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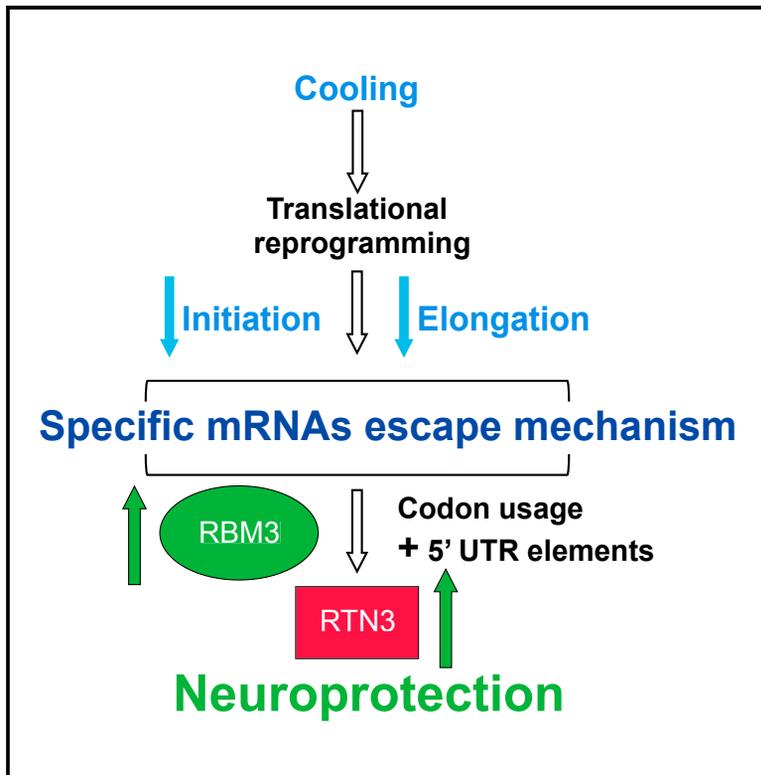
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Current Biology

RTN3 Is a Novel Cold-Induced Protein and Mediates Neuroprotective Effects of RBM3

Graphical Abstract



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In Brief

Therapeutic hypothermia is neuroprotective, and the cold shock protein RBM3 plays a critical role in mediating synaptic repair processes that accompany cooling. Bastide and Peretti et al. show that cooling selectively reprograms the translome and identify RTN3 as a cold-induced protein that acts downstream of RBM3 in the neuroprotection pathway.

Highlights

- Cooling-induced reprogramming of the translome increases synthesis of *RTN3*
- The neuroprotective protein RBM3 binds *RTN3* mRNA and drives its expression
- *RTN3* overexpression prevents synaptic loss in mice with prion disease
- *RTN3* expression is a mediator of RBM3-induced neuroprotection

Accession Numbers

E-MTAB-5437



RTN3 Is a Novel Cold-Induced Protein and Mediates Neuroprotective Effects of RBM3

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<http://dx.doi.org/10.1016/j.cub.2017.01.047>

SUMMARY

Cooling and hypothermia are profoundly neuroprotective, mediated, at least in part, by the cold shock protein, RBM3. However, the neuroprotective effector proteins induced by RBM3 and the mechanisms by which mRNAs encoding cold shock proteins escape cooling-induced translational repression are unknown. Here, we show that cooling induces reprogramming of the translome, including the upregulation of a new cold shock protein, RTN3, a reticulon protein implicated in synapse formation. We report that this has two mechanistic components. Thus, *RTN3* both evades cooling-induced translational elongation repression and is also bound by RBM3, which drives the increased expression of RTN3. In mice, knockdown of RTN3 expression eliminated cooling-induced neuroprotection. However, lentivirally mediated RTN3 overexpression prevented synaptic loss and cognitive deficits in a mouse model of neurodegeneration, downstream and independently of RBM3. We conclude that RTN3 expression is a mediator of RBM3-induced neuroprotection, controlled by novel mechanisms of escape from translational inhibition on cooling.

INTRODUCTION

Therapeutic hypothermia is a powerful neuroprotectant, acting through multiple mechanisms, although the underlying pathways are not fully understood [1, 2]. We recently showed that the cold shock RNA-binding protein, RBM3, plays a critical role in mediating synaptic repair processes essential for neuroprotection in mouse models of neurodegenerative disease [3]. Thus, the inability to induce RBM3 expression in early disease results in defective structural synaptic plasticity and hence reduced regenerative capacity, leading to synapse loss and eventually neuronal loss. Inducing endogenous RBM3 expres-

sion in vivo through cooling, or by lentiviral-mediated overexpression, prevented synapse loss in prion and Alzheimer-type mice, rescued memory deficits, protected against neurodegeneration, and significantly prolonged survival [3]. How RBM3 mediates these effects is unknown, although it is likely to be related to its RNA chaperone function, as it facilitates selective mRNA translation following a number of cellular stresses, including cooling [4–6]. RBM3 is also implicated in protection against cell death [7, 8] and increases local protein synthesis at dendrites [9].

Upon cooling, the changes to the protein synthesis machinery are similar to those observed with other stress inducers (e.g., UVB exposure) [10, 11] and include phosphorylation of eukaryotic initiation factor 2 (eIF2) on the alpha subunit [12, 13]. However, cold shock differs because relieving eIF2 α -mediated inhibition of translation is insufficient to restore protein synthesis rates [12]. Instead, the rapid reduction in protein synthesis that accompanies cooling [12–15] is a result of a decrease in translational elongation mediated by the phosphorylation of elongation factor 2 (eEF2) by elongation factor 2 kinase (eEF2K; a negative regulator of eEF2) [12]. Importantly, suppression of eEF2K and subsequent reactivation of eEF2 significantly increases the rate of protein synthesis rates in cooled cells [12], consistent with the concept that elongation is a major regulatory node under specific pathophysiological conditions [16, 17].

To examine the relationship between cold stress, RBM3 induction, and the modulation of mRNA translation for the synthesis of putative neuroprotective proteins, we investigated the post-transcriptional response to hypothermia in vitro and validated the data in vivo in a mouse model of neurodegeneration, in which we know cooling is protective, mediated by RBM3. We show that, following cold shock/cooling, the global decrease in protein synthesis rates is associated with selective reprogramming of the translome. We find enhanced synthesis of specific proteins: not only the cold shock protein RBM3 (as predicted) but also of a number of proteins with a role in development and function of the nervous system, including reticulon 3 (RTN3). RTN3 has a role in synaptic plasticity and synapse formation [18, 19] and is thus a compelling candidate for the neuroprotective effects mediated by increased RBM3 expression. We find that both *RBM3* and *RTN3* evade translational repression and that

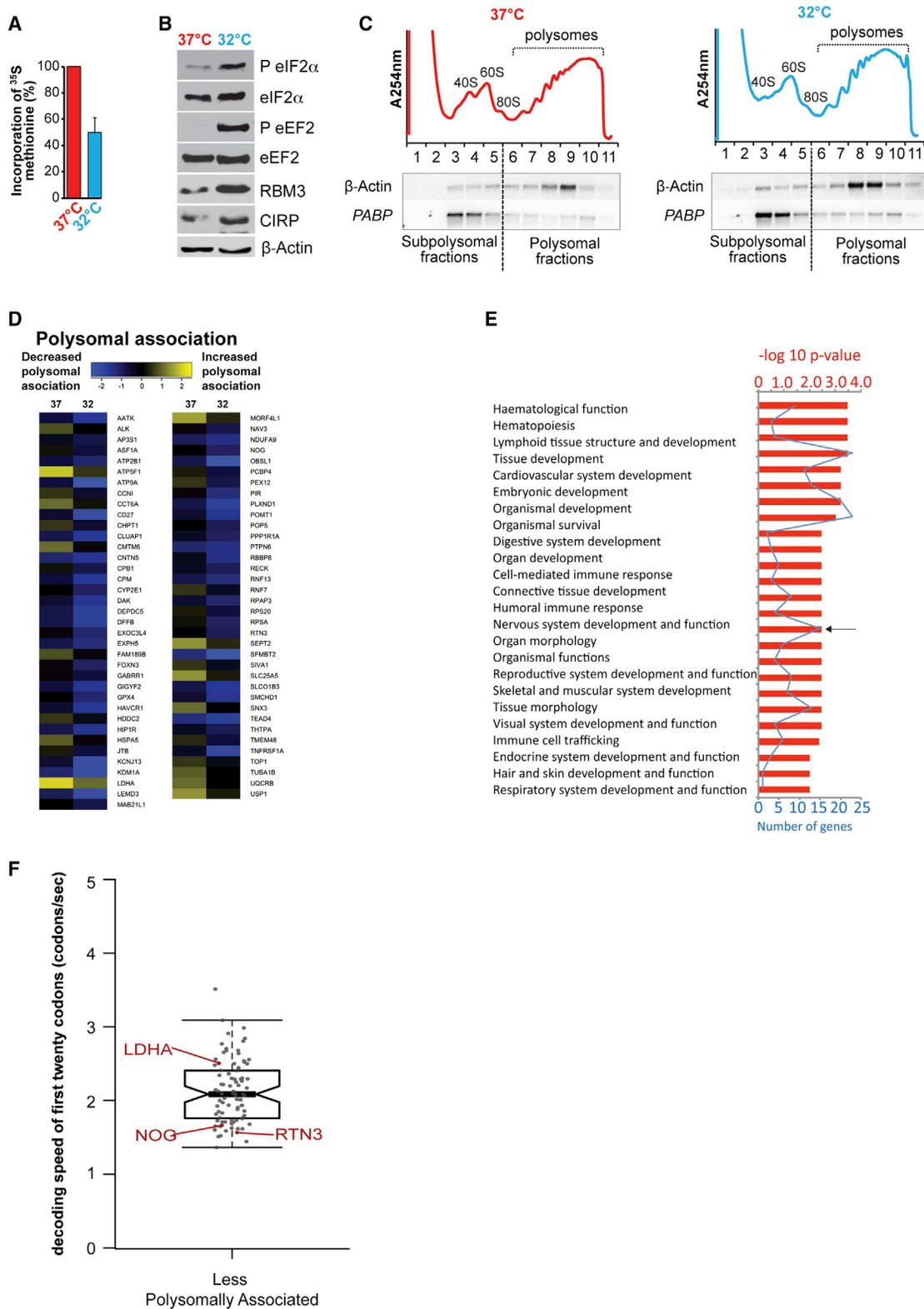


Figure 1. Mild Cooling Results in Translation Regulation of a Defined Set of Transcripts

(A) Protein synthesis rates determined by [^{35}S]-methionine label incorporation after 24 hr incubation of HEK293 cells at 32°C. Values were normalized to cells incubated at 37°C. Error bars represent SE within three independent experiments.

(B) HEK293 cells were incubated at 37°C or at 32°C for 24 hr and immunoblotted for RBM3 and CIRP, eIF2 alpha eEF2, and β -actin.

(legend continued on next page)

RBM3 binds *RTN3* mRNA and plays a major role in driving cooling-induced upregulation of *RTN3* expression. Finally, we show that *RTN3* expression, downstream of RBM3 induction, mediates cooling-induced neuroprotection in mice with neurodegenerative disease and importantly is neuroprotective even in the absence of cooling.

RESULTS

Cooling Induces Reprogramming of the Translatome

Both transcriptional and post-transcriptional control mechanisms are required for the overall response to cell stress [20]. In order to examine the genome-wide changes accompanying cooling, we incubated HEK293 cells at 32°C for 24 hr. This resulted in reduction in protein synthesis (Figure 1A) and phosphorylation of the translation initiation and elongation factors eIF2 α and eEF2 (Figure 1B; in agreement with previous studies [12]). We chose HEK293 cells because the response to cooling is well documented [12, 15, 21] and, in addition, they express many markers associated with neuronal lineage [22]; thus, using this cell line increases the potential for the identification of cold-induced putative neuroprotective proteins. Transcriptional analyses of the cooled HEK293 cells showed that 119 genes were downregulated at the transcriptional level, with no significant increases in transcription of any mRNAs (Figure S1; Table S1). In addition, no differences greater than 2-fold were identified in the expression of microRNAs (miRNAs) (Table S2). These data support regulation of protein synthesis as an important mechanism for control of gene expression following cooling. We have shown previously that cooling reduces global rates of protein synthesis and importantly that elongation repression is the driver of this process (Figures 1A and 1B [12]). We hypothesize that, during cooling, specific mRNAs are able to evade a global reduction in translation elongation so that the expression of the corresponding proteins is maintained or even increased. However, identifying such mRNAs represents a technical challenge. Under conditions in which the initiation of translation is inhibited, the number of actively translating ribosomes decreases [10, 23] and polysome profiling can be used to identify those mRNAs that remain polysomally associated; this generally correlates with increased synthesis of the corresponding proteins [20]. However, under conditions in which elongation is inhibited, the number of polysomally associated ribosomes will stay the same or increase, and therefore it is difficult to identify mRNAs that either escape or are relatively insensitive to elongation slow down. Therefore, to identify proteins whose synthesis was increased during cooling, computational modeling was used in conjunction with polysome profiling.

Sucrose density gradient analysis was carried out on cooled samples to separate polysomes and the associated transcripts (Figure 1C), which were subsequently purified and analyzed by cDNA microarray. By microarray 71, mRNAs were identified that were associated with a decreased number of polysomes at 32°C (Figures 1D and S2; Tables S3 and S4). Importantly, ingenuity pathway analysis showed that there was significant enrichment for mRNAs that encode proteins that function in the nervous system and its development (15/71; marked by an arrow, Figure 1E). To predict which of these neuronal-related mRNAs were translated in cooled cells, we used a computational model of elongation control [24] generated by defining the intracellular concentration of ribosomes, translation factors, and tRNAs (Table S5). The model allows the speed of decoding to be estimated for any given open reading frame [25], assuming that decoding speed is not significantly limited by tRNA-independent parameters, such as mRNA secondary structure or modifications. We have previously shown that, despite this assumption, the model can be used to rank expression levels from multiple elongation-controlled mRNAs reliably [25]. The model predicts that, under eEF2 ablation, fast codons (which are decoded by abundant tRNAs) change their elongation rate by an order of magnitude, whereas slow codons are relatively unaffected (see Figures S3A–S3C).

We applied the model to the transcripts that encoded mRNAs with roles in neuronal processes identified by polysome profiling (Figure 1D) to analyze elongation over the initial 20 codons (Figure 1F; Table S6). Our analysis showed that a subset of mRNAs, including *RTN3* and *Noggin*, contained codons that require less abundant tRNAs in the 5' end of the transcripts (Figure 1F), and our model predicted that these could escape the repression of elongation.

mRNAs Encoding a Subset of Neuronal Proteins Overcome Elongation Inhibition on Cooling

Western analysis showed that expression of *Noggin* and *RTN3*, and as expected *RBM3*, increased at 32°C, whereas *GGBR1* and *LDHA* levels, which are encoded by mRNAs that contain codons requiring abundant tRNAs, were unchanged in both cell lines (Figure 2A). To confirm a post-transcriptional response, we examined mRNA expression changes of *RTN3* and *Noggin* using qPCR (Figure 2B); there was a small reduction in the levels of *Noggin*, consistent with the transcriptional profiling data (Table S1; Figure S1) and no change for *RTN3*. There was an increase in the expression of *RBM3* mRNA, in agreement with previous studies [4].

To confirm escape of translation elongation repression and identify a strong candidate protein with a neuroprotective function,

(C) Sucrose density gradient ultracentrifugations were performed from HEK293 cells incubated at 37°C or 32°C for 24 hr. Plots show the distribution of RNA within subpolysomes (40S, 60S, and 80S) and polysomes. Northern analysis was carried out on individual fractions, which were probed for β -actin or PABP.

(D) mRNAs from gradient fractions were pooled and subjected to cDNA microarray. The color scale represents the ratio of mRNA in subpolysome and polysome fractions, normalized \log_2 (polysome/subpolysome) value; yellow is polysome- and blue subpolysome-associated mRNAs.

(E) mRNAs that showed significant change in polysome/subpolysome (P/S) ratio on cooling were clustered into functional groups. Biological functions associated with decreased polysomal-associated transcripts, obtained from the ingenuity pathways analysis. Fisher's exact test was used to calculate a p value (threshold $p < 0.05$) for each biological function represented in the red bar chart. The blue line represents number of proteins per category.

(F) Predictive modeling of transcript-decoding speed was performed on the initial 20 codons of human transcript sequences from those that showed decreased polysomal association. The boxplot shows mRNAs that have a decrease in polysomal association and contain an initial 20 "slow" codons (e.g., *RTN3* and *Noggin* [*NOG*]) compared to those that contain "fast" codons, such as *LDHA*.

See also Figures S1–S3 and Tables S1–S6.

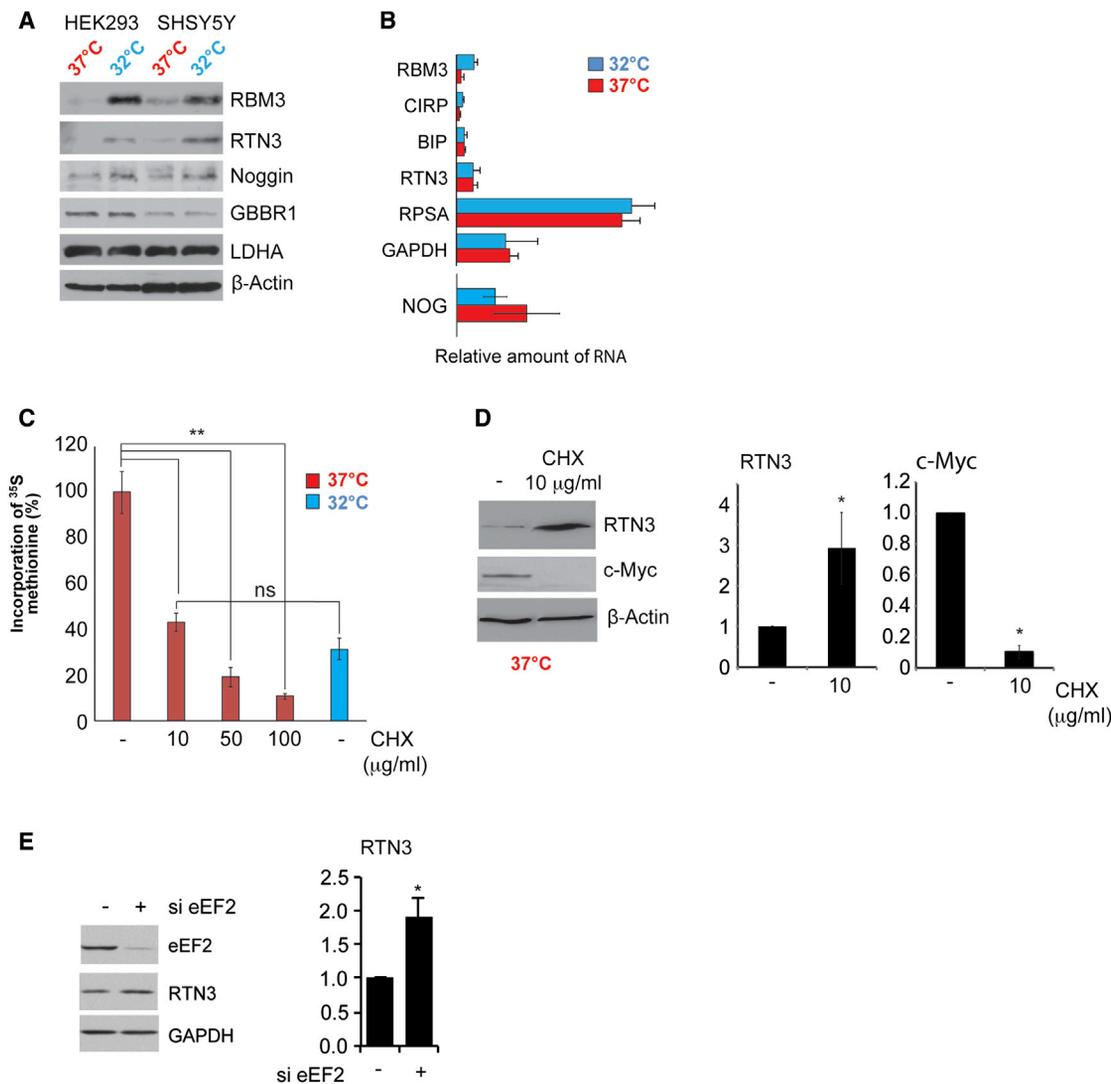


Figure 2. RTN3 Is Subject to Elongation Control

(A) Extracts from control or cooled HEK293 or SHSY5Y cells were immunoblotted for RTN3 and Noggin, GBBR1, and LDHA. β-actin is used as a loading control. (B) qRT-PCR was performed on corresponding transcripts. Error bars represent 1 SD from the mean within three independent experiments. *GAPDH* was used as a control.

(C) Protein synthesis rates determined by [³⁵S]-methionine label incorporation after 24 hr incubation of HEK293 cells at 37°C with cycloheximide. Values were normalized to untreated cells. A two-tailed paired Student's t test was used to calculate statistical significance. Error bars represent SE within three independent experiments. ***p* < 0.001, all three conditions.

(D) Extracts from cells exposed to 10 μg/mL cycloheximide at 37°C were immunoblotted for RTN3, c-Myc, and β-actin. Error bars represent 1 SD from the mean within three independent experiments. **p* < 0.01.

(E) eEF2 expression was reduced by siRNA, proteins extracted and immunoblotted with the antibodies shown. *GAPDH* was used as a loading control. Error bars represent 1 SD from the mean within three independent experiments. **p* < 0.01.

See also Figures S4 and S5A.

we focused on RTN3, a protein that has known function in synaptogenesis in the adult nervous system [26–28] and a role in neuroprotection. To reduce elongation by an alternative method, we treated cells grown at 37°C with cycloheximide, which stalls the translocation step in the elongation cycle [29]. As expected, incubation with cycloheximide decreased global protein synthesis rates (Figure 2C) and reduced expression of c-Myc, which is known to have a short half-life of 20 min. However, there was increased RTN3 expression, consistent with our model's

prediction that slowed elongation enhances synthesis of this protein (Figures 2D and S4).

To mimic the cooling-induced elongation block, we reduced eEF2 expression by RNAi (Figure 2E). This resulted in an increase in RTN3 levels, suggesting that the rate of elongation along *RTN3* mRNA is relatively unaffected by reduced availability of eEF2, in agreement with our model (Figure S3).

Whereas we have shown previously there is a small effect of cooling on the stability of specific proteins [21], RTN3 is

a relatively stable protein with a half-life of at least 24 hr, and therefore, any effects of turnover in the time frame of the experiment will be minimal (Figures S5A and S5B).

RBM3 Binds to RTN3 mRNA and Increases Its Translation through *trans*-Acting Effects on Initiation

Cooling also reduces the rate of translation initiation, via inhibitory phosphorylation of eIF2 α (Figure 1A) [12], which compensates for the reduction in translation elongation, as fewer ribosomes will be available for initiation while they are limited by globally reduced elongation speeds. A similar phenomenon has been suggested to occur previously in yeast [25, 30]. Thus, we predict that, in order to increase their translation, transcripts also overcome the cooling-induced initiation block. This is likely to be driven by *trans*-acting factors acting upon *cis* elements within the transcripts. Because RBM3 is an RNA chaperone whose expression is increased in cooled cells [31, 32] and is known to mediate the neuroprotective effects of cooling [3], we hypothesized that cooling-induced RBM3 may act as a *trans*-acting factor promoting *RTN3* translation and that some of the neuroprotective effects of RBM3 may be mediated through *RTN3*.

To address whether RBM3 interacted with *RTN3* mRNA, we carried out immunoprecipitation reactions, and data showed that RBM3 binds to *RTN3* mRNA in both HEK293 cells and in hippocampus of wild-type mice (Figures 3A and 3B). We then asked whether RBM3 expression affects *RTN3* levels in HEK293 cells and mouse brain by transfecting with RBM3-expressing constructs or lentiviruses, respectively. In each case (Figures 3C and 3D), the data show that overexpression of RBM3 resulted in a dramatic increase in *RTN3* protein expression, with no corresponding increase in *RTN3* transcript levels in vitro (Figure 3C) or in vivo (Figure 3D).

***cis*-Acting Elements in *RTN3* Contribute to Evasion of the Initiation Block**

To examine the role of *cis*-acting elements in 5' UTR of *RTN3* in its post-transcriptional regulation by RBM3, we used a luciferase reporter construct containing the *RTN3* 5' UTR (Figure 3E). At 25°C, there was a 5-fold increase in translation of messages containing the 5' UTR of *RTN3*, compared to the control (Figure 3F). Further, overexpression of RBM3 resulted in a 5-fold induction of luciferase expression at 37°C (Figure 3Gi). In contrast, RNAi of RBM3 produced a small but significant reduction in luciferase activity (Figure 3Gii). Taken together, the data support a role for RBM3 in controlling *RTN3* expression through the *RTN3* 5' UTR *cis*-regulatory sequence.

Cooling Induces *RTN3* Expression In Vivo through Post-transcriptional Mechanisms

Given the role of RBM3 in regulating *RTN3* (Figures 2 and 3), we focused on *RTN3* as a potential mediator of the neuroprotective effects of RBM3 induction. *RTN3* is a strong candidate for this role. It is a member of the reticulon family of proteins, with multiple functions in the nervous system, including axon and neurite outgrowth [18, 19] and synapse formation [26–28]. It also has an indirect role in synaptic plasticity through its inhibition of BACE1, a secretase involved in cleavage of APP and a negative modulator of pCREB levels [28, 33, 34].

We tested whether *RTN3* expression was increased in brain on cooling in vivo by inducing hypothermia in wild-type FVB mice using 5' AMP, as described [3, 35]. We found the levels of both RBM3 and *RTN3* increased on cooling (Figure 4Ai) without corresponding changes in respective mRNA levels (Figure 4Aii), consistent with post-transcriptional upregulation. Further, cooling induced a reduction in global protein synthesis rates to ~40% of control levels (Figure 4B), as observed in vitro (Figure 1A) (although the abundance of polysomes was not reduced to an equivalent extent [Figure 4C], again consistent with in vitro findings [Figure 1C] [12]). Knockdown of RBM3 in mice via lentivirally mediated RNAi significantly reduced the *RTN3* increase on cooling (Figure 4D), confirming physiological relevance of this functional interaction and suggesting that *RTN3* expression is downstream of RBM3 in cooling in vivo.

***RTN3* Mediates Cooling-Induced Neuroprotective Effects of RBM3**

We next asked to what extent *RTN3* is neuroprotective in mice with neurodegenerative disease, using mice with prion disease, specifically tg37 mice [36] inoculated with Rocky Mountain Laboratory (RML) strain as in our previous studies [3, 37–41]. These mice overexpress prion protein (PrP) and have a rapid disease course, succumbing to disease in 12 weeks [36]. In these mice, synaptic loss is associated with the failure to induce RBM3 on cooling early in the disease course at 6 weeks post-inoculation (w.p.i.) [3], developing behavioral deficits at 9 w.p.i., with neuronal loss from 10 w.p.i. In general, terminal clinical signs appear at around 12 w.p.i.

We first confirmed that increased *RTN3* expression is downstream of RBM3 in prion-diseased mice. Lentivirally mediated RNAi of *RTN3* reduced *RTN3* levels in hippocampi (Figure 5A), but not RBM3 levels (Figure 5A). Further, RNAi of *RTN3* did not affect high RBM3 levels induced by cooling while preventing, as predicted, the cold-induced rise in *RTN3* (Figure 5B). This allowed us to address whether and to what extent *RTN3* mediates the neuroprotective effects of RBM3. We found that lentivirally mediated RNAi of *RTN3* in prion-infected mice abolished the protective effects of cooling on behavioral impairments (Figure 5C), accelerated neuronal loss (Figure 5D), and abolished the cooling-associated increase in survival in prion-infected mice (Figure 5E). Thus, reducing levels of *RTN3* largely abolished the protective effects of high levels of cold-induced RBM3, supporting the conclusion that *RTN3* is a major mediator of the effects of RBM3. Even in the absence of cooling, RNAi of *RTN3* accelerated synapse loss in disease (Figures S5C and S5D), supporting a role for *RTN3* in synapse maintenance and formation/plasticity. However, the exact mechanism remains unknown.

***RTN3* Is Neuroprotective in Neurodegenerative Disease**

RBM3 induction is profoundly neuroprotective in both prion and 5XFAD mice, effects that are abrogated if animals undergo knockdown of RBM3 [3] or of *RTN3* (Figures 5C–5E). To address whether *RTN3* is neuroprotective in the absence of cooling, we injected prion-infected tg37 mice with lentiviruses overexpressing *RTN3* (LV-*RTN3*). This increased expression of *RTN3* as expected (Figure 6A), importantly without increasing RBM3 expression, consistent with *RTN3* being downstream of RBM3

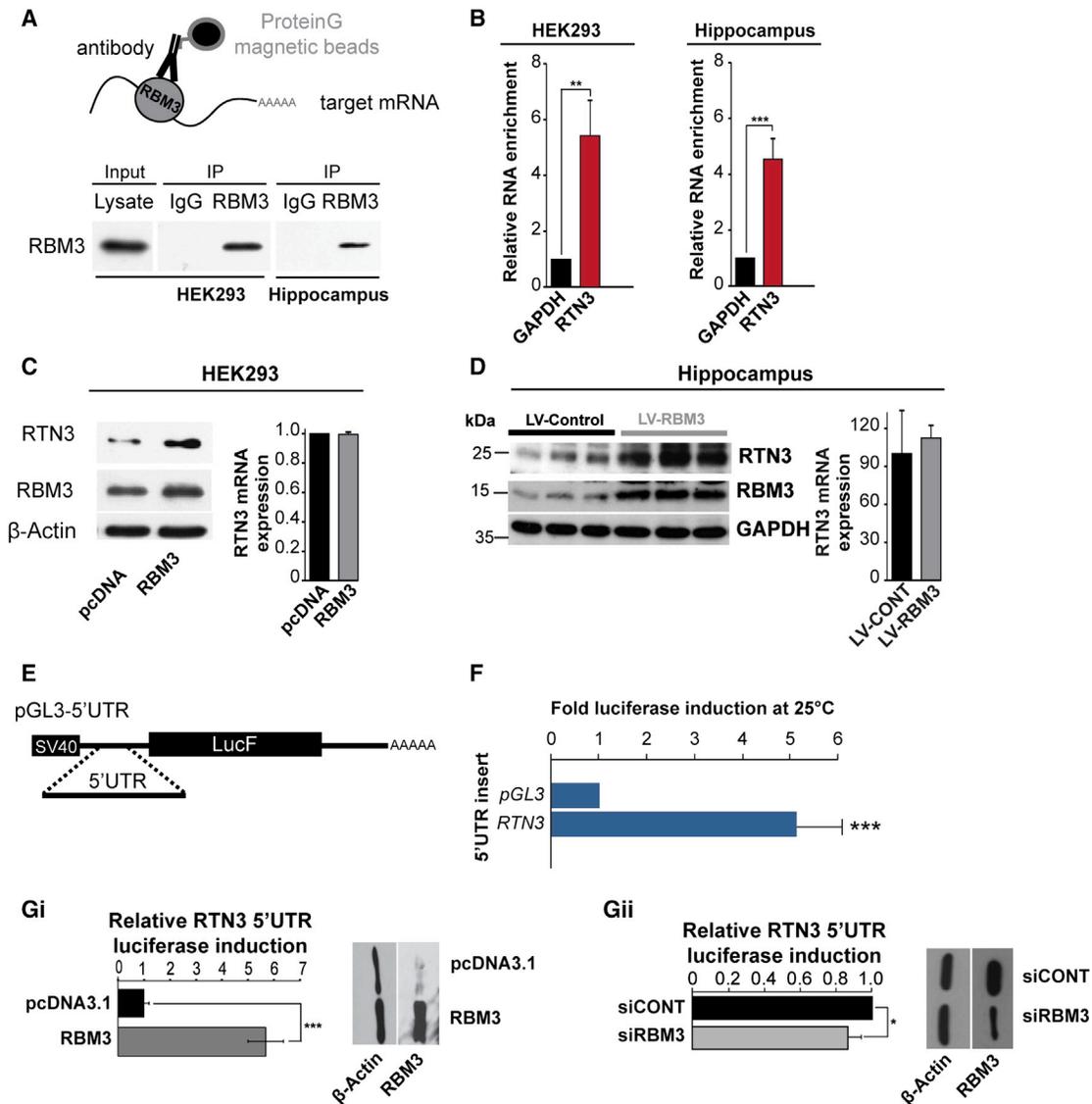


Figure 3. RTN3 Expression Is Downstream of RBM3

(A) Schematic representation of an RNA immunoprecipitation (RNA-IP) assay. Immunoblots of input lysate from HEK293 cells or hippocampus, immunoprecipitated with either rabbit IgG or RBM3 antibody, are shown.

(B) qRT-PCR was performed on RNA-IP samples using primers specific for human (HEK293) or mouse (hippocampus) samples. All values are normalized with respect to the initial RNA input material, and the enrichment is plotted relative to *GAPDH*. A two-tailed paired Student's *t* test was used to calculate statistical significance. Error bars represent 1 SD from the mean within three independent experiments. ***p* < 0.01; ****p* < 0.001.

(C) HEK293 cells were transfected with an expression plasmid construct encoding RBM3 or a control plasmid, and extracts were immunoblotted for RTN3. β -actin was used as a loading control. RNA expression of RTN3 was assessed by qRT-PCR.

(D) Mouse hippocampi stereotaxically injected with lentivirus containing a construct to overexpress RBM3 and extracts were immunoblotted for RTN3 and GAPDH. qRT-PCR was used to assess the expression of RTN3.

(E) Schematic representation of the RTN3 containing plasmid constructs encoding firefly luciferase.

(F) HEK293 cells were transfected with construct containing the 5' UTR of RTN3 and a Renilla luciferase control and incubated at either 37°C or 25°C for 24 hr. Firefly luciferase activity was calculated relative to Renilla luciferase for each condition and expressed as the fold induction from 37°C to 25°C.

(Gi) HEK293 cells were transfected with either control (pcDNA3.1) or RBM3 expression plasmid (pcDNA-RBM3) and then transfected with either RTN3 5' UTR pGL3 or pGL3 and Renilla luciferase constructs and luciferase activity determined. A two-tailed paired Student's *t* test was used to calculate error. Error bars represent 1 SD from the mean within three independent experiments. ****p* < 0.001.

(Gii) HEK293 cells were transfected with either control siRNA (siCONT) or *RBM3* siRNA (siRBM3) and then transfected with pGL3 and Renilla luciferase constructs. The fold repression from *RTN3* 5' UTR pGL3 compared to the control pGL3 was calculated and normalized to siCONT transfection. A two-tailed paired Student's *t* test was used to calculate statistical significance. Error bars represent 1 SD from the mean within three independent experiments. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

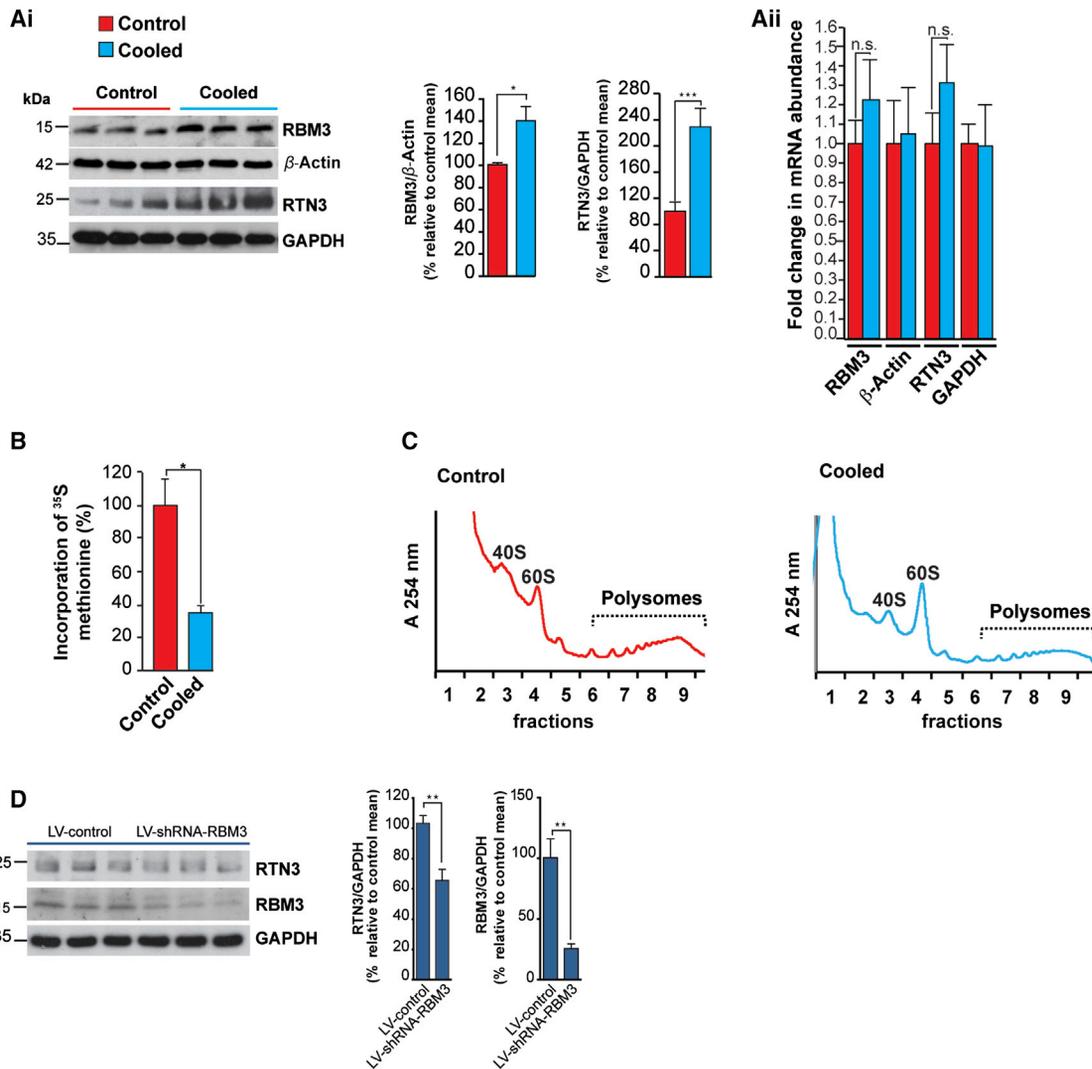


Figure 4. RBM3 Regulates RTN3 In Vivo

(Ai) Cooling increases RBM3 and RTN3 levels in hippocampi of wild-type mice. Representative western blots are shown. Bar graphs show quantification of RBM3 and RTN3 levels relative to β -actin and GAPDH, respectively. $n = 5$ control and 5 cooled mice for RBM3. $n = 9$ control mice and 9 cooled mice for RTN3. * $p < 0.05$; *** $p < 0.001$

(Aii) qRT-PCR of RNA isolated from hippocampi of cooled mice showed no significant change in the abundance of RBM3 or RTN3 mRNAs following cooling.

(B) Protein synthesis rates were determined by ^{35}S methionine incorporation into nascent protein using ex vivo hippocampus slices from cooled mice and control mice. A two-tailed paired Student's t test was used to calculate statistical significance. Error bars represent 1 SD from the mean within three independent experiments. * $p < 0.05$.

(C) Sucrose density gradient ultracentrifugation performed of cytoplasmic extracts from hippocampi from control and cooled mice. Absorbance plots show the distribution of RNA within subpolysomes (40S, 60S, and 80S) and polysomes.

(D) RTN3 induction on in vivo cooling is dependent on RBM3 protein expression. Knockdown of RBM3 resulted in a 38% decrease in RTN3 induction. $n = 6$ LV-control and 6 LV-shRNA-RBM3 mice. ** $p < 0.01$.

(Figure 6A). To assess neuroprotective effects of RTN3 overexpression, we measured synapse number (Figure 6B), burrowing activity (Figure 6C), and neuron number in CA1 of hippocampus from diseased animals treated with LV-RTN3 compared to controls (Figure 6D) over the time course of disease. All of these parameters decline in the course of prion disease in the absence of intervention. Importantly, RTN3 overexpression restored synapse number to wild-type levels, markedly above levels seen in untreated mice at the same and later time points (Figure 6B),

and prevented the decline in burrowing behavior (Figure 6C). RTN3 overexpression also conferred marked neuroprotection at the histological level, with very notable conservation of the CA1 pyramidal neuron layer (Figure 6D). Importantly, LV-RTN3 significantly increased survival of prion-infected mice (Figure 6E), recapitulating the effects of LV-RBM3 we previously described [3]. In our study of RBM3, we showed that onset of synaptic failure correlates with failure of induction of RBM3 at 6 w.p.i. [3]. And interestingly, RTN3 induction at this time point

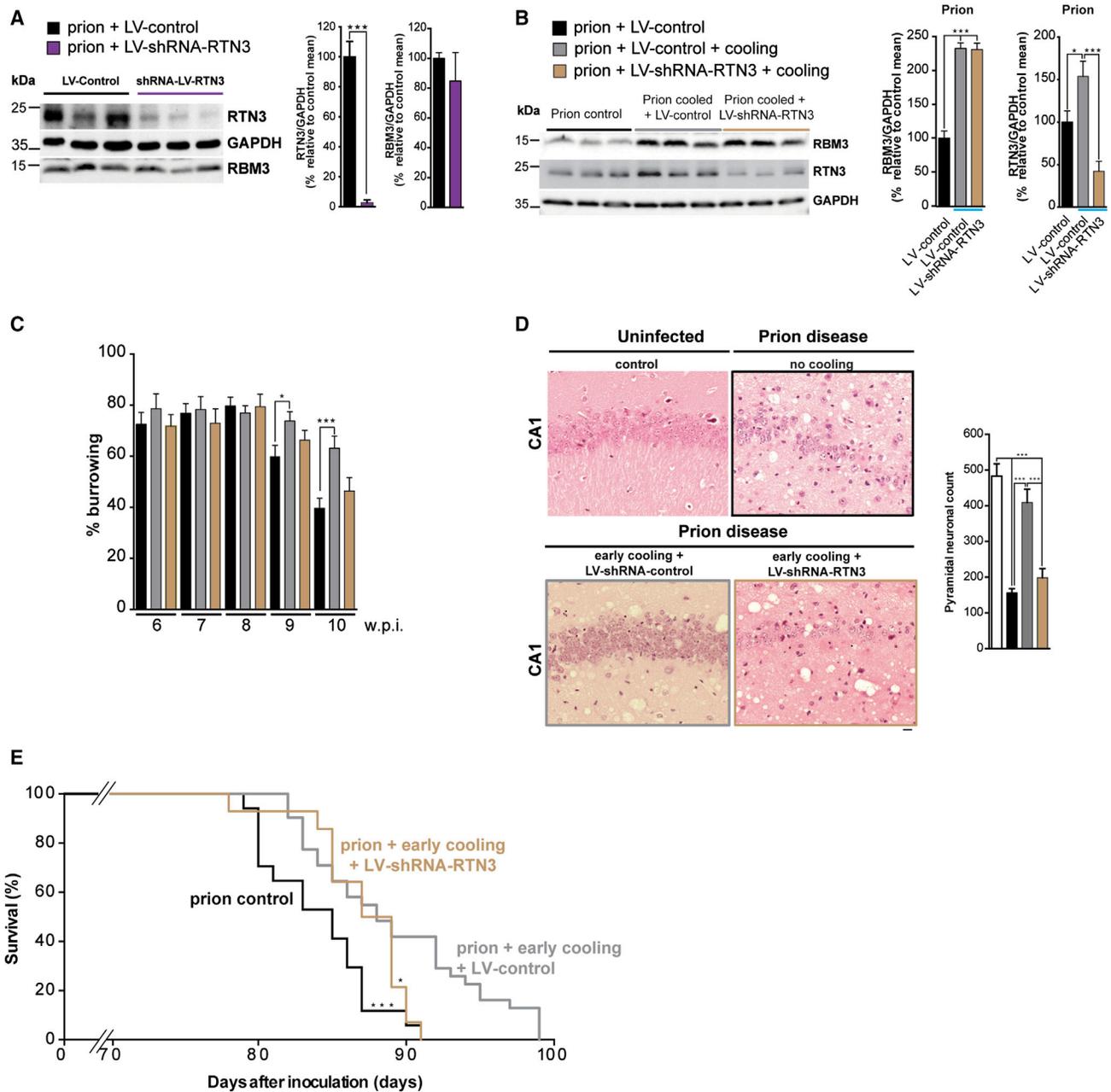


Figure 5. Lentiviral-Mediated Downregulation of Endogenous RTN3 Prevents Cooling-Induced RBM3-Mediated Neuroprotection

(A) LV-shRNA-RTN3 injected into hippocampi of prion-infected mice significantly reduces RTN3 protein levels compared to control shRNA (LV-shCONT). $n = 6$ prion+LV-control mice and 6 prion+LV-shRNA-RTN3 mice. Representative western blots and bar graphs quantification are shown.

(B) Western blot of RBM3 in LV-shRNA-RTN3-treated early-cooled prion mice shows no change in expression. $n = 6$ mice per experimental condition.

(C) The early-cooling-induced protection in burrowing behavior declines in LV-shRNA-RTN3 mice. Food pellet remaining in the tube measured after 2 hr is expressed in percentage burrowed. Graph bar with prion (black bars; $n = 12$ mice), prion + early cooling (gray bars; $n = 20$ mice), and prion + early cooling + LV-shRNA-RTN3 (light brown bars; $n = 12$ mice) is shown. One-way ANOVA with Tukey's post-test was used for multiple comparisons. $*p < 0.05$; $***p < 0.0005$.

(D) Representative images of H&E-stained hippocampal sections from uninfected control (top left-hand panel), prion-infected mice (top right-hand panel), prion-infected mice treated with early cooling and LV-control (bottom left-hand panel), and prion-infected mice treated with early cooling and LV-shRNA-RTN3 (bottom right-hand panel). Prion-infected mice show extensive neuronal loss, with associated spongiosis, whereas early cooling treatment prevents neurodegeneration. This protection is abrogated with LV-shRNA-RTN3. The graph bar shows quantification of the average number of neurons for each condition in the CA1 area of hippocampus. $n = 3$ mice (white bar), 7 mice (black bar), 7 prion mice (bar), and 9 mice (light brown bar). One-way ANOVA and Brown-Forsythe test with Tukey's post hoc analysis for multiple comparisons were used. $***p < 0.001$. The scale bar represents $50 \mu\text{m}$.

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is also lost (Figure 6F), supporting a functional relationship between these proteins in the context of disease and loss of synaptic structural plasticity.

Misfolded PrP levels were not affected by cooling or RTN3 expression, and levels were equivalent in all mice, precluding a mechanism of action via prion protein aggregation, consistent with our previous findings with RBM3 [3] (Figure S5D).

Thus, RTN3 overexpression results in neuroprotection at the level of synapse number, behavior, neuronal numbers, and increased survival, downstream and independently of cooling-mediated RBM3 induction.

DISCUSSION

The metabolic response to cooling is highly conserved [3, 35, 42]. The neuroprotective effects of hypothermia are essential for healthy brain function after hibernation and are widely exploited medically [43, 44]. However, relatively little is known about how global gene expression changes bring about these protective effects.

We have examined the genome-wide changes induced by cold stress by carrying out transcriptional, miRNA, and translational profiling on cells that were subjected to cooling. Our data show that specific induction of gene expression during cold stress is regulated at the level of translation with no significant transcriptional upregulation or changes in miRNA expression (Figures 1, S1, and S2; Tables S1 and S2).

Elongation rate control is the major determinant of global protein synthesis suppression upon cooling [12], but which transcripts are controlled in this way was unknown. We therefore generated a computational model to predict those messages that were particularly sensitive to regulation at this stage (Figure S3). According to our model, expression from transcripts that require abundant tRNAs would be dependent on eEF2 to maintain efficient elongation and protein expression. In contrast, mRNAs requiring rare tRNAs would be proportionally less affected by reductions in eEF2 availability and would be predicted to display either a small decrease or exhibit no net change in polysomal association upon cooling (Figure S3). In support of this hypothesis, the *RTN3* mRNA is decoded by rare tRNAs and, following cooling, exhibited reduced polysomal-associated yet increased protein expression (Figure 2). We showed that *cis*- and *trans*-acting factors were required for *RTN3* to overcome cooling-induced translation inhibition (Figure 3) and that the RNA chaperone RBM3 [6, 31, 32] was required (Figures 3 and 4).

We were interested in the functional consequence of RTN3 upregulation in response to cold shock. We have previously reported that RBM3 mediates the neuroprotective effects of cooling in mouse models of neurodegeneration and is necessary for maintenance of synaptic structural plasticity [3]. How it does this was not understood. We considered RTN3 to be a candidate neuroprotective protein specifically upregulated by RBM3 induction on cooling. In support of this, knockdown of RTN3 reduced synapse number at an earlier stage and abolished the

neuroprotective effects of cooling in prion-diseased mice, despite cooling-induced increase in RBM3 levels (Figures 5 and S5C). Conversely, lentivirally mediated overexpression of RTN3 prevented synapse loss and neurodegeneration in prion-diseased mice (Figure 6), recapitulating the neuroprotective effects observed during RBM3 expression [3].

In conclusion, we propose that, following cooling, there is translational reprogramming, leading to the overexpression of specific cold-inducible proteins, including the known cold shock protein, RBM3, but also of RTN3. Critically, we show that induction of RTN3 is downstream of RBM3 expression, and our data suggest that RTN3 is a mediator of the RBM3-driven neuroprotective effects of cooling in prion-diseased mice, most likely through its multiple roles in the regulation of neurite outgrowth and regulation of synaptic plasticity. It is likely that RTN3 induction would mediate a similar neuroprotective role in other neurodegenerative conditions. Further, its inhibition of BACE1-mediated cleavage of APP could also contribute to neuroprotection in Alzheimer's pathology. We propose that the control of RTN3 expression through escape from inhibition of translation on cooling at the levels of initiation and elongation provides new targets for neuroprotective therapies in neurodegenerative disease.

EXPERIMENTAL PROCEDURES

All animal work was conducted according to UK Home Office Regulations. For details of antibodies, plasmids, and oligonucleotides, please see the [Supplemental Experimental Procedures](#).

Cell Culture and Transfections

HEK293 cells were cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum (FBS). For induction of cold stress, cells were incubated at 32°C or at 25°C for 24 hr before harvesting. Small interfering RNAs (siRNAs) were transfected using DharmaFECT 1 and plasmids with Lipofectamine 2000 (Invitrogen).

SDS-PAGE and Western Blotting

Western blots were performed as described previously [45] with modifications described in the [Supplemental Experimental Procedures](#).

Determination of Protein Synthesis Rates

The rate of protein synthesis was measured by the incorporation of ³⁵S-methionine into trichloroacetic acid (TCA)-insoluble material as described previously [10]. Further details are provided in the [Supplemental Experimental Procedures](#).

Sucrose Gradient Density Centrifugation and RNA Detection

Sucrose density gradient analysis was carried out as described [10]. Full details are provided in the [Supplemental Experimental Procedures](#).

RNA Extraction

Total RNA extraction was performed using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Microarray Hybridization

The human cDNA microarrays were manufactured in Nottingham University. This custom cDNA microarray consists of 29,593 (32,448 total, including 2,855 control probes) oligonucleotide probes derived from MWG Human 30K slides A, B, and C. RNA from sucrose density gradient fractionation was

(E) Early cooling prolongs survival in prion-infected mice but is abolished by knockdown of RTN3. Kaplan-Meier plot; n = 25 cooled mice (gray line); n = 17 not cooled (black line); n = 14 cooled + shRNA of RTN3 (light brown line). One-way ANOVA with Tukey's post-test was used for multiple comparisons; not cooled versus cooled mice ***p < 0.001; not cooled versus cooled + shRNA of RTN3 n.s.; cooled mice versus cooled + shRNA of RTN3 *p < 0.05. See also [Figures 5C and 5D](#).

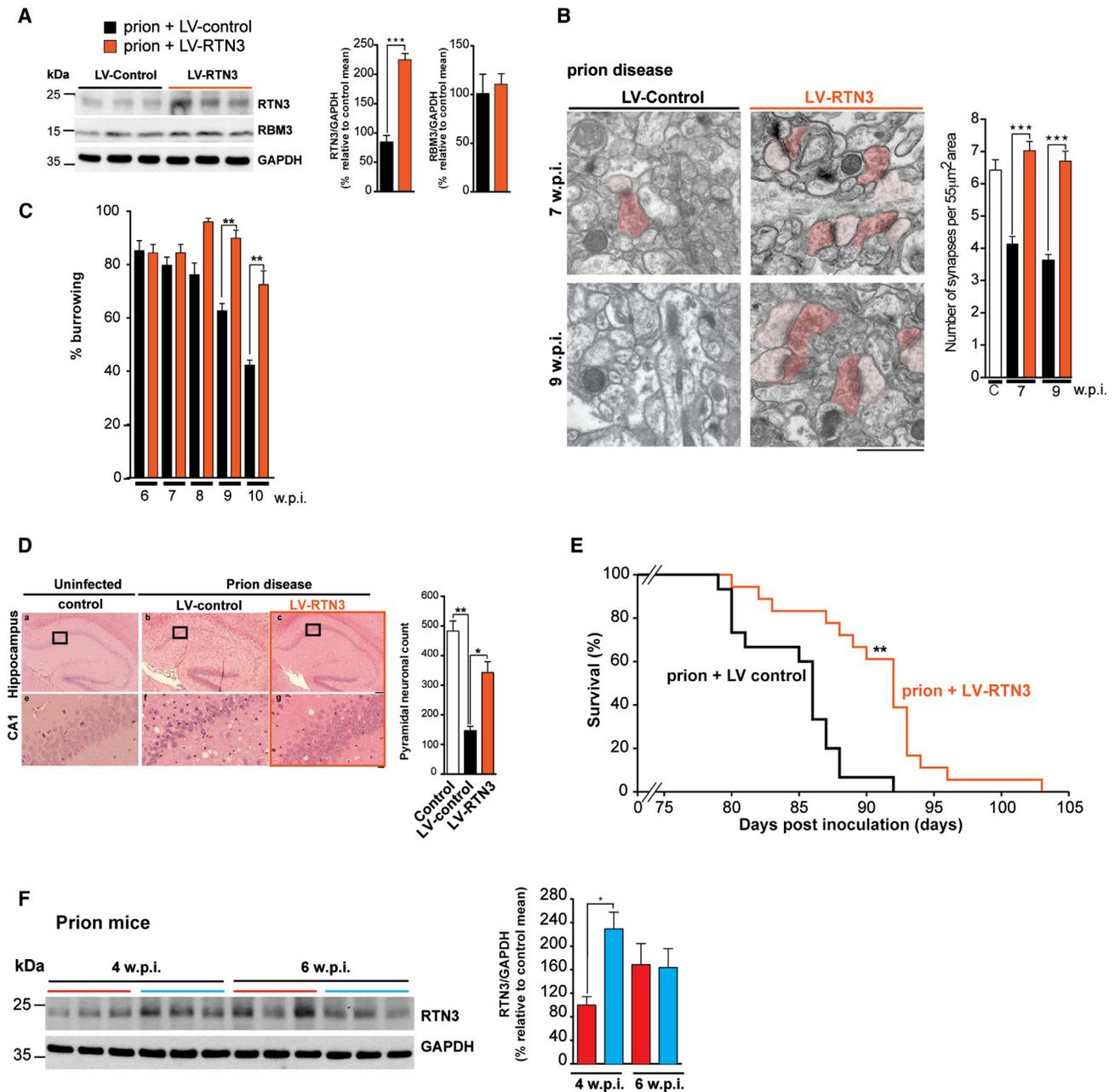


Figure 6. Enhanced RTN3 Expression Is Sufficient to Protect against Prion Disease in the Absence of Cooling

(A) LV-RTN3 delivery to hippocampi of prion-infected mice increases RTN3 in the absence of cooling compared to control lentiviral treatment (LV-control) and endogenous RBM3 (remain constant). $n = 6$ mice LV-control and 6 mice LV-shRNA-RTN3. Representative western blots and bar graphs quantification are shown.

(B) LV-RTN3 protected the deficit in synapse loss in prion-infected mice at 7 and 9 w.p.i. Representative electron micrographs are shown, pseudo-colored for ease of synapse identification: presynaptic, dark pink; postsynaptic, light pink. Bar chart quantification is shown. $n = 93$ images from three animals per condition. Data represent mean \pm SEM; t test; *** $p < 0.0001$. The scale bar represents 1 μ m.

(C) RTN3 overexpression prevented the decline in burrowing behavior of prion-infected mice. Food pellets remaining in the tube were measured after 2 hr and are expressed as percentage burrowed. Graph bar with prion (black bars; $n = 14$ mice) and prion + LV-RTN3 (orange bars; $n = 20$ mice) is shown. Kruskal-Wallis test with Dunn's multiple comparisons test; ** $p < 0.01$.

(D) Representative images of H&E-stained hippocampal sections from uninfected control, prion-infected mice with LV-control, and prion-infected mice with LV-RTN3. The graph bar shows quantification of the average number of neurons for each condition in the CA1 area of hippocampus. $n = 3$ mice (white bar), 5 mice (black bar), and 15 mice (orange bar). One-way ANOVA and Brown-Forsythe test with Tukey's post hoc analysis for multiple comparisons were used. *** $p < 0.001$. The scale bars represent 400 μ m (top row) and 50 μ m (bottom row).

(legend continued on next page)

pooled into subpolysomal or polysomal fractions, labeled, and hybridized to the arrays as described previously [10]. Microarray slides were scanned using a GenePix 4200B microarray scanner and GenePix Pro 6.0 software (Axon Instruments).

Analysis of Microarray Data

GenePix Pro 6.0 was used to quantify fluorescence intensities for individual spots on the microarray. All statistical analysis was performed in the statistical environment R, version 2.6.1, and the Limma package [46].

Northern Blot

Northern analysis was performed as described previously [10]. Visualization and quantification of northern blot analysis was performed using a Molecular Imager FX phosphorimager and ImageJ software.

RNA-Protein Complex Immunoprecipitation

Post-nuclear extracts were incubated with either anti-RBM3 antibody or immunoglobulin G (IgG)-coated protein G magnetic beads and processed as described in the [Supplemental Information](#).

Reverse Transcription and qPCR

Reverse transcription was carried out using random primers and Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer instructions. qPCR was carried out using SensiFAST SYBR Lo-ROX Kit (Bioline) according to manufacturer instructions. Primers used are in the [Supplemental Information](#).

Prion Infection of Mice

As described previously [37], hemizygous tg37 mice of both sexes were inoculated with 1% brain homogenate of Chandler/RML prions at 3–6 weeks of age. Control mice received 1% normal brain homogenate.

Induction of Hypothermia in Mice

FVB wild-type (WT) or hemizygous Tg37 mice weighing more than 20 g were cooled using 5' AMP as described [3]. Prion-infected mice were injected with lentiviruses at 2 w.p.i. and subsequently cooled at 3 and 4 w.p.i.

Lentiviruses and Mice Stereotaxic Surgery

GenTarget generated lentiviral plasmids. Viruses were injected stereotaxically into the CA1 region of the hippocampus as described [3]; additional information is provided in the [Supplemental Information](#).

Burrowing

Burrowing was performed as described [3]. Briefly, mice were placed in a cage with a tube full of pellets, which they “burrowed.” The extent of burrowing was assessed by the weight of pellets displaced in 2 hr.

Histology

Paraffin-embedded brains and pancreases were sectioned at 5 μ m and stained with H&E as described [38, 39]. Neuronal counts were determined by quantifying NeuN-positive pyramidal CA1 neurons as described [39]. Synapses were counted in electron microscopy (EM) images of the stratum radiatum of the hippocampal CA1 region, blind. A synapse was defined as a structure with synaptic vesicles, synaptic cleft, and post-synaptic density, as described [3].

Computational Modeling

Translation elongation rates on human mRNAs were estimated using a published computational model [24]. The model was re-parameterized for the human decoding system using relative total tRNA abundances from [47] and

a total tRNA concentration in HEK293 cells of 5.6 pg per cell, which was determined by comparing the staining intensity of the tRNA band in total RNA preps from HEK293 cells to the intensity of bands generated with known amounts of commercial yeast tRNA. Individual tRNA selection and translocation reactions were modeled in PRISM [48] using rate constants [49] and tRNA ratios for individual codons [24] as published.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is ArrayExpress: E-MTAB-5437.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2017.01.047>.

AUTHOR CONTRIBUTIONS

A.B., D.P., J.R.P.K., S.G., X.P., J.R., A.R., and D.V. conducted the experiments. R.V.S. and T.S. analyzed the data. T.v.d.H. generated the computational model and analyzed decoding speeds. A.E.W., M.B., G.R.M., and C.M.S. designed the experiments and wrote the paper.

ACKNOWLEDGMENTS

The work was funded by the BBSRC (BB/019790/1; A.B., A.R., J.R., J.R.P.K., and R.V.S.), MRC (D.P., programme grant, 5TR50 to G.R.M.; S.G., programme funding, 5TR00 to A.E.W.), and Wellcome Trust (2010847/Z/16/Z; S.G., collaborative award to A.E.W., G.R.M., C.M.S., and T.v.d.H.).

Received: October 17, 2016

Revised: December 21, 2016

Accepted: January 23, 2017

Published: February 23, 2017

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(E) LV-RTN3 significantly lengthened survival of prion-infected mice. Kaplan-Meier plot (orange; n = 18) compared to LV-control prion-infected mice (black; n = 15); t test; **p < 0.01.

(F) Induction of RTN3 fails at 6 w.p.i. in prion-diseased mice. Blue lines above the western blots are samples from cooled mice, whereas red lines denote control mice. Bar graphs show quantification of RTN3 levels relative to GAPDH at 4 and 6 w.p.i. prion disease, blue bars represent quantification from cooled mice, and red bars from control mice. n = 9 mice per condition. *p < 0.05. Data are mean \pm SEM.

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