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Review

Long Noncoding RNA in Cancer: Wiring Signaling Circuitry

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Long noncoding RNAs (lncRNAs), which are encoded by a vast less explored region of the human genome, may hold missing drivers of cancer and have gained attention recently as a potentially crucial layer of cancer cell regulation. lncRNAs are aberrantly expressed in a broad spectrum of cancers, and they play key roles in promoting and maintaining tumor initiation and progression, demonstrating their clinical potential as biomarkers and therapeutic targets. Recent discoveries have revealed that lncRNAs act as key signal transduction mediators in cancer signaling pathways by interacting with proteins, RNA, and lipids. Here, we review the mechanisms by which lncRNAs regulate cellular responses to extracellular signals and discuss their clinical potential as diagnostic indicators, stratification markers, and therapeutic targets of combinatorial treatments.

Highlights

Somatic mutations or disease-associated SNPs of lncRNAs implicate lncRNAs as cancer risk genes.

Through RNA–protein, RNA–RNA, and RNA–lipid interactions, lncRNAs modulate cancer signaling pathways and consequences.

Dysregulation of lncRNAs leads to hyper- or hypoactivation of cellular pathways, leading to resistance to current targeted therapies.

Targeting lncRNA alone or a cotargeting strategy may help overcome the targeted therapy resistance

lncRNAs as Diagnostic and Prognostic Markers for Human Cancer

It is being recognized that certain single-nucleotide polymorphisms (SNPs) are associated with cancer risk. Large-scale data analysis from cancer genome-wide association studies (GWASs) indicates that the majority of SNPs associate with noncoding genes [1,2]. The majority of recurrent somatic mutations, copy number alterations, and cancer-related SNPs are related to ncRNAs [3–5] (recently reviewed [6]), and the presence of risk SNPs may modulate the expression of corresponding ncRNAs. Among those noncoding genes, lncRNAs are emerging as a new class of indispensable players involved in the development and progression of cancer [7–9]. Indeed, SNPs of lncRNAs have been shown to be associated with risk for prostate cancer [10,11], lung cancer [12], breast cancer [13–15], among other cancer types [16,17]. Moreover, the dysregulation of a number of lncRNA targets has correlated with the stage and prognosis of several tumor types [16,17] including prostate cancer [10,11], lung cancer [12], and breast cancer [13–15], as well as being linked to resistance against chemotherapy and targeted therapy [18–21]. Correlation analyses indicate that numerous lncRNAs are upregulated in cancer cells that are resistant to DNA damage inducers [22–25], antihormone therapies [26–28], targeted therapies [29], and signaling pathway inhibitors [13] (Figure 1). Indeed, the expression of *HOTAIR* activates estrogen receptor (ER) target transcription program and contributes to resistance to tamoxifen [28]. Loss-of-function studies using small hairpin RNA-based knockdown and Clustered regularly interspaced short palindromic repeats (CRISPR)/cas9-mediated genetic depletion indicate that lncRNAs facilitate cancer cell growth, apoptosis resistance, and cell mobility [22,30,31]. Gain-of-function studies suggest that increased expression of lncRNAs enhances cell viability during drug treatment [24,28,32].

lncRNA derives from several sources including intergenic regions, the antisense strand of the protein coding sequence, intronic transcription, and alternative splicing (reviewed in [33,34]). A considerable percentage of known lncRNAs either reside within the cytosol or shuttle between

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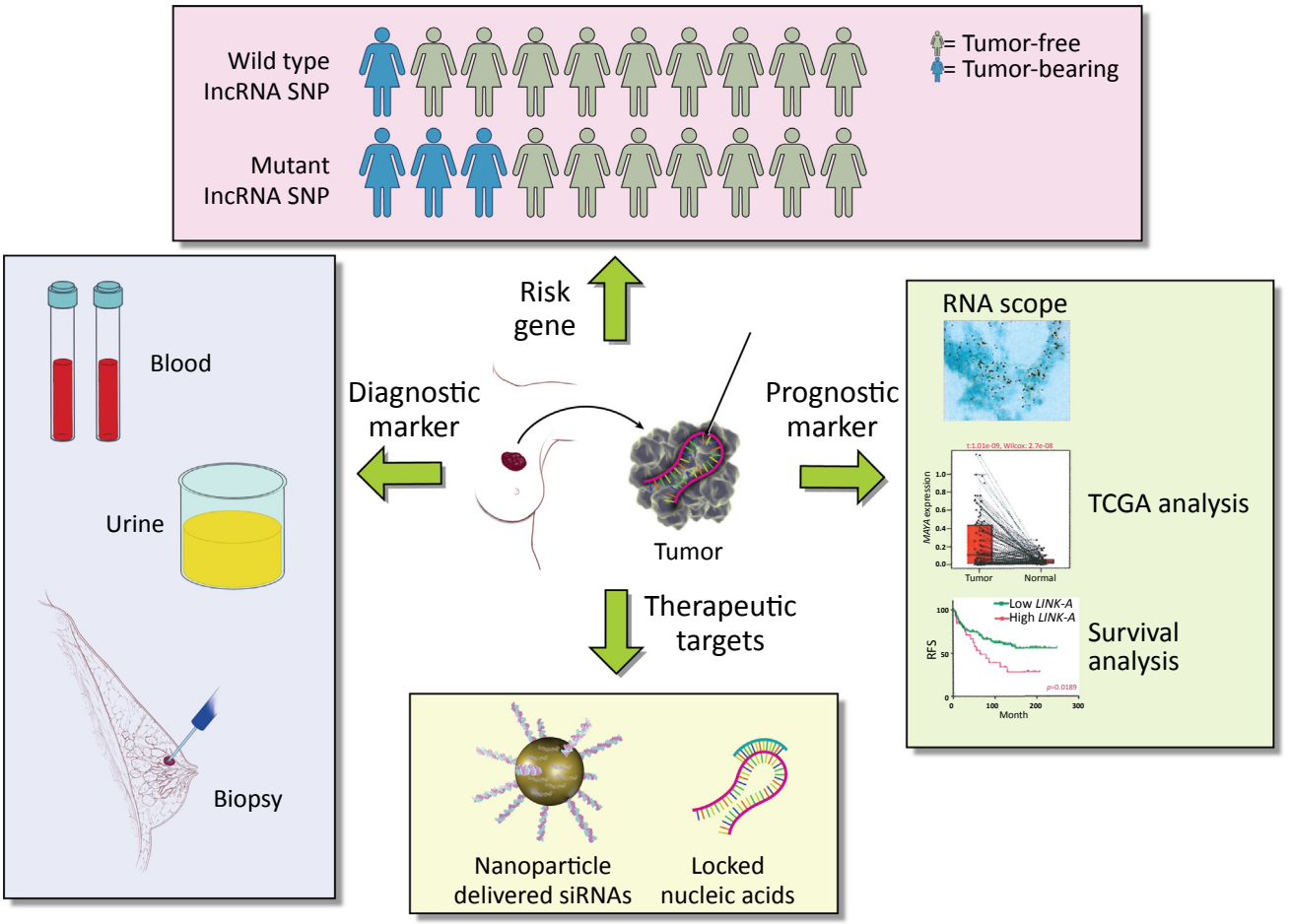

Figure 1. Value of lncRNAs as Breast Cancer Risk Genes, Diagnostic Markers, Prognostic Markers and Therapeutic Targets. lncRNAs as cancer risk genes: the search for genomic mutations related to lncRNAs suggests that disease-related SNP of lncRNAs may situate lncRNAs as cancer risk genes. Populations with the wild-type allele may exhibit low incidence of cancer; mutant allele carriers may show elevated incidence of cancer development. lncRNAs as diagnostic markers: the detection of lncRNAs in human blood, urine, or biopsy samples could be beneficial for risk detection in a gene carrier for early diagnosis of human cancer. lncRNAs as prognostic markers: the expression status of lncRNAs in human cancer tissues could be correlated with cancer stage, metastatic potential, resistance to target therapy, and patient outcome. The expression level of lncRNA could stratify cancer patients with target therapy. lncRNAs as therapeutic targets: antisense-oligonucleotide-based strategies are under development. siRNAs targeting lncRNAs could be encapsulated in nanoparticles for improved tissue distribution and pharmacodynamics. LNAs could also be the cargo of nanoparticles. Without delivery vehicles, LNAs exhibits adequate half-life in serum and tolerable toxicology. Abbreviations: LNAs, locked nucleic acids; lncRNA, long noncoding RNA; SNP, single-nucleotide polymorphism; TCGA, The Cancer Genome Atlas.

the nucleus and the cytoplasm [35]. Current studies indicate that these cytoplasmic lncRNAs play important functional roles in modulating mRNA translation and decay in a base-pairing-dependent manner [36–38] or by competing with a protein- or miRNA-mediated mRNA decoy [39]. In addition, cytoplasmic lncRNAs have been shown to regulate cytoplasmic protein trafficking from the cytosol to the nucleus for transcriptional activation [40]. Recent studies have also indicated that lncRNAs may associate with proteins, cellular lipids, and metabolic intermediates (Figure 2).

Although still largely unexplored, it has been suggested that lncRNAs are essential mediators of intracellular signaling pathways. Here, we describe emerging insights into the role of lncRNAs in

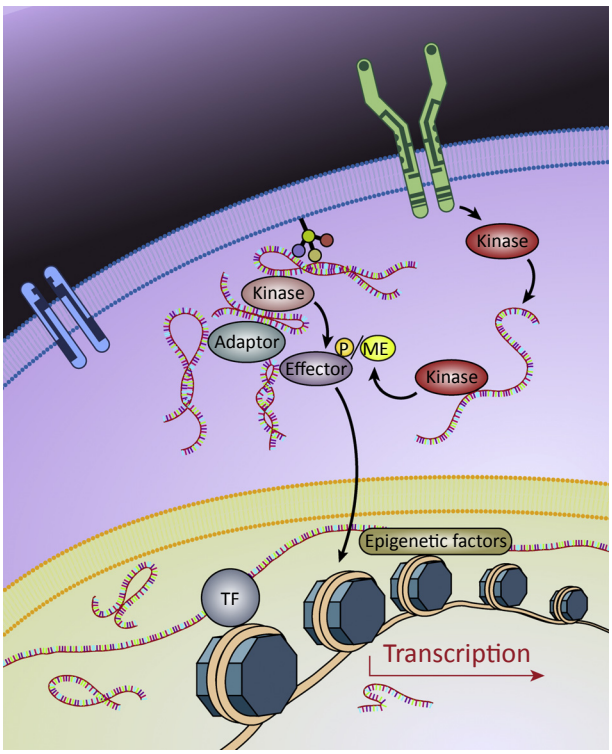

Figure 2. lncRNAs Are Key Regulators of Cancer Signaling. In response to extra- or intracellular signals, complicated signaling cascades are strictly regulated to achieve coordinate cellular activities. lncRNAs could be involved in all of the steps of signaling cascades: lncRNAs may associate with cellular receptors to modulate the hetero- and homodimers of the receptors; lncRNAs mediate the recruitment of kinases to receptors for activation/inactivation; lncRNAs intercede second-messenger-mediated signaling events; lncRNAs interacts with protein kinases to modulate the kinase-dependent post-translational modifications of effectors; lncRNAs facilitate the subcellular relocations of kinases/effectors, lncRNAs regulate epigenetics modifications and assemble/dissemble of transcriptional machinery. Abbreviations: lncRNA, long noncoding RNA; ME, methylation; P, phosphorylation; TF, transcription factor.

regulating signaling pathways in cancer. New insights into the regulatory roles of lncRNAs in cancer for governing novel mechanisms and pathways by which cancer cells acquire their invasiveness and metastatic properties serve as the basis of a new approach in the fight against cancer. This understanding of lncRNAs in cancer signaling should stimulate new directions for future research endeavors and therapeutic options that focus on lncRNAs as novel cancer prognostic markers and therapeutic targets.

lncRNA Directs the Outcome of Cell Signaling Pathways

In addressing the functional role of lncRNAs, one of the possible explanations is to identify the binding proteins of a given lncRNA (Box 1). Accumulating research has demonstrated that key signaling mediators, such as receptors, protein kinases, and transcription factors, directly associate with lncRNAs and that their enzymatic activities are regulated by those lncRNAs (Figure 2). For example, an lncRNA expressed exclusively in human dendritic cells (*lnc-DC*) associates with signal transducer and activator of transcription (STAT)3 to prevent Src homology region 2 domain-containing phosphatase (SHP)1-dependent dephosphorylation of STAT3, which leads to dendritic cell differentiation [41]. NF-KappaB interacting LncRNA (*NKILA*), the lncRNA that associates with IκB, inhibits IKK-mediated IκB phosphorylation at Ser32 and Ser36, via direct association with the IκB N terminus. Consequently, nuclear factor (NF)-κB signaling is negatively regulated by *NKILA* to suppress cancer metastasis [40]. The lncRNA *NBR2* (neighbor of BRCA1 gene 2) has been demonstrated to associate with AMP-activated protein kinase (AMPK) upon energy stress and to promote the kinase activity of AMPK [42]. Cardiac and apoptosis-related lncRNA (*Carlr*) associates with p65 NF-κB in macrophages cells, and knockdown of *Carlr* impairs the expression of NF-κB target genes [43]. A phosphoinositide 3-kinase (PI3K) p85 subunit-interacting lncRNA, *AK023948*, positively regulates

Box 1. Biochemical Approaches to Identify lncRNA–Protein Interactions

lncRNA Pulldown Combined with Mass Spectrometry

This is an unbiased and open-ended lncRNA pulldown assay for better understanding of the lncRNA-associated protein partners. Typically, the pulldown assay uses a RNA probe labeled with a high-affinity biotin tag that allows the probe to be recovered. After incubation, the biotinylated RNA probe can bind with a protein–protein complex in a cell lysate and then the complex is purified using magnetic streptavidin beads. The proteins are then eluted from the RNA and detected by western blotting or mass spectrometry. This assay has the advantages of enrichment of low-abundance protein targets, isolation of intact protein complexes, and being compatible with immunoblotting and mass spectrometry analysis. This methodology will drive the advances of studying lncRNA functions and the related mechanisms [13,27,45,46,123].

Capture Hybridization Analysis of RNA Targets (CHART)

This method has been used to identify RNA-bound DNA, RNA, or protein partners, mainly focusing on the lncRNA molecules within the nucleus [124,125]. Formaldehyde-crosslinked chromatin extracts are subjected to target RNA capture using biotinylated ASOs. The DNA–RNA hybrids are digested by RNase H and enriched by streptavidin beads. The CHART-enriched proteins can be subjected to mass spectrometry to identify proteins that associate with target RNA molecules [126].

Chromatin Isolation by RNA Purification (ChIRP)

Similar to the chromatin IP (ChIP) assay and CHART assay, the ChIRP method is based on the biotinylated oligonucleotides to immunopreciate endogenous lncRNAs from cell or tissues to identify the lncRNA-associated DNA or proteins [127]. The probe design requires prescreening and validation for maximum hybridization efficiency. Elutes from the ChIRP method using magnetic streptavidin beads are subjected to mass spectrometry or immunoblotting for protein analysis [128].

RNA Antisense Purification with Mass Spectrometry (RAP-MS)

Similar to the CHART and ChIRP methods, RAP techniques apply ASOs as probes for RNA capture [129]. Distinct from the two previous methods, RAP uses 90mer oligonucleotides to create stable DNA–RNA hybrids; applies UV-cross-linking to capture transient RNA–protein interaction by introducing covalent bonds; purifies RNA–protein complexes by stringent denaturing condition to limit nonspecific binding; and quantitatively determines RNA-binding proteins using stable isotope labeling with amino acids in cell culture (SILAC) [129]. The RAP-MS method has been used to identify RNA-associated proteins of nuclear lncRNA or mitochondria lncRNAs [74].

Crosslinking Immunoprecipitation (CLIP)-Coupled with High-throughput Sequencing (HTS)

After UV crosslinking, RNA is labeled with ^{32}P-γ-ATP and partially digested. The RNA–protein complex is immuno-precipitated using an antibody. The bound RNAs are ligated with an RNA linker (20 nucleotides) and the RNA–protein complex is subjected to SDS-PAGE and autoradiography. Under the +++RNase condition, the bound RNAs are overdigested, which exhibits a ~7-kDa molecular weight shift (roughly the molecular weight of the RNA linker). Under the +RNase condition, the 15–20-kDa molecular weight shift corresponds to the 26–41 nucleotide RNA fragments (minus the RNA linker). Therefore, the RNase over-digestion (+++RNase condition) is considered the negative control, and the 15–20-kDa molecular shift band is excised for the subsequent steps. The CLIP assay yields a mixture of RNA fragments of 25–55 nucleotides, plus 2–20-nucleotide RNA linkers, which is subjected to sequencing. HITS-CLIP has been used to demonstrate the binding RNA motif of a given protein [130].

In Vitro RNA Pulldown Followed by Dot-Blot Assay

The biotinylated RNA and recombinant proteins are incubated to promote binding. The bound RNA is subjected to partial RNase digestion (+RNase condition), and the remaining bound RNA is subjected to protease K digestion and RNA extraction. The RNA, in fragments, is hybridized to a dot-blot. The dot-blot is a nylon membrane spotted with 54-60 mer antisense DNA oligonucleotides tiling along the lncRNA targets. After stringent washing, the protein-bound RNA sequence is visualized by detection of streptavidin–horseradish peroxidase signals. Depending on the different sequence motifs (regions) bound and protected by interested proteins, these RNA sequence motifs are hybridized to different positions on the dot-blot. Using this method, it has been demonstrated that two distinct positions, corresponding to nucleotides 235–288 and 991–1044 of *BCAR4*, directly bind to SNIP1 and PNUTS, respectively [27]. Similarly, two regions of *LINK-A*, nucleotides 481–540 and 781–840, associate with the two domains of BRK at the SH3 domain and the C-terminal tail [45]. Another example is that the nucleotides 241–300 and 841–900 of *MAYA* are responsible for LLGL2 and NSUN6 binding, respectively [46]. *BCAR4*, Breast Cancer Anti-Estrogen Resistance 4; LLGL2, lethal(2) giant larvae protein homolog 2; *MAYA*, MST1/2-antagonizing for YAP activation; NSUN6, NOP2/Sun RNA methyltransferase family member 6; PNUTS, Phosphatase 1 Nuclear Targeting Subunit; SNIP1, Smad Nuclear Interacting Protein 1.

the AKT pathway in breast cancer [44]. In addition, long-intergenic noncoding RNA for kinase activation (*LINK-A*) directly associates with the non-receptor tyrosine kinase BRK (breast tumor kinase) and promotes the recruitment of BRK to the liganded receptor epidermal growth factor receptor (EGFR), leading to hyperactivation of the EGF-BRK-HIF (hypoxia-inducible factor 1) signaling pathway [45]. Moreover, lncRNA *MAYA* (*MST1/2-antagonizing for YAP activation*), via interaction with the scaffold protein LLGL2 (Lethal Giant Larvae Homolog 2) and the methyltransferase NSUN6 (NOP2/Sun RNA Methyltransferase Family Member 6), forms an RNA–protein complex for methylation of MST1 (Mammalian STE20-Like Protein Kinase 1), a master regulator of the Hippo–YAP pathway [46].

Recent observations indicate that certain lncRNAs associate with a variety of phospholipids [13]. Genome-wide identification of lncRNAs in cell lipid fraction indicates that about 1.6% of lncRNAs may associate with lipid directly or indirectly. By an open-ended screening, a cohort of lncRNAs, including *XLOC-002384*, *SNHG6*, *SNHG9*, *RP11-383G10.5*, and *LINC00607* exhibited particularly strong and specific interaction with lysophosphatidic acid (LPA), lysobisphosphatidic acids (LBPAs), phosphatidic acid (PA), cardiolipin, and phosphatidylethanolamine (PE), respectively, suggesting that lncRNAs play important roles in regulating lipid metabolism, lipid signaling, mitochondrial function, cholesterol transportation, or even the formation of multivesicular bodies [47–50].

In summary, the proposed functional roles of lncRNAs in regulating signaling cascades and crosstalk between pathways can be classified into six categories (Figure 3). (i) The Merge category refers to when an lncRNA conjoins the kinases of neighboring pathways, allowing the pathways to work in parallel to mediate a common cellular effect. Thus, the ligand of either receptor is able to activate the effector, accelerating the consequent cellular response. (ii) In the Switch group, lncRNA may couple one receptor to multiple kinases, creating a junction in the pathway. When the appropriate ligand binds to the receptor, the linked kinases are each able to activate their respective effectors in response to that one signal protein. (iii) In response to the cellular environment and specific ligand, an lncRNA could reroute and form a bridge between a kinase and one of many possible effectors. In this scenario, one receptor and its associated kinase are able to achieve an array of cellular effects in accordance to the cellular context. (iv) lncRNAs may also be categorized by their ability to yield. In the situation of antagonistic pathways, an lncRNA is able to mediate interaction between two kinases so that only one pathway is activated at a given time. Therefore, activation of a pathway is blocked when the other is already activated. (v) lncRNAs can also be classified into a shortcut group. In the presence of the lncRNA, the conventional kinase cascade could be bypassed in favor of a shortcut that enables a more direct route to activating the downstream effector. Consequently, the lncRNA allows for more rapid induction of the desired cellular response. (vi) lncRNAs may detour. In instances where the conventional pathway is disrupted or blocked, lncRNAs may facilitate the formation of an alternative pathway by way of recruiting substitute pathway components or mediators. In response to poisons or inhibitors, a cellular effect may still be achieved by the more circuitous route enabled by the lncRNA.

lncRNA–Protein Interactions: The Building Blocks of Cancer Signaling Cascades

The interactions between lncRNAs and binding proteins act as one of the major mechanisms for lncRNA-mediated signaling pathways. In addition to typical RNA binding domains, atypical RNA binding domains are less recognized, but they play important roles in mediating lncRNA–protein interaction. Indeed, recent evidence has indicated that classical RNA-binding domains, nonclassical RNA binding domains and domains with unknown RNA-binding domains all play critical roles in mediating RNA–protein interactions in given signaling pathways (Figure 4).

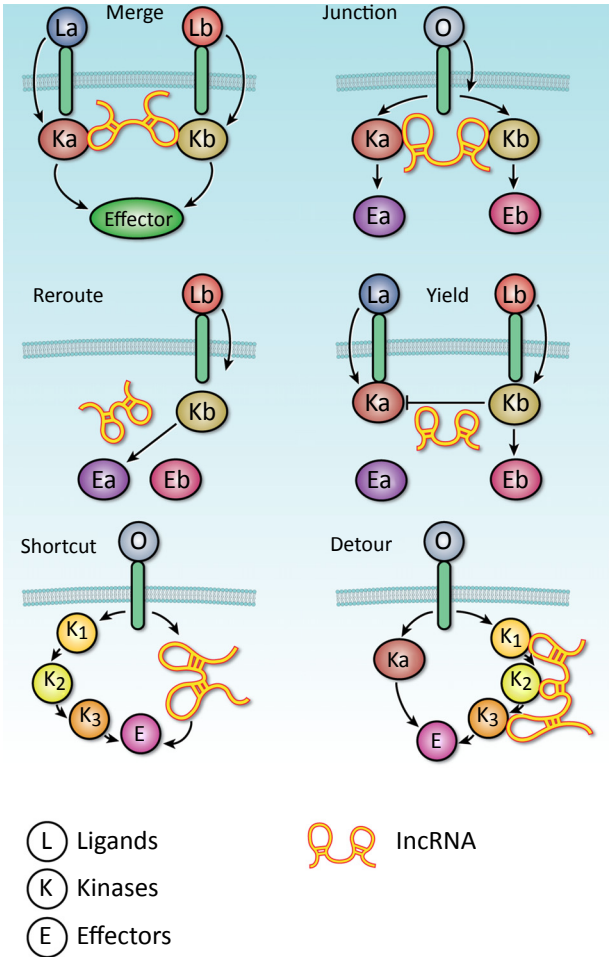

Figure 3. lncRNAs Direct the Outcome of a Signaling Network. The functional role of lncRNAs in regulating cancer signaling can be classified as following. Merge –lncRNA conjoins the kinases of neighboring pathways, allowing the pathways to work in parallel to mediate a common cellular effect. Thus, the ligand of either receptor is able to activate the effector, accelerating the consequent cellular response. Switch –lncRNA couples one receptor to multiple kinases, creating a junction in the pathway. When the appropriate ligand binds to the receptor, the linked kinases are each able to activate their respective effectors in response to that one signal protein. Reroute – in response to the cellular environment and specific ligand, the lncRNA forms a bridge between the kinase and one of many possible effectors. In this scenario, one receptor and its associated kinase are able to achieve an array of cellular effects in accordance to the cellular context. Yield – in the situation of antagonistic pathways, the lncRNA is able to mediate interaction between two kinases so that only one pathway is activated at a given time. Effectively, activation of a pathway is blocked when the other is already activated. Shortcut – in the presence of the lncRNA, the conventional kinase cascade is bypassed in favor of a shortcut that enables a more direct route to activating the downstream effector. Consequently, the lncRNA allows for more rapid induction of the desired cellular response. Detour – in instances where the conventional pathway is disrupted or blocked, lncRNAs can facilitate the formation of an alternative pathway by way of recruiting substitute pathway components or mediators. In response to poisons or inhibitors, a cellular effect may still be achieved by the more circuitous route enabled by the lncRNA. Abbreviations: lncRNA, long noncoding RNA.

Open-ended proteomic studies using oligo-dT capture to identify the interactome of mRNAs and potentially polyadenylated ncRNAs have indicated that classical RNA-binding domains, nonclassical RNA-binding domains, and domains with unknown RNA-binding all exhibit RNA-binding possibilities [51]. Proteins harbor noncanonical RNA-binding domains, such as the zinc finger, WD40, and SAM domains, which all have demonstrable RNA binding [51]. Structural analyses have demonstrated that WD40 repeats may form an RNA-binding surface that can interact with snRNAs via base-stacking interactions, which is required for the ribonucleic particle biogenesis [52,53]. In cancer signaling pathways, the WD40 domains of LLGL2 mediate LLGL2–*MAYA* interaction, which is required for the RNA–protein complex formation [46]. Another example of WD40 domain mediation of RNA–protein interaction is LRRK2, in

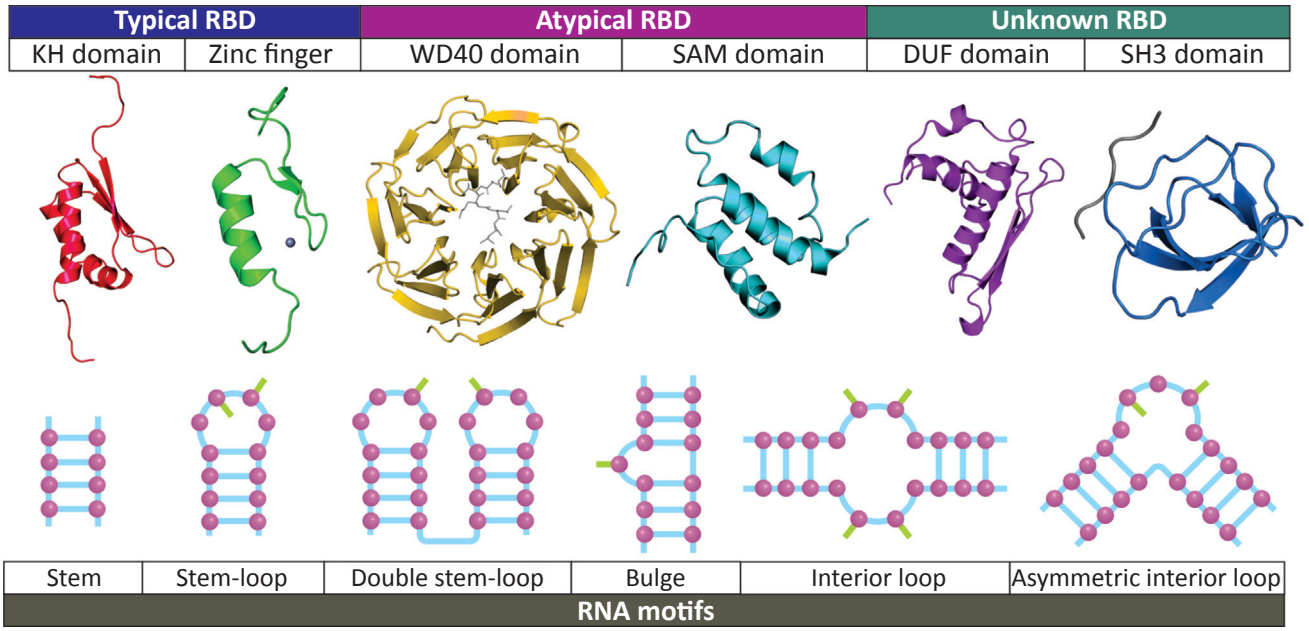

Figure 4. Protein Domains and RNA Motifs Involved in lncRNA–Protein Interactions. In addition to typical RBDs, atypical RBDs, and domains with unknown RNA binding mediate the interactions between lncRNAs and the key mediators of cancer signaling pathways. Noncanonical RNA-binding domains, such as the WD40 and SAM domains, are required for the RNA–protein complex formation upon ligand binding. Domains with unknown RNA-binding properties, including the DUF and SH3 domains, facilitate the recruitment of SNIP1 to lncRNA *BCAR4* and *LINK-A*–BRK interaction. The RNA binding domains may recognize nucleotide sequences or structures of RNA motifs, resulting in specific lncRNA–protein interactions. The single-stranded lncRNA molecule forms complex secondary and 3D structures. Stem, base-paring formed between two complimentary strands of the RNA molecule. The double helices are essential for RNA folding, and also provide docking sites for sequence-mediated interaction. Stem-loop, the RNA sequence forms a complimentary base paring with an unpaired loop. The nitrogenous bases within the single-stranded loop could face inside or outside of the loop to form additional hydrogen bonds with binding partners. Double stem-loop: two or more stem-loop structures side by side. The nitrogenous bases from both loops may coordinate the lncRNA–protein interaction. Bulge: the unpaired residues flanking with base pairs may be packed toward the helix or extrude to outside of the stem, which are all important for protein binding. Symmetric and asymmetric interior loops: the structure of separate RNA strands flanked with stacking base pairs can be found in the middle of RNA strands. The loops provide free nitrogenous bases for stacking interaction or hydrophobic interactions. Abbreviations: BCAR4, breast cancer anti-estrogen resistance 4; BRK, breast tumor kinase; DUF, domain of unknown function; LINK-A, long intergenic noncoding RNA for kinase activation; lncRNA, long noncoding RNA; RBDs, RNA-binding domains; SAM, sterile alpha motif; SH3, Src homology 3SNIP1, Smad nuclear interacting protein 1; WD40, tryptophan–aspartic acid 40.

which the WD40 domain of this protein serine/threonine kinase is required for the association between LRRK2 and *LINK-A* [45].

Domains with unknown RNA-binding properties, including the domain of unknown function (DUF) and Src homology 3 (SH3) domains, have also been suggested to be involved in RNA binding [51]. The DUF domain of Dicer has been shown to interact with single-stranded or double-stranded RNA [54,55]. Characterization of the DUF domain of Smad-interacting nuclear protein (SNIP)1 has revealed that it is essential for interaction with the lncRNA *BCAR4* [27]. The SH3 domain of BRK contributes to the BRK–*LINK-A* interaction and the consequent hyper-activation of BRK [45]. Evidence has also been reported that the kinase domain of protein kinases may be involved in RNA–kinase interactions. Characterization of the *NBR2*–AMPK interaction indicated that deletion of the C-terminal half of AMPK abolished the RNA–protein interaction [42]. Global characterization of unknown RNA-binding domains indicates that unstructured domains exhibited potential RNA binding, suggesting the diversity of lncRNA–protein interactions [56].

Unlike proteins, which contain domain structures that can provide pilot information on the potential function, cellular location, and regulation involved in RNA–protein interaction, RNA motifs involved in RNA–protein interaction are more challenging to define. Using crosslinking-based technologies (Box 1), the RNA motifs responsible for protein binding could be identified. It has been demonstrated that two distinct positions, corresponding to nucleotides 235–288 and 991–1044 of *BCAR4*, directly bind to SNIP1 and phosphatase 1 nuclear-targeting subunit (PNUTS), respectively [27]. Similarly, two regions of *LINK-A*, nucleotides 481–540 and 781–840, associate with the two domains of BRK at the SH3 domain and the C-terminal tail [45]. Another example is that the nucleotides 241–300 and 841–900 of *MAYA* are responsible for LLGL2 and NSUN6 binding respectively [46]. The requisition of the identified RNA motifs can be validated by deletion mutants and CRISPR/cas9-mediated genomic editing [13]. Using CRISPR/cas9 technology, the specific RNA motif could be engineered without affecting the flanking sequence and unlikely to affect the intrinsic expression status of the lncRNAs, which provide advantages in studying the cellular consequence of lncRNAs. The secondary and 3D structures of RNAs are likely to play important roles in RNA–protein interaction (Figure 4). Within our limited knowledge, the nucleotides located in the loop region of the RNA motif are critical for RNA–protein binding, and the hairpin loop is one of the more common structures found in RNA molecules [57]. Crystallographic dissection of RNA–protein interactions is urgently needed to pinpoint the underlying molecular mechanisms.

lncRNAs Directly Associate with Phospholipids and Other Small Molecules

The observation that RNA association with the cell membrane is involved in the formation of the *Escherichia coli* signal recognition particles [58] and regulation of *Saccharomyces cerevisiae* cell membrane permeability [59] supports the notion that RNA–lipid interactions are important to life. The association of polynucleotides with charged lipid membranes, including phosphatidylcholines (PCs), PE, phosphatidylinositol (PI), and phosphatidylserine (PS), have been observed in the cell nucleus [60] and RNA viruses [61]. Biophysical studies have suggested that the phospholipid bilayer can absorb single-stranded DNA and RNA [62,63]. Membranes composed of cationic or zwitterionic phospholipids with bivalent cations associate with RNA [62,64]. It is intriguing that lncRNAs have been shown to interact with phospholipids with strong binding affinity, which are comparable to lipid-binding proteins [13,65–67]. However, RNA–lipid interactions in their appropriate biological context need to be further demonstrated.

RNA nucleotides contain a negatively charged backbone and nitrogenous bases that possess a weak positive charge. The interaction between RNAs and phospholipids may be explained by the 3D structure of RNA. Structural analysis has shown that the loop structures of RNA molecules may be important for such binding [68]. It has been indicated that an 18-nucleotide stem-loop structure is required for *LINK-A*–PIP_3 interaction. Deletion of the loop region, or mutations of either 4′C to A or 6′C to A abolished RNA–PIP_3 interaction [13]. It is likely that the loop formed by the RNA molecules arranges the negatively charged backbone into a circle. It is possible that the cytosine bases at the 4′ and 6′ positions are arranged toward the center of the loop, allowing for potential hydrogen bonding with the three phospho groups of PIP_3. In addition to this particular stem-loop structure, other parts of *LINK-A* may also contribute to the *LINK-A*–PIP_3 interaction. Ultimately, to understand the 3D interaction between *LINK-A* and PIP_3 will require structural analysis using crystallography or NMR.

In addition to macromolecules, RNAs have been demonstrated to associate with small molecules, including mental complexes, amino acids, and other organic or inorganic complexes [69]. Structural studies indicate that RNA molecules associate with arginine, which

triggers a conformational change in the secondary structure of the RNA [70]. Hence, RNA molecules can likely serve as sensors for the regulation of metabolic pathways.

lncRNAs Modulate Enzyme Activity

The functional roles of lncRNA–protein interactions are diverse. One of the surprising, yet frequent, observations is that once a protein binds with an lncRNA, the enzymatic activity of the binding protein may be modulated. For instance, the lncRNA *NBR2* has been indicated as an AMPK activator upon energy stress [42]. It has been demonstrated that lncRNAs can modulate the activity of various enzymes (e.g., kinases) in the following ways (Figure 5).

lncRNAs can modulate the activity of various enzymes (e.g., kinases) by acting as chaperones, whereby lncRNA interacts with subunits of an enzyme and can induce a conformational change, altering its enzymatic activity. BRK is negatively regulated by its C terminus, which harbors a phosphorylated tyrosine [71]. Protease digestion assay suggest a potential conformational change in BRK upon *LINK-A* binding on both the SH3 domain and C terminus of BRK, which attenuates its self-inhibition, leading to enhanced enzymatic activity of BRK [45].

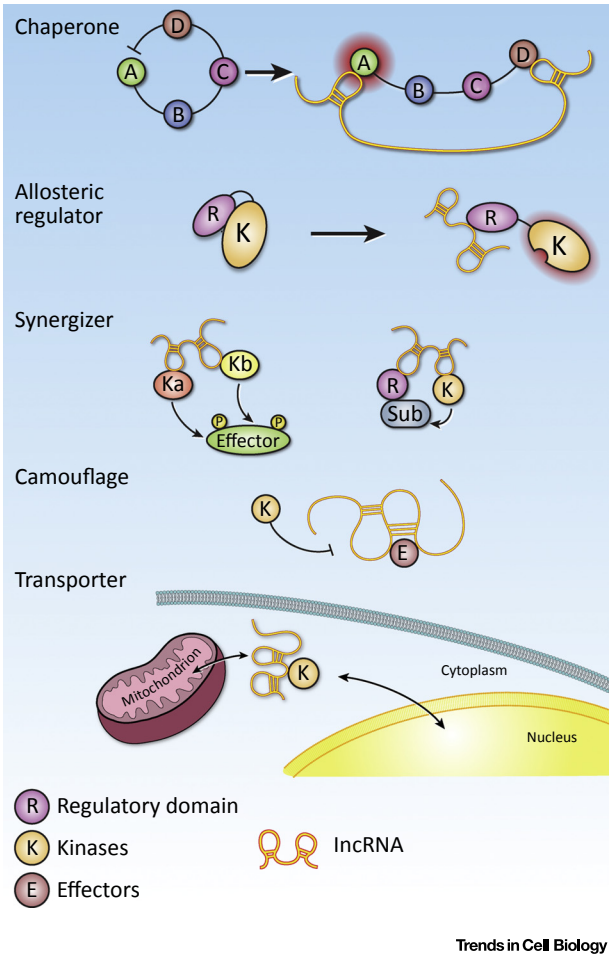

Figure 5. lncRNA Governs the Enzymatic Activities of Protein Kinases in Signaling Circuitry. Upon lncRNA–kinase interaction, the enzymatic activities of protein kinase can be modulated. Chaperones –lncRNA interaction with subunits of an enzyme can induce a conformational change, altering its enzymatic activity. A–D illustrate the domains of the protein kinases, by which the inhibitory effect of domain D is repressed upon lncRNA binding, leading to enzymatic activation of domain A. Allosteric regulators – when the lncRNA binds to the allosteric site of the enzyme, modifying the conformation of the regulatory domain (R) and kinase domain (K), the enzyme could be either inhibited or activated. Synergizers – lncRNA conjoins multiple kinases and/or regulators together, mediating cooperative action on its effectors or substrates. Left panel illustrate that two kinases are orchestrated by lncRNA phosphorylation of effector. Right panel illustrate the scenario that the regulatory unit (R) and kinase (K) are bridged by lncRNAs as a mega complex for substrate modification. Camouflage – presence of the lncRNA disguises the surface of target proteins, preventing the reorganization and post-translational modification of target proteins by upstream signaling events. Transporter – presence of the lncRNA facilities the relocation of binding proteins from cytosol to mitochondria or shuttling between cytosol, nucleus, and other subcellular compartments. Abbreviations: lncRNA, long noncoding RNA; Sub, substrate.

lncRNAs can modulate enzyme activity by acting as allosteric regulators, whereby lncRNA binds to the allosteric site of the enzyme, modifying the conformation of the active site. One example is that the PH domain of AKT serves as docking site to associate with PIP_3, which modulate the kinase activity of AKT [72]. The presence of *LINK-A* enhances the interaction between PH domain and PIP_3, leading to hyperactivation of AKT [13]. Another example is that lncRNAs that are upregulated in breast cancer brain metastasis associates with the negative regulatory domain of Janus kinase (JAK)2, leading to alleviation of the inhibitory role of JH2 domain, and enzymatic activation [73].

lncRNAs may act as synergizers, by which lncRNA conjoins multiple enzymes and/or regulators together, mediating cooperative action on its effectors or substrates. Recent studies indicate that the lncRNA *AK023948* associates with both DHX9 and p85 and that the presence of *AK023948* is required for the DHX9–p85 interaction, consequently resulting in enhanced PI3K activity and AKT phosphorylation [44]. Another example is that upon ligand binding, the signaling events trigger RNA–protein complex formation containing LLGL2–*MAYA*–NSUN6. LLGL2 recruits the substrate MST1 to the complex; NSUN6 acts as the methyltransferase; and *MAYA*, the lncRNA, serves as a scaffold that bridges the interaction between LLGL2 and NSUN6 upon extracellular stimulation, leading to MST1 methylation [46].

Alternatively, the lncRNA can also camouflage or disguise the surface of target proteins, preventing the reorganization and post-translational modification of target proteins by upstream signaling events. *Lnc-DC* interacts with STAT3 in the cytoplasm, preventing SHP1-dependent dephosphorylation of STAT3 (Tyr705). These events facilitate the differentiation of dendritic cells [41]. *NKILA* is upregulated in breast cancer and associates with IκB, preventing IKK-dependent IκB phosphorylation at Ser32/36 [40]. Hence, it is clear that signaling pathways in cancer and other cellular activities are tightly regulated by lncRNAs.

The diverse roles of lncRNAs may also contribute to the relocalization of binding proteins as transporters. Recent studies indicate that the lncRNA *SAMMSON* associates with p32, a mitochondria surface protein. The *SAMMSON*–p32 interaction facilitates relocalization of p32 to mitochondria, resulting in enhanced mitochondrial metabolism [74,75]. The relocation of the lncRNA component of the RNA processing endoribonuclease (RMRP) from the nucleus to the mitochondria facilitates the accumulation of RMRP in the mitochondria, which is required for mitochondrial respiration [76]. Hence, lncRNA may communicate between nucleus and mitochondria for fine-tuning the cellular and mitochondrial activities [75].

Targeting lncRNAs in Cancer Therapy

From a clinical perspective, lncRNAs serve as promising therapeutic targets. Multiple therapeutic strategies have been developed to target lncRNAs. Antisense oligonucleotide (ASO)-based strategies that downregulate the transcripts of lncRNAs via RNase H-dependent degradation are under active investigation (recently reviewed by Matsui and Corey [77]). Alternatively, liposome/nanoparticle-delivered siRNAs have been developed to knockdown lncRNAs *in vivo* via Dicer- and Agonaute-dependent RNA silencing [78–80], which have been evaluated in xenograft models and have been found to inhibit tumorigenesis and distant metastasis [79,81]. Small-molecule inhibitors to block lncRNA–protein interactions or interfere with lncRNA–protein complex formation are also on the rise [77]. Cancers frequently become resistant to administered chemotherapeutic agents. In these chemotherapy-resistant tumors, dysregulated lncRNAs can contribute significantly to the development of this resistance. Given the failure of certain pathway inhibitors during clinical trials, combinations of pathway-specific inhibitors integrated with an lncRNA-directed strategy may provide maximum efficacy in

treating human cancer, which is under active investigation. Targeting lncRNAs using a variety of technologies, including ASO-based strategies, siRNAs and small molecular inhibitors should be evaluated for their effects on cancer initiation, progression, metastasis and response to therapy.

ASOs

ASOs, including ASO gapmers [82], duplex RNA [83], and locked nucleic acids (LNAs) [84] binds to lncRNA transcripts via base pairing. The RNA–DNA duplex triggers RNase-H-dependent cleavage [85]. The newer generation of ASOs incorporates chemical modification of the sugar backbone to improve binding affinity and *in vivo* stability [86,87]. LNAs [84,88,89] and S-constrained ethyl (cEt) modifications [90] have been advanced to clinical trials [91–93]. LNAs are synthesized using modified RNA nucleotides, which contain an extra covalent bond between the 2′-O and 4′-C of the ribofuranose ring [94,95]. The mixed LNA–DNA–LNA gapmers can base pair with RNA targets, which can be used to silence RNA targets in cell-line-based experiments and animal models. The incorporation of bridged nucleic acid (BNA) monomers can serve a similar purpose as LNAs [96]. The application of ASOs to knockdown lncRNAs *in vivo* has been tested in a variety of cancer models, with noteworthy inhibitory effects on tumor growth and progression [97,98]. LNAs targeting *PVT1* (plasmacytoma variant translocation 1) have been shown to sensitize ovarian cancer cells to cisplatin, substantiating the effectiveness of combinatorial treatment [99]. Additionally, *lncARSR* promotes cancer cell resistance to sunitinib, but cancer cell sensitivity to sunitinib could be improved by targeting *lncARSR* using LNAs [100]. For lncRNAs that act as tumor suppressors, LNAs mimicking *GAS5* (growth arrest specific transcript 5) binding sequence to the hormone receptors is sufficient to induce cancer cell apoptosis [101]. Clinical trial using LNAs targeting Bcl-2 oncoprotein [91], hypoxia-inducible factor (HIF)1α (NCT00466583), and androgen receptor (AR) [102] have shown promise and are under evaluation. Beyond its uses in cancer, LNAs have also been proposed to benefit patients with cardiovascular diseases [103], kidney diseases [104], neuronal disorders [105], and other human pathological conditions.

Nanoparticle-Delivered siRNAs

Targeting lncRNAs using a siRNA-based strategy has been successfully applied in several preclinical models [106]. Recently, dioleoyl phosphatidylcholine (DOPC)-based nanoliposomes have been developed for the delivery of nucleotide based-therapeutics (siRNA, miRNA, lncRNA, and ASOs) for *in vivo* and clinical use [107–115]. Studies indicate that a single injection of DOPC–nanoliposomal siRNAs can inhibit the expression of target proteins for 3–5 days in mouse tumors [113,115]. DOPC nanoliposomes, with an average size of 50 nm, can be administered via a single intravenous or intraperitoneal injection to deliver select siRNAs and anti-miRs into tumor cells *in vivo*. This single administration produces a significant repression in the expression levels of the gene targets (e.g., Bcl2, eEF2K, FoxM1, Kras or miRs155, miR34a, and JAK2) and in tumor size in mouse models and preclinical models of human cancer, including subcutaneous xenografts and orthotopic tumor models [73,107–109,111,113,115–117].

Small-Molecule Inhibitors of lncRNAs

lncRNAs form complicated tertiary structures. Whether the secondary or 3D structure of lncRNAs molecules are conserved between species remain elusive [118]. One explanation is that a portion of the secondary or tertiary structures of lncRNA molecules, for example, key stem-loop structures for protein binding, are conserved; similar to the protein domain conservation that linker regions are variable. RNA molecules are potential targets for small-molecule inhibitors. High-throughput screening has been used to identify small-molecule compounds

that could potentially inhibit RNAs [119–121]. Efforts to establish platforms to aid the design and identification of small molecule inhibitors for oncogenic ncRNAs are under development [122], which will facilitate the large-scale development of pharmaceutical agents that target lncRNAs.

Concluding Remarks

The interwoven signaling pathways present in many human cancer types complicate the development of targeted therapies or chemotherapeutic agents. lncRNAs play a pivotal role in mediating the crosstalk between the various cellular components, including proteins, RNAs, and lipids, that are involved in cancer cell signal transduction. Due to the prevalence of lncRNA involvement in cancer pathways, numerous studies have elucidated the importance of lncRNAs in various disease processes of cancer, including initiation, progression, invasion, and metastasis. Identification of the specific lncRNAs that function in various human cancer types has led to pioneering efforts to develop lncRNA-based clinical applications such as biomarkers for diagnosis, prognostic indicators, stratification markers, drug sensitizers, and therapeutic targets. The lncRNA profile of each human cancer type should be systematically investigated to improve clinical outcomes for cancer patients by engendering a personalized approach to medicine.

Future studies on the regulatory and biological roles of lncRNAs in cancer signaling will define the future of the field (see Outstanding Questions). Although a huge list of lncRNAs has been identified thus far, it has been a strenuous task to demonstrate the functional relevance of lncRNAs in cancer. To answer this problem, thorough examinations of lncRNA candidates involved in cancer signaling pathway need to be conducted to reveal the physiological relevance of lncRNAs in cell apoptosis, survival, metastasis, and metabolism. Cellular and xenograft models have been the common means of studying the roles that lncRNAs play in cancer and are useful tools in cursory evaluations of their functions. However, conclusions that are more definitive will require representative *in vivo* models of cancer, such as genetic models that better recapitulate the tumor microenvironment. It will be crucial to determine if tissue-specific expression of lncRNAs, by genetic knock-in, can induce spontaneous tumor formation, which can then be blocked by targeting the lncRNAs.

Outstanding Questions

Can lncRNAs serve as regulatory components of cancer signaling pathways?

Do lncRNAs have *bona fide* biological functional roles in cancer?

Can effective therapeutic strategies be developed by targeting either lncRNAs alone or in combination with chemotherapeutic and targeted therapy agents?

Disclaimer Statement

None.

Acknowledgments

We apologize to colleagues whose work was not able to be included in this review due to space limitations. We thank Mr D. Aten for assistance with figure presentation. We are grateful to Mr Peter K. Park and Sergey Egranov for assistance with manuscript drafting. Research in the C.L. and L.Y. laboratories is funded by NIH R00 award (R00DK094981), R01 award (1R01 CA218025-01), and CPRIT (150094) to C.L., and the NIH R00 award (R00CA166527), R01 award (1R01 CA218036-01), CPRIT award (R1218), and DOD breakthrough (BC151465) grants to L.Y.

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