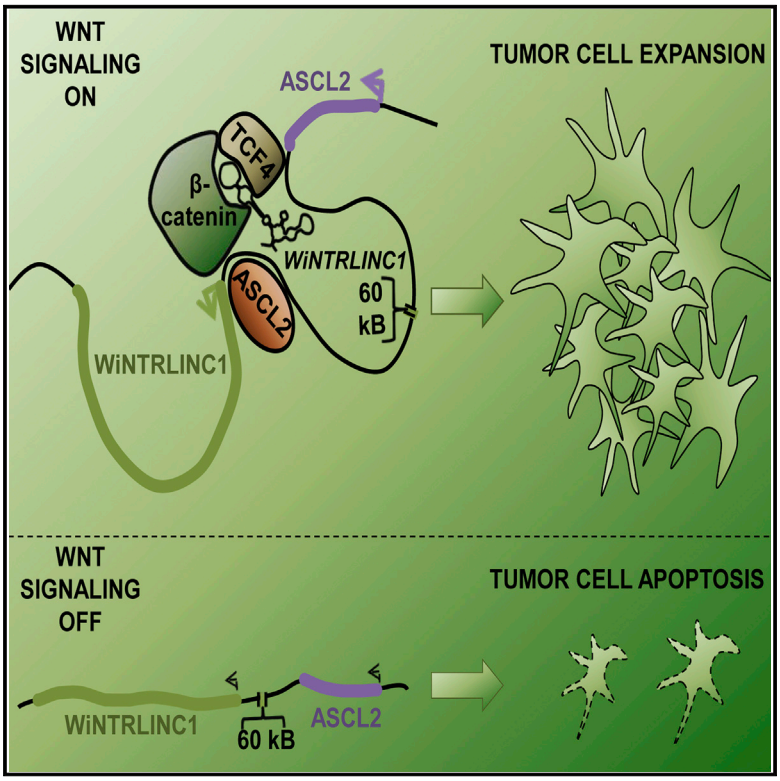
Report

Cell Reports

A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate

Graphical Abstract



Authors

Antonis Giakountis, Panagiotis Moulos, Vasiliki Zarkou, ..., Artemis G. Hatzigeorgiou, Martin Reczko, Pantelis Hatzis

Correspondence

hatzis@fleming.gr

In Brief

Giakountis et al. show that the Wnt-regulated long non-coding RNA WiNTRLINC1 promotes proliferation and survival of colon cancer cells by regulating its genomic neighbor ASCL2 through long-distance looping. The expression of both genes is amplified in colorectal cancer, providing potential new prognostic and drug targets.

Highlights

- We identify a Wnt-regulated long non-coding RNA, WiNTRLINC1
- Its downregulation in colon cancer cells causes growth defects and induces apoptosis
- It positively regulates ASCL2 through long-distance looping and chromatin changes
- The WiNTRLINC1-ASCL2 regulatory axis is amplified in colorectal cancer

Accession Numbers

GSE69036
KR736336

Giakountis et al., 2016, Cell Reports *15*, 1–9
June 21, 2016
http://dx.doi.org/10.1016/j.celrep.2016.05.038

CellPress

Please cite this article in press as: Giakountis et al., A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate, Cell Reports (2016), http://dx.doi.org/10.1016/j.celrep.2016.05.038

Cell Reports
Report

A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate

Antonis Giakountis,[1] Panagiotis Moulos,[1] Vasiliki Zarkou,[1,2] Christina Oikonomou,[1,4] Vaggelis Harokopos,[1] Artemis G. Hatzigeorgiou,[1,3] Martin Reczko,[1] and Pantelis Hatzis[1,*]
[1]Biomedical Sciences Research Center 'Alexander Fleming', 16672 Vari, Greece
[2]School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[3]DIANA-Lab, Department of Electrical and Computer Engineering, University of Thessaly, 38221 Volos, Greece
[4]Present address: Department of Tumor Cell Biology, St. Jude Children's Research Hospital and Integrated Program in Biomedical Sciences, University of Tennessee Health Science Center, Memphis, TN 38105, USA
*Correspondence: hatzis@fleming.gr
http://dx.doi.org/10.1016/j.celrep.2016.05.038

SUMMARY

The canonical Wnt pathway plays a central role in stem cell maintenance, differentiation, and proliferation in the intestinal epithelium. Constitutive, aberrant activity of the TCF4/β-catenin transcriptional complex is the primary transforming factor in colorectal cancer. We identify a nuclear long non-coding RNA, termed WiNTRLINC1, as a direct target of TCF4/β-catenin in colorectal cancer cells. WiNTRLINC1 positively regulates the expression of its genomic neighbor ASCL2, a transcription factor that controls intestinal stem cell fate. WiNTRLINC1 interacts with TCF4/β-catenin to mediate the juxtaposition of its promoter with the regulatory regions of *ASCL2*. ASCL2, in turn, regulates WiNTRLINC1 transcriptionally, closing a feedforward regulatory loop that controls stem cell-related gene expression. This regulatory circuitry is highly amplified in colorectal cancer and correlates with increased metastatic potential and decreased patient survival. Our results uncover the interplay between non-coding RNA-mediated regulation and Wnt signaling and point to the diagnostic and therapeutic potential of WiNTRLINC1.

INTRODUCTION

Stem cell maintenance and the proliferation/differentiation switch in the adult, self-renewing intestinal epithelium are controlled by the canonical Wnt pathway (Clevers and Nusse, 2012), which determines developmental fates by regulating the transcriptional output of TCF target genes. In the absence of Wnt ligands, a destruction complex encompassing, among others, AXIN, APC, and GSK3 tightly controls the cytosolic levels of β-catenin, the key molecular effector of the pathway (Behrens et al., 1998). Upon physiological or mutational Wnt pathway activation, ubiquitination of β-catenin is blocked; it translocates to the nucleus and displaces corepressors from TCF4. This leads to activation of a crypt stem/progenitor cell transcriptional program (Clevers and Nusse, 2012; van de Wetering et al., 2002). Mutations in APC, AXIN, or β-catenin lead to the constitutive nuclear localization of the latter. The subsequent aberrant transcriptional activity of the TCF4/β-catenin complex is the primary transforming factor in colorectal cancer (Polakis, 2000).

Despite great recent inroads, the targets of Wnt-dependent transcriptional regulation remain incompletely understood. The binding sites of the relevant regulators in the genome are distributed mostly in distal gene locations, in regions of the genome with few or no known nearby annotated genes (Bottomly et al., 2010; Hatzis et al., 2008; Mokry et al., 2012; Schuijers et al., 2014). This leaves open the possibility that other transcriptional components of the pathway remain to be uncovered.

Long non-coding RNAs are such putative Wnt pathway targets or regulators. Long intergenic non-coding RNAs (lincRNAs) identified in other systems frequently have been shown to act as important regulators of transcriptional responses, among other functions (Guttman et al., 2009; Mercer et al., 2009). For instance, HOTAIR represses transcription in *trans* of the *HOXD* locus. It does so by interacting with and linking repressive histone modification complexes, such as PRC2 and LSD1/CoREST/REST, thus modulating the chromatin structure of affected loci, promoting cancer metastasis, and affecting imprinting and developmental patterning (Gupta et al., 2010; Li et al., 2013; Rinn et al., 2007; Tsai et al., 2010). The p53-induced lincRNA-p21 activates p21 in *cis* to regulate the G1/S checkpoint (Dimitrova et al., 2014). Its other putative functions include the suppression of mRNA translation (Yoon et al., 2012), the modulation of the Warburg effect (Yang et al., 2014), and gene repression in *trans* (Huarte et al., 2010).

Long non-coding RNAs with enhancer-like functions also have been identified in human cells; they interact with components of the Mediator co-activator complex to mediate loop formation between non-coding RNA (ncRNA)-a loci and neighboring protein-coding genes and positively affect the expression of the

Please cite this article in press as: Giakountis et al., A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate, Cell Reports (2016), http://dx.doi.org/10.1016/j.celrep.2016.05.038

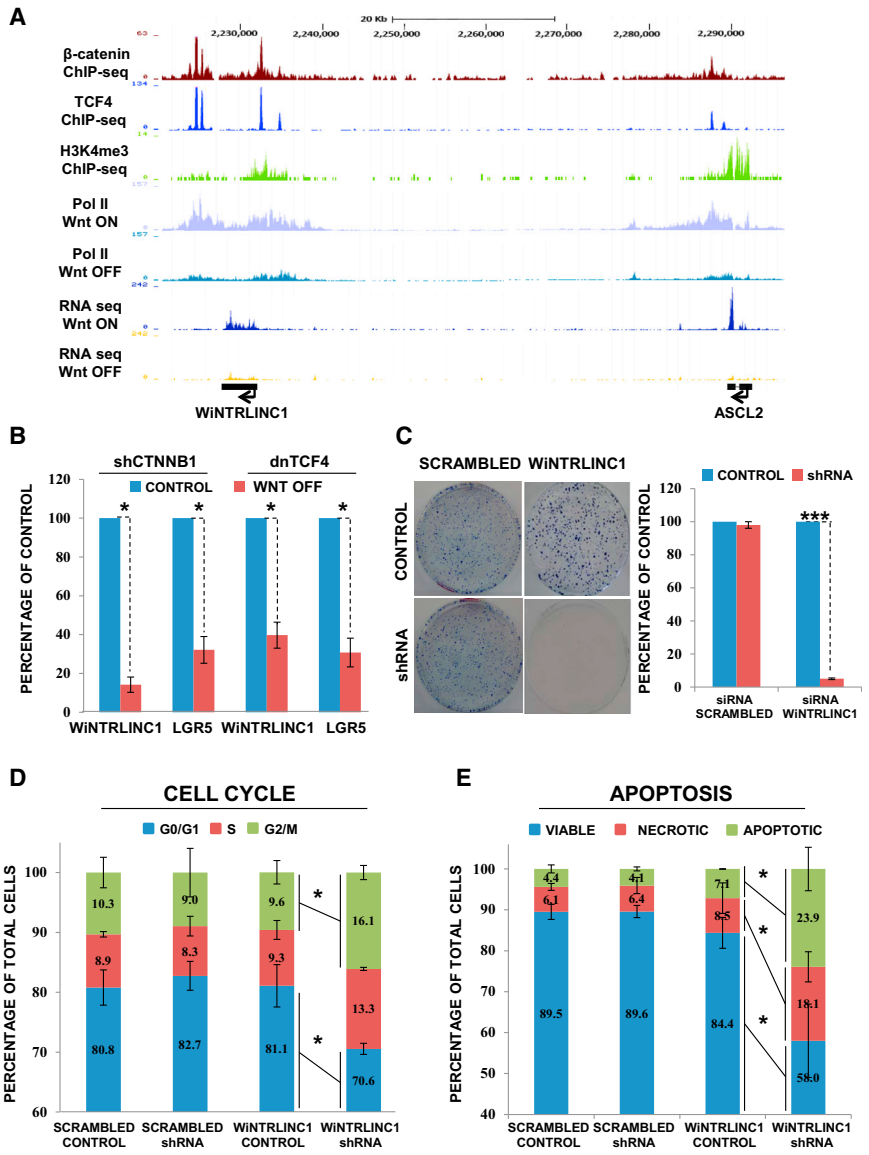


Figure 1. WiNTRLINC1 Controls the Proliferation and Survival of Cancer Cells

(A) Snapshots show ChIP-seq and RNA-seq reads at the genomic region encompassing WiNTRLINC1 and ASCL2.

(B) The qPCR analysis of WiNTRLINC1 (and LGR5 as control), with and without overexpression of shRNAs against β-catenin or of a dominant-negative form of TCF4, is shown.

(C) Colony formation assay with and without knockdown of WiNTRLINC1. Cells with inducible expression of scrambled shRNAs are shown as control.

(D) FACS analysis of cell-cycle profiles of Ls174T cells with and without knockdown of WiNTRLINC1. Analysis was performed 192 hr after the addition of doxycycline.

(E) FACS analysis of apoptotic and necrotic profiles of Ls174T cells with and without knockdown of WiNTRLINC1. Analysis was performed 192 hr after the addition of doxycycline (*p value < 0.05 calculated with ANOVA). Data are mean ± SD of three independent biological replicates.

latter (Lai et al., 2013; Ørom et al., 2010; Trimarchi et al., 2014). These and other recent examples highlight the importance of these RNA species in the regulation of the transcriptional and epigenetic landscapes in diverse tissues and disease and developmental contexts (Bonasio and Shiekhattar, 2014).

RESULTS

To identify Wnt-regulated long non-coding RNAs, we took advantage of previously generated colorectal carcinoma (CRC) cell lines (derived from LS174T) engineered to inducibly overexpress a small hairpin RNA (shRNA) against β-catenin or a dominant-negative mutant of TCF4 (van de Wetering et al., 2002, 2003). Doxycycline induction leads, in both cases, to robust abrogation of the TCF4/β-catenin-driven transcriptional program in these cells. Chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (ChIP-seq) with antibodies against RNA polymerase II (Pol II), before and after Wnt pathway abrogation, reveals sites of Wnt-dependent transcription (Mokry et al., 2012). This includes not only annotated long non-coding RNAs but also regions with no previously annotated genes or other transcripts. Many of these regions overlap with or are reminiscent of similar sites of transcription of lincRNAs identified previously on the basis of chromatin state marks (Guttman et al., 2009; Khalil et al., 2009).

One such region was located in the vicinity of the *ASCL2* locus, which encodes a transcription factor that controls intestinal stem cell fate (van der Flier et al., 2009): at a distance of ~60 kb from *ASCL2*, the region is marked by trimethylation of histone H3 at lysine 4 and TCF4- (Mokry et al., 2010) and β-catenin- (Schuijers et al., 2014) binding sites in the vicinity of the Pol II domain, and it transcribes a Wnt-regulated transcript, as revealed by RNA sequencing (RNA-seq) transcriptional profiling in cells inducibly overexpressing an shRNA against β-catenin (Mokry et al., 2012) (Figure 1A, labeled WiNTRLINC1, see below). We confirmed the TCF4/β-catenin dependency of the transcript with qRT-PCR experiments in cells with β-catenin knockdown and in cells inducibly overexpressing a dominant-negative mutant of TCF4 (Figures 1B and S1A). Importantly, the transcript was expressed and regulated by β-catenin in other CRC cell lines, such as CaCo2 and DLD1 (Figure S1A).

Rapid amplification of cDNA ends (RACE) allowed the cloning of a full-length transcript of 4,117 bp, which was produced from a single exon. Northern blot analysis of polyA-enriched RNA confirmed the presence of a long, polyadenylated, Wnt-regulated

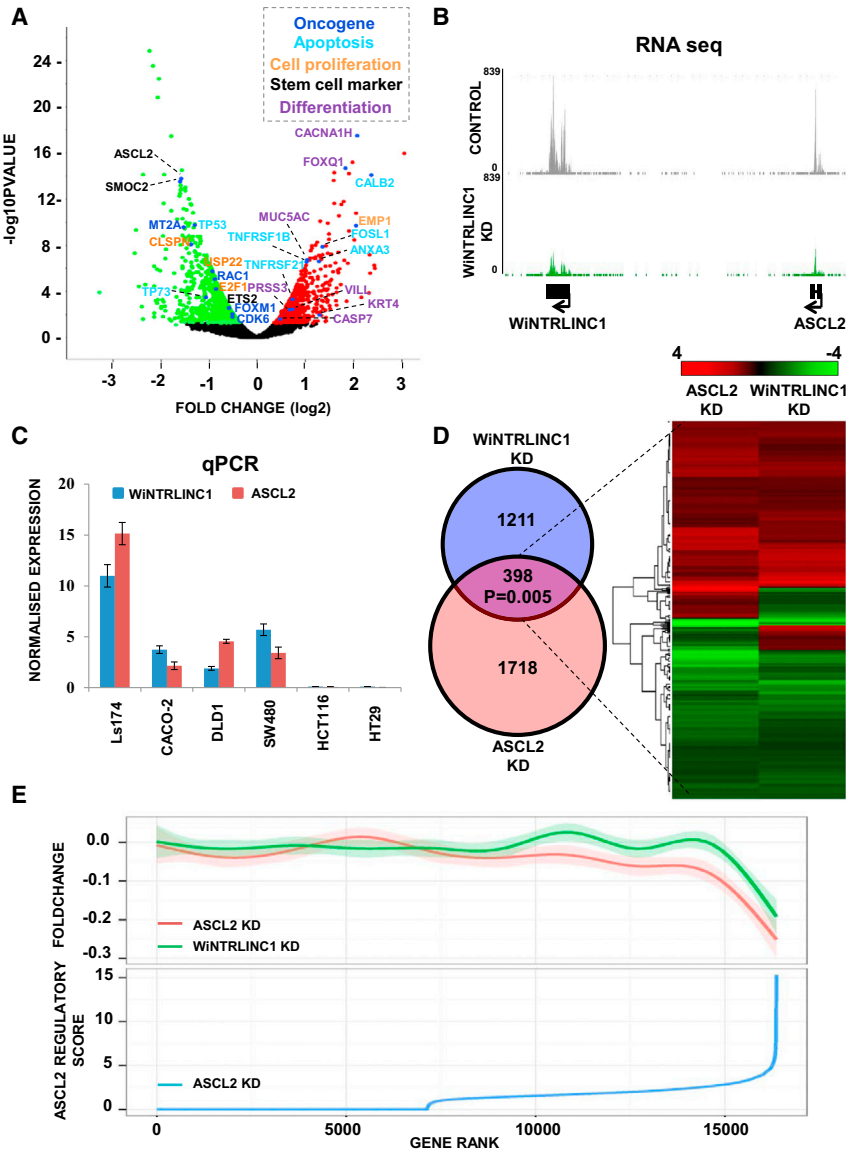


Figure 2. WiNTRLINC1 Positively Regulates ASCL2 Expression

(A) Volcano plot showing the differentially expressed genes upon WiNTRLINC1 knockdown. Green and red dots mark significantly down- and upregulated genes, respectively (fold change 1.5×, p value < 0.05). Blue dots mark selected examples of deregulated genes that are color coded according to their functional annotation.

(B) Snapshot of RNA-seq signal over the WiNTRLINC1 and ASCL2 loci, in control cells and upon WiNTRLINC1 knockdown. RNA harvesting was performed 72 hr after doxycycline addition.

(C) WiNTRLINC1 expression correlates with ASCL2 in a panel of colon cancer cell lines, with Ls174T cells showing the highest expression of both transcripts and HCT116 and HT29 showing no expression.

(D) Venn diagram and heatmap of the common differentially expressed genes after WiNTRLINC1 and ASCL2 knockdown are shown.

(E) Correlation between the deregulation of gene expression in the WiNTRLINC1 and ASCL2 knockdown datasets (upper panel) and the regulatory potential of ASCL2 (lower panel). Increased regulatory potential by ASCL2 of a gene correlates with increasingly deregulated expression upon WiNTRLINC1 and ASCL2 knockdown.

RNA species in CRC cells (Figure S1B). PhyloCSF (Lin et al., 2011) (Figure S1C) and InterProScan5 (Figure S1D) analyses revealed the transcript to have very low protein-coding potential, and in vitro transcription-translation assays produced no protein from the transcript (Figure S1E). Fractionation of cell extracts followed by qPCR analyses (Chalkiadaki and Talianidis, 2005) showed it to be predominantly chromatin associated (Figure S1F). We confirmed that the putative proximal promoter region of the transcript (the region surrounding the transcription start site [TSS], as defined by 5′ RACE) is bound by TCF4 and β-catenin in a ChIP-qPCR assay (Figure S1G) and that it can drive Wnt-dependent transcription of a luciferase reporter (Figure S1H). Based on the low protein-coding potential of the transcript, its predominant localization in chromatin, and its direct regulation by the TCF4/β-catenin complex, we have named it WNT-regulated lincRNA 1 (WiNTRLINC1).

Conditional shRNA-mediated knockdown of WiNTRLINC1 in LS174T cells reduced WiNTRLINC1 transcript levels to ~30% of control cells (Figure S2A) and dramatically affected cell growth, as demonstrated by colony formation assays (Figure 1C). WiNTRLINC1 knockdown caused a partial G2 arrest (Figure 1D) and a pronounced increase in apoptotic and necrotic cells (Figure 1E), as revealed by propidium iodide or Annexin V/propidium iodide staining, respectively, pointing to a role of WiNTRLINC1 in correct cell-cycle progression and protection of cells from apoptosis. Differential expression analysis after RNA-seq revealed ~1,609 genes deregulated after WiNTRLINC1 knockdown, with 890 genes downregulated and 719 genes upregulated >1.5× ($p < 0.05$) (Figure 2A; Table S1). The list of deregulated genes was enriched in genes involved in differentiation, cell proliferation, apoptosis, as well as stem cell markers, oncogenes, and tumor suppressors (Figure 2A; see below), consistent with the phenotype observed after WiNTRLINC1 abrogation.

Prominent among the most significantly deregulated genes was ASCL2, the closest *WiNTRLINC1* protein-coding neighbor, a Wnt-responsive gene whose product is required for stem cell maintenance in the intestine (Schuijers et al., 2015; van der Flier et al., 2009). Both shRNA- and small interfering RNA (siRNA)-mediated knockdown of WiNTRLINC1 led to the decrease of ASCL2 levels (Figures 2B and S2A). ASCL2 expression tightly followed WiNTRLINC1 levels in a panel of colon cancer cell lines.

Please cite this article in press as: Giakountis et al., A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate, Cell Reports (2016), http://dx.doi.org/10.1016/j.celrep.2016.05.038

Ls174T cells displayed maximum levels for both transcripts (Figure 2C). Based on this and the proximity of the two loci, we reasoned that WiNTRLINC1 may exert its functions on cell proliferation and viability predominantly through regulation of ASCL2 expression.

To investigate this hypothesis, we compared differentially regulated genes in WiNTRLINC1- and ASCL2-knockdown Ls174T cells. RNA-seq-based differential expression analysis after inducible shRNA-mediated ASCL2 knockdown (van der Flier et al., 2009) revealed ~2,116 genes to be deregulated, ~1,144 up- and ~972 downregulated >1.5× ($p < 0.05$) (Table S2). The two datasets demonstrated a highly significant overlap of ~398 common targets ($p = 0.0051$) (Figure 2D; Table S3). Interestingly, the common targets—mostly deregulated in the same direction—belong to relevant enriched gene ontology categories, such as the regulation of apoptosis, cell proliferation, differentiation, stemness, and oncogenesis (Table S4). We further took advantage of the ASCL2 genome-wide binding pattern in these cells (more than 8,000 genomic locations were bound by ASCL2 in Ls174T) (Schuijers et al., 2015) to calculate a regulatory score for ASCL2, which takes into account ASCL2 peak occurrence and binding pattern and reflects the probability of direct regulation of a gene by ASCL2. Interestingly, the regulatory score highly correlated with the extent of deregulation of gene expression, both after ASCL2 as well as after WiNTRLINC1 knockdown, as the genes most severely transcriptionally affected were the ones most prominently bound by ASCL2 (exhibiting the highest regulatory score) (Figure 2E). In addition, we complemented WiNTRLINC1-knockdown cells with exogenous ASCL2 (by generating a cell line that, upon doxycycline induction, simultaneously overexpresses FLAG-ASCL2 and shRNA against WiNTRLINC1) (Figure S2A). ASCL2 binding to target loci (Figure S2B), transcription of its target genes (Figure S2C), and cellular viability (Figure S2D) were restored in these cells, pointing to ASCL2 as the major target of WiNTRLINC1 function. Importantly, the molecular and phenotypic effects of WiNTRLINC1 extend beyond the Ls174T background to other colon cancer cell line models, as siRNA-mediated knockdown of WiNTRLINC1 in CaCo-2 and SW480 decreased transcription of ASCL2, affected the expression of common target genes (Figures S2E and S2F), and decreased cell survival (Figures S2G and S2H). These results suggest that WiNTRLINC1 exerts a significant part of its functions through controlling ASCL2 expression.

We reasoned that the mechanism through which WiNTRLINC1 exerts this regulatory function may involve the formation of a topological loop between the two loci. Chromosome conformation capture (3C) experiments demonstrated that the TSS region of *WiNTRLINC1* interacts with the region immediately downstream of the *ASCL2* locus (ASCL2 enhancer, a region characterized by the absence of H3K4 trimethylation and the presence of Mediator and H3K4 monomethylation and H3K27 acetylation, Figure S3A), in a WiNTRLINC1-dependent manner: knockdown of the latter results in disruption of this loop (Figure 3A). The potential involvement of the ASCL2 promoter in this loop, which would help explain its activation, could not be investigated because of a lack of suitable restriction sites. We employed chromatin isolation with RNA purification (ChIRP) (Chu et al., 2011) to examine the physical presence of the WiNTRLINC1 transcript on the regions determined by 3C to be topologically juxtaposed. ChIRP revealed the transcript to be localized to the WiNTRLINC1-transcribed region, as expected. This localization was abolished by WiNTRLINC1 downregulation after β-catenin knockdown (Figure 3B). Importantly, WiNTRLINC1 RNA also was recruited to the WiNTRLINC1 promoter and the ASCL2 enhancer regions (but not to the ASCL2 promoter region, data not shown) in a similar β-catenin-dependent manner (Figure 3B). Both these WiNTRLINC1-occupied, interacting genomic regions were bound by TCF4 and β-catenin (see Figure 1A and below).

We next explored the possibility that TCF4 and β-catenin might form a complex with WiNTRLINC1 to mediate loop formation. RNA immunoprecipitation (RIP) assays confirmed a specific interaction between WiNTRLINC1 and TCF4 and β-catenin (Figure 3C). WiNTRLINC1 knockdown led to dramatically decreased recruitment of TCF4, β-catenin (Figures S3B and S3C), and Pol II (Figure S3D), as well as decreased H3K4 monomethylation, H3K27 acetylation, and Mediator recruitment on the relevant genomic regions of *WiNTRLINC1* and *ASCL2* (Figures S3E–S3G). These data demonstrate that WiNTRLINC1 orchestrates factor recruitment, physical juxtaposition, and activity of the regulatory regions that maintain ASCL2 expression.

The eventuality of a reciprocal relationship between WiNTRLINC1 and ASCL2 became evident upon visual inspection of the ASCL2-binding profile around the relevant loci: it featured a prominent binding site on the promoter region of *WiNTRLINC1* (Figures 3D and S2B). The RNA-seq signal over *WiNTRLINC1* was concomitantly diminished upon ASCL2 knockdown (Figures 3D and S2A). A regulatory scheme that emerges from the above data involves the TCF4/β-catenin transcription complex binding to and activating the *WiNTRLINC1* promoter region; it then interacts with the transcribed RNA to stabilize a loop juxtaposing the *WiNTRLINC1* promoter and the 3′ *ASCL2* regulatory regions, driving high-level ASCL2 expression. This topological loop requires WiNTRLINC1 for its stability. The regulatory loop is enhanced and completed by reciprocal ASCL2 binding to the *WiNTRLINC1* promoter to drive transcription of the lincRNA (Figure S3I).

Additional support for this order of events was provided by experiments in the cell line where WiNTRLINC1 knockdown was complemented with exogenous ASCL2: overexpression of the latter restored ASCL2 binding to the WiNTRLINC1 promoter (Figure S2B) and WiNTRLINC1 transcription (as measured by Pol II occupancy, Figure S3D), but not, as expected, WiNTRLINC1 transcript levels (Figure S2A). This restored *transcription* of *WiNTRLINC1* was not sufficient to reestablish binding of TCF4 or β-catenin to the WiNTRLINC1 promoter or ASCL2 enhancer (Figures S3B and S3C) or the previous histone modification status of the latter (Figures S3E and S3F); neither did it reestablish loop formation between the *WiNTRLINC1* and *ASCL2* loci (Figure S3H) or endogenous ASCL2 transcription (Figure S3D) or RNA levels (Figure S2A). These results demonstrate that the WiNTRLINC1 RNA product is essential both for loop formation and for expression of ASCL2: the *act* of transcription of WiNTRLINC1 is not sufficient for either event. They also show that human ASCL2 cannot activate its own transcription without WiNTRLINC1, lending additional support to the regulatory scheme outlined in Figure S3I.

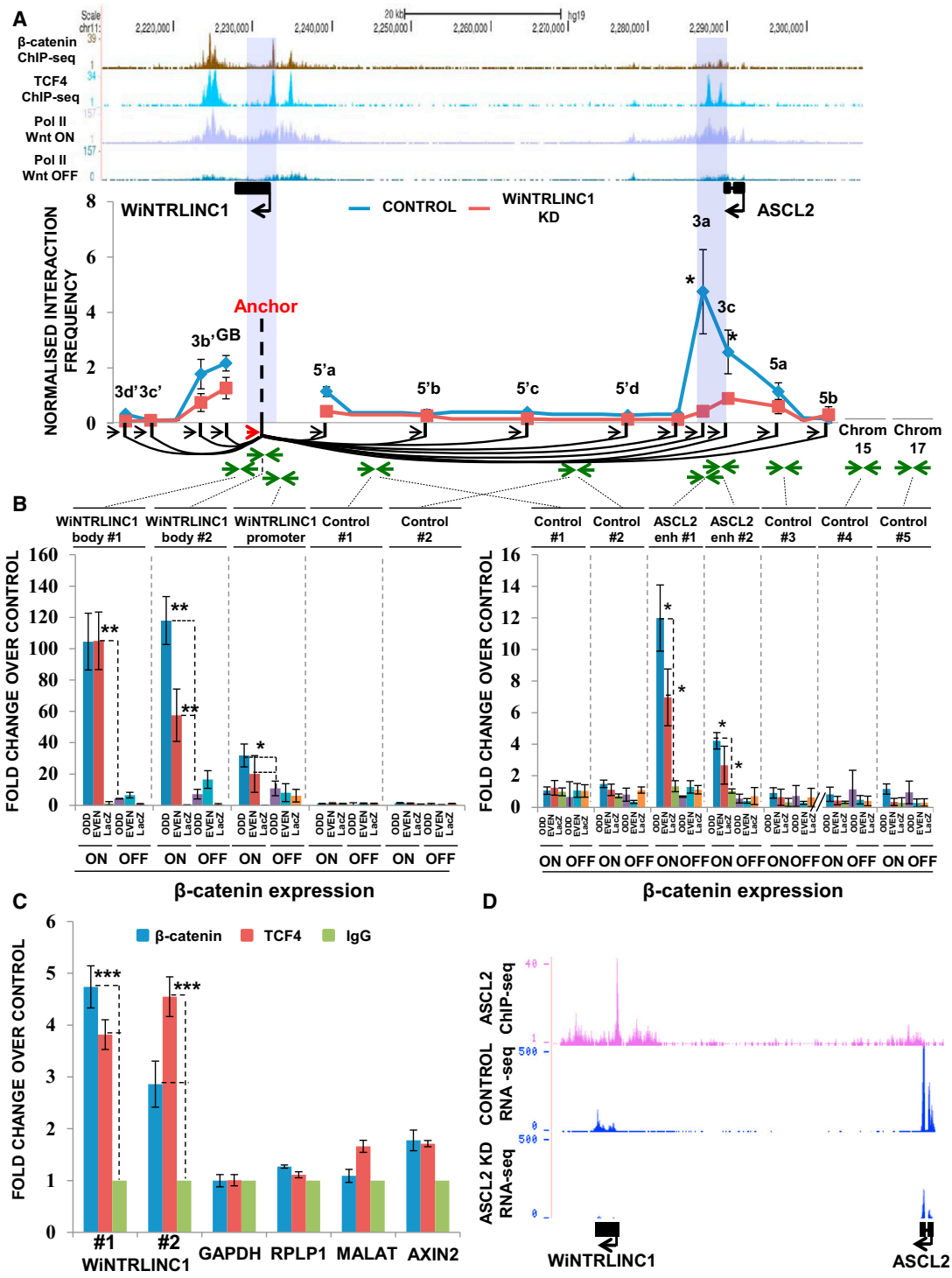


Figure 3. Mechanism of WiNTRLINC1-Mediated Control of ASCL2 Expression

(A) Normalized interaction frequencies between the WiNTRLINC1 and the ASCL2 loci, as revealed by 3C-qPCR, using an anchor primer (red arrowhead) and test primers (black arrowheads). ChIP-seq signals for TCF4, β-catenin, and Pol II over the region are highlighted at the top.

(B) ChIRP-qPCR analysis for WiNTRLINC1 over the WiNTRLINC1 and the ASCL2 loci using the indicated primer pairs (pairs of green arrowheads) is shown.

(C) RIP of β-catenin, TCF4, or IgG control coupled to qPCR with primers for the indicated RNAs is shown.

(D) Snapshots of ChIP-seq reads for ASCL2 and RNA-seq reads after ASCL2 knockdown, at the genomic region encompassing WiNTRLINC1 and ASCL2 (*p value < 0.05, **p value < 0.01, and ***p value < 0.001, calculated with ANOVA). Data are mean ± SD of three independent biological replicates. All experiments were performed 72 hr after doxycycline addition.

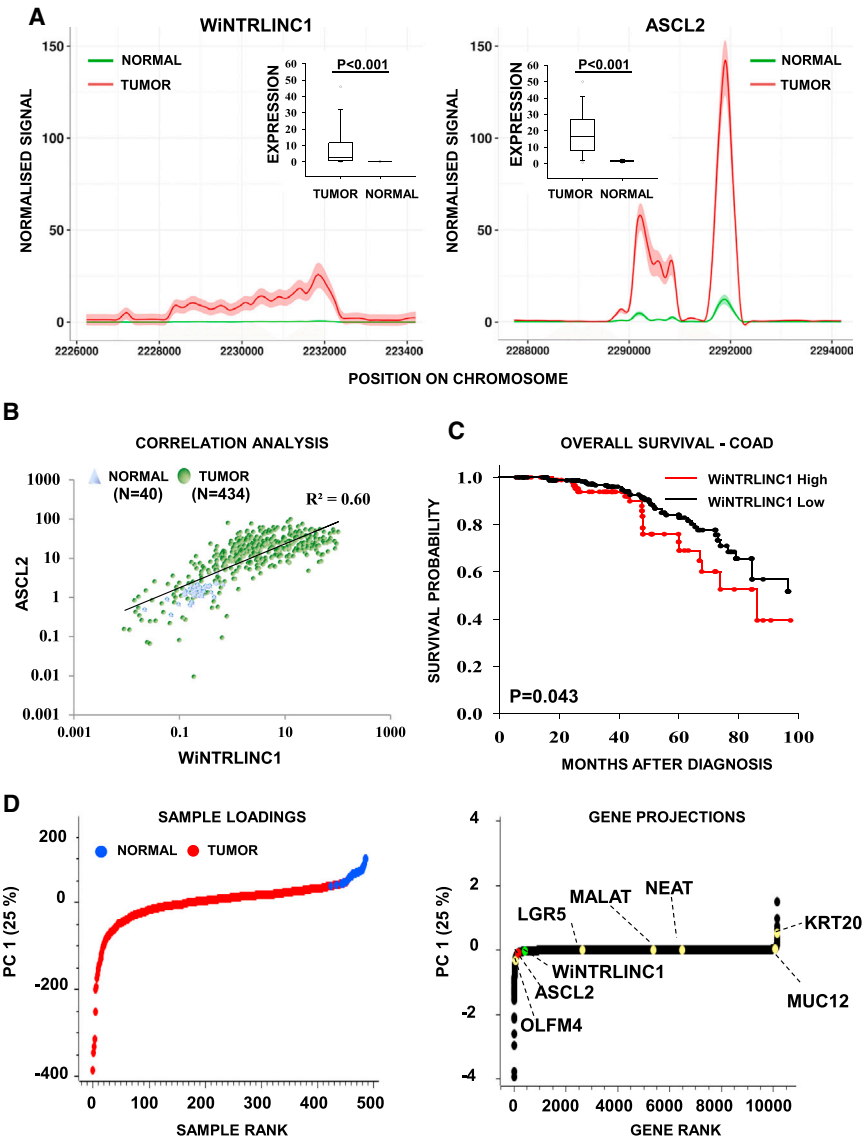


Figure 4. The WiNTRLINC1-ASCL2 Regulatory Loop Is Amplified in Colorectal Cancer

(A) Summarized expression of WiNTRLINC1 (left panel) and ASCL2 (right panel) in a set of 434 tumor and 40 normal samples from the colon cancer dataset of TCGA Network. Inset shows expression of each gene as boxplots (*p value < 0.05 calculated with ANOVA).

(B) Correlation analysis of WiNTRLINC1 and ASCL2 expression in the normal and tumor samples of (A) is shown.

(C) Kaplan-Meier analysis and log-rank p value for overall survival, comparing colon cancer patients with high and low WiNTRLINC1 expression, are shown.

(D) Principal-component analysis for normal versus tumor sample discrimination based on gene expression in the TCGA colon cancer patient panel. Left panel demonstrates sample loadings for component 1 (25% of total variability) effectively separating normal samples (positive loadings shown with blue marks) from tumor samples (negative loadings shown with read marks). Right panel illustrates gene loadings for the same component. WiNTRLINC1 is ranked at position 237 and ASCL2 at position 158 among 11,537 genes. Other known oncogenes and differentiation genes also are shown.

The genomic region proximal to *ASCL2*, at chromosome arm 11p.15.5, has recently been found to be focally amplified in ~7% of the colorectal tumors analyzed by The Cancer Genome Atlas (TCGA) Network (2012). The amplified region does not include *ASCL2*; we observed, however, that, in addition to genes such as *IGF2*, *INS*, *TH*, and *miR-483*, it frequently included the *WiNTRLINC1* locus; this served as an impetus for us to reanalyze the TCGA RNA-seq data to investigate WiNTRLINC1 expression levels in colorectal cancer. WiNTRLINC1 was robustly overexpressed in the tumor samples, coupled with concordant overexpression of ASCL2 (Figures 4A and 4B). We verified this overexpression by qPCR in a panel of normal and colorectal cancer patient samples; WiNTRLINC1 and ASCL2 expression were significantly correlated (Figure S4A), demonstrating the amplification of the WiNTRLINC1-ASCL2 regulatory loop in colorectal cancer. Hierarchical clustering of expressed genes in normal and tumor samples of the TCGA panel further illustrated this regulatory relationship, as WiNTRLINC1 and ASCL2 expression were tightly linked across 434 tumor and 40 normal samples; pairwise correlation analysis between WiNTRLINC1 and each of the 11,537 expressed genes in the TCGA panel, using different metrics (see Experimental Procedures), consistently ranked the WiNTRLINC1-ASCL2 pair in the top position in the order of correlation strength ($R^2 = 0.60$).

Stratification of the TCGA tumors into higher- and lower-expressing cohorts (Figure S4B) coupled to Kaplan-Meier analysis revealed that high-level WiNTRLINC1 expression correlates significantly with lower overall probability of survival (Figure 4C), lower disease-free survival (Figure S4C), worse disease progression (Figure S4D), higher probability for lymph node invasion (Figure S4E) and greater metastatic potential (Figure S4F). High ASCL2 expression significantly correlates only with lower disease-free survival (Figure S4G) and not with the other characteristics tested (Figures S4H–S4K); this is perhaps not surprising, given the already significant overexpression of ASCL2 (and WiNTRLINC1) in the "low" tumor category compared to normal samples (Figures S4L–S4O). The increased mRNA levels of ASCL2 in the low group may already be sufficient to lead to full phenotypic changes in tumor cells. In addition, the amounts of ASCL2 mRNA in the low group may already produce near-saturating levels of protein that are not further increased by an additional increase in Ascl2 mRNA in the high group.

Principal-component analysis of gene expression values across the TCGA patient panels allowed for the separation of

normal from tumor samples (Figure 4D). WiNTRLINC1 and ASCL2 exhibited high discriminatory power for tumor-normal classification; they were among the highest ranking genes (at positions 237 and 158, respectively) among 11,537 expressed genes across the TCGA samples. This reinforces our findings, highlighting the WiNTRLINC1-ASCL2 regulatory relationship and its amplification in colorectal cancer.

DISCUSSION

ASCL2 has been shown to function as a master regulator of the intestinal stem cell (van der Flier et al., 2009). It controls the expression of genes fundamental to the stem cell state, by synergizing with TCF4/β-catenin on target gene regulatory regions (Schuijers et al., 2015). In the mouse, it also has been reported to be regulated by an auto-activating loop, which maintains Ascl2 expression levels in a hysteretic manner, enabling it to maintain its expression even upon fluctuation of the initial activating Wnt signal (Schuijers et al., 2015). The most parsimonious explanation of the results presented here is that human ASCL2 is not directly regulated by Wnt: the direct Wnt target is WiNTRLINC1, a neighboring *cis*-acting lincRNA. Upon activation by TCF4/β-catenin, WiNTRLINC1 interacts with TCF4/β-catenin and mediates their recruitment/stabilization, through the formation of a topological loop, to the 3′ regulatory regions of *ASCL2*. The recruitment of ASCL2 to the *WiNTRLINC1* promoter, in turn, enhances its expression and closes a feedforward regulatory loop that is compatible with driving hysteretic expression of human ASCL2.

Our results also provide support for the role of WiNTRLINC1 in human colorectal cancer. ASCL2 is upregulated in human intestinal neoplasia (Jubb et al., 2006), and an ASCL2-related stem cell signature is expressed in colorectal cancer liver metastases with the 11p15.5 gain, which involves the *WiNTRLINC1*-containing genomic region (Stange et al., 2010). While a potential contribution of cytoplasmically localized WiNTRLINC1, possibly independent of ASCL2, could not be determined at this point, the feedforward regulatory loop we have uncovered here is robustly amplified in human colorectal cancer. The correlation of WiNTRLINC1 expression with patient survival and other clinical characteristics warrants the exploration of its diagnostic and therapeutic potential in colorectal cancer.

EXPERIMENTAL PROCEDURES

Cell Lines

Ls174T, CaCo2, DLD1, and SW480 cells were used in this study. Ls174T cells that inducibly overexpress a dominant-negative form of TCF4 or an shRNA against β-catenin or ASCL2 after treatment with doxycycline (1 μg/ml) have been described previously (van de Wetering et al., 2002, 2003; van der Flier et al., 2009). To generate Ls174T cells inducibly overexpressing an shRNA against WiNTRLINC1, oligonucleotides 5′-GATCCCCAGGGAGGCTGAAGAGCAATTCAAGAGATTGCTCTTCAGCCTCCCTGTTTTTGGAAA-3 and 5′-AGCTTTTCCAAAAACAGGGAGGCTGAAGAGCA ATCTCTTGAATTGCTCTTCAGCCTCCCTGGG-3′ were used in conjunction with the pTER system, as described (van de Wetering et al., 2003). The conditional ASCL2 overexpression (CASOX) cells, conditionally overexpressing ASCL2 after treatment with doxycycline and in parallel to overexpression of shRNA against WiNTRLINC1, were generated after transfection of a stable Ls174T clone overexpressing shRNAs against WiNTRLINC1, as described above, with a modified PCDNA3 vector overexpressing a FLAG-tagged version of ASCL2, under the control of a CMV promoter fused to Tet operator sequences.

RNA Isolation and Deep Sequencing

Total RNA was isolated with TRI reagent (Molecular Research Center) and 10 μg was treated with DNaseI (Promega) according to the manufacturer's protocols. DnaseI-treated RNA was extracted with phenol/chloroform, chloroform, and ethanol precipitation. RNA-seq was performed as described (Nikolaou et al., 2015).

RIP

Ls174T cells (5 × 10^6) were plated in 150 × 20-mm tissue culture dishes (Sarstedt) and were grown in DMEM for 72 hr. Cells were crosslinked with 1% formaldehyde for 30 min at room temperature and quenched with glycine. Chromatin was sonicated for 10 min using the Covaris S series SonoLAB single instrument with the following settings: duty cycle 20%, intensity 10, and cycles/burst 1,000. RIP was performed as described (Rinn et al., 2007) with 20 μg antibody against β-catenin (sc7199, Santa Cruz Biotechnology) and TCF4 (sc8631, Santa Cruz Biotechnology). RNA was eluted after incubation at 37°C for 20 min, de-crosslinked with 200 mM NaCl at 65°C for 5 hr, and extracted with TRI reagent (Molecular Research Center) as described above. Extracted RNA was DNaseI treated, and cDNA synthesis was performed with SuperScript II (Invitrogen) according to the manufacturer's recommendations. All primers are shown in Table S5. Data are from three independent biological replicates. ANOVA was performed in SigmaStat/Plot.

3C

Ls174T cells (5 × 10^6), conditionally overexpressing shRNAs against ASCL2 and WiNTRLINC1 with and without parallel ASCL2 overexpression, were plated in 150 × 20-mm tissue culture dishes (Sarstedt), and they were grown in DMEM for 72 hr with and without doxycycline. Cells were crosslinked with 1% formaldehyde for 10 min at room temperature, and 3C was performed as described (Naumova et al., 2012), using the BglII restriction endonuclease (Minotech). Digested chromatin was tested with electrophoresis in 1% agarose gel, and digestion efficiency was tested with qPCR using primers flanking the BglII restriction sites. Digested chromatin was ligated as described (Naumova et al., 2012) and tested again with qPCR with the same primers as for digestion efficiency. After ligation, the chromatin was de-crosslinked with 200 mM NaCl at 65°C, treated with RNase A, and extracted with phenol/chloroform, chloroform, and ethanol precipitation. 3C digestion and ligation conditions were optimized with a BAC clone containing both the *WiNTRLINC1* and the *ASCL2* loci (BACPAC resources RP11-542J6). 3C-qPCR also was performed with the BAC DNA, which was used as control for the digestion and ligation efficiency tests, as well as for quantifying the genomic 3C qPCR results. 3C amplicons were validated with BglII digests and Sanger sequencing. All primers are shown in Table S5. Data are from three independent biological replicates. ANOVA was performed in SigmaStat/Plot.

ChIRP

ChIRP was performed as described (Chu et al., 2011), with the following modifications: Ls174T cells, control or overexpressing shRNAs against β-catenin, were grown in DMEM in the absence or presence, respectively, of doxycycline for 72 hr, after which they were washed with PBS and crosslinked with 1% glutaraldehyde/PBS for 20 min at room temperature. Chromatin was sonicated for 30 min using the Covaris S series SonoLAB single instrument with the following settings: duty cycle 20%, intensity 10, and cycles/burst 1,000. Hybridization was performed overnight at 37°C, and oligo capturing was performed for 30 min at 37°C with 150 μl streptavidin-coated beads per sample (Dynabeads MyOne Streptavidin C1, Invitrogen). Odd and even pools of biotynilated oligonucleotides complementary to the mRNA sequence of WiNTRLINC1 were used to capture WiNTRLINC1-bound chromatin. A symmetrical set of probes against lacZ RNA also was generated as the mock control. All probe sequences are shown in Table S5. DNA was eluted; de-crosslinked with 15 μl proteinase K (10 mg/ml) for 45 min at 50°C; extracted with phenol/chroloform, chloroform, and ethanol precipitation; and subsequently diluted with ddH_2O to 240 μl for qPCR. Data are from three independent biological replicates. ANOVA was performed in SigmaStat/Plot.

Please cite this article in press as: Giakountis et al., A Positive Regulatory Loop between a Wnt-Regulated Non-coding RNA and ASCL2 Controls Intestinal Stem Cell Fate, Cell Reports (2016), http://dx.doi.org/10.1016/j.celrep.2016.05.038

ACCESSION NUMBERS

The accession numbers for the RNA-seq data and the WiNTRLINC1 cDNA sequence reported in this paper are GEO: GSE69036 and GenBank: KR736336, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.038.

AUTHOR CONTRIBUTIONS

P.H. and A.G. conceived and designed experiments. A.G., V.Z., C.O., and P.H. performed experiments. V.H. performed high-throughput sequencing. P.M., A.G., and M.R. performed bioinformatic analyses with assistance from A.G.H. P.H. and A.G. wrote the manuscript.

ACKNOWLEDGMENTS

This research was supported by Marie Curie Career Integration Grant (PCIG9-GA-2011-293968); a Fondation Santé Research Grant; the European Union (European Social Fund [ESF]); and Greek national funds through the Operational Program Education and Lifelong Learning of the National Strategic Reference Framework Research Funding Programs THALES (Thales656) and POST-DOC SUPPORT (LS2-3475) Investing in knowledge society through the ESF. We thank Dr. I. Talianidis for support and critical reading of the manuscript, Dr. S. Grammenoudi for assistance with fluorescence-activated cell sorting, and D. Lilakos for assistance with next-generation sequencing.

Received: November 16, 2015
Revised: April 4, 2016
Accepted: May 6, 2016
Published: June 9, 2016

REFERENCES

Behrens, J., Jerchow, B.A., Würtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kühl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science *280*, 596–599.

Bonasio, R., and Shiekhattar, R. (2014). Regulation of transcription by long noncoding RNAs. Annu. Rev. Genet. *48*, 433–455.

Bottomly, D., Kyler, S.L., McWeeney, S.K., and Yochum, G.S. (2010). Identification of beta-catenin binding regions in colon cancer cells using ChIP-Seq. Nucleic Acids Res. *38*, 5735–5745.

Cancer Genome Atlas Network (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature *487*, 330–337.

Chalkiadaki, A., and Talianidis, I. (2005). SUMO-dependent compartmentalization in promyelocytic leukemia protein nuclear bodies prevents the access of LRH-1 to chromatin. Mol. Cell. Biol. *25*, 5095–5105.

Chu, C., Qu, K., Zhong, F.L., Artandi, S.E., and Chang, H.Y. (2011). Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. Mol. Cell *44*, 667–678.

Clevers, H., and Nusse, R. (2012). Wnt/β-catenin signaling and disease. Cell *149*, 1192–1205.

Dimitrova, N., Zamudio, J.R., Jong, R.M., Soukup, D., Resnick, R., Sarma, K., Ward, A.J., Raj, A., Lee, J.T., Sharp, P.A., and Jacks, T. (2014). LincRNA-p21 activates p21 in cis to promote Polycomb target gene expression and to enforce the G1/S checkpoint. Mol. Cell *54*, 777–790.

Gupta, R.A., Shah, N., Wang, K.C., Kim, J., Horlings, H.M., Wong, D.J., Tsai, M.C., Hung, T., Argani, P., Rinn, J.L., et al. (2010). Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature *464*, 1071–1076.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P., et al. (2009). Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature *458*, 223–227.

Hatzis, P., van der Flier, L.G., van Driel, M.A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I.J., Koster, J., Santo, E.E., Welboren, W., et al. (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol. Cell. Biol. *28*, 2732–2744.

Huarte, M., Guttman, M., Feldser, D., Garber, M., Koziol, M.J., Kenzelmann-Broz, D., Khalil, A.M., Zuk, O., Amit, I., Rabani, M., et al. (2010). A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell *142*, 409–419.

Jubb, A.M., Chalasani, S., Frantz, G.D., Smits, R., Grabsch, H.I., Kavi, V., Maughan, N.J., Hillan, K.J., Quirke, P., and Koeppen, H. (2006). Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia. Oncogene *25*, 3445–3457.

Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., Thomas, K., Presser, A., Bernstein, B.E., van Oudenaarden, A., et al. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl. Acad. Sci. USA *106*, 11667–11672.

Lai, F., Orom, U.A., Cesaroni, M., Beringer, M., Taatjes, D.J., Blobel, G.A., and Shiekhattar, R. (2013). Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature *494*, 497–501.

Li, L., Liu, B., Wapinski, O.L., Tsai, M.-C.C., Qu, K., Zhang, J., Carlson, J.C., Lin, M., Fang, F., Gupta, R.A., et al. (2013). Targeted disruption of Hotair leads to homeotic transformation and gene derepression. Cell Rep. *5*, 3–12.

Lin, M.F., Jungreis, I., and Kellis, M. (2011). PhyloCSF: a comparative genomics method to distinguish protein coding and non-coding regions. Bioinformatics *27*, i275–i282.

Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. Nat. Rev. Genet. *10*, 155–159.

Mokry, M., Hatzis, P., de Bruijn, E., Koster, J., Versteeg, R., Schuijers, J., van de Wetering, M., Guryev, V., Clevers, H., and Cuppen, E. (2010). Efficient double fragmentation ChIP-seq provides nucleotide resolution protein-DNA binding profiles. PLoS ONE *5*, e15092.

Mokry, M., Hatzis, P., Schuijers, J., Lansu, N., Ruzius, F.P., Clevers, H., and Cuppen, E. (2012). Integrated genome-wide analysis of transcription factor occupancy, RNA polymerase II binding and steady-state RNA levels identify differentially regulated functional gene classes. Nucleic Acids Res. *40*, 148–158.

Naumova, N., Smith, E.M., Zhan, Y., and Dekker, J. (2012). Analysis of long-range chromatin interactions using Chromosome Conformation Capture. Methods *58*, 192–203.

Nikolaou, K.C., Moulos, P., Chalepakis, G., Hatzis, P., Oda, H., Reinberg, D., and Talianidis, I. (2015). Spontaneous development of hepatocellular carcinoma with cancer stem cell properties in PR-SET7-deficient livers. EMBO J. *34*, 430–447.

Ørom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Notredame, C., Huang, Q., et al. (2010). Long noncoding RNAs with enhancer-like function in human cells. Cell *143*, 46–58.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., and Chang, H.Y. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell *129*, 1311–1323.

Schuijers, J., Mokry, M., Hatzis, P., Cuppen, E., and Clevers, H. (2014). Wnt-induced transcriptional activation is exclusively mediated by TCF/LEF. EMBO J. *33*, 146–156.

Schuijers, J., Junker, J.P., Mokry, M., Hatzis, P., Koo, B.K., Sasselli, V., van der Flier, L.G., Cuppen, E., van Oudenaarden, A., and Clevers, H. (2015). Ascl2 acts as an R-spondin/Wnt-responsive switch to control stemness in intestinal crypts. Cell Stem Cell *16*, 158–170.

Stange, D.E., Engel, F., Longerich, T., Koo, B.K., Koch, M., Delhomme, N., Aigner, M., Toedt, G., Schirmacher, P., Lichter, P., et al. (2010). Expression of an ASCL2 related stem cell signature and IGF2 in colorectal cancer liver metastases with 11p15.5 gain. Gut *59*, 1236–1244.

Trimarchi, T., Bilal, E., Ntziachristos, P., Fabbri, G., Dalla-Favera, R., Tsirigos, A., and Aifantis, I. (2014). Genome-wide mapping and characterization of Notch-regulated long noncoding RNAs in acute leukemia. Cell *158*, 593–606.

Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689–693.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241–250.

van de Wetering, M., Oving, I., Muncan, V., Pon Fong, M.T., Brantjes, H., van Leenen, D., Holstege, F.C., Brummelkamp, T.R., Agami, R., and Clevers, H. (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep. *4*, 609–615.

van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., et al. (2009). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell *136*, 903–912.

Yang, F., Zhang, H., Mei, Y., and Wu, M. (2014). Reciprocal regulation of HIF-1α and lincRNA-p21 modulates the Warburg effect. Mol. Cell *53*, 88–100.

Yoon, J.-H.H., Abdelmohsen, K., Srikantan, S., Yang, X., Martindale, J.L., De, S., Huarte, M., Zhan, M., Becker, K.G., and Gorospe, M. (2012). LincRNA-p21 suppresses target mRNA translation. Mol. Cell *47*, 648–655.