CASC3 promotes transcriptome-wide activation of nonsense-mediated decay by the exon junction complex

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Abstract

The exon junction complex (EJC) is an essential constituent and regulator of spliced messenger ribonucleoprotein particles (mRNPs) in metazoans. As a core component of the EJC, CASC3 was described to be pivotal for EJC-dependent nuclear and cytoplasmic processes. However, recent evidence suggests that CASC3 functions differently from other EJC core proteins. Here, we have established human CASC3 knockout cell lines to elucidate the cellular role of CASC3. In the knockout cells, overall EJC composition and EJC-dependent splicing are unchanged, whereas mRNA isoforms targeted by nonsense-mediated decay (NMD) are upregulated on a transcriptome-wide scale. Mechanistically, recruiting CASC3 to reporter mRNAs by direct tethering or via binding to the EJC stimulates mRNA decay and endonucleolytic cleavage at the termination codon. Building on existing EJC-NMD models, we propose that CASC3 equips the EJC with the ability to communicate with the NMD machinery in the cytoplasm. Collectively, our results characterize CASC3 as a peripheral EJC protein that tailors the transcriptome by promoting the degradation of EJC-dependent NMD substrates.

47 ## *Introduction*

48 Messenger RNA-binding proteins (mRBPs) determine the stability, location, and fate of bound
49 mRNAs and are therefore important regulators of post-transcriptional gene expression (Hentze *et al.*,
50 2018). A central component of spliced mRNPs in metazoans is the exon-junction-complex (EJC), which
51 is deposited during splicing upstream of exon-exon boundaries (Boehm & Gehring, 2016, Le Hir *et al.*,
52 2016, Woodward *et al.*, 2017). The heterotetrameric core of the EJC is composed of the proteins
53 EIF4A3, MAGOH, RBM8A (Y14) and CASC3 (BTZ, MLN51) (Andersen *et al.*, 2006, Bono *et al.*, 2006).
54 Generally, EJCs serve on spliced mRNAs as a mark that act as a binding platform for peripheral EJC-
55 interacting factors (Singh *et al.*, 2012). The core and peripheral EJC components contribute to different
56 steps of post-transcriptional gene expression including splicing regulation, mRNA localization,
57 translation and nonsense-mediated mRNA decay (NMD) (Boehm & Gehring, 2016, Woodward *et al.*,
58 2017).

59 The EJC does not form spontaneously, but instead undergoes stepwise assembly in association
60 with the spliceosome, while it proceeds through different spliceosomal complexes (Gerbracht &
61 Gehring, 2018). As a first step, the splicing factor CWC22 recruits EIF4A3, to which the MAGOH/RBM8A
62 heterodimer binds later on (Gehring *et al.*, 2009, Alexandrov *et al.*, 2012, Barbosa *et al.*, 2012,
63 Steckelberg *et al.*, 2012, Steckelberg *et al.*, 2015). Unlike the three spliceosome-associated EJC
64 components EIF4A3, MAGOH and RBM8A, the fourth protein CASC3 is absent from the purified
65 spliceosomal C complex (Bessonov *et al.*, 2008). Furthermore, it cannot be detected in mRNPs formed
66 on splicing intermediates (Gehring *et al.*, 2009). Therefore, it was suggested that CASC3 binds to the
67 initially formed trimeric pre-EJC (consisting of EIF4A3, MAGOH and RBM8A) at a later stage.
68 Interestingly, CASC3 has also been shown to be a shuttling protein that is mainly located in the
69 cytoplasm, whereas the other EJC components are predominantly detected in the nucleus (Kataoka *et*
70 *al.*, 2000, Le Hir *et al.*, 2001, Degot *et al.*, 2002, Palacios *et al.*, 2004, Shibuya *et al.*, 2004). It has
71 therefore been suggested that CASC3 binds to the EJC in the nucleus and is transported with it into the
72 cytoplasm (Ballut *et al.*, 2005). A recent study demonstrated that EJCs undergo a compositional switch

73 and that the ASAP/PSAP component RNPS1 and the protein CASC3 bind to functionally different
74 mRNPs and exist in mutually exclusive EJCs (Mabin *et al.*, 2018). While EJCs of nuclear enriched
75 transcripts were found to interact with RNPS1, the EJCs of cytoplasmic enriched transcripts rather
76 contained CASC3. This observation is in line with the predominantly cytoplasmic localization of CASC3,
77 but would argue against a nuclear function.

78 Another aspect under debate is the involvement of CASC3 in the NMD pathway. According to the
79 EJC-dependent model of NMD, an EJC present more than 50-55 nucleotides downstream of a
80 premature termination codon (PTC) triggers degradation by the NMD machinery (Nagy & Maquat,
81 1998). This quality control mechanism rids the cells from aberrant transcripts that contain PTCs due to
82 mutations or mis-splicing. Additionally, it serves as a post-transcriptional mechanism of gene
83 expression, especially when coupled to alternative splicing (Nasif *et al.*, 2018). This is a common
84 feature of many genes coding for mRBPs, e.g. most SR proteins (Lareau *et al.*, 2007, Ni *et al.*, 2007).
85 The EJC triggers NMD by interacting with members of the SURF complex resulting in phosphorylation
86 of the central NMD factor UPF1 (Kashima *et al.*, 2006). Phosphorylated UPF1 then stimulates two
87 distinct degradation pathways of NMD: The SMG5/7-dependent pathway results in deadenylation and
88 decapping of the transcript followed by exonucleolytic decay from the 5' end by XRN1 and the 3' end
89 by the exosome (Loh *et al.*, 2013). Alternatively, the transcript can be cleaved in the vicinity of the PTC
90 by the endonuclease SMG6 which results in two mRNA fragments that can be exonucleolytically
91 degraded by XRN1 and the exosome (Huntzinger *et al.*, 2008, Eberle *et al.*, 2009). While these pathways
92 can act redundantly and in principle compensate for each other, SMG6-dependent endonucleolytic
93 cleavage (endocleavage) has been shown to be the dominant pathways for NMD in human cells
94 (Boehm *et al.*, 2014, Lykke-Andersen *et al.*, 2014, Colombo *et al.*, 2017). In cells depleted of CASC3 a
95 stabilizing effect on PTC-containing reporter mRNAs and selected endogenous targets was reported
96 (Palacios *et al.*, 2004, Gehring *et al.*, 2005, Gehring *et al.*, 2009). Furthermore, tethering CASC3 to an
97 mRNA results in UPF1-dependent degradation of the transcript (Gehring *et al.*, 2009). However, a

98 recent report has challenged these observations and showed that CASC3 plays a minor role in NMD
99 and only for certain endogenous targets in contrast to EIF4A3 or RNPS1 (Mabin *et al.*, 2018).

100 We were intrigued by the contrasting reports about the enigmatic role of CASC3 and decided to
101 investigate the function of CASC3 and its distinction to the other EJC core components in more detail.
102 For this purpose, we established HEK 293 CASC3 knockout (KO) cells using CRISPR-Cas9-mediated gene
103 editing. The CASC3 KO cell lines are unchanged in their composition of the EJC core and peripheral
104 interacting proteins. However, RNA-sequencing reveals an upregulation of transcript variants
105 containing premature-termination codons (PTC) as well as the differential expression of many known
106 NMD targets, indicating a perturbation of this decay pathway. Mechanistically, CASC3 stimulates
107 SMG6-dependent turnover of NMD targets and likely acts as a link from the EJC to the NMD machinery.
108 On the basis of these results we propose a revised model of EJC-dependent NMD in human cells.

109 # *Results*

110 ## *CASC3 is dispensable for nuclear EJC functions*

111 Previously, we and others have shown that depletion of EJC core components in human
112 cells leads to pervasive re-splicing of cryptic splice sites, resulting in aberrant splice variants lacking
113 exonic sequences (schematically depicted in Supplementary Figure 1) (Wang *et al.*, 2014, Boehm
114 *et al.*, 2018). Mechanistically, the EJC prevents the use of cryptic splice sites either by interaction
115 with the ASAP/PSAP component RNPS1 or by sterically masking splice sites (Boehm *et al.*, 2018).
116 In keeping with previous observations from HeLa cells, the knockdowns of the EJC core
117 components EIF4A3 or RBM8A in HEK293 cells resulted in exon-skipping of the mRNAs for RER1,
118 OCIAD1 and MRPL3 (Figure 1 A-C). Surprisingly, a knockdown of the EJC core factor CASC3 did not
119 result in mis-splicing of these three selected transcripts. (Figure 1A-C). However, the knockdown
120 of CASC3 increased the abundance of an alternative transcript isoform of the SR protein SRSF2,
121 which is known to be targeted by NMD (Figure 1D) (Sureau *et al.*, 2001, Wollerton *et al.*, 2004).
122 Together, these results indicate that CASC3 contributes to NMD activation by the EJC, but is not
123 required for EJC-dependent splicing regulation. Given that the generation of EJC-specific splice
124 isoforms is a readout for EJC assembly, the initial formation of EJCs does not seem to be affected
125 by the knockdown of CASC3.

126 ## *EJC assembly is unaffected in CASC3 knockout cells*

127 Although the knockdown efficiency of CASC3 was substantial (Figure 1E), we wished to
128 exclude that residual amounts of CASC3 prevented a reliable assessment of the protein's function.
129 Since CASC3 was found to be non-essential in multiple genome-wide screens of human
130 immortalized cell lines, we reasoned that knockout (KO) of CASC3 should be feasible (Hart *et al.*,
131 2015, Hart *et al.*, 2017). Accordingly, we obtained three cell lines by CRISPR-Cas9-mediated gene
132 editing, designated H, F, and T lacking the CASC3-specific 130 kDa band on a western blot (Figure
133 2A). In all cell lines we detected genomic insertions of different length and sequence at the

134 beginning of the coding region of CASC3, which resulted in frame shifts of the downstream coding
135 region or, in the case of cell line T, contained in-frame termination codons (Figure 2B,
136 Supplemental Figure 2A and B). For the cell lines H and F, we observed an additional band of 100
137 kDa on western blots with antibodies recognizing the C-terminal or central region of CASC3 (Figure
138 2A, red arrow, Supplementary Figure 2A and C). This cross-reactive protein interacted with FLAG-
139 tagged EIF4A3 and disappeared upon treatment with siRNAs against CASC3 (Figure 2C,
140 Supplementary Figure 2C and D). This suggests that the cell lines H and F produce an N-terminally
141 truncated form of CASC3, presumably representing a novel, non-canonical protein product. Such
142 aberrant proteins have been recently described in a systematic analysis to be common by-products
143 of CRISPR-Cas9 genome editing (Tuladhar *et al.*, 2019).

144 To assess the composition of exon junction complexes and their peripheral interacting
145 proteins in the absence of CASC3 KO, we analyzed the FLAG-tagged EIF4A3 interactome in the cell
146 line H and wild type cells using mass spectrometry. EIF4A3 was successfully enriched, together
147 with other known EJC complex members (Supplemental Figure 2E). Although co-precipitated
148 CASC3 was strongly reduced in the knockout cell line (Supplemental Figure 2G, log2 fold change =
149 -3.75), it was enriched relative to the control (Supplemental Figure 2F, log2 fold change = 2.43).
150 This confirmed that residual amounts of CASC3 protein are still present in the knockout cell line H.
151 When the cells were treated additionally with CASC3 siRNAs, the amount of CASC3 pulled down
152 by EIF4A3 was reduced to background levels (Figure 2D and E). Therefore, the cell lines H and F are
153 comparable to a constitutive knockdown of CASC3, whereas the cell line T (without further
154 treatment) and cell line H in combination with CASC3 siRNA treatment completely lack detectable
155 CASC3 protein. This set of cell lines enables a hitherto unfeasible analysis of CASC3's cellular
156 function as part of the EJC.

157

158 Strikingly, no other EJC core factor or splicing regulatory EJC component (e.g. ASAP/PSAP) was
159 significantly altered in the CASC3-depleted condition (Figure 2F). This is in line with our initial
160 conclusion that splicing-regulatory EJCs can assembly even in the absence of CASC3. Further
161 support comes from co-immunoprecipitation analysis of EIF4A3, which demonstrated unchanged
162 interaction with the EJC component MAGOH in the CASC3 knockout cell line H (Supplementary
163 Figure 2H). In agreement with the data obtained from the knockdown of CASC3 in Figure 1,
164 transcripts containing EJC-dependent splice sites were correctly spliced in CASC3 KO cells (Figure
165 2G and H, Supplementary Figure 2I and J), indicating that CASC3 is not necessary for correct and
166 stable deposition of the EJC during splicing and its subsequent nuclear splice function. The role of
167 CASC3 during EJC assembly was also characterized by performing *in vitro* splicing of a MINX intron
168 in cell lysates obtained from the cell line H (Figure 2I and Supplementary Figure 2K). Notably, the
169 overall splicing efficiency of the MINX transcript was slightly reduced in the input of CASC3 KO
170 lysates when compared to wild-type lysates. However, spliced RNA was pulled down to the same
171 extent by EIF4A3 (2.97% in KO vs. 3.01% in WT), suggesting that EIF4A3 is deposited on spliced
172 mRNA even when CASC3 is strongly depleted. In the absence of CASC3, the only other protein that
173 was significantly, although mildly reduced in EIF4A3-IPs was the NMD factor UPF3B (Figure 2F, log2
174 fold change = -1.52). UPF3B links the EJC to the NMD machinery via direct interactions (Buchwald
175 *et al.*, 2010) and was recently found to be enriched in cytoplasmic CASC3-loaded EJCs (Mabin *et*
176 *al.*, 2018). The reduction of NMD-competent EJCs could contribute to the NMD impairment that
177 we observed upon loss of CASC3.

178 *CASC3 regulates NMD-sensitive isoforms*

179 To investigate the global effects of CASC3 depletion on the transcriptome, we performed RNA-
180 sequencing (RNA-seq) of the cell lines H and T either treated with CASC3 or control siRNAs (Figure
181 3A) and identified differentially expressed genes (Supplementary Figure 3A and 3B, Supplementary
182 Table 4). To exclude clone-specific and siRNA treatment-related effects, we compared the
183 identified targets between the four conditions. Overall, the high number and the substantial

184 overlap of upregulated genes suggests that CASC3 KO mainly results in the accumulation of certain
185 transcripts (Figure 3B and C, Supplementary Figure 3C). This observation fits with the hypothesis
186 that CASC3 is required for efficient execution of NMD. Accordingly, several upregulated genes
187 belong to the class of small RNA (e.g. snoRNA) host genes, which are frequently NMD targets
188 (Figure 3C and D) (Lykke-Andersen *et al.*, 2014). We validated the upregulation of the snoRNA host
189 gene ZFAS1 by qPCR, which was even more pronounced in CASC3 KO than UPF1 knockdown cells
190 (Supplementary Figure 3D and E). Across the top 100 significantly upregulated genes in CASC3 KO,
191 15 small RNA host genes were identified (Figure 3D). Comparing the differentially expressed genes
192 to recent transcriptome-wide NMD screens, many of the top 100 significantly upregulated genes
193 were also differentially expressed in UPF1 and SMG6/7 KD (Figure 3D, 23% and 59%, respectively)
194 (Colombo *et al.*, 2017, Data ref: Colombo *et al.*, 2017). However, none of the identified targets
195 were present in an RNPS1 knockdown (Figure 3D) (Boehm *et al.*, 2018, Data ref: Boehm *et al.*,
196 2018). Collectively, our differential gene expression analysis strengthens the proposed link
197 between CASC3 and the NMD-machinery.

198 Next, we analyzed alternative splicing changes in CASC3 KO cells (Figure 3A). Since our earlier
199 assays showed that CASC3 was not involved in EJC-regulated splicing (Figures 1 and 2), we were
200 surprised to detect many altered splicing events in CASC3 depleted cells (Supplementary Figure 3F,
201 Supplementary Table 5). It is remarkable that hardly any alternative splicing events were shared
202 between RNPS1 knockdown and CASC3 KO cells (Figure 3E, Supplementary Figure 3F). Either
203 CASC3 regulates an RNPS1-independent set of alternative splice sites or the splicing changes are
204 due to impaired NMD, which fails to remove NMD-sensitive isoforms. To test these possibilities,
205 we investigated the functional consequence of CASC3-dependent alternatively splicing on the
206 transcript isoform level (Figure 3A, Supplementary Figure 4A and B, and Supplementary Table 6).

207 Strikingly, in all CASC3 KO conditions the majority of upregulated mRNA isoforms contained a
208 premature termination codon (PTC), rendering the transcripts susceptible to NMD (Figure 4A). On

209 the other hand, downregulated isoforms rarely contained a PTC. Among the identified isoform
210 switches was the NMD target SRSF2, which we confirmed earlier to be CASC3-dependent (Figure
211 1D). While overall SRSF2 gene expression varied only slightly between wild-type and CASC3 KO
212 cells, the isoform usage changed dramatically towards the accumulation of NMD-sensitive
213 transcripts in the CASC3 KO conditions (Figure 4B).

214 We next validated a set of transcript isoform switches by qPCR (Figure 4C-E, Supplementary Figure
215 C and D). In the transcript isoforms stabilized by the CASC3 KO and by a UPF1 KD, the inclusion of
216 intronic regions resulted in the inclusion of PTCs. For example, an NMD-sensitive transcript variant
217 of the gene TOE1 is produced alongside the canonical isoform by usage of an alternative 3' splice
218 site (Supplementary Figure 4D). This event was also prominently detected in the SMG6/7 and UPF1
219 dataset (Supplementary Figure 4E).

220 The shift of isoform usage from NMD-insensitive to PTC-containing transcripts was also observed
221 transcriptome-wide in CASC3 KO cells and was comparable to NMD-compromised SMG6/7 or UPF1
222 depleted cells (Figure 4F). These findings indicate that many transcript isoforms upregulated upon
223 depletion of CASC3 represent genuine endogenous NMD targets. On the gene level, nearly 50% of
224 CASC3-dependent alternatively used transcript isoforms were also found in the SMG6/7 or UPF1
225 conditions (Figure 4G and Supplementary Figure 4F). The incomplete overlap between CASC3 and
226 SMG6/7 or UPF1 could be due to technical differences of the data sets or because not all NMD
227 sensitive transcripts in the cell are affected by a knockout of CASC3. This could include transcripts
228 that are degraded by EJC-independent NMD (Buhler *et al.*, 2006, Metze *et al.*, 2013).

229 *CASC3 stimulates SMG6-dependent endocleavage*

230 To deepen the understanding of how a lack of CASC3 results in reduced NMD efficiency, we
231 stably integrated the well-established globin NMD reporter PTC39 in WT and CASC3 KO cell lines
232 (Figure 5A and B, Supplementary Figure 5A) (Thermann *et al.*, 1998). The analysis of a reporter
233 mRNA enables a read-out of multiple aspects of mRNA degradation: firstly, the total levels of the

234 full-length reporter, secondly the contribution of 5'->3' exonucleolytic decay by XRN1 (detection
235 of xrFrag due to an XRN1-resistant element (Boehm *et al.*, 2016, Voigt *et al.*, 2019); and thirdly the
236 amount of endonucleolytic cleavage by SMG6 (detection of 3' fragment stabilized by XRN1
237 knockdown). In both WT and CASC3 KO cell lines the reporter was efficiently degraded, showing
238 that the NMD pathway is still functional in CASC3 depleted cells. However, full-length reporter
239 levels in CASC3 KO were slightly higher when compared to wild-type cells (lane 2 vs. lane 5).
240 Notably, the accumulation of 3' fragments following XRN1 knockdown was clearly reduced in the
241 CASC3 KO condition (lane 3 vs. lane 6). This difference in endocleavage efficiency was also
242 observed when expressing a minigene reporter of the endogenous CASC3 target TOE1. While there
243 was a substantial upregulation of full-length reporter mRNA in CASC3 KO cells, the amount of the
244 3' fragment was strongly reduced, suggesting that SMG6-dependent endocleavage is inefficient in
245 CASC3 KO cells (Figure 5C and D, Supplementary Figure 5B-D). To further address this, a TPI
246 reporter was expressed in combination with knockdowns of XRN1, SMG6 and/or SMG7 (Figure 5E-
247 G). The degree of reporter and 3' fragment stabilization of the TPI reporter following XRN1
248 knockdown was comparable to the observations made for the globin reporter (lanes 1-3 vs. lanes
249 6-8). In both WT and CASC3 KO cells, SMG6 knockdown resulted in a drastic reduction of 3'
250 fragments, as expected (lanes 4 and 9). Notably, a knockdown of SMG7 together with XRN1
251 revealed a major difference between the cell lines. In WT cells the PTC-containing reporter was
252 only minimally stabilized by the SMG7/XRN1 knockdown and 3' fragments were unaffected.
253 Performing a SMG6/XRN1 knockdown in CASC3 KO cells lead to a more dramatic stabilization of
254 the full-length reporter and a decrease of 3' fragments compared to the XRN1 knockdown
255 condition (lanes 3 and 5 vs. lanes 8 and 10). Collectively, our results indicate that in CASC3 KO cells
256 SMG6-mediated endocleavage is impaired. This could explain why the CASC3 KO cells are more
257 sensitive to a knockdown of SMG7 when compared to wild type cells.

258 To identify, which part of CASC3 promotes NMD, we employed a tethering reporter that was
259 designed to monitor mRNA turnover as well as endocleavage at the termination codon (Figure 6A).

260 Tethering the full-length CASC3 protein to the MS2 stem loops downstream of the stop codon
261 resulted in degradation of the reporter compared to tethering of the negative control GST (Figure
262 6B and C, Supplementary Figure 6A). This degradation was accompanied by the production of 3'
263 fragments in XRN1 knockdown conditions, indicating that the mechanism of decay is comparable
264 to the PTC-containing reporter mRNAs (Figure 6B, lane 6). Surprisingly, C-terminally truncated
265 deletion mutants of CASC3 that contain the first 480 or even 137 amino acid residues were able to
266 induce degradation of the tethering reporter to a comparable extent as full-length CASC3 (Figure
267 6B, lanes 3 and 4, 7 and 8).

268 Finally, CASC3 deletion mutants were expressed in the CASC3 KO cells to identify the minimal
269 part necessary to rescue the effects on endogenous NMD targets (Figure 6D, Supplementary Figure
270 6B and C). As in the tethering experiment, expression of full-length CASC3 and the C-terminal
271 truncated variant 1-480 resulted in transcript isoform levels comparable to wild-type cells for the
272 targets CLN6 and TOE1 (Figure 6D lanes 1-4). An EJC binding-deficient mutant of CASC3 (188/218
273 double point mutation) was unable to rescue, supporting the notion that CASC3 is recruited to the
274 mRNA by binding to the EJC (Figure 6D, lane 5). Deleting the N-terminal 109 amino acids of CASC3
275 (110-480) did not alter the rescue ability (Figure 6D, lane 6). While in the tethering assay it was
276 sufficient to place the N-terminus downstream of a termination codon, this part of CASC3 was not
277 necessary to rescue NMD activity in the KO cells. This suggests that different domains of CASC3 act
278 in a redundant manner during the activation of NMD by the EJC.

279 ## *Discussion*

280 The role of CASC3 within the EJC has been the subject of scientific controversy for many years.
281 CASC3 has been initially described as an EJC core protein, because it was required for the assembly of
282 the EJC from recombinant protein components *in vitro* (Ballut *et al.*, 2005). However, it has been
283 demonstrated that the mechanism of EJC assembly using recombinant proteins is mechanistically
284 different from EJC assembly in splicing extracts or in living cell (Steckelberg *et al.*, 2012). Furthermore,
285 several recent publications challenged the view of CASC3 being an EJC core component. For instance,
286 CASC3 was reported to be present in substoichiometric amounts compared to the other three EJC core
287 proteins EIF4A3, RBM8A, and MAGOH in HEK293 (Singh *et al.*, 2012) and U2OS cells (Beck *et al.*, 2011).
288 Also, during mouse embryonic brain development CASC3 deficiency results in a different phenotype
289 than the other EJC core components (Mao *et al.*, 2017). By using CASC3 CRISPR-Cas9 knockout cells,
290 we unambiguously establish that CASC3 is not required for EJC assembly or EJC-regulated splicing in
291 the nucleus (Figure 7). Therefore, our molecular analyses fully support the recently emerging view of
292 defining CASC3 as a peripheral EJC component. As a mainly cytoplasmic component of the EJC we
293 propose that the principal role of CASC3 is to alter the efficiency by which NMD-sensitive transcript
294 isoforms are degraded.

295 Although NMD has been extensively studied in the past decades and many NMD factors have been
296 identified and characterized, no universal model exists that describes how they work together to elicit
297 NMD. While a function of CASC3 in NMD has been reported before, previous analyses did not show
298 consistent results, ranging from a substantial contribution of CASC3 to only a minor role in NMD
299 (Palacios *et al.*, 2004, Gehring *et al.*, 2005, Gehring *et al.*, 2009, Mabin *et al.*, 2018). In addition, none
300 of the previous publications performed transcriptome-wide analyses but concentrated on reporter
301 mRNAs of only a few selected endogenous NMD targets. We reasoned that the inconsistent results in
302 the literature may be influenced by variable CASC3 knockdown efficiency. By generating CASC3
303 knockout cell lines, we can for the first time analyze the global effects of a complete depletion of CASC3
304 on the transcriptome and can exclude that residual CASC3 protein masks these effects. Interestingly,

305 the CASC3 knockout had no deleterious effect on the viability of HEK293 cells, unlike the depletion of
306 the EJC components EIF4A3, RBM8A, or MAGOH. Nonetheless, since CASC3 is required for mouse
307 embryogenesis and involved in the transport of mRNAs in *D. melanogaster*, it is likely that CASC3
308 downregulation in highly specialized cell types such as neurons or in developing tissues would result
309 in a more severe phenotype compared to HEK293 cells.

310 In recent years, high-throughput RNA-sequencing became an increasingly important method for
311 the analysis of NMD. Several RNA-Seq datasets of cells with NMD-factor knockdowns have been
312 generated and analyzed (Tani *et al.*, 2012, Hurt *et al.*, 2013, Lykke-Andersen *et al.*, 2014, Schmidt *et*
313 *al.*, 2015, Colombo *et al.*, 2017). However, these datasets were obtained in different cell lines, with
314 different amounts of replicates and due to the rapid developments of next-generation sequencing, not
315 using the same technologies. Furthermore, batch effects and divergent approaches of data analyses
316 may contribute to the fact that only a minor overlap of NMD targets could be established so far
317 (Colombo *et al.*, 2017). We compared the results of our CASC3 KO RNA-sequencings to the most recent
318 and comprehensive NMD factor analysis performed by Colombo *et al.* (2017). The differential
319 expression analysis revealed that many of the top upregulated genes in the CASC3 KO datasets are also
320 significantly affected by UPF1 or SMG6/7 knockdowns (Colombo *et al.*, 2017) and/or encode for small
321 RNA (sRNA) host genes, a previously described class of NMD targets (Lykke-Andersen *et al.*, 2014).

322 We detected many alternative splicing events in the CASC3 KO data, which was unexpected given
323 that CASC3 was apparently dispensable for the nuclear EJC-related functions. However, we could
324 attribute these splicing patterns to dysfunctional NMD, since isoform-specific algorithms revealed that
325 predominantly PTC-containing transcripts accumulated. Using all the available bioinformatics analysis
326 pipelines and a systematic approach to detect affected transcripts under NMD factor
327 knockdown/knockout conditions could therefore be a crucial step to paint a complete picture of the
328 regulation of transcripts by NMD in the future.

329 How exactly CASC3 activates NMD when bound to an EJC, is not yet fully understood. Previously,
330 we reported that the presence of EJCs in the 3' UTR enhances endocleavage (Boehm *et al.*, 2014). In
331 line with the proposed role as a peripheral NMD-activating EJC component, we observed that CASC3
332 stimulates SMG6-dependent endocleavage, thereby promoting the degradation of NMD-targeted
333 transcripts. This effect can be recapitulated by tethering full-length CASC3, its N-terminal two thirds
334 (1-480) or just its N-terminal 137 amino acids to a reporter mRNA. How the small N-terminal region,
335 which cannot assemble into the EJC or contains any known protein domains or sequence motifs can
336 elicit NMD remains to be determined. It is also unclear, if the N-terminus activates translation-
337 dependent degradation, as it was previously shown for the full length CASC3 (Boehm *et al.*, 2016).
338 Since the N-terminal segment of CASC3 is a region of low-complexity it could hypothetically undergo
339 liquid-liquid phase separation (LLPS) and be present in condensates with mRNA decay factors, such as
340 processing bodies (P-bodies). In agreement with this idea CASC3 was shown to localize to cytoplasmic
341 granules when overexpressed (Cougot *et al.*, 2014).

342 Our data suggest that CASC3 activates NMD by potentially redundant mechanisms. This fits very
343 well into the general picture of NMD, which uses multiplexed degradation pathways to efficiently and
344 robustly remove mRNAs. Mechanistically, binding of CASC3 to the EJC could have an indirect effect on
345 NMD stimulation by increasing the stability of the bound EJC and thus maintaining the possibility of
346 efficient endonucleolytic cleavage of the transcript. An indication for this role comes from the initial *in*
347 *vitro* observation that CASC3 stabilizes recombinant EJCs (Ballut *et al.*, 2005). Additionally, the
348 moderately reduced pull-down of UPF3B with EIF4A3 could indicate that cytoplasmic NMD-competent
349 EJCs are less stable in CASC3 KO cells. Alternatively, CASC3 may directly contribute to the recruitment
350 of NMD factors. We therefore propose that CASC3 either alone or in conjunction with UPF3B links the
351 EJC with the NMD machinery. In particular, CASC3 influences the contribution of SMG6-mediated
352 endonucleolytic and SMG7-dependent exonucleolytic decay pathways to the overall degradation
353 efficiency of NMD. Accordingly, in wild type cells a knockdown of SMG7 only had a marginal effect on
354 the abundance of the analyzed NMD reporter mRNA, whereas it clearly impaired NMD in CASC3 KO

355 cells. Also, the amount of endocleavage-derived 3' fragments was reduced when CASC3 is depleted,
356 mirroring the SMG6-knockdown condition.

357 By integrating CASC3 as a specific NMD-activating factor we can now postulate a modified model
358 of EJC-dependent NMD, which is also compatible with several molecular properties of the EJC (Figure
359 7). Since CASC3 is only present in modest amounts in the cytoplasm, it will probably not immediately
360 associate with all EJCs on recently exported mRNPs. This would also not be necessary, since most EJCs
361 are located in the coding sequence and will therefore be removed by the first translating ribosome.
362 However, mRNAs containing PTCs will carry one or more EJCs in their 3' UTR, which are available for
363 binding of CASC3. The first few translating ribosomes may terminate upstream of CASC3-free EJCs,
364 which could preferentially trigger SMG7-dependent exonucleolytic degradation. Previously, NMD has
365 been proposed to occur primarily in the pioneering round of translation when newly synthesized
366 transcripts are bound to the cap-binding complex (Ishigaki *et al.*, 2001, Maquat *et al.*, 2010). This model
367 has been challenged and there is evidence that NMD can occur on already translating mRNAs and
368 possibly with a constant probability during every round of translation (Durand & Lykke-Andersen,
369 2013, Rufener & Muhlemann, 2013, Hoek *et al.*, 2019). Thus, CASC3 could bind to the EJC at a later
370 time point and then increase the probability to activate SMG6-mediated endonucleolytic degradation
371 after each round of termination. Important molecular targets of CASC3 may be mRNAs that escape
372 initial NMD activation, despite containing a PTC (Trcek *et al.*, 2013, Hoek *et al.*, 2019). CASC3 may help
373 to reduce the amount of these mRNAs by maintaining the NMD-activating function of the EJC, either
374 by increasing its stability on the mRNA or via direct interactions with the NMD machinery. This concept
375 would be consistent with the recent observation that NMD targets undergo several rounds of
376 translation before endocleavage occurs (Hoek *et al.*, 2019).

377 In summary, our data paint a picture, in which CASC3 has no essential EJC-related function in the
378 nucleus, but helps to turn EJCs into a persistent RNA degradation mode. We do not exclude the
379 possibility that CASC3 is already associated with the EJC in the nucleus. However, our model of delayed

380 binding of CASC3 to the EJC in the cytoplasm would explain why only a small amount of CASC3 is
381 sufficient to activate EJC-dependent NMD. In this model, CASC3 is an indispensable cytoplasmic
382 component of the EJC that helps to degrade mRNAs that failed to unload all their bound EJCs during
383 the initial rounds of translation. Thus, the binding of CASC3 to the EJC could signal the final round(s) of
384 translation of an mRNA.

385 # *Materials and Methods*

386 ## *Cell culture*

387 Flp-In 293 T-REx cells (Thermo Fisher Scientific) were maintained at 37° C, 5% CO_2 and
388 90% humidity in Dublecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) supplemented
389 with 9% fetal bovine serum (FBS) and Penicillin-Streptomycin (both Thermo Fisher Scientific).
390 Tethering experiments were performed in HeLa Tet-Off cells (Clontech) cultured in the same
391 conditions.

392 ## *siRNA-mediated knockdowns*

393 The cells were seeded in 6-well plates at a density of $2x10^5$ cells per well and reverse transfected
394 using 2.5 µl Lipofectamine RNAiMAX and 60 pmol of the respective siRNA(s) according to the
395 manufacturer's instructions. In preparation for mass spectrometry, the cells were reverse transfected
396 in 10 cm dishes using 10 µl Lipofectamine RNAiMAX and 300 pmol siRNA. siRNAs were targeted against
397 Luciferase (5'-CGTACGCGGAATACTTCGA-3'), EIF4A3 (5'-AGACATGACTAAAGTGGAA-3'), RBM8A (5'-
398 TTCGCAGAATATGGGGAAA-3'), CASC3 (5'-CTGATGACATCAAACCTCGAAGAAT-3', 5'-
399 CGTCATGAACTTTGGTAATCCCAGT-3'), UPF1 (5'-GATGCAGTTCCGCTCCATT-3'), XRN1 (5'-
400 AGATGAACTTACCGTAGAA-3'), SMG6 (5'-GGGTCACAGTGCTGAAGTA-3') or SMG7 (5'-
401 CGATTTGGAATACGCTTTA-3').

402 ## *Generation of knockout cells using CRISPR-Cas9*

403 The knockouts were performed using the Alt-R CRISPR-Cas9 system (IDT) and reverse transfection
404 of a Cas9:guideRNA ribonucleoprotein complex using Lipofactamine RNAiMAX (Thermo Fisher
405 Scientific) according to the manufacturer's protocol. The crRNA sequences to target CASC3 were
406 /AlTR1/rGrCrGrCrGrCrUrUrCrGrCrArArGrArCrArCrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2/
407 (clone H) and
408 /AlTR1/rGrUrUrCrGrGrCrCrUrCrCrGrCrGrCrUrGrUrGrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AlTR2/
409 (clones F and T). Reverse transfection was performed on $1.5x10^5$ cells per crRNA in 12-well dishes.

410 48 hours after transfection the cells were trypsinized, counted and seeded at a density of a single cell
411 per well in 96-well plates. Cell colonies originating from a single clone were then validated by Sanger
412 sequencing of the targeted genomic DNA locus and western blotting.

413 *Plasmid transfection*

414 All used plasmids are listed in Supplementary Table 1. To express FLAG-tagged protein constructs
415 and the reporter mRNAs detected by northern blotting, the cells were stably transfected using the Flp-
416 In T-REx system and the tetracycline inducible pcDNA5/FRT/TO vector (Thermo Fisher Scientific). The
417 constructs TPI-WT, TPI-PTC, β-globin WT and β-globin PTC are available on Addgene (IDs 108375-
418 108378). $2.5x10^5$ cells were seeded 24 h before transfection in 6-wells. Per well, 1 µg of reporter
419 construct was transfected together with 1 µg of the Flp recombinase expressing plasmid pOG44 using
420 the calcium phosphate method. 48 h after transfection, the cells were transferred into 10 cm dishes
421 and selected with 100 µg/ml hygromycin. After 10 days, the colonies were pooled. Expression of the
422 reporter mRNA was induced with 1 µg/ml doxycycline for 24 h.

423 Constructs that express V5-tagged and MS2V5-tagged proteins were stably integrated into the
424 cells using the PiggyBac (PB) Transposon system and the cumate-inducible PB-CuO-MCS-IRES-GFP-EF1-
425 CymR-Puro vector (System Biosciences). $2.5x10^5$ cells were seeded 24 h before transfection in 6-wells.
426 2.5 µg of the PB Transposon vector and 0.8 µg of PB Transposase were transfected per well using the
427 calcium phosphate method. After 48 h, the cells were pooled in 10 cm dishes and positive clones
428 selected with 2 µg/ml puromycin for a week. Expression of proteins was induced using 30 µg/ml
429 cumate for 72 h.

430 The tethering construct pSBtet-Hyg-TPI-4MS2-SMG5-4H was stably integrated into HeLa Tet-Off
431 cells using the Sleeping Beauty (SB) transposon system (Mates *et al.*, 2009, Kowarz *et al.*, 2015). pSBtet-
432 Hyg was a gift from Eric Kowarz (Addgene plasmid #60508; http://n2t.net/addgene:60508;
433 RRID:Addgene_60508). pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid
434 #34879; http://n2t.net/addgene:34879; RRID:Addgene_34879). $2.5x10^5$ cells were seeded 24 h before

435 transfection in 6-wells. Per well, 1 µg of the reporter construct was transfected together with 1.5 µg
436 of the SB Transposase using the calcium phosphate method. 48 h after transfection, the cells were
437 transferred into 10 cm dishes and selected with 100 µg/ml hygromycin. After 10 days, the colonies
438 were pooled. In absence of tetracycline the reporter was constitutively expressed.

439 *RNA-Sequencing and computational analyses*

440 RNA-Seq analysis was carried out with 293 WT cells transfected with Luciferase siRNA and the
441 CASC3 KO clones H and T transfected with either Luciferase or CASC3 siRNAs. Three biological
442 replicates were analyzed for each sample. RNA was isolated with the kit NucleoSpin RNA Plus
443 (Macherey-Nagel). The Lexogen SIRV Set1 Spike-In Control Mix (SKU: 025.03) that provides a set of
444 external RNA controls was added to the total RNA to enable performance assessment. Mix 0 was added
445 to replicate 1, mix 1 was added to replicate 2 and mix 3 to replicate 3. The Spike-Ins were not used for
446 analysis. The library preparation was performed with the TrueSeq Stranded Total RNA kit (Illumina).
447 First steps of the library preparation involve the removal of ribosomal RNA using biotinylated target-
448 specific oligos combined with Ribo-Zero gold rRNA removal beads from 1ug total RNA input. The Ribo-
449 Zero Human/Mouse/Rat kit depletes samples of cytoplasmic and mitochondrial rRNA. Following
450 purification, the RNA is fragmented and cleaved. RNA fragments are copied into first strand cDNA using
451 reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA
452 Polymerase I and RNase H. These cDNA fragments then have the addition of a single'A' base and
453 subsequent ligation of the adapter. The products are purified and enriched with PCR to create the final
454 cDNA library. After library validation and quantification (Agilent tape station), equimolar amounts of
455 library were pooled. The pool was quantified by using the Peqlab KAPA Library Quantification Kit and
456 the Applied Biosystems 7900HT Sequence Detection System and sequenced on an Illumina
457 NovaSeq6000 sequencing instrument and an PE100 protocol.

458 Read processing and alignment was performed as described previously (Boehm *et al.*, 2018). In
459 short, adaptor sequences and low quality bases were removed with Flexbar 3.0 (Dodt *et al.*, 2012).

460 Short reads from the rRNA locus were subtracted by mapping against the 45S precursor (Homo
461 sapiens, NR_046235.1) using Bowtie2 (Langmead & Salzberg, 2012). The remaining reads were aligned
462 against the human genome (version 38, EnsEMBL 90 transcript annotations) using the STAR read
463 aligner (version 2.5.3a) (Dobin *et al.*, 2013).

464 To compute gene differential expression analysis, reads covering exons were counted with
465 FeatureCounts (version 1.5.1) (Liao *et al.*, 2014) using the '—primary' and '—ignoreDup' parameters.
466 Differential gene expression analysis was performed with DESeq2 (Love *et al.*, 2014, Ignatiadis *et al.*,
467 2016) and IWH R packages. Significance thresholds were |log2FoldChange|> 1 and adjusted p-value
468 (padj) < 0.05. Genes were designated as small RNA (sRNA) host gene, if they contained other Ensembl-
469 annotated genes of biotypes snoRNA or miRNA within their genomic coordinates (Zerbino *et al.*, 2018).

470 Differential splicing was detected with LeafCutter (version 0.2.7) (Li *et al.*, 2018) with the
471 parameters min_samples_per_intron = 2 and min_samples_per_group = 2. Significance thresholds
472 were |deltapsi| > 0.1 and adjusted p-value (p.adjust) < 0.05.

473 Transcript abundance estimates were computed with Salmon (version 0.13.1) (Patro *et al.*, 2017)
474 using the the -validateMappings --gcBias parameters. Differential transcript usage was computed with
475 IsoformSwitchAnalyzeR (version 1.7.1) and the DEXSeq method (Robinson & Oshlack, 2010, Anders *et*
476 *al.*, 2012, Ritchie *et al.*, 2015, Soneson *et al.*, 2015, Vitting-Seerup & Sandelin, 2017, Vitting-Seerup &
477 Sandelin, 2019). Significance thresholds were |dIF| > 0.1 and adjusted p-value
478 (isoform_switch_q_value) < 0.05. For the Boxplot and Kolmogorov-Smirnoff test, the data were filtered
479 only for the adjusted p-value. PTC status of transcript isoforms with annotated open reading frame
480 was determined by IsoformSwitchAnalyzeR using the 50 nt rule of NMD (Weischenfeldt *et al.*, 2012,
481 Vitting-Seerup *et al.*, 2014, Huber *et al.*, 2015, Vitting-Seerup & Sandelin, 2017). Isoforms with no
482 annotated open reading frame in Ensembl were designated "NA" in the PTC analysis.

483 The UPF1 and SMG6/7 (Data ref: Colombo *et al.*, 2017) and RNPS1 (Data ref: Boehm *et al.*, 2018)
484 knockdown datasets were processed and analyzed with the same programs, program versions, and

485 scripts as the CASC3 dataset. All packages used are listed in the respective analysis table
486 (Supplementary Tables 4-6). Sashimi plots were generated using ggsashimi (Garrido-Martin *et al.*,
487 2018). Overlaps of data sets were represented via nVenn (Perez-Silva *et al.*, 2018), eulerr (Larsson,
488 2019) and Upset plots (Lex *et al.*, 2014). Heatmaps were generated using ComplexHeatmap (Gu *et al.*,
489 2016).

490 *SILAC, co-immunoprecipitation and mass spectrometry*

491 293 WT and 293 CASC3 KO clone H cells expressing either FLAG or FLAG-EIF4A3 were labeled by
492 maintaining them for 5 passages in DMEM for SILAC medium (Thermo Fisher Scientific) supplemented
493 with FBS (Silantes), Penicillin-Streptomycin (Thermo Fisher Scientific) and the respective amino acids
494 at a final concentration of 0.798 mmol/L (Lysine) and 0.398 (Arginine). Unlabeled proline was added
495 to prevent enzymatic Arginine-to-Proline conversion. The conditions were "light" (unlabeled
496 Lysine/Arginine), "medium" (Lysine 4/Arginine 6) and "heavy" (Lysine 8/Arginine 10). A label switch
497 was performed between the three replicates according to the experimental setup listed in
498 Supplementary Table 2. The expression of FLAG or FLAG-EIF4A3 was induced for 72 h with 1 µg/ml
499 doxycycline. The cells were lysed in buffer E with RNAse (20 mM HEPES-KOH (pH 7.9), 100 mM KCl,
500 10% glycerol, 1 mM DTT, Protease Inhibitor, 1 µg/ml RNAse A) and sonicated using the Bandelin
501 Sonopuls mini20 with 15 pulses (2.5 mm tip in 600 µl volume, 1s, 50% amplitude). 600 µl of a 1.6 mg/ml
502 total protein lysate were incubated with 30 µl Anti-FLAG M2 magnetic beads (Sigma) at 4° C while
503 rotating for 2 h. The beads were washed three times for 5 min with EJC-buffer (20 mM HEPES-KOH (pH
504 7.9), 200 mM NaCl, 2 mM $MgCl_2$, 0.2% Triton X-100, 0.1% NP-40, 0.05% Sodium deoxycholate) and
505 eluted in 43 µl of a 200 mg/ml dilution of FLAG peptides (Sigma) in 1x TBS. The samples were merged
506 according to Supplementary Table 2. 1 volume of 10% SDS was added and the samples were reduced
507 with DTT and alkylated with CAA (final concentrations 5 mM and 40 mM, respectively). Tryptic protein
508 digestion was performed using a modified version of the single pot solid phase-enhanced sample
509 preparation (SP3) (Hughes *et al.*, 2014). In brief, reduced and alkylated proteins were supplemented
510 with paramagnetic Sera-Mag speed beads (Thermo Fisher Scientific) and mixed in a 1:1-ratio with 100%

511 acetonitrile (ACN). After 8 min incubation protein-beads-complexes were captured using an in-house
512 build magnetic rack and two times washed with 70% EtOH. Afterwards, samples were washed once
513 with 100% ACN, air-dried and reconstituted in 5 µl 50 mM Triethylamonium bicarbonate
514 supplemented with 0.5 µg trypsin and 0.5 µg LysC and incubated overnight at 37°C. On the next day
515 the beads were resuspended and mixed with 200 µl ACN, incubated for 8 min and again placed on the
516 magnetic rack. Tryptic peptides were washed once with 100% ACN, airdried, dissolved in 4% DMSO
517 and transferred into 96-well PCR tubes. After acidification with 1 µl of 10% formic acid, samples were
518 ready for LC-MS/MS analysis

519 For the MS experiment combined with CASC3 knockdown, the 293 CASC3 KO clone H cells were
520 treated with siRNA against CASC3 24 h before expression of the FLAG-tagged construct. The cells were
521 lysed and samples prepared for MS as described above. However, the washing steps were performed
522 in a less stringent EJC buffer (20 mM HEPES-KOH (pH 7.9), 137 mM NaCl, 2 mM $MgCl_2$, 0.2% Triton X-
523 100, 0.1% NP-40).

524 Proteomics analysis was performed by data-dependent acquisition using an Easy nLC1200 ultra
525 high-performance liquid chromatography (UHPLC) system coupled via nanoelectrospray ionization to
526 a Q Exactive Plus instrument (all Thermo scientific). Tryptic peptides were separated based on their
527 hydrophobicity using a chromatographic gradient of 60 min with a binary system of buffer A (0.1%
528 formic acid) and buffer B (80% ACN, 0.1% formic acid). In-house made analytical columns (length: 50
529 cm, inner diameter: 75 µm) filled with 1.9 µm C18-AQ Reprosil Pur beads (Dr. Maisch) were used for
530 separation. Buffer B was linearly increased from 3% to 27% over 41 min followed by a steeper increase
531 to 50% within 8 min. Finally, buffer B was increased to 95% within 1 min and stayed at 95% for 10 min
532 in order to wash the analytical column. Full MS spectra (300 – 1,750 m/z) were acquired with a
533 resolution of 70,000, a maximum injection time of 20 ms and an AGC target of 3e6. The top 10 most
534 abundant peptide ions of each full MS spectrum were selected for HCD fragmentation (NCE: 27) with

535 an isolation width of 1.8 m/z and a dynamic exclusion of 10 seconds. MS/MS spectra were measured
536 with a resolution of 35,000, a maximum injection time of 110 ms and an AGC target of 5e5.

537 MS RAW files were analysed using the standard settings of the MaxQuant suite (version 1.5.3.8)
538 with the before mentioned SILAC labels (Cox & Mann, 2008). Peptides were identified by matching
539 against the human UniProt database using the Andromeda scoring algorithm (Cox *et al.*, 2011).
540 Carbamidomethylation of cysteine was set as a fixed modification, methionine oxidation and N-
541 terminal acetylation as variable modification. Trypsin/P was selected as digestion protein. A false
542 discovery Rate (FDR) < 0.01 was used for identification of peptide-spectrum matches and protein
543 quantification. Data processing and statistical analysis was done in the Perseus software (version
544 1.5.5.3) (Tyanova *et al.*, 2016). Significantly changed proteins were identified by One-sample t-testing
545 (H0 = 0, fudge factor S0 = 0.1). The results are listed in Supplementary Table 2 as "Results without
546 knockdown" (MAP conditions) and "Results with knockdown". Visualization was performed with the
547 Instant Clue software (version 0.5.3) (Nolte *et al.*, 2018).

548 Co-immunoprecipitation experiments followed by western blotting were performed as described
549 above except that a 15 min incubation step in SDS buffer (600 mM Tris pH 6.8, 100 mM DTT, 10%
550 Glycerol, 2% SDS, 0.002% Bromophenolblue) was used for elution from the beads.

551 *In vitro splicing assay*

552 FLAG-emGFP and FLAG-EIF4A3 was expressed in 293 WT and 293 CASC3 KO clone H cells for 72 h.
553 Whole cell lysate preparation and *in vitro* splicing was performed as previously described (Steckelberg
554 & Gehring, 2014), however the nuclear extract was substituted with whole cell extract from the
555 respective cell line. Pulldown efficiency of spliced mRNA was calculated by dividing the amount of
556 spliced mRNA in the pulldown by the amount in the respective input and dividing the results by 10
557 since 10% of the input was loaded.

558 *Semi-quantitative and quantitative reverse transcriptase (RT)-PCR*

559 RNA was extracted using peqGOLD TriFast reagent (VWR) according to the manufacturer's
560 instructions. Reverse transcription was performed with GoScript Reverse Transcriptase (Promega)
561 using 2 µg total RNA and oligo dT primers. Semi-quantitative PCR was carried out with MyTaq Red Mix
562 (Bioline). Quantitative real time PCR was performed with 16 ng of cDNA per reaction with GoTaq qPCR
563 Master Mix (Promega) and the CFX96 Touch Real-Time PCR Detection System (Biorad). The average cT
564 values were calculated from three technical replicates. The mean fold changes from three biological
565 replicates were calculated according to the ΔΔCt method (Schmittgen & Livak, 2008). When measuring
566 isoform switches, the fold change of the PTC-containing transcript was normalized to the canonical
567 transcript. When measuring differential expression, the fold change was normalized to GAPDH. For
568 each primer pair amplification efficiencies were measured by a 2-fold dilution curve and ranged
569 between 87 and 100.1%. The primer sequences are listed in Supplementary Table 3.

570 *Western blotting*

571 Protein extraction was performed with peqGOLD TriFast reagent (VWR), separated by SDS-PAGE
572 gel electrophoresis and transferred to a PVDF membrane (GE Healthcare Life Sciences). The following
573 antibodies were used: Anti-β-actin (Sigma-Aldrich, #A5441), anti-CASC3 amino acid residues 653-703
574 (Bethyl Laboratories, #A302-472A-M), anti-CASC3 amino acid residues 367-470 (Atlas Antibodies,
575 #HPA024592), anti-EIF4A3 (Genscript), anti-FLAG (Cell Signaling Technology, #14793), anti-MAGOH
576 (Santa Cruz Biotechnology, #sc-271365), anti-RBM8A (Atlas Antibodies, #HPA018403), anti-SMG6
577 (Abcam, #ab87539), anti-SMG7 (Elabscience, #E-AB-32926), anti-Tubulin (Sigma-Aldrich, #T6074), anti-
578 V5 (QED Bioscience, #18870), anti-XRN1 (Bethyl Laboratories, #A300-443A), anti-rabbit-HRP (Jackson
579 ImmunoResearch, #111-035-006), anti-mouse-HRP (Jackson ImmunoResearch, #115-035-003).
580 Detection was performed with Western Lightning Plus-ECL (PerkinElmer) or Amersham ECL prime (Ge
581 Healthcare Life Sciences) and the chemiluminescence imager Fusion FX6 EDGE (Vilber-Lourmat).

582 *Northern blotting*

583 The cells were harvested in peqGOLD TriFast reagent (VWR) and total RNA extraction was
584 performed as recommended by the manufacturer's protocol. 2.5 µg of total RNA were resolved on a
585 1% agarose/0.4 M formaldehyde gel using the tricine/triethanolamine buffer system (Mansour &
586 Pestov, 2013) followed by transfer on a nylon membrane (Roth) in 10x SSC. The blots were incubated
587 overnight at 65 °C in Church buffer containing [α-32P]-GTP body-labeled RNA probes for detection of
588 the reporter mRNA. Endogenous 7SL RNA was detected by a 5'-32P-labeled oligonucleotide (5'-
589 TGCTCCGTTTCCGACCTGGGCCGGTTCACCCCTCCTT-3'). The blots were visualized and quantified using a
590 Typhoon FLA 7000 (GE Healthcare) and ImageQuant TL 1D software.

591 *Data Availability*

592 The datasets produced in this study are available in the following databases. These data will be
593 made publicly accessible upon publication.

594 • RNA-seq data have been deposited in the ArrayExpress database (Kolesnikov *et al.*, 2015) at
595 EMBL-EBI under accession number E-MTAB-8461
596 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8461).
597 • The mass spectrometry proteomics data have been deposited to the ProteomeXchange
598 Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset
599 identifier PXD015754 (https://www.ebi.ac.uk/pride/archive/projects/PXD015754).

Author Contributions

Conceptualization, N.H.G., J.V.G. and V.B.;

Methodology, N.H.G., V.B., J.V.G., and C.K.F.;

Software, T.B.B., V.B., J.L.W., S.K. and C.D.;

Investigation, J.V.G, V.B., J.L.W., S.K., D.U.A. and S.C.;

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Conflict of Interest

The authors declare no competing interests.

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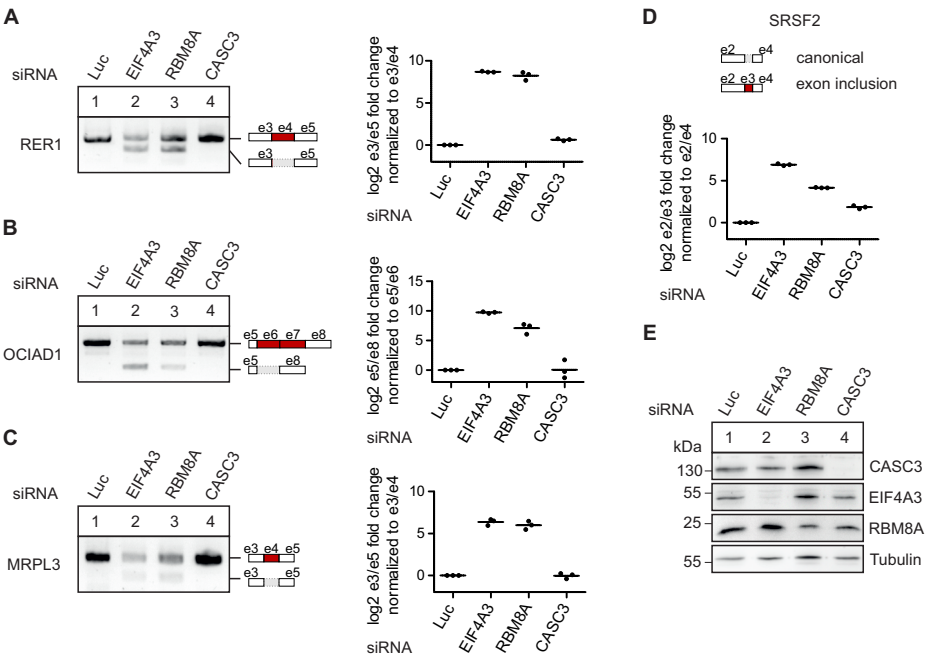

Figure 1 - CASC3 is not involved in the splicing regulation of known EJC-dependent targets.

A-C: RT-PCR- and quantitative RT-PCR-based detection (qPCR) of transcript isoforms of the genes RER1 (A), OCIAD1 (B), and MRPL3 (C) after siRNA-mediated knockdown of the indicated EJC components or Luciferase (Luc) as a negative control. Skipped exons are depicted schematically (e: exon). Data points and means from the qPCRs are plotted (n=3).

D: Relative quantification of the SRSF2 transcript isoforms by qPCR following knockdown of the indicated EJC components or Luciferase (Luc) as a negative control. The transcript variants at the position of the included exon are depicted schematically. Data points and means are plotted (n=3).

E: Confirmation of the knockdowns shown in A-D by western blotting.

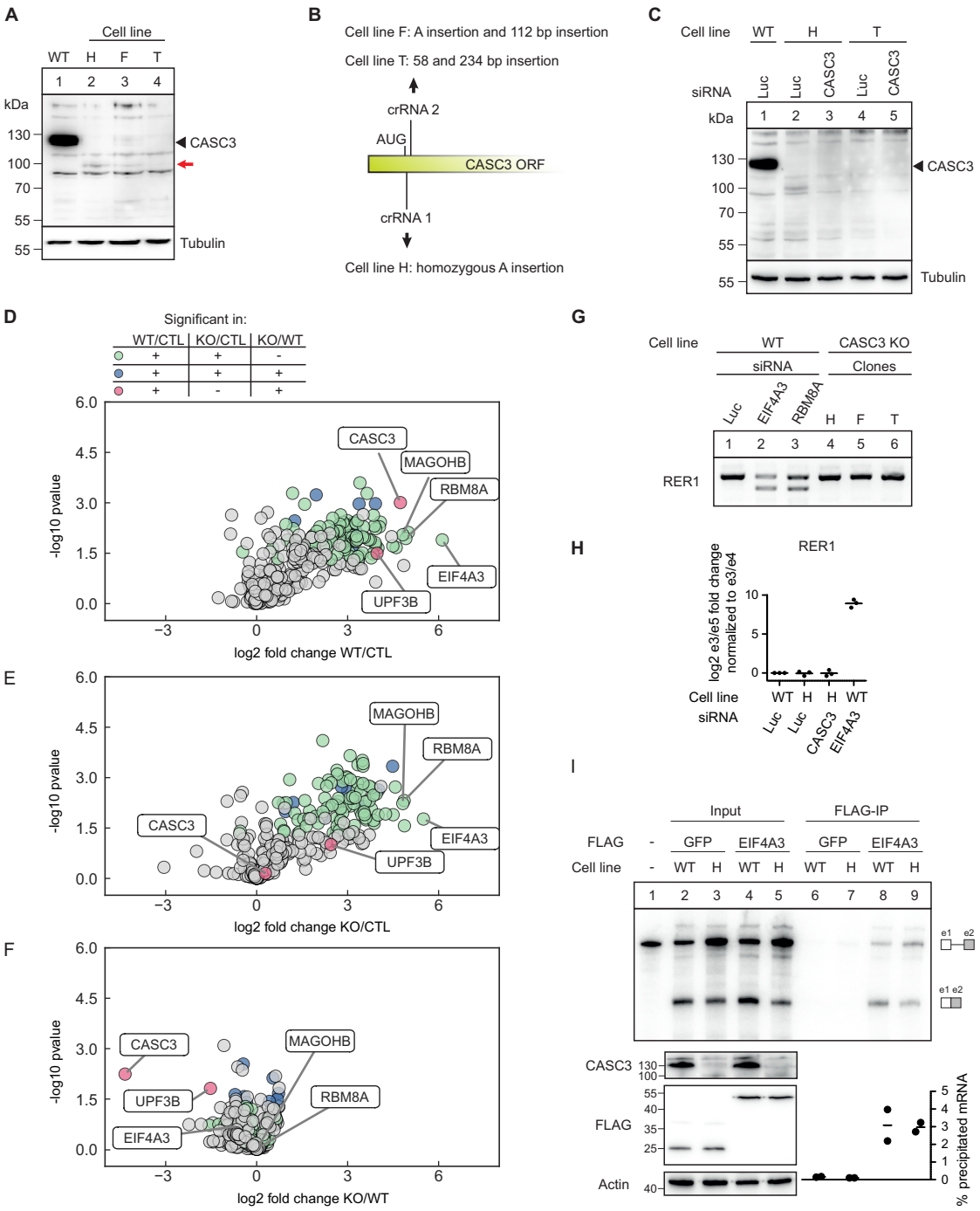

Figure 2 - Cells that lack CASC3 have intact EJCs and are splicing competent.

A: Total protein lysates from wild-type cells (WT) and CASC3 knockout (KO) cell lines H, F and T were separated by SDS-PAGE and CASC3 was detected by western blotting. The red arrow indicates an additional band detected in the cell lines H and F and which is not visible in the WT condition or in the cell line T.

B: Schematic depiction of the insertions resulting in a CASC3 KO or constitutive knockdown in the indicated clones.

C: Total protein lysates from WT and CASC3 KO cell lines H and T were separated by SDS-PAGE and CASC3 was detected by western blotting. In lanes 3 and 5 the cells have additionally been treated with siRNAs targeting CASC3.

D-F: Volcano plots of mass spectrometry-based analysis of the interaction partners of EIF4A3 in WT cells and in the CASC3 KO cell line H treated with siRNAs targeting CASC3. D: EIF4A3 against FLAG control in WT cells, E: EIF4A3 against FLAG control in KO cells, F: EIF4A3 in KO cells against EIF4A3 in WT cells. The color labeling indicates targets that are significant in the respective comparisons after one-sample t-testing.

G: RT-PCR of transcript isoforms of the gene RER1 after siRNA-mediated knockdown of the indicated EJC components or Luciferase (Luc) as a negative controle, compared to CASC3 KO cell lines H, F and T.

H: Relative quantification of the RER1 transcript isoforms by qPCR in WT cells treated with Luc siRNA as a negative control, CASC3 KO cell line H treated with Luc siRNA, CASC3 KO cell line H treated with CASC3 siRNAs and WT cells treated with EIF4A3 siRNA. Data points and means are plotted (n=3).

I: *In vitro* splicing assay of a MINX transcript in cell lysates obtained from WT cells or the CASC3 KO cell line H which stably expressed FLAG-GFP or FLAG-EIF4A3. The protein levels of CASC3 and the recombinant proteins in the cell lysates were analyzed by western blotting. The pulldown efficiencies of spliced mRNA were calculated and are plotted as individual data points and means (n=2).

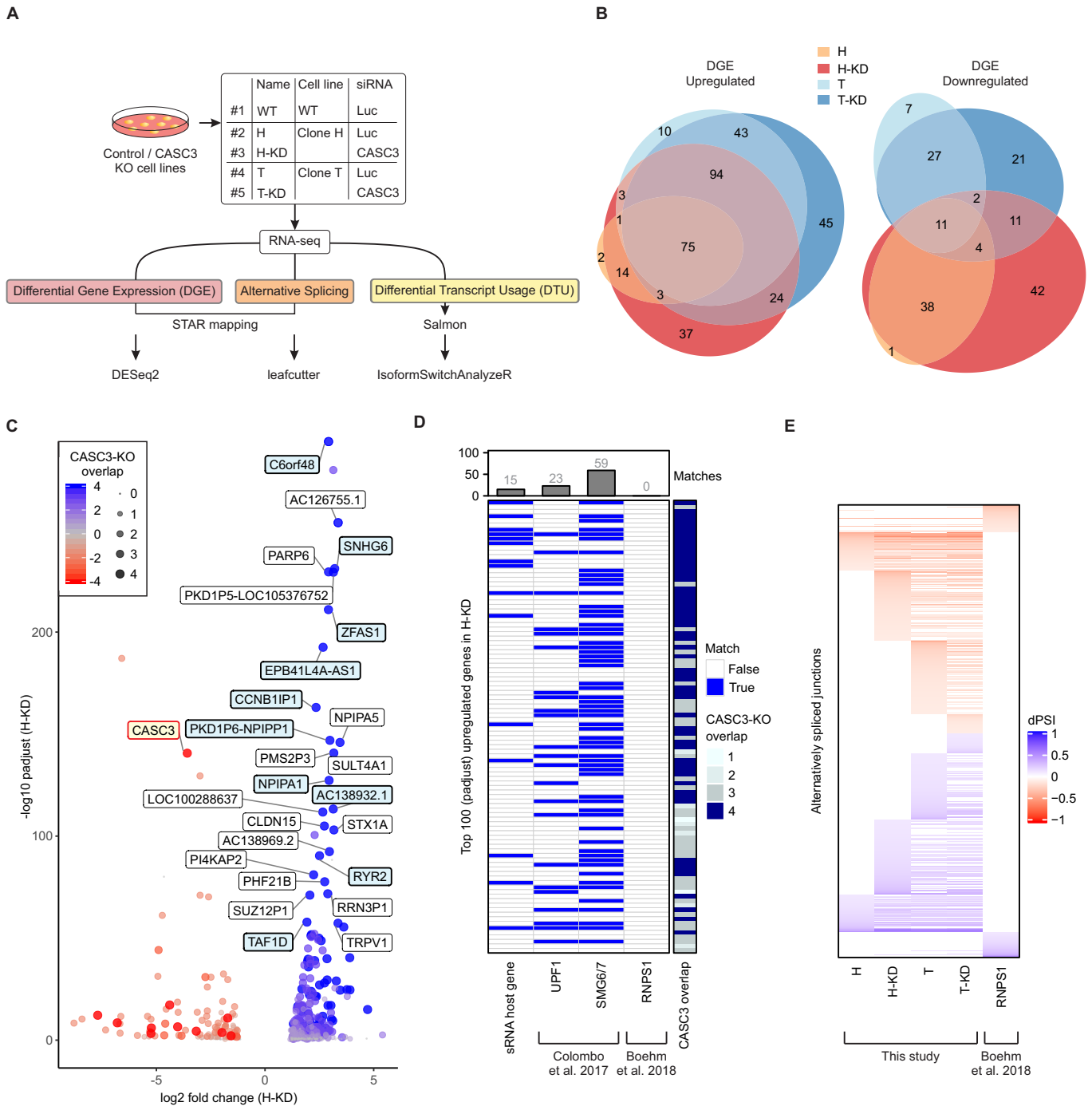

Figure 3 - CASC3-dependent targets are also affected by a perturbation of the NMD machinery.

A: Pipeline for RNA-sequencing analysis.

B: Overlap of up- and downregulated genes in the CASC3 KO cell lines H and T, +/- CASC3 siRNAs. DGE: Differential gene expression. Due to the visualization as Euler plot, some intersections cannot be plotted. All intersections are shown in Supplementary Figure 3C.

C: Volcano plot of differential gene expression analysis of the condition H-KD using overlap from B as color and point size definition. Gene symbols are indicated for the top 25 upregulated genes detected in all four conditions and for CASC3 (colored in light red). Labels of small RNA host genes are colored in light blue. Log2 fold change is plotted against –log10 padjust (adjusted p-value).

D: Matching of the top 100 upregulated genes sorted by padjust (adjusted p-value) in condition H-KD with small RNA (sRNA) host genes and comparison to knockdowns of UPF1, SMG6/7 and RNPS1.

E: Heatmap of all identified alternatively spliced junctions in the respective condition, measured in delta percent spliced in (dPSI).

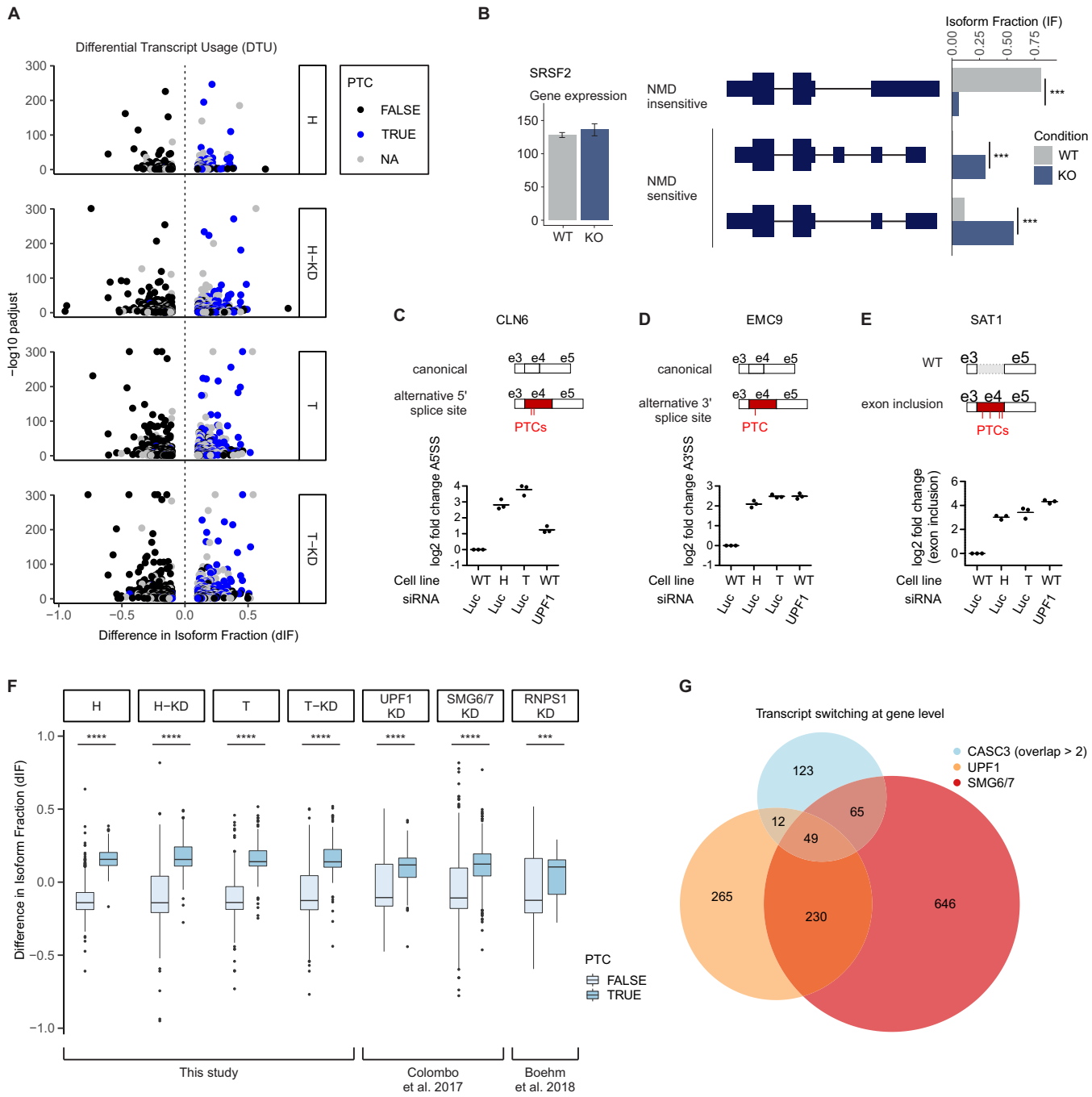

Figure 4 - Knockout of CASC3 leads to a global upregulation of NMD-sensitive transcript isoforms.

A: Results from IsoformSwitch analysis plotted as volcano plots. Transcript isoforms identified as NMD-sensitive are shown as blue dots. Isoforms with no annotated open reading frame are designated as “NA”. Difference in Isoform Fraction (dIF) is plotted against –log10 padjust (adjusted p-value).

B: Quantification of transcript isoforms from SRSF2 by IsoformSwitchAnalyzeR.

C-E: Relative quantification of the schematically depicted transcript isoforms of the genes CLN6 (C), EMC9 (D), and SAT1 (E) by qPCR in WT cells, CASC3 KO cell lines H and T and WT cells treated with siRNA targeting UPF1. PTC: premature termination codon. Individual data points and means are plotted (n=3).

F: Boxplot of PTC-containing vs. non-PTC-containing transcript isoforms after IsoformSwitch analysis for all CASC3 KO conditions compared to UPF1, SMG6/7 and RNPS1. A Kolmogorov-Smirnoff test was applied (p-value < 0.001 ***, p-value < 10-16 ****).

G: Euler plot showing overlaps of DTU-affected genes in the CASC3 (> 2 overlaps), UPF1-KD and SMG6/7-KD condition.

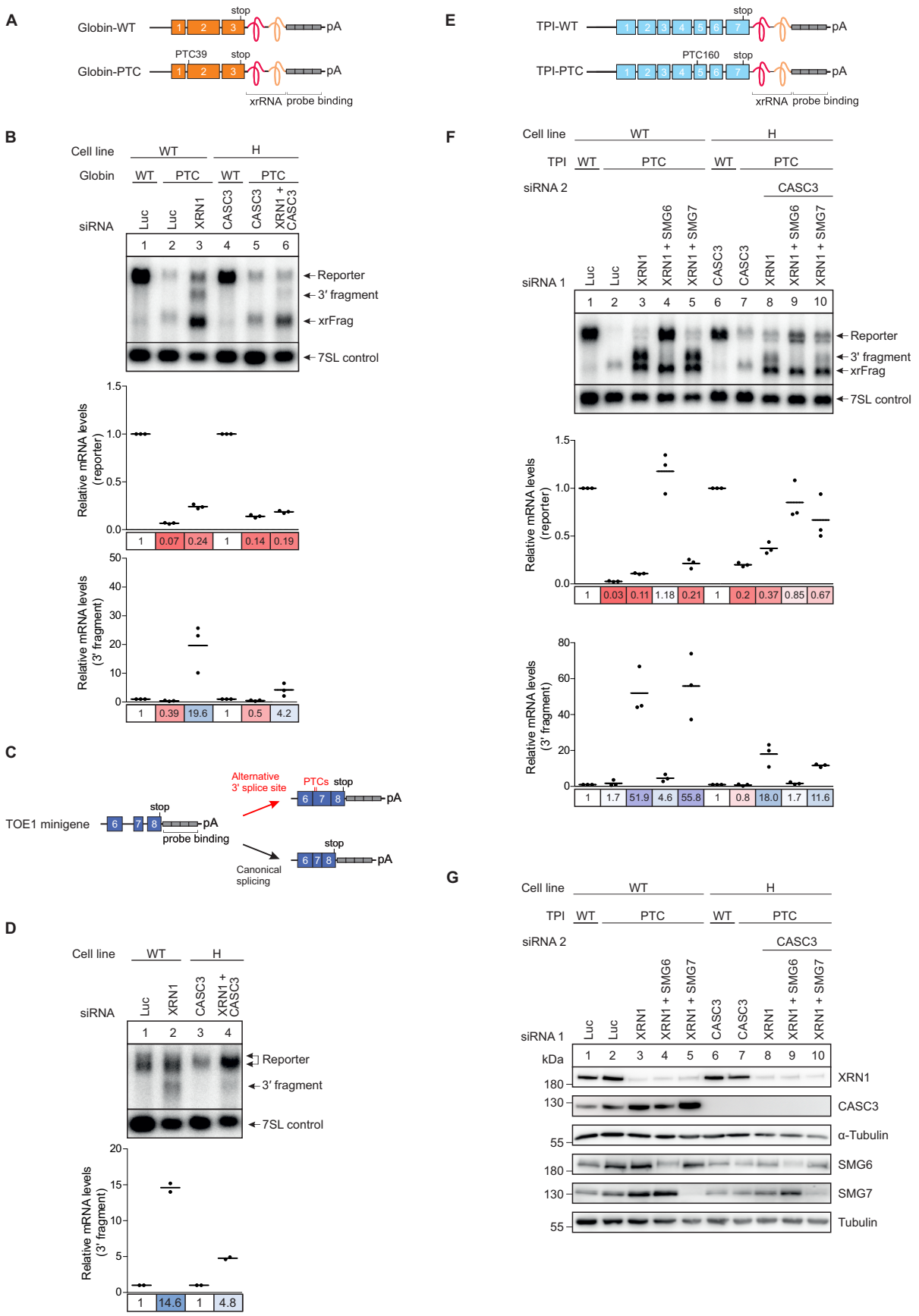
A
Globin-WT
stop
pA
Globin-PTC
PTC39
stop
pA
xrRNA
probe binding
B
Cell line
WT
H
Globin
WT
PTC
WT
PTC
siRNA
Luc
Luc
XRN1
CASC3
CASC3
XRN1 + CASC3
1 2 3 4 5 6
Reporter
3′ fragment
xrFrag
7SL control
Relative mRNA levels (reporter)
1.5
1.0
0.5
0.0
1 0.07 0.24 1 0.14 0.19
Relative mRNA levels (3′ fragment)
50
40
30
20
10
0
1 0.39 19.6 1 0.5 4.2
C
TOE1 minigene
stop
pA
probe binding
Alternative 3′ splice site
PTCs
stop
pA
Canonical splicing
stop
pA
D
Cell line
WT
H
siRNA
Luc
XRN1
CASC3
XRN1 + CASC3
1 2 3 4
Reporter
3′ fragment
7SL control
Relative mRNA levels (3′ fragment)
20
15
10
5
0
1 14.6 1 4.8
E
TPI-WT
stop
pA
TPI-PTC
PTC160
stop
pA
xrRNA
probe binding
F
Cell line
WT
H
TPI
WT
PTC
WT
PTC
siRNA 2
CASC3
siRNA 1
Luc
Luc
XRN1
XRN1 + SMG6
XRN1 + SMG7
CASC3
CASC3
XRN1
XRN1 + SMG6
XRN1 + SMG7
1 2 3 4 5 6 7 8 9 10
Reporter
3′ fragment
xrFrag
7SL control
Relative mRNA levels (reporter)
1.5
1.0
0.5
0.0
1 0.03 0.11 1.18 0.21 1 0.2 0.37 0.85 0.67
Relative mRNA levels (3′ fragment)
80
60
40
20
0
1 1.7 51.9 4.6 55.8 1 0.8 18.0 1.7 11.6
G
Cell line
WT
H
TPI
WT
PTC
WT
PTC
siRNA 2
CASC3
siRNA 1
Luc
Luc
XRN1
XRN1 + SMG6
XRN1 + SMG7
CASC3
CASC3
XRN1
XRN1 + SMG6
XRN1 + SMG7
kDa
1 2 3 4 5 6 7 8 9 10
180
130
55
180
130
55
XRN1
CASC3
α-Tubulin
SMG6
SMG7
Tubulin

Figure 5 - SMG6-mediated endocleavage is impaired when CASC3 is not present.

A: Schematic depiction of the globin mRNA reporter. The reporter consists of three exons (orange boxes) followed by an XRN1-resistant element (xrRNA) and a probe binding cassette (gray boxes). The PTC reporter contains a premature termination codon (PTC) in the second exon.

B: Northern blot of RNA extracted from the indicated cell lines that stably express the globin reporter. The xrFrag corresponds to the 3′ part of the reporter that is resistant to degradation by XRN1 due to the xrRNA. The cell lines in lane 3 and 6 were additionally treated with XRN1 siRNA which results in the appearance of a 3′ degradation fragment below the full-length reporter. Reporter and 3′ fragment mRNA levels were normalized to 7SL RNA which is shown as a loading control. For the relative mRNA quantification, in each condition (WT vs. CASC3 KO with KD) the reporter and 3′ fragment levels were normalized to the globin WT reporter (lanes 1 and 4). Individual data points and means are plotted from n=3 experiments.

C: Schematic depiction of the TOE1 minigene reporter consisting of exons 6-8 (purple boxes) followed by a probe binding cassette (gray boxes). The reporter can be spliced to either contain the canonical stop codon (bottom right) or, by usage of an alternative 3′ splice site, a PTC in exon 7 (top right).

D: Northern blot of RNA extracted from the indicated cell lines treated with the indicated siRNAs stably expressing the TOE1 minigene reporter. The 3′ fragment levels were first normalized to the 7SL RNA loading control and for every cell line the XRN1 knockdown condition to the condition without XRN1 knockdown (n=2).

E: Schematic depiction of the triose phosphate isomerase (TPI) mRNA reporter. The reporter consists of seven exons (blue boxes) followed by an XRN1-resistant element (xrRNA) and a probe binding cassette (gray boxes). The PTC reporter contains a premature termination codon (PTC) in the fifth exon.

F: Northern blot of RNA extracted from the indicated cell lines treated with the indicated siRNAs stably expressing the either the TPI WT or TPI PTC mRNA reporter. The reporter and 3′ fragment mRNA levels were normalized to the 7SL control. For each cell line, the mRNA levels were then normalized to the respective TPI WT reporter or 3′ fragment levels. Individual data points and means are plotted from n=3 experiments.

G: Western blot of samples shown in Figure 5F.

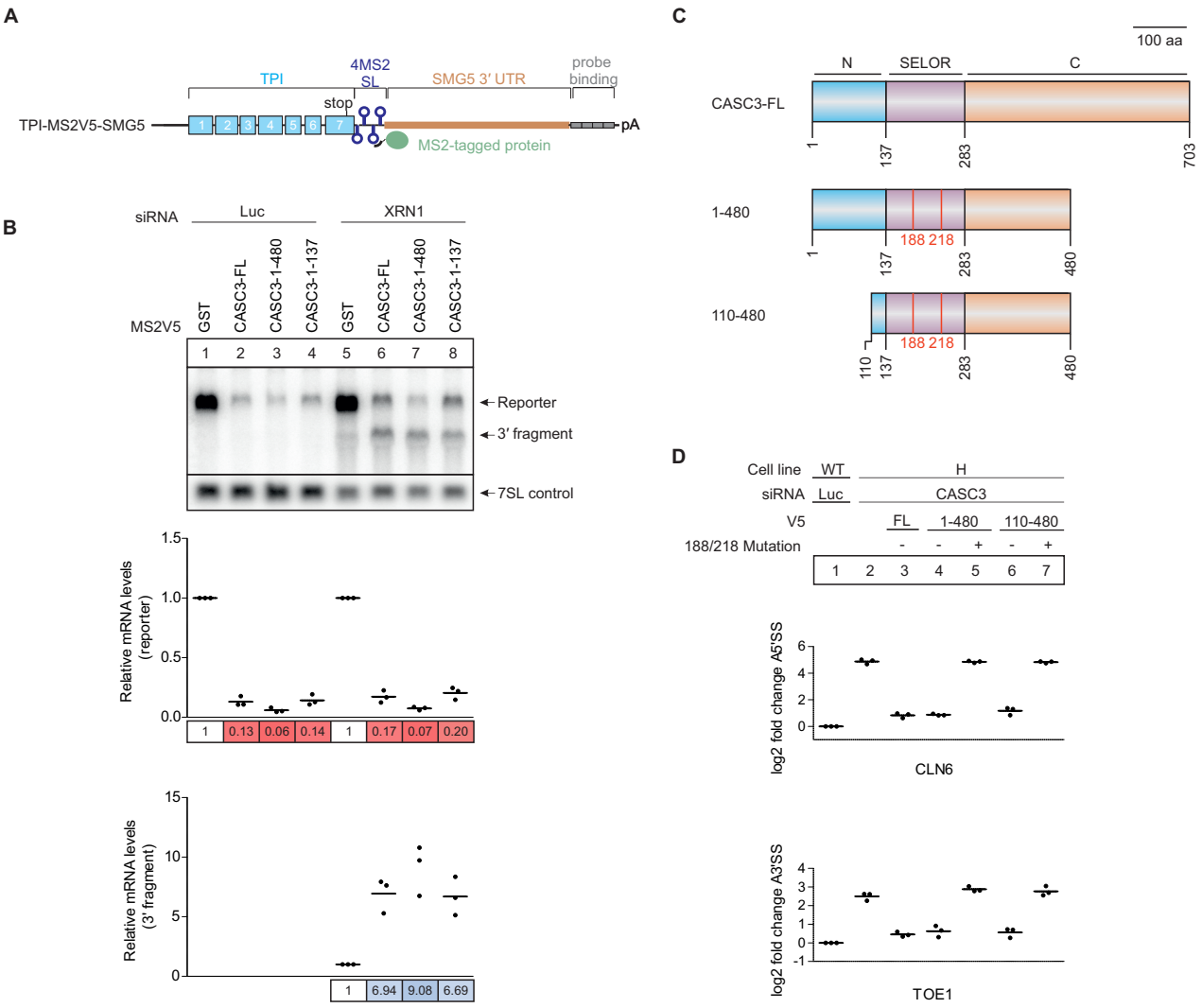

Figure 6 - The CASC3 N-terminus promotes but is not necessary to elicit NMD.

A: Schematic depiction of the TPI-MS2V5-SMG5 tethering reporter. The reporter consists of the TPI ORF (blue boxes) followed by 4 MS2 stem loops (SL). Downstream the SMG5 3′ untranslated region (UTR) is inserted to increase the size of 3′ fragments that result from cleavage at the termination codon. Reporter and 3′ fragment mRNAs can be detected via the probe binding cassette (gray boxes).

B: Northern blot of a tethering assay performed in HeLa Tet-Off cells. The cells stably express the tethering reporter shown in A together with the indicated MS2V5-tagged proteins. When the cells are additionally treated with XRN1 siRNA, a 3′ degradation fragment can be detected below the full-length reporter. The reporter and 3′ fragment mRNA levels are normalized to the 7SL RNA. For the calculation of the relative mRNA levels in each condition (Luc vs. XRN1) the levels were normalized to the MS2V5-GST control (lanes 1 and 5).

C: Schematic depiction of CASC3 rescue protein constructs. The full-length (FL) protein consists of an N-terminal (blue), C-terminal (orange) and central SELOR domain (purple). The construct 1-480 has a C-terminal deletion, whereas in the construct 110-480 both the N- and C-terminus are truncated. Both deletion constructs were also rendered EJC-binding deficient by mutating the amino acid residues 188 and 218 (F188D, W218D).

D: Relative quantification of the CLN6 (top) and TOE1 (bottom) transcript isoforms by qPCR in the indicated cell lines. The V5-tagged rescue proteins expressed in the KO condition are shown in C. Individual data points and means are plotted from n=3 experiments.

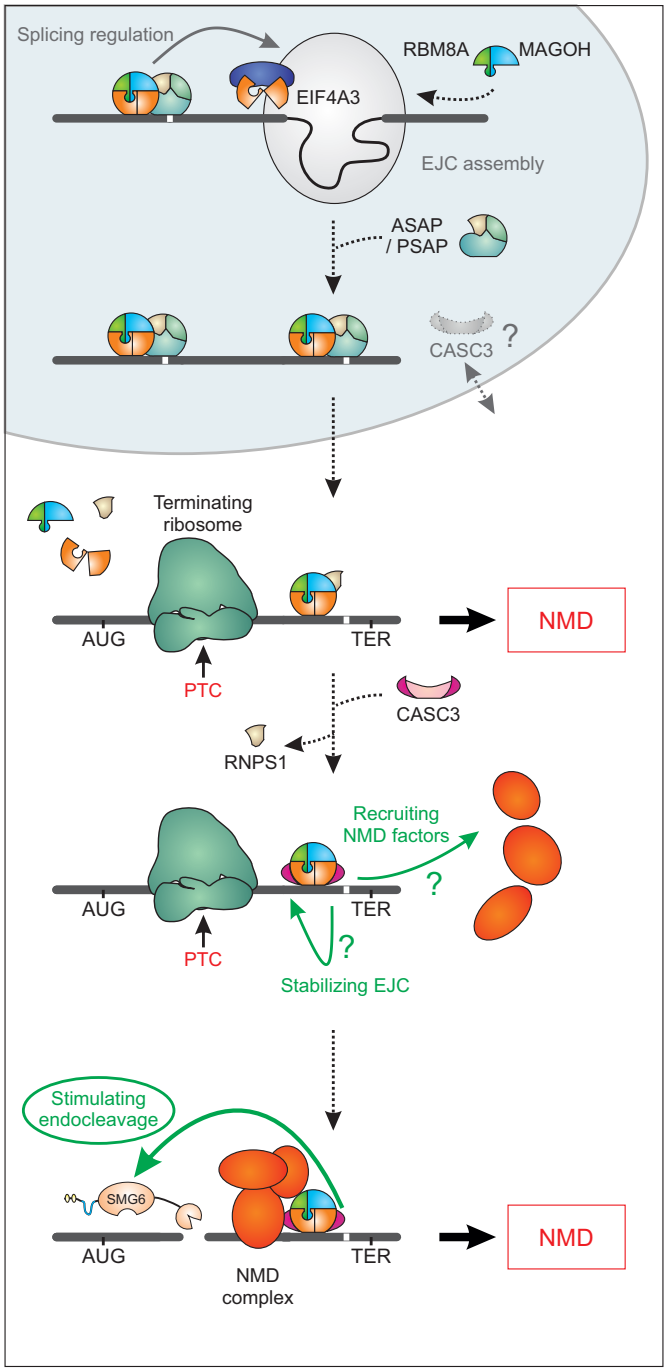

Figure 7 - Model of CASC3's cellular function.

Schematic depiction of the proposed function of CASC3 in the cell. We have found no evidence that CASC3 is necessary for EJC assembly in the nucleus, although CASC3 shuttles between cytoplasm and nucleus. Transcripts that require the deposition of the EJC to be correctly spliced were not affected by a lack of CASC3. In the cytoplasm, premature termination codon (PTC)-containing transcripts may still be degraded by NMD during the initial round(s) of translation in a CASC3-independent manner. CASC3 association with the EJC maintains and/or promotes the NMD-stimulating effect of the EJC, resulting in the degradation of transcripts that evaded initial NMD activation.