Title

Loss of PABPC1 is compensated by elevated PABPC4 and correlates with transcriptome changes

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Abstract

Cytoplasmic poly(A) binding protein (PABP) is an essential translation factor that binds to the 3' tail of mRNAs to promote translation and regulate mRNA stability. PABPC1 is the most abundant of several PABP isoforms that exist in mammals. Here, we used the CRISPR/Cas genome editing system to shift the isoform composition in HEK293 cells. Disruption of PABPC1 elevated PABPC4 levels. Transcriptome analysis revealed that the shift in the dominant PABP isoform was correlated with changes in key transcriptional regulators. This study provides insight into understanding the role of PABP isoforms in development and differentiation.

Keywords

PABPC1, PABPC4, c-Myc

1. Introduction

Cytoplasmic poly(A) binding protein (PABP) is a key component of the translational machinery; it is critical for the closed loop formation of mRNA and stimulates mRNA translation into protein (Sonenberg and Hinnebusch 2009). PABP plays a direct role in 60S subunit joining and is integral to the formation of the translation initiation complex on the mRNA (Kahvejian, Svitkin et al. 2005). PABP also protects mRNA transcripts from decay (Coller, Gray et al. 1998).

Most structural and functional studies of cytoplasmic PABPs are based on PABPC1. PABPC1 is the most abundant of several cytoplasmic poly(A) binding proteins (PABPs) found in vertebrates and has been known for four decades (Blobel 1973). PABPC1 consists of four RNA-binding domains (RRM1-4) followed by a linker region and a conserved C-terminal MLLE domain. The RRM domains mediate the circularization of mRNA through the binding of the 3' poly(A) tail and eIF4F complex on the mRNA 5' cap (Imataka, Gradi et al. 1998, Deo, Bonanno et al. 1999, Kahvejian, Svitkin et al. 2005, Safaee, Kozlov et al. 2012). The linker region may promote the self-association of PABPC1 on mRNA although the molecular details of the interaction are unknown (Melo, Dhalia et al. 2003, Simon and Seraphin 2007). The C-terminus of PABPC1 contains a MLLE domain that mediates binding of a peptide motif, PAM2, found in many PABP-binding proteins (Xie, Kozlov et al. 2014).

Five other less abundant cytoplasmic PABPs exist in higher vertebrate. The PABP isoforms are believe to fulfill functionally distinct roles in vertebrate development (Gorgoni, Richardson et al. 2011). PABPC3 (tPABP or PABPC2 in mouse) is testis-specific (Kleene, Mulligan et al. 1998). PABPC4 (iPABP) is inducible in activated T cells (Yang, Duckett et al. 1995) and serves a critical role in erythroid differentiation (Kini, Kong et al. 2014). PABPC1L (ePABP) functions in oocytes and early embryos (Voeltz, Ongkasuwan et al. 2001, Seli, Lalioti et al. 2005, Guzeloglu-Kayisli, Pauli et al. 2008). PABPC1L is substituted by PABPC1 later in development, but remains expressed in ovaries and testes of adult (Vasudevan, Seli et al. 2006). PABPC4L and PABPC5 (Blanco, Sargent et al. 2001) lack the linker and MLLE domain.

56 There are additional binding sites for PABPC1 on mRNA transcripts in addition to the 3'
57 poly(A) tail. Gel shift assays show that the RRM domains of PABPC1 bind various RNA
58 sequences other than poly(A); the RRM3-4 domains have broader specificity than RRM1-
59 2 (Sladic, Lagnado et al. 2004). It was shown by CLIP-seq that only a low percentage
60 (2.6%) of sequencing reads are pure poly(A) (Kini, Silverman et al. 2016). PABPC1 binds
61 to an auto-regulatory sequence in the 5'- UTR of its own mRNA, and controls its own
62 translation (de Melo Neto, Standart et al. 1995, Wu and Bag 1998, Hornstein, Harel et al.
63 1999). CLIP-seq study in mouse reveals that PABPC1 binds to a subset of A-rich sequences
64 in 5'-UTR, besides predominant binding to 3'-UTR of mRNAs (Kini, Silverman et al.
65 2016). These PABPC1 interactions at 5'-UTR can impact and coordinate post-
66 transcriptional controls on mRNAs (Kini, Silverman et al. 2016).

67 The functional specificity or redundancy of cytoplasmic PABPs is not well understood yet.
68 There have been increasing interests in PABPC4 in recent years. PABPC4, a minor isoform
69 of PABP, was first identified as an inducible protein in activated T-cells (Yang, Duckett et
70 al. 1995). Depletion of PABPC4 interferes with embryonic development of *Xenopus laevis,*
71 and cannot be rescued by isoforms PABPC1 or PABPC1L (Gorgoni, Richardson et al.
72 2011). PABPC4 plays an essential role in erythroid differentiation, and its depletion
73 inhibits terminal erythroid maturation (Kini, Kong et al. 2014). Motif analyses of PABPC4
74 affected mRNAs reveal a high-value AU-rich motif in the 3' untranslated regions (UTR)
75 (Kini, Kong et al. 2014).

76 A major difficulty in studying the roles of PABP isoforms is the abundance of PABPC1
77 compared with the minor isoforms. To investigate specific or redundant roles of PABP
78 isoforms, we disrupted PABPC1 in human cells with CRISPR/Cas9 gene editing system.
79 An elevated level of PABPC4 compensated the loss of PABPC1, which suggested certain
80 redundancy between the two isoforms. However, the transcriptome profile changed in
81 PABPC4 elevated cells. Gene set enrichment analysis indicated that c-Myc was the most
82 common gene in enriched pathways. Further, we showed correlated changes between
83 PABP isoforms and c-Myc levels. These studies expand our understanding of cytoplasmic
84 PABPs and suggest importance of a finely tuned network of PABP isoform usage.

85 **2. Materials and methods**

86 **2.1 Cell culture and plasmids**

87 Cells were cultured in DMEM supplemented with antibiotics and 10% fetal bovine serum.
88 10^5 cells per well were plated in 24-well plate the day before transfection. 0.8 μg DNA
89 plasmid was mixed with 2 μl Lipofectamine 2000 in Opti-MEM and then added to cells.
90 After 24 h, cells were trypsin digested and split onto cover slides. pFRT/TO/FLAG/HA-
91 DEST PABPC4 was from Thomas Tuschl (Addgene plasmid #19882) (Landthaler,
92 Gaidatzis et al. 2008). DNA fragment expressing PABPC1 (NM_002568) or
93 PABPC1ΔMLLE (1-542) were cloned into pCDNA3-EGFP between BamH I and Not I.

94 **2.2 CRISPR/Cas9 genome editing**

95 Target sequences were identified in PAPBC1 using CasFinder
96 (http://arep.med.harvard.edu/CasFinder/) (Mali, Yang et al. 2013). hCas9 was from George
97 Church (Addgene plasmid #41815). gBlocks expressing gRNA and the target sites were
98 synthesized at Integrated DNA Technologies. The synthesized gBlocks were PCR
99 amplified with primers gRNAforward/reverse for transfection into cells. 0.1×10^6
100 HEK293T cells were transfected with 1 μg Cas9 plasmid, 1 μg gRNA, and 0.5 μg linealized
101 NeoR gene fragment, using Lipofectamine 2000 as per the manufacturer's protocols. Cells
102 were split after 24 h, and selected in 400 μg/mL G418. Single cell-derived colonies were
103 screened by western blotting for PABPC1 null mutations.

104

105 **2.3 Primer and siRNA sequences**

Primer/siRNA	Sequence/catalog #
siPABPC1	AAGGUGGUUUGUGAUGAAAAU
siPABPC4-1	GCUUUGGCUUUGUGAGUUA
siPABPC4-2	GGUAAGACCCUAAGUGUCA
siControl	Qiagen (SI03650318)

gRNAForward	TGTACAAAAAAGCAGGCTTTAAAGGAACCA
gRNAReverse	TAATGCCAACTTTGTACAAGAAAGCTGGGT
Pabpc1_C_termR	AACACCGGTGGCACTGTTAACTGC
Pabpc1_midF:	ACTCCTGCTGTCCGCACCGTTCCA
Pabpc1_3UTRR	ATCAATTCTGTTACTTAAAACAGAA
Pabpc1_MLLER	GTTAACTGCTTTCTGGGCAGCCTCT
Pabpc1_L10	TCACCCAAGAAATGTGATTTTTATTAAGAAATCATTAAA
(genome)	TCCATACCTGTTGCATTGTAA
Pabpc1_U9	GCAAACCTCAGATCGAAGAAGACAGCATAAACACTTTT
(genome)	CACTCAGTAAGTTTTCCCAGTT

2.4 Genomic DNA extraction

Cells were pelleted and resuspended in 3 mL of TE buffer and 100 µL of 20% SDS. 20 µL of Proteinase K 20 mg/mL stock solution was added to mix gently by inversion and incubate overnight at 55 °C. 1 mL of saturated NaCl solution was added to the mixture. The solution was then precipitated overnight in 10 mL 100% EtOH (room temperature). DNA was transferred to 5 mL of 70% EtOH, and incubated overnight on rocker. DNA was then moved to a new Eppendorf tube and left air-dry. The DNA was dissolved in water and stored at -20 °C.

2.5 RNA extraction and cDNA library preparation

Total RNA was extracted from confluent 10-cm dishes using Trizol (Thermo-Fisher 15596-026) as per manufacturer's instructions. RNA was air-dried and dissolved in distilled water. Dissolved RNA was further purified using Qiagen miRNeasy micro kit (Cat #217084). RNA from replicates of HEK293 or clone-c1c4 cells were aliquoted and stored at -80 °C for later quantitative RT-PCR analysis or sent for mRNAseq library preparation at Genome Quebec Innovation Center.

2.6 RNA-seq and Differential gene expression analysis

Pair-ended RNA sequencing with read lengths of 100 bases was performed at the Genome Quebec Innovation Center using the Illumina Hiseq 2000 sequencer. Reads were trimmed

127 from the 3' end to have a phred score of at least 30. Illumina sequencing adapters were
128 removed from the reads, and all reads were required to have a length of at least 32.
129 Trimming and clipping were done with Trimmomatic
130 (http://www.usadellab.org/cms/index.php?page=trimmomatic). The filtered reads were
131 aligned to reference genome b37. The alignment was done with the combination of
132 tophat/bowtie software to generate a Binary Alignment Map file (Trapnell, Pachter et al.
133 2009). Read counts were obtained using HTSeq as input. The differential gene expression
134 analysis was done using DESeq (Anders and Huber 2010) and edgeR (Robinson, McCarthy
135 et al. 2010) Bioconductor package. The results of the differential transcript expression
136 analysis were generated using cuffdiff. FPKM values calculated by cufflinks were used as
137 input (Trapnell, Roberts et al. 2012).
138

139 **2.7 Preranked gene set enrichment analysis**

140 Differential genes from Deseq were ranked by their fold of changes (FC) and p-values
141 (Ranking score = $\log_2$FC ×(−$\log_{10}$(p-value)) (Supplemental Table 3) (Plaisier, Taschereau
142 et al. 2010). The ranked gene list was used as input for GSEA and leading edge analysis
143 (Mootha, Lindgren et al. 2003, Subramanian, Tamayo et al. 2005). The detailed GSEA
144 parameters were as follows: the number of permutations is 1000, and the permutation type
145 was configured to the gene set.
146

147 **2.8 Immunofluorescence and confocal microscopy**

148 Cells were fixed with 4% PFA in PBS, and penetrated by cold methanol (-20 °C) or 0.1%
149 Triton X-100 in PBS for 10 min. Cells were blocked with 5% goat serum (Millipore S26)
150 in PBS for 1 h. Then cells were incubated in PBS, supplemented with anti-PABPC1 (Santa
151 Cruz sc32318, 1:200) and anti-PABPC4 (PTGlab AP-14960, 1:200). Cells were washed in
152 PBS three times, before incubation with second antibodies conjugated with Alexa488 or
153 Alexa647 (Sigma-Aldrich A31620, A31628, A31571) at 1:200 – 1:500 dilutions. DAPI
154 (Roche) was added to the first wash at 0.5 μg/ml for 10 min. Cover slides were mounted in
155 ProLong Gold anti-fade reagent (Life Technology P36930). Images were collected on a
156 Zeiss LSM 310 confocal microscope in the McGill University Life Sciences Complex
157 Advanced BioImaging Facility (ABIF).

158

159 **2.9 Quantitative RT-PCR**

160 Total RNA was extracted from cells with Trizol (Life Technology). cDNA libraries were
161 prepared using SuperScript First-Strand Synthesis System for RT-PCR (Life Technology).
162 Validated Taqman assays were purchased for quantification of GAPDH (Applied
163 Biosystems Hs 02758991), c-Myc (Applied Biosystems Hs00153408), and 18sRNA
164 (Applied Biosystems Hs 99999901). qRT-PCR were run and analyzed in Stepone Plus PCR
165 system (Applied Biosystems).

166

167 **2.10 Western blotting**

168 Protein samples were heated at 95°C and separated by SDS-PAGE. Proteins were then
169 transferred to PVDF membrane (Millipore) in Tris/Glycine buffer with 20% methanol in
170 cold room. PVDF membrane was blocked in TBST (pH 7.5), containing 0.05% Tween-20
171 and 5% skim milk powder or bovine serum albumin. The membrane was then incubated
172 with primary antibodies, including anti-PABPC1 (Abcam ab21060, Cell signaling 4992 or
173 Santa Cruz sc32318 1:1000), anti-PABPC4 (Abcam ab76763), anti-c-Myc (Santa Cruz sc
174 40), anti-tubulin (Sigma-Aldrich T9028 1:5000) and anti-GFP (Clontech 632381 1:2000).
175 The membrane was then washed three times in TBST and incubated with goat-anti-rabbit
176 (Jackson ImmunoResearch 111-035-046 1:5000) or goat-anti-mouse (Jackson
177 ImmunoResearch 115-035-071 1:5000) for 0.5 h, washed again, developed with
178 Amersham ECL prime kit (GE healthcare RPN2236), and imaged on an Alpha Innotech
179 imaging system.

180

181 **3. Results**

182 **3.1 PABPC4 compensates partial loss of PABPC1 in HEK293**

183 PABPC1 is the predominant isoform of cytoplasmic PABP in cells. We chose to edit
184 endogenous PABPC1 to check for changes in expression of other isoforms. We selected
185 two target sites in exon 10 of the *Pabpc1* locus, and incorporated the sequences into
186 gBlocks expressing guide RNA scaffolds (Fig. S1). The two scaffold RNAs were
187 separately transfected into HEK293, together with Cas9 plasmid and a linearized NeoR
188 gene for antibiotic selection. Random deletion, insertion or mutation was introduced

189 around the targeted sites. Single cell colonies were screened with an antibody recognizing
190 the C-terminal of PABPC1, for mutant cell-lines expressing PABPC1 mutants altered after
191 the target sites. About 20% of the colonies had insertion or deletions leading to a shortened
192 or disrupted PABPC1 (Fig. 1A). Two typical cell-lines, clone-c1 expressing an exon-
193 skipped PABPC1 (Fig. 2 and Fig. S2), and clone-c1c4 (from target sequence 2) expressing
194 a truncated PABPC1 (Fig. 1A&D) were selected for genomic DNA sequencing (Fig. 1C).
195 Deletion of 3 base pairs in clone-c1 resulted in skipping of the whole exon 10 (Fig. S2).
196 The 2 base pair insertion in clone-c1c4, led to truncated *Pabpc1* mRNA (Fig. 1D) and a
197 corresponding PABPC1 protein truncates due to early termination after the two base pair
198 insertion (Fig. 1B and Fig. 2). Clone-c1c4 was selected for further study, as its PABPC1
199 protein was totally disrupted and greatly decreased. An approximate two-fold elevation of
200 PABPC4 in protein and mRNA levels was observed in clone-c1c4, where the PABPC1 is
201 truncated and decreased (Fig. 1A & E). The major PABP isoform in clone-c1c4 is
202 PABPC4, instead of PABPC1 as in HEK293.
203
204 **3.2 Overexpression of PABPC1 represses elevated PABPC4 in clone-c1c4 cells**
205 The shift of dominative PABP isoform from PABPC1 to PABPC4 did not change cell
206 proliferation (data not shown) or morphology (Fig. 6B). This suggests that the two isoforms
207 are redundant in maintaining basic cellular activities. We then asked whether the elevated
208 PABPC4 in clone-c1c4 was reversible by expression of PABPC1. Clone-c1c4 cells were
209 overexpressed with PABPC1 or PABPC1ΔMLLE (Fig. 3A). PABPC1ΔMLLE
210 overexpression repressed PABPC4 more in clone-c1c4 cells. It is not clear why deletion of
211 the MLLE domain enhances the repression activity of PABPC1. However, it indicates that
212 the repression comes from the RNA binding ability of the RRM domains.
213
214 **3.3 Overexpression of PABPC4 represses endogenous PABPC1 in HEK 293**
215 We next overexpressed PABPC4 in HEK293, and found a reduction of endogenous
216 PABPC1 (Fig. 3B). This further confirms the redundancy of isoforms PABPC1 and
217 PABPC4. The total amount of PABP isoforms may be well regulated for cellular
218 operations. Thus the elevation of PABPC4 in clone-c1c4 cells is due to loss of PABPC1.
219

220 **3.4 Differential gene expression analysis in clone-c1c4 cells**

221 The clone-c1c4 cell-line offered us a platform to study PABP isoform specific functions.
222 To investigate the effects of PABP isoform usage shift on the transcriptome, we submitted
223 clone-c1c4 and HEK293 cells for RNA-seq. Read counts were obtained using HTSeq.
224 Differential gene expression analysis was done with edgeR (Robinson, McCarthy et al.
225 2010) and DESeq (Anders and Huber 2010) R bioconductor packages. Differential
226 expressed (DE) genes were ranked according to the p-values. The gene expression heat
227 map (Fig. S3) indicated profile changes in clone-c1c4 cells. The top 300 DE genes were
228 labeled red on MA plot (Fig. 4A). Most of the top 300 DE genes were relatively highly
229 expressed according to the counts. The top ten DE genes were shown in table (Fig. 4B).
230 Representative wiggle track views of the ten genes displayed mRNA profiles, confirming
231 the DE gene calling (Fig. 4C).

232

233 **3.5 c-Myc is central to differential gene expression in clone-c1c4**

234 To infer biologically important genes underlying the transcriptome changes, we used Gene
235 Set Enrichment Analysis to examine the enrichment of 50 hallmark signature gene sets
236 (Liberzon, Birger et al. 2015). Enriched gene sets were plotted against their normalized
237 enrichment scores. Gene sets with p-values lower than 0.10 are marked red (Fig. 5A). The
238 leading edge subset analysis of the most enriched gene sets revealed c-Myc as the most
239 overlapped gene (Fig. 5B and Fig. S4). The increase of c-Myc in clone-c1c4 was confirmed
240 by western blotting and qRT-PCR (Fig. 5 C & D).

241

242 **3.6 c-Myc mRNA half life is not changed in clone-c1c4**

243 To check whether the increased c-Myc mRNA level in clone-c1c4 was transcriptional or
244 post-transcriptional, we treated HEK293 or clone-c1c4 cells with 10 μg/mL actinomycin-
245 D to inhibit new transcription. Samples were collected at different time points to determine
246 c-Myc mRNA half-life by quantitative RT-PCR. C-Myc mRNA levels measured by
247 taqman assay were normalized to 18s RNA (Fig. 6A). This suggests that the increase of c-
248 Myc mRNA level is transcriptional.

249

250 **3.7 PABPC4 is predominantly cytoplasmic in clone-c1c4**

251 Although role of PABPC4 in transcriptional control is not known, there is increased nuclear
252 distribution of PABPC4 in response to stress (Burgess, Richardson et al. 2011) or PABPN1
253 depletion (Bhattacharjee and Bag 2012). We stained PABPC4 in HEK293 and clone-c1c4
254 cells, and found PABPC4 are predominantly cytoplasmic in both cell-lines (Fig. 6B). Thus
255 the increased PABPC4 in clone-c1c4 doesn't lead to significant nuclear relocation.
256
257 **3.8 Correlation of PABPC4 and c-Myc changes**
258 We then knocked-down *Pabpc4* expression in clone-c1c4 cells with siRNAs. c-Myc
259 mRNA and protein levels decreased correlated to PABPC4 depletion (Fig. 7A & B). We
260 asked whether substitution of PABPC1 by PABPC4 led to the c-Myc increase.
261 Overexpression of PABPC1-GFP in clone-c1c4 repressed endogenous PABPC4 protein
262 (Fig. 3A). However, the increased PABPC1-GFP in clone-c1c4 did not decrease the c-
263 Myc mRNA level. We reasoned that it might take longer for the PABPC1 to indirectly
264 affect the c-Myc mRNA level, or the relatively large GFP tag might interfere with certain
265 PABPC1 functions. Nonetheless, the relative usage of PABPC4 isoform correlates with the
266 c-Myc level and can potentially affect the transcriptome.
267
268 **3.9 Isoform usage of PABP and c-Myc levels**
269 To test the effects of PABP isoform usage on c-Myc levels, we depleted PABPC1 or
270 PABPC4 with siRNAs in HEK293 cells (Fig. 8A). Depletions of the two isoforms affect
271 c-Myc mRNA levels in opposite directions. Decrease of PABPC4 lowered c-Myc mRNA,
272 while decrease of PABPC1 raised c-Myc mRNA level. This confirms that the usage of the
273 PABPC1 or PABPC4 isoform can influence the transcriptome through c-Myc.
274

275 **4. Discussion**

276 Here, we reported compensation of PABPC4 for the partial loss of PABPC1 in HEK293
277 cells. The cells are viable suggesting a functional overlap of the two isoforms. Analysis of
278 the transcriptome profile revealed differential gene expression correlated with usage of
279 PABPC4 and PABPC1. The study helps us understand isoform specific functions of PABP
280 in development or different tissues.
281
282 Recent genomic studies support PABPC1 recognition of RNA sequences other than pure
283 poly(A) in mouse (Kini, Silverman et al. 2016) and yeast (Baejen, Torkler et al. 2014). The
284 overall structures of RRM domains in PABP isoforms are likely to be similar, due to high
285 sequence similarities between domains. However, conserved differences are found in
286 PABP isoforms across species. Some of the differences are located at interfaces critical for
287 RNA recognition, especially in the RRM3-4 domains (Fig. S5). These differences do not
288 alter the overall structure of thee RRM domains, but may contribute to specificity in RNA
289 recognition. There is a growing realization that PABP isoforms are functionally different
290 in vertebrate development (Gorgoni, Richardson et al. 2011) and other contexts. PABPC4
291 depletion impacts steady-state expression of a subset of mRNAs and affects erythroid
292 differentiation (Kini, Kong et al. 2014). PABPC1L (ePABP) regulates translation and
293 stability of maternal mRNAs (Vasudevan, Seli et al. 2006), and is substituted by PABPC1
294 after onset of zygotic transcription (Cosson, Couturier et al. 2002).
295
296 The recently developed *c*ross*l*inking *i*mmuno*p*recipitation coupled high-throughput
297 *seq*uencing technique (CLIP-seq) provides a powerful tool to map association of RNA-
298 binding proteins with RNA. Mapping the interactions of PABP isoforms with mRNA
299 would greatly facilitate understanding of the functions of those isoforms. It is likely that
300 PABP isoforms function through recognition of separate subsets of mRNAs, besides
301 binding to a common mRNA pool. So far, such mapping data is only available for PABPC1
302 in higher eukaryotes (Kini, Silverman et al. 2016). The lack of CLIP data from other PABP
303 isoforms makes biological inferences of differential gene expression profiles difficult.
304

305 In this study, we identified c-Myc as a central player in remodeling the transcriptome of
306 clone-c1c4 cells. C-Myc is a transcription factor that can shape the cellular transcriptome
307 (Kress, Sabo et al. 2015). PABP isoforms may act through a subset of mRNAs to indirectly
308 upregulate c-Myc transcription. In the Gene Set Enrichment Analysis, WNT pathway is
309 enriched (Fig. 5) and can induce c-Myc transcription (Kress, Sabo et al. 2015). Mechanisms
310 underlying correlation of PABPC4 and c-Myc remain to be revealed by further studies.
311
312 The significant increase (10-fold) of c-Myc mRNA is tolerated in clone-c1c4. Cell cycle
313 analysis by propidium iodide DNA staining and flow cytometry showed similar
314 distribution in HEK293 and clone-c1c4 cells in different phases of the cell cycle (data not
315 shown). This may be because the increase in c-Myc protein levels were smaller,
316 approximately three-fold. Elevation of genes like Axin1 (Supplemental Table 1) may
317 enhance c-Myc protein turnover (Arnold, Zhang et al. 2009), which balances the sharp
318 increase of c-Myc mRNA. Nonetheless, c-Myc targets are upregulated in clone-c1c4
319 extensively (Fig. 5B, Supplemental Tables 3 & 4 GSEA sets). Variant calling on the RNA-
320 seq data reveals no insertion, deletion or mutation in c-Myc, or genes we know to affect c-
321 Myc transcription. Blast search returns no other genomic sequence for the first 13 base
322 pairs of the target sequence 2 (Fig. S1), which was used for generation of clone-c1c4.
323 Modulation of PABPC4 or PABPC1 levels support a correlation of PABPC4 and c-Myc
324 levels (Fig. 7&8). One interesting observation is that PABPC4 overexpression in HEK293
325 decreased endogenous PABPC1 by ~50% (Fig. 3B), but did not affect the level of c-Myc
326 mRNA (data not shown). The may reflect the dominant role PABPC1 compared to other
327 PABP isoforms. Other isoforms can only function significantly when PABPC1 is at very
328 low levels, as in clone-c1c4 (Fig. 7A) or PABPC1 depleted HEK293 (Fig. 8A).
329 In summary, we created a human cell-line where the predominant PABPC1 is stably
330 substituted by PABPC4. This opens a window to observe functional differences between
331 the two isoforms. Validations and further investigations in different approaches will help
332 us understand the mechanistic details of PABP regulation.

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Competing interests

No competing interests declared.

Author contributions

J.X. designed and carried out the experiments. X.W. and Y.C. assisted J.X. with experiments. J.X. did the bioinformatics analysis and wrote the manuscript. K.G. revised the manuscript.

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356 **Figure legends**

357

358 Figure 1. PABPC1 disruption in HEK293 cells by CRISPR/Cas9 based genome editing is
359 compensated by elevated PABPC4. (A) CRISPR/Cas9 genome editing of PABPC1
360 generated multiple mutations. Antibody recognizing C-terminal MLLE domain of
361 PABPC1 (PABPC1 C-domain, Santa Cruz 32318) labels null mutations of PABPC1.
362 PABPs N-domain antibody (New England Biolabs 4992), which recognizes multiple
363 cytoplasmic PABP, reveals PABP isoform distribution. Tubulin is stained as a loading
364 control. Anti-PABPC4 shows an elevated level of PABPC4 protein in clone-c1c4, when
365 PABPC1 is truncated to about 40 kD and less expressed. Percentages are relative protein
366 levels after normalized to tubulin. (B) PABPC1 was knocked-down by siRNA, in 293,
367 clone-c1, or clone-c1c4 cells. The decreased lower bands in clone-c1 and clone-c1c4 were
368 PABPC1 mutations. Sequences of siRNAs used are listed in materials section. (C)
369 Genomic DNA sequences at *Pabpc1* gene loci of 293, clone-c1, and clone-c1c4. Deletion
370 of three base pairs in clone-c1 leads to skipping of exon 10 in mRNA (Fig. S2) and a shorter
371 PABPC1 protein (Fig. 2). Clone-c1c4 has a two base pair insertion, which results in
372 significant reduction of mRNA reads after the targeted region (D). Genomic DNA was
373 extracted and amplified with gene specific primers. PCR products were gel purified and
374 cloned into PCR2.1 vector for sequencing. Only one sequence was read in multiple clones,
375 indicating the homogeneity of cell-lines. (D & E) Wiggle tracks showing representative
376 read alignment of *Pabpc1* or *Pabpc4* genes in HEK293 or clone-c1c4 cells. Track files
377 were generated from the aligned reads using BedGraphToBigWig.

378

379 Figure 2. Description of PABPC1 protein in HEK293, clone-c1, and clone-c1c4. Wild-type
380 PABPC1 is 68 kD in molecular weight. A shortened form of PABPC1 was generated in
381 clone-c1 with residues 447-483 deleted. The mRNA sequence of clone-c1 is shown in
382 Figure S2. In clone-c1c4, the major PABPC1 was about 42 kD in agreement with the size
383 of the major mRNA species (Fig. 1E).

384

385 Figure 3. Mutual repression of PABPC1 and PABPC4. (A) Over-expression of pCDNA3-
386 EGFP (Ctrl), pCDNA3-PABPC1-EGFP, or pCDNA3-PABPC1ΔMLLE-EGFP in clone-

387 c1c4 cells. Western blotting shows decrease of PABPC4 protein level in clone-c1c4 cells,
388 due to over expression of PABPC1-EGFP or PABPC1ΔMLLE-EGFP. (B) Over-
389 expression of PABPC4 (construct in materials) in HEK293 reduces endogenous PABPC1
390 protein.
391
392 Figure 4. Differential gene expression in HEK293 and the modified clone-c1c4 cells. (A)
393 MA plot of differential expression magnitude (log2 of fold change (clone-c1c4/HEK293)
394 versus expression levels (log2 of counts per million). The red dots are the top 300
395 differential genes ranked by p-values. (B) The top 10 differential genes. Ensembl gene id,
396 gene symbol, log2 (fold of changes), and log2 (counts per million) are shown in the table.
397 (C) Representative view of the mRNA profile of the top 10 differential genes. The figure
398 is generated with Integrative Genomics Viewer.
399
400 Figure 5. Gene expression in clone-c1c4 cells. (A) Gene set enrichment analysis of selected
401 hallmark gene sets (Subramanian, Tamayo et al. 2005). The size of circles indicates the
402 number of significant genes. Gene sets with p-values lower than 0.1 are labeled red. (B)
403 Leading edge overlap for the modified clone-c1c4 cells. The significantly enriched gene
404 sets from (A) are aligned to indicate common genes. c-Myc is the most significant
405 overlapped gene. Other overlapped genes are shown in figure S4. The intensity of color
406 indicates fold of changes in expression. Only a subset of the genes are displayed for
407 visibility. (C) Increased c-Myc protein in clone-c1c4 cells. The percentage indicates
408 relative quantifications of c-Myc protein after normalization. Cells were lysed in SDS-
409 loading buffer and boiled. c-Myc protein level was probed by antibody (Sant Cruz sc-40).
410 Tubulin is used as loading control. (D) Increased c-Myc mRNA level. Taqman assay
411 (Applied Biosystems Hs 00153408) reveals an increase of about 10-fold in c-Myc mRNA
412 in clone-c1c4. GAPDH (Applied Biosystems Hs 02758991) is used as loading control.
413
414 Figure 6. (A) c-Myc mRNA half life is similar in HEK293 and clone-c1c4. Cells were
415 treated by (10 μg/ml) antinomycin-D for indicated time before Trizol extraction of total
416 RNA. Taqman assays were used to measure c-Myc levels normalized to 18s RNA (Applied
417 Biosystems Hs 99999901). The c-Myc mRNA levels of cells without treatment are

418 normalized to 100 for comparison. (B) PABPC4 in clone-c1c4 is predominantly
419 cytoplasmic. HEK293 or clone-c1c4 cells were stained with DPAI, anti-PABPC4 (Abcam
420 ab76763), and anti-PABPC1 (Abcam ab21060). The PABPC1 antibody recognizes the C-
421 terminal tail of PABPC1.
422
423 Figure 7. (A) Decrease of c-Myc protein level correlates with depletion of PABPC4.
424 Representative blots of *Pabpc4* knocked-down clone-c1c4 cells. Corresponding antibodies
425 were used to probe PABPC1, PABPC4, c-Myc, and tubulin levels. (B) Correlated c-Myc
426 mRNA decrease after siRNA mediated knock-down of PABPC4.
427
428 Figure 8. Differential effects of PABPC1 or PABPC4 depletion on c-Myc mRNA level in
429 HEK293 cells. (A) HEK293 cells were transfected with control siRNA, si*Pabpc1*, or
430 si*Pabpc4* for 48 hrs. PABPC1 and PABPC4 were probed to examine knock-down effects.
431 (B) Changes of c-Myc mRNA levels in siRNA treated HEK293 cells. Depletion of
432 PABPC4 reduces c-Myc mRNA level, while depletion of PABPC1 increases c-Myc
433 mRNA. Both differences were significant at a p-value of 0.05 in a two-tail t-test for samples
434 with identical variance.
435
436
437
438
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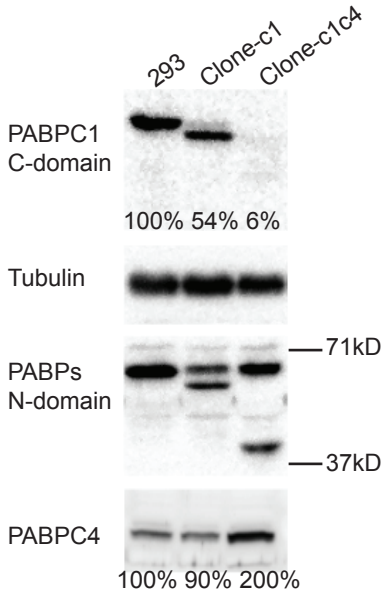

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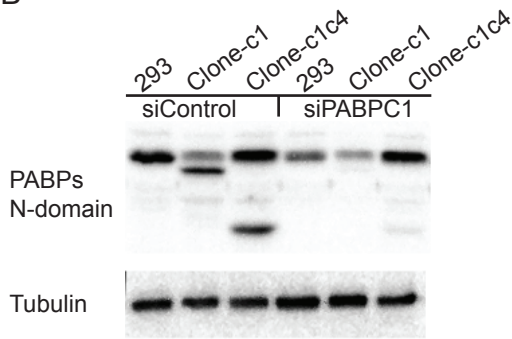

C

```
PABPC1 DNA     TTTAAAATGCAGCATTCCAAAA--TATGCCCGGTGCTATCCGCCCAGCTGCT
PABPC1 mRNA    ------------CATTCCAAAA--TATGCCCGGTGCTATCCGCCCAGCTGCT
Clone-c1 DNA   TTTAAAATGCAGCATTCCAAAA--TATGCCCGG---TATCCGCCCAGCTGCT
Clone-c1c4 DNA TTTAAAATGCAGCATTCCAAAAAATATGCCCGGTGCTATCCGCCCAGCTGCT
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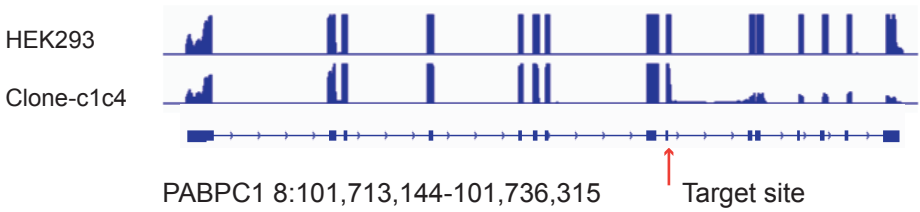
D



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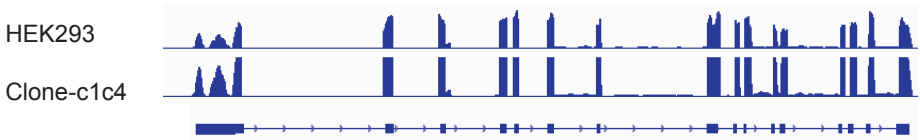
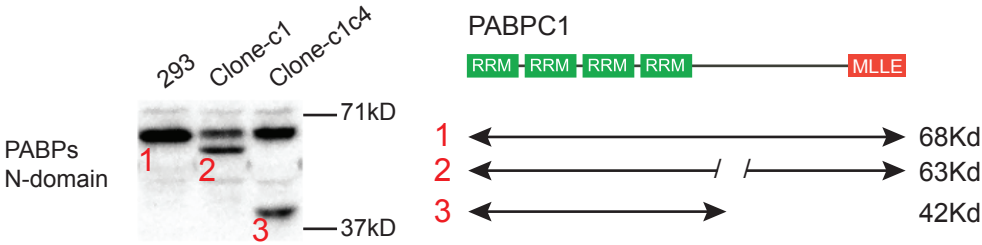
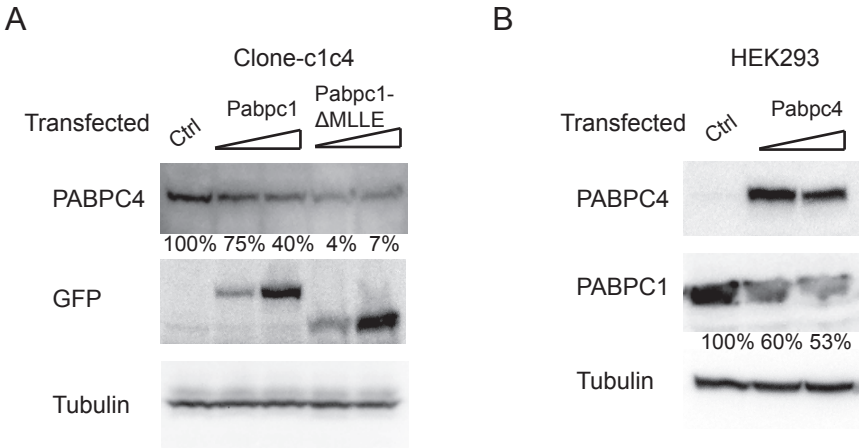
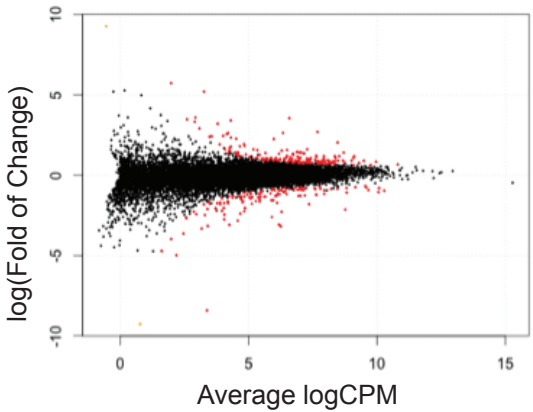


PABPC4 1:40,025,339-40,045,375
Fold Change (c1c4/293) after normalization = 1.8


293
Clone-c1
Clone-c1c4
PABPs
N-domain
71kD
37kD
1
2
3
PABPC1
RRM
RRM
RRM
RRM
MLLE
1
2
3
68Kd
63Kd
42Kd


A
Clone-c1c4
Transfected
Ctrl
Pabpc1
Pabpc1-
ΔMLLE
PABPC4
100% 75% 40% 4% 7%
GFP
Tubulin
B
HEK293
Transfected
Ctrl
Pabpc4
PABPC4
PABPC1
100% 60% 53%
Tubulin

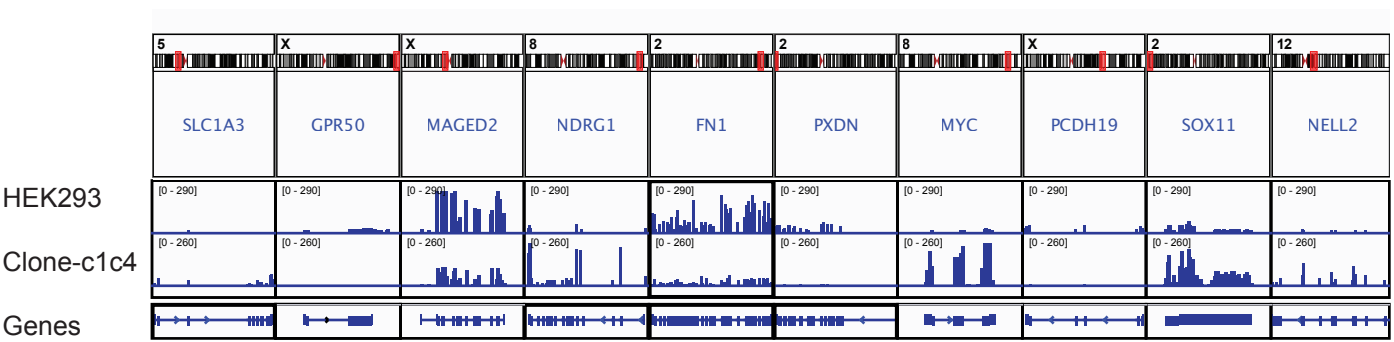
A



B

	gene_symbol	log_FC	log_CPM
ENSG00000079215	SLC1A3	2.268	5.975
ENSG00000102195	GPR50	-8.407	3.371
ENSG00000102316	MAGED2	-1.774	7.347
ENSG00000104419	NDRG1	2.711	7.685
ENSG00000115414	FN1	-2.113	8.757
ENSG00000130508	PXDN	-3.052	6.177
ENSG00000136997	MYC	3.559	6.587
ENSG00000165194	PCDH19	-3.157	6.248
ENSG00000176887	SOX11	1.631	7.282
ENSG00000184613	NELL2	2.652	6.333

C



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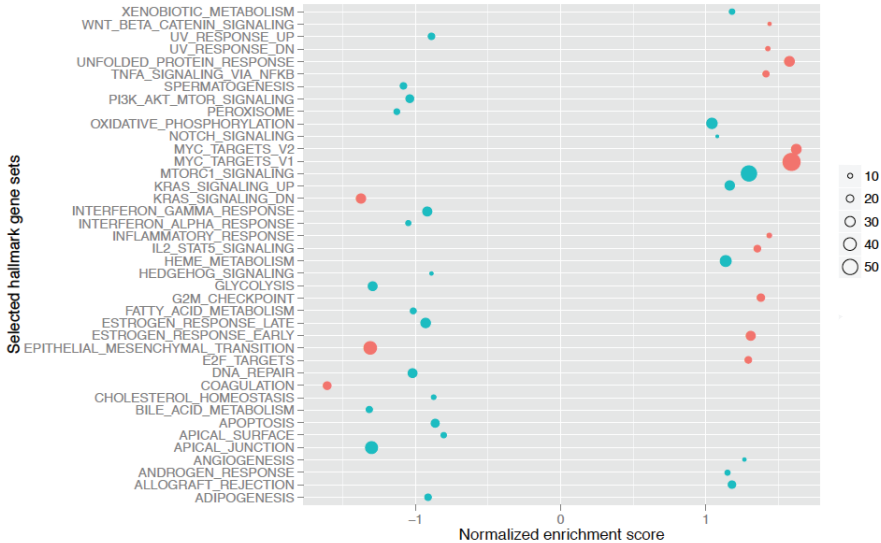
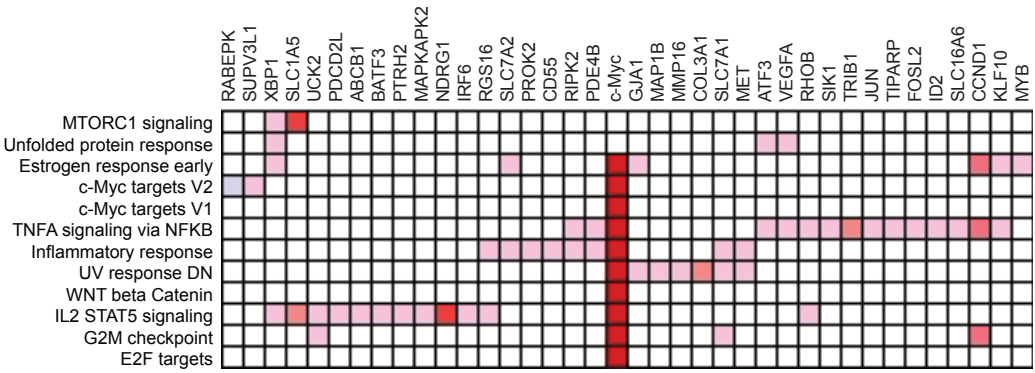
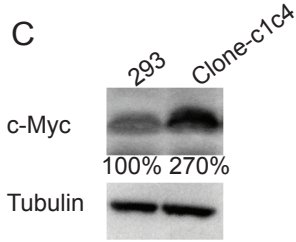

Selected hallmark gene sets
XENOBIOTIC_METABOLISM
WNT_BETA_CATENIN_SIGNALING
UV_RESPONSE_UP
UV_RESPONSE_DN
UNFOLDED_PROTEIN_RESPONSE
TNFA_SIGNALING_VIA_NFKB
SPERMATOGENESIS
PI3K_AKT_MTOR_SIGNALING
PEROXISOME
OXIDATIVE_PHOSPHORYLATION
NOTCH_SIGNALING
MYC_TARGETS_V2
MYC_TARGETS_V1
MTORC1_SIGNALING
KRAS_SIGNALING_UP
KRAS_SIGNALING_DN
INTERFERON_GAMMA_RESPONSE
INTERFERON_ALPHA_RESPONSE
INFLAMMATORY_RESPONSE
IL2_STAT5_SIGNALING
HEME_METABOLISM
HEDGEHOG_SIGNALING
GLYCOLYSIS
G2M_CHECKPOINT
FATTY_ACID_METABOLISM
ESTROGEN_RESPONSE_LATE
ESTROGEN_RESPONSE_EARLY
EPITHELIAL_MESENCHYMAL_TRANSITION
E2F_TARGETS
DNA_REPAIR
COAGULATION
CHOLESTEROL_HOMEOSTASIS
BILE_ACID_METABOLISM
APOPTOSIS
APICAL_SURFACE
APICAL_JUNCTION
ANGIOGENESIS
ANDROGEN_RESPONSE
ALLOGRAFT_REJECTION
ADIPOGENESIS
−1
0
1
Normalized enrichment score
10
20
30
40
50

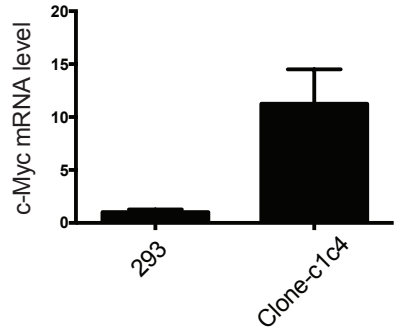
B


RABEPK
SUPV3L1
XBP1
SLC1A5
UCK2
PDCD2L
ABCB1
BATF3
PTRH2
MAPKAPK2
NDRG1
IRF6
RGS16
SLC7A2
PROK2
CD55
RIPK2
PDE4B
c-Myc
GJA1
MAP1B
MMP16
COL3A1
SLC7A1
MET
ATF3
VEGFA
RHOB
SIK1
TRIB1
JUN
TIPARP
FOSL2
ID2
SLC16A6
CCND1
KLF10
MYB
MTORC1 signaling
Unfolded protein response
Estrogen response early
c-Myc targets V2
c-Myc targets V1
TNFA signaling via NFKB
Inflammatory response
UV response DN
WNT beta Catenin
IL2 STAT5 signaling
G2M checkpoint
E2F targets

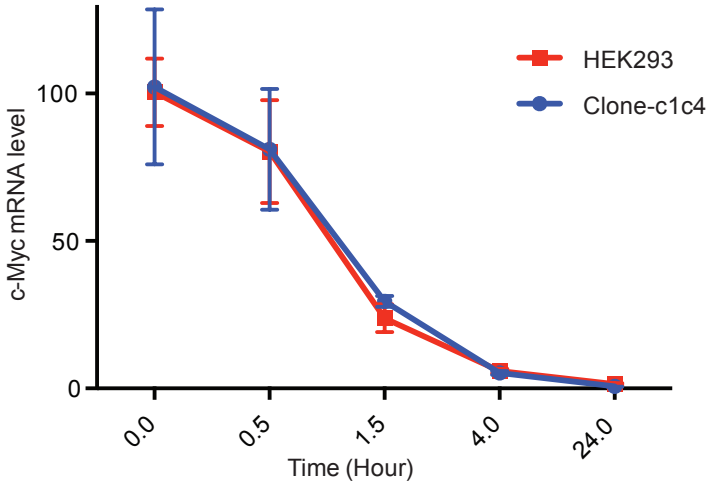
C


293
Clone-c1c4
c-Myc
100%
270%
Tubulin

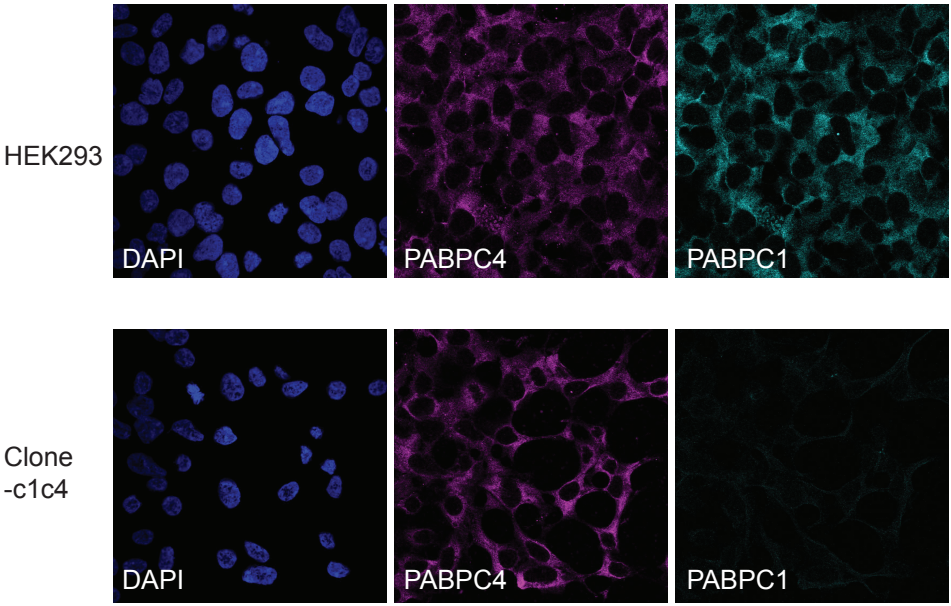
D

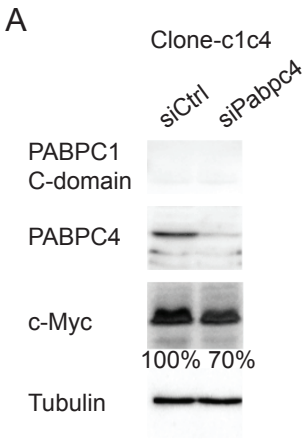

c-Myc mRNA level
20
15
10
5
0
293
Clone-c1c4

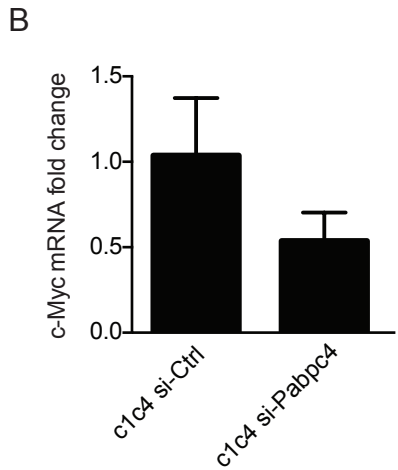
A

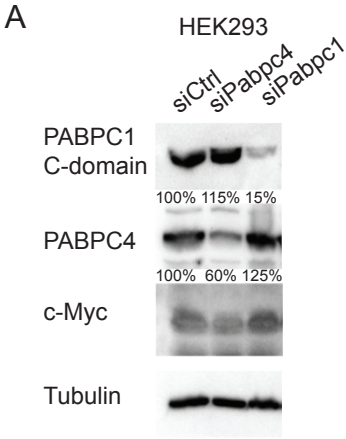

HEK293
Clone-c1c4
c-Myc mRNA level
100
50
0
0.0
0.5
1.5
4.0
24.0
Time (Hour)

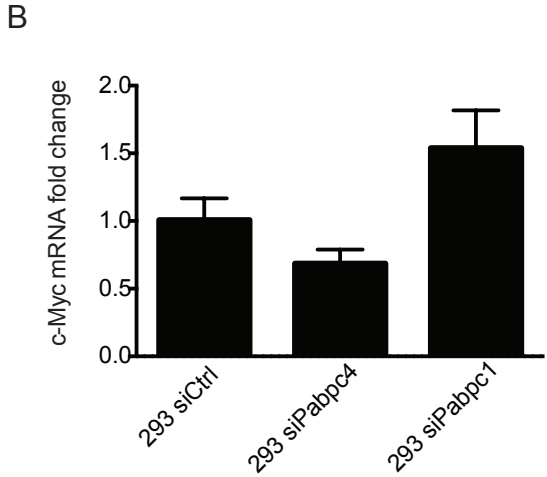
B


HEK293
Clone
-c1c4
DAPI
PABPC4
PABPC1
DAPI
PABPC4
PABPC1


A
Clone-c1c4
siCtrl
siPabpc4
PABPC1
C-domain
PABPC4
c-Myc
100% 70%
Tubulin


B
c-Myc mRNA fold change
1.5
1.0
0.5
0.0
c1c4 si-Ctrl
c1c4 si-Pabpc4


A
HEK293
siCtrl
siPabpc4
siPabpc1
PABPC1
C-domain
100% 115% 15%
PABPC4
100% 60% 125%
c-Myc
Tubulin


B
c-Myc mRNA fold change
2.0
1.5
1.0
0.5
0.0
293 siCtrl
293 siPabpc4
293 siPabpc1