Figures and figure supplements

MAF1 represses CDKN1A through a Pol III-dependent mechanism

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**Figure 1.** MAF1 knockdown strongly upregulates CDKN1A expression and arrests MCF-7 cells at the G₀/G₁ phase. Analysis of MAF1 expression after MAF1 knockdown using three different siRNAs in MCF-7 cells by quantitative RT-PCR (**A**) and immunoblot analysis (**B**). The immunoblot results were quantified (left panel) using α-tubulin as a loading control on a representative gel (right panel). **(C)** MAF1 knockdown arrested the MCF-7 cell cycle at the G₀/G₁ phase. At 72 hr after knockdown, cells were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry (top panel). The quantification results show that MAF1 knockdown increased cells arrested at the G₀/G₁ phase by 16.4% ± 1.76% (bottom panel). **(D)** Quantitative RT-PCR of genes in MCF-7 cells subjected to siRNA knockdown for 72 hr. CDKN1A expression was upregulated 10-fold, and upregulation was abolished by double knockdown of MAF1 and POLR3A. Relative expression normalized to GAPDH is displayed. **(E)** Immunoblot analysis of CDKN1A expression after MAF1 knockdown in MCF-7 cells. The results were quantified (left panel) using α-tubulin as a loading control on a representative gel (right panel). All data shown represent mean ± SD, n ≥ 3, **p < 0.01, ***p < 0.001 (t-test). DOI: 10.7554/eLife.06283.003
Figure 1—figure supplement 1. MAF1 knockdown upregulates CDKN1A and GDF15 expression in HCT116<sup>p53<sup>++/+</sup></sup> (wild-type), HCT116<sup>p53<sup>−/−</sup></sup> (p53-null), MCF-10A, and MDA-MB-231 cell lines. (A) Immunoblot analysis of p53 expression in wild-type and p53-null HCT116. Quantitative RT-PCR of genes in HCT116 wild-type (B) and HCT116<sup>p53<sup>−/−</sup></sup> (C) cells subjected to siRNA knockdown for 72 hr. (D) Immunoblot analysis of MAF1, CDKN1A, and p53 expression in p53-null HCT116 subjected to MAF1 knockdown. Quantitative RT-PCR of genes in MCF-10A (E) and MDA-MB-231 (F) cells subjected to siRNA knockdown for 72 hr. Expression of CDKN1A and GDF15 was upregulated independent of p53 after MAF1 knockdown. POLR3A expression analysis after POLR3A knockdown using three different siRNAs in MCF-7 cells by quantitative RT-PCR (G) and immunoblot analysis (H). The results were quantified (left panel) using α-tubulin as a loading control on a representative gel (right panel). Relative expression normalized to GAPDH is displayed for all quantitative RT-PCR. All data shown represent mean ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test).

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MAF1 knockdown upregulates CDKN1A at the transcriptional level. (A) For run-on assay, MCF-7 cells were subjected to siRNA knockdown of MAF1 (KD MAF) or simultaneous knockdown of MAF1 and Pol III for 72 hr (KD P/M). Nuclei were prepared, and a run-on reaction was performed. Run-on biotin-labeled newly transcribed RNA (Run-on) was affinity purified and subjected to RT-PCR (left panel). Input indicates total RNA before affinity purification, and a negative control was performed by omitting biotinylated nucleotides and subjected to RT-PCR (right panel). (B) The run-on results were quantified, and the data shown represent mean ± SD, n = 3, *p < 0.05, **p < 0.01 (t-test). (C) Schematic diagram of the CDKN1A promoter, including locations of exon 1 (black rectangle), SINE (MIR3), CpG island (green rectangle), guanine-cytosine (GC) skew, and R-loop foot-printing region (blue rectangle). (D) Each vertical black line indicates the position of a cytosine on the sense DNA strand. (E) Analysis of R-loop foot-printing was performed by native sodium bisulfite treatment followed by PCR amplification and cloning. A total of at least 10 clones were obtained for each knockdown condition (knockdown control, ‘KD Ctrl’; knockdown MAF1, ‘KD MAF1’; and simultaneous knockdown of MAF1 and Pol III, ‘KD MAF1/Pol III’). Each vertical red line represents a converted cytosine to thymine in the sense direction (CDKN1A mRNA) for the knockdown control, knockdown MAF1, and simultaneous knockdown of MAF1 and Pol III. Percentage indicates how many clones at a particular cytosine were converted. Knockdown MAF1 extended the length of R-loop formation in CDKN1A, whereas simultaneous knockdown of MAF1 and Pol III abolished the extension. This indicates that regulation of CDKN1A expression by MAF1 and Pol III occurs at the transcriptional level. Background conversion (approximately 5% of cytosine) may be seen because of DNA breathing during the prolonged incubation at 37°C in our data and data produced by others (Yu et al., 2003). (F) Schematic diagram of ACTB, including locations of exons, CpG island, GC skew, and R-loop foot-printing region. (G) Each vertical black line indicates the position of a cytosine on the sense DNA strand. (H) Each vertical red line represents a converted cytosine to thymine in the sense direction (ACTB mRNA) for knockdown control and knockdown MAF1. Knockdown MAF1 did not affect the length of R-loop in ACTB, which correlates with the expression data from Figure 1A.

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Figure 3. MAF1 knockdown enhanced binding of Pol III and Pol II at the CDKN1A promoter. ChIP was performed in MCF-7 cells subjected to siRNA knockdown for 72 hr. DNA isolated from immunoprecipitated chromatin was subjected to qPCR and calculated as indicated in the ‘Materials and methods’. Significant binding of Pol III was detected at two tRNA genes, tRNAArg (A) and tRNALeu (B), after MAF1 knockdown (KD MAF1). The enhance binding of Pol III was diminished when there was simultaneous knockdown of MAF1 and Pol III (KD M/Pol III). (C) Diagram of the CDKN1A promoter, including locations of exon 1 (long form: L-Ex1, and short form: Ex1), SINEs (AluSx and MIR3), and ChIP–qPCR amplicons (p21-L, p1, p2, and p3). (D) Binding of MAF1 was detected at the CDKN1A promoter, which diminished after MAF1 knockdown. (E) Enhanced binding of Pol III was detected at the CDKN1A promoter after MAF1 knockdown. (F) MAF1 knockdown indicates enhanced binding of Serine-5-phosphorylated Pol II, which was abolished when there was simultaneous knockdown of Pol III and MAF1. (G) Enhanced binding of BRF1 was detected at the CDKN1A promoter after MAF1 knockdown. (H) Top panel: diagram of the ACTB promoter, including locations of each exon (Ex1 to Ex4) and ChIP–qPCR amplicons (p1, p2, and p3). Bottom panel: neither MAF1 nor Pol III was detected at the ACTB promoter. Only binding of Pol II was detected at the ACTB promoter. (I) Top panel: diagram of the TAF5 promoter, including locations of exon 1 and ChIP–qPCR amplicons (p1, p2, and p3). Bottom panel: neither MAF1 nor Pol III was detected at the TAF5 promoter. Only binding of Pol II was detected at the TAF5 promoter. All data shown represent the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test).

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Figure 3—figure supplement 1. Expression and promoter activity of CDKN1A transcripts in the MCF-7 cell line. (A) Representative diagram of NM_001220777 (CDKN1A-L, long form) and NM_001220778 (CDKN1A-S, short form). (B) cDNA samples used in Figure 1C were used to analyze CDKN1A transcript expression. Expression of CDKN1A-S was measured relative to that of CDKN1A-L. (C) Expression of both CDKN1A transcripts was upregulated after MAF1 knockdown, and the upregulation was abolished by simultaneous knockdown of MAF1 and Pol III. A relative expression normalized over GAPDH is displayed. (D) CDKN1A-L and CDKN1A-S promoter regions were constructed and cloned into a pGL3-basic reporter plasmid, as indicated in the ‘Materials and methods’. Luciferase reporter assays were performed in MCF-7 cells, and the results were normalized with those for β-galactosidase. Promoter activity was measured relative to CDKN1A-L. All data shown represent mean ± SD, n ≥ 3, *p < 0.05, **p < 0.01 (t-test).

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Figure 4. In vitro binding and transcription assays demonstrate MAF1-regulated Pol III-mediated activation of Pol II-regulated genes. (A) Diagrams of Pol II promoters (CDKN1A, ACTB, RPPH1, and GDF15) with locations of exon 1, SINEs (red), and constructed DNA template (green arrow) for the in vitro MAF1 binding assay. (B) An in vitro DNA binding assay was performed as described in the ‘Materials and methods’. In brief, DNA template, MAF1 protein (His-tagged), and Anti-6×His tag antibody were added to the binding reaction (Protein + Ab). A negative control was performed by substituting IgG antibody for Anti-6×His tag antibody (Protein + IgG) or with only the Anti-6×His tag antibody for the MAF1 protein (Ab only). DNA isolated from the immunoprecipitated protein–DNA complex was subjected to qPCR. Deletion of a SINE in the CDKN1A template as well as deletion or mutation of the Pol III A-box element in the CDKN1A and GDF15 template depleted MAF1 binding. Binding of MAF1 to RPPH1 or ACTB promoters was not detected. Data shown are the mean ± SD, n ≥ 3, **p < 0.01, ***p < 0.001 (t-test). (C) An in vitro DNA–protein binding assay was performed using a colorimetric assay kit (ab117139). The assayed DNA template ‘p21’ (DNA template with a Pol III A-box element obtained from CDKN1A) was labeled with biotin (a probe). Purified MAF1 protein (His tag) (80R-1955, Fitzgerald) was used for the binding assay. Different competitors (described below) were added to the mixture to demonstrate the specificity of binding of MAF1 at the Pol III promoter element. Competitors: ‘self’ indicates the same DNA template without the biotin label, ‘GDF’ indicates the non-labeled DNA template that contained the Pol III promoter element obtained from the GDF15 promoter, and ‘Mut’ indicates the Pol III A-box element was mutated in the DNA template. A blank control was performed without the addition of protein, and the degree of enrichment was calculated by subtracting the value of the blank control. MAF1 directly bound to the Pol III promoter element, but the mutant form did not. Data shown are the mean ± SD, n = 3, ***p < 0.001 (t-test). (D) In vitro transcription assays were performed on CDKN1A and TAF5 using the HeLaScribe® Nuclear Extract in vitro Transcription System (Promega), as indicated in the ‘Materials and methods’. Inhibition of Pol II transcription was performed by addition of α-amanitin during in vitro transcription of CDKN1A and TAF5. The MAF1 protein was pre-incubated with template DNA before addition of nuclear extract to enable binding of MAF1 to the template DNA. (E) Different antibodies, as indicated, were pre-incubated with nuclear extract before adding template DNA to perform in vitro transcription to deplete the target protein of interest. For the control, no antibody was added prior to in vitro transcription. In vitro transcription performed on Pol III-transcribed RPPH1 and Pol II-transcribed TAF5 served as controls. In vitro transcription performed on CDKN1A and GDF15 revealed that removal of MAF1 promoted transcription, whereas A-box-deleted GDF15, denoted as ‘GDF15 (Del)’, did not. The Figure 4. continued on next page
degree of enrichment of all performed in vitro transcription was calculated relative to the ratio of signals obtained from the input RNA after subtraction of the negative control (no biotin labeling). All data shown represent the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test).

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Figure 4. Continued

Figure 5. MAF1 knockdown induces Pol II initiation, active histone marks (H3K4me3, H3K9Ace, and H3K27Ace), and binding of CFP1, p300, PCAF, TBP, and POLR2E at the CDKN1A promoter. (A) Diagram of the CDKN1A promoter, including locations of exon 1 (Ex1), SINEs (AluSx and MIR3), and ChIP qPCR amplicons (p21-L, p1, p2, and p3). (B) Knockdown coupled with ChIP assays with antibodies for H3K27me3, H3K4me3, H3K27Ace, and H3K9Ace were performed in MCF-7 cells subjected to siRNA knockdown for 72 hr. DNA isolated from immunoprecipitated chromatin was subjected to qPCR and calculated as described in the ‘Materials and methods’. Knockdown MAF1 (KD MAF1) enhanced active histone marks H3K4me3, H3K27Ace, and H3K9Ace, whereas simultaneous knockdown of Pol III and MAF1 (KD M/Pol III) abolished the enhanced histone marks. ChIP with anti-CFP1 (IP: CFP1) (C), anti-p300 (IP: p300) (D), anti-PCAF (IP: PCAF), (E) anti-TBP (IP: TBP) (F), and anti-POLR2E (IP: POLR2E) (G) antibodies were performed as described in (B). Knockdown MAF1 (KD MAF1) enhanced binding of CFP1, p300, PCAF, TBP, and POLR2E, whereas simultaneous knockdown of Pol III and MAF1 (KD M/Pol III) abolished the enhanced binding. All data shown are the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test).

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Figure 5—figure supplement 1. Enhanced gene expression by MAF1 knockdown is abolished by simultaneous knockdown of MAF1 with TBP or CFP1. (A) Quantitative RT-PCR of genes in MCF-7 cells subjected to siRNA knockdown of MAF1, CFP1, or simultaneous knockdown of both for 72 hr. CDKN1A expression was upregulated after MAF1 knockdown, and the upregulation was abolished by simultaneous knockdown of MAF1 and CFP1. (B) Quantitative RT-PCR of genes in MCF-7 cells subjected to siRNA knockdown of MAF1, TBP, or simultaneously knockdown of knockdown of MAF1 and TBP for 72 hr. Expression of CDKN1A and TBP was upregulated after MAF1 knockdown, and the upregulation was abolished by simultaneous knockdown of MAF1 and TBP. Relative expression normalized to 18S is displayed. All data shown represent the mean ± SD, n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test). DOI: 10.7554/eLife.06283.010
Figure 6. Pol III is required for chromatin looping at the CDKN1A promoter after MAF1 knockdown. (A) Schematic diagram of CDKN1A with the orientation of 3C primers (arrows: 5r, 4r, 3r, 2r, and 2f) and location of exon 1 (long form: L-Ex1; short form: Ex1). (B) MCF-7 cells were subjected to siRNA knockdown of MAF1 (KD MAF1) for 72 hr. 3C assay was performed as indicated in the ‘Materials and methods’, and DNA was subjected to PCR. Chromatin looping was detected after MAF1 knockdown from 2r to 2f (top panel) and are shown by a representative gel (bottom panel). (C) The induced chromatin looping after MAF1 knockdown was diminished when either Pol III (KD M/Pol III) or BRF1 (KD M/BRF1) underwent simultaneous knockdown with MAF1 (top panel) and are shown by a representative gel (bottom panel). All data shown represent the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test).

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Figure 7. Pol III is required for chromatin looping at the GDF15 promoter after MAF1 knockdown. (A) Schematic diagram of GDF15 with ChIP-qPCR amplicons (AluSx, 3C, MIR, p1, p2, and p3), the orientation of 3C primers (arrows: 3C-3r, 3C-2r, and 3C-1f), and locations of exons (Ex1 and Ex2). (B) ChIP with anti-MAF1 antibody (IP: MAF1) was performed in MCF-7 cells subjected to siRNA knockdown of MAF1 (KD MAF1) or simultaneous knockdown of MAF1 and Pol III (KD M/Pol III) for 72 hr. Binding of MAF1 was detected at the GDF15 promoter, which diminished after MAF1 knockdown. (C) A 3C assay was performed as indicated in the ‘Materials and methods’, and DNA was subjected to PCR. Chromatin looping was detected after MAF1 knockdown from 3C-3r to 3C-1f (top panel) and is shown by a representative gel (bottom panel). (D) The induced chromatin looping after MAF1 knockdown (KD MAF1) was diminished when MAF1 underwent simultaneous knockdown with either Pol III (KD M/Pol III) or BRF1 (KD M/BRF1) (top panel) and is shown by a representative gel (bottom panel). (E) ChIP with anti-Pol III antibody (IP: Pol III) or anti-Pol II antibody (IP: Pol II) was performed in MCF-7 cells subjected to siRNA knockdown. Enhanced binding of Pol III was detected at the GDF15 promoter after MAF1 knockdown, which was abolished when there was simultaneous knockdown of Pol III and MAF1 (KD M/Pol III). (F) MAF1 knockdown indicates enhanced binding of serine 5-phosphorylated Pol II, which was abolished when there was simultaneous knockdown of Pol III and MAF1. All data shown represent the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test). DOI: 10.7554/eLife.06283.012
Figure 8. Demonstration of MAF1- and Pol III-mediated transcription regulation using a reporter gene assay. (A) Promoter regions of Pol II genes were constructed and cloned into pGL3-basic reporter plasmids, as indicated in the ‘Materials and methods’. Luciferase reporter assays were performed in MCF-7 cells subjected to siRNA knockdown of MAF1 or simultaneous knockdown of Pol III and MAF1. Results are normalized with β-galactosidase and presented relative to knockdown control cells transfected with pGL3-basic. (B) The consensus sequence of the A-box (−447 to −437) in the GDF15 promoter (−889 to +110) was either deleted or mutated. Reporter assays were performed in MCF-7 cells subjected to siRNA knockdown of MAF1 (KD MAF1). All data shown represent the mean ± s.e.m., n ≥ 3, *p < 0.05, **p < 0.01, ***p < 0.001 (t-test). DOI: 10.7554/eLife.06283.013