCH-L1 I93M and protein involved in chaperon mediated autophagy (CMA) machinery: LAMP2A Hsp70 and Hsp90 (Kabuta et al. 2008). UCH-L1 I93M was proposed to be a negative regulator of the CMA pathway and to have also a role in the inhibition of α-synuclein shunting to lysosomes.

Recent studies have shown classical Lewy pathology in a deceased sibling of a family affected by the 193M UCHL1 mutation who developed, in addition to DOPA-responsive Parkinsonism, marked cognitive deficits (Leroy et al. 1998).

From a clinical point of view, UCH-L1 has been extensively studied in post-mortem brains of neurodegenerative diseases. In Alzheimer's Diasease (AD) brains UCH-L1

was also commonly found in neurofibrillary tangles with decreased levels of soluble protein (Choi et al. 2004). Down regulation and extensive oxidative modifications have been observed in post-mortem brains of both AD and PD idiopathic forms (Butterfield et al. 2002; Castegna et al. 2002; Choi et al. 2004). Aberrant ubiquitin hydrolase and or ligase activity occurring after oxidative modifications and/or downregulation of UCHL1 might lead to dysfunction of the neuronal ubiquitination/de-ubiquitination machinery, thus causing synaptic deterioration and neuronal degeneration. Furthermore, oxidative modification can make UCH-L1 itself more resistant to proteolysis and promote aggregation into hallmark lesions of AD and PD brains (Choi et al. 2004; Lowe et al. 1990). In fact UCH-L1 has been found in neurofibrillary tangles and the level of soluble UCH-L1 protein was inversely proportional to tangle number. The association of UCH-L1 with such structures in AD brains together with the lower hydrolytic activity shown by oxidized forms of UCH-L1 and the inverse correlation with tangles number raise the possibility that its role may be the de-ubiquitination of posphorylated tau protein and prevention of its aggregation *in vivo*. Importantly, transduction of UchL1 protein restored enzymatic and synaptic activity in hyppocampal slices treated with oligomeric Ab42 peptides and in APP/Ps1 mice model of AD (Gong et al. 2006).

A potential link of UCH-L1 with alpha-synuclein pathology is supported by the observation that inhibition of UCH-L1 activity in foetal rat ventral mesencephalic cultures is associated with alpha-synuclein aggregates (McNaught et al. 2002). Increased intracellular aggregates containing ubiquitinated proteins have been found after UCH-L1 inhibition by prostaglandins in human SK-N-SH cells (Li et al. 2004). These findings suggest that reduced UCH-L1 activity impairs UPS function and protein degradation, thus facilitating, under appropriate conditions, the accumulation of abnormal protein aggregates. In line with this, reduced UCHL1 mRNA and protein is found in PD and in DLB, but only in brain regions in which aggregated proteins occur in Lewy bodies and neuritis (Barrachina et al. 2006).

1.5 Molecular mechanism of translational control

The mammalian translational machinery is a tightly regulated system composed by eukaryotic initiation and elongation factors that are responsible for the recuiment of ribosomes to the 5’ cap structure of cytoplasmic RNAs and for the following step of polypeptide chain synthesis.

Two predominant pathways translate mammalian mRNA throught cap-dependent and independent mechanism. The capping of the 5' end of mRNA by 7metil-GTP allows the recruitment of eIF4F complex eIF3 and 40S ribosomal subunit to the 5' mRNA cap. Cap independent translation is mediated by internal RNA structure called internal ribosomal entry site (IRES), which recruits the ribosome independently of both the cap structure and the entire eIF4F complex.

1.5.1 Cap-dependent translation

The rate-limiting step of protein synthesis is translation initiation. During this process the small ribosome subunit 40S is loaded on the 5' of mRNA (7metil-GTP-capped) and scans toward the 3' for the start codon AUG where the complete ribosome is subsequently assembled to begin polypeptide formation (**Figure 9**).

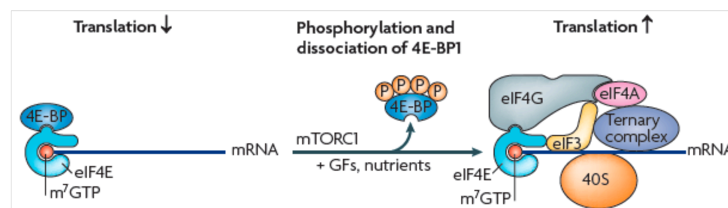


Figure 9. Regulating cap-dependent translation initiation. Hypophosphorylated 4E-binding proteins (4E-BPs) bind tightly to eIF4E, thereby preventing its interaction with eIF4G and thus inhibiting translation. Mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation of 4E-BPs releases the 4E-BP from eIF4E, resulting in the recruitment of eIF4G to the 5′ cap, and thereby allowing translation initiation to proceed.

The recruitment of small ribosomal subunit requires the assembly of the eukaryotic initiation factor 4F (eIF4F) complex on the 5' cap structure of mRNA. EIF4E (cap-recognizing protein) binds to the 5' 7mG of mRNAs and promotes the assembly of eIF4A and eIF4G. The inhibitory 4E-binding protein (4E-BP1), when hyperphosphorylated, releases eIF4E, allowing eIF4F complex to form (**Figure 9**).
4E-BP is target of a regulated signaling pathway that controls its phosphorilation status and thus its ability to bind to eIF4E.
Some mRNAs contain inhibitory secondary structures at their 5' UTRs. They may encode proteins that are involved in promoting cell-growth and proliferation. An initiation factor belonging to the family of RNA helicases eIF4A has the proper function to unwind the RNA as long as the initiation complex is forming on the cap of

mRNAs. eIF4A activity can be significantly enhanced when associated to its regulatory factor eIF4B, that is target of phosphorilation pathways as well as 4E-BP1.

1.5.1.1 The mammalian target of rapamycin (mTOR)-regulated pathway

Cells respond to changes in environmental conditions by altering gene expression and proteins are produced as a consequence of new mRNA synthesis. However, translation is a tightly regulated molecular step and it has a fundamental role in forming the proteome of a cell.
To grow and proliferate cells must ensures that sufficient resources are available to drive protein production. When amino acids availability becomes limiting, protein production has to be down-regulated, keeping the spare energetic resources to survive. Mammalian cells have evolved a fine mechanism of translational control in response to nutrient availability, cellular energy, stress, hormones and growth factors stimuli.
Translational control often targets translational initiation. A key pathway that responds to environmental cues and integrates protein synthesis rate with external conditions involves target of rapamycin (TOR). TOR is part of mammalian TOR complex 1 (mTORC1) along with raptor (regulatory associated protein of TOR (Fig) and LST8. mTORC1 phosphotransferase activity is elicited toward 4E-BP and 40S ribosomal protein S6 kinase, that are its major downstream targets.

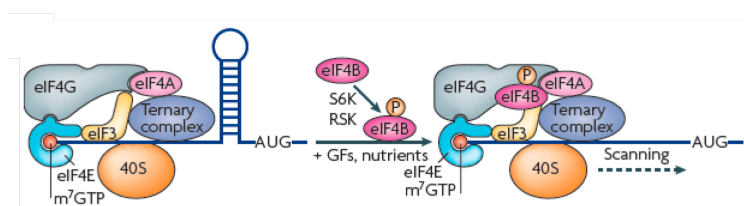


Figure 10. Regulating cap-dependent translation initiation. Following 40S ribosomal protein S6 kinase (S6K)-mediated phosphorylation, eIF4B is recruited to the translation pre-initiation complex and enhances the RNA helicase activity of eIF4A. This is particularly important for translating mRNAs that contain long and structured 5′ untranslated region sequences, because the unwinding of these RNA structures is required for efficient 40S ribosomal subunit scanning towards the initiation codon. GF, growth factor.

Normal processivity of translational machine requires that highly structured 5’ UTR are first linearized. This is achieved by eIF4A helicase enzymatic complex. EIF4A

exhibits basically low activity when not stimulated by the binding of cofactor eIF4B, which in turn enhances eIF4A affinity to ATP. S6K phosphorilates eIF4B near its RNA-binding site thus promoting its association to eIF4A (**Figure 10**). Mutant eIF4B that cannot be modified by S6K is inactive *in-vitro*, confirming that phosphorilation is both sufficient and necessary for its recruitment to the translation-initiation complex.

1.5.2 Cap-independent translation

RNA genomes of picornaviruses, such as encephalomyocarditis virus (EMCV) and poliovirus, have properties that are incompatible with initiation by 5′ end–dependent scanning. In 1988, it was discovered that picorna viral mRNAs are translated by a mechanism, distinct from shunting, that enables ribosomes to initiate translation effectively on highly structured regions located within the 5' non-translated region (NTRs) (Pelletier et al. 1988). These regions were named IRESs.

Picornaviral 5’ NTRs can range in length from 610 to 1500 nucleotides, are highly structured, and contain multiple nonconserved AUG triplets upstream of the initiation codon that should act as strong barriers to scanning ribosomes. Interestingly, several oncogenes, growth factors and proteins involved in the regulation of programmed cell death, cell cycle progression and stress response contain IRES elements in their 5' UTRs. Internal initiation escapes many control mechanisms that regulate cap-dependent translation. Thus, a distinguishing hallmark of IRES-mediated translation is that it allows for enhanced or continued protein expression under conditions where normal, cap-dependent translation is shut-off or compromised. For instance, IRES elements were found to be active during irradiation (Gu et al. 2009), hypoxia (Lang et al. 2002), angiogenesis (Nagamachi et al. 2010), apoptosis (Spriggs et al. 2005) and amino acid starvation (Gilbert et al. 2007).

The repression of global protein synthesis associated with such stresses is in part a consequence of phospholylation of Ser51 on the α subunit of the translation initiation factor eIF2A. During one of the first step of translation, eIF2A binds to GTP and the initiator Met-tRNA$_i^{Met}$ to form the ternary complex which subsequently binds to the 40S ribosomal subunit. In a second step GTP is hydrolyzed. For recurring initiation, the GDP must be released and eIF2A charged with fresh GTP.

This reaction is catalyzed by eIF2B. After phosphorylation, eIF2A is turned into an eIF2B competitive inhibitor, leading to a global repression of protein synthesis

(**Figure 11**).

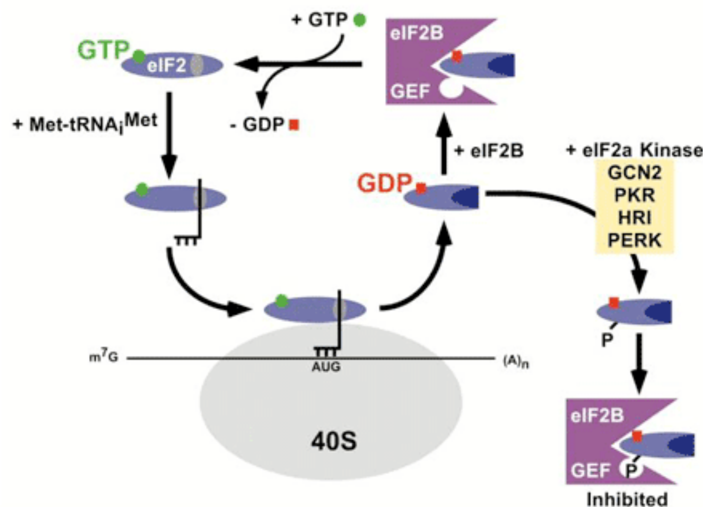


Figure 11. Recycling of EiF2 by EiF2B and regulation by Eif2A kinases. The g subunit of eIF2 binding to GTP is essential for the ternary complex of eIF2, GTP and Met-tRNA$_i^{Met}$. During the course of translation initiation the GTP bound by eIF2 is hydrolyzed to GDP and eIF2 is released from the ribosome in a binary complex with GDP. As eIF2 has a much higher affinity for binding GDP than GTP, a guanine-nucleotide exchange factor termed eIF2B is required to recycle eIF2•GDP to eIF2•GTP.

Paradoxically, inhibition of eIF2B by phosphorylated eIF2A leads to upregulated translation of mRNAs with particular motifs in their 5' UTRs; these motifs are recognizable as upstream open reading frames (uORFs) and IRES (Komar and Hatzoglou 2005; Stoneley and Willis 2004; Tzamarias et al. 1989; Vattem and Wek 2004). In the genome of *Saccharomyces cerevisiae* a single eIF2α kinase has been found: general control non-derepressing (GCN)2. GCN2 is activated in response to nutrient deprivation and in particular to amino acids starvation (Kimball 2001). Mammals have 4 different eIF2α kinases, the mammalian ortholog to GCN2, the double-standed RNA activated kinase (PKR), the heme-controlled inhibitor and the PKR-like endoplasmic reticulum (ER) resident kinase (PERK). Each of these kinases are activated upon distinct stresses: GCN2 after deprivation of essential amino acids, PKR after viral infection and interferon response, HRI during heme limitation and PERK during ER stress or unfolded protein response (UPR).

Phosphorylation of eIF2 α is essential for both the shut-off of global translation and recovery from stress. For instance, when unfolded protein accumulates in the lumen of ER, activated PERK mediates the phosphorylation of eIF2α. PERK-mediated phoshorylation of eIF2α represses the synthesis of proteins targeted to the ER thereby

minimizing further accumulation of unfolded proteins. Lower cellular availability of eIF2A-GTP bound complexes increases the chance that mRNAs with internal AUG are translated, as for ATF4 and ATF5 (**Figure 12**).

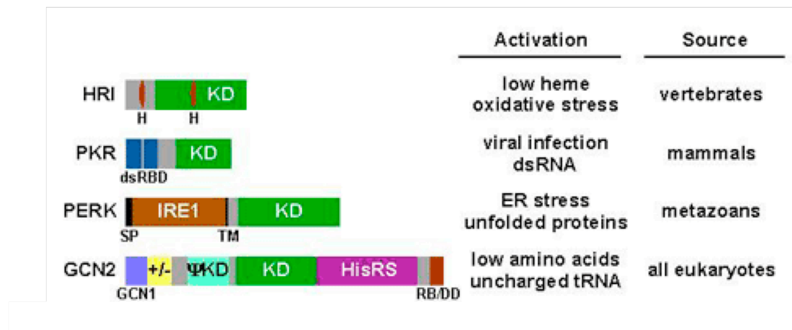


Figure 12. These kinases are sensors of cellular stress. These 4 shown protein kinases are activated following heme-deprivation (HRI), virus infection (PKR), ER stress (PERK), and amino acid starvation (GCN2). Subsequent phosphorylation of eIF2a on Ser-51 inhibits eIF2B and thus impairs general translation.

Together, these observations suggest that IRES and uORF mediate the translation initiation of certain mRNAs representing a regulatory mechanism that helps the cell cope with transient stress. It is thus reasonable that each of the eIF2α kinases would be required for a recovery program specific to its activating stress, hence influencing the translation efficency of different subsets of mRNAs. Moreover, IRES activity may also participate in the maintenance of normal physiological processes such as adequate synthesis of some proteins during cell cycle progression (Fingar et al. 2004).

1.5 Rapamycin

The ability of a cell to respond to environmental stress is a fundamental property that allows survival. mTOR signaling is activated downstream to numerous growth stimuli responding mainly to the Akt/PI3K pathway. Thus, TOR signaling coordinates cell growth and metabolism in response to physiological changes and elicits its control mainly via phosphorilaton of its target 4E-BP as previously described.

Inhibition of cap-dependent translation is essential for survival under stress conditions. Many cellular stressors result in the rapid cessation of overall cap-dependent translation and promotion of cap-independent translation of several pro-survival factors. Overexpression of the translational inhibitor 4E-BP1 or treatment

with mTOR inibitor rapamycin (that diminishes 4E-BP1 molecules able to exert the translational inhibition itself) has been shown to possess viable therapeutic potential for PD.

Mutation in two genes, PARK1, which encodes for an E3 ubiquitin ligase (Parkin) and PARK2, which encodes for a mitochondrial targeted kinase (Park2), result in autosomal recessive parkinsonism. PINK1 and Parkin are component of a common pathway whose function is essential to maintain mitochondrial integrity (Clark et al. 2006; Exner et al. 2007).

In *Drosophila PINK1* and *parkin* mutants exhibit dopaminergic neurodegeneration, locomotor deficits and mitochondrial dysfunction, representing a reliable animal model of PD.

A genetic screening on mutants has identifyied in the *Drosophila Thor* a modifier gene capable to influence the *Parkin-/-* genotype. *Thor* encodes for the sole ortholog of the mammalian 4E-BP1. 4E-BP1 as described before, is a known translational repressor that sequester eIF4E and prevents its binding to the 5'-cap of cytoplasmic mRNAs.

It has been proved that 4E-BP1 mediates the survival response of cells exposed to various stresses (Clemens 2001; Richter and Sonenberg 2005). A regulated control of translation is believed to be the strategy used by cells to elicit a rapid response to toxic insults aiming to immediately change protein synthesis from pre-existing mRNAs pools.

Loss of 4E-BP markedly reduced the viability of double mutants for Parkin and PINK 1. On the contrary, overexpression of 4E-BP1 was sufficient to suppress all the pathologic phenotype in *Parkin/PINK1* double mutants, included neurodegeneration (Tain et al. 2009). The same protective effect *in-vivo* is achieved by rapamycin-driven pharmacological inhibition of TOR.

Rapamycin is a macrolide antibiotic produced by *Streptomyces hygroscopicus*, which binds FKBP-12 (FK506 binding protein). Thereby, the rapamycin–FKBP12 complex can inhibit mTOR preventing further phosphorylation of S6K, 4E-BP1 and, indirectly, other proteins involved in transcription, translation and cell cycle control.

Rapamycin has been recently found to provide neuroprotection in PD models (Malagelada et al. 2010). Primary neurons pre-exposed to Rapamycin are protected by different neurotoxins that are known to recapitulate PD pathogenesis. More importantly, the infusion of rapamycin in the brain of mice treated with the PD

mimicking drug MPTP (1-metil 4-phenil 1,2,3,6-tetrahydro-piridin) rescues neurons from undergoing degeneration.

The neuroprotection is exerted at different levels: 1) Rapamycin promotes autophagy in neurons; protein aggregates and organelles damaged by PD-mimicking neurotoxins would be cleared by autophagic process; 2) Rapamycin suppresses the expression of pro cell death proteins known to induce neuronal apoptosis. One of these proteins is RTP801, a transcription factor that is induced during Parkinson's disease and neuronal oxidative stress (Malagelada et al. 2006a; Ryu et al. 2002). 3) Rapamycin maintains the activation status of neuroprotective signaling pathways in neurons such as the pro-survival Akt pathway (Malagelada et al. 2008). Importantly, rapamycin treatment is also able to rescue mitochondrial defects in *parkin*-mutant PD patients-cells. Other studies have shown that rapamycin significantly reduces the toxic build-up of protein aggregates such as amyloid beta and huntigntin proteins *in vivo* and *in vitro* models of AD and HD (Sarkar and Rubinsztein 2008; Yu et al. 2005).

2. METHODS

Plasmids

Full length AS Uchl1: Full length DNA sequence of AS Uchl1 was amplified via fusion PCR starting from RACE fragment and FANTOM clone Rik6430596G22 with the following primers:

For mAS Uchl1 fl: 5'-ACAAAGCTCAGCCCACACGT-3'

Rev mAS Uchl1fl: 5'-CATAGGGTTCATT -3'

Uchl1: Mouse Uchl1 mRNA was cloned from FANTOM 2900059O22 with the following primers: For mUchl1: 5'-ATGCAGCTGAAGCCGATG-3'

Rev mUchl1: 5'-TTAAGCTGCTTTGCAGAGAGC-3'

AS Uchl1 shRNA: Oligo containing the sequence -14/+4 around the TSS of AS Uchl1 CGCGCAGTGACACAGCACAAA was cloned into *pSUPERIOR.Neo.GFP* vector (OligoEngine). Scrambled sequence was also cloned and used as control.

AS Uchl1 Δ5': 5' deletion mutant was generated by PCR using the following oligonucleotides:

For mAS Uchl1 fl

Rev Δ 5'AS Uchl1: 5'-TACCATTCTGTGCGGTGCA-3'

AS Uchl1Δ3': 3' deletion mutant was generated by PCR using the following primers:

For mAS Uchl1: 5'-GACCTCCTCTAGCACTGCACA-3'

Rev mAS Uchl1 fl

AS Uchl1 Δ(Alu+SINEB2): This mutant was obtained by subsequent cloning of PCR fragment I (NheI-EcoRI site) and PCR fragment II (EcoRI-HindII site) into pcDNA3.1. Primers used to amplify AS Uchl1 to generate fragments I and II were the following:

PCR fragment I:

For mAS Uchl1 fl

Rev pre-SINE B2 5'-CAATGGATTCCATGT-3'

PCR fragment II:

For post-ALU 5'-GATATAAGGAGAATCTG-3'

Rev mAS fl

AS Uchl1 ΔAlu: This mutant was generated with a similar strategy than AS Uchl1 ΔAlu+SINEB2. Below are the primers to obtain PCR fragment I and II.

PCR fragment I:

For mAS Uchl1 fl

Rev pre-SINE B2 5'-CAATGGATTCCATGT-3'

PCR fragment II:

For post-ALU 5'-GATATAAGGAGAATCTG-3'

Rev mAS fl

AS Uchl1 ΔSINEB2: This mutant was obtained as described before. Oligonucleotides used for the PCR:

PCR fragment I:

For mAS Uchl1 fl and

Rev pre-SINE B2: 5'-CAA TGGATTCCATGT-3'

PCR fragment II:

For post-SINE B2: 5'-GAATTCCTCCAGTCTCTTA-3'

Rev mAS fl

AS Uchl1 (Alu+SINEB2) flip: PCR fragment obtained with the following primers was cloned in the unique EcoRI site of AS Uchl1 ΔAlu+SINEB2:

For SINE B2 inside: 5'-TGCTAGAGGAGG-3'

Rev Alu flip: 5'-GTCAGGCAATCC -3'

AS Uchl1 SINEB2 flip: PCR fragment obtained with following primers was cloned in the unique EcoRI site of AS Uchl1 ΔSINEB2:

For SINE B2 inside: 5'-TGCTAGAGGAGG-3'

Rev SINE flip: 5'-AAAGAGATGGC-3'

Cells

MN9D cells were obtained from Prof Michael J. Zigmond at University of Pittsburg. Cells were seeded in 10 mm dishes in Dulbecco's modified Eagle's (DMEM) medium containing 10% fetal bovine serum (Invitrogen) supplemented with penicillin (50 units/ml) and streptomycin (50 units/ml). When required, cells were treated with 1μg/ml Rapamycin (R0395, Sigma) for 45 minutes.

For the establishment of stable cell lines (siRNA -15/+4, siRNA scrambled, pcDNA 3.1- and AS Uchl1ΔSINEB2), MN9D cells were seeded in 100mm petri-dishes and

transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. Stable clones were selected by 500μM Neomycin (N1142, Sigma). HEK cells (SIGMA) were cultured under standard condition in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with antibiotics. Transient transfections were done with Lipofectamine (Invitrogen).
MN9D-Nurr1$^{Tet\text{-}On}$ cells(Hermanson et al. 2003) were obtained from Dr. Perlmann (Ludwig Institute for Cancer Research, Stockholm, Sweden). Cells were maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum, in presence of penicillin (50 units/ml), streptomycin (50 units/ml) and 250 mM Neomycin. Nurr1 expression in MN9D-Nurr1$^{Tet\text{-}On}$ cell lines was induced by addition of 3 μg/ml doxycycline hyclate (Sigma) to the culture medium. Cells were grown as above except that the 10% serum was changed to 5% Fetal Bovine Serum.

RACE and multiplex RT-PCR

The 5' UTR of AS Uchl1 was amplified by RACE PCR (GeneRacer, Invitrogen) by MN9D total RNA and cloned into pGEM®-T Easy vector (Promega).
Multiplex RT-PCR was performed with SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen). 500 μg of total RNA was first DNAse-treated and then retro-transcribed in presence of reverse primers for Gapdh, Uchl1, AS Uchl1 for 60 minutes at 60 degrees. For PCR amplification forward primers were then added at final concentration of 200nM to the reaction.

qRT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. Purified RNA was subjected to DNAse I treatment (Ambion) and 1μg was retro-transcribed using iScript cDNA Synthesis Kit (BioRad). Real Time qRT-PCR was carried out using Sybr green fluorescence dye (2X iQ5 SYBR Green supermix, BioRad). Actin and Gapdh were used as internal standard. Relative quantification was performed with the comparative Ct method. Briefly, the results were normalized to β-actin and /or GAPDH. The initial amount of the template of each sample was determined as relative expression versus one of the samples chosen as reference. Relative expression in each sample was calculated by the

formula $2x^{\Delta\Delta Ct}$ (User Bulletin 2 of the ABI Prism 7700 Sequence Detection System). Below are the primers used:

Gapdh F: 5'-GCAGTGGCAAAGTGGAGATT-3'

Gapdh R: 5'-GCAGAAGGGGCGGAGATGAT-3'

Beta-actin F: 5'- CACACCCGCCACCAGTTC-3'

Beta-actin R: 5'- CCCATTCCCACCATCACACC-3'

AS Uchl1 F: 5'CTGGTGTGTATCTCTTATGC-3'

AS Uchl1 R: 5'CTCCCGAGTCTCTGTAGC-3'

TH qPCR F: 5'- CCGTCTCAGAGCAGGATACC-3'

TH qPCR R: 5'- CGAATACCACAGCCTCCAATG-3'

AS Uchl1 overlap F: 5'-GCACCTGCAGACACAAACC-3',

AS Uchl1 overlap R: 5'-TCTCTCAGCTGCTGGAATCA-3'

Uchl1 F: 5'-CCCGCCGATAGAGCCAAG-3'

Uchl1 R: 5'-ATGGTTCACTGGAAAGGG-3'

ASUchl1 pre RNA F: 5'-CCATGCACCGCACAGAATG-3'

AS Uchl1 R: 5'-GAAAGCTCCCTCAAATAGGC-3'

Pre_ribosomal RNA F: 5'-TGTGGTGTCCAAGTGTTCATGC-3'

Pre ribosomal RNA R: 5'-CGGAGCACCACATCGATCTAAG-3

AS_Uxt F: 5'-CAACGTTGGGGATGACTTCT-3'

AS Uxt R: 5'-TCGATTCCCATTACCCACAT-3'

Uxt F: 5'-TTGAGCGACTCCAGGAAACT-3'

Uxt R: 5'-GAGTCCTGGTGAGGCTGTC-3'

Laser Capture Microdissection technology (LCM)

For LCM, regions of midbrain from TH-GFP/21-31 mice were dissected and incubated in 1X Zincfix solution for 6 hours. They were then cryoprotected in 30% sucrose solution at 4˚C overnight, embedded in Neg-50 section medium, snap-frozen and left to equilibrate in a cryostat chamber at -21˚C for 1 hour before sectioning, as described earlier(Biagioli et al. 2009). Cryostat 14 μm midbrain coronal sections were thaw-mounted on Superfrost plus glass slides (Mezzle-Glasser) and dopaminergic GFP^{+} cells were harvested via LCM and collected in microfuge (PALM adhesive caps). RNA was immediately extracted using Absolutely RNA® Nanoprep Kit (Stratagene), eluted in RNAse /DNAse free water (Ambion) and retro-transcribed.

Double Fluorescent *in situ* hybridization (D-FISH)

After perfusion with 4% formaldehyde, mouse brain was cryoprotected overnight in 30% sucrose. *In situ* hybridization was performed on cryostat slices (16 μm). Sense and antisense probes were generated by *in vitro* transcription from the cDNA encoding the distal 600 bps of mouse Uchl1 cDNA and the last 1000 bps of mouse AS Uchl1. The probes for Uchl1 and AS Uchl1 were labeled with digoxigenin (DIG labelling, Roche) and biotin (BIO-labelling mix, Roche), respectively. Incorporation of biotin and digoxigenin was checked via Northern Blot. ISH was performed as described previously(Ishii et al. 2004). Slices were pre-treated with hydrogen peroxide 3% for 30 minutes. Hybridization was performed with probes at a concentration of 1 μg/ml (Uchl1) and 3 μg/ml (AS Uchl1) at 60°C for 16 h. For biotinilated RNA detection, streptavidin-HRP (Amersham Bioscience) was used (1:250) for 2 hours in TNB buffer (Tris HCl PH 7.5 100mM, NaCl 150 mM, 0,5% Blocking Reagent), and signals were visualized using the *TSA Cy3 system* (Perkin Elmer) after washing in TNT buffer (Tris HCl PH 7.5 100mM, NaCl 150 mM, 0.05% Tween-20)
ISH on DIG-labeled probe was performed with monoclonal anti-DIG antibody after TSA reaction. To combine RNA ISH with immunofluorescence, slices were incubated with anti-TH antibody 1:1000 (Chemicon). Signals were then detected with fluorescent dye-conjugated secondary antibody goat anti-rabbit 405 and goat anti-mouse 488. Sections were then washed, mounted with Vectashield (Vector lab) mounting medium and observed at confocal microscope (Leica).

CHIP Assay

Chromatin immunoprecipitation was performed with magnetic beads (Dynabeads, Invitrogen) following the protocol as described(Schmidt et al. 2009). For each ChIP, one confluent 100mm plate of MN9D-Nurr1$^{Tet\text{-}On}$ cells (Hermanson et al. 2003) was treated with doxycicline 3 μg/ml. Upon doxycicline treatment, Nurr1 expression was monitored by western blot. 1 μg of ChIP-grade anti-Nurr1 antibody was used (sc-990 X). Rabbit IgG were used as negative control (Cell signalling #2729).

qPCR was performed with primers for DNA binding regions of indicated targets and distal primers were designed for an unrelated region 6000 bps upstream the AS Uchl1 TSS:
VMAT NBRE F: 5’-ATTGTGCTAACATTTATTCCAGAG-3’
VMAT NBRE R: 5’-AGGGCTTCCTACGTGACC-3’
OCN NBRE F: 5’-CCACAACACGCATCCTTT-3’
OCN NBRE R: GGACTTGTCTGTTCTGCA-3’
AS Uchl1 NBRE F: 5’ CTTCCCATACAGCTTAGTTCC-3’
AS Uchl1 NBRE R 5’-TTGCGTCTCTGCCAGATG-3’
Distal F 5’-TCATCCAGCCACAAGGTCAGAG-3’
Distal R 5’- CCAGCAGGCACACTGTTGAAC-3’
Enrichment of chromatin binding was calculated relative to total input, as described previously(Guccione et al. 2006).

MPTP treatment

Eight-week-old, male, C57BL mice (Charles River Laboratories) were subjected to a sub-acute MPTP regimen(Kuhn et al. 2003). Mice used in this study were treated according to the NIH guidelines for Care and Use of Laboratory Animals. MPTP use and safety precautions were as described previously(Przedborski et al. 2000). Animal handling and experimental procedures were performed according to guidelines of the University of Trieste. Animals received one intraperitoneal injection of MPTP-HCl (20 mg/kg free base suspended in saline; Sigma-Aldrich) or saline every 2 h for a total of four doses over an 8 h period. When required, animals were sacrificed.

Post-mortem human brain samples

Brain samples were obtained from the brain bank at the Institute of Neuropathology, Bellvitge Hospital (University of Barcelona, Spain). Samples were dissected at autopsy with the informed consent of patients or their relatives and the institutional approval of the Ethics Committee of the University of Barcelona. Brains were obtained from Caucasian, pathologically confirmed PD cases and age-matched controls. The time between death and tissue dissection was in the range of 3 to 5 hours. *Substantia nigra* was excised and immediately frozen for RNA extration.

Western blot

Cells were lysed in SDS sample buffer 2X. Proteins were separated in 15% SDS-polyacrilamide gel and transferred to nitrocellulose membrane. Immunoblotting was performed with the following primary antibodies: anti-UchL1 (#3524 Cell Signalling) 1:300, anti-Uxt (11047-1-AP Proteintech Group) and anti-β actin (A5441, Sigma) 1:5000. Signals were revealed after incubation with recommended secondary antibodies conjugated with horseradish peroxidase by using enhanced chemioluminescence for UchL1 (#WBKLS0500 Immobilion Western Chemioluminescent HRP substrate) and ECL detection reagent (RPN2105, GE Healthcare).

Cellular fractionation

Nucleo-cytoplasmic fractionation was performed using Nucleo-Cytoplasmic separation kit (Norgen) according to manufacturer's instruction. RNA was eluted and treated with DNAse I. The purity of the cytoplasmic fraction was confirmed by Real Time qRT-PCR on Pre-ribosomal RNA.

Polysomes profiles

Polysomes profiles were obtained using sucrose density gradients. MN9D cells were treated with 1 mg/ml rapamycin for 35 min, then with 100 mg/ml cycloheximide for 10 min prior lysis in 150 ml lysis buffer (50mM Tris-HCl pH7.5, 100mM NaCl, 30mM $MgCl_2$, 100 mg/ml cycloheximide, 0.1% NP-40, 40U/ml RNasin®, protease inhibitors cocktail). Whole cell extracts were clarified at 4°C for 10 min at 15000g. The equivalent of 5-10 absorbance units at 254 nm of the clarified cell extract was layered onto 15%-55% (w/v) sucrose gradient (50mM Tris/acetate pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$ and 1 mM DTT) and centrifuge for 3h30 at 39000 rpm in a Beckman SW41Ti rotor at 4°C. The gradient was pump out by upward displacement and absorbance at 254mn was monitored using BioLogic LP software (Bio-Rad). 1ml fractions were collected, 1ml Trizol reagent (Invitrogen) was added and, RNA was extracted following manufacturers instructions. A fixed volume of each RNA sample was then retro-transcribed and percentage of mRNA in each fraction was calculated as relative Ct value to total RNA.

Bioinformatic analysis

For ChIP experiments, identification of NGFI-B binding elements was performed with the Genomatrix program (http://genomatrix.de) and the TRANSFAC database (Wingender et al. 1997). The mouse *AS Uchl1* genome region from kb −3000 to +1000 was the reference sequence. Transcriptional binding factor motifs were chosen on the basis of core similarity (score 1.0) and matrix similarity (above 0.80).

For the identification of a cadidate human hortologue of AS Uchl1, conservation between human and mouse in the orthologus region of AS uchl1 was performed using VISTA genome browser. We selected parameters for conserved sequence tags (CTS) that have a minimum of 75% identity between the mouse and human genome. For each conserved element a primer on the homologus human region was designed.

For the identification of additional translational activator candidates, we searched for FANTOM3 full-length cDNAs that were non-coding RNAs and overlap the 5' end of coding transcripts in a head to head configuration [PMID: 16141072]. The filtered set of 8535 FANTOM3 ncRNA transcripts described previously(Nordstrom et al. 2009) was used as our starting point. Genomic locations of these ncRNA transcripts and REFSEQ(Maglott et al. 2000) coding transcripts were extracted from the alignments in the UCSC Genome browser(Kent et al. 2002) to identify a set of 788 coding-sense/non-coding-antisense pairs. ncRNAs were then checked by RepeatMasker to identify SINEB2-related sequences (Smit, AFA, Hubley, R & Green, P. RepeatMasker Open-3.0.1996-2010 <http://www.repeatmasker.org>). This analysis reduced the number of pairs to 127 protein coding transcripts with overlap at the 5' end (60 with a sense strand version of the repeat, 53 with an antisense version and 14 with both sense and antisense versions).

Alignment of the SINEB2-related elements was then carried out using Clustalw (http://www.ebi.ac.uk/Tools/clustalw2/index.html). From this analysis the antisense overlapping transcripts with a repeat most similar to the one of AS Uchl1, as well as in the same orientation were chosen for experimental testing (AS Uxt).

3. RESULTS

3.1 Identification of a noncoding antisense transcript at Uchl1 locus in the mouse.

The mouse synthenic genomic loci of 10 genes associated to familiar cases of PD were analyzed to identify putative noncoding antisense transcripts. By using the Ensembl browser (http://www.ensembl.org) ESTs and/or cDNAs were scored for being transcribed in the antisense direction with respect to the known gene as well as for its overlap at the 3' or 5' UTR. Furthermore, transcripts were required to be highly conserved across mammalian genomes. As a result, we identified Rik6430596G22 as a putative spliced ncRNA in the locus of the mouse Uchl1 gene that maps in antisense fashion to its coding counterpart. It is a typical 5' head to head transcript that initiates within the second intron of Uchl1 (**Figure 13**). It overlaps the first 72 bps of the sense gene including the ATG codon. 5′ rapid amplification of cDNA ends (RACE) was then carried out on total RNA from MN9D cells to map the precise transcriptional start site (TSS) of the AS Uchl1 gene. As shown in **figure 13**, its TSS lies 250 bps upstream the previously annotated sequence and it is localized in the second intron of Uchl1. Two embedded repetitive sequences, SINEB2 and Alu, were revealed by Repeatmasker within the 3' half of the transcript. The FANTOM2 clone spans a genomic region of 70kb identifying four exons whose intron-junctions follow the traditional GT-AG rule.

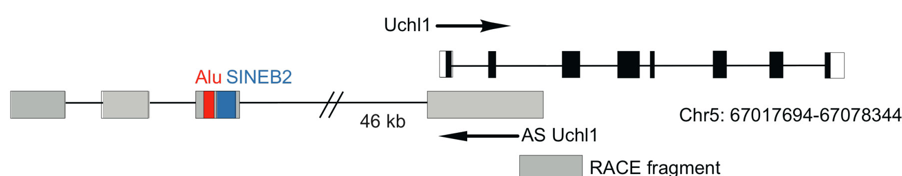


Fig. 13 Genomic organization and expression of Uchl1 and AS Uchl1
Genomic structure of Uchl1/AS Uchl1 locus (Chr5: 67017713-67078563) is represented in the cartoon. Uchl1 exons are depicted in black, transcription occurs on the genomic strand +. AS Uchl1 is transcribed from a TSS (Transcriptional Start Site) that lies within the second intron of Uchl1 and is transcribed on the strand -. The two repetive elements within the antisense are depicted in colours: alu in purple, SINEB2 in green.

3.2 AS Uchl1 is expressed in DA neurons of the SN and nuclear enriched.

Multiplex RT-PCR and double in situ-hybridizations were carried out to study the expression pattern of the antisense gene and to compare it with its sense pair. The analysis of a panel of mouse adult tissues, macroscopically dissected brain regions and neuronal cell lines proved that AS Uchl1 expression was restricted to ventral midbrain and cortex in the mouse adult brain as well as in MN9D dopaminergic cells while it was absent in non dopaminergic cell lines like N2A and Sdh striatal cells.

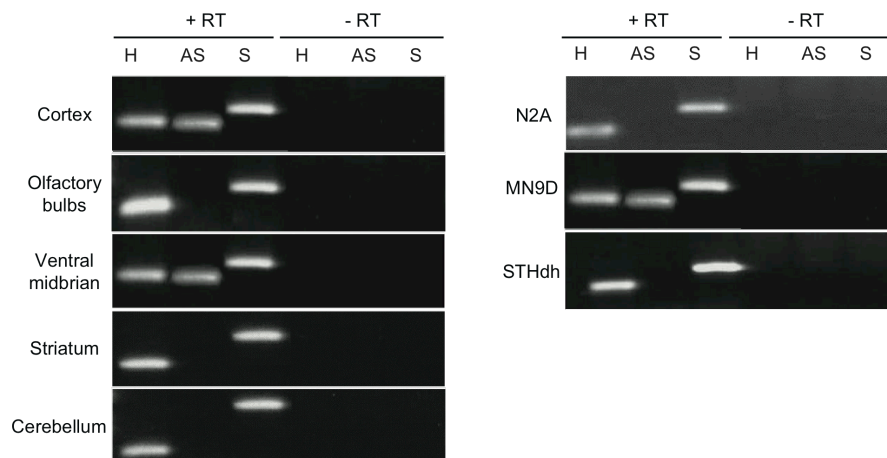


Figure 14. AS Uchl1 is selectively expressed in dopaminergic cells and in Cortex. Multiplex RT-PCR carried on 500ng of total RNA extracted from different parte of adult mouse brain (left) and different cell lines (right). H: Housekeeping Gapdh, AS: AS Uchl1, S: Uchl1.

By taking advantage of the TH-GFP mouse line where dopaminergic (DA) cells are selectively labeled, Laser Capture Microdissection (LCM) technology was used to harvest 300 A9 DA cells from the *Substantia Nigra* (SN). The expression levels of Uchl1 and AS Uchl1 in A9 cells were then monitored by qRT-PCR and compared to macroscopically dissected ventral midbrain. As shown in **Figure 14**, while Uchl1 mRNA was only modestly enriched when compared with total ventral midbrain (3.2 fold), AS Uchl1 transcripts was increased in DA cells an average of 180 fold proving

it is selectively enriched in these neurons (**Figure 15**).

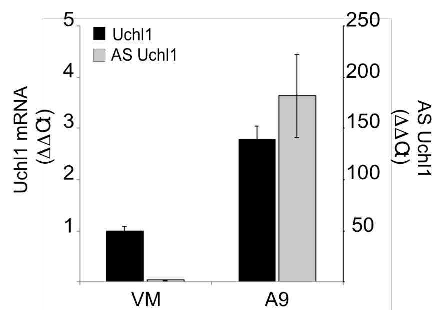


Figure 15. AS Uchl1 is enriched in DA neurons. qPCR starting from 300 LCM-isolated neurons from A9 region of the ventral midbrain of TH-GFP mice. Uchl1 and AS Uchl1 were amplified with intron spanning primers on three biological replica ($P<0.01$).

In situ-hybridization was then performed on coronal sections of ventral midbrain of mouse adult brains with riboprobes corresponding to 600 bps of the 3' end of Uchl1 mRNA and to 1000 bps of the 3' end of AS Uchl1. Both probes did not encompass the region of overlap. Uchl1 mRNA, as expected by comparison with the Allen Brain Atlas, was prevalent in the cytoplasm of cells of the hippocampus, cortex and subcortical regions as well as of the dorsal and ventral midbrain. In situ-hybridization for AS Uchl1 decorated similar structures including cortex, hippocampus and the midbrain (Data not shown).

A combination of Double Fluorescent *in situ* hybridization (D-FISH) with anti-tyrosine hydroxylase immunfuorescence was then carried out on the SN of the midbrain. mRNAs for Uchl1 and its AS transcript were proved to be expressed in the very same DA neurons of the SN (**Figure 16**). Intriguingly, transcripts for the sene/antisense pair were prevalently localized in two different subcellular compartments: mature Uchl1 mRNA mainly stained the cytoplasm, while the AS Uchl1 was nuclear, accumulating in specific subnuclear regions .

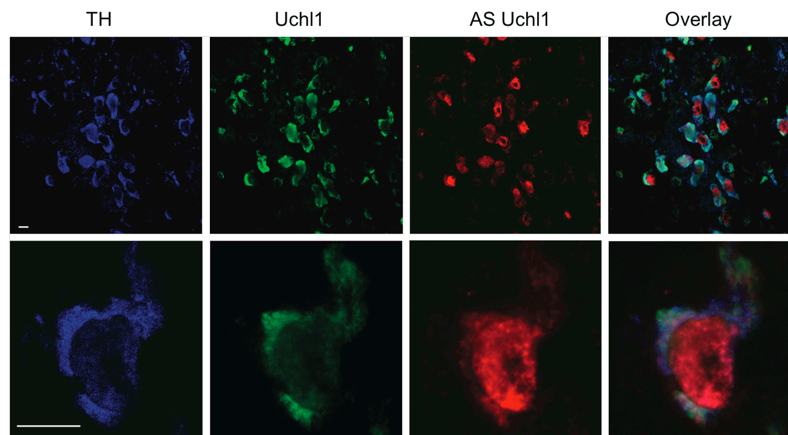


Figure 16. AS Uchl1 transcript is nuclear-enriched in A9 DA neurons. Ventral midbrain slices were processed with antisense probe for Uchl1 (digoxigenin labelled, revealed with FITC) and AS Uchl1 (biotin labelled, revealed with Cy3). DA neurons were visualized by immunohistochemistry using an anti-TH antibody (blue). The overlay (merge) shows co-localization of the transcripts of Uchl1 and AS Uchl1 respectively in the cytoplasm and in the nucleus of A9 neurons. The zoom offers magnifications of the overlay images. Scale bars indicate 10 μm.

3.3 Nurr1 activity regulates AS Uchl1 expression

The genomic region -3000/+1000 around its TSS was then scanned for Transcription Factor Binding Sites (TFBSs) known to be involved in DA-specific transcriptional networks. By taking advantage of MatInspector (www.genomatrix.de), TRANSFAC database (Kel et al. 1995) was analyzed for a core similarity of 1.0 and matrix similarity above 0.90. A NGFI-B element was thus identified in position -1230/-1222 to the AS Uchl1 TSS. This TFBS is the target of the Nurr subfamily of nuclear receptors including Nurr-1, a key dopaminergic transcription factor required for late-dopaminergic differentiation and crucial for the expression of several dopaminergic-specific genes like VMAT2, AADC, DAT and TH (Castro et al. 2001). Nurr-1 binds DNA in an orientation-dependent manner on the NBRE site AAAGGTCA (Wilson et al. 1991) with a possible variation around the core motive responding to RxR/Retinoids Nurr subfamily of nuclear receptors AGGTCA. To validate the Nurr-1 dependency of AS Uchl1 expression, we took advantage of a stable MN9D cell line overexpressing Nurr-1 under a doxycicline inducible promoter MN9D-Nurr1^{Tet-On} (Hermanson et al. 2003). Upon drug treatment, AS Uchl1 underwent a rapid upregulation starting from 12 hours, with kinetics comparable to the one observed for VMAT2, a well-known primary target of Nurr-1. Within 48 hours, both AS Uchl1

and VMAT2 mRNAs reached their peak of induction (**Figure 17a**).

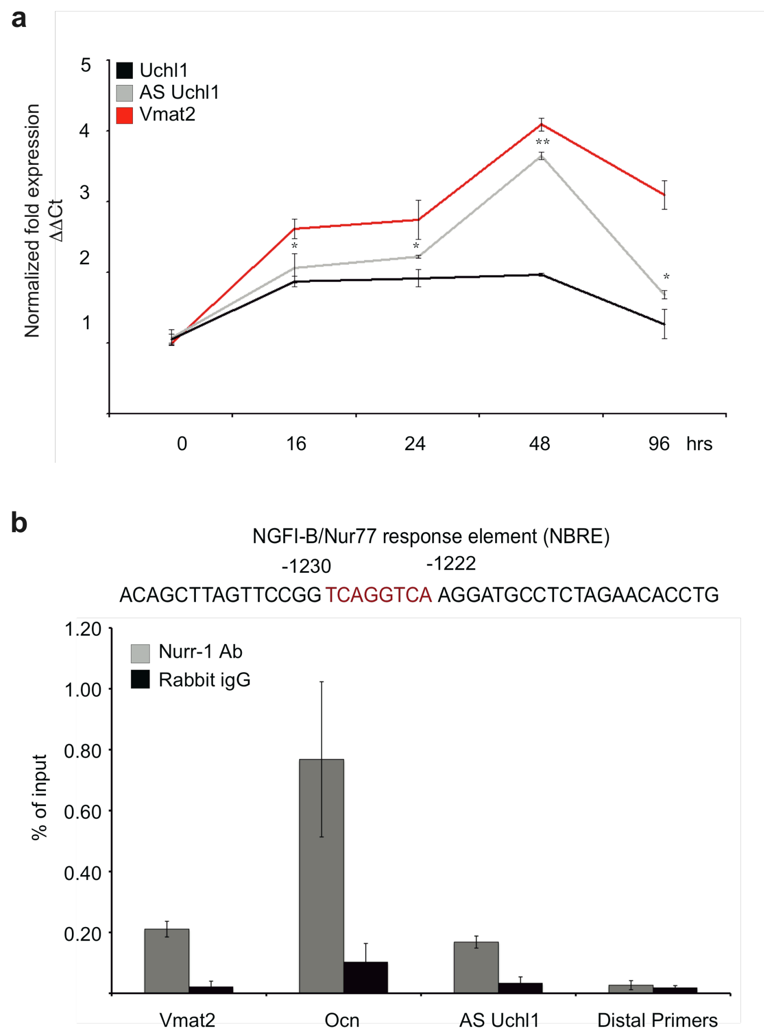


Figure 17. AS Uchl1 is primary target of a dopaminergic specific TF Nurr-1 a, Quantification of Uchl1, AS Uchl1 and VMAT2 RNA expression during *in-vitro* dopaminergic differentiation of MN9D cells stably expressing Nurr-1 under doxycicline-inducible promoter (on the left). Cells are harvested at the following time points after drug exposure: 16, 24, 48, 96 hrs * P<0.05, ** P<0.01. **b,** Chromatin immunoprecipitation of the NGFI-B response element (NBRE) present on VMAT2, OCN and AS Uchl1 promoters (on the right), % of the bound DNA is shown on the Y axes, evaluated as Dct of Nurr-1 (Ct Nurr-1 IP-Ct Imput) compared with Dct IgG control (Ct IgG IP-Ct Imput).

The physical binding of Nurr-1 to the NGFI-B element was then proved by chromatin immunoprecipitation (ChIP) of Mn9D cells after 12 hours of doxycicline induction, when Nurr-1 protein concentration is the highest. Chromatin-protein complexes were immunoprecipitated with anti-Nurr-1 or control (IgG) antibodies and bound genomic DNA was quantified by qPCR using primers for Nurr-1 response elements in AS Uchl1, Osteocalcin and Vescicular Monoamine Transporter 2 gene promoters. PCR

reactions generated only the expected specific amplicon, as detected by gel electrophoresis and melting curve analysis (data not shown). As in **Figure 17b**, Nurr-1 binding was significantly enriched relative to IgG control.

These data strongly suggest that AS Uchl1 is a primary target of Nurr-1 activity and a component of its dopaminergic-specific gene network. On the contrary, its sense, protein encoding counterpart is not since no Nurr-1 binding site was found in the Uchl1 promoter and the kinetics and strength of Uchl1 mRNA upregulation was different from VMAT2.

3.4. AS Uchl1 and Uchl1 are downregulated in neurochemical models of PD.

Since Uchl1 protein is downregulated and inactivated in sporadic PD *post-mortem* brains, the behaviour of the sense/antisense pair was investigated in neurochemical models of the disease.

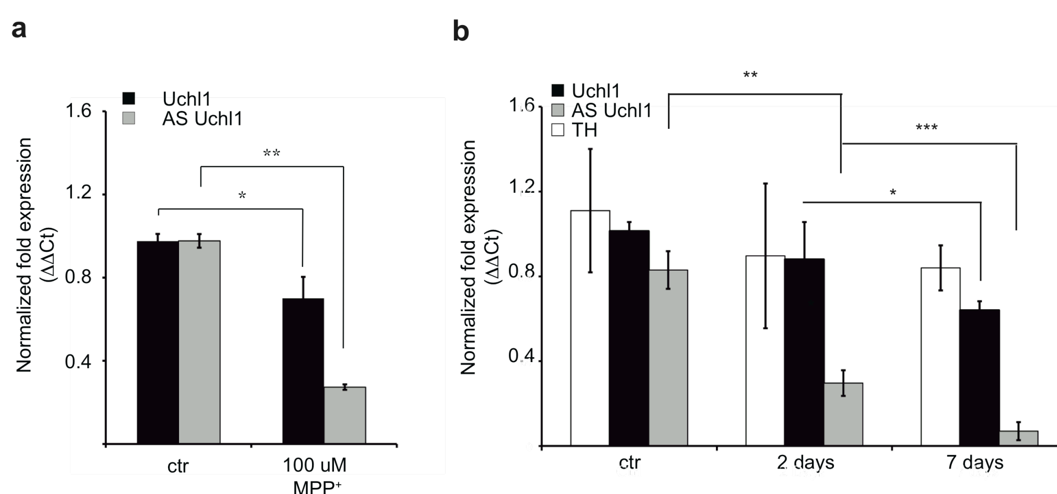


Figure 18. Uchl1/AS Uchl1 SAP are downregulated by PD-like stimuli *in-vitro* and *in-vivo* mouse models and in *post-mortem* SN of PD patients. a, qRT-PCR of Uchl1 and AS Uchl1 transcript levels in MN9D cells treated overnight with 100 uM of MPP^+ (on the left). **b,** qRT-PCR of Uchl1 and AS Uchl1 transcripts perfomed on 300 A9 dopaminergic cells harvested with LCM from mice treated with MPTP (2 days and 7 days after last MPTP injection). Control mice are injected with saline control solution. n=3 mice per time point. * P<0.05, ** P<0.01, *** P<0.001.

When MN9D neuronal cells were exposed to 100 uM MPP+ for 16 hours, Uchl1 mRNA was reduced to 0.72 of its physiological level, while the antisense transcript was reduced to 0.27 (**Figure 18a**). Sub-chronic administration of MPTP was then carried out on 8 months old TH-GFP mice and 300 A9 neurons were purified by LCM in three biological replicates. While modest but significant (P>0.05) downregulation

of Uchl1 was observed (0.67 at day 7 compared to the control), expression of AS Uchl1 was almost completely abolished after 7 days (**Figure 18b**).

3.5 Cloning of human AS UCHL1 and analysis of its expression in *post-mortem* brains of PD patients.

A 70-kb region of the mouse genome encompassing the *AS UchL1* locus was compared to the corresponding human genomic sequence using Genome Vista alignment (http://genome.lbl.gov/cgi-bin/GenomeVista).

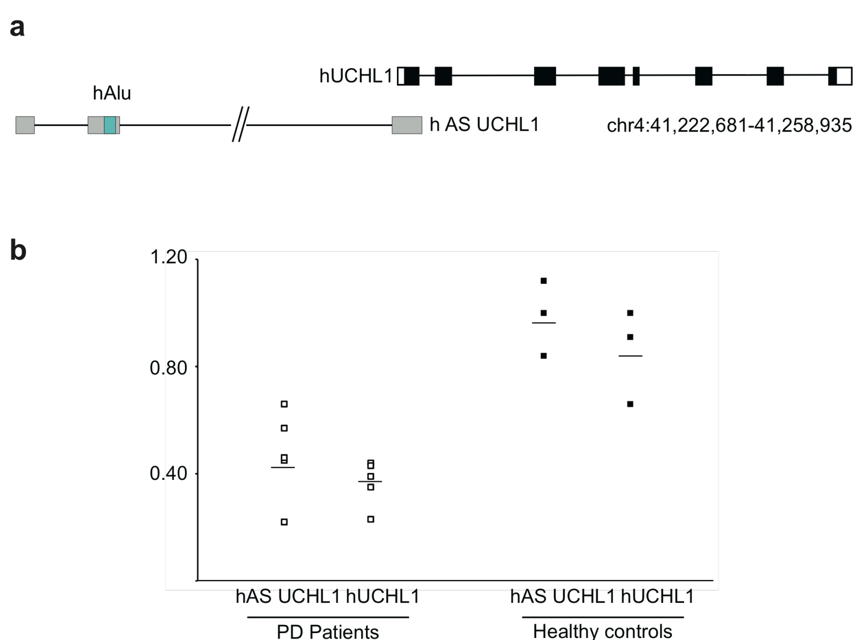


Figure 19. hAS UCH-L1/UCH-L1 are downregulated in in *post-mortem* SN of PD patients. a, Cartoon representing the structure of the human S/AS pair locus (chr4: 41,222,681-41,258,935) including the partial AS UCHL1 human clone, amplified from human total brain RNA. **b,** Total RNA was extracted from SN of five PD and three control brains. hUCHL1 and hAS UCHL1 levels were measured by qPCR. Open (PD cases) and full (controls) squares represent single expression values, dashes between the squares represent the average expression. Significance between PD and control group was calculated with *t*-test.

By the use of primers designed on the human sequence in correspondence to the regions of highest conservation (Conserved Sequence Tags peaks), a 1.2 kb noncoding transcript, 5' head to head antisense to human UCHL1 gene, was cloned from human brain RNA. The anatomical organization of hAS UCHL1 gene was very similar to its mouse counterpart including the extension of the sense/antisense pair overlap region as well as the presence of embedded repetitive elements (Alu element in blue, (**Figure 19a**). As its murine counterpart, hAS UCHL1 expression was highly

restricted to human brain (data not shown). Since UCHL1 espression was reported to be downregulated in PD patients, 5 SN *post-mortem* samples with a clinical diagnosis of PD versus 3 age-matched healthy controls were used in RT-PCR to evaluate hAS UCHL1 mRNA levels. Both UCHL1 and its AS UCHL1 underwent a significative downregulation in all the patients, which is an average of 0.47 of the control level for AS UCHL1 and 0.36 for UCHL1 (**Figure 19b**).

3.5 AS Uchl1 causes Uchl1 protein upregulation in an embedded SINEB2-dependent fashion.

The full length cDNA of AS Uchl1 was then cloned from Mn9D cells and used in the subsequent functional analysis. The interplay between the sense and the antisense transcript was then examined by transiently overexpressing AS Uchl1 in MN9D dopaminergic cells and monitoring endogenous Uchl1 mRNA and protein levels by qRT-PCR and western blotting.

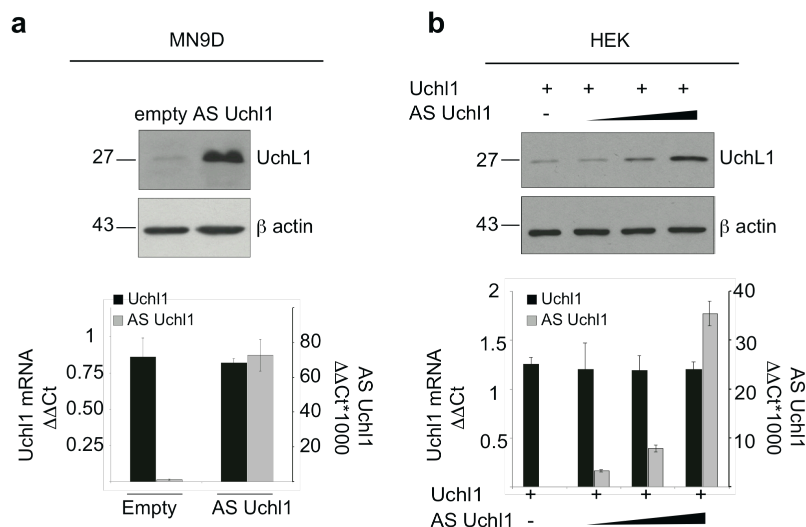


Figure 20. AS Uchl1 regulates Uchl1 translation. AS Uchl1-transfected dopaminergic MN9D cells show increased levels of endogenous Uchl1 protein relative to empty vector control, with unchanged mRNA quantity. **b,** Increasing doses of transfected AS Uchl1 titrate quantity of translated Uchl1 protein in HEK 293T cells. No changes in Uchl1 mRNA levels.

While no significant change in Uchl1 mRNA endogenous levels was observed if compared with empty vector-transfected cells, a strong and reproducible upregulation of UchL1 protein product was detected within 24 hours (**Figure 20a**). We thus resorted to Hek T cells that do not express both transcripts to assess whether the

cotransfection of both cDNAs was able to recapitulate what observed on the endogenous Uchl1 transcript in MN9D cells. When increasing amounts of AS Uchl1 was co-transfected with murine Uchl1, a dose-dependent UchL1 protein upregulation was observed (**Figure 20b**) in absence of any significant change in exogenous Uchl1 mRNA level.

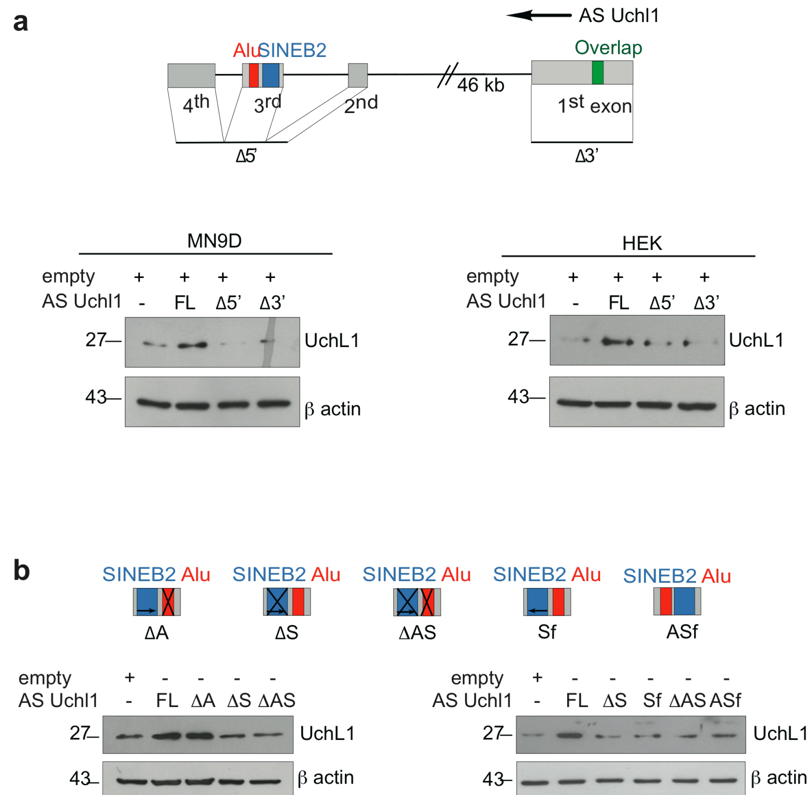


Figure 21. AS Uchl1 regulates Uchl1 translation via embedded SINEB2. a, Full length (FL) AS Uchl1 is required for regulating endogenous (MN9D cells, left panel) and overexpressed (HEK 293T cells, right panel) Uchl1 protein levels. Scheme of D5' or D3' deletion mutants is shown. **b,** Inverted SINEB2 is sufficient to control Uchl1 protein levels. Scheme of mutants is shown.

To identify sequences and/or structural elements of AS Uchl1 mRNA that elicit its functional activity on UchL1 protein, deletion mutants were produced and tested in MN9D cells as well as in co-transfection in Hek cells. AS Uchl1 Δ5' lacked the 5' sequence overlapping Uchl1 first exon while AS Uchl1 Δ3' did not contained the last three exons of the antisense gene. Neither the Δ3' nor the Δ5' transcripts were able to upregulate endogenous UchL1 protein levels in dopaminergic MN9D (**Figure 21a**) and in cotransfection in Hek cells (**Figure 21a**). Titration of the endogenous level of

Uchl1 in transfected MN9D cells was evaluated with qPCR, and no significant change in its mRNA expression was noticed. Additional deletion mutants were then synthesized to assess the role of the embedded repetitive sequences in UchL1 protein upregulation: the ΔSINEB2+Alu (ΔAS), the ΔSINE B2 (ΔS) (764-934) and the ΔAlu ΔA (1000-1045). While the ΔAlu mutant showed a comparable effect on UchL1 protein upregulation as full length AS Uchl1, the ΔSINEB2+Alu and the ΔSINEB2 mutants were unable to do so proving a functional role of the embedded SINEB2 (**Figure 21b**). No change in Uchl1 mRNA level was observed. Since the deletion mutant ΔSINEB2 lacks 170 nucleotides potentially impairing AS Uchl1 RNA secondary structure, a mutant was produced with the SINEB2 sequence flipped in between nucleotide 764-934. Interestingly, SINEB2 flip was unable to increase UchL1 protein levels in transient transfection thus proving the orientation-dependent activity of the SINEB2 domain (**Figure 21b**, right).

3.6 Rapamycin induces shuffle of AS Uchl1 mRNA into the cytoplasm.

Several stressors that have been implicated in PD pathogenesis were then assayed for their ability to shuffle the nucleus-retained AS Uchl1 mRNA into the cytoplasm.

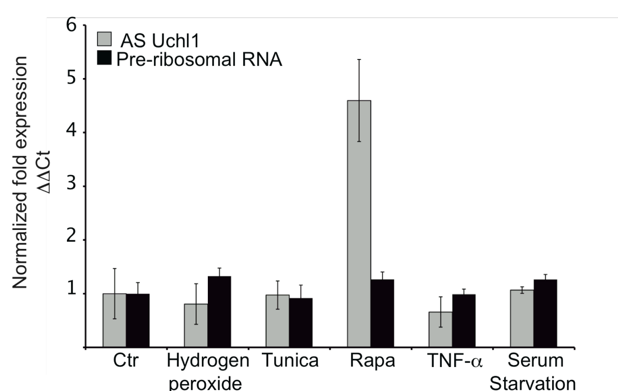


Figure 22. Rapamycin induces AS Uchl1 accumulation in the cytoplasm. qPCR for AS Uchl1 carried on cytoplasmic RNA samples of MN9D cells exposed to hydrogen peroxide 1mM, serum starvation, rapamycin 1 ug/ml, tunycamycin 20nM and TNFalpha 20 nM for 45 minutes. Differential contamination of nuclear RNA was evaluated with pre-ribosomal RNA titration in each sample.

MN9D cells were exposed to hydrogen peroxide 1mM, serum starvation, rapamycin 1 ug/ml, tunycamycin 20nM and TNFalpha 20 nM for 45 minutes and AS Uchl1 mRNA content was independently measured in the cytoplasm and nucleus by qRT-PCR. While for the majorities of treatments no effect was detected, a strong

upregulation of 4-5 fold for AS Uchl1 cytoplasmic mRNA occurred upon exposure to Rapamycin (**Figure 22**).

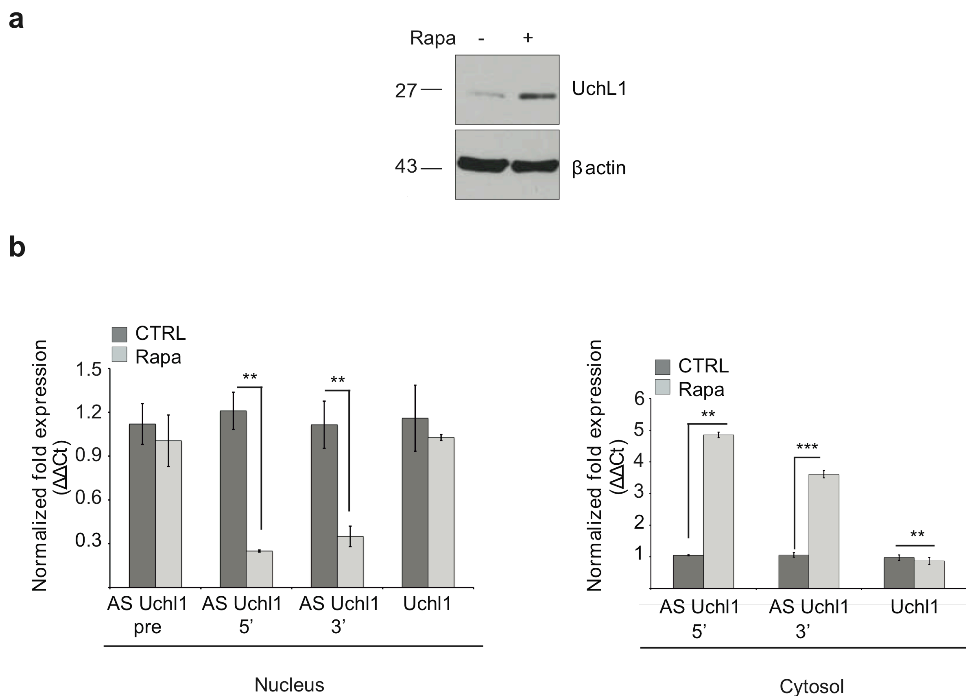


Figure 23. AS Uchl1-embedded SINEB2 induces translation of Uchl1 upon rapamycin treatment. **a,** Uchl1 protein level is increased in rapamycin-treated MN9D cells. **b,** AS Uchl1 translocates to the cytoplasm upon rapamycin treatment in MN9D cells. mRNA levels measured with primers spanning 5' overlapping or 3' distal portions of the transcript. Data indicate mean ± s.d., n≥3 (3). $^{**}p<0.01$; $^{***}p<0.005$.

Rapamycin is a well-known inhibitor of cellular translation through its activity on mTOR and it is currently included in clinical trials for neurodegenerative diseases. The effects of rapamycin were thus studied in more details confirming the redistribution of AS Uchl1 from the nucleus to the cytoplasm in the absence of changes in total cellular content of the transcript (**Figure 23b**, right). A concomitant analysis of Uchl1 mRNA showed no changes in subcellular distribution, de novo transcription and total cellular content (**Figure 23b**, left). However, in the very same samples UchL1 protein level increased several fold upon rapamycin treatment (Figure 23a).

3.7 AS Uchl1 mediates rapamycin-induced UchL1 protein upregulation.

Since the effects of rapamycin on UCH-L1 protein levels were concomitant to AS Uchl1 mRNA redistribution from the nucleus to the cytoplasm, we assessed whether AS Uchl1 was required for UchL1 induction by interfering with AS Uchl1 mRNA expression and function. Small 21-25 nucleotides RNAs directed to gene promoters have been reported to silence gene transcription in a methylation-dependent manner or causing steric interference to the transcriptional apparatus by triple helix formation (Morris et al. 2004).

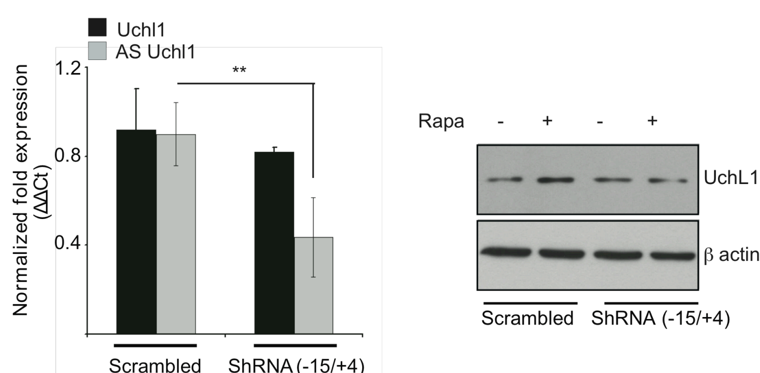


Figure 24. a, Silencing AS Uchl1 transcription in MN9D cells (shRNA, encompassing -15/+4 position of target sequence) doesn not affect mRNA levels. Scramble, shRNA control sequence **b,** In silenced cells rapamycin-induced Uchl1 protein translation is inhibited.

Stable MN9D cell lines were then established expressing constitutively shRNA for AS Uchl1. This sequence targeted AS Uchl1 promoter region from -4 to + 15 nt around the RACE-validated TSS. A single clone showing more than 50% silencing with respect to the scrambled control was chosen for the analysis of the effects of rapamycin treatment (1ug/ml). While scrambled cells showed UchL1 protein upregulation as in MN9D parental line, cells expressing the shRNA for AS Uchl1 presented a strong downregulation of UchL1 protein proving a causal link between rapamycin induction of Uchl1 protein and AS Uchl1 mRNA expression (**Figure 24**).

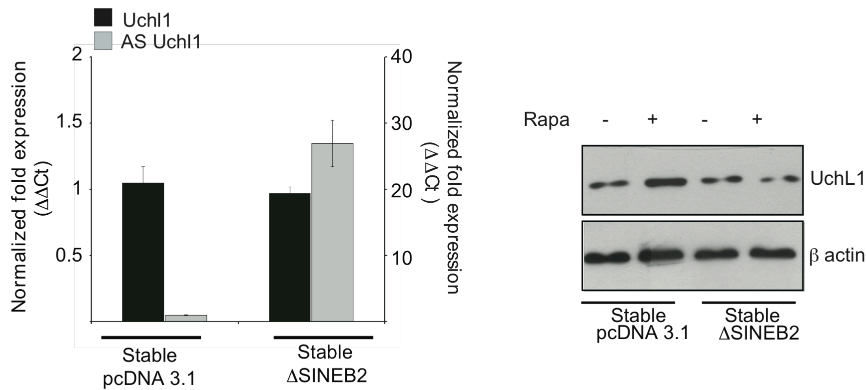


Figure 25. Deletion of embedded SINEB2 (DSINEB2) inhibits rapamycin-induced Uchl1 protein up-regulation. As in Figure 24 **a,** overexpression of AS uchl1 ΔSINEB2 does not affect Uchl1 mRNA levels. pcDNA 3.1 control clone **b,** In ΔSINEB2 cells rapamycin-induced Uchl1 protein translation is inhibited.

As an indipendent model, we established stable cell lines constitutively expressing AS Uchl1 ΔSINEB2 and empty vector as control to take advantage of the dominant negative properties of this construct on the activity of full length AS Uchl1. As expected, when polyclonal stable MN9D cells for empty vector pcDNA 3.1 were treated with rapamycin, UchL1 protein was found increased. In presence of the dominant negative form of AS Uchl1 ΔSINEB2, this upregulation was no longer visible (**Figure 25**, right).

3.8 Analysis of association of polysomes upon rapamycin treatment.

Association of Uchl1 mRNA to polisomes was then monitored upon rapamycin treatment to assess the role of translation in UchL1 protein induction. MN9D cells were treated with Rapamycin 1ug/ml for 45 minutes and with vehicle DMSO as control. Cytoplasmic extracts were prepared and fractionated through sucrose gradients. Thirteen fractions were collected from each gradient while recording the absorbance profile. Distribution of specific transcripts was assayed with qRT-PCR. Uchl1 mRNA was found increased in fraction 12 of 1.54 fold upon rapamycin treatment, showing an enhanced polysome association (**Figure 26a**). mRNAs of β-actin show a pattern similar for DMSO and Rapamycin treated cells since no variation of polysomal association can be observed in the different growth conditions (**Figure 26b**). Rack1 transcript distribution was also studied as representative of TOP mRNAs which translation is specifically suppressed by rapamycin (Jefferies et al. 1997). As

expected, Rack-1 mRNA was mostly associated with polysomes in growing cells (DMSO), but shifted to subpolysomal particles after rapamycin treatment (**Figure26c**).

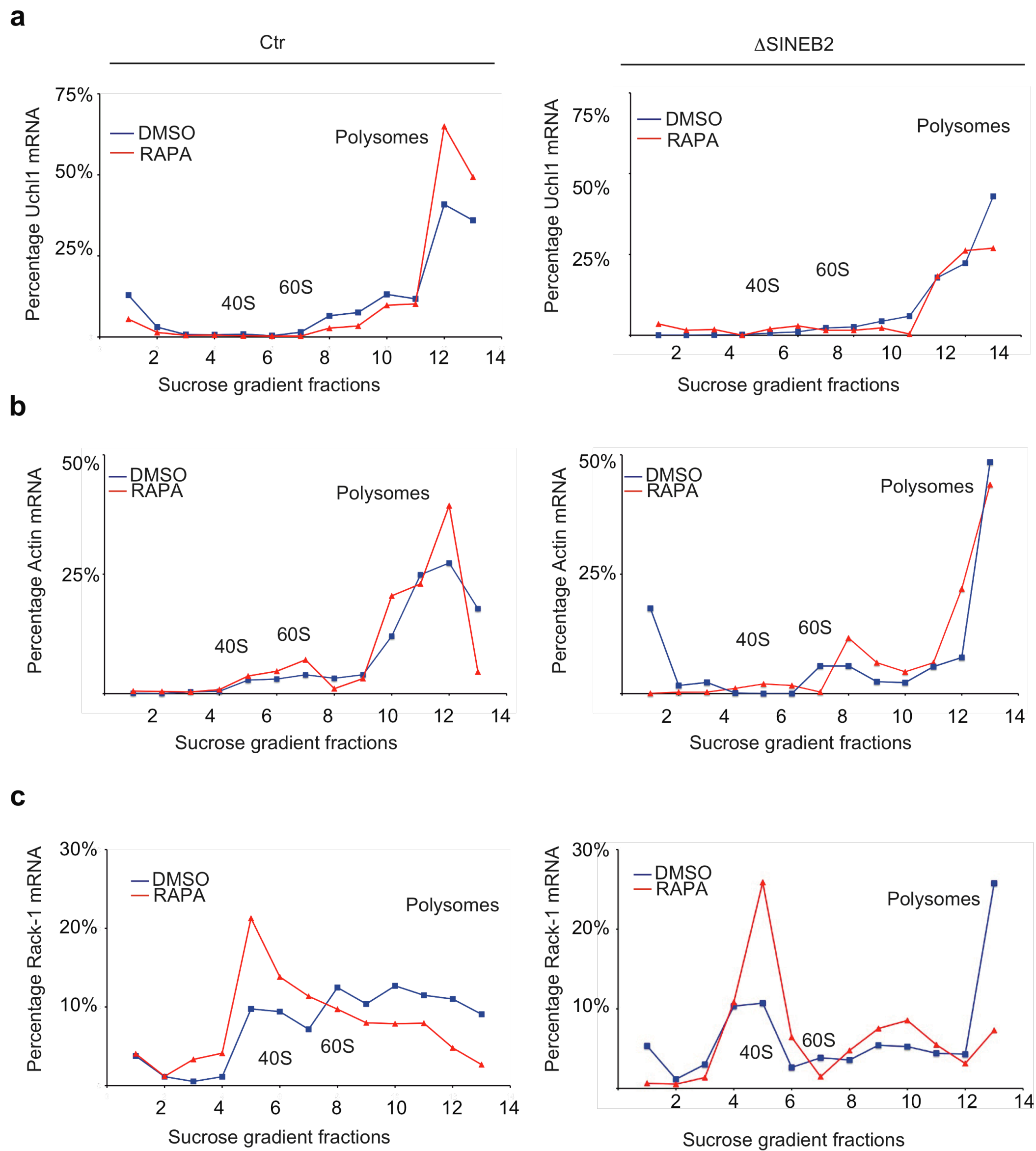
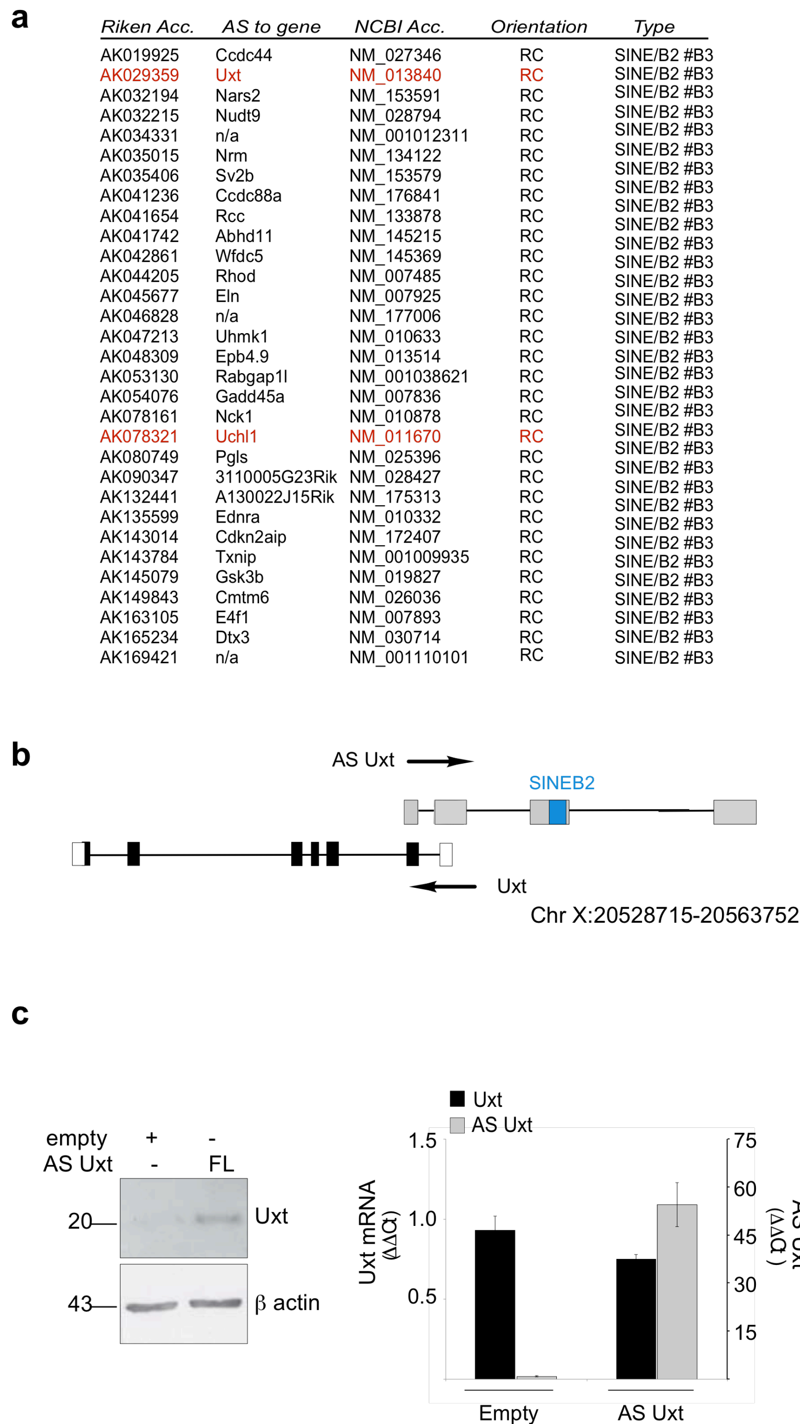


Figure 26. Uchl1 associated with polysomal fraction upon rapamycin treatment. qRT-PCR for **a,** Uchl1 **b,** β-actin and **c,** Rack-1 was performed on RNA extracted from 14 sucrose gradient fractions of MN9D cells (Ctr) and stable AS Uchl1 DSINEB2 mutant (DSINEB2) treated with rapamycin or vehicle alone (DMSO), as indicated. Association with each fraction is shown as percentage of total mRNA.

3.9 AS Uchl1 identifies a new functional class of noncoding antisense RNA.

The collection of FANTOM3 non-coding cDNAs was bioinformatically screened for clones representing natural antisense transcript 5' head to head overlapping to protein coding genes. This list was subsequently analyzed for the presence of embedded SINEB2 of the B3 type in reverse complement orientation in the 3' half of mRNA. 31 transcripts and SAP were thus identified (**Figure 27a**). On the bases of the expression of their protein coding mRNA counterpart in MN9D dopaminergic cells (Biagioli et al. 2009), an antisense RNA Rik4833404H03 to the Ubiquitously expressed transcript Uxt (NM_013840) was then chosen (**Figure 27b**) and tested for its ability to induce upregulation of the sense protein coding overlapping gene. Transfection of AS Uxt in MN9D dopaminergic cell shows transient upregulation of Uxt protein product with no change in the total mRNA levels. (**Figure 27c**).



Riken Acc.	AS to gene	NCBI Acc.	Orientation	Type
AK019925	Ccdc44	NM_027346	RC	SINE/B2 #B3
AK029359	Uxt	NM_013840	RC	SINE/B2 #B3
AK032194	Nars2	NM_153591	RC	SINE/B2 #B3
AK032215	Nudt9	NM_028794	RC	SINE/B2 #B3
AK034331	n/a	NM_001012311	RC	SINE/B2 #B3
AK035015	Nrm	NM_134122	RC	SINE/B2 #B3
AK035406	Sv2b	NM_153579	RC	SINE/B2 #B3
AK041236	Ccdc88a	NM_176841	RC	SINE/B2 #B3
AK041654	Rcc	NM_133878	RC	SINE/B2 #B3
AK041742	Abhd11	NM_145215	RC	SINE/B2 #B3
AK042861	Wfdc5	NM_145369	RC	SINE/B2 #B3
AK044205	Rhod	NM_007485	RC	SINE/B2 #B3
AK045677	Eln	NM_007925	RC	SINE/B2 #B3
AK046828	n/a	NM_177006	RC	SINE/B2 #B3
AK047213	Uhmk1	NM_010633	RC	SINE/B2 #B3
AK048309	Epb4.9	NM_013514	RC	SINE/B2 #B3
AK053130	Rabgap1l	NM_001038621	RC	SINE/B2 #B3
AK054076	Gadd45a	NM_007836	RC	SINE/B2 #B3
AK078161	Nck1	NM_010878	RC	SINE/B2 #B3
AK078321	Uchl1	NM_011670	RC	SINE/B2 #B3
AK080749	Pgls	NM_025396	RC	SINE/B2 #B3
AK090347	3110005G23Rik	NM_028427	RC	SINE/B2 #B3
AK132441	A130022J15Rik	NM_175313	RC	SINE/B2 #B3
AK135599	Ednra	NM_010332	RC	SINE/B2 #B3
AK143014	Cdkn2aip	NM_172407	RC	SINE/B2 #B3
AK143784	Txnip	NM_001009935	RC	SINE/B2 #B3
AK145079	Gsk3b	NM_019827	RC	SINE/B2 #B3
AK149843	Cmtm6	NM_026036	RC	SINE/B2 #B3
AK163105	E4f1	NM_007893	RC	SINE/B2 #B3
AK165234	Dtx3	NM_030714	RC	SINE/B2 #B3
AK169421	n/a	NM_001110101	RC	SINE/B2 #B3

Figure 27. Family of AS transcripts with embedded SINEB2. a, Family of FANTOM 3 non-coding clones that are AS to protein coding genes and contain embedded SINEB2 in inverted orientation. **b,** Schematic diagram of Uxt/AS Uxt genomic organization. **c,** AS Uxt increases endogenous Uxt protein levels in transfected MN9D cells (left), without affecting its transcription (right).

4 DISCUSSION

Recently several long non-coding RNAs (lncRNAs) have been found in the mouse genome (Carninci et al. 2006). This set of transcripts displays a clear evolutionary conservation, thus suggesting a potential biological role. Correlation studies show that nuclear lncRNAs are involved in several biological processes from stem cell pluripotency to cell cycle regulation (Chen and Carmichael 2010; Guttman et al. 2009; Huarte et al. 2010). Numerous lncRNAs are physically associated with chromatin-modifying complexes at the promoter of repressed genes (Huarte et al. 2010) or they can either function as enhancer elements permitting the transcription of neighbouring protein coding genes (Feng et al. 2006). In the widest class of long non-coding RNAs, antisense RNAs represent a powerful subclass of ncRNAs for one main reason: antisense RNAs contain both the information necessary for target recognition (the overlapping sequence with protein coding transcript) and additional analogical or digital features for interaction with RNA binding proteins and/or co-factors. Antisense RNAs can then be the perfect candidate for post-transcriptional regulation of gene expression.

Here we report a novel function for a nuclear lncRNAs AS Uchl1 in the post-transcriptional control of gene expression of its sense mRNA Uchl1.

4.1 AS Uchl1 is expressed in dopamnergic cells of the substantia nigra.

By taking advantage of two indipendent approaches: LCM and Fluorescent *in-situ* hybridization, we detected the expression of AS Uchl1 in the A9 neurons of the Substantia Nigra. Interestingly, while Uchl1 is widespreadly expressed in mammalian brain, AS Uchl1 is found to be selectivly expressed in two sites of the brain, the Cortex and the Substantia Nigra. Spacial or temporal restricted expression pattern for ncRNAs is a very important element to address its functional role.

We revealed that AS Uchl1 is target of specific dopaminergic network responding to Nurr-1 activation, by using an in-vitro model of dopaminergic differentiation MN9D dopaminergic cells MN9D-Nurr1^{Tet-On} cells.

4.2 AS Uchl1 is stress-responding lncRNA.

From transient transfection experiments it emerged that AS Uchl1 was able to drive Uchl1 protein upregulation and that this activity was dependent on the presence of a SINEB2 repetitive element.

As shown by Fluorescent in-situ hybridization and nucleo-cytoplasmic franctionation, the Uchl1 antisense was enriched in the nucleus. Rapamycin treatment is able to mobilize endogenous AS Uchl1 from nucleus to the cytoplasm, where concomitant increase of Uchl1 mRNA association to polysomes takes place.

Taking advantage of two cell lines, the siRNA AS Uchl1 and the AS Uchl1 ΔSINEB2 we determined the functional role of AS Uchl1 in rapamicin-induced UchL1 protein upregulation.

Rapamycin impairs cap-dependent translation, by blocking mTORC1 kinase, which normally activates the eIF4F complex (Andrei et al. 2005; Holcik and Sonenberg 2005). A similar regulatory system occurs upon stress, when regular cap-dependent translation of most proteins is greatly reduced (Patel et al. 2002) and the expression of a small group of proteins such as heat shock proteins or genes involved in survival or apoptosis is greatly enhanced (Lu et al. 2006; Rhoads and Lamphear 1995). The mechanisms that account for the selective translation of certain mRNAs, while overall translation is momentaneously halted, are still incompletely understood but they may include internal ribosome entry sites and ribosome shunting (Gilbert 2010; Rubtsova et al. 2003). They do not require IF4F. Indeed, IRES-mediated translation is prominent in conditions of stress or growth factor inhibition and its alteration affects processes such as tumorigenesis (Holcik and Sonenberg 2005; Yoon et al. 2006). These findings are consistent with the idea that acute stress response does not increase production of new transcripts, but affects the post-transcriptional regulation of previously synthesized mRNAs that are in the cytoplasmic cohort.

In mice rapamycin shows both *in-vitro* and *in-vivo* protection for DA neurons upon neurochemical intoxication and alleviates L-DOPA induced diskynesia in humans (Malagelada et al. 2010; Santini et al. 2009). The protective effect exerted by rapamycin on neurons has been explained by both induction of autophagy (Sarkar et al. 2009) and suppression of RTP801 translation (Malagelada et al. 2006b). We know that after rapamycin treatment AS Uchl1 re-localize into the cytoplasm and rescues Uchl1 from occurring block of translation.

Interestingly, UchL1 is a pro-survival protein for neurons, reason why the manipulation of its expression has been proposed for intervention in neurodegenerative diseases, including PD and AD.
We propose that the antisense-driven elevation of UchL1 in dopaminergic cells that occurs upon rapamycin might be an additional molecular event to explain the protective effect previously recognized for this drug in neurons.

4.3 New class of lncRNAs

The mechanism proposed for AS Uchl1 suggest the intriguing idea that it might exist a pool of cellular nuclear-retained RNAs, normally kept latent in the nucleus that are ready to respond to acute stimuli by changing their subcellular location. We already know that stress-dependent nuclear-cytoplasm shuttling of lncRNAs seems to be a common strategy to regulate translation since CTN-RNA, another nuclear-retained lncRNA, unveiled a cryptic protein coding sequence at its 3'end when in the cytoplasm (Prasanth et al. 2005). By changing location, RNA molecules can be able to exert their specific function, i.e. for AS Uchl1 using the SINEB2 module to increase protein translation and the 5' overlaps to target the sense mRNA.
We thus clustered several antisense transcripts that are overlapping 5' head to head to RefSeq genes in mouse and have a SINEB2 element in reverse complement within their sequence. From this list we experimentally found that a second antisense RNA (AS Uxt) is able to drive protein upregulation of its protein-coding overlapping partner. These results suggests that AS Uchl1 and AS Uxt may be a representative member of a new functional class of lncRNAs that are associated to S/AS pairs in the mammalian genome . They require an overlap at the 5' end and the action of a SINEB2 repeat. The region at the 5' provides specificity to a protein-encoding mRNA partner transcribed from the complementary strand. An inverted SINEB2 element at the 3' is required for translational activation.
It will be interesting in the future to capture all the transcripts that contain a reverse complement SINEB2 element via Deep Sequencing of 5'RACE products obtained from SINEB2 internal primer, thus profiling all the cellular pool of non-coding RNAs that can use this SINEB2 module to enhance translation.
It is also reasonable that all the ncRNA molecules capable to reprogram translation of cognate protein-coding mRNAs via a SINEB2 module may represent a new potential

pharmacological target for pathologies where these proteins are down-regulated and/or inactivated.

4.4 LncRNAs as fine-tuners of gene expression in the brain

Besides the role that ncRNAs have in several biological systems, they have emerged as particularly relevant players in a sophisticated place like the CNS, where the major diversity of ncRNAs is found (in particular lncRNAs (Mercer et al. 2008).

LncRNAs generally play a role by modulating the transcription, post-transcriptional processing, and translation of mRNAs. In the central nervous system they play additional roles in mediating nervous system development, homeostasis, stress responses and plasticity (Qureshi et al. 2010). Moreover, lncRNAs are involved in the pathophysiology of a spectrum of CNS pathologies including neurodevelopmental, neurodegenerative, neuro-oncological and psychiatric diseases.

LncRNAs are implicated in the pathophysiology of neurodevelopmental disorders associated with genomic imprinting, such as Prader–Willi syndrome (PWS) and Angelman syndrome (AS) (Koerner et al. 2009).

As an example of neurodegenerative disorder, deregulation of lncRNAs has been assessed for AD, where the levels of lncRNA AS BACE1 are correlated with higher BACE1 protein which, in turn, leads to increased production of Aβ peptide (Faghihi et al. 2008). Another study utilized human AD brain tissue to link alterations in levels of lncRNA BC200 with AD pathogenesis. Increased levels of BC200 were found in brain regions that are preferentially affected in AD (Mus et al. 2007).

Pathways related to lncRNA are altered in CNS tumors, where a subset of lncRNAs are associated to p53 cis-regulatory elements on the promoter of genes that are specifically induced in response to DNA damage (Guttman et al. 2009). These lncRNAs have probably a fundamental role in the p53-mediated induction of cell cycle arrest, DNA repair and apoptosis that protect neural cells from DNA damage and transformation (Tedeschi and Di Giovanni 2009).

Moreover lncRNAs are known targets of the master epigenetic regulator REST/NRSF (repressor element-1 silencing transcription factor/neuron-restrictive silencing factor) in both mouse and human (Johnson et al. 2009). Deregulation of REST and CoREST functions is linked to a range of CNS pathologies that include cancer (glioblastoma, medulloblastoma, and neuroblastoma), neurodegenerative disease (Huntington's

disease), neurodevelopmental disorder (Down syndromeand X-linked mental retardation), epilepsy, and Ischemia (Qureshi and Mehler 2009).
Neurological disorders like epileptogenesis involve BC1/BC200 brain-specific lncRNAs and Evf2, since they modulate neural network plasticity and excitability (Mercer et al. 2008; Qureshi et al. 2010).
In addition to neurological diseases, a number of psychiatric disorders have also been associated with lncRNAs. For example, the disruption of the DISC sense/antisense genomic locus, which encodes both the DISC1 protein-coding gene and the DISC2 lncRNA, has been linked to the risk of developing schizophrenia, schizoaffective disorder, bipolar disorder, major depression, and autistic spectrum disorders (Chubb et al. 2008; Williams et al. 2009).
Previous genetic linkage studies on brain diseases have revealed that a significant percentage of loci map to non-protein-coding regions of the genome. If we now consider the abundance of antisense and other lncRNAs in the brain it will be interesting to define if those previously identified disease association signals are linked to lncRNAs.
This might be relevant for understanding in depth the genetic program of a complex system like the brain in its development as well as in its ability to learn and to adapt.
Moreover the discovery of new non-coding molecules in the brain can constitute a starting point for the development of novel diagnostic and/or therapeutical approaches directed for example, to those lncRNAs whose down-regulation (or over-expression) is aberrant in CNS disorders.

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