University of Lisboa
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PhD PROJECT

The interplay between nonsense-mediated mRNA decay (NMD) and the unfolded protein response (UPR): implications for physiology and myocardial infarction

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

PhD in Biology *(specialty: Systems Biology)*
SFRH/BD/114392/2016

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Contents

Page

1. Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that recognizes and degrades mRNAs carrying premature translation-termination codons (PTCs), protecting the cell from potentially harmful truncated proteins (1). Recent studies demonstrated that NMD also targets mRNAs transcribed from a large subset of wild-type genes, arising as a mechanism of gene expression regulation (2,3). This raised the possibility that NMD is a controlled mechanism, an idea that was confirmed by recent studies, where NMD activity was seen to be modulated in specific cell types (4) and auto regulated through its intrinsic mechanism of mRNA degradation (5). Cellular stress, such as endoplasmic reticulum (ER) stress, hypoxia, reactive oxygen species, and nutrient deprivation were also seen to modulate the magnitude of NMD by mechanisms that are beginning to be understood (6). For example, the activation of kinases, as part of the cell-stress corrective pathways, induces the phosphorylation of the eukaryotic initiation factor 2 alpha (eIF2α), reducing protein translation and thus impairing NMD activity (7,8). In contrast, this eIF2α phosphorylation-dependent inhibition of NMD in stress conditions is responsible for the upregulation of many stress-related transcripts that are responsible for allowing the cell to cope with stress (7,9–11).

There is currently great interest in decoding the mechanisms that couple stress signaling to human pathology. Only recently has ER stress been considered a potential contributor to cardiac and vascular diseases (12). Myocardial infarction is a pathological state that occurs during ischemia, where there is nutrient and oxygen deprivation in the heart, causing aggregation of proteins in the ER. This aggregation triggers ER stress and the three arms (ATF6, IRE1α and PERK) of the unfolded protein response (UPR), to mitigate or eliminate the stress (12). Ultimately, if the stimulus is continued, cell death is activated (13,14). NMD plays a role in the regulation of the UPR, establishing a threshold for its activation and its time-dependent attenuation, that is accomplished, in part, through degradation of the IRE1α mRNA (9). NMD also protects the cell from death in response to stress, but the mechanism for this remains unclear (9).

Despite being a very well-studied mechanism, NMD and its role in cell physiology needs to be further explored. Given this and the all above-mentioned, the main goal of this project is to understand the role of NMD in the PERK-mediated response to ER stress induced by ischemia during myocardial infarction, and its impact to the pathophysiology of this disease. For this purpose, H9C2 cell line will be used as a model of cardiomyocytes, which will help us to dissect the crosstalk communication between NMD and UPR, through the PERK pathway, in myocardial infarction-mimicking conditions.

2. State of the Art

Nonsense-mediated mRNA decay (NMD) is a translation-dependent surveillance pathway that recognizes and selectively degrades mRNAs carrying premature translation-termination codons (PTCs) that would otherwise lead to the production of potentially harmful truncated proteins (1). During translation termination PTCs are recognized by the "SURF" complex formed by the essential NMD factor UPF1, its kinase SMG1, and the eukaryotic release factors eRF1 and eRF3. The subsequent phosphorylation of UPF1 by SMG1 triggers a sequence of events that ultimately lead to fast decay of the mRNA molecule (15,8). How NMD machinery distinguishes PTCs from normal termination codons and induce degradation of target mRNAs continues to be the focus of intense study. To date, there are three main mRNA features that have been implicated in NMD induction (16). The first one is the presence of a set of proteins collectively called the exon-junction complex (EJC), that is placed upstream of almost all exon-exon junctions after RNA splicing (17,9,18). If the EJC resides at least at 50-55 nucleotides downstream of the PTC, its interaction with UPF1 mediated by the NMD factors UPF2 and UPF3 will render the mRNA to NMD (15,17). Upstream open reading frames (uORFs) in the 5'-untranslated region (UTR) of the transcripts can also trigger NMD, possibly because the stop codon in the uORFs is at the 5' end of the mRNA with downstream EJCs, and thus can be recognized as 'premature' (19). A third NMD-inducing feature is a long 3'-UTR (15,9,20,21). How NMD targets these transcripts to decay is not completely understood, but it may involve the physical distance between the stop codon and the cytoplasmic poly(A)-binding protein 1 (PABPC1) at the poly(A) tail (9,20,21), which has been presumably related to translation termination (22,23) and to NMD efficacy (24,25). These NMD-inducing features can be intrinsic to the transcripts or arise from random DNA mutations or programmed genomic rearrangements that generate PTCs, alternative splicing processes that produce PTC-harboring isoforms, or, as mentioned above, from alternative translation of ORFs (15).

The development of high-throughput technologies in the past years, such as microarrays and genome-wide sequencing, increased the interest in NMD as it was found that this mechanism also plays a role in the regulation of gene expression (19). Genome-wide analysis studies have demonstrated that NMD, in addition to PTC-harboring transcripts, also targets physiological wild-type mRNAs that have some of the described NMD-inducing features or others not yet known, being responsible for the regulation of up to 10% of the mammalian transcriptome (2,3,26–28). This ability to regulate normal gene expression, in turn, raises the possibility that NMD itself is under regulatory control. Indeed, some reports have shown that NMD activity is modulated in specific cell types (19,5) and that key components of the NMD pathway are regulated by several pathways, including microRNA circuits (29) and NMD itself (19,28,5). Apart from this, NMD was also shown to be repressed by cellular stress. The first evidence of this appeared in 2004 by the work of Mendell *et al.* where amino acid deprivation induced NMD inhibition (2,30). Later on, it was demonstrated that a variety of other stresses could also inhibit NMD, including hypoxia (7,11), endoplasmic reticulum (ER) stress (8,9,11,10), double-stranded RNA and reactive oxygen species (ROS) (10). During these stress conditions eukaryotic initiation factor 2α (eIF2α) is phosphorylated by specific kinases, namely GCN2 kinase (amino acid deprivation), PERK (ER stress and

ROS), HRI kinase (hem deprivation) and PKR kinase (double-stranded RNA through viral infection), which in turn suppress global protein translation as part of the cell integrated stress response (ISR) (31,32). This suppression provides a likely common mechanism by which cellular stress inhibits NMD since it strongly depends on translation to recognize and degrade PTC-harboring transcripts (9,30,7,11,10). Indeed, it has been proven that NMD inhibition during ER stress and hypoxia is dependent on the phosphorylation of eIF2α (9,7,10). However, suppression of translation is unlikely to be the only mechanism by which eIF2α phosphorylation represses NMD since there is not an absolute blockage of translation and stress does not completely prevent NMD mRNA targets from being translated (30,7).

Apart from inducing the ISR through the PERK pathway, ER stress also triggers the unfolded protein response (UPR), which is a survival stress response that enables a cell to cope with the accumulation of misfolded proteins in the ER lumen (33). ER stress is initially sensed by the three ER-transmembrane proteins, PERK, ATF6 and IRE1α, which serve as the primary proximal effectors of the UPR. The subsequent activation of specific transcription factors by these proteins, induces the expression of downstream, stress-corrective factors, such as chaperones like BIP (induced by the ATF6 and IRE1α branches of the UPR), or intermediates of autophagy and cell death, like CHOP (induced by the PERK branch of the UPR) (8,12). Interestingly, many of these stress-related proteins were seen to be NMD targets, including PERK and IRE1α, ATF4 (a stress-related transcription factor which mRNA is selectively translated when eIF2α phosphorylation occurs) and CHOP, being stabilized by NMD inhibition during stress conditions (8,9,7,4). Furthermore, a recent proteomic study revealed that NMD inhibition during ER stress results in the upregulation of genes that are targets of the PERK and IRE1α pathways, suggesting that the UPR is shaped by NMD in a branch-specific manner (8). In fact, there are evidences showing that NMD modulates the UPR in part by degrading IRE1α mRNA, establishing a threshold for UPR activation that prevents an exuberant response and cell death in innocuous ER stress conditions (9). On the other hand, NMD inhibition during strong ER stress allows the stabilization of stress-related transcripts, inducing an adequate UPR and promoting cell survival (8,9).

Response to stress, specifically to ER stress, has been linked to both physiological and pathological states, including diseases of the cardiovascular system, like myocardial infarction (12,14). This disease is caused by ischemia, a condition of severe abrogation of the blood supply to a part of the heart that results in ER stress and ROS production. When protracted, these stress conditions can induce cell death and tissue damage. The induction of the UPR is one of the stress-corrective strategies that cardiomyocytes use to restore energy and nutrient homeostasis, and to avoid cell death during ischemia (12). Since NMD was shown to respond and modulate the UPR and the ISR, and nothing is known about the impact of NMD in cardiomyocytes, it is pertinent to further study the feedback loops that are established between NMD and cell stress, and their physiological/pathological impact to the cardiomyocyte. As chronic ER stress occurs not only in cardiovascular diseases, but also in many other diseases, including cancer, diabetes, pro-inflammatory disorders and neurodegenerative diseases involving abnormal protein aggregation, this kind of studies have even more biomedical impact (9,34). Understanding the detailed molecular mechanisms on which a disease relies on is a long but essential step for the development and implementation of new strategies for disease prevention and treatment.

3. Objectives

During this doctoral work, we aim to study the role and relevance of NMD in the PERK-mediated response to ER stress induced by ischemia during myocardial infarction. To accomplish this, five main objectives have been established:

1. Assess the influence of NMD in the expression pattern of PERK pathway factors during chemical- and ischemia-induced ER stress:
   - 1.1. Determine the expression levels of PERK pathway factors regarding the time/intensity of the stress stimulus.
   - 1.2. Study the impact of NMD inhibition in the expression levels of PERK pathway factors during chemical ER stress and ischemia.
   - 1.3. Determine which of the PERK pathway factors are NMD targets.
2. Investigate which NMD-inducing feature(s) (presence of uORFs in the 5'UTR and/or long 3'UTR) is(are) operating in the identified targets.
3. Study the relevance of uORFs and the influence of the possible coding peptides in translational regulation of the PERK pathway factors.
4. Test if NMD acts through the PERK pathway to shape the UPR.
5. Thesis writing and manuscript(s) preparation.

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

4. Experimental approach

Objective 1 – *Assess the influence of NMD in the expression pattern of PERK pathway factors during chemical- and ischemia-induced ER stress.*

So far, nothing is known about the connection between NMD and the stress-response in cardiomyocytes, and neither about its impact in the physiology of cardiac tissue. Given this, and since the communication between IRE1α branch of the UPR and NMD has already been explored by Karam and co-workers (9), this work will focus on the PERK branch of the UPR, and H9C2 cell line will be used as a model of cardiomyocytes. This cell line (myoblasts) will be differentiated into cardiomyocytes with retinoic acid, accordingly to Branco and co-workers (35). HeLa cells will be also used as a control.

In this particular aim, we intend to evaluate the relevance of NMD in the PERK-mediated response to ER stress. We will determine how and on what extent PERK and its downstream targets (eIF2α, eIF2B, ATF4, CHOP, GADD34) are regulated by NMD during stress conditions.

Objective 1.1 – *Determine the expression levels of PERK pathway factors regarding the time/intensity of the stress stimulus.*

UPR has a dual effect in the cell that is dictated by the extent and intensity of the stress stimulus. At first, the induction of stress-related genes allows attenuation of the stress, but then, if not resolved, UPR activates pro-apoptotic cascades that results in cell death. PERK is one of the UPR players responsible for inducing cell death, mostly through induction of the transcription factor, CHOP (13). Having this in mind, the mRNA and/or protein levels of PERK and its downstream targets will be determined by reverse transcription-quantitative PCR (RT-qPCR) and Western-blot (WB), respectively, in H9C2 and HeLa cells exposed to chemical ER stress and ischemic conditions. ER stress will be directly induced by Tunicamycin (TM), using the experimental conditions described by Karam and co-workers as reference (9). Ischemia-induced ER stress will be accomplished through incubation of the cells in hypoxic pouches with a glucose-free media, as described by Sousa and co-workers (36). Cell viability will be measured by cell counting via MTT assays, and programmed cell death (apoptosis) by fluorescence-activated cell sorting (FACS) analysis of cells stained with annexin-V/propidium iodide. This will allow us to define the experimental stress conditions that drive the adaptive and apoptotic responses of UPR. The expression levels of XBP1s (marker of IRE1α activation) and BIP (marker of ATF6 activation) will be quantified in order to assess efficiency and magnitude of UPR activation in all the stress conditions.

Objective 1.2. – *Study the impact of NMD inhibition in the expression levels of PERK pathway factors during chemical ER stress and ischemia.*

In order to understand the role of NMD in controlling the expression levels of the mentioned factors, H9C2 and HeLa cells will be transiently depleted of NMD central factor, UPF1, using siRNAs. Then, chemical ER stress and ischemia will be induced (and not induced, in the case of control cells) accordingly to the experimental conditions determined in Objective 1.1., and the mRNA and/or protein levels of PERK and its downstream targets will be determined. Cell viability and apoptosis will also be evaluated. The comparison of these results with the ones obtained in the previous objective will highlight the impact of NMD on the course of the PERK pathway during UPR.

Objective 1.3. – *Determine which of the PERK pathway factors are NMD targets.*

PERK, ATF4 and CHOP were previously proven to be direct NMD targets in HeLa and U2OS cells (9,7). However, eIF2α, its activator, the guanine nucleotide exchange factor, eIF2B, and GADD34, which reverses eIF2α phosphorylation during the stress response, were out of the focus of these studies. Given this, we intend to test if these three intermediates of the PERK pathway are direct targets of NMD, and further confirm that PERK, ATF4 and CHOP are targeted by NMD in H9C2 cells. To know if they are direct NMD targets, the analysis of RNA half-life will be determined for those transcripts that appear upregulated in response to non-stress conditions and UPF1 knockdown in Objective 1.2., as previously described by Gardner and co-workers (7). Shortly, control and UPF1-depleted H9C2 and HeLa cells will be incubated with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). RNA will be isolated at the time of DRB addition and at several subsequent time points, and the level of the transcripts will be measured by RT-qPCR. If the concerned mRNAs are direct NMD targets, UPF1 knockdown is predicted to stabilize them.

Objective 2 – *Investigate which NMD-inducing feature(s) (presence of uORFs in the 5'UTR and/or long 3'UTR) is(are) operating in the identified targets.*

Here we aim to test which features are responsible for NMD commitment of the identified targets in Objective 1.3. To accomplish this, the following tasks are proposed:

a) We will look for the presence of uORFs in the 5'UTR and for the length of the 3'-UTR of the selected transcripts, using databases like *RefSeq* and *Ensembl*. An online uORF database (available at: http://www.compgen.uni-muenster.de/tools/uorfdb/index.hbi) can also be used to look for already described uORFs.

b) We will determine the beginning and the sequence of the 5'-UTRs and 3'-UTRs through Rapid Amplification of cDNA Ends (RACE), using the circularized method described by McGrath (37), in order to confirm the presence of non-annotated uORFs and the length of the 3'-UTR in the transcripts of interest.

c) Using plasmids containing the human β-globin gene, we will make constructs of the wild type β-globin [known non-NMD target (38)] coding region with the 5'-UTRs and/or the 3'-UTRs in study. H9C2 and HeLa cells will be transiently transfected with these constructs and the levels and half-life of the β-globin mRNA will be quantified by RT-qPCR, as described in Objective 1.3.

Objective 3 – *Study the relevance of uORFs and the influence of the possible coding peptides in translational regulation of the PERK pathway factors.*

Over 40% of mammalian mRNAs contain uORFs in the 5’UTR that can serve as major regulators of translation (39,40). Typically, uORFs are considered to be inhibitors of downstream translation initiation. This inhibitory effect is attributed to the fact that the preinitiation complex initiates translation at the first encountered initiation codon in optimal context, which can be the uORF initiation codon. When the translating ribosome encounters the stop codon of the uORF the translation machinery disassembles, a fact that can avoid translation of the main ORF if the ribosome cannot reinitiate at the main initiation codon (39). The mRNAs of ATF4, CHOP and GADD34 are proven examples of uORF-harboring transcripts with translation repression activity (41–43). This translation inhibition can be counteracted in stress conditions, where the increased amount of phosphorylated eIF2α provides the necessary conditions for uORF translation leaky scanning and translation initiation at the main ORF (39). Given this, in this section we intend to study if the uORFs identified in Objective 2 play a role in the translational regulation of their respective transcripts. To accomplish this, we will construct luciferase reporter plasmids carrying the cDNA sequence of the 5’UTR containing the uORFs of interest fused to the *Firefly* luciferase (Fluc) ORF. H9C2 and HeLa cells, cultured in normal and stress conditions induced as described in Objective 1.1, will be transiently co-transfected with this construct and with another expressing *Renilla* luciferase (Rluc) ORF, as an internal control. Relative luciferase activity will be measured by luminometry assays and the mRNA levels quantified by RT-qPCR to normalize the results and obtain the translation efficiencies as described in previous studies conducted by our group (44,45). Translation efficiencies will be compared to those obtained from a construct in which uORF initiation codon is mutated by site-directed mutagenesis. This will allow us to understand if translation of the PERK pathway factors is regulated by uORFs in normal and stress conditions.

Furthermore, to unequivocally prove that the uORFs of interest are in fact translated, a site-directed mutagenesis approach will be applied to mutate the uORF stop codon and the luciferase ORF start codon, leaving both in frame. The obtained constructs will be expressed in both cell lines as above and the detection of a longer luciferase protein will be performed by WB.

Finally, since the polypeptide sequences coded by uORFs can be involved in gene expression regulation, whether by inducing ribosome stalling and dissociation during uORF translation (39,43,46) or by functioning in a *trans*-acting manner (47,48), we will assess if the translational regulation of main ORFs is dependent on the peptide sequence of the uORFs previously seen to be actively translated. For this, we will use the same constructs described above, but they will be modified by shifting the uORF reading frame to get a different amino acid sequence. These constructs will be transiently transfected in H9C2 and HeLa cells, and luciferase activity and mRNA levels will be measured as referred.

Objective 4 – *Test if NMD acts through the PERK pathway to shape the UPR.*

Once we have uncovered how NMD regulates PERK-mediated response to stress, we will turn our attention to the impact of that regulation in the UPR. As said before, in a recent study of Karam and co-workers, NMD was proven to shape the UPR, and its ability to degrade the IRE1α UPR sensor transcript is, at least in part, one implicated mechanism (9). However, it does not explain how NMD protects cells from death in response to stress. Using this information as reference, in this final section we aim to test if NMD also acts through PERK to shape the UPR, and assess its involvement in cell death/survival during chemical- and ischemia-induced ER stress. The experimental procedure will be carried out as follows:

a) Using a gain-of-function approach, we will force the expression of PERK mRNA to a level similar to that in UPF1-knockdown H9C2 and HeLa cells, using an expression vector (if necessary, a NMD-resistant PERK mRNA can be expressed). Chemical ER stress and ischemia will be induced using the experimental conditions determined in Objective 1.1., and the magnitude of UPR activation will be measured by quantification of XBP1s, BIP and CHOP mRNAs through RT-qPCR. This will provide direct evidence if NMD acts through PERK to supress the UPR (9).

b) SiRNA-mediated UPF1 knocked down H9C2 and HeLa cells will be simultaneously transfected with a siRNA against PERK, in order to prevent the upregulation of PERK that usually occurs in response to NMD impairment. Chemical ER stress and ischemia will be induced, and the magnitude of UPR activation will be measured by quantification of XBP1s and BIP mRNAs through RT-qPCR. This will allow us to test if NMD acts through its ability to degrade PERK mRNA to shape the UPR (9).

c) H9C2 and HeLa cells transfected in the same conditions as in task b) will be treated in chemical ER stress and ischemic conditions for a specific period of time. Then, apoptosis will be determined by FACS analysis as described in Objective 1.1., in order to understand if NMD acts through PERK to supress cell death during stress.

Objective 5 – *Thesis writing and manuscript(s) preparation.*

5. Workflow and Timeline

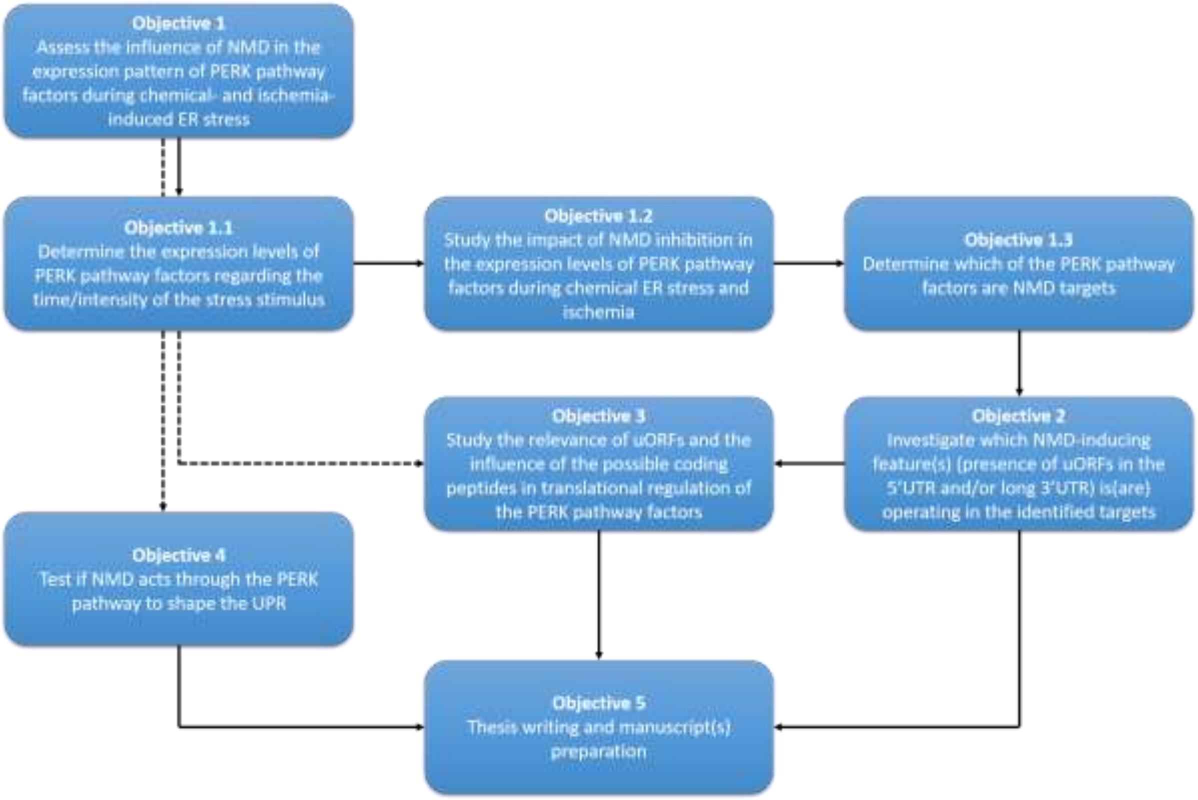
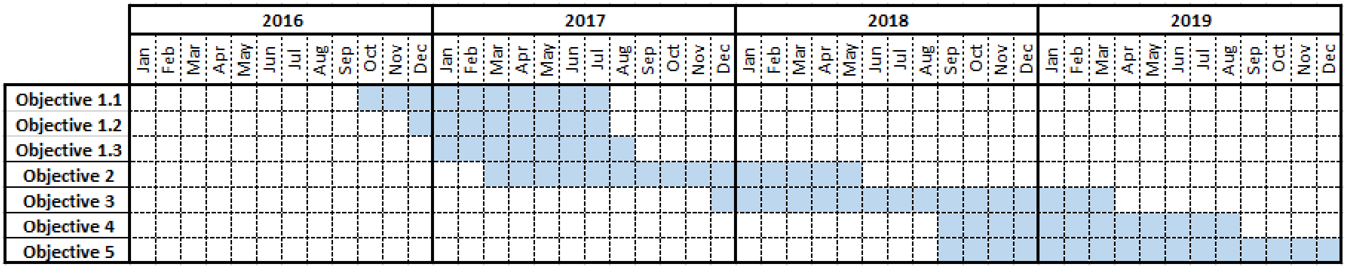


Figure 1 – Workflow of the objectives proposed to study the role of NMD in the PERK-mediated response to stress in cardiomyocytes.



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	2017											
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Figure 2 – Timeline for execution of the objectives proposed to study the role of NMD in the PERK-mediated response to stress in cardiomyocytes.

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