The Cold-Inducible RNA-Binding Motif Protein 3 (RBM3) Prevents Cell Death Through Various Mechanisms

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Nanjing, Volksrepublik China

Basel, 2016

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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Preface

Parts of the INTRODUCTION (1.1 and 1.3.3) have been prepared as a literature review on RBM3 and CIRP, which is submitted to *Cell Mol Life Sci* for publication. The section 1.2.1 of the INTRODUCTION, parts of the MATERIALS AND METHODS section, chapter 3.1.3 and 3.2 of the RESULTS and parts of the DISCUSSION have been published in *FASEB J* (Zhu X et al., 2015).

Abstract

Moderate hypothermia promotes the survival of neural cells in a wide range of brain disorders. During hypothermia, a subgroup of proteins including two homologues, RNA-binding motif protein 3 (RBM3) and cold-inducible RNA-binding protein (CIRP), is substantially induced when global translation is attenuated. Both RBM3 and CIRP do not only enhance cell viability and suppress apoptosis under lower temperature, but also exert multiple functions in euthermic environment. Since CIRP has a dual role in either cytoprotection or inflammation-mediated cell damage, we concentrated on RBM3, which has been shown so far as a beneficial factor for cell survival.

The goal of the thesis was to figure out the mechanisms of RBM3 action in cytoprotection when cells are exposed to various stressors. In an *in vitro* hypoxic-ischemic model, we noticed that hippocampal neural cells were protected by moderate hypothermia, accompanied with an induction of RBM3. In HEK293 cells, RBM3 enhanced cell viability and suppressed oxidative stress-induced apoptosis. In particular, we focused on endoplasmic reticulum (ER) stress because recent studies suggest that ER stress activates unfolded protein response (UPR) and eventually causes apoptosis in brain injuries and neurodegenerative diseases. In order to examine the hypothesis that RBM3 influences cell fate via regulating UPR, we challenged organotypic hippocampal slice cultures with ER stress inducers and observed exacerbated PERK-eIF2α-CHOP signaling in RBM3 knockout mice compared to wildtype mice. Furthermore, in HEK293 cells RBM3 attenuated PERK-eIF2α-CHOP signaling by inhibiting PERK phosphorylation. However, RBM3 neither interacted with PERK directly, nor altered the expression of ER stress sensor BiP. An interactome analysis of RBM3 in HEK293 cells revealed nuclear factor NF90 as a novel binding partner of RBM3 and PERK. Notably, NF90 was required for RBM3-mediated regulation of PERK activity. In addition, during our research we noticed that RBM3 downregulated a subset of pro-apoptotic miRNAs while upregulated a group of anti-apoptotic miRNAs, which may also contribute to its anti-apoptotic activity. In

summary, this study confirmed the cytoprotective role of RBM3, and revealed underlying molecular mechanisms including the prevention of oxidative stress-induced apoptosis, the inhibition of PERK-eIF2α-CHOP signaling and the modulation of apoptosis-related miRNAs.

Key words: RBM3; cell death; oxidative stress; ER stress; miRNA

1. Introduction

1.1 Cold responsive proteins

Decreased body temperature is a key feature of seasonal hibernation, which is an entrained state of slowed metabolism that widely exists in amphibians and mammals to endure food austerity (Carey HV et al., 2003; Storey KB, 2010; Milsom WK and Jackson DC, 2011). As a direct cellular consequence of decreased body temperature, global protein synthesis is repressed, thereby switching the cellular program from cell growth to cell preservation. In contrast to the general decrease in protein synthesis, the production of a small group of proteins, including cold-inducible RNA-binding protein (CIRP; alternative abbreviation: CIRBP; synonymous: heterogeneous ribonucleoprotein A18, hnRNP A18) and RNA binding motif protein 3 (RBM3), increases in hibernating animals (Williams DR et al., 2005; Sano Y et al., 2015).

In clinical practice, therapeutic hypothermia (32-34°C) has been proven a potent tool to alleviate neurological deficits in infants with hypoxic-ischemic encephalopathy (HIE) (Committee on Fetus and Newborn et al., 2014) and in adults with acute brain injuries (Yenari MA and Han HS, 2012). Whereas much deeper hypothermia is used during cardiac and transplant surgery (Lampe JW and Becker LB, 2011), CIRP and RBM3 synthesis peaks in a range of mild to moderate temperatures (32-34°C) (Tong G et al., 2013). Because clinical hypothermia is associated with various life-threatening side effects (Choi HA et al., 2012), CIRP and RBM3 are promising research candidates for new therapies.

Apart from their functions under hypothermia, various studies have indicated that CIRP and RBM3 also have important functions in cell protection under general endogenous and environmental stresses at normal temperatures (Lleonart ME, 2010). Here, we provide a comprehensive and systematic overview of biological functions mediated by CIRP and RBM3, within and outside the context of hypothermia by systematically considering most of the papers published on CIRP and RBM3 thus far.

1.1.1 Structure, evolution and spatial distribution

RBM3 was first identified as an X-chromosome linked gene that mapped to region Xp11.23 on the short arm (Derry JM et al., 1995). *RBM10* gene locus is in close proximity, while another RNA-binding motif protein (RBM) gene, *RBMX*, is located on region Xq26 (Martínez-Arribase F et al., 2006). These three X chromosome-linked genes all encode proteins belonging to the RBM family, and their expressions are often analyzed together, such as in breast cancer and astrocytoma (Martínez-Arribase F et al., 2006; Martín-Garabato E et al., 2008; Schneider J et al., 2011; Zhang HT et al., 2013). Surprisingly, in male rats, the expression level of RBM3 in sexually dimorphic nucleus of the preoptic area (SDN-POA) neurons is almost 2-fold that in female rats, although it declines 50% upon NMDA receptor inactivation. In contrast, the expression level of RBM3 in females is unaffected by an NMDA receptor inhibitor, indicating a dose-dependent mechanism related to X-chromosome inactivation (Hsu HK et al., 2005).

The gene coding for CIRP, the homologue of RBM3, is localized on chromosome 19p13.3 (Nishiyama H et al., 1997a), which may indicate the different evolutionarily origins of CIRP and RBM3.

Both of CIRP and RBM3 belong to a group of stress-responsive proteins sharing high sequence similarity (Figure 1.1 and Table 1.1). Two highly conserved RNA-recognition motifs (RRMs), namely RNP1 and RNP2, are located at the N terminal protein end and are moderately conserved in eukaryotes, the consensus sequences of RNP1 and RNP2 are (K/R)G(F/Y)(G/A)FVX(FY) and (L/I)(F/Y)(V/I)(G/K)(G/N)L, respectively (Ciuzan O et al., 2015). Notably, the two RRMs of CIRP and RBM3 show functional and partially sequence similarities to the two RNA-recognition motifs of cold shock proteins (CSP) present in almost all organism from bacteria to higher eukaryotes and accordingly are called cold shock domains (CSD), with the consensus sequence (K/S)G(F/K/Y)G(F/L)IXX and (L/I/V)(F/Q)(V/A/L)HX(STR), respectively (Horn G et al., 2007). Prokaryotic CSPs exert important functions in cold response due to drastic drops in temperature, *e.g.*, from 37°C to 10°C, and in other euthermic stress

responses, indicating common features as well as differences between CIRP/RBM3 and CSPs (Horn G et al., 2007; Ciuzan O et al., 2015). Because the C-terminal end of both CRIP and RBM3 proteins contains a less conserved arginine-glycine--rich domain (RGG), CIRP and RBM3 belong to the large family of glycine-rich proteins (GRP) and because they are featured with RRMs they belong to the subfamily Class IVa of GRPs (Mangeon A et al., 2010; Ciuzan O et al., 2015).

The evolution of Class IVa GRP subfamily to which CIRP and RBM3 belong is highly conserved across vertebrates and higher plants (Ciuzan O et al., 2015) (Figure 1.1). The evolutionary conservation occurs at not only the primary amino acid sequence level but also the functional level. For example, in *Arabidopsis*, the well-characterized member of this subfamily, AtGRP7, is indispensable in cold adaption and drought/osmotic stress response (Cao S et al., 2006; Kim JS et al., 2007; Yang DH et al., 2014). AtGRP7 also regulates a number of post-transcriptional and translational events (Streitner C et al., 2010; Streitner C et al., 2012; Köster T et al., 2014; Löhr B et al., 2014), functions as circadian oscillator (Heintzen C et al., 1997), and is involved in pathogen defense (Lee HJ et al., 2012). In poikilotherm animals such as fish, CIRP homologues are also elevated upon environmentally osmotic or severe cold stresses (<10°C), but may not change at normal ambient temperature (20-25°C) (Pan F et al., 2004; Hsu CY and Chiu YC. 2009; Verleih M et al., 2015). The well-studied amphibian and mammalian CIRP and RBM3 possess biological functions highly similar to AtGRP7 and fish CIRPs, implying their preservation of biological activities (see below for further details).

An interesting finding from the alignment of mammalian RBM3 sequences is that the RGG domain is almost absent in some RBM3 isoforms in species including *Capra hircus*, *Bos taurus* and *Pongo abelii* (Zargar R et al., 2015; Figure 1.1 and Table 1.1).

The spatial distribution of CIRP and RBM3 in major organs differs. In humans, RBM3 expression is low or absent in thyroid and heart, where CIRP is abundant in these organs (Danno S et al., 1997; Wellmann S et al., 2010). In hibernating bears, RBM3 is upregulated in all tissues, including muscle, liver and heart (Fedorov VB et al., 2009; Fedorov VB et al., 2011), whereas CIRP fails to be stimulated in muscle and liver

tissues in rats with chronic intermittent cold exposure (Wang X et al., 2015).

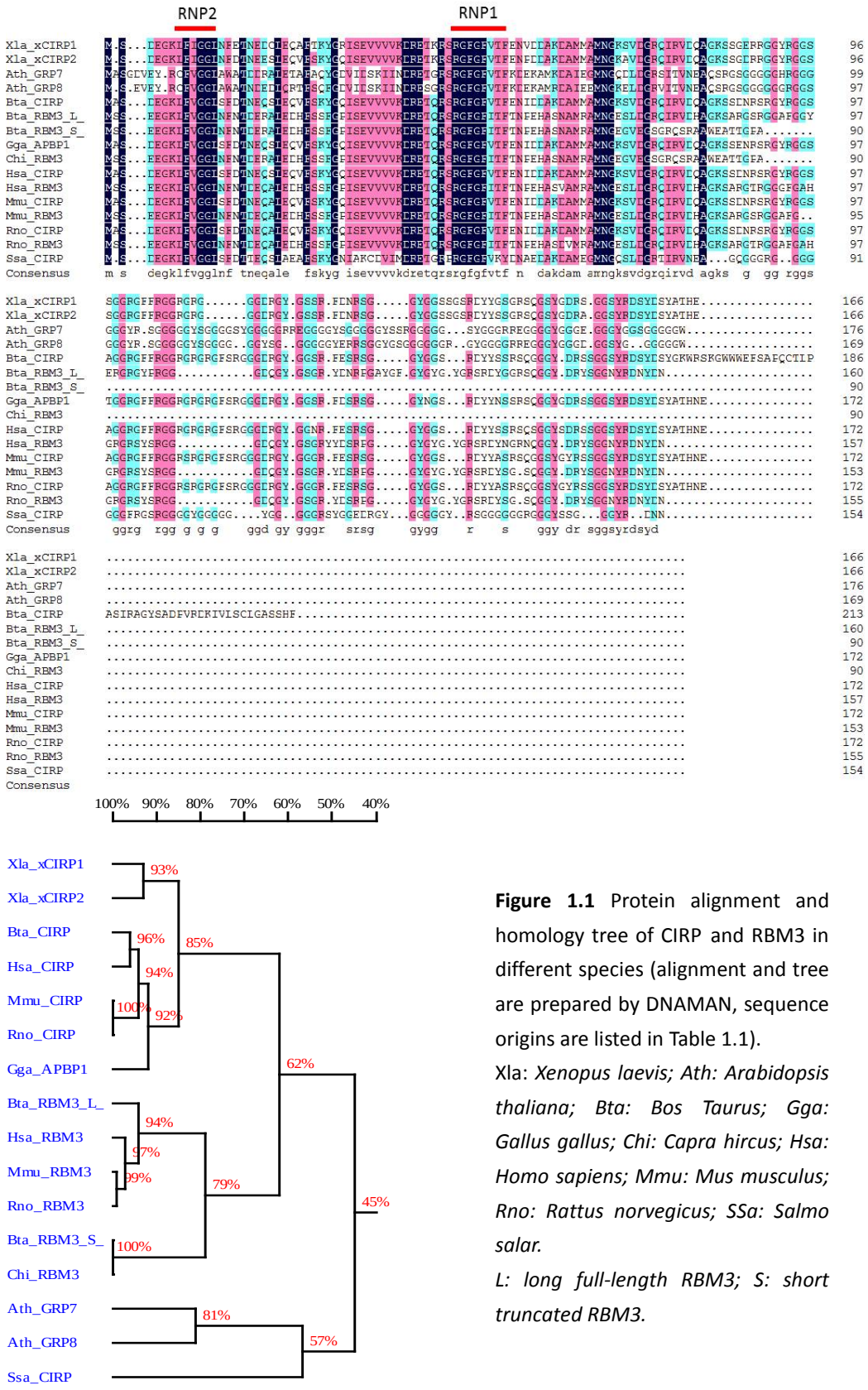

Figure 1.1 Protein alignment and homology tree of CIRP and RBM3 in different species (alignment and tree are prepared by DNAMAN, sequence origins are listed in Table 1.1).

Xla: *Xenopus laevis; Ath: Arabidopsis thaliana; Bta: Bos Taurus; Gga: Gallus gallus; Chi: Capra hircus; Hsa: Homo sapiens; Mmu: Mus musculus; Rno: Rattus norvegicus; SSa: Salmo salar.*

L: long full-length RBM3; S: short truncated RBM3.

Table 1.1 The proteins used for alignment in Figure 1.1

	Species	Name	Reference No. in Uniprot	Full Name
Plant	*Arabidopsis thaliana*	Ath_GRP7	Q03250	Glycine-rich RNA-binding protein 7
	Arabidopsis thaliana	Ath_GRP8	Q03251	Glycine-rich RNA-binding protein 8
Fish	*Salmo salar*	Ssa_CIRP	B5DGC5	Cold-inducible RNA-binding protein
Amphi-bian	*Xenopus laevis*	Xla_xCIRP1	O93235	Cold-inducible RNA-binding protein A
	Xenopus laevis	Xla_xCIRP2	Q9DED4	Cold-inducible RNA-binding protein B
Bird	*Gallus gallus*	Gga_CIRP	Q45KQ2	Aggrecan promoter binding protein (CIRP homologue)
Mammal	*Bos Taurus*	Bta_CIRP	Q3SZN4	Cold inducible RNA binding protein
	Bos Taurus	Bta_RBM3_L	F6RBQ9	Uncharacterized protein
	Bos Taurus	Bta_RBM3_S	Q3ZBA4	RNA binding motif (RRM) protein 3
	Capra hircus	Chi_RBM3	W8E7I1	RBM3
	Mus musculus	Mmu_CIRP	P60824	Cold-inducible RNA-binding protein
	Mus musculus	Mmu_RBM3	O89086	RNA-binding protein 3
	Rattus norvegicus	Rno_CIRP	P60825	Cold-inducible RNA-binding protein
	Rattus norvegicus	Rno_RBM3	Q925G0	RNA-binding protein 3
	Homo sapiens	Hsa_CIRP	Q14011	Cold-inducible RNA-binding protein
	Homo sapiens	Hsa_RBM3	P98179	RNA-binding protein 3

1.1.2 Induction

1.1.2.1 Inducers

Hypothermia: *CIRP* and *RBM3* are general stress-responsive genes; their expressions can be induced by a variety of stressful conditions, including cold stress, which is the first identified condition that increases CIRP and RBM3 expression (Nishiyama H et al., 1997b; Danno S et al., 1997). In mammalian cells, the expressions levels of both CIRP and RBM3 reach their peaks upon mild to moderate hypothermia (28-34°C) and drop significantly upon deep hypothermia (15-25°C) (Nishiyama H et al., 1997b; Tong G et al., 2013; Rzechorzek NM et al., 2015). The dose response kinetics of CIRP and RBM3 are somehow different depending on the systems studied (Danno S et al., 1997; Tong G et al., 2013.; Neutelings T et al., 2013; Rzechorzek NM et al., 2015). In an *in vivo* mouse model, cooling at 16-18°C for 45 min is sufficient to stimulate the expression of RBM3, but not CIRP, in the brain (Peretti D et al., 2015). In contrast, hyperthermia (39-42°C) causes substantial decreases in CIRP and RBM3 either in cultured cells *in vitro* (Nishiyama H et al., 1997b; Danno S et al., 1997) or under pathological or experimental conditions *in vivo* (Nishiyama H et al., 1998; Danno S et al., 2000). Notably, RBM3 induction is extremely sensitive to temperature change; even a 1°C drop from 37°C to 36°C is sufficient (Jackson TC et al., 2015).

Hypoxia: Under natural circumstances when breathing air containing 21% oxygen at sea level, the oxygen concentrations in different tissues of the body are considerably heterogeneous (Sharp FR and Bernaudin M, 2004). Reduced oxygen tension compared to physiological tension (hypoxia) occurs during diverse acute and chronic injuries or diseases in brain, lung, blood vessels and other organs or tissues as well as in cancers (Harris AL, 2002). Experimentally, both moderate (8%) and severe (1%) hypoxia can drastically induce CIRP and RBM3 expression to a comparable level by a mechanism that involves neither hypoxia-inducible factor-1 nor mitochondria (Wellmann S et al., 2004). Oxygen-regulated expression of CIRP and RBM3 may

indicate their roles in response to pathological changes such as hypoxic-ischemia, carcinogenesis, and inflammation.

Radiation: Independent of the first characterization of CIRP from Nishiyama et al., in 1997, Sheikh et al., identified the UV light-induced heterogeneous nuclear ribonucleoprotein A18 (hnRNP A18) in the same year, which was soon proven to be a CIRP homologue in hamster and which may play a role in DNA damage repair (Sheikh MS et al., 1997). Similarly, ionizing radiation can also stimulate a number of hnRNPs, including CIRP; induced hnRNPs are involved in the repairing of radiation-induced DNA damage (Haley B et al., 2009). However, whether radiations can induce RBM3 overexpression remains unclear. In addition, spaceflight increases CIRP and RBM3 expression (Baba T et al., 2008; Lebsack TW et al., 2010), which may result from radiations in space.

Miscellaneous: Toxins and drugs can also promote CIRP and RBM3 induction. For instance, the neurotoxin domoic acid elevates CIRP and RBM3 expression at a late stage (Ryan JC et al., 2005). Moreover, CIRP responses to lipopolysaccharide (LPS) treatment (Prieto-Alamo MJ et al., 2009) and positively regulates IκB in the NF-κB signaling pathway (Brochu C et al., 2013). Likewise, RBM3 is present in the regulatory profile of LPS-induced gene expression (Cok SJ et al., 2004). Growth factors, such as insulin-like growth factor-1 (IGF-1) and fibroblast growth factor 21 (FGF21), can induce CIRP and RBM3 expressions, respectively (Pan Y et al., 2015; Jackson TC et al., 2015). Furthermore, a well-studied hormone with both endogenous and exogenous sources, melatonin, may augment the induction of RBM3 upon mild hypothermia in young neurons but not in mature neurons (Jackson TC et al., 2015). Of note, very recently RBM3 has been shown to be suppressed by metformin and AMP analog AICAR, probably via the inference of cell metabolism and the activation of AMPK (Laustriat D et al., 2015). This indicates that stress is not always an inducer, but can also be an inhibitor of cold responsive proteins.

1.1.2.2 Molecular mechanisms

The precise mechanism by which hypothermia and other stresses modulate the transcription and translation of CIRP and RBM3 is poorly understood, although several models that address various regulatory levels have been suggested. A core promoter and an alternative promoter have been identified in mouse *CIRP* gene; both promoters are activated upon mild hypothermia (Al-Fageeh MB and Smales CM, 2013).

Furthermore, alternative splicing is one important route in response to cold stress. Hamsters, which are non-hibernating animals, express a long *CIRP* transcript in their hearts. This transcript has an extra insert containing a stop codon inside of the open reading frame (ORF), which probably leads to a truncated translational product and aberrant function. In contrast, hibernating animals predominantly express the short isoform with complete ORF. Artificial hypothermia can partially promote a shift from the long isoform to the short functional isoform (Sano Y et al., 2015). In contrast, in mouse fibroblasts, the 5'-UTR and full-length ORF of *CIRP* are present in two transcripts that are generated under hypothermic conditions, whereas the isoform under euthermic condition lacks the 5'-UTR and the code for initial methionine (Al-Fageeh MB and Smales CM, 2009). The mechanism by which a *CIRP* transcript with alternative splicing is generated is versatile in different organisms. In addition, cold stress upregulates the level and stability of the longest *CIRP* transcript which contains a putative internal ribosome entry site (IRES) (Al-Fageeh MB and Smales CM, 2009). In general, IRES can provide a cap-independent mechanism for approximately 10% protein translation when global translation is attenuated during hypothermia or other stresses (Mitchell SA et al., 2005). In *RBM3* transcript, the presumed discovery of an IRES in the 5'- leader sequence (Chappell SA et al., 2001; Chappell SA and Mauro VP, 2003) has led to additional experiments with transgenes in some biotechnological models until very recently (Fux C et al., 2004; Wang Z et al., 2013; Cheng X et al., 2014). However, the presumed RBM3 IRES was unmasked as a cloning artifact derived from the *thoc1* gene on chromosome 18 and not from the authentic

X-chromosome-located *RBM3* gene (Baranick BT et al., 2008). Interestingly, a short isoform of *RBM3* is more abundant in sleep deprivation (SD) mouse, while a long isoform with different 3'-untranslated region (UTR) predominantly exists in control mice, suggesting that these isoforms may play different roles in circadian oscillation (Wang H et al., 2010).

In addition to alternative splicing, transcription factors may contribute to the modulation of cold-responsive gene transcription. At 32°C, a great number of the transcription factor Sp1 is recruited to the mild-cold responsive element (MCRE) in the 5'-flanking region of *CIRP* gene than at 37°C, leading to increased CIRP expression (Sumitomo Y et al., 2012).

Post-translational modifications are important component for cell signaling. Harboring the C-terminal RGG domain, arginine methylation is widely distributed in CIRP and RBM3, as well as their plant homologues AtGRP7 and AtGRP8. Arginine methylation of RNA-binding proteins is performed by protein arginine methyltransferases (PRMTs) and associates with various functions including germ cell reproduction and neural development (Blackwell E et al., 2012). Experimentally, PRMT5 was found to modify arginine residues in AtGRP7 and AtGRP8 (Deng X et al., 2010). In frog, xPRMT1 mediates arginine methylation in xCIRP2, which guides nuclear-cytoplasmic migration of xCIRP2 (Aoki K et al., 2002). In mammalian neurons, two RBM3 splicing isoforms differ by a single arginine and behave distinct subcellular localization, which may be due to the methylation of the differentiated arginine residue (Smart F et al., 2007) (see below for nuclear-cytoplasmic shuttling). Bioinformatic analysis also reveals potential phosphorylation sites in CIRP and RBM3, but they remain to be validated experimentally.

Overall, versatile mechanisms exist by which CIRP and RBM3 expression are altered and modified in response to stresses, suggesting a wide-range of organism adaption to various external and internal challenges.

1.1.3 Molecular and cellular activities

1.1.3.1 Regulation of post-transcriptional and translational events

As RNA-binding proteins, CIRP and RBM3 have the capacity to bind RNAs, and modulate them at the post-transcriptional levels (Danno S et al., 1997; Morf J et al., 2012; Xia Z et al., 2012). In general, such post-transcriptional interactions by RNA-binding proteins and other regulatory elements, involve binding to the target regions within the 3'-UTR, which spans between a stop codon and poly(A) tail (Schwerk J and Savan R, 2015). Upon UV radiation, CIRP binds to the 3'-UTR of two stress responsive transcripts, *replication protein A (RPA)* and *thioredoxin (TRX)*, thereby stabilizing the bound mRNA and promoting their translation (Yang C and Carrier F, 2001; Yang R et al., 2006). Both CIRP RRM domains and the RGG domain are required for the maximal binding to *TRX* mRNA; these domains bridge 5'- and 3'-UTR of *TRX* transcript via eIF4G, a key component in the translational machinery to enhance TRX translation (Yang R et al., 2006). A CIRP-binding motif found in *RPA* and *TRX* 3'-UTR also exists in the 3'-UTR of *ataxia telangiectasia mutated and Rad3-related (ATR)* mRNA, a key regulator of the DNA damage response (Yang R et al., 2010). Thus, CIRP repair of UV-induced DNA damage is mediated at least partially by ATR (Yang R et al., 2010).

Similar to CIRP, RBM3 can also bind to and alter the translation of mRNA, as shown for *cyclooxygenase-2 (COX-2)*, *interleukin-8(IL-8)* and *vascular endothelial growth factor*(*VEGF)* in macrophages or cancer cells (Cok SJ et al., 2004; Sureban SM et al., 2008), presumably in a cell type-specific manner (Wellmann S et al., 2010). RBM3 is also involved in alternative splicing. In prostate cancer cells, RBM3 represses the variant *v8-v10* of *CD44* mRNA to inhibit the stemness and tumorigenesis but enhances the standard spliced *CD44* transcript (Zeng Y et al., 2013).

In addition to the 3'-UTR, poly(A) tail is an important regulatory elements for CIRP and RBM3 mediated post-transcriptional modulation. Actually, both CIRP and RBM3 are enriched in poly(A) sites and control alternative polyadenylation of a variety of

genes, including circadian genes (Liu Y et al., 2013; Hu W et al., 2014). Moreover, in the regulation of *TRX* mRNA, poly(A) tail can strengthen the binding of CIRP to *TRX* 3'-UTR and enhances its stability (Yang C and Carrier F, 2001).

Another RNA-binding protein, human antigen R (HuR), which is known to bind to AU-rich element of 3'-UTR (Brennan CM and Steitz JA, 2001), can strengthen CIRP- and RBM3-mediated regulation. In African clawed frog, the CIRP homologue xCIRP2 interacts with ElrA, a HuR homologue, which may assist xCIRP2 in inhibiting mRNA deadenylation (Aoki K et al., 2003). Interactions of HuR with CIRP or RBM3 have also been discovered in mammalian cells (Guo X et al., 2010, Sureban SM et al., 2008).

CIRP and RBM3 also modulate the translational process in several ways. In general, RBM3 enhances global protein translation (Dresios J et al., 2005; Smart F et al., 2007). The underlying mechanisms of this enhancement include (1) binding to 60S ribosomal subunits in an RNA independent manner; (2) increasing the formation of active polysomes; (3) inactivating eukaryotic initiation factor 2 alpha (eIF2α) via dephosphorylation, which initiates the assembly of translational machinery; (4) and facilitating the phosphorylation of eukaryotic initiation factor 4E (eIF4E) (Dresios J et al., 2005; Smart F et al., 2007). However, whether RBM3 can regulate translation via microRNAs remains controversial (see 1.3.3). Moreover, our recent study revealed many ribosomal proteins that are associated with RBM3 (Zhu X et al., 2015), supporting its role in promoting translation.

In contrast, the function of CIRP in protein translation is unclear. Similar to RBM3, CIRP can associate with ribosomes (Mastumoto K et al., 2000). The RGG domain of CIRP tethers a specific 3'-UTR and suppresses translation (De Leeuw F et al., 2007; Gonçalves Kde A et al., 2011). Additionally, a few evidences have shown that CIRP may also inhibit gene transcription and translation by targeting regulatory elements in the gene. For example, APBP-1, the chicken homologue of CIRP, binds to the *cis*-element of the gene encoding for aggrecan and represses its expression (Pirok EW 3rd et al., 2005). However, overexpression of CIRP at 37°C in an engineered CHO cell line improves recombinant interferon gamma protein production (Tan HK et al., 2008). How CIRP affects protein translation remains to be discussed. The molecular

functions of CIRP and RBM3 were summarized by a previous review (Figure 1.2).

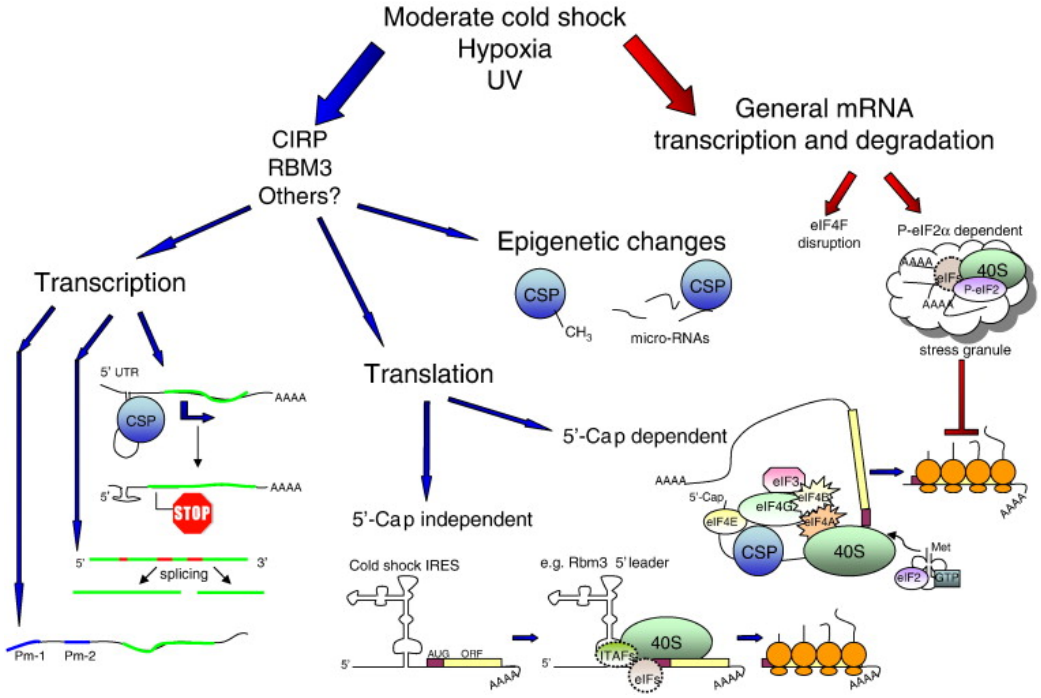

Figure 1.2 RBM3 and CIRP regulate transcription, translation and epigenetic modifications.

(LLeonart ME, 2010)

1.1.3.2 Signaling pathways

As regulatory proteins, CIRP and RBM3 are involved in complex signaling pathways related to cellular physiological processes such as cell growth, senescence and apoptosis. For example, CIRP activates ERK pathway by increasing the phosphorylation of ERK1/2 (Sakurai T et al., 2006; Artero-Castro A et al., 2009; Liu J et al., 2015). In the Wnt/β-catenin signaling, CIRP and RBM3 have been identified as a downstream target and an upstream regulator, respectively. The frog XCRIP is a target of XTcf-3 downstream of β-catenin (van Venrooy S et al., 2008), whereas in mammalian cancer cells, RBM3 increases β-catenin and enhances TCF/LEF-targeted gene transcription (Venugopal A et al., 2015). Accordingly, the kinase and endogenous inhibitor of β-catenin, GSK-3β, is downregulated by RBM3 (Venugopal A et al., 2015). GSK-3β itself upregulates *CIRP* transcription, phosphorylates CIRP protein and promotes its cytosolic translocation (Liu GP et al., 2008; Yang R et al.,

2006; Yang R et al., 2010). In the unfolded protein response, RBM3 represses the phosphorylation of PERK and eIF2α, which leads to a decrease in CHOP expression (Zhu X et al., 2015). Notably, although RBM3 and CIRP are both induced by hypothermia, hypothermia itself can enhance the ER stress response without inducing apoptosis (Rzechorzek NM et al., 2015). Upon ischemia-induced ER stress, hypothermia has a protective effect, suppressing the ER stress response (Poone GK et al., 2015) in accordance with Zhu et al.,'s report (Zhu X et al., 2015). Moreover, CIRP is involved in MAPK (Xia ZP et al., 2012), NF-κB (Brochu C et al., 2013) and p53 signaling pathways (Lee HN et al., 2015). The biological meaning of the involvement of CIRP and RBM3 in these pathways will be elucidated in the following paragraphs.

1.1.3.3 Nuclear-cytoplasmic shuttling

The RGG domain is a nuclear localization signal (Heine MA et al., 1993); because both CIRP and RBM3 contain such a domain, they should localize in the nucleus. Experimentally, as summarized above, CIRP and RBM3 are predominantly found in the nucleus (Rzechorzek NM et al., 2015), regulate gene expression or bind to mRNA for posttranscriptional regulation. In addition, many GRPs including the plant homologue AtGRP7 are able to shuttle to the cytoplasm under physiological or stressful conditions (Kim JS et al., 2008; Lummer M et al., 2011) so are CIRP and RBM3. In *Xenopus laevis*, xCIRP2 serves as a major cytoplasmic protein in oocytes (Mastumoto K et al., 2000). An RG4 region in the RGG domain has been identified as a nucleocytoplasmic shuttling signal of xCIRP2 (Aoki K et al., 2002), which is important for guiding the accumulation of xCIRP2 in the cytoplasm when the RG4 region is methylated by the methytransferase xPRMT1 (Aoki K et al., 2002). In response to cytoplasmic stresses and endoplasmic reticulum (ER) stress, CIRP is relocalized from the nucleus to cytoplasmic stress granules (SGs) in a manner independent of TIA-1, the major mediator of SG formation (De Leeuw F et al., 2007). Both RRM and RGG can independently promote CIRP migration into SGs, and methylation of arginine in RGG is required for exiting the nucleus, consistent with

findings in frogs (De Leeuw F et al., 2007). In RBM3, the absence of a single arginine residue in RGG leads to more preferentially localization in dendrites of neurons rather than in nuclei (Smart F et al., 2007). A pool of RBM3 proteins can also shuttle to the ER upon ER stress and regulate the activity of ER membrane-bound protein PERK, albeit the majority of RBM3 remains in the nucleus (Zhu X et al., 2015). Collectively, these studies reveal the critical role of the arginine residue of the RGG domain in cytoplasmic shuttling and indicate the diverse nuclear and cytolasmic functions of CIRP and RBM3.

1.1.3.4 Cell cycle

Hypothermia is known to slow cell proliferation and to cause cell cycle arrest. In contrast to early studies, which have shown an inhibitory role of CIRP in cell growth upon hypothermia (Nishiyama H et al., 1997b) and no effect of RBM3 (Danno S et al., 2000), a more recent series of mechanistic investigations revealed that both CIRP and RBM3 positively modulate the cell cycle at different stages. CIRP has been shown to bind RNA-binding protein human antigen R (HuR) and to upregulate its expression. Elevated HuR further increases cyclin E1, a key positive regulator for G1/S transition, and promotes mitosis (Guo X et al., 2010, Wu Y et al., 2011). Furthermore, CIRP accelerates G0/G1 and G1/S transitions by inhibiting the phosphorylation of cyclin D1 and p27 via the kinase Dyrk1b/Mirk (Masuda T et al., 2012). In another report, CIRP appears to promote progression through G2/M phase (Liu J et al., 2015). Additionally, activation of ERK pathway by CIRP downregulates cyclin inhibitors p16, p21 and p19, which leads to enhanced proliferative activity and bypassed senescence (Artero-Castro A et al., 2009). RBM3 also interacts with HuR, overcomes G2/M transition, and prevents mitotic catastrophe by downregulating cyclin B1 and phosphorylated checkpoint proteins Chk1/Chk2 as well as Cdc25c (Sureban SM et al., 2008; Ehlén A et al., 2010). An RBM3 knock-out mouse model confirmed that cells are arrested in G2/M phase in the absence of RBM3 (Matsuda A et al., 2011). Hence, the current understanding is that CIRP and RBM3 possess very similar roles in facilitating cell

proliferation.

1.1.3.5 Anti-apoptosis

Apoptosis is induced by numerous exogenous and endogenous signals, involves various signaling pathways, and occurs in a broad range of diseases (Reed JC, 2002). RBM proteins are largely known as a family that modulates apoptosis (Sutherland LC et al., 2005). Many studies have revealed that both CIRP and RBM3 mediate, at least partially, the hypothermic protection of cells from apoptosis, especially in neuronal cells. Specifically, CIRP suppresses apoptosis in neural stem cells (Saito K et al., 2010) and cortical neurons probably through mitochondrial pathways (Zhang HT et al., 2015). Inhibition of p53, Fas and caspase-3 pathways also contribute to CIRP-mediated anti-apoptotic effects (Zhou KW et al., 2009; Lee HN, 2015; Li S et al., 2012). RBM3 inhibits staurosporine-induced apoptosis in neuronal or neuron-like cells by repressing PARP cleavage (Chip S et al., 2011). The induction of Bcl-2 and suppression of caspase expression may also be involved in RBM3-mediated survival against apoptosis (Zhu X et al., 2015; Ferry AL et al., 2011).

1.1.4 Biological functions and diseases

1.1.4.1 Brain disorders

Therapeutic hypothermia can not only efficiently reduce primary injury and prevent secondary injury in acute ischemia (Yenari MA and Han HS, 2012) and spinal cord injury (SCI) (Alkabie S and Boileau AJ, 2015) but may also slow down the progression of chronic neurodegenerative diseases (Salerian AJ and Saleri NG, 2008). *In vitro*, the two cold-responsive proteins CIRP and RBM3 both function against apoptosis in cultured primary neurons or neuron-like PC12 cells (Kita H et al., 2002; Chip S et al., 2011; Zhang HT et al., 2015), indicating their indispensable roles in therapeutic hypothermia.

The role of CIRP in brain ischemic injury is controversial. In animal ischemic models,

CIRP expression kinetics after ischemic injury is still uncertain. *CIRP* mRNA levels measured by Northern blot were found to decrease 3-6 h after transient ischemia in rat hippocampus but to remain unchanged in cerebral cortex during a 48 h observation period (Xue JH et al., 1999). However, in the same ischemic model, real-time RT-PCR showed a gradual increase in *CIRP* mRNA by approximately 5-fold until 24 h after cerebral ischemia in rat cortex (Liu A et al., 2010). In contrast to ischemia, hypothermia considerably induced CIRP expression by approximately 30-fold until 24 h, and the combination of hypothermia and ischemia did not further enhance the *CIRP* level from 30-fold (Liu A et al., 2010).

An elevated level of reactive oxygen species (ROS) is one important detrimental factor in the induction of oxidative stress during ischemia-reperfusion injury in the brain (Sanderson TH et al., 2013). In PC12 cells, CIRP expression has been observed to be downregulated upon H_2O_2 treatment (Xue JH et al., 1999), unlike its plant homologues AtGRP7 and AtGRP8 (Schmidt F et al., 2010). When CIRP is induced endogenously or overexpressed airtificially, H_2O_2-induced apoptosis in cultured neural cells is dramatically inhibited, indicating a neuroprotective role of CIRP (Li S et al., 2012; Liu J et al., 2015). In contrast to this beneficial intracellular action of CIRP, release of CIRP into the blood system is associated with the activation of detrimental immune responses. Zhou et al., reported secretion of CIRP from microglia after cerebral ischemia with subsequent CIRP-mediated TNF-α expression and activation of signaling pathways leading to neuroinflammation and causing neuronal damage both *in vivo* and *in vitro* (Zhou M et al., 2014). An investigation of alcohol-induced brain inflammation also demonstrated that extracellular CIRP critically mediates neuroinflammation by upregulating TNF-α and IL-1β (Rajayer SR et al., 2013). To summarize, CIRP exerts opposing functions during brain ischemia-reperfusion injury. On one hand, as long as CIRP remains intracellularly localized, CIRP protects neurons from apoptosis; on the other hand, once CIRP is released, e.g., from microglia, it mediates devastating neuroinflammation from cellular level in the early stage. However, in a long term, the damaged neurons are eliminated by inflammation and regenerated neurons can substitute the dysfunctional ones (Kizil C et al., 2015).

RBM3 also responds to spinal cord injury (SCI) by increasing the number of RBM3-positive cells after SCI in a rat model (Cui Z et al., 2014; Zhao et al., 2014) with varying temporal dynamics reported: in one report, RBM3 expression peaked at one day after SCI (Zhao et al., 2014), while in the other, RBM3 significantly increased 1 day post-SCI but did not reach the maximal expression level until 5 days post-SCI (Cui Z et al., 2014). The spatial expression of RBM3 in these two reports is also inconsistent. Zhao et al., reported that neurons and only a few astrocytes were positive for RBM3 under normal conditions and that RBM3 was induced in both neurons and astrocytes (Zhao et al., 2014). In the other study, RBM3 expression was even higher in astrocytes than in neurons in the sham group, and only astrocytic RBM3 can response to SCI-induced stress (Cui Z et al., 2014). This discrepancy may result from the instable surgery conditions, although both reports generally support the hypothesis that RBM3 is inducible upon SCI and may exert important pathophysiological functions.

Until now, studies have supported the notion that RBM3 is a general neuroprotective effector, while CIRP can either protect neuronal cells or induce massive neuronal death by mediating neuroinflammation once released. To the best of our knowledge, in contrast to CIRP, no data published thus far suggest that RBM3 might be released.

1.1.4.2 Cancers

Deduced from the above-summarized features, CIRP and RBM3 are both involved in cell cycle regulation and cell proliferation, and are both present in proliferating and malignant cells. Therefore, both are considered proto-oncogenes, promoting cancer cell proliferation and transformation *in vitro* (Sureban SM et al., 2008; Tang C et al., 2015; Wellmann S et al., 2010) and are differentially expressed in a variety of different cancers compared to normal tissues (Table 1.2). Despite these common features, their roles in clinical cancer development seem to be opposite. RBM3 expression always correlates with good prognosis and reduced risk of disease progression and recurrence, whereas CIRP seems to be an indicator for poor

prognosis (Table 1.2).

Breast cancer is the leading cancer type in women, and RBM3 is overexpressed in this cancer (Martínez-Arribase F et al., 2006), with a direct correlation of RBM3 expression level and improved clinical outcome (Jögi A et al., 2009).

In female genital organ, RBM3 correlates with favorable cisplatin sensitivity and good prognosis in epithelial ovarian cancer (EOC) (Ehlén A et al., 2010), presumably via a mechanism by which RBM3 suppresses poor prognostic markers MCM3, Chk1 and Chk2, which are all involved in DNA integrity and the cell cycle (Ehlén Å et al., 2011).

In males, prostate cancer is one of the most common cancer types in the male genital system. Very similar to breast cancer and EOC, a high level of RBM3, as an independent biomarker in prostate cancer, predicts a low risk of disease progression and recurrence (Josson L et al., 2011a). Interestingly, high RBM3 expression is found in poorly differentiated prostate tumor (Shaikhibrahim Z et al., 2013), whereas experimental downregulation of RBM3 and CIRP in prostate cancer cells attenuates cell survival and enhances chemosensitivity (Zeng Y et al., 2009). These observations are consistent with the role of RBM3 in promoting cell proliferation and survival but cannot explain the favorable prognosis, indicating the involvement of other mechanisms. One study suggests that the activation of v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) and the depletion of PTEN may contribute to RBM3-mediated good prognosis in prostate cancer (Grupp K et al., 2014). Another study indicates that RBM3 attenuates the stemness and tumorgenesis of prostate cancer cells by inhibiting *CD44* variant splicing (Zeng Y et al., 2013), although this finding is in contrast to findings in colorectal cancer (Venugopal A et al., 2015). Specific cancer types likely differentially affect RBM3 signaling as is known for other proteins such as oestrogen receptors (Thomas C and Gustaffson JÅ, 2011). Moreover, low RBM3 in testicular non-seminomatous germ cell cancer correlates with high risk of treatment failure (Olofsson SE et al., 2015). In addition, in urinary organs, reduced RBM3 levels in urothelial bladder cancer are associated with tumor progression and poor prognosis (Boman K et al., 2013); while high RBM3 expression correlates with

lower stage tumors and decreased risk of lymphovascular invasion (Florianova L et al., 2015).

Colorectal cancer is the most common type of cancer of the digestive system, and a high level of RBM3 expression is associated with improved prognosis (Hjelm B et al., 2011), whereas a loss of RBM3 expression is associated with poor prognosis and right-sided localization (Melling N et al., 2015). Therefore, RBM3 has been proposed as a potential prognostic biomarker, especially in young patients (Wang MJ et al., 2015).

RBM3 is downregulated in HPV-negative oropharyngeal squamous cell carcinoma compared to normal oral mucosa (Martinez I et al., 2007). Instead, high nuclear expression of RBM3 in esophageal and gastric adenocarcinoma correlates with intestinal metaplasia-associated tumors and predicts low risk of recurrence and death independently (Jonsson L et al., 2014).

Furthermore, low RBM3 expression in other cancer types is associated with poor survival as well. RBM3 is expressed in malignant melanoma (Badli A et al., 2003), and low expression of RBM3 is associated with tumor progression and poor prognosis (Jonsson L et al., 2011b). High MCM3 expression is observed with a reduced RBM3 level similar to epithelial ovarian cancer (Nodin B et al., 2012). The only exception published so far is astrocytoma, where higher expression of RBM3 is associated with a higher grade and may promote astrocytic carcinogenesis (Zhang HT et al., 2013).

CIRP is differentially expressed in many cancer types (Artero-Castro A et al., 2009). In hepatocellular carcinoma (HCC), CIRP has been proposed to promote carcinogenesis by controlling ROS accumulation and cancer stem/progenitor cell expansion (Sakurai T et al., 2015). In addition, as a novel inflammation mediator, CIRP links chronic inflammation and colorectal tumorgenesis by stimulating cytokines, including TNF-α and IL-23 (Sakurai T et al., 2014), and by increasing IL-1β and IL-6 levels in resident liver macrophages (Sakurai T et al., 2015). Furthermore, CIRP is upregulated by chronic inflammation, which subsequently inhibits apoptosis and promotes cancer progression, indicating that CIRP expression could be a marker for predicting the risk

of HCC (Sakurai T et al., 2015) and that inhibiting CIRP could be a therapeutic value in cancer treatment (Lee HN et al., 2015). In pituitary adenoma, high CIRP expression correlates with proliferating invasive and recurrent tumor (Wang M et al., 2015), and in oral squamous cell carcinoma, CIRP is co-expressed with TLR4 and is associated with a short survival rate (Ren WH et al., 2014). Additionally, CIRP is thought to be involved in the progression of ductal carcinoma into invasive breast cancer (Mangé A et al., 2012). At the molecular level, CIRP increases the expression of CyclinE1, an important cell cycle regulator, thereby promoting proliferation and tumor progression in breast cancer (Guo X et al., 2010; Wu Y et al., 2011). In endometrial carcinoma and some endometrial hyperplasia, CIRP expression is absent or markedly decreased compared to normal endometrium, indicating a role of CIRP in normal proliferative events (Hamid AA et al., 2003), although the clinical outcome is uncertain. In summary, increasing numbers of clinical studies have demonstrated that CIRP is linked to poor clinical outcome in cancer.

A pivotal feature of most tumors is hypoxia (Wilson WR and Hay MP. 2011). The hypoxic cancer stem cell niche provides a microenvironment for the maintenance of immature cancer cells (Kise K et al., 2015), and hypoxia triggers the induction of CIRP and RBM3 (Wellmann S et al., 2004). In fact, experiments in colorectal cancers showed that RBM3 suppresses GSK3β activity and enhances β-catenin signaling to induce stemness of cancer cells (Venugopal A et al., 2015), in contrast to findings in prostate cancer cells (Zeng Y et al., 2013). However, the clinical data gathered on RBM3 in various tumor types (Table 1.2) with RBM3 as a marker of better outcome point towards a more complex network of RBM3 interaction than considered thus far. Various specific cell types likely affect RBM3 baseline expression and functions differentially; the cell environment also has an important impact on RBM3.

In conclusion, whereas both CIRP and RBM3 show common features of proto-oncogenes at the cellular level, their roles in the clinical tumor setting are diverse, with CIRP as a marker of worse prognosis and RBM3 as a marker of good prognosis. Among a variety of possible mechanisms that may explain this disparity, we would like to highlight the following consideration based on the available above-described

data. Major inflammatory pathways are involved in carcinogenesis (Elinav E et al., 2013). In contrast to RBM3, CIRP is secreted from microglia cells after ischemia (Zhou M et al., 2014) and functions as an inflammatory mediator during sepsis (Qiang X et al., 2013) (see also below). This extracellular action of CIRP has been linked to detrimental consequences. Albeit no data have yet been published regarding similar extracellular action in cancers, it is hypothesized that extracellular CIRP signaling in cancers may contribute to tumor progression and worse outcome through cytokine activation, as supported by the works from Sakurai T et al., (Sakurai T et al., 2014; Sakurai T et al., 2015). Moreover, reduced CIRP level is believed to associate with the enhanced chemosensitivity and selectivity of cancer cells (Zeng Y et al., 2009; He H et al., 2015). However, decreased RBM3 leads to higher chemosensitivity as well (Zeng Y et al., 2009), making it difficult to explain the correlation of RBM3 with beneficial prognosis in cancer patients.

Table 1.2 The roles of CIRP and RBM3 in cancer

	Cancer type	CIRP or RBM3 involved		Proposed mechanism	Prognosis with high expression	References
Urogenital cancer	Breast cancer	both	RBM3	-	Good	Jögi A et al., 2009
			CIRP	increase Cyclin E1	Poor	Guo X et al., 2010; Mangé A et al., 2012
	Epithelial ovarian cancer	RBM3		inhibit MCM3, Chk1 and Chk2	Good	Ehlén A et al., 2010; Ehlén Å et al., 2011
	Endometrial carcinoma	CIRP		-	Unclear	Hamid AA et al., 2003
	Prostate cancer	RBM3		involve ERG and PTEN; enhance chemo-sensitivity; CD44 splicing on stemness	Good	Josson L et al.,2011a; Grupp K et al., 2014; Zeng Y et al., 2009; Zeng Y et al., 2013; Shaikhi-brahim Z et al., 2013

	Testicular non-seminomatous germ cell cancer	RBM3		-	Good	Olofsson SE et al., 2015
	Urothelial bladder cancer	RBM3		-	Good	Boman K et al., 2013
Gastro-intestinal cancer	Oropharyngeal squamous cell carcinoma	both	RBM3	-	Down-regulated, outcome unknown	Martinez I et al., 2007
			CIRP	induce TLR4-related inflamma-tion	Poor	Ren WH et al., 2014
	Esophageal and gastric adenocarcinoma	RBM3		-	Good	Jonsson L et al., 2014
	Liver cancer	CIRP		increase ROS, IL-1β and IL-6; suppress p53	Poor	Sakurai T et al., 2015; Lee HN et al., 2015
	Colorectal cancer	both	RBM3	suppress GSK3β activity and enhance β-catenin signaling	Good	Hjelm B et al., 2011; Melling N et al., 2015; Wang MJ et al., 2015; Venugopal A et al., Mol Carinog. 2015
			CIRP	Induce TNF-α and IL-23	Poor	Sakurai T et al., 2014
Other cancers	Melanoma	RBM3		Inhibit MCM3	Good	Nodin B et al., 2012; Jonsson L et al., 2011b; Badli A et al., 2003
	Astrocytoma	RBM3		-	Unknown	Zhang HT et al., 2013
	Pituitary adenoma	CIRP		-	Poor	Wang M et al., 2015

1.1.4.3 Other functions

The biological activities of CIRP and RBM3 are versatile. Novel functions have been identified rapidly in recent years, including the regulation of circadian rhythm, reproduction and development, immune response and so on.

Circadian rhythm: CIRP and RBM3 show high homology with the two glycine-rich RNA-binding proteins, AtGRP7 and AtGRP8, in *Arabidopsis thaliana*; both of these proteins are pivotal components in a circadian-regulated feedback loop (Heintzen C et al., 1997; Schmal C et al., 2013). It is assumed that CIRP and RBM3 may also modulate circadian rhythm in animals. In mammals, the central clock in the suprachiasmatic nucleus (SCN) synchronizes the body temperature cycles to environmental light-dark cycles (Morf J and Schibler U, 2013) and to peripheral clocks (e.g., liver and pancreas) systematically (Mohawk JA et al., 2012; Gerber A et al., 2015). CIRP expression is diurnally regulated by light signal in the SCN of mice (Nishiyama H et al., 1998) as well as in amphibian brain (Saito T et al., 2000; Sugimoto K and Jiang H. 2008). Abnomal diet which affects peripheral clocks in liver and pancreas can also alter CIRP expression level (Oishi K et al., 2013; Li XM et al., 2010). In sleep-wake cycle, CIRP (Bellesi M et al., 2015) and RBM3 are differentially expressed in distinct stages and dysregulated situations (Costa M et al., 2015; Wang H et al., 2010). In 2012, the molecular mechanism of CIRP in the regulation of circadian rhythm was described: it modulates a variety of circadian oscillator genes, including *CLOCK* gene, in a post-transcriptional pattern in mammals (Morf J et al., 2012). Therefore, CIRP and RBM3 are both believed to be components of mammalian circadian oscillation, which not only is regulated by body temperature and responds to the change in the environment such as light and food in a subtle manner but also controls the expression of downstream circadian genes.

Reproduction and development: In the very early beginnings of CIRP and RBM3 research, both were discovered with high expression in mammalian testis but with

different spatial pattern, CIRP was predominantly in germ cells (Nishiyama H et al., 1998), whereas RBM3 was mainly in Sertoli cells (Danno S et al., 2000). The different spatial expression patterns of CIRP and RBM3 may indicate their distinct functions in spermatogenesis. Several mechanisms have been proposed to explain the temperature-sensitive function of CIRP in spermatogenesis and testicular injury protection, including mRNA stabilization (Xia Z et al., 2012), DNA integrity maintenance (Banks S et al., 2005), the suppression of pro-apoptotic proteins p53 and Fas (Zhou KW et al., 2009), as well as the inhibition of oxidative stress-induced apoptosis in germ cells (Xia Z et al., 2013). Furthermore, CIRP regulates cell cycle and promotes the proliferation of immature germ cells by interacting with Dryk1b/Mirk to inhibit its binding to p27 (Masuda T et al., 2012), and modulates p44/p42, p38 and SAPK/JNK MAPK pathways (Xia ZP et al., 2012).

Oocytes and embryo cryopreservation is of great importance in reproductive medicine (Edgar DH and Gook DA, 2012). To freeze an egg or embryo, a novel flash-freeze process called vitrification has replaced the traditional slow-cooling method with more benefits (Edgar DH and Gook DA, 2012). RBM3 and CIRP are both involved in the protection against cold stress or crystallization during vitrification of oocytes (Jo JW et al., 2012; Wen Y et al., 2014; Jo JW et al., 2015) and embryos (Boonkusol D et al., 2006), as well as in the cryopreservation of genital organs of livestock (Devi L et al., 2014; Du T et al., 2015).

Both CIRP and RBM3 are key factors during development. The investigations of the roles of CIRP in development focus on amphibians, which are well-described models for developmental study. In *X. laevis*, XCIRP-1 is transiently expressed in developing kidney and brain (Uochi T and Asashima M, 1998) and is required for embryonic kidney formation (Peng Y et al., 2000) and anterior brain development (van Venrooy S et al., 2008). The regulatory mechanisms of CIRP in development include the maintenance of adhesion molecules and cell movement (Peng Y et al., 2006). Furthermore, the expression of a CIRP homologue in *Mexico axolotl* peaks in the neural plate and fold at stage 15 during development (Bhatia R et al., 1999). All the collective data support the notion that CIRP is indispensable in versatile events

during development. Under euthermic conditions, the RBM3 level peaks during the early postnatal periods and then decreases to a basal level in most regions of the brain except for areas where proliferation is active, such as the subventricular zone (SVZ) and the rostral migration stream (RMS) (Pilotte J et al., 2009; Chip S et al., 2011), indicating a pivotal role of RBM3 in the maintenance of stemness and proliferation in neuronal cells.

Immune Response: In 2013, CIRP was identified as a novel inflammatory mediator released from the heart and liver into the circulatory system stimulating TNF-α during hemorrhagic shock and sepsis (Qiang X et al., 2013), and thus accelerating the imflammation phase to improve wound healing (Idrovo JP et al., 2016). Interestingly, CIRP expression can be also impaired by TNF-α (Lopez M et al., 2014), suggesting a negative feedback loop. Similar to the dual role of CIRP in the brain, as discussed above, CIRP does not only protect hepatocytes from oxidative stress (Sakurai T et al., 2013), but also stimulates inflammation in liver (Godwin et al., 2015). Today, CIRP is suggested as a potential diagnostic marker for sepsis (Zhou Y et al., 2015). In RBM3 knockout mice, no obvious change of cytokine expression has been found in DNA-mediated immune response (Matsuda A et al., 2011). Hence, the role of RBM3 in inflammation is still unclear and remains to be clarified. Thus far, whether RBM3 can be secreted has remained unknown.

Miscellaneous: Other studies suggest RBM3 is involved in viral infection (Wright CF et al., 2001; Dellis S et al., 2004) and skeletal muscle regulation (Dupont-Versteegden EE et al., 2008; Ferry AL et al., 2011). In addition, CIRP was found to inhibit transient outward potassium (Ito) channel, consequently modulates cardiac repolarization (Li J et al., 2015), and to regulate telomerase activity (Zhang Y et al., 2015). These studies open new avenues to the understanding of multiple functions of CIRP and RBM3. However, all these studies require more evidence for confirmation and elucidation. The common and distinct functions of CIRP and RBM3 are summarized in Figure 1.3.

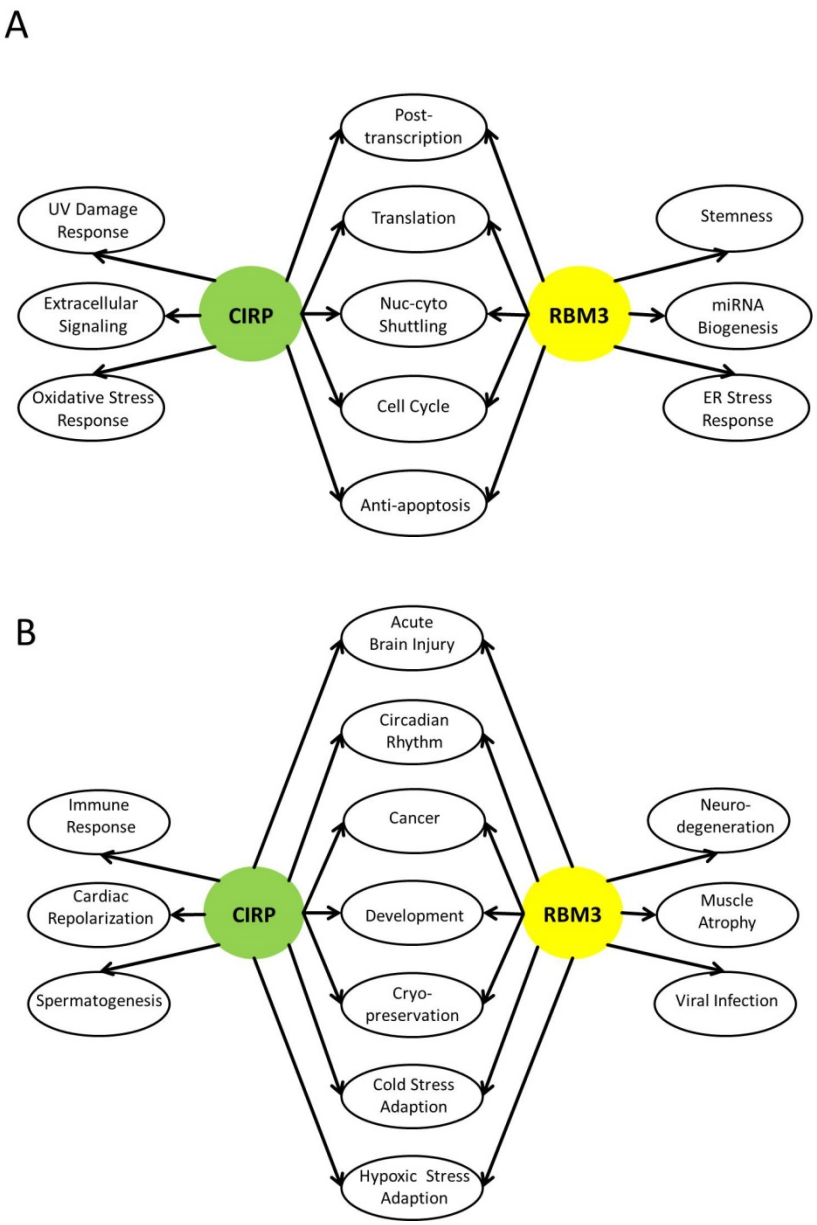

Figure 1.3 Common and distinct functions of CIRP and RBM3. A: molecular and cellular functions; B: physiological and pathological functions. Nuc-cyto: nuclear-cytoplasmic. The distinct functions of CIRP or RBM3 may not be exclusive for respective protein, but due to insufficient investigations on its homologue.

1.2 Unfolded protein response (UPR)

1.2.1 Main signaling pathways of UPR

The ER is an important cellular compartment for both protein synthesis/folding and calcium homeostasis. A variety of physiological and pathological insults can disturb normal ER function, causing an accumulation of misfolded or unfolded proteins in the ER, referred to as ER stress. ER stress subsequently triggers a specific set of intracellular signaling pathways collectively known as the unfolded protein response (UPR). Based on the type of transmembrane sensors for misfolded/unfolded proteins, such as the chaperone glucose regulated proteins 78 (GRP78, also known as Immunoglobulin Binding protein, BiP), the UPR comprises three canonical signaling pathways: the PRKR-like ER kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α)-CCAAT/enhancer-binding protein homologous protein (CHOP) pathway, the inositol-requiring protein 1α (IRE1α)-X-box-binding protein 1 (XBP1) pathway and the activating transcription factor 6α (ATF6α) pathway (Figure 1.4; Ron D and Walter P, 2007; Wang M and Kaufman RJ, 2014).

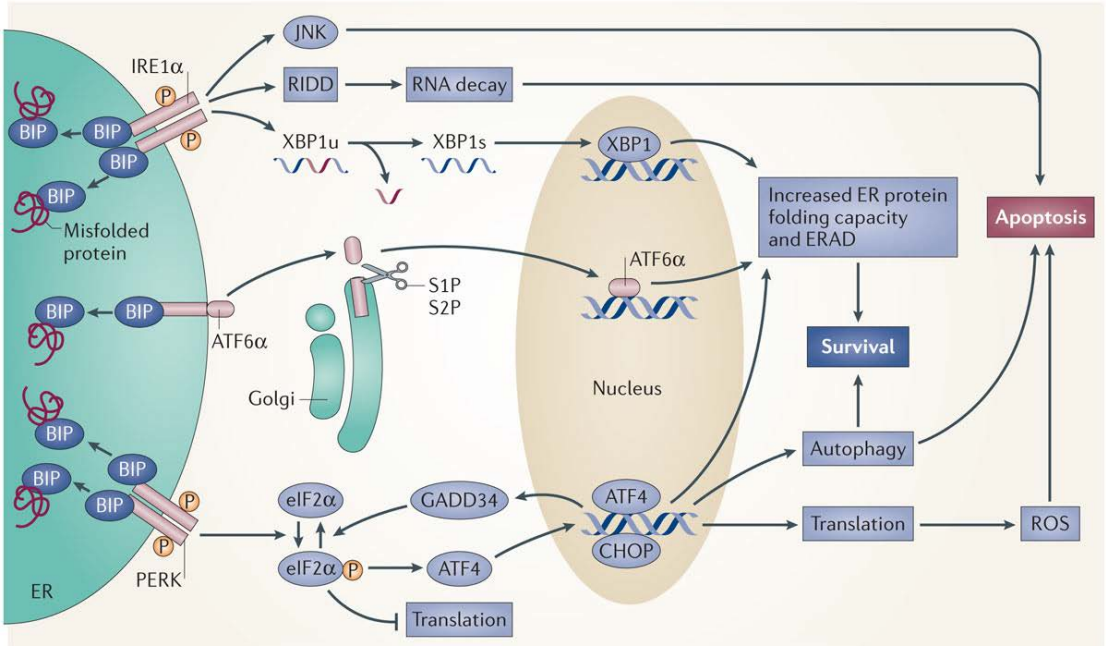

Figure 1.4 Three main branches of unfolded protein response (UPR) signaling pathways (Wang M and Kaufman RJ, 2014)

The three branches behave at different time points during ER stress. At early stage, all the three arms are activated and cells are protected via several mechanisms including the formation of stress granule and the halting of translational process. Instead, upon prolonged ER stress, IRE1α pathway is soon attenuated, followed by ATF6α pathway. The PERK pathway, however, is persistent and ultimately initiates apoptosis (Lin JH et al., 2007).

1.2.2 UPR and brain disorders

Both ER stress and UPR activation are involved in the pathology of many, if not all, acute and degenerative diseases of the brain (Yang W and Paschen W, 2009; Halliday M and Mallucci GR, 2014; Xin Q et al., 2014). In hypoxic-ischemic insults, intracellular calcium load disturbances play an important role in causing ER stress (Bodalia A et al., 2013), while ER stress subsequently provokes inflammation and mitochondrial dysfunction (Xin Q et al., 2014). In contrast, each neurodegenerative disorder is characterized by the accumulation of disease-specific misfolded proteins, resulting in ER stress (Halliday M and Mallucci GR, 2014). Notably, PERK-eIF2α-CHOP signaling is highly associated with neurodegenerative diseases like Alzheimer's disease and prion disease, serving as potential therapeutic targets (Halliday M and Mallucci GR, 2014).

1.2.3 Hypothermia and UPR

As mentioned above, early UPR activation can protect cells unless ER stress is prolonged. Hypothermia itself can stimulate all the three main arms of UPR without initiating apoptosis, partially explaining the advantages of pre-conditioning with hypothermia (Rzechorzek NM et al., 2015). In parallel, both RBM3 and CIRP are induced during preconditioning (Rzechorzek NM et al., 2015), suggesting their involvement in the regulation of UPR. On the contrary, upon ischemia-induced ER stress, hypothermia is protective by suppressing CHOP, the effector protein leading to apoptosis (Poone GK et al., 2015). These studies addressed the model that, therapeutic hypothermia can activate UPR to make cells adapt to subsequent cellular

stress, but repress UPR-induced apoptosis upon persistent ER stress signal.

1.3 MicroRNA (miRNA) biology

1.3.1 Biogenesis of miRNA

MiRNAs are small non-coding RNA molecules with around 21 nucleotides. They are widely found in different organisms and regulate numerous developmental and cellular processes post-transcriptionally. Most of miRNAs are generated *in vivo* from canonical pathway: transcribed primary precursors (pri-miRNA) are processed by Drosha/DGCR8 complex into 70-nucleotide precursor (pre-miRNA) in the nucleus; pre-miRNA is further exported into cytoplasm and processed by Dicer complex in order to generate mature miRNA duplex; finally, one strand of the duplex is incorporated into RISC complex to guide the degradation of specific mRNA (Figure 1.5, Krol J et al., 2010; Lin S and Gregory RI, 2015).

1.3.2 miRNA and brain disorders

An increasing number of researches demonstrate that miRNA play important roles in both acute brain injuries and neurodegeneration (Saugstad JA, 2010). Microarray data reveals massive changes in miRNA expression profile upon spinal cord injury (SCI), traumatic brain injury (TBI) and ischemic stroke (Bhalala OG et al., 2013). Specific miRNAs have been verified and analyzed functionally and mechanistically. For instance, miR-15a and miR-497 can both target *Bcl-2* transcript thus promote apoptosis during ischemic injury (Yin KJ et al., 2010a; Yin KJ et al., 2010b). In the cases of neurodegenerative diseases, the expressions of key proteins in disease progression are regulated by one or more specific miRNAs (Bushati N and Cohen SM, 2008; Eacker SM et al., 2009). To name a few, BACE1 in Alzheimer's disease and PITX3 in Parkinson's disease are modulated by miR-29a/b-1 and miR-133b, respectively (Hébert SS et al., 2008; Kim J et al., 2007).

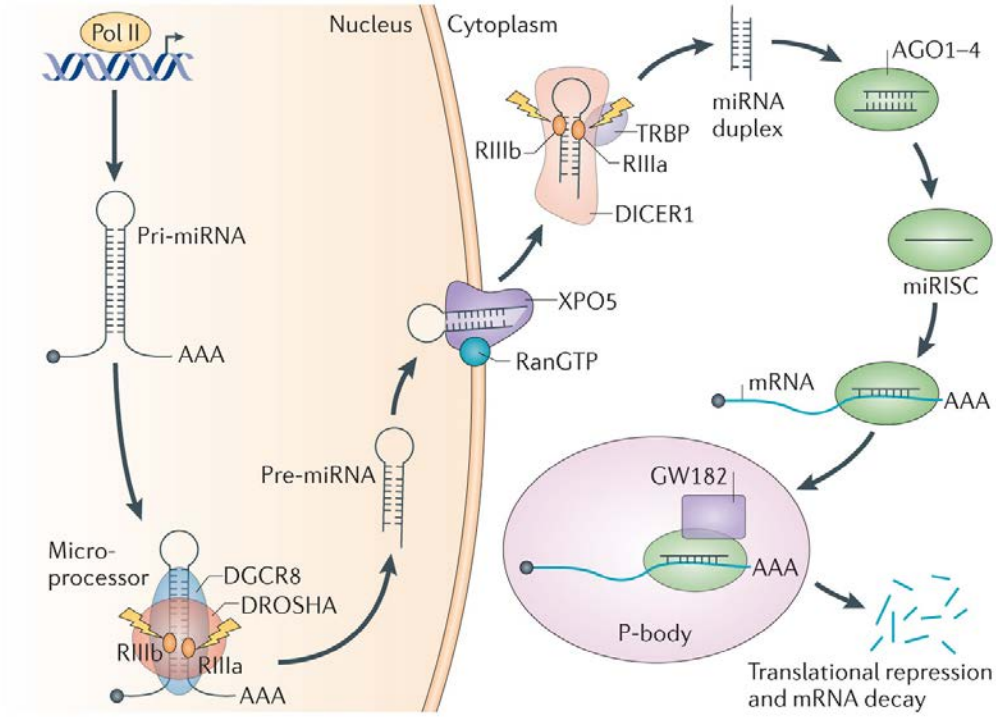

Figure 1.5 Canonical biogenesis of miRNA (Lin S and Gregory RI, 2015)

1.3.3 Hypothermia, RBM3 and miRNA

Moderate hypothermia can change the miRNA expression profile (Potla R et al., 2015). It may not only contribute to its protective function in injuries and diseases including traumatic brain injury (TBI) (Truettner JS et al, 2011; Truettner JS et al, 2013) and cardiac arrest (Gilje P et al., 2014), but may also correlate with deleterious consequences such as in intestinal hypothermic arrest (Lin WB et al., 2015) and unintentional hypothermia after surgery (Billeter AT et al., 2012). RBM3 is considered to alter miRNA levels as well and contributes thereby to global protein translation under hypothermia (Dresios J et al., 2005). A few years later, Pilotte J et al., reported that RBM3 positively modulates a majority of miRNAs, and negatively regulates only a minority. They also observed that RBM3 binds to 70-nucleotide and facilitates its processing to Dicer complex (Pilotte J et al., 2011). However, the finding that RBM3 upregulates most of miRNAs is conflicting to the fact that RBM3 increases overall translation (Dresios J et al., 2005). Particularly, miR-125b shows a decreasing tendency when RBM3 is overexpressed as shown by Dresios J et al., but the opposite

is demonstrated by Pilotte J et al.,, making the RBM3-mediated modulation of miRNA controversial (Dresios J et al., 2005; Pilotte J et al., 2011). Therefore, it is believed that RBM3 executes a regulatory function in miRNA expression, while the exact role remains largely unclear.

1.4 Scope of the thesis

The two cold-responsive proteins CIRP and RBM3 mediate the protective functions of moderate hypothermia, and regulate a variety of physiological processes beyond the context of cold. Recent studies have revealed a dual-role of CIRP: on one hand it protects cells in various stressful conditions; on the other hand it exacerbates stress-induced injuries via stimulating inflammation, and promotes cancer progression. Instead, RBM3 is generally beneficial for cell survival without detrimental effects. Therefore, we focused our study on the actions of RBM3 in cytoprotection.

The first objective was to confirm the protective effects of RBM3 in diverse stressful conditions relating to brain acute and chronic disorders. Oxidative stress plays a vital role in brain ischemia/reperfusion (Sanderson TH et al., 2013). Endoplasmic reticulum (ER) stress is associated with various brain disorders, and ER stress-activated UPR promotes acute damage exacerbation or chronic disease progression (Yang W and Paschen W, 2009). We aimed to discover the consequences on cell fate when manipulating RBM3 level upon oxidative stress or ER stress.

Secondly, our purpose was to investigate the regulatory role of RBM3 in PERK-eIF2α-CHOP pathway of UPR under ER stress. Among the three branches of UPR, PERK-eIF2α-CHOP signaling is the dominant pathway under persistent ER stress ultimately resulting in apoptosis. We hypothesized that RBM3 prevents cell apoptosis by impairing PERK-eIF2α-CHOP pathway. We intended to examine this assumption in different models.

In the third part, we made efforts to figure out whether RBM3 can modulate pro- and anti-apoptotic miRNAs. As RBM3 can up- or downregulate distinct groups of miRNAs (Pilotte J et al., 2011), we suggested that RBM3 might decline pro-apoptotic

miRNA level and simutaneously promotes anti-apoptotic miRNA expression, by which it enhances the possibility of cell survival.

2. Materials and Methods

2.1 Animals and organotypic hippocampal slice cultures

All animal experiments were performed with permission of the local animal care committee and in accordance with international guidelines on handling laboratory animals and current swiss law. RBM3 knockout (KO) mice were kindly provided by Prof. Tadatsugu Taniguchi (University of Tokyo). Hippocampi from C57BL/6J wildtype (WT) and RBM3 KO mice were isolated from postnatal day 3 (P3) mice and organotypic slice cultures were prepared as described previously (Chip S et al., 2014). Briefly, transversal hippocampal slices were sectioned using a McIlwain tissue chopper (Ted Pella, Redding, CA, USA) under aseptic conditions with a thickness of 350 µm. Slices were carefully separated and laid over 0.4 µm Millicell-CM culture inserts with 30 mm diameter (Millipore) in six-well plates, and cultured in 1 mL per well medium containing HEPES-buffered minimal essential medium (50%), Hank's buffered salt solution (HBSS) (25%), and heat-inactivated horse serum (25%) supplemented with glutamax (2 mM, Life Technologies), glucose (1 g/L), pH 7.3, in humidified incubator with 5% CO_2 at 37°C. Medium was changed 24 h after the slices were prepared and then every other day until the harvest of the cultures. At 5 *days in vitro* (DIV), the cultured slices were challenged with oxygen–glucose deprivation (OGD) or ER stress at either 32°C (moderate hypothermia) or 37°C (normothermia) as described below.

To induce ER stress, 0.3 µM thapsigargin was added to the medium and slices were used after 0.5 h for phos-eIF2α detection, after 6 h for *CHOP* mRNA quantification, after 8 h for CHOP protein detection and after 24 h of thapsigargin exposure for propidium iodide (PI, Sigma-Aldrich) staining. Slices (n=4-6) were homogenated and proceeded to standard total protein or mRNA extraction protocol as described below. PI (1 µg/mL) was added to the slices for 30 min, then the samples were observed under inverted microscope with 10X objective lens (Olympus IX51, Olmpus,

Volketswil, Switzerland). Photographs were taken with Olympus U-CMAD3 camera (Olympus).

2.2 Cell culture and manipulation

Human embryonic kidney cells, HEK293, were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco/LuBioScience, Lucerne, Switzerland), unless indicated elsewhere. Cells were treated with H_2O_2, thapsigargin or tunicamycin (all Sigma-Aldrich, Buchs, Switzerland) at indicated concentrations and exposure times. PERK inhibitor (3 μM, Millipore/Merck Millipore, Darmstadt, Germany) was used for 6 h prior to thapsigargin treatment. For target gene knock-down, 2×10^5 cells were cultured for 24 h followed by transfection of scrambled or gene-specific siRNAs (30 pM, Qiagen, Hombrechtikon, Switzerland) with lipofectamine 2000 (Life Technologies, Zug, Switzerland). Flp-In T-Rex 293 cell line (Life Technologies) was used to construct inducible RBM3 overexpressing cells according manufacturer's recommendations, and RBM3 overexpression was induced with 4 ng/mL doxycycline for 24 h. Episomal mammalian expression vector pCEP4 (Life Technologies) was applied for the construction of constitutive RBM3 overexpressing cell line as published previously (Chip S et al., 2011).

2.3 Oxygen-glucose deprivation (OGD)

OGD experiment with organotypic hippocampal slice cultures was modified following previous report (Rytter A et al., 2003). In brief, 10X OGD medium supplement was prepared as follows: 3 mM $CaCl_2$, 700 mM NaCl, 52.5 mM $NaHCO_3$, 700 mM KCl, 12.5 mM NaH_2PO_4, 20 mM $MgSO_4$ and 100 mM sucrose, pH adjusted to 6.8. Six-well plates containing 1 mL 1X OGD medium (glucose-free Hank's buffered salt solution plus 1X OGD medium supplement) in each well were placed in anaerobic chamber (Elektrotek Ltd., Keighley, UK) with the 10% H_2, 5% CO_2 and 85% N_2 for 1 h. The equilibrated 1X OGD medium was kept in the anaerobic chamber at 37℃ overnight.

At 5 DIV, cultured slices were washed once in fresh 1X OGD medium and transferred to balanced 1X OGD medium in the anaerobic chamber. OGD was performed with 10 H_2, 5% CO_2 and 85% N_2 for 15 min and the tissues were further transferred to 37℃or 32℃ incubator for up to 48 h. During post-OGD incubation, 60 µL medium from each culture was taken for CellTox™ Green Cytotoxicity Assay (Promega) at 0 h, 6 h, 24 h and 48 h under manufacturer's instructions. After 48 h incubation, cultures were subjected to PI staining and RBM3 immunostaining.

2.4 Protein extraction and Western blot

Cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris, 150 mM NaCl, 1X Roche Protease Inhibitor Cocktail, pH 8.0), separated by NuPAGE Novex 4-12% Bis-Tris protein gels (Life Technologies) and transferred to Amersham Hybond-P PVDF membranes (Amersham/GE Healthcare Life Sciences, Glattbrugg, Switzerland). Membranes were incubated with primary antibodies at 4℃ overnight: anti-phos-PERK Thr981 diluted 1:250 (sc-32577), anti-PERK 1:1000 (sc-13073), anti-phos-PKR Thr446 1:250 (sc-101783), anti-PKR 1:1000 (sc-708), anti-GADD153/CHOP 1:1000 (sc-575), anti-NF45 1:5000 (sc-271718) (all from Santa Cruz Biotechnology, Dallas, TX, USA); anti-phos-eIF2α Ser51 1:500 (#9721), anti-eIF2α 1:5000 (#9722), anti-Bip 1:1000 (#3177), anti-HuR 1:1000 (#12582), anti-Bcl-2 1:1000 (#2870), and anti-β-actin 1:2000 (#4967) (all from Cell Signaling/Merck Millipore, Darmstadt, Germany); anti-RBM3 1:1000 (14363-1-AP, Proteintech, Manchester, United Kingdom); anti-NF90 1:1000 (anti-DRBP76, 612154, BD Biosciences, Allschwil, Switzerland). After washing, membranes were incubated with the following secondary antibodies conjugated with horseradish peroxidase (HRP) and diluted 1:10000 for 1 h at room temperature: anti-rabbit IgG (#7074, Cell Signaling), anti-mouse IgG (4759, Carl Roth, Karlsruhe, Germany), and finally the signals were detected with enhanced chemiluminescence system (Clarity ECL Western Blotting Substrate, Bio-Rad Laboratories, Cressier, Switzerland).

2.5 Real-time RT-PCR

Total RNA was isolated using ReliaPrepTM RNA Cell Miniprep System (Promega, Dübendorf, Switzerland). cDNA was prepared by GoScriptTM Reverse Transcription System (Promega) to measure mRNA levels. In order to measure mature microRNAs, cDNA was synthsized by NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). Real-time PCR was performed with GoTaq qPCR Master Mix (Promega) in 15 µL reaction volume. *β-actin* and *β-2 microgloblin* (*β2m*) were used as reference genes for mouse and human mRNA samples, respectively. *U6* was used as a reference gene for human miRNA measurement. Primer sequences for real-time PCR were listed below (mmu: *Mus musculus*; hsa: *Homo sapiens*):

mmu-β-actin-F: GGCCAACCGTGAAAAGATGA

mmu-β-actin-R: CACAGCCTGGATGGCTACGT (Al Chawaf A et al., 2007)

hsa/mmu-CHOP-F: CATCACCACACCTGAAAGCA

hsa/mmu-CHOP-R: TCAGCTGCCATCTCTGCA (Bettaieb A and Averill-Bates DA. 2015)

hsa-β2m-F: GATGAGTATGCCTGCCGTGTG

hsa-β2m-R: TCCAATCCAAATGCGGCATCT (Wellmann S et al., 2004)

hsa/mmu-Bcl-2-F: TGGGATGCCTTTGTGGAACT

hsa/mmu-Bcl-2-R: GAGACAGCCAGGAGAAATCAAAC (Pugazhenthi S et al., 2000)

hsa-Bip-F: GGAAAGAAGGTTACCCATGC

hsa-Bip-R: AGAAGAGACACATCGAAGGT (Kosakowska-Cholody T et al., 2014)

hsa-pre-miR-16-F: GCAGCACGTAAATATTGGCGT

hsa-pre-miR-16-F: CAGCAGCACAGTTAATACTGGAGA (Suzuki HI et al., 2009)

hsa-pre-let-7a-F: TGAGGTAGTAGGTTGTATAGT

hsa-pre-let-7a-R: TGTATAGTTATCTCCCAGTG (Trabucchi M et al., 2009)

hsa-miR-16-F: TAGCAGCACGTAAATATTGGCG (Gao SM et al., 2012)

hsa-let-7a-F: GCCGCTGAGGTAGTAGGTTGTA (Zhang HH et al., 2007)

hsa-miR-106b-F: TAAAGTGCTGACAGTGCAGAT (Zhao ZN et al., 2012)

hsa-miR-93-F: CGGCGGCAAAGTGCTGTTCGTG (Zhao ZN et al., 2012)

hsa-miR-25-F: CATTGCACTTGTCTCGGTCTGA (Zhao ZN et al., 2012)

hsa-U6-F: CGCAAGGATGACACGCAAATTC (Gao SM et al., 2012)

For miRNA relative quantification, universal reverse primer was provided by NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). Thermal cycles were carried out on StepOne Real-Time PCR Systems (Applied Biosystems/Life Technologies, Zug, Switzerland) at 95℃ for 5 min, followed by 45 cycles of 95℃ for 15 s and 60℃ for 1 min. Values were normalized to respective reference gene expressions and quantified with comparative C_T method.

2.6 Cell viability assay

Cell viability was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). The assay was performed by adding 20 µL assay reagent containing MTS tetrazolium compound to 5×10^4 cells in 100 µL volume for 2 h. The absorbance was recorded by 96-well plate reader under 450 nm wavelength.

2.7 Fluorescence-activated Cell Sorting (FACS)

Episomal mammalian expression vector pCEP4 (Life Technologies) was used to overexpress RBM3 in HEK293 cells for FACS analysis. To knock down RBM3, RBM3 specific siRNA was transfected into HEK293 cells for 48 h. Apoptosis was induce by 5 mM H_2O_2 treatment for 1 h, then the stressed cells were transferred to fresh DMEM medium without H_2O_2 but containing only 1% fetal bovine serum. The percentages of apoptotic cells were determined with Annexin V Apoptosis Detection Kit APC (eBioscience). In brief, 1×10^6 cells were suspended in 200 µL Annexin Binding Buffer and stained with 10 µL Annexin V-APC (AnnV) for 15 min in dark at room temperature. Subsequently, cells were washed with 200 µL Annexin Binding Buffer again and stained with 5 µL propidium iodide (PI). Analysis was performed by FACSCalibur FL3/FL4 at the speed of 400 cells/second (BD Biosciences) according to manufacturer's instructions. The proportion of AnnV(+) and PI (-) cells indicate cells undergoing apoptosis.

2.8 Co-Immunoprecipitation (CoIP)

4 µg of primary antibodies (the same as used for Western blot) or control normal mouse IgG (sc-2025), and normal rabbit IgG (sc-2027) (both from Santa Cruz Biotechnology) were conjugated to 50 µL Dynabeads Protein G 50% slurry (Life Technologies) for 45 min at room temperature. HEK293 cells were harvested and 5 mg cell lysates were incubated overnight at 4℃ with antibody-coupled Dynabeads Protein G. For RNase treated group, cell lysates were pre-treated with 10 U/µL RNaseT1 (Fermentas/ Life Technologies, Zug, Switzerland) for 15 min at room temperature before subjected to the beads. Proteins were eluted from beads in NuPAGE LDS Sampler Buffer (Life Technologies) containing 50 mM DTT at 70℃ for 10 min). In CoIP experiments, when detecting antibody and IP antibody were generated from the same species, HRP conjugated Protein G (P-21041, Life Technologies) was used as secondary antibody to minimize the interfering signals from heavy chains and light chains.

2.9 Mass Spectrometry

RBM3 overexpressing cells were derived from Flp-In T-Rex 293 cell line. Both RBM3 non-induced and induced cells were lysed, purified by affinity purification and digested into peptides to screen RBM3 interaction partners. The complex peptides mixture was separated by C18 HPLC column and directly analyzed by LC/MS-MS (LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, MA, USA). RBM3 interacting proteins were identified by comparing the experimentally acquired fragment spectra and theoretically fragmented protein database (SwissProt/UniProt), and validated by subtracting background protein. Searching was applied with Sorcerer search tool and Sequest search algorithm. The experiment and data analysis were performed by CaptiVate Dualsystems Biotech (Dualsystems Biotech, Schlieren, Switzerland) as published previously (Glatter T et al., 2009).

2.10 Immunostaining

HEK293 cells were cultured on BioCoat Poly-D-Lysine Coverslips (BD Biosciences), treated with or without 3 μM thapsigargin for 6 h, and fixed in freshly made 4% paraformaldehyde for 10 min at 37°C. After washing with phosphate-buffered saline, cells were permeabilized with 0.5% TritonX-100 and blocked with 5% normal goat serum. Primary antibodies against NF90 (diluted 1:100), RBM3 (1:50), and PERK (1:50) were incubated with cells at 4℃ overnight and then washed 3 to 4 times. Then, secondary antibodies conjugated with fluorescent dye Alexa Fluor 488 or Alexa Fluor 568 (Life Technologies) were added and incubated at room temperature for 1 h. Mounting was performed with mounting medium containing DAPI. Images were acquired with Olympus AX-70 microscope using 40X objective lens and SPOT Insight CCD camera. For organotypic hippocampal slice cultures, the fixation with 4% paraformaldehyde were performed overnight at 4°C, and the dilution for RBM3 antibody was 1:100. Then the staining procedure was the same as for HEK293 cells.

2.11 Proximity ligation assay (PLA)

For proximity ligation assay (PLA), HEK293 cells were prepared as the steps for immunostaining until the incubation with primary antibodies was completed. Subsequently, incubation with PLA probes, probe ligation and amplification steps were conducted following the manufacturer's manual (Duolink/ Sigma-Aldrich, Buchs, Switzerland). For primary antibody pairs which were generated from the same species, Duolink In Situ Probemaker was used to pre-conjugate primary antibodies to PLA oligos. Cells were mounted with DAPI-containing mounting medium. Microscopy was performed as for normal immunostaining.

2.12 Statistics

Quantificational data were presented as mean ±S.D. from at least three independent experiments. Significance was assessed by two-tailed student's t-test. The values with $P<0.05$ was considered as significant in statistics.

3. Results

3.1 Hypothermia and RBM3 promote cell survival

3.1.1 Hypothermia prevents neural cell death in OGD model

OGD is an *in vitro* model for ischemia/reperfusion injury in cultured tissues or cells (Rytter A et al., 2003). We used organotypic hippocampal slice cultures from postnatal day 3 (P3) animals as described previously (Chip S et al., 2011). Organotypic hippocampal slices were cultured 5 days *in vitro*, and challenged with OGD for 15 min. Subsequently, the slices were transferred to fresh incubation medium containing dye for CellTox™ Green Cytotoxicity Assay, and incubated at either normothermic or hypothermic conditions. The dye binds to naked DNA molecular and gives a fluorescent signal, indicating the disruption of membrane integrity and cell death. 60 µL medium of each culture was taken for the measurement of the fluorescent signal at 0, 6, 24 and 48 h after OGD (Figure 3.1).

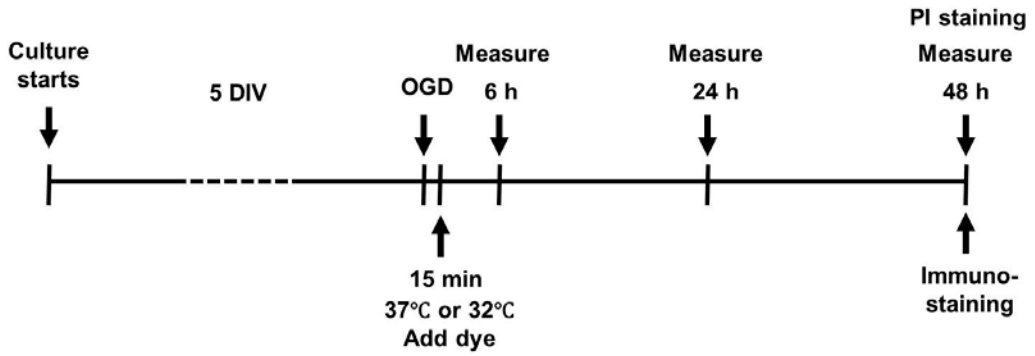

Figure 3.1 Schematic experimental procedure of OGD model in organotypic hippocampal slice culture from post-natal day 3 C57BL/6J WT mice. DIV, days *in vitro*; OGD, oxygen-glucose deprivation; PI staining, propidium iodide staining.

The results showed a gradual increase in cell death after OGD in both normothermic and hypothermic groups, indicated by fluorescent signal strengths obtained from DNA-binding indicator dye (Figure 3.2). No significant difference of cell death was observed between the two groups until 24 h after OGD, while cell death was dramatically prevented by hypothermia between 24 and 48 h after OGD (Figure 3.2).

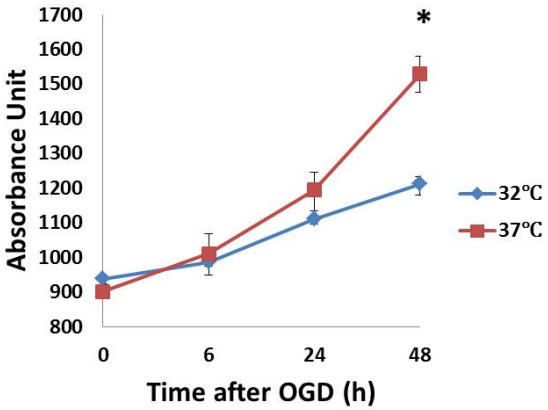

Figure 3.2 Moderate hypothermia protects neural cells from OGD. Organotypic hippocampal slice cultures from C57BL/6J WT mice were challenged with 15 min OGD, and transferred to 37°C or 32°C incubator in five independent experiments for up to 48 h. 60 µL medium was taken from each culture for CellTox™ Green Cytotoxicity Assay at indicated time point. 0 h is defined as immediately after OGD treatment (*P<0.05).

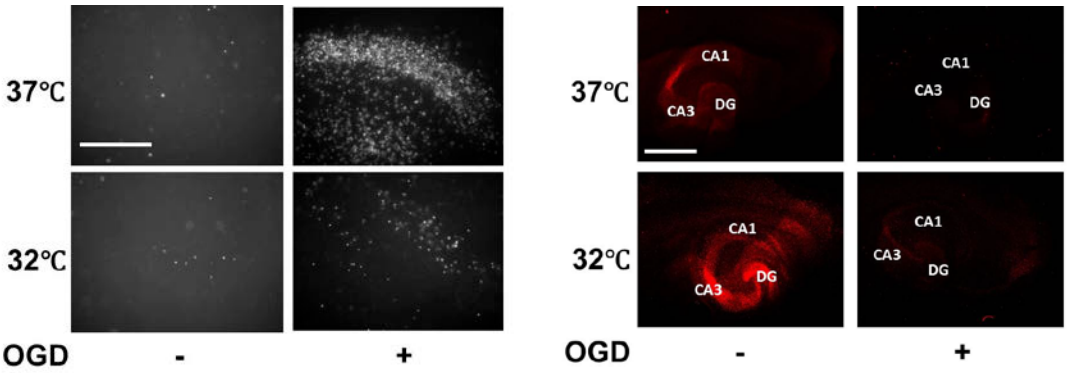

Figure 3.3 Representative PI staining (left panel) and immunostaining of RBM3 (right panel) from C57BL/6J WT organotypic hippocampal slice cultures. Cultures were incubated at 37°C or 32°C after OGD or mock treatment for 48 h. Representative results are shown from three independent experiments. Scale bar: 500 µm.

The principle of propidium iodide (PI) staining is similar to CellTox™ Green Cytotoxicity Assay. In brief, PI molecule can be excited with fluorescent signal only when binding to DNA released from dying cells, indicating necrotic and late apoptotic cells, but impermeant to living cells. PI staining signal confirmed an elevated dead cell number after 48 h post-OGD incubation at 37°C, and a reduced dead cell number when hypothermia was performed (Figure 3.3). Immunostaining revealed increased RBM3 expression under hypothermia, especially in dentate gyrus (DG) and CA3 region of hippocampus (Figure 3.3). After OGD treatment RBM3 level decreased, but recovered to some extent by hypothermic treatment (Figure 3.3), presenting a negative correlation with cell death.

In addition, the anti-apoptotic protein Bcl-2 was found to be upregulated by moderate hypothermia in cultured hippocampus, while in the absence of cold-inducible RBM3, Bcl-2 expression is attenuated (Figure 3.4). These results support the notion that moderate hypothermia favors neural cell survival upon hypoxic-ischemic injury, and RBM3 may play a role in the protection.

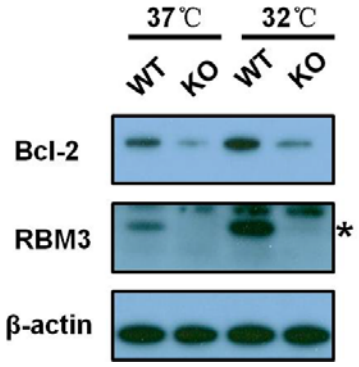

Figure 3.4 Moderate hypothermia and RBM3 are both positively associated with Bcl-2 expression in organotypic hippocampal slice culture. Organotypic hippocampal slices from RBM3 WT or KO mice were cultured at 37°C or 32°C for 48 h and subsequently homogenated to detect Bcl-2, RBM3 and β-actin by Western blot. Representative results are shown. Asterisk indicates RBM3 band.

3.1.2 RBM3 protects cells from H_2O_2-induced oxidative stress

Oxidative stress is an important deleterious factor in ischemia/reperfusion injury (Sanderson TH et al., 2013). In order to figure out the role of RBM3 in cell death provoked by oxidative stress, we used human embryonic kidney 293 (HEK293) cells for the manipulation of RBM3 expression. Recombinant RBM3 was overexpressed by the episomal mammalian expression vector pCEP4, and RBM3 downregulation was achieved by specific siRNA targeting human *RBM3* gene. Similar to that observed in organotypic hippocampal slice culture model, the expression of Bcl-2 was also elevated in cells overexpressing RBM3 but decreased in RBM3 knock-down HEK293 cells (Figure 3.5).

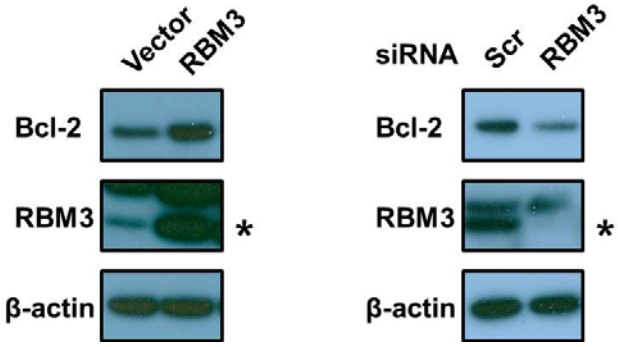

Figure 3.5 RBM3 promotes Bcl-2 expression in HEK293 cells. RBM3 was constitutively expressed in pCEP4 system (left panel) or downregulated by siRNA (right panel) in HEK293 cells. After 24 h culture at 37°C, cells were harvested and western blot was performed to measure Bcl-2, RBM3 and β-actin expressions. Representative results are shown. Asterisks indicate RBM3 band. Scr: scrambled.

In the next step, we used hydrogen peroxide (H_2O_2) to induce oxidative stress in HEK293 cells with constitutive RBM3 overexpression or transient *RBM3* gene silencing. Cell proliferation assay was performed to measure cell viability, and fluorescence-activated cell sorting (FACS) analysis with Annexin V/PI double-staining was used to determine the percentage of apoptotic cells. Our results revealed reduced cell viability and increased apoptotic cell numbers in the hydrogen peroxide-

treated group compared with the control group (Figure 3.6 and 3.7). The lack of RBM3 led to fewer viable cells and aggravated apoptosis. Conversely, when RBM3 was overexpressed, cell viability was enhanced and apoptosis was substantially prevented compared with the empty vector control (Figure 3.6 and 3.7). To conclude, RBM3 is beneficial for cell viability and survival during oxidative stress-induced apoptosis.

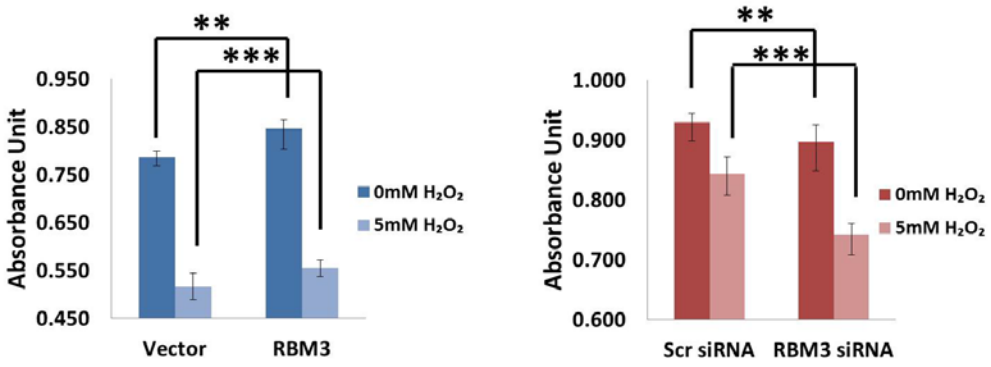

Figure 3.6 RBM3 enhances cell viability in HEK293 cells. Oxidative stress was induced in the manipulated cells from Figure 3.5 by 5 mM H_2O_2 for 1 h following by 24 h incubation in fresh DMEM medium containing 1% fetal bovine serum. Left panel is for RBM3 overexpression and right panel is for RBM3 knock-down. Non-treated cells were used as controls. Cell viability was determined by proliferation assay. Data from six independent experiments was analyzed for significance evaluation (**P<0.01, ***P<0.001).

3.1.3 Both hypothermia and RBM3 prevent ER stress-induced cell death

ER stress is widely involved in both acute and neurodegenerative brain disorders. Unfolded protein response is activated upon ER stress and attempts to rescue cells by halting protein translation at early stage, but prolonged ER stress can lead to cell death eventually (Yang W and Paschen W. 2009; Xin Q et al., 2014; Halliday M and Mallucci GR. 2014). Therefore, we examined whether hypothermia and RBM3 can rescue the neural cells upon sustained ER stress by challenging organotypic hippocampal slice cultures with 0.3 µM ER stress inducer thapsigargin (Tg) for 24 h.

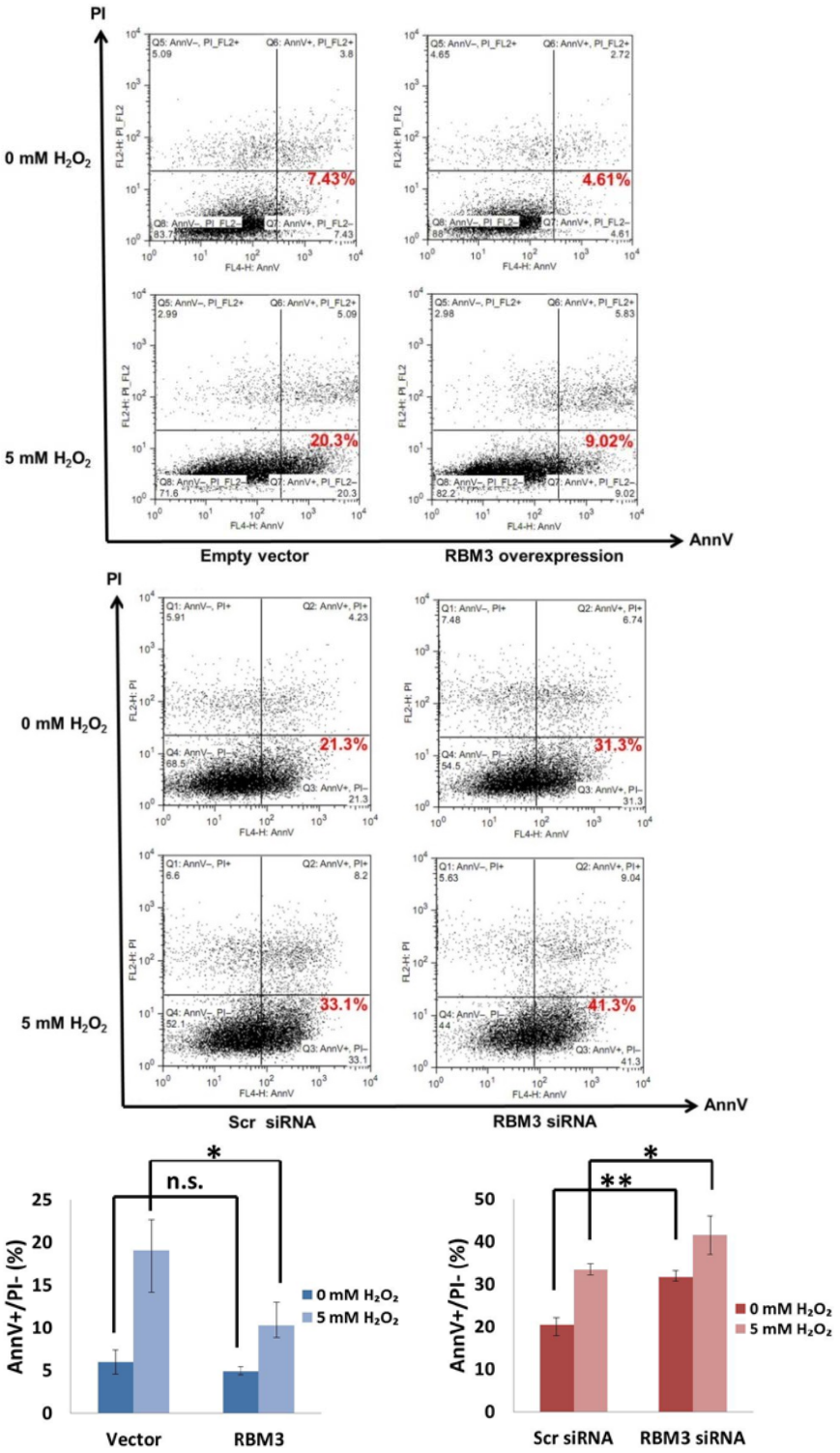

Figure 3.7 RBM3 prevents apoptosis in HEK293 cells. The same batch of cells from Figure 3.6 was analyzed for apoptotic proportion by FACS. Annexin V positive (AnnV+) and propidium idodide negative (PI-) cells were considered as apoptotic cells at early or middle stages. One representative FACS result from three independent experiments was shown (upper and middle panels). Average values from triplicates were used for statistical analysis (lower panel, left for RBM3 overexpression and right for silencing. n.s.=not significant, *P<0.05, **P<0.01).

After 24 h treatment with Tg, necrotic and late apoptotic cell number was significantly higher than non-treated samples as analyzed by propidium iodide (PI) staining (Figure 3.8). Instead, cell death was alleviated in hypothermic group when compared to the normothermic group (Figure 3.8). Accordingly, PI staining revealed more severe cell death in RBM3 KO mice under sustained ER stress at normothermia compared with WT mice (Figure 3.8). However, cell death did not obviously differ between RBM3 WT and KO samples within hypothermic group (Figure 3.8). This indicates that RBM3 may not be the sole factor mediating hypothermic effect on neuroprotection. Other compensating pathways may be activated when RBM3 is depleted.

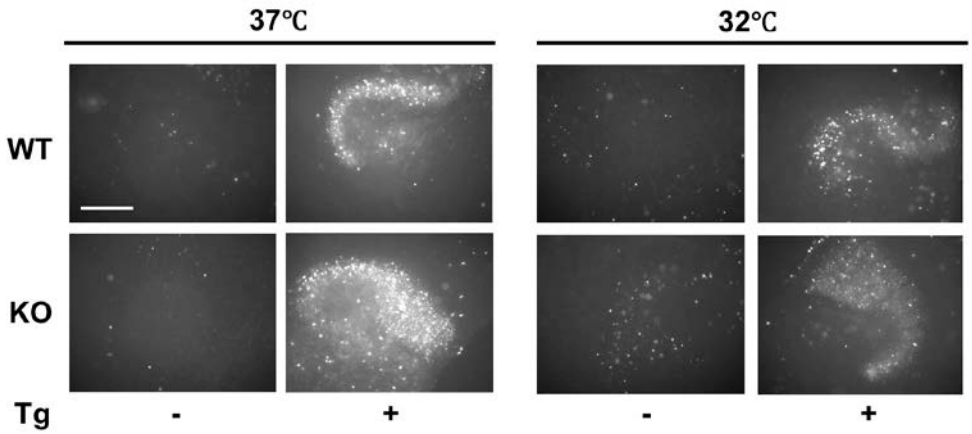

Figure 3.8 Both moderate hypothermia and RBM3 prevent neural cell death from ER stress-induced cell death. Organotypic hippocampal slice cultures were prepared from RBM3 WT or KO mice and challenged with or without 0.3 μM thapsigargin (Tg) for 24 h at 37°C or 32°C, and then subjected to PI staining. Five independent experiments were performed. Representative results are shown. Scale bar: 500 μm.

3.2 Hypothermia and RBM3 suppress PERK-eIF2α-CHOP signaling pathway under sustained ER stress

3.2.1 Hypothermia and RBM3 inhibit PERK-eIF2α-CHOP pathway

Since moderate hypothermia and RBM3 were proved to attenuate ER stress-induced cell death in 3.1.3, we attempted to find out the underlying mechanisms. Among the three main signaling pathways of unfolded protein response, PERK-eIF2α-CHOP pathway is the dominant one contributing to cell apoptosis provoked by prolonged ER stress signals (Lin JH et al., 2007). In order to examine whether hypothermia and RBM3 affect the key components of PERK-eIF2α-CHOP pathway, we performed Western blot and real-time RT-PCR at different time points in thapsigargin-stressed organotypic hippocampal slice culture as described in Figure 3.9.

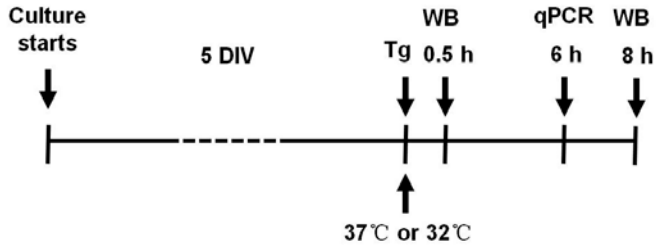

Figure 3.9 Schematic experimental procedure of organotypic hippocampal slice culture from post-natal day 3 C57BL/6J mice (RBM3 WT or KO). Slices were collected 0.5 h after thapsigargin treatment for phos-eIF2α and total-eIF2α protein detection, 6 h for *CHOP* mRNA quantification, and 8 h for CHOP and β-actin protein detection. DIV, days *in vitro*; Tg, thapsigargin; WB, Western blot; qPCR, quantitative real-time RT-PCR.

When organotypic hippocampal slices were stressed with thapsigargin, the increase of phos-eIF2α, *CHOP* mRNA and CHOP protein levels was prevented in hypothermic group (Figure 3.10). Interestingly, in the controls without thapsigargin, hypothermia exposure increased phos-eIF2α as compared to normothermia, whereas CHOP

expression remained at low levels (Figure 3.10). Next, we examined ER stress response by comparing RBM3 KO and WT mice. In thapsigargin-treated hippocampal slice cultures eIF2α phosphorylation and *CHOP* mRNA as well as protein expression were elevated in RBM3 KO mice, indicating exuberant ER stress signaling via the PERK-eIF2α-CHOP pathway in the absence of RBM3 (Figure 3.10). Under hypothermic conditions we noted no differences in ER stress response between RBM3 KO and WT mice (Figure 3.10), consistent with the result in Figure 3.8 and supporting the involvement of compensating mechanisms.

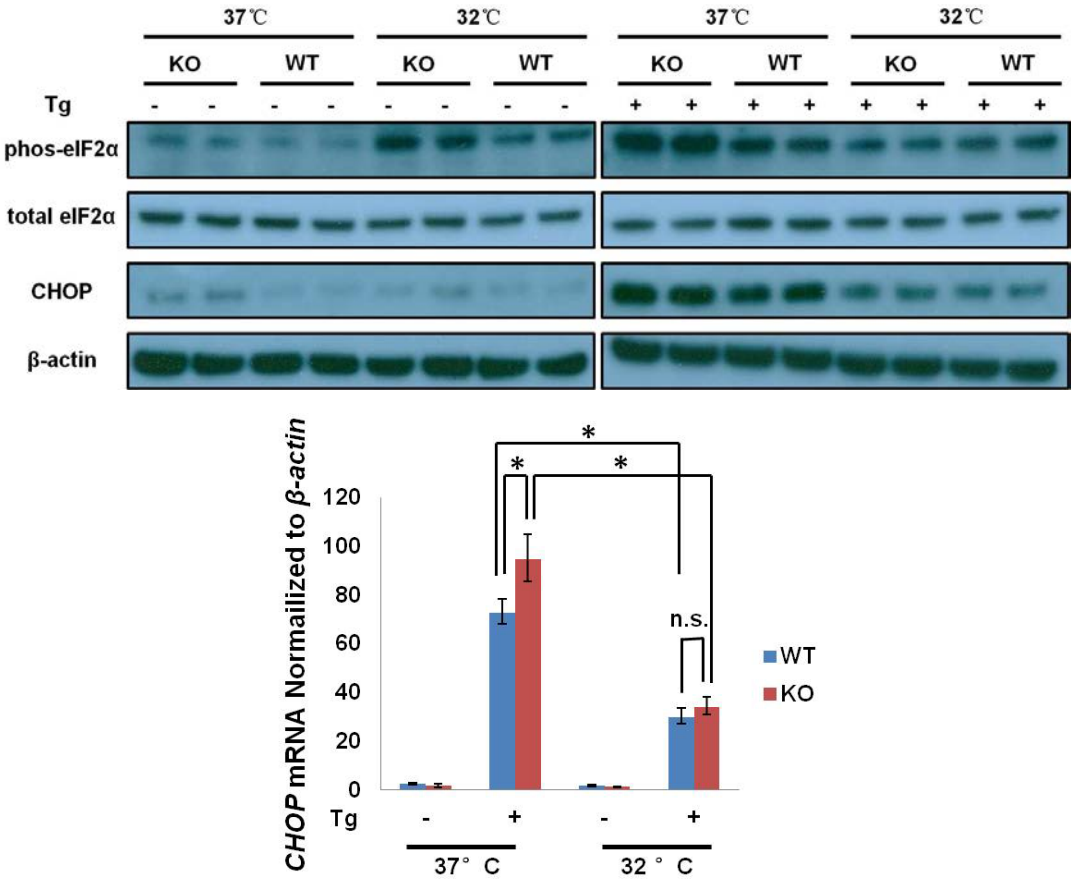

Figure 3.10 Both hypothermia and RBM3 suppress PERK-eIF2α-CHOP pathway in neural cells. Organotypic hippocampal slice cultures were prepared from RBM3 WT or KO mice and challenged with or without 0.3 μM Tg at 37°C or 32°C in five independent experiments. Slices were homogenated to detect phosphorylated eIF2α, total eIF2α, CHOP, and β-actin (upper panel, representative Western blot with samples from two independent experiments is presented), and to analyze *CHOP* mRNA analysis in triplicates (lower panel, *P<0.05, n.s. =not significant).

To investigate the effect of moderate hypothermia and RBM3 on the PERK-eIF2α-CHOP ER stress signaling pathway *in vitro*, we treated HEK293 cells with increasing concentrations of thapsigargin (0, 0.3 and 3 μM) and incubated them under either normothermic or hypothermic conditions. As shown in Figure 3.11, the key components of the PERK-eIF2α-CHOP pathway, including phosphorylated PERK, phosphorylated eIF2α and CHOP protein, were activated in a dose-responsive fashion to thapsigargin treatment and were attenuated by hypothermia (Figure 3.11). Notably, in groups without thapsigargin treatment, eIF2α phosphorylation was higher upon hypothermia (Figure 3.11), which is consistent with the findings in the hippocampal slice culture model (Figure 3.10). Simultaneously, RBM3 expression was significantly increased under hypothermic conditions (Figure 3.11).

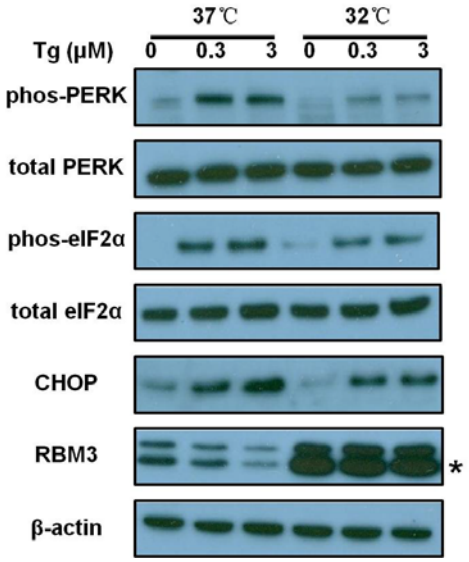

Figure 3.11 Moderate hypothermia inhibits PERK-eIF2α-CHOP pathway in HEK293 cells. HEK293 cells were incubated at 37°C or 32°C for 48 h and treated in the following 24 h with thapsigargin (Tg) at indicated concentrations without changing temperature. Cells were subjected to Western blot with indicated primary antibodies. The specific band of RBM3 is labeled with an asterisk.

Since compensating mechanisms during moderate hypothermia may interfere with the revealing of RBM3 function (Figure 3.8 and Figure 3.10), we aimed to study exclusively on RBM3 instead of general hypothermia. To focus on the effect of RBM3,

we overexpressed human RBM3 in HEK293 cells using the Flp-In 293 T-Rex system. When recombinant RBM3 was expressed upon addition of 4 ng/ml doxycycline, endogenous RBM3 expression was not altered (Figure 3.12). Whereas thapsigargin treatment strongly increased PERK and eIF2α phosphorylation in Flp-In 293 T-Rex cells compared to the negative control, PERK and eIF2α phosphorylation were inhibited in cells expressing recombinant RBM3 (Figure 3.12), indicating that RBM3 has an inhibitory effect on the PERK-eIF2α-CHOP signaling pathway.

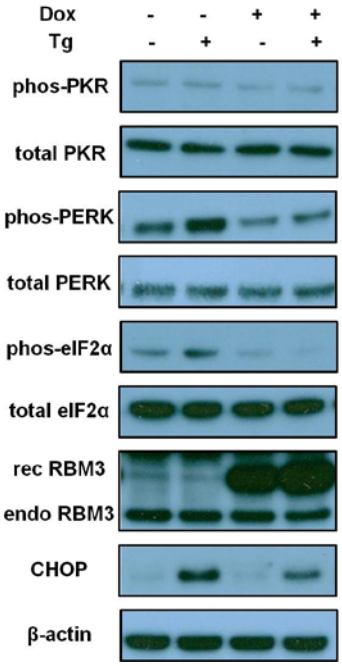

Figure 3.12 Overexpressed RBM3 inhibits PERK-eIF2α-CHOP pathway in HEK293 cells. RBM3 expression was induced by 4 ng/mL doxycycline (Dox) in Flp-In T-Rex 293 cells and subsequently challenged with 0.3 µM Tg for 6 h or 24 h. Incubation with Tg was terminated after 6 h for phos-PKR, total PKR, phos-PERK and total PERK; 24 h for phos-eIF2α, total-eIF2α, CHOP, RBM3 and β-actin. A representative Western blot is shown, primary antibodies are indicated, protein bands corresponding to endogenous (endo, 17 kDa) and recombinant (rec, 25 kDa) RBM3. Of note, in the Flp-In T-Rex 293 cell system RBM3 is fused with a strep tag resulting in a higher molecule weight.

To determine the effect of reduced RBM3 expression, RBM3 knock-down was performed using specific siRNAs. Two siRNA oligos specifically targeting RBM3 were transfected into HEK293 cells, and both exhibited efficient silencing of RBM3 expression (Figure 3.13). In cells treated with RBM3 siRNA oligos, PERK phosphorylation was enhanced upon ER stress induction by thapsigargin in contrast to mock-treated cells (Figure 3.13). Accordingly, eIF2α phosphorylation and CHOP expression were sharply elevated upon RBM3 silencing (Figure 3.13). Although PERK, the PKR-like ER kinase, exhibits high similarity with PKR in the kinase domain and shares similar function with PKR to phosphorylate eIF2α (Yan W et al., 2002), RBM3 overexpression or silencing did not affect PKR phosphorylation with or without thapsigargin treatment (Figure 3.12 and Figure 3.13, respectively).

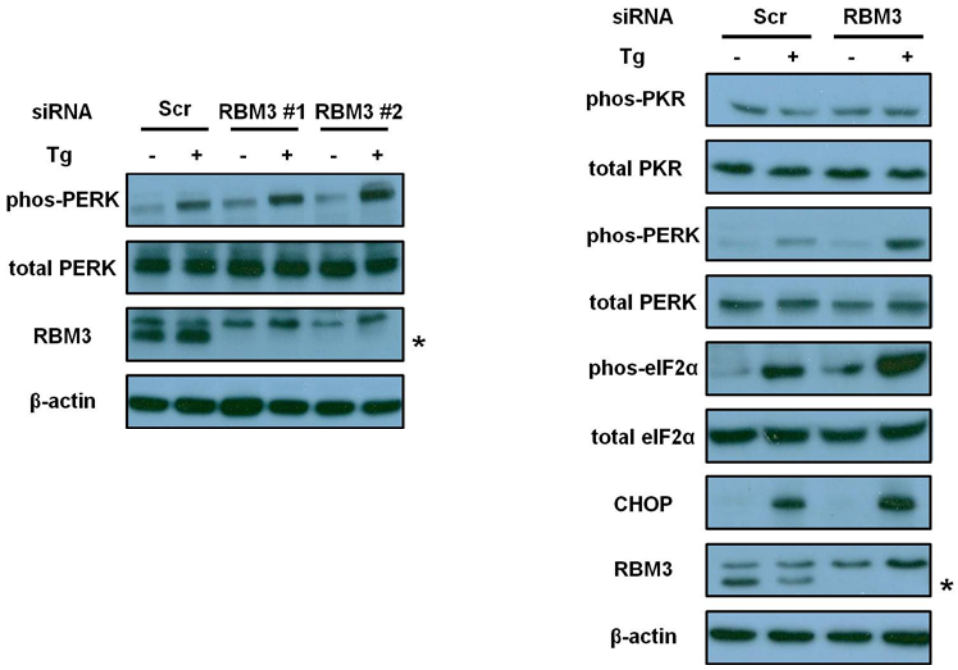

Figure 3.13 Reduced RBM3 level exacerbates PERK-eIF2α-CHOP signaling in HEK293 cells. RBM3 expression was silenced by transfection of two different specific siRNAs (RBM3#1 and RBM3#2) in HEK293 cells as compared to scrambled (Scr) siRNA for 48 h and then stressed with 3 μM Tg for 6 h (left panel). Cells transfected with Scr or RBM3#2, followed by 3 μM Tg treatment were analyzed by Western blot. Incubation with Tg was terminated after 6 h for phos-PKR, total PKR, phos-PERK and total PERK; 24 h for phos-eIF2α, total-eIF2α, CHOP, RBM3 and β-actin (right panel). Asterisks indicate specific RBM3 band.

To exclude drug-specific effects, we used an additional ER stress inducer tunicamycin and compared its effect with thapsigargin. Mechanistically, tunicamycin elicits ER stress by blocking N-linked glycosylation while thapsigargin inhibits ER Ca^{2+} pump (Lin JH et al., 2007). Similar results of the activated PERK-eIF2α-CHOP signaling were found in tunicamycin-treated cells (Figure 3.14). Whereas the pathway activation occurred faster after treatment with thapsigargin as compared to tunicamycin at given concentration (Figure 3.14), cell viability was comparable for both compounds (Figure 3.15).

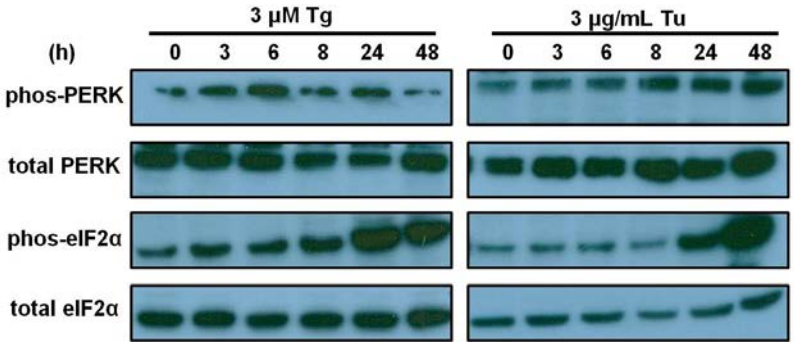

Figure 3.14 The ER stress inducers thapsigargin (Tg, left panel) and tunicamycin (Tu, right panel) increase both the level of phos-PERK and phos-eIF2α in HEK293 cells. Western blot was performed with protein samples from HEK293 cells treated with 3 µM Tg or 3 µg/mL Tu for 0, 3, 6, 8, 24, and 48 hours (h) as indicated, primary antibodies are indicated.

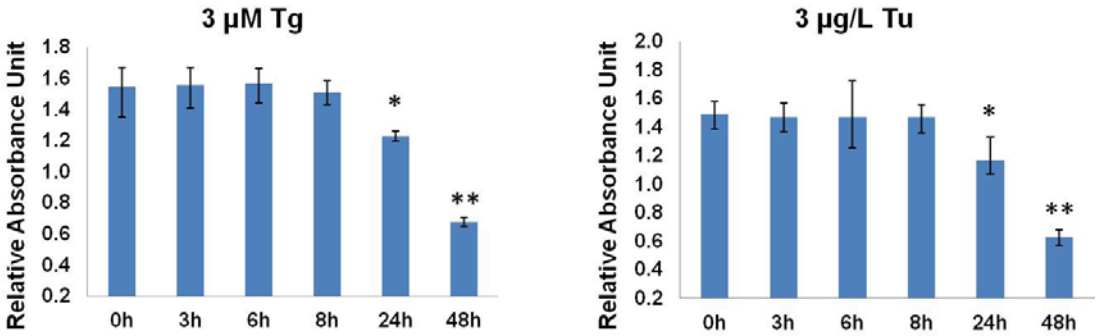

Figure 3.15 Cell viability with samples from Figure 3.14 was determined by cell proliferation assay. HEK293 cells were treated with thapsigargin (Tg, left panel) or tunicamycin (Tu, right panel) at given concentrations for 24 h. Data from three independent experiments was analyzed (*P<0.1, **P<0.01).

Similar effects were observed with RBM3 silencing in tunicamycin treated cells as observed upon thapsigargin treatment. RBM3 knock-down led to an increase of PERK and eIF2α phosphorylation, indicating that the effect was related to ER stress in general (Figure 3.16).

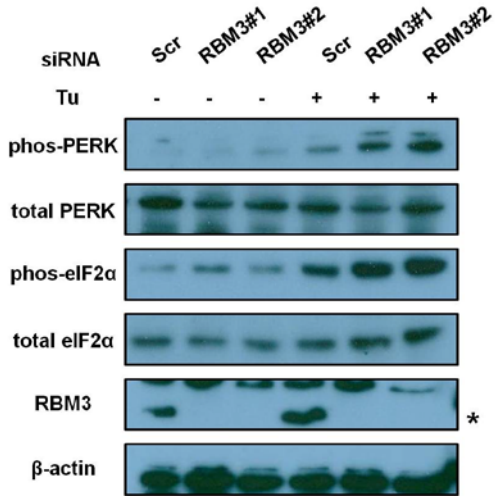

Figure 3.16 Silenced RBM3 expression also enhances PERK-eIF2α-CHOP signaling upon tunicamycin-induced ER stress. HEK293 cells were transfected with two RBM3 siRNAs (RBM3#1 and RBM3#2) or scrambled (Scr) siRNA as used in Figure 3.13, and then stressed with 3 μg/mL tunicamycin (Tu) for 48 h. Cell lysates were subjected to Western blot, primary antibodies are indicated. Asterisk indicates specific RBM3 band.

3.2.2 Screening of RBM3 interacting partners

Given the observation that RBM3 influences the PERK-eIF2α-CHOP signaling pathway at its most upstream point, we were interested in understanding how PERK activity is regulated by RBM3. First, we tested the hypothesis that RBM3 binds to PERK and thereby regulates its phosphorylation. In co-immunoprecipitation (CoIP) experiments, we used HuR and Bip as positive controls. HuR is known to interact with RBM3 (Sureban SM et al., 2008), and Bip binds to PERK in physiological condition but dissociates from PERK under ER stress (Bertolotti A et al., 2000). However, we failed to demonstrate a direct interaction between RBM3 and PERK by either CoIP or reverse CoIP, although the positive controls were successful (Figure 3.17). This may

imply that, either the interaction between RBM3 and PERK is transient or too weak to be captured by this technique, or they do not interact with each other.

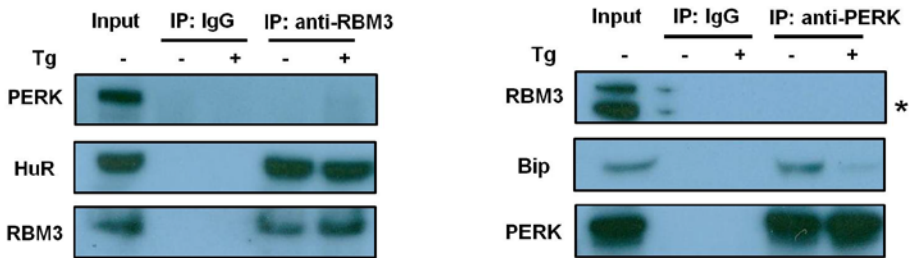

Figure 3.17 RBM3 does not show direct binding to PERK. HEK293 cells were treated with or without 3 µM Tg for 6 h, then lysed gently, and lysates were subjected to RBM3 or PERK antibody coupled magnetic beads. Immunoglobulin G (IgG) was used as negative control. Representative Western blots for PERK and RBM3 are given. HuR and Bip were used as positive controls for RBM3 and PERK interactors, respectively.

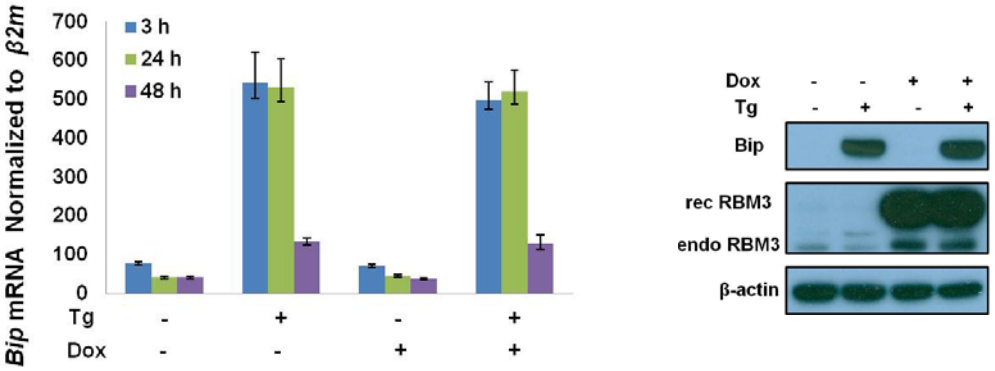

Figure 3.18 Overexpression of RBM3 does not regulate Bip expression. RBM3 overexpressing Flp-In T-Rex 293 (4 ng/mL doxycycline, Dox) or control cells (no Dox) were treated without or with 0.3 µM Tg for 3, 24, and 48 h. *Bip* transcripts were measured by real-time RT-PCR. Data from three independent experiments show no statistical significant differences comparing results from doxycycline treated and untreated cells when stressed or unstressed. Representative Western blot for Bip and β-actin is given. rec, recombinant; endo, endogenous.

Second, we examined whether RBM3 regulates the key folding sensor BiP, which binds to and inactivates PERK in unstressed conditions (Bertolotti A et al., 2000). Bip expression was dramatically elevated upon ER stress, but neither the overexpression nor the silencing of RBM3 affected *Bip* mRNA or its protein expression (Figure 3.18 and Figure 3.19).

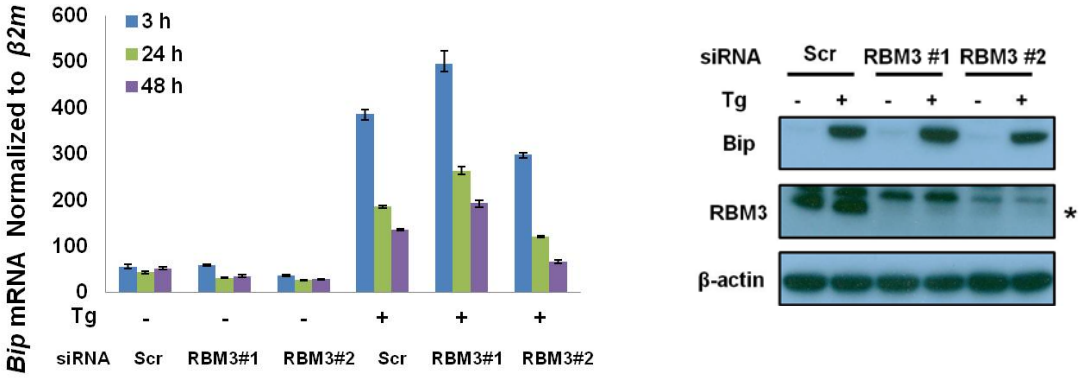

Figure 3.19 Absence of RBM3 does not affect Bip expression. HEK293 cells transfected with RBM3 specific siRNAs (RBM3#1 and RBM3#2) or scrambled (Scr) siRNA were treated without or with 3 μM Tg for 3, 24, and 48 h. Real-time RT-PCR was performed to examine *Bip* mRNA expression. No statistical significant differences were noted when comparing data from three independent experiments as given between respective RBM3 and Scr siRNA groups. Representative Western blot for Bip and β-actin is shown.

Therefore, we suspected the involvement of additional factors in the regulation of RBM3 and PERK interaction. To test this possibility, we screened for putative RBM3-binding proteins. Recombinant human RBM3 fused to streptavidin tag was overexpressed in HEK293 cells, and baits from 4 independent experiments were purified via affinity tags and digested into peptides. The peptides were identified by mass spectrometry (Figure 3.20). Sixtyfive candidate RBM3-binding proteins were identified, over 50% of them with functions in transcription and translation (Figure 3.21 and Appendix II). Among these proteins, two members of the nuclear factors

associated with dsRNA (NFAR) family, namely NF45 and NF90, exhibited the highest screening scores in all independent experiments performed and were consequently chosen for further study.

NF45 and NF90/NF110 (NF110 is an NF90 long isoform) form a heterodimer and are involved in a variety of events, including DNA break repair, transcriptional and post-transcriptional regulation (Parker LM et al., 2001). In further, NF90 binds to PKR and regulates PKR phosphorylation (Parker LM et al., 2001). Of interest, similar to PERK, PKR also phosphorylates eIF2α in response to distinct types of stress (Donnelly N et al., 2013), although its activity is not regulated by RBM3 (Figure 3.12 and Figure 3.13).

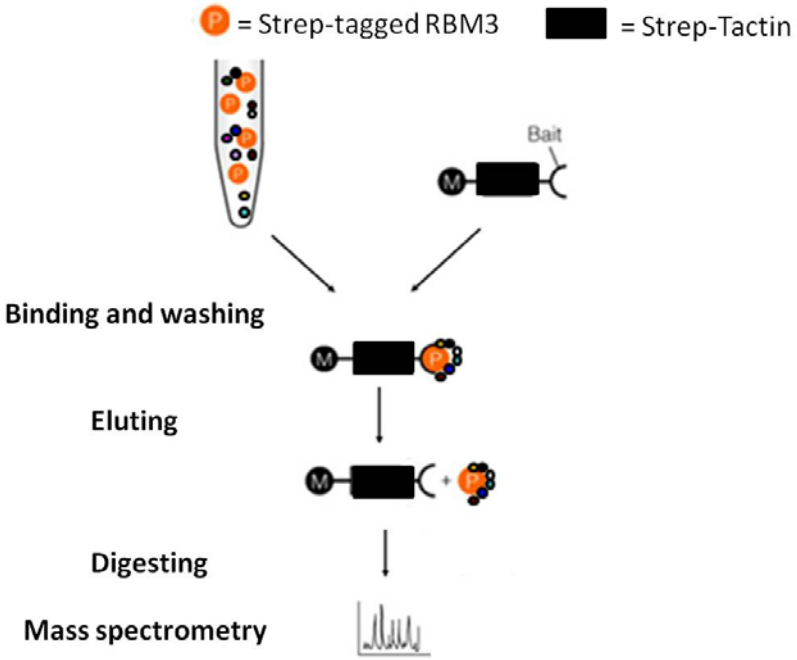

Figure 3.20 The flowchart of screening RBM3-interacting proteins. Strep-tagged RBM3 overexpression was induced by doxycycline in Flp-In T-Rex 293 cells. Cell lysates were subjected to Strep-Tactin beads and washed with phosphate-buffered saline (PBS). Eluted RBM3 and its interactors were digested into peptides and identified by mass spectrometry. (The flowchat was modified from Promega website "Protein Purification and Analysis" Figure 11.16.
https://ch.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/protein-purification-and-analysis/)

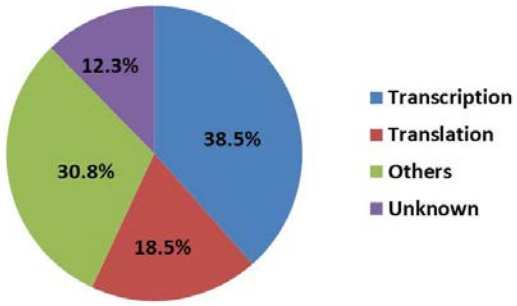

Figure 3.21 Classification of candidate RBM3-interacting proteins according their function. The complete list with detailed screening results and protein names is provided in Appendix II.

Interaction of RBM3 with NF45 or NF90 in HEK293 cells was confirmed using CoIP (Figure 3.22). Pretreatment of cell lysates with RNase T1 indicated that the interaction between RBM3 and NF45 or NF90 is mediated by RNA (Figure 3.22), whereas NF45 binds to NF90 in an RNA-independent manner (Figure 3.23) as published elsewhere (Guan D et al., 2008).

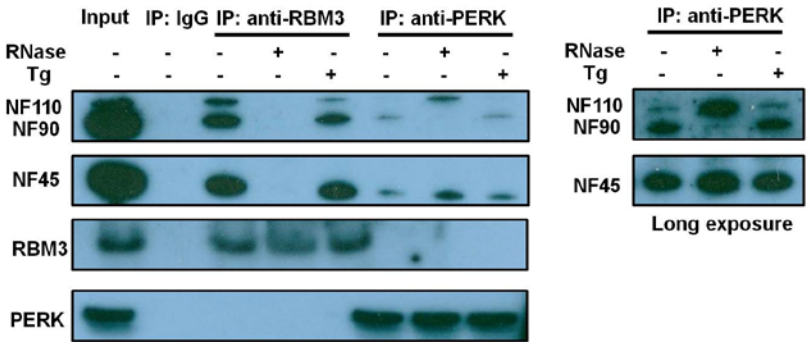

Figure 3.22 RBM3 shows an interaction with NF45 and NF90 in a RNA dependent manner, whereas NF90 and NF45 bind to PERK independently of RNA mediation. A similar procedure for CoIP was performed as described in Figure 3.17 with additional RNase T1 pre-treatment as indicated, and representative Western blots for NF110/NF90, NF45, and PERK are given (left panel). Samples precipitated with PERK antibody and detected with NF90 or NF45 antibodies were exposed longer for a better resolution (right panel).

Results from CoIP unambiguously supported the notion that NF90 directly binds to PERK, as the interactions were generally unaffected by RNase treatment (Figure 3.22 and Figure 3.23). However, a shift from NF90 short isoform to NF110 long isoform upon RNase digestion was observed in NF90-PERK interaction but not in NF45-PERK interaction (Figure 3.22 and Figure 3.23). This probably indicates a different but not distinct role of NF110 compared to NF90, and/or the involvement of some RNA species in the context of NF90-PERK interaction. Moreover, we noted also the NF45-PERK interaction (Figure 3.22) did not appear in reverse CoIP (Figure 3.23), indicating a weaker or less indirect interaction as compared to NF90-PERK interaction.

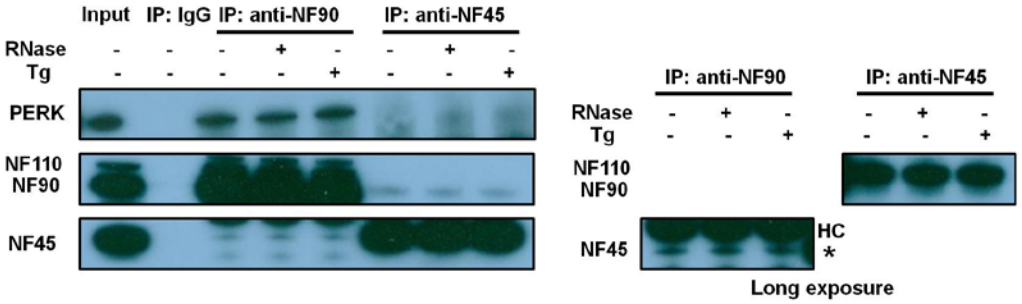

Figure 3.23 NF90 interacts with PERK and NF45 independently of RNA in reverse CoIP. A similar procedure for CoIP was performed as in Figure 3.17 with additional RNase T1 pre-treatment as indicated. Representative Western blots for PERK, NF110/NF90 and NF45 are given. The interaction of NF90 and PERK was confirmed by reverse CoIP. NF45-PERK interaction was not observed in reverse CoIP (left panel). Samples precipitated with NF90 or NF45 antibodies and detected with NF45 or NF90 antibodies respectively were exposed longer for a better resolution to show NF45-NF90 interaction (right panel). Asterisk indicates NF45. HC: antibody heavy chain.

Comparing the expression of NF45, NF90/ NF110 as well as the CoIP products, there was no difference noted between normothermia and hypothermia (Figure 3.24). We suggested that, only a small proportion of the proteins are involved in the interactions and exert related functions.

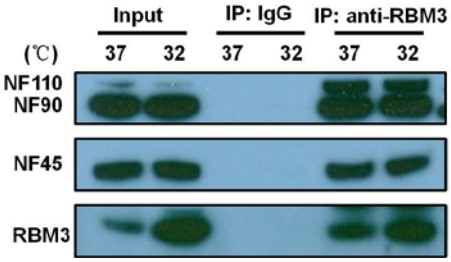

Figure 3.24 Hypothermia does not change the interactions between RBM3 and NF45/NF90. A similar CoIP procedure was performed as described in Figure 3.17 with cells cultured at 37°C or 32°C for 24 h. Western blots for RBM3, NF45 and NF90 are given.

In parallel, we studied the distribution patterns of RBM3, NF90, NF45 and PERK *in situ* in HEK293 cells. Immunostaining revealed that RBM3, NF90 and NF45 localize mainly in nucleus in both thapsigargin untreated or treated samples (Figure 3.25 and Figure 3.26). Whereas the ER membrane-bound protein, PERK, was mainly cytoplasmic localized on ER membranes (Figure 3.25 and Figure 3.26).

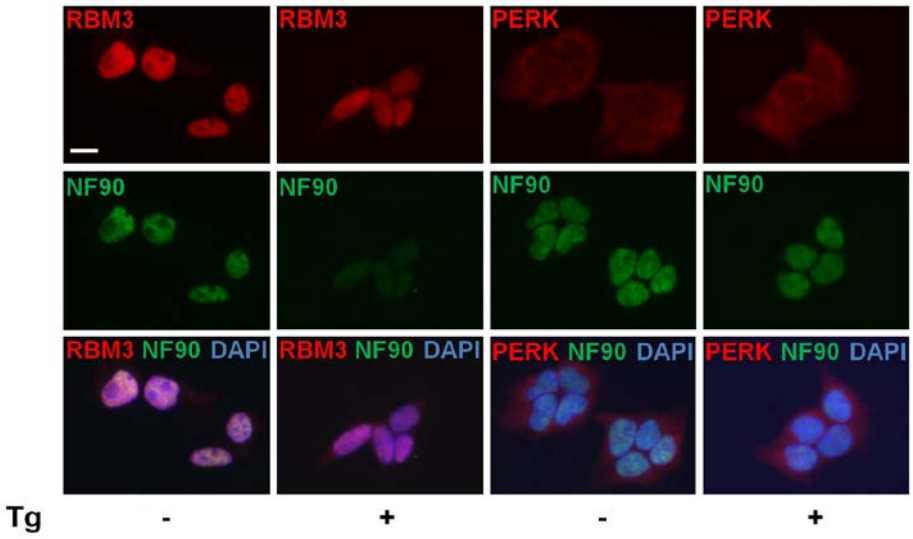

Figure 3.25 Subcellular localization of NF90, PERK, and RBM3. HEK293 cells were cultured on glass surface for 48 h, challenged with 3 µM Tg for 6 h, and stained with antibodies against RBM3, NF90 and PERK. The nuclei were stained with DAPI. Scale bar: 10 µm.

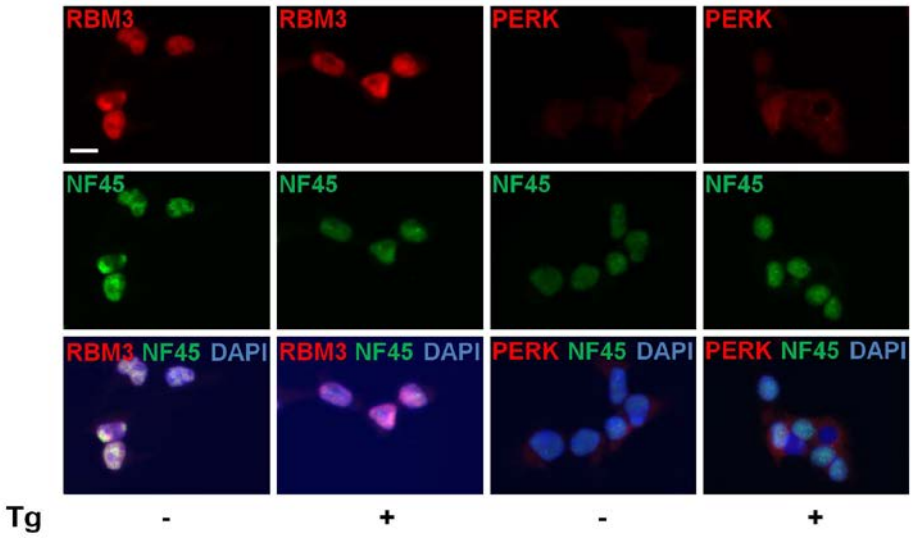

Figure 3.26 Subcellular localization of NF45, PERK, and RBM3. The same procedure was executed as Figure 3.25. NF45 was stained instead of NF90. The nuclei were stained with DAPI. Scale bar: 10 µm.

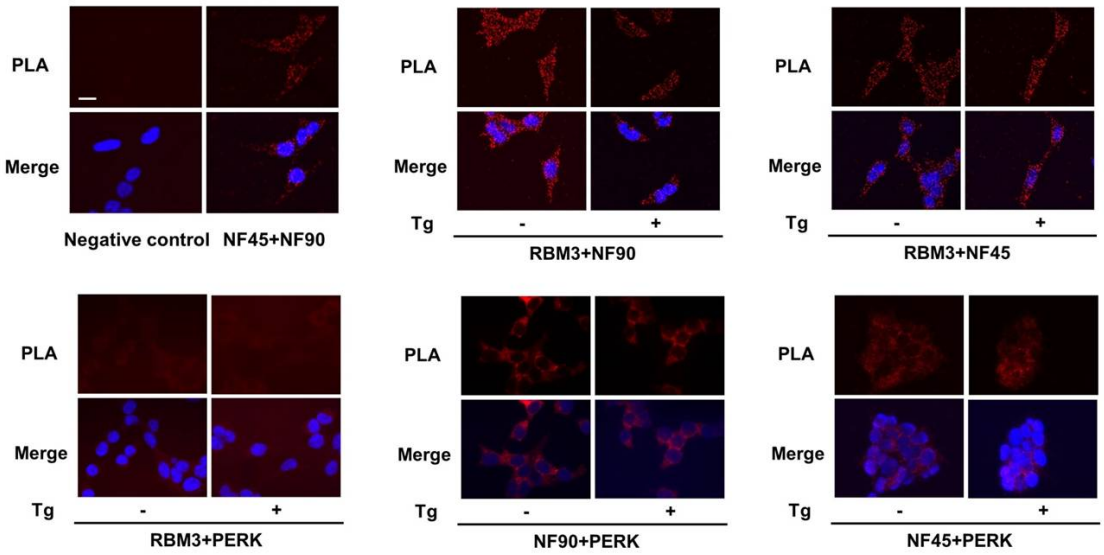

Figure 3.27 Proximity ligation assay (PLA) was performed in HEK293 cells with or without ER stress induction as described in Figure 3.26. Antibodies used for control samples without ER stress: RBM3 only (negative control), NF45 and NF90 (positive control). The following interactions *in situ* were studied, RBM3 and PERK, RBM3 and NF90, RBM3 and NF45, NF90 and PERK, and NF45 and PERK. DAPI stained nuclei in blue. Scale bar in all panels: 10 µm.

Proximity ligation assay (PLA) is a method demonstrating protein interactions in situ. With the method of PLA, we confirmed that RBM3 is located in proximity to both NF45 and NF90, co-localizing in both the nucleus and cytoplasm (Figure 3.27). The PERK and NF90 interaction occurred in a non-nuclear pattern (Figure 3.27). A similar interaction pattern was observed for NF45 and PERK (Figure 3.27). Combining with the immunostaining data, the results support the idea that only a small pool of proteins are involved in these interactions and mainly in the cytoplasm, in accordance with that observed in CoIP experiments.

3.2.3 NF90 is required for RBM3-mediated PERK inhibition

Having demonstrated the direct physical interactions of PERK with RBM3 and NF90, we next investigated whether these interactions are of functional importance. For this purpose, NF90 gene expression was silenced using two different siRNAs in HEK293 cells treated for 24 h with thapsigargin. RBM3 siRNA was used as a positive control, and scrambled siRNA served as a negative control. PERK phosphorylation was reduced with both NF90-specific siRNAs and was increased upon RBM3 knock-down (Figure 3.28).

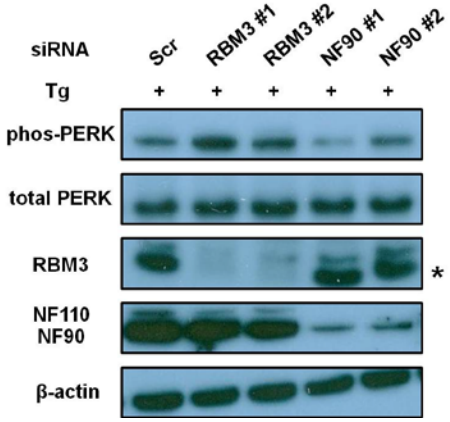

Figure 3.28 PERK phosphorylation was blunted in HEK293 cells exposed to 3 μM Tg for 24 h when transfected with NF90 siRNAs (NF90#1 or NF90#2), and enhanced when transfected with RBM3 siRNAs (RBM3#1 or RBM3#2) as compared to control scrambled siRNA (Scr). A representative Western blot for phos-PERK, total PERK, NF90, RBM3, and β-actin is shown. RBM3 is indicated with asterisk.

Furthermore, co-transfection of RBM3 and NF90 siRNAs followed by ER stress induction with thapsigargin led to a remarkable reduction of phos-PERK and phos-eIF2α compared to the negative control, very similar to samples treated with a combination of RBM3 siRNA and a specific PERK inhibitor (Figure 3.29).

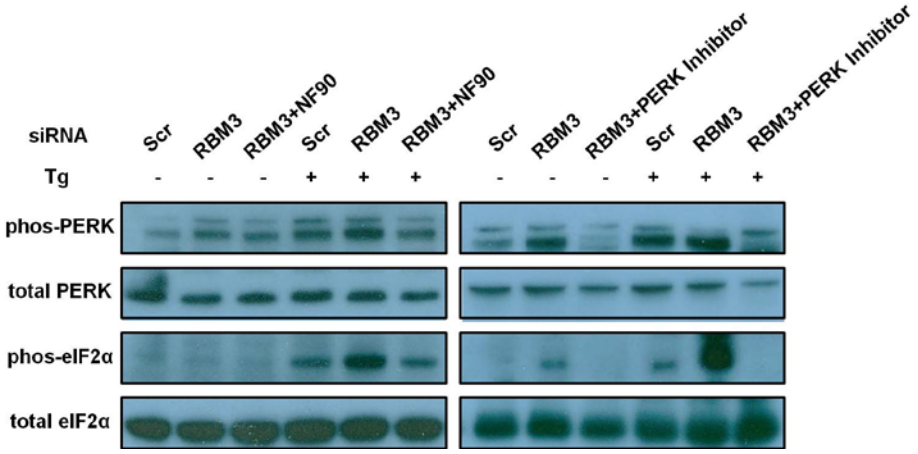

Figure 3.29 NF90 is indispensable for RBM3 mediated PERK activity. HEK293 cells were transfected with RBM3 and NF90 specific siRNAs in either separate or combined settings and then treated with or without Tg (3 μM for 24 h) as indicated. Cells treated with siRBM3 combined with PERK inhibitor prior to Tg were used as controls. Phos-PERK, total PERK, phos-eIF2α, and total eIF2α were analyzed by Western blot.

Finally, we checked if the protein interactions are involved in the NF90-mediated activity as discovered above. Whereas RBM3 knock-down alone did not influence the interaction between PERK and NF45/NF90, a double knock-down of RBM3 and NF90 led to a significant decrease of PERK and NF45/NF90 interaction (Figure 3.30). Surprisingly, even a small proportion of RBM3 is sufficient to maintain RBM3-NF90 and RBM3-NF45 interactions, consisting with the hypothesis that only the small amount of RBM3 in cytoplasm but not the major nucleic RBM3 is responsible for the interactions and functions in regulating ER stress response, as discussed above. Taken together, these data support the notion that NF90 alone regulates PERK phosphorylation, and is necessary for mediating RBM3 function on PERK activation.

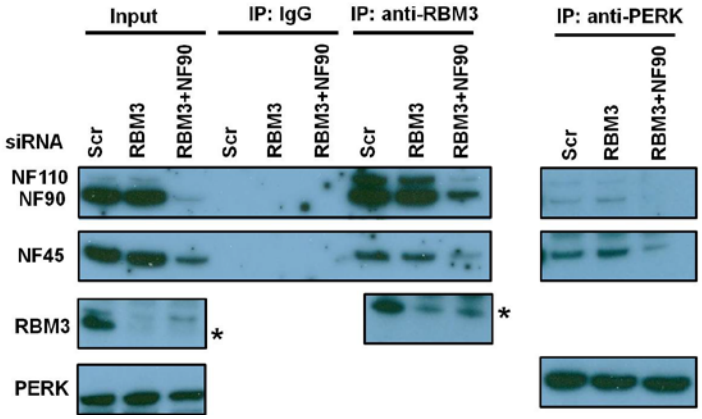

Figure 3.30 PERK and NF45/NF90 interaction in the absence of RBM3 and NF90. HEK293 cells were treated with RBM3 and NF90 siRNA in a separate or combined way as in Figure 3.29. CoIP was performed with RBM3 and PERK antibodies as in Figure 3.17. NF90 and NF45 were detected by Western blot. RBM3 and PERK inputs are also given. Asterisks indicate RBM3 band.

3.3 Hypothermia and RBM3 regulate miRNA expression

3.3.1 Hypothermia reduces the expression of pro-apoptotic miRNA

Several groups of pro-apoptotic and anti-apoptotic miRNAs are conserved in mammals (Garofalo M et al., 2010). As a therapeutic approach, moderate hypothermia can alter miRNA expression upon brain injuries and reduce neural apoptosis (Truettner JS et al., 2011). To investigate if moderate hypothermia modulates pro-apoptotic miRNAs, we selected miR-16 and let-7a, two well-characterized pro-apoptotic miRNAs as putative targets. In HEK293 cells, both expressions of mature miR-16 and let-7a were attenuated by hypothermia treatment, while their respective precursors were accumulated accordingly (Figure 3.31), indicating that hypothermia suppresses the shift from precursor miRNA to mature miRNA at least for miR-16 and let-7a. In addition, we examined *Bcl-2* mRNA, the target for miR-16 (Cimmino A et al., 2005). Moderate hypothermia dramatically elevated the level of *Bcl-2* transcript (Figure 3.32), at least partially due to the decreased level of miR-16. This finding is also consistent the increasing Bcl-2 protein level under hypothermia (Figure 3.4), and supports the anti-apoptotic function of moderate hypothermia.

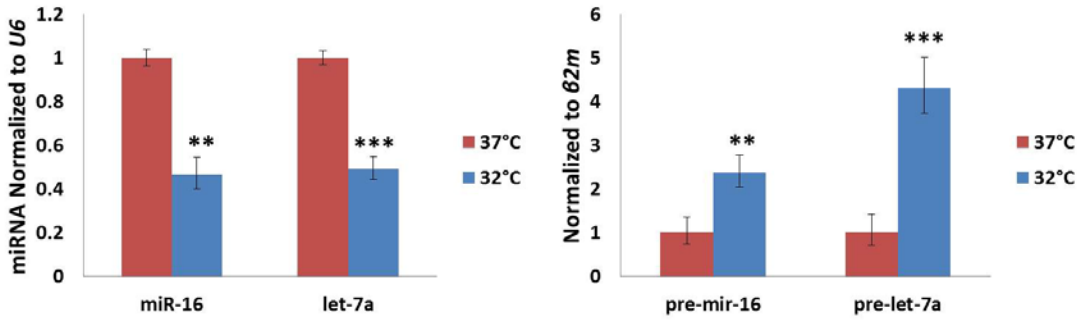

Figure 3.31 Moderate hypothermia inhibits the expression of pro-apoptotic miR-16 and let-7a, and increases their respective precursors. HEK293 cells were cultured at 37°C or 32°C for 24 h and harvested for mature miRNA (left panel) as well as pre-miRNA (right panel) analysis by real-time RT-PCR. Average values from triplicates were used for statistical analysis (**P<0.01, ***P<0.001).

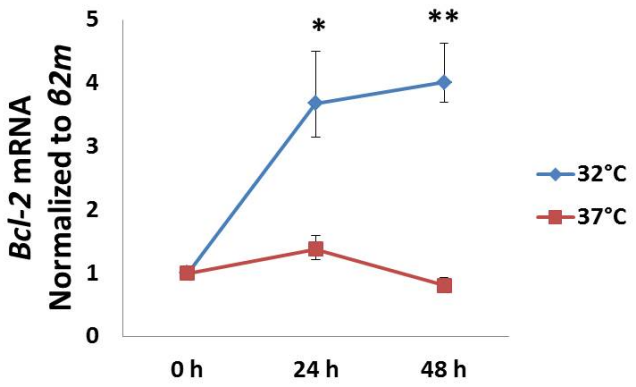

Figure 3.32 Moderate hypothermia elevates *Bcl-2* expression. HEK293 cells were cultured at 37°C or 32°C for 0, 24 and 48 h. Cells were collected for the analysis of *Bcl-2* mRNA by real-time RT-PCR. Three independent experiments were performed (*P<0.05, **P<0.01).

3.3.2 RBM3 negatively regulates pro-apoptotic miRNAs

Previous research has demonstrated that RBM3 positively regulates a large group of miRNAs, while negatively regulates a smaller pool (Pilotte J et al., 2011). To unravel whether RBM3 inhibits pro-apoptotic miRNAs, we used constitutively expressed RBM3 in pCEP4 system in HEK293 cells. Empty vector was used as negative control. We used miR-16 and let-7a as putative targets of RBM3 as that for hypothermia. The overexpression of RBM3 resulted in a reduction of mature miR-16 and let-7a in comparison with the empty vector, and the precursors of miR-16 and let-7a were accumulated (Figure 3.33). On the contrary, downregulated RBM3 level significantly enhanced miR-16 and let-7a level with decreasing precursor miRNAs (Figure 3.34). In further, miR-16 targeted *Bcl-2* transcripts decreased when RBM3 was silenced (Figure 3.35), in accordance with the protein data observed in HEK293 cells (Figure 3.5). These data suggested that RBM3 inhibits the maturation of miR-16 and let-7a from their respective precursors and prevents apoptosis, in accordance to the findings with hypothermia. However, our data is contradict to Pilotte's report, in which miR-16 and let-7a are downregulated in the absence of RBM3 in mouse B-104 cell line

(Pilotte J et al., 2011). As their work is also inconsistent to the fact that RBM3 promotes global translation (Dresios J et al., 2005; Smart F et al., 2007) and anti-apoptotic function (Chip S et al., 2011), we suspect that their inconsistent results derive from cell-specific effects.

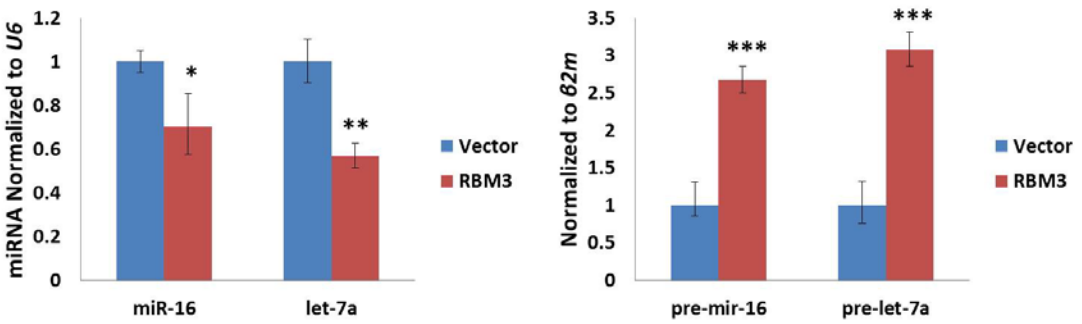

Figure 3.33 Overexpressed RBM3 represses the expression of pro-apoptotic miR-16 and let-7a, and increases their respective precursors. RBM3 was constitutively expressed in pCEP4 system in HEK293 cells. Cells were cultured at 37°C for 24 h, then collected for mature miRNA (left panel) and pre-miRNA (right panel) analysis by real-time RT-PCR in triplicates (*P<0.05, **P<0.01, ***P<0.001).

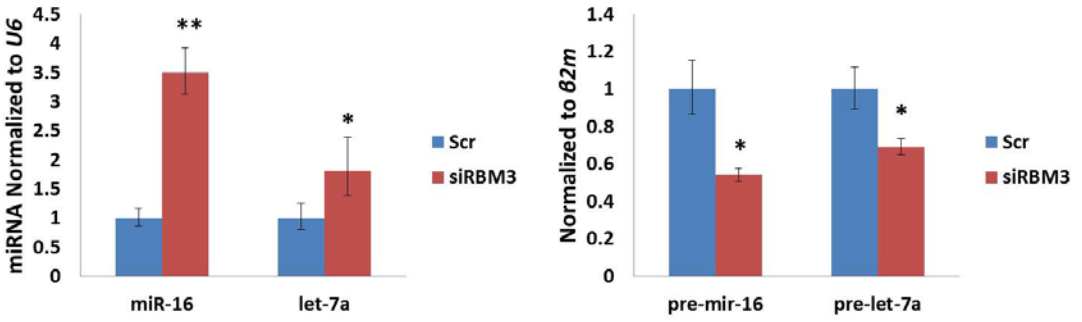

Figure 3.34 Reduced RBM3 level upregulates the expression of pro-apoptotic miR-16 and let-7a, and downregulates their respective precursors. RBM3 was knocked down by specific siRNA (RBM3#2) for 24 h in HEK293 cells. Real-time RT-PCR was taken to detect mature (left panel) and precursor (right panel) miR-16 and let-7a. Three independent experiments were performed for statistic analysis (*P<0.05, **P<0.01).

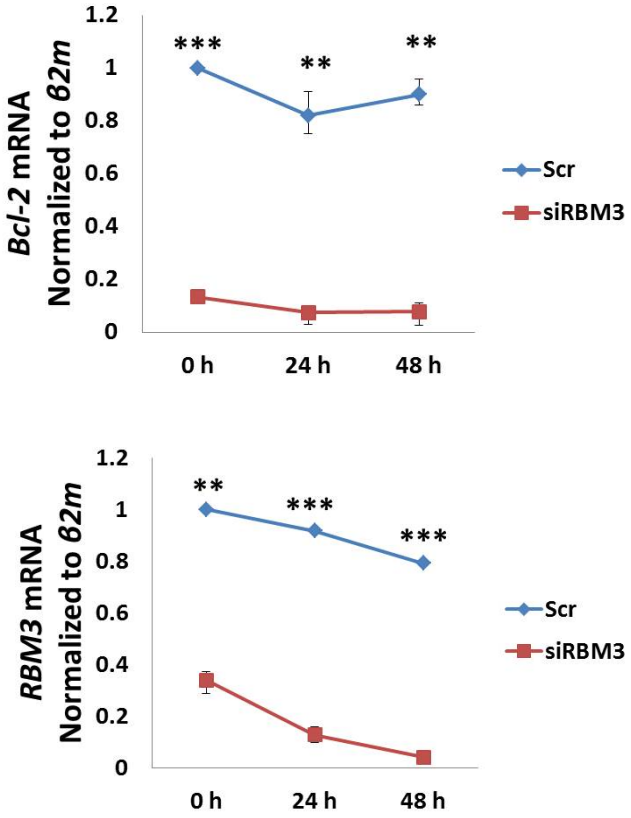

Figure 3.35 The absence of RBM3 attenuates *Bcl-2* expression. RBM3 was downregulated by siRNA (RBM3#2) for 24 h, and then incubated for additional 0, 24 and 48 h. *Bcl-2* (upper) *and RBM3* (lower) mRNAs were analyzed by real-time RT-PCR in triplicates (**P<0.001, ***P<0.01).

3.3.3 RBM3 positively regulates anti-apoptotic miRNAs

Several groups of miRNAs have been described to contribute to UPR-dependent activities (Chitnis N et al., 2013). In previous study, the anti-apoptotic miR-106b-93-25 cluster was found to be under control of PERK and further influence cell destiny (Gupta S et al., 2012). To examine whether the expression of the miR-106b-93-25 cluster is subject to RBM3, we measured the expression of miR-106b and miR-93, as well as miR-25 in RBM3 knock-down HEK293 cells. As expected, both miR-106b and

miR-93 were downregulated by thapsigargin induced ER stress, when PERK was activated (Figure 3.36). Silencing of RBM3 declined miR-106b and miR-93 expression with or without PERK activation (Figure 3.36). Instead, miR-25 remained unchanged rather than a decreasing tendency upon ER stress in HEK293 cells, whereas miR-25 expression dropped when RBM3 was absent in unstressed cells (Figure 3.36). Therefore, we conclude that the lack of RBM3 results in the reduction of the miR-106b-93-25 cluster in general and probably contributes to exacerbated apoptosis. However, the regulation of miR-106b-93-25 by RBM3 may not be related to PERK activation.

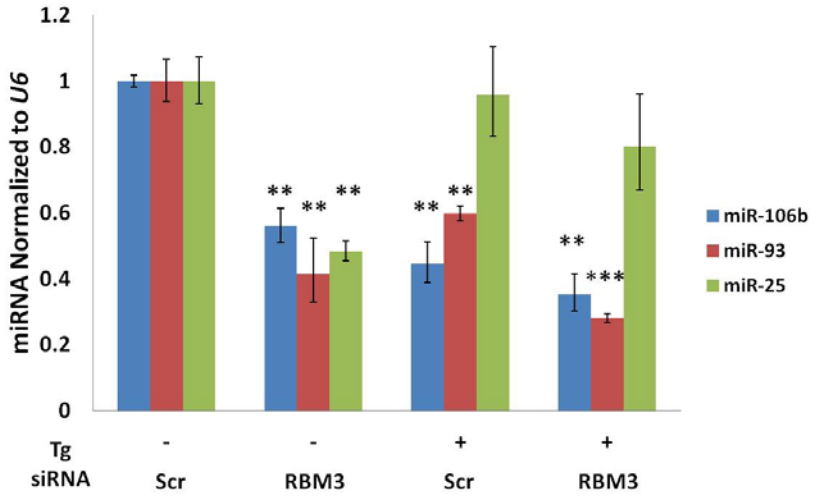

Figure 3.36 RBM3 knock-down leads to the inhibition of anti-apoptotic miR-106b-93-25 cluster. HEK293 cells were transfected with scrambled (Scr) or RBM3 specific siRNA (RBM3#2), and challenged with 3 μM thapsigargin (Tg) for 6 h. Then the cells were harvested for mature miRNA analysis, the expressions of miR-106b, miR-93, and miR-25 were measured from three independent experiments by real-time RT-PCR (**P<0.01, ***P<0.001).

4. Discussion

Former studies displayed a high expression level of RBM3 in neuron precursors and young neurons in the subventricular zone (SVZ) and the rostral migratory stream (RMS), suggesting a pivotal role in neuron stem cell proliferation and brain development (Pilotte J et al., 2009; Chip S et al., 2011). In brain injury and neurodegenerative diseases, neuron stem cell-based therapy has become an important approach to regenerate the damaged or lost neurons and recover their functions (George PM and Steinberg GK, 2015; Barker RA et al., 2015). Hypothermia displays a critical role in protecting neuron stem cells in the region of dentate gyrus (DG) upon ischemic brain injury (Kwak M et al., 2015). In our study, RBM3 was dramatically increased by hypothermia in DG of cultured hippocampal slices (Figure 3.3), in accordance with its activity in proliferation of neuron precursors and immature neurons. In OGD model which mimics hypoxic-ischemia, RBM3 was globally downregulated (Figure 3.3) and may contribute to the failure of neuron regeneration after ischemic injury. The therapeutic hypothermia after OGD treatment can rescue neural cells, probably in part by reactivate RBM3 in DG to some extent, although not fully recovered (Figure 3.3). Altogether, our data support the hypothesis that hypothermia maintains neuron stem cell numbers and functions through elevating RBM3 level in brain disorders.

The provocation of oxidative stress is one of the main characteristics in cerebral ischemia/reperfusion injury and neurodegeneration (Pradeep H et al., 2012; Dasuri K et al., 2013). The overproduction of reactive oxygen species (ROS) results primarily from dysfunctional mitochondria as well as from other organelles or complexes like peroxisomes, disrupting oxidant/anti-oxidant balance in neural cells (Pradeep H et al., 2012; Dasuri K et al., 2013). Oxidative stress activates multiple downstream events including calcium imbalance, excitotoxicity, neuroinflammation, protein modification and degradation, which all lead to neural cell death (Pradeep H et al., 2012; Dasuri K et al., 2013). Our data suggested that the expression of RBM3 prevents oxidative stress-induced apoptosis (Figure 3.6 and Figure 3.7), similar to the effects of its

homologue CIRP as reported by other groups (Liu J et al., 2015; Sakurai T et al., 2015). We assumed there is an overlapping function of these two sequence-conserved cold responsive proteins against oxidative stress.

A hallmark of hypothermia in mammalian cells is the general inhibition of protein synthesis, which is mainly regulated through an increase in phosphorylated eIF2α (Hofmann S et al., 2012). The same protein synthesis brake is activated upon ER stress causing eIF2α phosphorylation through the kinase PERK (Donnelly N et al., 2013). It is important to note that either hypothermia alone or ER stress alone can elevate eIF2α phosphorylation, indicating that moderate hypothermia itself is a cell stressor but does not provoke apoptosis (Rzechorzek NM et al., 2015). However, in the presence of additional and considerably stronger stress factors such as prolonged ER stress, hypothermia is protective. For example, hypothermia attenuates ER stress-induced apoptosis through CHOP in rat models of cerebral ischemia (Liu X et al., 2013; Poone GK et al., 2015). This finding was confirmed by our data, which indicate that moderate hypothermia attenuates PERK and eIF2α phosphorylation and subsequent apoptosis in the presence of sustained ER stress (Figure 3.10 and Figure 3.11). This peculiar overlap attracted our attention and, together with the known facts about RBM3, propelled our research to investigate RBM3 signaling in detail.

RBM3 has been shown to trigger translation, and it has been deduced that RBM3 may prevent a more dramatic reduction of protein synthesis under conditions of hypothermia (Dresios J et al., 2005; Smart F et al., 2007). In our experimental settings, RBM3 was proposed as a PERK and eIF2α inhibitor both in the absence and presence of ER stress (Figure 3.10; Figure 3.12; Figure 3.13 and Figure 3.16), assuming that RBM3 could be exclusively advantageous for cells to overcome stressful conditions without exerting detrimental adverse effects associated with hypothermia.

We found an increased ER stress response in hippocampal organotypic slice cultures from RBM3 KO mice as well as in cell cultures with transient RBM3 knockdown (Figure 3.10 and Figure 3.13). Forced expression of RBM3 in cell cultures, either endogenously by hypothermia or exogenously by recombinant RBM3, blocked the thapsigargin triggered ER stress response (Figure 3.11 and Figure 3.12). Very recently,

RBM3 has been identified as an important mediator of structural plasticity and protective effects in rodent models of neurodegeneration. RBM3 overexpression, achieved either endogenously through hypothermia before the loss of the RBM3 response in advancing neurodegeneration or by lentiviral delivery, resulted in sustained neuroprotection by preventing neuronal loss and restoring synapse reassembly (Peretti et al., 2015). Notably, only RBM3, but not the RBM3 homologue CIRP was significantly induced in their settings (Peretti et al., 2015), implying distinct induction patterns of RBM3 and CIRP *in vivo*. Although the underlying mechanism is unknown, one hypothesis may involve the suppression of eIF2α kinase PERK, which has been shown as a potential therapeutic target in Alzheimer's disease-related deficits of synapse plasticity (Ma T et al., 2013) and has been linked by our findings to RBM3. Based on our findings of increased ER stress response in RBM3 KO mice it might be speculated that RBM3 KO mice are more susceptible to neurodegeneration. In our organotypic hippocampal slice culture model of ER stress the lack of RBM3 did not impair the cytoprotective effect of hypothermia (Figure 3.10), posing the question if other cold response mechanisms may counteract the lack of RBM3, such as CIRP. In fact, the potential functional overlap of RBM3 and CIRP demands further experiments in RBM3 KO mice for example by applying hypothermia after stroke and by cross-breading RBM3 with CIRP KO mice. Additionally, even though deep hypothermia was used in experimental rodents to mimic hibernation (Peretti D et al., 2015), moderate cooling temperature is considered to be safer and more favorable for human to avoid increasing side effects and risks of deep hypothermia, which is in accordance with induced RBM3 expression pattern at different cooling temperature.

In addition to PERK, three other kinases, PKR, GCN2 and HRI, also phosphorylate eIF2α in response to distinct types of stress known as integrated stress response (ISR) (Harding HP et al., 2003; Donnelly N et al., 2013). ISR is characterized by the formation of cytoplasmic non-membrane RNA granules, known as stress granules (SGs), which are composed of initiation factors, ribosomal proteins, translationally-stalled mRNAs and RNA binding proteins. Upon stress, the assembly of SGs is initiated by phosphorylated eIF2α in a rapid dynamic manner to stall global

translational process. Once the stress has been removed, SGs disperse and protein translation recovers (Anderson P et al., 2015). Our results demonstrated that RBM3 regulates eIF2α activity in a stress-responsive pattern. Many of the putative RBM3 interacting partners identified in our RBM3 interactome screening are described to exist in stress granules and to modulate transcriptional and translational processes (Figure 3.21 and Appendix II). For example, NF90 has been shown to co-localize with stress granule markers in cytoplasm in infected cells although most of NF90 proteins remain in the nucleus (Wen X et al., 2014). Our data support this finding, RBM3, NF45 and NF90 predominantly localize in the nucleus (Figure 3.25 and Figure 3.26), but interaction occurs in a non-nuclear pattern together with the ER membrane bound protein PERK (Figure 3.27). Thus, we suppose that a pool of RBM3 proteins can shuttle to ER upon ER stress, and regulates the activity of ER membrane-bound protein PERK, albeit the majority of RBM3 remains in the nucleus. Silencing of RBM3 expression by siRNA cannot abolish RBM3-NF45 and RBM3-NF90 interactions, confirming the notion that only a small pool of RBM3 in cytoplasm is involved in these interactions (Figure 3.30). Meanwhile, the RBM3-NF45/90-PERK interactome exists in a ribonucleoprotein complex, which is mainly located in cytosol, and thereby exerts its function on ER stress response and presumably on stress granule formation, where the RBM3 interactome could be involved in protecting mRNAs as a chaperone, promoting cell growth, and preventing apoptosis. Supportively, a previous report has demonstrated that CIRP is localized predominantly in nucleus as well, and migrates to stress granules in RGG motif-dependent manner upon ER stress and other cellular stresses (De Leeuw F et al., 2007). The high similarity of RGG domain between RBM3 and CIRP provides the structural basis of their comparable migration behavior. However, as a component of stress granules, CIRP represses protein translation (De Leeuw F et al., 2007), which is opposite to the role of RBM3 in global translation (Dresios J et al., 2005; Smart F et al., 2007), indicating the distinct regulatory function of CIRP and RBM3 in translational events (see also 1.1.3.3).

In general, our data provide evidence for a key role of RBM3 in the interplay among hypothermia, ER stress, and cell protection (Figure 4.1). We identified RBM3 as an

inhibitor of the canonical PERK-eIF2α-CHOP ER stress pathway. Specifically, RBM3 inhibits PERK phosphorylation in an NF90-dependent manner, ultimately resulting in reduced apoptosis. Our findings were confirmed in a KO mouse model of RBM3 and establish a signaling mechanism by which RBM3 prevents the dysfunction and apoptosis of cells under stressful conditions.

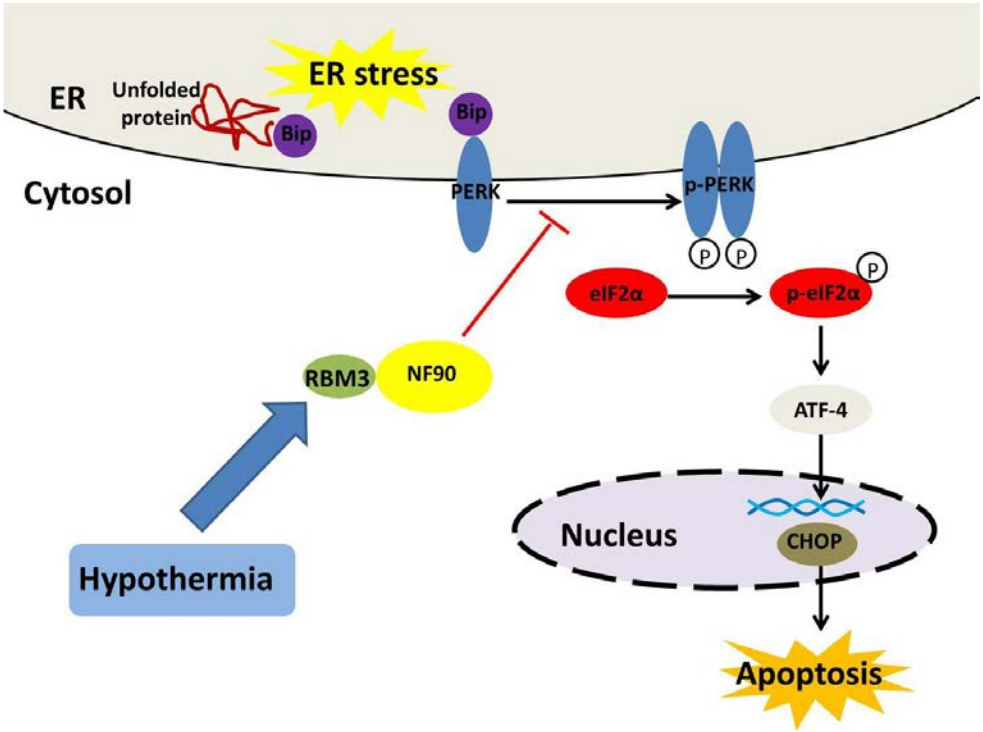

Figure 4.1 Model for RBM3-regulating ER stress response by acting as PERK inhibitor together with NF90.

As summarized in 1.3.3, RBM3 can either up- or downregulate miRNA expression, but to specific miRNA such as miR-125b, the regulatory tendencies are contradict (Dresios J et al., 2005; Pilotte J et al., 2011). The inconsistence may associate with different models or cell types as well as culturing conditions, indicating a complicated context of RBM3 in regulating miRNA biogenesis. Our findings support the assumption that RBM3 regulates the transition from precursor miRNA to mature miRNA (Pilotte J et al., 2011). However, it is quite surprising to see both pro- and anti-apoptotic miRNAs are positively regulated by RBM3 (Pilotte J et al., 2011), which makes it ambiguous to explain the anti-apoptotic role of RBM3. In our settings, instead, the pro-apoptotic miRNAs, miR-16 and let-7a, were inhibited by RBM3

(Figure 3.33 and Figure 3.34), which is opposite to Pilotte's findings (Pilotte J et al., 2011) but supports the effects of hypothermia (Figure 3.31) and anti-apoptotic activity by increased *Bcl-2* transcripts (Figure 3.35). We assume, at least in part, RBM3 prevents cell apoptosis by negatively modulating pro-apoptotic miRNAs.

The expression of mir-106b-93-25 cluster is suppressed by PERK activation and their reduction results in ER stress-related apoptosis (Gupta S et al., 2012). In our model, we observed that RBM3 positively modulates miR-106b-93-25 cluster in general (Figure 3.36), which supports its effects on PERK activity in ER stress response (Figure 3.10; Figure 3.12; Figure 3.13 and Figure 3.16) and Pilotte's report (Pilotte J et al., 2011). Interestingly, although the primary precursors are transcribed simultaneously, miR-25 expression pattern behaves differently compared to miR-106b and miR-93 under ER stress (Figure 3.36). In the aspect of sequence similarity, miR-25 belongs to miR-17 family while miR-106b and miR-93 are both miR-92 family members (Tan W et al., 2014). This probably explains the inconsistent mature miRNA expression patterns due to related functional discrepancy. Actually, the differential expression of miR-25 compared to miR-106b and miR-93 was also observed by other study (Liang WC et al., 2015). Besides of the anti-apoptotic functions, the expression of miR-106b-93-25 cluster is present in neural stem cells and promotes their proliferation (Brett JO et al., 2011). Thereafter, the positively modulated miR-106b-93-25 by RBM3 is consistent with the association RBM3 with neurogenesis after neural ischemic damage (Figure 3.3).

In summary, RBM3 appears to be generally cytoprotective in stressful conditions relating to brain injuries and diseases. First, our work unraveled that RBM3 prevents oxidative stress-induced cell apoptosis. Second, the protective mechanism of RBM3 includes attenuating of ER stress response by inhibiting PERK phosphorylation, which involves the assistance of RBM3 interactor NF90. Third, the modulation of pro-apoptotic and anti-apoptotic miRNA subsets can also contribute to RBM3-mediated effects against apoptosis. Our study provides evidences to demonstrate that RBM3 promotes cell survival under stress through versatile mechanisms. RBM3 represents

a unique cellular tool present in many cell types, that can be activated by various cellular stressors including hypothermia and that can be utilized by the cells depending on the specific cellular context to adapt stressful environment.

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Appendix I

Abbreviations

AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide

AnnV: annexin V

APBP-1: aggrecan promoter-binding protein-1

ATF6: activating transcription factor 6α

ATR: ataxia telangiectasia mutated and Rad3-related

β2m: β-2 microgloblin

Bcl-2: B-cell lymphoma 2

BiP: binding-immunoglobulin protein

cDNA: complementary deoxyribonucleic acid

CHOP: CCAAT/enhancer-binding protein homologous protein

CIRP: cold-inducible RNA-binding protein

CoIP: co-immunoprecipitation

COX-2: cyclooxygenase-2

CSD: cold shock domain

CSP: cold shock protein

DAPI: 4',6-diamidino-2-phenylindole

DG: dentate gyrus

DMEM: Dulbecco's modified Eagle's medium

Dox: doxycyclin

eIF2α: eukaryotic translation initiation factor 2α

eIF4E: eukaryotic initiation factor 4E

EOC: epithelial ovarian cancer

ER: endoplasmic reticulum

ERG: v-ets avian erythroblastosis virus E26 oncogene homolog

FACS: fluorescence-activated cell sorting

FGF21: fibroblast growth factor 21

GRP: glycine-rich protein

HBSS: Hank's buffered salt solution

HCC: hepatocellular carcinoma

HEK293: human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HuR: human antigen R

IGF-1: insulin-like growth factor-1

IRE1α: inositol-requiring protein 1α

IRES: internal ribosome entry site

KO: knockout

LPS: lipopolysaccharide

MCRE: mild-cold responsive element

miRNA: microRNA

MS: mass spectrometry

NF45/90/110: nuclear factor 45/90/110

n.s.: not significant

OGD: oxygen-glucose deprivation

ORF: open reading frame

PBS: phosphate-buffered saline

PERK: PRKR-like ER kinase

PKR: protein kinase R

PI: propidium iodide

PLA: proximity ligation assay

pre-miR16/let-7a: miR-16/let-7a precursor

RBM3: RNA-binding motif protein 3

Real-time RT-PCR: real-time reverse transcriptase polymerase chain reaction

RGG: arginine/glycine-rich domain

RMS: rostral migration stream

RNase: ribonuclease

RNP1/2: ribonucleoprotein 1/2

RPA: replication protein A

RRM: RNA recognition motif

ROS: reactive oxygen species

SCI: spinal cord injury

SCN: suprachiasmatic nucleus

Scr: scrambled siRNA

SD: sleep deprivation

SDN-POA: sexually dimorphic nucleus of the preoptic area

SG: stress granule

SVZ: subventricular zone

TBI: traumatic brain injury

Tg: thapsigargin

TRX: thioredoxin

Tu: tunicamycin

UPR: unfolded protein response

UTR: untranslated region

WB: western blot

WT: wildtype

xCIRP: *Xenopus* homologue of cold-inducible RNA-binding protein

XBP1: X-box-binding protein 1

Appendix II

Screening data for RBM3 interactors in HEK293 cells

CaptiVate™ Services: Experimental Results

Bait Name: RBM3

Purification: Strep-tag affinity purification

Experiment (Exp) L and M: 37°C

Experiment (Exp) N and O: 32°C

		Nr. of unique peptides			Nr. of unique peptides			
Swiss prot Acc	Overal rank	Exp L	Exp M	Rank Exp L and M	Exp N	Exp O	Rank Exp N and O	Protein name
P98179	A	58	58	A	44	48	A	RNA-binding protein 3
Q12906	**A**	**36**	**39**	**A**	**11**	**15**	**A**	**NF90, Interleukin enhancer-binding factor 3**
O00425	A	29	26	A	11	7	A	Insulin-like growth factor 2 mRNA-binding protein 3
Q12905	**A**	**14**	**18**	**A**	**9**	**10**	**A**	**NF45, Interleukin enhancer-binding factor 2**
Q9Y6M1	A	16	17	A	1	1	C	Insulin-like growth factor 2 mRNA-binding protein 2
Q9NUD5	A	9	14	A	4	3	B	Zinc finger CCHC domain-containing protein 3
Q9UKM9	A	8	7	A	2	1	C	RNA-binding protein Raly
Q15369	A	4	4	B	4	3	B	Transcription elongation factor B polypeptide 1
Q86SE5	A	1	1	C	1	1	C	RNA-binding Raly-like protein
A6NLC5	A	1	1	C	1	1	C	UPF0524 protein C3orf70
Q6PID8	B	10	7	A	2	0	D	Kelch domain-containing protein 10
Q15370	B	4	5	B	3	0	D	Transcription elongation factor B polypeptide 2
P62891	B	1	0	D	1	1	C	60S ribosomal protein L39
Q9HCE1	C	24	26	A	0	0	-	Putative helicase MOV-10
Q92900	C	7	8	A	0	0	-	Regulator of nonsense transcripts 1
Q9NZB2	C	6	8	A	0	0	-	Constitutive coactivator of PPAR-gamma-like protein 1
Q9Y2Q9	C	3	5	B	0	0	-	28S ribosomal protein S28
P27694	C	0	0	-	3	5	B	Replication protein A 70 kDa DNA-binding subunit
P61326	C	3	4	B	0	0	-	Protein mago nashi homolog
O15234	C	2	3	B	0	0	-	Protein CASC3
Q86TS9	C	1	4	C	0	0	-	39S ribosomal protein L52
O75569	C	1	4	C	0	0	-	Interferon-inducible double stranded RNA-dependent protein kinase activator A
Q12926	C	2	2	B	0	0	-	ELAV-like protein 2
Q00577	C	1	3	C	0	0	-	Transcriptional activator protein Pur-alpha
Q9Y676	C	1	3	C	0	0	-	28S ribosomal protein S18b
Q9Y5S9	C	1	2	C	0	0	-	RNA-binding protein 8A
Q92665	C	1	2	C	0	0	-	28S ribosomal protein S31
P10745	C	1	0	D	1	0	D	Retinol-binding protein 3

P82932	C	1	1	C	0	0	-	28S ribosomal protein S6
P52298	C	1	1	C	0	0	-	Nuclear cap-binding protein subunit 2
Q8TCC3	C	1	1	C	0	0	-	39S ribosomal protein L30
P51398	C	1	1	C	0	0	-	28S ribosomal protein S29
P82933	C	1	1	C	0	0	-	28S ribosomal protein S9
P10155	C	1	1	C	0	0	-	60 kDa SS-A/Ro ribonucleoprotein
Q86VH2	C	1	1	C	0	0	-	Kinesin-like protein KIF27
Q9UKY7	C	0	0	-	1	1	C	Protein CDV3 homolog
P52815	D	0	3	C	0	0	-	39S ribosomal protein L12
Q8WZ42	D	0	0	-	0	2	D	Titin
P14923	D	0	0	-	2	0	D	Junction plakoglobin
Q9BYD6	D	0	2	D	0	0	-	39S ribosomal protein L1
Q15428	D	0	2	D	0	0	-	Splicing factor 3A subunit 2
P62258	D	1	0	D	0	0	-	14-3-3 protein epsilon
P49368	D	1	0	D	0	0	-	T-complex protein 1 subunit gamma
P50991	D	1	0	D	0	0	-	T-complex protein 1 subunit delta
Q96HQ2	D	1	0	D	0	0	-	CDKN2AIP N-terminal-like protein
Q8N5N7	D	1	0	D	0	0	-	39S ribosomal protein L50
P06881	D	1	0	D	0	0	-	Calcitonin gene-related peptide 1
Q9NPE2	D	1	0	D	0	0	-	Neugrin
Q6RFH5	D	1	0	D	0	0	-	WD repeat-containing protein 74
O75843	D	1	0	D	0	0	-	AP-1 complex subunit gamma-like 2
Q96CP2	D	0	0	-	0	1	D	FLYWCH family member 2
O75127	D	0	0	-	0	1	D	Pentatricopeptide repeat-containing protein 1
Q9BYH8	D	0	0	-	0	1	D	NF-kappa-B inhibitor zeta
Q5H9U9	D	0	0	-	0	1	D	Probable ATP-dependent RNA helicase DDX60-like
Q70CQ2	D	0	0	-	0	1	D	Ubiquitin carboxyl-terminal hydrolase 34
Q01469	D	0	0	-	1	0	D	Fatty acid-binding protein
Q15517	D	0	0	-	1	0	D	Corneodesmosin
P50570	D	0	0	-	1	0	D	Dynamin-2
Q6ZT12	D	0	0	-	1	0	D	E3 ubiquitin-protein ligase UBR3
A6NLC8	D	0	0	-	1	0	D	Putative TAF11-like protein ENSP00000332601
P24386	D	0	0	-	1	0	D	Rab proteins geranylgeranyltransferase component A 1
Q12884	D	0	0	-	1	0	D	Seprase
O76094	D	0	0	-	1	0	D	Signal recognition particle 72 kDa protein
O75940	D	0	0	-	1	0	D	Survival of motor neuron-related-splicing factor 30
Q12767	D	0	0	-	1	0	D	KIAA0195
Q04844	D	0	1	D	0	0	-	Acetylcholine receptor subunit epsilon

Bait: Protein of interest (RBM3) used as bait in the affinity purification

Overall ranking (ranking not respecting the different treatments of the samples)

Class A Interactors:	Interactors which have been identified in all 4 biological replicates. They represent highly likely interactors of RBM3 bait
Class B Interactors:	Interactors which have been identified in 3 biological replicates. They represent likely interactors of RBM3 bait
Class C Interactors:	Interactors which have been identified in two biological replicates. These proteins may represent weak , transient and/or substoichiometric interactors of RBM3 bait
Class D Interactors:	Interactors which have been identified in only one of the biological replicates. These proteins may still represent real interactors of your protein of interest. Proteins within these group rather represent weak, transient and/or substoichiometric interactors of RBM3 bait

Note:

Data filtering:	All identified proteins were filtered against a contaminant database to subtract common background proteins
Unique Peptides:	Unique peptides of a particular protein that allows its clear identification (non redundant)

Acknowledgement

After over four years study, eventually it comes to the end of my PhD. During this tough but fruitful period, many people supported my work and life and I am sincerely grateful to their efforts.

Firstly, I have to express the gratitude to my supervisor, Dr. Sven Wellmann, who gave me the opportunity to join in his lab and work on RBM3 project. Although busy with clinical business and clinical studies, you were always enthusiastic with the data interpretations, discussions and suggestions on RBM3 project, and your guidance on the general orientation both in my work and my career is of great importance for me.

Thanks to Prof. Dr. Josef Kapfhammer and Dr. Sophorn Chip in Institute of Anatomy. Both of you introduced the concepts and methods of neuroscience study to me. I can never forget your guidance at the bench and at the microscope.

Thanks to Prof. Dr. Anne Spang in Biozentrum, your critical assessment is definitely beneficial for the improvement of my work.

I am also thankful to Andrea Zelmer and Markus Saxer, the lab technicians in UKBB and Institute of Anatomy respectively, who provide substantial assistances to my experiments. And the PhD students Etsuko Shimobayashi, Pradeep Sherkhane,Sinoy Sugunan and Sabine Winkler in Institute of Anatomy, thanks for your scientific discussions and your career advices. I am happy to share the interesting and funny things in science and life with you.

Finally, I would like to thank my parents. You gave me your best care and supported me for my career all these years. You always encouraged me whenever I met difficulties. Thanks again!

Curriculum Vitae