



## A novel translational control mechanism involving RNA structures within coding sequences

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1 **A novel translational control mechanism involving RNA structures within coding sequences**

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21  
22 **Running title:** Dhh1 drives translation of structured mRNA CDSs

23 **Keywords:** Dhh1 / helicase / viral RNA / BMV / translational regulation / RNA secondary structure

29 **Abstract**

30 The impact of RNA structures in coding sequences (CDS) within mRNAs is poorly understood. Here  
31 we identify a novel and highly conserved mechanism of translational control involving RNA  
32 structures within coding sequences and the DEAD-box helicase Dhh1. Using yeast genetics and  
33 genome-wide ribosome profiling analyses we show that this mechanism, initially derived from studies  
34 of the *Brome Mosaic virus* RNA genome, extends to yeast and human mRNAs highly enriched in  
35 membrane and secreted proteins. All Dhh1-dependent mRNAs, viral and cellular, share key common  
36 features. First, they contain long and highly structured CDSs, including a region located around  
37 nucleotide 70 after the translation initiation site, second, they are directly bound by Dhh1 with a  
38 specific binding distribution and third, complementary experimental approaches suggest that they are  
39 activated by Dhh1 at the translation initiation step. Our results show that ribosome translocation is not  
40 the only unwinding force of CDS and uncover a novel layer of translational control that involves RNA  
41 helicases and RNA folding within CDS providing novel opportunities for regulation of membrane and  
42 secretome proteins.

43 **Introduction:**

44 Structural features in messenger RNA (mRNA) regulate its localization, translation and degradation.  
45 The dynamic folding of mRNA into secondary and tertiary structures guides the interaction with  
46 proteins and RNAs that ultimately directs mRNA fates. Yet, the elucidation of such structures and  
47 their functional implications remain elusive. Recent advances in next-generation sequencing (NGS) in  
48 combination with nuclease treatments or chemical probing allowed, for the first time, experimental  
49 genome-wide measurements of RNA secondary structures. These seminal studies have uncovered  
50 novel evolutionary conserved structural patterns and their nexus to translational control (Mortimer et  
51 al. 2014). A surprising discovery was that the CDSs of *Saccharomyces cerevisiae* mRNAs present a  
52 higher degree of secondary structures than untranslated regions (UTRs) *in vitro* (Kertesz et al. 2010).  
53 Subsequent studies demonstrated that CDSs are significantly less structured or, alternatively, more  
54 structurally dynamic *in vivo* than *in vitro* (Rouskin et al. 2014). This remodeling is energy-dependent,  
55 as depletion of ATP results in recovery of the structure, mimicking the *in vitro* results. How this *in*  
56 *vivo* remodeling is achieved and what functional implications it represents are poorly understood.  
57 Ribosome translocation during translation elongation has been traditionally considered to drive CDS  
58 unwinding since strand separation activity is inherent to the ribosome, requiring no exogenous  
59 helicases (Takyar et al. 2005). However, this activity might not be the only unwinding force *in vivo*, as  
60 high ribosome occupancy within coding regions is not associated with lower structure propensities  
61 (Rouskin et al. 2014).

62

63 Strong candidates for factors directing mRNA unfolding *in vivo* are ATP-dependent helicases. From  
64 these, the large DEAD-box family of RNA helicases is involved in all cellular processes that require  
65 RNA remodeling, such as transcription, pre-mRNA splicing, ribosome biogenesis and RNA decay  
66 (Martin et al. 2013; Jarmoskaite and Russell 2014). In addition to ATP-dependent RNA unwinding  
67 activities, DEAD-box RNA helicases promote RNA duplex formation, serve as assembly platforms  
68 for the formation of large RNP complexes and displace proteins from RNA (Linder and Jankowsky  
69 2011). Dhh1 is a highly evolutionary conserved member of the family of DEAD-box RNA helicases.  
70 The conservation is such that, in yeast, Dhh1 can be functionally replaced by its counterparts from

71 *Drosophila melanogaster* (Me31B), *Xenopus laevis* (Xp54) or humans (DDX6) (Maekawa et al. 1994;  
72 Tseng-Rogenski et al. 2003; Westmoreland et al. 2003; Alves-Rodrigues et al. 2007). Dhh1 functions  
73 as a translational repressor, and as a decapping activator in the major deadenylation-dependent 5'-3'  
74 mRNA decay pathway (Presnyak and Coller 2013). Former *in vitro* studies suggested that Dhh1  
75 represses mRNA translation by acting on translation initiation and translation elongation (Coller and  
76 Parker 2005; Franks et al. 2008; Sweet et al. 2012). In humans, DDX6 functions as well in promoting  
77 miRNA-mediated repression via association with AGO1, AGO2 and the CCR4-NOT complex (Chu  
78 and Rana 2006; Chen et al. 2014; Mathys et al. 2014).

79

80 In contrast to the well-established Dhh1 repressing functions, we and others have found that Dhh1  
81 activates translation of viral RNA genomes. By using a model system that allows the replication of the  
82 plant *Brome mosaic virus* (BMV) in yeast, we have previously shown that Dhh1 depletion  
83 dramatically inhibits BMV RNA translation (Alves-Rodrigues et al. 2007). Moreover, this role is  
84 extended to its human homolog DDX6 since first, in the yeast model system it replaces Dhh1 to  
85 promote BMV RNA translation and second, it promotes translation of the human *Hepatitis C virus*  
86 (HCV) RNA genome in hepatoma cell lines (Alves-Rodrigues et al. 2007; Scheller et al. 2009; Huys  
87 et al. 2013). The mechanism for translational activation by Dhh1 is unclear, but it should not involve  
88 the cap structure because unlike BMV RNA, HCV RNA is translated via an IRES-dependent  
89 mechanism. BMV and HCV belong to the large group of positive-strand RNA viruses, whose RNA  
90 genomes are highly structured. Throughout infection, positive-strand RNA genomes display three  
91 mutually exclusive functions. They first function as mRNAs for expression of the viral proteins, later  
92 as templates for RNA replication and subsequently as genomes for encapsidation of new particles.  
93 Profound remodeling steps of the viral ribonucleoprotein complex are temporarily required to  
94 coordinate these essential transitions that are poorly understood. Given the dynamic folding properties  
95 of viral RNA genomes and the fact that viruses hijack processes already existing in the host, we  
96 hypothesized that the positive role of Dhh1 in viral RNA translation is linked to specific RNA folding  
97 regulations that may be extended to a specific set of cellular mRNAs. By combining viral studies in  
98 the BMV/yeast system with high-throughput RNA-seq, ribosome profiling and CRAC (UV

99 crosslinking and analysis of cDNA) of host mRNAs, here we uncover an additional layer of  
100 translational control involving an RNA helicase and RNA folding within CDSs that is seemingly  
101 conserved from yeast to humans and hijacked by viruses.

102 **Results:**103 **Dhh1 ATPase activity promotes translation of BMV RNA2**

104 Dhh1 has been well described as a translational repressor and a decapping activator, however, by  
105 using the ability of BMV RNA to translate and replicate in the yeast *Saccharomyces cerevisiae* we  
106 have previously shown that Dhh1 promotes translation of the BMV RNA2 (Mas et al. 2006). BMV  
107 RNA2 is 5'capped and contains a tRNA-like structure instead of a poly(A)-tail at the 3'end (Noueiry  
108 and Ahlquist 2003). To elucidate which Dhh1 features are required for this unexpected function, we  
109 introduced several characterized point mutations in multiple conserved motifs (Cheng et al. 2005;  
110 Dutta et al. 2011) and studied their *in vivo* effect on both yeast viability and BMV RNA2 translation  
111 (Fig. 1). The mutants were named based on the mutated motif (described in Fig. 1A and Sup. Fig.  
112 S1A). A single-copy plasmid expressing each mutant from the endogenous *DHH1* promoter was  
113 transformed into the *dhh1Δ* yeast strain. Expression levels of all Dhh1 mutant proteins were similar to  
114 that of wild type Dhh1 (Sup. Fig. S1B). First, we examined the effect of Dhh1 mutations in cell  
115 viability by studying cell growth at 30°C and 36°C (Fig. 1B). Confirming previous results for other  
116 yeast genetic backgrounds (Cheng et al. 2005; Dutta et al. 2011), all mutants failed to complement the  
117 *dhh1Δ* thermosensitive phenotype, except mutant 1A, 3 and 6B, which are described not to affect  
118 Dhh1 remodeling activity (Cheng et al. 2005; Dutta et al. 2011). Importantly, a similar pattern was  
119 observed when we tested the effect of the Dhh1 mutants on BMV RNA2 translation (Fig. 1C). As  
120 steady-state BMV RNA2 levels were not affected, we conclude that the observed differences in 2a  
121 expression are due to translation defects. Together, these results indicate that similar Dhh1  
122 features, including the ATPase activity, are required for both cell viability and BMV RNA2 translation.

123

124 A plausible explanation for these effects is that Dhh1 promotes translation of BMV RNA2 by  
125 remodeling its structure. If so, Dhh1 and RNA2 must interact. To test this possibility we carried out  
126 RNA-co-immunoprecipitation experiments (RIP) using a Flag-tagged Dhh1 (Fig. 1D). Addition of the  
127 Flag-tag did not affect Dhh1 function in BMV RNA2 translation (Sup. Fig. S1C). As a control, Pat1, a  
128 known Dhh1-interacting protein (Coller et al. 2001; Nissan et al. 2010), was co-immunoprecipitated  
129 (Fig. 1D). BMV RNA2, detected by quantitative PCR, was enriched ~9-fold in Dhh1-Flag eluates

130 compared to the untagged Dhh1 control, indicating that Dhh1 interacts with viral RNA2 (Fig. 1D). To  
131 test whether the detected interaction is direct or mediated by another protein we used CRAC analysis  
132 (Bohnsack et al. 2009; Bohnsack et al. 2012). This method allows for a rapid and accurate  
133 identification of protein binding sites on RNA. Dhh1 directly bound at three sites in the CDS and one  
134 in the 3'UTR, namely within the tRNA-like structure (Sup. Fig. S1D). Given that Dhh1 is a decapping  
135 activator and BMV RNA2 is 5' capped, the detected binding might be related to decapping and not to  
136 the translation activation mechanism. To rule this out, we determined whether Dhh1 affects the  
137 stability of RNA2, as expected should decapping be operative. BMV RNA2 was expressed in WT and  
138 *dhh1Δ* strains from an inducible *GAL* promoter whose activity is repressed in the presence of glucose  
139 in the media. After adding glucose, RNA2 levels were followed over 60 minutes by Northern blot  
140 analysis (Fig. 1E). The half-life of RNA2 in *dhh1Δ* cells was not significantly affected when compared  
141 to that in WT cells, indicating that Dhh1 is not involved in BMV RNA2 decapping or decay.  
142 Altogether, the data suggest a direct role of Dhh1 and its ATPase activity in BMV RNA2 translation.

143

#### 144 **Depletion of Dhh1 shifts BMV RNA2 towards fractions containing single ribosomal subunits**

145 To determine which step of BMV RNA2 translation is promoted by Dhh1, we carried out polysome  
146 profiling analyses in WT and *dhh1Δ* cells expressing BMV RNA2. Absorbance measurements  
147 indicated that the average monosome to polysome (M/P) ratio in WT and *dhh1Δ* cells was similar,  
148 showing that the absence of Dhh1 does not affect polysome profiles, as previously reported (Fig. 2A)  
149 (Coller and Parker 2005). Northern blot of RNA2 along the polysome profile showed that depletion of  
150 Dhh1 shifted RNA2 towards monosomal, 60S and 40S fractions (Fig. 2B and Sup. Fig. S2).

151 Puromycin, a drug that releases elongating ribosomes, is routinely used to confirm that mRNAs  
152 located in polysomal fractions are associated to ribosomes and not to merely heavy RNP that do not  
153 contain ribosomes (Thermann and Hentze 2007). As puromycin and similar drugs do not work in yeast  
154 due to poor uptake (Melcher 1971; Schindler and Davies 1975) we inhibited global translation with 15  
155 min of glucose withdrawal to test whether RNA2 is associated to ribosomes (Fig. 2C). Glucose  
156 deprivation inhibits translation initiation, thus if RNA2 is associated with elongating ribosomes a shift  
157 of RNA2 out of polysomes should be observed due to ribosome run-off and the inhibition of initiation.

158 Glucose withdrawal led in WT and *dhh1* $\Delta$  cells to a comparable shift of RNA2 out of the polysomal  
159 fractions towards the free fractions and the monosomal fractions (Fig. 2D). Together, these results  
160 suggest that Dhh1 promotes translation initiation of RNA2. In agreement with this conclusion,  
161 immunoprecipitation analyses showed that Dhh1 binds to the translation initiation factors eIF4E,  
162 eIF4A and eIF4G in an RNase-resistant manner (Fig. 2E).

163

#### 164 **A stem-loop within the CDS of RNA2 confers dependence on Dhh1 for translation**

165 To identify which BMV RNA2 regions confer Dhh1-dependence for translation we replaced different  
166 RNA2 segments by alternative sequences and quantified 2a and RNA2 levels in the presence and  
167 absence of Dhh1 (Fig. 3A). The 5' and 3'UTRs of BMV RNA2 are highly structured sequences with  
168 overlapping *cis*-acting signals essential for RNA2 translation and replication (Noueiry and Ahlquist  
169 2003). The BMV 5'UTR was replaced by that of the *GALI* transcript and the non-polyadenylated  
170 3'UTR by the polyadenylated 3'UTR of the *ADHI* transcript. Translation of the natural RNA2  
171 exhibited a 12-fold dependence on Dhh1. Replacing either the 5'UTR or the 3'UTR decreased RNA2  
172 Dhh1-dependence to two-fold (Fig. 3A). Similarly, the concurrent replacement of both UTRs  
173 decreased Dhh1-dependence to two-fold. Interestingly, replacing the CDS by that of GFP also resulted  
174 in a two-fold Dhh1-dependence (Fig. 3A). An RNA derivative lacking RNA2 sequences did not  
175 depend on Dhh1 for translation, strengthening the specificity of Dhh1 for the viral RNA. Thus, full  
176 dependence on Dhh1 for RNA2 translation requires the concerted action of 5'UTR, 3'UTR and CDS.

177 To investigate how replacements of the different RNA2 segments affect Dhh1 binding, we carried out  
178 RIP in *dhh1* $\Delta$  cells expressing the different RNA2 constructs plus Dhh1-Flag or Dhh1. In line with the  
179 CRAC results indicating crosslinking of Dhh1 to the CDS and the 3'UTR of BMV RNA2,  
180 replacement of the CDS or the 3'UTR region by the CDS of GFP or by the polyadenylated 3'UTR of  
181 the *ADHI* transcript reduced the amount of RNA2 co-immunoprecipitated while replacement of the  
182 5'UTR region by that of the *GALI* transcript did not have any significant effect (Fig. 3B and Sup. Fig.  
183 S3). Given that Dhh1 interacts with components of the cap binding complex (Fig. 2E), it is likely that  
184 Dhh1 binds to both the 5'UTR of BMV RNA2 and that of *GALI* mRNA through interactions with the

185 cap-binding complex. However, such interaction would be only required for translation in the context  
186 of RNA2 sequences.

187

188 We next focused on the CDS because *cis*-acting sequences inside the CDS regulating translation have  
189 been rarely described. To investigate further which region of the CDS is responsible for Dhh1-  
190 dependence, we generated a construct in which the CDS of RNA2 was fused to that of *Renilla*  
191 Luciferase (RLUC) and obtained derivatives with successive deletions in the RNA2 CDS. The  
192 reporter construct containing the complete CDS of RNA2 (FL-RLUC) exhibited a five-fold Dhh1-  
193 dependence for RLUC activity (Fig. 3C). Given the very low expression level of 2a protein in *dhh1Δ*  
194 cells, the difference with the 12-fold Dhh1-dependence observed for WT RNA2 is likely related to the  
195 higher sensitivity of luciferase measurements when compared to western blot analysis. The 330-RLUC  
196 derivative shows a similar Dhh1-dependence to FL-RLUC, indicating that the *cis*-element conferring  
197 dependence on Dhh1 is located within these first 330 nucleotides. Given that Dhh1 is a helicase, we  
198 explored whether there were structured elements within these 330 nucleotides. Indeed, the Vienna  
199 RNA-fold package predicted the formation of a stem-loop structure at nucleotide 42-85 after the  
200 initiation codon ( $\Delta G = -11.1$  kcal/mol) (Fig. 3D, left). To determine whether this stem-loop was  
201 responsible for Dhh1-dependence, we shortened the RNA2 CDS to 87 and 42 nucleotides to generate  
202 constructs containing or lacking the stem-loop (Fig. 3C). While 87-RLUC maintained full Dhh1-  
203 dependence, this dependence was decreased to two-fold in 42-RLUC (Fig. 3C). This two-fold  
204 difference was also observed in a construct in which the complete RNA2 CDS was deleted (0-RLUC)  
205 and is mediated by the UTRs (Fig. 3C). The importance of nucleotides 42 to 87 in translational  
206 regulation was also visualized when comparing RLUC activity of the different RNA2 derivatives in  
207 WT cells (Fig. 3E). Deletion of the 42-87 sequence led to a large increase of RLUC activity indicating  
208 its repressing function. Nucleotides 87 to 330 played also an important role in translation, however  
209 this effect was independent from Dhh1 activity (Fig. 3C). Collectively, these data indicate that  
210 nucleotides 42 to 87 repress translation, a repression that is moderated by Dhh1. To determine whether  
211 the sequence or the predicted structure within nucleotides 42-87 mediates Dhh1-dependence, we  
212 generated two RNA2 derivatives in which (i) the stem-loop was disrupted by replacing all bases on

213 one side of the stem with complementary ones, and (ii) the stem-loop was replaced by a structurally  
214 equivalent stem-loop with no sequence-homology which was designed using RNAiFold (Fig. 3D,  
215 right) (Garcia-Martin et al. 2013). Disruption of the stem-loop decreased Dhh1-dependence to that  
216 found in 0-RLUC (Fig. 3F). Importantly, Dhh1-dependence was recovered when the stem-loop was  
217 replaced by the designed one, as similar values were obtained with this and 87-RLUC RNA2, the  
218 derivative containing the minimal sequence conferring full Dhh1-dependence. These data indicate that  
219 the stem-loop structure within the RNA2 CDS strongly inhibits translation and mediates the  
220 dependence on Dhh1 for translational stimulation.

221

## 222 **Dhh1 drives translation of a selected set of cellular mRNAs**

223 Viruses are powerful tools to uncover cellular processes. In fact, most post-transcriptional processes  
224 were first described in viral studies and then confirmed in cellular mRNAs (Cullen 2009). To  
225 investigate whether Dhh1 can also drive translation of specific cellular mRNAs, we combined three  
226 high-throughput analyses in WT and *dhh1* $\Delta$  strains: RNA-seq, ribosome profiling and CRAC (Fig.  
227 4A). Ribosome profiling combined with RNA-seq provides a snapshot of the translational status of the  
228 genome on a transcriptome-wide level and is based on isolating and sequencing ribosome-protected  
229 fragments (Ingolia et al. 2012). We performed three independent ribosome profiling and RNA-seq  
230 experiments in *dhh1* $\Delta$  cells and were able to align sequence reads to 5424 genes of *S.cerevisiae*. Both  
231 ribosome profiling and RNA-seq results were highly reproducible between biological replicates for  
232 each strain ( $r \approx 0.99$ ). We compared these data to RNA-seq and ribosome profiling data from WT cells  
233 (Nedialkova and Leidel 2015). Regarding the mRNA levels, in the absence of Dhh1 1467 genes did  
234 not exhibit significant changes, whereas 2177 have increased and 1780 decreased levels (Fig. 4B).  
235 Given the long established function of Dhh1 on mRNA decay, an increase in mRNA levels is expected  
236 but a decrease might seem paradoxical. However, it is important to note that Dhh1 also plays a direct  
237 role in transcription of some mRNAs (Haimovich et al. 2013). The ribosome protected fragment  
238 (RPF) analysis shows that 1841 genes did not exhibit significant changes, while 1775 have decreased  
239 and 1808 increased ribosome occupancy (Fig. 4B). To study translation effects we focused on  
240 transcripts where RPF levels but not mRNA levels are changed or where changes in the RPF and

241 mRNA levels go in opposite directions. With this we ensure that the selected mRNAs are  
242 translationally regulated. Following these criteria we identified 492 mRNAs with increased translation  
243 rates in *dhh1* $\Delta$  cells, indicating that Dhh1 represses their translation (Sup. Table S1). The  
244 identification of these mRNAs was not surprising as Dhh1 has been widely studied as a repressor of  
245 translation. Excitingly, we identified 538 additional mRNAs with decreased translation rates in *dhh1* $\Delta$   
246 cells, indicating that Dhh1 drives their translation in WT cells (Fig. 4B) (Sup. Table S1). Selected  
247 mRNAs from these two groups were validated to be translationally regulated by Dhh1 by Western blot  
248 and qPCR analysis (Sup. Fig. S4A-C).

249

250 Dhh1 may drive translation of the identified mRNAs by a direct or indirect mechanism. To select the  
251 mRNAs directly interacting with Dhh1 we performed two independent CRAC experiments that  
252 showed a high correlation ( $r = 0.90$ ) (Sup. Fig. S5). In total 183 (37%) of the translationally repressed  
253 mRNAs and 245 (46%) of the translationally activated mRNAs were specifically crosslinked to Dhh1  
254 (Fig. 4C, Sup. Table S1). Most mRNAs were crosslinked in the CDS and additionally in the 5' and/or  
255 3'UTR (Fig. 4D, Sup. Table S2). To test whether the Dhh1 crosslinking position defines its function in  
256 translation regulation we determined the specific crosslinking sites for all crosslinked genes in the  
257 different subsets by calculating the CRAC read distribution across a virtual CDS (Fig. 4E) or per  
258 nucleotide (Sup. Fig. S6). The mRNAs not translationally regulated by Dhh1 exhibit a low and largely  
259 uniformly distributed binding pattern. In contrast, mRNAs regulated by Dhh1 exhibit higher Dhh1  
260 crosslinking particularly around the initiation codon and before the stop codon, with crosslinking  
261 around the initiation codon much higher in translationally-repressed mRNAs. These different  
262 crosslinking patterns may reflect the versatile function of Dhh1 in translational control.

263

264 Ribosome profiling enables to detect defects in specific translation steps. Ribosome pausing increases  
265 the likelihood of capturing a footprint by sequencing and hence defects in translation elongation will  
266 result in a peak in ribosome density. In contrast, defects in translation initiation will result in a lower  
267 RPF count throughout the complete CDS with no peaks in ribosome densities. We monitored  
268 ribosome pausing in different mRNA subsets by calculating the observed versus expected RPF reads

269 (Ingolia et al. 2009) across a virtual CDS and plotted its distribution for the different sets of genes in  
270 WT and *dhh1Δ* cells (Fig. 4F). From hereafter, “activated” only includes mRNAs translationally  
271 activated and crosslinked to Dhh1, “repressed” only mRNAs translationally repressed and crosslinked  
272 to Dhh1 and “unchanged” mRNAs with no changes at the mRNA and RPF level regardless of the  
273 crosslinking to Dhh1. In agreement with a role of Dhh1 on translation initiation, no significant  
274 differences were observed in WT and *dhh1Δ* cells for none of the different subsets. Similar results  
275 were obtained when focusing on the first 120 nucleotides versus the last ones (Sup. Fig. S7).

276

### 277 **Cellular mRNAs translationally activated by Dhh1 contain long, highly structured CDSs**

278 To further understand the underlying mechanism of Dhh1 function in translation regulation, we  
279 studied the physical properties of the specific mRNA subsets. First, we calculated the average length  
280 of the CDS and the 5' and 3'UTRs for mRNAs translationally activated, repressed or not affected by  
281 Dhh1. Translationally activated mRNAs have on average a two-fold longer CDS than repressed or not  
282 affected mRNAs (Fig. 5A). However, no significant length differences were detected between mRNA  
283 subsets for 5' and 3'UTRs. It was previously shown that longer CDSs present lower ribosome density  
284 (Arava et al. 2003). This decreased translation efficiency might result from the fact that the folding  
285 probability increases with the length of the CDS. To explore this possibility we calculated the intrinsic  
286 tendency to form secondary structures using the published parallel analyses of RNA structure (PARS)  
287 (Kertesz et al. 2010). In this pioneering study, PARS scores were obtained by coupling deep  
288 sequencing with enzyme probing of paired and unpaired nucleotides of isolated yeast total mRNAs.  
289 We calculated the mean PARS score for the CDS and the UTRs of the different mRNA subsets and  
290 “all” genes. “All” includes all mRNAs for which the PARS score has been published. Importantly,  
291 translationally activated mRNAs present higher mean PARS values in the CDS (0.49) than all genes  
292 (0.37) and than those mRNAs translationally repressed (0.33) or not affected (0.27), with these  
293 differences being highly significant ( $p \leq 0.0001$ ) (Fig. 5B). Noteworthy, both repressed and not affected  
294 genes have a lower mean PARS score in the 5'UTR (0.05 and 0.08, respectively) than all genes and  
295 translationally activated mRNAs (both 0.17). A plausible explanation for this observation would be  
296 that mRNAs not affected by Dhh1 do not require other RNA helicases whereas the majority of all

297 cellular mRNAs does require the action of RNA helicases. No significant differences were observed  
298 among PARS values of 3'UTRs.

299

300 Next, we analyzed the PARS score distribution across a virtual gene in which values are averaged over  
301 percentage bins (25% in the UTRs and 5% in the CDS) for the three subsets of mRNAs and all  
302 mRNAs (Fig. 5C). Although mRNAs translationally activated by Dhh1 had significantly higher PARS  
303 values throughout the complete 5'UTR and CDS, one of the biggest and more statistically significant  
304 differences between activated and repressed or not affected mRNAs was observed at the 7.5-15%  
305 region of the metagenome (Fig. 5C and Sup. Table S3 A). To identify the nucleotides involved we  
306 plotted the PARS score versus the nucleotide position (Fig. 5D). One of the biggest and more  
307 statistically significant differences between the activated and the repressed/unchanged mRNAs was  
308 found between nucleotides 50-120 after the initiation codon (Sup. Table S3 B). Remarkably, this area  
309 overlaps with the location of the mapped stem-loop in BMV RNA2 (nucleotides 42 to 87).

310

311 Together these results show that Dhh1 is required to drive translation of a specific subset of cellular  
312 mRNAs that as the BMV RNA harbor highly structured 5'UTRs and long and highly structured CDSs.

313

#### 314 **Dhh1 drives translation of mRNAs coding primarily membrane and secreted proteins**

315 Specific mRNA features might allow co-regulation of functionally related proteins. To determine  
316 whether the mRNAs translationally regulated by Dhh1 are functionally linked we performed Gene  
317 Ontology (GO) analysis (Fig. 5E and 5F). Genes translationally activated by Dhh1 were highly  
318 enriched for intracellular transport processes. Accordingly, GO terms under cellular components were  
319 enriched in ER and membranes (Fig. 5E). In contrast, translationally repressed genes were enriched  
320 for proteins involved in DNA-dependent DNA replication and vacuole fusion. Accordingly, the GO  
321 term enriched regarding the cellular component of this subset was the nuclear nucleosome (Fig. 5F).

322

323 The ER and the cytosol represent distinct biological environments for translation with different  
324 regulatory factors affecting protein expression. Secreted proteins are preferentially translated at the ER

325 by mechanisms that are still under intense study (Ast and Schuldiner 2013; Cui and Palazzo 2014; Jan  
326 et al. 2014; Reid and Nicchitta 2015). The canonical route is based on the co-translational recognition  
327 of a hydrophobic region in the mRNA by the signal recognition particle (SRP). This results in a  
328 repression of translation until the RNP reaches the ER. Less understood SRP-independent routes  
329 include ribosome-mediated mRNA targeting, post-translational targeting and RNA-based localization  
330 (Ast and Schuldiner 2013). Based on published predictions (Ast et al. 2013), from the identified Dhh1-  
331 dependent mRNAs, 45% utilize the SRP-dependent and 55% a SRP-independent pathway, indicating  
332 that Dhh1-mediated translational control functions alongside the SRP-dependent and -independent  
333 pathways.

334 A previous study described that mRNAs encoding secreted proteins display lower PARS scores in the  
335 5'UTR and the first 30 nucleotides of the CDS (Kertesz et al. 2010). This seems to be contradictory to  
336 our results as the subset of activated genes consists to ~30% of secreted proteins and is characterized  
337 by a higher PARS score. When we analyzed the PARS score distribution per metagene and per  
338 nucleotide of the secreted genes in the activated gene set (Sup. Fig. S8 A and B), we observed that  
339 these mRNAs exhibit an even higher PARS score in 5'UTR and CDS, including the first 30  
340 nucleotides, than those from the complete subset of translationally activated mRNAs. Thus, this  
341 specific group of secreted proteins harbors unique features that might correlate with their dependence  
342 on Dhh1 for translation.

343

#### 344 **Dhh1 functions as a translational activator in humans.**

345 Dhh1 is highly conserved from yeast to humans. We have previously observed that DDX6, the Dhh1  
346 human counterpart, drives translation of *Hepatitis C virus* RNA in human hepatoma cell lines  
347 (Scheller et al. 2009). To approach whether this function is extended to human mRNAs, we first  
348 studied the physical properties of the human homologs of the yeast mRNAs translationally activated  
349 by Dhh1. They contained as their yeast counterparts long CDSs. In contrast to yeast, human mRNAs  
350 have been described not to contain on average highly structured CDSs (Wan et al. 2014). Interestingly,  
351 when we calculated the mean PARS score distribution using the PARS data set of the child described  
352 in (Wan et al. 2014), we identified in the human mRNA homologs of the yeast mRNAs

353 translationally-activated by Dhh1, but not in the translationally-repressed ones, structured regions in  
354 the CDSs. Interestingly, one of them is located 60-100 nucleotides after the start codon (Fig. 6A), a  
355 location that overlaps with the one identified for yeast and viral mRNAs. Together, this suggests that  
356 DDX6 might drive translation of human mRNAs by similar mechanisms. As a proof of principle we  
357 focused on the *PTCH1* mRNA, the human counterpart of the yeast *NCR1* mRNA, because of its  
358 clinical importance, as its deregulation is a hallmark of pancreatic cancer. The *PTCH1* mRNA exhibits  
359 a strong peak in the PARS score distribution after the starting codon (Fig. 6B) and has a very long  
360 CDS, features we defined to be typical for mRNAs translationally activated by Dhh1. Indeed, *PTCH1*  
361 mRNA depended on DDX6 for translation. DDX6 depletion by siRNA-mediated silencing resulted in  
362 a decrease in PTCH1 protein level without affecting *PTCH1* mRNA level, indicating a positive role of  
363 DDX6 in translation of the *PTCH1* mRNA (Fig. 6C).

364 **Discussion:**

365 The impact of RNA secondary and tertiary structures within the CDS on gene expression is poorly  
366 understood. Here we describe a novel translation control mechanism conserved from yeast to humans  
367 and hijacked by viruses that involves the DEAD-box helicase Dhh1 and RNA structures within the  
368 UTRs and coding regions. Our results, first observed in a viral RNA and then extended to cellular  
369 mRNAs enriched in secreted proteins, are based in four major lines of evidence. First, the mRNAs  
370 present highly structured 5'UTRs and CDSs including a structured region located around 70  
371 nucleotides after the translation initiation site (nucleotides 42 to 87 for BMV RNA, nucleotides 50 to  
372 120 for yeast mRNAs and nucleotides 60 to 100 for human mRNAs). This was observed with PARS  
373 analyses and validated with mutational assays. Second, polysome and ribosome profiling suggest that  
374 Dhh1 drives translation at the translation initiation step. Consistent with a role in translation initiation,  
375 Dhh1 interacts with translation initiation factors, as observed in immunoprecipitation assays. Third, in  
376 contrast to its established role as a translational repressor and decapping activator, Dhh1 does not  
377 affect the steady-state level of the identified mRNAs, indicating that the role of Dhh1 in translation is  
378 not linked to its role in translation repression and decapping. Fourth, Dhh1 binds to mRNAs that  
379 depend on Dhh1 for translation with a different specificity than to those mRNAs that do not depend on  
380 Dhh1 for translation, as found with CRAC analyses, suggesting a link between binding and mode of  
381 action. Together, these results uncover a novel translational regulation mechanism driven by Dhh1 that  
382 involves RNA folding within CDSs.

383

384 Using the ability of BMV RNA to translate and replicate in yeast we find that not only the CDS but  
385 also both UTRs confer Dhh1-dependence for translation. Full dependence was only achieved when the  
386 three regions were present. This suggests a scenario in which Dhh1 remodels tertiary contacts  
387 involving the three regions. Our CRAC data identified crosslinking of Dhh1 to sequences in the  
388 3'UTR and CDS but not to the identified stem-loop (Sup. Fig. S1D). The low number of reads  
389 observed for the stem-loop sequence could be due to transient unwinding and therefore low steady-  
390 state interaction due to short residence of the helicase. Alternatively, Dhh1 might be required to  
391 remodel only some of the RNA helices involved in these interactions. In agreement with this model, a

392 recent study shows that DEAD-box helicases can disrupt tertiary contacts by binding a secondary  
393 structure only after it spontaneously loses its tertiary contacts. After binding, they use ATP to unwind  
394 the helix (Pan et al. 2014). Interestingly, the requirement for spontaneous dynamics implies a  
395 preference of DEAD-box helicases for less stable RNA structures, which are likely to experience  
396 greater dynamic fluctuations (Pan et al. 2014). One would expect such low stable structures to exist in  
397 mRNA CDSs given that vastly fewer structures are identified by RNA probing *in vivo* than *in vitro*  
398 (Rouskin et al. 2014). Thus, a similar Dhh1 mode of action involving tertiary contacts might be  
399 operating Dhh1-dependence of cellular mRNAs, given that they present high PARS values not only in  
400 the regions located around 70 nucleotides after the initiation codon but also across the CDSs and  
401 UTRs. Moreover, as for BMV RNA, we did not detect by CRAC analyses a direct Dhh1 binding to  
402 this region but to other sequences in the mRNA. Besides CDS RNA remodeling, Dhh1 might exert  
403 alternative or additional functions in translation. Our interesting observations that Dhh1 interacts with  
404 members of the eIF4F complex (Fig. 2E) and that both UTRs are involved in Dhh1-dependence  
405 suggest a model in which Dhh1 would help forming a 5'-3' closed loop to favor translation. This  
406 might be of special importance for BMV RNA2 as it contains a tRNA-like structure at its 3' end  
407 instead of a poly(A) tail, a major element that mediates circularization via PAB1 binding.

408

409 Given the high conservation of Dhh1 from yeast to mammals, it is plausible that its human counterpart  
410 DDX6 activates translation of certain human mRNAs by similar mechanisms. Indeed, we show that  
411 the human homologs of the yeast mRNAs translationally activated by Dhh1 share the highly  
412 structured region around nucleotide 70 of the CDS and as a proof of principle we demonstrated that  
413 DDX6 promotes translation of the *PTCH1* mRNA. Given the role of DDX6 in *PTCH1* mRNA  
414 translation, DDX6 overexpression would result in *PTCH1* overexpression, a feature typical of  
415 pancreatic cancer. Interestingly, DDX6 is overexpressed in several cancers (Nakagawa et al. 1999;  
416 Hashimoto et al. 2001; Miyaji et al. 2003; Lin et al. 2008; Sen et al. 2015; Taniguchi et al. 2015)  
417 opening the possibility that translational regulation by DDX6 plays an important role in malignant  
418 transformations.

419

420 The cellular mRNAs translationally activated by Dhh1 encode mainly secreted and membrane  
421 proteins. Many of these mRNAs have in common that they are preferentially translated at the ER.  
422 Curiously, BMV RNA2 has been described to accumulate at the ER in the presence of the viral  
423 recruitment protein 1a, and Dhh1 assists in this recruitment process (Chen et al. 2001; Mas et al.  
424 2006). Furthermore, deletion of a region in the RNA2 CDS containing the identified stem-loop  
425 decreases the recruitment rate (Chen et al. 2001). Similar to BMV, multiple positive-strand RNA  
426 viruses, including serious human pathogens such as HCV and the emerging *Dengue virus* and  
427 *Chikungunya virus*, replicate their RNA genome in ER-derived double membranes termed spherules  
428 (den Boon and Ahlquist 2010). The spherules function as organelle-like compartments that scaffold,  
429 protect and coordinate multiple facets of genome replication, expression and encapsidation. Given that  
430 most viral infection steps are associated to ER membranes, it will seem plausible that these viruses  
431 would ensure expression of viral proteins in the ER to facilitate their function. Besides the presence of  
432 transmembrane domains in some viral proteins, little is known about how viral mRNAs would be  
433 preferentially translated at the ER. Our results suggest that positive-strand RNA viruses hijack Dhh1  
434 not only to help in the recruitment process to the ER, but also to promote translation of viral  
435 messengers through RNA remodeling.

436

437 Translation at the ER is under intense study as neither the targeting nor the translation mechanism is  
438 completely understood (reviewed in Cui and Palazzo, 2014; (Ast and Schuldiner 2013; Reid and  
439 Nicchitta 2015)). In our analyses we identified 65 mRNAs coding secretome proteins (Ast et al. 2013)  
440 whose translation is promoted by Dhh1. Moreover, proximity-specific ribosome profiling analyses  
441 demonstrate that all these mRNAs are co-translationally targeted to and translated at the ER (Jan et al.  
442 2014). The targeting pathways involve SRP-dependent and SRP-independent mechanisms, suggesting  
443 that Dhh1 can function across pathways. Notably, the highly structured AUG-proximal CDS sequence  
444 located between nucleotides 50 to 120 in cellular mRNAs translationally activated by Dhh1 partially  
445 overlaps with two *cis*-acting ER targeting signals. One is the hydrophobic core domain recognized by  
446 SRP to halt translation (Meyer and Dobberstein 1980; Walter and Blobel 1981a; Walter and Blobel  
447 1981b; Walter et al. 1981; Meyer et al. 1982) and the other a non-optimal codon cluster of 35-40

448 codons located downstream of the SRP-binding site that promotes nascent-chain recognition by  
449 slowing down translation (Pechmann et al. 2014). The location of these three features (SRP  
450 recognition site, non-optimal codon cluster and highly structured region) within the initial portion of  
451 the CDS highlights the complexity of the information stored in the mRNA sequence for translation. As  
452 secreted and membrane proteins often contain aggregation-prone hydrophobic domains, this  
453 complexity might be required to ensure appropriate local translation and to avoid accumulation of  
454 toxic aggregates in the cytosol. Interestingly, additional structural RNA *cis*-signals in CDS might be  
455 guiding mRNA localization as genome-wide structurome studies (Kertesz et al. 2010) identified an  
456 increased structure in the CDS of mRNAs encoding proteins that localize in specific subcellular  
457 compartments, such as cell wall, vacuole and ER or that function in distinct metabolic pathways, such  
458 as glycolysis, organic acid and amine metabolic processes.

459

460 In our study we also identified a group of mRNAs that are translationally repressed by Dhh1 and  
461 enriched in mRNAs coding proteins involved in nuclear processes. We were surprised by the  
462 relatively low number of translationally repressed mRNAs, as Dhh1 is well-known as a general  
463 translational repressor. However, of note is that in those studies Dhh1 was overexpressed or tethered  
464 to the 3'UTR of an mRNA or cells were stressed (Caroll 2011, Sweet 2015, Collier 2005) while in our  
465 study Dhh1 was depleted and cells were grown to log phase under normal growth conditions.  
466 Likewise, previous Dhh1 crosslinking and immunoprecipitation (CLIP) analyses, a UV crosslinking  
467 technique similar to CRAC, were carried out under stress conditions (Mitchell et al. 2013). Despite  
468 these differences, 58% of the identified Dhh1-bound mRNAs are common in both conditions.

469

470 In conclusion, our findings bring to light a novel and layer of translational control that involves the  
471 DEAD-box RNA helicase Dhh1 and RNA folding within the CDS. Two other DEAD-box helicases,  
472 Ded1 and eIF4A, have recently been shown to jointly facilitate ribosome scanning through secondary  
473 structures at the 5'UTR of mRNAs (Sen et al. 2015). Thus, a complex cooperation between distinct  
474 DEAD-box helicases seems to be required for translation to proceed possibly allowing tight control  
475 over specific mRNA fates.

476 **Materials and methods:**

477

478 Yeast strains, molecular and cell biology techniques, list of plasmids, high-throughput methods and  
479 detailed computational analysis are described in Supplemental Methods. Unless indicated, all yeast  
480 growth and translation experiments were carried out under permissive conditions (30°C).

481

482 **Polysome profiling**

483 Polysome profiling was carried out as previously described (Noueiry et al. 2003) with slight  
484 modifications. Briefly, cells were grown from  $OD_{600} = 0.02$  to mid-exponential phase ( $OD_{600} \sim 0.4$ ) at  
485 30°C and treated for 1min with 100 µg/ml cyclohexamide (CHX). Cells were harvested by vacuum  
486 filtration, frozen in liquid nitrogen and pulverized under cryogenic conditions at 5 cps in a SPEX 6750  
487 Freezer/Mill (SPEX SamplePrep) in 20 mM Tris-HCl (pH=7.5), 100 mM NaCl, 10 mM  $MgCl_2$ , 1%  
488 Triton X100, 0.5 mM DTT, 100 µg/ml CHX. Extracts were purified by several centrifugation steps. 12  
489  $OD_{260}$  were applied to linear 10%-50% sucrose gradients and spun for 3h at 35000 rpm in a Beckman  
490 SW41 rotor. Fractions were collected and 30 ng of purified BMV RNA3 were added to each fraction  
491 before further processing to normalize for technical variations during the purification. RNA was  
492 purified and analyzed by Northern blot.

493

494 **Ribosome profiling**

495 Ribosome profiling was carried out as previously described (Ingolia et al. 2009; Ingolia et al. 2012;  
496 Nedialkova and Leidel 2015). Detailed information of the experimental procedure and details on  
497 ribosome profiling data analysis is available in the Supplemental Methods.

498

499 **CRAC experiments and analysis**

500 CRAC experiments were performed as previously described (Bohnsack et al. 2009; Bohnsack et al.  
501 2012) and analyzed using pyicoclip  
502 (<http://regulatorygenomics.upf.edu/Software/Pyicoteo/pyicoclip.html>). Detailed processing  
503 information is available in the Supplemental Methods.

504 **RNA secondary structure prediction**

505 All RNA secondary structure prediction and base pairing probability calculations were carried out by  
506 running RNAfold and RNAfold -p from the Vienna package, version 2.1.7 (Lorenz et al. 2011).  
507 Design of the mutant RNA2-BMV stem-loop was carried out using RNAiFold (Garcia-Martin et al.  
508 2013).

509

510 **Data access:**

511 The ribosome profiling, RNA-seq and CRAC data from this study have been submitted to the NCBI  
512 Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers  
513 GSE87888 and GSE87892, respectively. Ribosome profiling and RNA-seq data from WT have  
514 been uploaded under accession number GSE67387 (Nedialkova and Leidel 2015).

515

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527

528 **Disclosure declaration:**

529 The authors have no conflicts to declare.

530

531

532 **Figure legends**

533 **Figure 1.** Effect of multiple Dhh1 mutations on cell growth and on viral RNA translation. (A) Motifs  
534 and functional domains of the DEAD-box RNA helicase Dhh1. Number 1A to 6B indicate the location  
535 of the point mutations. (B) Growth at 30°C and 36°C of WT and *dhh1Δ* expressing various Dhh1  
536 mutant alleles. (C) Effect of Dhh1 mutants on BMV RNA2 translation. The *dhh1Δ* strain was  
537 transformed with a plasmid expressing RNA2 together with an empty plasmid or a plasmid expressing  
538 *DHH1* or the different *dhh1* mutant alleles. RNA2 and protein 2a were analyzed by Northern and  
539 Western blot, respectively. As a control for equal loading and sample quality 18S rRNA and  
540 phosphoglycerate kinase protein 1 (Pgk1) were also analyzed. The average 2a expression value from  
541 at least three independent experiments is shown. The average value obtained for cells expressing  
542 *DHH1* was set to 100. The SEM values are indicated below. (D) Dhh1 binds BMV RNA2. Western  
543 blot analysis of immunoprecipitation assays carried out in *dhh1Δ* cells expressing RNA2 and Dhh1 or  
544 Dhh1-Flag. Input corresponds to 100 μg of total protein present in lysates prior to precipitation and IP  
545 corresponds to the corresponding eluates from the anti-Flag matrix following precipitation. Diagram  
546 shows the relative amount (+/-SEM) of precipitated RNA2 detected by qPCR after  
547 immunoprecipitation, the input amount of RNA2 was set to 100. (E) The half-life of RNA2 is not  
548 significantly altered in *dhh1Δ* cells. RNA2 was expressed from a *GALI* promoter in WT and *dhh1Δ*  
549 cells. Transcription was stopped by adding glucose at time point t=0 min. Samples were taken at  
550 different time points and RNA2 accumulation analyzed by Northern blot. Numbers below indicate the  
551 average half-life of RNA2 (+/-SEM) based on three independent experiments. T-test analysis shows  
552 that the half-life is not significantly altered in *dhh1Δ* cells.

553

554 **Figure 2.** Depletion of Dhh1 shifts BMV RNA2 towards single ribosomal subunit fractions. (A)  
555 Global translation is unaffected in a strain expressing RNA2. UV absorbance profile at 254 nm of an  
556 extract from WT and *dhh1Δ* cells expressing RNA2 after sedimentation on a 10-50% sucrose gradient.  
557 The monosome to polysome ratio (M/P +/- SEM) is not significantly affected. (B) Sucrose density  
558 gradient analysis of BMV RNA2 in WT and *dhh1Δ* yeast. Below a representative UV absorbance

559 profile is the distribution of normalized RNA2 levels in the specific fractions. Bars represent the  
560 average +/-SEM from three independent experiments. Fractions were grouped into free (1-5), single  
561 ribosomal subunits (6-11), monosomes (12-15), light polysomes (16-21) and heavy polysomes (22-  
562 26). The total amount of RNA2 recovered over the gradient was set to 100%. (C) UV absorbance  
563 profile at 254 nm after 15 min glucose withdrawal of an extract from WT and *dhh1Δ* cells expressing  
564 RNA2. (D) Distribution of normalized RNA2 levels in the specific fractions after 15 min glucose  
565 withdrawal. Like in (B). (E) Dhh1 interacts with translation initiation factors. Western blot analysis of  
566 immunoprecipitation assays. Extracts were either treated (+) or not treated (-) with RNase A prior to  
567 the washing steps.

568

569 **Figure 3.** A stem-loop in the 2a CDS confers dependence on Dhh1 for translation. (A) Northern and  
570 Western blot analysis of WT and *dhh1Δ* yeast expressing different BMV and *GFP* mRNA constructs  
571 (schematic diagrams of the constructs depicted on top, in blue native BMV RNA2 sequences). Dhh1-  
572 dependence is calculated from the relative expression of 2a and GFP normalized to Pgk1 in WT and  
573 *dhh1Δ* cells. Dhh1-dependence +/- SEM is given below. (B) Diagram shows the amount (+/-SEM) of  
574 co-immunoprecipitated RNA2-construct relative to that of WT RNA2 (BMV/2a/BMV) detected by  
575 qPCR. The amount of co-immunoprecipitated WT RNA2 was set to 100. (C) Schematic diagrams of  
576 RNA2-RLUC constructs containing the complete or a part of the 2a CDS fused to RLUC. Translation  
577 was determined by measuring luciferase activity corrected by the amount of RNA determined by  
578 qPCR. (D) Left, RNA-fold model of the secondary structure of nucleotides 42-85. Nucleotides marked  
579 in yellow have been replaced by complementary ones to disrupt the stem-loop. Right, designed  
580 structurally equivalent stem-loop. (E) The region between nucleotides 42-87 strongly inhibits RLUC  
581 activity. RLUC activity was measured and corrected by the amount of the RNA2 construct determined  
582 by qPCR and is represented relative to the activity in the presence of FL-RLUC which was set to 1.  
583 (F) The structure and the position of the stem loop is important for the dependence of RNA2  
584 translation on Dhh1. WT and *dhh1Δ* yeast cells transformed with RNA2-RLUC constructs in which  
585 the stem-loop structure has been disrupted by point mutations (“disrupted loop”) or replaced by a  
586 designed stem-loop structure (“designed loop”, depicted in Fig. 3D). The relative Dhh1 dependence

587 refers to the RLUC ratio between WT and *dhh1* $\Delta$  cells and was set to 1 for FL-RLUC to facilitate  
588 comparison. Mean values +/- standard error of the mean were obtained from at least three independent  
589 experiments throughout the figure.

590

591 **Figure 4.** Dhh1 binds and regulates translation of distinct sets of cellular mRNAs. (A) Experimental  
592 strategy to identify cellular mRNAs translationally controlled by Dhh1. (B) Changes in mRNA, RPF  
593 and translation comparing WT to *dhh1* $\Delta$  cells. Distribution of significant mRNA and RPF level  
594 changes is given for all genes and distribution of translation efficiency changes is given for genes with  
595 no changes at the mRNA level and for genes with opposite changes at the mRNA and RPF level. For  
596 RNA-seq data “up” includes genes with a log<sub>2</sub>-fold change > 0.433 and an adjusted p-value < 0.1.  
597 “Down” includes genes with a log<sub>2</sub>-fold change < -0.433 and an adjusted p-value < 0.1. For RPF data  
598 “up” and “down” includes genes with a log<sub>2</sub>-fold change > 0 and < 0, respectively, and an adjusted p-  
599 value < 0.1. (C) Histogram showing the number of translationally activated and repressed genes  
600 crosslinked and not crosslinked by Dhh1. (D) Venn diagram depicting Dhh1 binding targets identified  
601 by CRAC in different transcript regions. (E) Metagene analysis as indicated by CRAC data. Y-axis  
602 shows average number of reads in significant Dhh1 crosslinking sites (peaks) in the corresponding  
603 region. Reads that were not part of a significant peak are not considered. Dotted lines mark start and  
604 stop codon. (F) Metagene analysis of ribosome density in WT and *dhh1* $\Delta$  cells for the different sets of  
605 mRNAs. Y-axis shows average of observed RPF reads related to expected ones.

606

607 **Figure 5.** Cellular mRNAs translationally activated by Dhh1 contain highly structured CDSs. From  
608 the total number of mRNAs translationally activated, translationally repressed and unchanged, only  
609 the given numbers were analyzed as for the rest the UTRs and CDSs are not annotated. (A) Genes  
610 translationally activated by Dhh1 have a significant longer CDS ( $p < 0.0001$ ). P-values were  
611 computed after permuting randomly 1000 times the labels of the genes in the two groups (detailed in  
612 Supplemental Methods). Distribution of the average length of 5'UTR, CDS and 3'UTR for genes  
613 translationally activated, repressed and not affected by Dhh1 represented in a Tukey plot. Numbers  
614 give number of transcripts per subset. (B) Genes translationally activated by Dhh1 have significant

615 higher PARS scores in the CDS ( $p < 0.0001$ ) and the 5'UTR ( $p < 0.01$ ). P-values as in (A).  
616 Distribution of the average PARS score of 5'UTR, CDS and 3'UTR for genes translationally  
617 activated, repressed and not affected by Dhh1 represented in a Tukey plot (band inside the box  
618 represents median). Cross indicates mean PARS score of each subset and green line marks mean  
619 PARS of all genes. Numbers give number of transcripts per subset. (C) Metagene PARS score analysis  
620 of the different sets of genes. Y-axis shows the average of smoothed PARS score on the  
621 corresponding region. Smoothing is achieved by calculating the average PARS scores over a window  
622 of size 20 centered on the corresponding nucleotide. Dotted lines mark start and stop codon. Green  
623 box marks the 7.5-15% region, one area with an especially high difference between the PARS scores.  
624 Numbers in the legend indicate the number of analyzed genes in each subset. (D) Like in (C) but for  
625 the 5'UTR and the first 300 nucleotides. Green box marks nucleotides 50-120. (E) GO term analysis  
626 for genes translationally activated and bound by Dhh1 regarding biological process and cellular  
627 component. Numbers in brackets indicate (B/n/b). B = total number of genes associated with  
628 functional category (often comprising several GO terms grouped by REVIGO); n = number of genes  
629 in input list; b = number of genes in intersection. (F) As in (E) but for genes translationally repressed  
630 and bound by Dhh1.

631

632 **Figure 6.** Dhh1-mediated translational control is seemingly conserved from yeast to humans. (A)  
633 PARS score distribution analysis of the human mRNA counterparts for the 5'UTRs and the first 300  
634 nucleotides of the CDS. Axis and smoothing like in Fig. 5 C. Green box marks nucleotides 60-100.  
635 (B) PARS score of the 5'UTR and the first 300 nucleotides of *PTCHI* mRNA. (C) DDX6 drives  
636 translation of *PTCHI*. Western blot, qPCR and translation rates of *PTCHI* after DDX6 silencing.  
637 Translation was determined by correcting protein levels by the amount of the corresponding RNA. As  
638 control for equal sample size tubulin protein levels and *HPRT1* mRNA levels were examined.  
639 Experiments have been carried out at least three times independently. **\*\***( $p < 0.05$ , TTEST).

640

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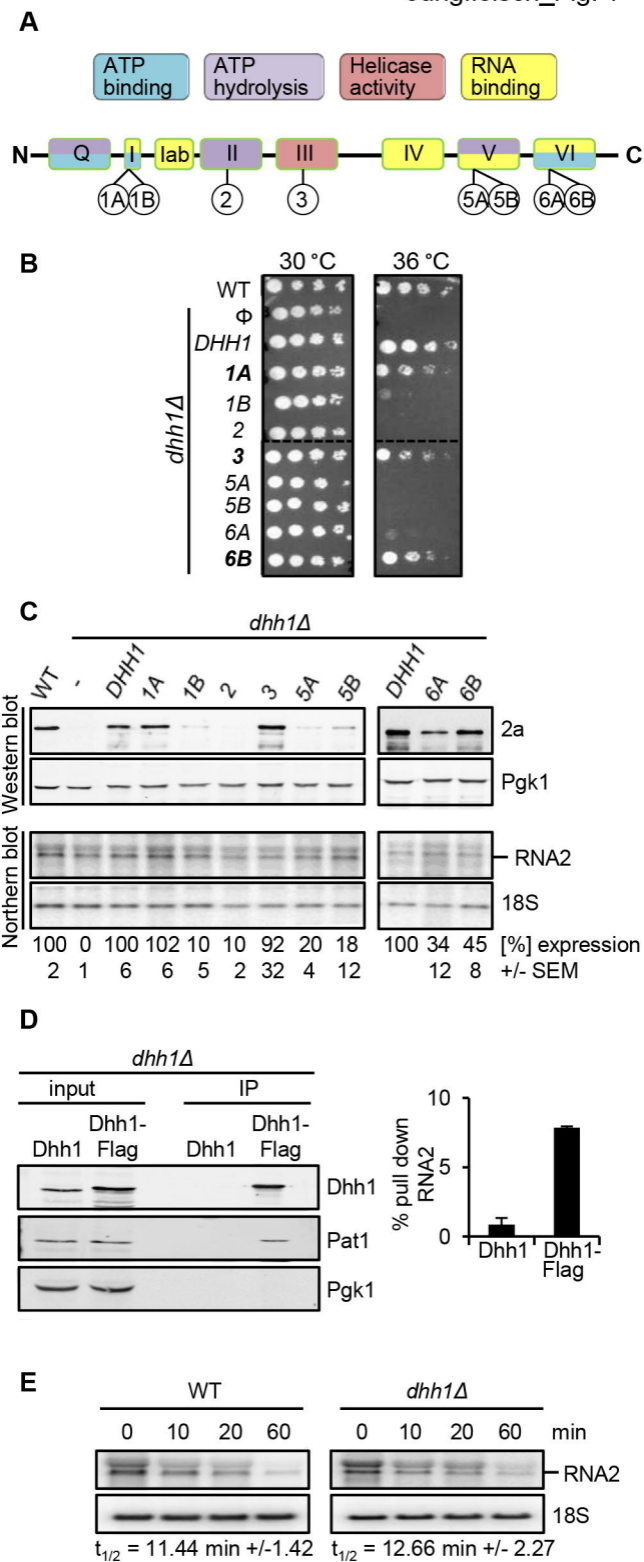
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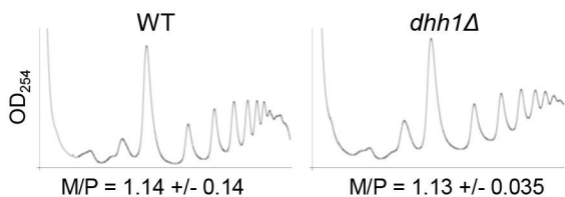
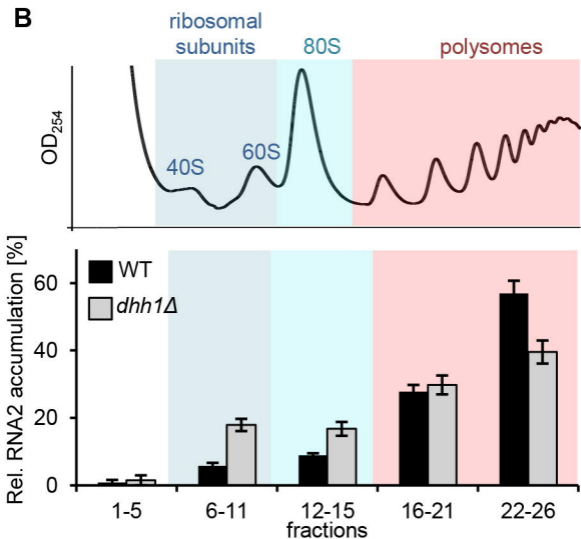
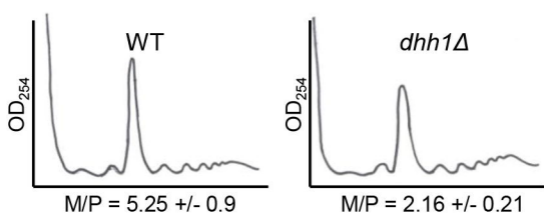
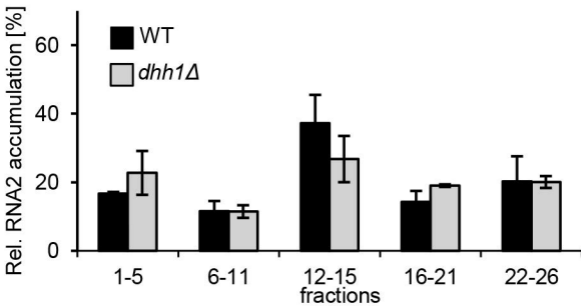
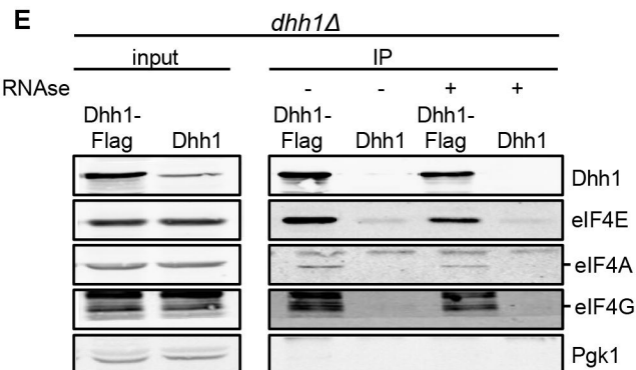
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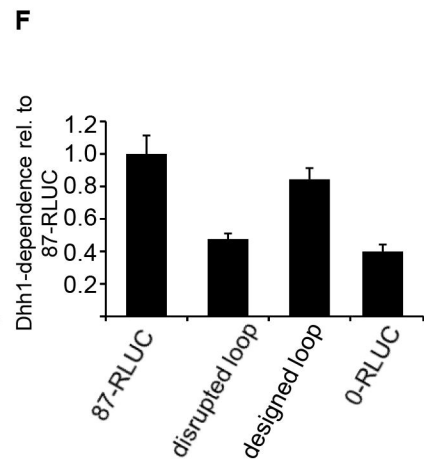
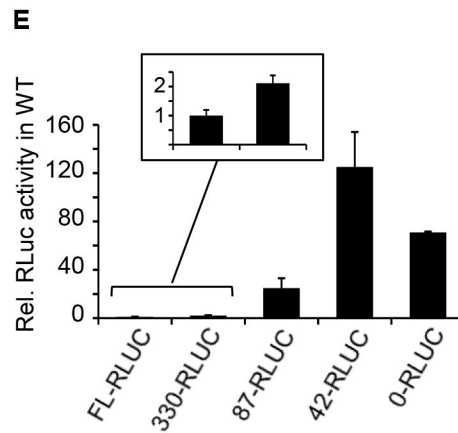
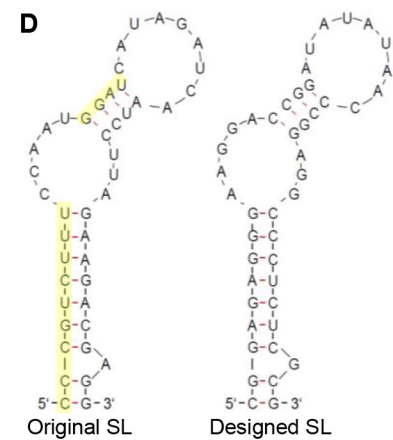
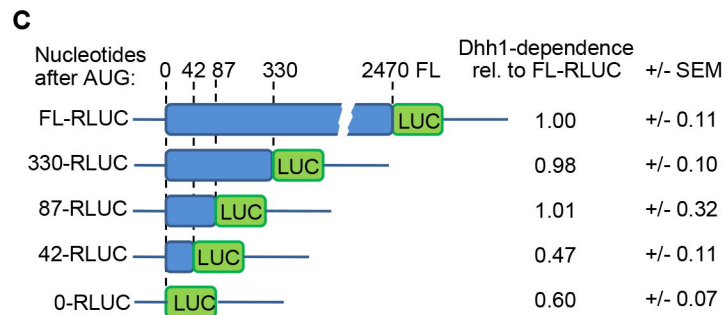
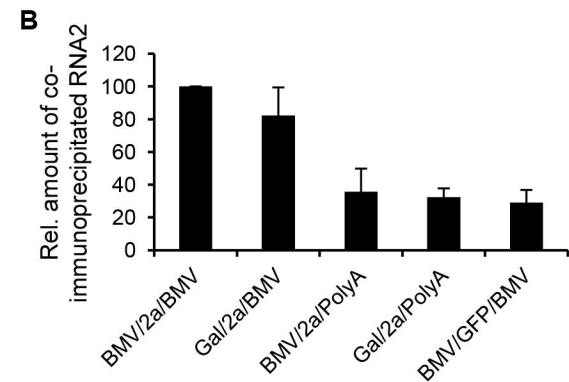
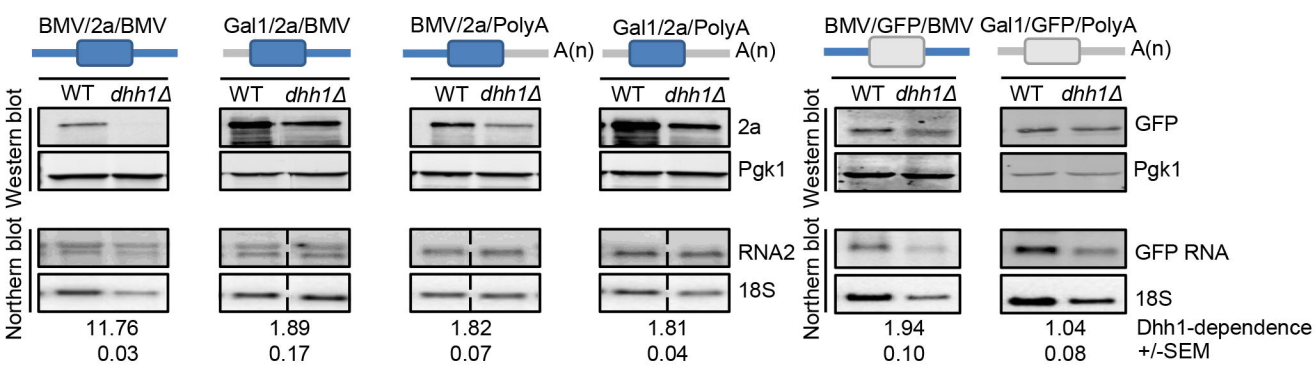
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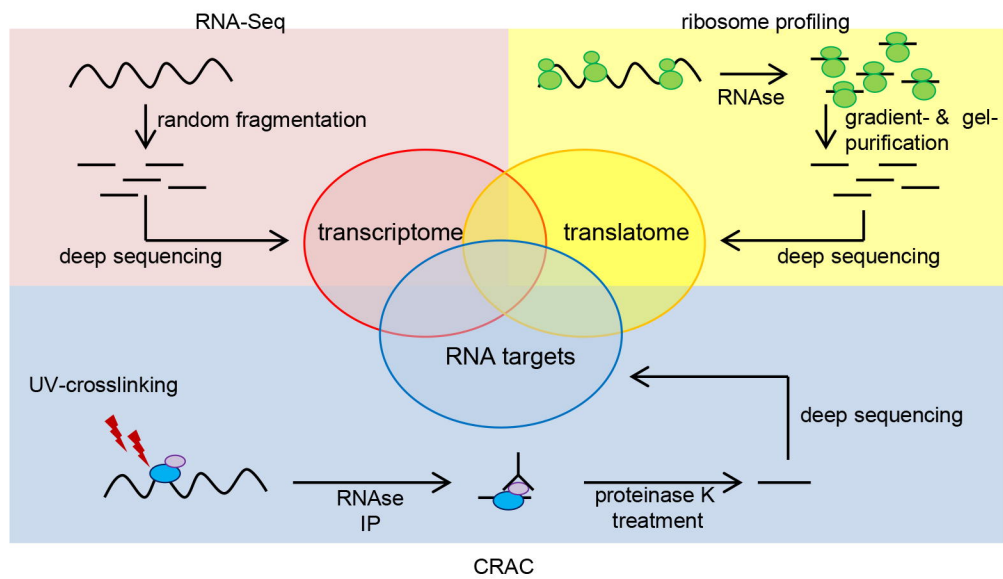
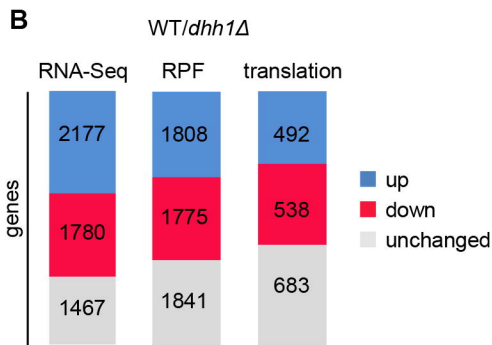
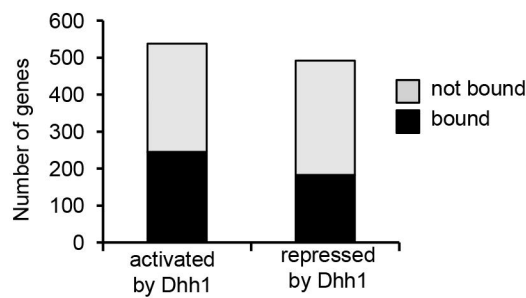
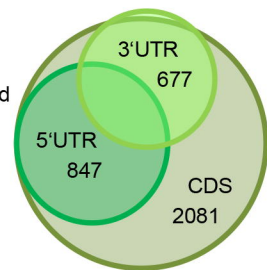
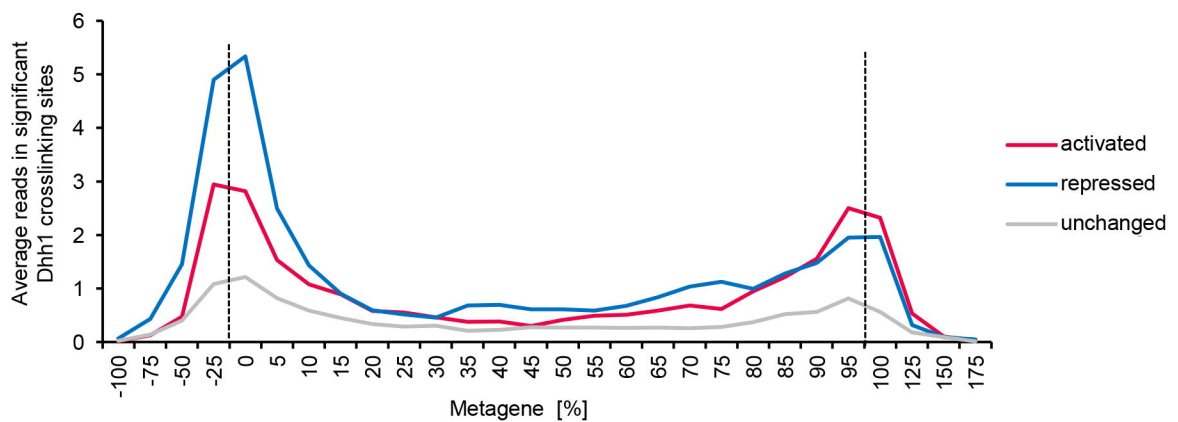
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