

Supplementary Note 1, Bridge et al.

Plasmid construction (normal cloning)

The U6 promoter cassette was cloned into the XbaI/SalI sites in pUC19 following PCR from human genomic DNA using primers 5'-atctagagcttcgaatcgtccttccacaag-3' and 5'-tgaagtcgacaaggtcgggcaggaag-3' to give pSP-81. Oligonucleotides encoding both strands of the targeting sequence were annealed and ligated into the BglII/HindIII sites in pSUPER¹, or into the BstBI site of pSP-81. The sequence was verified on a Licor sequencer and the cassette containing the RNA pol III promoter plus the hairpin sequence was transferred to the lentiviral vector (pAB286) on a BamHI/SalI fragment. pAB286 is derived from pHR'² and contains BamHI and SalI cloning sites followed by the SV40 promoter-puromycin acetyl transferase cassette from pBabe-puro (fig 1a).

The H1 promoter plasmids contain the following inserts:

Gene	pSUPER	lenti vector	oligo sequence
blank	pSUPERpAB303	none	(no shRNA insert)
<i>MORF4L1</i>	pAB314	pAB319	agatccccGTAAAGATTCCCTGAAGAGCttcaagagaGCTCTTCAGGAATCTTTACTtttttggaaaagcctt
<i>MORF4L1</i>	pAB324	pAB334	agatccccGAGCAGAGTACTCAAATACttcaagagaGTATTTGAGTACTCTGCTCtttttggaaaagcctt
<i>MORF4L1</i>	pAB326	pAB336	agatccccGATGGTGGCAGTACCAGTGttcaagagaCACTGGTACTGCCACCATCtttttggaaaagcctt
<i>MORF4L1</i>	pAB327	pAB337	agatccccTGTGGATTCCATTCTTGAGttcaagagaCTCAAGAATGGAATCCACAtttttgaaaagcctt
<i>MORF4L1</i>	pAB328	pAB338	agatccccGAGCCTTGCTTTATTACTCttcaagagaGAGTAATAAAGCAAGGCTCtttttggaaaagcctt
<i>MORF4L2</i>	pAB317	pAB322	agatccccGGAATATGCGGTTAATGAAttcaagagaTTCATTAACCGCATATTCCTtttttggaaaagcctt
<i>LMNA</i>	pAB287	pAB295	agatccccGGACTTCCAGAAGAACATCttcaagagaGATGTTCTTCTGGAAGTCCTtttttggaaaagcctt

The siRNA target sequences are shown in upper case. pAB319 is the lentiviral vector that induces interferon. In fig 1C, the vectors were labeled as follows: *MORF4L1* shRNA 1-pAB319, 2-pAB334, 3-pAB336, 4-pAB337, 5-pAB338.

To restore *MORF4L1* expression, the site targeted in pAB314 was mutated by inverse PCR using primers 5'-accagaggaactaaaaccgtggttggatgatgac-3' and 5'-attttcactttaactcaactctgttcatgaatg-3'. The final lentiviral expression vector (pAB349) contains the *MORF4L1* cDNA expressed from the EF1 α promoter and the hygromycin phosphotransferase gene expressed from the SV40 promoter (from pBabe-hygro, pBH2) for selection of transduced cells with hygromycin. The sequence around the cut site is shown below (upper strand mutant, lower strand wild type, siRNA sequence in capitals):

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cattcatgaacagagttgaagttaaagtgaaaataaccagaggaactaaaaccgtggttggatgatgac
|||||
cattcatgaacagagttgaagttaaaGTAAAGATTCCCTGAAGAGCtaaaaccgtggttggatgatgac
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The U6 promoter plasmids contain the following inserts:

Gene	pSP-81	Lenti vector	oligo sequence
Blank	pSP-81	pSP-108	none (no shRNA insert)
<i>EP300</i>	pSP-109	pSP-134	ttcgaaaaaGGACCGCTTTGTCTACACttcaagagaGGTGTAGACAAAGCGGTCCttttcgaa
<i>EP300</i>	pSP-110	pSP-135	ttcgaaaaaTGACACAGGCAGGCTTGACTtcaagagaGTCAAGCCTGCCTGTGTCAttttcgaa
<i>EP300</i>	pSP-111	pSP-136	ttcgaaaaaGCAAACATGCAAGATGAACtcaagagaGTTTCATCTTGCATGTTTGCttttcgaa
<i>EP300</i>	pSP-112	pSP-137	ttcgaaaaaCTAGGCCTTGGCTTAGATGttcaagagaCATCTAAGCCAAGGCCTAGttttcgaa
<i>EP300</i>	pSP-113	pSP-138	ttcgaaaaaGTGTGCACTGGGAGGAGGCTtcaagagaGCCTCCTCCCAGTGCACACTttttcgaa
<i>CREBBP</i>	pSP-114	pSP-139	ttcgaaaaaCTGTCCGAGCTTCTACGAGttcaagagaCTCGTAGAAGCTCCGACAGttttcgaa
<i>CREBBP</i>	pSP-115	pSP-140	ttcgaaaaaCTGCGACTTCCGAGCCATGttcaagagaCATGGCTCGGAAGTCGCAGttttcgaa
<i>CREBBP</i>	pSP-117	pSP-142	ttcgaaaaaGACATCCCAGTCTATAAGtcaagagaCTTATAGACTCGGGATGTCTttttcgaa
<i>CREBBP</i>	pSP-118	pSP-143	ttcgaaaaaATATCTGGTCTCTCTTTGGtcaagagaCCAAAGAGAGAGACCAGATATttttcgaa
<i>PCAF</i>	pSP-119	pSP-144	ttcgaaaaaGGATTATGAAGGAGCCACTtcaagagaAGTGGCTCCTTCATAATCCTttttcgaa
<i>PCAF</i>	pSP-121	pSP-146	ttcgaaaaaCTCTCCCATCTGGGATCAGGAttcaagagaTCTTGATCCCAGATGGGAGAGttttcgaa
<i>PCAF</i>	pSP-122	pSP-147	ttcgaaaaaGAAATTATTCATGGCAGACTtcaagagaGTCTGCCATGAATAATTTCTttttcgaa
<i>PCAF</i>	pSP-123	pSP-148	ttcgaaaaaGCAGTGTGCCTAAAGCAAGtcaagagaCTTGCTTTAGGCACACTGCTttttcgaa
<i>GCN5L2</i>	pSP-124	pSP-149	ttcgaaaaaGAGATCATCAAGAAGCTGAttcaagagaTCAGCTTCTTGATGATCTCTttttcgaa
<i>GCN5L2</i>	pSP-125	pSP-150	ttcgaaaaaGCTTGAGAAGCTAGGGGCTtcaagagaGACCCCTAGCTTCTCAAGCttttcgaa
<i>GCN5L2</i>	pSP-126	pSP-151	ttcgaaaaaGACCATGACTGAGCGGCTGttcaagagaCAGCCGCTCAGTCATGGTCTttttcgaa
<i>GCN5L2</i>	pSP-127	pSP-152	ttcgaaaaaGGGTCTCCGACCCGATCttcaagagaGATCGGGTCCGAGGACCCttttcgaa
<i>GCN5L2</i>	pSP-128	pSP-153	ttcgaaaaaTCCCTTAGAGGGAATAATtcaagagaTATTATCCCTCTAAGGGAtttttcgaa
<i>TRRAP</i>	pSP-129	pSP-154	ttcgaaaaaTAGACTCAACCCGAGATGttcaagagaCATCTCGGGGTTGAGTCTAtttttcgaa
<i>TRRAP</i>	pSP-130	pSP-155	ttcgaaaaaCACCTCACAGCAGCTTCGTGCTtcaagagaGCACGAAGCTGCTGTGAGGTGttttcgaa
<i>TRRAP</i>	pSP-131	pSP-156	ttcgaaaaaGCCCCATAGTTTCACTGGGtcaagagaCCCAGTGAACTATGGGGCttttcgaa
<i>TRRAP</i>	pSP-132	pSP-157	ttcgaaaaaGTTCACGTCATCTCCTCAGTAttcaagagaTACTGAGGAGATGACGTGACAtttttcgaa
<i>TRRAP</i>	pSP-133	pSP-158	ttcgaaaaaACAAGAGGACACGTCCTCttcaagagaGAGGACGTGTCCTCTTGTGttttcgaa

The siRNA target sequences are shown in upper case. pSP-135, 136, 138, 147, 149, 157 and 158 are the lentiviral vectors that induce interferon.

Plasmid construction (Gateway vectors)

To facilitate production of shRNA vectors, a lentiviral vector (pSP-161) was constructed with an attP cassette for direct cloning of PCR products with attB sites (“Gateway” cloning, Invitrogen). pSP-161 contains the ApaI-BspHI attP cassette from pDONR201 (Invitrogen) inserted into the BamHI/BstXI sites of pAB286. The U6-shRNA cassettes from pSP-81, 122 and 124 were amplified by PCR with attB-modified primers 5’-ggggacaagttgtacaaaaagcaggctcaaggtcgggcaggaag-3’ and

5’-ggggaccactttgtacaagaaagctgggtgatcctctagagcttcg-3’. The PCR products were cloned into pSP-161 using lambda integrase and *E coli* IHF (“BP Clonase”, Invitrogen). The resulting plasmids pSP-177 (no shRNA insert), pSP-179 (*PCAF* shRNA from pSP-122) and pSP-181 (*GCN5L2* insert from pSP-124) contain the U6 promoter in the sense orientation relative to the LTRs.

Cell culture

The lentiviral vector plasmid (10 μ g) and second generation packaging plasmids (3.5 μ g pMD2-VSVG, 6.5 μ g pCMV Δ R8.91²) were cotransfected into 293T cells (ATCC CRL-11268) using calcium phosphate. Lentivirus-containing supernatants of transfected cells were collected at 24 hours, polybrene was added to 8 μ g/ml, the solution was 0.2 μ m filtered and snap frozen at -70°C. The viral titer was estimated by selecting for puromycin resistant human embryonic lung fibroblasts (HLFs) following infection with three-fold dilutions of virus. The 293T cells were shown to be negative for mycoplasma contamination by PCR ³.

For the microarray and Taqman experiments, HLFs were grown in DMEM plus 10% FCS and infected at passage 6 at a multiplicity of infection of 10. Cells were infected for 24 hours, selected in puromycin for 24 hours, replated, then harvested after 24 hours. HeLa cells (4 x 10⁵ cells per 6 cm dish) were transfected using 5 μ g of pBluescript or pSUPER plasmid DNA in 20 μ l lipofectamine 2000 (Invitrogen) in OptiMEM (Invitrogen), the medium was changed to DMEM 10% FCS after 4 hours, and cells were harvested after 48 hours. siRNA duplexes were purchased from Dharmacon (Lafayette, CO), resuspended at 20 μ M in RNase-free water and transfected at the indicated concentrations into HLFs (3 x 10⁵ cells per 6 cm dish) using 20 μ l oligofectamine in OptiMEM. FCS was added to 10% after 4 hours, the medium was changed to DMEM 10% FCS after 16 hours and cells were harvested after 24 hours. Total RNA was extracted using RNeasy columns according to the manufacturer's instructions (Qiagen).

Northern blotting

HLFs (3 x 10⁶ cells per 15 cm dish) were infected as described above, selected for 48 hours in puromycin and harvested in 20ml Trizol (Invitrogen) according to the manufacturer's instructions. 25 μ g total RNA per lane was resolved on a 12% polyacrylamide, 1 x TBE, urea gel. RNA was electro-transferred to Hybond N and UV cross-linked to the membrane in a Stratalinker. The *MORF4L1* (5'-aagtaaagattcctgaagagc-3') and *MORF4L2* (5'-aaggaatatgctgtaataa-3') oligonucleotide probes were end-labeled with γ ³²P dATP using T4 polynucleotide kinase. The filters were prehybridized in 5x SSC, 7% SDS, 100 μ g/ml sonicated salmon sperm DNA, 1x Denhardt's solution, 20mM Na₂HPO₄ pH 7.2 for 1 hour at 50°C, hybridized in the same conditions overnight, washed twice in 5x SSC, 5% SDS at 50°C, then once in 1x SSC, 1% SDS at 50°C and exposed to phosphorimager screens and scanned using a Fuji BAS-1000 phosphorimager.

Quantitative PCR

Quantitative PCR was performed on a PE 5700 PCR machine using SYBR green PCR master mix (Perkin Elmer no. 4312704) for *MORF4L1* and *OAS1*, and Taqman PCR master mix (Perkin Elmer no. 4305719) for 18S ribosomal RNA (Perkin Elmer no. 4310875). The *MORF4L1* and *OAS1* values were normalized to 18S. The primers used for *OAS1* amplification were 5'-aggtggtaaagggtgctcc-3' and 5'-acaaccaggtcagctcagat-3' and for *MORF4L1* 5'-acagaaaacacctggaatggag-3' and 5'-catgaatgttctcatttcaac-3'.

Microarray protocol

Microarrays were printed by the ISREC Microarray Core Facility essentially as described by Brown and colleagues⁴. Excluding blank and failed spots, the ISREC Hu10Kb microarray contains 8884 spots: 8515 Incyte (IC) ready-to-spot PCR products and 369 Resgen (RG) PCR products. Each slide was hybridized to Stratagene human reference RNA labeled with Cy3 and the test sample labeled with Cy5 (a detailed protocol is given below). Slides were scanned using Scanarray software on an SA4000 scanner (Packard Biochip Technologies) and numerical data were extracted from the image using Scanalyze version 2.44⁵. 80 spots were manually flagged for high background and deleted. Normalization was performed using the S option without background subtraction in the sma package in R (<http://www.R-project.org/>) using a web interface developed by the ISREC Bioinformatics Core Facility (M Delorenzi). The NCBI GEO platform accession number is GPL247.

MORF4L1 and *MORF4L2* were simultaneously targeted with shRNA because of concerns that the gene products might act redundantly. The shRNA slide (Hu10Kb480) was hybridized to RNA from HLFs infected with lentiviruses pAB319 and pAB322. The control slide (Hu10Kb482) was hybridized to RNA from HLFs infected with lentivirus pAB303. The induction was calculated as the difference between the log to the base 2 of the ratios on slides Hu10Kb480 and Hu10Kb482. The magnitude of changes in gene expression is smaller when measured by array than by quantitative PCR for all genes tested. The NCBI GEO slide accession numbers are GSM3891 and GSM3892.

Supplementary Table 1 gives the 100 most induced and the 100 most repressed genes. Induced genes are labeled as interferon targets if the Gene Card (Weizmann Institute) or Unigene (NCBI) entry lists the gene as interferon inducible. 7 out of the top 10, and 27 out of the top 100 genes are interferon inducible by this criterion. The mechanism of interferon induction by shRNA in HLFs is unknown, but by analogy with other systems may involve induction of interferon- β (*IFNB1*) secretion by dsRNA followed by autocrine stimulation of interferon receptors. *IFNB1* is the 208th gene in the list of induced genes. The genes most strongly repressed on the entire array are the targeted genes, *MORF4L1* and *MORF4L2*. Many of the cell cycle genes in the repressed list are also seen on arrays expressing a *MORF4L1* shRNA that does not induce interferon (AB and RI, unpublished).

Amplification, Labeling and Hybridization protocol

Reagents: Human Universal Reference RNA, cat. no. 740000, Stratagene, La Jolla, CA
MessageAmp aRNA Kit, cat. no. 1750, Ambion, Austin, TX
Random Primers 3µg/µl, cat. no. 48190-011, Invitrogen, Carlsbad, CA
Superscript II RT 200U/µl, 5xbuffer, 0.1 M DTT, cat. no. 18064-14, Invitrogen
Cy3-dCTP(1mM), cat. no. PA53021, Amersham, Little Chalfont, UK
Cy5 dCTP (1mM), cat. no. PA55021, Amersham
dNTPs (10mM dGTP, dATP, dTTP; 4mM dCTP)
Rnasin 40U/µl, cat. no. N251B, Promega, Madison, Wisconsin
Plasmid Mini Kit, cat. no. 12123, Qiagen, Hilden, Germany
Microcon YM-30, Millipore, Billerica, MA
Cot 1 DNA 1 µg/µl, cat. no. 1581074, Roche, Rotkreuz, Switzerland
Poly A RNA 10µg/µl
Yeast tRNA 10 µg/µl
Human 10K chips, DNA Array Facility, ISREC Epalinges
LifterSlips 22x50, Erie Scientific, Portsmouth, NH
CMT Hybridization chambers, cat. no. 2551, Corning, USA

3µg total RNA was amplified once according to the MessageAmp aRNA Kit protocol. Amplified RNA (aRNA) was labeled by incorporation of Cy3 or Cy5 dCTP during reverse transcription as follows. To 3µl random primers, add 6µg aRNA and sterile distilled water to 19µl. Heat to 70°C for 5 min. then chill on ice. Add 8µl 5x Superscript buffer, 4µl 0.1 M DTT, 4µl Cy3 dCTP or Cy5 dCTP, 2µl dNTPs and 1µl Rnasin. Incubate at 25°C for 10 min., then 42°C for 2 min. In the PCR machine, add 2µl Superscript II and incubate at 42°C for 1 h., then add another 1µl Superscript II and incubate at 42°C for 1 h. Add 2µl 500mM EDTA, mix by pipetting, add 4.5µl 1M NaOH, mix by pipetting and incubate at 65°C for 15min, then place at room temperature. Add 4.5µl 1M HCl and 2.5µl 1M Tris pH6.8.

The Cy3 and Cy5-labelled cDNAs were then mixed together and purified according to the Plasmid Mini Kit protocol, and eluted in a final volume of 800µl. This was concentrated with a Microcon YM-30 as follows. To prewash the cartridge add 400µl 10mM Tris pH 7.5 and spin at 14,000 g for 10 min. Add the 800µl of labeled cDNA, spin 14,000g until only a few µl remain, add 380µl 10mM Tris pH 7.5, 20µl Cot1 DNA, 1.25µl Poly A RNA and 2.5µl Yeast tRNA. Spin at 14,000g and adjust final volume to 60.7µl. Add 11.3µl 20x SSC and 3µl 10% SDS for a final volume of 75µl.

Hybridization was performed as follows. Position a LifterSlip over the printed array on the slide and place the slide in a Corning hybridization chamber. Add 15µl 3xSSC in the two grooves of the chamber to humidify. Heat the labeled cDNA mix to 98°C for 2min, spin briefly and slowly pipette the mix under the LifterSlip. Close the chamber and leave overnight at 64°C in a water bath without agitation.

After hybridization, open the chamber, remove the LifterSlip and wash the slide as follows: 5min 2xSSC 1% SDS; 5min 2xSSC 1% SDS; 1min 0.2xSSC; 1min 0.2xSSC; 1min 0.1xSSC; 1min 0.1xSSC, 0.1% TritonX100. Dry the slide by spinning for 2 min at 800 rpm. Store in a light-tight box until scanning. Slide Hu10Kb480 was scanned at laser power - gain Cy3: 77% - 77%; Cy5: 72% - 72%. Slide Hu10Kb482 was scanned at laser power - gain Cy3: 78% - 78%; Cy5: 73% - 73%.

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