TDP-43 regulates the Microprocessor complex activity
during *in vitro* neuronal differentiation

Dottorando
Valerio Di Carlo

Docente guida
Dr.ssa Elisa Caffarelli

Tutore
Prof.ssa Francesca Cutruzzolà

Coordinatore
Prof. Marco Tripodi
Contents:

Abstract 4

1. Introduction 5-20
   1.1 TAR DNA-binding protein 43 (TDP-43) 7
      1.1.1 Physiological functions of TDP-43 7
      1.1.2 Pathological functions of TDP-43 9
   1.2 Microprocessor Complex 9
      1.2.1 microRNA processing 9
      1.2.2 hairpin-containing mRNA regulation 10
   1.3 TDP-43 and the Microprocessor complex 13

2. Aims 21

3. Results 22-39
   3.1 Knockdown of TDP-43 globally affects miRNA production in NB differentiating cells 24
   3.2 Knockdown of TDP-43 leads to a strong reduction of Drosha protein in NB differentiating cells 25
   3.3 Drosha protein level is rescued in TDP-43 depleted cells after proteasome inhibition 27
   3.4 TDP-43 controls Neurog2 but not Dgcr8 expression in NB differentiating cells 28
4. Discussion and perspectives

5. Materials and methods
   5.1 Cell culture and transfections
   5.2 Stable cell lines generation
   5.3 Plasmid construction
   5.4 Oligonucleotides (sequences complementary to exonic regions)
   5.5 RNA extraction and analysis
   5.6 miRNA high-throughput analysis
   5.7 RNA ImmunoPrecipitation Assay
   5.8 Immunoblotting
   5.9 Co-immunoprecipitation
   5.10 GST pull-down
   5.11 Statistics

6. Glossary

7. Acknowledgments

8. List of publications

9. References
Abstract

TDP-43 is an RNA-binding protein implicated in RNA metabolism at several levels. Even if ubiquitously expressed, it is considered as a neuronal activity-responsive factor and a major signature for neurological pathologies, making the comprehension of its activity in the nervous system a very challenging issue. TDP-43 has also been described as an accessory component of the Drosha-DGCR8 microprocessor complex, which is crucially involved in basal and tissue-specific RNA processing events.

In the present study, we exploited in vitro neuronal differentiation systems to investigate the TDP-43 demand for the microprocessor function, focusing on both its canonical microRNA biosynthetic activity and its alternative role as a post-transcriptional regulator of gene expression.

Our findings reveal a novel role for TDP-43 as an essential factor that controls the stability of Drosha protein during neuronal differentiation, thus globally affecting the production of microRNAs. We also demonstrate that TDP-43 is required for the Drosha-mediated regulation of Neurogenin 2, a master gene orchestrating neurogenesis, whereas post-transcriptional control of Dgcr8, another Drosha target, resulted to be TDP-43-independent. These results implicate a previously uncovered contribution of TDP-43 in regulating the abundance and the substrate-specificity of the microprocessor complex and provide new insights onto TDP-43 as a key player in neuronal differentiation.
1. Introduction

1.1 TAR DNA-binding protein 43 (TDP-43)

TAR DNA-binding protein 43 (Transactive response DNA-binding protein 43 or TDP-43) is a 43 kDa multifunctional protein which is encoded by the 6-exon gene TARDBP (on human chromosome 1p36.2) (Banks et al, 2008). From a structural point of view, TDP-43 is a component of the heterogeneous nuclear Ribonucleoprotein (hnRNP) family: as the other members, it contains two RNA recognition motifs (RRMs), which are involved in binding to UG repeats (Kuo et al, 2009), followed by a C-terminal glycine-rich domain. This region is essential for the interaction with other proteins and is, thus, required for modulating its function (Lagier-Tourenne et al, 2010). TDP-43 is mainly nuclear; however, it is able to shuttle between nucleus and cytoplasm due to its Nuclear Localization Signal (NLS) and to its predicted Nuclear Export Signal (NES) (Lagier-Tourenne & Cleveland, 2009) (Figure 1.1).

![Figure 1.1 Schematic representation of TDP-43 protein.](image)

Schematic representation of TDP-43 domains: this protein contains five known functional domains. Many dominant mutations in TDP-43 have been identified in sporadic (red) and familial (black) ALS patients; almost all of them reside in the C-terminal glycine-rich region of TDP-43. Modified from (Lagier-Tourenne & Cleveland, 2009).

TDP-43 is able to bind DNA and RNA and has been reported to play a crucial role in RNA metabolisms, regulating transcription, pre-mRNA splicing, mRNA stability, mRNA transport and microRNA (miRNA) biosynthesis. Even if it is an ubiquitously expressed protein, it is widely considered a neuronal activity responsive factor that may function as a modulator of neuronal plasticity (Wang et al, 2008).
Since 2006, many groups have been working on TDP-43 since mutations in its gene have been associated to many neurological disorders. In particular, TDP-43 has been described as a major component of the protein aggregates in patients’ brains with neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS) and FrontoTemporal Lobar Degeneration (FTLD), and has also been linked to Alzheimer, Parkinson and Huntington diseases (Kokoulina & Rohn, 2010; Schwab et al, 2008; Wilson et al, 2011). However, it is not clear yet whether protein aggregation per se causes or reflects a consequence of the neurodegenerative pathologies. Therefore, uncovering TDP-43 physiological function is crucial for a better understanding of its contribution to the onset of such pathologies.

1.1.1 Physiological functions of TDP-43

TDP-43 was originally identified in 1995, as a transcriptional factor involved in the regulation of the HIV trans-activation response (TAR) element, that is required for the trans-activation of the viral promoter and virus replication. The authors demonstrated, both in vitro and in vivo, that TDP-43 was unable to bind TAR RNA, whereas it specifically bound to the pyrimidine-rich sequence of TAR DNA (Ou et al, 1995). More recently, TDP-43 has also been found to play a role in the mouse spermatogenesis progression, by regulating the acrosomal protein SP10: by binding to the promoter region of the gene encoding SP10, TDP-43 regulates its expression (Acharya et al, 2006).

It was hypothesized that TDP-43 can repress transcriptional factor recruitment on these two promoter regions, even if, to date, there are no genome-wide data concerning TDP-43 binding site.

So far, the better characterized role for TDP-43 is its involvement in pre-mRNA splicing regulation. The protein can act both as a positive and negative regulator of exon inclusion, by binding to splicing sites as well as to intronic regions far from the splicing sites (>2kb). The first example of splicing regulation is that of human cystic fibrosis transmembrane conductance regulator (CFTR). When mutated, CFTR gene causes Cystic fibrosis (CF), an autosomal recessive genetic disorder. In this case, TDP-43 acts as a negative splicing regulator that, by binding to its preferred UG-repeated elements, drives exon 9 skipping. For this reason, TDP-43 could become a therapeutic target to correct aberrant splicing of exon 9 in CF patients (Buratti et al, 2001).
TDP-43 controls the splicing reaction also by the association with many regulative proteins involved in alternative splicing, such as hnRNPs (A1/A2/B1/C) (Buratti et al, 2005; Freibaum et al, 2010).

Another function that has been attributed to TDP-43 concerns the microRNA biosynthesis. It has, in fact, found associated to the nuclear RNase type III Drosha, which is involved in the first step of microRNA processing (Gregory et al, 2004), and to Dicer, the enzyme that mediates the second step of microRNA maturation (Kawahara & Mieda-Sato, 2012; Kim et al, 2009). A growing body of evidence indicates that TDP-43 could affect miRNA biogenesis at various levels (Buratti et al, 2010; Kawahara & Mieda-Sato, 2012).

TDP-43 is also able to shuttle between nucleus and cytoplasm, possibly regulating the transport of some RNA targets (Colombrita et al, 2012). In the cytoplasm, it has been found associated to the 3’UTR of distinct mRNAs, suggesting that it can affect RNA stability and transport to specific districts (Colombrita et al, 2012). It has also been found associated to mRNA-binding proteins (mRBPs), involved in repression of the local translation in neurons, suggesting a role in the modulation of neuronal plasticity upon neuronal stimuli (Fallini et al, 2012) (Wang et al, 2008).

Moreover, TDP-43 is a component of RNA stress granules (SGs), transient cytoplasmic structures that are formed in response to cellular stress and act as sorting stations for mRNAs, even if it is neither essential for their formation nor a neuroprotective factor in stress conditions (Anderson & Kedersha, 2006; Anderson & Kedersha, 2008; Buchan & Parker, 2009; Freibaum et al, 2010; Nishimoto et al, 2010; Nover et al, 1989; Wang et al, 2008b; (Colombrita et al, 2009) (Figure 1.2).
Figure 1.2
Physiological roles of TDP-43 in RNA Processing
(A) TDP-43 associates with promoter regions, thus regulating transcription.
(B) TDP-43 regulates alternative splicing binding to UG repeats and recruits other hnRNPs.
(C) Is found in a complex with Drosha (and also Dicer) thus regulating microRNA processing.
(D) TDP-43 is involved in mRNA transport to dendritic spines and to the axonal terminal and facilitates local translation.
(E) Is able to shuttle between nucleus and cytoplasm.
(F) Is found incorporated in stress granules associated with other proteins/RNAs.
Modified from (Lagier-Tourenne et al, 2010).
1.1.2 Pathological functions of TDP-43

In pathological conditions, TDP-43 loses its nuclear localisation and delocalises to the cytoplasm where it forms insoluble inclusions, in which the protein is hyperphosphorylated, ubiquitinated, and cleaved into small fragments (Figure 1.3).

![Figure 1.3](image)

**Figure 1.3**

*TDP-43 in physiology and pathology.*
The progressive loss of Motor Neurons (MN) led to death in ALS patients. TDP-43 is one of the most important proteins in ALS: on the left is represented its physiological contribution to cellular metabolism, on the right is represented the effect of TDP-43 mutation in ALS-patients. Modified from (Chen-Plotkin et al, 2010; Fallini et al, 2012).
Such TDP-43 inclusions are characteristic of several neurological disorders, such as Amyotrophic Lateral Sclerosis (ALS) and FrontoTemporal Lobar Degeneration (FTLD) and have been identified in post-mortem Motor Neurons (MN), that are the most affected cell type in ALS, but also in neurons and glia cells (Lagier-Tourenne et al, 2010). Recently, these two diseases have been proposed to be a continuum of a broad neurodegenerative disorder: different studies highlighted that about 15% of FTLD patients developed motor neuron disease and approximately 50% of ALS patients developed cognitive problems (Ringholz et al, 2005; Synofzik et al, 2013; Wheaton et al, 2007). Moreover, TDP-43 pathology seems to be a secondary feature of other neurodegenerative pathologies, including Alzheimer, Parkinson and Huntington diseases (Kokoulina & Rohn, 2010; Schwab et al, 2008; Wilson et al, 2011) (Figure 1.4).

Figure 1.4
TDP-43 is the main protein found in inclusions both in FTLD and in ALS.
Since 2006, FTLD cases were divided in two major subtypes: FTLD-tau, that present an abnormal accumulation of tau protein, and FTLD-U, an heterogeneous second group characterized by ubiquitin-positive inclusions. With the discovery of TDP-43 as the major signature found in ALS pathology, it became clear that TDP-43-proteinopathy was also common to FTLD-U (approximately 50% of cases of FTLD). This evidence suggests a link between these two diseases (Chen-Plotkin et al, 2010).
So far, about 40 different missense mutations in TARDBP gene have been linked to ALS (Borroni et al, 2009; Dewey et al, 2004; Gitcho et al, 2009; Kovacs et al, 2009; Luquin et al, 2009). Almost all of them reside in the C-terminal Glycine-rich domain, suggesting that this region could be responsible for the protein mislocalization and subsequent induction of neurodegeneration (Pesiridis et al, 2009).

To understand whether the pathological contribution of TDP-43 is due to the loss of its nuclear function or to the gain of toxic cytoplasmic function is one of the major challenge in this field.

With regard to the gain of function, it is not clear if TDP-43 aggregation is a cause or a consequence of the pathology (Lee et al, 2011).

It is known that TDP-43 is intrinsically aggregation-prone and that it forms aggregates both in vitro and in vivo. Additionally, it has been highlighted that several mutations within the C-terminal domain (Q331K, M337V, Q343R, N345K, R361S, and N390D) cause a strong increase of inclusions and TDP-43-dependent toxicity (Johnson et al, 2009).

The fact that the C-terminal glycine-rich domain plays a key role in aggregate formation is also supported by the discovery that in post-mortem ALS brain and MNs it is cleaved (with an unknown mechanism) and forms aggregates in the cytoplasm, following a prion-like mechanism (lack of NLS domain) (Cushman et al, 2010; Polymenidou & Cleveland, 2011; Wang et al, 2012; Winton et al, 2008).

In TDP-43 proteinopathies, the inclusions are also phosphorylated and ubiquitinated. TDP-43 phosphorylation is reported to make protein aggregates resistant to degradation, therefore enhancing their insolubility (Zhang et al, 2010). On the other hand, TDP-43 ubiquitination might represent an effort by the cell to target pathological TDP-43 toward proteosomal degradation (Schwab et al, 2008).

Multiple transgenic approaches over-expressing wild type and mutated human TDP-43 have been utilised to clarify the relevance of TDP-43 aggregates in disease onset and progression; however, to date, no satisfying animal models are available, since the ectopic expression of the protein leads, at the same time, to nuclear clearance and to cytoplasmic inclusion formation. Furthermore, it has been demonstrated that reduction of TDP-43 aggregation did not protect cells from death, suggesting that TDP-43 inclusions are not the major cause of citotoxicity (Liu et al, 2013).

In the last few years it became clear that nuclear TDP-43 clearance is a very early event in ALS-TDP, occurring in ubiquitin-negative inclusion cells (Davidson et al, 2007; Giordana et al, 2010; Mori et al, 2008; Pamphlett et al, 2009). It has also been shown that the
consequent TDP-43 loss of function widely affects RNA processing; in fact, in sporadic ALS patients, splicing alterations and editing errors have been reported (Kawahara et al, 2004; Lin et al, 1998; Rabin et al, 2010).

Knock-down experiment of the protein, which mimics the pathological nuclear clearance, highlighted changes in the splicing of about 1500 transcripts. In particular, the most affected are those containing introns longer than 100 kb (Tollervey et al, 2011).

In the absence of TDP-43 many genes linked to ALS are misregulated such as ALSIN, charged multivesicular body protein 2B (CHMP2B), FIG4 (FIG4 homolog, SAC1 lipid phosphatise domain containing protein), synatobrevin-associated protein B (VAPB), vasolin-containing protein (VCP), sortilin (that is a receptor for Progranulin) (Hu et al, 2010; Polymenidou & Cleveland, 2011).

However, TDP-43 nuclear clearance is not only involved in RNA mis-metabolism but has been also associated to alteration of protein homeostasis, since it affects several factors involved in autophagosome maturation and in protein degradation (Ling et al, 2013).

So far, many cellular and animal models have been designed to clarify if the loss of TDP-43 nuclear functions could be sufficient to cause degeneration. In cellular models, depletion of TDP-43 determines defects in processes like proliferation, apoptosis and neuronal differentiation. In mouse models, constitutive depletion causes embryonic lethality; therefore, recently, a mouse model was designed with targeted depletion of TDP-43 expression in the spinal cord motor neurons. This mouse model underwent significant motor neuron loss, reactive astrocytosis, microglia activation, and accumulation of poly-ubiquitinated proteins, all of which were characteristic of ALS-TDP. These results demonstrated a crucial role for TDP-43 in the survival and functioning of the mammalian spinal cord motor neurons and, more interestingly, they provide direct evidence that loss of functions of TDP-43 in motor neurons is sufficient and could be one of the major causes leading to the pathogenesis in ALS-TDP patients (Wu et al, 2012).
1.2 Microprocessor Complex

The “Microprocessor” complex is a huge multi-protein complex of about 650 kDa in humans, which is responsible for the processing of two distinct types of targets (Gregory et al, 2004; Han et al, 2004):

i) it is involved in the first step of microRNA processing, being responsible for the pri-microRNA (pri-miRNA) endonucleolytic cleavage in the nucleus, that leads to the production of pre-microRNAs (pre-miRNA).

ii) it is involved in the control of some hairpin-containing RNA stability.

The two main components of the Microprocessor are the endoribonuclease Drosha and its cofactor DGCR8 (DiGeorge syndrome critical region gene 8) (Pasha in D.melanogaster and C.elegans) (Denli et al, 2004). It has been demonstrated that neither the recombinant DGCR8/Pasha nor Drosha alone are able to process pri-microRNAs in vitro, indicating that the two proteins are necessary and sufficient for this process (Gregory et al, 2004; Han et al, 2004) (Figure 1.5).

![Figure 1.5](image-url)

**Microprocessor complex’s targets**

Drosha and DGCR8 complex can process both pri-microRNAs and hairpin-containing mRNAs since they contain conserved secondary structures.
Drosha is a 160 kDa Ribonuclease III enzyme (RNase III). It is characterized by two RNase III domains (RIIIDa and RIIIDb), necessary for substrate cleavage, and a double-stranded RNA binding domain (dsRBD) responsible for the substrate recognition. It also contains other two domains of unknown function, a proline-rich and a serine/arginine rich one, at the N-terminal region (Figure 1.6b).

DGCR8 has two highly conserved double-stranded RNA binding domains (dsRBD1 and 2) that are crucially involved in the substrate binding. Recently, it has been demonstrated that the Microprocessor complex’s specificity does not rely on DGCR8, since it displays an unspecific RNA binding activity (Roth et al, 2013).

This complex was first identified in 2004 thanks to Drosha purification experiments (Gregory et al, 2004). The authors demonstrated that Drosha is associated with several proteins denominated Drosha-Associated Polypeptides (DAP), 19 of which were identified. These proteins included specific classes of RNA-associated proteins with common structural domains: the DEAD-box and DEAH-box family of RNA helicases, proteins with domains that bind double-stranded RNA, heterogeneous nuclear ribonucleoproteins (hnRNPs) and the Ewing’s sarcoma family of proteins containing an RNA recognition motif (RRM) and a zinc-finger domain. Notably, some of these proteins, such as p68, p72, two RNA helicases and TDP-43, have been shown to modulate the Microprocessor activity (Fukuda et al, 2007); (Kawahara & Mieda-Sato, 2012).

### 1.2.1 microRNA processing

The “canonical” and the best characterized function of the Microprocessor complex is the pri-microRNA processing in the nucleus, leading to the generation of microRNAs. Such molecules are one of the most abundant and important class of small non-coding RNAs, crucially involved in post-transcriptional gene regulation (Aalto & Pasquinelli, 2012). These small RNAs, of about 19-25 nucleotides, through base-pairing, drive the RNA-induced silencing complex (RISC) onto their mRNA targets repressing their expression (Doench & Sharp, 2004). The importance of this type of gene regulation depends on the flexibility and on the specificity of microRNA:mRNA interactions. In fact, one microRNA can target distinct mRNAs; furthermore, one mRNA can be targeted, in a combinatorial manner, by many microRNAs (Balaga et al, 2012).

Pri-microRNAs are usually transcribed by RNA Polymerase II as a several hundred nucleotides long RNA containing a 60-80 nucleotide stem-loop structure. These precursors
are processed co-transcriptionally by the Microprocessor: Drosha cleaves both RNA strands, near the base of the stem-loop structure, leading to the release of a 60-80 nucleotide long pre-microRNA. Pre-microRNAs are characterized by a 5’ phosphate and 2 nucleotide 3’ overhang (Lee et al, 2003); they are transported to the cytoplasm by Exportin-5 where a second RNase III endonuclease, Dicer, produces the double stranded miRNA. Subsequently, only one strand of the miRNA duplex is incorporated into the effector complex RISC that mediates target gene silencing (Figure 1.6a).

Figure 1.6
microRNAs processing.
(A) Pri-miRNAs processing by the Microprocessor complex.
(B) Structural organization of Drosha, DGCR8 and Dicer proteins. Modified by (Han et al, 2006).
Notably, microRNAs are key players in several distinct biological processes and their mis-regulation has been linked to cancer, heart ailments, diabetes and neurological defects in humans. Interestingly, the brain expresses the highest variety of microRNAs species, suggesting an important role played by these molecules in the regulation of brain development, cell type differentiation and maintenance etc (Johnston et al, 2005). Several microRNAs:mRNA regulatory circuits have also been characterized in neuronal tissue. For example, the transcriptional neuronal repressor REST (RE1-silencing transcription factor) and its cofactors are targets of multiple brain-related miRNAs, including miR-124, miR-9 and miR-132, thus allowing neuronal gene expression in neuronal precursor cells (Laneve et al; Wu & Xie, 2006).

1.2.2 hairpin-containing RNA regulation

The “alternate” function of the Microprocessor complex concerns the post-transcriptional regulation of several hairpin-containing mRNAs, thus regulating the cellular proteome (Chong et al, 2010; Kadener et al, 2009; Knuckles et al, 2012; Macias et al, 2012). Many groups clarified that this effect is mediated by Drosha and is miRNA independent, since Dicer depletion does not affect these transcripts. These RNAs contain evolutionarily conserved hairpins similar to those recognized by the Microprocessor complex in pri-miRNAs (Kadener et al, 2009).

Notably, this regulatory mechanism is cell type-specific, suggesting its involvement in the regulation of differentiation-development processes (Chong et al, 2010). Among them, there are ubiquitously expressed mRNAs, such as the Drosha cofactor, DGCR8, and tissue-specific genes, as Neurogenin 2, a proneural gene directing neurogenesis. DGCR8 is the first identified target. Distinct groups reported that the Microprocessor complex negatively regulates DGCR8 expression through the cleavage of hairpin structures localized in the 5’ UTR region leading to mRNA destabilization and degradation (Han et al, 2009; Triboulet et al, 2009). The recognised hairpins are highly conserved “pre-miRNA-like” structures cleaved by the Microprocessor (Pedersen et al, 2006); in fact, it has also been demonstrated that DGCR8 5’UTR confers Microprocessor-dependent repression of reporter genes (Triboulet et al, 2009).

This regulatory event is even more intriguing in the light of the DGCR8-mediated regulation of Drosha protein stability. In fact, it has been reported that DGCR8 protein stabilizes Drosha
protein through interaction of DGCR8 C-terminal region with the central portion of the enzyme (Han et al, 2009; Yeom et al, 2006). Altogether this evidence demonstrate a cross-regulation between the two main components of the Microprocessor complex that guarantee the integrity and stoichiometry of the complex itself (Han et al, 2009; Triboulet et al, 2009) (Figure 1.7).

**Figure 1.7**

**Cross-regulation between Drosha and DGCR8**

The correct stoichiometry of the complex is guaranteed by the reciprocal control between Drosha and DGCR8. Drosha protein controls DGCR8 at the RNA level whereas DGCR8 protein controls Drosha at the protein level Modified from (Winter et al, 2009).

Neurogenin 2 is a tissue-specific gene whose transcript is regulated by the Microprocessor complex in mouse (Knuckles et al, 2012). Neurogenin 2 (Ngn2) is a bHLH transcription
factor that promotes neuronal differentiation by increasing the expression of proneural genes and inhibiting glial genes in neural progenitor cells (NPC) (Kageyama et al, 1997). It has been demonstrated that its messenger RNA contains four distinct hairpins in the 3’UTR, that are cleaved by Drosha/DGCR8 complex thus regulating Ngn2 protein level in a microRNAs-independent manner (Figure 1.8). Therefore, the Microprocessor, by preventing aberrant accumulation of Ngn2, plays a key role in neurogenesis (Knuckles et al, 2012).

![Figure 1.8](image)

**Figure 1.8**
Post-transcriptional regulation of Neurog 2.
(A) Post-transcriptional regulation of Neurog 2 is microRNAs-independent since it is not upregulated in Dicer knockout mice.
(B) Schematic structure of Neurog 2 mRNA.
Modified from (Knuckles et al, 2012).
1.3 TDP-43 and the Microprocessor complex

TDP-43 was identified as a component of the 650kDa Microprocessor complex in 2004 by Gregory. It was described, thanks to the affinity purification of epitope-tagged Drosha, as a Drosha-associated polypeptide (DAP) and was supposed to be responsible for the regulation of Microprocessor complex functions (Gregory et al, 2004) (Figure 1.9a).

![Figure 1.9](image)

**Figure 1.9**
**TDP-43 is associated to the Microprocessor complex.**
(A) Drosha immunoprecipitation reveal several DAP, including TDP-43. Modified from (Gregory et al, 2004).
(B) Superose 6 chromatography of FLAG-Drosha containing complexes revealed the presence of TDP-43 in active Drosha/DGCR8 complex. Modified from (Kawahara & Mieda-Sato, 2012).

SILAC–TAP experiments also demonstrated its association with several Drosha/DGCR8 complex components included interleukin-enhancer binding factor 2/nuclear factor 45 KDa
(ILF2/NF45), interleukin-enhancer binding factor 3/nuclear factor 90 KDa (ILF3/NF90), DEAD (Asp-Glu-Ala-Asp) box polypeptide 17, also known as p72 (DDX17), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 5, also known as p68 (DDX5) (Ling et al, 2010). In 2012, Kawahara and Mieda-Sato described TDP-43 as a component of nuclear Drosha complexes by Co-ImmunoPrecipitation and size-exclusion chromatography experiments (Figure1.9b). TDP-43 interacts with Drosha complex, in an RNA-dependent and -independent manner, thanks to its Glycine-rich domain (in particular amino acids 316–402). Moreover, they highlighted that TDP-43 facilitates the binding of the Drosha complex to a subset of pri-miRNAs (pri-miR-132, pri-miR-143, pri-miR-558, and pri-miR-574) in proliferating condition, which results in their efficient cleavage into pre-miRNAs (Buratti et al, 2010; Kawahara & Mieda-Sato, 2012). Finally they clarified that, in the absence of TDP-43, the same microRNAs were down-regulated; among them, miR-132-3p was responsible for correct neurite outgrowth during neuronal differentiation (Kawahara & Mieda-Sato, 2012).
2. Aims

For its nature of neuronal activity-responsive factor and hallmark of neurodegenerative diseases, TDP-43 has recently become the focus of intense interest in the fields of neuronal physiology and pathology. Mutations within the gene encoding TDP-43 have been linked to neurodegenerative disorders, such as Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Dementia (FTLD). In these pathologies, TDP-43 is mis-localised from its predominant nuclear localization to the cytoplasm where it forms ubiquitin-positive inclusions leading, at the same time, to loss of normal nuclear TDP-43 function and to toxic cytoplasmic gain of function. Recently, targeted depletion of TDP-43 expression in the mouse spinal cord motor neurons has shown that loss of TDP-43 function is sufficient and could be one of the major causes leading to TDP-43-linked ALS.

Originally identified as a transcription factor, TDP-43 was subsequently described to regulate RNA splicing, stability, transport and translation. In the last few years, a role in microRNA (miRNA) biogenesis has also been highlighted. In particular, as a factor associated with the nuclear Drosha/DGCR8 Microprocessor complex, TDP-43 has been shown to promote the production of a subset of miRNAs in undifferentiated cells. However, since miRNAs are crucially implicated in neural differentiation and diseased neurons show dramatic loss of nuclear TDP-43 staining, studying the nuclear contribution of TDP-43 to the miRNA biogenesis during in vitro neuronal differentiation was the main goal of my PhD research program.

Utilizing in vitro model systems and RNAi as a technique to operate TDP-43 cell depletion, which mimics the pathological TDP-43 nuclear clearance, I highlighted a previously uncovered function for the protein as an essential factor that controls both the abundance and the substrate-specificity of the Microprocessor complex during the differentiation process. In fact, the loss of TDP-43 caused the destabilization of a conspicuous amount of Drosha protein, with the consequent downregulation of the entire miRNA repertoire. Furthermore, I showed that, in the same condition, TDP-43 directs the Microprocessor complex on a master neurogenic gene, Neurogenin 2, thus contributing to neuronal differentiation process.
3. Results

3.1 Knockdown of TDP-43 globally affects miRNA production in NB differentiating cells

TDP-43 knockdown was carried out in human neuroblastoma (NB)-derived SK-N-BE cell line, both in proliferating and in differentiating conditions. Neuronal differentiation was triggered by treatment with Retinoic Acid (RA) (Laneve et al. 2007), which induces cell growth arrest and the modulation of several neuronal differentiation markers (Figure 3.1 a and b).

![Figure 3.1](image)

**Figure 3.1**
RA treatment of SK-N-BE cells modulates differentiation markers.
(a) Immunoblotting of REST, ID2, N-Myc and Vgf in NB cells treated with RA for 3 and 6 days. GAPDH was used as a loading control.
(b) Northern blot analysis of miRNAs induced in NB cells treated with RA for 3 and 6 days. U2 snRNA was used as a loading control.

Untreated or RA-treated NB cells were transfected with siRNAs against TDP-43 mRNA (Figure 3.2 a, lanes +) or control scrambled siRNAs (Figure 3.2 a, lanes -), and TDP-43 levels were assessed by Western blot. As shown in Figure 3.2 a, a considerable reduction of TDP-43 protein level both in untreated and in RA-treated NB cells (65% and 90% reduction, respectively) was obtained.
RNA from the same samples was then used for miRNA expression profiling by Real-Time PCR (qRT-PCR). Figure 3.2 b shows that the levels of the tested miRNAs were unaffected or slightly affected in untreated cells; conversely, they all were significantly reduced (from 45% to 80%) in RA-treated cells. The same profile was obtained in RA-treated cells when TDP-43 knockdown efficiency was lower (about 60%; data not shown). After confirming these results in SH-SY5Y, another human-derived neuroblastoma cell line (data not shown) (Annibali et al, 2012; Schwab, 2004; Tweddle et al, 2001), the role of TDP-43 in miRNA biogenesis was further investigated through a global analysis of miRNA population. This analysis, carried out by the high-throughput qRT-PCR in SK-N-BE cells induced to neuronal differentiation, revealed that 90% knockdown of TDP-43 caused a global downregulation of miRNAs (about 98%, Figure 3.2 c).
Figure 3.2
TDP-43 knockdown affects miRNA expression in differentiating SK-N-BE cells.
(a) Immunoblotting of TDP-43 in untreated or 6 day RA-treated NB cells transfected with siRNAs against TDP-43 (lanes siTDP-43 +) or control scrambled siRNAs (lanes siTDP-43 -). GAPDH was used as a loading control. The densitometric analysis on the right shows the relative amounts of TDP-43 in sample cells (grey bars) versus control cells (black bars, set as 1). Data are presented as mean values ± s.e.m. from three independent experiments. *: p value < 0.05; **: p value < 0.01, ***: p value < 0.001.

(b) The histograms show the relative amount of miRNA levels, as determined by qRT-PCR analysis, in both untreated (left panel) or 6 day RA-treated (right panel) NB cells transfected with siRNAs against TDP-43 (grey bars) or control scrambled siRNAs (black bar, set as 1). SCARNA17 was used as a loading control. Statistics as in panel (a).

(c) Volcano plot reporting fold change (log\textsubscript{10}[RQ]) versus p-value for each microRNA, according to high-throughput qRT-PCR expression analysis. 6 day RA-treated NB cells depleted for TDP-43 are compared to control cells. For this group of microRNAs, p-values are <0.05.

3.2 Knockdown of TDP-43 leads to a strong reduction of Drosha protein in NB differentiating cells

The levels of Drosha, DGCR8 and TDP-43 were evaluated at specific time points during the RA-induced differentiation of human SK-N-BE cells (Figure 3.3).

![Figure 3.3](image_url)

Drosha and DGCR8 but not TDP-43 expression are modulated in RA-treated SK-N-BE cells.
Western blot analysis of Drosha, DGCR8 and TDP-43 levels during NB cell differentiation (timepoints of RA treatment reported above each lane). GAPDH levels were detected for calibration. Relative Drosha (black bars) and DGCR8 (grey bars) decrease are quantified in the histogram aside. Untreated cells are set as “1”.

Notably, we found that, while the levels of TDP-43 did not change upon RA treatment, those of both the microprocessor components strongly decreased at 3 days (a reduction of about 60% for Drosha and 40% for DGCR8). This profile suggested that the amount of the microprocessor complex may be limiting in differentiating versus proliferating cells. We then addressed whether TDP-43 knockdown may alter the levels of the proteins involved in miRNA biogenesis. We found that the amount of both Drosha and DGCR8, as well as Dicer, were unaffected in undifferentiated SK-N-BE cells (Figure 3.4). By contrast, knockdown of TDP-43 in RA-treated cells was accompanied by a strong reduction of Drosha levels (66% decrease) and a concomitant slighter downregulation of DGCR8 (24% decrease), whereas the levels of Dicer were not affected (Figure 3.4).
Figure 3.4
Drosha protein level is reduced upon TDP-43 knockdown in differentiating SK-N-BE cells.
(a) Left panel: immunoblotting for TDP-43, Drosha, DGCR8 and Dicer in untreated NB cells transfected with siRNAs against TDP-43 (lane siTDP-43 +) or with control scrambled siRNAs (lane siTDP-43 -). GAPDH was used as a loading control. Right panel: densitometric analysis showing the relative amounts of Drosha, DGCR8 and Dicer in sample cells (grey bars) versus control cells (black bar, set as 1). Data are presented as mean values ± s.e.m. from three independent experiments. *: p value < 0.05; **: p value < 0.01, ***: p value < 0.001.
(b) Immunoblotting for TDP-43, Drosha, DGCR8 and Dicer in 6 day RA-treated NB cells depleted for TDP-43. Legend details as in panel (a).

The same effect of TDP-43 depletion on Drosha and DGCR8 protein levels was observed in SH-SY5Y cells (data not shown).

To verify whether the observed Drosha protein reduction was due to an alteration of transcription and/or RNA stability, the levels of Drosha transcripts were evaluated in RA-treated SK-N-BE cells knocked down for TDP-43 relative to control cells. Based on the annotated Drosha splicing isoforms, derived from UCSC genome browser (http://genome.ucsc.edu), forward and reverse primers common to all the annotated transcripts or specific for the individual mRNA isoforms were designed. As shown in Figure 3.5 a, qRT-PCR on total RNA using the primers common to all transcripts indicated that the strong reduction of Drosha protein was not accompanied by a corresponding reduction of Drosha mRNA level, which decreased by only 15% in cells depleted for TDP-43. This result was also confirmed in SH-SY5Y cell line (data not shown).

In agreement, also the deeper analysis of the individual splicing isoforms, carried out by RT-PCR, did not show any qualitative or quantitative difference between cells knocked down for TDP-43 and control cells (Figure 3.5 b-d), suggesting that TDP-43-dependent Drosha downregulation does not occur at the RNA level. This conclusion was further supported by the lack of binding between TDP-43 and Drosha transcript, as demonstrated by RNA Immunoprecipitation (RIP) assay (Figure 3.5 e).
**Figure 3.5**

*Drosha* mRNA level is not affected by TDP-43 depletion in differentiating SK-N-BE cells.

(a) The histogram shows the relative amount of *Drosha* mRNA levels, as determined by qRT-PCR, in 6 days RA-treated NB cells depleted for TDP-43 (grey bars) versus control cells (black bar, set as 1). *Gapdh* was used as a loading control. Data are presented as mean values ± s.e.m. from three independent experiments. *: p value < 0.05; **: p value < 0.01, ***: p value < 0.001.

(b) Genomic organization of human Drosha locus as reported in UCSC genome browser. Exons represented as boxes, introns as lines. Capital letters (A, B, C, D) indicate different Drosha isoforms. The oligonucleotides utilized for Drosha isoform amplification are indicated like arrows placed above the target sequences. The region amplified by the oligos Drosha-fw1 and Drosha-rev1 is common to the A, B and C RNAs. The oligos Drosha-fw2 and Drosha-rev2 amplify four putative PCR products, corresponding to A, B, C and D isoforms.
(c) RT–PCR analysis of mRNAs isolated from 6 day RA-treated cells transfected with siRNAs against TDP-43 (lane siTDP-43) or control scrambled siRNAs (lane siCTRL). Reverse Transcriptase – reaction was loaded as a control in lane –RT. Gapdh mRNA level was used as a loading control.

(d) Quantification of the RNA levels of Drosha isoforms in 6 day RA-treated cells depleted for TDP-43 (grey bars) relative to control cells (black bars, set as 1). Error bars in the histogram represent the standard error of the mean obtained from two independent experiments.

(e) qRT-PCR quantification of Drosha RNA immunoprecipitated by TDP-43 (grey bars) or IgG (black bars) in NB cells treated with RA (4 days). See Legend to Fig. 6c for details about positive and negative experimental controls. Histogram reports the mRNA fold enrichment relative to Input.

The possibility that miRNA-mediated translation repression could be responsible for Drosha protein decrease was also considered. Since a very small fraction of miRNAs was upregulated following TDP-43 depletion, we searched for their putative target sites on Drosha transcripts interrogating several miRNA databases. In particular, miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), DIANA microT (http://diana.cslab.ece.ntua.gr) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw) were utilized to analyze the 3’ untranslated region (3’UTR), whereas the 5’UTR and coding sequences were tested through miRWalk database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). These in silico analyses showed the absence of putative target sites for the upregulated miRNAs on Drosha transcripts.

Altogether, these results indicate that TDP-43 may control Drosha levels during in vitro NB cell differentiation, and that this control is mainly exerted at the protein level. This conclusion, consistent with the reported physical association of TDP-43 with the Drosha complex (Gregory et al, 2004; Kawahara & Mieda-Sato, 2012) that we confirmed in our model system (Figure 3.6 a), is further supported by the demonstration of a direct interaction between TDP-43 and Drosha, as shown by the GST pull-down assay reported in Figure 3.6 b.
TDP-43 physically interacts with the microprocessor complex in SK-N-BE cells and directly binds Drosha in vitro.

(a) Western blot analysis of Drosha levels in untreated or 6 day RA-treated NB cells nuclei immunoprecipitated with anti-TDP-43 antibodies. Immunoprecipitation quality was assessed by evaluating TDP-43 enrichment as well. Inputs indicate the native nuclear lysates.

(b) Pull down of GST-TDP-43 incubated with in vitro translated Drosha. As a negative control, interaction was assayed between GST-TDP-43 and an in vitro translated unrelated endoribonuclease (Laneve et al, 2003). Input samples are 10%, Resin and Resin-GST were used to exclude unspecific bindings.

3.3 Drosha protein level is rescued in TDP-43 depleted cells after proteasome inhibition

The observed decrease of Drosha protein levels after TDP-43 knockdown may be due to the microprocessor complex destabilization, followed by protein degradation. According to this hypothesis, we should expect that inhibition of the protein degradation pathway would lead to a rescue of Drosha protein levels.

Since the bulk of proteins in mammalian cells are degraded by the ubiquitin-proteasome pathway (Lee & Goldberg, 1998), we analyzed Drosha protein level in RA-treated cells knocked down for TDP-43 after proteasome inhibition (Figure 3.7). To this aim, we treated cells with MG-132, one of the most commonly used inhibitor of the proteasome degradative pathway (Lee & Goldberg, 1998). As control, we analyzed the levels of p27, known to significantly increase upon MG-132 treatment (Bagui et al, 2003).
In RA-treated cells transfected with control scrambled siRNAs (Figure 3.7, lanes siCTRL) the level of the Drosha protein increased after MG-132 treatment with respect to mock-treated cells, suggesting a proteasome-dependent Drosha degradation. In the same conditions, the level of TDP-43 did not increase; this is due to the already described insolubility of TDP-43 after MG-132 treatment (van Eersel et al, 2011), as confirmed by the recovery of TDP-43 by denaturing extraction of the insoluble pellet (Figure 3.8).

**Figure 3.7**

**Drosha level is rescued upon proteasome inhibition in differentiating SK-N-BE cells depleted for TDP-43.** Left panel: immunoblotting of TDP-43 and Drosha in mock (DMSO)-treated NB cells or in MG-132-treated cells, transfected with siRNAs against TDP-43 (lanes siTDP-43) or control scrambled siRNAs (lanes siCTRL) and also stimulated by RA for 4 days. p27 protein was used as a positive control. Normalization relative to GAPDH protein level. Right panel: histogram displaying the relative quantities (means ± s.e.m.) of the proteins (TDP-43: black bars, Drosha: grey bars) in sample (MG-132) versus control (mock) cells, from three different experiments.
Figure 3.8
Proteasome inhibition treatment induces TDP-43 precipitation.
Immunoblotting of TDP-43 recovered from insoluble pellets derived from 4 days RA-treated SK-N-BE cells, transfected with siRNAs against TDP-43 (lanes siTDP-43) or with control scrambled siRNAs (lanes siCTRL) and treated with MG-132 (lanes MG-132) or with its vehicle DMSO (lanes Mock). Relative protein expression levels are reported below each lane. As a loading control GAPDH was revealed.

Notably, Figure 3.7 shows that the Drosha protein levels, which dropped down in mock-treated cells depleted for TDP-43 (lane siTDP-43/mock), were significantly rescued when cells were grown in the presence of MG-132 (lane siTDP-43/MG-132). These results led us to conclude that Drosha turnover relies on the proteasome degradative pathway and that its stability is strongly affected by TDP-43 depletion during in vitro neuronal differentiation.

3.4 TDP-43 controls Neurog2 but not Dgcr8 expression in NB differentiating cells

Apart from miRNA processing, an alternate function for Drosha and the microprocessor complex in the regulation of the cellular transcriptome has been underscored. Several hairpin-containing mRNAs whose levels are negatively regulated by Drosha have been identified (Chong et al, 2010; Macias et al, 2012). Besides the first detected mRNA substrate, Dgcr8 (Han et al, 2009), that is involved in an autoregulatory feedback circuit, novel endogenous sets of RNA targets were identified in non-neuronal cell types (Chong et al, 2010; Macias et al, 2012). In addition, a crucial role for Drosha complex in regulating neurogenesis, by modulating mouse Neurogenin 2 RNA (Neurog2) expression through 3’UTR cleavage, has been highlighted (Knuckles et al, 2012). A computational prediction of human Neurog2 3’UTR structure strongly suggested the occurrence of a similar control mechanism (Figure 3.9 a-b-c, Zuker mFold webserver 2003).
Figure 3.9
**Neurog 2 3’UTR sequence and structure are predicted to be conserved between mouse and humans.**
(a) Neurog2 transcripts are conserved among Mammals (from UCSC database). The dotted circle highlights the terminal 300nt of Neurog2 3’UTR region, hosting the microprocessor entry sites as described in mouse.
(b) Sequence alignment between mouse (upper string) and human (lower string) Neurog2 3’UTR termini described in (a).
(c) Predicted conformation of the 3’UTR sequences aligned in panel (b) according to the Mfold web server (http://www.bioinfo.rpi.edu/applications/mfold).

However, since it has been shown that the mRNA-cleaving activity of Drosha may vary between cell types (Chong et al, 2010), we first addressed whether Drosha exerted its regulative function in our model system. We analyzed the expression of 12 different mRNA species in RA-treated NB cells depleted for Drosha. Notably, we found that only two mRNAs, *Dgcr8* and *Neurog2*, were derepressed by Drosha deficiency while the other 10 species tested were not expressed or not upregulated (Figure 3.10).

Figure 3.10
**Reported Microprocessor target genes are not regulated by Drosha in differentiating SH-SY5Y cells.**
Relative expression of 10 mRNAs reported as Drosha substrates was assessed by qRT-PCR analysis in RA-treated, Drosha-depleted NB cells (grey bars) compared to RA-treated control cells (black bars, set as 1).
Data are presented as mean values ± s.e.m. from at least three independent experiments. *: p value < 0.05; **: p value < 0.01; ***: p value < 0.001.

In particular, the levels of Dgcr8 increased by about 2.5-folds and those of Neurog2 by about 2.9-folds in RA-treated cells depleted for Drosha with respect to control cells (Figure 3.11a). Based on these results and on the previous observation that Drosha protein was regulated by TDP-43 only during differentiation (Figure 3.4), we performed RNAi against TDP-43 in RA-treated cells and analyzed Neurog2 and Dgcr8 expression levels. Notably, a 2.8-fold increase of Neurog2 mRNA was observed in TDP-43 knockdown cells relative to control cells, whereas the levels of Dgcr8 mRNA were not affected at all (Figure 3.11b).

**Figure 3.11**
Neurog2 expression is modulated in differentiating SH-SY5Y cells depleted for Drosha or TDP-43.
(a) Neurog2 and Dgcr8 mRNA expression (left and right panel respectively) in NB RA-treated cells, depleted (lanes +) or not (lanes -) for Drosha. Relative mRNA expression levels are quantified in flanking histograms (siDrosha: grey bars, scrambled siCTRL: black bars).

(b) Neurog2 and Dgcr8 mRNA expression (left and right panel respectively) in NB RA-treated cells, depleted (lanes +) or not (lanes -) for TDP-43. Relative mRNA expression levels are quantified in flanking histograms (siTDP-43: grey bars, scrambled siCTRL: black bars).

We excluded that TDP-43-dependent microRNA abatement was responsible for Neurog2 induction since the depletion of Dicer did not affect its mRNA levels, suggesting that this kind of regulation is microRNAs-independent (data not shown). As a supporting evidence, the ability of TDP-43 to bind Neurog2 and Dgcr8 mRNAs was verified through a RIP assay, carried out using a nuclear lysate from differentiating SH-SY5Y cells.

We employed tubulin-β1 mRNA as a negative control and TDP-43 mRNA, whose concentration is auto-regulated by TDP-43 itself (Avendano-Vazquez et al, 2012; Ayala et al, 2011), as a positive control. Remarkably, we found that TDP-43 was physically associated with Neurog2 transcript, whereas Dgcr8 mRNA was not bound (Figure 3.12a). This suggests that TDP-43 may drive the Microprocessor complex to distinct RNA substrates.

We performed a RIP assay for DGCR8 in stable cell lines expressing shRNAs against TDP-43: in agreement with our hypothesis, we found that the binding of the Microprocessor to Neurog2 mRNA occurred only in the presence of TDP-43, whereas the binding of Dgcr8 mRNA was TDP-43 insensitive (Figure 3.12b). These results demonstrate that the Microprocessor requires TDP-43 for specific target recognition.
The Microprocessor complex requires TDP-43 for specific target recognition.

(a) qRT-PCR quantification of RNA immunoprecipitated by TDP-43 (grey bars) or IgG (black bars) in NB cells treated with RA (4 days). Neurog2 and Dgcr8 transcripts were analyzed in parallel to Tubulin-β1 and Tdp-43.
RNAs representing the negative and positive controls, respectively. Histogram reports the mRNA fold enrichment relative to Input. Data are presented as mean values ± s.e.m. from three technical replicates. *: p value < 0.05; **: p value < 0.01, ***: p value < 0.001.

(b) qRT-PCR quantification of RNA immunoprecipitated by DGCR8 (grey bars) or IgG (black bars) in control NB cells treated with RA for 4 days (left histogram) or in RA-treated NB cells expressing shRNAs against TDP-43 (right histogram). Neurog2 and Dgcr8 transcripts were analyzed in parallel to pre-Gapdh and Tubulin-β1 RNAs representing the negative controls. Histograms report the mRNA fold enrichment relative to Input. Statistics as in panel (a).

An increase of about 2-folds of the Ngn2 protein levels (Figure 3.13a) was also highlighted in RA-treated cells knocked down for TDP-43.

We next asked whether such Ngn2 upregulation caused the activation of its target and downstream effector NeuroD1 (Knuckles et al, 2012). We found that in differentiating cells, TDP-43 knockdown caused an increase of Neurod1 mRNA levels by about 70% (Figure 3.13b), even though such regulation did not rely on physical association between TDP-43 and Neurod1 mRNA (data not shown).

Figure 3.13
Ngn2 and Neurod1 expression is upregulated in differentiating SH-SY5Y cells depleted for TDP-43.
(a) Western blot of Ngn2 in NB RA-treated cells, depleted (lanes +) or not (lanes -) for TDP-43. GAPDH was used as a loading control. Relative quantity (RQ) is expressed in respect of control cells, set as 1. Data are presented as mean values ± s.e.m. from three independent experiments. *: p value < 0.05; **: p value < 0.01, ***: p value < 0.001.

(b) Neurod1 mRNA expression in NB RA-treated cells, depleted (lanes +) or not (lanes -) for TDP-43. Quantifications and statistics as in panel (a).
4. Discussion and perspectives

Despite its nature of nuclear, ubiquitously expressed protein, TDP-43 is considered as a neuronal activity-responsive factor that may function as a modulator of neuronal plasticity (Wang et al, 2008). Analysis of its distribution in mouse brain showed that it is enriched in the hippocampal neurons, where it resides in the neuronal dendrites, and that it increases in the cultured neurons upon specific stimuli simulating the learning/memory process (Wang et al, 2008).

In line with its physiological role in the nervous system, TDP-43 has also been underscored as a major signature of neurodegeneration, occurring in diseases such as ALS and FTLD. The common feature of these pathologies is the mislocalization of the mutated protein to the cytoplasm, with consequent loss of its normal nuclear function. A similar behaviour has also been demonstrated for FUS, another ALS-associated RNA-binding protein (Iguchi et al, 2013; Morlando et al, 2012; Wu et al, 2012). Among its nuclear activities, and besides the established role in RNA splicing, TDP-43 has been involved in miRNA biogenesis (Buratti et al, 2010; Kawahara & Mieda-Sato, 2012) as an auxiliary component of the microprocessor complex (Gregory et al, 2004). Such association seems to be even more intriguing in the light of the recent discovery of an alternate function for Drosha complex in recognizing and cleaving many stem-loop structure-containing protein-coding messenger RNAs (Chong et al, 2010; Macias et al, 2012). In particular, the microprocessor controls neurogenesis by regulating the expression of Neurog2 mRNA, a proneural transcription factor critical for neurogenic differentiation (Knuckles et al, 2012). Therefore, unveiling the functional link between TDP-43 and the microprocessor will advance the understanding of the regulatory and disease mechanisms in which TDP-43 is involved.

In this study, carried out during my PhD research program, TDP-43 was demonstrated for the first time to crucially intervene in neuronal differentiation by controlling Drosha complex at different levels. This neuronal regulatory role was highlighted through loss-of-function experiments carried out in a model system of in vitro neuronal differentiation, a condition where the levels of both Drosha and DGCR8 strongly decrease, which makes the amount of the microprocessor complex limiting. In this context, the absence of TDP-43 led to the proteasome-dependent destabilization of a considerable amount of Drosha protein. This may be due to a different protein composition (absence or presence of stem or neuronal factors) of the microprocessor in growing vs differentiating cells. In turn, this would lead to a
differential susceptibility of Drosha to the depletion of TDP-43, which may justify its role onto Drosha stability only upon differentiation stimulus.

The direct interaction between TDP-43 and Drosha induces us to speculate that TDP-43 might affect Drosha by protecting it from ubiquitin-dependent degradation. Drosha destabilization, in turn, elicited a global downregulation of microRNA biosynthesis that, in combination with the ongoing microRNA degradation and with their dilution upon cell division (data not shown), may explain the substantial decrease of microRNA levels.

Besides this first level of regulation, exerted on the microprocessor machinery abundance, TDP-43 is also implicated in Drosha substrate recognition. In fact, during neuronal differentiation, TDP-43 was dispensable for the Drosha-mediated DGCR8 regulation, whereas it was essential for Neurog2 expression control. These findings, together with the physical association of TDP-43 with Neurog2 mRNA and not with Dgcr8, led us to propose that, in cells undergoing neuronal differentiation, TDP-43 may be the factor that regulates the affinity of Drosha complex for Neurog2 mRNA. This result perfectly agrees with the recent discovery that DGCR8 alone is not responsible for specific RNA target recognition by the microprocessor (Roth et al, 2013).

*Neurog2* expression occurs early in proliferating neural precursor cells and, upon loss of stem-cell status, the protein induces the expression of multiple downstream direct targets. Among these, *NeuroD1* represents a key player in driving neuronal differentiation, since it induces the expression of neuronal differentiation-related genes (Ali et al, 2011). *NeuroD1*, whose expression decreases as neurogenesis proceeds (Katayama et al, 1997), is regulated not only at the transcriptional level by Ngn2, but also post-transcriptionally as a Drosha complex substrate (Knuckles et al, 2012). Here we demonstrate that, during differentiation, TDP-43 contributed to modulate *NeuroD1* expression by controlling the cellular levels of both its regulators, Ngn2 and Drosha (Figure 4.1).
Microprocessor component levels progressively reduced along in vitro neuronal differentiation: in such sensitized context, TDP-43 plays a role in the stabilization of Drosha protein (1), which impacts onto global microRNA biosynthesis (Kadener et al). TDP-43 also participates in post-transcriptional regulation of the Drosha target Neurog2 mRNA both directly, by association with Neurog2 mRNA (3), and indirectly, by controlling Drosha stability (4). In turn, Ngn2 transcriptionally controls NeuroD1 expression (5), whose steady state levels are also modulated by the microprocessor itself (6). Finally, NeuroD1 induces expression of downstream neuronal-related genes driving neuronal differentiation (7).

In conclusion, we found that, while in growing cells TDP-43 cooperates with the microprocessor by selecting a subset of pri-miRNAs for Drosha cleavage (Buratti et al, 2010; Kawahara & Mieda-Sato, 2012), upon triggering of neuronal differentiation it behaves as an essential regulatory factor. It controls the abundance and the substrate-specificity of the microprocessor complex, thus guaranteeing the homeostatic control of miRNA biogenesis and the correct expression of Neurog2. By regulating Neurog2, a master neurogenic gene orchestrating a transcriptional regulatory cascade that directs undifferentiated neural precursor
cells towards a neuronal fate, TDP-43 contributes to proper neuronal differentiation, possibly preventing aberrant accumulation of neurogenic factors. Finally, our study, whose experimental loss-of-function design mimicked the TDP-43 nuclear clearance reported in ALS, would provide a toehold for understanding TDP-43 normal cellular function and suggest potential consequences of its deregulation in neurodegenerative disease.
5. Materials and Methods

5.1 Cell culture and transfections
SK-N-BE (Kadener et al)-C cells, from ATCC (Cat. No. CRL-2268), were cultured in RPMI medium 1640 (Gibco) supplemented with 10% fetal bovine serum, l-glutamine and penicillin/streptomycin (Gibco). SH-SY5Y cells were cultured in DMEM-F12 medium (Invitrogen) supplemented with 10% fetal bovine serum, l-glutamine and penicillin/streptomycin (Gibco). Both cell lines were induced to differentiate with 10 μM all-trans-Retinoic acid (Sigma-Aldrich). TDP-43 siRNAs (5’-AGGCUCAUCUUGGCUUUGCTT-3’), Drosha siRNAs (5’-AACGAGTAGGCTTCGTGACTT-3’) and control siRNAs (AllStars Neg. Control siRNAs - Cat. No. 1027281) were purchased from Qiagen. Dicer siRNAs (D-003483-01) are from Dhharmacon. SK-N-BE and SH-SY5Y cells were transiently transfected, in absence or in presence of RA, by using Lipofectamine and Plus Reagent (Invitrogen) in OPTI-MEM I medium (Gibco) according to manufacturer’s directions. Cells were transfected for 3, 4 or 6 days with siRNAs at a final concentration of 100 nM.
For the proteasome inhibition studies, siRNAs transfected cells (3 days) were also treated for 24 h with 10 μM MG-132 (Z-Leu-Leu-Leu-al) (Sigma) or its vehicle Dimethyl Sulfoxide (DMSO) (Sigma). Cell counting was performed by using an Countess Automated Cell Counter (Invitrogen).

5.2 Stable cell lines generation
TDP-43 knockdown was obtained by Mission Lentiviral shRNA clone targeting TDP-43 (TRCN000016040). Mission Lentiviral Non-Targeting shRNA clone SHC002 was used as a control (Sigma-Aldrich). Lentiviral particles were prepared according to the manufacturer’s specifications. Infection of SK-N-BE cell lines was performed as previously described (Follenzi & Naldini, 2002a; Follenzi & Naldini, 2002b).

5.3 Plasmid construction
GST-TDP-43: TDP-43 ORF was PCR amplified from total SK-N-BE cell cDNA with the oligonucleotides
TDP-43_Up_WT (CGCGGATCCATGTCTGAATAATTCGGGTAACCAGAAG)
TDP-43_Dw_WT (TTGCGGCGCTACATTCGCCAGCCAGAAGACTTA) and inserted between BamHI and NotI restriction sites of pGEX-4T-1 vector (Amersham Biosciences).
5.4 Oligonucleotides (sequences complementary to exonic regions)

qRT-PCR analysis:

Drosha_fw  5’-TAGGCTGTGGGAAAGGACCAAG-3’
Drosha_rev 5’-GTTCGATGAACCGCTTCTGATG-3’
DGCR8_fw  5’-GTTAGCCTCACAGAAGATCC-3’
DGCR8_rev 5’-CTTTGGAGCTTGCTGAGGAT-3’
GAPDH_fw  5’-CACCATCTTCCAGGAGTGAG-3’
GAPDH_rev 5’-CCCTTCTCCCATGGTGGAAGAC-3’
TUBB1_fw  5’-AAGGACACACTAGGTTGGAGA-3’
TUBB1_rev 5’-CCAGCTGATTGAGAATGCA-3’
GAPDH INTR2_fw  5’-CTGGGGGTAAGGAGATGCT-3’
GAPDH EX3_rev  5’-TTACCAGATTTAAAAGCAGCCC-3’

Oligonucleotides used for Neurog2 and pre-GAPDH (for RIP analysis), TDP-43 and Dicer mRNA and microRNAs amplification were purchased from Qiagen.

RT-PCR analysis:

Drosha_fw1  5’-CTATATGGACTGCCATTGTT-3’
Drosha_rev1 5’-CCCCGTGAGCATACAGCATAA-3’
Drosha_fw2  5’-TATGACCGAGGGAGAACACC-3’
Drosha_rev2 5’-CCATGGGGTCACTGGAGTAG-3’
Neurog2_fw  5’-GAGAGACAGACAGACCACCAC-3’
Neurog2_rev 5’-CTTCAACTCCAAGGTCTCGG-3’
Neurod1_fw  5’-ACGACCTCGAACCACATGAC-3’
Neurod1_rev 5’-CTTCCAGGTCCTCATCTTTGC-3’
Lsm1_fw  5’-GAAAGGGTGCTCCCCAACC -3’
Lsm1_rev  5’-CTCGAAGCAAACCAAGTG-3’

Oligonucleotides for Dcp1a, Dlg5, Snx12, Ino80e, Hoxa7 were as described (Macias et al, 2012); oligonucleotides for Eno1, Brd2, Mbnl1, Wipi2 were as described (Chong et al, 2010).

5.5 RNA extraction and analysis

Total RNA was extracted using miRNeasy Mini Kit (Qiagen). Northern blot analyses were performed as previously described (Annibali et al, 2012) employing 32P-labeled DNA oligonucleotides complementary to the sequence of mature miR-124, miR-9, miR125b, let-7b, miR-181a and U2 snRNA as probes.
For quantitative real-time PCR (qRT-PCR) assays, cDNA was obtained using miScript Reverse Transcription Kit (Qiagen) or SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen) for miRNA or mRNA expression studies, respectively. The qPCR detection was performed using miScript SYBR-Green PCR Kit (Qiagen) on a 7500 Fast Real-Time PCR (Applied Biosystem). The relative expression levels of miRNAs and mRNAs were normalized over SCARNA17 and GAPDH levels, respectively.

For Drosha isoforms determination and Neurog2 expression analysis, the same cDNAs were analyzed through semi-quantitative reverse transcriptase-PCR (RT-PCR), using the specific oligonucleotides listed above. Unsaturated amplification reactions were agarose-separated, the fractionation pattern was revealed by ChemiDoc XRS+ Molecular Imager (Biorad) and band intensity was estimated through the Image Lab (Beta1) software.

5.6 miRNA high-throughput analysis
The Applied Biosystems TaqMan® microRNA Arrays A and B (Applied Biosystems) were used to measure miRNA expression according to the manufacturer’s instructions. cDNA synthesis was carried out with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) by using 750 ng of total RNA as a template. Amplification signal detection was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System with all samples run in duplicate. The values obtained were normalized for mammalian U6 snRNA (MammU6–4395470). Two independent high-throughput qRT-PCR experiments were carried out and replicates were omitted when undetermined (Ct ≥ 40). Average fold differences below 0.8 and above 1.5 were considered to represent a significant difference between treated and untreated cells.

5.7 RNA ImmunoPrecipitation Assay
SH-SY5Y cells were grown with or without RA as described above. Nuclei were isolated in PBS 0.2X (supplemented with 0.25M sucrose, 8mM Tris-HCl pH 7.5, 4mM MgCl2, 0.8% Triton X-100) and disrupted in RIP buffer (150mM KCl, 25mM Tris pH 7.4, 5mM EDTA, 0.5mM DTT, 0.5% NP-40 1X PIC, 1mM PMSF, 100U/ml RNAsin [Promega]). Nuclear extracts were incubated with TDP-43 (10 μg) of or DGCR8 (5 μg) antibody (or IgG, as control) and immunoprecipitated with ProteinA/G-sepharose resin (Pierce). Immunoprecipitated RNA was extracted by QIAZOL (Qiagen) and aliquots were retrotranscribed and analyzed by qRT-PCR using specific oligos for Neurog2, TDP-43, Dgcr8
and Tubulin-β1. Enrichments were reported as difference between Immunoprecipitation and Bead values, normalized over the corresponding input.

### 5.8 Immunoblotting

Whole-cell protein extracts were prepared from cells lysed in RIPA buffer (50 mM Tris HCl (pH 8), 150 mM EGTA, 150 mM NaCl, 50mM NaF, 10% Glycerol, 1.5 mM MgCl2, 1% Triton and a protease inhibitor mixture Roche Diagnostics), whereas insoluble pellet was treated with denaturing buffer (100 mM Tris HCl pH 7.4), 1 mM EDTA, 2% SDS and a protease inhibitor mixture Roche Diagnostics). Immunoblotting analyses were performed as previously described (Annibali et al, 2012). Dicer, GAPDH and Actinin antibodies were from Santa Cruz Biotechnology (sc-30226, sc-32233 and sc-15335, respectively), REST antibody from Millipore (07-579), NGN2, Drosha, DGCR8 antibodies from Abcam (ab109236, ab12286 and ab90579, respectively), ID2, N-Myc and Vgf antibody were used as previously described (Annibali et al, 2012)), p27 antibody was from BD Biosciences and TDP-43 antibody was kindly provided by Dr. E. Buratti (ICGEB, Trieste, Italy). Each immunoblot reported constitutes a representative example of at least three biological replicates. All the experiments were quantified through the Image Lab Software (release 3.0.1) launched over “.scn” files, generated directly during the acquisition procedure (by ChemiDoc XRS+ scanner), without any image conversion and/or manipulation. Possible saturated signals (out of linear range) leading to erroneous measurements are recognized and highlighted during the chemiluminescence revelation process by the instrument itself, thus allowing to discard over-exposed bands from quantifications.

### 5.9 Co-immunoprecipitation

Co-immunoprecipitation was performed using Immunoprecipitation kit-Dynabeads Protein G (Invitrogen) according to manufacturer’s instructions. To obtain the nuclear extracts, the cell pellets were resuspended with Buffer A (Tris–HCl pH 8 20 mM, NaCl 10 mM, MgCl2 3 mM, Igepal 0.1%, glycerol 10%, EDTA 0.2 mM) supplemented with protease inhibitor (Roche) and after centrifugation the nuclei were resuspended in Buffer C (Tris–HCl pH 8 20 mM, NaCl 400 mM, glycerol 20%, DTT 1 mM) supplemented with protease inhibitor (Roche). After three cycles of incubation in liquid nitrogen followed by incubation at 37°C the nuclear extract was recovered by centrifugation.
5.10 GST pull-down
Drosha ORF for *in vitro* translation (performed by TnT-coupled Reticulocyte Lysate System kit, Promega) was amplified from SK-N-BE cell cDNA using oligos T7-DroshaCDS_fw (GAATTAATACGACTCACTATAGGGATGATGCAGGGAAACAC) and DroshaCDS_rev (TTATTTCTTGATGTCTTCAGTCTCTC). The GST pull-down experiment was carried out as described in (Morlando et al, 2004).

5.11 Statistics
Statistical significance was determined by two-tailed Student’s t-tests. P<0.05 was considered significant. Data shown here are the mean±s.e.m. from at least three experiments unless otherwise indicated.
6. Glossary:

TDP-43: TAR DNA-binding protein 43
ALS: Amyotrophic Lateral Sclerosis
FTLD: Frontotemporal Lobar Dementia
DGCR8: DiGeorge syndrome critical region gene 8
miRNA or miR: microRNA
Neurog2: Neurogenin 2 mRNA
Ngn2: Neurogenin 2 protein
Neurog2: Neurogenin 2
NB: neuroblastoma
RA: Retinoic Acid
siRNA: short interfering RNA
mRNA: messenger RNA
qRT-PCR: Real-Time PCR
RT-PCR: Reverse transcriptase-polymerase chain reaction
UTR: untranslated region
RIP: RNA-immunoprecipitation
FUS: fused in sarcoma
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
SCARNA17: small Cajal body-specific RNA 17
snRNA: small nuclear RNA
REST: RE1-silencing transcription factor
ID2: inhibitor of DNA binding 2
N-Myc: v-myc myelocytomatosis viral related oncogene neuroblastoma derived
Vgf: nerve growth factor inducible
7. Acknowledgments

I would like to thank the following people who collaborated in different parts of the project: Elena Grossi, Dr. Pietro Laneve, Dr. Mariangela Morlando, Dr. Stefano Dini Modigliani, Dr. Monica Ballarino, Prof. Irene Bozzoni and Dr. Elisa Caffarelli.
I would like to thank M. Marchioni and M. Arceci for technical support.
8. List of publications

Impact Factor (JCR 2012): 5.471

Impact Factor (JCR 2011): 9.205
9. References


