

# Regulation of proto-oncogene transcription, cell proliferation, and tumorigenesis in mice by PSF protein and a VL30 noncoding RNA

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We describe the role of PSF protein and VL30–1 RNA, a mouse retroelement noncoding RNA, in the reversible regulation of proto-oncogene transcription, cell proliferation, and tumorigenesis in mice. The experiments involved increasing expression of PSF or VL30–1 RNA in NIH/3T3 fibroblast cells and B16F10 melanoma cells by transfecting the respective coding genes under control of a strong promoter or decreasing expression by transfecting a shRNA construct that causes degradation of PSF mRNA or VL30–1 RNA. The results are as follows: (i) PSF binds to the proto-oncogene *Rab23*, repressing transcription, and VL30–1 RNA binds and releases PSF from *Rab23*, activating transcription; (ii) increasing expression of PSF or decreasing expression of VL30–1 RNA suppresses cell proliferation in culture and tumorigenesis in mice; and (iii) decreasing expression of PSF or increasing expression of VL30–1 RNA promotes cell proliferation in culture and tumorigenesis in mice. These results indicate that PSF is a major tumor-suppressor protein and VL30–1 RNA is a major tumor-promoter RNA in mice. Although VL30–1 RNA can integrate into the cell genome, tumor promotion by VL30–1 RNA involves a *trans* effect rather than a *cis* effect on gene transcription. Expression of VL30–1 RNA is 5- to 8-fold higher in mouse tumor lines than in mouse fibroblast or myoblast lines, whereas expression of PSF mRNA does not decrease in the tumor lines, suggesting that tumorigenesis is driven by an increase of VL30–1 RNA rather than a decrease of PSF. A similar regulatory mechanism functions in human cells, except that human PSF-binding RNAs replace VL30–1 RNA, which is not encoded in the human genome. We propose that PSF protein and PSF-binding RNAs have a central role in the reversible regulation of mammalian cell proliferation and tumorigenesis and that increasing PSF expression or decreasing PSF-binding RNA expression in tumor cells is a potential therapeutic strategy for cancer.

cancer therapy | *Rab23* | regulatory RNA | tumor suppression

Preceding studies have described a reversible mechanism controlling gene transcription that involves PSF protein (1) and VL30–1 RNA, a member of the VL30 family of mouse retroelement noncoding RNAs (2, 3). PSF contains a DNA-binding domain (DBD) that binds and represses transcription of genes that have a PSF-binding site (4–6) and 2 RNA-binding domains (RBDs) that bind VL30–1 RNA, forming a PSF-RNA complex that dissociates from a gene and activates transcription (5–7). Increasing expression of PSF in a human tumor cell suppressed tumorigenesis (6), and ectopic expression of VL30–1 RNA in a human tumor cell promoted metastasis (3). VL30 RNAs are expressed in virtually all tissues of adult mice (8) and are associated with Ras-mediated transformation of mouse fibroblast cells (9). Here, we extend our studies of the function of PSF and VL30–1 RNA to the regulation of proto-oncogene transcription, cell proliferation, and tumorigenesis in mice. The results indicate that PSF is a major tumor-suppressor protein and VL30–1 RNA is a major tumor-promoter RNA in mice.

## Results

**Expression of PSF and VL30–1 RNA in NIH/3T3 and B16F10 Cell Lines.** NIH/3T3 and B16F10 cells were transfected with a transgene encoding PSF (NIH/3T3-PSF ↑ and B16F10-PSF ↑ lines) or VL30–1 RNA (NIH/3T3-VL30 ↑ line) or with a plasmid encoding a shRNA that causes degradation of PSF mRNA (NIH/3T3-PSF ↓ line) or VL30–1 RNA (NIH/3T3-VL30 ↓ and B16F10-VL30 ↓ lines). The control lines were transfected with an empty plasmid pcDNA3.1(+) (NIH/3T3-pcDNA3.1 and B16F10-pcDNA3.1 cell lines) or a plasmid encoding sh-Luciferase (NIH/3T3-shLuc and B16F10-shLuc lines). The NIH/3T3 and B16F10 cell lines were assayed for expression of PSF mRNA and VL30–1 RNA (Fig. 1 and Table 1) and PSF protein (Fig. 1).

**Binding of PSF to the Regulatory DNAs of Mouse Genes.** Chromatin fragments from NIH/3T3 cells were coimmunoprecipitated with anti-PSF antibody, and the DNAs in the fragments were tested for hybridization to a mouse gene-promoter chip (NimbleGen; Roche) that contains 385,000 regulatory DNAs from the mouse genome. A total of 57 DNA fragments were identified by chip assay, of which 12 mapped to characterized mouse coding genes [supporting information (SI) Tables S1 and S2]. The 12 DNAs bound to PSF in NIH/3T3 and B16F10 cells, as shown by a ChIP assay (Fig. 2); the *P450scc* gene had been identified as a PSF-binding gene in earlier studies (4, 6). We chose the *Rab23* gene, a member the RAS gene family (10, 11), to analyze the role of PSF and VL30–1 RNA in the regulation of proto-oncogene transcription in mice.

**Binding of PSF to *Rab23* Regulatory DNA and the Effect on Transcription of the *Rab23* Gene in NIH/3T3 and B16F10 Cell Lines.** (i) The binding assays were done by ChIP in NIH/3T3 and B16F10 lines expressing high or low levels of PSF (Table 1). Binding of *Rab23* DNA to PSF is higher in NIH/3T3-PSF ↑ and B16F10-PSF ↑ cells and lower in NIH/3T3-PSF ↓ cells than in NIH/3T3 or B16F10 control cells (Fig. 3A). (ii) Transcription of *Rab23* was analyzed in the same cell lines by RT-PCR. Transcription is lower in NIH/3T3-PSF ↑ and B16F10-PSF ↑ cells and higher in NIH/3T3-PSF ↓ cells than in NIH/3T3 or B16F10 control cells (Fig. 3B). The results indicate that PSF binds and represses transcription of the *Rab23* gene in all the NIH/3T3 and B16F10 cell lines.

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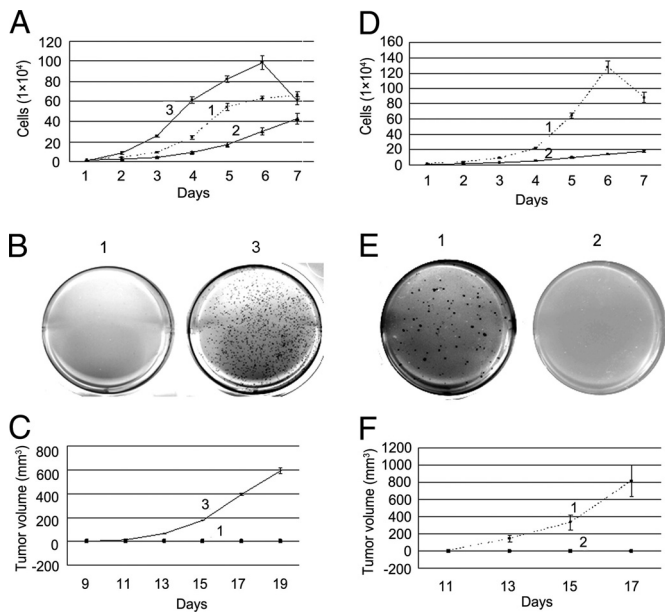
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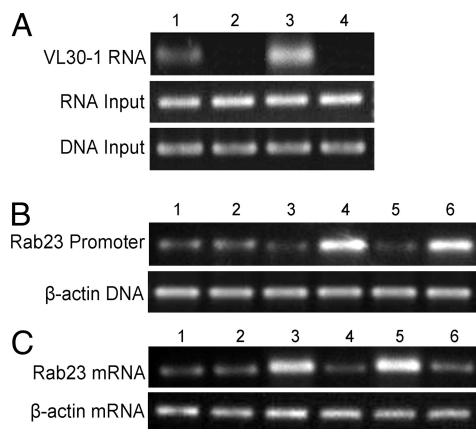




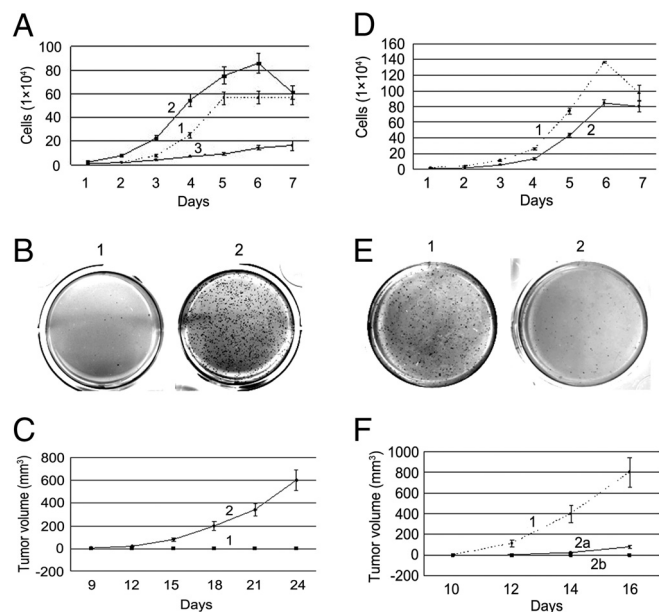
**Fig. 4.** Effect of PSF on cell proliferation and tumorigenesis in NIH/3T3 and B16F10 cell lines. (A–C) 1, NIH/3T3 control cells (NIH/3T3-pcDNA3.1 and NIH/3T3-shLuc); 2, NIH/3T3-PSF  $\uparrow$  cells; 3, NIH/3T3-PSF  $\downarrow$  cells. (D–F) 1, B16F10-pcDNA3.1 cells; 2, B16F10-PSF  $\uparrow$  cells. (A and D) Rates of cell proliferation. The error bars for each point show the variation in 3 experiments. (B and E) Colony formation in soft agar. (C and F) Tumor formation following injection of the cells into two sites in the necks of 3 nude mice (C) or 3 C57BL/6J mice (F). The error bars for each point show the variation at 6 sites.

proliferation and tumorigenesis in the NIH/3T3 and B16F10 cell lines.

**Expression of PSF mRNA and VL30–1 RNA in Differentiated Cells and Tumor Cells.** The purpose of this experiment was to determine whether tumorigenesis in mouse tumor lines is driven by de-



**Fig. 5.** Binding of VL30–1 RNA to PSF and the effect on binding of PSF to *Rab23* regulatory DNA and transcription of the *Rab23* gene. (A) Binding of VL30–1 RNA to PSF protein by RIP assay. 1 and 2, NIH/3T3-pcDNA3.1 cells; 3 and 4, NIH/3T3-VL30 RNA  $\uparrow$  cells. PSF antibody was added to cell extracts in 1 and 3 but not in 2 and 4. DNA and RNA inputs of  $\beta$ -actin were used to normalize the amount of RIP products. (B and C) 1, NIH/3T3-pcDNA3.1 cells; 2, NIH/3T3-shLuc cells; 3, NIH/3T3-VL30  $\uparrow$  cells; 4, NIH/3T3-VL30  $\downarrow$  cells; 5, B16F10-shLuc cells; 6, B16F10-VL30  $\downarrow$  cells. (B) ChIP assay for binding of PSF to *Rab23* regulatory DNA. Upper shows the amount of *Rab23* promoter DNA, and Lower shows the amount of  $\beta$ -actin DNA used to normalize the total DNA in the samples. (C) Semi-quantitative RT-PCR assays for transcription of the *Rab23* gene. Upper shows the amount of *Rab23* mRNA, and Lower shows the amount of  $\beta$ -actin mRNA used to normalize the total RNA in the samples.

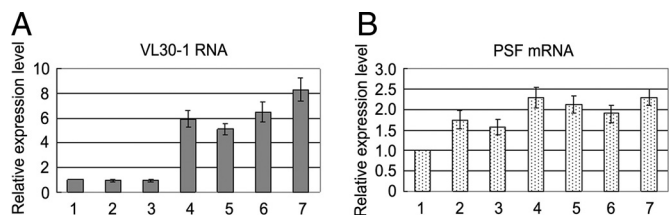


**Fig. 6.** Effect of VL30–1 RNA on cell proliferation and tumorigenesis in NIH/3T3 and B16 F10 cell lines. (A–C) 1, NIH/3T3 control cells (NIH/3T3-pcDNA3.1 and NIH/3T3-shLuc); 2, NIH/3T3-VL30  $\uparrow$  cells; 3, NIH/3T3-VL30  $\downarrow$  cells. (D–F) 1, B16F10-shLuc cells; 2, B16F10-VL30  $\downarrow$  cells. (A and D) Rates of cell proliferation. The error bars for each point show the variation in 3 experiments. (B and E) Colony formation in soft agar. (C and F) Tumor formation following injection of the cells into two sites in the necks of 3 nude mice (C) or 3 C57BL/6J mice (F). The error bars for each point show the variation at 6 sites. Curve 2a in F shows the tumors size at 3 sites, and curve 2b shows the other 3 sites that had no visible tumor.

creased expression of PSF, increased expression of VL30–1 RNA, or both. The expression levels of PSF mRNA and VL30–1 RNA were assayed by real-time RT-PCR in differentiated fibroblast and myoblast lines and 4 tumor lines. The level of VL30–1 RNA is 5 to 8 times higher in the tumor lines than in the fibroblast and myoblast lines, whereas the level of PSF mRNA does not decrease in the tumor lines (Fig. 7A and B). The results suggest that increased expression of VL30–1 RNA, rather than decreased expression of PSF protein, drives tumorigenesis in mouse tumor cells.

### Discussion

Previous studies showed that PSF binds and represses transcription of multiple genes in a human tumor cell line, mostly proto-oncogenes that drive the cell cycle, and VL30–1 RNA binds and releases PSF from a proto-oncogene and activates transcription (5–7). Here, we analyzed the regulatory functions



**Fig. 7.** Expression of VL30–1 RNA and PSF mRNA in mouse fibroblast cells, myoblast cells, and tumor cells. Cell lines: 1, primary fibroblast; 2, NIH/3T3 fibroblast; 3, C2C12 myoblast; 4, TC-1 lung tumor; 5, Y-1 adrenal tumor; 6, EMT6 mammary tumor; 7, B16F10 melanoma tumor. RNA expression levels were assayed by real-time RT-PCR and normalized to the level in primary fibroblasts.

**Table 2. Regulatory functions of PSF and VL30-1 RNA in NIH/3T3 and B16F10 cell lines**

Cell line	<i>Rab23</i>		Cell proliferation	Tumorigenesis
	DNA binding to PSF	transcription		
NIH/3T3-pcDNA3.1	++	++	++	0
NIH/3T3-shLuc	++	++	++	0
NIH/3T3-PSF ↑	+++	+	+	Not tested
NIH/3T3-PSF ↓	+	+++	+++	+++
NIH/3T3-VL30 ↑	+	+++	+++	+++
NIH/3T3-VL30 ↓	+++	+	+	Not tested
B16F10-pcDNA3.1	+	+++	+++	+++
B16F10-shLuc	+	+++	+++	+++
B16F10-PSF ↑	+++	+	+	0
B16F10-VL30 ↓	+++	+	++	0 to +

The scale 0/+/++/+++ indicates relative levels of activity.

of PSF and VL30-1 RNA in mouse NIH/3T3 fibroblast and B16F10 melanoma cell lines. The experiments involved transfecting a coding gene for PSF or VL30-1 RNA to increase expression (PSF ↑ and VL30 ↑ cell lines) or a shRNA construct that causes degradation of PSF mRNA or VL30-1 RNA to decrease expression (PSF ↓ and VL30 ↓ cell lines). The cell lines were compared for binding of PSF to the proto-oncogene *Rab23*, transcription of *Rab23*, rate of cell proliferation in culture, and tumorigenesis by colony formation in agar and tumor formation in mice (Table 2).

The results indicate that PSF is a tumor-suppressor protein and VL30-1 RNA is a tumor-promoter RNA in mice. All cell lines express PSF and VL30-1 RNA; a high molar ratio of PSF/VL30-1 RNA suppresses and a low molar ratio promotes proto-oncogene transcription, cell proliferation, and tumorigenesis. The tumor-promoter function of VL30-1 RNA involves binding to PSF (Fig. 5A) and forming a PSF-RNA complex that dissociates from a repressed proto-oncogene (4, 5, 9). PSF and VL30-1 RNA appear to have a primary role in tumorigenesis, because a decrease of PSF expression or increase of VL30-1 RNA expression transforms NIH/3T3 cells, whereas an increase of PSF expression or decrease of VL30-1 RNA expression suppresses tumorigenesis of B16F10 cells. Although VL30-1 RNA can integrate into the cell genome (3, 12), the tumor-promoter function of VL30-1 RNA involves a *trans* effect rather than a *cis* effect on gene transcription. A similar regulatory mechanism functions in human cells with human PSF-binding RNAs replacing VL30-1 RNA (13), which is not encoded in the human genome.

To determine whether tumorigenesis in mice is associated with decreased expression of PSF, increased expression of VL30-1 RNA, or both, we compared expression of PSF mRNA and VL30-1 RNA in 4 tumor lines with fibroblast and myoblast lines (Fig. 7). Expression of VL30-1 RNA increased 5- to 8-fold in the tumor lines compared with the fibroblast and myoblast lines, whereas expression of PSF mRNA did not decrease in the tumor lines, suggesting that tumorigenesis is driven by an increase in VL30-1 RNA expression rather than a decrease in PSF expression. Tumorigenesis also can result from mutations affecting the synthesis or function of PSF, as indicated by the human cervical tumor line HeLa, which has a mutation in the PSF gene that deleted the coding region for the DBD of PSF and was the probable cause of the HeLa tumor (5).

Tumorigenesis can progress via multiple pathways controlled by proto-oncogenes (14). The results reported here and in other studies (5-7, 12) suggest that tumorigenic pathways are initiated and driven by increased expression of PSF-binding RNAs, which

reverse repression of proto-oncogenes by PSF. The regulation of tumorigenesis by PSF and PSF-binding RNAs identifies 2 previously undescribed markers for tumor cells: a reduced level of proto-oncogene promoters bound to PSF and an elevated level of PSF-binding RNAs. The former can be assayed by ChIP, and the latter can be assayed by RT-PCR. Both markers could be the basis of therapeutic procedures for cancer that increase the amount of PSF or deplete the amount of PSF-binding RNAs in tumor cells.

## Materials and Methods

**Cell Lines.** The mouse fibroblast line NIH/3T3, myoblast line C2C12, melanoma line B16F10, and mammary carcinoma line EMT6 were cultured in DMEM; the mouse lung tumor line TC-1 was cultured in RPMI 1640; and the mouse adrenal line Y-1 was cultured in F12 medium. Mouse primary fibroblast cells were isolated from the dorsal skin of 15-day C57BL/6J embryos. All cells were grown as attached monolayers in the indicated medium supplemented with 10% (vol/vol) FBS, penicillin, and streptomycin in a CO<sub>2</sub> incubator at 37 °C.

**Plasmids Encoding a shRNA.** A shRNA target site in PSF mRNA or VL30-1 RNA was identified, and complementary sense and antisense oligonucleotides encoding a shRNA with a 19-mer stem derived from the target site were synthesized as follows: (i) for PSF mRNA, the plasmid was called pGenesil-shPSF: 5'-GATCCGTTAAGGCGCATGGAGAACTTCAAGAGAGTTCTCCATGCGCCTTAAATTTTTTA-3' and 5'-AGCTTAAAAAATTAAGGCGCATGGAGAACTTCTTGAAGTCTCCATGCGCCTTAAACG-3' and (ii) for VL30-1 RNA, the plasmid was called pGenesil-shVL30: 5'-GATCCGcagaaaaactgtcttctactTCAAGAGAAGTGAACGAGTTTTCTGTTTTTTA-3' and 5'-AGCTTAAAAAacagaaaaactgtcttctactTCTTCTTGAAGTGAAGGAAAGCAGTTTTCTGCG-3'.

The sense oligonucleotide contained the *Bam*H1 3' residues (5'-gatcc-3'), followed by the 19-nucleotide target sequence, a 9-nucleotide loop (5'-ttcaagaga-3'), a 19-nucleotide reverse sequence complementary to the target sequence, a TTTTTT tract to terminate transcription, and a HindIII 5' residue. The antisense oligonucleotide was complementary to the sense oligonucleotide except for the upstream HindIII 3' residue (5'-agctt-3') and the downstream *Bam*H1 5' residues -g. To facilitate transcription by mouse U6 promoter, an additional guanine was added to the target sequence that began with a pyrimidine. The sense and antisense oligonucleotides were annealed and inserted into the *Bam*H1/HindIII sites of the plasmid pGenesil-1 (Genesil Company). The control plasmid pGenesil-shLuc targeted the luciferase mRNA sequence GTAGCGGGTGATTATAC.

**Stably Transfected Cell Lines.** (i) The NIH/3T3-VL30 ↑ cell line was constructed by transfecting NIH/3T3 cells with the plasmid pcDNA-VL30 encoding the 319-bp VL30-1 RNA fragment containing the PSF-binding sequences (5). (ii) The NIH/3T3-PSF ↑ or B16F10-PSF ↑ cell line was constructed by transfecting NIH/3T3 or B16F10 cells with plasmid pcDNA-PSF (5). (iii) The NIH/3T3-VL30 ↓ or B16F10-VL30 ↓ cell line was constructed by transfecting NIH/3T3 or B16F10 cells with plasmid pGenesil-shVL30. (iv) The NIH/3T3-PSF ↓ cell line was constructed by transfecting NIH/3T3 cells with the plasmid pGenesil-shPSF. (v) NIH/3T3-pcDNA3.1 or B16F10-pcDNA3.1, the control cell line for VL30 ↑ or PSF ↑, was constructed by transfecting NIH/3T3 or B16F10 cells with the empty plasmid pcDNA-3.1(+). (vi) NIH/3T3-shLuc or B16F10-shLuc, the control cell line for VL30 ↓ or PSF ↓, was constructed by transfecting NIH/3T3 or B16F10 cells with the plasmid pGenesil-shLuc. The transfection reagent Lipofectamine 2000 (Invitrogen) was used for all transfections. Two days after transfection, transfected cells were selected with 800 μg/mL G418 for 10 days and maintained with 500 μg/mL G418. Cell clones derived from a single cell were isolated, and the expression levels of target genes were analyzed by semi-quantitative RT-PCR.

**Semiquantitative RT-PCR.** Cells were cultured to approximately 80% confluence in 6-well plates, washed with ice-cold PBS, and lysed in 1 mL of TRIzol (Invitrogen), and RNA was isolated according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA in a 20-μL reaction volume containing 4 μL of 5× reaction buffer, 1 μL of dNTP mix (10 mM each), 1 μL of random hexamers (10 μM), 2 μL of 0.1 M DTT, 0.5 μL of RNaseOUT Recombinant Ribonuclease Inhibitor (40 U/μL; Invitrogen), 0.5 μL of M-MLV reverse transcriptase (200 U/μL; Invitrogen), and H<sub>2</sub>O. β-actin mRNA was used to normalize the amounts of RNA in the samples. The forward and reverse PCR primers were as follows: β-actin: 5'-CTCCTCCCTGGAGAAGACTA-3' and 5'-CCTTCTGCATCCTGTGCGCAA-3', PSF: 5'-CAAGATCTGATGAGACGCCAGGAA-3' and 5'-CCATCTCAGTTGGCGAATCATCA-3', VL30: 5'-GAGAGCAGCCAGCGGTCA-CAGT-3' and 5'-GGCGTCTTGCCGGCTACTCG-3', and *Rab23*: 5'-

