The IncRNA SLNCR Recruits the Androgen Receptor to EGR1-Bound Genes in Melanoma and Inhibits Expression of Tumor Suppressor p21

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In Brief
Long non-coding RNA function can be understood by defining their interacting proteins. Schmidt et al. demonstrate that the melanoma IncRNA SLNCR binds to AR and complexes with different transcription factors to mediate invasion or proliferation. Thus, IncRNAs regulate distinct transcriptional programs based on specific protein interactions.

Highlights
- SLNCR-AR complexes drive melanoma growth and invasion
- SLNCR-AR complexes are recruited to EGR1-bound loci
- SLCNR-AR-EGR1 complexes regulate transcription of proliferative genes
- EGR1 normally activates p21; SLNCR-AR-EGR1 complexes repress p21
The IncRNA SLNCR Recruits the Androgen Receptor to EGR1-Bound Genes in Melanoma and Inhibits Expression of Tumor Suppressor p21

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SUMMARY

Melanoma is the deadliest form of skin cancer, affecting men more frequently and severely than women. Although recent studies suggest that differences in activity of the androgen receptor (AR) underlie the observed sex bias, little is known about AR activity in melanoma. Here we show that AR and EGR1 bind to the long non-coding RNA SLNCR and increase melanoma proliferation through coordinated transcriptional regulation of several growth-regulatory genes. ChIP-seq reveals that ligand-free AR is enriched on SLNCR-regulated melanoma genes and that AR genomic occupancy significantly overlaps with EGR1 at consensus EGR1 binding sites. We present a model in which SLNCR recruits AR to EGR1-bound genomic loci and switches EGR1-mediated transcriptional activation to repression of the tumor suppressor p21Wnt/Cip1. Our data implicate the regulatory triad of SLNCR, AR, and EGR1 in promoting oncogenesis and may help explain why men have a higher incidence of and more rapidly progressive melanomas compared with women.

INTRODUCTION

The worldwide incidence of melanoma has been on the rise for the past 30 years. In the United States, there are ∼73,000 new cases diagnosed and ∼10,000 deaths annually attributed to melanoma (Siegel et al., 2015). Of these deaths, approximately two-thirds occur in males, the result of a well-established sex bias disfavoring males in melanoma etiology (Bidoli et al., 2012; de Vries et al., 2007; Fisher and Geller, 2013; Geller et al., 2002; Joosse et al., 2011; Schwartz et al., 2002; Sweetter et al., 2009). In addition to a significant survival advantage compared with males (38%), females demonstrate fewer metastases, a longer delay before relapse, and higher curable rates, strongly suggesting a biological basis for the observed sex bias (Gamba et al., 2013; Joosse et al., 2011, 2012). The androgen receptor (AR) regulates tumorigenesis in many human cancers, including prostate, breast, kidney, lung, bladder, and liver cancer (Chang et al., 2014). AR may function as a tumor suppressor or oncogene, likely dependent on cellular context and the presence or absence of AR-modulating factors. For example, AR activity in prostate cancer cells may be modulated by RNAs, including the long non-coding RNA (IncRNA) HOTAIR (Zhang et al., 2015), or by proteins, such as the transcriptional repressor RE1-silencing transcription factor (REST; also called neuron-restrictive silencer factor [NRSF]) (Svensson et al., 2014). Early studies suggested that AR has oncogenic functions in melanoma and that differences in AR function or expression might explain the observed melanoma gender differences (de Vries et al., 2000; Joosse et al., 2011; Li et al., 2013b; Micheli et al., 2009; Morvillo et al., 1995, 2002; Spanogle et al., 2010). In direct support of an oncogenic function for AR, we recently showed that AR increases melanoma invasion through transcriptional upregulation of the matrix metalloproteinase MMP9 (Schmidt et al., 2016). Interestingly, this regulation occurs independently of canonical AR activation, in which an androgen (such as testosterone) binds to the receptor to elicit downstream transcriptional patterns. Instead, AR-mediated invasion requires a novel IncRNA, SLNCR; specifically, the abundant SLNCR1 isoform that directly binds to and recruits AR to the MMP9 promoter.

IncRNAs are transcripts of more than 200 nt that lack an open reading frame and exhibit cell type and tissue-specific expression. IncRNAs are important regulators of tissue physiology and disease processes and may function either as oncogenes or tumor suppressors (Li and Chen, 2013; Serviss et al., 2014). Although the fundamental mechanism of many IncRNAs remains unknown, many (like SLNCR) function through direct interactions with proteins. Using a highly sensitive technique developed for the identification of RNA-associated transcription factors called
RATA (RNA-associated transcription factor array), we showed that SLNCR binds to multiple transcription factors, possibly regulating their downstream transcriptional activities (Schmidt et al., 2016, 2017). In addition to AR and Brn3a, both of which are required for SLNCR1-mediated regulation of MMP9, we also identified early growth response 1 (EGR1) as a candidate SLNCR1-interacting transcription factor (Schmidt et al., 2016).

EGR1 is a zinc-finger transcription factor implicated in many human cancers, likely functioning as a tumor suppressor. In prostate cancer, EGR1 is a critical regulator of AR function (Yang and Abdulkadir, 2003). In melanoma, EGR1 has been implicated in apoptosis, cell growth, and fibronectin matrix synthesis (Ahmed et al., 1996; Gaggioli et al., 2005; Muthukumar et al., 1995; Sells et al., 1995). Other roles for EGR1 in melanogenesis, including biological consequences of possible physical interactions with SLNCR and/or AR, have not been described.

Because SLNCR imparts oncogenic function to AR in the absence of canonical androgen-induced signaling, we sought a more complete mechanistic understanding of AR function as a possible explanation for the observed melanoma sex bias. Here we examine ligand-independent, SLNCR-regulated AR function in melanoma and find that AR directly binds many SLNCR-regulated genes. Our data suggest that SLNCR recruits AR directly to EGR1-bound chromatin. AR and SLNCR appear to act as a transcriptional switch, reversing EGR1-mediated upregulation of the p27Waf/Cip1 tumor suppressor gene. These results suggest that SLNCR, AR, and EGR1 form a novel regulatory triad that regulates melanoma proliferation. These data demonstrate that a comprehensive evaluation of AR function in melanoma is critical for understanding the mechanistic underpinnings of the melanoma sex bias.

RESULTS

SLNCR Isoforms Exhibit Both Unique and Overlapping Functions

Melanomas express at least 3 isoforms of SLNCR, SLNCR1–SLNCR3 (Figure S1A). SLNCR1 is the shortest and most prevalent isoform, whereas SLNCR2 and SLNCR3 differ only in the inclusion of an additional exon of varying length. We demonstrated previously that SLNCR1 binds to AR and recruits it to the MMP9 promoter and that SLNCR1 and AR are required for transcriptionally upregulating MMP9 expression and promoting melanoma invasion (Schmidt et al., 2016). Surprisingly, unlike SLNCR1, neither SLNCR2 nor SLNCR3 upregulate MMP9 or increase melanoma invasion, suggesting that SLNCR isoforms have at least partially unique functions (Figures S1B and S1C). Isoform-specific functions cannot be explained by differences in AR binding because all 3 isoforms contain the RNA region required for AR binding and consistently bind AR in vivo (Figures S1A and S1D). Thus, all three SLNCR isoforms likely regulate AR activity.

To investigate the role of isoform-overlapping SLNCR function in melanoma development, we designed small interfering RNAs (siRNAs) to knock down all SLNCR isoforms (hereafter, SLNCR refers to SLNCR1–SLNCR3) (Figure S1A). These siRNAs knocked down SLNCR by ~60%–80% in two patient-derived melanoma short-term cultures (MSTCs), WM1976 and WM858, and by 50%–70% in the immortalized malignant melanoma cell line A375 (Figure S2A). Importantly, MSTCs have undergone minimal passages outside of the patient and provide an accurate genetic model of melanoma (Lin et al., 2008). WM1976 and WM858 exhibit moderate to high levels of SLNCR expression and are amenable to genetic studies requiring transfection of DNA or RNA (Schmidt et al., 2016). Using RNA sequencing (RNA-seq), we transcriptionally profiled melanoma cells before and after siRNA-mediated knockdown of SLNCR in WM1976, the MSTC expressing the highest levels of SLNCR. Depletion of SLNCR significantly dysregulated 222 genes compared with a scramble siRNA control, upregulating 131 genes and downregulating 91 genes (p < 0.01; Figure 1A; Table S1).

Analysis of the full melanoma dataset from The Cancer Genome Atlas (TCGA) revealed that SLNCR expression is significantly correlated with expression of 120 candidate target genes (p < 0.05; Table S1; Schmidt et al., 2016). Moreover, expression of SLNCR and 62 of these target genes is significantly correlated, even when correcting for multiple-hypothesis testing (Bonferroni correction, p < 0.00023), strongly suggesting that our RNA-seq analysis faithfully identified many SLNCR-regulated genes.

We next compared SLNCR differentially expressed genes (DEGs) to isoform-specific SLNCR1 DEGs identified previously (Schmidt et al., 2016). Of the 222 SLNCR DEGs, 41 genes (18.5%) were also dysregulated upon knockdown of SLNCR1 (Table S1; p < 0.01 in both RNA-seq experiments). Moreover, the majority of SLNCR and SLNCR1 DEGs (35 of 41) displayed concordant dysregulation (Pearson r = 0.27, p < 0.0001; Figure S2B), reflecting that SLNCR1 and SLNCR similarly regulate these genes.

Importantly, 6 DEGs are discordantly regulated upon SLNCR or SLNCR1 knockdown, supporting our conclusion that SLNCR isoforms have unique functions. Two of these discordantly regulated genes, fibronectin (FN1) and integrin subunit beta-1 (ITGB1), regulate cell matrix adhesion (Gene Ontology [GO] enrichment analysis, GO category 0007161, p = 1.02 × 10⁻⁶, false discovery rate = 1.58 × 10⁻⁶), whereas another two of these genes, transmembrane protein 45A (TMEM45A) and Ras-related protein RAB31, have been implicated in cancer invasion and cell adhesion (Grismayer et al., 2012; Guo et al., 2015). Combined with the findings that (1) SLNCR1, but not SLNCR2 or SLNCR3, increases melanoma invasion (Figure S1) and (2) knockdown of SLNCR1, but not SLNCR, significantly decreases MMP9 (Table S1; data not shown), we conclude that SLNCR isoforms uniquely regulate melanoma invasion. Because knockdown of SLNCR or SLNCR1 only similarly dysregulates the majority of DEGs, and all three SLNCR isoforms bind AR, we focused on isoform-overlapping regulation of AR.

SLNCR and Ligand-Independent AR Increase Melanoma Cell Proliferation

GO Enrichment analysis of the 222 DEGs upon SLNCR knockdown identifies significant enrichment of genes involved in multiple cancer-relevant processes, including cell adhesion and/or motility, apoptosis, differentiation, response to stress, and proliferation (Figure 1B). We were particularly interested in possible roles of SLNCR in melanoma proliferation because SLNCR has been implicated in proliferation of pancreatic, gastric, and
non-small-cell lung cancers (Huang et al., 2017; Lu et al., 2017b; Shi et al., 2016; Zheng et al., 2016). Consistent with enrichment of cell proliferation genes identified with GO analysis (22% or 49 of 222 DEGs, p = 2.54 × 10−48; Figure 1B), interrogation of TCGA data reveals that SLNCR expression is significantly correlated with the mitotic growth rate of primary melanomas (Spearman r = 0.20, p = 0.0083; Figure S2C), with slow-growing melanomas expressing significantly lower levels of SLNCR (mitotic growth rate ≤ 1 versus > 1 mitosis/mm², p = 0.014; Figure 1C). SLNCR knockdown significantly decreased proliferation of both MSTCs compared with a scramble siRNA control (p < 0.0001; Figure 1D). Proliferation of the melanoma cell line A375 was slightly reduced upon SLNCR knockdown (p < 0.0001), likely because of a lower endogenous levels of SLNCR, which limits the fold depletion and range of knockdown-related phenotypes (Figure 1D; Schmidt et al., 2016). Importantly, knockdown of SLNCR did not alter the percentage of apoptotic cells in either MSTCs or in A375 melanoma cells (Figure S2D). Collectively, these experiments indicate that SLNCR increases melanoma proliferation. Because depleting SLNCR1 alone did not affect cell growth, SLNCR isoforms likely share an overlapping role in regulation of melanoma proliferation, with SLNCR2 and SLNCR3 able to functionally compensate in the absence of SLNCR1 (Schmidt et al., 2016).

Consistent with previous studies implicating AR in melanoma cell proliferation (Morvillo et al., 1995, 2002), the anti-androgen flutamide, which competes with androgen for binding to AR, significantly decreased melanoma cell proliferation (p < 0.0001; Figure S3A). Although this suggests an androgen-dependent role of AR in melanoma proliferation, SLNCR and AR interact even in the absence of canonical ligand-induced AR activation (Schmidt et al., 2016). Although standard cell culture conditions use fetal bovine serum, which contains exogenous hormones, it is unknown whether these standard cell culture conditions accurately reflect the natural hormone state of the melanoma tumor microenvironment. To test whether AR regulates melanoma proliferation in the absence of androgens, we quantified melanoma cell proliferation in hormone-deprived medium (phenol-red free medium supplemented with charcoal-stripped medium) before and after AR depletion. Two AR-targeting siRNAs resulted in 60%–90% of knockdown of AR in 3 melanoma cells (Figures S3B and S3C). AR knockdown significantly decreased proliferation of hormone-deprived melanoma cells (p < 0.0001; Figure 2A). These results confirm that
AR also regulates melanoma proliferation in an androgen-independent manner.

To test whether SLNCR and AR cooperatively regulate melanoma proliferation, we introduced short, single-stranded RNA oligonucleotides designed to sterically block the interaction of SLNCR and AR. These oligonucleotides either mimic the SLNCR sequence required for AR binding and dominantly repress AR binding to SLNCR (mimic 1 or 2) or are the reverse complement to the SLNCR sequence required for AR binding, which generates double-stranded RNA incapable of binding AR (antisense 1 or 2). The antisense oligonucleotides are specifically designed to bind to SLNCR without eliciting RNase H-mediated degradation of SLNCR. Gymnotic delivery (i.e., delivery without the use of transfection reagents) of either AR- or SLNCR-binding 2'-deoxy-2'-fluoro-D-arabinonucleic acid (2'-FANA)-modified oligonucleotides significantly decreased melanoma proliferation without decreasing SLNCR expression (Figures 2B and S3D). We note that the steric blocking oligonucleotides occasionally upregulated SLNCR expression 2- to 3-fold, possibly resulting from a feedback loop regulating SLNCR expression that is initiated upon inhibition of SLNCR function (the mechanism of which is beyond the scope of this manuscript). Decreased cell proliferation upon inhibition of the SLNCR-AR interaction, despite increased SLNCR expression, further indicates that SLNCR and AR cooperatively regulate melanoma proliferation.

**SLNCR and AR Cooperatively Regulate Melanoma Gene Expression**

We next used AR chromatin immunoprecipitation and massively parallel sequencing (ChiP-seq) to identify global genomic loci bound by ligand-free AR (i.e., hormone-deprived cells). Performing AR ChiP-seq from MSTCs is technically challenging because of low AR expression; thus, we performed AR ChiP-seq from higher AR-expressing A375 melanoma cells. Because SLNCR1 regulates AR occupancy at at least one genomic region (Schmidt et al., 2016), cells were transfected with either an empty or SLNCR1-expressing vector, representing either endogenous SLNCR levels or SLNCR1 overexpression conditions. AR ChiP-seq identified a total of 9,974 AR binding regions (referred to as “active regions”); 5,717 for the empty vector and 8,239 for the SLNCR1-expressing vector) in hormone-deprived A375 melanoma cells (Table S2). The majority of the binding events in empty vector and SLNCR1-expressing cells occurred within 10,000 bp of annotated genes as defined by the NCBI (including both coding and non-coding, as defined by NCBI; 4,522 [78.66%] and 6,547 [79.13%] active regions, respectively; Table S2), hereafter referred to as “active genes,” suggesting that AR regulates gene expression even in the absence of canonical androgen signaling. Our AR ChiP-seq analysis faithfully identified several known AR target genes, including C15orf40, POLR2A, and WDR70 (average peak intensities, 40–175; Figure 3A) (Lin et al., 2009; Massie et al., 2007; Wang et al., 2013).

Several lines of evidence indicate that SLNCR1 regulates AR chromatin occupancy. SLNCR1 overexpression (1) increased the number of AR active regions (from 5,717 to 8,239 active regions with 4,257 unique sites) without increasing expression or altering localization of AR (Figure 3B; Schmidt et al., 2016), (2) increased tag density at transcriptional start sites (Figure 3B), (3) increased AR occupancy at 101 of 112 sites with altered AR binding, as identified by differential binding analysis (using AR also regulates melanoma proliferation in an androgen-independent manner.

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model-based analysis of ChIP-seq (MACS; Table S2), and (4) dysregulated 9 (9.2%) of the possible 98 associated genes (included in our RNA-seq analysis) exhibiting differential AR binding ($p < 0.05$) (Schmidt et al., 2016). Collectively, these data suggest that SLNCR1 recruits AR to particular genomic loci. Because AR binds multiple SLNCR1- and SLNCR-regulated genes, even in the absence of ectopically expressed SLNCR1 (Table S2), we considered all identified AR-bound genes in subsequent analyses.

To identify candidate AR- and SLNCR-regulated genes, we searched for genes that are both AR-bound (AR binds within 10,000 bp of gene annotation), as determined by AR ChIP-seq (Table S2), and SLNCR-regulated, as determined by RNA-seq (Table S1). Consistent with a functional relationship between SLNCR and AR, AR binding is enriched among SLNCR-regulated genes. For example, 25.3% of genes (9,139 of 36,074 NCBI-defined genes) were bound by AR, but 43.2% of SLNCR-regulated genes (96 of 222) were bound by AR (binomial test, $p < 0.0001$; Figure 3C; Table S2). Additionally, 45.5% of genes (50 of 110) dysregulated by SLNCR1 overexpression were bound by AR (binomial test, $p = 0.0001$), and 43.8% (53 of 121) of genes dysregulated by SLNCR1 knockdown were bound by AR (binomial test, $p = 0.0003$), further supporting that AR binding is enriched on SLNCR1- and SLNCR1-regulated genes (Schmidt et al., 2016). Consistent with SLNCR and AR cooperatively regulating melanoma cell proliferation, AR binding is enriched among SLNCR-regulated proliferative genes ($-37\%$, 18 of 49; Figure S4A). Interestingly, 53 AR-bound genes were up-regulated upon SLNCR knockdown, whereas 43 genes are downregulated, suggesting that the directional effect of cooperative AR/SLNCR function depends on genomic context.

Many of the AR-bound, SLNCR-regulated genes are known or believed to play important roles in melanoma etiology, including the GRO oncogene and chemokine ligand CXCL2 (log2 fold change, $-2.0$), JUN oncogene (log2 fold change, 0.9), STAT3 transcription factor (log2 fold change, $-0.9$), interleukin-24 (IL-24; log2 fold change, 2.5), and melanoma cell adhesion molecule (MCAM; log2 fold change, 1.2). qRT-PCR of several AR-bound, SLNCR-regulated genes (JUN, CXCL2, and STAT3) before and after siRNA-mediated knockdown of either SLNCR or AR confirms that SLNCR and AR regulate expression of these target genes in both WM1976 and A375 cells (Figure S4B). Contrary to decreased levels upon SLNCR knockdown, AR knockdown increased the levels of CXCL2 (1.25- to 1.75-fold), suggesting that SLNCR and AR may regulate the expression of certain target genes in an opposing manner. Integrative analysis of SLNCR RNA-seq and AR ChIP-seq datasets reveals that AR binding is enriched on SLNCR-regulated genes and suggest that AR and SLNCR similarly regulate the expression of many of these target genes both in vitro and in vivo.

Analysis of TCGA expression data reveals that AR is significantly correlated with expression of over half of SLNCR-regulated genes (148 of 222), 66 of which are also bound by AR based on our ChIP-seq analysis (Table S2). Correcting for multiple-hypothesis testing (Bonferroni correction) maintained the significance of AR correlation with 92 SLNCR-regulated genes, 43 of
Figure 4. **SLNCR and AR Inhibit Expression of CDKN1A/p21 Independent of p53**

(A and B) Knockdown of SLNCR or AR increases CDKN1A levels. Shown is relative expression of the indicated transcripts 72 h post-transfection of the indicated cells with 10 nM of either scramble or SLNCR-targeting (A) or AR-targeting (B) siRNAs. qRT-PCR data are represented as the fold change compared with the scramble control, normalized to GAPDH. Error bars represent SD calculated from 3 reactions.

(C and D) Knockdown of SLNCR (C) or AR (D) increases p21 protein levels. Protein levels were quantified using ImageJ and are presented as a fold change of p21 levels normalized to GAPDH levels. Bars represent mean ± SD from 3 independent biological replicates.

(E) AR and SLNCR inhibit CDKN1A expression independent of p53. Shown is relative expression of the indicated transcripts 72 h post-transfection of the p53-deficient SK-MEL-28 melanoma cell line with 10 nM of either scramble or SLNCR- or AR-targeting siRNAs as in (A and B).

(F) AR and SLNCR inhibit p21 expression independent of p53. The same as in (C) and (D), using the p53-deficient SK-MEL-28 melanoma cell line. Significance was calculated using Student’s t test: *p < 0.05, **p < 0.005, ***p < 0.0005.

(legend continued on next page)
which are bound by AR. There is a significant concordance between target gene correlations with SLNCR and AR expression (Spearman r = 0.2, p = 0.003 overall), further supporting our hypothesis that SLNCR and AR cooperatively regulate the expression of many of these target genes in vivo.

**SLNCR and AR Cooperatively Inhibit Expression of the Cyclin-Dependent Kinase Inhibitor p21 in a p53-Independent Manner**

We next examined the mechanism of AR- and SLNCR-mediated regulation of one representative gene. We focused on the SLNCR-mediated regulation of CDKN1A, the gene encoding the tumor-suppressive cyclin-dependent kinase (CDK) inhibitor 1A (p21\(^{G1p1/Waf1}\)), for several reasons: (1) p21 is an important regulator of cell cycle progression and anti-proliferative pathways, inducing G1 or G2 cell cycle arrest (Gire and Dulic, 2015; Giuliani et al., 2011; Yanagi et al., 2017); (2) it is commonly dysregulated in multiple tumors, including melanoma (Abbas and Dutta, 2009; Jiang et al., 1995; Vidal et al., 1995); and (3) it is transcriptionally regulated by other non-coding RNAs ( Dimitrova et al., 2014; Léveillé et al., 2015; Morris et al., 2008). Additionally, p21 inhibits melanoma proliferation because depletion of p21 increases proliferation of A375 cells (Figures 4C and S4D, p < 0.0001) (Yanagi et al., 2017). In agreement with our RNA-seq analysis, knockdown of SLNCR in WM1976 and A375 cells significantly upregulated p21 mRNA (~1.5- to 2.5-fold increase; Figure 4A). Moreover, knockdown of SLNCR has been shown to increase p21 mRNA in lung cancer cells (Roth et al., 2018). AR knockdown also increased p21 mRNA levels (1.3- to 2.5-fold increase; Figure 4B). Furthermore, knockdown of either AR or SLNCR increased p21 protein levels (~1.4- to 3-fold; Figures 4C and 4D). Thus, SLNCR and AR transcriptionally repress p21 expression.

p21 can be regulated by p53-dependent mechanisms or through less well-characterized p53-independent mechanisms. Neither p53 mRNA (TP53) nor protein are significantly altered upon knockdown of AR or SLNCR in WM1976 or A375 (wild-type p53 [p53\(^{WT}\)]; cells (Figures S5A–S5C). Additionally, knockdown of SLNCR or AR increased p21 mRNA and protein levels in the p53 mutant (p53\(^{143R}\), inactive) primary malignant melanoma cell line SK-MEL-28 (~1.5- to 4-fold increase in both mRNA and protein; Figures 4E and 4F). These data indicate that AR and SLNCR repress p21 in a p53-independent manner.

We next tested whether SLNCR depletion mimics p21-induced melanoma phenotypes. SLNCR knockdown in WM858 (p53\(^{Mut}\)) and WM1976 (p53\(^{WT}\)) cells led to an increased percentage of cells in G2/M and a decreased percentage of cells in G1/G0 (Figure 4G; Gire and Dulic, 2015; Giuliani et al., 2011; Yanagi et al., 2017). These data demonstrate that SLNCR depletion phenocopies p21 induction of G2/M melanoma cell cycle arrest.

In addition to inducing cell cycle arrest, p21 binds to and regulates the activity of many transcription factors (Abbas and Dutta, 2009). We therefore quantified nuclear transcription factor binding to specific DNA motifs in WM1976 cells before and after depletion of SLNCR (Figures 4H and S5D). SLNCR knockdown reduced DNA binding of two transcription factors bound to and regulated by SLNCR1 (AR and Brn3a) by 60%, as measured by transcription factor activation array. Decreased DNA binding by AR occurred independently of altered AR expression or localization (Figure S5E; Schmidt et al., 2016). SLNCR knockdown also decreased DNA binding by other candidate SLNCR-interacting proteins, including EGR1 (70%), E2F-1 (30%), ATF2 (70%), and the ATF2-containing activator protein 1 (API) transcription factor heterodimer (60%) (Schmidt et al., 2016).

SLNCR knockdown decreases DNA binding of known p21 targets, including the estrogen receptor (ER) and C/EBP (both ~40% activity) (Fritah et al., 2005; Harris et al., 2001), and increases DNA binding by SMAD (~270%) (Dai et al., 2012). E2F-1 is a candidate SLNCR-interacting protein (Schmidt et al., 2016) that is inhibited by p21 (Dimri et al., 1996; Isaeva and Mitov, 2009; Jung et al., 2010; Teplyuk et al., 2015). SLNCR knockdown downregulates E2F-1 DNA binding by 30%. These data suggest that SLNCR directly (through protein-RNA interactions) and indirectly (through p21-mediated regulation) regulates the activity of multiple transcription factors. Collectively, SLNCR knockdown phenocopies p21-mediated cell cycle arrest and transcription factor regulation, suggesting that SLNCR knockdown induces biologically relevant upregulation of p21.

Because SLNCR knockdown dysregulated the activity of multiple transcription factors (Figure 4H), we hypothesized that altered transcription factor activity might explain transcriptional effects of SLNCR not directly attributed to AR binding. When limiting our analysis to the 126 SLNCR-regulated genes not bound by AR (Figure 3C), we observed significant enrichment of genes within SLNCR-regulated transcription factor networks, as identified by the transcription factor activation array (Figure 4I), including ER, AR, C/EBP, EGR1, and E2F1 (Figure 4I). Interestingly, SLNCR knockdown also altered the expression of STAT3-regulated genes, a transcription factor whose expression is regulated by both SLNCR and AR (Table S2; Figure S4B). However, depletion of SLNCR does not appear to affect STAT3 activity (Figure S5D), warranting further investigation into the nature of STAT3 regulation. Collectively, these studies suggest that, in addition to cooperative transcriptional regulation (G) SLNCR knockdown induces G2 cell cycle arrest. The cells were stained with propidium iodide (PI) 72 h post-transfection with the indicated siRNAs. Left panel: cell populations of one representative analysis. Right panels: cell populations were analyzed using FlowJo software, and significance was calculated using GraphPad Prism software. Bars represent the average percentage of total cells in the indicated stage of the cell cycle, and error bars represent SD from 3 independent replicates. Significance was calculated using a two-tailed Student’s t test via GraphPad Prism. \( *p < 0.05. \) See also Figures S1 and S2.

(h) SLNCR regulates the activity of multiple transcription factors. Nuclear fractions were isolated from WM1976 cells 72 h post-transfection with either scrambled or si-SLNCR (1) siRNA and entered directly in Signosis Transcription Factor Activation Array I. The ratio of relative luminescence units (RLUs) corresponds to the indicated transcription factor signals of si-SLNCR (1) versus the scramble control. Bars represent mean ± SD from 2 independent biological replicates. Shown are only transcription factors with significantly altered activity. (i) Transcription factor networks enriched among SLNCR-regulated genes that are not bound by AR. The analysis was performed using MetaCore (Thompson Reuters). See also Figure S5.
of AR-bound genes, SLNCR regulates the expression of additional non-AR bound genes through modulation of transcription factor activity, possibly by inhibition of p21.

**SLNCR Recruits AR to EGR1-Bound Loci**

To investigate how SLNCR and AR regulate gene expression, we searched for DNA sequence motifs enriched in AR ChIP-seq datasets. Multiple EM for Motif Elicitation (MEME) and TOOMTOOM analysis (Bailey et al., 2009) identified enrichment of the DNA binding motif of REST (or NRSF), a transcriptional repressor that regulates AR activity in prostate cancer (p = 1e−191; Figure S6A; Svensson et al., 2014). However, the REST motif was not enriched in AR binding sites among SLNCR-regulated genes. Instead, the DNA binding site of the EGR1 transcription factor was enriched (p = 2.24e−05; Figure 5A), suggesting that AR binds to SLNCR-regulated genes through a distinct mechanism, perhaps in cooperation with EGR1.

Our previous (Schmidt et al., 2016) and current (Figures 4G and 4H) work suggest that EGR1 and SLNCR interact directly and functionally. Incubation of biotinylated, full-length SLNCR1 with A375 melanoma cell lysate followed by streptavidin pull-down enriched AR and EGR1 (Figure 5B). This interaction was independently validated using RNA immunoprecipitation (RIP) assays, which enriched SLNCR (~4- to 10-fold) in RNAs immunoprecipitating with EGR1 (Figure 5C). These data confirm that endogenous levels of SLNCR and EGR1 interact in A375 cells.

To distinguish between direct interaction of SLNCR and EGR1 versus an indirect interaction mediated by secondarily associated macromolecules, we performed RNA electrophoretic mobility shift assays (REMSAs). Recombinant EGR1 protein corresponding to amino acids 282–433 altered the mobility of full-length, in vitro-transcribed and biotinylated SLNCR1 in a protein concentration-dependent manner (Figure 5D). Interestingly, EGR1 binding increased RNA mobility (sub-shifted complex), as opposed to more commonly observed decreased RNA mobility (super-shifted complex), possibly as a consequence of altered RNA secondary structure upon protein binding. Unlabeled SLNCR1 competed for EGR1 binding, observed as a loss of increased mobility (i.e., upward shift). Collectively, these data further support the conclusion that endogenous SLNCR and EGR1 directly interact in vitro and at endogenous levels in melanoma cells.

Because SLNCR1 binds to EGR1, and the EGR1 motif is enriched in AR-bound, SLNCR-regulated genes, we hypothesized that AR binds to a subset of SLNCR-regulated genes in cooperation with EGR1. To globally identify EGR1 binding sites in A375 cells, we performed EGR1 ChIP-seq. EGR1 binds a total of 8,373 active regions (Table S3) corresponding to a total of 6,960 active genes (Table S3). Consistent with the expected genomic occupancy, EGR1 ChIP-seq analysis identified many known EGR1-regulated genes, including CCDC28B, ATAD2, and the promoter of EGR1 itself (Figure S6B; Arora et al., 2008; Kubsasaki et al., 2009; Subbaramaiah et al., 2004). Unlike AR, EGR1 appears to bind its known DNA binding sequence in A375 cells because a sequence resembling this motif is the most significantly enriched in EGR1 ChIP-seq peaks (p < 1 × 10−10; Figure S6D).

Surprisingly, we observed a significant overlap between AR and EGR1 binding sites. Additionally, AR and EGR1 frequently co-bound at SLNCR-regulated genes. Although AR and EGR1 bound only 25.3% (9,139 of 36,074) and 19.3% (6,960 of 36,074) of all genes, respectively, AR bound to 58.8% of EGR1-bound genes (4,091 of 6,960 total EGR1 active genes; binomial test, p < 0.0001; Figure S6E). It is important to note that co-bound genes were identified through a stringent analysis of overlapping ChIP-seq reads. This was accomplished by directly integrating AR and EGR1 ChIP-seq reads (spanning an average of only 747 bp) rather than extrapolating binding events occurring within 10,000 bp of an annotated gene. EGR1 bound to 31% of SLNCR-regulated genes (68 of 222 genes; binomial test, p = 0.0003) and 46% of SLNCR-regulated, AR-bound genes (44 of 96 genes; Table S3; Fisher’s exact test, p < 0.0001; Figure 5E). Consistent with cooperative transcription factor binding, AR and EGR1 ChIP-seq peak read intensities overlapped within many of the 44 SLNCR-regulated AR- and EGR-bound genes, including PSAT1, SHF, SLC36A11, and SSU72, and the divergently transcribed SLNCR-regulated gene pair NAASG and ATP6V1A (Figure 5F). Collectively, these data reveal that AR and EGR binding sites overlap more frequently than expected by chance and that these sites are enriched among SLNCR-regulated genes. Because AR and EGR1 binding occurs at known or predicted EGR1 DNA binding motifs, these data suggest that EGR1 is required for regulation of at least a subset of AR- and SLNCR-regulated genes.

Because SLNCR binds to both AR and EGR1, and AR and EGR1 co-bind EGR1 motifs within SLNCR-regulated genes, we postulated that SLNCR recruits AR to EGR1-occupied genomic regions. If true, then EGR1 should regulate the expression of these genes, and SLNCR- and AR-based regulation would require an intact EGR1 DNA binding site. In support of EGR1-mediated regulation, EGR1 expression is significantly correlated with expression of over half of SLNCR-regulated genes (65.3%, 145 of 222), whereas significant correlation is maintained for 71 of these genes after correcting for multiple hypothesis testing (Bonferroni correction, p < 0.00023; Table S1). EGR1 positively regulates the expression of p21 because EGR1 knockdown decreased p21 mRNA and protein levels (Figures 6A–6C). Moreover, EGR1 regulates p21 independent of p53 because EGR1 knockdown in p53 mutant SK-MEL-28 cells decreased p21 levels (Figures 6B, 6C, and S7A). Thus, in contrast to SLNCR and AR, which repress p21, EGR1 activates p21 expression in a p53-independent manner.

To test whether SLNCR- and AR-mediated p21 regulation requires an intact EGR1 binding site, we generated a firefly luciferase reporter construct containing 4,663 nt of the CDKN1A promoter, spanning from the transcription start site to 2,966 nt upstream of the translation start codon and containing the AR- and EGR1-bound consensus EGR1 DNA binding site (Figure 6D). In contrast to regulation of the endogenous CDKN1A gene (Figure 4), knockdown of SLNCR or AR decreased expression of the ectopic CDKN1A luciferase reporter. This discrepancy is likely due to inherent differences between genomic and ectopically expressed plasmid DNA, which may lack the proper chromatin architecture and/or additional proximal or distant enhancer sequences required for recruitment of specific chromatin remodelers. Consistent with ligand-independent AR activation, SLNCR and AR knockdown also
decreased expression of the CDKN1A reporter, even in the absence of exogenous hormones (Figure S7B). Importantly, mutation of the EGR1 binding site negated AR- or SLNCR-mediated regulation of the CDKN1A promoter, confirming that the EGR1 DNA binding site is required for SLNCR- and AR-based regulation of CDKN1A. Together, these data strongly suggest that AR and SLNCR associate with the CDKN1A promoter through DNA-bound EGR1.
Figure 6. EGR1 Increases p21 Expression and Is Required for AR- and SLNCR-Mediated p21 Regulation

(A–C) Knockdown of EGR1 decreases CDKN1A levels independent of p53.

(A and B) Relative expression of the indicated transcripts 72 h post-transfection in (A) A375 or (B) SK-MEL-28 cells with 10 nM of either scramble or EGR1-targeting siRNAs. qRT-PCR data are represented as the fold change compared with the scramble control, normalized to GAPDH. Error bars represent SD calculated from 3 reactions.

(C) Left panel: representative western blot analysis of A375 (top) or SK-MEL-28 (bottom) cell lysates probed for EGR1, GAPDH, or p21 levels. Center and right panels: protein levels were quantified using ImageJ and are presented as relative expression of the indicated protein, normalized to GAPDH levels. Bars represent mean ± SD from 3 independent biological replicates.

(D and E) Mutation of the EGR1 DNA binding site negates AR- and SLNCR-mediated CDKN1A regulation.

(D) Schematic of the CDKN1A locus, highlighting the sequence incorporated into the firefly luciferase reporter. The EGR1/AR binding site is denoted, with the wild-type and mutant sequences shown below, with mutated bases shown in red.

(E) A375 cells were transfected with the indicated siRNAs and, 24 h later, were subsequently transfected with the wild-type (top panel) or mutant (bottom panel) CDKN1A firefly (FL) reporter plasmid and a CMV-RL (cytomegalovirus-Renilla luciferase) control. Relative FL activity was calculated as a fold change compared with vector-only control cells after normalization to RL activity. Shown is one representative assay from four independent biological replicates. Error bars represent SD from four reactions within one biological replicate. Significance was calculated using Student’s t test: *p < 0.05, **p < 0.005, ***p < 0.0005.

See also Figure S7.
expression. When SLNCR
vival (data not shown). Here we comprehensively interrogated
expression is not associated with worse overall melanoma sur-
melanoma oncogene has been confounded by the fact that AR
etiology. Moreover, the interpretation that AR acts as a
have been implicated previously in melanoma invasions, this
and severe melanomas than females (Joosse et al., 2011; Micheli
mediated transcriptional activation of p21. The “tethering” mechanism described
SLNCR-mediated regulation of MMP9, in which AR and
SLNCR can both induce novel transcriptional activity (as in the
cases of the cooperative recruitment of AR and Brm3a) as well
as modulate preexisting regulatory networks (as for Egr1).

To further explore the relationship between p21 and known
regulatory proteins and RNAs (identified here and elsewhere),
we investigated possible associations between SLNCR, AR,
EGR1, p53, and gender with p21 expression within the TCGA
melanoma dataset (using available protein expression for AR,
p53, and p21; n = 354). Using hierarchical multiple regression,
we identified a model containing a significant three-way interac-
tion between EGR1 mRNA, AR, and p53 expression associated
with p21 expression (estimate [95% confidence interval (CI)] =
0.12 [0.03, 0.21]; p = 0.008; Table S5). This finding is in line
with data we and others have presented, indicating that EGR1
and p53 upregulate p21 (Figures 6A–6C; Alemu et al., 2011;
Schmidt et al., 2016; Wang et al., 2017). Collectively, our data are consistent with a model in which
SLNCR recruits AR to chromatin-bound EGR1 to inhibit EGR1-
mediated transcriptional activation of p21 (Figure 7). Under
normal physiological conditions, EGR1 binds directly to an
EGR1 consensus motif located within the CDKN1A promoter,
increasing p21 expression. These findings agree with previous
reports identifying EGR1 as an important activator of p21 in gli-
oma and gastric, colon, prostate, and breast cancer (Escou-
bet-Lozach et al., 2009; Kim et al., 2007, 2014; Parra et al.,
2011; Shin et al., 2010, 2012). During melanogenesis, SLNCR
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with data we and others have presented, indicating that EGR1
and p53 upregulate p21 (Figures 6A–6C; Alemu et al., 2011;
Parra et al., 2011; Riley et al., 2008) and that AR and p53 are transcriptionally and functionally linked (Table S2; Alimirah et al., 2007; Fu et al., 2003; Kang et al., 2009; Mooslehner et al., 2012; Shenk et al., 2001; Zhu et al., 2016). However, this association does not necessarily imply a direct, functional relationship between these variables, and further mechanistic studies are required to understand the biological implications of this association. Although three-way interaction models may be difficult to interpret without additional biological information, they are well suited to cope with dynamic co-expression relations and can capture complex biological associations (Bowers et al., 2004; Khayer et al., 2017). Our regression model also identified a borderline, nonsignificant three-way interaction between EGR1, AR, and SLNCR (estimate [95% CI] = −0.12 [−0.25, 0.001], p = 0.052) and a significant two-way interaction between SLNCR and EGR1 (estimate [95% CI] = −0.10 (−0.19, −0.01), p = 0.024), suggesting an inverse association between SLNCR and p21 expression dependent on the levels of EGR1 and AR. This is consistent with our proposed mechanism where EGR1 is required for SLNCR-mediated repression of p21. Considering our data indicating that (1) SLNCR binds EGR1 and AR (Figures 7B–7D; Schmidt et al., 2016), (2) SLNCR or AR knockdown increased p21 expression (Figure 4), (3) AR and EGR1 bind to an EGR1 DNA binding site in the p21 promoter, and (4) the EGR1 binding site is required for SLNCR and AR-mediated p21 regulation (Figures 6D and 6E), identification of this possible interaction further supports our hypothesis that SLNCR-mediated repression of p21 requires AR and EGR1.

According to our working model, AR is recruited to EGR1-bound loci through SLNCR, not through direct interaction with DNA. AR ChIP-seq peaks at SLNCR-regulated genes display, on average, a lower peak intensity than known AR-targets, consistent with proximal recruitment (as in our model) rather than direct DNA binding. Although current models suggest that trans-recruitment of IncRNA-associated chromatin modifiers occurs through triplex helix formation between the IncRNA and target DNA or through direct base-pairing between the IncRNA and proximally expressed RNAs (Daviddovich and Cech, 2015), multiple lines of evidence argue against direct interaction of SLNCR with either DNA or nascent RNA transcripts. Specifically, (1) sequences enriched among SLNCR-regulated, AR-bound genes do not show significant similarity or complementarity to SLNCR; (2) the CDKN1A locus does not express proximal RNAs in the cells studied here (data not shown); and (3) knockdown of EGR1 does not phenocopy SLNCR knockdown (i.e., increasing p21 levels), which would be expected if SLNCR-mediated the EGR1 association to the CDKN1A promoter. Rather, our data are most consistent with a model in which the IncRNA interacts with a protein bound independently to the target loci (Daviddovich and Cech, 2015). This model is strongly supported by our data indicating that SLNCR binds EGR1 (Figures 5B–5D) and that the EGR1 consensus DNA binding sequence in the CDKN1A promoter is required for SLNCR- and AR-mediated transcriptional regulation of CDKN1A (Figures 6D and 6E).

Because EGR1 is required for SLNCR and AR recruitment to the CDKN1A promoter, our working model anticipates that knockdown of EGR1 would mimic SLNCR and AR knockdown and increase p21 expression. However, this requires that all cells within a heterogeneous cell population express both SLNCR and AR in excess of EGR1-bound DNA. If not, then SLNCR or AR levels are limited in some cells of the heterogeneous cell population, and knockdown of EGR1 would affect normal EGR1 transcriptional upregulation (Figure 7). Indeed, the melanoma cells used in this study likely express lower levels of AR and SLNCR than EGR1, likely explaining why knockdown of EGR1 decreases p21 (Table S1; Figures 6A–6D; Schmidt et al., 2016).

SLNCR- and AR-regulated gene expression appears to be gene-specific because SLNCR-mediated recruitment of AR may either increase or decrease gene expression (Figure S4B). Intriguingly, SLNCR and AR can have opposing effects on a single locus, as seen with CXCL2 (Figure S4B). Although our data indicate that SLNCR and AR are minimally required for transcriptional regulation at many of these sites, it is important to consider that other transcriptional regulators or chromatin modifiers may be recruited by SLNCR or that unique transcriptional regulators can be bound independently to genomic loci. These additional regulators may influence SLNCR and AR activity, and the presence or absence of these factors likely explains the observed complexity of SLNCR- and AR-mediated gene regulation. The fundamental mechanisms of gene regulation by SLNCR and/or AR as well as the identity of additional recruited or precluded factors may need to be empirically determined for each individual target gene.

The observations described here provide the first global characterization of a role of AR in melanoma biology and confirm that AR binds to many melanoma-relevant genes. Remarkably, AR is associated with these regions even in the absence of canonical hormone-mediated activation, indicating that traditional androgen therapies are unlikely to inhibit the oncogenic activities of AR. Instead, SLNCR likely recruits AR to many loci. It is important to note that SLNCR but not AR expression is associated with shorter overall melanoma survival (Schmidt et al., 2016) and may be required for mediating gender-specific differences in AR activity. Collectively, our data implicate SLNCR-mediated AR function as a novel oncogenic pathway, resulting in gender-specific differences in target gene expression. Moreover, this work is further proof that non-coding RNAs are critical regulators of human gene expression. Detailed mechanistic studies of the fundamental actions of IncRNAs and identification of their associated protein partners is critical for the design and implementation of novel therapeutic agents.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
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  - RNA immunoprecipitation and chromatin immunoprecipitation and sequencing
  - RNA-sequencing and analysis
  - Transcription factor activation array
  - Protein extraction and analysis

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○ RNA electrophoretic mobility shift assays
○ TCGA informatics and statistical analyses
● QUANTIFICATION AND STATISTICAL ANALYSIS
● DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.04.101.

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AUTHOR CONTRIBUTIONS

K.S., J.S.C., and E.Y. conducted experiments. K.S., D.D.T., and C.D.N. designed experiments. Bioinformatic analyses were performed by G.S., and statistical analyses were performed by J.R., S.C.N., and L.W.-L. The manuscript was written by K.S. and C.D.N.

DECLARATION OF INTERESTS

K.S. and E.Y. are current employees of Aynlam Pharmaceuticals. J.C. is a current employee of Rubius Therapeutics. K.S. and C.N. are inventors of two patents based on work presented in this manuscript (PCT/US2016/041343 and PCT/US2018/050597).

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## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-AR H-280       | Abcam  | cat# sc-13062; RRID:AB_633881 |
| anti-EGR1 44D5      | Cell Signaling | cat# 4154; RRID:AB_591737 |
| anti-p53 DO-1       | Santa Cruz | cat# sc-126; RRID:AB_628082 |
| anti-AR M-20        | Santa Cruz | cat# sc-816; RRID:AB_1563391 |
| anti-p21 Waf1/Cip1 12D1 | Cell Signaling | cat# 2947; RRID:AB_823586 |
| anti-EGR1 44D5      | Cell Signaling | cat# 4154; RRID:AB_2097035 |
| anti-S6 Ribosomal protein 5G10 | Cell Signaling | cat# 2217; RRID:AB_331355 |
| anti-GAPDH 44C10    | Cell Signaling | cat# 2118; RRID:AB_561053 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| recombinant EGR1 amino acids 282-433 | Aviva Systems Biology | cat# OPCD02876 |
| M-PER Mammalian Protein Extraction Reagent | Thermo Fisher Scientific | cat# 78501 |
| WST-1 proliferation reagent | Roche, available from Sigma-Aldrich | cat# 5015944001 |
| DMEM, high glucose, no glutamine | Invitrogen | cat# 11960044 |
| GibCO FBS | Thermo Fisher Scientific | cat# 26140 |
| DMEM, high glucose, no glutamine, no phenol red | Invitrogen | cat# 31053028 |
| Charcoal-stripped FBS | Sigma/Millipore | cat# F6765 |
| FxCycle PI/RNase Staining Solution | LifeTech/Thermo Fisher Scientific | cat# F10797 |
| Dynabeads Protein A for Immunoprecipitation | Invitrogen | cat# 10008D |
| Any kD Mini-PROTEAN™ TGX Precast Protein Gels | BioRad | cat# 4569033 |
| 5% Mini-PROTEAN™ TBE Gel, 10 well, 30 ml | BioRad | cat# 4565013 |
| Amersham Hybond-N+ | GE Healthcare Lifesciences | cat# RPN203B |
| **Critical Commercial Assays** |        |            |
| LightShift Chemiluminescent RNA EMSA (REMSA) Kit | Thermo Fisher Scientific | cat# 20148 |
| TF Activation Profiling Plate Array I | Signosis | cat# FA-1001 |
| TruSeq RNA sample preparation kit (v2) | Illumina | cat# rs-122 |
| Pacific Blue Annexin V Apoptosis Detection Kit with 7-AAD | Biologend | cat# 640926 |
| Dual-Glo® Luciferase Assay System | Promega | cat# E2920 |
| NE-PER Nuclear and Cytoplasmic Extraction Kit | Thermo Fisher Scientific | cat# 78833 |
| **Deposited Data** |        |            |
| AR ChIP-seq, EGR1 ChIP-seq, WM1976 RNA-seq | This study. NCBI's Gene Expression Omnibus | GEO: GSE116191 |
| A375 RNA-seq | NCBI's Gene Expression Omnibus | GEO: GSE77903 |
| National Cancer Institute Genomic Data Commons | N/A | https://gdc.cancer.gov/ |
| cbioPortal | Cerami et al., 2012; Gao et al., 2013 | https://www.cbioportal.org/ |
| The Cancer Proteome Atlas | Li et al., 2013a | https://tcpaportal.org/tcpa/ |
| TP53 database | Leroy et al., 2014 | http://p53.fr/ |
| **Experimental Models: Cell Lines** |        |            |
| WM1976 human melanoma cell line | Wistar Institute | WM1976 |
| WM858 human melanoma cell line | Wistar Institute | WM858; RRID:CVCL_283 |
| A375 | American Type Culture Collection | cat# ATCC® CRL-1619; RRID:CVCL_0132 |
| SK-MEL-28 | American Type Culture Collection | cat# ATCC® HTR-72; RRID:CVCL_0526 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Carl Novina (Carl_Novina@dfci.harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

WM1976 (p53 wild-type) and WM858 (female, BRAFV600E, p53MUT) are from the Wistar Institute collection, A375 (female, BRAFV600E, p53 wild-type, CDKN2AE61*) and SK-MEL-28 (male, BRAFV600E, EGFRP753S, P53L145R) cells were purchased from ATCC. The A375 and WM858 cell lines were authenticated via short tandem repeat profiling at the American Tissue Culture Repository on May 19, 2016. WM1976 and SK-MEL-28 cells were not subject to additional authentication. Unless otherwise indicated, cells were grown in DMEM (Invitrogen) without glutamine supplemented with 10% fetal bovine serum (FBS). Hormone-deprived cells were cultured in phenol-red free DMEM without glutamine (Invitrogen) with 5% charcoal-stripped FBS (Sigma-Aldrich).

METHOD DETAILS

Cell culture and cell-based assays

All siRNAs were transfected using RNAiMAX (Thermo Fisher). AR and SLNCR targeting siRNAs were used at 10 nM final concentration. EGR1 targeting siRNA was used at 20 nM. For knockdown and flutamide proliferation assays, cells were seeded at 0.4 × 10^4 cells/well in a 96-well plate. For assays using FANA-modified oligos (AUM Technologies), cells were seeded at 0.3 × 10^5 cells/well in a 96-well plate. Cells were treated with FANA oligos, flutamide, or transfected with the indicated siRNAs 24 hours (hr) post seeding, and proliferation was measured using a 1:10 dilution of WST-1 proliferation reagent (Roche) at the indicated time points. Cells were incubated for one hour at 37°C, and absorbance at 450 nm was measured. For cell cycle analyses, cells were harvested and washed 72 hours post-transfection, fixed in cold 70% ethanol for 2 hours, and incubated in LifeTech PI/RNaseA solution for 30 minutes at 37 degrees. For analysis of apoptosis, cells were seeded at 30 × 10^4 cells/well in 6-well plate, harvested 72 hours post-transfection, and stained using Biolegends Pacific Blue™ Annexin V Apoptosis Kit with 7-AAD. Cells were analyzed on a Fortessa X-20 and populations were identified and quantified using FlowJo software. Luciferase assays, A375 cells were seeded in 96-well plates at 0.75 × 10^5 cells/well, harvested 24 hours later with the indicated siRNAs. Fifty micrograms of either wild-type or mutated CDKN1A reporter plasmid and 50 μg of a pCMV-renilla luciferase control vector were transfected Lipofectamine® 2000 (Life Technologies) 24 hours post-transfection of siRNAs. Luciferase activity was measured another 24 hours later using Promega Dual-Glo® Luciferase Assay system. The CDKN1A reporter plasmid was generated by replacing the MMTV promoter in pGL4.36 vector (Promega) using Gibson Cloning (Gibson et al., 2009). Sequences for all siRNAs and oligos used in this study can be found in Table S6.

RNA immunoprecipitation and chromatin immunoprecipitation and sequencing

AR and EGR1 RIP assays were performed as previously described with minor modifications (Schmidt et al., 2016). For EGR1 RIP, IgG or α-EGR1 antibody was added to A375 cell lysate at a final concentration of 0.5 μg and rotated at 4°C for 2 hours. Lysate was then incubated with Protein A Dynabeads® (Life Technologies) (25 μL slurry) for 1 hour at 4°C and samples were processed as described. Fold enrichment of SLNCR was calculated as the fold enrichment in the IgG or EGR1 IP compared to input control after normalization to the indicated mRNA transcript (18S, GAPDH, ACTIN).

REAGENT or RESOURCE SOURCE IDENTIFIER

Oligonucleotides

See Table S6 N/A

Recombinant DNA

pGL4.36[luc2P/MMTV/Hygro] Vector, 20ug Promega cat# E1360

Software and Algorithms

FlowJo Software

FlowJo https://www.flowjo.com/

MACS

Trappnell et al., 2012 http://cole-trapnell-lab.github.io/cufflinks/

MetaCore

Thompson Reuters, now Clarivate https://clarivate.com/products/metacore/

GraphPad Prism version 7.00 for Windows

GraphPad Software https://www.graphpad.com/

Integrated Genome Viewer

Robinson et al., 2011; Thorvaldsdóttir et al., 2013 https://www.broadinstitute.org/igv/
For AR ChIP-seq, A375 cells were cultured in phenol-red-free DMEM without glutamine (Invitrogen), supplemented with 5% charcoal-stripped FBS, and transfected with the indicated plasmid 24 hour post-seeding. For EGR1 ChIP-seq, A375 cells were cultured in DMEM without glutamine (Invitrogen), supplemented with 10% FBS, and grown to ~80% confluency. Cells were crosslinked in 1% formaldehyde for 15 min 48 hour post-transfection, and the reaction was quenched by addition of 0.125 M glycine. ChIP-seq was performed by Active Motif using Santa Cruz AR (H-280), or Cell Signaling EGR1 (44D5). After chromatin isolation and fractionation, 75-nt reads were generated by Illumina sequencing (using NextSeq 500) and were mapped to human reference genome (hg19) using the BWA algorithm with default settings. The 3' ends of aligned reads were extended in silico using Active Motif software to a length of 150-200 bp. Fragment density was determined based on the number of reads corresponding to 32-nucleotide genomic bins. Peak calling, to identify intervals with local enrichment in reads, was performed using MACS (Zhang et al., 2008). MACS default cutoff for narrow peaks and 1e-1 for broad peaks. Peak filtering was performed by removing false ChIP-Seq peaks as defined within the ENCODE blacklist. Active regions were defined by the start coordinate of the most upstream interval and the downstream coordinate of the most downstream interval. Active genes are defined as any active region present within 10,000 bps upstream or downstream of an annotated gene.

RNA-sequencing and analysis
Total RNA was isolated from WM1976 transfected with either scramble, si-SLNCR (1) or si-SLNCR (2) siRNAs, in duplicate, using Trizol. Sequencing cDNA libraries were prepared from 1ug of total RNA using the Illumina TruSeq RNA sample preparation kit (v2). Libraries were pooled and sequenced on the Illumina HiSeq 2500 platform. Normalized read counts (FPKM) were generated in Cufflinks v2.1.1 (http://cole-trapnell-lab.github.io/cufflinks/) by mapping onto the hg19 build of the human transcriptome (https://support.illumina.com/sequencing/sequencing_software/igenome.html). Raw FASTQ sequence was mapped using Bowtie (Langmead et al., 2009), and differentially expressed genes were identified using CuffDiff (http://cufflinks.cbcb.umd.edu/), comparing duplicate scramble controls against duplicate conditions of both SLNCR-specific knockdowns. Values represented in the heatmaps were generated by CuffDiff comparison of duplicate scramble controls versus duplicates of only one siRNA duplicate. GeneOntology Enrichment Analysis was performed in MetaCore (Thompson Reuters), against a control background set of genes expressed in skin cells.

Gene expression of AR- and SLNCR-target genes were accessed using cBioPortal (Cerami et al., 2012; Gao et al., 2013). All statistics (including t tests, ANOVAs, and correlations) were calculated using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA (https://www.graphpad.com/). Bonferroni correction for multiple hypothesis testing was performed by defining the significance threshold as the critical p = 0.05 divided by the total number of comparisons. BAM files from RNA-seq and ChIP-seq were visualized using the Integrated Genome Viewer (https://www.broadinstitute.org/igv/) (Robinson et al., 2011; Thorvaldsdottir et al., 2013).

Transcription factor activation array
WM1976 cells were seeded in 6-well tissue culture treated dishes, transfected 24 hours later with either scramble or si-SLNCR (1) siRNA, and were harvested and fractionated using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit, according to the manufacturer’s instructions, 3 days after transfection. Ten micrograms of nuclear lysate was used directly as input into the Signosis TF Activation Profiling Plate Array I.

Protein extraction and analysis
Unless otherwise indicated, lysate was prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), according to manufacturer’s instructions. Samples were separated on BioRad Any kD Mini-PROTEAN® TGX Precast Protein Gels and transferred to LF-PVDF using the mixed MW protocol on the BioRad Transblot Turbo. The following antibodies were used: Santa Cruz p53 (DO-1) sc-126 at 1:200, AR (M-20) sc-816 at 1:200, Cell Signaling P21 Waf1/Cip1 (12D1) at 1:1000, Cell Signaling EGR1 (44D5) at 1:1000, Cell Signaling S6 Ribosomal Protein (5G10) at 1:1000, and Cell Signaling GAPDH (14C10) at 1:5000.

RNA electrophoretic mobility shift assays
REMSAs were performed using Thermo Fisher Scientific LightShift Chemiluminescent RNA EMSA (REMSA) Kit, according to manufacturer’s instructions. Briefly, 20 µl binding reactions were assembled in low-adhesion tubes in 1X binding buffer (10mM HEPES pH 7.3, 20 mM KCl, 1 mM MgCl2, 1 mM DTT), with 2 µg of yeast tRNA, the indicated amount of recombinant EGR1 corresponding to amino acids 282-433 (Aviva Systems Biology, catalog number OPCD02876), 0.5 nM final concentration of the biotinylated SLNCR1, and 10 µM of unlabelled SLNCR1 where indicated. Reactions were incubated at room temperature for 20 minutes, 5 µl of loading dye was added, and 20 µl was electrophoresed on Bio-Rad’s 5% Mini-PROTEAN® TBE Gel, 10 well, 30 µl. RNA and protein/RNA complexes were transferred to GE Healthcare Amersham Hybond –N+ Membrane in 0.5x TBE at 400 mAh for 30 minutes in 0.5x TBE on Bio-Rad’s Trans Blot Turbo Transfer System. Detection was performed according to LightShift REMSA kit, using Bio-Rad’s ChemiDoc XRS+ System.
TCGA informatics and statistical analyses

Gene Ontology Enrichment Analysis was performed in MetaCore (Thompson Reuters), against a control background set of genes expressed in skin cells. Gene expression of AR- and SLNCR-target genes, p21 protein levels, and p53 mutational status were accessed using cBioPortal (Cerami et al., 2012; Gao et al., 2013). SLNCR RNA expression values were derived from normalized read coverage across the SLNCR genomic range. Raw RNA-Seq data for cutaneous melanoma (SKCM) was downloaded from the NCI Genomic Data Commons (GDC). The RNA-Seq data was in the format of BAM files representing an alignment, using the STAR aligner, of raw reads to hg38. Human gene models were downloaded from RefSeq on November 3rd, 2017, and the SLNCR ranges were defined from the range of LINC00673. For each patient, the total number of reads aligning the SLNCR genomic region was obtained by parsing the output of samtools flagstat and SLNCR-specific counts were normalized by the total number of reads aligning to hg38. The expression values for non-SLNCR genes were obtained by downloading a results table from Xenabrowser (https://toil.xenahubs.net/download/tcga_rsem_isoform_tpm.gz). TPM expression values were computed from a reanalysis of the TCGA dataset under the TOIL framework using RSEM. Hierarchical multiple regression analysis was performed using R v3.2.2, where the model estimates and p values, based on the t-statistic, were calculated using the ‘lm’ function. SLNCR and EGR1 mRNA expression values were log2 transformed to account for non-normal distributions. Expression of AR, p53 and p21 protein was accessed from Level 4 cross-batch normalized data in The Cancer Proteome Atlas (Li et al., 2013a). All continuous variables were converted to z-scores in order to improve interpretability of the model output. To determine the set of parameters present in the final model, standard reduction techniques were used, including iterative removal of the least significant parameter along with evaluation of the Akaike’s information criterion (AIC) and ANOVA comparisons of model fit. Bonferroni correction for multiple hypothesis testing was performed by defining the significance threshold as the critical P value (0.05) divided by the total number of comparisons. BAM files from RNA-seq and ChIP-seq were visualized using the Integrated Genome Viewer (https://www.broadinstitute.org/igv/) (Robinson et al., 2011; Thorvaldsdottir et al., 2013).

QUANTIFICATION AND STATISTICAL ANALYSIS

t tests, ANOVAs, and correlations were calculated using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA (https://www.graphpad.com/). In proliferation assays error bars represent the mean ± SD of 3 technical replicates. Significance was calculated using the two-way analysis of variance (ANOVA), with the Dunnett test for multiple comparison testing. For binding enrichments, statistical significance was calculated using a either a Binomial test (two-sided) by comparing the observed versus expected probabilities under independence or using a Fisher Exact test of the co-bound targets, using GraphPad Prism software. In cell cycle assays, cell populations were analyzed using FlowJo software, and significance was calculated using GraphPad Prism software. Bars represent the average percent of total cells in the indicated stage of the cell cycle, and error bars represent SD from 3 independent replicates. RT-qPCR data is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. Protein levels were quantified using ImageJ, and are presented as a fold change normalized to GAPDH levels. Bars represent mean ± SD from 3 independent biological replicates. Significance for RNA and protein quantification were calculated using the Student’s t test. Transcription Factor Activation Array is represented by relative luminescence mean ± SD from 2 independent biological replicates.

DATA AND SOFTWARE AVAILABILITY

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series: GSE116191.
Supplemental Information

The IncRNA \textit{SLNCR} Recruits the Androgen Receptor to EGR1-Bound Genes in Melanoma and Inhibits Expression of Tumor Suppressor p21

Karyn Schmidt, Johanna S. Carroll, Elaine Yee, Dolly D. Thomas, Leon Wert-Lamas, Steven C. Neier, Gloria Sheynkman, Justin Ritz, and Carl D. Novina
Figure S1: *SLNCR1*, but not *SLNCR2* nor *SLNCR3*, increase melanoma invasion, related to Figure 1. (A) Schematic of the exons (numbered) of the 3 *SLNCR* isoforms previously identified in melanomas (not drawn to scale). Denoted is the sequence required for AR binding, and the regions targeted by the siRNAs used in this study. (B) Quantification from 3 independent replicates, represented as mean ± SD, of matrigel invasion assays of A375 cells transfected with the indicated empty or *SLNCR*-expressing plasmid. Invasion is calculated as the percent of invading cells compared to mobile cells as counted in 8 fields of view. (C) Relative *MMP9* (left) or *SLNCR* (right) expression in A375 cells transfected with the indicated empty or *SLNCR*-expressing plasmid. RT-qPCR data is represented as the fold change compared to scramble control, normalized to *GAPDH*. Error bars represent standard deviations calculated from 3 reactions. Significance was calculated using the two-tailed Student’s t-test: ** p < 0.005. (D) RNA immunoprecipitations from HEK293T cells transfected with GFP-tagged AR, using α-AR antibody or a matched IgG nonspecific control. Left panel: western blot analysis of input or bound proteins following IP with either IgG or α-AR (AR) antibodies. Right panel: relative enrichment of the indicated transcript measured via RT-qPCR compared to IgG nonspecific control.
Figure S2: siRNA-mediated knockdown of SLNCR does not affect melanoma apoptosis, related to Figure 1.
(A) Relative SLNCR expression in the indicated cells transfected with either scramble or SLNCR-targeting siRNAs.
RT-qPCR data is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. Significance was calculated using the two-tailed Student’s t-test: * p < 0.05, ** p < 0.005, *** p < 0.0005, n.s. = not significance. (B) Scatter plot of the log2 fold change of genes significantly dysregulated (p < 0.01) upon knockdown of both SLNCR1 and SLNCR (all isoforms). Genes that are dysregulated in an opposing manner by SLNCR1 versus SLNCR are labeled and denoted in red. Pearson’s correlation (r) was calculated using GraphPad Prism. (C) Scatter plot of primary melanoma mitotic growth rate versus Log2 SLNCR expression for 172 melanomas from the TCGA (mitotic growth rate was available for only 172 melanomas). (D) Representative scatter plots of annexin V and 7-AAD staining, as measured via fluorescence-activated cell sorting (FACS) analysis, of the indicated melanoma cells transfected with the indicated siRNAs. Cells are classified as “viable” (Q4, bottom left), “apoptotic” (Q1, top left), or “necrotic” (Q4, top right).
Figure S3: AR increases melanoma cell proliferation, related to Figure 2. Twenty-four hours after the indicated cells were seeded in 96-well plates, vehicle-control or the indicated concentration of flutamide was added. Cell proliferation was quantified using WST-1 reagent, as in Figure 1D. (B) Relative AR expression in the indicated cells transfected with either scramble or SLNCR-targeting siRNAs. (C) Western blot of A375 cell lysates following transfection with the indicated siRNAs. Left panel: representative blot probed with α-AR and α-GAPDH antibodies. Right panel: quantification from three independent replicates, normalized to GAPDH. (D) Relative SLNCR expression in the indicated cells following addition of the indicated FANA-modified oligos. RT-qPCR data ((A) and (C)) is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. Significance was calculated using the Student’s t-test: * p < 0.05, ** p < 0.005, *** p < 0.0005.
Figure S4: SLNCR and AR regulate many overlapping genes, including genes implicated in melanoma proliferation, related to Figure 3. (A) Heat map of SLNCR-regulated genes (as in Figure 1A) that are also AR-bound. The shading represents the log2 fold change compared to scramble siRNA control. Genes are clustered with Euclidean distance and average linkage clustering. Red arrows denote genes implicated in cell proliferation. (B) Relative expression of the indicated genes from indicated cells transfected with either scramble, SLNCR-, or AR-targeting siRNAs. (C) Knockdown of CDKN1A in A375 cells. Left panel: Relative CDKN1A expression following transfection of the indicated siRNAs, represented as the fold change compared to scramble control, normalized to GAPDH. Middle panel: western blot of A375 cell lysates following transfection with the indicated siRNAs probed with α-p21 and α-GAPDH antibodies. Right panel: quantification from three independent replicates, normalized to GAPDH. (D) Cell proliferation was quantified using WST-1 reagent following transfection with the indicated siRNAs. Significance was calculated using the two-way analysis of variance (ANOVA), with the Dunnett test for multiple comparison testing, **** p < 0.0001. RT-qPCR data is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. Significance was calculated using the Student’s t-test: * p < 0.05, ** p < 0.005, *** p < 0.0005.
Figure S5: *SLNCR* and AR do not directly regulate expression of p53, related to Figure 4. (A and B) Relative expression of TP53 72 hours post-transfection of the indicated cells with 10 nM of either scramble or *SLNCR* (A) or AR (B) targeting siRNAs. (C) Knockdown of *SLNCR* or AR in A375 melanoma cells does not affect p53 expression. Protein levels were quantified using ImageJ, and are presented as a fold change of p21 levels, normalized to GAPDH levels. Bars represent mean ±SD from 3 independent biological replicates. (D) Transcription factor activity in WM1976 nuclear lysate following transfection with either scramble or si-*SLNCR* (1) siRNA was measured by Signosis’ Transcription Factor Activation Array I. The raw relative luminescence units (RLUs) are shown for all transcription factor probes included in the array. (E) Knockdown of *SLNCR* does not affect AR expression. Relative expression of AR 72 hours post-transfection of the indicated cells with 10 nM of either scramble or *SLNCR* targeting siRNAs. RT-qPCR data is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. Significance was calculated using the Student’s t-test and is only indicated where determined to be significant: ** p < 0.005.
Figure S6. AR and EGR1 cobind EGR1 consensus DNA motifs in melanoma cells, related to Figure 5. (A) A motif resembling the consensus REST DNA binding motif (top panel) is significantly enrichment in AR ChIP-seq peaks with vector (middle) or SLNCR-expression plasmid (bottom). (B) Integrated Genome Viewer plot displaying EGR1 ChIP-seq read intensities for the indicated transcripts. Numbers on the left indicated plot height. (C) Venn diagram representing the total regions bound by either AR in A375 cells transfected with vector (light blue) or SLNCR1-expressing plasmid (blue), or bound by EGR1 (green). (D) TOMTOM analysis identified a significant enrichment of a motif in EGR1 ChIP-seq peaks (bottom) showing significant similarity to the consensus EGR1 DNA binding motif (top panel). (E) Venn diagram representing the genes bound by either AR (blue) or EGR1 (green) within 10,000 bp of an annotated gene in A375 cells.
Figure S7: AR and SLNCR-mediated regulation of p21 does not require p53 or androgens and occurs in a gender-specific manner, related to Figures 6 and 7. (A) Knockdown of EGR1 does not affect TP53 levels. Relative expression of TP53 72 hours post-transfection of either A375 (left) or SK-MEL-28 (right) cells with 10 nM of either scramble or EGR1-targeting siRNAs. RT-qPCR data is represented as the fold change compared to scramble control, normalized to GAPDH. Error bars represent standard deviations calculated from 3 reactions. (B) SLNCR and AR regulate the CDKN1A promoter in the absence of androgens. Same as in Figure 5D, using A375 cells grown in hormone-starved conditions. (C) Box plot of relative AR protein expression, determined via reverse phase protein array (RPPA), in male and female TCGA primary melanomas. Data are represented as mean ± SEM. (D) Box plot of relative p21 (left) and AR (right) expression but for only p53-deficient melanomas. Significance was calculated using the Student’s t-test: * p < 0.05, ** p < 0.005, *** p < 0.0005, ns = not significant.
| PATIENT ID   | Gender | TP53 mutational status | P21 (RPPA) | AR (RPPA) |
|--------------|--------|------------------------|------------|-----------|
| TCGA-EE-A29M | FEMALE | F134L                  | 0.14587    | -0.86133  |
| TCGA-DA-A1HW | FEMALE | K120E                  | 0.040554   | -0.9723   |
| TCGA-GF-A6C8 | FEMALE | P278S                  | 0.052421   | -0.5473   |
| TCGA-BF-A3DL | FEMALE | R196*                  | 0.065464   | -0.95115  |
| TCGA-DA-A115 | FEMALE | R196*, E286K           | 0.052141   | -0.98755  |
| TCGA-EB-A5FP | FEMALE | R213*                  | 0.25405    | -0.55538  |
| TCGA-EE-A3AE | FEMALE | R213*                  | -0.063335  | -1.0698   |
| TCGA-DA-A1HV | FEMALE | R280K                  | 0.062088   | -0.70658  |
| TCGA-EE-A181 | FEMALE | V143E                  | 0.053279   | -0.91168  |
| TCGA-EE-A2MM | FEMALE | V143G                  | -0.25283   | -0.46138  |
| TCGA-ER-A199 | FEMALE | X126_splice            | -0.13843   | -0.87842  |
| TCGA-D3-A51T | FEMALE | X187_splice            | 0.10025    | -1.0643   |
| TCGA-ER-A19E | FEMALE | X224_splice            | 0.059999   | -0.27543  |
| TCGA-FS-A4F9 | MALE   | A276Lfs*29             | -0.47049   | -0.6793   |
| TCGA-EB-A3XC | MALE   | C275Y                  | 0.11002    | -0.78656  |
| TCGA-EE-A2MU | MALE   | G266E                  | 0.4328     | -0.011376 |
| TCGA-D3-A1QA | MALE   | H214Qfs*7              | -0.28437   | -0.20244  |
| TCGA-EE-A29E | MALE   | L330R                  | -0.2328    | -0.87094  |
| TCGA-EE-A3AA | MALE   | P151L, P27S, P151S     | 0.12717    | -0.73382  |
| TCGA-EE-A29L | MALE   | P177_C182del           | -0.22706   | -0.9179   |
| TCGA-EE-A2GC | MALE   | P177L                  | -0.43232   | -0.68729  |
| TCGA-GN-A266 | MALE   | R290C, A159V           | 0.090022   | -0.30487  |
| TCGA-GF-A6C9 | MALE   | S127F                  | -0.43199   | -0.79911  |
| TCGA-EE-A3AD | MALE   | S241F                  | -0.23497   | -0.9065   |
| TCGA-EE-A3J7 | MALE   | S241F                  | -0.20028   | -0.6418   |
| TCGA-FS-A1Z4 | MALE   | V97Sfs*26              | 0.1418     | -0.59557  |
| TCGA-D9-A6EC | MALE   | X187_splice, R213*     | -0.39416   | -0.8732   |

Table S4: AR and p21 protein expression of p53-deficient TCGA melanomas, related to Figure 7. P53-deficient melanomas were defined as (i) primary melanomas or metastases of known melanoma origin, (ii) patients with no prior treatment, and (iii) harboring nonfunctional p53 mutations, as defined by the TP53 database (p53.fr) (Leroy et al., 2014). Three additional patients containing R248W or Y220C gain of function p53 mutations were excluded based on reported regulation of p21 (Di Fiore et al., 2014; Song et al., 2007; Xu et al., 2014).
| Variable or interaction | Estimate (95% CI) | p-value |
|------------------------|-------------------|---------|
| Intercept              | -0.05 (-0.15, 0.06) | 0.38    |
| SLNCR                  | -0.07 (-0.17, 0.03) | 0.19    |
| EGR1 mRNA              | -0.12 (-0.23, -0.02) | 0.023   |
| AR protein             | 0.04 (-0.07, 0.15) | 0.49    |
| P53 protein            | -0.13 (-0.24, -0.01) | 0.027   |
| SLNCR * EGR1           | -0.10 (-0.19, -0.01) | 0.024   |
| SLNCR * AR             | 0.002 (-0.11, 0.11) | 0.96    |
| EGR1 * AR              | -0.05 (-0.16, 0.06) | 0.34    |
| EGR1 * p53             | <0.001 (-0.11, 0.11) | 0.99    |
| AR * p53               | 0.12 (0.05, 0.18) | 0.0004  |
| EGR1 * AR * SLNCR      | -0.12 (-0.25, 0.001) | 0.052   |
| EGR1 * AR * p53        | 0.12 (0.03, 0.21) | 0.008   |

Table S5: Final model determined from hierarchical multiple regression analysis of p21 protein expression in TCGA melanomas (n=354), related to Figure 7. Adjusted R-squared = 0.06, Model (F-statistic) p = 0.0009. Variables assessed as part of the multiple regression analysis of p21 expression included patient gender, SLNCR and EGR1 mRNA log2 expression, and AR and p53 protein expression (from TCPA) and all possible interactions. All continuous variables were converted to z-scores in order to improve interpretability of the model output. In order to determine the set of parameters present in the final model, standard reduction techniques were used, including iterative removal of the least significant parameter along with evaluation of the Akaike's information criterion (AIC) and ANOVA comparisons of model fit. Non-significant parameters remain in the final model due to the presence of significant two- and three-way interactions. P-values have not been adjusted for multiple hypothesis testing.
| Sequence                          | Name  | Type                      |
|----------------------------------|-------|---------------------------|
| TTAGGGTCAAATAGGATCTAAA (targeting) | si-SLNC (1) | siRNA                    |
| AAAGGACGTCTACCCGAGAAA (targeting) | si-SLNC (2) | siRNA                    |
| CAGGAATTCCTGTGAGATCAAA (targeting) | si-AR (1) | siRNA                    |
| CAGGGAAAGTGGAGAGCTA (targeting)  | si-AR (2) | siRNA                    |
| CTGCTACTCTTCAGCATTATT (targeting) | si-AR (3) | siRNA                    |
| GAAGGTGAAGTGGATTTCTCCAAGA       | GAPDH Forward | qPCR                     |
| GAAGATGGTGTGGATTTCTCCAAGA       | GAPDH Reverse | qPCR                    |
| GTGGAGCGATTTGTCTGTTTGGTAGAAG    | 18S Forward | qPCR                     |
| CGCTGAGGACTGTGAGAGTAAGAAG      | 18S Reverse | qPCR                     |
| AAACCTTAATGAGCTGTACAGGAAG      | β-ACTIN Forward | qPCR                    |
| CCGCTAGACATGACATGACAGGAAG      | β-ACTIN Reverse | qPCR                    |
| GAGAAAGGTGTGGATTTCTCCAAGA       | SLNCR Forward (all isoforms) | qPCR                |
| TCCCCTCTCTTTTCTTTCTTA          | SLNCR Reverse (all isoforms) | qPCR                |
| GGTGACACCAAAAGGCTAGAA          | AR Forward | qPCR                     |
| GACTTGTAGAGACAGCGGATGAAG        | AR Reverse | qPCR                     |
| TGTCAGTCTCTGTACCCTCTG          | CDKN1A Forward | qPCR                    |
| GGGCGTGAGTGGTAGAAGAAG          | CDKN1A Reverse | qPCR                    |
| GCCATGATCAACGTACATAGCAG         | TP53 Forward | qPCR                     |
| TACATCAATACATCCACACGC          | TP53 Reverse | qPCR                     |
| GTGTTGTCTCCGGGCTGGAAGCTTGGCAATCCGGTAC | Remove MMTV from pGL4.36 | Gibson cloning primer |
| TCTTCTATGCCAGGCGCAGTCTGGCAATCCGGTAC | Remove MMTV from pGL4.36 | Gibson cloning primer |
| CTGGCATAGAAGAGGCTGGT           | CDKN1A promoter Forward | PCR amplification |
| CAAGCGAGACACACTGATGTAT         | CDKN1A promoter Reverse | PCR amplification |
| GTCAAGTCCGCGCGTGATTTCTGAG      | EGR1 binding site mutant | Site-directed mutagenesis |
| GTGCGTGGGCGAGACCTCCGGAC        | EGR1 binding site mutant | Site-directed mutagenesis |

Table S6: siRNA targets and oligo sequences used in this study, related to Figures 1-7. Red font denotes mutated nucleotides.