Coordinated control of senescence by IncRNA and a novel T-box3 co-repressor complex

Pavan Kumar P1,2, Uchenna Emechebe3†, Richard Smith4†, Sarah Franklin5,6, Barry Moore7, Mark Yandell7, Stephen L Lessnick2,4,8, Anne M Moon1,2,7*

1Weis Center for Research, Geisinger Clinic, Danville, United States; 2Department of Pediatrics, University of Utah, Salt Lake City, United States; 3Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, United States; 4The Centre for Children’s Cancer Research, Huntsman Cancer Institute, University of Utah, Salt Lake City, United States; 5Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, United States; 6Department of Internal Medicine, University of Utah, Salt Lake City, United States; 7Department of Human Genetics, University of Utah, Salt Lake City, United States; 8Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, United States

Abstract Cellular senescence is a crucial tumor suppressor mechanism. We discovered a CAPERα/TBX3 repressor complex required to prevent senescence in primary cells and mouse embryos. Critical, previously unknown roles for CAPERα in controlling cell proliferation are manifest in an obligatory interaction with TBX3 to regulate chromatin structure and repress transcription of CDKN2A-p16INK and the RB pathway. The IncRNA UCA1 is a direct target of CAPERα/TBX3 repression whose overexpression is sufficient to induce senescence. In proliferating cells, we found that hnRNPA1 binds and destabilizes CDKN2A-p16INK mRNA whereas during senescence, UCA1 sequesters hnRNPA1 and thus stabilizes CDKN2A-p16INK. Thus CAPERα/TBX3 and UCA1 constitute a coordinated, reinforcing mechanism to regulate both CDKN2A-p16INK transcription and mRNA stability. Dissociation of the CAPERα/TBX3 co-repressor during oncogenic stress activates UCA1, revealing a novel mechanism for oncogene-induced senescence. Our elucidation of CAPERα and UCA1 functions in vivo provides new insights into senescence induction, and the oncogenic and developmental properties of TBX3.

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Introduction Senescence is defined as irreversible arrest of cell growth and loss of replicative capacity (Hayflick, 1965). Senescent cells have a large, flattened morphology and a characteristic secretory phenotype. They may be multinucleate, exhibit nuclear distortion, and contain senescence-associated heterochromatin foci (SAHFs) (Kosar et al., 2011). Senescence can be induced by various stimuli such as DNA damage, metabolic or oxidative stress, or expression of oncoproteins (Larsson, 2005; Kuilman et al., 2010; Coppé et al., 2011).

The p16/retinoblastoma protein (RB) and p53 tumor suppressor pathways are key regulators of senescence induction and maintenance in many cell types (Narita et al., 2003). p14ARF-p53 activates p21, whereas the p16INK4a-RB pathway culminates in EZF transcriptional target repression and senescence (DeGregori, 2004). Expression of CDKN2A-p14ARF and CDKN1A-p21CIP is repressed by the related transcription factors TBX2 and TBX3; this is the postulated mechanism for senescence bypass...
Cell division and growth are essential for survival. But it is equally important that cells can stop dividing, because failing to do so can lead to the uncontrolled tumor growth seen in cancer. One such quality control mechanism is called senescence, which stops the growth and multiplication of cells that are old, damaged or behaving in ways that may harm the organism. All cells eventually stop dividing and undergo senescence, but a number of factors may trigger the process early, such as DNA damage, stress or the appearance of cancer-causing proteins.

Senescence can be harmful if it occurs too early in life and interferes with normal growth. Severe birth defects—including fatal heart problems and limb malformations—occur if senescence is inappropriately triggered early in development. Mutations in a gene encoding a protein called TBX3 have been linked to these severe birth defects.

Normally, TBX3 stops the production of other proteins that trigger senescence in early development, and helps to maintain stable conditions in adult cells. Understanding how it does so could help scientists understand normal cell function and aging, and also help to find ways to trigger senescence in cancerous cells.

Kumar et al. found that a protein called CAPERα—for short Coactivator of AP1 and Estrogen Receptor—forms a complex with TBX3 that stops cells dividing in living organisms in at least two different ways. One way is by altering how DNA is folded. The other way involves a non-coding strand of RNA from a gene called UCA1: this RNA prevents the degradation of proteins that stop cell division.

In normal proliferating cells, the CAPERα/TBX3 protein complex prevents the production of UCA1 RNA. In contrast, in cells that received a cancer causing stimulus, TBX3 and CAPERα physically separate: this activates production of UCA1 RNA and causes senescence. Further studies will be required to establish exactly how the CAPERα/TBX3 protein complex interacts with DNA and RNA to control senescence and prevent cancer.

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(IP'd) proteins identified CAPERα (Figure 1A). Since TBX3 functions in mammary development and may contribute to the pathogenesis of breast and other hormone responsive cancers (Douglas and Papaioannou, 2013), its interaction with an ERα co-activator drove further investigation.

To determine if Tbx3 and Capera interact in vivo, we IP’d endogenous Capera from embryonic day (e)10.5 mouse embryo lysates (Figure 1B). Immunoblotting for Tbx3 confirmed its interaction with Capera (Figure 1C, lane 5) and in vitro pull down assays revealed that their interaction is direct (Figure 1D, lane 6). Capera is very broadly expressed during mouse embryonic development (Moon, unpublished), whereas Tbx3 expression is very tissue specific and dynamic. We thus questioned whether the endogenous proteins interact in mouse tissues relevant to malformations seen in humans with UMS. Immunohistochemistry on sectioned e10.5 embryos showed that Tbx3 and Capera proteins are co-expressed and have distinct localization patterns in different tissues: Capera is detected in all dorsal root ganglia nuclei (Figure 1E), some of which contain co-localized Tbx3; in proximal limb mesenchyme, Tbx3 and Capera co-localize in nuclei (Figure 1F) while in some distal cells and the ectoderm, Capera is nuclear and Tbx3 is cytoplasmic (Figure 1G, white arrowheads). Such tissue specificity suggests that functions of the Capera/Tbx3 complex are context dependent.

TBX3 DNA binding and repressor domains (DBD, RD) independently mediate interactions with partner proteins (Carlson et al., 2001; Coll et al., 2002; Kumar et al., 2014). To identify domains required for CAPERα interaction, we used a series of overexpression plasmids encoding mouse Tbx3 proteins with different mutations and functional domains (Figure 1H). The DBD, deleted repressor domain (ARD) and exon7 missense mutants are untagged proteins, whereas the C-terminal deletion mutants are Myc-tagged.

To assay the interactions of the untagged exogenous proteins with endogenous CAPERα in HEK293 cells, we needed to knockdown endogenous TBX3 with shRNA (Figure 1I). We previously demonstrated that mutant Tbx3 proteins produced from the overexpression plasmids are present in TBX3 knockdown HEK293 cells (Figure 2 in Kumar et al. 2014). CAPERα is present and can be IP’d in the context of knockdown of endogenous TBX3 and subsequent overexpression of mutant mouse Tbx3 proteins (Figure 1J). Immunoblot of anti-CAPERα IP’d samples shows that the endogenous CAPERα interacts with Tbx3 DBD mutant proteins (Figure 1J, lanes 2 and 3 are L143P and N227D, respectively).

The Tbx3 deletion constructs encode Myc-tagged mutants that can be distinguished from endogenous Tbx3, so interactions were assayed in wild-type HEK293 cells. Myc-tagged deletion mutants are IP’d by the anti-Myc antibody (Figure 1K), and probing anti-Myc IP’d material for CAPERα reveals that deletions more proximal than amino acid 655 disrupt the CAPERα/Tbx3 interaction (Figure 1K).

The observation that deletions of the Tbx3 C-terminus disrupt the CAPERα/Tbx3 interaction led us to test whether the C-terminal repressor domain, which is crucial for the ability of Tbx3 to function as a transcriptional repressor and immortalize fibroblasts (Carlson et al., 2001), plays a role. Although the untagged ARD mutant is produced in Tbx3 shRNA knockdown cells and IP’d by the anti-Tbx3 antibody (Figure 1L and Kumar et al., 2014) it does not interact with CAPERα (Figure 1L). CAPERα also fails to interact with a C-terminal Tbx3 frameshift mutant similar to one identified in humans with UMS (Bamshad et al., 1999) (Figure 1—figure supplement 1).

**CAPERα and TBX3 are required to prevent premature senescence of primary human and mouse cells**

Roles for TBX3 in cell cycle regulation and senescence of primary cells have not been reported. We employed loss-of-function to test whether TBX3 is required for sustained proliferation of primary cultured human foreskin fibroblasts (HFFs) and to determine if CAPERα functions in this process. We tested two different CAPERα and TBX3 shRNAs (please see ‘Materials and methods’ for sequences and location in target mRNAs). Both CAPERα and TBX3 shRNAs effectively decreased the amount of CAPERα mRNA (Figure 2—figure supplements 1A and 2A,B). Knockdown of either protein resulted in a dramatic increase in senescence associated β-galactosidase activity (SA-βgal, Figure 2A–D; Figure 2—figure supplements 1 and 2C–H). This effect is specific because it occurs with two different shRNAs and is rescued by overexpression of CAPERα (Figure 2—figure supplement 1B, E, G, H) and Tbx3 (Figure 2—figure supplement 2B, E, G, H). For all subsequent experiments, CAPERα shRNA ‘A’ and TBX3 shRNA ‘A’ were used to perform knockdown (KD) in HFFs (protein knockdowns are shown in Figure 2—figure supplements 1 and 2, l panels).

The effects of CAPERα and Tbx3 KD on HFF cell growth, and SA-βgal activity suggest induction of premature senescence. Consistent with this, both KDs dramatically influenced nuclear structure,
Figure 1. CAPERα and TBX3 directly interact via the TBX3 repressor domain. (A) Representative spectrum for CAPERα identified in anti-TBX3 co-IP of HEK293 cell lysates. Mass spec analysis identified six specific CAPERα peptides, providing 8.5% sequence coverage of the protein. This spectrum shows fragmentation of one of these peptides, C*PSIAAAIAAVNALHGR, with diagnostic b- and y-series ions shown in red and blue, respectively. * indicates Figure 1. Continued on next page
carbamidomethylation. (B) Anti-CAPERα immunoblot (IB) analysis of anti-CAPERα immunoprecipitated (IP’d, lane 2) e10.5 mouse embryo lysates. Black arrowheads indicate IgG heavy chain and red indicate protein of interest (CAPERα or TBX3). (C) Anti-Tbx3 IB of anti-Tbx3 (lane 4) and anti-Caperα (lane 5) IP’d mouse embryo lysates. Rabbit (r)-IgG (lanes 1, 6) and mouse (m)-IgG (lane 7) are negative controls. (D) In vitro MBP pull down assay: MBP and MBP-Tbx3 bound amylose affinity columns were incubated with GST or GST-CAPERα. Bound proteins were eluted, subjected to SDS-PAGE followed by IB with anti-CAPERα antibody. (E–G) Co-localization of Tbx3 and Caperα in vivo shown by immunohistochemical analysis of sectioned e10.5 mouse embryo: embryonic dorsal root ganglion (DRG, E), proximal (F), and distal (G) limb bud with anti-Tbx3 (red) and anti-Caperα (green) antibodies and DAPI (blue). White arrowheads in G label representative ectodermal and mesenchymal cells with cytoplasmic Tbx3 and nuclear Caperα. (H) Schematic representation of mouse Tbx3 overexpression constructs. Tbx3 DNA binding domain (DBD) point, △RD and exon7 missense proteins are untagged and the C-terminal deletion mutants are Myc-tagged. (I) Anti-Tbx3 IB of HEK293 cell lysates transfected with control or anti-TBX3 shRNA. (J) Anti-CAPERα IB of anti-CAPERα IP’d samples from HEK293 cells transfected with anti-TBX3 shRNA and expressing mouse Tbx3 proteins listed at top. Production and IP of endogenous CAPERα is not affected by production of mutant Tbx3 proteins. (J) Anti-Tbx3 IB of anti-CAPERα IP’d samples from HEK293 cells transfected with anti-TBX3 shRNA and expressing Tbx3 proteins as in J. The DBD point mutant proteins (lanes 2, 3) interact with CAPERα as efficiently as wild type Tbx3 (lanes 1, 4). (K) Anti-Myc IB of anti-Myc IP’d samples from HEK293 cell lysates expressing Myc-tagged mouse Tbx3 C-terminal deletion mutants. The mutant proteins are expressed and efficiently IP’d. These cells were not treated with anti-TBX3 shRNA because the expression constructs produce a Myc-tagged mutants that can be IP’d independently of endogenous Tbx3. (K) anti-CAPERα IB of anti-Myc IP’d samples from HEK293 cell lysates expressing Myc-tagged mouse Tbx3 C-terminal deletion mutants. These cells were not treated with anti-TBX3 shRNA because the expression constructs produce a Myc-tagged mutants that can be IP’d independently of endogenous Tbx3. (L) Anti-Tbx3 IB of anti-Tbx3 IP’d samples from HEK293 cells transfected with anti-TBX3 shRNA and expressing wt or repressor domain deletion mutant (△RD) mouseTbx3. The shRNA does not prevent production of the overexpression proteins. (L) Anti-CAPERα IB of HEK293 cells transfected with anti-TBX3 shRNA and expressing mouse wt or △RD Tbx3 proteins and IP’d with anti-Tbx3 or IgG. Loss of the repressor domain prevents interaction with CAPERα. Black arrowheads indicate IgG heavy chain and red indicate protein of interest (CAPERα or TBX3). Tbx3, CAPERα = human; Tbx3, Caperα = mouse.

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The following figure supplements are available for figure 1:

Figure supplement 1. Missense mutation of the C-terminus of Tbx3 disrupts interaction with CAPERα.

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chromatin organization and formation of SAHFs (Figure 2G–J). Expression of senescence mediators was increased and conversely, expression of cell growth and cell cycle promoting genes was similarly decreased by CAPERα and TBX3 KD (Figure 2K–M). Increased expression of CDKN2A-p16INK (henceforth referred to as p16\(^{INK}\)) and decreased PCNA, E2F1 and 2, CDK2, CDK4, CDC2 transcripts indicate that CAPERα/TBX3 represses the p16/RB pathway in proliferating HFFs. PMAIP1, CDKN1A-p21, and other p53 pathway members were also increased. Collectively, these data indicate that CAPERα and TBX3 are required to prevent senescence of primary HFFs and act upstream of major cell cycle and senescence regulatory pathways.

**Tbx3 null murine embryonic fibroblasts undergo p16/RB-mediated premature senescence, Caperα mislocalization and nuclear disruption**

Tbx3 deficiency in mice causes lethal embryonic arrhythmias and limb defects however, these phenotypes are not due to increased apoptosis (Frank et al., 2012 and Emechebe and Moon, unpublished). We hypothesized that Tbx3 may prevent senescence of embryonic cells, and so examined murine embryonic fibroblasts (MEFs) from e13.5 wild type (WT) and Tbx3 null mice (−/−) embryos. WT MEFs undergo ~10 passages with regular, 20 hr doubling times. In contrast, Tbx3−/− MEFs had increased SA-βgal activity and ceased proliferating after only four passages (Figure 2N–Q). Most Tbx3−/− MEFs had distorted or ruptured nuclei (Figure 2—figure supplement 3A–C) and laminB1 staining was already altered at passage 1 (Figure 2—figure supplement 3B). Caperα null mutant embryos do not survive long enough to generate MEFs for complementary experiments (Emechebe and Moon, unpublished) however, Caperα localization is markedly abnormal in Tbx3−/− MEFs after only 1 passage (Figure 2—figure supplement 3D–F). These data suggest that Tbx3 is required for preservation of nuclear architecture and to tether Caperα in its normal nuclear domains in proliferating cells.

Consistent with premature senescence seen in Tbx3−/− MEFs, key pro-senescence pathways are activated after loss of Tbx3 in vivo: in protein lysates from Tbx3−/− embryos, RB was hypophosphorylated on multiple serine residues, consistent with increased p16 and decreased Cdk2 and Cdk4 protein levels relative to control (Figure 2R). The levels of p21 and other senescence markers were increased, while numerous Cyclins and other Cdns were decreased (Figure 2R, Figure 2—figure supplement 3G). All of these findings are consistent with a requirement for Tbx3 to prevent senescence in embryonic mice and MEFs.
Figure 2. Knockdown of endogenous CAPERα and TBX3 in primary human fibroblasts and mouse embryos induces premature senescence and disrupts expression of cell cycle and senescence regulators. (A–C) Representative bright field images of senescence associated β-galactosidase (SA-βG) assays of HFFs transduced with control, TBX3 shRNA A or CAPERα shRNA A. Only occasional cells in the control transduction have detectable lacZ staining (blue).
whereas knockdown of either TBX3 or CAPERα results in marked changes in cell morphology and increased lacZ staining. (D) Bar graph quantitating % beta-galactosidase positive cells from four replicate plates of SA-βgal assays. * indicates p<0.01 compared to control. (E and F) 3T5 cell proliferation assay (Lessnick et al., 2002) of cumulative population doublings in HFFs transduced at passage 30 with control, TBX3 or CAPERα shRNAs. These are representative curves of duplicate experiments; each point on the curve is a measurement of cell count from a single plating followed over the course of the experiment as described in methods. (G–J) Immunohistochemical analysis of H3K9me3 immunoreactivity (red) and DAPI (blue) in HFFs after knockdown with control (G and I), TBX3 (H), or CAPERα (J) shRNAs. Individual channels are shown and the merged image is on the right. Note increased nuclear punctate staining consistent with Senescence-associated heterochromatin foci (SAHFs) in both channels and evidence of nuclear disruption (white arrowheads in red channel) after loss of either TBX3 or CAPERα. (K–M) Analysis of cell cycle and senescence marker transcript levels in HFFs transduced with control, TBX3, or CAPERα shRNAs. (K) Relative transcript levels assessed by quantitative real-time-PCR (qPCR) of cDNA. Values reflect fold change in knockdown HFFs relative to control after normalization to HPRT levels. Note general pattern of expression changes are similar in TBX3 (blue) and CAPERα (red) knockdowns. Data are plotted as fold change mean ± standard deviation. * indicates p<0.05 relative to control. (L and M) Agarose gel of PCR amplicons of cDNAs reverse transcribed from TBX3 (L) or CAPERα (M) shRNA knockdown HFF. RNA reveals similar decreases in cell cycle promoting genes CDK2 and 4 in TBX3 and CAPERα knockdowns and increased p21 levels. (N and O) SA-βgal assay of wild type and Tbx3 null MEFS reveals that Tbx3 is required to prevent premature senescence of primary murine embryonic fibroblasts (MEFs). (P) Quantitation of % beta-galactosidase positive cells from five replicate experiments exemplified in O, P. * indicates p<0.01. (Q) 3T5 cell proliferation assay of cumulative population doublings in wild-type and Tbx3 null MEFS. These are representative curves from duplicate experiments; each point on the curve is a measurement of cell count from a single plating followed over the course of the experiment as described in ‘Materials and methods’. (R) IBs to assay levels of cell cycle and senescence proteins in wild type and Tbx3 null embryo lysates. Tubulin loading control is at top left (Tub). The changes at the protein level correlate with those observed at the RNA level (K–M) and RB is hypophosphorylated on multiple serine residues consistent with increased p16 and decreased CDK activity. TBX3, CAPERα = human; Tbx3, Capera = mouse.

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The following figure supplements are available for figure 2:

**Figure supplement 1.** Effective knockdown of endogenous CAPERα in primary human foreskin fibroblasts using viral shRNA transduction.
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**Figure supplement 2.** Effective knockdown of endogenous TBX3 in primary human foreskin fibroblasts using viral shRNA transduction.
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**Figure supplement 3.** Tbx3 null murine embryonic fibroblasts (MEFS) have altered lamin B1 localization, nuclear disruption and mislocalized Capera.
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**CAPERα/TBX3 regulates chromatin status of the p16INK promoter**

Increased p16 protein and RB hypophosphorylation in Tbx3−/− embryos and p16/RB-mediated senescence after CAPERα and Tbx3 KD could result from loss of direct repression of p16INK by CAPERα/TBX3 in proliferating cells. We screened 7 amplicons spanning ~6 kb upstream of p16INK by ChIP-PCR of HFF chromatin (Figure 3—figure supplement 2); 3 amplicons were bound by CAPERα and Tbx3 (Figure 3O, lanes 7, 10). Loss of either protein decreased the heterochromatic marks H3K9me3 (Figure 3O, lanes 14, 15) and H3K27me3 (Figure 3—figure supplement 3) and increased the euchromatic mark H3K4me3 (Figure 3O, lanes 17, 18). Notably, less CAPERα occupied p16INK elements after Tbx3 KD (Figure 3O, lanes 11) while the amount of Tbx3 bound post-CAPERα KD was comparable to control (Figure 3O, lanes 9 vs 7). This is consistent with the abnormal localization of CAPERα seen in Tbx3−/− MEFS (Figure 2—figure supplement 3D′–F) and indicates that CAPERα requires Tbx3 to occupy p16INK regulatory chromatin.

We examined whether CAPERα and/or TBX3 associate with promoters of other cell cycle genes that are transcriptionally disregulated after CAPERα/Tbx3 loss-of-function (Figure 2K–M). Antibodies against TBX3 and CAPERα ChIP’d the p14ARF initiator (Lingbeek et al., 2002) (Figure 3—figure supplement 4A);
Figure 3. RB and p16 mediate senescence after CAPERα/TBX3 loss of function and CAPERα/TBX3 regulates chromatin structure of CDKN2A-p16. (A–F) SA-βgal assays of HFFs stably transduced with control (Ctl) or p53 (Masutomi et al., 2003) or RB (Boehm et al., 2005) shRNAs subsequently transduced with CAPERα or TBX3 shRNAs. (G) % Quantitation of A–F from three replicate experiments. * indicates p<0.05 relative to Control or p53 shRNAs. (H) Cell proliferation assayed by crystal violet incorporation (OD units) in HFFs treated as in A–F. * indicates p<0.001 relative to Ctl or p53 shRNAs. (I–L) SA-βgal. Figure 3. Continued on next page
Figure 3. Continued

assays of HFFs stably transduced with control or p16 (Haga et al., 2007) shRNAs subsequently transduced with CAPERα or TXB3 shRNAs. (M) % Quantitation of I-L from three replicate experiments. * indicates p<0.05 relative to Ctl shRNA. (N) Cell proliferation assay by crystal violet incorporation (OD units) in HFFs treated as in I–L. * indicates p<0.01 relative to Ctl shRNA. (O) ChIP-PCR with antibodies listed at top on three regions upstream of the CDKN2A-p16 transcriptional start site (TSS); position relative to (TSS) is indicated in parentheses at left of panels. PCR of input material used for the ChIP is shown under ‘Input’. The shRNA transduced is listed above each lane (HFF Tx). TBX3 knockdown decreases binding of TBX3 (lanes 11) to all three regions. CAPERα knockdown has minimal effect on TBX3 binding (lanes 9). Knockdown of either TBX3 or CAPERα decreases the repressive chromatin mark H3K9me3 (lanes 14, 15) and increases the activating chromatin mark H3K4me3 (lanes 17, 18). TBX3, CAPERα = human; Tbx3, Caperα = mouse.

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The following figure supplements are available for figure 3:

Figure supplement 1. Effective knockdown of p53, RB and p16 in HFFs.
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Figure supplement 2. UCSC Genome Browser view of the CDKN2A locus and 5′ regions screened for binding by CAPERα and TBX3.
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Figure supplement 3. CDKN2A-p16 H3K27 trimethylation markedly decreases in HFFs after knockdown of CAPERα or TBX3 consistent with activation of CDKN2A-p16 expression.
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Figure supplement 4. Testing CAPERα and TBX3 binding to p14, p21, CDK2, CDK4, and CDKN1B regulatory elements.
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here too, TBX3 KD disrupted CAPERα binding (Figure 3—figure supplement 4A′, red arrowhead). Neither CAPERα nor TBX3 associated with amplicons scanning 1.8 kb upstream of CDKN1A-p21 or elements reportedly bound by TBX2 or TBX3 in other cell types (Figure 3—figure supplement 4B) (Prince et al., 2004; Saramaki et al., 2006; Hoogaars et al., 2008). Testing for association with known regulatory elements of CDK2, CDK4, CDKN1B was also negative (Figure 3—figure supplement 4C–E) (Baksh et al., 2002; Wang et al., 2005; Louie et al., 2010). These data indicate that in proliferating primary cells, CAPERα/TBX3 specifically and directly repress the CDKN2A locus by binding multiple regulatory sequence elements and regulating chromatin marks.

Expression of the IncRNA UCA1 is repressed by CAPERα/TBX3 and sufficient to drive senescence of primary cells

To identify novel genes repressed by CAPERα/TBX3, we employed differential display to detect transcripts that increased in response to KD of TBX3 and CAPERα in HEK293 cells (Figure 4A–C). Although most transcripts were unaffected by either KD, or changes were not shared (Figure 4—figure supplement 1A), DUSP4 and UCA1 were upregulated (Figure 4D, Figure 4—figure supplement 1B). DUSP4 is known to regulate cell survival and tumor progression, and overexpression induces senescence downstream of RB/E2F (Torres et al., 2003; Wang et al., 2007), thus placing CAPERα/TBX3 upstream of another p16/RB effector. Little is known about the function of the IncRNA UCA1 (Wang et al., 2006, 2008), so we investigated it further.

We found that shRNA KD of CAPERα or TBX3 in primary HFFs recapitulated the increase in UCA1 transcripts seen in HEK293 cells (Figure 4E–H). We then tested whether CAPERα/TBX3 directly control transcription of UCA1 by interacting with potential regulatory elements. Public ChIP data (http://genome.ucsc.edu/) indicate that the 2 kb upstream of UCA1 may contain such elements. We assayed 3 amplicons in this region (Figure 4I: A1, A2, A3) by ChIP-PCR of TBX3 and CAPERα: only region A3 was bound (Figure 4J,K, lanes 18, red arrowheads).

We next determined whether increased UCA1 expression in response to KD of CAPERα or TBX3 was associated with altered chromatin structure (as seen with p16 (Figure 3O)). UCA1/A3 is normally in a heterochromatin configuration in HFFs, with repressive marks H3K9me3 and H3K27me3 (Figure 4L, lanes 12, 14) and little H3K4me3 (Figure 4L, lane 18). After TBX3 KD, activating chromatin marks replaced repressive ones (Figure 4L, lanes 13, 15 and 19) and markedly less CAPERα was bound (Figure 4L, lane 17, red arrowhead). CAPERα KD also led to loss of repressive marks on UCA1/A3 (Figure 4M lanes 9, 16), although TBX3 remained bound (Figure 4M, lane 11, red arrowhead). Combined with previous findings, we conclude that: (1) TBX3 recruits CAPERα to UCA1/A3 chromatin, (2) TBX3 alone is insufficient to repress UCA1 and, (3) the default state of UCA1 in proliferating HFFs is repression conferred by CAPERα/TBX3.
Figure 4. CAPERα/TBX3 directly represses expression of the long noncoding RNA UCA1.  

(A–C) Gel showing RT-PCR analysis of TBX3, CAPERα, and HPRT expression in control, TBX3 and CAPERα siRNA-transfected HEK293 cells. The siRNAs effectively decreased transcript levels of their targets. (D) Differential display: representative PAGE gel of cDNAs derived from random primed, RT-PCR’d mRNAs from CAPERα, TBX3 and control siRNA transfected HEK293 cells. Blue arrowheads denote upregulated transcripts subsequently identified by sequencing as DUSP4 and UCA1.  

(E and F) qPCR analysis of TBX3 and CAPERα transcript levels in control and TBX3 or CAPERα shRNA transfected HEK293 cells. Results confirm differential display result that KD of TBX3 or CAPERα results in increase in UCA1 transcript levels. (I) Schematic representation of the UCA1 locus with primer sets employed for ChIP-PCR amplification of denoted regions 5′ of gene (A1, A2, A3). (J) Anti-TBX3 ChIP-PCR of regions of the UCA1 promoter in HFFs; only A3 is ChIP’d by TBX3 (lane 18, red arrowhead). (K) Anti-CAPERα ChIP-PCR of regions of the UCA1 promoter in HFFs; only A3 chromatin is ChIP’d (lane 18, red arrowhead). (L) ChIP-PCR analysis of UCA1/A3 chromatin from in HFFs transfected with control (C) or TBX3 (KD) shRNA; ChIP antibodies are listed at top. Note decreased CAPERα binding after TBX3 KD (lane 17, red arrowhead), gain of activating mark H3K4me3 and loss of repressive marks H3K9me3 and H3K27me3. (M) ChIP-PCR analysis of UCA1/A3 with antibodies listed at top of panel in HFFs transfected with control (C) or CAPERα shRNAs. Note continued TBX3 binding despite CAPERα KD (lane 11, red arrowhead) and changes in chromatin marks parallel those seen in with TBX3 KD in panel L. TBX3, CAPERα = human; Tbx3, Caperα = mouse.

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The following figure supplements are available for figure 4:

**Figure supplement 1.** Validation of differential display findings. 

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UCA1 modulates behavior of bladder cancer cell lines (Wang et al., 2008), but there are no data on its function in primary cells; our results suggest that UCA1 may be involved in premature senescence. UCA1 transcripts are low in proliferating HFFs, but 4 days after overexpression of UCA1 (Figure 5A), a robust SA-βgal response is evident (Figure 5B–D). Cells constitutively expressing UCA1 ceased proliferating during selection and accumulated SAHFs (Figure 5E,F). Cell proliferation decreased in a UCA1 dosage-sensitive manner (Figure 5G–I), consistent with reduced levels of cell cycle promoting transcripts and increased levels of pro-senescent ones (Figure 5J). These transcriptional changes were manifest at the protein level (Figure 5—figure supplement 1). Premature senescence resulting from overexpression of UCA1 in HFFs reveals that this lncRNA is a novel regulator of cell proliferation and may function as a tumor suppressor in some contexts.

**Loss of UCA1 delays the onset of oncogene-induced senescence**

We tested the hypothesis that UCA1 is required for induction of oncogene-induced senescence (OIS) in primary cells (‘RAS’: HFFs transduced with constitutively active G12V-RAS [Serrano et al., 1997]). There are markedly more UCA1 transcripts in RAS compared to presenescent ‘PS’ HFFs (Figure 5K). Knockdown of UCA1 in RAS HFFs reduced SA-βgal activity (Figure 5L–Q) and improved RAS cell growth: the number of Ki67 + RAS cells was increased at days 3 and 6 after UCA1 KD (Figure 5R, P0 and P1). However, by passage 2, the number of Ki67 + cells was not statistically different in UCA1 KD cells from control, despite persistently low levels of UCA1 (Figure 5S) and decreased levels of pro-senescent transcripts (Figure 5T). Overall, this indicates that senescence can occur in the absence of high levels of UCA1 but that timely execution of the OIS program requires UCA1.

We next investigated whether increase in UCA1 transcripts in OIS is a manifestation of loss of CAPERα/TBX3 occupancy/repression of UCA1/A3. Indeed, the repressor dissociates from UCA1/A3 in RAS HFFs and UCA1/A3 chromatin switches from heterochromatic to euchromatic marks (Figure 5U). This is consistent with the senescence-inducing effects of CAPERα/TBX3 loss-of-function (Figure 2) and resulting upregulation of UCA1 (Figure 4), and establishes CAPERα/TBX3 regulation of UCA1 in an independent model of senescence.

**UCA1 promotes senescence by sequestering hnRNP A1 to stabilize p16INK mRNA**

Some lncRNAs influence transcription by recruiting chromatin modifiers to target genes (Fatica and Bozzoni, 2014). We tested whether the increased levels of prosenescent transcripts occurring in response to UCA1 (Figure 5J) were the result activating chromatin changes however, ChIP-PCR assay for H3K9 acetylation of the p16INK, p14ARF, CDKN1A-p21 (and other) promoters did not reveal changes in this activating mark in response to UCA1 (Figure 5—figure supplement 2). We thus tested whether altered mRNA stability contributed to the observed changes. HFFs were transfected with UCA1 expression or control plasmid and after 2 days, treated with Actinomycin D. Total RNA was collected at 0–4 hr post-treatment and mRNA levels assayed using RT-PCR. Remarkably, overexpression of UCA1 resulted in the stabilization of mature p16INK, p14ARF, E2F1, and TGFβ1 mRNAs: in the time frame examined, p16INK, p14ARF, and E2F1 mRNAs do not decay and their t_{1/2} values are therefore denoted as ‘n’ (no decay). The half-life estimates shown were calculated using linear regression; those best fit lines, their equations and R values are shown in Figure 6—figure supplement 1. t_{1/2} of p16INK mRNA in control cells was 3.9 hr vs n in UCA1 overexpressing cells; p14ARF, 2.4 vs n; E2F1, 7.2 vs n; TGFβ1, 1.9 vs 2.9. In marked contrast, MYC, CDKN1A-p21, CDKN2D and RB mRNAs decayed at rates indistinguishable from control (Figure 6A; Figure 6—figure supplement 1). The effects of UCA1 overexpression on p16INK mRNA stability were confirmed by Northern blot (Figure 6—figure supplement 2).

Regulation of p16INK transcript stability is a critical mechanism for control (Wang et al., 2005; Chang et al., 2010; Zhang et al., 2012) and hnRNP A1 has been postulated to stabilize p16INK mRNA (Zhu et al., 2002), but this has not been tested. To this end, we treated HFFs with siRNA to hnRNP A1 and used Actinomycin D to assess stability of p16INK transcripts. Loss of hnRNP A1 (Figure 6—figure supplement 3) stabilized both p16INK (t_{1/2}=2.1 in control vs 12.3 after HNRNP A1 knockdown) and p14ARF mRNAs (t_{1/2}=1.5 in control vs 6.9 after HNRNP A1 knockdown) but not those of E2F1 or MYC (Figure 6B). Half-life estimates were obtained as described for panel A and the best fit lines, their equations and R values are shown in Figure 6—figure supplement 3B. The differences in control half-lives between Figure 6A,B are likely attributable to the different treatments used: in A, control cells were transfected with pcDNA3.1 plasmid, while in B, control cells were transfected with
Figure 5. UCA1 expression is sufficient to induce senescence and required for normal execution of oncogene-induced senescence. (A) UCA1 and HPRT transcripts assessed by RT-PCR in control and UCA1-overexpressing HFFs. (B and C) Representative bright field images of SA-βgal assay of cultured HFFs transfected with control and UCA1 overexpression plasmids. (D) % quantitation of SA-βgal cells from five replicates in control and UCA1 overexpressing HFFs. * indicates p<0.05. (E) and (F) Immunohistochemical analysis reveals co-localization of H3K9me3 and DAPI in SAHFs in HFFs transfected with UCA1 overexpression plasmid (F) but not control plasmid (E). (G) Cell count of control and UCA1 overexpressing HFFs 3 days post transfection. Mean ± SD of 3 plates is shown at each time point. * indicates p<0.05 relative to control. (H) Crystal violet assay of cell growth in control and UCA1 overexpressing HFFs transfected with 2 μg of expression or control vector and assayed daily for 3 days post transfection. * indicates p<0.01 relative to control. (I) Crystal violet assay of HFFs cultured for 3 days after transfecting 0, 1, 2, or 4 μg of control or UCA1 overexpression plasmid. * indicates p<0.01 relative to control. (J) Transcript levels assessed by qPCR; values reflect fold change in UCA1-overexpressing HFFs relative to control after normalization to HPRT levels. * indicates p<0.05 relative to control. (K) qPCR analysis of UCA1 expression in untransduced, presenescent (PS) HFFs and HFFs transfected with constitutively active G12VRAS (RAS). * indicates p<0.05 relative to PS. (L) Efficient knockdown of UCA1 transcripts in RAS HFFs with UCA1 shRNA (quantitated in panel T). (M–P) SA-βgal assays of RAS HFFs transfected with either control or UCA1 shRNA at 3 days post transduction. (Q) % quantitation of SA-βgal cells from six replicate experiments as represented in panels M–P. * indicates p<0.001 relative to control. (R) % quantitation of Ki67 + cells from three replicates in control vs UCA1 shRNA transduced RAS HFFs. * indicates p<0.001 relative to control. (S) RT-PCR for UCA1 transcripts shows persistent knockdown of UCA1 in RAS HFFs with increasing passage (P0–P2). (T) qPCR analysis of fold changes in transcript levels of cell cycle and senescence genes after UCA1 shRNA knockdown in RAS HFFs. * indicates p<0.05 relative to control. (U) ChIP-PCR analysis of UCA1 region A3 with antibodies listed at top in PS and RAS HFFs. Note gain of activating (H3K4me3, H3K9ace, H4K5ace) and loss of repressive marks (H3K9me3, H3K27me3) at the UCA1 locus after oncogene-induced senescence by RAS. TBX3, CAPERα = human; Tbx3, Capera = mouse.

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The following figure supplements are available for figure 5:

Figure supplement 1. Western blots showing changes in protein levels in response to UCA1 overexpression in HFFs.
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Figure supplement 2. ChIP-PCR assay for H3K9 acetylation of known regulatory elements of prosenescence and cell cycle genes whose expression is dysregulated after UCA1 overexpression.
DOI: 10.7554/eLife.02805.018
Figure 6. UCA1 stabilizes CDKN2A-p16 mRNA levels during senescence by sequestering hnRNP A1. (A) Graphs of transcript levels assayed by RT-qPCR in HFFs transfected with control (blue) or UCA1 (red) expression plasmids and treated with Actinomycin D. Y axis shows % mRNA level relative to time zero and X axis shows time in hours assayed post treatment. The estimated half-lives ($t_{1/2}$) were obtained using linear regression, the best fit lines, their

Figure 6. Continued on next page
control siRNA. The half-life of an mRNA is cell/context specific (as evident in the differences in control half-lives in 6A vs 6B) and in general, cell cycle regulatory genes have short half-lives (Sharova et al., 2009). The $t_{1/2}$ of $p16^{INK}$ mRNA we observed in HFFs transfected with either control plasmid ($t_{1/2}=3.9$) or control siRNA ($t_{1/2}=2.1$) is similar to that reported in HeLa cells ($t_{1/2}=2.9$) (Chang et al., 2010). The results we obtained were also similar to those reported for MYC mRNA (Herrick and Ross, 1994; Sharova et al., 2009), CDKN1A mRNA in HT29-tsp53 cells (Melanson et al., 2011) and ES cells (Sharova et al., 2009), and E2F1 mRNA in ES cells (Sharova et al., 2009). The half-lives of Rb and TGFβ1 are mRNAs extremely variable and those we obtained in HFFs were shorter than reported in ES cells (Sharova et al., 2009).

We next used RNA-IP (RIP) to determine if hnRNP A1 binds $p16^{INK}$ and $p14^{ARF}$ mRNAs in proliferating cells and found that this was indeed the case (Figure 6C, lane 6 and Figure 6—figure supplement 4). Remarkably, hnRNP A1/$p16^{INK}$ binding was lost in RAS HFFs (Figure 6C, lane 7), despite an overall increase in the number of $p16^{INK}$ transcripts (Figure 6C, lane 3). As shown previously, UCA1 RNA levels also increase with RAS (Figure 6D, lane 3). UCA1 is bound by hnRNP A1 in PS cells (Figure 6D, lanes 6, 7; Figure 6—figure supplement 5), but unlike $p16^{INK}$, the hnRNP A1/UCA1 interaction increases in RAS cells (Figure 6D, lane 7). TUG1 IncRNA serves as a negative control (Figure 6E). Protein levels for hnRNP A1 are shown in Figure 6F. The interaction between UCA1 and hnRNP A1 is specific, as UCA1 does not bind hnRNP K, C1/C2, H, U, or D (Figure 6—figure supplement 5). Although hnRNP A1 binds MYC and $p14^{ARF}$ mRNAs (Figure 6—figure supplement 4), it does not bind RB, p21, or CDK6 mRNAs under the numerous conditions tested (Figure 6—figure supplement 6).

The opposite binding properties of UCA1 and $p16^{INK}$ mRNA with hnRNP A1 in PS vs RAS HFFs led us to postulate that UCA1 stabilizes $p16^{INK}$ mRNA during OIS by disrupting the interaction between hnRNP A1 and $p16^{INK}$ mRNA. In control transfected proliferating cells, there is robust binding of $p16^{INK}$ to hnRNP A1 (Figure 6C, lane 13), but direct overexpression of UCA1 (Figure 6D, lane 10) or that resulting from TXB3 or CAPERa KD (Figure 6D, lanes 17, 18) disrupts the hnRNP A1/$p16^{INK}$ mRNA interaction (Figure 6C, lanes 14, 23, 24, red arrowheads). These findings support the hypothesis that loss of hnRNP A1/$p16^{INK}$ mRNA interaction in OIS (Figure 6C, lane 7) is the result of increased UCA1 expression and its binding and sequestration of hnRNP A1 (Figure 6D, lane 7). To further test this, we used shRNA to KD UCA1 in RAS HFFs (Figure 6D, lane 27). UCA1 KD restored the interaction between
hnRNP A1 and p16α mRNA (Figure 6C, lane 31) and led to lower levels of total p16α mRNA (Figure 6C, lane 27), a finding consistent with the negative effects of hnRNP A1/ p16α interaction on stability of p16α transcripts. The effects of UCA1 on p16α mRNA stability are specific, because hnRNP A1 interactions with MYC or p14ARF mRNAs are unaffected by UCA1 (Figure 6—figure supplement 1).

In total, these findings indicate that in proliferating cells, the very low quantity of UCA1 transcripts is insufficient to disrupt hnRNP A1/p16α binding, and levels of p16α mRNA are low due to: (1) direct repression by CAPERα/TBX3 and, (2) p16α mRNA instability conferred by hnRNP A1. When UCA1 levels increase during OIS, by UCA1 overexpression, or via KD of CAPERα/TBX3, UCA1 binds and sequesters hnRNP A1, preventing it from destabilizing p16α mRNA.

The CAPERα/TBX3 co-repressor dissociates during oncogene-induced senescence leading to activation of UCA1 and pro-senescence pathways

Increased p16 protein is required for RAS-induced senescence in MEFS and some human cell types (Serrano et al., 1997), leading us to determine whether OIS affects CAPERα/TBX3 occupancy of p16α chromatin. CDKN2A-p16α genomic regulatory elements bound in PS HFFs (Figure 4I) were not occupied by either TBX3 or CAPERα in RAS HFFs (Figure 7A). Chromatin marks on these regions switched from heterochromatic to euchromatic (Figure 7B, Figure 7—figure supplement 1A). This was also observed with UCA1/A3 (Figure 5U) and DUSP4 chromatin (Figure 7—figure supplement 1B).

We investigated the possibility that altered quantity of either CAPERα or TBX3 could disrupt the stoichiometry of their interaction and cause dissociation from p16α and UCA1 regulatory elements in OIS. Surprisingly, both TBX3 and CAPERα protein levels were increased in RAS HFFs (Figure 7C), but they no longer co-ip’d (Figure 7D, red box). Immunocytochemistry of endogenous TBX3 and CAPERα in PS and RAS HFFs confirmed increased protein levels in OIS (Figure 7F–M), and revealed dramatic changes in CAPERα localization: CAPERα immunoreactivity became concentrated in large intranuclear foci (Figure 7L,M), as we previously observed in early passage Tbx3−/− MEFS (Figure 2—figure supplement 2D–F). These foci are distinct from SAHFs and PML bodies (Figure 7M and Figure 7F—figure supplement 2).

To further investigate the molecular basis of senescence initiation after loss of CAPERα/TBX3, we performed genome-wide transcriptional profiling 2 days post CAPERα, TBX3 and control KD in HFFs. More than half of the transcripts with expression altered 1.5-fold or more by CAPERα KD were similarly affected by loss of TBX3 (N = 2375 CAPERα KD, 2188 TBX3 KD; 1157 co-regulated, p<<<<0.0001, Figure 7—source data 1–3, Figure 7N,O). Gene ontology-biologic process (GO-BP) analysis with DAVID (Huang da et al., 2009a, 2009b) showed highly significant co-regulation of ‘transcription regulation’ (increased expression) and ‘cell-cycle’ (decreased expression) transcripts (Figure 7N,O). We tested a subset of these with known roles in senescence by qPCR: 100% validated and were similarly altered by RAS (Figure 7—figure supplement 3). Further interrogation of this group revealed that IL6 and HDAC9 are CAPERα/TBX3 direct targets and their upregulation in RAS is associated with loss of CAPERα/TBX3 binding (Figure 7—figure supplement 4).

We compared CAPERα/TBX3 co-regulated transcripts to a published data set comparing PS and G12V RAS fibroblasts (Loayza-Puch et al., 2013). This revealed that 11% of CAPERα/TBX3 up-regulated transcripts were also increased by RAS (Figure 7N′); among these, GO-BP ‘programmed cell death’ (31%) and ‘transcription regulation’ (34%) were highly overrepresented. 30% of CAPERα/TBX3 downregulated transcripts were also in the RAS data set; >1/3 of these were cell cycle genes (Figure 7O). In all comparisons, the number of transcripts common to both groups was greater than predicted by chance and highly statistically significant (Figure 7—source data 3). KEGG pathway analyses revealed overrepresented pathways that were common to both CAPERα/TBX3 and RAS data sets (Figure 7N′–O′, pie charts), but notably fewer pathways were shared in the upregulated data sets: JAK/STAT, TLR and TGFβ signaling pathways were only significantly overrepresented in the CAPERα/TBX3 data set.

**Discussion**

Our knowledge of the regulatory mechanisms that govern the onset and maintenance of senescence in different contexts must be considered fragmentary (Wang and Chang, 2011; Fatica and Bozzoni, 2014). In this study, we provide compelling evidence for critical and novel functions of CAPERα, the IncRNA UCA1 and TBX3 in the regulation of cell proliferation and senescence. We have discovered a CAPERα/TBX3 complex that is required to prevent senescence of primary human and mouse cells in
Disruption of the CAPERα/TBX3 repressor by OIS activates CDKN2A-p16 and UCA1 to trigger a senescence transcriptional response.

(A) ChIP-PCR of regions upstream of the CDKN2A-p16 transcriptional start site (position relative to TSS in parentheses) in PS and RAS HFFs; the −3706–3308 amplicon is a negative control. OIS disrupts binding of p16 regulatory elements (initially identified in Figure 3O) by TBX3 and CAPERα. (B) ChIP-PCR of p16 −4855 element shown in A. Decreased TBX3 and CAPERα binding in RAS correlates with loss of repressive chromatin marks and gain of activating...
marks. Evaluation of chromatin marks on the other CDKN2A-p16 CAPERα/TBX3- responsive regulatory elements is shown in Figure 7—figure supplement 1A. (C) IBS for TBX3, CAPERα, and actin loading control show increased amount of both proteins in RS compared to PS HFFs. (D) Anti-TBX3 and anti-CAPERα IBS of IP’d proteins from PS and RS HFFs. (F–M) Immunocytochemical staining of PS (F, G, J, K) and RS (H, I, L, M) HFFs for TBX3 (F and H), Hoechst (DNA; G and I), CAPERα (J and L). Panels K and M are merged Hoechst/CAPERα. Scale bar for all panels is sown at lower right of panel I. (N–O) Functional analyses of genome wide transcriptional profiles of TBX3 KD, CAPERα KD, and control HFFs. All comparisons were statistically significant with p values <<<<0.0001; see Figure 7—source data 3 for hypergeometric test, as implemented in the R statistical language, used to test significance of the number of genes found to be co-regulated between samples. (N) Venn diagrams show highly significant number of CAPERα/TBX3 co-upregulated transcripts (446 total), especially in the GO biologic process (BP) category of transcriptional regulation (122 transcripts) as assayed with qPCR validation of coregulated genes. Pie chart in S. Figure 6A. Pie chart shows KEGG pathway analysis of co-regulated genes. (O) Venn diagram showing 48 CAPERα/TBX3 co-upregulated transcripts also upregulated by RAS/OIS (Loayza-Puch et al., 2013), especially in BP categories of transcriptional regulation and programmed cell (pc) death. qPCR validation of coregulated genes is in S. Figure 7A. (P) As in N and O but for downregulated genes. Pie chart in O’ shows KEGG pathway analysis of OIS dataset. (Q and O) As in N and O but for downregulated genes. Pie chart in O’ shows KEGG pathway analysis of OIS dataset.

The following source data and figure supplements are available for figure 7:

Source data 1. Differentially expressed genes after knockdown of CAPERα in HFFs detected by RNA-Seq.
DOI: 10.7554/eLife.02805.027

Source data 2. Differentially expressed genes after knockdown of TBX3 in HFFs detected by RNA-Seq.
DOI: 10.7554/eLife.02805.028

Source data 3. Determining the statistical significance of shared differentially expressed genes using the hypergeometric test, as implemented in the R statistical language (phyper).
DOI: 10.7554/eLife.02805.029

Figure supplement 1. Repression of CDKN2A-p16 and DUSP4 by CAPERα/TBX3 correlates with chromatin architecture and is relieved during oncogene induced senescence.
DOI: 10.7554/eLife.02805.030

Figure supplement 2. CAPERα relocalization due to oncogene-induced senescence is independent of PML bodies.
DOI: 10.7554/eLife.02805.031

Figure supplement 3. Validation of RNA-Seq identified expression changes induced by CAPERα and TBX3 KD.
DOI: 10.7554/eLife.02805.032

Figure supplement 4. IL6 and HDAC9 are direct targets of CAPERα/TBX3.
DOI: 10.7554/eLife.02805.033

vivo and that functions as a master regulator of cell proliferation by directly repressing transcription of IncRNA UCA1, p16INK4a and other tumor suppressor genes (Figure 7P). Overexpression of UCA1 occurs after loss of TBX3/CAPERα and in OIS (Figure 7Q), and is itself sufficient to induce senescence at least in part, by disrupting the interaction of p16INK4a mRNA with hnRNP A1 leading to increased p16INK4a mRNA stability (Figure 7R,Q). Disrupting the CAPERα/TBX3 complex by decreasing the amount of either TBX3 or CAPERα, or by CAPERα mislocalization during OIS, coordinately increases activity of multiple pro-senescence targets at both the transcriptional and post-transcriptional levels in a reinforcing mechanism.

Increased CAPERα has been reported in human breast cancers and a shift from cytoplasmic to nuclear localization correlates with transition from pre-malignant to malignant lesions (Mercier et al., 2009). In contrast, CAPERα co-activates vRel mediated transcription but inhibits vREL transforming activity in vitro (Dutta et al., 2008). It is likely that anti- or pro- oncogenic activity of CAPERα is determined by cell type and the interacting protein(s) present in a given context; our results suggest that CAPERα has oncogenic potential in primary cells since loss of CAPERα/TBX3 induces premature senescence, a vital tumor suppressor mechanism. CAPERα binds to regulatory chromatin domains via TBX3 but dissociates from these domains and becomes concentrated in large intranuclear foci prior to senescence induced by loss of TBX3 or during OIS. Future efforts will define the composition of CAPERα + nuclear foci and the role of this nuclear subdomain during senescence induction.

The TBX3 RD is required for TBX3 to interact with CAPERα (this study), immortalize primary fibroblasts and confer senescence bypass (Carlson et al., 2001). Since loss of CAPERα activates target gene transcription despite continued TBX3 occupancy, it is the CAPERα/TBX3 complex (interacting via
TBX3 RD) that represses pro-senescent target loci. It will be important to determine if previously identified targets of TBX3 transcriptional repression are actually regulated by this complex.

Additional studies are warranted to determine the precise mechanisms whereby histone status is regulated by CAPERα/TBX3: TBX3 is known to interact directly with HDACs (Yarosh et al., 2008), but there are no reports of it or CAPERα interacting with histone methyltransferases or demethylases. Our recently published Mass Spec screen for Tbx3/TBX3 interactors did not identify such factors however, the screen cannot be considered exhaustive as we did not reproducibly detect HDACs or transcription factors previously reported to interact with Tbx3. Future studies to specifically determine whether TBX3 and/or CAPERα interact with, recruit, or modify the function of EZH2, SUV39 and other methyltransferases will be informative.

Previous studies showed that TBX3 represses transcription of p14ARF (upstream of p53) (Bamshad et al., 1997; Fan et al., 2009; Kumar et al., 2014), yet embryonic lethality and mammary phenotypes of Tbx3 mutants are p53-independent (Jerome-Majewska et al., 2005). Our findings reconcile these observations because CAPERα/TBX3 represses p16INK, the p16/RB pathway is activated in Tbx3−/− embryos, and knockdown of either RB or p16 (but not p53) prevents senescence after loss of CAPERα/TBX3. Furthermore, Tbx3−/− and Cdk2−/−;Cdk4−/− mutant embryos share multiple phenotypes including RB hypo-phosphorylation, reduced E2F-target gene expression, decreased proliferation and premature senescence of MEFs (Berthet et al., 2012; Frank et al., 2012, 2013). Our discoveries of multiple CAPERα/TBX3 binding sites across the CDKN2A locus, and altered chromatin marks after Tbx3 and CAPERα KD, indicate that the complex directly represses transcription by regulating chromatin structure. In total, the data conclusively demonstrate that p16 elevation, CDK2 and CDK4 down-regulation, and RB hypophosphorylation mediate senescence downstream of CAPERα/TBX3 loss of function in primary human cells and Tbx3 null mutant embryos. When combined with the pleiotropic effects of CAPERα/TBX3 on UCA1, DUSP4, IL6, HDAC9 and other pathways, it is clear why loss of this repressor induces senescence.

TBX3 may function in nuclear organization and structure: severe changes in nuclear morphology and mislocalization of both CAPERα and laminB1 are apparent in Tbx3−/− MEFs after just one passage, prior to other signs of senescence. Progeria is a rare disease in which LMNA mutations induce cellular and organismal senescence in part by altering stoichiometry and interactions of type A and B Lamins. Progeria fibroblasts have decreased expression of TBX3, TBX3 interacting proteins, and TBX3 targets (Csoka et al., 2004). LMNβ1 is a TBX3 interacting protein (Kumar et al., 2014) and expression of LMNA, LMNβ1, and LMNβ2 is disrupted by Tbx3/CAPERα KD (Figure 7-source data 1–3 and Figure 7—figure supplement 3). Tbx3 may regulate LMN gene expression and physically interact with LaminB to influence nuclear homeostasis.

There are many downregulated genes common to the senescence responses triggered by RASG12V and loss of CAPERα/TBX3 however, upregulated transcripts and pathways are largely distinct (Figure 7N). This is likely attributable to the presence of direct targets of CAPERα/TBX3 repression in the upregulated data set. It will be informative to determine which Jak-STAT, TLR, and TGFβ pathway members (Figure 7N) are direct CAPERα/TBX3 targets, as the complex roles of these pathways in the senescence associated secretory phenotype, inducing or enforcing autocrine and paracrine senescence, and tumor progression are emerging (Hubackova et al., 2010; Senturk et al., 2010; Hubackova et al., 2012; Davalos et al., 2013).

Recent discoveries of the pervasive functions of lncRNAs as ‘signals, decoys, guides and scaffolds’ (Wang and Chang, 2011), conferred by their ability to interact with other nucleic acids and as protein ligands, has added new layers of complexity to regulation of transcriptional and post-transcriptional gene expression and translation. Although there has been a logarithmic increase in studies exploring lncRNA expression and activity, potential senescence-regulating activities are still largely unexplored. LncRNA HOTAIR functions as a scaffold to regulate ubiquitination of Ataxin-1 and Snuportin-1 to prevent premature senescence (Yoon et al., 2013). Global alterations in lncRNA expression have been reported in association with replicative senescence (Abdelmohsen et al., 2013), and telomere-specific lncRNAs that regulate telomere function during this process have been identified (Yu et al., 2014). As this manuscript was in revision, regulation of H4K20 trimethylation of rRNA genes by interaction of quiescence-induced lncRNAs PAPAS and Suv4-20h2 was reported (Bierhoff et al., 2014). To our knowledge, UCA1 is the first lncRNA sufficient to induce senescence.

UCA1 is expressed in bladder transitional cell carcinomas (Wang et al., 2006) and influences tumorigenic potential of bladder cancer cell lines (Wang et al., 2008; Yang et al., 2012). A very recent
study identified hnRNP I as a UCA1 interacting protein that stabilizes UCA1 RNA; this interaction was postulated to decrease translation of p27 to support growth of the MCF7 breast cancer line (Huang et al., 2014). In contrast, our results support a tumor suppressor/proliferon function for UCA1 in primary cells. UCA1 increases stability of p16\textsuperscript{INK} mRNA by sequestering hnRNP A1, employing a decoy mechanism that is in some aspects reminiscent of lncRNA PANDA sequestering NF-YA transcription factor to prevent activation of proapoptotic p53 targets and promote cell cycle arrest in the DNA damage response (Wang and Chang, 2011). In the case of UCA1 and hnRNP A1 however, the sequestration has a very specific effect: even though UCA1 expression stabilizes (and hnRNP A1 destabilizes) both p16\textsuperscript{INK} and p14\textsuperscript{ARF} mRNAs (Figure 6A,B), UCA1 only disrupts the association of hnRNP A1 with p16\textsuperscript{INK} mRNA (Figure 6C and Figure 6—figure supplement 4). In proliferating cells, abundant hnRNP A1 binds with p16\textsuperscript{INK} mRNA resulting in p16\textsuperscript{INK} degradation. In senescent cells, p16\textsuperscript{INK} mRNA levels increase via reinforcing mechanisms of increased transcription and stability: loss of CAPER\textsubscript{α}/TBX3 activates transcription of p16\textsuperscript{INK} and UCA1, in turn, UCA1 sequesters hnRNPA1.

We recognize that the systems we employed (primary HFFs, mouse embryos and MEFs), while very informative, provide limited information directly applicable to aging or tumorigenesis without further experimentation. Our data support an important role for CAPER\textsubscript{α}/TBX3 in regulation of senescence in developmental contexts and, since the CAPER\textsubscript{α}/TBX3 complex regulates known critical tumor suppressors and there is an increasing literature supporting roles for both TBX3 and CAPER\textsubscript{α} in tumor biology, this is another worthy area for future investigation. As noted above, expression of CDKN2A-p14\textsuperscript{ARF} and CDKN1A-p21\textsuperscript{CIP} are repressed by TBX2 and TBX3 and this is postulated to confer the ability of overexpressed TBX2 and TBX3 to permit senescence bypass of Bmi1\textsuperscript{−/−} and SV40 transformed mouse embryonic fibroblasts, respectively (Jacobs et al., 2000; Brummelkamp et al., 2002; Prince et al., 2004). Numerous overexpression studies have suggested a role for TBX3 in breast cancer (Liu et al., 2011) and references therein) and recent papers have reported the tumorigenic and proinvasive effects of overexpressed TBX3 in melanoma cells (Peres et al., 2010; Peres and Prince, 2013) which may derive in part from TBX3 repression of E-cadherin expression (Rodriguez et al., 2008). More relevant to our work on the importance of the CAPER\textsubscript{α}/TBX3 complex to prevent senescence and regulate cell proliferation are reports that Tbx3 improves the pluripotency of iPS cells (Han et al., 2010) and prevents differentiation of mouse ES cells (Ivanova et al., 2006).

In conclusion, CAPER\textsubscript{α}/TBX3 acts as a master regulator of cell growth and fate, exerting pleotropic effects by at least two modes of action: (1) regulating chromatin structure and transcription of both coding and non-coding genes and, (2) modulating mRNA stability by altering the association of RNA binding proteins with target transcripts via UCA1. Further exploration will identify tissue-specific UCA1 targets and binding proteins, and determine whether the ability of TBX3 to confer senescence bypass in other contexts requires CAPER\textsubscript{α} interaction and/or UCA1 repression. Mining the pathways regulated by UCA1 and CAPER\textsubscript{α}/TBX3 will reveal factors that control cell proliferation and fate during development and disease and thus constitute novel cancer therapeutic targets.

**Material and methods**

**Mass spectroscopy**

Mass spectroscopy as in Kumar et al., (2014)

**Protein extraction and immunoprecipitation**

Dignam lysates were prepared and incubated for 4 hr at 4°C with the appropriate antibody followed by 2 hr at 4°C with the pre equilibrated Dynabeads Protein G (Invitrogen). Immune complexes were collected and washed three times with lysis buffer. Pelleted beads were resuspended in 6X Laemmli buffer and subjected to SDS-PAGE analysis followed by immunoblotting with specific antibodies.

Input lanes contain 5% of protein lysate used for IP; the rest was used in the IP and of the IP’d material, 25% was loaded onto the gel for immunoblotting.

**Antibodies**

Tbx3 (Frank et al., 2012, 2013), TBX3 (SC-17871,MAB10089,A300-098A), CAPER\textsubscript{α} (A300-291A), GST (SC-33613), LaminB1 (SC-56144), C-Myc (SC-40), R-IgG (SC-2027), m-IgG (SC-2025), Anti-Flag (Sigma, F3165), H3K9me3 (Cell Signaling, 9754), H3K4me3 (Cell Signaling, 9751), H3K27me3 (Cell Signaling, 9733), H3K9ace (Cell Signaling, 9649), H4K5ace (Cell Signaling, 9672), H3K14ace (Cell Signaling, 4353), p-RB -Ser 810–811 (SC-16670), p-RB -Ser 795 (SC-7986), p-RB -Ser 780 (SC-12901), Rb1
MBP pull down assay

Amylose bound MBP and MBP-tagged TBX3 affinity columns were prepared as per the procedure (E8022S, NEB) described in the manufacturer’s protocol. These beads were incubated with 5 and 10 μg of GST and GST-CAPER at 4°C for 8 hr. Bound proteins were eluted with reduced glutathione and analyzed by Western blotting with anti-CAPER antibody.

Cell transfection

Transfections were performed in HEK293 or EBNA-293 cells with Lipofectamine 2000 (Invitrogen) or in Human fibroblasts with X-tremeGENE HP DNA transfection Reagent (Roche) as per the manufacturer’s recommendations.

Plasmids

Wild-type Tbx3 and exon 7 missense, deleted repressor domain (Tbx3ΔRD1), and Tbx3ΔNLS were generated by PCR amplification and cloned into pcDNA3.1. C-terminal deletion constructs Tbx3 1-655, Tbx3 1-623, Tbx3 1-565, Tbx3 1-470 were generated by PCR amplification and cloned into pCS2 with an N-terminal Myc tag. Tbx3 L143P and N277D point mutants were kind gifts of Phil Barnett. UCA1 and CAPERΔ cDNAs were cloned into pCDN3.1 and PQCXIH for over-expression studies, respectively. Sequence of all plasmids was confirmed. Tbx3 L143P and N277D point mutants plasmids were kind gifts of Phil Barnett. Wild-type CAPERΔ was generated by PCR amplification and then cloned into pQCXIH retroviral vector; sequence was confirmed. Full length UCA1 was amplified by PCR and then cloned into pcDNA3.1 vector; sequence was confirmed.

TBX3 shRNA A: targets TBX3 exon 7

TBX3 shA FP: CCGG GACCATGGAGCCCGAAGAA ttcaagaga TTCTTCGGGCTCCATGGTC TTTTTG
TBX3 shA RP: AATTCAAAAA GACCATGGAGCCCGAAGAA tctcttgaa TTCTTCGGGCTCCATGGTC

TBX3 shRNA B: targets TBX3 exon 5

TBX3 shB FP: CCGG CAGCTCACCCTGCAGTCCA ttcaagaga TGGACTGCAGGGTGAGCTG TTTTTG
TBX3 shB RP: AATTCAAAAA CAGCTCACCCTGCAGTCCA tctcttgaa TGGACTGCAGGGTGAGCTG

Immunofluorescence

E10.5 embryos were harvested in PBS followed by overnight fixation at 4°C in 4% paraformaldehyde and processed for 7 μm cryosections. For cell lines, human fibroblasts were cultured on 8-well chamber slides (BD Flacon) and processed for Immunohistochemistry. Immunohistochemistry was performed using primary antibodies listed above and detected using donkey anti-goat or anti-rabbit Alexa fluor 594 (1:500) and goat anti-mouse Alexa fluor 488(1:500) from Invitrogen. Nuclei were stained with Hoechst or DAPI. Slides were imaged with a Nikon ARI inverted confocal microscope at the University of Utah Imaging Core.
CAPERα shRNA A: targets CAPERα (gene name RBM39) exon 5
CAPERα shA FP: CCGG GACAGAAATTCAAGACGTTCgagaaAAGGCTTTGAAAAATTTCTGCTTTT
CAPER shA RP: AATTCAAAAA GACAGAAATTCAAGACGTTC tctcttgaa AAGGCTTTGAAAAATTTCTGCTTTT
CAPERα shRNA B: targets CAPERα exon 1
CAPER shB P: CCGG AAGGCAAGAGCAAGATCGTA attcagagaTACGACTTTCTGCTTTT
CAPER shB RP: AATTCAAAGG AAAAGCAAGAGCAAGACGTTC tctcttgaa TACGACTTTCTGCTTTT

The pMKo.1 puro RB and pMKo.1 puro p53 shRNA vectors were a kind gift of William Hahn obtained via Addgene.

pRB shRNA: Addgene #10670
p53 shRNA: Addgene #10672
p16 shRNA: Addgene #22271

Efficacy and specificity of the pRb, p53, and p16 shRNAs was validated with second shRNAs, and these reagents have been used extensively by many investigators in the years since their initial publication (Masutomi et al., 2003; Stewart et al., 2003; Boehm et al., 2005; Haga et al., 2007; Hong et al., 2009; Elzi et al., 2012).

UCA1 shRNA: targets UCA1 exon 3
UCA1 shA FP:
GATCCGTTAATCCAGGAGACAAAGA tcagagaTCTTTGTCTCCTGGATTAACTTTTTTGGA
UCA1 shA RP:
AGCTTCCAAAAAAGTTAATCCAGGAGACAAAGActcttgaTCTTTGTCTCCTGGATTAACG
Senescence associated β-galactosidase assay
Performed as per the manufacturer’s protocol (9860, Cell Signaling).
Population doubling assay/3T5 growth curves (Figure 2E,F,R)

Primary HFFs were plated in a 10-cm dish and transduced with retrovirus. After 24 hr, cells were cultured with antibiotic selection (puromycin or blasticidin) for an additional 24–72 hr. On day 0 of the 3T5 growth curve, cells were trypsinized, counted and 500,000 cells were then plated per 10-cm dish. This procedure was repeated every 3 days for 15 days. Population doublings were calculated by (logN1/log2) − (logN0/log2) N1 = current cell count, N0 = initial cell count. Curves shown in Figure 2 are representative of two independent experiments.

Cell count (Figure 5C)
Primary HFFs were plated in 6-well dishes and transfected at 70% confluence. At days noted in the figure, cells were trypsinized and counted using a hemocytometer.

Crystal violet assay/optical density method of cell quantitation
5 × 10^5 cells were plated per well in 6-well tissue culture plates. At times indicated, medium was removed and cells were washed with PBS, and fixed for 10 min in 10% formalin solution. Cells were rinsed 5X with distilled water, and then stained with 100 μl 0.1% crystal violet solution for 30 min, rinsed 5X in water and dried. Cell-associated crystal violet dye was extracted with 500 μl of 10% acetic acid. Aliquots were collected and optical density at 590 nm measured. Each point on the curve shown represents three independent plates.

Senescence marker gene expression in TBX3 and CAPERα KD fibroblasts
Primary HFFs were incubated with TBX3 or CAPERα or Control shRNA encoding retrovirus medium with fresh virus added every 8 hr for 48 hr, followed by antibiotic selection for 6 days. 6 days after selection, floating cells were discarded and adherent cells were utilized for senescence associated β-gal assay or preparation of RNA.

RNA isolation and reverse transcription-PCR analysis
Total RNA was prepared using the RNeasy RNA isolation kit (Qiagen) or NucleoSpin RNA II Kit (Clontech) and cDNA was synthesized by cDNA EcoDry Premix Double Primed (Clontech) kit. Q-RT-PCR was performed with SoFast Evagreen Supermix (Bio-Rad) as per manufacturer’s protocol.
### RT-PCR primer sequences

| Gene       | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|------------|------------------------|------------------------|
| TBX3       | TGAGGCCCTTTGAAGGAAATCG | TCAGCAGCTATAATGTCACATC |
| CAPERα     | CGGAACAGGGCTTTAGGAAGAG | TGCCACTGCTCAACTTGGTC   |
| CDK2       | GCTTTCTGGCCATTCTCATG   | GCCTCAGCCGATCCGAAAGAT |
| CDK4       | ACGGGTGTTAAGTGCCATCTGG| TGTTGTCGGTGCCCTATGGAAG |
| P21        | TCAGAGGGGCCGCTGTTGCTGT | TGTCAGGTGGAGTGTCAGTC   |
| CDC2       | GGGGATTCAGAAATTGATCA   | TGTCAGAAGGCTATGCTAAG   |
| MDM2       | ACTTCTACAGATTTTCCATGT | TTCTCATGTAATAGTGTCATTT |
| MAPK14     | TCTGTTTGACCTCCCTGACTG | ACACACATGACACGAGCACCTAC |
| CDKN2C     | TGGGATTCAGAAATTGATCA   | TGTCAGAAGGCTATGCTAAG   |
| P16        | CAACGCACCAAGAATAGTCAG | GCACAAGGCTATGCTAAG     |
| SerpinE1   | CCGGAACAGGCTGAAAGATGG | GTGTTTCTCGAGGTTGGGCC   |
| P14ARF     | CCCTCCTGCTATGCTACTG    | ACCTGTTCTTGGAGGACC     |
| MCM3       | CTTTTTCCTCCAGCTGCTGC  | CTCTGAGGATCTTGGGTGTTCT |
| TGFβ       | GGACGGGATTAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| EGR1       | CCGGAACAGGCTGAAAGATGG | GTGTTTCTCGAGGTTGGGCC   |
| E2F1       | AGTTTTTCTCTGGCCTCGAG  | ATCTGTGAGGAGGATGAGG    |
| IL6R       | GGGCAAGAAACCACATCAG   | CCGGAGTTATGCGGTTGTA    |
| CHK2       | TTTTTTCCGAAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| MYC        | ACTTCTACAGATTTTCCATGT | TTCTCATGTAATAGTGTCATTT |
| CDKN2D     | TTTTTTCCGAAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| RB         | TGTTAGGGAAGCTGATGCA    | TCTGTTGAGGTTTTCTGCTG  |
| CXCL10     | GAATATTTCTTGGCAAACGAAT | TCACCTGGCTTGGATGACCA   |
| IFNB1      | GAGAGGAGGAGGAGGAGGAG  | TGGTGAATAAGGAGGAGGAG  |
| CH2K2      | TTTTTTCCGAAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| PMAIP1     | GTTTTTTCCGAAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| MYC        | ACTTCTACAGATTTTCCATGT | TTCTCATGTAATAGTGTCATTT |
| CDKN2D     | TTTTTTCCGAAGAATCTCGAG | AGTTCGAGGCTAGCTAGAGCT |
| P53        | CCTTCCACTCCAGCTGCTGC  | CTCTGAGGATCTTGGGTGTTCT |
| RB         | TGTTAGGGAAGCTGATGCA    | TCTGTTGAGGTTTTCTGCTG  |
| HMGA2      | GTCCTTTCTTTGGCAGCTCAA | TCTCCTGGCTTAAAAGATCCTAC |
| BIRC5      | CATGGGAAGGAGGAGGAGGAG  | TGGTGAATAAGGAGGAGGAG  |
| ASF1       | GGTGGCGAGGATCTTCCGAG  | CATGGTAGGGTGAGGTGAGT   |
| WD6R6      | CCGAGAAGCAACAGGAGGAAG | CTGTGTCCTCAGACACAGTCA |
| CDC25C     | GCACAGGAGGAGGAGGAGGAG  | TGGTGAATAAGGAGGAGGAG  |
| CENPF      | CAGAACATGGGACACCTCAGTG | TTCTGGAGGAGGCTAGTGAATT |
| LAM2A      | ATTAATCTGGCCTGTCTGAT   | TTCTCCAATAGGCGAATCTTC |
| LMBNB1     | AAGCAGGCTGAGTGTGTTTTG | TTGGAGTGCCTTTGGGGTGC  |
| LMNB2      | GCTCTGGACCAAACGACAAGAG | CCCAGCATCTTCCGAGACTTG |
| CDC20      | TCTAAGGCTTGGGACACCCCTC | GCATTGCCAGGCAAGCAGCAG |
| DUSP5      | GCTGGTGATGATGATGATGATG | GCATTGCCAGGCAAGCAGCAG |
| DUSP4      | GCTGGTGATGATGATGATGATG | GCATTGCCAGGCAAGCAGCAG |
| mTbx3      | TGAGGCCCTTTGAAGGAAATCG | TCAGCAGCTATAATGTCACATC |
| mSerpinE1  | AGCCAACAGAGGAGCAGAC   | GGATTTCTCGAGGAGGAAAG   |
| mIL6       | GATGGGATGCTGACTAAGCAGT | GCAGAGGATGCTGACTAAGCAG |
| mP21       | TCCACAGGCGATACCCAGACA | GGCACACTTGGCTCTGCTG    |
mCdc2: CTGCAATTGGAAAATCTCT, TCCATGGCAGGAACTCAA
mReprimo: CTTACGGACCTGGGACTTTG, CCAGCAGTGAATCCACAC

**MEF isolation from WT and Tbx3 null embryos**

All steps were performed under aseptic conditions. Pregnant female mice were euthanized and 13.5-day-old embryos were isolated from the uterus. Embryos were washed in sterile PBS in 60-mm tissue culture dish at room temperature and transferred into 15-ml sterile falcon tube containing 1 ml of 50% trypsin in DMEM medium. Embryos were minced using fine scissors followed by gentle pipetting with 1 ml pipette tips and dispersed into cell suspensions in 5 min. Suspensions were plated into 10-cm plates in 10 ml of DMEM with 5% FBS and penicillin/streptomycin and incubated for 8 hr in CO₂ incubator. Culture medium was replaced with fresh medium every day for 3 days. Passage 0 refers to the stage when cell suspension from the embryos was put into cell culture and subsequent passages are numbered.

**Chromatin immunoprecipitation (ChIP)**

Performed as per the manufacturer’s protocol (9003S, Cell Signaling).

ChIP primers

UCA1 FP1: GGCTCTCGAGTCAAGATAATTCACTTAC
UCA1 RP1: GGCACATCTTTTGTCTGTGAAAGGAT
UCA1 FP2: CACCTCTTCTGCTCCTTGATATT
UCA1 RP2: CACTTACTAATTTAATAGGATCGGCTCT
UCA1 FP3: CAGGAGGCTGATATCGCCCTC
UCA1 RP3: CTGGGCTCTGTGACCCACCTGGACAT
DUSP4 FP: CGAGGGGACCAGTGCTACCAGGCGGCCTTTG
DUSP4 RP: GGGACGAGGAGCGAGGCTTCTCC
P16 1A FP: CGACCCTGAAACTTACGCTGGGAGCAG
P16 1A RP: GCTCTGAGGAGGGCTGGCCCTTGCTGAGC
P16 2A FP: GAGCAGGAGGGGCCTGCTACCTCAATAT
P16 2A RP: GAGCAGGAGGGGCCTGCTACCTCAATAT
P17 1A FP: GACCGATGCGCCACACGAGCCTCTATT
P17 1A RP: CCGTTGGAACGGTGTGCTGGCAAGAG
P17 2A FP: GAGCAGGAGGGGCCTGCTACCTCAATAT
P17 2A RP: GAGCAGGAGGGGCCTGCTACCTCAATAT
P17 3A FP: CCTCGGGGTACCTCTTAATTAGCTGTGTA
P17 3A RP: AGTTCGAAAGAAAAGCCAACATCTGGT
P17 4A FP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 4A RP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 5A FP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 5A RP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 6A FP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 6A RP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 7A FP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P17 7A RP: GGGTGGAAGGGCTGAGAGGGCATGGGC
P14ARF FP: GCCGAATCCGGAGGGGTACCCAGCCGGAGG
P14ARF RP: GTGCCGAGGCTGAGGCTGAGGCTGAGGCT
CDK2 FP: GATGGAAAGCGAGATATCTCT
CDK2 RP: AAAAAAGAATCCGGCCGG
P21 −324 to −676 FP: CCGGGAAGCATGTGACAATCT
P21 −324 to −676 RP: CCGGGAAGCATGTGACAATCT
P21 −677 to −981 FP: CCGGGAAGCATGTGACAATCT
P21 −677 to −981 RP: CCGGGAAGCATGTGACAATCT
P21 −964 to −1340 FP: CCGGGAAGCATGTGACAATCT
P21 −964 to −1340 RP: CCGGGAAGCATGTGACAATCT
P21 −1335 to −1688 FP: CCGGGAAGCATGTGACAATCT
P21 −1335 to −1688 RP: CCGGGAAGCATGTGACAATCT
CDKN1B FP: CGGCCCTTGGCTGAGGCTTGGCTGAGGCT
CDKN1B RP: CGGCCCTTGGCTGAGGCTTGGCTGAGGCT
siRNA knockdown
For differential display (Figure 4), HEK293 cells were transfected with control siRNAs (Sense; 5′-CAGCGACUAAACACUAAC-3′ Antisense; 5′-UUGAUGUGUUAGUCGUGTT-3′) or TBX3 specific siRNA A (Sense: GACCGAGGCCCGAAGAA, Antisense: UUCUUCGGCUCCAUGGU) or CAPEPa-specific siRNA (Sense: CAGACAGUAAUCAAGCAGGUU, Antisense: AACGUCUUGAAUUCUGUC) using lipofectamine 2000 (Invitrogen) or X-treme GENE HP DNA transfection reagent as per manufacturer’s instructions.

HNRNP A1 siRNA for knockdown in HFFs (Figure 6) was obtained from Cell Signaling (cat. #7668).

Oncogene-induced senescence with constitutively active RAS
V12G RAS virus was produced with pBABE-V12G RAS as per the procedure described above. HFFs were transduced with RAS virus and incubated with antibiotic selection medium (puromycin 2 μg/ml) for 4–5 days.

RNA immunoprecipitation (RIP) and RIP-PCR
For RNA immunoprecipitation, 10 million cells were lysed in 1 ml of NP-40 lysis buffer (50 mM Tris HCl, ph 7.4, 150 mM NaCl, 1% NP-40 and Protease inhibitor cocktail). Lysate was cleared by centrifugation at 12,000 RCF for 15 min. Cleared lysate was immunoprecipitated independently with 5 μg of anti-hnRNP A1, anti-hnRNP D, Anti-hnRNP A2/B1, Anti-hnRNP C1/C2, Anti-hnRNP K, mIgG and R-IgG antibodies. Immune complexes were incubated with 30 μl of pre-equilibrated Dynabeads for 4 hr at 4°C. Dynabead purified immune complexes were subjected to Proteinase K digestion at 37°C for 1 hr followed by NucleoSpin RNA II purification kit and cDNA was prepared by RNA-to-cDNA EcoDry Premix kit (Clontech). cDNA was used as a template in PCR amplifications with gene specific primers.

mRNA stability assays
TBX3, CAPEPa, or Control shRNA KD, PS and RAS HFFs were cultured in 6-well culture dishes for 2 days to 80% confluence. Then Actinomycin D was added to a final concentration of 5 mg/ml to suppress transcription. At 0, 1, 2, and 4 hr after addition of Actinomycin D, equal numbers of cells were harvested from each sample and mRNA was prepared by nucleoSpin RNA II purification kit and cDNA was prepared by RNA-to-cDNA EcoDry Premix kit (Clontech) followed by qRT-PCR for specific transcripts.

P16INK mRNA northern blot
HFFs were transfected with pcDNA3.1 control or UCA1 expression plasmids as described above, incubated +/- Actinomycin D, and total cellular RNA was harvested at 0, 1, 2, and 4 hr post treatment. For
northern blot analysis, 5 µg total RNA from each time point was electrophoresed through a 1% agarose gel. The RNA was blotted onto Hybond-N+ membrane (Amersham Pharmacia), and membranes were UV crosslinked. Membranes were hybridized for 18 hr with (Torres et al., 2003) P-labeled probes. Probes were generated by end-labeling DNA oligonucleotides containing following sequences complementary to p16^{INK} mRNA:

1. 5′ GAGGAGGTTGCTATTAATCCGAGCTATTAGCGAATGTGGC
2. 5′ AATCCCTCTGAGGCGGACCGTATCTTTCCAGGCAAGGG
3. 5′ AAGGCTCCATGCTGCCTCCCAGGCCGCGGCCGCTCAGGCTC

End-labeling reactions were performed using T4 polynucleotide Kinase (NEB) according to the manufacturer's directions. The hybridized blots were washed, and autoradiographs were developed as per standard procedure. Band intensities were measured by Image J analysis, and densitometric values were plotted as bar graphs.

**RNA-Seq analysis of TBX3 and CAPERα KD HFFs**

HFFs were incubated with TBX3 or CAPERα shRNA encoding retrovirus for 48 hr followed by incubation for an additional 48 hr in selection medium. Total RNA was isolated and purity was assessed. Poly-A RNA was purified, fragmented, primed with random hexamers and used to generate first strand cDNA using reverse transcriptase. Samples that passed quality control steps were used for Illumina library preparation using the Illumina TruSeq RNA Sample Prep protocol. All libraries were sequenced (with barcoding) on a single lane of an Illumina HiSeq instrument for 50 cycles from a single end. A total of 177,155,781 reads were produced in total for all 10 libraries (median 17,348,374 reads). Base calling was performed using Illumina software.

**Bioinformatics analysis**

Sequence reads were aligned (98.5% mapped) to the human genome build 37.2 with Tophat (v2.0.8b) using default parameters. Aligned reads were assembled into transcripts and their relative abundance was measured using Cufflinks (v2.1.1) with fragment bias correction (frag-bias-correct) and multi-read correction (multi-read-correct). Cufflinks transcript assemblies were based on transcripts of NCBI Homo sapiens annotation release 104 and miRBase release 19 as provided in the Illumina iGenomes data set. Cuffdiff was used to test for differential expression between samples and control and expression differences were taken as significant if the FDR adjusted p-value was less than 0.05 (Source Data Files 1 and 2). Statistically overrepresented gene ontology/biological process categories and KEGG pathways were determined using DAVID (Huang da et al., 2009a, 2009b). The hypergeometric test, as implemented in the R statistical language (phyper), was used to test significance of the number of genes found to be co-regulated between samples (Figure 7—source data 3).

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**Author contributions**

PKP, AMM, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article, Contributed unpublished essential data or reagents; UE, Conception and design, Acquisition of data, Contributed unpublished essential data or reagents; RS, Acquisition of data, Analysis and interpretation of data, Contributed unpublished essential data or reagents; SF, BM, Conception and design, Acquisition of data, Analysis and interpretation of data; MY, Bioinformatics, Analysis and interpretation of data, Contributed unpublished essential data or reagents; SLL, Training, Acquisition of data, Analysis and interpretation of data
References

Abdelmohsen K, Panda A, Kang MJ, Xu J, Selimyta R, Yoon JH, Martindale JL, De S, Wood WH III, Becker KG, Gorospe M. 2013. Senescence-associated lncRNAs: senescence-associated long noncoding RNAs. Aging Cell 12:890–900. doi: 10.1111/ace.12115.

Baksh S, Widlund HR, Frazer-Abel AA, Du J, Fosmire S, Fisher DE, DeCaprio JA, Modiano JF, Burakoff SJ. 2002. NFATc2-mediated repression of cyclin-dependent kinase 4 expression. Molecular Cell 10:1071–1081. doi: 10.1016/S1097-2765(02)00701-3.

Bamshad M, Le T, Watkins WS, Dixon ME, Kramer BE, Roeder AD, Carey JC, Root S, Schinkel A, Van Maldergem L, Gardner RJ, Lin RC, Seidman CE, Seidman JG, Wallerstein R, Moran E, Sutphen R, Campbell CE, Jorde LB. 1999. The spectrum of mutations in TBX3: genotype/phenotype relationship in ulnar-mammary syndrome. American Journal of Human Genetics 64:1550–1562. doi: 10.1086/302417.

Bamshad M, Lin RC, Law DJ, Watkins WC, Krakowiak PA, Moore ME, Franceschini P, Lala R, Holmes LB, Gebuhr TC, Bruneau BG, Schinkel A, Seidman JG, Seidman CE, Jorde LB. 1997. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. Nature Genetics 16:311–315. doi: 10.1038/ng0979-311.

Berthet C, Klarmann KD, Hilton MB, Suh HC, Keller JR, Kiyokawa H, Kaldis P. 2006. Combined loss of Cdk2 and Cdk4 results in embryonic lethality and Rb hypophosphorylation. Developmental Cell 10:563–573. doi: 10.1016/j.devcel.2006.03.004.

Bierhoff H, Dammert MA, Brocks D, Dambacher S, Schotta G, Grummt I. 2014. Quiescence-induced lncRNAs trigger H4K20 trimethylation and transcriptional silencing. Molecular Cell 54:675–682. doi: 10.1016/j.molcel.2014.03.032.

Boehm JS, Hession MT, Bulmer SE, Hahn WC, 2005. Transformation of human and murine fibroblasts without viral oncogenes. Molecular and Cellular Biology 25:6464–6474. doi: 10.1128/MCB.25.15.6464-6474.2005.

Brummelkamp TR, Kortlever RM, Lingbeek M, Trettel F, MacDonald ME, van Lohuizen M, Bernards R. 2002. TBX-3, the gene mutated in ulnar-mammary syndrome, is a negative regulator of p19ARF and inhibits senescence. The Journal of Biological Chemistry 277:6567–6572. doi: 10.1074/jbc.M110492200.

Carlson H, Ota S, Campbell CE, Hurlin PJ. 2001. A dominant repression domain in Tbx3 mediates transcriptional repression and cell immortalization: relevance to mutations in Tbx3 that cause ulnar-mammary syndrome. Human Molecular Genetics 10:2403–2413. doi: 10.1093/hmg/10.21.2403.

Chang N, Yi J, Guo G, Liu X, Shang Y, Tong T, Cui Q, Zhan M, Gorospe M, Wang W. 2010. HuR uses AUF1 as a cofactor to promote p16INK4 mRNA decay. Molecular and Cellular Biology 30:3875–3886. doi: 10.1128/MCB.00169-10.

Coll M, Seidman JG, Muller CW. 2002. Structure of the DNA-bound T-box domain of human TBX3, a transcription factor responsible for ulnar-mammary syndrome. Structure 10:343–356. doi: 10.1016/S0969-2126(02)00722-0.

Coppé JP, Rodier F, Patil CK, Freund A, Desprez PY, Campisi J. 2011. Tumor suppressor and aging biomarker p16INK4a induces cellular senescence without the associated inflammatory secretory phenotype. The Journal of Biological Chemistry 286:36396–36403. doi: 10.1074/jbc.M111257071.

Csoka AB, English SB, Simkevich DP, Ginzinger DG, Butte AJ, Rothman FG, Sedivy JM. 2004. Genome-scale expression profiling of Hutchinson-Gilford progeria syndrome reveals widespread transcriptional misregulation leading to mesodermal/mesenchymal defects and accelerated atherosclerosis. Aging Cell 3:235–243. doi: 10.1111/j.1474-9728.2004.00105.x.

Davalos AR, Kawahara M, Malhotra GK, Schaan N, Huang J, Ved U, Beausejour CM, Coppe JP, Rodier F, Campisi J. 2013. p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes. The Journal of Cell Biology 201:613–629. doi: 10.1083/jcb.201206006.

DeGregori J. 2004. The Rb network. Journal of Cell Science 117:3411–3413. doi: 10.1242/jcs.011189.

Douglas NC, Papaioannou VE. 2013. The t-box transcription factors Tbx2 and Tbx3 in mammary gland development and breast cancer. Journal of Mammary Gland Biology and Neoplasia 18:143–147. doi: 10.1007/s10911-013-9282-8.

Dowhan DH, Hong EP, Auboeuf D, Dennis AP, Wilson MM, Berget SM, O’Malley BW. 2005. Steroid hormone receptor coactivation and alternative RNA splicing by U2AF65-related proteins CAPERalpha and CAPERbeta. Molecular Cell 17:429–439. doi: 10.1016/j.molcel.2004.12.025.

Dutta J, Fan G, Gelinas C. 2008. CAPERalpha is a novel Rel-TAD-interacting factor that inhibits lymphocyte transformation by the potent Rel/NF-kappaB oncoprotein v-Rel. Journal of Virology 82:10792–10802. doi: 10.1128/JVI.00903-08.

Elzi DJ, Song M, Hakala K, Weintrab ST, Shioy Y. 2012. Wnt antagonist SFRP1 functions as a secreted mediator of senescence. Molecular and Cellular Biology 32:4388–4399. doi: 10.1128/MCB.06203-11.

Fan C, Chen Q, Wang QK. 2009. Functional role of transcriptional factor TBX5 in pre-mRNA splicing and Holt-Oram syndrome via association with SC35. The Journal of Biological Chemistry 284:25653–25663. doi: 10.1074/jbc.M109.041368.

Fatica A, Bozzi I. 2014. Long non-coding RNAs: new players in cell differentiation and development. Nature Reviews Genetics 15:7–21. doi: 10.1038/nrg3606.

Frank DU, Carter KL, Thomas KR, Burr RM, Bakker ML, Coetzee WA, Tristani-Firouzi M, Bamshad MJ, Christofkofs VM, Moon AM. 2012. Lethal arrhythmias in Tbx3-deficient mice reveal extreme dosage sensitivity of cardiac conduction system function and homeostasis. Proceedings of the National Academy of Sciences of the United States of America 109:E154–E163. doi: 10.1073/pnas.1115165109.

Frank DU, Emechebe U, Thomas KR, Moon AM. 2013. Mouse tbx3 mutants suggest novel molecular mechanisms for ulnar-mammary syndrome. PLOS ONE 8:e67841. doi: 10.1371/journal.pone.0067841.
Haga K, Ohno S, Yugawa T, Narisawa-Saito M, Fujita M, Sakamoto M, Galloway DA, Kyono T. 2007. Efficient immortalization of primary human cells by p16INK4a-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT. *Cancer Science* **98**:147–154. doi: 10.1111/j.1349-7006.2006.00373.x.

Han J, Yuan P, Yang H, Zhang J, Soh BS, Li P, Lim SL, Cao S, Tay J, Orlov YL, Lufkin T, Ng HH, Tam WL, Lim B. 2010. Tbx3 improves the germ-line competency of induced pluripotent stem cells. *Nature* **463**:1096–1100. doi: 10.1038/nature08735.

Hartwell L. 1965. The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research* **37**:614–636. doi: 10.1016/0014-4827(65)90211-9.

Herrick DJ, Ross J. 1994. The half-life of c-myc mRNA in growing and serum-stimulated cells: influence of the coding and 3′ untranslated regions and role of ribosome translocation. *Molecular and Cellular Biology* **14**:2119–2128.

Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, Okita K, Yamanaka S. 2009. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* **460**:1132–1135. doi: 10.1038/nature08235.

Hoogaars WM, Barnett P, Rodriguez M, Clout DE, Moorman AF, Godding CR, Christoffels VM. 2008. Tbx3 and its splice variant Tbx3 + exon 2a are functionally similar. *Pigment Cell & Melanoma Research* **21**:379–387. doi: 10.1173/j.1755-148X.2008.00461.x.

Huang da W, Sherman BT, Lempicki RA. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research* **37**:1–13. doi: 10.1093/nar/gkn923.

Huang da W, Sherman BT, Lempicki RA. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**:44–57. doi: 10.1038/nprot.2008.211.

Huang J, Zhou N, Watabe K, Lu Z, Wu F, Xu M, Mo YY. 2014. Long non-coding RNA UCA1 promotes breast tumor growth by suppression of p27 (Kip1). *Cell Death & Disease* **5**:e1308. doi: 10.1038/cddis.2013.541.

Hubackova S, Novakova Z, Krejcikova K, Kosar M, Dobrovolna J, Duskova P, Hanakova H, Vancurova M, Barath P, Bartek J, Hodny Z. 2012. Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. *Cell Cycle* **9**:3085–3099. doi: 10.4161/cc.9.15.12521.

Hubackova S, Krejcikova K, Bartek J, Hodny Z. 2012. IL1- and TGFbeta-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine ‘bystander senescence’. *Aging* **4**:932–951.

Imai H, Chan EK, Kiyosawa K, Fu XD, Tan EM. 1993. Novel nuclear autoantigen with splicing factor motifs identified with antibody from hepatocellular carcinoma. *The Journal of Clinical Investigation* **92**:2419–2426. doi: 10.1172/JCI116848.

Ivantsova V, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Saha X, Lyn M, Lemiacka IR. 2006. Dissecting self-renewal in stem cells with RNA interference. *Nature* **442**:533–538. doi: 10.1038/nature04915.

Jacobs JJ, Keblusek P, Robanus-Maandag E, Kristel P, Lingbeek M, Nederlof PM, van Welsem T, van de Vijver MJ, Jenkins GP, Ernstoff E, Zindy F, Sherr CJ, Papaioannou VE. 2005. Tbx3, the ulnar-mammary syndrome gene, and Tbx3 interact in mammary gland development through a p19Arf/p53-independent pathway. *Developmental Dynamics* **234**:922–933. doi: 10.1002/dvdy.20575.

Jung DJ, Na SY, Na DS, Lee JW. 2002. Molecular cloning and characterization of CAPER, a novel coactivator of activating protein-1 and estrogen receptors. *The Journal of Biological Chemistry* **277**:1229–1234. doi: 10.1074/jbc.M110417200.

Kosar M, Bartkova J, Hubackova S, Hodny Z, Lukas J, Bartek J. 2011. Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). *Cell Cycle* **10**:457–468. doi: 10.4161/cc.10.3.14707.

Kuillman T, Michaloglou C, Mooi WJ, Peepers DS. 2010. The essence of senescence. *Genes & Development* **24**:2463–2479. doi: 10.1101/gad.1971610.

Kumar PP, Franklin S, Emechebe U, Hu H, Moore B, Lehman C, Yandell M, Moon AM. 2014. Tbx3 regulates splicing in vivo: a novel molecular mechanism for ulnar-mammary syndrome. *PLOS Genetics* **10**:e1004247. doi: 10.1371/journal.pgen.1004247.

Kumar P et al. *eLife* 2014;3:e02805. DOI: 10.7554/eLife.02805. 27 of 28.
Melanson BD, Bose R, Hamill JD, Marcellus KA, Pan EF, McKay BC. 2011. The role of mRNA decay in p53-induced gene expression. RNA 17:2222–2234. doi: 10.1261/rna.030122.111.

Mercier I, Casimiro MC, Zhou J, Wang C, Pymiere C, Bryant KG, Daumer KM, Sotgia F, Bonuccelli G, Witkiewicz AK, Lin J, Tran TH, Milliman J, Frank PG, Jasmin JF, Rui H, Pestell RG, Lisanti MP. 2009. Genetic ablation of caveolin-1 drives estrogen-hypersensitivity and the development of DCIS-like mammary lesions. The American Journal of Pathology. 174:1172–1190. doi: 10.2339/ajpath.2009.080882.

Narita M, Núñez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113:703–716. doi: 10.1016/S0092-8674(03)00401-X.

Peres J, Davis E, Mowla S, Bennett DC, Li JA, Wansleben S, Prince S. 2010. The highly Homologous t-box transcription factors, TBX2 and TBX3, have distinct roles in the oncogenic process. Genes Cancer 1:272–282. doi: 10.1177/1947619109336616.

Peres J, Prince S. 2013. The T-box transcription factor, TBX3, is sufficient to promote melanoma formation and invasion. Molecular Cancer 12:117. doi: 10.1186/1476-4598-12-117.

Prince S, Carreira S, Vance KW, Abrahams A, Goding CR. 2004. Tbx2 directly represses the expression of the p21(WAF1) cyclin-dependent kinase inhibitor. Cancer Research 64:1669–1674. doi: 10.1158/0008-5472.CAN-03-3286.

Rodriguez M, Aladovicz E, Lanfrancone L, Goding CR. 2008. Tbx3 represses E-cadherin expression and enhances melanoma invasiveness. Cancer Research 68:7872–7881. doi: 10.1158/0008-5472.CAN-08-0301.

Saramaki A, Banwell CM, Campbell MJ, Carlberg C. 2006. Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. Nucleic Acids Research 34:543–554. doi: 10.1093/nar/gkl466.

Senturk S, Mumcuoglu M, Gursoy-Zuzugullu O, Cingoz B, Akcali KC, Ozturk M. 2010. Transforming growth factor-beta induces senescence in hepatocellular carcinoma cells and inhibits tumor growth. Hepatology 52:966–974. doi: 10.1002/hep.23679.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602. doi: 10.1016/S0092-8674(00)81902-9.

Sharova LV, Sharov AA, Nedorezov T, Piao Y, Shaik N, Ko MS. 2009. Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. DNA Research 16:45–58. doi: 10.1093/dnares/dsn030.

Stewart SA, Dykhooom DM, Palliser D, Mizuno H, Yu EY, An DS, Sabatini DM, Chen IS, Hahn WC, Sharp PA, Weinberg RA, Novina CD. 2003. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 9:493–501. doi: 10.1261/rna.2192803.

Torres C, Francis MK, Lorenzini A, Tesrini M, Cristofalo VJ. 2003. Metabolic stabilization of MAP kinase phosphatase-2 in senescence of human fibroblasts. Experimental Cell Research 290:195–206. doi: 10.1016/S0014-4827(03)00309-4.

Wang KC, Chang HY. 2011. Molecular mechanisms of long noncoding RNAs. Molecular Cell 43:904–914. doi: 10.1016/j.molcel.2011.08.018.

Wang C, Hou X, Mohapatra S, Ma Y, Cress WD, Pledger WJ, Chen J. 2005. Activation of p27Kip1 Expression by E2F1. A negative feedback mechanism. The Journal of Biological Chemistry 280:12339–12343. doi: 10.1074/jbc.C400536200.

Wang F, Li X, Xie X, Zhao L, Chen W. 2008. UCA1, a non-protein-coding RNA up-regulated in bladder carcinoma and embryo, influencing cell growth and promoting invasion. FEBS Letters 582:1919–1927. doi: 10.1016/j.feblet.2008.05.012.

Wang J, Shen WH, Jin YJ, Brandt-Rauf PW, Yin Y. 2007. A molecular link between E2F-1 and the MAPK cascade. The Journal of Biological Chemistry 282:18521–18531. doi: 10.1074/jbc.M610538200.

Wang XS, Zhang Z, Wang HC, Cai JL, Xu QW, Li MQ, Chen YC, Qian XP, Lu TJ, Shen WH, Lanfrancone L, Goding CR. 2008. Tbx3 represses E-cadherin expression and enhances melanoma invasiveness. Cancer Research 68:693–699. doi: 10.1158/0008-5472.CAN-07-5012.

Yoon JH, Abdelmohsen K, Kim J, Yang X, Martindale PJ, Tominaga-Yamanaka K, White EJ, Orjalo AV, Rinn JL, Abdelmohsen K, Kim J, Yang X, Martindale JL, Tominaga-Yamanaka K, White EJ, Orjalo AV, Rinn JL, Cristofalo VJ. 2014. Epigenetic silencing of the tRNA methyltransferase STMN2 by the non-coding RNA STMN2-UTR1. Nature Communications 5:3939. doi: 10.1038/ncomms3939.

Yu TY, Kao YW, Lin JJ. 2014. Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase. Proceedings of the National Academy of Sciences of the United States of America 111:3377–3382. doi: 10.1073/pnas.1307415111.

Zhang X, Liu Z, Yu J, Tang H, Xing J, Yu M, Tong T, Shang Y, Gorospe M, Wang W. 2012. The RNA methyltransferase NSun2 stabilizes p16INK4a mRNA by methylating the 3′-untranslated region of p16. Nature Communications 3:712. doi: 10.1038/ncomms1692.

Zhu D, Xu G, Ghandhi S, Hubbard K. 2002. Modulation of the expression of p16INK4a and p14ARF by hnRNP A1 and A2 RNA binding proteins: implications for cellular senescence. Journal of Cellular Physiology 193:19–25. doi: 10.1002/jcp.10147.