Supplementary materials and methods

Plasmids. The pS2f-lMCg-F3 plasmid, carrying a tet-responsive element flanked by a firefly luciferase (FL) under the control of a minimal MMTV promoter and a GFP under a CMV promoter, was obtained from Dr. K. Schönig and is described in [1]. Using standard subcloning techniques, we replaced the GFP cassette by a Renilla luciferase (RL), and the MMTV promoter by a CMV promoter to create the pSF3 plasmid. The 3'UTRs of validated human miRNA targets were introduced downstream of the RL cDNA. *hmga2* WT and MUT cDNAs were obtained from Dr. G. Meister, University of Regensburg [2], *reck* WT and MUT cDNAs from Dr. A. Krichevsky, Harvard medical school [3]. The MUT cDNAs contained three point mutations within the seed region of the miRNA binding sites, preventing binding to the corresponding miRNAs [2, 3]. For testing weaker promoters, both CMV promoters in pSF3 were replaced by MMTV promoters to create pSF4 plasmids. pPGKFLPobpA, encoding an optimized FLP recombinase [4] was obtained from Addgene (plasmid number 13793). Plasmids encoding dominant negative mutants of Pan2 and CNOT7 (aka Caf1a) were obtained from Dr. A.B. Shyu, University of Texas Medical School, Houston.

Antibodies. The following antibodies were used for Western blot analysis: anti-Ago2 (11A9, Ascenion, 1:5000), anti-CNOT1 (kind gift of M. Collart, 1:250), anti-CNOT7 (Abnova 2F6, 1:1000), anti-CNOT8 (Lifespan biosciences C99242, 1:500), anti-α-tubulin (Sigma T6074, 1:10000).

Cell culture. HeLa-11ht cells [5], a derivative of HeLa-CCL2 stably expressing the reverse tet-controlled transactivator gene and containing a retargetable genomic locus selected for tight regulation of dox-regulated reporters, was obtained from Dr. K. Schönig (Central Institute of Mental Health, Mannheim, Germany). Construction, selection and characterization of clonal stable cell lines were performed as described [5]. Cells were grown in DMEM medium containing 10% tet-free bovine serum (Sigma) and induced by adding doxycycline (Sigma) at 200 $ng.mL^{-1}$. For transcription pulse-chase experiment, actinomycin D (Sigma) was added to 5 $\mu g.mL^{-1}$.

Luciferase assay. Cells were seeded at 10^3 cells per well of 96-well plates or 0.3 x 10^6 cells per well of 6-well plates. After 24 h, expression of the reporters was induced with dox as indicated. For kinetic analysis, induction was started at different time-points and, after washing with ice-cold PBS buffer, cells were lysed simultaneously on ice with cytoplasmic RNA lysis buffer (see below). A fraction of lysates was used in the Dual luciferase reporter assay system (Promega). RL activities of different WT reporters were expressed as percentages of values for matching MUT reporters. RL activity was always normalized to FL activity.

RNA extraction and qPCR. The isolation of cytoplasmic RNA was similar to that described previously in [6]. Briefly, cells were lysed using cytoplasmic RNA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40). After centrifugation at 10,000 *g* for 10 min to remove nuclei, the supernatants were treated with Trizol LS (Invitrogen). For each sample, 1 μg of purified RNA was reverse-transcribed using the Transcriptor first strand cDNA synthesis kit (Roche) with random hexamers (poly(A) tail length measurements) or oligo-dT primers (other experiments). Subsequently, cDNA was used as a template for qPCR with SYBR Green qPCR Mix (Roche) using gene-specific primers in a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative expression levels were calculated using the formula $2^{-(\mathrm{DeltaCt})}$, where DeltaCt is Ct(RL or FL)–Ct(GAPDH) and Ct is the cycle at which the threshold is crossed. The primer pair for detection of the RL reporters amplified their first 5'-proximal 120 nucleotides, ensuring that only full-length mRNAs were quantified when the cDNA was synthesized from oligo-dT primers. Cytoplasmic mRNA levels of different WT reporters were expressed as percentages of values for matching MUT reporters. The cytoplasmic WT and MUT reporter mRNA levels were normalized to the cytoplasmic levels of either FL mRNA (all experiments except the ones shown in Fig. 2A and 3A) or endogenous GAPDH mRNA (experiments shown in Fig. 2A and 3A). We routinely verified that, at each kinetic time point, levels of FL mRNA expressed from WT or MUT cell lines were equivalent when compared to endogenous GAPDH mRNA levels. Primers used for detection:

GAPDH -FW: CGCTCTCTGCTCCTCCTGTT
-RV: CCATGGTGTCTGAGCGATGT

FL: -FW: GAGCACGGAAAGACGATGACGG

-RV: GGCCTTTATGAGGATCTCTCTG

RL: -FW: ATGGCTTCCAAGGTGTAC

-RV: TAGTTGATGAAGGAGTCCA

Transfection of siRNAs and anti-miRs oligonucleotides. For transfection of siRNAs, the duplexes described in [7] were transfected at a concentration of 100 nM with Hiperfect (Qiagen) following manufacturer's recommendations. For silencing CNOT7 and CNOT8, pre-designed siRNAs were ordered from Ambion (#s26638 and #s17849 respectively). For transfection of anti-miRs (Anti-let-7a: UCUUCACUAUACAACCUACUACCUCAACCUU, Anti-miR-21: UCAACAUCA-GUCUGAUAAGCUA, Anti-miR-30a: CUUCCAGUCGAGGAUGUUUACA, anti-miR-590: CUGGCACUUUUAUGAAUAAGCUC), the 2'-*O*-methyl oligoribonucleotides were used at a final concentration of 200 nM and transfected with Hiperfect (Qiagen), following manufacturer's recommendations. 48h after transfection, the cell lines were induced for 4h with dox, and then processed for dual luciferase assay.

Poly(A) tail length measurements. Cytoplasmic RNA was fractionated according to poly(A) tail length according to [8]. Briefly, at the indicated time points, cytoplasmic RNA was isolated as described in the main methods section. 20 μg of cytoplasmic RNA was then resuspended in GTC buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.1, 100 mM β-mercaptoethanol). 10 μL of biotinylated oligo(dT) (50 μM, Promega) were added. The mixture was then diluted with 821 μL dilution buffer (6x SSC, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25% SDS, 100 mM β-mercaptoethanol) and incubated at 70°C for 5 min. After centrifugation at 12,000 *g* for 10 min, the supernatant was mixed with 600 μL streptavidin-coupled paramagnetic beads (Promega) for 15 min at room temperature. The unbound fraction was removed and the beads were washed three times with 0.5x SSC at room temperature. Polyadenylated RNA was then eluted by successively incubating the beads with 300 μL buffer containing decreasing amount of SSC (0.2x, 0.1x, 0.075x, 0.05x, 0.025x, and finally water; the collected fractions correspond to fractions E1 through E6 in Fig. S2). All fractions were collected and precipitated with 10 μg yeast

tRNA and 15 μg Glycoblue (Ambion). For the quantification of fully polyadenylated reporters shown on Fig. 3C, samples were then analyzed by RT-qPCR using random hexamers for the cDNA synthesis step.

The length of poly(A) tails eluting at different SSC concentrations was determined by using an *in vitro* transcript. A RL *in vitro* transcript was generated using Ambion's Megascript kit as described in [9]. Aliquots of this transcript were polyadenylated using different concentrations of *E. coli* poly(A) polymerase (Ambion) as described in [8], and mixed together, resulting in mixture of RNAs bearing poly(A) tails of different lengths. This mixture was fractionated using the procedure described above. Direct poly(A) tail measurements to qualitatively define the population of polyadenylated reporters (as shown on Fig. 3B and 4D) were performed based on a polyG/I extension method [10] using the Poly(A) Tail-Length Assay Kit (Affymetrix) following manufacturer's instructions. Specific primers used to determine poly(A) tail length were: TCTAGAAAGTATAGGAACTTCAGATC (forward) and TTTATTTTATCTATGGCTCGTACTCT (reverse). The latter primer anneals to the reporter sequence directly upstream of the polyadenylation site. The resulting amplification product corresponds to the Ao fragment in Fig.3 and 4. A reverse primer annealing at the poly(A)/(G/I) track junction was provided in the kit. PCR products from a 35 cycles reaction were purified using Qiagen's PCR purification kit and analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (5067-4626).

Supplemental references

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Supplementary figure legends

Figure S1. Characterization of MMTV-RL-hmga2 and CMV-RL-reck cell lines.

A. MMTV-RL-hmga2-WT and -MUT cell lines were seeded at the same density and induced for the indicated time. Cells were then harvested, counted, and same number of cells were lysed and processed for dual luciferase assay. The control FL reporters expressed in WT and MUT cell lines yielded similar readings at each time-point (left panel), while the RL-hmga2-WT reporter was expressed at lower levels than the RL-hmga2-MUT reporter (right panel).

B. Same as in (A) with the CMV-RL-reck cell lines.

Figure S2. Linearity and accuracy of the qPCR-based quantification.

CMV-RL-hmga2-MUT and parental HeLa-11ht cell lines were induced for 2h with doxycycline. RNA was then extracted from cytoplasmic extracts. Dilutions of the cytoplasmic RNA extracted from the CMV-RL-hmga2-MUT cell line were then prepared by combining it with RNA extracted from the parental cell line so that samples containing 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% of the starting amounts of reporters were obtained. Relative amounts of RL (right) and FL (left) reporters were then determined by qPCR as in Figures 2-4. Calculated amounts are plotted against theoretical amounts. The black line indicates the corresponding linear correlation curve, the pink dashed line indicates the theoretical perfect correlation. Error bars are 95% confidence intervals from four independent experiments performed from three biological replicates by two different operators.

Figure S3. Relative RL reporter mRNA levels accumulation over time.

A. CMV-RL-hmga2-WT and -MUT cell lines were seeded at the same density and induced for the indicated time. Cells were then harvested, lysed and processed for cytoplasmic mRNA analysis. RL mRNA levels were normalized to GAPDH and shown as percentage of the MUT RL-hmga2 reporter mRNA levels at 48h.

B. Same as in (A) with the MMTV-RL-hmga2 cell lines

C. Same as in (A) with the CMV-RL-reck cell lines.

Figure S4. Poly(A) fractionation of cytoplasmic RNA.

A. Calibration of the fractionation procedure. Aliquots of an *in vitro* transcribed RL reporter were polyadenylated using different dilutions of the *E. coli* poly(A)

polymerase, resulting in a mixture of RNAs bearing poly(A) tails of various lengths. The mixture was used as a calibration sample for the fractionation protocol. After annealing to biotinylated oligo(dT) and binding to streptavidin-coupled paramagnetic beads, polyadenylated RNA was eluted with decreasing concentration of SSC buffer. Six fractions were collected (E1 through E6, with E1 corresponding to highest salt) and used for G/I tail extension. cDNAs were prepared using either specific primers amplifying the 166 bp region directly upstream the poly(A) tail of the reporter (S lanes), or the same forward primer as for (S) samples and a reverse primer annealing at the poly(A)-G/I tail junctions (T lanes). Poly(A) tail lengths were estimated as the size difference between T and S lanes. For each elution, the approximate poly(A) tail length was estimated from the slowest and fastest migrating bands in each fraction.

B. Distribution of the CMV-RL-hmga2-WT and -MUT reporters in different elution fractions at various time points post-induction. CMV-RL-hmga2-WT and MUT cell lines were induced for 1 h, transcription was then stopped with actinomycin D (see Fig. 3A), and material collected at the indicated time points. Cytoplasmic RNA was then prepared and fractionated according to poly(A) tail length. The fractionated RNA samples were used as a template for reverse transcription using random hexamers, and RL reporters were quantified by qPCR. The bars indicate the percentage of RL reporters found in each fraction at each time-point. 100% represents the total amount (i.e the sum of all fractions) of RL-hmga2-MUT reporter at each time point. The total amount of RL-hmga2-WT at each time-point is the related to the total amount of MUT reporter at the same time-points. No RL reporter was detected in fractions E5 and E6, hence fraction E4 was considered as containing fully polyadenylated RL mRNA. Accordingly, the percentage of fully polyadenylated WT reporter shown in figure 3C is calculated as the ratio of WT over MUT reporter in fraction E4.

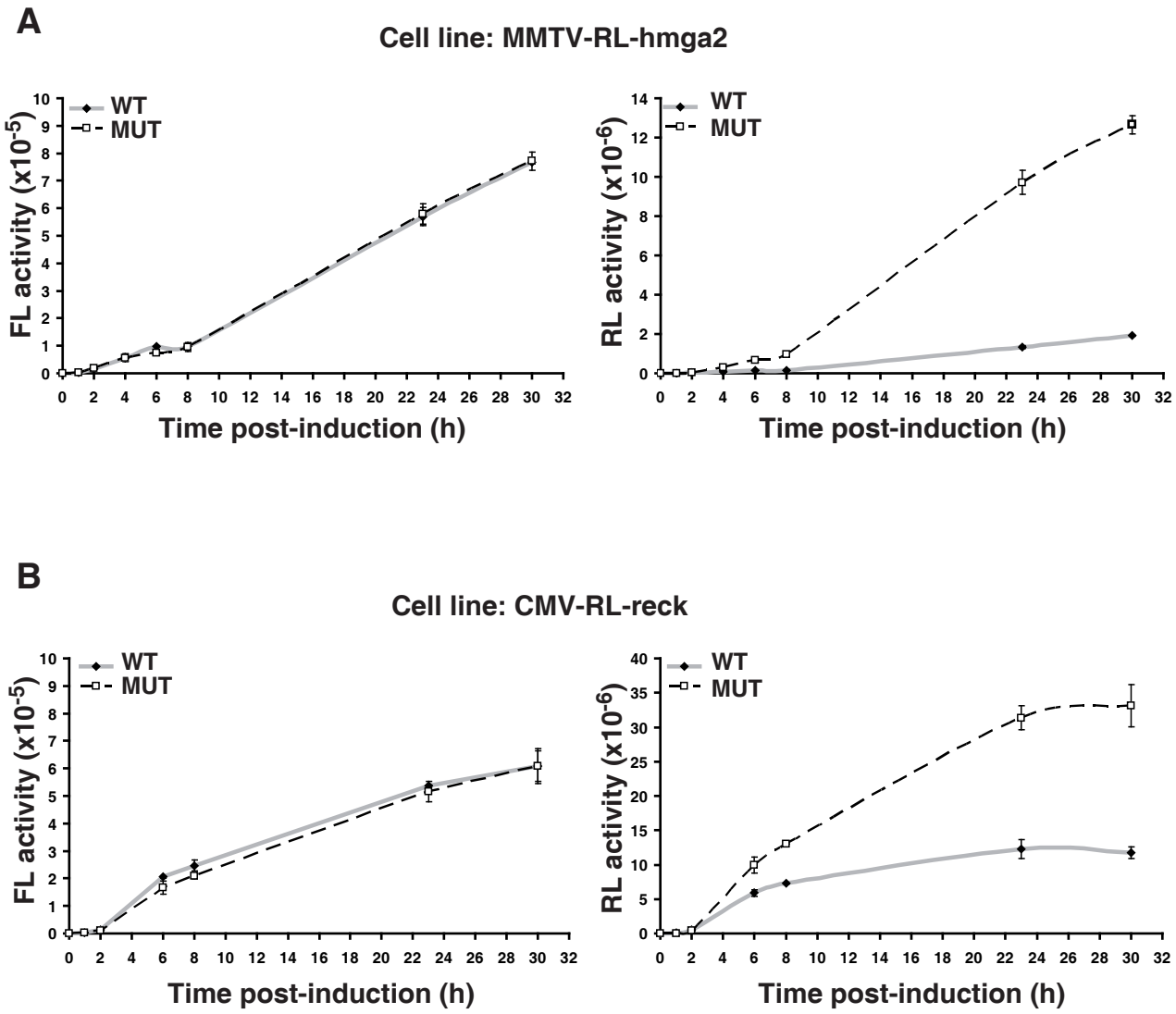
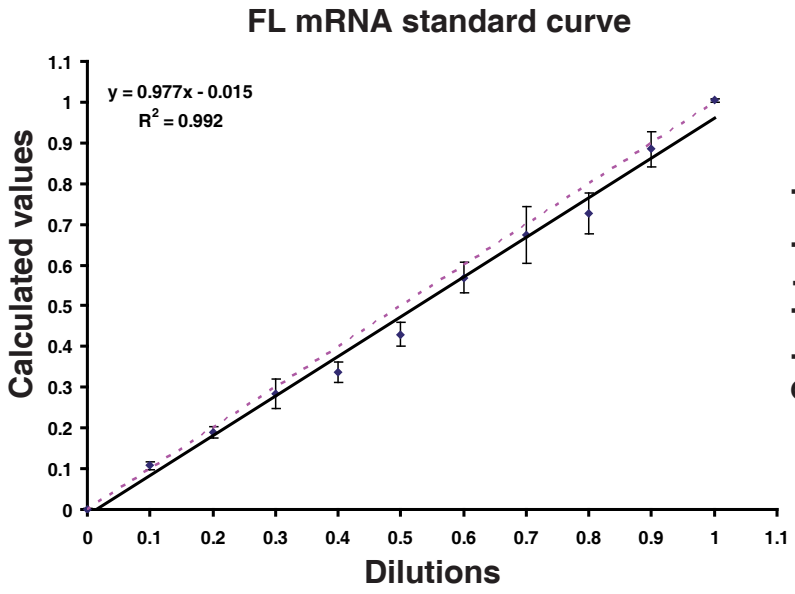

A
Cell line: MMTV-RL-hmga2
WT
MUT
FL activity (x10-5)
Time post-induction (h)
RL activity (x10-6)
Time post-induction (h)
B
Cell line: CMV-RL-reck
WT
MUT
FL activity (x10-5)
Time post-induction (h)
RL activity (x10-6)
Time post-induction (h)

Figure S1


FL mRNA standard curve
y = 0.977x - 0.015
R2 = 0.992
Calculated values
Dilutions
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1

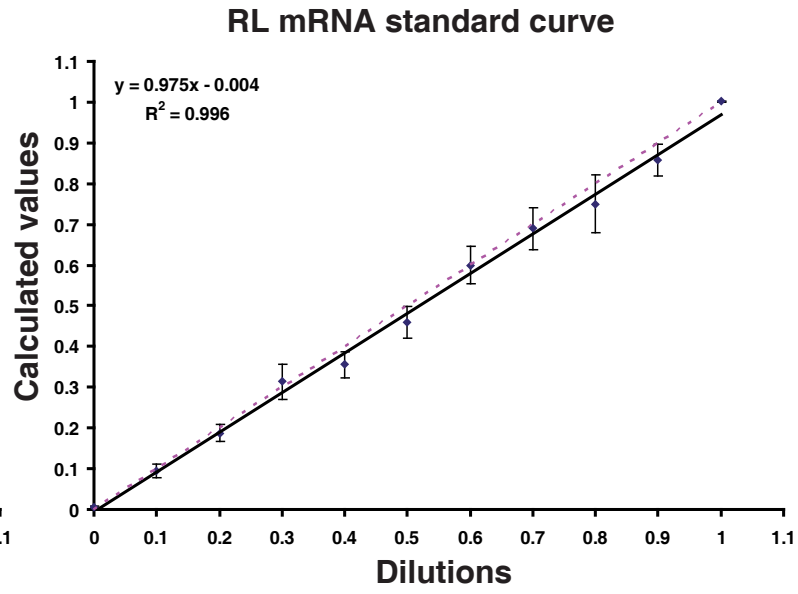

RL mRNA standard curve
y = 0.975x - 0.004
R2 = 0.996
Calculated values
Dilutions
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1

Figure S2

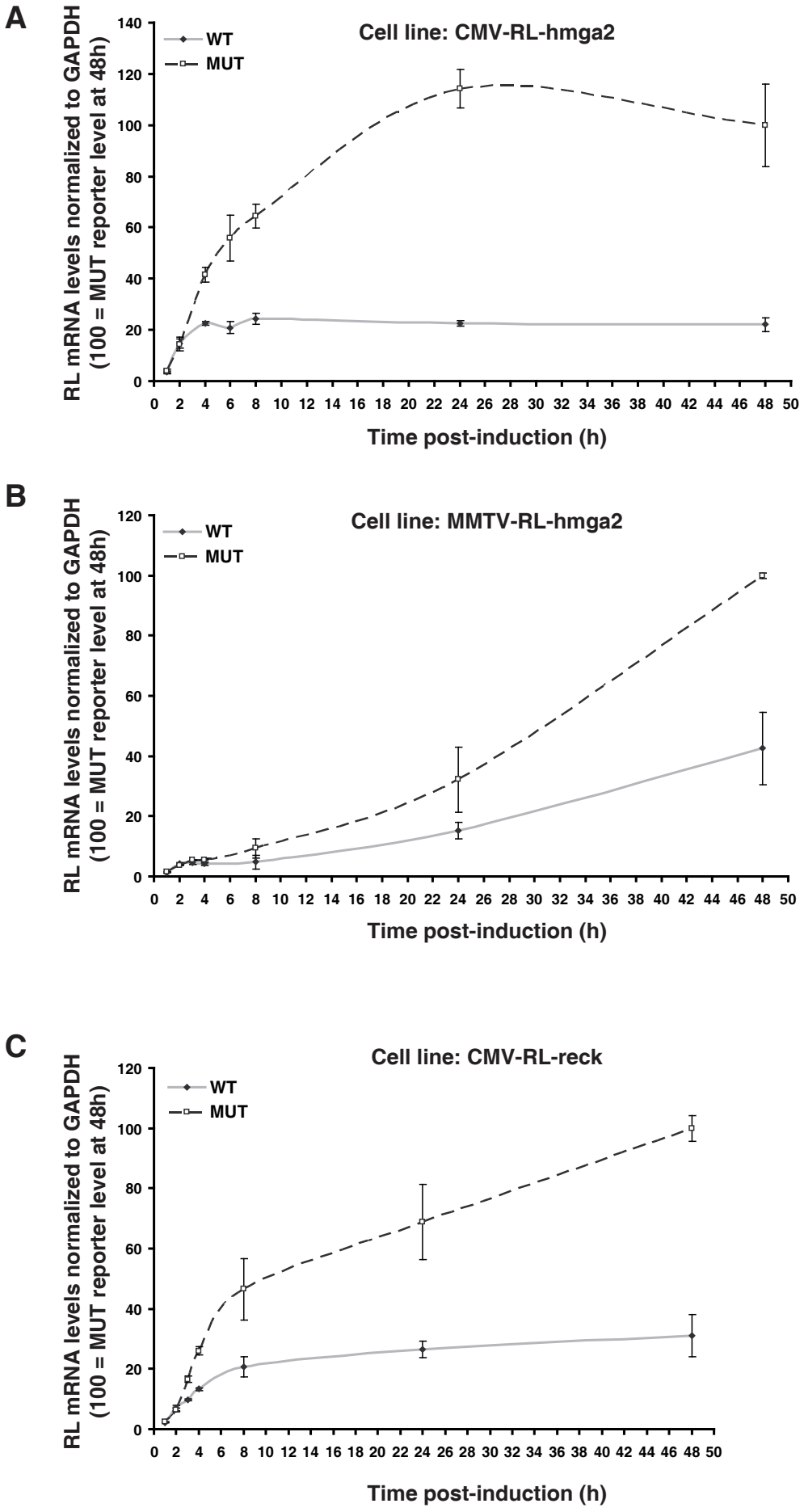
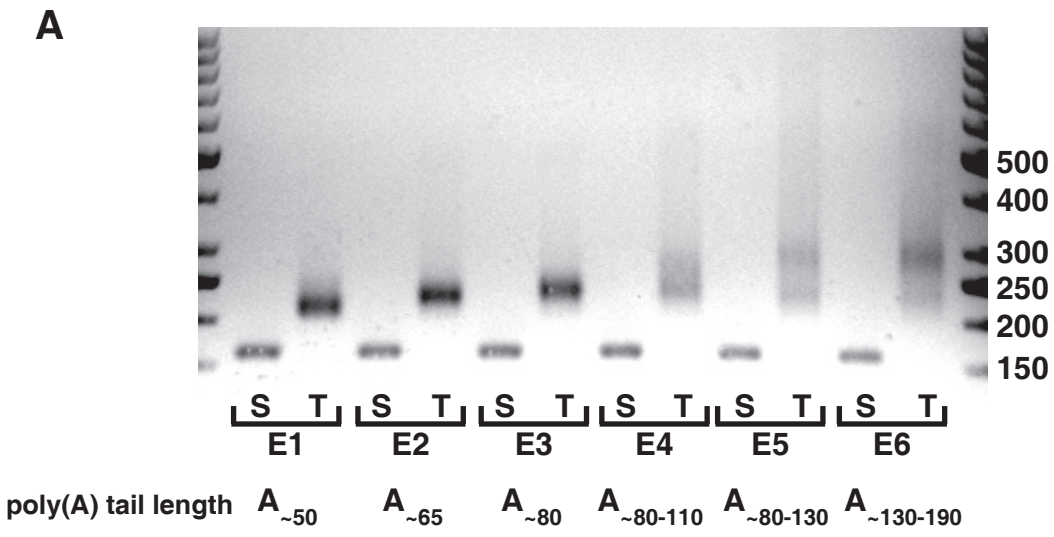

A
Cell line: CMV-RL-hmga2
WT
MUT
RL mRNA levels normalized to GAPDH
(100 = MUT reporter level at 48h)
Time post-induction (h)
B
Cell line: MMTV-RL-hmga2
WT
MUT
RL mRNA levels normalized to GAPDH
(100 = MUT reporter level at 48h)
Time post-induction (h)
C
Cell line: CMV-RL-reck
WT
MUT
RL mRNA levels normalized to GAPDH
(100 = MUT reporter level at 48h)
Time post-induction (h)

Figure S3


A
500
400
300
250
200
150
S T S T S T S T S T S T
E1 E2 E3 E4 E5 E6
poly(A) tail length A~50 A~65 A~80 A~80-110 A~80-130 A~130-190

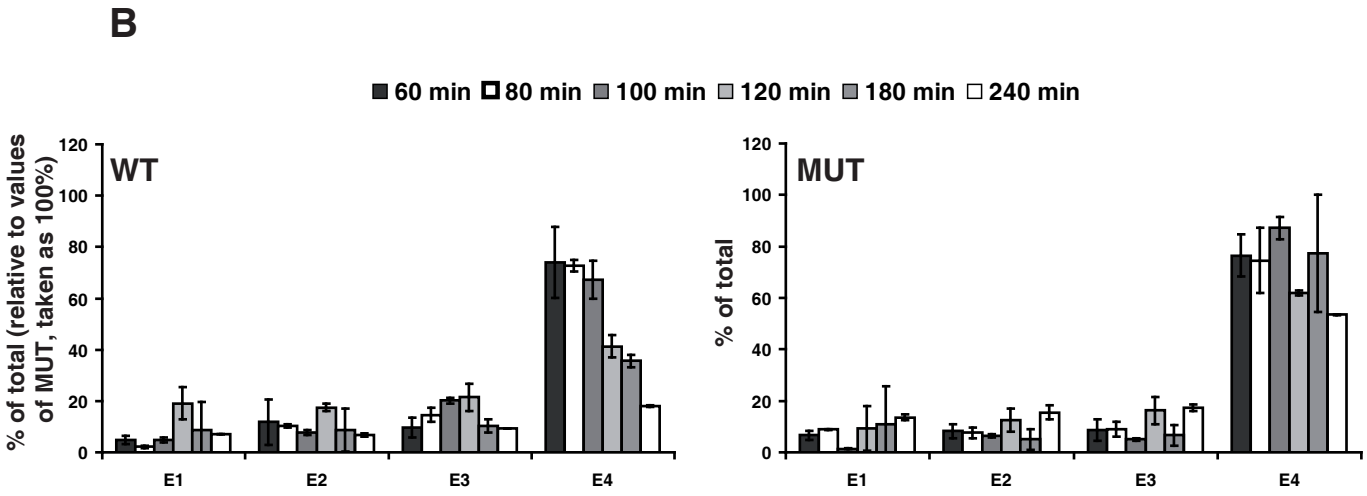

B
60 min 80 min 100 min 120 min 180 min 240 min
WT
MUT
% of total (relative to values of MUT, taken as 100%)
% of total
0 20 40 60 80 100 120
E1 E2 E3 E4

Figure S4