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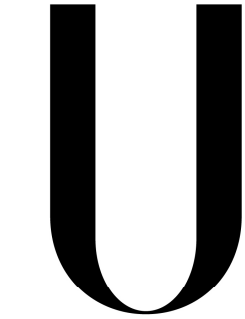

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PROGRESS REPORT
YEAR 2016
(1 January 2016 – 31 September 2016)

Transcriptomic screen for DIS3, DIS3L1 and DIS3L2-associated functional networks in colorectal cancer

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Biological Systems: Functional and Integrative Genomics
PhD Programme in Biology/Biochemistry

PhD in Biology *(specialty: Systems Biology)*
SFRH/BD/52495/2014

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1. Abstract

The final step of cytoplasmic mRNA degradation proceeds in either a 5'-3' direction, catalyzed by XRN1, or in a 3'-5' direction catalyzed by the exosome. In yeast, DIS3/Rrp44 protein is the catalytic subunit of the exosome. In humans, there are three known paralogues of this enzyme: DIS3, DIS3L1, and DIS3L2. Important findings over the last years have shed a new light onto the.mechanistic details of RNA degradation by these exoribonucleases. In addition, it has been shown that they are involved in growth, mitotic control and important human diseases, including cancer. For example, DIS3L2 inactivation was associated with mitotic abnormalities and altered expression of mitotic checkpoint proteins (Astuti et al., 2012). In another study, DIS3 was found to be highly expressed in colorectal cancer (CRC), suggesting an oncogenic function (Camps et al., 2013).

A major challenge in systems biology is to reveal the cellular networks that give rise to specific phenotypes (Lan et al., 2013). In this project, we aim to analyze how DIS3 and DIS3L1 regulate the human transcriptome, and how their functional interactions modulate the transcriptional reprogramming of colorectal cancer cells. We will perform an extensive characterization of the DIS3 and DIS3L1 mRNA targets, using DIS3 and DIS3L1 knockdown and microarray analysis, in normal colorectal cells, and in different CRC cell lines, in the presence and absence of stress stimuli, such as hypoxia.

Differential expression and gene set enrichment analyses of collected data will elucidate new cellular pathways regulated by DIS3 and DIS3L1 and/or by their targets, as well as how they can be involved in CRC. In addition, this analysis may reveal novel functional networks through which the RNA exosome modulates the eukaryotic transcriptome.

2. State of the Art

In eukaryotes, mRNAs are first synthesized in the nucleus as pre-mRNAs that are subject to 5'-end capping, splicing, 3'-end cleavage, and polyadenylation. Once pre-mRNA processing is complete, mature mRNAs are escorted by a host of associated factors and exported to the cytoplasm, where they serve as blueprints for the protein synthesis by ribosomes and then are degraded. In the last years, it has been shown that the post-transcriptional control of eukaryotic gene expression is much more elaborate and extensive than previously thought, with essentially every step of mRNA metabolism being subject to regulation and quality control. The most studied surveillance mechanism is the nonsense-mediated mRNA decay (NMD) mechanism. NMD is an mRNA surveillance mechanism that rapidly degrades mRNAs carrying premature translation termination codons (PTCs) (Popp & Maquat, 2014). However, it has been shown that NMD also targets mRNAs transcribed from a large subset of wild-type genes (Mendell et al., 2004; Wittmann et al., 2006). Many of the "NMD-inducing features" in normal transcripts that cause them to be degraded by NMD have been elucidated, most of which place stop codons in a premature context (Mendell et al., 2004; Wittmann et al., 2006; Rebbapragada & Lykke-Andersen, 2009). Thus, it is now known that natural NMD substrates include transcripts containing upstream open reading frames in the 5' untranslated region (UTR), transcripts with long or intron-containing 3' UTRs, transcripts containing selenocysteine codons, and products of alternative splicing that contain PTCs (Mendell et al., 2004; Wittmann et al., 2006; Martins et al., 2012).

Besides NMD there are other relevant mRNA-surveillance mechanisms like nonstop decay (NSD) that is a mechanism for rapid turnover of transcripts lacking an in-frame stop codon, that was found in yeast (Frischmeyer et al., 2002; Van Hoof et al., 2002). These transcripts are recognized by GTPase Ski7 in yeast, that bridges the recognition process to its decay by directly interact with cytoplasmic exosome. (Frischmeyer et al., 2002; Araki et al., 2001). Despite could be originated by various processes as point mutations or stop codon read-through, premature polyadenylation events are the most commom cause of NSD (Frischmeyer et al., 2002; Graber et al., 1999). NSD is triggered by a ribosome stalling when it reaches the end of the polyA tail and promotes a endonucleolytic cleavage (Tsuboi et al., 2012, Kuroha et al., 2010, Ito-Harashima et al., 2007). Some studies suggested that the stalling is due to a charge-charge interaction between the negatively charged exit tunnel of the ribosome and the positively charged lysine residues decoded by the polyA. (Dimitrova et al., 2009; Lu & Deutsch, 2008) However, more recent studies have argued that stalling also depends on the mRNA sequence. In particular, poly(AAA) sequences are more likely to promote stalling than poly(AAG) ones even though both code for the same polypeptide sequence of poly(Lys). (Arthur et al., 2015; Koutmo et al., 2015)

The ribonucleases (RNases) are the key players in RNA decay mechanisms. Studies in yeast and mammals indicate that mRNA degradation inherent to the normal mRNA turnover, or by means of the NMD pathway, can occur from both the 5' end of the message, involving decapping and 5'-to-3' degradation by the XRN1 RNase, and the 3' end through deadenylation and exosome-mediated 3'-to-5' decay (Reis et al., 2013; Popp & Maquat, 2014). The only catalytic subunit of the cytoplasmic exosome has been the RNaseII-family exoribonuclease DIS3. More recently, it was shown that another human protein from the same family of exoribonucleases (DIS3L1) could also interact with the core exosome (Staals et al., 2010; Tomecki et al., 2010). Actually,humans have three DIS3 homologues: DIS3, DIS3L1 and DIS3L2. DIS3 and DIS3L1 interact with the exosome ring. DIS3L1 is a strictly cytoplasmic exoribonuclease, while DIS3 is mainly nucleoplasmic and also displays endonuclease activity (Staals et al, 2010; Tomecki et al, 2010). Despite being homologues, the three proteins seem to have different roles in the cell. DIS3 and DIS3L1 are exosome partners while DIS3L2 does not

interact with exosome (Malecki et al., 2013; Lubas et al., 2013). DIS3L2 is a 3'-to-5' cytoplasmic active exonuclease and represents a novel RNA decay pathway alternative to degradation by XRN1 or the exosome, since it does not interact with the exosome ring (Astuti et al., 2012; Malecki et al., 2013; Lubas et al., 2013). Lubas and colleagues have shown that DIS3L2 is a 3'-5' exosome-independent cytoplasmic ribonuclease, which can degrade structured substrates, contributing to the maintenance of cellular RNA homeostasis. This work is one of the few that present a genome-wide view of the effects of DIS3L1 and DIS3L2 depletion on cellular RNA metabolism. To achieve this goal, they made RNA-seq experiments of total RNA isolated from HeLa cells. The most affected transcripts by DIS3L1 depletion were upregulated, as expected for an exonuclease depletion phenotype, while some showed decreased expression, most probably representing secondary effects. Interestingly, RNA degration pathways involving DIS3L2 and DIS3L1 show some degree of redundancy (Lubas et al., 2013).

Mutations in the DIS3 locus have been associated with aberrant accumulation of processing intermediates and aberrant forms of some mRNAs and rRNAs (Schaeffer et al., 2009; Lebreton et al., 2008; Suzuki et al., 2001; Mitchell et al., 1997). Regardless of their known importance, only recently studies have associated RNases, like DIS3 and DIS3L2, with human disease (Astuti et al., 2012; Chapman et al., 2011; Ding et al., 2012; Rose et al., 2011).

The number of publications associating DIS3 and its homologues with human disease are increasing. Thus, it is important to understand how deregulation of these exoribonucleases can trigger human disease. A few number of studies has associated DIS3 with cancer development and progression. In one of them, Lim and colleagues suggest that DIS3 might be associated with malignant phenotypes of some colorectal cancers and colorectal and liver metastasis (Rose et al., 2011; Lim et al., 1997; Liang et al., 2007; Rozenblum et al., 2002). Silencing of DIS3 showed a slight enrichment for MYC gene targets, which is a transcription factor involved in cell cycle progression, apoptosis and cellular transformation, associated with CRC (Astuti et al, 2012; Camps et al., 2013). According to the World Health Organization (WHO), in 2008, CRC had the highest mortality rate for malignant neoplasms (40.1/100,000 deaths) in Portugal. Worldwide, in 2008, about 608,000 people died from colorectal cancer, making it the fourth most common cause of death from cancer. This makes the mechanisms behind this disease very important to know and understand. DIS3 was found to be upregulated in a highly metastatic CRC cell line (SW620 cell line) and to be involved in cell growth and differentiation, transcription, apoptosis, tumorigenesis, and signal transduction (Liang et al., 2007). Mutations in the DIS3 gene that result in a loss of function of the protein have also been described in acute myeloid leukemia and multiple myeloma (Chapman et al., 2011; Ding et al., 2012). In addition to this, DIS3-like proteins have been shown to be involved in genetic diseases. For example, DIS3L2 is associated with Pearlman syndrome (a rare congenital overgrowth and cancer susceptibility disorder) and Wilms tumor susceptibility. Mutations in DIS3L2 gene were suggested to inhibit the exonucleolytic activity of the protein, inducing deregulation of cell-cycle genes leading to a faster cell growth. Moreover, the same work demonstrated that when DIS3L2 is overexpressed there is a suppression of the growth of human cancer cell lines. In the case of DIS3L2, this agrees with published data showing that its knockdown leads to increased cell proliferation and decreased chromosomal stability (Astuti et al, 2012).

In summary, the DIS3 family of exoribonucleases seem to play a crucial role in different mRNA degradation pathways, whether in normal mRNA decay, NMD, or non-stop decay (NSD) (Nicholson, et al., 2010), although the precise mechanisms are not yet completely understood. Several lines of evidence implicate this family of proteins in oncogenic processes, particularly in the context of CRC. However, little is known about DIS3L1 function, identified as an exoribonuclease specifically associated with the cytoplasmic exosome (Staals et al., 2010; Tomecki et al., 2010) and to our knowledge, no studies addressing its potential association to human oncogenic processes have been performed so far.

3. Objectives

According to the proposed workplan, this doctoral work had four main objectives, namely:

1) To investigate whether DIS3 and DIS3L1 are involved in the mRNA surveillance mechanism of NMD and in the regulation of natural NMD targets.

2) To perform an extensive characterization of the DIS3 and DIS3L1 mRNA targets using DIS3 and DIS3L1 siRNA-mediated knockdown coupled to microarray profiling assays in normal colorectal cells (NCM460 cell line) and in different CRC cell lines (HCT116, HT29, DLD-1 Caco-2, SW480), subjected to different stress stimuli.

3) To elucidate new cellular pathways regulated by DIS3 and DIS3L1 and/or by their targets, as well as how they can be involved in CRC by performing differential gene expression and gene set enrichment analyses of previously collected data (task 2).

4) To elucidate novel functional networks through which the RNA exosome modulates the eukaryotic transcriptome.

Given some constraints and the results that we have obtained, we adapted our plan, and thus it presents the following adjusted objectives:

1) To directly investigate whether DIS3, DIS3L1 and DIS3L2 are involved in the mRNA surveillance mechanisms of NMD and NSD, and/or in the regulation of natural NMD targets.

2) To perform an extensive characterization of the DIS3L1 and DIS3L2 mRNA targets using DIS3L1 and DIS3L2 siRNA-mediated knockdown assays.

3) To elucidate how DIS3L2 modulates the eukaryotic transcriptome and its role in modulating mRNA levels of natural NMD targets.

4) To test if the DIS3L2 and/or their targets function in tumorigenesis, proliferation and survival.

The work carried out towards each of these objectives is described in the following section.

4. Summary of Activities

1) Objective 1 – To investigate whether DIS3, DIS3L1 and DIS3L2 are involved in the mRNA surveillance mechanisms of NMD and NSD, and/or in the regulation of natural NMD targets.

Studies in yeast and mammals indicate that mRNA degradation by means of the NMD pathway can occur from both the 5' end of the message, involving decapping and 5'-to-3' degradation, and the 3' end through deadenylation and exosome-mediated 3'-to-5' decay (Lejeune & Maquat, 2003). Even though DIS3L1 and DS3L2 localizes the same compartment in where NMD occurs (Bhuvanagiri et al., 2010; Lubas et al., 2013; Popp & Maquat, 2014), nothing is known about their specific role in this process.

To discriminate the role of DIS3, DIS3L1 and DIS3L2 in NMD and in regulation of natural NMD targets, we performed siRNA-mediated knockdowns for these proteins in HeLa cells. We also performed a knockdown of XRN1, the major 5'-to-3' exoribonuclease as a control experiment. In addition, cells were transiently transfected with constructs containing different human β-globin variants: a wild-type (βWT), an NMD-resistant (β15), two NMD-sensitive (β26 and β39) and an NSD-sensitive (βNS) variant. (Silva & Romão, 2009) Then, we assessed the changes in the mRNA levels upon the different exoribonucleases knockdown.

Our results show that DIS3L1 and DIS3 are involved in NMD, NSD and in normal mRNA turnover, as their knockdown (Figure 1A and 2A) significantly increases mRNA levels of all β-globin variants (Figure 1B and 2B). Nevertheless, DIS3L2 knockdown (Figure 3A) does not have a significant impact on the β-globin mRNAs levels (Figure 3B). As expected, XRN1 knockdown (Figure 4A) induces an increase in mRNA levels of all β-globin variants (Figure 4B).

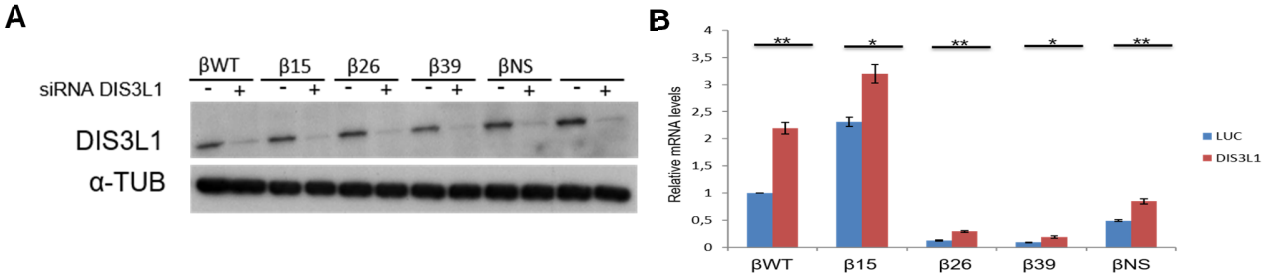

Figure 1| DIS3L1 is involved in the mRNA decay of all β-globin variants. (A) Western blotting analysis of protein samples obtained from HeLa cells transiently transfected with constructs expressing wild-type (βWT), NMD resistant (β15), NMD sensitive (β26, β39) or NSD sensitive (βNS) human β-globin mRNAs. Cells were subjected to single knockdown of DIS3L1 (+DIS3L1 siRNA) or treated with control siRNA targeting firefly Luciferase (-DIS3L1 siRNA). Anti-DIS3L1 and anti-α-tubulin (α-TUB) antibodies were used as indicated. (B) DIS3L1 knockdown increases mRNA levels of all β-globin transcripts tested. mRNA levels were determined by RT-qPCR using primers specific for human β-globin mRNA and for the GAPDH mRNA (endogenous control). Histogram represent fold-change of each condition relative to the control, arbitrarily set to 1. *$p < 0.05$; **$p < 0.01$.

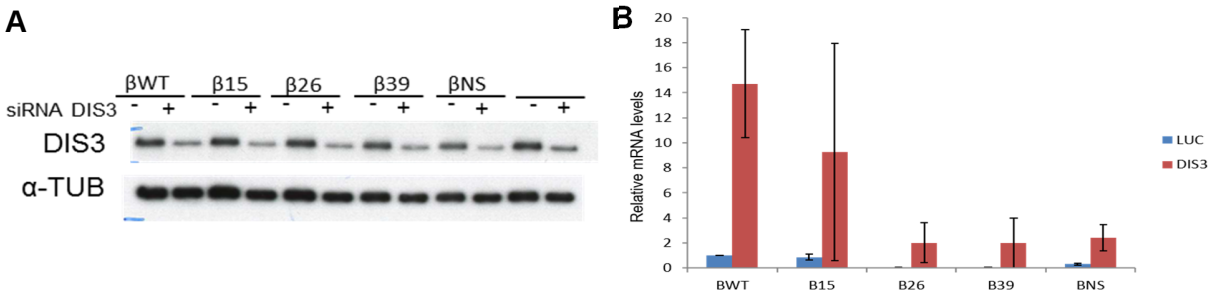

Figure 2| DIS3 is involved in the mRNA decay of all β-globin variants. (A) Western blotting analysis of protein samples obtained from HeLa cells transiently transfected with constructs expressing wild-type (βWT), NMD resistant (β15), NMD sensitive (β26, β39) or NSD sensitive (βNS) human β-globin mRNAs. Cells were subjected to single knockdown of DIS3 (+DIS3 siRNA) or treated with control siRNA targeting firefly Luciferase (-DIS3 siRNA). Anti-DIS3 and anti-α-tubulin (α-TUB) antibodies were used as indicated. (B) DIS3 knockdown increases levels of all β-globin transcripts. mRNA levels were determined by RT-qPCR using primers specific for human β-globin mRNA and for the GAPDH mRNA (endogenous control). Histogram represent fold-change of each condition relative to the control, arbitrarily set to 1. *p < 0.05; **p < 0.01.

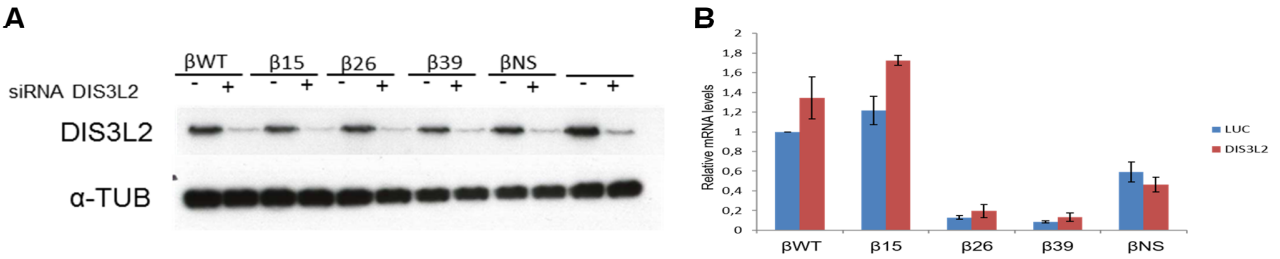

Figure 3| DIS3L2 is not involved in β-globin variants mRNA decay. (A) Western blotting analysis of protein samples obtained from HeLa cells transiently transfected with constructs expressing wild-type (βWT), NMD resistant (β15), NMD (β26, β39) or NSD sensitive (βNS) human β-globin mRNAs. Cells were subjected to single knockdown of DIS3L2 (+DIS3L2 siRNA) or treated with control siRNA targeting firefly Luciferase (-DIS3L2). Anti-DIS3L2 and anti-α-tubulin (α-TUB) antibodies were used as indicated. (B) DIS3L2 knockdown does not significantly affect mRNA levels of all variants. mRNA levels were determined by RT-qPCR using primers specific for human β-globin mRNA and for the GAPDH mRNA (endogenous control). Histogram represent fold-change of each condition relative to the control, arbitrarily set to 1.

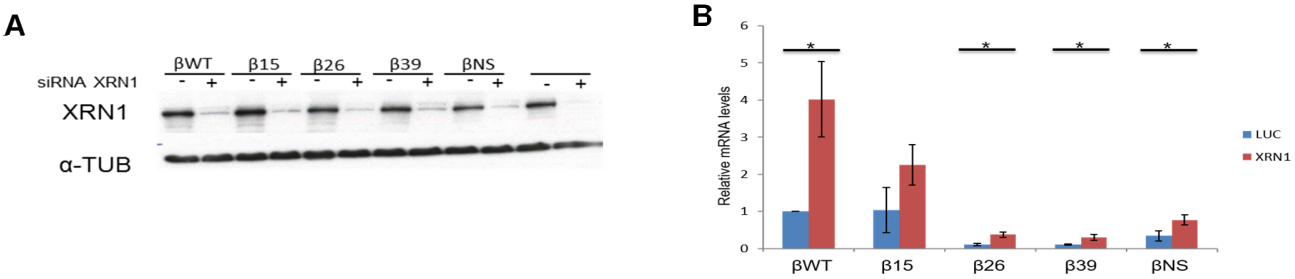

Figure 4| XRN1 is involved in the mRNA decay of all β-globin variants. (A) Western blotting analysis of protein samples obtained from HeLa cells transiently transfected with constructs expressing wild-type (βWT), NMD resistant (β15), NMD (β26, β39) or NSD sensitive (βNS) human β-globin mRNAs. Cells were subjected to single knockdown of XRN1 (+XRN1 siRNA) or treated with control siRNA targeting firefly Luciferase (- XRN1 siRNA). Anti-XRN1 and anti-α-tubulin (α-TUB) antibodies were used as indicated. (B) XRN1 knockdown increases levels of all β-globin transcripts. mRNA levels were determined by RT-qPCR using primers specific for human β-globin mRNA and for the GAPDH Mrna (endogenous control). Histogram represent fold-change of each condition relative to the control, arbitrarily set to 1. *p < 0.05; **p < 0.01.

Trying to confirm the lack of specificity of DIS3, DIS3L1, DIS3L2 and XRN1 for some mRNA decay mechanism, we then investigated whether these proteins are involved in the modulation of mRNA levels of well-known natural NMD targets (SMG5, SLC7A11, GADD45A, GABARAPL1). (Mendell et al., 2004; Wittmann et al., 2006; Yepiskoposyan et al., 2011; Martin and Gardner, 2014; Huang et al, 2011; Tani et al. 2012; Toma et al., 2015). For that, we analysed the effect of depleting each one of the nucleases (Figure 5A) on the mRNA levels of these targets (Figure 5 B-E).

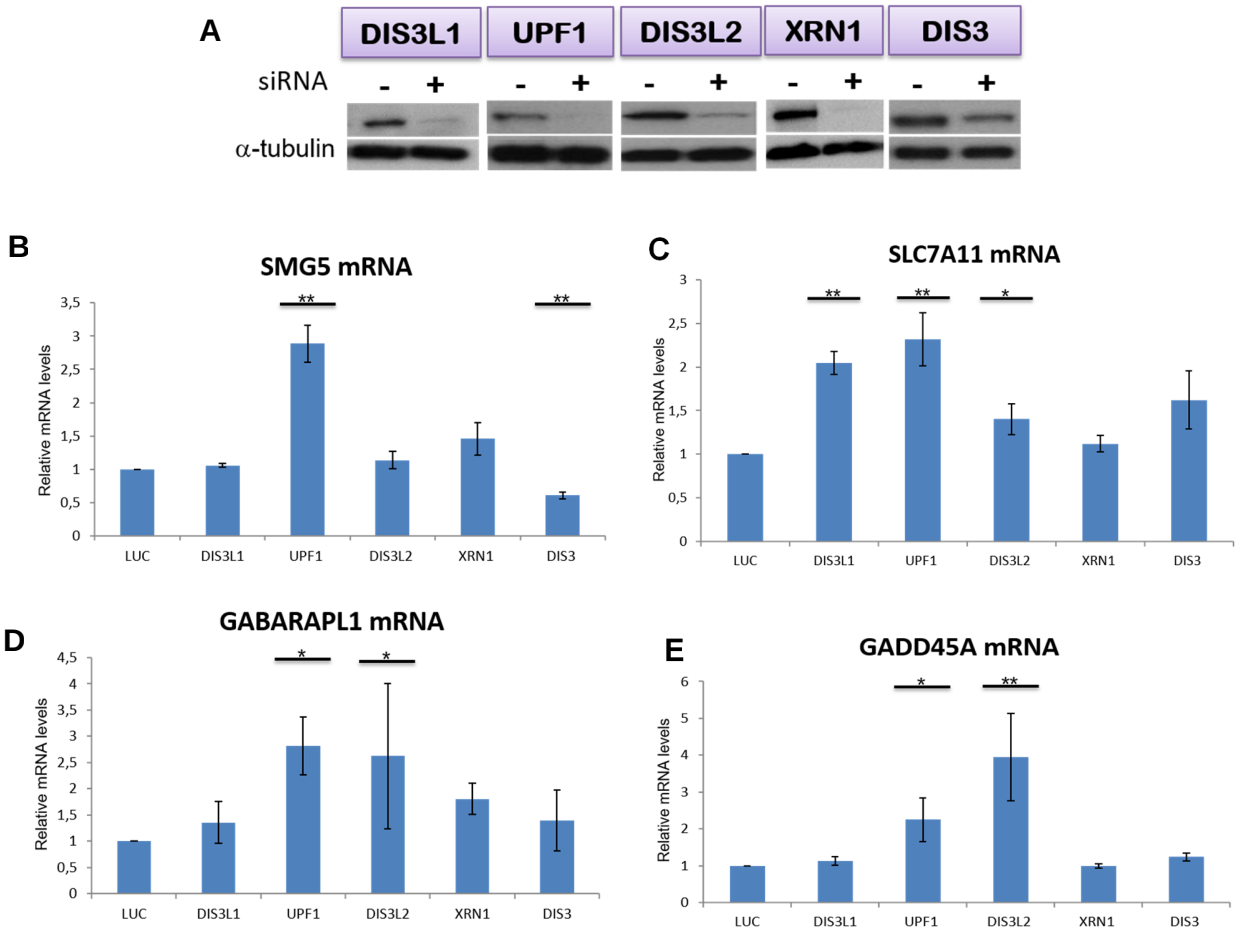

Figure 5| The role of DIS3, DIS3L1 and DIS3L2 in the degradation of the natural NMD targets is target specific. (A) Western blotting analysis of protein samples obtained from HeLa cells subjected to single knockdown of DIS3, DIS3L1, UPF1 and DIS3L2 or treated with control siRNA targeting firefly Luciferase (LUC). Anti-DIS3, anti-DIS3L1, anti-UPF1, anti-DIS3L2 and anti-XRN1 and anti-α-tubulin (α-tubulin) antibodies were used as indicated. (B), (C), (D) and (E) SMG5, SLC7A11, GABARAPL1 and GADD45A mRNA levels were determined using specific primers for each mRNA and for the GAPDH mRNA (endogenous control). Histogram represent fold-change of each condition relative to the control, arbitrarily set to 1. *$p < 0.05$; **$p < 0.01$.

2) Objective 2 – To perform an extensive characterization of the DIS3L1 and DIS3L2 mRNA targets using DIS3L1 and DIS3L2 siRNA-mediated knockdown assays.

Our initial goal was to perform a transcriptomic approach to extensively characterize the DIS3L1 and DIS3L2 mRNA targets and establish a connection between the corresponding proteins and tumorigenis.

Due to finantial constraints we did not perform the microarray profiling assays. Instead, we took advantage of the published datasets in which, authors either analysed by RNA deep sequencing the genes diferentially expressed upon UPF1 knockdown, a protein with crucial role in NMD (Tani et al., 2012) or upon DIS3L1, DIS3L2 and XRN1 knockdown (Lubas et al., 2013). In collaboration with Prof. Margarida Gama-Carvalho group we got a list of genes that was upregulated in both datasets. The results (Figure 6) show very few overlap between both datasets not allowing us to retrieve much conclusions. However, these results indicate that the exoribonucleases DIS3L1 and DIS3L2 are neither specific nor exclusive of natural NMD targets, which is in accordance with our previous data. Even so, our bioinformatic analysis revealed a small number of NMD targets mRNAs being degraded by DIS3L1 (34) and/or DIS3L2 (28). Nevertheless, since it is the first time DIS3L2 is implicated in NMD, we will pursue this finding to explore the mechanism through which this exoribonuclease functions in NMD.

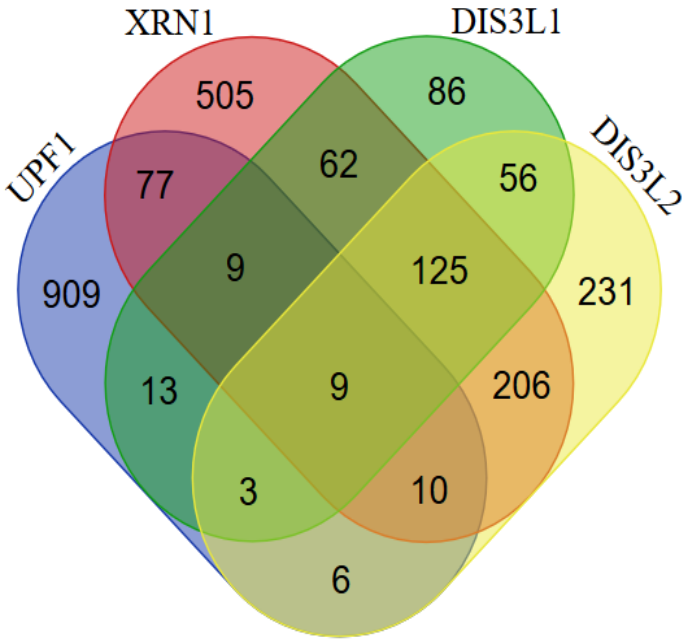

Figure 6 | There are little overlap of genes upregulated upon DIS3L1, DIS3L2 and UPF1 knockdown. **Venn diagram showing the overlap between upregulated genes upon UPF1, XRN1, DIS3L1 an DIS3L2 knockdown. Data retrieved from Tani et al., 2012 (UPF1) and Lubas et al., 2013 (XRN1, DIS3L1 and DIS3L2).**

5. Publications and Conference Communications

5.1. Papers in scientific peer-reviewed journals

5.2. Communications at scientific conferences

5.2.1. Poster presentations

Costa P. Transcriptomic screen for XRN1 associated functional networks in colorectal cancer. Post-transcriptional gene regulation. 2016.Institut Curie. Orsay.

Onofre C, Menezes J, Gomes-Duarte A, Peixeiro I, Barbosa C, Costa P, Romão L. The interface between mRNA translation and nonsense-mediated decay in AUG-proximal nonsense-mutated transcripts. The EMBO/EMBL symposium "Complex Life of mRNA". 2016. Heidelberg.

Costa P, Santos H, Gama-Carvalho M, Romão L. Transcriptomic screen for DIS3, DIS3L1 and DIS3L2-associated functional networks in colorectal cancer. BioSys Retreat. 2016. Santa Cruz.

5.2.2. Oral communication

Costa P, Santos H, Gama-Carvalho M, Romão L. Transcriptomic screen for DIS3, DIS3L1 and DIS3L2-associated functional networks in colorectal cancer. BioSys Retreat. 2016. Santa Cruz.

6. Attendance at Scientific Meetings, Courses and Training Visits

6.1. Conferences

I participated in the following conferences during this year:

International Conference on Systems Biology. Barcelona. 16-20th September, 2016

The EMBO/EMBL symposium "Complex Life of mRNA". 2016. Heidelberg. 5-8th October, 2016

6.2. Courses and Training Visits

I participated in the following courses during this year:

2nd Course on post-transcriptional gene regulation. 7-11th March, 2016. Institut Curie. Orsay.

Hands-on tutorial on software tools for logical modelling. 21th September, 2016. Universitat Pompeu Fabra. Barcelona.

7. Future Work and Timeline

Within the next year of doctoral work, we propose to continue with the studies described here aimed at studying the function of DIS3L2 in NMD and how it shapes the human transcriptome. We propose:

Objective 3 – To elucidate how DIS3L2 modulates the eukaryotic transcriptome and its role in modulating mRNA levels of natural NMD targets.

a) Experimental validation of up to 20 candidates identified in objective 2 as being upregulated upon UPF1 and DIS3L2 knockdown, will be achieved by RT-qPCR. We will perform single and double knockdown of UPF1 and DIS3L2 to test whether the effect is additive or cooperative.

b) To validate whether these candidates are directly degraded by DIS3L2 we will calculate the mRNA half-lives of some candidates' with and without DIS3L2 knockdown.

c) To unveil whether the 3'UTRs of DIS3L2 targets are responsible for DIS3L2 targeting specificity, we will clone the 3'UTR of validated targets into a vector expressing a DIS3L2 resistant transcript and test whether these UTRs are enough to make the transcript DIS3L2 sensitive.

Objective 4 - To test if the DIS3L2 and/or their targets function in tumorigenesis, proliferation and survival, we will knockdown and overexpress the relevant proteins and then analyse the survival and proliferative phenotypes.

The planed tasks are expected to follow the subsequent schedule:

Time	Year 1 (2014)		Year 2 (2015)		Year 3 (2016)		Year 4 (2017)	
	Months 1-6	Months 7-12	Months 13-18	Months 19-24	Months 25-30	Months 31-36	Months 37-42	Months 43-48
PhD courses								
Objective 1								
Objective 2								
Objective 3								
Objective 4								

Accomplished
Ongoing

8. References

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