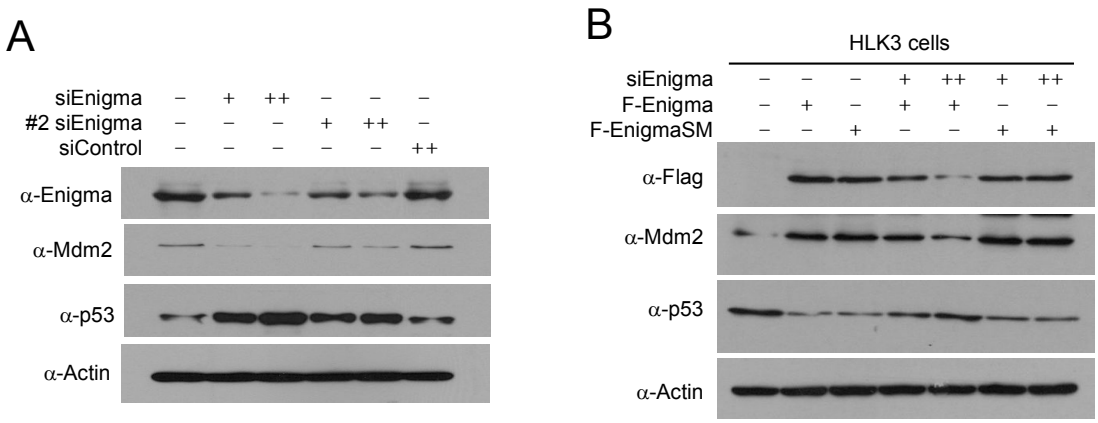
Supplementary data

Supplemental Figure 1

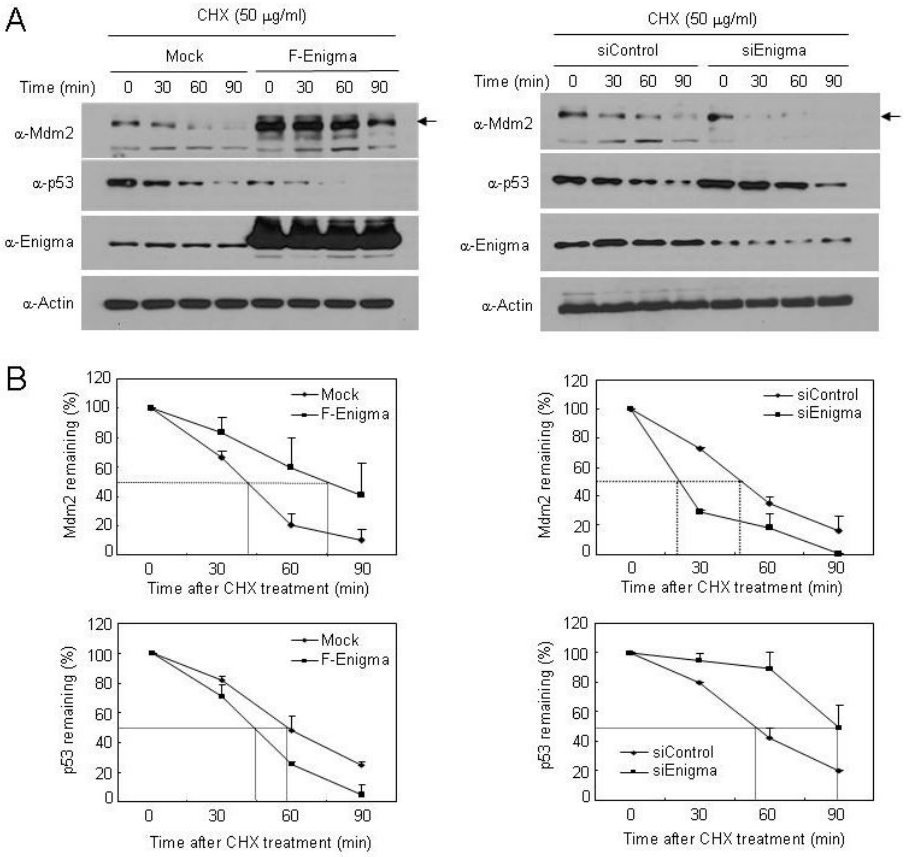


Supplemental Figure 1: The target-specificity of Enigma-siRNAs.

(A) We synthesized a second Enigma-specific siRNA (5'-AATGCCATGGCTGTGACTTCA-3') oligonucleotide duplex that targets Enigma mRNA at nucleotide position different from siEnigma and cloned it into pSuper vector to generate #2 siEnigma expression vector. We transfected HCT116*p53*$^{+/+}$ cells with or without siEnigma (5, 10 μg), #2 siEnigma (5, 10 μg), or siControl (10 μg) vectors, prepared cell lysates at 48 h posttransfection before IB as indicated.

(B) siEnigma does not target a codon-optimized non-degradable F-Enigma mutant (F-EnigmaSM). To construct an F-EnigmaSM construct which has multiple codon mutations within the targeting region of siEnigma (AA**A** AC**G** TT**T** TA**T** TC**G** AA**A** AA**A**), we PCR-amplified F-Enigma cDNA by using relevant primers and cloned amplified PCR product into the *BamH*1-*Hind*II sites of pCMV-Tag2, and isolated a correct mutant to confirm by sequencing. We transfected HLK3 cells with F-Enigma (5 μg) or F-EnigmaSM constructs (5 μg) and with or without siEnigma vector (10, 15 μg), and incubated them for 48 h before IB as indicated.

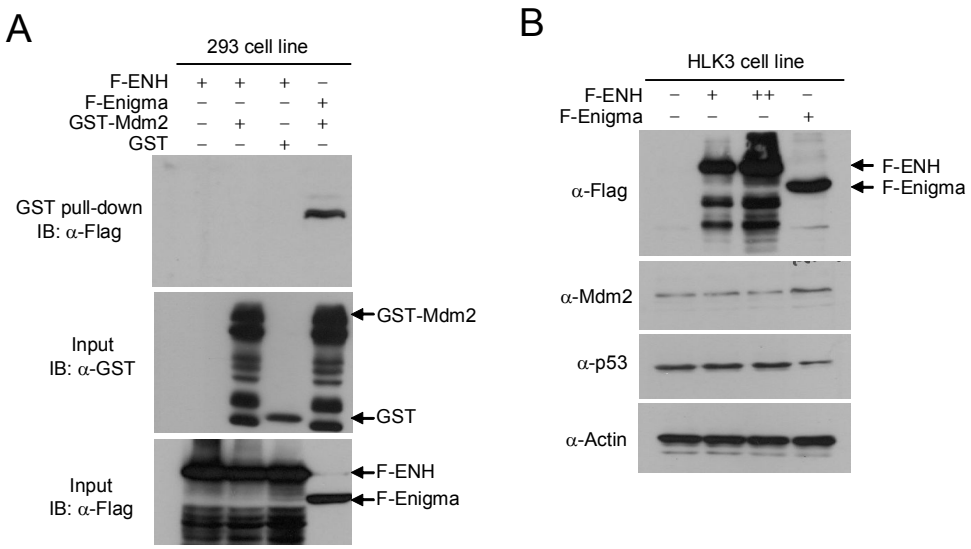
Supplemental Figure 2



Supplemental Figure 2: Enigma increases the half-life of Mdm2 and decreases that of p53.

(A, B) We transfected HLK3 cells with F-Enigma (10 μg), siEnigma (15 μg), or control vectors as indicated. At 36 h posttransfection, the cells were incubated with cycloheximide (CHX) for the indicated times. Endogenous Mdm2 and p53 levels were determined by Western blot analysis (A). We repeated these experiments three times and representative ones are shown. Levels of Mdm2 and p53 were quantified by densitometry. Their mean values are relatively expressed (B).

Supplemental Figure 3

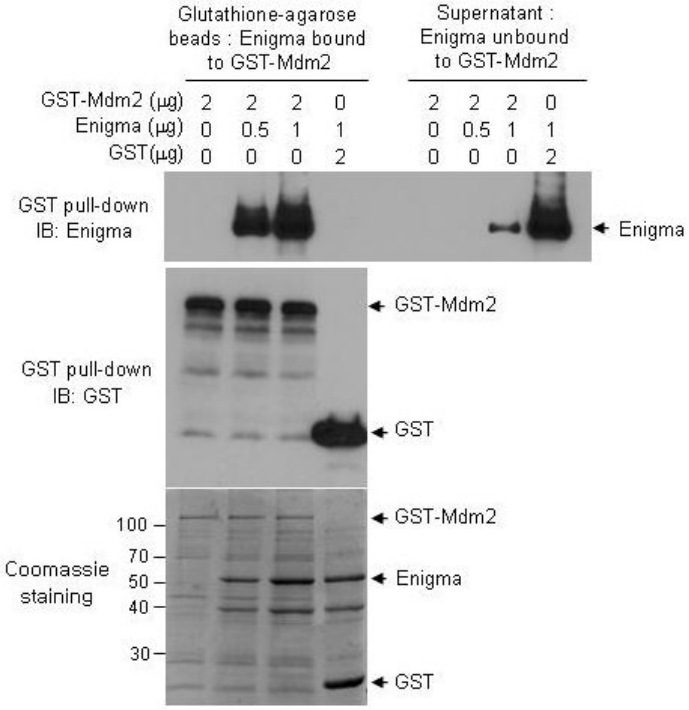


Supplemental Figure 3: Enigma specifically interacts with Mdm2.

(A) Enigma, but not Enigma homolog (ENH) binds to Mdm2. We transfected 293 cells with or without F-ENH (5 μg), F-Enigma (5 μg), and/or GST-Mdm2 (5 μg) vectors, and incubated them for 48 h. Cell lysates were incubated with glutathione agarose beads. The beads-bound proteins and cell lysates were immunoblotted as indicated.

(B) ENH has no significant effects on Mdm2 and p53 protein levels. We transfected HLK3 cells with or without F-ENH (2.5 or 5 μg) or F-Enigma (5 μg) vectors, and incubated them for 48 h. Cell lysates were immunoblotted as indicated.

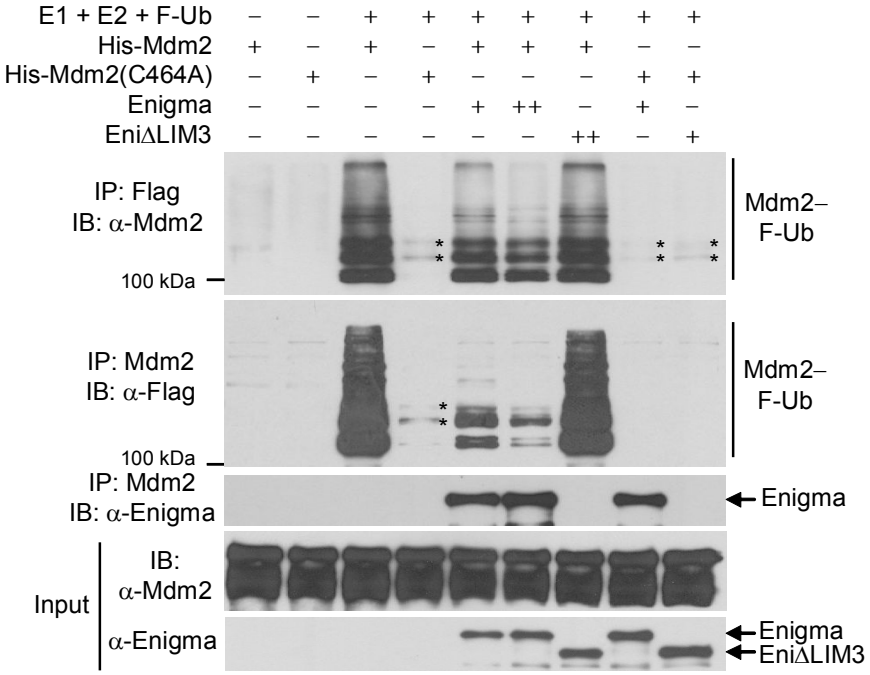
Supplemental Figure 4



Supplemental Figure 4: Almost all the Mdm2 molecules are bound to Enigma in vitro self-ubiquitination assay condition.

We purified GST-Mdm2, Enigma, and GST proteins from bacteria, and mixed them in 200 μl of IP buffer at the indicated concentrations. Each mixture was incubated with glutathione agarose beads (20 μl) at 4°C for 2 h or protein components in the mixtures were visualized by Coomassie blue staining following SDS-PAGE (3rd panel). After incubation, we obtained pellets (beads) and supernatants by centrifugation. We washed the beads with IP butter five times and re-suspended them in 200 μl of SDS sample buffer. Twenty μl of the beads-suspensions and the supernatants were analyzed as indicated. Enigma was not detected in the supernatant from the mixture of 2 μg of GST-Mdm2 plus 0.5 μg of Enigma, and was found to be mostly associated with the beads-bound Mdm2. When 1 μg of Enigma was incubated with 2 μg of GST-Mdm2, most of Enigma was bound to Mdm2 and a small portion of Enigma was present in the supernatant. GST-Mdm2 was not detected in the supernatants (data not shown).

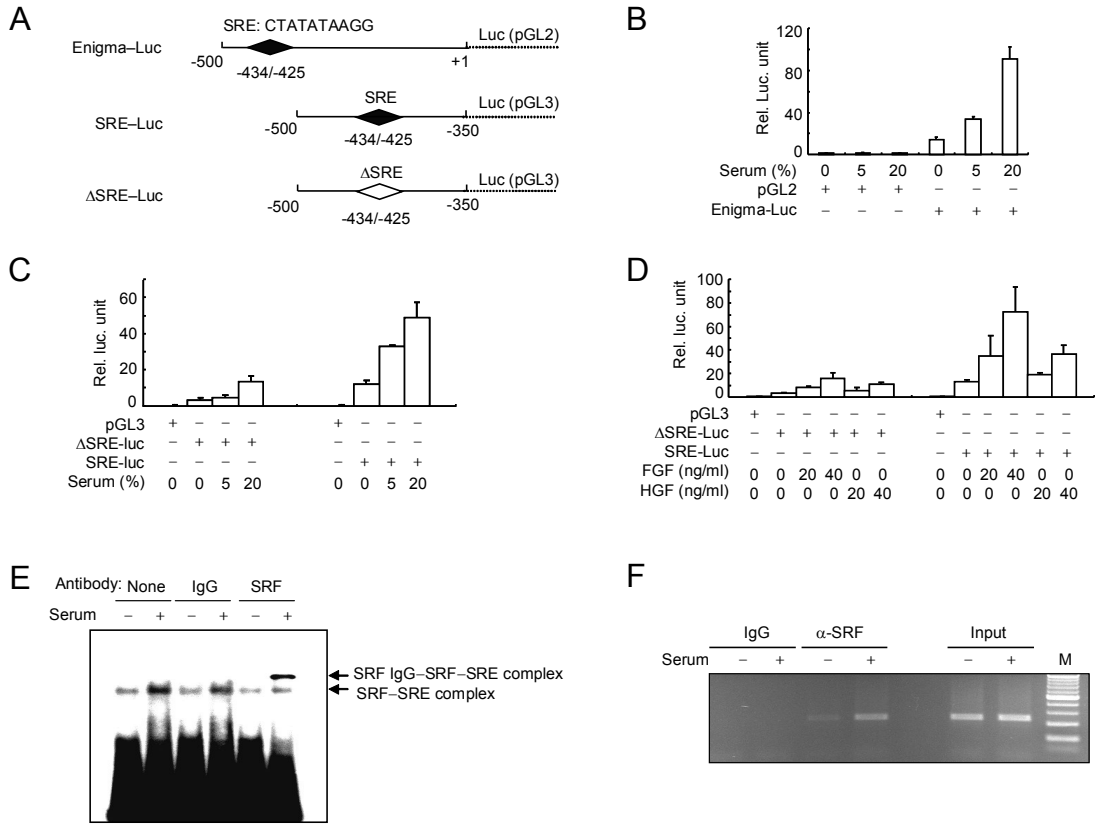
Supplemental Figure 5



Supplemental Figure 5: Enigma inhibits self-ubiquitination of Mdm2 in vitro.

We performed in vitro ubiquitination assay with His-Mdm2 (2 μg), His-Mdm2(C464A) (2 μg), Enigma (0.5, 1 μg), or EniΔLIM3 (1 μg), and analyzed the reaction mixtures as indicated. Protein components in the assay were purified from bacteria. Protein bands marked by an asterisk are nonspecific. F-Ub, Flag-tagged ubiquitin.

Supplemental Figure 6



Supplemental Figure 6: Growth factors activate the Enigma promoter through serum response element (SRE).

(A) Schematic illustration of the Enigma promoter reporter constructs. An Enigma promoter region encompassing 500 bp upstream from putative transcription initiation site of the human *Enigma* gene was PCR-amplified using relevant primers from HLK3 genomic DNA. Amplified PCR product was cloned into the *Sac*I–*Bgl*II sites of pGL2 that contains a promoter-less firefly luciferase (luc) reporter gene (Promega). The resulting plasmid was named Enigma-luc. SRE-luc reporter plasmid was constructed by PCR-amplification of the Enigma promoter region from –500 to –350 and by inserting the amplified PCR product into the *Sac*I–*Bgl*II sites of pGL3 (Promega), in which the luc reporter gene is under the transcriptional control of SV40 minimal promoter. ΔSRE-Luc, in which SRE (5'-CTATATAAGG-3') in the SRE-luc was deleted, was constructed by PCR- amplification using relevant primers and a standard cloning procedure.

(B) We transfected HLK3 cells with Enigma-luc construct (0.5 μg) or pGL2 (0.5 μg) as a control, incubated them in DMEM with 0.1% fetal bovine serum (serum) for 24 h,

further incubated them in DMEM with 5% or 20% serum for 4 h, and performed luciferase assay.

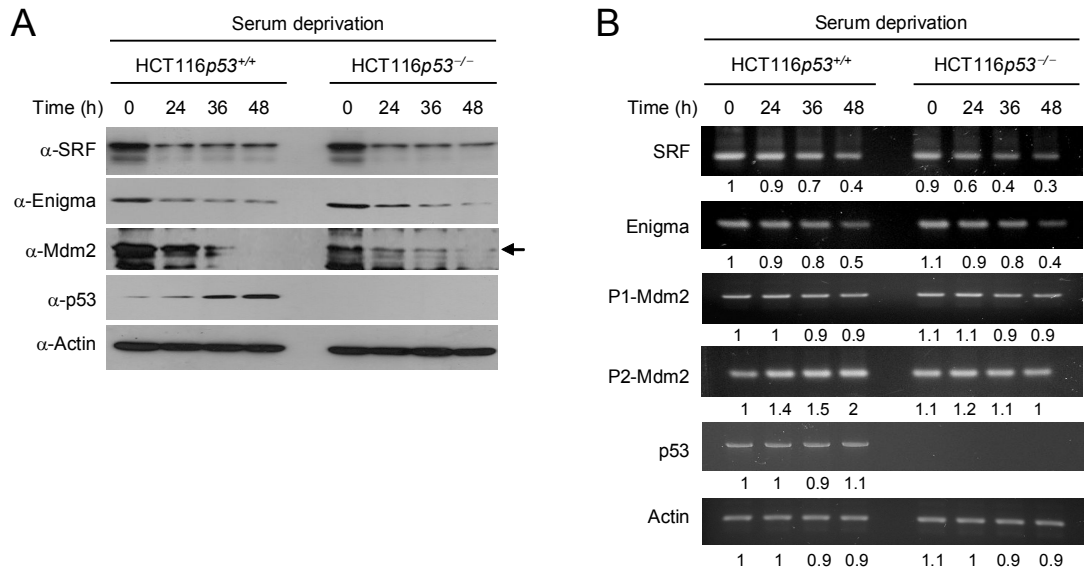
(C) We transfected HLK3 cells with SRE-luc (0.5 μg), ΔSRE-luc (0.5 μg), or pGL3 (0.5 μg) as a control, incubated them in DMEM for 24 h, further incubated them in DMEM with or without 5% or 20% serum for 6 h, and performed luciferase assay.

(D) We transfected HLK3 cells with the reporter constructs under the conditions described in (C), treated them with or without FGF or HGF, and performed luciferase assay. We repeated the experiments in (B, C, D) three times, each in duplicate.

(E) Electrophoretic mobility shift assay (EMSA) showing that serum induces the specific binding of SRF to the SRE in the Enigma promoter. We prepared nuclear extracts from HLK3 cells cultured in DMEM with or without serum for 2 h. We synthesized 3 × SRE oligonucleotide duplex (5'-GGATGTCTATATAACGCCCTATATAACGCCCTATATAACGTCT-3') based on the SRE in the Enigma promoter. Nuclear extracts (10 μg each) were incubated with the ^{32}P-labeled SRE oligonucleotide duplex for 15 min. The reaction mixtures were further incubated in the presence or absence of nonspecific antibody (IgG) or anti-SRF antibody for 30 min. The reaction products were resolved by a 6% native polyacrylamide gel electrophoresis and visualized by a PhosphorImager.

(F) Chromatin immunoprecipitation (ChIP) assay showing that SRF transcription factor binds sequence-specifically to the SRE of the Enigma promoter in cells. We incubated serum-starved HLK3 cells in DMEM with 10% serum for 2 h, cross-linked protein–chromatin complex by incubation of the cells with 1% formaldehyde at 37°C for 10 min, completely lysed the cells by sonication, and cleared cell lysates by centrifugation. We immunoprecipitated protein-chromatin complex with rabbit-IgG or anti-SRF antibodies, recovered DNA from protein-DNA complex by phenol/chloroform extraction, and performed PCR-amplification with chromatin DNA and the forward primer 5'-AGCTTACACGCCTTATATAGTCCGAGCA-3' and the reverse primer 5'-CCTGAATCCAGGCTTGAGGT-3'. Input corresponds to PCR products directly amplified from the SRF-chromatin complex cross-linked by formaldehyde treatment before IP. The reaction products were resolved by a 1.5% agarose gel electrophoresis and stained with EtBr.

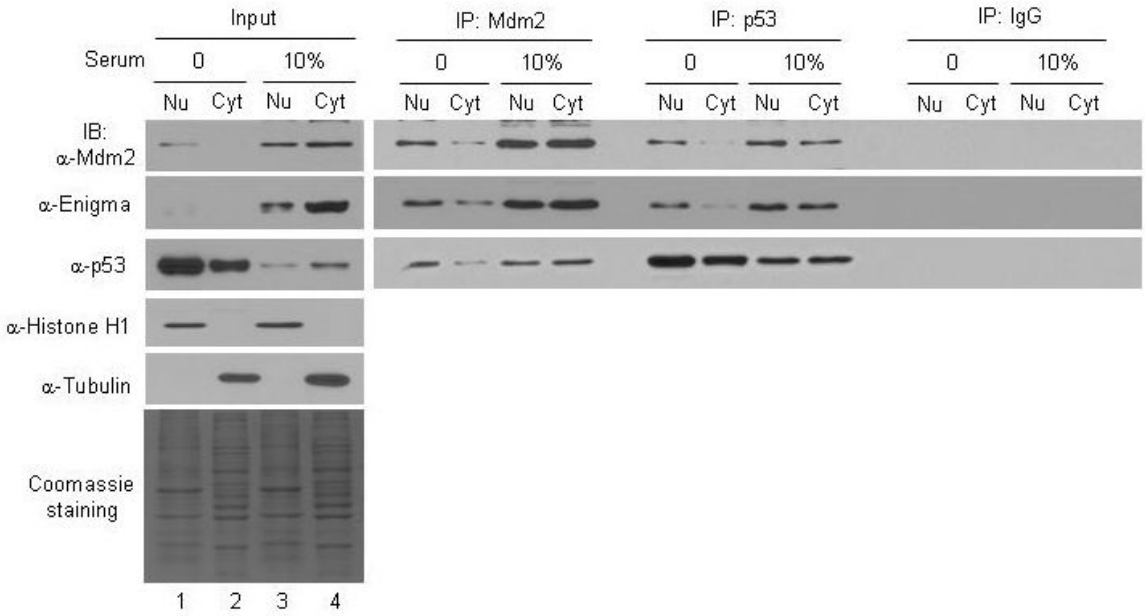
Supplemental Figure 7



Supplemental Figure 7: Effects of serum removal on protein and mRNA levels of SRF, Enigma, Mdm2, and p53.

(A, B) After the cells were grown in DMEM with 10% serum, we incubated them in DMEM without serum for the indicated times, and prepared cell lysates or total RNAs. Cell lysates were immunoblotted as indicated (A). An arrow indicates Mdm2 band. We amplified indicated mRNAs by RT-PCR using total RNAs and relevant primers. RT-PCR products were resolved by a 1.5% agarose gel electrophoresis, and visualized by EtBr staining (B). Each mRNA level was quantified by densitometry and is expressed relatively.

Supplemental Figure 8



Supplemental Figure 8: Enigma is localized in the cytosol and the nucleus.

We incubated HLK3 cells in DMEM without 10% serum for 48 h, and further incubated them in DMEM with or without serum for 2 h. After incubation, cells were suspended in a low salt buffer (10 mM Hepes, pH 7.5, 10% sucrose, 0.1% NP40, 1 mM $MgCl_2$, one tablet/100 ml proteinase cocktail inhibitor), were allowed to swell on ice for 10 min, and centrifuged for 1 min at 3,000*g*. Supernatants were cleared by centrifugation for 15 min at 12,000*g* and were considered cytoplasmic fractions. Pellets were suspended in an ice-cold, high salt buffer (10 mM Hepes, pH 7.5, 0.45 M NaCl, 0.1% NP40, 1 mM $MgCl_2$, one tablet/100 ml proteinase cocktail inhibitor), incubated on ice for 20 min, and centrifuged for 10 min at 10,000*g* for preparation of nuclear fractions. We diluted nuclear fractions by the addition of 3 volumes of low salt buffer, and then analyzed both fractions by IB or IP/IB as indicated. We resolved 2 μg of proteins of each fraction, which had been quantified by Bradford assay, by SDS-PAGE, and stained them with Coomassie blue to indicate equivalent loading. Enigma was mainly cytoplasmic and was detected in nuclear fraction at lower level (lanes 3 and 4). Enigma-Mdm2-p53 ternary complex was detected in both nuclear and cytoplasmic fractions from cells with or without 10% serum.

Supplemental Figure 9: Primers used for RT-PCR, Northern blotting, and construction of reporter plasmids.

Gene	Forward primer	Reverse primer
Human *Enigma*	ATGGATTCCTTCAAAGTAGT	GATCTTGTTCTGAGCTTCGA
Human *p53*	ATGGAGGAGCCGCAGTCAGA	CTGGCATTCTGGGAGCTTCA
Human *SRF*	TGAAGCCGGAGCCTGAGCGAGAT	TGTGTCCCTGTCAGCGTGGA
Human *actin*	ATGGATGATGATATCGCCGC	AGGGTGAGGATGCCTCTCTT
Human P1-*Mmd2*	GACTCCAAGCGCGAAAACC	CAGTAGGTACAGACATGTTGGT
Human P2-*Mdm2*	CGGACGCACGCCACTT	CAGTAGGTACAGACATGTTGGT
Human *p21*	ATGTCAGAACCGGCTGGGGA	AAGGTAGAGCTTGGGCAGGC
Murine *Enigma*	ATGGATTCCTTCAAGGTGGT	TTGTTCTGGGCTTCGATGTG
Murine *p53*	ATGACTGCCATGGAGGAGTC	AGGAGCTCCTGACACTCGGA
Murine *SRF*	TCAAGCGGAGCCTGAGCGAGAT	TGCCCGTCTTCCTCTTGCTG
Murine *actin*	ATGGATGACGATATCGCTGC	TCAGGGTCAGGATACCTCTC
Murine P1-*Mdm2*	CGTGAAGGGTCGGAAGATGC	GCTCCAACGGACTTTAACAACTTC
Murine P2-*Mdm2*	TGGGCGAGCGGGAGACCGAC	GCTCCAACGGACTTTAACAACTTC