Wnt-regulated IncRNA discovery enhanced by in vivo identification and CRISPRi functional validation

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Abstract

Background: Wnt signaling is an evolutionarily conserved developmental pathway that is frequently hyperactivated in cancer. While multiple protein-coding genes regulated by Wnt signaling are known, the functional IncRNAs regulated by Wnt signaling have not been systematically characterized.

Results: We comprehensively mapped IncRNAs from an orthotopic Wnt-addicted pancreatic cancer model, identifying 3,633 IncRNAs, of which 1,503 were regulated by Wnt signaling. We found IncRNAs were much more sensitive to changes in Wnt signaling in xenografts than in cultured cells. To functionally validate Wnt-regulated IncRNAs, we performed CRISPRi screens to assess their role in cancer cell proliferation. Consistent with previous genome-wide IncRNA CRISPRi screens, around 1% (13/1,503) of the Wnt-regulated IncRNAs could modify cancer cell growth in vitro. This included CCAT1 and LINC00263, previously reported to regulate cancer growth. Using an in vivo CRISPRi screen, we doubled the discovery rate, identifying twice as many Wnt-regulated IncRNAs (25/1,503) that had a functional effect on cancer cell growth.

Conclusions: Our study demonstrates the value of studying IncRNA functions in vivo, provides a valuable resource of IncRNAs regulated by Wnt signaling and establishes a framework for systematic discovery of functional IncRNAs.

Keywords: functional IncRNAs, Wnt signaling, cancer, CRISPRi screen
Background

IncRNAs play key roles in diverse biological processes, ranging from development, such as *XIST* for dosage compensation (Brown et al., 1991) and *H19* for imprinting (Brannan et al., 1990), to different diseases including cancer (Huarte, 2015). IncRNAs have been shown to play important roles in fundamental biological signaling pathways regulated by P53, Notch and TGF-β (Huarte et al., 2010; Trimarchi et al., 2014; Yuan et al., 2014). IncRNAs can contribute to the development of cancer through aberrant expression or mutation, altering their normal physiological functions in signaling pathways (Schmitt & Chang, 2016). Advancements in transcriptomics have greatly expanded the number of long noncoding RNAs (lncRNAs) annotated in the human genome (Hon et al., 2017; Iyer et al., 2015), but only a small fraction have been characterized at a functional level.

Wnt/β-catenin signaling is an important evolutionarily conserved signaling pathway that is crucial for embryonic development and tissue regeneration (Nusse & Clevers, 2017). After Wnt ligands binding to Frizzled and other co-receptors on the cell surface, β-catenin is stabilized and translocates into the nucleus, where it interacts with TCF/LEF transcription factors in a context-dependent manner to regulate the expression of multiple protein-coding genes such as *MYC* and *AXIN2*. Dysregulation of Wnt signaling is found in multiple cancers. The most common mutations activating Wnt/β-catenin signaling occur in colorectal cancer, where truncations of APC cause abnormal stabilization of β-catenin and constitutive transcriptional activation (Polakis, 2012; Zhan et al., 2017; Zhong & Virshup, 2019). A different class of mutations confer cancer dependency on Wnt ligands. For example, *RNF43* and *RPSO3* mutations cause increased abundance of Wnt receptors on the cell surface, making the cancer cells addicted to Wnt signaling (X. Jiang et al., 2013; Koo et al., 2012; Seshagiri et al., 2012).
RNF43 mutations are found in 5 – 10% of pancreatic cancers, while RPSO3 translocations are found in 10% of colorectal cancers (Bailey et al., 2016; Cancer Genome Atlas Research Network, 2017; Giannakis et al., 2014; Seshagiri et al., 2012; Waddell et al., 2015).

Wnt addiction in cancer presents a therapeutic opportunity (Madan & Virshup, 2015). All Wnts require palmitoleation in the endoplasmic reticulum by the enzyme PORCN for their secretion and function (Willert et al., 2003). Small molecule PORCN inhibitors block this modification and hence the activity of all Wnts. We and others have demonstrated that PORCN inhibitors such as ETC-159 suppress the growth of Wnt-addicted cancers in multiple preclinical models (B. Chen et al., 2009; X. Jiang et al., 2013; Madan et al., 2016). Due to its efficacy, the PORCN inhibitor ETC-159 has advanced to clinical trials (Ng et al., 2017). ETC-159 is also a useful research tool to study Wnt dependent genes. We found that more than 75% of the transcriptome responded to PORCN inhibition by ETC-159 in Wnt-addicted cancers, with significantly more genes changing in vivo than in vitro (Madan et al., 2018, 2016). Thus, PORCN inhibition is a powerful tool to study Wnt-regulated genes, and these Wnt-regulated genes are best studied in vivo in the presence of the appropriate microenvironment.

To date, only a few individual lncRNAs have been linked to Wnt signaling. For example, MYU (VPS9D1-AS1) is a target of Wnt/c-Myc signaling involved in colon cancer (Kawasaki et al., 2016). However, currently there are no systematic studies on functional lncRNAs regulated by Wnt signaling in vivo. Here, we comprehensively mapped Wnt-regulated lncRNAs from an orthotopic Wnt-addicted pancreatic cancer model and determined their wider roles in other cancers. To functionally validate the Wnt-regulated lncRNAs, we performed CRISPRi screens both in vitro and in vivo. Notably, we found multiple Wnt-regulated lncRNAs that had functional effects on cancer
cell growth only in a xenograft model, demonstrating the value of studying IncRNA functions \textit{in vivo}. This study provides a valuable resource of functional IncRNAs regulated by Wnt signaling. It also establishes a framework that can be broadly adapted for systematic discovery and functional annotation and validation of IncRNAs \textit{in vivo}.

**Results**

**Discovery of Wnt-regulated IncRNAs**

The HPAF-II pancreatic cancer cells contain a \textit{RNF43} missense mutation that makes them addicted to Wnt signaling. As previously reported, mice with established orthotopic HPAF-II xenografts were treated with the PORCN inhibitor ETC-159 for 7 days. Tumors were harvested for transcriptomic analysis at indicated time points (0, 3, 8, 16, 32, 56 and 168 hours) after starting ETC-159 treatment. The data were previously analyzed with a focus on protein-coding genes and splice variants (Idris et al., 2019; Madan et al., 2018). To comprehensively identify Wnt-regulated IncRNAs in pancreatic cancer \textit{in vivo}, we reanalyzed this time-course transcriptomic dataset (Figure 1A). We first used \textit{de novo} assembly to comprehensively identify all the putative transcripts in this Wnt-addicted pancreatic xenograft model. These transcripts were then compared with the Ensembl build 79 transcriptome to identify putative novel IncRNAs. The putative novel IncRNAs were filtered based on their length (> 200 bp), and we eliminated those with coding potential called by any of three computational tools: CPAT (L. Wang et al., 2013), CPC (Kong et al., 2007) and Slncky (J. Chen et al., 2016) (see Methods for details). The novel IncRNAs were combined with previously annotated IncRNAs from Ensembl build 79 to establish a comprehensive list of IncRNAs present in our RNA-seq dataset. We next selected all the IncRNA genes with TPM > 1. Using these stringent criteria, we identified a set of 3,633 IncRNAs in an orthotopic \textit{RNF43}-mutant pancreatic
cancer model (Figure 1A). Amongst these 3,633 IncRNAs, we found that the expression of 1,503 IncRNAs changed over time upon Wnt inhibition (false discovery rate (FDR) < 5%), therefore we refer to these IncRNAs as “Wnt-regulated IncRNAs” (Table S1).

Among the 1,503 Wnt-regulated IncRNAs, 325 IncRNAs were not annotated in Ensembl build 79. We further compared these novel IncRNAs with FANTOM5 IncRNA annotations (Hon et al., 2017) and found 172 IncRNAs that have not been previously annotated either in Ensembl or FANTOM5 (Figure 1B).

We found that twice as many IncRNAs were upregulated (976 Wnt-repressed IncRNAs) than downregulated (527 Wnt-activated IncRNAs) following PORCN inhibitor treatment (Figure 1C). Among them, 240 Wnt-repressed and 85 Wnt-activated IncRNAs are not annotated in Ensembl build 79. The 527 Wnt-activated IncRNAs responded as early as 3 hours after the first dose of ETC-159, consistent with direct regulation by Wnt/β-catenin signaling. Conversely, the 976 Wnt-repressed IncRNAs responded more slowly to Wnt inhibition (Figure 1C), which could be due to indirect Wnt regulation. For example, VPS9D1-AS1, a previously reported target of Wnt/MYC signaling (Kawasaki et al., 2016), was down-regulated rapidly after PORCN inhibitor treatment and the inhibition was sustained for 7 days. Similarly, a previously unannotated IncRNA XLOC_017401 was also downregulated shortly after Wnt inhibition. In contrast, XLOC_045229, another previously unannotated IncRNA, was upregulated after ETC-159 treatment, but the effect was only observed after 32 hours of treatment (Figure 1D). Taken together, we identified 1,503 IncRNAs whose expression is regulated either directly or indirectly by Wnt signaling in vivo in an RNF43-mutant pancreatic cancer.

Genes that are important in cancer pathogenesis can be regulated by multiple pathways. For example, the well-known proto-oncogene MYC can be activated by pathological Wnt signaling in Wnt-driven cancers, and also by diverse additional
pathways in other cancers (Gabay et al., 2014). Similarly, we postulated that if a specific Wnt-regulated IncRNA is important in cancer, the same IncRNA might also be dysregulated by other mechanisms in other cancer types. To test this, we analyzed gene expression data from TCGA (Goldman et al., 2019), comparing tumors with their paired normal samples. We found that many Wnt-regulated IncRNAs were also dysregulated in different and Wnt-independent types of cancers (Figure S1A). For example, 1,150 of the 1,503 Wnt-regulated IncRNAs were found in lung adenocarcinoma samples (LUAD) in TCGA, and 435 of these were significantly upregulated compared to paired normal samples (Figure 1A). We also found 253 Wnt-regulated IncRNAs exclusively upregulated or downregulated across different cancer types (Table S1). For example, VPS9D1-AS1, a known Wnt/MYC target, was both Wnt-activated in our study and also upregulated in 11 different types of cancers (Figure S1B), consistent with its established role as a IncRNA with oncogenic function (Kawasaki et al., 2016). Together, these analyses suggest that a subset of Wnt-regulated IncRNAs can act as mediators of oncogenic processes in both Wnt-dependent and Wnt-independent cancers.

LncRNAs respond to Wnt inhibition more robustly in vivo, especially in orthotopic xenograft model

Tumor microenvironment is important for tumor pathogenesis (Miller et al., 2017; Muir & Vander Heiden, 2018; Whiteside, 2008). To examine how the response of IncRNAs to Wnt inhibition is affected by the stromal microenvironment, we compared the effect of ETC-159 on IncRNAs expression in HPAF-II orthotopic or subcutaneous xenografts (in vivo) and in cultured cells (in vitro). Nearly twice as many IncRNAs responded to the PORCN inhibitor treatment in the subcutaneous xenograft (541/3,633) compared to those that responded in vitro (341/3,633) (Figure 1E). A further increase in the number of IncRNAs responding to Wnt inhibition was observed in the orthotopic
xenografts (1,191/3,633) (Figure 1F). This is consistent with our previous observation that Wnt-regulated gene expression changes are more robust in vivo (Madan et al., 2018). Interestingly, between the two in vivo models, many more lncRNAs responded to Wnt inhibition in the orthotopic than subcutaneous xenograft (Figure 1G). This is consistent with our previous observation that the overall changes in gene expression following Wnt inhibition were most marked in the orthotopic model (Madan et al., 2018). Taken together, this indicates that in vivo models can substantially enhance the discovery of Wnt-regulated genes, including lncRNAs.

A subset of Wnt-regulated lncRNAs are co-expressed with their nearest protein-coding gene in the same TAD

Most of the Wnt-regulated lncRNAs identified here have not previously been described or functionally characterized. Since lncRNAs can be important regulators of nearby genes (Engreitz et al., 2016; Gil & Ulitsky, 2019; Luo et al., 2016), we set out to explore their potential cis functions. If a lncRNA and its nearby protein-coding gene (PCG) are positively co-expressed after Wnt inhibition, it suggests that the lncRNA may enhance the expression of its neighbor. To test this, we analyzed the expression changes of lncRNAs and PCGs in response to PORCN inhibitor treatment. We found that on average, Wnt-regulated lncRNAs exhibited stronger co-expression with their nearest PCG after Wnt inhibition compared to their co-expression with all PCGs (Figure S2A). This stronger co-expression can be partially explained by the fact that some of the Wnt-regulated lncRNA–nearest PCG pairs are within the same topological associated domain (TAD) (Figure S2B), where they may functionally interact with each other more frequently, as previously suggested (Dixon et al., 2012). Interestingly, for these Wnt-regulated lncRNA–nearest PCG pairs encoded within the same TAD, the PCGs were significantly enriched for Gene Ontology (GO) biological processes such as organ
development and cell fate specification (Figure S2C). This suggests that these highly co-
expressed Wnt-regulated lncRNAs that are proximal to PCGs and co-localized within the
same TAD, are likely to be involved in the same cellular processes.

Wnt signaling affects the cis functional interaction between lncRNAs and protein-
coding genes

Expression quantitative trait loci (eQTLs) analysis that links DNA sequence variation
with changes in gene expression has been a powerful approach for understanding the
functional effects of common SNPs (Consortium & GTEx Consortium, 2017). The
underlying regulatory mechanisms of the eQTL SNPs on gene expression depend on
the genomic functional element perturbed by the genetic variant. For example, an eQTL
SNP within a lncRNA might modify its interaction with transcription factors or epigenetic
modifiers, thereby altering the expression of nearby PCGs (Gao et al., 2018). SNPs
within lncRNA loci that are associated with the mRNA abundance of nearby genes (<1
Mbp apart), i.e., cis-acting regulation, have been systematically annotated by the
FANTOM5 consortium to establish lncRNA-mRNA pairs linked by these eQTL SNPs
(Hon et al., 2017). This lncRNA-mRNA interaction mediated by an eQTL suggests that
these lncRNAs loci might potentially regulate the expression of nearby mRNAs. The
FANTOM5 dataset contains genome-wide transcriptome profiles of 1,829 samples from
more than 173 human primary cell types and 174 tissues across the human body, 276
cancer cell lines and 19 time courses of cellular treatment. If the eQTL linked lncRNA-
mRNA are co-expressed in FANTOM5 samples, it further suggests a functional
interaction between the lncRNA and its eQTL-linked mRNA. Here, to identify Wnt-
regulated lncRNAs with potential regulatory effects on nearby PCG mRNAs, we
overlapped 1,503 Wnt-regulated lncRNAs with all of the lncRNA-mRNA pairs annotated
by the FANTOM5 consortium. We found 1,486 lncRNA PCG mRNA (lncRNA-PCG) pairs
linked by eQTL SNPs involving 602 Wnt-regulated lncRNAs. (Some of the lncRNAs were linked to multiple PCGs, Figure 2A and Table S2). Among them, 587 lncRNA-PCG pairs were also significantly co-expressed ($p < 0.05$) in FANTOM5 samples. This co-expression across FANTOM5 samples suggests a functional interaction between the Wnt-regulated lncRNA and its eQTL-linked PCG broadly across cell types.

We examined if Wnt signaling altered the functional interaction (co-expression) between Wnt-regulated lncRNAs and their eQTL-linked PCGs. To do this, we compared the co-expression detected in response to Wnt inhibition to the co-expression observed in the FANTOM5 dataset. First, we found 260 lncRNA-PCG pairs that were significantly co-expressed in both our dataset and FANTOM5, irrespective of Wnt signaling status (Figure 2B). One illustrative example of this consistent co-expression pattern is VPS9D1-AS1 (lncRNA) and FANCA (eQTL-linked PCG) in Figure 2C. Here, the lncRNA-PCG co-expression was significant ($p < 0.05$) and had the same direction, i.e., positive in response to Wnt inhibition in our model of pancreatic cancer and positive in all FANTOM5 samples. In this set of lncRNA-PCG pairs, their functional interactions were not directly dependent on Wnt signaling. Second, there were 327 lncRNA-PCG pairs significantly co-expressed in the FANTOM5 dataset that were either not significantly co-expressed or co-expressed in the opposite direction after Wnt inhibition (Figure 2B). For example, VPS9D1-AS1 was also linked to CKD10 through 9 eQTL SNPs. VPS9D1-AS1 and CKD10 were positively co-expressed in FANTOM5 samples ($\beta = 0.42$), but in response to Wnt inhibition, they were negatively co-expressed ($\beta = -0.49$) (Figure 2D). This suggests that their functional interaction is affected by Wnt signaling inhibition. Finally, a third group of 407 Wnt-regulated lncRNA-PCG pairs (Figure 2B), although linked by eQTL SNPs, were not significantly co-expressed across FANTOM5 samples. However, they were significantly co-expressed in response to Wnt inhibition in our model
of pancreatic cancer. For example, *MALAT1* and *LTBP3* are not correlated in FANTOM5 but they are similarly regulated by Wnt signaling, Figure 2E. Thus, these lncRNAs and PCGs are co-regulated in a Wnt-dependent manner. Taken together, these analyses demonstrate that Wnt signaling can affect the functional interaction between Wnt-regulated lncRNAs and their eQTL-linked PCG. Therefore, Wnt signaling is important for both the regulation and the function of a subset of Wnt-regulated lncRNAs.

We then investigated the diseases associated with the Wnt-regulated lncRNA-PCG pairs linked by eQTLs, with a focus on cancer. eQTLs that co-localize with disease risk loci identified by genome-wide association studies (GWAS) are candidates for the regulation of complex traits and diseases, including SNPs associated with cancer susceptibility by GWAS (Q. Li et al., 2013). Thus we further examined the eQTL SNPs overlapping with the Wnt-regulated lncRNAs loci and matched these SNPs with those curated by FANTOM5 for 56 cancer GWAS traits. Among the 1,486 eQTL-linked Wnt-regulated lncRNA-PCG pairs, a subset of 115 pairs involving 49 Wnt-regulated lncRNAs were linked by eQTL SNPs that colocalize with cancer GWAS loci (Figure 2F, Table S3). For example, *LINC00035* was linked to *CLDN3* (Figure 2G) through 10 distinct *CLDN3* eQTL SNPs that were also associated with leukemia by GWAS (Table S3). In addition, *LINC0035* showed functional interaction with *CLDN3* in FANTOM5 (*p* = 2.10e-9), however, this functional interaction disappeared when we inhibited Wnt signaling (*p* = 0.74). This might suggest that *LINC0035* is involved in susceptibility to leukemia through its regulation of *CLDN3* in a Wnt-dependent manner. Integrating eQTL-linked Wnt-regulated lncRNA-PCG pairs with cancer GWAS data suggests that 3% (49/1,503) of the Wnt-regulated lncRNAs may confer cancer susceptibility through their *cis*-regulation of eQTL-linked PCGs (Gao et al., 2018; Tan et al., 2017).
Wnt-regulated IncRNAs and protein-coding genes form gene networks that are
dysregulated in cancers

Beside *cis* regulatory functions, IncRNAs can also participate in gene networks that
regulate diverse biological processes (Guttman et al., 2011; Kopp & Mendell, 2018). To
investigate which gene networks the various Wnt-regulated IncRNAs may be involved in,
we performed time-series clustering on the differentially expressed Wnt-regulated
IncRNAs and PCGs. This analysis closely paralleled a similar time-series clustering of
PCGs that we reported previously (Madan et al., 2018). The IncRNAs and PCGs fell into
63 distinct clusters based on their pattern of expression change following Wnt inhibition
(Figure S3A). The similar and coherent dynamic response of each cluster to Wnt
inhibition suggests the presence of a common regulatory process within each cluster
(Rotival & Petretto, 2014). As many Wnt-regulated IncRNAs and PCGs were also
dysregulated in different types of cancers as determined by differential expression
between tumors and their paired normal samples using the TCGA dataset (Goldman et
al., 2019)(Figure S1A), we tested if the IncRNA-PCGs clusters were enriched for
dysregulated genes in different cancer types. We found that 46 out of the 63 clusters
were enriched (FDR < 5%) for genes dysregulated in at least one type of cancer (Figure
S3B). In addition, most of these clusters (38/46) were enriched for genes consistently
either up- or down-regulated in several different cancer types (Figure S3B and Figure 3A
and 3B). For example, cluster 9 contained 67 Wnt-activated IncRNAs and 357 PCGs,
including well established Wnt target genes (e.g., *NKD1*, *AXIN2*, *LGR5*, *MYC*, *BMP4*,
*FGF9*) (Figure 3E). This cluster was enriched for genes upregulated in 6 cancer types
and was significantly enriched for ncRNA metabolic process, Wnt signaling and cell
differentiation. Many of the genes associated with ncRNA metabolic process (*NOP56*,
*METTL1*, *RRP1*, *AIMP2*, *EXOSC5*) were also overexpressed in multiple cancers. With a
few notable exceptions such as IncRNA *LINC00511* (J. Zhang et al., 2019), most of the IncRNAs in this cluster do not have established biological functions. One the other hand, cluster 2 contained mainly Wnt-repressed genes, the majority of which were downregulated in eight cancer types. The PCGs from this cluster were enriched for processes related to vesicle organization, vesicle transport and immune response. This last finding is consistent with recent studies demonstrating that Wnt signaling prevents anti-tumor immunity and suppresses immune surveillance (Holtzhausen et al., 2015; Spranger et al., 2015). Although most of the IncRNAs from cluster 2 have not been characterized before, *LINC00910* was previously identified as a IncRNA highly connected to other gene promoter regions and was proposed to be involved in lymphocyte activation (Cai et al., 2016). Taken together, this IncRNA-PCG network analysis suggests specific Wnt-regulated IncRNAs in gene networks are involved in distinct biological processes that contribute to the pathogenesis of cancers.

**CRISPRi screens identify Wnt-regulated IncRNAs that modify HPAF-II cell growth in a context-dependent manner**

Our analysis identified multiple Wnt-regulated IncRNAs, a subset of which might be important in cancer progression. To specifically identify the IncRNAs that play functional roles in the pathogenesis of *RNF43*-mutant pancreatic cancer *in vivo*, we performed CRISPRi screens. This approach utilizes dCas9-KRAB, where a catalytically inactive Cas9 is fused to a Krüppel associated box (KRAB) transcriptional repressor domain (Gilbert et al., 2013). dCas9-KRAB is recruited to the transcription start site (TSS) of IncRNAs by single guide RNAs (sgRNAs) to repress the transcription of the IncRNA of interest. CRISPRi screens have been demonstrated to be an efficient and specific approach for genome-wide loss-of-function studies of IncRNAs (S. J. Liu et al., 2017),
which can not reliably be inactivated by indels introduced by the standard CRISPR-Cas9 system.

We chose to perform this CRISPRi screen in vivo because we have shown that both lncRNAs and PCGs respond to Wnt inhibition more robustly in vivo (Figure 1E-G and (Madan et al., 2018)), and that in vivo screening identifies dependencies not seen in tissue culture (Zhong et al., 2019). To capture the difference of Wnt-regulated lncRNA functions in vivo and in vitro, the CRISPRi screen was conducted both using xenograft tumor in vivo as well as cultured cells in vitro (Figure 4A).

We designed five sgRNAs to target the transcription start site (TSS) of each of the 1,503 Wnt-regulated lncRNAs (Horlbeck et al., 2016). We divided the sgRNAs into 3 lentiviral sub-libraries to allow for full representation of the sgRNAs throughout the in vivo screen, due to the limited number of cells that can be implanted and engrafted in each tumor. For each sub-library, we also included 55 sgRNAs targeting 11 genes involved in cell survival or Wnt signaling as positive controls, and 50 non-targeting controls (Table S4).

We transduced an HPAF-II cell line stably expressing dCas9-KRAB with the lentiviral sgRNA sub-libraries at a low multiplicity of infection (MOI < 0.3) to ensure that each cell was only infected by one virus with a single sgRNA. The transduced cells were selected with puromycin for 3 days (T0 population) and then maintained in culture for two weeks (the in vitro screen) with ≥ 3 x 10⁶ cells to allow for 1000-fold coverage of each sgRNA throughout the in vitro screen. Alternatively, the transduced cells were injected subcutaneously into immunocompromised mice. To get a good representation of each guide in the subcutaneous tumor, a total of 10⁷ cells were injected per mouse flank to allow for 3000-fold coverage of each sgRNA. The tumors were harvested after 3 weeks (the in vivo screen). Integrated lentiviruses encoding sgRNAs (i.e., barcodes)
from the T0 population, the in vitro screen end population and the in vivo screen end population were then recovered by PCR and quantified by next-Gen sequencing (see Methods for additional details).

We first assessed the technical quality of the CRISPRi screen. There was a high correlation of sgRNA frequencies between independent experimental replicates (Figure S4), suggesting the robustness of the screen. We used the MAGeCK algorithm (W. Li et al., 2014) to analyze the in vitro and in vivo screens, using the non-targeting control sgRNAs for normalization. The statistical determination that a lncRNA gene regulated cancer proliferation was calculated based on the performance of all its sgRNAs compared to the non-targeting controls, as previously reported (W. Li et al., 2014). Each lncRNA gene was also scored based on the fold change of its second best performing sgRNA (W. Li et al., 2014). We classified a gene as a hit if its associated FDR was less than 10% (Figure 4B). First, our screen was able to identify important positive controls as gene hits. For example, all 5 sgRNAs targeting POLR2A (RNA polymerase II subunit A) were depleted in both in vitro and in vivo screens, consistent with its essential role for cell growth (Figure S5). As expected for a Wnt-addicted cancer, sgRNAs targeting CTNNB1 were also depleted in both in vitro and in vivo screens (Figure S5). Thus, the screen appears to function well both in vitro and in vivo.

We next compared the lncRNA hits from the in vivo and in vitro screens. We identified 4 Wnt-regulated lncRNA loci as hits in both screens, 21 lncRNA loci as hits only in the in vivo screen and 9 lncRNA loci as hits only in the in vitro screen (Figure 4B and 4C, and Table 1). Since CRISPRi acts within a 1 kb window around the targeted TSS to repress gene expression (Gilbert et al., 2014), we also included in our sgRNA library guides designed to suppress the expression of the protein-coding genes that also had a TSS within 1 kb of the TSS of lncRNA hits. We found that for 6 lncRNA hits,
protein-coding genes were nearby that could be suppressed by CRISPRi in the screen. However, CRISPRi suppression of these protein neighbors did not produce a phenotype in a separate screen library (Table S5). This indicates that the lncRNA hits identified through CRISPRi screen are likely due to the functions of lncRNA loci themselves. Taken together, around 1% (13/1,503) of the Wnt-regulated lncRNAs can modify cancer cell growth in the \textit{in vitro} screen, which is consistent with previous genome-wide CRISPRi screens for functional lncRNAs in cell lines (S. J. Liu et al., 2017). Notably, using the \textit{in vivo} CRISPRi screen, we identified twice as many Wnt-regulated lncRNAs (25/1,503) that had a functional effect on cancer cell growth.

We found that the four Wnt-regulated lncRNA loci that were hits in both screens were essential for HPAF-II cancer cell growth (Figure 4B and 4C). For example, 3 out of 5 sgRNAs targeting \textit{LINC00263} were depleted in both screens, suggesting that it was an essential lncRNA for HPAF-II growth both \textit{in vivo} and \textit{in vitro} (Figure 4D). Interestingly, \textit{LINC00263} has previously been reported to be a cell type specific lncRNA essential for the growth of U87 cells but not K562, HeLa or MCF7 cells (S. J. Liu et al., 2017). 21 Wnt-regulated lncRNA loci were hits only in the \textit{in vivo} screen and would not have been identified in an \textit{in vitro} screen. Of these, 2 lncRNAs can promote cancer cell growth, while 19 lncRNAs appear to have suppressive effects on cell proliferation \textit{in vivo}. For example, 4 sgRNAs targeting \textit{ABGD11-AS1} were only enriched at the end of the \textit{in vivo}, but not the \textit{in vitro} screen (Figure 4E). Among the 9 Wnt-regulated lncRNA loci that were hits only in the \textit{in vitro} screen, we found 3 of them promoted, while 6 suppressed HPAF-II proliferation in culture. For example, all 5 sgRNAs targeting \textit{AP000487.1} were enriched at the end of the \textit{in vitro} screen, however, none of the 5 sgRNAs showed significant change after the \textit{in vivo} screen (Figure 4F). This suggests that \textit{AP000487.1} may have tumor suppressive function only \textit{in vitro}. Taken together, using CRISPRi
screens both *in vivo* and *in vitro*, we identified Wnt-regulated IncRNAs loci that modify HPAF-II growth in a context-dependent protein-coding genes that also had a TSS within 1 kb of the TSS of IncRNA hits manner. It also suggests that IncRNA loci identified *in vitro* may not have important functions *in vivo*.

To further validate the CRISPRi screen results, we focused on *LINC00263*, which was an essential IncRNA for HPAF-II cell growth both *in vivo* and *in vitro* (Figure 4D). We cloned the top two sgRNAs targeting *LINC00263* into doxycycline-inducible lentiviral sgRNA vectors. After confirming the doxycycline-inducible knockdown of *LINC00263* expression in the HPAF-II cell lines (Figure 4G), we verified that knocking down *LINC00263* reduced HPAF-II cell growth *in vitro* (Figure 4H). Interestingly, we found that knocking down *LINC00263* also reduced the expression of its nearest protein-coding gene stearoyl-CoA desaturase (*SCD*) (Figure 4G), similar to what was reported in U87 cells (S. J. Liu et al., 2017). To test if *SCD* regulates the growth of HPAF-II cells, we next targeted the TSS of *SCD* using CRISPRi with two independent sgRNAs. Knockdown of *SCD* reduced *SCD* mRNA abundance (Figure S6) and inhibited HPAF-II cell growth similar to that observed after knockdown of *LINC00263* (Figure 4I). However, sgRNAs targeting the TSS of *SCD* did not reduce the expression of *LINC00263* (Figure S6). Based on these results, we hypothesize that *LINC00263* is essential for HPAF-II cell growth through *cis*-regulation of *SCD*.

**Discussion**

LncRNAs play important roles in diverse biological processes. Here we present a systematic study to identify and functionally assess IncRNAs regulated by Wnt signaling. Using an orthotopic Wnt-addicted pancreatic cancer model treated with a potent and effective PORCN inhibitor, we identified 1,503 IncRNAs regulated by Wnt signaling *in...
Many of these IncRNAs were also dysregulated in different cancer types and may function in gene networks that contribute to the pathogenesis of cancers. Our eQTL-IncRNA interactions analysis identified Wnt-regulated IncRNAs that may regulate nearby protein-coding genes. Using CRISPRi screens, we found that 34 Wnt-regulated IncRNAs could modify cell growth in a context-dependent manner with a higher hit rate in the in vivo model. This pipeline for IncRNA discovery and functional validation may be broadly applicable.

We previously reported that Wnt-regulated protein-coding genes were more robustly regulated in an orthotopic model than in cultured cells. We find that this holds true for IncRNAs as well. More than twice as many IncRNAs responded to Wnt inhibition in the in vivo xenografts than in cells cultured in vitro. These differences in the number and magnitude of gene expression changes will be influenced by a variety of local and experimental factors including tumor microenvironment, culture conditions, doubling times in different environments, local nutrients versus culture medium ingredients, the presence of stromal and other host cells, and variations in extracellular matrix. Overall, our findings are consistent with the large body of literature showing that the expression of genes is regulated by interaction with the relevant environment (Killion et al., 1998).

Cancer cells show differential dependencies on protein-coding genes for their growth and survival in vivo versus in vitro (Miller et al., 2017; Possik et al., 2014; Yau et al., 2017; Zhong et al., 2019). Our CRISPRi screen results indicate that cancer cells also have different requirements for IncRNAs when grown in vivo vs in vitro conditions. Multiple IncRNAs exhibit different phenotypes when studied in cell culture compared to animal knock-out models and in vivo systems (Bassett et al., 2014; Goudarzi et al., 2019; Han et al., 2018; Kohtz, 2014; Ruan et al., 2020). Our results highlight the importance of studying IncRNAs in vivo with the relevant microenvironment in order to
better understand their functions in cancer pathogenesis. This has implications for the identification of IncRNAs as potential therapeutic targets for cancer treatment. For instance, it has been shown that drugs identified through high-throughput screening of cell culture in vitro have limited success in patient care (Letai, 2017; Sharma et al., 2010). The same might be true for drugs identified to target IncRNAs.

Despite the large number of IncRNAs annotated in the human genome (Hon et al., 2017; Iyer et al., 2015), only a very small fraction of them have been either validated or characterized at a functional level. This is due to the complex nature of the IncRNA loci and a prior lack of tools to study them at a large scale (Bassett et al., 2014; Kopp & Mendell, 2018). In recent years, CRISPR screens have been shown to be an efficient and specific approach to investigate IncRNA functions genome-wide in cultured cells (Esposito et al., 2019; Joung et al., 2017; S. J. Liu et al., 2017; Zhu et al., 2016). In this study, we perform a CRISPRi screen not only in cultured cells, but also in xenograft tumors to assess the ability of 1,503 Wnt-regulated IncRNAs to influence cancer cell proliferation. Validating this approach, among the 4 Wnt-regulated IncRNAs that we found to be functional both in vivo and in vitro, 3 were identified to promote cell growth in prior CRISPRi screens (S. J. Liu et al., 2017). Furthermore, consistent with what has been reported for genome-wide IncRNA CRISPRi screens in cell lines (S. J. Liu et al., 2017) 1% (13/1,503) of the Wnt-regulated IncRNAs in our in vitro screen modified cancer cell growth. Notably, our in vivo CRISPRi screen identified twice as many Wnt-regulated IncRNAs (25/1,503) that had a functional effect on cancer cell growth. 21 Wnt-regulated IncRNAs had functional effects on cancer cell growth only in the xenograft model and would not have been identified in an in vitro screen, demonstrating the value of studying IncRNA functions in vivo. This is also demonstrated in a recent study that an in vivo
system is essential for understanding the biological role of a human lncRNA in metabolic regulation that cannot be recapitulated in vitro (Ruan et al., 2020).

The CRISPR based approach can produce different results than those based on RNA interference. *LINC00176*, found in our screen as a functional Wnt-regulated lncRNA locus, has also been identified in four other publications. Two groups used different CRISPR approaches (paired-sgRNAs (Zhu et al., 2016) or sgRNA targeting splice site (Y. Liu et al., 2018)) and found, as we did, that *LINC00176* has a tumor-suppressive effect in vivo. Two additional studies used RNA interference and concluded, conversely, that *LINC00176* has a pro-proliferative role in ovarian and hepatocellular carcinoma cell lines (Dai et al., 2020; Tran et al., 2018). These differences could be due to differences in cell type or experimental approach, as RNA interference is known to suffer from significantly more off-target effects compared to the CRISPR approach and is less effective for targeting nuclear lncRNAs (Smith et al., 2017; Stojic et al., 2018). Together, the comparisons here further support the identification of Wnt-regulated lncRNA loci that can modify cancer cell growth and the importance of choosing a loss-of-function strategy to characterize lncRNAs.

Nevertheless, there are some limitations to using CRISPRi to target lncRNAs. First, recruiting dCas9-KRAB to the TSS of a lncRNA can suppress the transcriptional activity and local regulatory sequence (enhancer) of the lncRNA locus; second, it results in decreased production of the lncRNA transcript, inhibiting potential cis or trans function of the lncRNA transcript (S. J. Liu et al., 2017). Both the repressive effect on chromatin and the lack of lncRNA transcripts can cause biological consequences that cannot be differentiated by CRISPRi knock-down alone. Thus, additional studies are needed to dissect how the Wnt-regulated lncRNA loci identified in our screen regulate cell proliferation.
GWAS studies have identified thousands of common genetic variants that are associated with complex traits and diseases, but 90% of these fall into noncoding regions of the genome (Hindorff et al., 2009). This has made it difficult to dissect the underlying molecular mechanisms. eQTLs that co-localize with GWAS SNPs suggest the effect of the SNPs on diseases and traits is mediated by changes in gene expression. IncRNAs overlapping with these GWAS associated cis-eQTL SNPs are potential candidates to explain the underlying mechanisms of risk loci because IncRNAs can be important cis regulators of nearby genes (Engreitz et al., 2016; Gil & Ulitsky, 2019; Luo et al., 2016). When we mapped Wnt-regulated IncRNAs-mRNA pairs linked by eQTL SNPs using the annotation from FANTOM5 (Hon et al., 2017) we found previously unappreciated regulatory effects of Wnt-regulated IncRNAs in disease. For example, Wnt-regulated IncRNA \textit{LINC00339} was linked to \textit{CDC42} through five eQTL SNPs, suggesting the \textit{LINC00339} locus may regulate the expression of \textit{CDC42}. Supporting this, knocking-down \textit{LINC00339} expression has been reported to increase \textit{CDC42} expression (X.-F. Chen et al., 2018). Consistent with the importance of Wnt regulation, \textit{LINC00339} and its linked gene \textit{CDC42} are involved in both endometriosis and bone metabolism (X.-F. Chen et al., 2018; Powell et al., 2016), two Wnt-regulated biological processes (Krishnan, 2006; Yongyi Wang et al., 2009). Thus, identifying eQTL-linked Wnt-regulated IncRNA-PCG pairs helps to prioritize the potential cis-regulatory targets of Wnt-regulated IncRNAs. Further integrating the disease risk information based on GWAS SNPs co-localizing with eQTL, the Wnt-regulated IncRNA-PCG pairs may help explain the underlying mechanisms of risk loci in the context of disease, which is potentially affected by Wnt signaling.

Although the 1,503 Wnt-regulated IncRNAs were discovered in the orthotopic \textit{RNF43}-mutant pancreatic cancer xenograft model, many of them were also...
dysregulated in different types of cancers in TCGA (Figure S1A). 253 Wnt-regulated
lncRNAs were exclusively upregulated or downregulated across different cancer types
(Table S1). This suggests the fundamental roles of Wnt-regulated lncRNAs in cancer
pathogenesis in a broader context beyond Wnt-addicted pancreatic cancer. For
example, CCAT1, identified as a Wnt-activated lncRNA, was also upregulated in 9
cancer types (Table S1). Our CRISPRi screens indicated that it is an essential lncRNA
both in vivo and in vitro (Table 1). This suggests that CCAT1 is a Wnt-activated lncRNA
with oncogenic function, which is consistent with previous studies showing that CCAT1
can promote the progression of different types of cancers (Y. Jiang et al., 2018; Xiang et
al., 2014; E. Zhang et al., 2017). Integrating Wnt-regulated lncRNAs with their
expression profiles in TCGA and CRISPRi functional screens can better distinguish their
oncogenic or tumor suppressive functions in cancer pathogenesis.

Conclusions

This study comprehensively identified 1,503 lncRNAs regulated by Wnt signaling in
vivo and determined their wider roles in other cancers. We found more than twice as
many lncRNAs responded to Wnt inhibition in the in vivo xenografts than in cells cultured
in vitro. With CRISPRi screens both in vivo and in vitro, we found two fold (21/1503) as
many Wnt-regulated lncRNAs have functional effects on cell growth only in vivo,
suggesting the importance of studying lncRNA function with relevant microenvironment.
Thus, this study provides a valuable resource of functional Wnt-regulated lncRNAs in
vivo. It also establishes a framework for integrating orthogonal transcriptomics dataset
with functional CRISPRi screening which can be broadly adapted for systematic
discovery, functional annotation and validation of lncRNAs in vivo.
Methods

De novo IncRNA discovery

The polyA+ RNA-seq dataset contains the transcriptional response to PORCN inhibitor ETC-159 treatment at seven time points (0, 3, 8, 16, 32, 56 and 168 hours) using an orthotopic model of RNF43-mutant pancreatic adenocarcinoma (HPAF-II). The data was previously published (Madan et al., 2018) under accession number GSE118041. RNA-seq reads were assessed for quality with FASTQC. Reads originating from mouse genome (mm10) were removed with Xenome (Conway et al., 2012). All the reads among replicates from each time point were pooled to achieve deep coverage for novel IncRNA discovery. Each time point generated between 160 million to 237 million reads. The reads were aligned to hg38 (Ensembl version 79) using TopHat v2.0.10 (Kim et al., 2013). De novo transcriptome assembly was performed separately for each time point with Cufflinks v2.1.1 (Trapnell et al., 2010). Transcriptome assemblies at each time point were merged and compared with Ensembl build 79 as reference, using Cuffmerge. The novel transcripts were selected using Cuffcompare class code for novel intergenic and novel antisense transcripts. All the novel transcripts were then merged with Ensembl build 79 to establish a full reference transcriptome. RNA-seq reads from each sample were also individually aligned to hg38 (Ensembl version 79) using TopHat v2.0.10 (Kim et al., 2013). Gene level reads counts for each sample were computed with HTSeq 0.6.0 (Anders et al., 2015), which were then converted to gene expression in Transcripts per Million (TPM). To identify putative novel IncRNAs transcripts, the novel transcripts were filtered using the following criteria: length longer than 200 bp and estimation to be non-protein coding based on three methods: CPAT with threshold less than 0.364 (L. Wang et al., 2013), CPC with threshold less than 0 (Kong et al., 2007) and Snclky defined as...
“lncRNA” (J. Chen et al., 2016). Known lncRNAs from Ensembl build 79 were obtained based on their transcript biotype: “lincRNA”, “antisense”, “sense_intronic”, “sense_overlapping”. All the genes were also filtered based on their expression to make sure that the median expression level of each gene at every time point had TPM > 1. This analysis yielded 16,160 genes, including 12,527 protein-coding genes, 2,846 annotated lncRNAs and 787 novel lncRNAs that were expressed in RNF43-mutant pancreatic adenocarcinoma (HPAF-II).

**Identification of Wnt-regulated lncRNAs**

To identify genes regulated by Wnt signaling, DESeq2 (Love et al., 2014) was used to perform differential expression analysis on 16,160 genes across time points with likelihood ratio test (LRT). Adjusted \( P \) value < 0.05 was used to select genes significantly responded to Wnt inhibition across time points. This led to 10,554 Wnt-regulated genes, including 9,051 protein-coding genes and 1,503 lncRNAs (1,178 annotated lncRNAs and 325 novel lncRNAs).

**Comparison of lncRNAs response to Wnt inhibition across models**

Two RNA-seq datasets contain transcriptional response of *in vitro* model (48 h ETC and 48 h Veh) and subcutaneous model (0h and 56h) of RNF43-mutant pancreatic adenocarcinoma (HPAF-II) to PORCN inhibitor ETC-159 treatment. The data was previously published (Madan et al., 2018) under accession number GSE118190 and GSE118179, respectively. RNA-seq reads from these datasets were assessed for quality with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads originating from mouse genome (mm10) were removed with Xenome (Conway et al., 2012) and aligned to hg38 (Ensembl version 79) using TopHat v2.0.10 (Kim et al., 2013) for each sample. Gene level reads counts were computed with HTSeq 0.5
(Anders et al., 2015). DESeq2 (Love et al., 2014) was used to perform differential gene expression analysis on 16,160 genes between the time points with Wald test for each of the models, namely \textit{in vitro} model (48 h ETC and 48 h Veh), subcutaneous model (0h and 56h) and orthotopic model (0h and 56h). An adjusted $P$ value < 0.1 was used to select genes that significantly responded to Wnt inhibition between the two time points.

\textbf{Wnt-regulated lncRNA co-expression with PCGs}

The degree of co-expression between Wnt-regulated lncRNAs and either all PCGs or their nearest PCG in response to Wnt inhibition in the orthotopic HPAF-II cancer model was calculated with cor function (spearman correlation) in R. The TAD data from the PANC-1 cell line mapped to hg38 was downloaded from the 3D Genome Browser (Yanli Wang et al., 2018). The Wnt-regulated lncRNA and nearest PCG pair were classified into two groups, the pair in the same TAD versus the pair in different TADs based on the PANC-1 TAD information. The correlation distributions between the two groups were tested for difference by using a two-sample nonparametric Mann–Whitney U test using the R function wilcox.test.

\textbf{Analysis of TCGA dataset}

HTSeq - Counts data of all the TCGA cancers were downloaded from the UCSC Xena platform (Goldman et al., 2019). The cancer types were selected for further analysis if at least 5 tumor-normal pairs were present, and there was a clear separation between the tumor and normal samples in the dataset based on PCA analysis. This yielded 14 cancer types. Genes with less than 10 reads mapped across the samples within each cancer type were removed. Differential expression analysis between the paired tumor-normal samples for each cancer type was performed using DESeq2 (Love
et al., 2014). An adjusted $P$ value < 0.05 was used to select genes significantly differentially expressed between tumor and normal sample.

**Integrative analysis of FANTOM5 dataset**

Wnt-regulated IncRNAs were mapped to FANTOM5 IncRNA annotations as follows:

1. If the IncRNA was annotated with the same Ensembl Gene ID in FANTOM5, it's considered the same IncRNA. 2. The remaining IncRNAs were overlapped with FANTOM5 IncRNA assembly (hg38) to identify the corresponding FANTOM5 CAT_geneID. Among the 1,503 Wnt-regulated IncRNAs, 1,073 were also annotated in FANTOM5 and 430 were novel previously unannotated IncRNAs. The eQTL linked IncRNA protein-coding gene (PCG) pairs for these 1,073 annotated Wnt-regulated IncRNAs were extracted from FANTOM5 annotation eQTL_linked_IncRNA_mRNA_pair (Hon et al., 2017). This yielded 1,486 IncRNA-PCG mRNA pairs linked by eQTL SNPs involving 602 Wnt-regulated IncRNAs (Figure 2A and Table S2). The gene expression profiles of all the pairs in 1,829 FANTOM5 samples were downloaded from the FANTOM_CAT.expression_atlas.gene.lv3_robust.rle_cpm curated by FANTOM5 (Hon et al., 2017). The IncRNA-PCG pair was identified as significantly co-expressed in FANTOM5 samples if it passed the threshold used in (Hon et al., 2017), i.e., that their co-expression is greater than 75th percentile of the matched background correlation ($binom_p < 0.05$ compared to the background). The IncRNA-PCG pair co-expression in response to Wnt inhibition was calculated using Spearman correlation $rho$ on gene expression TPM across time points. The associated $p$ value was also calculated using cor.test function in R. To identify the eQTL that are co-localizing with GWAS SNP, eQTLs linking Wnt-regulated IncRNA and protein-coding genes were first mapped to SNP id using biomart in R. These SNPs were overlapped with trait-associated SNPs curated by FANTOM5 to subest the SNPs associated with cancer by GWAS. In total,
271 eQTL SNPs were found to be associated with cancer by GWAS, linking 115 Wnt-regulated IncRNA-PCG pairs involving 49 Wnt-regulated IncRNAs (Table S3).

**Time series clustering**

Time series clustering on 10,554 Wnt-regulated genes was performed using GPClust (Hensman et al., 2013) as previously described (Madan et al., 2018). Gene expression TPM were converted to z-scores and time points were square root transformed. Genes were clustered with GPClust (Hensman et al., 2013) using the Matern32 kernel with a length scale of 6 and a concentration (alpha) parameter of 0.001, 0.01, 0.1, 1, and 10. Genes were assigned to a cluster based on the highest probability of being a member of that cluster. Clustering was performed 10 times for a specified set of parameters, with the best clustering taken as the one with the lowest distance to the other clusterings, i.e. the most representative.

**Functional enrichment analysis**

Gene Ontology (GO) enrichment analysis was performed with g:Profiler (Reimand et al., 2016) using all the Wnt-regulated protein-coding genes as background. Significantly enriched GO terms were selected with FDR < 5%.

**Enrichment analysis for dysregulated genes from different cancers**

Genes significantly differentially expressed (adjusted \( P \) value < 0.05) between tumor-normal pairs were defined as dysregulated genes. To test whether the clusters were enriched for dysregulated genes in each cancer type, genes from each of the 63 clusters were intersected with dysregulated genes from each cancer separately by carrying out a Fisher’s exact test. The gene background used for the test were Wnt-regulated genes that were dysregulated in the specific cancer. Upregulated genes and
downregulated genes were examined separately for enrichment. The Fisher’s exact test was performed with fisher.test in R for overrepresentation. Nominal $p$ values were adjusted for multiple testing using the Benjamini-Hochberg method. Clusters significantly enriched for dysregulated genes were selected with FDR < 5%. The significance of the enrichment was clustered for each cluster and its enriched cancer type.

**CRISPRi sgRNA library design**

CRISPRi single guide RNA (sgRNA) library was designed to target the transcription start site (TSS) of each of the Wnt-regulated lncRNAs. 1,503 Wnt-regulated lncRNAs were selected for the CRISPRi screen, which contained 3,151 transcripts including different isoforms. To avoid redundancy of different TSSs located in close proximity, if TSSs of transcripts belonging to the same gene were within 100bp, they were grouped together. A total set of 2,337 TSSs were obtained for Wnt-regulated lncRNAs, which were then converted to hg19 with the liftover function in R. These TSSs were furthered refined with FANTOM based TSS annotation and 5 sgRNAs were designed to target each of the TSS using hCRISPRi-v2.1 algorithm (Horlbeck et al., 2016). Since some TSSs could not be uniquely targeted, in total 8,560 sgRNAs were designed to target 1,486 Wnt-regulated lncRNAs. The sgRNAs were then divided into 3 sub-libraries. Protein-coding genes whose TSSs were within 10 kb of Wnt-regulated lncRNAs were selected. sgRNAs targeting these protein-coding genes were extracted from the hCRISPRiv2 library (Horlbeck et al., 2016) to constitute a 4th sub-library. For each sub-library we also included 55 sgRNAs targeting 11 genes ($PCNA$, $POLR2A$, $PSMA7$, $RPS27$, $SF3A3$, $CTNNB1$, $FZD5$, $APC$, $AXIN1$, $CSNK1A1$, $PORCN$) involved in cell survival and Wnt signaling as positive controls and 50 non-targeting controls (Table S4). The sgRNAs libraries were synthesized by CustomArray (Bothell, WA, USA).
sgRNA cloning and lentiviral packaging

The sgRNA libraries were cloned into pCRISPRia-v2 sgRNA expression vector (Horlbeck et al., 2016) by Gibson assembly (NEB). They were then amplified using electroporation in Endura electrocompetent cells (Lucigen), to achieve at least 250 colonies per sgRNA in the library. For individual CRISPRi knockdown, top 2 performing sgRNAs targeting LINC00263 and SCD were selected with protospacer sequences: sgLINC00263_1 (GACCTCAGTCTGCCCTACCC), sgLINC00263_2 (GGGTAGGGCAGACTGAGGTC), sgSCD_1 (GCTTGGCAGCGGATAAAAGG), sgSCD_2 (GCACATTCCCAACTCACGGA). The sgRNAs were cloned into doxycycline-inducible lentiviral sgRNA expression vector FgH1tUTG as previously described (Aubrey et al., 2015). The sgRNA plasmid was packaged into lentiviral particles with psPAX2 and pMD2.G packaging plasmids. The virus supernatant was harvested 48 and 72 hours after transfection, filtered through 0.45 µm filter and stored at -80 °C.

Cell lines

The HPAF-II cell line was obtained from the Duke Cell Culture Facility. An HPAF-II stable cell line expressing dCas9-KRAB was generated by lentiviral transduction with pMH0001 plasmid (UCOE-SFFV-dCas9-BFP-KRAB) (Adamson et al., 2016) and sorting for the top 20% - 30% BFP expressing cells. All cell lines were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% penicillin/streptomycin, maintained in 5% CO₂. Cells were regularly tested for mycoplasma.
CRISPRi screens

The HPAF-II-dCas9-KRAB stable cell line was infected with sgRNA lentiviral libraries at a multiplicity of infection (MOI) < 0.3 with 8 µg/ml polybrene. The infected cells were selected with 2 µg/ml puromycin for 3 days (T0 population). 3 x 10^6 cells from the T0 population were harvested and stored as a cell pellet at -20 °C for sequencing. For the in vitro screen, cells from T0 population were passaged with a seeding density of 3 x 10^6 cells at each passage to allow for 1000 times coverage of each sgRNA, and cultured for 2 weeks. 3 x 10^6 cells at the end of the in vitro screen were harvested and stored as a cell pellet at -20 °C for sequencing. The in vitro screen was performed in duplicates for each sub-library. For the in vivo screen, cells from the T0 population were mixed with Matrigel (BD Biosciences) and injected subcutaneously into the flanks of NOD-scid gamma (NSG) mice. 10^7 cells were injected per flank to allow for library coverage of 3000 cells/sgRNA at the time of implantation. A group of 3 mice were injected per sub-library. Mice were sacrificed 3 weeks after injection, and tumors were harvested and stored at -80 °C. Genomic DNA from the frozen cell pellets and homogenized tumors was extracted with high salt precipitation. The sgRNA region was amplified by PCR. A second round of PCR was performed to append Illumina sequencing adaptors and barcodes for each sample. PCR products were purified and quantified with a Bioanalyzer, and sequenced on the Illumina MiSeq platform.

CRISPRi screens analysis

Reads from sequenced screening sgRNA libraries were demultiplexed based on sample barcodes with FASTX-Toolkit. The reads were then counted against individual sub-libraries using MAGeCK count function (W. Li et al., 2014) with non-targeting control sgRNA for normalization. sgRNA counts were used for quality control using PCA and
clustering analysis with DESeq2 (Love et al., 2014) to exclude outlier samples. Robust
Rank Aggregation analysis (RRA) was performed with MAGeCK (W. Li et al., 2014) test
function to detect sgRNAs significantly depleted or enriched from the screens. Gene
level significance was calculated based on the performance of all its sgRNAs compared
to non-targeting controls, as previously shown (W. Li et al., 2014). Each gene was also
scored based on the fold change of its second best performing sgRNA (W. Li et al.,
2014). We classified genes as hits if their associated FDR < 10%.

Inducible CRISPRi knockdown

1 µg/ml doxycycline final concentration (dox) (from a stock of 10 mg/ml dissolved in
DMSO) was used to induce sgRNA expression from the inducible lentiviral sgRNA
expression vector, while DMSO was used as the control. After 48 hours induction, total
RNA was isolated from the CRISPRi knockdown cells. RT-qPCR was performed to
assess the knockdown efficiency for LINC00263 and SCD with HPRT gene as an
internal control. RT-qPCR primers were: LINC00263_Forward
(AAAGATTGGGCAGTCACTGG),
LINC00263_Reverse
(TGGGTCTTCAGCACAAATG), SCD_Forward (TTCCTACCTGCAAGTTACCC),
SCD_Reverse (CCAGCTTTGTAAGAGCGGT). The effect of CRISPRi knockdown on
cell growth was assessed with internally controlled, relative growth assays. Cells were
seeded in duplicates and treated with either 1 µg/ml dox or DMSO. Cells were counted
every 3-4 days after the initial dox treatment.
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Figure legends

Figure 1: Identification of Wnt-regulated lncRNAs from orthotopic RNF43-mutant pancreatic cancer model. (A) Computational pipeline to identify 1,503 Wnt-regulated lncRNAs from orthotopic RNF43-mutant pancreatic cancer. (B) Comparison of Wnt-regulated lncRNAs with Ensembl build 79 and FANTOM5 lncRNA annotations. (C) Expression profiles of 1,503 Wnt-regulated lncRNAs across time points after Wnt inhibition. (D) Gene expression of selected Wnt-regulated lncRNAs, including annotated lncRNAs (VPS9D1-AS1 and ABHD11-AS1) and novel lncRNAs (XLOC_017401 and XLOC_045229). TPM, transcripts per million. (E, F, G) Fold change of lncRNAs after Wnt inhibition compared across models. More lncRNAs respond to Wnt inhibition in the HPAF-II subcutaneous (E) and orthotopic models (F) than in HPAF-II cells cultured in vitro. FC, fold change. (G) More lncRNAs respond to Wnt inhibition in HPAF-II orthotopic model than in the subcutaneous model.

Figure 2: Wnt signaling affects the cis functional interaction between lncRNAs and protein-coding genes. (A) Wnt-regulated lncRNA is linked to its nearby protein-coding gene (PCG) if the eQTL SNP of a PCG overlaps with a lncRNA locus, as annotated by FANTOM5 consortium (Hon et al., 2017). The co-expression between Wnt-regulated lncRNA and its eQTL-linked PCG is examined both in FANTOM5 across cell types and in our dataset after Wnt inhibition. (B) Functional interaction between Wnt-regulated lncRNA and its eQTL-linked PCG is affected by Wnt signaling. Red, functional interaction between lncRNA-PCG pair implicated in FANTOM5 is not directly dependent on Wnt signaling; blue, functional interaction between lncRNA-PCG pair implicated in FANTOM5 is dependent on Wnt signaling; yellow, no significant functional interaction between lncRNA-PCG pair but they are co-regulated in response to Wnt-signaling; grey, lncRNA-PCG pair is neither co-regulated in response to Wnt-signaling nor functionally
interacting. (C) Wnt-dependent lncRNA VPS9D1-AS1 and its eQTL-linked PCG FANCA are functionally-interacting ($\beta = 0.6$, $p = 3.21\times 10^{-9}$), and the interaction is not dependent on Wnt signaling ($\beta = 0.95$, $p < 1.00\times 10^{-9}$). CPM, counts per million. (D) Functional interaction between Wnt-dependent lncRNA VPS9D1-AS1 and its eQTL-linked PCG CDK10 suggested by their co-expression ($\beta = 0.42$, $p = 2.99\times 10^{-6}$) across FANTOM5 samples, is dependent on Wnt signaling, as they are co-expressed in the opposite direction after Wnt inhibition ($\beta = -0.49$, $p = 1.42\times 10^{-3}$). Functional interaction ($\beta = 0.43$, $p = 1.10\times 10^{-11}$) between Wnt-dependent lncRNA DHRS4-AS1 and its eQTL-linked PCG SDR39U1 is also dependent on Wnt signaling, as they are not co-expressed after Wnt inhibition ($\beta = 0.12$, $p = 0.46$). (E) Wnt-dependent lncRNA MALAT1 and its eQTL-linked PCG LTBP3 are co-regulated in response to Wnt-signaling ($\beta = 0.72$, $p = 5.82\times 10^{-7}$), but not functionally-interacting ($\beta = 0.16$, $p = 0.99$). (F) 115 Wnt-regulated lncRNA-PCG pairs are linked by eQTL SNPs that are associated with cancer by GWAS. (G) Representative Wnt-regulated lncRNA LINC0035 associated with leukemia has functional interaction with CLDN3 ($\beta = 0.5$, $p = 2.10\times 10^{-9}$), and the functional interaction is dependent on Wnt signaling ($\beta = -0.054$, $p = 0.74$).

**Figure 3: Wnt-regulated lncRNA and protein-coding genes form gene networks that are dysregulated in different cancer types.** (A) Clusters enriched for genes upregulated in different cancer types. The top 5 clusters, cluster 1, 5, 7, 9 and 12 are enriched with the most number of cancers for genes upregulated. Normalized gene expression of these 5 clusters with number of PCGs and lncRNAs from each cluster are shown (left). (B) Clusters enriched for genes downregulated in different cancer types. The top 5 clusters, cluster 2, 3, 6, 11 and 24 are enriched with the most number of cancers for genes downregulated. Normalized gene expression of these 5 clusters with number of PCGs and lncRNAs from each cluster are shown (left). (C, D) GO Biological
Processes enrichments (FDR < 5%) of the top 5 clusters enriched for genes upregulated (C) or downregulated (D) in different cancer types. The top 3 significantly enriched GO terms for each cluster are shown. (E) Wnt-regulated lncRNAs are part of gene networks that are upregulated in different cancers. PCGs from cluster 9 are enriched for ncRNA metabolic processes, negative regulation of cell differentiation and positive regulation of Wnt signaling. Wnt-regulated lncRNAs from cluster 9 are shown in the inner circle. (F) Wnt-regulated lncRNAs are part of gene networks that are downregulated in different cancers. PCGs from cluster 2 are enriched for immune response, vesicle-mediated transport and vesicle organization. Wnt-regulated lncRNAs from cluster 2 are shown in the inner circle.

Figure 4: CRISPRi screens identify Wnt-regulated lncRNAs loci that modify cell growth in a context-dependent manner. (A) Schematic representation of CRISPRi screens conducted using xenograft tumors in vivo and in cultured cells in vitro to identify functional Wnt-regulated lncRNAs in RNF43-mutant pancreatic cancer. (B) Comparison of FDR from in vivo and in vitro screens. The dashed lines represent the threshold (FDR = 10%) for calling hits by gene-associated FDR. IncRNA hits are colored based on their FDR from both in vivo and in vitro screens. (C). Comparison of sgRNA fold change after in vivo and in vitro screens. Each gene is colored based on hits calling from B. (D) sgRNAs targeting LINC00263 are significantly depleted from both in vivo and in vitro screens. (E) sgRNAs targeting ABHD11-AS1 are significantly enriched only from the in vivo screen. (F) sgRNAs targeting AP000487.1 are significantly enriched only from the in vitro screen. The normalized counts of 5 sgRNAs targeting the TSS of LINC00263, ABHD11-AS1 and AP000487.1 are shown before and after both screens in D, E, F. (G) sgRNAs targeting the TSS of LINC00263 reduce the expression of LINC00263 and SCD. (H) sgRNAs targeting LINC00263 reduce HPAF-II cell growth in vitro. Cell
numbers were counted at days 6, 10, 14 and 16 after seeding and normalized to the seeding density. (I) sgRNAs targeting SCD reduce HPAF-II cell growth \textit{in vitro}. sgNTC does not affect cell growth. Cell numbers were counted at day 6, 10 and 14 after seeding and normalized to the seeding density. NTC, non-targeting control.

Additional Files

Additional file 1: Table 1. Wnt-regulated lncRNAs that affect HPAF-II cell growth \textit{in vivo} and \textit{in vitro}. (DOCX 26 kb)

Additional file 2: Figure S1. Wnt-regulated lncRNAs and PCGs are dysregulated in TCGA cancers (A) Wnt-regulated lncRNAs and PCGs, defined as genes changed over time upon Wnt inhibition (FDR < 5%) in the orthotopic RNF43-mutant pancreatic cancer model (Figure 1A). Wnt-regulated lncRNAs and PCGs are dysregulated in different types of cancers as determined by differential expression between tumors and their paired normal samples using the TCGA dataset. (B) \textit{VPS9D-AS1} is upregulated in 11 different types of cancers. (PDF 946 kb)

Additional file 3: Figure S2. Subset of Wnt-dependent lncRNAs co-express with its nearest PCG in the same TAD. (A) Wnt-regulated lncRNAs exhibit stronger co-expression with their nearest PCG after Wnt inhibition compared to their co-expression with all PCGs. (B) Wnt-regulated lncRNA–nearest PCG pairs within the same TAD exhibit stronger co-expression than the pairs in different TADs. P for significance was calculated by Mann–Whitney U test. (C) For the Wnt-regulated lncRNA–nearest PCG pairs encoded within the same TAD, the PCGs are significantly (FDR < 5%) enriched for GO biological processes. (1076 kb)
Additional file 4: Figure S3. Clusters are enriched for genes dysregulated in different cancers. (A) The Wnt-regulated lncRNAs and PCGs fall into 63 distinct clusters based on their pattern of expression change following Wnt inhibition. (B) 46 out of the 63 clusters are enriched (FDR < 5%) for genes dysregulated in at least one type of cancer. (PDF 5287 kb)

Additional file 5: Figure S4. A high correlation of sgRNA counts between independent experimental replicates in CRISPRi screens. (A) Correlation of sgRNA counts between experimental replicates in the in vitro screens. (B) Correlation of sgRNA counts between experimental replicates in the in vivo screens. (PDF 959 kb)

Additional file 6: Figure S5. CRISPRi screens are able to identify important positive controls as gene hits. (PDF 909 kb)

Additional file 7: Figure S6. Knockdown of SCD with CRISPRi reduce SCD mRNA abundance, but not the expression of LINCO0263. (PDF 876 kb)

Additional file 8: Table S1. 1,503 Wnt-regulated lncRNAs. (XLSX 139 kb)

Additional file 9: Table S2. Wnt-regulated lncRNA-PCG pairs linked by eQTL SNPs involving 602 Wnt-regulated lncRNAs. (XLSX 158 kb)

Additional file 10: Table S3. Wnt-regulated lncRNAs were linked by eQTL SNPs that colocalize with cancer GWAS loci. (XLSX 63 kb)

Additional file 11: Table S4. sgRNA libraries used in CRISPRi screens. (XLSX 767 kb)

Additional file 12: Table S5. CRISPRi screens results on protein-coding gens that have TSS within 1 kb of the TSS of Wnt-dependent lncRNAs. (XLSX 135 kb)
Declarations

Ethics approval and consent to participate

This work has been approved by the Duke-NUS Institutional Animal Care and Use Committee.

Consent for publication

Not applicable

Availability of data and materials

The RNA-seq data for HPAF-II orthotopic, subcutaneous and in vitro model is available at NCBI GSE118041, GSE118179, GSE118190. Detailed results for the Wnt-regulated IncRNAs can be found in supplementary tables.

Competing interests

BM and DMV have a financial interest in ETC-159.

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Authors' contributions

SYL, DMV, EP conceived the project. SYL performed the data analysis with assistance from NH. SYL performed the CRISPRi screens with assistance from YKW, BM and ZZ. SYL and TLG performed the inducible CRISPRi knockdown validation with assistance from ZZ. SYL, DMV, EP wrote the manuscript with inputs from NH and BM. All authors read and approved the final manuscript.

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Table 1 Wnt-regulated IncRNAs that affect HPAF-II cell growth in vivo and in vitro

| Group          | Ensembl Gene ID | Gene Symbol | Gene Type | log2FC (in vitro) | Fold change (in vitro) | log2FC (correlation) | Fold change (correlation) | Wnt-dependence | Nearest PCG | Correlation (nearest PCG) | p-value of correlation | IncRNA-PCG Distance (bp) | Up-regulated No. cancers | Down-regulated No. cancers |
|----------------|----------------|-------------|-----------|-------------------|------------------------|----------------------|--------------------------|-----------------|-------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Significant in vitro and in vivo | ENSG00000276131 | RP11-481J2.3 | antisense | -4.42             | 0.001                  | -1.16                | 0.001                    | Wnt-Activated | GINS3      | 0.67                     | 3.65E-06                 | 97726                    | 6                        | 0                        |
| | ENSG00000188525 | LINC00910     | IncRNA     | -1.99             | 0.027                  | -1.22                | 0.045                    | Wnt-Repressed | ARL4D      | 0.39                     | 4.50E-02                 | 9759                     | 1                        | 4                        |
| | ENSG00000235823 | CULMALINC     | IncRNA     | -1.85             | 0.029                  | -2.67                | 0.001                    | Wnt-Repressed | SC2D       | 0.54                     | 4.04E-04                 | 26480                    | 6                        | 1                        |
| | ENSG00000247844 | CCAT1         | IncRNA     | -1.20             | 0.030                  | -0.99                | 0.033                    | Wnt-Activated | MYC        | 0.80                     | 3.42E-08                 | 51624                    | 8                        | 3                        |

**log2FC:** the enrichment/depletion of sgRNAs targeting a lncRNA, calculated based on log2 transformed fold change of read counts of the best sgRNA targeting the lncRNA. Positive FC means sgRNA targeting increased cell growth in the screen; negative FC means sgRNA targeting decreased cell growth in the screen.

**Fold change:** false discovery rate, calculated based on the fold change of all sgRNAs targeting the lncRNA compared to the non-targeting controls; FDR = 10% is highlighted in bold (see Methods).

**Correlation (nearest PCG):** spearman correlation coefficient of Wnt-regulated IncRNA with its nearest PCG in response to Wnt inhibition in the orthotopic HPAF-II cancer model.

**IncRNA-PCG distance (bp):** the distance in base pair between the TSS of Wnt-regulated IncRNA and its nearest PCG; Distance less than 1kb is highlighted in italic, as the PCG may be suppressed by sgRNA targeting the lncRNA.

**Up-regulated No. cancers:** number of TCGA cancer types the lncRNA is upregulated, as determined by differential expression between tumors and their paired normal samples.

**Down-regulated No. cancers:** number of LNCVA cancer types the IncRNA is downregulated, as determined by differential expression between tumors and their paired normal samples.

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bFDR: false discovery rate, calculated based on the fold change of all sgRNAs targeting the lncRNA compared to the non-targeting controls; FDR <10% is highlighted in bold (see Methods).

cCorrelation (nearest PCG): spearman correlation coefficient of Wnt-regulated IncRNA with its nearest PCG in response to Wnt inhibition in the orthotopic HPAF-II cancer model.

dIncRNA-PCG distance (bp): the distance in base pair between the TSS of Wnt-regulated IncRNA and its nearest PCG; Distance less than 1kb is highlighted in italic, as the PCG may be suppressed by sgRNA targeting the lncRNA.

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Figure 1: Identification of Wnt-regulated lncRNAs from orthotopic \textit{RNF43}-mutant pancreatic cancer model.

A) Tumors harvested at different time points

B) Ensembl 79 IncRNAs (n = 14,330)
- FANTOM IncRNAs (n = 27,887)
- Wnt-dependent IncRNAs (n = 1,503)

C) Mouse reads removed with Xenome
- Reads aligned to hg38
- \textit{de novo} assembly
- Novel transcript discovery
- Merge with Ensembl 79 to build a full reference
- Size filter (>200 bp)
- Coding potential filter (CPAT, CPC, Slncky)
- Expression filter (TPM>1)
- 3633 IncRNA genes
- 1503 Wnt-dependent IncRNA genes (ANOVA, FDR < 0.05)

D) VPS9D1-AS1
- FDR = 2.46e-14

E) Subcutaneous tumor log\textsubscript{2}FC (56h vs. 0h)
- FDR < 10\% in both (3.9\%)
- FDR < 10\% only in subcutaneous tumor (11.0\%)
- FDR < 10\% only in cell line (5.4\%)
- FDR > 10\% in both (79.7\%)

F) Orthotopic tumor log\textsubscript{2}FC (56h vs. 0h)
- FDR < 10\% in both (6.8\%)
- FDR < 10\% only in orthotopic (26.0\%)
- FDR < 10\% only in cell line (2.6\%)
- FDR > 10\% in both (64.6\%)

G) Subcutaneous tumor log\textsubscript{2}FC (56h vs. 0h)
- FDR < 10\% only in subcutaneous tumor (5.8\%)
- FDR < 10\% only in orthotopic (23.6\%)
- FDR > 10\% in both (61.5\%)

Figure 1: Identification of Wnt-regulated lncRNAs from orthotopic \textit{RNF43}-mutant pancreatic cancer model.
Figure 2: Wnt signaling affects the cis functional interaction between lncRNAs and protein-coding genes.
Figure 3: Wnt-regulated IncRNA and protein-coding genes form gene networks that are dysregulated in different cancer types.
**Figure 4: CRISPRi screens identify Wnt-regulated lncRNAs loci that modify cell growth in a context-dependent manner.**

A. sgRNA oligo pools targeting Wnt-dependent lncRNAs

B. Pooled sgRNA plasmids

C. Lentiviral sgRNA library

D. HPAF-II cells stably expressing dCas9-KRAB

E. Lentiviral infection

F. Puromycin selection

G. Cells seeded in flask in vitro

H. In vitro End population

I. In vivo End population

- **T0 population**
- **In vivo screening**
- **In vitro screening**

- **Comparison of sgRNA abundance via deep sequencing**

**FDR**

- **N = 4**
- **FDR < 10% Both**
- **N = 9**
- **FDR < 10% in vitro**
- **N = 21**
- **FDR < 10% in vivo**
- **FDR > 10%**

**In vivo End population**

- **In vivo screening**
- **FDR**

**In vitro End population**

- **In vitro screening**
- **log2FC**

**In vitro screen**

**In vivo screen**

**Positive control**

| sgRNA | Normalized Counts | log2 FC |
|-------|-------------------|---------|
| 1     | 2                 | 3       | 4       | 5       |
|       |                  |         |         |         |
|       |                  | 0.66    | 8.5e−04 |
| LINC00263 | log FC = −2.67 (FDR = 6.31e−04) |

**In vivo screen only**

| sgRNA | Normalized Counts | log2 FC |
|-------|-------------------|---------|
| 1     | 2                 | 3       | 4       | 5       |
|       |                  |         |         |         |
|       |                  | 0.79    | 1.79e−02 |
| ABHD11-AS1 | log FC = 0.04 (FDR = 8.49e−01) |

**In vitro screen only**

| sgRNA | Normalized Counts | log2 FC |
|-------|-------------------|---------|
| 1     | 2                 | 3       | 4       | 5       |
|       |                  |         |         |         |
|       |                  | 0.79    | 1.79e−02 |
| AP000487.1 | log FC = −0.73 (FDR = 6.82e−01) |

**LINC00263 expression**

**SCD expression**

**Relative growth**

- **Control**
- **Dox**

**sgLINC00263_1**

**sgLINC00263_2**

**sgSCD_1**

**sgSCD_2**

**sgNTC**

- **Days 0 5 10 15 20**
- **Relative growth**
- **Control**
- **Dox**

**LINC00263 expression**

**log2 FC = −0.73 (FDR = 6.82e−01)**

**LINC00263**

**log2 FC = 0.66 (FDR = 8.5e−04)**

**AP000487.1**

**log2 FC = 0.04 (FDR = 8.49e−01)**

**lncRNA hit from both in vivo and in vitro screen**

**lncRNA hit from in vivo screen only**

**lncRNA hit from in vitro screen only**

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**Figure 4** CRISPRi screens identify Wnt-regulated lncRNAs loci that modify cell growth in a context-dependent manner.