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The Mitotic Checkpoint Gene, *SIL* is Regulated by *E2F1*

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Abstract

The *SIL* gene expression is increased in multiple cancers and correlates with the expression of mitotic spindle checkpoint genes and with increased metastatic potential. *SIL* regulates mitotic entry, organization of the mitotic spindle and cell survival. The *E2F* transcription factors regulate cell cycle progression by controlling the expression of genes mediating the G1/S transition. More recently *E2F* has been shown to regulate mitotic spindle checkpoint genes as well. As *SIL* expression correlates with mitotic checkpoint genes we hypothesized that *SIL* is regulated by *E2F*. We mined raw data of published experiments and performed new experiments by modification of *E2F* expression in cell lines, reporter assays and chromatin immunoprecipitation. Ectopic expression or endogenous activation of *E2F* induced the expression of *SIL*, while knockdown of *E2F* by shRNA, downregulated *SIL* expression. *E2F* activated *SIL* promoter by reporter assay and bound to *SIL* promoter in-vivo. Taken together these data demonstrate that *SIL* is regulated by *E2F*. As *SIL* is essential for mitotic entry, *E2F* may regulate G2/M transition through the induction of *SIL*. Furthermore, as silencing of *SIL* cause apoptosis in cancer cells, these finding may have therapeutic relevance in tumors with constitutive activation of *E2F*.

Keywords

SIL; *E2F*; Mitosis

The *SIL* gene, (*SCL* Interrupting Locus), located on chromosome 1, encodes a cytosolic protein that have a critical role in cell growth and proliferation^{1,2,3}. *SIL* is tightly regulated during the cell cycle. Its mRNA expression is higher in rapidly proliferating cells⁴ and tissues, and it decreases rapidly during terminal differentiation⁵. The *SIL* protein is phosphorylated upon transition into mitosis and is degraded upon exit from mitosis^{4,6}. *SIL* is necessary for mitotic entry and proper organization of the mitotic spindle^{3,7}.

SIL critical role in cell growth and proliferation lead us to examine its role in tumorigenesis. Increased expression of *SIL* is observed in multiple types of cancer and is associated with metastatic spread⁸. *SIL* is essential for cancer cells survival as knockdown of *SIL* resulted in marked delay in mitotic entry coupled with apoptosis, in-vitro and in-vivo³. *SIL* expression in tumors is correlates significantly with the expression pattern of the mitotic spindle checkpoint genes and with, high histopathological mitotic index. We were interested in elucidating the transcriptional basis for the co-regulation of *SIL* with the expression of other mitotic spindle checkpoint genes.

Numerous studies have shown the E2F family of proteins to be aberrantly activated in multiple cancers. The E2F transcription factors have been demonstrated to control gene expression necessary for cell cycle progression, particularly for the G1/S transition. Recently a role for E2F in regulating mitosis has emerged. Specifically, *E2F1* was shown to induce the transcription of genes coding mitotic checkpoint regulator proteins, such as *MAD2*. As *SIL* expression is highly correlated with the expression of *MAD2*,³⁸, we hypothesized that *SIL* may be a target of *E2F*.

Materials and Methods

SIL regulation by *E2F* induction

WI38 human embryonic lung fibroblasts stably expressing E2F1 fused to the ligand binding domain of estrogen receptor were induced for *E2F1* activation with 4-hydroxytamoxifen (OHT) (300 nM) as published⁹.

PCR—In each 50 μ L PCR reaction; 50–100 ng DNA sample, 15 pmol of the 5' and 3' oligonucleotide primers, and 1.5 U Super-Therm DNA polymerase were used. Conditions: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 60 seconds at 95°C, 60 seconds at 58°C, and 90 seconds at 72°C using a MJ Research PTC-100 thermal cycler. After the last cycle, an additional extension step of 10 minutes at 72°C was performed. Appropriate positive and negative controls were included in all experiments

The oligonucleotides used for amplification:

Hu-*SIL*—F- 5'-GACTACTTCAGGCACAGATTC-3' R- 5' – ATGCATGCCAACACACTG-3'

Cell culture: *Drosophilla melangogaster* SL2 cells (obtained from A.Black) were cultured at room temperature in Schneider's *Drosophilla* medium (Life Technologies) supplemented with 10% fetal calf serum (Rehatuin Intergen). 293T cell line was grown in DMEM supplemented with 10% heatinactivated fetal bovine serum, penicillin–streptomycin (100U and 100 mcg/ml, respectively) and 2mM glutamine (Life Technologies). WI38 human embryonic lung fibroblasts as published⁹.

Infection of the shRNA for *E2F1*—293T cell line was transfected with shRNA for *E2F1* (5' GACGTGTCAGGACCTTCGT)⁹. in pSuper reterovirus plasmid together with the gag pole of the Maloni virus, The viral supernatant was harvested 48 hours later, filtered through 0.45 micron mixed with u 8 mcg/ml polybrene and added to a T-Cell leukemic cell- line, plated a day before on a 6 well plate at a concentration of 2×10^5 . The cells were centrifuged 45 minutes, 1800 RPM at room temperature. The procedure was repeated 24 hours later. The infected cells were selected with Puromycin 10 mcg/ml for 10 days before the extraction of RNA.

Reporter Assay

Plasmids—a 2.3 kb fragment encompassing the *SIL* transcription initiation site was subcloned into the *Xba* I site of the chloramphenicol acyltransferase (CAT) reporter plasmid, pCAT Basic (Promega) to generate pCATSIL. PMINCATSIL was prepared by exonuclease III digestion.

Transfection of SL2 Cells—SL2 cells were transfected by calcium- phosphate coprecipitation with five mcg of DNA from each plasmid (with the exception of E2F1- O.2 mcg). A truncated adenovirus¹⁰ was cotransfected as control for transfection efficiency. The total amount of transfected DNA was adjusted to 20 mcg with pBLUESCRIPT II. Cells were harvested after 2 days, pelleted at 1000 × g and resuspended in Reporter Lysis Buffer (Promega). The lysates were analyzed according to the protocol. All CAT data was normalized to luciferase activity to control for transfection efficiency. The fold activation was calculated based on pCAT Basic expression.

Chromatin immunoprecipitation

was performed as described⁹ The PCR was performed using these oligos:

SIL promoter

F- 5'- CCGCAGTTCTCCAAGAAGAC- 3' R-5'GAACTGAGGCGGCAAAC- 3'

Exon 12 of *SIL*

F- 5'GAGACACTGCAAAGTAAGACAG3' R-5'GTGGAGGGTCTTATAGGATACTC3'

Results

Bioinformatic analysis of published data suggests that *SIL* is an E2F target gene

The finding that *MAD2*, a well characterized mitotic check point gene that is co-expressed with *SIL* in cancer cells, is a target of *E2F1*¹¹ lead us to hypothesize that *SIL* might be an *E2F1* target gene. We interrogated the raw microarray data of several studies done on both human and mice cell lines, designed to find *E2F1* direct target genes¹²¹³¹⁴. We found that either ectopic overexpression of *E2F1* or silencing the *Rb* gene in several cell lines, result in a rise of both mouse and human *SIL*, together with other known *E2F1* target genes. Table 1 summarizes the results from Semizarov paper focusing on E2F induced mitotic genes in human cell lines together with *SIL* results retrieved by us from the supplementary data (table 1)¹³. Thus, mining of publicly available data reveals that *SIL* is induced in a similar pattern of response with known *E2F1* induced genes necessary to insure a proper G2/M transition.

SIL is induced by E2F in vitro

To examine if *SIL* is induced by activation of E2F we have utilized our previously published human WI-38, embryonic lung fibroblasts engineered to conditionally express E2F1 after treatment 4-hydroxytamoxifen (OHT)⁹. Activation of E2F by OHT was associated with increased mRNA levels of *SIL* (Figure 1A). As an alternative to ectopic expression of E2F we wished to examine the effect of activation of the endogenous E2F1 on *SIL*. E1A is a viral oncoprotein that, when expressed, binds to and inhibits *Rb* and consequently activates the endogenous *E2Fs*. As we have previously shown, *SIL* is not expressed in serum starved growth arrested fibroblasts (Figure 1B, lane 1). Transfection of E1A resulted in marked expression of *SIL* mRNA, despite the serum starvation (Figure 1B, lane 2). Similarly the expression of *SIL* markedly increased in proliferating fibroblasts after activation of the endogenous E2F1 by E1A (Figure 1B, lane 4 in comparison to lane 3). To further substantiate the relationship between *E2F1* and *SIL* we infected T- cell leukemic cell line with either shRNA for *E2F1* or with a

nonspecific shRNA. As can be seen in Figure 1C knockdown of E2F was associated with reduction in SIL expression. Taken together, these results suggest that expression of *SIL* is regulated by *E2F1*.

E2F binding sites in *SIL* promoter are functional in vitro and in vivo

We next wanted to examine if E2F induce *SIL* expression directly through activation and binding to its promoter. *SIL* promoter¹⁵ has two potential *E2F1* binding sites in a region conserved between human and mouse at -162 and -26 from the transcription initiation site. The upstream site is a 6/8 match to the murine sequence while the downstream site is not well conserved. However, this binding site is a 7/8 match with E2F binding site in N-MYC.¹⁶

To determine if these binding sites are functionally active on *SIL* promoter, we first performed a reporter assay using either *SIL* whole promoter, pCATSIL, or a deleted construct possessing only -80 nucleotide before the start transcription initiation site, pMINCATSIL (Figure 2A). Only one *E2F* site is positioned within the *SIL* minimal promoter vector. To determine whether *E2F* could modulate *SIL* promoter activity we transfected either pCATSIL or pMINCATSIL into D. melanogaster SL2 cells either alone or with an *E2F1* coexpression vector. The *SIL* vectors alone had very low activity in SL2 cells in the absence of *E2F*. After correcting for the fold activation by the promoterless construct, pCATBasic, the coexpression of *E2F1* with pCATSIL resulted in a 6-fold increased activation while coexpression of *E2F1* with pMINCATSIL lead to 12-fold increased in CAT activity (Figure 2B).

To examine if E2F transcription factors are bound to the *SIL* promoter in-vivo we performed a chromatin immunoprecipitation assay (ChIp). Chromatin from Jurkat cells (human T lymphocyte cell line) crosslinked and immunoprecipitated with antibodies against E2F1, E2F2, E2F3, E2F4, and HA was used as a template for PCR amplification of *SIL*. Negative (no DNA) and positive (input DNA representing 0.2% of total input chromatin) control amplifications are shown (Figure 2C). *SIL* primers were constructed from the promoter containing an *E2F1* binding site, and also from exon 12, not containing an *E2F1* binding site (negative control). All E2F factors, in particular E2F1 and E2F4 were found to be bound to the *SIL* promoter. The results show that *E2F* binding sites in *SIL* promoter are functional in vitro and in-vivo, especially for *E2F1* and *E2F4*.

Discussion

The E2F transcription factors are aberrantly activated in cancer. Recent studies have revealed an expanded role of the E2F in cell cycle regulation beyond its function in regulating G1 to S transition and suggest it plays a role also in regulating G2/M transition^{17,18}. Specifically it has been shown to regulate the MAD2 spindle checkpoint gene. MAD2 is an essential component of the spindle checkpoint that blocks premature sister chromatid separation during metaphase. E2F1 activation of MAD2 expression has been shown to be critically important for the cancer phenotype. We have recently shown that *SIL* is overexpressed in a large variety of cancers and that its expression is essential for mitotic entry and cell survival³⁸. *SIL* expression is highly correlated with the expression of other mitotic checkpoint genes, such as *MAD2*. Here we confirmed the hypothesis that E2F regulates the expression of *SIL*.

Analysis of raw databases of published micro-array experiments revealed that *SIL* could be a target gene of E2F1 together with other genes upregulated during the G2/M phase of the cell cycle^{12,13,14,8}. We then experimentally demonstrated that *SIL* is regulated by E2F1 and that *E2F1* and E2F4 bind to *SIL* promoter.

Although *SIL* general role in growth and development has been observed in mouse and zebra fish mutants^{2, 19}, its function has been unknown. We have recently shown that *SIL* is an

important mitotic regulator³⁶. It is phosphorylated during mitosis and degraded upon transition to G1. Silencing of *SIL* demonstrated that it is needed for mitotic entry and activation of the CDK1/Cyclin B complex. A recent study by Pfaff et al,⁷ demonstrated that *SIL* localizes to the mitotic spindle poles during metaphases and is essential for proper organization of the mitotic spindle. Together these studies establish *SIL* as a major mitotic regulator.

SIL is the second mitotic regulator, after *MAD2*, which has been unequivocally shown to be regulated by *E2F1*. Co-regulation of S phase genes and G2/M genes by the same transcription factor ensures proper coordination of DNA replication with preparation of mitosis. Accordingly, deregulation of this activity in cancer leads to enhanced DNA replication and defects in the mitotic checkpoint. *SIL* regulation by *E2F1* could have implications in many types of cancers, since most cancers display an aberrant expression of the *E2F* pathway. Since *SIL* is required for survival of cancer cells³, drugs targeting *E2F1* may mediate their anticancer activity through silencing of *SIL*. More specifically, in T-Cell-ALL where *SIL* promoter causes aberrant expression of the oncogenic *SCL*, blockage of *E2F1* may reverse the leukemia by counteracting the activity of the *SIL* promoter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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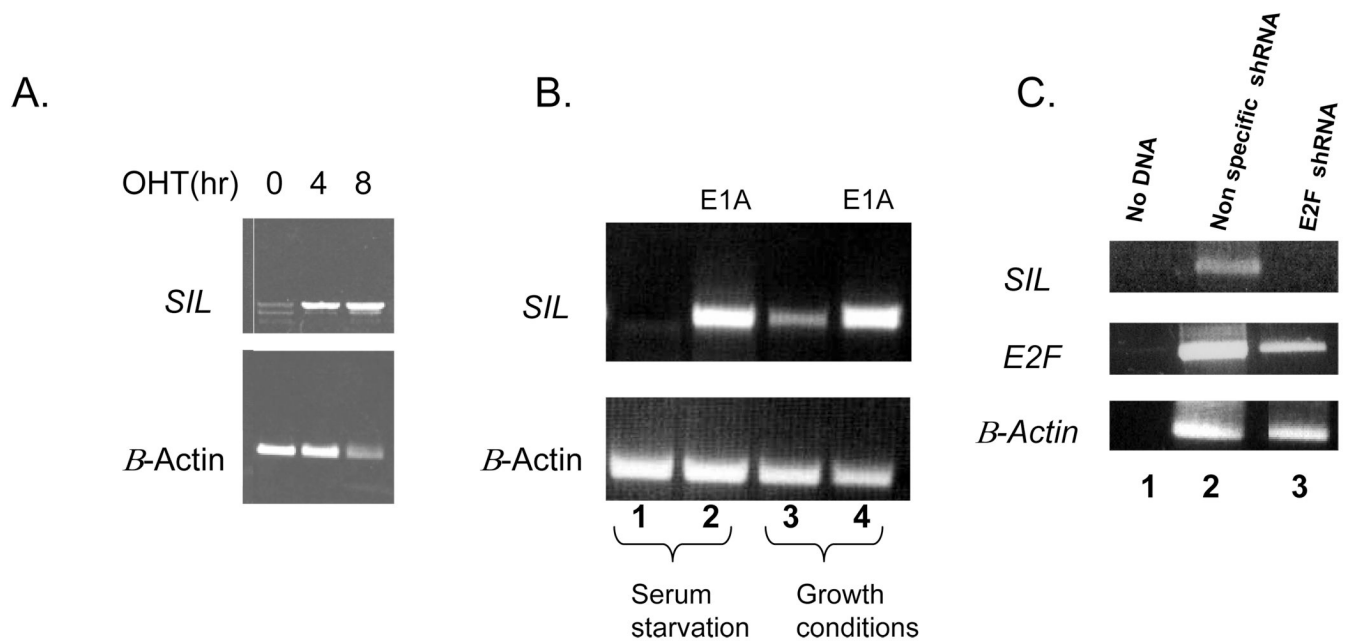


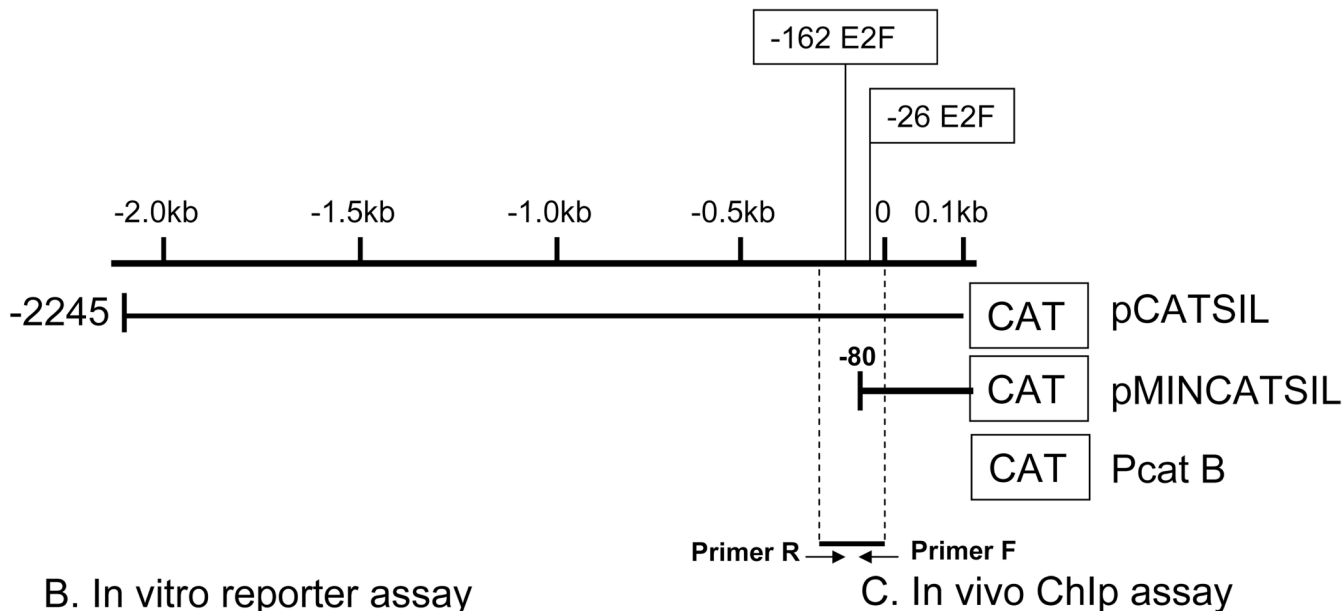
Figure 1. SIL is regulated by E2F1 in vitro

A. SIL levels rise post induction with ectopic E2F1; PCR results WI38, embryonic lung fibroblasts, expressing ER-E2F1 were kept in serum starvation in 0.1% FCS containing medium for 24 hours after which E2f1 E2F1 was activated for the indicated times-0, 4hr, 8hr.

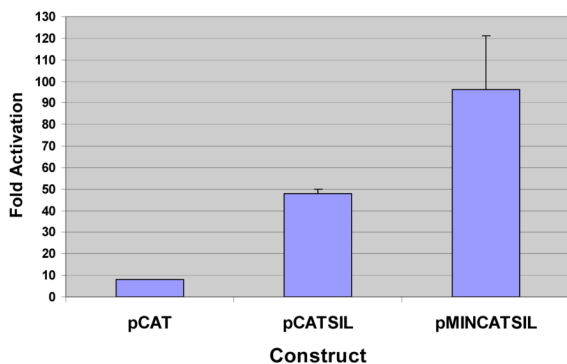
B. SIL levels rise post induction of endogenous E2F by E1A; In the first two lanes, WI38 cells were grown under serum starvation conditions; in 0.1% FCS containing medium, while in lanes 3 and 4, cells were grown under growth conditions, (MEM plus 7.5% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate). Lane 2 and 4 show SIL levels in WI-38 post induction with E1A.

C. shRNA for E2F1 downregulates SIL 1. No DNA 2. Control- unspecific shRNA 3. shRNA for E2F1.

A: SIL Promoter Constructs



B. In vitro reporter assay



C. In vivo ChIP assay

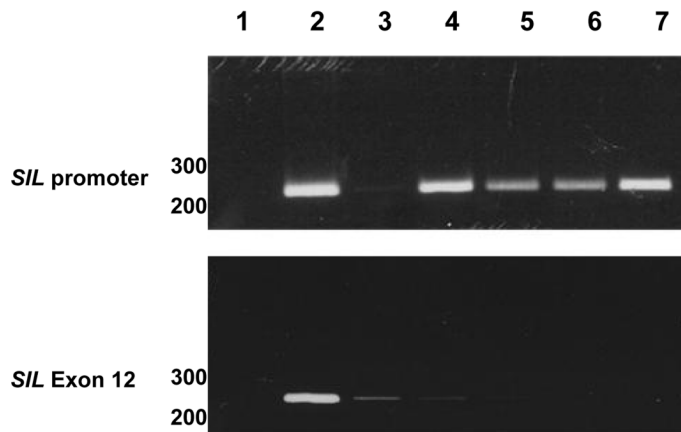


Figure 2. SIL Promoter is regulated by E2F1 In vitro and In vivo

A. SIL promoter constructs and designed primers

B. Reporter Assay- E2F1 overexpression activates SIL promoter. 5 mcg of either pCATSIL or pMINCATSIL with the minimal promoter were transfected in *Drosophila melanogaster* SL2 cells alone or with 0.2 mcg of an E2F1 coexpression vector. CAT activity was measured, background activity from mock transfection controls was subtracted and the adjusted activity was normalized to luciferase activity. Each bar represents the mean of at least three transfections.

C. Chromatin immunoprecipitation - E2F transcription factors bind SIL promoter in vivo; PCR for SIL promoter and exon 12 as control 1. no DNA 2. Input before IP 3. anti-HA 4. anti- E2F1 5. anti- E2F2 6. anti- E2F3 7. anti- E2F4.

Table 1**Selected Genes induced upon *E2F* Activation**

Selected genes induced upon Rb knockdown: The table is based on the analysis of microarray data from the Semizarov paper 2004, concerning genes induced upon Rb knockdown and focusing specifically on SIL and known mitotic genes. The table shows the increase in gene expression levels represented by the fold change (FC), and the standard deviation, (SD) post suppression of Rb. The last column indicates the presence of an E2F binding site in the promoter as analyzed by Semizarov [9].

Name	FC	SD	E2F site
SIL	2.93	0.36	+
ZWINT	3.64	0.24	+
TTK	3.6	0.44	+
MAD2	4.16	0.95	+
Cyclin B	3.66	0.22	-
Cyclin E	5.27	0.81	+