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

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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.

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 E2F 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.

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of Bmi1−/− and SV40 transformed mouse embryonic fibroblasts by overexpressed TBX2 and TBX3, respectively (Jacobs et al., 2000; Brummelkamp et al., 2002; Prince et al., 2004).

Mutations in human TBX3 cause a constellation of severe birth defects called ulnar-mammary syndrome (Bamshad et al., 1997). Efforts to understand the molecular biogenesis of this developmental disorder uncovered additional functions for TBX3 beyond transcriptional repression (Fan et al., 2009; Frank et al., 2013; Kumar et al., 2014) as well as critical roles in adult tissue homeostasis (Frank et al., 2012). The pleiotropic effects of TBX3 gain and loss of function suggest its molecular activities are context and cofactor dependent.

Despite the biologic importance of TBX3, few interacting proteins or target genes have been discovered, and the mechanisms underlying its regulation of cell fate, cell cycle, and carcinogenesis are obscure. We found that TBX3 associates with CAPERα (Coactivator of AP1 and Estrogen Receptor), a protein identified in a liver cirrhosis patient who developed hepatocellular carcinoma (Imai et al., 1993). CAPERα regulates hormone responsive expression and alternative splicing of minigene reporters in vitro (Jung et al., 2002; Dowhan et al., 2005) but its in vivo functions are unknown.

We show that a CAPERα/TBX3 repressor complex is required to prevent premature senescence of primary cells and regulates the activity of core senescence pathways in mouse embryos. We discovered co-regulated targets of this complex in vivo and during oncogene-induced senescence (OIS), including a novel tumor suppressor, the IncRNA UCA1. UCA1 is sufficient to induce senescence and does so in part by sequestering hnRNP A1 to specifically stabilize CDKN2A-p16INK mRNA. Our finding that CAPERα/TBX3 regulates p16 levels by dual, reinforcing mechanisms position CAPERα/TBX3 and UCA1 upstream of multiple members of the p16/RB pathway in the regulatory hierarchy that controls cell proliferation, fate and senescence.

Results

CAPERα interacts with TBX3 in vivo

We recently discovered that TBX3 (human) and Tbx3 (mouse) interact with RNA-binding and splicing factors (Kumar et al., 2014). Among these, mass spectrometry of anti-TBX3 immunoprecipitated
(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 10.5 mouse embryo lysates (Figure 1B). Immunoblotting for Tbx3 confirmed its interaction with Caperα (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 (ΔRD) 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-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 ΔRD 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*PSIAAAIAVNALHGR, 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) Colocalization 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, ΔRD 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 (ΔRD) 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 ΔRD 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 interactions with CAPERα.

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chromatin organization and formation of SAHF (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 p16INK) 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 (−/−) 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 Cdk5s were decreased (Figure 2, 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) Figure 2. Continued on next page
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 HPRT7 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 assay of 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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Previous studies have suggested that overexpression of TBX3 permits senescence bypass by directly repressing CDKN2A-p14ARF (p14ARF) to activate p53 (Brummelkamp et al., 2002), but a role for TBX3 in regulating p16INK and the RB pathway has not been demonstrated. Thus, we expected that loss of p53 would rescue senescence resulting from TBX3 or CAPERα KD. To test this, we transduced TBX3 and CAPERα KD HFFs with shRNA to p53 (Masutomi et al., 2003) and assayed SA-βgal activity and growth. Surprisingly, although p53 shRNA effectively decreased p53 (Figure 3—figure supplement 1A), it did not rescue SA-βgal activity or growth arrest due to absence of TBX3 or CAPERα (Figure 3B,E,G,H). In contrast, shRNA-mediated KD of either RB (Boehm et al., 2005) or p16 (Haga et al., 2007) (Figure 3—figure supplement 1B,C) rescued these phenotypes in TBX3 and CAPERα KD cells (Figure 3C,F–H,I–N). These rescue experiments demonstrate that the p16/RB pathway mediates senescence downstream of CAPERα and TBX3 loss-of-function in primary cells.

**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, loss of 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 dysregulated 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 CAPERa/TBX3 loss of function and CAPERa/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 CAPERa 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
assays of HFFs stably transduced with control or p16 (Haga et al., 2007) shRNAs subsequently transduced with CAPERα or TBX3 shRNAs. (M) % Quantitation of I-L from three replicate experiments. * indicates p<0.05 relative toCtl shRNA. (N) Cell proliferation assay by crystal violet incorporation (OD units) in HFFs treated as in I-L. * indicates p<0.01 relative toCtl 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 8) and CAPERα (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 (lanes14, 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.
DOI: 10.7554/eLife.02805.010

Figure supplement 2. UCSC Genome Browser view of the CDKN2A locus and 5′ regions screened for binding by CAPERα and TBX3.
DOI: 10.7554/eLife.02805.011

Figure supplement 3. CDKN2a-p16 H3K27 trimethylation markedly decreases in HFFS after knockdown of CAPERα or TBX3 consistent with activation of CDKN2a-p16 expression.
DOI: 10.7554/eLife.02805.012

Figure supplement 4. Testing CAPERα and TBX3 binding to p14, p21, CDK2, CDK4, and CDKN1B regulatory elements.
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Expression of the lncRNA 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 lncRNA UCA1 (Wang et al., 2006, 2008), so we investigated it further.

We found that shRNA KD of UCA1 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 p16Rb, 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 transduced HFFs (repeat of experiment shown in Figure 2—figure supplements 1A and 2A). (G) RT-PCR analysis of UCA1 and HPRT gene expression in control, TBX3 or CAPERα shRNA-transduced HFFs. (H) qPCR analysis of UCA1 transcript levels in control, TBX3 or CAPERα shRNA transduced HFFs. 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 transduced 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 transduced 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 p16\(^{INK}\) 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 p16\(^{INK}\), p14\(^{ARF}\), 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 p16\(^{INK}\), p14\(^{ARF}\), E2F1, and TGF\(^{B1}\) mRNAs in the time frame examined, p16\(^{INK}\), p14\(^{ARF}\), 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 p16\(^{INK}\) mRNA in control cells was 3.9 hr vs n in UCA1 overexpressing cells; p14\(^{ARF}\), 2.4 vs n; E2F1, 7.2 vs n; TGF\(^{B1}\), 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 p16\(^{INK}\) mRNA stability were confirmed by Northern blot (Figure 6—figure supplement 2).

Regulation of p16\(^{INK}\) transcript stability is a critical mechanism for growth control (Wang et al., 2005; Chang et al., 2010; Zhang et al., 2012) and hnRNP A1 has been postulated to stabilize p16\(^{INK}\) 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 p16\(^{INK}\) transcripts. Loss of hnRNP A1 (Figure 6—figure supplement 3) stabilized both p16\(^{INK}\) (\(t_{1/2}=2.1\) in control vs 12.3 after HNRNP A1 knockdown) and p14\(^{ARF}\) 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-β-gal assay of cultured HFFs transfected with control and UCA1 overexpression plasmids. (D) % quantitation of SA-β-gal 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<0.005 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-β-gal assays of RAS HFFs transduced with either control or UCA1 shRNA at 3 (M and O) and 5 (N and P) days post transduction. (Q) % quantitation of SA-β-gal 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 shRNA cells 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, Caperα = 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.
DOI: 10.7554/eLife.02805.017

**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 (t1/2) were obtained using linear regression, the best fit lines, their
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\(^{Nk}\) 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\(^{Nk}\) 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\(^{Nk}\) binding was lost in RAS HFFs (Figure 6C, lane 7), despite an overall increase in the number of p16\(^{Nk}\) 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\(^{Nk}\), the hnRNP A1/UCA1 interaction increases in RAS cells (Figure 6D, lane 7). TUG1 IncRNA serves as a negative control (Figure 6F). 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\(^{Nk}\) mRNA with hnRNP A1 in PS vs RAS HFFs led us to postulate that UCA1 stabilizes p16\(^{Nk}\) mRNA during OIS by disrupting the interaction between hnRNP A1 and p16\(^{Nk}\) mRNA. In control transfected proliferating cells, there is robust binding of p16\(^{Nk}\) to hnRNP A1 (Figure 6C, lane13), but direct overexpression of UCA1 (Figure 6D, lane 10) or that resulting from TBX3 or CAPERα KD (Figure 6D, lanes 17, 18) disrupts the hnRNP A1/p16\(^{Nk}\) mRNA interaction (Figure 6C, lanes 14, 23, 24, red arrowheads). These findings support the hypothesis that loss of hnRNP A1/p16\(^{Nk}\) 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 p16\textsuperscript{INK} mRNA (Figure 6C, lane 31) and led to lower levels of total p16\textsuperscript{INK} mRNA (Figure 6C, lane 27), a finding consistent with the negative effects of hnRNP A1/ p16\textsuperscript{INK} interaction on stability of p16\textsuperscript{INK} transcripts. The effects of UCA1 on p16\textsuperscript{INK} mRNA stability are specific, because hnRNP A1 interactions with MYC or p14\textsuperscript{ARF} 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/p16\textsuperscript{INK} binding, and levels of p16\textsuperscript{INK} mRNA are low due to: (1) direct repression by CAPERα/TBX3 and, (2) p16\textsuperscript{INK} 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 p16\textsuperscript{INK} 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 p16\textsuperscript{INK} chromatin. CDKN2A-p16\textsuperscript{INK} 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 p16\textsuperscript{INK} 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\textsuperscript{−/−} MEFS (Figure 2—figure supplement 2D–F). These foci are distinct from SAHF's and PML bodies (Figure 7M and Figure 7—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 G\textsuperscript{1210} 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 down-regulated 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 set: 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
Figure 7. 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 in senescent cells. (C) Immunoblot analysis of TBX3 expression in RAS HFFs compared to PS HFFs. (D) Immunoblot analysis of CAPERα expression in RAS HFFs compared to PS HFFs. (E) Immunoblot analysis of TBX3 expression in RAS HFFs compared to PS HFFs. (F) Immunofluorescence staining of H2A.K11ub, CAPERα, and H3K4me3 in RAS HFFs compared to PS HFFs. (H) Nuclear localization of Hoachme/CAPERα in RAS HFFs compared to PS HFFs. (J, K) Immunofluorescence staining of CAPERα in RAS HFFs compared to PS HFFs. (N, Q) Venn diagrams of up-regulated and down-regulated genes in RAS HFFs compared to PS HFFs. (N’) Venn diagram of genes with higher expression in RAS HFFs compared to PS HFFs. (Q’) Venn diagram of genes with lower expression in RAS HFFs compared to PS HFFs.
Intranuclear foci. Pro-senescence genes including UCA1 and are converted to euchromatin and their expression/products induce senescence. In the IL6 and HDAC9 are direct targets of CAPER.

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 by DAVID. Pie chart shows KEGG pathway analysis of co-regulated genes. (N') 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 6A. Pie chart shows KEGG pathway analysis of OIS dataset. (O) As in N and N' but for downregulated genes. Pie chart in O' shows KEGG pathway analysis of OIS data set; note most pathways are the same as in TBX3/CAPERα (P and Q). Models of CAPERα/TBX3 repressor and UCA1 function in proliferating (PS) HFFs vs RAS HFFs. In PS cell nuclei, CAPERα/TBX3 represses UCA1, p16, p14, and DUSP4 promoters in heterochromatin which permits ongoing cell proliferation. RAS disrupts the CAPERα/TBX3 complex and CAPERα relocates to dense intranuclear foci. Pro-senescence genes including UCA1 and p16 are converted to euchromatin and their expression/products induce senescence. In the cytoplasm of PS cells, hnRNP A1 binds and destabilizes p16 mRNA, but activation of UCA1 expression in OIS allows UCA1 to sequester hnRNP A1 and stabilize p16 mRNA. TBX3, CAPERα = human; Tbx3, Caperα = mouse.

Source data 1. Differentially expressed genes after knockdown of CAPERα in HFFs detected by RNA-Seq.
Source data 2. Differentially expressed genes after knockdown of CAPERα in HFFs detected by RNA-Seq.
Source data 3. Determining the statistical significance of shared differentially expressed genes using the hypergeometric test, as implemented in the R statistical language (phyper).

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

Figure supplement 2. CAPERα relocalization due to oncogene-induced senescence is independent of PML bodies.

Figure supplement 3. Validation of RNA-Seq identified expression changes induced by CAPERα and TBX3 KD.

Figure supplement 4. IL6 and HDAC9 are direct targets of CAPERα/TBX3.

vivo and that functions as a master regulator of cell proliferation by directly repressing transcription of IncRNA UCA1, p16ink 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 p16ink mRNA with hnRNP A1 leading to increased p16ink mRNA stability (Figure 7R). 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 prosenescence 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-senescence 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). LMNB1 is a TBX3 interacting protein (Kumar et al., 2014) and expression of LMNA, LMNB1, and LMNB2 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 Lamins 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 IncRNAs 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 IncRNA expression and activity, potential senescence-regulating activities are still largely unexplored. IncRNA HOTAIR functions as a scaffold to regulate ubiquitination of Ataxin-1 and Snpurportin-1 to prevent premature senescence (Yoon et al., 2013). Global alterations in IncRNA expression have been reported in association with replicative senescence (Abdelmohsen et al., 2013), and telomere-specific IncRNAs 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 IncRNAs PAPAS and Suv4-20h2 was reported (Bierhoff et al., 2014). To our knowledge, UCA1 is the first IncRNA 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/prosenescent function for UCA1 in primary cells. UCA1 increases stability of p16INK mRNA by sequestering hnRNP A1, employing a decoy mechanism that is in some aspects reminiscent of IncRNA 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 p16INK and p14ARF mRNAs (Figure 6A, B), UCA1 only disrupts the association of hnRNP A1 with p16INK mRNA (Figure 6C and Figure 6—figure supplement 4). In proliferating cells, abundant hnRNP A1 binds with p16INK mRNA resulting in p16INK degradation. In senescing cells, p16INK mRNA levels increase via reinforcing mechanisms of increased transcription and stability: loss of CAPERα/TBX3 activates transcription of p16INK and UCA1, in turn, UCA1 sequesters hnRNPA1.

We recognize that the systems we employed (primary HFFs, mouse embryos and MEFs), while very informative models, provide limited information directly applicable to aging or tumorigenesis without further experimentation. Our data support an important role for CAPERα/TBX3 in regulation of senescence in developmental contexts and, since the CAPERα/TBX3 complex regulates known critical tumor suppressors and there is an increasing literature supporting roles for both TBX3 and CAPERα in tumor biology, this is another worthy area for future investigation. As noted above, expression of CDKN2A-p14ARF and CDKN1A-p21CIP 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−/− 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 CAPERα/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, CAPERα/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 CAPERα interaction and/or UCA1 repression. Mining the pathways regulated by UCA1 and CAPERα/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,A303-098A), CAPERα (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

Kumar P et al. eLife 2014;3:e02805. DOI: 10.7554/eLife.02805

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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 CAPERα 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 CAPERα was generated by PCR amplification and then cloned into pQCPXIH retroviral vector; sequence was confirmed. Full length UCA1 was amplified by PCR and then cloned into pcDNA3.1 vector; sequence was confirmed.

UCA1 Cloning FP: AGTTGCGGCCGCTGACATTCTTCTGGACAATGAG
UCA1 Cloning RP: TCTTCGTCGACCGGAGGAGGTATAGCTTTTATGAG
CAPERα Cloning FP: CATCGCGGCCGCAGCTGACCTGCTAGATTTAGATGTG
CAPERα Cloning RP: ACGTGGATCCTCATCGTCTACTTGAAGCACAGTAG

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 flour 594 (1:500) and goat anti-mouse Alexa flour 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.

Retroviral transduction and selection of stable cells

shRNA oligonucleotides (see sequences below) were annealed and cloned into the pGFP-B-RS, pRFP-C-RS (Origene) vector and PMKO.1 vector. shRNA against luciferase served as a negative control. High-titer retrovirus was produced by transfection of shRNA retroviral construct along with gag/pol and VSVG encoding plasmids into EBNA-293 cells by lipofectamine 2000 reagent as per the manufacturer’s protocol. Virus containing supernatant was collected after 48 hr of transfection and filtered through 0.45-μM filters (Fisher 09-720-4). HEK293 or HFFs were incubated with DMEM containing polybrene (8 mM) and 500 μl of TBX3 or CAPERα shRNA encoding retrovirus. 24 hr post infection, cells were split to lower densities and blasticidin or puromycin antibiotic selection applied for 2 days. Stably integrated colonies were selected and analyzed for knock down efficiency by western analysis using Tbx3 or CAPERα antibody.

TBX3 shRNA A: targets TBX3 exon 7
TBX3 shA FP: CCGG GACCATGGAGCCCGAAGAA tccaagaga TCTTTGCGCTCTGCTGGTTCTCTT
TBX3 shA RP: AATTCAAAAA GACCATGGAGCCCGAAGAA ttcaagaga TCTTTGCGCTCTGCTGGTT
TBX3 shRNA B: targets TBX3 exon 5
TBX3 shB FP: CCGG CAGCTCACACCTGCAGTCCA tcaagaga TGGACTGCAGGGTGAGCTG TTTTTG
TBX3 shB RP: AATTCAAAAA CAGCTCACACCTGCAGTCCA tcaagaga TGGACTGCAGGGTGAGCTG
CAPERα shRNA A: targets CAPERα (gene name RBM39) exon 5
CAPERα shA FP: CCGG GACAGAAATTCAAGACGTtcaagagaAACGTCTTTGAATTTCTCTTTTTG
CAPER shA RP: AATTCAAAAA GACAGAAATTCAAGACGTT tctcttgaA AACGTCTTTGAATTTCTCTTGTC
CAPERα shRNA B: targets CAPERα exon 1
CAPER shB P: CCGG AAGCAAGAGCAGAAGTCTCGTAttcaagagaTACGACTTTCTCTCTTTTTG
CAPER shB RP: AATTCAAAAA AAGCAAGAGCAGAAGTCTCGTA tctcttgaA TACGACTTTCTCTCTT

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:
GATCCGTTAATCCAGGAGACAAAGAtcaagagTCTTTGTCTCCTGGATTAACTTTTTTGGA
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 \( \log(N_1)/\log(2) \) – \( \log(N_0)/\log(2) \), \( N_1 = \) current cell count, \( N_0 = \) 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**

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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

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HDAC9CHIPFP: GGCTCAGGCGACCATTGTTATTTTCCTGT
HDAC9CHIPRP: CCTGAGAAGAAGCAGCAGAAGGATCAAC
IL6CHIPFP: GAACCAAGTGCGCTTGAATTTCAGG
IL6CHIPRP: CATTGAGCTTCTGTTGCTGCTTC
P14ARFP: CAGCTCTGCCTGATGCTACTG
P14ARRP: ACCTGGTCTTCTAGGAAGCGG
TGFB1 FP: GATGGCACAGTGGTCAAGAGC
TGFB1 RP: GAAGGATGGAAGGGTCAGGAG
RB FP: GCGCGAAGTTGACGTTTTC
RB RP: CCGACTCTCCGTACAAATAAT
MYC FP: AAGATCCCTTCCTCGTTACATTC
MYC RP: AGAAGCCCTTCCTGCTTCTC
E2F1 FP: GGCTACAGGTGAGGGTCACG
E2F1 RP: GAGCGCCGCCACAATTCGCT
CDKN2DFP: TCCCTTTCTTCACGGTGCTT
CDKN2DRP: GCGTCGCTCCTGATTGGTC
CDK2 FP: AAGCAGGTACTTGGGAAGAGTGTTCAGC
CDK2 RP: CAACTTGAAACAATGTTGCCGCCTC
MDM2 FP: GGCCTACCCAAAGTGATGGGATTACAAG
MDM2 RP: GCCGCTGGAGTTGTACCCAAATGAGTTA

siRNA knockdown
For differential display (Figure 4), HEK293 cells were transfected with control siRNAs (Sense; 5'-CAGCGACUAACACAUCA-3' Antisense; 5'-UUGAUGUGUUUGAGUCUGT-3') or TBX3 specific siRNA A (Sense: GACCAUGGAGCCCGAAGAA, Antisense: UUCUUCGGCUCAAUGGU) or CAPERα-specific siRNA (Sense: GACAGAAAUUCAAGACGUU, Antisense: AACGUCUUGAAUUUCUGUC) 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
V12GRAS virus was produced with pBABE-V12GRAS 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, pH7.4, 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, CAPERα, 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 μg/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 p16INK mRNA:

1. 5′GAGGGAGTGCTATATACTCCGAGCATTAGCGAATGTGGC
2. 5′AATCCTCTGAGGGACCGGTATCTTTCCAGGCAAGGGG
3. 5′AAGGCTCCATGCTGCTCCCCGCCGCCGGCTCCATGCTGCT

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 CAPER α KD HFFs**

HFFs were incubated with TBX3 or CAPER α 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 controls 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/biologic 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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**Additional information**

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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
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