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Cancer cells and normal cells differ in their requirements for *Thoc1*

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Abstract

The evolutionarily conserved TREX complex physically couples transcription, mRNP biogenesis, RNA processing, and RNA export for a subset of genes. *HPRI* encodes an essential component of the *S. cerevisiae* TREX complex. *HPRI* loss compromises transcriptional elongation, nuclear RNA export, and genome stability. Yet, *HPRI* is not required for yeast viability. *Thoc1* is the recently discovered human functional orthologue of *HPRI*. *Thoc1* is expressed at higher levels in breast cancer than in normal epithelia, and expression levels correlate with tumor size and metastatic potential. Depletion of *Thoc1* protein (pThoc1) in human cancer cell lines compromises cell proliferation. It is currently unclear whether *Thoc1* is essential for all mammalian cells, or whether cancer cells may differ from normal cells in their dependence on *Thoc1*. To address this issue, we have compared the requirement for *Thoc1* in the proliferation and survival of isogenic normal and oncogene transformed cells. Neoplastic cells rapidly lose viability via apoptotic cell death upon depletion of pThoc1. Induction of apoptotic cell death is coincident with increased DNA damage as indicated by the appearance of phosphorylated histone H2AX. In contrast, the viability of normal cells is largely unaffected by pThoc1 loss. Normal cells lacking *Thoc1* cannot be transformed by forced expression of E1A and Ha-ras, suggesting that *Thoc1* may be important for neoplastic transformation. In sum, our data are consistent with the hypothesis that cancer cells require higher levels of pThoc1 for survival than normal cells. If true, pThoc1 may provide a novel molecular target for cancer therapy.

Keywords

transcription; RNA processing; mRNP; apoptosis; cancer

Introduction

The accumulation of genetic and epigenetic alterations in cancer cells endows them with unwanted proliferative and metastatic potential. However these alterations can also handicap cancer cells with unique vulnerabilities such that it is possible to identify particular genes whose function is more critical for the viability of cancer cells than for normal cells(1). Mutations in such genes are formally synthetic lethal with the genetic and epigenetic alterations present in cancer cells. Proteins encoded by such synthetic lethal genes identify molecular targets for therapy since antagonizing their function will be more toxic to cancer cells than to normal cells, thus yielding superior therapeutic index. The inability to perform synthetic lethal screens in cultured human cells has limited the number of genes identified whose inactivation is uniquely

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toxic to human cancer cells, although this is likely to change with the advent of high throughput gene silencing technologies.

The human *Thoc1* gene, also known as hHpr1 or p84, encodes a protein that was originally identified as a nuclear matrix (NM) component that binds the retinoblastoma tumor suppressor protein(2). Alterations in the NM and resulting changes in nuclear structure have long been recognized to correlate with tumor progression, prompting their use as biomarkers for the diagnosis of cancer(3). Indeed, overexpression of *Thoc1* has recently been documented in human breast cancer with pThoc1 levels correlating with tumor size and metastases(4). We and others have recently identified pThoc1 as a functional orthologue of the *S. cerevisiae* *HPR1* gene(4–6). The *HPR1*-encoded protein (Hpr1p) is a component of the TREX (Transcription/Export) complex that physically couples the elongating RNA polymerase II with factors important for messenger ribonucleoprotein particle (mRNP) formation, RNA processing, and nuclear RNA export(7–9). Yeast TREX is composed of the salt resistant THO sub-complex containing the four proteins Hpr1p, Tho2p, Mft1p, and Thp2p, all of which are essential for efficient transcriptional elongation of a subset of yeast genes(10). THO associates with two proteins involved in nuclear RNA export, Sub2p and Yra1p, to form the larger TREX complex(9). Sub2p is the yeast orthologue of human UAP56 that has been implicated in RNA splicing(11). Hpr1p genetically and physically interacts with RNA Polymerase II(12,13) and is essential for recruitment of Sub2p to genes regulated by TREX(14). Loss of Hpr1p impairs both transcriptional elongation and nuclear RNA export(4,8,9,15–18). While Hpr1p is an essential component of the TREX complex, it is not essential for yeast viability(19).

The human TREX complex is composed of proteins encoded by *Thoc1* (yeast *HPR1*), *Thoc2* (yeast *THO2*)(20), *hTex1* (yeast *TEX1*), *UAP56* (yeast *SUB2*)(21), *Aly* (yeast *YRA1*)(22), and other genes that are not evolutionarily conserved with yeast(4,6). Depletion of pThoc1 compromises both transcriptional elongation and cell proliferation in the cancer cell lines that have been tested (4,5). *Thoc1* is also required for early embryonic development in the mouse (23). Cells of the blastocyst inner cell mass that include embryonic stem cells rapidly die upon loss of maternally supplied pThoc1. Interestingly, conditional ablation of *Thoc1* in adult mammary epithelia has no detectable effect on viability or gland function (Wang and Goodrich, unpublished). These observations suggest the hypothesis that *Thoc1* may be required for the viability of indefinitely self-renewing cells, like cancer cells, but not for normal differentiated cells. We have tested this possibility by comparing the sensitivity of isogenic normal and oncogene transformed cells to loss of pThoc1. We have also tested whether *Thoc1* is required for oncogene mediated neoplastic transformation in vitro.

Materials and Methods

Cell Culture and viruses

HeLa, HEK293, WI38, IMR90 (passage 12), and HCT116 cell lines have been obtained from American Type Culture Collection (Manassas, VA). The p53^{-/-} HCT116 subline has been previously described(24). The IMR5C, IMR90, and oncogene transduced IMR90 derivative cell lines have also been described previously(25). Mouse embryo fibroblast cultures were isolated from F^{-/-} and F^{+/+} littermate embryos at 13.5 days of gestation by standard procedures. The construction and genotyping of the floxed *Thoc1* allele has been previously described (26). Cells are cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.5% penicillin and streptomycin and grown at 37°C in a humidified atmosphere of 5% CO₂.

The Ad-*lacZ* adenoviral stock is from the M.D. Anderson Cancer Center Vector Core Facility. The Ad-Cre adenoviral stock is purchased from the Gene Transfer Vector Core of the University of Iowa(27). Expression of the transgene in each recombinant adenovirus is driven

by the early promoter of cytomegalovirus. Infections are typically performed at a multiplicity of infection (MOI) of 25–50 infectious units per cell.

Recombinant retroviruses designed to express E1A (E1A.12S.Lpc) or Ha-*ras* (c-H-RasV12.pWzl) have been previously described(25). Early passage MEFs (passage $\leq$ 5) were infected with freshly prepared retroviral stocks and subsequently selected for puromycin and hygromycin to enrich for E1A and Ha-*ras* expressing cells.

Small interfering RNA (siRNA)

The control (SC and 2M) and *Thoc1* (N52, N54) siRNA have been described previously(5). For siRNA transfections, cells were plated at 1×10^5 per well in DMEM supplemented with 10% FBS (without antibiotics) in 6-well plates. Twenty-four hours later, the siRNAs (synthesized by Xeragon Inc., Huntsville, AL) were transfected at 10nM by using lipofectamine 2000 as described by the manufacturer (Invitrogen, Carlsbad, CA). Typically, cells were collected or treated as indicated seventy-two hours after transfection.

Cell viability, cell cycle, apoptosis, and β -galactosidase assays

Cell viability was measured by direct counting of trypan blue excluding cells using a hemacytometer or by 23-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay performed as recommended (Roche Applied Science, Indianapolis, IN) with similar results. Optical density readings for the XTT assays were read at 490 nm using an Elx808 micro-plate reader (BIO-TEK Instruments Inc., Winooski, VT).

For cell cycle distribution analysis, single cell suspensions at 1×10^6 /ml in 1 ml phosphate buffered saline (PBS) were prepared. Cells were fixed by adding 3 ml ice-cold 70% ethanol and kept at -20°C at least over-night. Cells were then washed with PBS and incubated for 15 minutes at 37°C in 1 ml propidium iodide solution (50 $\mu\text{g}/\text{ml}$ PI, 20 $\mu\text{g}/\text{ml}$ RNase in PBS). The signals were collected on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by ModFit LT software (Verity Software House, Inc. Topsham, ME).

The fraction of apoptotic cells was measured by using the Annexin V-PE apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's recommendations. Briefly, the treated cells were collected by trypsinization, washed, and resuspended in 1x Annexin V binding buffer at a concentration of 1×10^6 cells/ml. Annexin V-Phycoerythrin (Annexin V-PE) and 7-amino-actinomycin D (7-AAD) was added and cells incubated for 15 min at 25°C in the dark. Stained cells were diluted in 1 x binding buffer and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) within one hour. WinList software was used to calculate the fraction of apoptotic cells (Verity Software House, Inc. Topsham, ME).

An Ortho-nitrophenyl-b-D-galactopyranoside (ONPG) assay was used according to manufacturer's recommendations (Promega, Madison, WI) to measure β -galactosidase activity. Cells were trypsinized, washed, and lysed in 1x Reporter Lysis Buffer for 15 minutes. The extract was clarified by centrifugation at 16,000g for 2 minutes at 4°C and protein concentration of the extracts quantitated using the Bradford assay (Bio-Rad, Hercules, CA). Thirty microgram of protein per sample was diluted in 1X Reporter Lysis Buffer and 2X Assay Buffer (200 mM sodium phosphate, pH 7.3, 2 mM MgCl_2 , 100 mM β -mercaptoethanol, 1.33 mg/ml ONPG). The mixture was incubated at 37°C for 30–60 minutes. The optical density of samples was measured at 405 nm using an Elx808 micro-plate reader (BIO-TEK Instruments Inc., Winooski, VT).

Western Blotting

Cells were harvested, washed in ice-cold PBS, and extracted in Lysis250 buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40), supplemented with a cocktail of protease and phosphatase inhibitors (Sigma, St. Louis, MO). Extracts were clarified by centrifugation and protein concentration measured by Bradford assay (Bio-Rad, Hercules, CA). Twenty microgram of total protein for each sample was resolved by 7.5% SDS-PAGE, transferred to Polyvinylidene Difluoride (PVDF) membrane, and the blot was blocked with 5% non-fat dry milk in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST) for 1 hour at room temperature. The blot was incubated with primary antibody diluted in fresh TBST-5% milk and the bound antibody detected using horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Amersham Biosciences Corp, Piscataway, NJ). The following antibodies were used: mouse monoclonal anti-pThoc1 (GeneTex, San Antonio, TX); mouse monoclonal anti- β -actin (Calbiochem, San Diego, CA); mouse monoclonal anti-Hsp70 (Stressgen, Victoria, BC, Canada); mouse monoclonal anti-Poly ADP-ribose polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-phospho-Histone H2AX (Upstate, Temecula, CA);.

Results

Loss of Thoc1 compromises the viability of oncogene-transformed fibroblasts, but not normal fibroblasts

We have previously generated oncogene transformed derivatives of normal, diploid IMR90 human fibroblasts(25). An *E1A/Ha-ras* transduced IMR90 derived line is immortal, but not tumorigenic. An *E1A/Ha-ras/c-myc* transduced IMR90 line is immortalized and tumorigenic in vivo. We have used this panel of human fibroblast cells to compare the effects of pThoc1 depletion on normal and oncogene transformed human cells. Examination of pThoc1 levels in the cell panel indicates that the oncogene transformed lines express significantly higher levels of pThoc1 than the normal, parental cells (Figure 1A, left panel), similar to previously published data comparing breast cancer cells and normal mammary epithelia(4).

We transfected siRNA directed against *Thoc1* into each cell line to deplete pThoc1 and assessed the effects on accumulation of viable cells over time. The targeted siRNA oligonucleotides used (N52 and N54) were previously verified to be specific for *Thoc1*(5). *Thoc1* siRNA, but not a mismatch control siRNA, was able to deplete pThoc1 from each of the cell lines (Figure 1A, right panels). To verify that siRNA mediated pThoc1 depletion inhibited TREX activity, we assayed the expression of a bacterial β -galactosidase reporter gene. *Thoc1* was previously noted to be required for efficient transcriptional elongation of the G/C rich bacterial β -galactosidase gene in both yeast and mammalian cells(5,16). Cells transfected with *Thoc1* or control siRNA were infected with a recombinant, β -galactosidase expressing adenovirus and then assayed for β -galactosidase activity. Consistent with western blot analysis, *Thoc1* siRNA treatment reduced β -galactosidase activity in all three cell lines relative to cells transfected with control siRNA (Figure 1B). Depletion of pThoc1 activity was relatively greater in normal IMR90 cells than in the transformed derivatives (see below). Loss of pThoc1 in parental IMR90 cells had no detectable effect on cell accumulation compared to control siRNA treated cells (Figure 1C and D). In contrast, *Thoc1* siRNA caused a significant decrease in the accumulation of *E1A/Ha-ras* or *E1A/Ha-ras/c-myc* transformed IMR90 cells.

We investigated whether the decrease in the accumulation of transformed cells might be due to induction of apoptotic cell death since a significant number of pThoc1 depleted cells stained positively for trypan blue. *Thoc1* siRNA treatment of *E1A/Ha-ras/c-myc* transformed cells caused an approximately two-fold increase in the fraction of cells staining positively for the apoptosis marker annexin V, relative to control siRNA treated cells (Figure 2A). *Thoc1* siRNA

treatment had no detectable effect on apoptosis in IMR90 cells. Similarly, *Thoc1* siRNA caused a significant increase in the fraction of *E1A/Ha-ras/c-myc* transformed cells containing fragmented DNA, as assessed by terminal transferase dUTP nick end labeling (TUNEL) assay, relative to control siRNA treated cells (Supplementary Figure 1A). We also examined whether there were changes in cell cycle distribution upon pThoc1 loss. No significant difference in cell cycle distribution was observed in either IMR90 or *E1A/Ha-ras/c-myc* transformed cells upon pThoc1 depletion (Figure 2B). These data suggested that loss of cell accumulation in transformed cells was due to apoptotic cell death rather than changes in the cell cycle, and that oncogene transformed human fibroblasts were significantly more sensitive to pThoc1 depletion than normal human fibroblasts.

We have investigated two additional normal, diploid human fibroblast cell lines to ensure that insensitivity to pThoc1 depletion was not unique to IMR90 cells. *Thoc1* siRNA efficiently depleted pThoc1 in both WI38 and IMR5C normal diploid human fibroblasts (Figure 3A), yet loss of pThoc1 had no detectable effect on the growth rate of these cells in culture (Figure 3B and C). Coincident with the decrease in *Thoc1* protein levels, *Thoc1* siRNA treatment also decreased the level of β -galactosidase activity by more than two-fold compared to control siRNA transfected cells (Figure 3D). Thus efficient depletion of pThoc1 protein and TREX activity had no detectable effect on the growth of normal human fibroblasts in vitro. Since the oncogene transformed cell lines lose viability upon pThoc1 loss, the modest siRNA mediated depletion of pThoc1 observed in these cells was probably due to selection for viable cells that escaped siRNA mediated *Thoc1* gene silencing.

We have recently generated a conditionally excisable allele of the murine *Thoc1* gene that facilitates stable depletion of pThoc1(26). The modified *Thoc1* allele (*Thoc1^F*) contains *lox* sites within introns 5 and 7. Mice homozygous or hemizygous for this allele are normal. Cre mediated recombination between the sites excises intervening exons 6 and 7, deleting evolutionary conserved coding sequence and creating a premature stop codon by reading frame shift. Mice homozygous for the Cre-excised allele phenocopy mice homozygous for a previously characterized *Thoc1* null allele(23). To test the effects of complete genetic loss of Thoc1, relative to intermediate levels of depletion achievable by siRNA, and to ensure that the results described above were not caused by off-target effects of siRNA mediated gene silencing, we have used murine embryonic fibroblasts (MEF) containing the conditional *Thoc1* allele to compare the effects of *Thoc1* loss on the viability of normal and transformed cells.

Early passage (<5) MEFs from littermate embryos heterozygous (*Thoc1^{F/+}*) or hemizygous (*Thoc1^{F/-}*) for the conditional allele were transformed into neoplastic cells by retroviral mediated transfer of the *E1A* and *Ha-ras* oncogenes(28). Both *Thoc1^{F/+}* and *Thoc1^{F/-}* MEFs were efficiently transformed by *E1A/Ha-ras* as indicated by the large number of drug selection resistant cell colonies that exhibited classic transformed morphology (data not shown). To test whether *Thoc1* is required for the viability of these oncogene transformed cells, we infected them with a Cre expressing recombinant adenovirus (AdCre) to convert the *Thoc1^F* allele to a null allele. AdCre caused a significant reduction in the accumulation of *Thoc1^{F/-}* transformed cells, but not of *Thoc1^{F/+}* transformed cells (Figure 4A). We have so far failed to recover viable, *E1A/Ha-ras* transformed cell clones that lack pThoc1. We also investigated whether normal, primary MEFs required *Thoc1* for viability. Early passage MEFs isolated from *Thoc1^{F/-}* and *Thoc1^{F/+}* mice were treated with AdCre and monitored for accumulation of viable cells. In contrast to *E1A/Ha-ras* transformed cells, there was no statistically significant difference in the relative accumulation of *Thoc1^{F/-}* or *Thoc1^{F/+}* cells subsequent to AdCre infection. These observations suggested that, similar to human fibroblasts, *Thoc1* was required to support the viability oncogene-transformed MEFs, but not normal primary MEFs.

To test this, we treated cells with AdCre and assayed for induction of apoptosis by annexin V staining. AdCre caused a significant increase in the percentage of apoptotic cells in transformed *Thoc1*^{F/-} cell cultures, but not in transformed *Thoc1*^{F/+} cell cultures that retain a wild type *Thoc1* allele (Figure 4B). We did not observe significant apoptosis in primary MEFs of either genotype subsequent to AdCre treatment. There was a small increase in apoptosis observed in *E1A/Ha-ras* transformed *Thoc1*^{F/+} cells as well as in primary MEFs of both genotypes, probably caused by nonspecific toxicity of adenovirus infection or Cre mediated DNA damage.

AdCre infection efficiently depleted pThoc1 from both primary and *E1A/Ha-ras* transformed *Thoc1*^{F/-} cells (Figure 4C). As expected, AdCre treatment had no detectable effect on pThoc1 levels in either primary or *E1A/Ha-ras* transformed *Thoc1*^{F/+} cells. Thus despite equally efficient pThoc1 depletion, induction of apoptosis and loss of viability only occurred at detectable levels in oncogene transformed fibroblasts, not normal MEFs. We also noted that *E1A/Ha-ras* transformed MEFs expressed significantly higher levels of pThoc1 than primary MEFs of the same genotype (compare E/R and MEF samples in Figures 4C relative to the loading control). This observation was consistent with the increased pThoc1 expression observed in oncogene transformed human fibroblasts. In sum, this data suggested that cancer cells may be dependent on higher levels of pThoc1 for survival than normal cells.

If true, *Thoc1* may be required for neoplastic transformation. To test this possibility, we have attempted to transform early passage primary *Thoc1*^{F/-} and *Thoc1*^{F/+} MEFs with *E1A/Ha-ras* subsequent to AdCre mediated excision of the *Thoc1* floxed allele. We have verified excision of the floxed allele by western blot analysis of MEF protein extracts prior to *E1A/Ha-ras* transduction (Figure 5A). As expected, *Thoc1*^{F/-} MEFs treated with AdCre exhibit significant depletion of pThoc1 relative to mock treated controls. *Thoc1*^{F/+} MEFs, both Cre or mock pretreated, generate numerous colonies of morphologically transformed cells upon retroviral mediated *E1A/Ha-ras* gene transfer and drug selection (Figure 5B). Mock treated *Thoc1*^{F/-} MEFs from two independent MEF isolates also generate abundant transformed colonies upon transduction of *E1A/Ha-ras*. However, very few transformed cell colonies are recovered from *Thoc1*^{F/-} primary MEFs lacking pThoc1. The few transformed colonies that are recovered have escaped AdCre mediated excision of *Thoc1* since all of those cell clones tested still express pThoc1 (Figure 5C). Similarly, *Thoc1*^{F/-} primary MEFs treated with Cre and spontaneously immortalized by continuous in vitro culture under the 3T3 protocol also retain pThoc1. We have been unable to isolate any *E1A/Ha-ras* expressing or spontaneously immortalized cell clones that lack pThoc1. These observations suggest that *Thoc1* is required for spontaneous immortalization and *E1A/Ha-ras* mediated transformation of primary MEFs.

Depletion of pThoc1 compromises the viability of human epithelial cancer cell lines

The data presented above demonstrated that neoplastic fibroblasts are more dependent on pThoc1 for survival than normal fibroblasts, and that neoplastic fibroblasts deprived of pThoc1 die via apoptotic cell death. We wished to determine whether human epithelial cancer cell lines also undergo apoptotic cell death upon pThoc1 loss, and to examine possible mechanisms that might trigger this apoptotic response. *Thoc1* siRNA efficiently mediated depletion of pThoc1 in a number of different human cancer cell lines including HeLa, 293, HCT116, a p53 null derivative of HCT116, and MCF-7 cells (Figure 6A). Depletion of pThoc1 reduced the accumulation of cells in each of these lines (Figure 6B), consistent with the data presented above and previously published observations(4, 5).

To ascertain if pThoc1 loss also induced apoptotic cell death in human epithelial cancer cells, we assayed pThoc1 depleted HeLa cells for apoptosis. HeLa cells depleted of pThoc1 and exhibiting the typical two-fold reduction in cell accumulation showed a greater than two-fold increase in the percentage of cells stained for the apoptosis marker annexin V, relative to control siRNA transfected cells (Figure 6C). Increased apoptosis was verified by assaying cleavage of

PARP, a known substrate of caspase proteases, in siRNA transfected HeLa cells. Specific depletion of pThoc1 caused an increase in PARP cleavage (Figure 6D). *Thoc1* siRNA treated HeLa, MCF-7, and HCT116 cells also showed an increase in the fraction of cells positive in the TUNEL assay relative to control siRNA treated cells (Supplementary Figure 1A). Finally, treatment of pThoc1 depleted HeLa cells with the pan-caspase ZVAD peptide inhibitor diminished the fraction of annexin V positive cells (Supplementary Figure 1B). In sum, these data indicated that depletion of pThoc1 in human epithelial cancer cell lines inhibited proliferation by induction of apoptosis and subsequent loss of cell viability. It should be noted that sensitivity to loss of pThoc1 was independent of p53 and caspase-3 status. HCT116 cells that retain wild type p53 and a p53 null HCT116 derivative cell line were both sensitive to *Thoc1* siRNA. We also failed to observe consistent increases in p53 and p21 levels in pThoc1 depleted HeLa cells, suggesting that the p53 pathway was not activated (data not shown). MCF-7 cells that lack caspase-3 were also sensitive to *Thoc1* siRNA, and we did not detect caspase-3 activation upon pThoc1 depletion.

How pThoc1 deprivation triggers apoptotic cell death in cancer cells is currently unknown, but a number of potential mechanisms are conceivable. Loss of pThoc1 compromises transcriptional elongation due to improper incorporation of nascent RNA into mRNPs. Nascent RNA can then hybridize to the single stranded DNA template emerging from the RNA polymerase complex thus generating stable RNA:DNA R-loop structures that are known to be recombinagenic and may compromise DNA integrity(17). *Thoc1* loss, therefore, may directly lead to DNA damage. To test this possibility, we have examined the accumulation of phosphorylated histone H2AX, a known marker for DNA damage, in pThoc1 depleted normal and cancer cells. Phosphorylated histone H2AX is undetectable in *Thoc1* or control siRNA treated normal IMR90 cells (Figure 7). In contrast, *Thoc1* siRNA treatment increases the levels of phosphorylated histone H2AX in *E1A/Ha-ras/c-myc* transformed IMR90 cells as well as in HeLa cells, compared to control siRNA treated cells. These results are consistent with the possibility that *Thoc1* loss may lead to accumulation of DNA damage.

Discussion

We describe a number of observations that suggest that cancer cells may differ from normal cells in their requirements for *Thoc1*. *Thoc1* expression is higher in oncogene transformed human and mouse fibroblasts compared to normal fibroblasts. This mimics the increased *Thoc1* expression observed in primary human breast cancer compared to normal mammary epithelium(4), and the abundant levels of pThoc1 typically observed in human cancer cell lines that have been tested(5). However, higher pThoc1 levels are not merely a reflection of the faster proliferation rate typical of cancer cells. Loss of pThoc1 in cancer cell lines and oncogene transformed human or mouse fibroblasts inhibit cell accumulation through induction of apoptosis and subsequent loss in cell viability. In contrast, loss of pThoc1 in normal human or mouse fibroblasts has no detectable effect on viability. Thus the requirements for *Thoc1* to support viability are different in the tested normal and cancer cells. We have been unable to recover viable, *E1A/Ha-ras* transformed or spontaneously immortalized MEFs that lack pThoc1. Thus cells undergoing neoplastic transformation or immortalization are unable to adapt to the absence of *Thoc1*, suggesting *Thoc1* may be required.

There are potential caveats in interpreting these experiments. Since we cannot recover viable *E1A/Ha-ras* expressing cells that lack pThoc1, we cannot exclude the possibility that pThoc1 loss inhibits neoplastic transformation by compromising expression of *E1A/Ha-ras* or the drug resistance genes. However, experiments have been performed in the absence of drug selection with similar results. *E1A/Ha-ras* transduction of *Thoc1*^{F/-} MEFs, but not Cre pre-treated *Thoc1*^{F/-} MEFs lacking pThoc1, generate cultures of immortal, transformed cells without drug selection (Y. Li and A. Lin, unpublished observation). Thus the inability to recover transformed

cells in the absence of pThoc1 is independent of drug selection. In addition, MEFs lacking pThoc1 are viable, yet we do not recover viable cells upon transduction of these cells with *E1A/Ha-ras*. This suggests that *E1A/Ha-ras* must be expressed sufficiently in the absence of pThoc1 to trigger a biological response. We note that pThoc1 loss also inhibits spontaneous immortalization of MEFs and the viability of many different human cancer cell lines. The effects of *Thoc1*, therefore, are not specific to *E1A/Ha-ras* transduction and likely reflect a general requirement for *Thoc1* in cancer cells.

We have previously observed that *Thoc1* is required for the viability of blastocyst stage mouse embryos(23). Blastocyst stage embryos are comprised largely of stem cells that, like cancer cells, have an extended potential for replication and self-renewal. Thus, *Thoc1* may be required more generally for the maintenance of extended replicative potential. Consistent with this hypothesis, we have observed that primary MEFs genetically ablated for *Thoc1*, while viable and able to proliferate in culture, undergo premature conversion to a senescence-like state (Y. Li and D.W. Goodrich, unpublished observation). Similarly, loss of the yeast orthologue *HPRI* is not lethal, but compromises lifespan(29). It is conceivable that lack of sufficient pThoc1 limits replicative potential in normal cells by induction of a cellular senescence program, thereby inhibiting immortalization and neoplastic transformation. Upregulation of *Thoc1* may be required, therefore, to facilitate immortalization and neoplastic transformation. Since cancer cells are unable to sustain cell viability upon acute loss of pThoc1 activity, the effects of pThoc1 loss must be dominant to the effects of deregulated *E1A/Ha-ras* expression studied here, as well as to other alterations that facilitate neoplastic transformation in a variety of different human cancer cell lines tested here.

How *Thoc1* loss triggers apoptosis in cancer cells is unknown. Since all detectable pThoc1 is apparently within the TREX complex(6), the simplest explanation is that loss of pThoc1 compromises TREX function. Loss of TREX function could have several conceivable effects on cells. Loss of TREX activity may adversely affect the generation of translatable mRNA from a subset of genes required to maintain viability. Loss of TREX activity may also compromise normal telomere maintenance. In yeast, loss of the *Thoc1* orthologue *HPRI* is associated with defects in telomere maintenance(30). Defects in telomere maintenance would be expected to influence replicative potential and viability. Alternatively, deficient mRNP biogenesis in the absence of *Thoc1* may trigger R loop formation and DNA strand breaks (31). Such DNA lesions could trigger apoptotic cell death in cancer cells if they are unable to efficiently repair them. The accumulation of phosphorylated histone H2AX upon pThoc1 depletion observed here is consistent with this possible mechanism, but additional studies will be required to identify and verify the mechanism underlying loss of cancer cell viability.

Irrespective of the precise mechanism, mutational inactivation of *Thoc1* is synthetic lethal with the genetic and epigenetic alterations associated with a number of cancer cell lines of different type and origin. Thus *Thoc1* may represent a novel molecular target for cancer therapy. Therapy that blocks pThoc1 activity is expected to preferentially compromise the viability of cancer cells, potentially yielding superior therapeutic index. Since the mechanism of pThoc1 action is novel, utilization of pThoc1 as a therapeutic target may yield unique clinical responses and opportunities for novel combination therapy. For example, yeast deficient in the *Thoc1* orthologue *HPRI* are synthetic lethal with topoisomerase mutations(32) and are more sensitive to DNA damage(33). Depletion of pThoc1 in human cancer cell lines renders them more sensitive to camptothecin and cisplatin(5). These observations suggest that therapeutic inhibition of pThoc1 in human cancer will increase sensitivity to topoisomerase poisons and possibly other forms of genotoxic therapy.

Thoc1 protein functions in the newly discovered TREX complex, a representative of a class of complexes that regulate gene expression subsequent to transcriptional initiation. This class of

protein complexes may specify post-transcriptional “operons” that facilitate protein expression from coordinately regulated genes of diverse size and structure(34). Although there is increasing appreciation for the importance of such complexes, they are understudied relative to the transcription factors that govern the initiation of transcription. As such, their relevance to carcinogenesis is largely undocumented. However, the von Hippel-Lindau tumor suppressor protein is a known inhibitor of the elongin transcription elongation factor(35). The interaction of the retinoblastoma tumor suppressor protein and pThoc1 may reflect an analogous interaction between a tumor suppressor gene product and a transcription elongation/RNA processing factor. Such interactions suggest that complexes that regulate gene expression at the level of mRNP formation and RNA processing may provide a largely untapped source of novel molecular targets for cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Loss of pThoc1 compromises the accumulation of oncogene transformed human fibroblasts, but not normal fibroblasts

A) IMR90, *E1A/Ha-ras* (E/R), or *E1A/Ha-ras/c-myc* (E/R/M) transformed human fibroblast extracts were analyzed for pThoc1 levels by western blotting (left panel). The indicated cells were transfected with *Thoc1* (N52) or mismatch control (2M) siRNA and protein extracts analyzed three days later for relative pThoc1 levels by western blotting (right panels). The protein loading control is β-actin. B) The indicated cells were transfected with siRNA as above, and then infected with a recombinant adenovirus designed to express the bacterial β-galactosidase gene. Cells were subsequently extracted and β-galactosidase activity quantitated by ONPG assay. The data is representative of three independent experiments, each done in

triplicate. Asterisks indicate differences between N52 and 2M siRNA treated samples that are statistically significant by Student's T-test ($P < .01$). C) The indicated cells transfected as in A with were photographed under phase contrast microscopy using a 10X objective. D) The indicated, trypan blue dye excluding cells transfected as in A with *Thoc1* (N52, open circles) or control (2M, solid squares) siRNA were counted at the indicated days post-transfection. The data reflect the mean and standard deviation of at least two transfections for each data point. Untreated E/R cells rapidly lose viability as cell density increases, accounting for the decline in cell numbers observed at later times for these cells.

Figure 2. Depletion of pThoc1 causes induction of apoptosis, but not changes in cell cycle distribution, in oncogene transformed human fibroblasts

A) IMR90 or the E/R/M transformed cells were transfected with the indicated siRNA and analyzed two days later for apoptosis by annexin V staining and flow cytometry. The percentage of annexin V positive, propidium iodide negative cells is indicated. The histograms are representative of two independent experiments. Asterisks indicate differences between N52 and 2M siRNA treated samples that are statistically significant by Student's T-test ($P < .01$). B) The indicated cells were treated with *Thoc1* (N52) or control (2M) siRNA then fixed and stained for propidium iodide three days later. Flow cytometry was used to analyze the cells and the histograms of a representative experiment (of at least three replicates per genotype) are shown.

The percentage of cells in each phase of the cell cycle, as estimated by ModFit software, is shown.

Figure 3. Depletion of pThoc1 does not affect the growth and viability of normal fibroblasts

A) The two additional diploid human fibroblast cell lines indicated are transfected with control (SC) or *Thoc1* siRNA (N52 or N54) and total protein extracted 3 days later. Protein extracts are analyzed for pThoc1 levels by western blotting with β -actin staining serving as a loading control. B) Cultures analyzed above in A are photographed under phase contrast microscopy using a 10X objective. C) The number of trypan blue dye excluding cells, transfected as above, is counted at the indicated times post-transfection with a hemacytometer. The data reflect the mean and standard deviation of three transfections. D) Extracts from IMR5C cells transfected with *Thoc1* (N52) or control (SC) siRNA and subsequently infected with a β -galactosidase expressing recombinant adenovirus are analyzed for β -galactosidase activity by ONPG assay. The OD405 readings are normalized to the control siRNA transfection. The data represent the mean and standard deviation of two experiments performed in duplicate. Asterisks indicate differences between the mean OD405 readings from N52 and SC siRNA treated samples that are statistically significant by Student's T-test ($P < .01$).

Figure 4. *E1A/Ha-ras* transformed MEFs, but not normal MEFs, require *Thoc1* for viability
 A) MEFs isolated from littermate embryos heterozygous (F/+) or hemizygous (F/-) for the floxed *Thoc1* allele (MEF), or MEFs transformed by stable retroviral mediated transduction of the *E1A* and *Ha-ras* genes (E/R), were infected with a recombinant adenovirus expressing the Cre recombinase (AdCre) at a multiplicity of infection (MOI) of 40. Two days following Cre treatment, accumulation of trypan blue dye excluding cells was counted with a hemacytometer. Since the growth rate of the untreated cell isolates varied, the data is expressed as cell number relative to a mock adenovirus treated (MOI 0) control. The data represent the mean and standard deviation of two experiments with the same MEF isolates, but are representative of multiple isolates of each genotype for the transformed cells. The data

represent the mean and standard deviation of four experiments, using two independent MEF isolates for each genotype for the primary cells. B) Cells of the indicated genotype were treated with AdCre or mock treated as above, and analyzed three days later for apoptosis by annexin V staining and flow cytometry. The fraction of annexin V positive, propidium iodide negative cells in each of the indicated cultures is indicated. The data are the mean and standard deviation of three experiments with the same MEF isolates, but are representative of multiple MEF isolates of each genotype for transformed cells. The data represent the mean and standard deviation of two independent experiments using a single MEF isolate for each genotype for the primary cells. C) Cells of the indicated genotype were treated with AdCre as above and analyzed three days later for pThoc1 levels by western blotting. β -actin serves as a loading control. Asterisks in panels A and B indicate differences between F/+ and F/- samples that are statistically significant by Student's T-test ($P < .01$).

Figure 5. *Thoc1* is required for efficient oncogene mediated neoplastic transformation

A) Early passage primary MEFs isolated from different littermate embryos of the indicated genotypes were treated with AdCre or mock treated. Three days after AdCre treatment, protein extracts were analyzed for relative pThoc1 levels by western blot analysis. The first two samples represent the same samples analyzed in Figure 1F. The last lane represents an additional isolate of *Thoc1*^{F/-} MEFs. β-actin serves as a loading control. B) Two passages after AdCre treatment, cultures were transduced with *E1A/Ha-ras* and drug selected to enrich for *E1A/Ha-ras* containing cells. One week after initiating drug selection, dishes were fixed and stained with crystal violet to visualize surviving cell colonies. The data shows one experiment, but is representative of three independent experiments. C) Primary *Thoc1*^{F/-} MEFs were

pretreated with AdCre or mock treated as indicated. Cells were analyzed for relative pThoc1 levels by western blotting as in A (left panel). Cells were subsequently transduced with *E1A/Ha-ras* or immortalized by passage under the 3T3 protocol. Viable cells recovered following oncogene transduction or spontaneous immortalization were expanded and analyzed for pThoc1 levels as above (right panel). The immortalized or transformed *Thoc1*^{F/-} cells retain *Thoc1* expression despite treatment with AdCre.

Figure 6. Depletion of pThoc1 compromises the viability of human epithelial cancer cell lines

A) The indicated cell lines are transfected with a *Thoc1* specific siRNA (N52) or scrambled control siRNA (SC). Total protein extracts are prepared from transfected cells and analyzed for pThoc1 levels by western blotting. Blots are stained for Hsp70 as a protein loading control. B) Transfected cell cultures analyzed above in A are photographed under phase contrast microscopy using a 10X objective. C) HeLa cells transfected with the indicated siRNA are analyzed for the fraction of apoptotic cells 3 days posttransfection by annexin V staining and flow cytometry. The fraction of annexin V positive cells is indicated. Thresholds were set by comparison to untransfected cells, thus the apoptotic cells in the control sample reflect the toxicity of the transfection procedure itself. The histograms shown are representative of three experiments. D) HeLa cells transfected with the indicated siRNA are extracted and analyzed for the indicated protein levels by western blotting. The asterisk indicates a band migrating at the expected apparent molecular weight of a caspase cleaved form of PARP. The protein loading control is β -actin.

Figure 7. Depletion of pThoc1 is associated with increased levels of phosphorylated histone H2AX in transformed, but not normal, human cells

The indicated cells are treated with Thoc1 (N54) or control (SC) siRNA and protein extracts prepared three days later. The extracts are analyzed for the phosphorylated form of H2AX by western blotting. β -actin serves as a loading control. The data are representative of two independent experiments.