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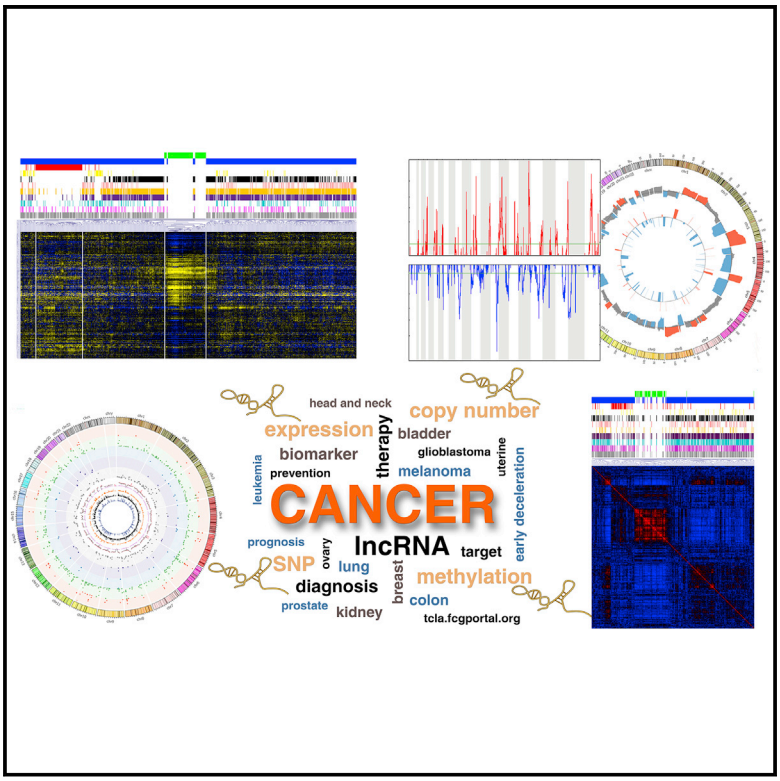
Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers

Graphical Abstract



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In Brief

Yan et al. analyze long non-coding RNA (lncRNA) alterations at transcriptional, genomic, and epigenetic levels across multiple cancer types from TCGA datasets and cancer cell lines. They also present a screening strategy and "co-expression" approach using the integrative data to identify cancer driver lncRNAs.

Highlights

- lncRNA dysregulation was characterized in 5,037 tumor samples across 13 cancer types
- lncRNAs are altered in cancers at transcriptional, genomic, and epigenetic levels
- The expression and dysregulation of lncRNAs are strikingly cancer-type specific
- This study provides a resource to systematically identify cancer driver lncRNAs

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Comprehensive Genomic Characterization of Long Non-coding RNAs across Human Cancers

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SUMMARY

The discovery of long non-coding RNA (lncRNA) has dramatically altered our understanding of cancer. Here, we describe a comprehensive analysis of lncRNA alterations at transcriptional, genomic, and epigenetic levels in 5,037 human tumor specimens across 13 cancer types from The Cancer Genome Atlas. Our results suggest that the expression and dysregulation of lncRNAs are highly cancer type specific compared with protein-coding genes. Using the integrative data generated by this analysis, we present a clinically guided small interfering RNA screening strategy and a co-expression analysis approach to identify cancer driver lncRNAs and predict their functions. This provides a resource for investigating lncRNAs in cancer and lays the groundwork for the development of new diagnostics and treatments.

Significance

The discovery of long non-coding RNA (lncRNA) has dramatically changed our understanding of the biology of diseases. Recent studies have identified lncRNAs with tumor-suppressive and oncogenic activities. We conducted comprehensive analyses of lncRNA profiles at transcriptional, genomic, and epigenetic levels in 5,037 tumor specimens across 13 cancer types from The Cancer Genome Atlas and in 935 cancer cell lines from the Cancer Cell Line Encyclopedia. Our large-scale analyses revealed that lncRNA alterations are highly tumor and lineage specific and are often associated with somatic copy number alterations, promoter hypermethylation, and/or cancer-associated SNPs. Here we provide a rich resource to the research community for further investigating lncRNAs functions and identifying lncRNAs with diagnostic and therapeutic potentials.

INTRODUCTION

Cancer is a genetic disease involving multi-step changes in the genome. The human genome contains ~20,000 protein-coding genes (PCGs), representing less than 2% of the total genome (Ezkurdia et al., 2014), whereas up to 70% of the human genome is transcribed into RNA, yielding many thousands of non-coding RNAs (Derrien et al., 2012; Mattick and Rinn, 2015). Long non-coding RNAs (lncRNAs) are operationally defined as transcripts that are larger than 200 nt that do not appear to have protein-coding potential (Kapranov et al., 2007; Mattick and Rinn, 2015). Similar to protein-coding transcripts, transcriptional control of lncRNAs is subject to typical histone modification-mediated regulation, and lncRNA transcripts are processed by the canonical spliceosome machinery (Cabili et al., 2011; Derrien et al., 2012; Guttman et al., 2009; Ravasi et al., 2006). Compared with their protein-coding counterparts, lncRNA genes are composed of fewer exons, are under weaker selective constraints during evolution, and are present in relatively lower abundance. Notably, the expression of lncRNAs is strikingly cell type and tissue specific (Cabili et al., 2011; Mercer et al., 2008; Ravasi et al., 2006) and, in many cases, even primate specific (Derrien et al., 2012). LncRNAs can serve as scaffolds or guides to regulate protein-protein or protein-DNA interactions, as decoys to bind proteins or microRNAs (miRNAs), and as enhancers to influence gene transcription, when transcribed from enhancer regions or their neighboring loci (Batista and Chang, 2013; Guttman and Rinn, 2012; Karreth and Pandolfi, 2013; Lee, 2012; Mattick and Rinn, 2015; Mercer et al., 2009; Morris and Mattick, 2014; Ørom and Shiekhattar, 2013; Prensner and Chinnaiyan, 2011; Ulitsky and Bartel, 2013). Importantly, rapidly accumulating evidence indicates that lncRNAs are associated with chromatin-modifying complexes and guide epigenetic regulations in both physiological and pathological conditions (Mercer and Mattick, 2013).

Recent studies suggested that lncRNA is involved in the initiation and progression of cancer. In addition to the fact that they are highly deregulated in tumors (Akrami et al., 2013; Calin et al., 2007; Du et al., 2013; Iyer et al., 2015; Kim et al., 2014; Li et al., 2015; Ling et al., 2013; Prensner et al., 2011; Trimarchi et al., 2014; Xing et al., 2014), lncRNAs have been found to act as tumor suppressors or oncogenes. Therefore, a comprehensive genomic characterization of lncRNA alterations across major cancers not only is urgently needed but may lead to new diagnostic and therapeutic strategies for cancer. The Cancer Genome Atlas (TCGA) project is a coordinated effort to accelerate our understanding of the molecular basis of cancer through the application of genomic analysis technologies. Here, we performed a multiplatform integrative analysis of lncRNA alterations in 5,037 of cancers from 13 tumor types in TCGA project.

RESULTS

The Expression of lncRNAs Is Dysregulated in Cancer

We analyzed RNA sequencing (RNA-seq) profiles from 5,037 tumors across 13 cancer types as well as 424 normal specimens from nine matching tissue types in TCGA (Table S1). An evidence-based lncRNA transcript annotation that contains 13,562 manually annotated lncRNA genes from the GENCODE consortium (V18) was used to define lncRNAs. To evaluate the analysis reliability of the workflow for RNA-seq data in the present study, we compared 520 breast specimens whose RNA expression had been analyzed by both RNA-seq and microarray in TCGA. The transcriptomic correlations of RNA expression determined by RNA-seq (reads per kilobase per million mapped reads [RPKM]) and by microarray were calculated in a total of 13,318 PCGs and lncRNAs. In more than 96.7% of genes analyzed, significant and positive correlations were observed between the RPKM- and microarray-derived RNA expression levels (Figures S1A and S1B). To ensure detection reliability and reduce background noise, we applied two filters in each cancer type: the first eliminates any gene whose 50th-percentile RPKM value is equal to 0, and the second selects only genes whose 90th-percentile RPKM value is greater than 0.1. On average, 4,409 lncRNAs (32.51% of those annotated by GENCODE) were detected in each cancer type. Of these, 2,316 lncRNAs (17.08%) were commonly detected in all 13 cancer types, and 8,179 lncRNAs (60.31%) were detected in at least one cancer type (Table S2 and Figure S1C). The lncRNAs detected in each cancer type are listed in Table S2.

To characterize tumor-associated dysregulation of lncRNA expression, we analyzed lncRNA expression in seven cancer types for which the number of corresponding normal tissue samples analyzed by RNA-seq was greater than 20 (Figure 1A). Compared with their normal counterparts, the seven cancer types had on average 15.00% and 11.18% of lncRNAs significantly up- and downregulated, respectively (Figure 1B). The lncRNAs whose RNA expression was significantly altered in each cancer type are listed in Table S2. Using the same pipeline, we also calculated the percentages of dysregulated PCGs and found that lncRNAs and PCGs have similar percentages of tumor-associated dysregulation of expression (Figure 1B). By comparing the dysregulated lncRNAs in different cancer types, we found that ~60% of these altered lncRNAs were cancer-type specific, and the rest were shared by at least two cancer types (Figures 1C, 1D, and S1D). We identified only five lncRNAs whose RNA expression was significantly altered in all seven cancer types (Figure 1E). The expression of many previously identified tumor-associated lncRNAs was found to be significantly dysregulated in multiple cancer types. For example, the oncogenic lncRNAs *PCAT7*, *PVT1*, and *HOTAIR* were significantly upregulated in six, five, and four cancer types, respectively. The lncRNAs whose dysregulated expression was shared or unique among different cancer types are listed in Table S2. Importantly, the percentage of cancer type-unique dysregulated lncRNAs was remarkably higher than that of PCGs (Figures 1C–1F), although lncRNAs and PCGs have similar percentages of global dysregulation. Together, this demonstrates that the dysregulation of expression of lncRNA is common in cancer. Although most lncRNAs showing dysregulated expression are cancer type unique, a small number of alterations are shared among different cancer types.

Somatic Copy Numbers of lncRNA Genes Are Altered in Cancer with Different Frequencies

We analyzed the somatic copy number alterations (SCNAs) of lncRNAs in cancer via SNP microarray analysis of 5,860 tumors in 13 cancer types from TCGA. For each cancer type, the SCNA

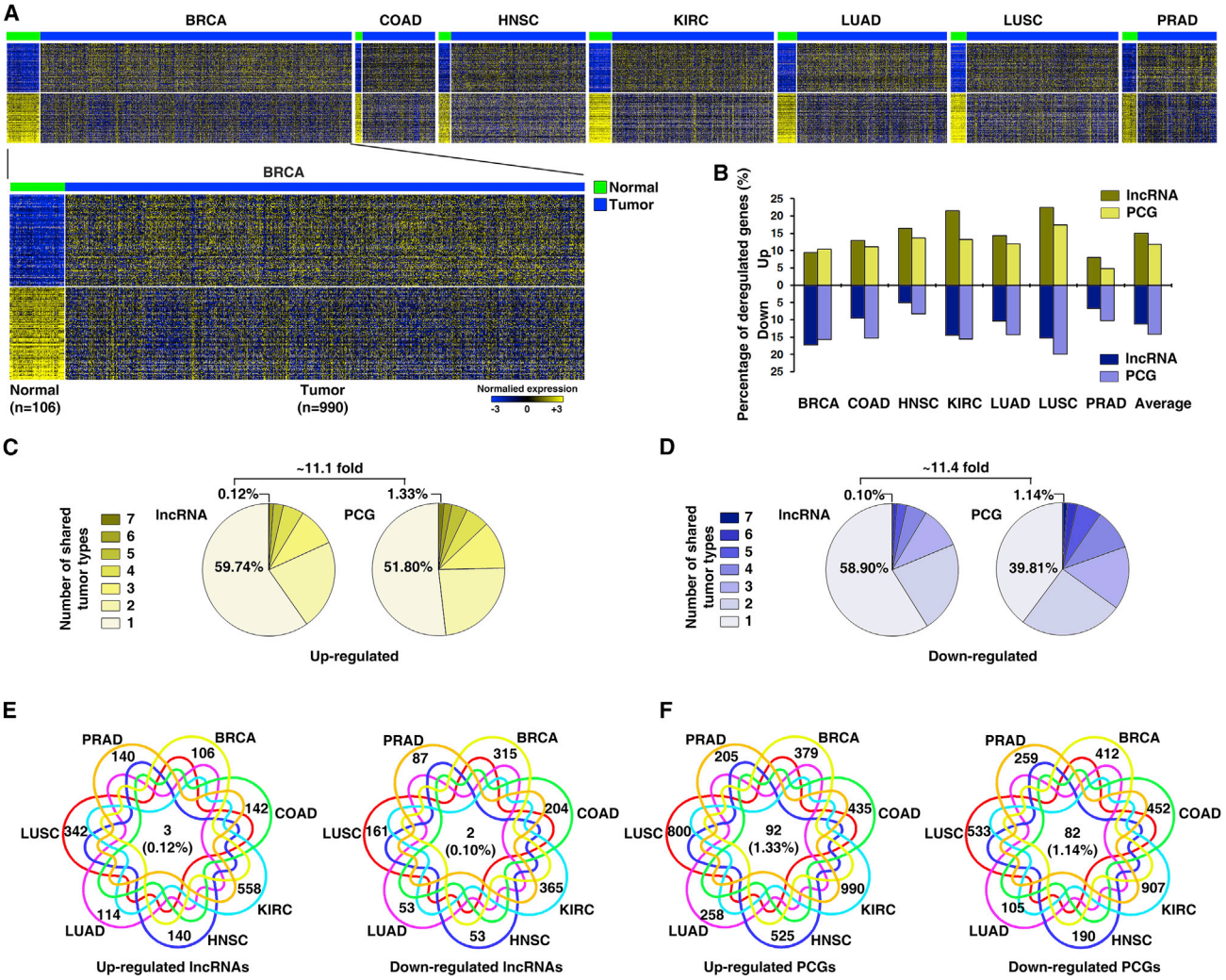

Figure 1. The Expression of lncRNAs Is Dysregulated in Cancer

(A) Heatmap of lncRNAs whose expression is significantly dysregulated. The top 100 most significantly dysregulated lncRNAs from each individual tumor type are presented.

(B) The percentages of the dysregulated lncRNAs and PCGs.

(C and D) The percentages of the upregulated (C) and downregulated (D) lncRNAs (left) and PCGs (right) that were shared among the seven cancer types.

(E and F) Venn diagrams of the upregulated (left) and downregulated (right) lncRNAs (E) and PCGs (F) shared among the seven cancer types.

See also Figure S1 and Tables S1 and S2.

frequencies of the lncRNA-containing loci were calculated (Figures 2A and 2B). When "high-frequency alteration" was defined as an alteration that occurs in more than 25% of the specimens in a given cancer type, few lncRNA gene loci had concurrent high-frequency gain and loss in the same type of cancer (Figure S2A). Across all 13 cancer types, there were on average 13.16% and 13.53% of lncRNA genes with high-frequency gain and loss, respectively (Figures 2A–2C; Table S3). Although ovarian serous cystadenocarcinoma (OV) and lung squamous cell carcinoma (LUSC) had the most lncRNAs with high-frequency SCNAs, very few lncRNAs in prostate adenocarcinoma (PRAD) and acute myeloid leukemia (LAML) had high-frequency alterations (Figures 2A and 2C).

To characterize the focal SCNAs that harbor lncRNA genes, we retrieved the location information of focal genomic alteration peaks from the Firehose project and mapped the lncRNA-containing loci to these focal alteration regions in each cancer type (Figure S2B and Table S3). In LUSC, for example, totals of 435 and 1,811 lncRNA genes were mapped to regions with focal gains and losses, respectively (Figure 2D). The lncRNA genes located in the focal alteration regions in other cancer types are shown in Figure S2B. Many previously identified tumor-associated lncRNAs were found to be associated with focal SCNAs in multiple cancer types. For example, the oncogenic lncRNAs *FAL1(FALEC)* and *PVT1* were focally amplified in seven and six cancer types, respectively.

To estimate the contribution of SCNAs to lncRNA dysregulation in cancer, we analyzed the correlation between lncRNA copy number and RNA expression level for all detectable

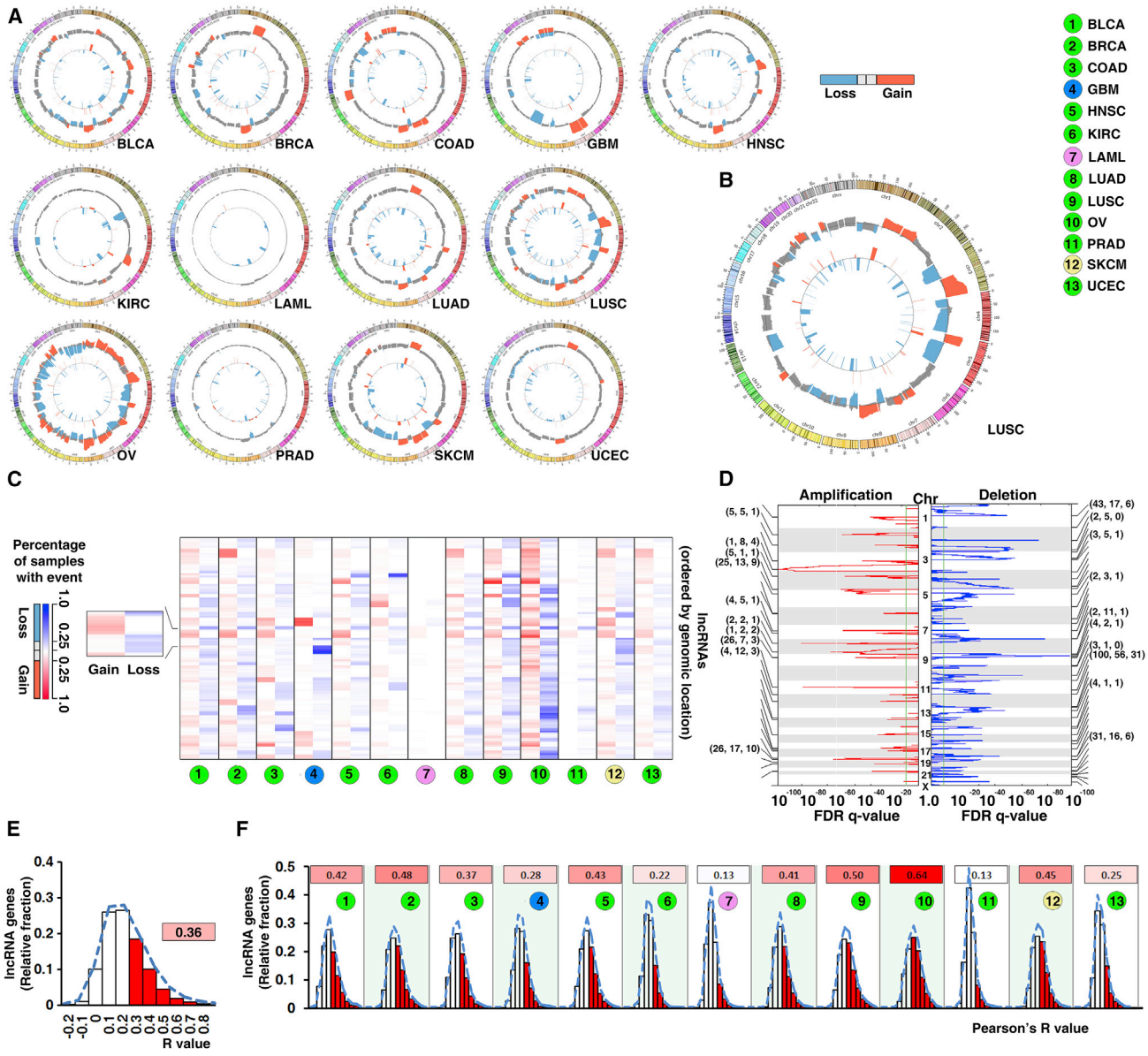

Figure 2. Somatic Copy Numbers of lncRNA Genes Are Altered in Cancer with Different Frequencies

(A) A genome-wide view of SCNAs in cancers. The outer track shows the frequencies of SCNAs from the lncRNA-containing loci, and the inner track shows the focal alteration regions.

(B) An enlarged view of SCNAs in LUSC.

(C) Heatmap of somatic copy number gain and loss for lncRNA genes. The rows, each of which represents an lncRNA gene locus, are arranged according to the genomic locations of the lncRNA genes. Left: frequency of gain (red); right: frequency of loss (blue).

(D) The lncRNA and PCGs in the top 20 focal gain (left) or loss (right) peaks in LUSC. The numbers of PCGs (left), annotated lncRNAs (middle), and detectable lncRNAs (right) in each peak are indicated in parentheses.

(E and F) Histogram of percentage of lncRNAs whose RNA-SCNA correlation coefficients are in specific ranges across 13 cancer types (E) and in each cancer type (F). The number and red color intensity in the insets indicate the percentage of the detectable lncRNAs whose Pearson's R values were $\geq$0.2 in a given cancer type.

See also Figure S2 and Tables S3.

lncRNAs in each cancer type. In summary, for 36.27% of the lncRNAs, there were positive correlations ($R \geq 0.2$) between their RNA expression levels and their gene copy numbers (Figure 2E). Importantly, cancer types that had higher levels of SCNAs (such as OV and LUSC) demonstrated stronger RNA-SCNA correlations than the cancer types with fewer SCNAs (such as LAML and PRAD) (Figure 2F). This suggests that SCNAs are an important mechanism that leads to the dysregulation of lncRNAs in cancer, especially for those cancer types whose genomes contain abundant SCNAs.

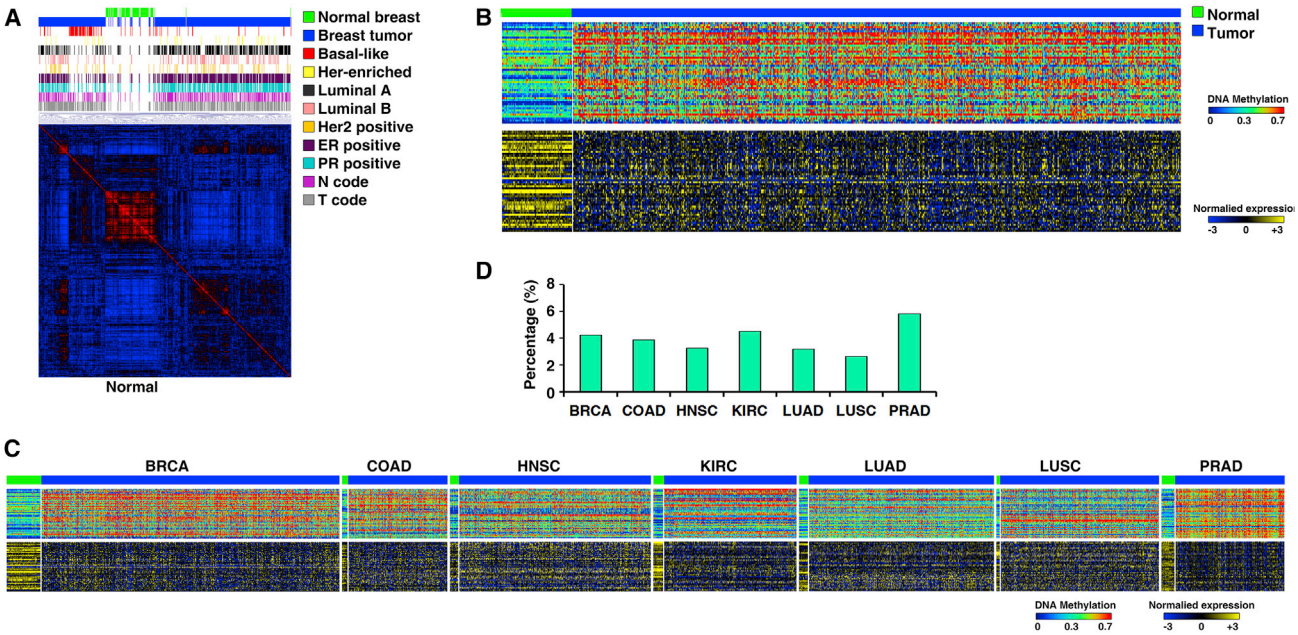

Figure 3. DNA Methylation Patterns in the Promoter Regions of lncRNA Genes Are Altered in Cancer

(A) NMF clustering of DNA methylation probes that are located in lncRNA promoters and whose methylation β values had the largest variations across all breast specimens.

(B) Heatmaps of the methylation status (β value, top) in the promoter regions and the RNA expression level (bottom) of the corresponding lncRNAs in breast specimens.

(C) Heatmaps of the methylation status of the lncRNA promoter regions and the RNA expression levels.

(D) A summary of the percentage of the CAESLG.

See also Figure S3 and Tables S4 and S5.

DNA Methylation Patterns in the Promoter Regions of lncRNA Genes Are Altered in Cancer

We analyzed DNA methylation alterations in the promoter regions of lncRNAs in cancers. DNA methylation microarray profiles on 2,791 tumor and 467 normal specimens across seven cancer types were obtained from TCGA. A total of 35,696 probes corresponding to the promoter regions of the 2,435 lncRNA genes whose expression was analyzed by RNA-seq were identified (Table S4). On average, the promoter region of each lncRNA gene was covered by 15 probes. We first used consensus nonnegative matrix factorization (NMF) clustering analysis to cluster samples according to their methylation profiles in each cancer type. This revealed that for all seven cancer types studied, the DNA methylation profiles of lncRNA genes from normal samples were very similar within the cancer type, while the DNA methylation patterns of lncRNA genes from tumor samples were quite diverse (Figures 3A and S3). It suggests that the promoter regions of lncRNAs are subjected to DNA methylation-mediated epigenetic alterations during tumorigenesis. Next, we applied four separate filtering criteria to screen for *c*ancer-*a*ssociated *e*pigenetically *s*ilenced *l*ncRNA *g*enes (CAESLG) (Figures 3B and 3C). On average, 3.92% of lncRNA genes had both hypermethylated promoters and reduced RNA expression in tumors compared with their normal counterparts (Figure 3D). The CAESLG candidates of each cancer type are listed in Table S4. These findings suggest that epigenetic silencing of lncRNA genes may be a mechanism that contributes to the dysregulation of expression of lncRNAs in cancer. Because the probes for many lncRNA genes were not available in the DNA methylation microarray platform, some lncRNAs that are epigenetically regulated may not be identified in our analysis.

Many Cancer-Associated SNPs Are Located in lncRNA Loci

Using 5 kb as the cutoff distance between an annotated transcript and a cancer-associated SNP, we re-mapped all cancer-associated SNPs reported by the National Human Genome Research Institute Catalog of Published Genome-Wide Association Studies (GWAS) (Table S5) to genes annotated by the Encyclopedia of DNA Elements. We found that 11.75% of the index SNPs were near loci harboring lncRNA genes (Table S5). The percentages of index SNPs close to PCGs, pseudogenes, and other genes were 54.75%, 3.75%, and 3.38%, respectively (Figure 4A). We further reasoned that only genes expressed in tumor tissues have the potential to be functionally involved in cancer development. By analyzing RNA-seq profiles from TCGA in the nine cancer types for which both GWAS SNP and TCGA RNA-seq information were available and combining the expression analysis with the above findings regarding SNP-associated lncRNA, we identified lncRNAs that are both close to index SNPs and that express detectable transcripts in tumors (Table S5). In PRAD, for example, 24 lncRNAs were found to reside near 28 index SNPs. Among these 24 lncRNAs, 6 were detected in prostate tumors (Figure 4B).

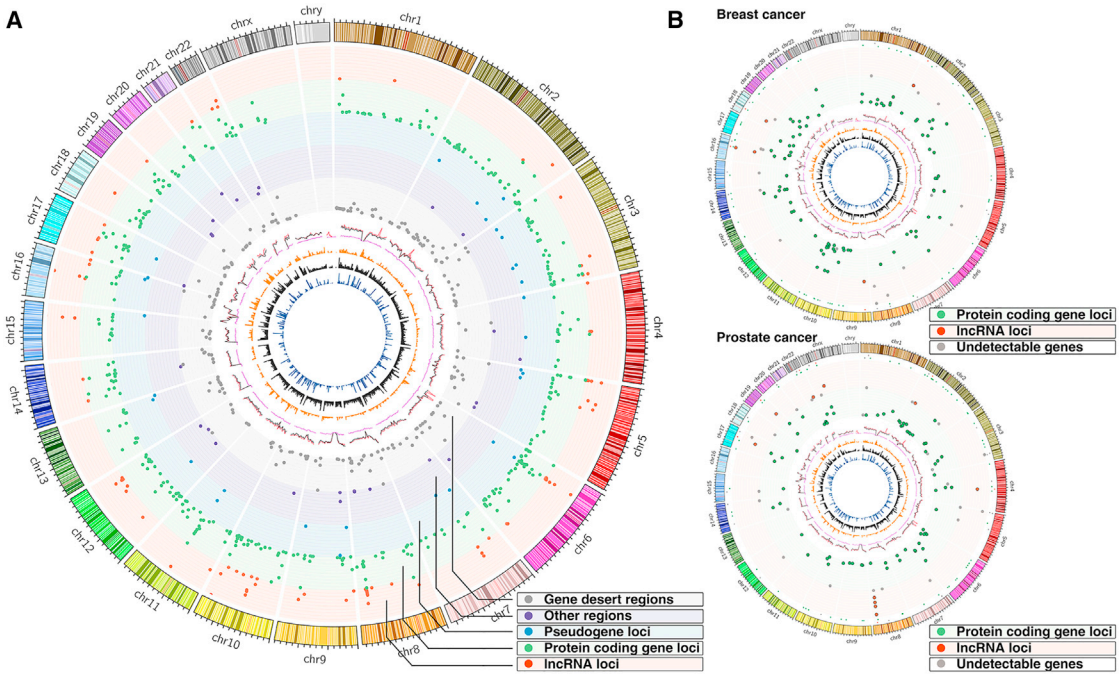

Figure 4. Many Cancer-Associated SNPs Are Located in lncRNA Loci

(A) A genome-wide view of the most significant cancer-associated index SNPs. The peaks in each track are proportional to the p values between the chromosomal locations of the index-SNPs.

(B) Genome-wide view of the breast (top) and prostate (bottom) index SNPs in lncRNA (red) and PCG loci (green).

The Expression of lncRNAs Is a Specific Biomarker in Cancer

To evaluate the potential value of lncRNAs as biomarkers in cancer, we first asked whether the expression signature of lncRNAs can differentiate between tumors and their corresponding normal tissues. In all nine tumor types in which both tumor and normal tissues were available, we were able to use unsupervised cluster analysis to differentiate normal tissues from tumors. Although the expression of lncRNAs in tumor demonstrated diverse patterns, the expression in normal tissue was relatively homogeneous and could be clearly separated from the expression patterns in tumor tissues (Figures 5A, 5B, and S4A). To further examine the value of lncRNAs as biomarkers, we chose to study breast cancer, because it is a heterogeneous cancer type with well-characterized pathological and molecular subtypes. We selected 817 breast tumors for which the molecular subtype had been defined by the University of California, Santa Cruz, Cancer Genome Browser. A cluster analysis showed that the unsupervised lncRNA expression subtypes demonstrated a high correlation with the defined PAM50 subtypes and also had a high correlation with clinical subtypes (Figure 5C). In particular, almost all of the basal-like/triple-negative breast tumors were clustered together and clearly separated from other tumor and normal tissue samples. Importantly, it has been reported that lncRNA expression is strikingly tissue and cell type specific compared with PCGs in normal tissues (Cabili et al., 2011; Mercer et al., 2008; Ravasi et al., 2006). We decided to compare the tissue specificity among lncRNAs, PCGs, and pseudogenes in cancer. We used an entropy-based metric that relies on Jensen-Shannon (JS) divergence to calculate specificity scores (Cabili et al., 2011) for each gene in breast specimens and found that the expression of lncRNA demonstrated the highest subtype specificity, followed by pseudogenes, while PCGs demonstrated the least subtype specificity (Figure 5D). About 18.27% of lncRNAs showed subtype specificity, whereas only 10.55% of PCGs were subtype specific (Figure 5E). To rule out the possibility that the higher specificity of lncRNAs is a result of their lower abundance, we calculated the specificity scores of highly expressed transcripts from these three different types of genes. Again, lncRNA showed a higher tissue specificity than PCG and pseudogenes (Figure 5D).

We also sought to determine if the expression signatures of lncRNAs are also cancer type specific using RNA-seq profiles from the Cancer Cell Line Encyclopedia (CCLE) in 935 human tumor cell lines (Table S6). As shown in Figure 5F, tumors of epithelia, melanoma, hematological, and neurological origins formed distinctive clusters on the basis of lncRNA expression. Sarcoma tumors displayed a diffuse lncRNA expression pattern, which may be explained by the fact that this type of tumor arises from various tissues. Using the JS divergence calculation, we compared the tissue specificity of lncRNAs, PCGs, and pseudogenes. Similar to our findings regarding subtype specificity in TCGA, the JS divergence measurements across cell lines of different origins revealed that lncRNA are more tissue specific than PCGs and pseudogenes (Figure 5G). Finally, we compared cancer type specificity across cell lines from 22 cancer types, and consistent results were observed (Figure S4B). These studies suggest that lncRNAs have the potential to serve as

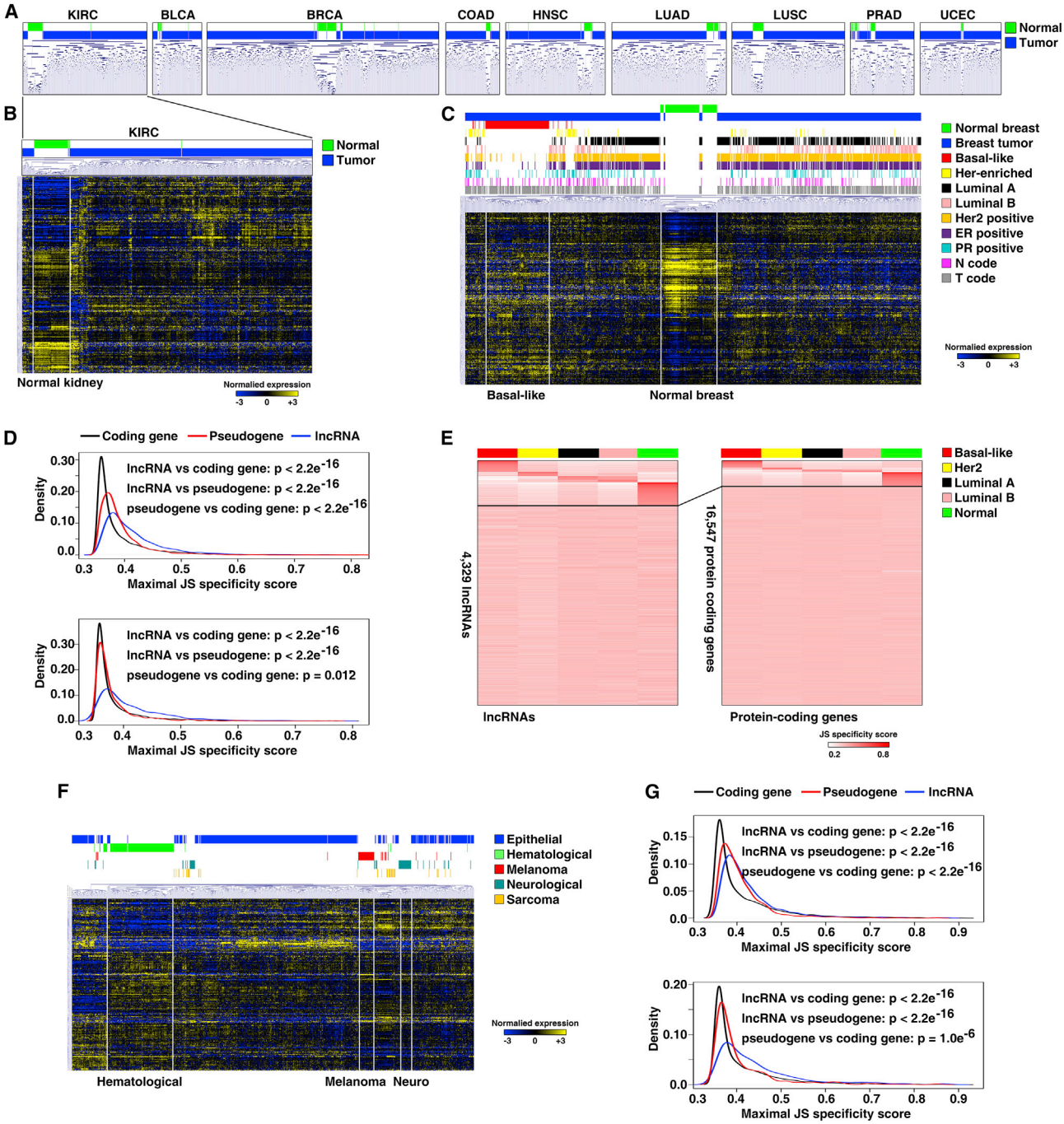

Figure 5. The Expression of lncRNAs Is a Specific Biomarker in Cancer

(A) Unsupervised hierarchical cluster analyses on the expression of the top 10% lncRNAs whose expression levels varied the most across all samples within each cancer type.

(B) Heatmap generated by unsupervised cluster analysis of lncRNAs with the largest expression variation in kidney cancer.

(C) Heatmap of unsupervised hierarchical cluster analysis using lncRNA signatures from breast cancer.

(D) Distribution of maximal subtype specificity scores calculated for each gene across the breast cancer specimens for all expressing transcripts (top) or high expressers (bottom) for lncRNA (blue), pseudogenes (red), and PCGs (black).

(E) Heatmap of lncRNA (left) and PCG (right) expression (JC scores) sorted on the basis of tissue-specific expression. Top: tissue specific; bottom: ubiquitously expressed.

(F) Heatmap of unsupervised hierarchical cluster analysis using lncRNA signatures from the CCLE RNA-seq dataset.

(G) Distributions of maximal cancer type specificity scores calculated for each gene across the CCLE major cancer types and across all expressing genes (top) or high expressers (bottom) for lncRNAs (blue), pseudogenes (red), and PCGs (black).

See also Figure S4 and Tables S6.

specific biomarkers with potential applications in cancer prediction, early detection, and diagnosis. Notable, unknown primary origin tumors account for 3%–5% of all new cancer cases and are aggressive diseases with poor prognosis. Our data indicate that lncRNAs may serve as informative biomarkers to determine the origin of these tumors.

lncRNome Profiles Provide a Resource to Functionally Identify Cancer Driver lncRNAs

We hypothesized that, using the TCGA lncRNome information as a clinical filter, we would be able to generate a concentrated and clinically relevant lncRNA list that could be used for a candidate-oriented functional screening. To test the concept, we chose breast cancer as an example and evaluated a four-step procedure to identify for potential driver lncRNAs (Figure 6A). In summary, we identified 19 lncRNAs that have cancer-associated genomic alterations and are also correlated with patient survival (Table S7). In a proof-of-concept screening, we found that all four small interfering RNAs (siRNAs) specifically targeted *ENSG00000253738* (*b*reast *c*ancer *a*ssociated *l*ncRNA*8* [*BCAL8*]) significantly reduced the proliferation of MDA-MB-231 cells (Figure 6B). *BCAL8* is the neighbor transcript of *OTUD6B* (Xu et al., 2011), and they share overlapping promoter regions. Further analysis of SNP arrays revealed that the *BCAL8* gene was gained in 49.7% of breast cancer (Figure 6C). Importantly, both higher expression of *BCAL8* RNA and genomic gain of the *BCAL8* gene were significantly associated with decreased survival in breast cancer (Figure 6D). There was also a strong positive correlation between *BCAL8* RNA expression and its genomic copy number in the breast tumors (Figure 6E). With the vast amount of data available in TCGA lncRNome, we had the resources to expand our characterization of *BCAL8* from breast cancer to other cancer types. Interestingly, we found that higher expression of *BCAL8* RNA was also significantly correlated with poor clinical outcome in OV, uterine corpus endometrial carcinoma (UCEC), and LAML (Figure S5A). Although *BCAL8* was significantly gained in OV and UCEC, this was not the case for LAML (Figure S5B). To further validate the function of *BCAL8*, we suppressed *BCAL8* expression by small hairpin RNA (shRNA) in breast and ovarian cancer cell lines. We consistently found that the expression of *BCAL8*-shRNAs significantly reduced growth rates in all cell lines tested (Figure 6F). Moreover, downregulating *BCAL8* expression also significantly reduced anchorage-independent growth in cells (Figures 6G and 6H). Finally, we injected cells expressing control and *BCAL8*-specific hairpins into nude mice and found that the expression of the *BCAL8*-shRNAs significantly suppressed tumor growth in vivo (Figure 6I). Together, this describes a strategy to integrate multidimensional molecular profiles with clinical annotations to generate clinical parameter-specific candidates for genetic screening.

lncRNome Profiles Provide a Resource to Infer lncRNA Functions

Predicting the biological functions of lncRNAs is challenging. Guilt-by-association (GBA) analysis has been proposed that the function of a poorly characterized lncRNA gene can be inferred on the basis of known functions of PCGs with which it is co-expressed (Huarte et al., 2010). Because TCGA provides multi-omic profiles in large scale, it may serve as an excellent resource for GBA-based lncRNA function prediction. To test this concept, we conducted GBA analysis for *BCAL8*. The RNA-seq profiles were analyzed to identify PCGs whose expression was significantly correlated with *BCAL8* expression in three cancer types (Figure 7A). We found that 38.2% (958 of 2,500) of *BCAL8*-associated PCGs were shared by all three cancer types (Figure 7B). Next, we performed gene ontology analysis on the *BCAL8*-associated PCGs that were common across the three cancer types and found that the most over-represented pathway in *BCAL8*-associated genes was the cell cycle pathway (Figures 7C and 7D). We also performed a GBA analysis for *BCAL8* using a protein expression profile (reverse phase protein array [RPPA]) of breast cancer from TCGA and identified 37 proteins (antibodies) whose expression levels were significantly and positively correlated with *BCAL8* expression (Figure 7E and Table S8). Consistent with the above RNA-based GBA analyses, many *BCAL8*-associated proteins were key regulators in cell-cycle pathways. For example, we found that *BCAL8* expression was significantly and positively correlated with cyclin E2 at both the mRNA and protein levels. We knocked down *BCAL8* expression in cancer cell lines and analyzed cell-cycle profiles. Consistent with our GBA prediction, knocking down *BCAL8* dramatically inhibited the G1-S transition of the cell cycle (Figure 7F). Finally, supporting our GBA analysis, suppressing *BCAL8* expression significantly reduced both CCNE2 mRNA and cyclin E2 protein levels (Figures 7G and 7H). In summary, using *BCAL8* as an example, we described an integrated bioinformatic approach to elucidate the function of given lncRNAs using information from the lncRNome dataset of TCGA (Figure S6).

DISCUSSION

Before the discovery of non-coding RNAs, the search for cancer drivers was focused on PCGs that resided in recurrent alterations in cancer genomes. However, many of these recurrent alterations were found to either be located in "gene desert" regions, or they contained no cancer-linked PCGs. The lack of PCGs in cancer-associated genetic alterations is further supported by the fact that only 2% of the human genome encodes proteins. These findings, in combination with the recent revelation that about 70% of the human genome is transcribed into RNA, strongly suggest that non-coding RNAs play significant roles in tumor development. Our study represents the one of largest analyses so far of lncRNA dysregulation at transcriptional, genomic, and epigenetic levels across cancers, substantially expanding our knowledge of non-coding RNAs in the cancer genome (the data generated from this study are available at http://tcla.fcgportal.org). Given that the majority of the human genome is transcribed to RNA, while only a small portion of these transcripts encode proteins, the number of lncRNA genes may be very large. An important challenge is that the genome-wide annotation and functional characterization of lncRNAs is still in its infancy. Further efforts will be needed to de novo annotate and characterize cancer unique lncRNA transcripts (Iyer et al., 2015; Trimarchi et al., 2014).

The expression of lncRNAs is strikingly cell type specific in normal tissues (Cabili et al., 2011; Mercer et al., 2008; Ravasi et al., 2006). Our results indicate that the expression of lncRNA

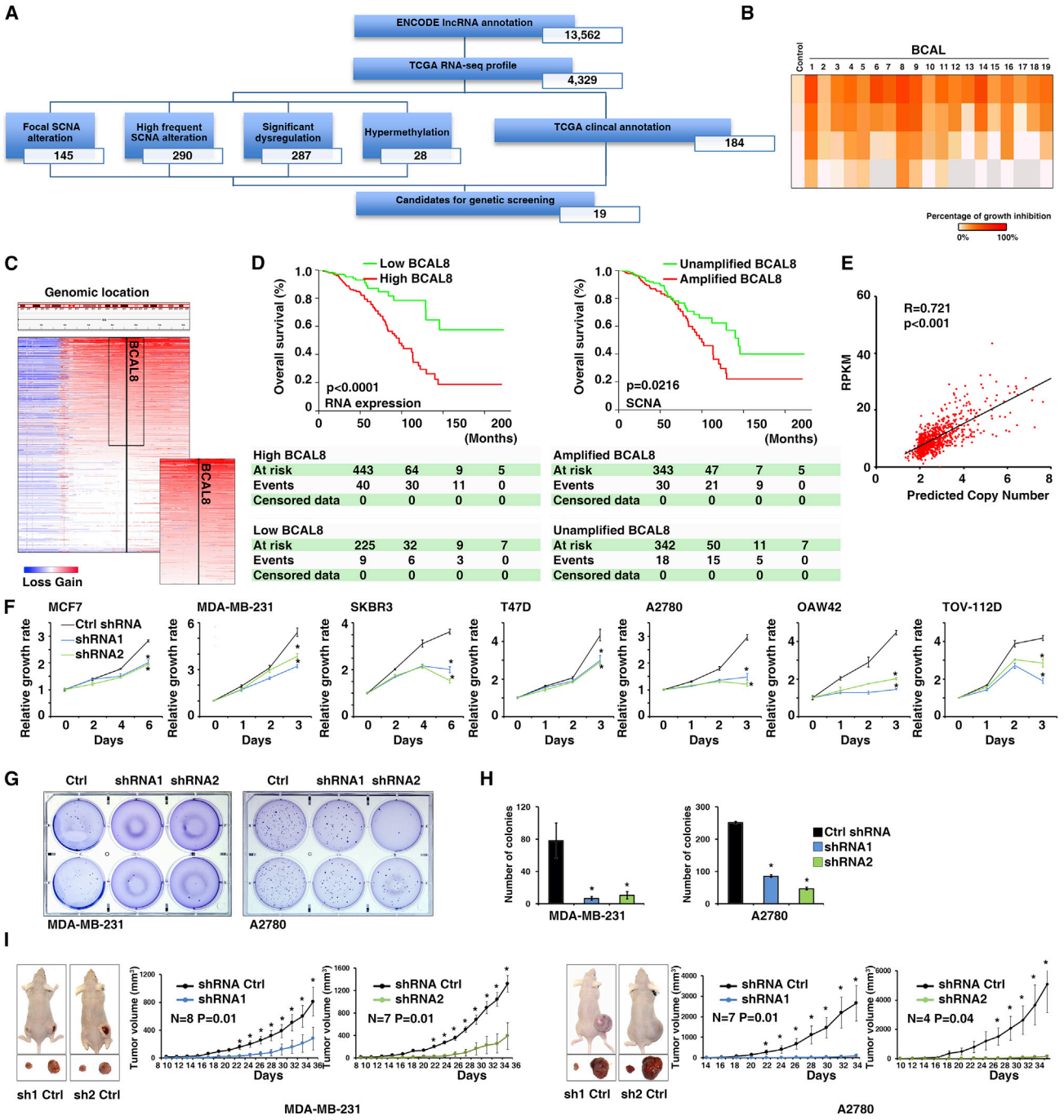

Figure 6. An Effective Strategy to Integrate Multidisciplinary Information from TCGA to Identify Cancer Driver lncRNAs

(A) Flowchart describing the process of candidate gene selection in breast cancer.

(B) The summary of the proof-of-concept siRNA screening in MDA-MB-231 cells.

(C) Copy number profiles of *BCAL8* locus from breast tumor specimens.

(D) Survival curves of breast cancer patients with high and low *BCAL8* RNA expression (left) and differing genomic SCNA status (right). The numbers of patients who were alive (at risk), deceased (event), and censored during the course of surveillance are indicated in the table under the corresponding time points.

(E) The correlation between *BCAL8* gene copy number and RNA expression in breast cancer.

(F) The growth curves of cells expressing control or *BCAL8* shRNAs.

(G) Soft-agar assays (in six-well plates) on cells expressing control or *BCAL8* shRNAs.

(H) Quantification of the number of colonies from the softer agar assays.

(I) Xenograft tumor growth of cells expressing control or *BCAL8* shRNAs. The error bars indicate SD. *p < 0.05.

See also Figure S5 and Table S7.

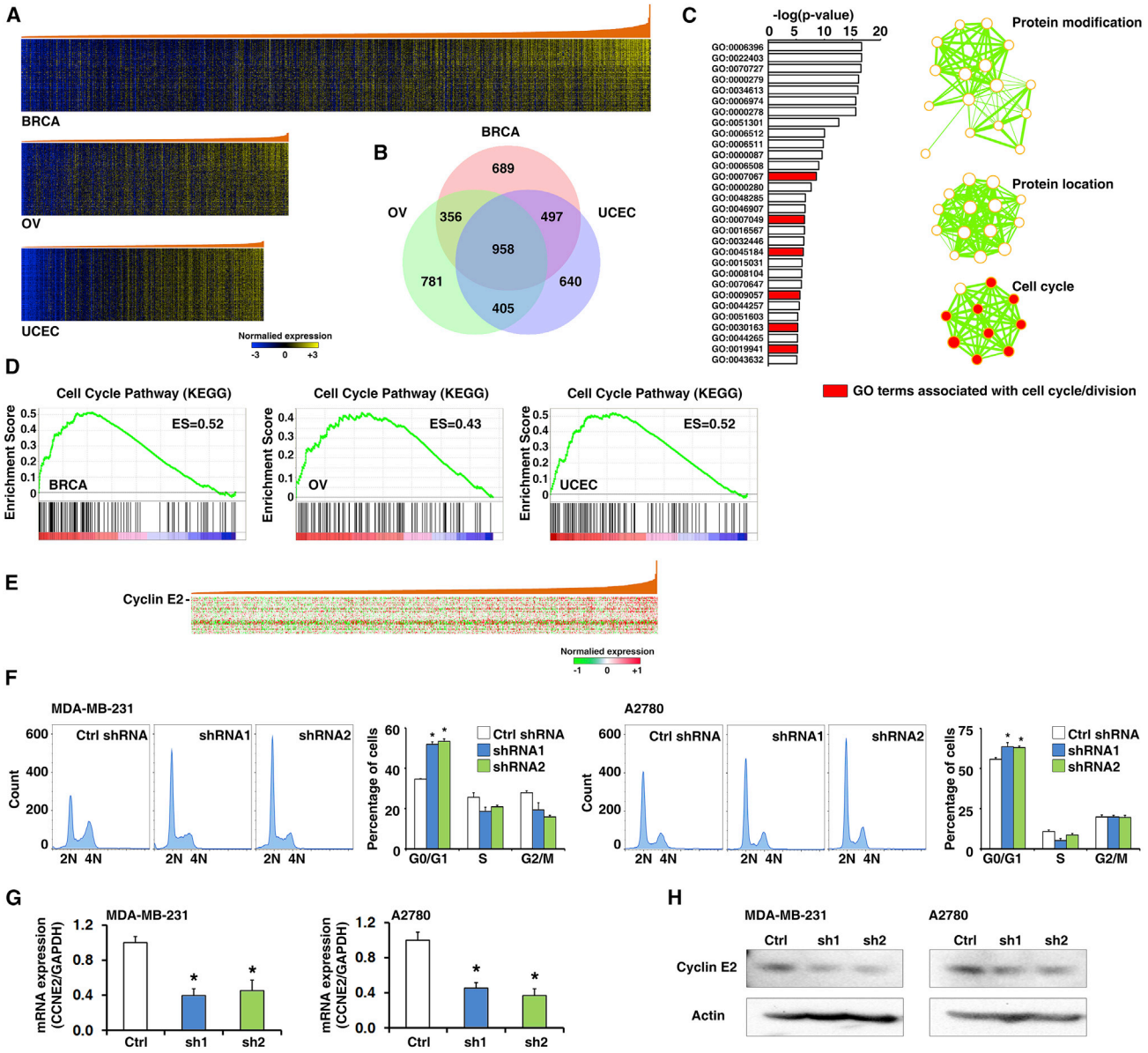

Figure 7. Inferring the Functions of *BCAL8* by Integrative Bioinformatics Analyses

(A) Heatmap of PCGs that were significantly and positively co-expressed with *BCAL8*. The genes were arranged from top to bottom in ascending order of their correlation with *BCAL8*.

(B) Venn diagrams of *BCAL8*-associated genes among breast, ovarian, and endometrial cancers.

(C) Pathways over-represented by *BCAL8*-associated PCGs in all three cancer types according to DAVID analysis on the basis of gene ontology term.

(D) Enrichment of cell-cycle pathway genes in cancer specimens with high levels of *BCAL8*.

(E) Heatmap of PCGs whose protein expression (RPPA) is significantly correlated with *BCAL8* expression in breast cancer. The proteins are arranged from top to bottom in ascending order of their correlation with *BCAL8* expression.

(F) Cell-cycle profiles of cells expressing control and *BCAL8* shRNAs.

(G) Quantitative RT-PCR (qRT-PCR) of CCNE2 mRNA expression in cells expressing control or *BCAL8* shRNAs.

(H) Western blot of cylin E2 in cells expressing control or *BCAL8* shRNAs. The error bars indicate SD. *p < 0.05.

See also Figure S6 and Table S8.

has the highest cancer type specificity, followed by pseudogenes, and then PCGs, which were the least subtype specific. The expression of lncRNAs is frequently dysregulated in cancer. There are sensitive, rapid, low-cost methods readily available for lncRNA quantification. Additionally, lncRNAs often form secondary structures that are relatively stable, thereby facilitating their detection as free RNAs in body fluids such as urine and blood. Therefore, lncRNAs may be an ideal class of biomarkers with potential applications in cancer prediction, early detection, diagnosis and classification.

The TCGA project has profiled large numbers of tumors to identify molecular aberrations at multi-omic levels. Extracting valid information from TCGA can deepen our understanding of tumorigenesis and lead to the development of therapeutics. However, because cancer genomes are highly unstable, many cancer-associated alterations are not the causes but instead the consequence of tumorigenesis. The main challenge in developing effective therapies is to identify cancer driver genes, which once targeted by therapeutic agents can suppress or eliminate tumor growth. Analyses of genome-wide molecular profiles using various bioinformatics approaches can reveal genomic alterations during cancer initiation and progression but cannot distinguish "causal" from "bystander" genetic alterations. Genome-wide functional screening approaches have been used with some success in identifying cancer driver genes; however, this approach can be time and labor intensive and, more important, susceptible to finding false positives and fraught with large numbers of false negatives. Here, we have developed a clinically guided genetic screening approach to identify functional lncRNAs in cancer. Using the cancer lncRNome resource generated in our study as biological and clinical filters, we were able to generate a relatively short list of lncRNA candidates for more extensive testing using candidate-oriented genetic screening. Predicting the biological functions of a given lncRNA is challenging. A "co-expression" approach has been used as one approach to begin to achieve an understanding of lncRNA function (Huarte et al., 2010). Because the level of lncRNA expression may directly represent its biological function in cancer, we proposed predicting lncRNA functions by co-expression analysis, that is, by identifying the PCGs whose expression are significantly correlated with the expression of a given lncRNA. TCGA contains multi-omic profiles of large-scale samples, serving as an excellent resource for co-expression analysis. Taken together, the lncRNome database generated in the present study provides a resource to effectively identify cancer driver lncRNAs and predict their functions in cancer, which will lead to a greater understanding of molecular mechanisms of cancer, and should lead to clinical applications in oncology.

EXPERIMENTAL PROCEDURES

Annotation of lncRNAs, PCGs, and Pseudogenes

The GENCODE lncRNA annotation (V18), a manually curated and evidence-based lncRNA annotation containing 13,562 genes and 23,105 transcripts, was used to define lncRNA genes. The GENCODE whole annotation (V18) was used to define PCGs and pseudogenes, resulting in a PCG set containing 20,318 genes and 81,673 transcripts, a pseudogene set containing 14,181 genes and 17,517 transcripts, and an "other genes" set containing 9,384 genes and 73,289 transcripts.

RNA-seq Data Processing

RNA-seq files were downloaded from the Cancer Genomics Hub (http://cghub.ucsc.edu). We imported the aligned reads of each BAM file to the Partek Genomic Suite (http://www.partek.com) to obtain the expression levels for genes by summarizing the RPKM values. For each cancer type, we applied two filters to eliminate unreliability in the measurements of genes: (1) the 50th percentile of the RPKM values is larger than 0, and (2) the 90th percentile of the RPKM values is larger than 0.1. The genes that passed these two filters were defined as detectable in a given cancer type. Please see Supplemental Experimental Procedures for a detailed discussion of procedures.

Xenograft Model In Vivo

6- to 8-week-old female nude mice were used for the xenograft assays. A2780 cells and MDA-MB-231 cells were trypsinized and harvested in PBS, then a total volume of 0.1 ml PBS containing A2780 cells (1×10^6) or MDA-MB-231 cells (1.5×10^6) were injected subcutaneously into the flanks of the animals. The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Please see Supplemental Experimental Procedures for a detailed discussion of procedures.

Statistical Analysis

Statistical analysis was performed using SPSS and SAS software. All results were expressed as mean ± SD, and $p < 0.05$ indicated significance. The survival curves were constructed according to the Kaplan-Meier method and compared with the log rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.09.006.

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