Down-Regulation of T-STAR, a Growth Inhibitory Protein, after SV40-mediated Immortalization

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Abstract
Normal human cells can undergo a limited number of divisions, whereas transformed cells may have an extended life span and can give rise to immortal cells. To isolate genes involved in the immortalization process, gene expression in SV40-transformed preimmortal human fibroblasts was compared with expression in SV40-transformed immortalized fibroblasts using an mRNA differential display. We found that the growth-inhibitory protein testis-signal transduction and activation of RNA (T-STAR) is strongly down-regulated in immortalized cells. Overexpression of T-STAR in the SV40-transformed immortalized cells resulted in a strong reduction of colony formation, whereas deletion of the RNA-binding domain of T-STAR abrogated this effect. Down-regulation of testis-signal transduction and activation of RNA (T-STAR) expression is found only in immortal cells isolated after a proliferative crisis accompanied with massive cell death. The strict correlation of down-regulation of T-STAR expression only in those immortal cells that arose after a clear proliferative crisis suggests that the loss of T-STAR might be necessary to bypass crisis.

Introduction
Normal human cells can perform a limited number of cell divisions in vitro, the so-called “Hayflick limit” (1, 2). At the end of their replicative life span, cells go into an irreversible arrest in the G1 phase of the cell cycle (3). This nonproliferative state is called “senescence” or M1. Telomeres are thought to play a critical role in the onset of senescence. The telomeres are the structures that cap the chromosomes, thereby stabilizing the chromosome ends and preventing end-to-end fusions. During the life span of the cells, telomeres become increasingly shorter after each cell doubling. When the telomeres reach a certain minimal threshold length, a signal is triggered leading to senescence (4, 5).

Transformation of human fibroblasts with viral oncogenes, such as SV40 Large T or human papillomavirus 16 E6 and E7, inactivates cellular proteins involved in the regulation of senescence, such as p53 and pRb, resulting in escape from senescence (6, 7). These transformed cells show an extension of life span which can amount to up to 40 population doublings (8, 9). During the extended life span, the telomeres continue to shorten beyond the threshold length, resulting in an increased reactivity of the chromosomal ends and hence decreased chromosomal stability. This results in a strong increase in the amount of end-to-end fusions in cells during the extended life span (10, 11). At the end of the extended life span, massive cell death occurs, probably caused by the high chromosomal instability, a state referred to as “crisis” or M2. Rare clonal populations can arise from cells undergoing crisis that have acquired an immortal phenotype (reviewed in Ref. 12).

To gain more knowledge about the process of immortalization and the genes involved, we performed mRNA differential display on SV40-transformed preimmortal and immortal cells to identify genes that play a role in this process. mRNA differential display is a PCR-based method that can be used to detect differences in expression between different cell populations (13, 14). Here we report that T-STAR2/Sam68-like mammalian protein 2 (SLM-2)/Sam68-like protein α (Salpø), a recently cloned gene (15–17), is strongly down-regulated in SV40-transformed immortal fibroblasts compared with preimmortal cells. T-STAR was identified in a yeast-two-hybrid screen as a protein interacting with the protein RNA-binding motif (RBM) in spermatogenesis (18). T-STAR is highly expressed in testis, muscle, and brain (15–17). The protein contains a STAR domain, an RNA-binding domain present in a growing family of proteins involved in developmental processes (18). T-STAR is highly homologous to Sam68, a STAR domain-containing protein found in human and mouse. Sam68 is a protein with an as-yet-unknown function that binds to a variety of signal-transducing proteins and may act as an adaptor in signal-transduction pathways (15, 17). Inactivation of Sam68 in mouse fibroblasts is associated with neoplastic transformation and tumorigenesis (19). We found that whereas T-STAR expression is down-regulated in SV40-transformed immortal fibroblasts, Sam68 expression is not affected. When T-STAR was reintroduced into SV40-transformed immortalized fibroblasts, a clear reduction in the number of colonies compared with controls was seen. Our data suggest that down-regulation of T-STAR

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2 The abbreviations used are: T-STAR, testis-signal transduction and activation of RNA; Sam68, 68-kDa Src substrate associated during mitosis; NLS, nuclear localization signal; aa, amino acid(s); wt, wild type; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
might be important for cells to escape from crisis and confirm the role of T-STAR as a growth-inhibitory protein.

Results

**mRNA Differential Display on Preimmortal and Immortal Cells.** In an attempt to identify genes that are involved in the immortalization of human fibroblasts, we performed mRNA differential display, an assay to compare gene expression in different cell populations (13, 14), on precrisis, nonimmortal and postcrisis, immortal SV40-transformed fibroblasts. Normal skin fibroblasts were transformed with an origin-defective SV40 early region (VH10/SV) and after proliferative crisis, immortal clones were isolated. These immortal clones have an unlimited proliferative capacity (9). In the mRNA differential display assay, two independently isolated RNA preparations of preimmortal VH10/SV cells were compared with two independently isolated RNA fractions of immortal VH10/SV cells to reduce detection of differences caused by experimental procedures. Furthermore, it was verified, using FACS analysis, that the preimmortal and immortal cells showed the same cell-cycle distribution at the time of RNA isolation to avoid differences caused by cell cycle-dependent gene expression (data not shown). Using primer combination T12/MC and a random decamer, a band was detected in the preimmortal fractions that was absent in the immortalized fractions (Fig. 1). The 250-nt fragment was isolated from the filter, cloned into a vector, and sequenced. The cloned fragment was 98% identical to part of the mRNA of a recently cloned gene called 7-STAR/SLM-2/Salpα (GenBank accession no. AF069681; Ref. 15–17).

**T-STAR Down-Regulated in Immortal Cells Compared with Preimmortal Cells.** On the basis of the T-STAR mRNA sequence, PCR primers were designed to amplify a 474-bp fragment of the T-STAR mRNA by PCR to use as a probe for Northern blot analysis. Hybridization of this probe to a Northern blot of RNA from preimmortal and immortal VH10/SV cells showed two bands of about 2.4 kb, which were strongly reduced in the immortal cells (Fig. 2A). These bands were running just above and under the 18S rRNA band, as was seen on the ethidium bromide staining of the gel, suggesting that the two bands detected by the T-STAR probe represent one mRNA divided by the rRNA running at the same height. When poly(A) RNA isolated from preimmortal and immortal VH10/SV cell was used for Northern blotting, only one band was detected using the same probe (Fig. 2B). Therefore, we conclude that the two bands on the total RNA Northern blot represent one mRNA of the same length that is split into two bands by the abundant 18S rRNA. Again, a clear down-regulation of the T-STAR gene was detected in the immortal cells.

Next, we examined expression of T-STAR in nontransformed VH10 fibroblasts and the SV40 transformed cells (Fig. 2C). In nontransformed cells, a clear level of T-STAR RNA is present, and this level does not change significantly upon SV40 transformation. Thus, SV40-transformation does not affect T-STAR RNA levels, but after immortalization the levels of T-STAR RNA clearly decrease.

**Down-Regulation of T-STAR Occurs Only in Immortalized Cells Isolated after a Clear Proliferative Crisis.** To investigate whether down-regulation of T-STAR is a common event in SV40-transformed immortalized cells, we isolated RNA from seven different panels of preimmortal SV40-transformed fibroblasts and their immortalized counterparts. After Northern blotting, we examined T-STAR expression. Five of seven panels showed a clear reduction in the amount of T-STAR in the immortalized cells. In LF2859/SV and XP20 MA/SV postcrisis cells, no reduction in expression was visible (Fig. 3A). The SV40-transformed immortal cell-lines that were isolated in our laboratory can be divided into two groups as follows: (a) cell lines that were isolated after a clear proliferative crisis in which massive cell death took place and nearly all cells died before clones appeared which had gained immortality; and (b) immortal cells that arose without crisis and massive cell death taking place. T-STAR is only down-regulated in cells from the first group, whereas cells from the second group do not show down-regulation of T-STAR expression (Table 1). This correlation has been confirmed in other immortal cell lines recently isolated in our laboratory (data not shown). Thus, loss of T-STAR seems to be specific for immortal cells that have passed through a clear crisis.

**Sam68 Expression Levels Are Not Affected by Immortalization.** Two groups isolated the T-STAR gene on the basis of its high homology to the Sam68 gene (15, 17). This high similarity might indicate similar roles of these proteins in the cell and thus involvement of Sam68 in immortalization. Therefore, expression levels of Sam68 were determined in the panels discussed above. As shown in Fig. 3B, the expression levels of Sam68 do not change significantly after immortalization. This indicates that, despite their homology, down-regulation after immortalization is specific for the T-STAR gene.

**T-STAR Is Down-Regulated in Immortal Cell Lines of Complementation Groups A, B, and D.** In the past, four different complementation groups, A, B, C and D, were identified by cell fusion of different immortal cell lines and sub-
sequent screening for loss of immortality of the fused cells (20). This suggests that at least four different genetic pathways exist for cells to become immortal. Nearly all SV40-transformed immortal cells were classified into complementation group A. We examined T-STAR expression levels in cell lines representative for the four different complementation groups (Fig. 3C). In EJ, a bladder carcinoma cell line from complementation group A, T-STAR expression was comparable with the low expression levels in VH10/SV postcrisis cells. In HeLa (group B) and A1698 (group D) no T-STAR expression was detectable, and only CMV-Mj-HEL-1 (group C) cells showed clearly high levels of T-STAR. Thus, a low level of T-STAR expression is found in immortal cells from three different complementation groups, but is not a general feature of immortalized human cells.

Cellular Localization of T-STAR and Its Derivatives. To test whether reintroduction of T-STAR leads to the loss of the immortal phenotype, three different constructs were cloned into the pcDNA3.1 expression vector: (a) pcT-STAR, containing the wild-type T-STAR; (b) a deletion mutant lacking the NH₂-terminal 150 aa of T-STAR containing most of the STAR domain (ΔSTAR), and (c), ΔNLS, a mutant lacking the last 20 aa of the COOH terminus containing a putative nuclear localization signal based on the homology to Sam68 (Ref. 21 and Fig. 4A). These cell lines were taken as representatives of the complementation groups (20). RNA samples of VH10/SV pre- and postcrisis cells were loaded as controls. A probe recognizing GAPDH was used as a control for equal loading.

Fig. 3. Down-regulation of T-STAR in SV40-transformed immortal cells and tumor cell lines. A, T-STAR expression was examined in preimmortal and immortal cells of various SV40 transformed fibroblasts (9). B, a probe recognizing the Sam68 mRNA was hybridized to a filter containing the same samples as in A to examine Sam68 expression. C, cell lines from the four different complementation groups were examined for T-STAR expression: HeLa, a cervical carcinoma cell line (complementation group B); A1698, a bladder carcinoma cell line (complementation group D); EJ, also a bladder carcinoma cell line (complementation group A); and CMV-Mj-HEL-1, a cytomegalovirus-transformed fibroblast cell line (complementation group C). These cell lines were taken as representatives of the complementation groups (20). RNA samples of VH10/SV pre- and postcrisis cells were loaded as controls. A probe recognizing GAPDH was used as a control for equal loading.

Table 1 Correlation between the occurrence of a proliferative crisis before immortalization and down-regulation of T-STAR expression

<table>
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<tr>
<th>Cell line</th>
<th>Crisis</th>
<th>Down-regulation of T-STAR</th>
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<tr>
<td>VH10/SV</td>
<td>+</td>
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<td>XP2CS/SV</td>
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<td>XP8/SV</td>
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Exogenous T-STAR Expression Reduces Colony Formation in VH10/SV Immortal Cells. Next, the constructs described above were used to transfect VH10/SV immortal cells. After transfection, cells were cultured on selective medium to obtain clones containing the plasmid. Also, cells were transfected with the empty expression vector. After clones had formed, they were stained with Giemsa stain to determine the number of colonies (Fig. 5A). On the dishes transfected with the wt T-STAR plasmid a strong reduction in the number of colonies is observed compared with cells transfected with the empty vector. This is in agreement with results presented by Lee and Burr (17), who found that exogenous T-STAR expression has antiproliferative capacity in chicken embryo fibroblasts. Also, the dishes containing the cells transfected with the ΔNLS mutant clearly show less colonies compared with the empty vector. This reduction is in the same order of magnitude as the reduction obtained with the wt protein. Apparently, although the mutant protein is not localized in the nucleus, it still shows growth-inhibitory effects. On the dishes with cells transfected with ΔSTAR, little reduction is visible, suggesting that the mutant protein lacking the STAR domain does not inhibit growth. However, because expression of the ΔSTAR construct seems lower than expression of the other constructs (see above), it cannot be excluded that higher levels of the ΔSTAR protein might also exert an effect.

After selection, 14 colonies of the dishes transfected with the plasmid containing wt T-STAR were picked and cultured further. Of these, 12 survived and were checked for the ectopic T-STAR protein with Western blotting. Only 4 of 12 clones showed expression of T-STAR (Fig. 5B).

To investigate whether the four clones expressing T-STAR might be selected by virtue of mutations in the transfected gene, mRNAs expressed from the construct were sequenced. In all four clones, the expressed mRNA was wild type. Thus, although there is a selection against T-STAR expression in these immortal cells, a subset of the cells can still proliferate in the presence of T-STAR.

Discussion

In an attempt to identify genes which are differentially regulated in preimmortal and immortal human cells, we isolated part of a cDNA that turned out to be identical to T-STAR, a recently cloned gene with high homology to Sam68 (15, 17). T-STAR is down-regulated in SV40-transformed immortalized fibroblasts (VH10/SV) compared with the corresponding nonimmortal SV40-transformed cells. In normal cells, T-STAR expression is clearly present and comparable with expression levels in SV40-transformed precrisis cells. No changes are observed in T-STAR expression as cells progress toward senescence (data not shown). The downregulation in the immortalized cells was not restricted to VH10/SV, but was also observed in other fibroblast cell lines transformed by SV40 Large T, with the notable exception of two cell lines. Interestingly, the common feature of the SV40-transformed cell lines showing down-regulation of T-STAR is that they displayed a distinct proliferative crisis before immortal clones arose. In contrast, both immortal cell lines that do not show abrogation of T-STAR expression, arose without a clear crisis. These data suggest that loss of T-STAR expression might be a prerequisite to escape from crisis. If immortalization occurs in earlier stages after SV40 transformation, these immortal cells will gradually overgrow the population before crisis and apparently do not need the downregulation of T-STAR. This could indicate that T-STAR plays a role during crisis, and that immortalization cannot occur unless T-STAR expression is down-regulated. Recently, the T-STAR rat homologue, rSLM-2, was described to regulate the selection of alternative splice sites during pre-mRNA processing (23). Therefore, down-regulation of T-STAR might result in the formation of an alternative splicing pattern. These alternatively spliced mRNAs might encode proteins that play a role in the escape from crisis.

In most immortal cell lines, telomerase activity can be detected, leading to stabilization of the telomeric length required for indefinite growth (24). In addition, exogenous expression of human telomerase reverse transcriptase, the catalytic subunit of telomerase, is able to immortalize normal
human fibroblasts (25). Notably, T-STAR and telomerase are both described to be involved in spermatogenesis, and expression levels of T-STAR increase in the later stages of spermatogenesis, whereas telomerase activity decreases in the later stages (16, 26). As telomerase apparently seems to play such a pivotal role in immortalization, we tested whether there was a correlation between T-STAR expression and telomerase activity. In our experiments, however, we did not find a relation between presence of telomerase activity and T-STAR expression (data not shown).

Next, we asked whether down-regulation of T-STAR would be a prerequisite for the maintenance of the immortalized phenotype in the cells that went through a clear crisis. Re-introduction of T-STAR into the VH/SV postcrisis cells led to a profound reduction in the number of colonies formed, indicating that T-STAR has a negative effect on cellular growth. This is in accordance with findings by Lee and Burr (17) in chicken embryo fibroblasts. When cells were transfected with a mutant lacking the NLS (ΔNLS), the mutant protein is located mainly in the cytoplasm but does not lead to abrogation of this effect. Because T-STAR is involved in pre-mRNA processing and regulation of the selection of specific splice sites (23), overexpression of T-STAR might cause a different splicing pattern. If T-STAR is involved in the formation of specific mRNAs, overexpression of T-STAR might lead to an increase in the formation of these mRNAs and a reduction of the formation of other mRNAs. This might reduce or enhance the levels of mRNAs encoding proteins involved in cellular growth. When T-STAR lacking the NLS, ΔNLS, is not properly located in the nucleus but resides in the cytoplasm, an analogous mechanism can be envisioned. Because of a lack of T-STAR in the nucleus, its target mRNAs are not spliced as they would be normally. The lack of these properly spliced mRNAs might prevent the production of proteins essential to proliferation, resulting in inhibited growth.

Alternatively, if the negative effect on growth of T-STAR acts via its STAR domain and thus via RNA binding, T-STAR, when located in the cytoplasm, might still bind to its target and hence could prevent proper translation of mRNA(s). A similar effect might occur when other nuclear RNA-binding proteins are prevented from entering the nucleus, but seems not to be a general feature of nuclear proteins retained in the cytoplasm per se (27, 28).

Previously, it was reported that overexpression of T-STAR results in lower protein levels of Sam68 in chicken embryo fibroblasts (17), suggesting that Sam68 mRNA might be a substrate for T-STAR. In human cells, however, we did not observe lower Sam68 protein levels in the clones stably expressing T-STAR protein compared with cells not expressing T-STAR (data not shown). Low expression or lack of expression of T-STAR was found not to be restricted to SV40-transformed and immortalized cell lines but was observed also in cell lines belonging to other complementation groups (20), with the exception of the CMV-Mj-HEL-1 cells, which have considerable levels of T-STAR. It should be noted that because we do not have precrisis CMV-Mj-HEL-1 cells, we cannot determine whether down-regulation of T-STAR upon immortalization in these cells took place or not. In short, although the preimmortalized counterparts of these cells were not available for comparison, these results suggest that T-STAR down-regulation is a more general event occurring also during immortalization mediated by other transforming agents than SV40 Large T. By inference, the tested representatives for the complementation groups could have originated from clones that arose after a crisis-like event.
Despite the growth-suppressive effect of exogenous T-STAR expression, we were able to isolate four clones which stably express nonmutated forms of T-STAR. At present, we cannot exclude that in these clones T-STAR is inactive because of mutations in other genes. In combination with the fact that loss of T-STAR is not observed in SV40-transformed immortal lines that have not gone through crisis, this result indicates that down-regulation of T-STAR is not a prerequisite for immortalization per se. Rather, loss of T-STAR could be a factor that enables cells to survive during crisis, thus providing cells time to accumulate additional mutations needed to become immortal. Identifying the targets of T-STAR during pre-mRNA processing, or proteins binding to T-STAR, could provide insight into immortalization in general and crisis in particular.

**Materials and Methods**

**Cell Culture and Treatment.** The following cells were used in this study: VH10 primary human foreskin fibroblasts; SV40-transformed human fibroblasts (VH10/SV, XP2CS/SV, XP3MA/SV, XPB/SV, LF2859/SV, XP20 MA/SV, and LF317/SV); EJ (bladder carcinoma cell line); A1698 (bladder carcinoma cell line); CMV-Mj-HEL-1 (cytomegalovirus-transformed HeLa cell line); SV; EJ (bladder carcinoma cell line); XP3MA/SV, XPB/SV, LF2859/SV, XP20 MA/SV, and LF317/SV; VH10 primary human foreskin fibroblasts; XPB/SV, LF2859/SV, XP20 MA/SV, and LF317/SV; EJ (bladder carcinoma cell line); CMV-Mj-HEL-1 (cytomegalovirus-transformed HeLa cell line); and HeLa cells (the latter four kindly provided by Dr. Olivia Pereira-Smith, Baylor College of Medicine, Houston, TX). Cells were grown on DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 8% FCS (Life Technologies, Inc., Paisley, United Kingdom) or supplemented with Fetal X (Life Technologies, Inc., Paisley, United Kingdom). Colonies were stained using Giemsa stain (Merck, Darmstadt, Germany).

**Reverse Transcription, Differential Display and Fragment Isolation.** Differential display was essentially performed as described (13, 14) with a few modifications (30). In brief, cytoplasmic RNA was isolated from growing cells and treated with DNase. cDNA was made of 2.5 μg of RNA using SuperScript II reverse transcriptase (Life Technologies, Inc.) in the presence of a 5'-DIG-labeled T<sub>12</sub>MC primer, where M is a 3-fold degenerate for C, A, or G (dNTP). Low stringency amplification reactions were performed using the same downstream primer and arbitrary random decamer primers under the following PCR protocol: 30° C for 95°C, 60° C for 38°C, 60° C for 40 cycles using ULTMA DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Primers used to identify T-STAR were downstream primer DIG-T<sub>12</sub>MC and upstream primer 5'-CAAGCGAGGT-3'. Part of the reaction was resolved on a denaturing 6% polyacrylamide gel. After blotting onto Hybond N membrane (Amersham Life Science, Buckinghamshire, United Kingdom), DIG-labeled products were visualized as described (Roche Diagnostics, Mannheim, Germany). Bands of interest were cut from the filter and eluted overnight at 65°C in 100 μl of 1.2 N NaCl, and 1% SDS. The eluent was purified over a Sephadex G50 column, and 5 μl of the eluent was used for a reamplification step of the DNA fragments with the T<sub>12</sub>MC primer (not DIG-labeled) and the upstream random decamer primer. PCR fragments were cloned into the pCR2.1 cloning vector (Invitrogen Corporation, Leek, the Netherlands). Colonies were screened for inserts with PCR using the appropriate primers. Using Spot-blots, colonies were screened for the correct insert (30).

**Sequencing.** Double-stranded DNA from the cloned fragments was sequenced with M13 forward and reverse primers and a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden). The fragment was identified using the BLAST program (National Center for Biotechnology Information; Bethesda, MD).

**RNA Isolation and Northern Blot Analysis.** RNA isolation and Northern blotting was performed as described (31). Twenty μg of total RNA were loaded for Northern blotting. Poly(A)<sup>+</sup> RNA was isolated using an mRNA Isolation Kit (Boehringer Mannheim, Mannheim, Germany). Poly(A) RNA (2.5 μg) were used for Northern blotting. Probes used for analysis of expression were labeled by random priming with <sup>32</sup>PdATP. A 474-bp probe for T-STAR was amplified from a cDNA made from VH10/SV precrisis cells using 5'-GGTTGAGTTTTCAAAATAG-3' as upstream primer and 5'-TTTCTACCTCCAGAGCAAG-3' as reverse primer. A rat GAPDH probe was used as a control for equal loading. As a probe for Sam68, a cloned 530-bp fragment, amplified by PCR using 5'-TGGTACACAGTAAGGGGAG-3' as an upstream primer and 5'-TAAGGTTGGAGAGAAAGG-3' as a downstream primer, was used.

**Plasmids.** The T-STAR open reading frame was amplified by PCR using pIX DNA polymerase (Stratagene Cloning Systems, La Jolla, CA) from pACT::T-STAR (kindly provided by Julia P. Venables, University of Leicester, Leicester, UK) containing most of the mRNA for T-STAR (16). As upstream primer STA 5'-CGGAAATTCGGCCGGATCCGAGAAGAGTCCTGCCCC-3' was used containing an EcoRI restriction site upstream of the start codon and a Kozak sequence just upstream of the start codon. The reverse primer STB 5'-CGCGGATCCGGGATCTGCCCATAATGGGTTCTCT-3' contains a BamHI restriction site downstream of the last coding codon, the BamHI site was used to clone the open reading frame in-frame with the myc-tag into plasmid pcDNA3.1(−)/Myc-His A (Invitrogen). The ΔSTAR mutant was constructed by PCR amplification using as upstream primer 5'-CGGAATTCCGGCCGGATCCGAGAAGAGTCCTGCCCC-3' and STB as a downstream primer and cloned into pcDNA3.1 using the EcoRI and BamHI sites. The ΔNLS mutant was constructed in the same way using as upstream primer STA and as a downstream primer 5'-CGGGATCCGGGATCTGCCCATAATGGGTTCTCT-3'.

**Western Blotting, Immunofluorescence.** Lysates were made using radioimmunoprecipitation assay buffer, and 15 μg of protein/sample were loaded on gel. For immunofluorescence, cells were fixed in 80% acetone. The 9E10 anti-myc (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA) antibody was used in a 1:1000 dilution to detect the myc-tagged proteins. For immunofluorescence the nuclei of the cells were stained using 4',6-diamidino-2-phenylindole.
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